

Construction of Infectious Disease
Resistant Animals by Manipulation
of the Acute Phase Response.

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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October 2000

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ACKNOWLEDGEMENTS.

I would like to thank my supervisor Prof. Tim Mitchell for all his help throughout my project and particularly during the period of writing up this thesis.

Also thanks to Dr Bernard Burke for all his practical help throughout my project, the many discussions about the direction of the project and his eternal optimism.

Thanks must go to Dr Andrew Collick and Ms Jane Brown (Leicester University Transgenic Unit) for all their hard work to create the line of transgenic mice. Also to Dr Robin Reid (Pathology, Glasgow Western Infirmary) and Dr Alan Mowat (Immunology, Glasgow University) for their help with the pathology and histology work. And to Prof. Chris Cannings (MGM, Sheffield University)

Thanks also to the many colleagues and friends from the labs both in Leicester and Glasgow who have made this a most enjoyable time; and for the practical help they have offered me (particular mention must go to Neill Gingles and Alison Kerr for the many discussions of the animal work).

Finally thanks to my parents, John and Liz, and to David for all their support (both emotional and financial) during this project and for always being at the end of a phone with words of encouragement.

Quote.

SIR RALPH BLOOMINGFIELD BONINGTON : Nature has provided in the white corpuscles as you call them - in the phagocytes as we call them - a natural means of devouring and destroying all disease germs. There is at the bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes.....The phagocytes are stimulated; they devour the disease; and the patient recovers.

From "The Doctor's Dilemma" by Bernard Shaw.

SIR PATRICK : Opsonin? What the devil is opsonin?

RIDGEON : Opsonin is what you butter the disease germs with to make your white corpuscles eat them.....The phagocytes won't eat the microbes unless the microbes are nicely buttered for them.

From "The Doctor's Dilemma" by Bernard Shaw.

ABBREVIATIONS.

Bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius
CAT	Chloramphenicol acetyl transferase
cfu	Colony forming units
CRP	C-reactive protein
dATP	2'- deoxyadenosine 5' -triphosphate
dCTP	2'-deoxycytosine 5' -triphosphate
dGTP	2'- deoxyguanosine 5' -triphosphate
dTTP	2'- deoxythymidine 5' -triphosphate
dNTP	2'- deoxynucleoside 5' -triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ES cell	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
x g	Acceleration in the earths gravitational field
g	Gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hr(s)	Hour(s)
IFN (α , β or γ)	Interferon (α , β or γ)
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneum
i.v.	Intravenous
kb	Kilobase pairs
l	Litre
LPS	Lipopolysaccharide
M	Molar

mAb	Monoclonal antibody
mg	Milligrams
min(s)	Minute(s)
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
nm	Nanometer
OD	Optical Density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec(s)	Second(s)
SEM	Standard error of the mean
TEMED	N,N,N',N-tetramethylethylenediamine
TNF	Tumour necrosis factor
Tris	2-amino-2-(hydroxymethyl)1,3-propanediol
UV	Ultraviolet light
V	Volts
µg	microgram
µl	microlitre

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APPENDIX I : Addresses of Suppliers

Chapter 1: Introduction.

The aim of this project was to produce transgenic animals that are resistant to infectious disease, with possible commercial applications in the production of disease resistant livestock. In order to achieve this we have used the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) which has many effects on the immune response including increasing the proliferation and activation of white blood cells. Constitutive expression of this cytokine leads to many deleterious effects and results in the mice dying prematurely (Lang *et al.*, 1987), so it was reasoned that to prevent these harmful effects it would be necessary to express the cytokine in a controlled manner. In order to achieve this controlled expression of GM-CSF we have utilised the pattern of expression of the acute phase response, so that high levels of GM-CSF are produced at the onset of infection or inflammatory stimulus. Acute phase expression of GM-CSF was predicted to rapidly increase the proliferation and activation of white blood cells and therefore confer an increased resistance of these animals to infectious disease.

In the introduction to this thesis I will discuss the use of transgenic technologies in the study of disease resistance, and the application of transgenic techniques in species other than the mouse. I will also discuss the two aspects of the immune system we have exploited in this study - the acute phase response, and the cytokine system - and will concentrate specifically on the acute phase protein C-reactive protein (CRP) and the cytokine GM-CSF.

1.1 Transgenic Approaches to Disease Resistance.

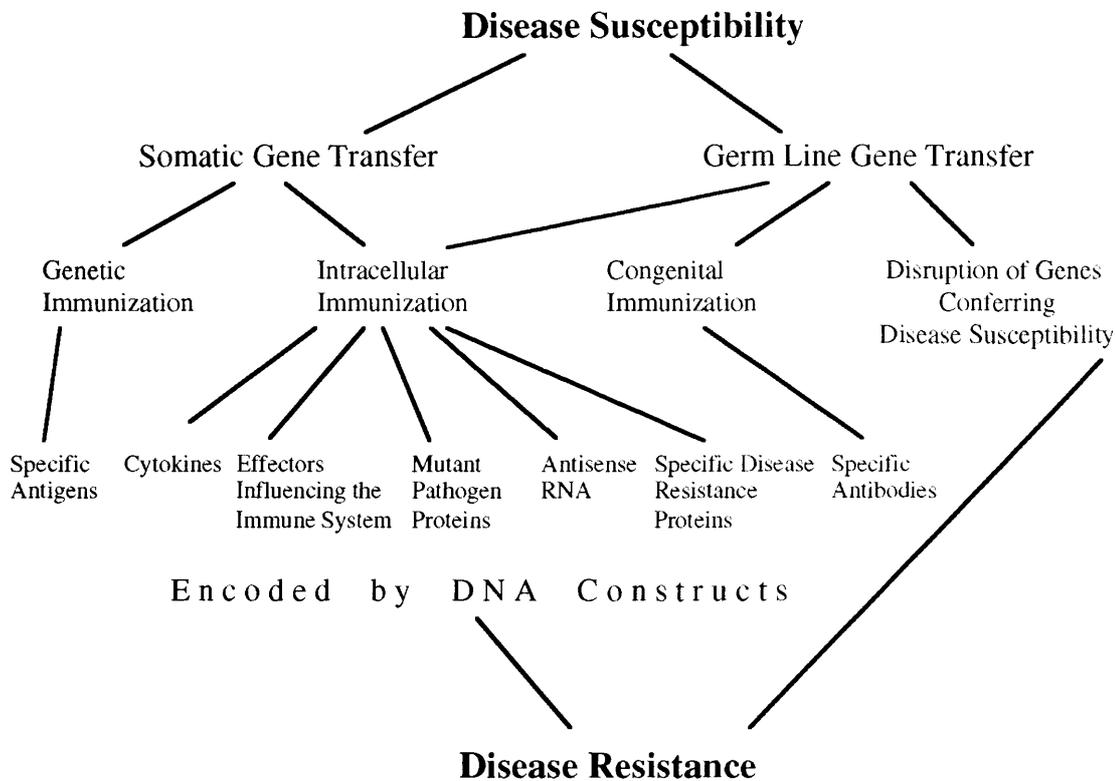
There are many transgenic approaches being followed to create animals that are resistant to disease; and these are discussed below (see figure 1.1).

1.1.1 Congenital Immunisation.

This is transgenic expression, and germ line transmission, of a gene encoding an immunoglobulin specific for a pathogen, therefore providing resistance to that pathogen without previous exposure.

Following infection of mice with lymphocytic choriomeningitis virus (LCMV), virus-neutralising antibodies appear and these play an important role in protection against reinfection. In order to try and prevent initial infection, transgenic mice were created that express the immunoglobulin μ heavy chain of an LCMV neutralising monoclonal antibody (Seiler *et al.*, 1998). On challenge with virus, the mice produce

Figure 1.1: Strategies to improve disease resistance by transgenic means. Adapted from Muller and Brem (Muller and Brem, 1996).



neutralising IgM within 8 days after infection and this significantly improved the ability of the animals to clear the infection.

Because the production of antibody to various polysaccharide antigens can be protective against pathogenic bacteria, transgenic pigs were made that carried genes coding for the mouse α and κ chains for antibodies against phosphorylcholine (PC) (Lo *et al.*, 1991). Two transgenic lines were created but only the IgA transgene was integrated in both lines. Both lines expressed high serum levels of mouse IgA despite the absence of any mouse L chain, indicating that it was able to form complexes with the endogenous pig L chain. Because of this the serum IgA was unable to specifically bind to PC. The results, however, show that immunoglobulin genes can be expressed in transgenic animals and that congenital immunisation is a viable proposition.

1.1.1.1 Expression of Protective Antibody in Milk.

Another approach utilising transgenic animals producing antibodies, is the direction of antibody production to the milk of the animals with the aim of protecting offspring from enteric infections.

Transgenic mice secreting a recombinant monoclonal antibody that neutralised transmissible gastroenteritis coronavirus (TGEV), under the control of the whey acidic protein regulatory sequences, have been made (Castilla *et al.*, 1998). The antibody was produced at high levels throughout lactation and the antibody reduced TGEV infectivity 10^6 fold.

1.1.2 Intracellular Immunisation.

This involves the intracellular expression in the host of transgene products that are able to interfere with the pathogen.

1.1.2.1 Genes Modulating the Immune Response.

Cytokines contribute directly to the development of pathology during infectious disease and tumourigenesis. Different cytokines can both positively and negatively influence host defence mechanisms.

Mice transgenic for interferon- β were shown to have increased resistance to viruses (Chen *et al.*, 1988). The human β 1-interferon gene (HuIFN β) was fused to the metallothionein I promoter region, and this transgene used to make transgenic mice. The sera of these mice protected human WISH cells against vesicular stomatitis virus infection, and this activity could be neutralised by preincubation with anti-HuIFN β antibody. The transgenic mice were also more resistant to pseudorabies virus infection than the non-transgenic littermate controls. Two different strains of transgenic mice were examined and the mean survival time, after pseudorabies injection, correlated with the concentration of HuIFN- β 1 in the serum.

However the insertion of cytokine transgenes can have harmful side effects (see discussion of GM-CSF transgenic mice section 1.6.8).

1.1.2.2 Specific Disease Resistance Genes.

Only a few single genetic loci responsible for disease resistance are known. A well characterised example of this is the Mx1 gene product of mice, and this is discussed in more detail below.

1.1.2.2.1 Mx1 Protein.

Resistance of A2G mice to influenza A and B viruses was recognised by Lindenmann (Lindenmann, 1962). Resistance was shown to be inherited as a single, dominant trait, it was specific for orthomyxoviruses, effective against high virus doses and independent of the route of infection. More recently resistance was shown to be the result of a single gene, Mx1, which is induced on viral infection through interferons α and β , but not γ . The Mx1 protein is a nuclear GTPase which interferes with the accumulation of primary influenza viral transcripts. Almost all animals possess interferon-inducible Mx genes. These show sequence similarity to dynamins, a family of GTPases involved in endocytosis. It is therefore possible that Mx proteins are involved in normal cellular processes and inhibition of viruses is a consequence of this.

1.1.2.2.2 Mx1 Transgenic Mice.

Using a constitutive promoter to drive the expression of Mx1 in transgenic mice only resulted in transgenic mice that expressed very little or no Mx1 protein (Arnheiter *et al.*, 1990). It was reasoned that constitutive expression of Mx1 protein was harmful and mice expressing high levels were eliminated before birth. This also highlights the importance of the expression of transgenes in a controlled manner in order to create viable offspring, and the fact that unregulated expression of transgenes can lead to many harmful effects (see also section 1.6.8).

Transgenic mice were therefore created using the Mx1 cDNA linked to the interferon-inducible Mx1 promoter. After intravenous introduction of interferon these mice were shown to produce Mx1 protein in the liver with maximal levels at 3-6 hours and levels falling after this time (Arnheiter *et al.*, 1990). These mice were then challenged intracerebrally with the neurotropic mouse-adapted human influenza A/NWS virus. Almost all transgenic mice survived the infection at all doses (10^{-2} to 10^{-7}) compared to non-transgenic littermates where all mice were killed at the higher doses (10^{-2} to 10^{-5}) and most died at the lower doses (10^{-6} and 10^{-7}) (Arnheiter *et al.*, 1990). The same pattern of survival was seen when the mice were infected intranasally with a pneumotropic mouse-adapted human influenza virus (A/PR/8/34). The transgenic mice survived infection with all doses (10^{-1} to 10^{-5}) compared to non-transgenic littermates where all mice were killed by infection with the higher doses (10^{-1} to 10^{-4}) and only survived the lowest dose given (10^{-6}) (Arnheiter *et al.*, 1996).

The brains of the intracerebrally infected animals were examined using immunocytochemistry to determine the pattern of expression of viral antigens and Mx1 protein-producing cells. This showed viral antigen in a few ependymal cells, which are

the first cells to support viral replication. Immediately surrounding these cells were many Mx1 protein-producing cells with fewer Mx1 expressing cells the greater the distance from the infected cells. This indicates that interferon induction, and therefore Mx1 protein expression, occurs locally; which results in the virus-infected cells becoming surrounded by a barrier of protected cells (Arnheiter *et al.*, 1990). With the intranasally infected animals the same pattern of Mx1 expressing cells surrounding the virus infected cells was seen in the lung. The mechanism of resistance in these mice is therefore thought to be virus induced production of Mx1 at the sites of initial infection which blocks the spread of the virus to surrounding cells and provides time for other defence mechanisms to clear the virus infected cells.

1.1.2.2.3 Resistance of Mx1 Mice to Murine Viruses.

Mice are not natural hosts of influenza viruses, so reasons for the evolution of a system of resistance to these viruses are unclear. It may be that they are not natural hosts because they possess this system of resistance; there are however tick-borne orthomyxoviruses, Thogoto (THO) and Dhori viruses, that the animals would be likely to encounter in the natural environment.

Mx1 positive congenic and A2G (natural carriers of the Mx1 gene) mice were infected intraperitoneum with a lethal dose of THO virus and compared with Mx1 negative controls. The Mx1 positive mice survived infection without symptoms compared to the negative controls which became progressively ill and succumbed to the infection (Haller *et al.*, 1995). The same pattern of survival of Mx1 positive mice was seen when the virus was introduced intracerebrally and subcutaneously (Haller *et al.*, 1995). To determine that this effect was due to the Mx1 protein alone, and not another interferon dependent mechanism, transgenic Mx1 mice (Arnheiter *et al.*, 1990) were analysed. These were infected with a lethal dose of THO virus and survival compared with that of non-transgenic littermates. As expected all the Mx1 transgenic mice survived compared to the non-transgenic littermates that succumbed to the infection (Haller *et al.*, 1995).

Mx1 positive congenic and A2G mice were also infected intraperitoneum with a lethal dose of Dhori virus and compared with Mx1 negative controls. The Mx1 positive mice survived infection without symptoms compared to the negative controls which became progressively ill and succumbed to the infection (Thimme *et al.*, 1995).

1.1.2.2.4 Mx1 Transgenic Pigs.

Pigs are affected by swine influenza epidemics and are also thought to act as 'mixing vessels' for the creation of new pandemic strains. It would therefore be desirable to improve the resistance of pigs to influenza viruses. One approach being followed is to create transgenic pigs expressing Mx1 which could promote resistance to influenza.

Transgenic pigs carrying the Mx1 gene controlled by constitutive promoters have been made. The efficiency of gene transfer was very low and all transgenic animals had rearrangements in the transgene copies that abolished protein synthesis (Muller *et al.*, 1992). This may be because constitutive expression of Mx1 is deleterious therefore no offspring expressing permanently high levels were produced. Using an interferon inducible promoter resulted in normal gene transfer efficiency. On treatment with interferon an increase in transgene mRNA was detectable but no increase in Mx1 protein was detected (Muller *et al.*, 1992).

1.1.2.3 Antibacterial Products.

Staphylococcal infection of the mammary gland in lactating ruminants (mastitis) is a commercially important disease, and it is difficult to treat with antibiotics. A novel idea for treatment of this disease would be to produce transgenic animals which could secrete the antistaphylococcal enzyme lysostaphin in their milk. Lysostaphin is a cell wall degrading enzyme which is active against staphylococcal species. It is a good candidate for treatment of mastitis as it has activity against both dividing and non-dividing bacteria, it has no oral toxicity and is digested by intestinal proteases.

It has been shown that treatment of mice with lysostaphin resulted in a significant reduction in the number of viable *Staphylococcus aureus* in the mammary gland (Bramley and Foster, 1990). The use of lysostaphin in the treatment of mastitis in cattle has also been studied (Bramley and Dodd, 1984), however to be effective at eliminating bacteria it must be present within and around host cells. It has been shown that regulatory sequences from milk protein genes can be used to direct the expression of other proteins to the mammary glands (Clark *et al.*, 1989); therefore it should be possible to direct the production of lysostaphin to the mammary glands.

The lysostaphin gene has been successfully expressed and processed in an eukaryotic system (Williamson *et al.*, 1994). This shows there is no problem in expressing this prokaryotic gene in mammalian cells. As active lysostaphin can be secreted in a eukaryotic system, lysostaphin is effective in clearing *S. aureus* from the mammary gland and vectors exist to target expression to the mammary glands it should be possible to create transgenic animals that are resistant to mastitis.

Transgenic mice have been created that express the human antimicrobial enzyme lysozyme in the mammary gland under the control of the bovine gene promoter for alpha(s1)-casein (Maga *et al.*, 1995). These mice secreted human lysozyme into the milk at an average concentration of 0.38 mg/ml. This concentration of lysozyme in the milk was shown to be bacteriostatic, *in vitro*, against a mastitis causing strain of *S. aureus* and the food-spoiling organisms *Pseudomonas fragi* and *Lactobacillus viscosus* (Maga *et al.*, 1998). This suggests that expression of this enzyme in transgenic animals may be able to prevent mastitis and also that milk containing lysozyme would be more resistant to spoilage.

1.1.2.4 Antisense RNA.

Antisense RNA functions by binding in a highly specific manner to complementary sequences to produce a duplex, which blocks the ability of the bound RNA to be processed and translated.

Transgenic mice with antisense RNA targeted to the retroviral packaging sequences of Moloney murine leukaemia virus did not develop leukaemia following challenge with infectious virus (Han *et al.*, 1991).

Ernst (Ernst *et al.*, 1990) described the generation of transgenic rabbits expressing an antisense construct complementary to adenovirus h5 RNA. Primary cells from these transgenic animals were found to be 90-98% more resistant to adenovirus infection than cells from control animals.

Because some RNA molecules (ribozymes) have enzymatic self-cleaving activity another approach is to create antisense molecules that also cleave the target RNA strand. Transgenic mice expressing a glucokinase (gk) antisense RNA containing a ribozyme element under the control of the insulin promoter have been made. Ribozyme-mediated attenuation of pancreatic β -cell gk expression in these mice resulted in approximately 30% of normal islet gk activity and impaired glucose-induced insulin secretion (Efrat *et al.*, 1994).

Bovine leukaemia virus (BLV) is a retrovirus that causes persistent lymphocytosis and B-lymphocyte lymphoma in cattle and sheep. A hammerhead ribozyme (the smallest (40-50 nucleotides) structural motifs that has the ability to cleave RNA, and so named because its secondary structure resembles the shape of the head of a hammerhead shark) flanked by antisense sequences directed against regulatory proteins of BLV has been shown to inhibit BLV expression in permanently transfected cells (Cantor *et al.*, 1993). This suggests the possibility of creating transgenic cattle or sheep that will be resistant to BLV.

A comparison of the effectiveness of antisense and ribozyme containing antisense constructs was done by creating transgenic lines that expressed either antisense mRNA directed against bacterial chloramphenicol acetyl transferase (CAT) (construct ACAT) or antisense mRNA containing 4 catalytic ribozyme structures (construct rbz-ACAT). The ability of these constructs to downregulate CAT protein levels was looked at in double CAT expressing and antisense/ribozyme transgenic mice. The transgenic mice expressing the ACAT construct downregulated CAT protein levels by 90% compared to the CAT expressing mice. The transgenic mice expressing the rbz-ACAT construct downregulated CAT protein levels by 87%. This result suggests that the expression of antisense mRNA effectively downregulates protein production, but the incorporation of ribozymes into the antisense construct does not further enhance the downregulation of protein production, at least in this system (Sokol *et al.*, 1998).

1.1.2.5 Pathogen Proteins.

This is based on the overexpression in the host of an aberrant form of a protein from a pathogenic organism which is able to interfere strongly with the replication of the wild type virus.

One example is resistance to avian leukosis virus (ALV) in chickens. Infection of chickens with ALV results in a variety of tumours and it is a commercially important disease, with subgroup A ALV being the most common cause of infection. Subgroup E ALV is an endogenous virus and chickens carrying the subgroup E envelope genes *ev3* and *ev6* in the genome are resistant to infection with subgroup E virus (Robinson *et al.*, 1981). It was therefore reasoned that insertion of subgroup A envelope genes into the germline of chickens would make them resistant to infection with subgroup A ALV.

Transgenic chickens were made that contained the *alv6* proviral insert which expressed the subgroup A envelope protein (Crittenden *et al.*, 1989). *In vitro* interference assays showed that chick embryo fibroblasts carrying the *alv6* proviral insert were resistant to subgroup A but not to subgroup B infection. The *in vivo* protection of *alv6* chickens was demonstrated by infection of week-old chicks with subgroup A ALV. None of the 6 chicks containing the *alv6* insert had tumours 2 weeks post infection; compared to 14 out of 17 of the chicks lacking the insert (Salter and Crittenden, 1989).

Mice transgenic for a murine leukaemia virus envelope gene (Fv4) resist infection and do not become immunosuppressed when inoculated with Friend virus, compared to non-transgenic littermates that succumb to the infection (Nihrane and Silver, 1997).

1.1.3 Targeted Disruption of Genes Causing Disease.

Targeted disruption of genes can be achieved by the use of embryonic stem cells. These cells can be manipulated *in vitro* to disrupt target genes by homologous recombination then injected into developing embryos to create chimeric animals with the gene disrupted (knockout animals).

An example of this is mice that are deficient for the IL-1 receptor gene. These mice showed decreased mortality when challenged with *E. coli* 0111:B4, and were less sensitive to lethal doses of LPS (Acton *et al.*, 1996). Another example is mice that have the gene for CD28 knocked out, which makes them resistant to the *Staphylococcus aureus* TSS toxin-1 (Saha *et al.*, 1996).

It should be noted that the disruption of target genes may have an effect on the progression of a disease, but this may also have consequences in terms of the health of the animals as these genes may play an important role in the normal system.

1.2 Nonmurine Transgenics.

Most transgenic work has been carried out in the mouse, this is because of: (a) the short gestation time of the mouse (19-21 days), (b) the early onset of fertility (6-8 weeks), (c) the large litter sizes (5-12 offspring), (d) and the space requirements for maintenance of a large breeding stock. The mouse is not always the ideal model for some situations and therefore transgenic technology is being expanded to create transgenic animals of many other species. For example, one of the first reports of a study using transgenic rats showed that the introduction of HLA-B27 and human β_2 -m into rats induced an inflammatory disease (Hammer *et al.*, 1990). When mice were made with these transgenes they failed to show an adequate phenotype, so in this case transgenic rats were a much more informative model of disease.

Most work on transgenic farm animals has focused on the improvement of productivity traits: production of proteins of high value (such as human health products), provision of animals models for human diseases, and organs for xeno-transplantation. The reduction of disease susceptibility in livestock is an important consideration for both animal welfare and economically, as the costs of disease account for 10-20% of the total production costs. Examples of nonmurine transgenics are given in table 1.1.

Table 1.1: Nonmurine transgenics.

Species	Transgene	Reference
Rat	HLA-B27 and human β_2 -m	Hammer <i>et al.</i> , 1990
Rabbit	Fusion between the mouse metallothionein promoter and the human growth hormone structural gene	Hammer <i>et al.</i> , 1985
Sheep	Metallothionein-human growth hormone fusion	Hammer <i>et al.</i> , 1985
Sheep	Human α_1 -antitrypsin directed to the mammary gland	Wright <i>et al.</i> , 1991
Goat	Human tissue-type plasminogen activator directed to the mammary gland	Ebert <i>et al.</i> , 1991
Pig	Human haemoglobin	Swanson <i>et al.</i> , 1992
Cow	Human α -lactalbumin directed to the mammary gland	Eyestone <i>et al.</i> , 1998

The development of embryonic stem (ES) cell and homologous recombination technologies allows the insertion of the transgene into a specific location within the genome, and controls the number of copies of the transgene (only a single copy is inserted). This prevents the problems of the effect of the insertion site and the copy number of the transgene that are associated with microinjection of embryos. ES cells from mice are routinely cultured, but the development of ES cell lines for other animals will be of great importance in the production of transgenic and knockout animals.

ES cells from pigs have now been isolated (Wheeler, 1994), and have been shown to be maintained as pluripotent cells for up to 44 passages. Differentiation of pluripotent ES cells *in vivo* was tested by their ability to participate in the formation of chimaeric offspring. Embryos at the blastocyst stage were injected with ES cells and transferred to recipient foster mothers. Chimaeric offspring were detected by coat colour (black hair ES cells were injected into red-brown hair embryos), and 72% of the offspring generated showed coat colour chimaerism. This showed that the ES cells were able to contribute to all aspects of development. Once these ES cells can be successfully transformed with foreign DNA the potential exists to produce chimaeric pigs from cultured ES cells.

Another method which may prove useful in the generation of transgenic livestock is the development of sperm mediated transfer of DNA. Over 25 years ago it was shown that, after exposure of rabbit sperm to ^3H -thymidine labelled SV40 DNA, radioactive material could be detected in the heads of the sperm. When these sperm were used to inseminate rabbits, the fertilised eggs obtained were able to produce infectious virus (Brackett *et al.*, 1971). These findings suggested that sperm cells are able to carry exogenous DNA into the egg at fertilisation to create transgenic animals.

The first report of the successful production of transgenic animals using this method showed that mouse epididymal sperm cells coincubated with a 5 kb pSV2-CAT gene construct spontaneously took up the DNA and transferred it into eggs during fertilisation (Lavitrano *et al.*, 1989). Of the offspring produced approximately 30% were transgenic.

Because rejection of transplanted organs is thought to be associated with human complement activation, and hDAF prevents the assembly of C3 and C5 convertases and accelerates the decay of C3 convertase, it is thought that expression of hDAF on pig cells may help prevent rejection of xenografts. Pigs transgenic for human decay accelerating factor (hDAF) have also been generated using sperm mediated gene transfer (Lavitrano *et al.*, 1997). Of 42 piglets born 12 of these were transgenic for hDAF. This is much higher than the frequency of generation of transgenics by standard microinjection procedures. This technique could also be an important addition to livestock transgenesis as it could be easily incorporated into routine breeding programmes (through artificial insemination).

1.3 The Acute Phase Response.

Our approach to the generation of transgenic animals that are resistant to disease has been to develop a controlled expression system that could be used to express a wide variety of proteins capable of contributing to disease resistance. This controlled expression system was based on the kinetics of expression of the acute phase proteins.

The local response of tissue to injury and infection is acute inflammation, and this inflammation induces a wide range of systemic and metabolic changes termed the acute phase response. This has been reviewed by, among others (Kushner, 1982)(Baumann and Gauldie, 1994)(Kushner and Rzewnicki, 1994)

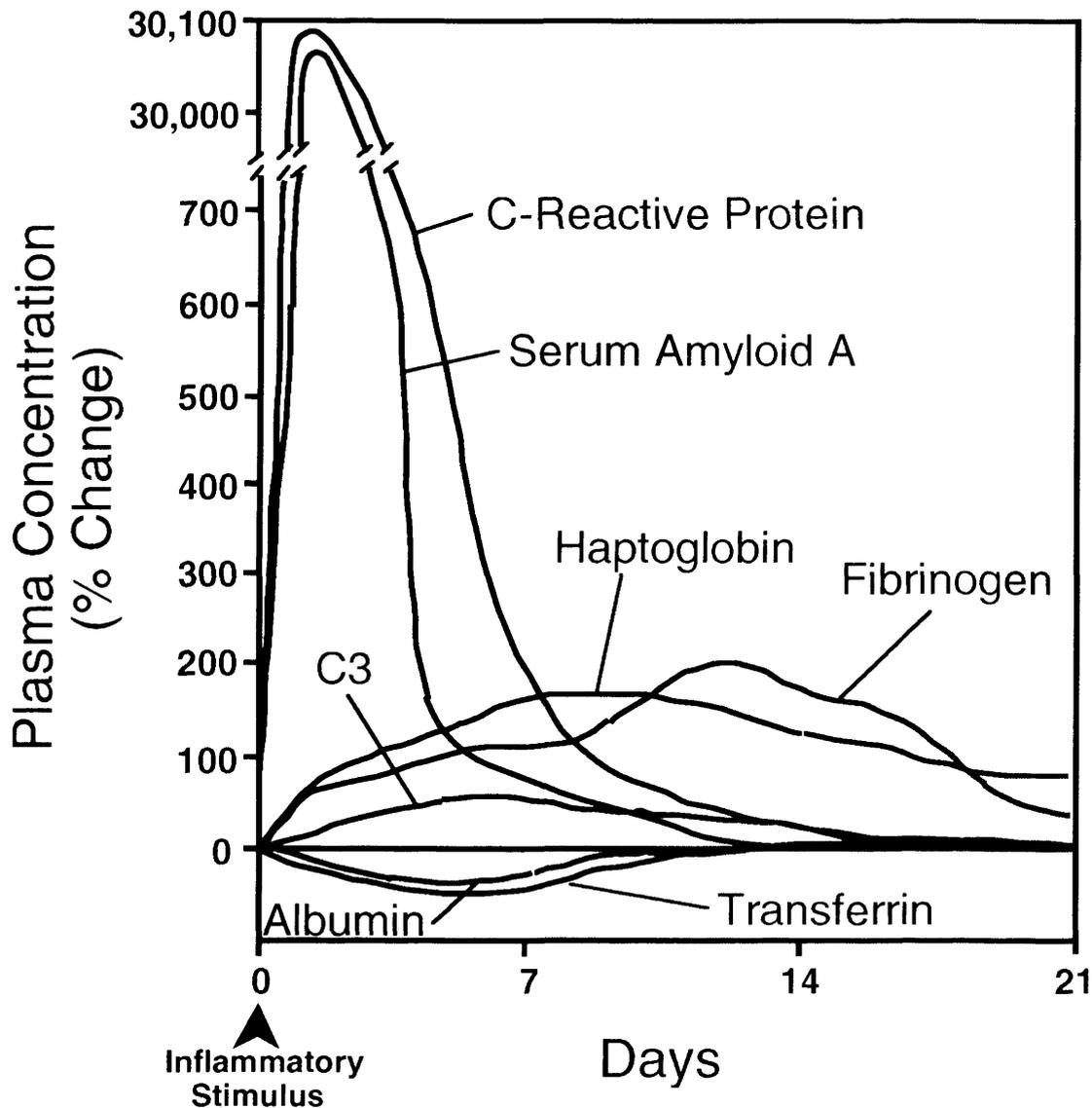
One of the earliest recognised changes was fever which is the result of endogenous pyrogen elevating the body temperature 'set point'. The febrile response seen during the acute phase is induced by the cytokines IL-1, IL-6 and TNF; which mediate fever in many ways including through the induction of prostaglandin E₂ (Dinarello *et al.*, 1991). Another long-recognised change is an increased granulocyte count in the blood due to increased release from bone marrow storage pools.

Changes in the rate of synthesis of various endocrine hormones are also seen in response to injury and infection; including increased synthesis of glucagon, insulin, ACTH (adrenocorticotrophic hormone), cortisol, adrenal catecholamines, growth hormone, thyroid stimulating hormone, thyroxin, aldosterone and vasopressin.

Changes in body metabolism include increased protein catabolism, increased gluconeogenesis, inhibition of bone formation and negative nitrogen balance (mainly due to proteolysis and decreased protein synthesis in skeletal muscle). Serum concentrations of copper are increased, due to an increase in the concentration of the copper binding protein ceruloplasmin. While serum concentrations of zinc and iron are decreased during the acute phase response. Decreased concentrations of zinc are thought to be due synthesis of hepatic zinc-binding metallothionein (Sobocinski *et al.*, 1979); while decreased iron concentrations are thought to be due to increased storage of iron in the tissue ferritin stores (Konijn and Hershko, 1977).

The term 'acute phase response' is however usually used to describe the change in concentration of a large number of plasma proteins. Both increases and decreases in protein synthesis are seen, and changes in different proteins occur at differing rates and to different degrees. See figure 1.2

Figure 1.2: The human plasma protein changes following an inflammatory stimulus.
 Taken from Kushner and Rzewnicki (Kushner and Rzewnicki, 1994).



It is assumed that the acute phase response is functionally useful because of the wide variety of changes that are induced in response to infection and injury, and the known functions of many of the acute phase proteins. For example many of the complement components are acute phase reactants and the complement system, when activated, can affect chemotaxis, opsonization and vascular permeability. Other acute phase changes such as fever and hypoferraemia are thought to provide a suitable environment for coping with tissue injury or infection.

1.3.1 Acute Phase Proteins.

The acute phase proteins differ greatly in their synthesis following an inflammatory stimulus and, in man, can be classified into four main groups; see table 1.2.

Table 1.2 : Expression of acute phase proteins in man.

Group	Examples
Concentration usually increases by about 50%	Ceruloplasmin C3
Concentration usually increases two to four-fold	α_1 -acid glycoprotein α_1 -antitrypsin α_1 -antichymotrypsin Fibrinogen Haptoglobin
Concentration usually increases several hundred-fold	C-reactive protein (CRP) Serum amyloid A-protein (SAA)
Concentration usually decreases (negative acute phase reactants)	Plasma albumin Transferrin α_2 -HS glycoprotein

Other human plasma proteins have also been reported to increase in concentration following an inflammatory stimulus including the complement components C2, C4, C5, C9 and factor B.

There are also significant species differences among the acute phase proteins. For example, α_2 -macroglobin in man does not behave as an acute phase protein; but the homologous protein in the rat, α_2 -macrofetoprotein, is a significant acute phase protein. Also, CRP is the major acute phase protein in man but not in the mouse.

1.3.2 The Liver in the Acute Phase Response.

The liver is known to be an important organ during the acute phase response as its weight increases dramatically during the first few days following infection (Little, 1978). The liver consists of two main cell types, the hepatocytes and the sinusoidal cells with the hepatocytes making up about 70% of the total hepatic volume. The hepatocytes are divided into three subpopulations that are morphologically identical but differ in their position in the liver; periportal hepatocytes are found around the portal tracts, centrilobular hepatocytes are found adjacent to the centrilobular vein, and mediolobular hepatocytes are found between these two populations. Sinusoidal cells consist of three different populations: the endothelial cells, Kupffer cells and Ito cells.

The main site of acute phase protein synthesis is the hepatocyte, although synthesis of some proteins has been reported in other cell types (see table 1.3).

Table 1.3: Site of production of acute phase proteins.

Acute Phase Protein	Site of Production	Reference
C-reactive protein	Hepatocyte	Kushner and Feldmann, 1978
Fibrinogen	Hepatocyte	Courtoy <i>et al.</i> , 1981
α_2 -macroglobin	Hepatocyte	Courtoy <i>et al.</i> , 1981
α_1 -antitrypsin	Hepatocyte	Lamri <i>et al.</i> , 1986
α_1 -acid glycoprotein	Hepatocyte	Courtoy <i>et al.</i> , 1981
Ceruloplasmin	Hepatocyte	Feldmann, 1973
Haptoglobin	Hepatocyte	Courtoy <i>et al.</i> , 1981
α_1 -inhibitor 3	Hepatocyte	Geiger <i>et al.</i> , 1987
Serum amyloid A protein	Hepatocyte	Benson and Kleiner, 1980
α_2 -macroglobin	Ito cells	Andus <i>et al.</i> , 1987
Fibrinogen	Kupffer cells	Hamashima <i>et al.</i> , 1964
α_1 -antitrypsin	Kupffer cells	Millward-Sadler, 1981 Lamri <i>et al.</i> , 1986
Various acute proteins - α_1 -antitrypsin α_1 -antichymotrypsin Transferrin Complement Factor B	Intestinal epithelial cell lines Caco2 and T84	Molmenti <i>et al.</i> , 1993

The amount of acute phase reactant mRNA and protein in individual hepatocytes differs according to the cell location, in both induced and uninduced liver (Feldmann *et al.*, 1989). This may be due to the different micro-environment surrounding each cell due to the unidirectional blood flow through liver which could result in different concentrations of acute phase mediators throughout the lobule. Another explanation could be different concentrations of receptors for the mediators on the hepatocytes according to their lobular location.

1.3.3 Initiation of the Acute Phase Response.

The cells involved in initiating the acute phase response are the tissue macrophages or the blood monocytes. Activated macrophages release a broad spectrum of mediators including the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF). These cytokines act both locally and distally to cause the release of a secondary wave of cytokines.

At the local inflammatory site IL-1 and TNF act on stromal cells including fibroblasts and endothelial cells to initiate this second wave of cytokines. This results in the production of molecules that are chemotactic for neutrophils (for example IL-8) and

for mononuclear cells (for example monocyte chemoattractant protein, MCP). The attracted leukocytes are activated within the site of inflammation and begin to release other cytokine mediators of the acute phase response.

The febrile response seen during the acute phase is induced by the cytokines IL-1, IL-6 and TNF; which mediate fever in many ways including through the induction of prostaglandin E₂ (Dinarello *et al.*, 1991).

Another systemic response to inflammation is an alteration in metabolism and gene regulation in the liver. The response of the liver is induced by four main groups of mediators. These are: IL-6 type cytokines such as IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF); the IL-1 type cytokines such as IL-1 α , IL-1 β , TNF- α and TNF- β ; the glucocorticoids; and growth factors such as insulin, hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and tissue growth factor- β (TGF- β)

The cytokines are the main stimulators of acute phase gene expression and the glucocorticoids and growth factors act to modulate the action of the cytokines.

One group of acute phase proteins (APP), type 1, are regulated by the IL-1 type cytokines. IL-1 and TNF enhance the production of α_1 -acid glycoprotein, SAA, CRP, complement component C3 and haptoglobin. In most cases IL-6 type cytokines synergistically enhance stimulation of type 1 APP.

The type 2 APP are stimulated by IL-6 type cytokines specifically. The IL-1 type cytokines do not stimulate any of the type 2 APP; they also do not enhance the effects of IL-6, in fact any influence they have is inhibitory. This group of APP, in most species, includes fibrinogen, haptoglobin and at least one of the major antiproteases (α_1 -antichymotrypsin, α_1 -antitrypsin, thioesterase and α_2 -macroglobin).

Glucocorticoids are able to stimulate expression of some APP directly, but the main action of these is to synergistically enhance the effect of IL-1 and IL-6 type cytokines, for example regulation of α_1 -acid glycoprotein.

Insulin attenuates IL-1 and IL-6 type stimulation of most APP in cell lines. TGF- β suppresses IL-1 stimulation, but enhances the effects of IL-6 on type 2 APP.

1.3.4 Resolution of the Acute Phase Response.

The acute phase response subsides over 24 to 48 hours following infection or injury, and within a few days the system returns to normal function. Because the cytokines involved in activation of the acute phase have a short half life there may not be active inhibitory mechanisms involved in the resolution of the acute phase response, just a rapid decay of the initiating factors.

However, it may be that there are other factors that influence the resolution of the acute phase response. For example glucocorticosteroid hormone which can stimulate the acute phase response in the liver also inhibits the production of initiating and secondary cytokines by macrophage and stromal cells (Ray *et al.*, 1990). This negative feedback loop may therefore play an important role in controlling the acute phase response.

Inhibitory antibodies or antagonists to IL-1 and TNF are also capable of interfering with the cascade of inflammation. Administration of recombinant IL-1 receptor antagonist (IL-1Ra) to mice protected these mice from the effects of lethal TNF injection (Everaerd *et al.*, 1992). Administration of rIL-1Ra to rats also suppressed the development of experimental autoimmune encephalomyelitis (Badovinac *et al.*, 1998). These results indicate that IL-1Ra can play a role in prevention of toxicity associated with the acute phase response. This would suggest that naturally occurring antagonists, such as IL-1Ra and soluble TNF receptor (sTNFR), may play a role in regulation of the acute phase response.

Cytokines may also be involved in regulation of inflammation. IL-4 is produced by Th2 lymphocytes and has been shown to downregulate production of IL-1 and TNF in isolated macrophages; it also upregulates the expression of IL-1Ra (Colotta *et al.*, 1992). IL-4 has also been shown to induce apoptosis of stimulated monocytes which could lead to a reduced number of these cells in inflammatory lesions and therefore to reduced initiation of the acute phase response (Mangan *et al.*, 1992).

IL-10 is produced by Th2 lymphocytes, monocytes, macrophages and B cells; and it inhibits synthesis of IL-1, TNF and IL-6 by activated monocytes and macrophages (Fiorentino *et al.*, 1991), it also upregulates the expression of IL-1Ra. It has also been shown that administration of IL-10 can protect mice from the lethal effects of endotoxic shock, suggesting that this cytokine has an important role in resolution of the acute phase response (Howard *et al.*, 1993).

IL-6 is one of the cytokines that initiates the acute phase response but it is also thought to have many anti-inflammatory and immunosuppressive effects. It is thought that IL-6 results in protective effects through multiple mechanisms including the reduction of TNF production. Mice given an intravenous injection of concanavalin A (Con A), which activates T cells, develop an acute hepatic injury characterised by

production of TNF. Administration of recombinant IL-6 protects these mice from induction of hepatitis correlating with a reduction in TNF levels (Mizuhara *et al.*, 1994). Work with IL-6 knockout mice showed that, when an inflammatory response was induced using LPS, the mice produced three times more TNF than IL-6 sufficient controls (Fattori *et al.*, 1994); again indicating the role of IL-6 as a mediator of inflammation through reduction of TNF production.

Another way in which IL-6 may mediate anti-inflammatory responses is through induction of IL-1Ra synthesis. Oral infection of mice with *Yersinia enterocolica* results in synthesis of IL-1Ra in Peyer's patches, the site of initial infection. Antibody mediated neutralization of IL-6 in this system caused a suppression of IL-1Ra synthesis in both the Peyer's patches and circulating neutrophils (Jordan *et al.*, 1995). Synthesis of IL-1Ra by cultures of macrophages and PMN was also shown to be induced by rIL-6 and blocked by anti-IL-6 antiserum (Jordan *et al.*, 1995). This suggests that IL-6 is important in regulating the anti-inflammatory response through stimulation of the synthesis of IL-1Ra.

There are also reports that acute phase proteins themselves can exert anti-inflammatory effects, see table 1.4.

Table 1.4 : Anti-inflammatory effects of acute phase proteins.

Protein	Function	Reference
α_2 -macroglobulin	Inhibition of superoxide production by macrophages Inhibition of delayed type hypersensitivity	Hoffman <i>et al.</i> , 1983 Hunter <i>et al.</i> , 1991
α_1 -acid glycoprotein	Protection against toxic effects of LPS and TNF α	Libert <i>et al.</i> , 1994 Molle <i>et al.</i> , 1997
α_1 -antitrypsin	Protection against TNF and LPS lethality	Libert <i>et al.</i> , 1996 Molle <i>et al.</i> , 1997
α_1 -antichymotrypsin	Inhibition of superoxide anion production by neutrophils	Kilpatrick <i>et al.</i> , 1992
CRP	Reduced neutrophil infiltration and vascular permeability in C5a induced alveolitis Inhibition of superoxide production	Heuertz <i>et al.</i> , 1993 Foldes-Filep <i>et al.</i> , 1992

1.3.5 Acute Phase Response in the Mouse.

Analysis of the secreted products of primary mouse hepatocytes isolated from mice that had been treated with turpentine to induce an inflammatory response show that four proteins, α_1 -acid glycoprotein (α_1 -AGP), haptoglobin, hemopexin and SAA show an increase in synthesis (Baumann *et al.*, 1983). Whereas the production of albumin and apolipoprotein A-I were shown to be reduced (Baumann *et al.*, 1983). Treatment of

primary hepatocytes from unstimulated mice with medium from activated monocytes also resulted in a similar pattern of expression of acute phase proteins with a fivefold increase in SAA, a twofold increase in haptoglobin, increased production of both hemopexin and α 1-AGP, and a 50-75% reduction in albumin production (Baumann *et al.*, 1983). Another group looked at the changes in liver mRNAs in inbred mouse strains following treatment with LPS, and again showed increases in SAA, haptoglobin and hemopexin; reduction of albumin, apolipoprotein A-I and major urinary proteins; and no change in α 1-antichymotrypsin and α 1- antitrypsin (Baumann *et al.*, 1984). Two mRNAs for AGP were detected in the mouse following treatment with LPS, encoding two forms of AGP (Baumann *et al.*, 1984). There was no detectable variation between the different mouse strains tested (Baumann *et al.*, 1984).

Although murine CRP is not a major acute phase protein, liver specific expression of mCRP can be detected (Ku and Mortensen, 1993). Monocyte conditioned medium and IL-1 were shown to increase mCRP mRNA in primary murine hepatocytes, but IL-6 did not increase mCRP mRNA levels (Ku and Mortensen, 1993). The mCRP gene contains two IL-1 response elements in the 5' flanking region but these are not functional *in vitro* when linked to a CAT reporter gene (Ku and Mortensen, 1993).

Because the human acute phase protein CRP has been well characterised and has been shown to be expressed as an acute phase protein in transgenic mice this is the acute phase protein upon which we have based our controlled expression system.

1.4.1 C-Reactive Protein (CRP).

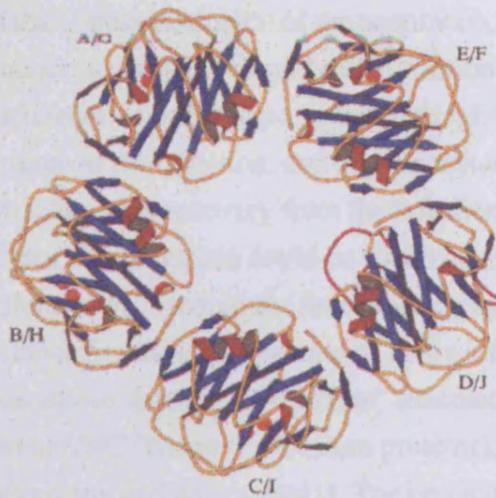
CRP is the most abundant acute phase protein in man. The level of CRP expression increases from basal serum levels of less than 1 µg/ml to more than 300 µg/ml during the acute phase response. It is classified as a pentraxin on the basis of its structure and calcium dependent binding specificities (Osmand *et al.*, 1977). On the basis of *in vitro* and *in vivo* experiments, it has been proposed that the function of CRP relates to its ability to specifically recognise foreign pathogens (through the ligand phosphocholine)(Volanakis and Kaplan, 1971) and damaged cells of the host and initiate their elimination by interacting with the complement (through C1q)(Kaplan and Volanakis, 1974) and cellular effector systems (through CRP receptors on the surface of phagocytes)(Tebo and Mortensen, 1990)(Zeller *et al.*, 1989) in the blood .

1.4.2 Structure of CRP, and its Binding Sites.

Human CRP is a pentameric protein composed of five identical, noncovalently bound subunits arranged in cyclic symmetry in a single plane (Osmand *et al.*, 1977). The gene for the CRP subunit in humans is encoded on chromosome 1 (Whitehead *et al.*, 1983). Sequence analysis of genomic clones has shown that each subunit consists of a linear chain of 206 amino acids with a single intrachain disulphide bond and an estimated molecular weight of 23,017 D (Woo *et al.*, 1985)(Lei *et al.*, 1985). The protein is not glycosylated or phosphorylated. The structure of CRP at 3.0 Å resolution was shown to consist of five protomers, each of 206 amino acids, arranged in cyclic symmetry (Shrive *et al.*, 1996); confirming what was seen in earlier studies. See figure 1.3.

The main ligand for CRP binding is phosphocholine (discussed in section 1.4.3.1), and the phosphocholine-binding site on CRP has been explored using site-directed mutagenesis. A number of amino acid residues are highly conserved between CRPs from various species and these were considered as candidates for CRP related functional sites. Substitution of these amino acids showed that the residue Trp-67 plays a critical role in the formation of the PC binding site of CRP and that residues Lys-57 and Arg-58 also contribute to the structure of the binding site (Agrawal *et al.*, 1992).

Figure 1.3: Representation of the five subunits of CRP. Taken from Shrive et al (Shrive et al., 1996).



The C1q-binding site of human IgG complexes is thought to be a Glu-Xxx-Lys-Xxx-Lys motif (Tao *et al.*, 1993). Based on this, a similar region of CRP was studied to determine the role of this region in C1q binding. The results of this study suggested that the residue Asp-112 (which is conserved in all mammalian CRPs) is important in CRP binding to C1q and therefore complement activation. The residues Lys-114 and Arg-116 also play a role in the binding of CRP to C1q and the activation of the complement system (Agrawal and Volanakis, 1994).

The activation of complement by CRP requires it to be bound to both a ligand and C1q. Recent X-ray crystallography studies showed that all the PC-binding sites are on the same face of the CRP pentamer (Shrive *et al.*, 1996). It would therefore follow that the C1q-binding site of CRP is on the face of CRP opposite from the ligand binding site. This side of the CRP pentamer has the clefts of the structural subunit displayed containing the residue Asp-112 (Shrive *et al.*, 1996). As this residue is important in C1q binding, it would therefore seem that the C1q-binding site of CRP lies within this cleft.

1.4.3 CRP Interactions.

CRP is thought to act *in vivo* as an opsonin because it is able to interact with many different organisms and antigens; and these are discussed below.

1.4.3.1 Interaction of CRP with Antigenic Determinants.

C-reactive protein (CRP) was first recognised, and so named, because of its ability to bind to the C-polysaccharide of the pneumococcal cell wall. A carbohydrate from the pneumococcus was isolated and called fraction C. Serum from patients with acute pneumonia infection was shown to contain a high titre of precipitins for fraction C during the acute stage of the infection, and this precipitation reaction could not be detected in serum taken after recovery from the infection (Tillet and Francis, 1930). It was also noticed that this precipitin could be found in serum from patients suffering from other infections such as rheumatic fever and staphylococcal osteomyelitis; indicating a substance produced in response to many infections not just streptococcal pneumonia. It was shown that the "C-Reactive" substance in serum was inactivated by heating the serum to 65°C (known to denature proteins), indicating that this substance was a protein (Abernethy and Avery, 1941). The precipitation reaction between CRP and fraction C was shown to be dependent on the presence of calcium (Abernethy and Avery, 1941). When calcium was removed from the serum using potassium oxalate the precipitation of fraction C by the serum was abolished, but this precipitation could be restored by the re-introduction of small amounts of calcium.

The precipitation of C-polysaccharide by serum is due to the interaction of CRP with the phosphocholine (PC) groups of the C-polysaccharide (Volanakis and Kaplan, 1971). Quantitative inhibition studies of CRP-C polysaccharide precipitation using choline phosphate and other organic phosphate monoesters showed that choline phosphate is the most active inhibitor of the precipitation reaction. This suggested that the choline phosphate residues in C-polysaccharide are the major site of binding of CRP to C-polysaccharide.

This choline phosphate (or phosphocholine) group is also found in extracts from *Lactobacillus* species, fungi and parasites, and in membrane phospholipids (Kaplan and Volanakis, 1974). It is also thought that phosphocholine-containing allergens may contribute to the allergenicity of mites, fungi and parasites; and CRP has been shown to react with many commercial allergen prick test solutions including whole body extracts of house dust mites, culture filtrates and cell extracts of many fungi (including *Aspergillus fumigatus*, *A. niger* and *Penicillium notatum*) (Baldo *et al.*, 1979), which may indicate a role for CRP in allergic reactions.

Phosphocholine has been reported to be a structural component of nematodes such as *Ascaris suum*, *Nippostrongylus brasiliensis* and *Haemonchus contortus* (Pery *et al.*, 1974). It has also been shown to be a structural component of trematodes and cestodes (*Toxocara canis*, *Diphyllobothrium latum*, *Paragonimus westermani*,

Trichinella spiralis and *Anisakis simplex*), in extracts of eggs, larvae and adult worms (Sugane and Oshima, 1983).

CRP binding has also been reported with cationic molecules, including histones and leukocyte cationic proteins (DiCamelli *et al.*, 1980)(Siegel *et al.*, 1975); also with galactose containing polymers.

Mouse erythrocytes coated with pneumococcal C-polysaccharide (PnC) to provide a binding site for CRP were shown to have an altered pattern of organ sequestration although the rate of clearance was not altered (Nakayama *et al.*, 1982). The PnC-CRP coated erythrocytes were shown to have a marked increase in splenic sequestration and a decrease in hepatic sequestration. This pattern of sequestration was not dependent on complement, as the pattern was not altered in mice pretreated with cobra venom factor (to deplete C3 and therefore inactivate the complement system). CRP therefore has a role in the *in vivo* clearance of cells to which it is bound. Because substances that react with CRP are found on bacteria and damaged cells the role of CRP *in vivo* may be to direct splenic clearance of these.

A feature of CRP that may be important in the interaction with antigen leading to precipitation may be the fact that it consists of five subunits. For precipitation to occur it is necessary to have more than one antigen binding site (such as in antibodies) so that a complex of antigen and immune molecule can be formed.

1.4.3.2 Interaction of CRP with *Streptococcus pneumoniae*.

Type 27 *Streptococcus pneumoniae* (Pn27) differs from most other serotypes in that it contains CRP-reactive phosphocholine determinants in its capsular polysaccharide (Bennett and Bishop, 1977). Radiolabelled CRP mixed with log phase cultures of type 27 *S. pneumoniae* was shown to bind to the pneumococci, in the presence of calcium, with a binding saturation of about 10^7 CRP molecules to each bacterium (Edwards *et al.*, 1982). This is about five to ten times more binding of CRP than is seen to serotypes 3 and 6. The role of CRP in the opsonophagocytosis of type 27 pneumococci was studied using a chemiluminescent assay where light emission was related to the metabolic stimulation of phagocytes by phagocytosis. The bacteria were opsonized with normal human serum, mixed with polymorphonuclear leukocytes (PMN) and the increase in the chemiluminescent response was related to the amount of serum added. When increasing amounts of CRP were introduced into the system, an increased chemiluminescent response to the Pn27 was seen (Edwards *et al.*, 1982). This enhancement was not seen with heated serum or with serum deficient in C2, suggesting that the classical complement pathway was necessary for CRP to stimulate opsonophagocytosis of Pn27. Using serum deficient in immunoglobulin, the

chemiluminescent response to Pn27 was reduced compared to normal serum, indicating that normal serum contains antibody to Pn27. When CRP was introduced to the immunoglobulin deficient serum the chemiluminescent response was again enhanced (Edwards *et al.*, 1982). This indicates that the CRP interaction is independent of antibody binding to the Pn27 and suggests that CRP may play a role early in pneumococcal infection when the antibody response has not had time to develop, that is it acts as a natural opsonin in a nonimmune host.

The ability of CRP to opsonize and induce phagocytosis of clinically important strains was also studied. Serotype 3 is an highly encapsulated and immunogenic strain that is an important cause of otitis media in children, and serotype 6 is a poorly encapsulated and weakly immunogenic strain that is the most frequent cause of pneumococcal bacteraemia and meningitis in children, a nonpathogenic unencapsulated strain (R36a) was also studied. Binding of radiolabelled CRP to all three strains studied was seen with a binding saturation between $0.9 - 1.2 \times 10^6$ molecules of CRP per bacterium (Mold *et al.*, 1982b), this was lower than the binding saturation of Pn27. Because serotypes 3 and 6 and the unencapsulated R36a all bound similar amounts of CRP it suggests that the common cell wall C-polysaccharide provides the binding site for CRP, whereas serotype 27 binds more CRP due to its capsule containing phosphocholine. The opsonophagocytosis of these serotypes was studied using the same chemiluminescent assay as for Pn27. Serotypes 3 and 6 were both efficiently opsonized by normal serum but the addition of CRP had no effect on the chemiluminescent response to type 6 and inhibited the response to type 3 (Mold *et al.*, 1982b). In immunoglobulin deficient serum CRP slightly enhanced the chemiluminescent response to types 3 and 6 but did not restore it to the level of normal serum, indicating that antibody is a more effective opsonin than CRP for these serotypes. The phagocytosis of R36a was not increased by CRP in normal serum but immunoglobulin deficient serum alone effectively opsonized this strain indicating that complement alone is sufficient for a chemiluminescent response to this strain.

1.4.3.3 Protection Against Pneumococcal Infection.

Antibodies to phosphocholine have been shown to be protective against fatal infection with *S. pneumoniae* (Briles *et al.*, 1981)(McDaniel *et al.*, 1984)(Szu *et al.*, 1983). Because CRP shares this binding specificity to phosphocholine and opsonic activity, it was reasoned that it could confer a similar protection to pneumococcal infection as anti-phosphocholine antibody. Mice were pretreated with an intravenous injection of 200 μg of purified CRP in order to achieve levels of CRP similar to those in acute phase serum. The mice were then infected intravenously with *S. pneumoniae* type

3 and survival of the mice monitored. Mice pretreated with CRP showed a significant increase in the percentage of mice surviving at every dose tested compared to mice pretreated with saline (Mold *et al.*, 1981). CRP treatment of mice increased the LD₅₀ from 4×10^4 to 2×10^5 cfu. To test that protection was due to CRP binding to cell wall phosphocholine and not due to CRP reacting with the type specific polysaccharide of type 3 pneumococci, the experiment was repeated using type 4 pneumococci. Pretreatment of the mice with 150 µg of CRP significantly increased survival of the infected mice compared to saline pretreated controls (Mold *et al.*, 1981). Because antibody to phosphocholine had been shown to be protective against pneumococcal infection, tolerance to C-polysaccharide was induced in neonatal mice and this was shown to increase their susceptibility to infection with type 3 pneumococci. These mice were pretreated with 200 µg CRP and challenged intravenously with type 3 pneumococci; and pretreatment with CRP was shown to increase survival in these mice (Mold *et al.*, 1981). This shows that protection against infection can be conferred by CRP alone. Human CRP has been shown to interact with both murine complement (Nakayama *et al.*, 1982) and Fc receptors on murine macrophages (Mortensen and Duszkiwicz, 1977), suggesting that protection of these mice against fatal pneumococcal infection is mediated by the human CRP.

Another study confirmed the finding that CRP is protective against fatal infection using a different *S. pneumoniae* type 3 strain and a different strain of mouse (Yother *et al.*, 1982). This group also showed that protection could be elicited by a dose of CRP as low as 9 µg and that no protection was seen with SAP (a protein with similar structure but no binding capacity for pneumococci).

The effect of CRP in protection against *Salmonella typhimurium* infection was examined as binding of radiolabelled CRP to these bacteria can not be detected. Mice were pretreated with 200 µg of CRP i.v. followed by infection with four times the LD₅₀ of *Salmonella typhimurium*. Pretreatment with CRP had no effect on the time of death or percentage death of these mice (Nakayama *et al.*, 1983). This indicates that CRP is not stimulating a non-specific host defence mechanism (such as macrophage activation), and that protection to pneumococcal infection is specific because of the specific opsonization of pneumococci by CRP.

Because clearance of C-polysaccharide coated erythrocytes results in an altered pattern of organ sequestration, that is an increased sequestration by the spleen (Nakayama *et al.*, 1982), the role of the spleen in the clearance of *S. pneumoniae* was examined. Radiolabelled pneumococci (type 3) were injected into mice and the site of sequestration looked at in CRP and saline pretreated mice. Pretreatment with CRP resulted in increased splenic localisation and decreased hepatic localisation, although the total number of bacteria measured in the spleen and liver did not differ between the two

groups of mice (Nakayama *et al.*, 1983). Because this suggested an important role for the spleen in CRP protection against *S. pneumoniae* the ability of CRP to protect splenectomized mice was examined. Splenectomy greatly increased the susceptibility of these mice to infection with *S. pneumoniae*, but pretreatment of splenectomized mice with CRP increased survival after challenged (Nakayama *et al.*, 1983). This therefore suggests that the spleen is not the only organ involved in protection against infection with *S. pneumoniae*.

Protection against pneumococci has been shown to be mediated in part by increased blood clearance in the case of anti-phosphocholine antibodies (McDaniel *et al.*, 1984). Blood clearance of pneumococci mediated by CRP was examined in *xid* mice that express an X-linked immune deficiency trait that makes them fail to make an antibody response to polysaccharide antigens (such as pneumococcal capsular antigens and cell wall phosphocholine). The protective effects of CRP could therefore be looked at in the absence of anti-pneumococcal antibodies. Mice were pretreated with different doses of CRP an hour before intravenous injection of type 3 *S. pneumoniae* and the effect on blood clearance and survival monitored. Marked blood clearance of the bacteria was seen and this was dependent on the dose of CRP given with the greatest clearance seen in the mice given 300 µg of CRP (Horowitz *et al.*, 1987). At this dose of CRP the number of bacteria in the blood was lower than the inoculation dose until 48 hours post infection, whereas with lower doses of CRP (90 or 30 µg) the number of bacteria in the blood was reduced compared to non-treated controls but did not fall below the inoculation dose. CRP treatment also significantly delayed the mortality of these mice compared to non-treated controls although it did not protect them from infection altogether (Horowitz *et al.*, 1987). The inability of CRP to protect these mice completely may be due to the short half life of CRP or to the absence of a protective antibody response developing after infection. To address the question of the short half life of CRP, repeated injections of CRP were given over the course of the infection. This regime protected the mice during the course of the repeated injections of CRP but did not protect them from eventual fatal infection (Horowitz *et al.*, 1987), suggesting that an intact antibody response is necessary for complete protection but CRP may be important at the onset of infection to clear bacteria from the blood until this antibody response has been established. The protective effects of CRP in *xid* mice appears to require an intact complement system as treatment of these mice with cobra venom factor abolished the protective effects of CRP in these mice (Horowitz *et al.*, 1987).

1.4.3.4 Binding of CRP to Other Bacteria.

An early report (Patterson and Higginbotham, 1965) showed that mouse CRP agglutinated *Staphylococcus aureus*, *S. pneumoniae*, alpha- and beta-haemolytic Streptococci and *Bacillus subtilis*, but no agglutination was seen with *Escherichia coli* suggesting a general reactivity of CRP with gram-positive organisms. Another report (Kindmark, 1972) showed human CRP binding to *S. pneumoniae*, a smaller amount of binding to *E. coli* and no binding to *Staphylococcus aureus*. Kindmark (Kindmark, 1971) also showed that CRP enhanced phagocytosis *in vitro* of *S. pneumoniae*, *Staphylococcus aureus*, *E. coli* and *Klebsiella aerogenes*.

More recently the binding of human CRP to clinical isolates of different bacterial species was studied to assess the potential role of CRP in defence against bacterial infection (Mold *et al.*, 1982c). CRP binding was consistently seen with different serotypes of *S. pneumoniae* but no CRP binding was seen with group A or B streptococci. See table 1.5.

The results show that human CRP binds to all *S. pneumoniae* serotypes tested, some other streptococcus isolates and one *Staphylococcus aureus* isolate. No binding of CRP was found with any of the gram-negative organisms or the other gram-positive organisms tested. This indicates that CRP binding is limited to a small group of potentially pathogenic gram-positive bacteria, and that the ability of CRP to contribute to host defence may be specific for infection with *S. pneumoniae*.

Table 1.5: Binding of CRP to bacteria. Taken from Mold et al (Mold *et al.*, 1982c).

Bacterial Strain	CRP bound ($\mu\text{g}/10^8$ cfu)
<i>Streptococcus pneumoniae</i>	
type 3	16.6
type 4	19.6
type 6	17.5
type 27	166.9
R36a	20
<i>Streptococcus pyogenes</i> (group A)	<0.05
<i>Streptococcus agalactiae</i> (group B)	<0.05
<i>Streptococcus mitis</i>	<0.25
<i>Streptococcus anginosus-constellatus</i>	9.2
<i>Streptococcus salivarius</i>	<0.05
<i>Streptococcus intermedius</i>	<0.05
<i>Streptococcus sanguis I</i>	<0.25
<i>Streptococcus sanguis II</i>	1.6
<i>Staphylococcus aureus</i>	2.5
<i>Staphylococcus aureus</i>	<0.02
<i>Staphylococcus aureus</i>	<0.02
<i>Staphylococcus epidermidis</i>	<0.05
<i>Escherichia coli</i>	<0.05
<i>Salmonella</i> group D	<0.02
<i>Campylobacter fetus</i> subsp. <i>jejuni</i>	<0.12
<i>Pseudomonas aeruginosa</i>	<0.01
<i>Neisseria meningitidis</i>	<0.01
<i>Neisseria gonorrhoeae</i>	<0.02
<i>Klebsiella pneumoniae</i>	<0.05
<i>Haemophilus influenzae</i> , type b	<0.01

1.4.3.5 Binding of CRP to Fungi.

CRP has been shown to react with extracts of fungi of the *Aspergillus* species (Baldo *et al.*, 1979), and the effect of CRP on phagocytosis of dormant and activated *Aspergillus fumigatus* conidia has been studied. Human serum containing a high level of CRP was heated (to inactivate complement) and used to opsonize the conidia before being added to neutrophil monolayers to analyse phagocytosis. It was shown that human CRP increases phagocytosis of both dormant and activated conidia by neutrophils (Richardson *et al.*, 1991b). Increased phagocytosis of the activated conidia compared to dormant conidia was also seen indicating a greater number of CRP binding sites on the surface of activated conidia.

The effect of CRP on phagocytosis of a virulent and an attenuated strain of *Candida albicans* was also studied. High levels of CRP increased phagocytosis of the attenuated strain but not the virulent strain and intracellular killing of CRP opsonized

blastospores of the attenuated strain was also higher (Richardson *et al.*, 1991a). This may explain the virulence of the strain in that it is more able to resist phagocytosis and therefore cause disease.

1.4.3.6 Binding of CRP to Parasites.

Leishmania are protozoal parasites that have two major developmental forms. CRP has been shown to bind to promastigotes (the form in the sandfly vector) with binding increasing during development (Culley *et al.*, 1996). These binding studies show saturable binding of CRP reaching a maximum by 10 µg/ml, which is comparable to binding of CRP to C-polysaccharide of *S. pneumoniae*. The surface of the promastigote consists of a glycocalyx that contains lipophosphoglycan (LPG) which is structurally modified during development. No binding of CRP was seen to LPG-negative mutants of *Leishmania donovani* which suggests that this is the ligand for CRP on promastigotes; and when purified LPG was used in direct binding assays specific CRP binding was demonstrated (Culley *et al.*, 1996). When promastigotes were preincubated with CRP then added to human macrophages, a greatly increased uptake of the parasites into the macrophages was seen compared to untreated promastigotes (Culley *et al.*, 1996). The establishment of a successful *Leishmania* infection involves suppression of the macrophage to allow amastigote survival. It is thought that CRP enhances the activation of macrophages, so increased uptake of promastigotes through CRP opsonization and subsequent activation of macrophages may be a host defence mechanism to prevent the establishment of a successful infection.

There is detectable phosphorylcholine in the eggs, larvae and adult worms of *Toxocara canis* (Sugane and Oshima, 1983). When this PC-containing component was isolated from the larvae it was shown to react strongly with CRP positive serum and not with serum from which the CRP had been adsorbed with pneumococcal C-polysaccharide (Sugane and Oshima, 1983). Activation of the complement system was also seen due to interaction of CRP with the PC-containing component. Complement consumption of 60% was seen when the PC-containing component was mixed with CRP-positive serum, but no complement consumption occurred when it was mixed with normal serum (Sugane and Oshima, 1983).

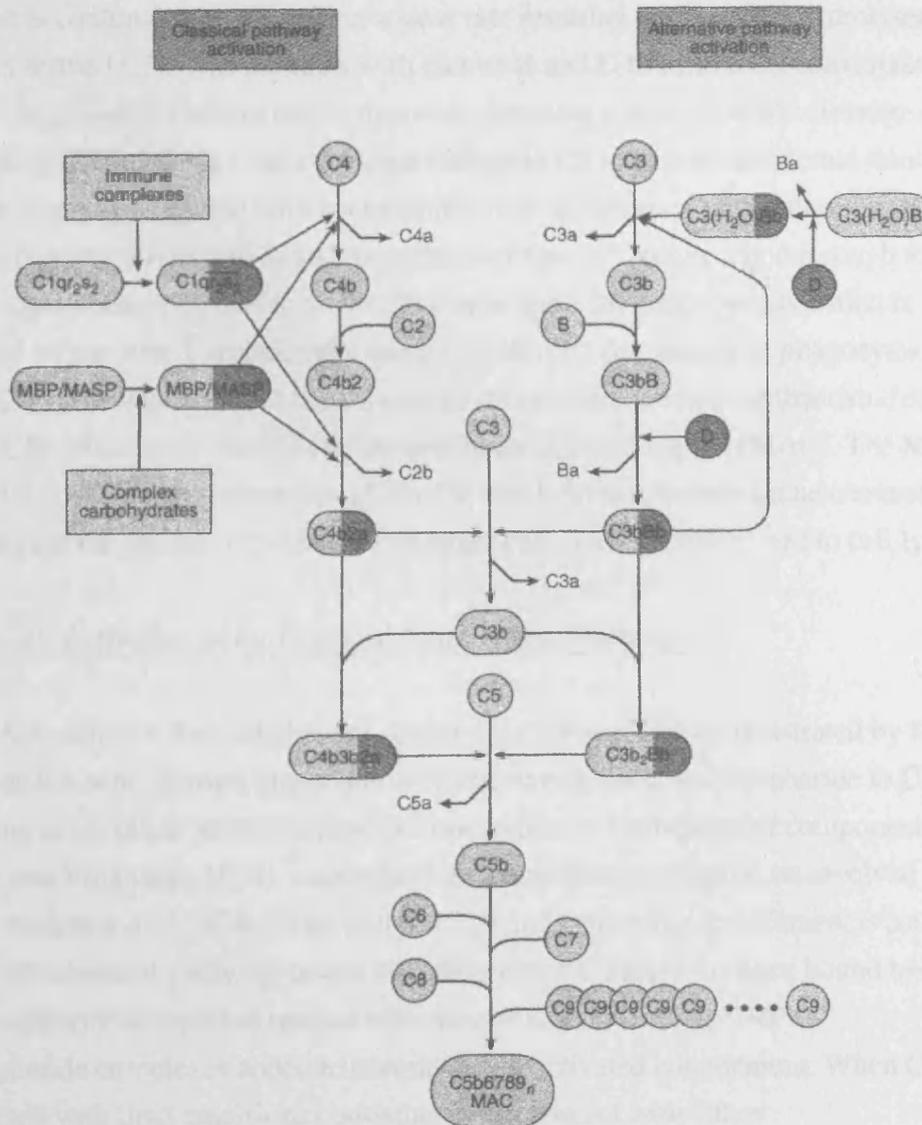
1.4.4 CRP and the Complement System.

CRP also has the ability to activate the complement system; and this is discussed below.

1.4.4.1 The Complement System.

The complement system is a major defence and clearance system in the bloodstream. Complement can be activated by two routes, the classical and alternative pathways, see figure 1.4. This topic has been extensively covered elsewhere (see Law and Reid, 1995) so only a brief overview is given in this thesis.

Figure 1.4: The complement system.



There are nine components of the complement system, designated C1-C9, and also other factors involved in the activation of the pathways.

The classical pathway is activated primarily by the interaction of the C1 complex with immune complexes or aggregates containing IgG or IgM. The C1 complex consists of three glycoproteins; C1q and two molecules each of C1r and C1s. Activation of the C1 complex is brought about by binding of C1q to the Fc region of immunoglobulins in immune complexes. Multiple binding of C1q to Fc regions is necessary such as in immune complexes of multiple IgG molecules bound to antigen or a single IgM molecule. It is thought that this binding induces a conformational change in C1q which results in activation of C1r and subsequently C1s. The activated C1 complex then interacts with C4 and C2 to form a C3 convertase.

Activation of the alternative pathway does not depend on antibody recognition of specific antigen, it relies on molecular structures on the target cell to upset the balance of proteins involved so that their activation and deposition are focused on its surface. C3 in the blood is continuously activated at a slow rate resulting in C3 with a hydrolysed thiolester termed C3i. This interacts with factors B and D to form a C3 convertase.

The classical and alternative pathways therefore converge on the cleavage of C3. Cleavage of C3 induces a conformational change in C3 to expose an internal thiolester which is extremely reactive with nucleophiles such as hydroxyl and amino groups. C3b can then become covalently bound to surfaces of host or foreign origin through these groups. Opsonization of surfaces by C3b marks these for phagocytosis which is facilitated by the type 1 complement receptor (CR1) on the surface of phagocytes.

C3b also interacts with the C3 convertases to form an enzyme that can cleave C5 to form C5b which goes on to form the membrane attack complex (MAC). The MAC consists of complement components C5b-C9 which form transmembrane channels. These disrupt the phospholipid bilayer of target cells and eventually lead to cell lysis.

1.4.4.2 CRP Activation of the Classical Complement Pathway.

Activation of the complement system by CRP was first demonstrated by Kaplan and Volanakis who showed that addition of pneumococcal C-polysaccharide to CRP-containing acute phase serum resulted in consumption of complement components (Kaplan and Volanakis, 1974). Analysis of the complement components involved showed depletion of C1, C4, C2 as well as C3-9, indicating that complement is activated through the classical pathway. It was also shown that C3 and C1q were bound to CRP-C-polysaccharide complexes reacted with normal serum, and that CRP-C-polysaccharide complexes added to normal serum activated complement. When CRP was reacted with lipid emulsions consisting of cholesterol with either

phosphatidylcholine or sphingomyelin, the complexes formed were also shown to activate the classical complement pathway (Kaplan and Volanakis, 1974). CRP-C-polysaccharide complexes fail to activate guinea pig complement although this defect could be overcome by the addition of human C1q (Volanakis and Kaplan, 1974). This indicates that CRP complexes activate the classical complement pathway in a similar way to antibody-antigen complexes, that is through an interaction with C1q.

Binding inhibition assays showed that preincubation of immobilised C1q with CRP did not block the binding of IgG aggregates, and similarly IgG aggregates did not block the binding of CRP (Jiang *et al.*, 1991). This indicates that CRP binds to a region of C1q that is different to the region of binding of antibody. To localise this region of binding, preparations of the globular region of C1q and the collagen-like region were made. The globular region preparation also contained a small residual collagen-like tail of 12 amino acid residues. Whereas IgG aggregates bound only to the globular region preparation, CRP bound to both the collagen-like and the globular preparations (Jiang *et al.*, 1991). This showed that CRP binds to the collagen-like region of C1q, and suggests that it binds to an epitope at the connecting strands of the collagenous portion of C1q just below the globular heads.

Further analysis of the binding of CRP to C1q showed that CRP bound preferentially to the A chain of C1q, in contrast to IgG aggregates which bind to the C chain (Jiang *et al.*, 1992). Synthetic peptides around the C1q regions thought to be involved in binding to CRP were made and their effect on binding to CRP, CRP binding to C1q and complement activation by CRP examined. The results identified two regions, within peptides corresponding to residues 14-26 and 76-92 of the C1q A chain collagen-like region, as sites through which CRP binds and activates the classical complement pathway (Jiang *et al.*, 1992). The exact mechanism of C1 activation by CRP is not known but is thought to involve a conformational change of the C1q which results in activation of C1r and C1s; or CRP may interact directly with C1r and C1s to activate these enzymes.

The CRP binding site for C1q has also been explored and this is discussed in section 1.4.2.

CRP has also been shown to activate the complement system under mildly acidic conditions (Miyazawa and Inoue, 1990), with maximum activity of CRP activation of complement at pH 6.3. CRP was shown to significantly enhance the consumption of C1, C4, C2 and C3 at pH 6.3, but CRP had little effect on C5 consumption. This indicates that the complement system is activated by the classical pathway, but that activation is restricted to the early part of the system. Activation of the early part of the system would still allow deposition of C3b on surfaces, thereby marking them for clearance by phagocytosis. Looking at the circular dichroism (CD) spectra of CRP

revealed that the CD spectrum in the aromatic region showed an appreciable difference at pH 6.3 compared to that at pH 7.4, indicating a conformational change of CRP at the acidic pH (Miyazawa and Inoue, 1990). It may therefore be this pH-dependent conformational change that induces the complement activating activity of CRP at an acidic pH. A feature of a site of inflammation is local acidosis which is attributed to the local increase of lactic acid production by the activity of infiltrated neutrophils (Menkin, 1956). A pH of 6.3 is often found at sites of inflammation, and the acidity of inflammatory sites reaches a maximum 72 to 96 hours after an inflammatory stimulus. This supports the hypothesis that CRP produced during the acute phase of inflammation plays a role in the inflammatory process, and that this role is possibly involvement in clearance of damaged tissues due to opsonization with C3b.

The formation of an effective C3 convertase by interaction of CRP-C-polysaccharide complexes with C1, C4 and C2; and binding of C4 to CRP has also been demonstrated (Volanakis and Narkates, 1983). These results suggest that CRP can interact with the complement system in many ways to induce activation through the classical pathway.

The ability of CRP-C-polysaccharide interactions to stimulate formation of MAC was shown using sheep erythrocytes with PnC on the surface. When CRP was added to the PnC-erythrocytes, in serum, complement mediated haemolysis of the blood cells was seen, and the amount of lysis was proportional to the amount of CRP added (Osmand *et al.*, 1975).

However other experiments using Hep-2 cells treated with protamine sulphate to allow binding of CRP exhibited no lysis when incubated with normal human serum (Berman *et al.*, 1986). Measurement of the consumption of complement components showed consumption of over 80% of C1, C4 and C2, about 40% of C3 and no consumption of C5-C9. This indicated, that in this system, activation of the complement system occurred, but that this activation was limited to the early part of the pathway.

It would seem that one of the main roles of CRP is to opsonize bacteria and disrupted cells, by initiating the early part of the complement pathway leading to deposition of C3b on the surface. This would then lead to phagocytosis of the opsonized organisms or cells and therefore clearance of these from the body.

1.4.4.3 Effect of CRP on the Alternative Pathway.

Although antibody is not required for activation of the alternative complement pathway, it has been found to enhance alternative pathway activation. When liposomes are treated with CRP, so that the CRP becomes bound to the liposomes, and then added

to C2 deficient serum (i.e. lacking the classical pathway), complement activation is inhibited (Mold and Gewurtz, 1981). CRP has also been shown to inhibit alternative pathway activation by *S. pneumoniae* to which it is bound (Mold *et al.*, 1982a). Two strains of *S. pneumoniae* (type 6 and R36a) were examined by mixing the bacteria with C2 deficient serum and adding CRP, and the amount of C3 consumption determined. It was shown that adding 25 or 50 µg of CRP to the reaction inhibited the amount of C3 consumption compared to the addition of no CRP.

The mechanism by which CRP blocks alternative pathway activation, and the significance of these observations has not been determined.

1.4.5 CRP and Immune Cells.

It has been shown that CRP binds to monocytes through a distinct receptor (CRP-R) (Tebo and Mortensen, 1990)(Zeller *et al.*, 1989).

IgG inhibits CRP binding to monocytes by direct competition or receptor modulation, whereas CRP does not inhibit IgG binding to monocytes.

CRP was shown to bind to the high affinity receptor for IgG, the FcγRI, on the monocytic cell line U-937 (Crowell *et al.*, 1991). CRP induced precipitation of FcγRI from lysates of surface radiolabelled cells, and receptor cross-linking studies also indicated CRP binding to FcγRI. In this study, CRP was also found to bind to a 43 to 45 kD protein on U-937 cells that may have been the specific receptor previously described (Tebo and Mortensen, 1990). Using COS-7 cells, which express no surface receptors for CRP, transfected with the cDNA for FcγRIa1 resulted in COS-7 surface expression of the FcγRI. CRP was shown to bind to these cells, and also competitively inhibited binding of IgG to these cells (Marnell *et al.*, 1995). Comparing the sequence of the IgG region known to be involved in binding to the FcγRI with the sequence of CRP revealed a similar amino acid motif which was suggested as the FcγRI-binding site on CRP. Site-directed mutagenesis of this motif produced a mutant CRP which was comparable to wild type CRP in binding to phosphocholine and activation of complement. The mutant CRP did not show reduced binding to FcγRI indicating that the mutations created did not affect the binding of CRP to FcγRI; or that this region is not actually involved in binding to FcγRI (Marnell *et al.*, 1995).

1.4.5.1 Influence of CRP on Monocytic Cells.

CRP has been shown to affect the activation of monocytic cells. After CRP binds to its receptor on U-937 cells (cells of monocytic origin), it is internalised and degraded to form a series of peptides which were shown to stimulate the cells to

differentiate (indicated by the ability of the cells to produce H_2O_2) (Tebo and Mortensen, 1991). Heat aggregated CRP has also been shown to increase the production of intracellular superoxide and H_2O_2 by monocytes in response to stimulation with IgG (Zeller and Sullivan, 1992). This would suggest that CRP plays a role in increasing the microbicidal activities of monocytes.

Synthetic peptides derived from CRP have also been utilised to study the effect of CRP on immune cells. One of these CRP derived peptides has been shown to stimulate adherent monocytes to produce monocyte chemoattractant protein-1 (MCP-1), which implicates CRP in the recruitment of monocytic cells to sites of infection or injury (Zhou *et al.*, 1995). Further work with this peptide integrated into multilamellar vesicles showed that alveolar macrophages isolated from mice treated with this peptide exhibited an increased expression of both MCP-1 and $TNF\alpha$, indicating that CRP is also involved in activation of alveolar macrophages *in vivo* (Barna *et al.*, 1996).

Study of the production of superoxide and nitric oxide (NO) by activated macrophages in response to CRP has revealed that CRP differentially regulates the expression of these in the cell (Ratnam and Mookerjee, 1998). Simultaneous production of superoxide and NO (both of which are utilised by the macrophages to kill microbes) could result in reaction of these to produce harmful intermediates (such as peroxynitrite, nitrogen dioxide and hydroxyl radicals); therefore CRP may play an important role in the regulation of production of superoxide and NO so they are not produced at the same time in the cells.

1.4.5.2 Influence of CRP on Neutrophils.

While CRP appears to increase superoxide production in monocytic cells, it seems to inhibit the production of superoxide in neutrophils. CRP has been shown to inhibit the production of superoxide after stimulation of the cells with phorbol ester (Dobrinich and Spagnuolo, 1991). Also, peptides derived from CRP have been shown to inhibit neutrophil function, including the production of superoxide (Yavin *et al.*, 1995).

CRP has also been shown to inhibit neutrophil chemotaxis. Treatment of neutrophils with CRP reduced their chemotactic movement towards C5a (Kew *et al.*, 1990). The *in vivo* action of CRP on neutrophil chemotaxis was examined by looking at the neutrophil influx to the lung in response to intratracheal administration of C5a or FMLP (a bacterial chemotactic peptide) in mice that had been treated with CRP-derived peptides. Mice that had been treated with CRP were shown to have significantly reduced numbers of neutrophils in the bronchoalveolar lavage fluid (Heuertz *et al.*, 1996).

Purified CRP was also shown to inhibit the chemotactic response of neutrophils to IL-8 (Zhong *et al.*, 1998).

1.4.5.3 Influence of CRP on Platelets.

CRP has also been shown to interact with platelets and to affect their cellular function. In rats infected with *Schistosoma mansoni*, the concentration of CRP in serum increases after the lung stage of infection and is at its highest at the time of terminal worm rejection. The development of platelet cytotoxic activity was found to correlate with the concentration of CRP, so the role of CRP in protection against schistosomiasis was examined. Acute phase rat serum was unable to kill immature worms alone, but rat platelets added to the acute phase serum were larvicidal *in vitro* (Bout *et al.*, 1986). Activation of platelets by purified CRP showed that cytotoxicity *in vitro* was CRP dose dependent. Passive transfer of platelets treated with acute phase serum or purified CRP into normal recipients prior to infection with *Schistosoma mansoni* resulted in protection of the rats compared to controls that received untreated platelets (Bout *et al.*, 1986). This suggests that CRP is involved in schistosome killing by platelets in the rat model.

1.4.6 CRP Transgenic Mice.

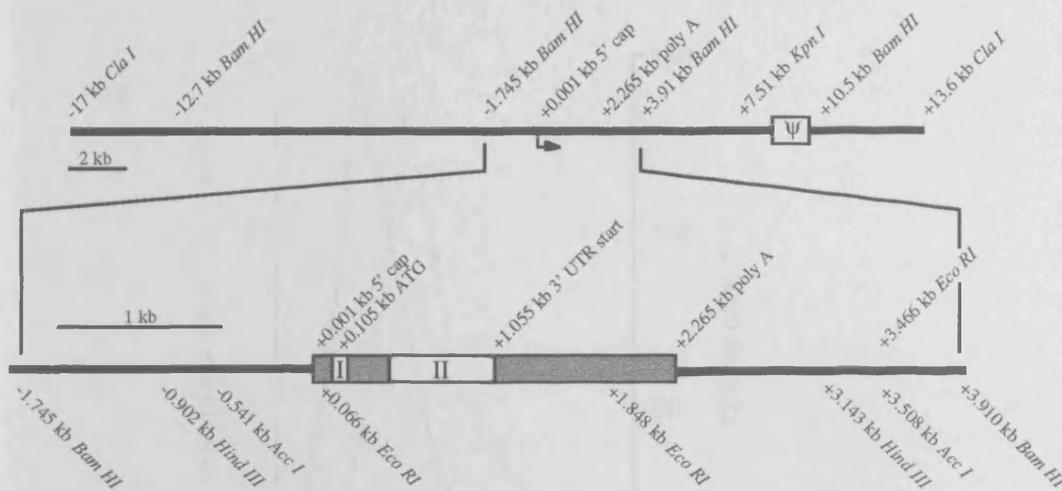
In order to examine the role of CRP *in vivo* transgenic mice expressing the human CRP gene have been constructed.

1.4.6.1 Generation of CRP Transgenic Mice and Initial Characterisation.

Using a full length cDNA for human CRP as a probe (Woo *et al.*, 1985), a clone (pCos-CRP1) was isolated from a human genomic library (Ciliberto *et al.*, 1987b). The total length of the insert was about 35.2 kb. See figure 1.5.

A 30 kb *Cla*I DNA fragment was isolated from this clone in order to eliminate most of the vector sequences. The CRP gene was located approximately in the centre of the isolated fragment with more than 16 kb of 5' flanking sequence and about 10 kb of 3' flanking sequence. This isolated fragment was used to create transgenic mice by microinjection into the eggs of C57/BL6 x SJL hybrids. Four transgenic offspring were detected, but only two were able to transmit the transgene to offspring (Ciliberto *et al.*, 1987a).

Figure 1.5: The pCosCRP1 clone. Taken from Murphy *et al.* (Murphy *et al.*, 1995).

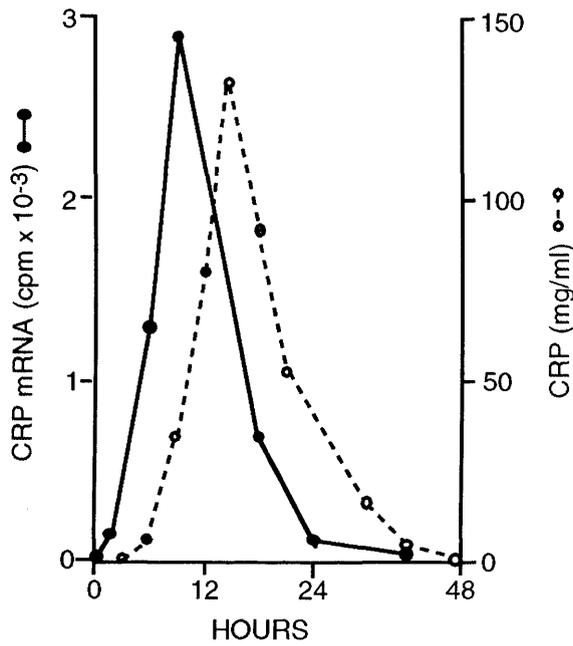


Human CRP could not be detected in the blood of the mice from the two transgenic lines under normal conditions. However, after stimulation of the mice with irritating agents such as LPS or croton oil, the amount of human CRP in the blood of the transgenic mice is greatly increased (Ciliberto *et al.*, 1987a). Analysis of human CRP mRNA expression in 11 different tissues, both before and after induction with LPS, showed that hCRP mRNA was not detectable in any tissue before induction. After induction with LPS total RNA analysis showed that a large amount of hCRP mRNA was detectable in the liver of transgenic mice. CRP mRNA could not be detected in any other tissues examined, indicating that expression of this transgene is restricted to the hepatocytes (Ciliberto *et al.*, 1987a).

Human CRP mRNA can be detected in the liver of transgenic mice 2 hours after LPS injection with the peak of RNA production at 9-10 hours, and the RNA disappears rapidly so that it is almost undetectable by 24 hours. Looking at the appearance in the blood of hCRP showed that CRP could not be detected in mice before LPS injection, and the highest level of hCRP in the serum is reached 15 hours after LPS injection, with a decline to basal levels by 48 hours. The kinetics of mRNA and protein production are therefore shifted by about 5 hours. See figure 1.6.

These results (Ciliberto *et al.*, 1987a) show that when the human CRP gene is transferred to mice it is expressed in the same way as in man; that is that its expression is highly inducible and tissue specific. As discussed later, this expression of the human CRP gene in transgenic mice as a major acute phase protein forms the basis of the controlled expression system that was developed during this project.

Figure 1.6: The acute phase kinetics of CRP mRNA and protein production in CRP transgenic mice following induction with LPS. Taken from Ciliberto et al (Ciliberto *et al.*, 1987a).



1.4.6.2 Sex Difference in CRP Expression by Transgenic Mice.

A distinct sexual dimorphism was seen with respect to expression of the transgene (Szalai *et al.*, 1995). Both constitutive and induced levels of CRP were significantly lower in transgenic females than transgenic males (see table 1.6).

Constitutive and induced levels of mSAP did not differ between the sexes; indicating that this is not caused by low level stimulation of male mice, but is an effect of the transgene.

Table 1.6: Comparison of CRP levels in transgenic mice. Information from Szalai et al (Szalai *et al.*, 1995)

	Basal Serum Concentration of CRP (µg/ml)	Induced Serum Concentration of CRP (µg/ml)
Males	22.5 ± 2.7	91.8 ± 7.4
Females	1.3 ± 0.1	60.6 ± 5.2
Castrated Males	0.6 ± 0.2	approx. 70
Testosterone Implanted Males	approx. 28	approx. 95

To investigate the effect of testosterone on the transgene, males were castrated then induction of the transgene by LPS examined. Constitutive serum CRP in castrated mice was significantly lower than that measured before castration, and lower than in sham castrated controls (Szalai *et al.*, 1995). Replacement of testosterone, with a subcutaneous pellet designed to release testosterone, fully restored the ability of castrated mice to express constitutive levels of CRP similar to those measured before castration. Both castration and testosterone replacement had no significant effect on the levels of CRP expressed after LPS stimulation. It can therefore be concluded that testosterone is required for constitutive expression of CRP, but does not affect the acute phase expression induced by LPS.

Interleukin-6 is the main inducer of the hCRP gene *in vitro* and using CRP transgenic / IL-6 deficient (CRPtg/IL-6^{-/-}) mice it was shown that the CRP transgene was not induced by LPS, but a slight increase in serum CRP was seen after administration of LPS and IL-6 (Weinhold *et al.*, 1997). It was concluded that IL-6 is necessary, but not sufficient, for induction of the CRP gene.

To evaluate the effect of IL-6 on the sexual dimorphism shown by the CRP transgene, CRP expression in the CRPtg/IL-6^{-/-} mice was looked at, using CRPtg/IL-6^{+/-} littermates as controls. The IL-6^{+/-} and IL-6^{-/-} CRP transgenic male mice expressed equal amounts of CRP constitutively, but acute phase levels were induced by LPS only in the presence of a functional IL-6 gene (IL-6^{+/-}) (Szalai *et al.*, 1998). Neither the IL-6^{+/-} or the IL-6^{-/-} female mice expressed CRP constitutively, and the LPS induced levels in IL-6^{+/-} females were much lower than in the corresponding males.

When these mice were given IL-6, the male IL-6^{-/-} and IL-6^{+/-} CRP transgenic mice expressed CRP at the same level. The female IL-6^{-/-} and IL-6^{+/-} both failed to express CRP in response to IL-6, indicating that IL-6 alone is sufficient for CRP induction in male mice but not in females. IL-1 also failed to produce a CRP response

in female IL-6^{-/-} mice either alone or in combination with IL-6. When female mice were implanted with testosterone pellets, the testosterone treated IL-6^{-/-} mice expressed CRP constitutively and administration of IL-6 induced higher levels of CRP (Szalai *et al.*, 1998).

The authors hypothesis to explain this is that the CRP gene is under negative control of two elements, and constitutive expression of CRP requires the release, or partial release, of one of these elements. One of the elements may be controlled by LPS-induced mediators and the other by testosterone or another mediator with a sexually dimorphic pattern of expression (Szalai *et al.*, 1998).

Like the CRP-transgene, several hepatically expressed endogenous genes display sexual dimorphism in rodents; including sex-limited protein (Slp). Expression of the Slp gene depends on the presence of testosterone; however this is not a direct effect but mediated through changes in the secretion of growth hormone (Georgatsou *et al.*, 1993). In males, testosterone induces high-amplitude, low-frequency pulses of growth hormone resulting in the levels of growth hormone in males to be double those in female mice. It is thought that a negative control on the Slp gene is relieved by growth hormone. A similar testosterone / growth hormone mediated mechanism could explain the sexually dimorphic pattern of constitutive expression of the CRP transgene.

No acute phase proteins have been shown to exhibit a sexually dimorphic pattern of expression in humans, so it is not clear whether this is an effect of expression of the human transgene in mice.

1.4.6.3 Pneumococcal Infection of CRP Transgenic Mice.

As discussed (section 1.4.3.3), administration of human CRP to mice increases the survival of mice subsequently infected with *S. pneumoniae* (Mold *et al.*, 1981)(Yother *et al.*, 1982)(Horowitz *et al.*, 1987). The effect of endogenously synthesised CRP on pneumococcal infection was studied using mice transgenic for CRP. Mice were infected intraperitoneally with 1x10⁴ cfu type 3 *S. pneumoniae* and the pattern of CRP expression, bacteraemia and survival monitored (Szalai *et al.*, 1995). The transgenic mice survived significantly longer than the non-transgenic controls, median survival time of transgenics was 67 hours compared to 56.8 hours for controls. When survival of male and female mice was examined, it was seen that only male transgenics survived significantly longer than controls, and the increased survival of transgenic mice as a whole was attributable to the prolonged survival of the males. By 24 hours after infection, a significant difference in levels of bacteraemia was seen between transgenic and control mice; this difference was maintained throughout the experiment. Both male and female transgenic mice had decreased bacteraemia compared

to controls, but the decreased bacteraemia in female mice did not enhance survival. The transgenic mice exhibited an acute phase CRP response to the infection, with higher serum concentrations of CRP in the male mice than in the females throughout the experiment; as expected from the results of LPS stimulation of these mice (section 1.4.6.2) (Szalai *et al.*, 1995).

When male CRP transgenic and control mice were given a lower dose of *S. pneumoniae* intraperitoneum, the effect of the transgene on survival was more marked. Approximately twofold difference in survival was seen with 61% of transgenics surviving compared to 33% of control animals (Szalai *et al.*, 1995). The expression of the transgene in infected mice is therefore closely associated with reduced bacteraemia and increased survival. Because only male transgenic mice lived significantly longer than controls following infection, it suggests that the higher constitutive expression of CRP gives the males a 'head start' in defence against pneumococcal infection.

The role of complement in the CRP-mediated protection of CRP transgenic mice from pneumococcal infection was investigated using mice decomplicated with cobra venom factor (CoVF). Mice were treated with CoVF, or saline as a control, then infected with type 3 *S. pneumoniae*. Twenty-four hours after infection, bacteraemia was significantly lower in both normal and decomplicated transgenic mice compared to non-transgenics (Szalai *et al.*, 1996). Also, bacteraemia was lower in both transgenic and non-transgenic mice compared to decomplicated mice. The median survival time of both transgenic and non-transgenic normal mice was significantly extended compared to their decomplicated counterparts. The presence of the transgene also extended the survival of normal mice but not of those decomplicated. This indicates that an intact complement system is required for CRP-mediated protection from pneumococcal infection, consistent with the known ability of CRP to activate the complement pathway.

1.4.6.4 Mice Transgenic for Rabbit CRP.

Mice transgenic for rabbit CRP expressed from the phosphoenolpyruvate carboxykinase promoter have been made (Lin *et al.*, 1995). These express rCRP in response to gluconeogenic stimuli; that is, mice kept on a high carbohydrate diet suppress rCRP expression and mice given an high protein diet are induced to express rCRP.

When these mice were challenged with a high dose of LPS (16-27 mg/kg), 70% of the mice expressing rCRP survived the challenge compared to 35% survival of the mice not expressing rCRP (Xia and Samols, 1997). Non-transgenic mice kept on the same diet regimes were equally sensitive to LPS challenge. This suggests that the expression of rCRP in transgenic mice is responsible for the resistance to endotoxic

shock induced by LPS challenge. Protection of rCRP expressing transgenic mice against challenge with platelet-activating factor, TNF- α and IL-1 β (which are mediators of endotoxic shock) was also demonstrated.

The role of CRP in malarial infection is controversial with some reports of CRP binding to the surface of *Plasmodium* and others reporting that this does not occur. One paper reports the binding of rCRP to *Plasmodium yoelii* and *P. berghei* and goes on to look at the role of rCRP expressed in transgenic mice on infection with *P. yoelii* (Renia *et al.*, 1993). Expression of rCRP was modulated by diet as described above, then the mice challenged with *P. yoelii* sporozoites. No difference was seen in the pattern of infection of either group indicating that rCRP does not play a role in the infection of mice with malaria.

1.4.7 CRP Expression.

The regions of the human CRP gene necessary for acute phase inducibility have been studied both in tissue culture (using human derived cell lines) and using transgenic mouse lines.

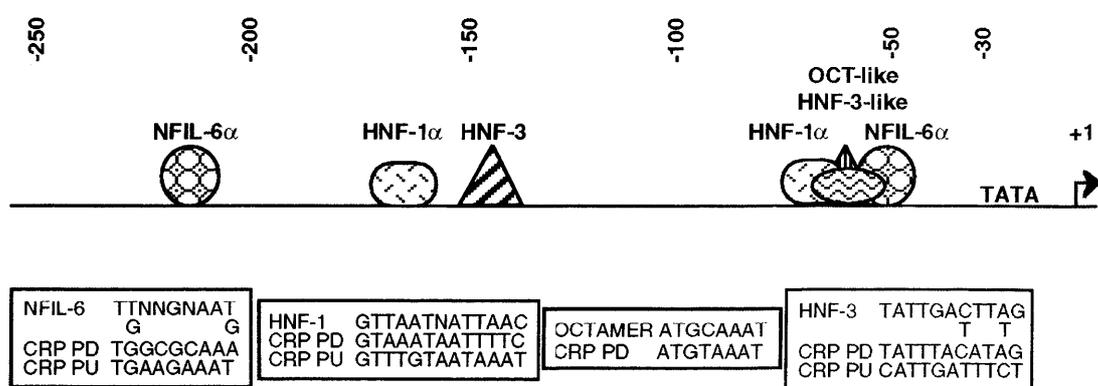
1.4.7.1 Promoter Sequences Necessary for Acute Phase Expression of CRP in Tissue Culture.

The regions involved in acute phase expression in the 5' region of the gene were studied using DNA transfer into the Hep3B cell line. A 2.5 kb region of the DNA 5' to the CRP gene was fused to the reporter gene chloramphenicol acetyl transferase (CAT). This construct was transfected into Hep3B cells and the cells stimulated with monocyte conditioned medium (MoCM). The CAT gene was shown to be activated only after stimulation with MoCM, and only in cell lines of hepatic origin (Arcone *et al.*, 1988). A series of 5' deletions of this construct was then made and inducibility of CAT expression by MoCM was seen down to a deletion of 94 bp of 5' DNA (Arcone *et al.*, 1988). A further series of deletions of the CRP promoter working from the 3' end was made and the section from nucleotides -121 to -50 was capable of induction of MoCM stimulated CAT expression (Arcone *et al.*, 1988). These results show that the CRP promoter is silent under conditions of no stimulation and that the region from -94 to -50 bp of the 5' flanking DNA contains the sites responsible for acute phase expression in this tissue culture system.

Further analysis of the *cis*-acting elements of the CRP promoter in the 5' region of the promoter was studied in a similar way by another group. This group used the human hepatoma cell line PLC/PRF/5 and stimulated the CRP promoter with IL-6.

Deletion mutants of the promoter (5', 3' and internal deletions) fused to the CAT reporter gene were made and transfected into the cell line before stimulation with IL-6 alone (which had been shown to be a sufficient stimulus of acute phase expression in this cell line). It was shown that in the 1 kb 5' to the CRP gene there are two regions of IL-6 inducible elements, two constitutive enhancer-like regions, and a negative regulatory region (Li *et al.*, 1990). See figure 1.7.

Figure 1.7: Schematic representation of the regulatory elements of the CRP promoter. Taken from Li et al (Li *et al.*, 1990).



One of the IL-6 inducible elements was seen to be located between -86 and -60 from the CRP gene; thereby confirming the previous results (Arcone *et al.*, 1988), and narrowing the boundaries of the responsible region. It was also shown that 3' deletions to -144 were still IL-6 inducible, and further internal deletions indicated that the second IL-6 inducible element lay between -234 and -200 (Li *et al.*, 1990). These two IL-6 inducible regions were also shown to be able to function independently of each other. Within one of the IL-6 inducible regions a heptamer sequence was repeated three times, and this motif was also seen in the other IL-6 inducible region. This motif is similar to that found in the rabbit CRP promoter, in the three IL-6 responsive elements of human haptoglobin (Oliviero and Cortese, 1989) and in the regulatory element for rat α_1 -acid glycoprotein (Prowse and Baumann, 1988).

One of the negative regulatory regions consisted of two negative elements (-144 to -125 and -107 to -88) and two positive elements (-178 to -144 and -125 to -107). Deletion of one of the negative elements resulted in a four-fold increase in inducible CAT expression and deletion of the other negative element resulted in a ten-fold increase, however there was no increase in uninducible CAT expression after deletion of either negative regulatory element. This negative regulatory region has an organisation

similar to that described for the region controlling β -interferon induction (Keller and Maniatis, 1988).

1.4.7.2 Promoter Sequences Necessary for Acute Phase Expression of CRP in Transgenic Mice.

The results of Ciliberto (Ciliberto *et al.*, 1987a) show that when the 30 kb cosmid clone of the human CRP gene is transferred to mice it is regulated as the major acute phase protein. The sequences required for the correct regulation of the hCRP in transgenic mice must therefore be contained within this 30 kb fragment of genomic DNA. In order to determine the flanking regions of DNA that are responsible for the *in vivo* regulation of the hCRP gene, transgenic mice have been made using various constructs that incorporated the CRP gene and different sections of the flanking DNA (Murphy *et al.*, 1995). See figure 1.8 for schematic representation of the different constructs used to make transgenic mice.

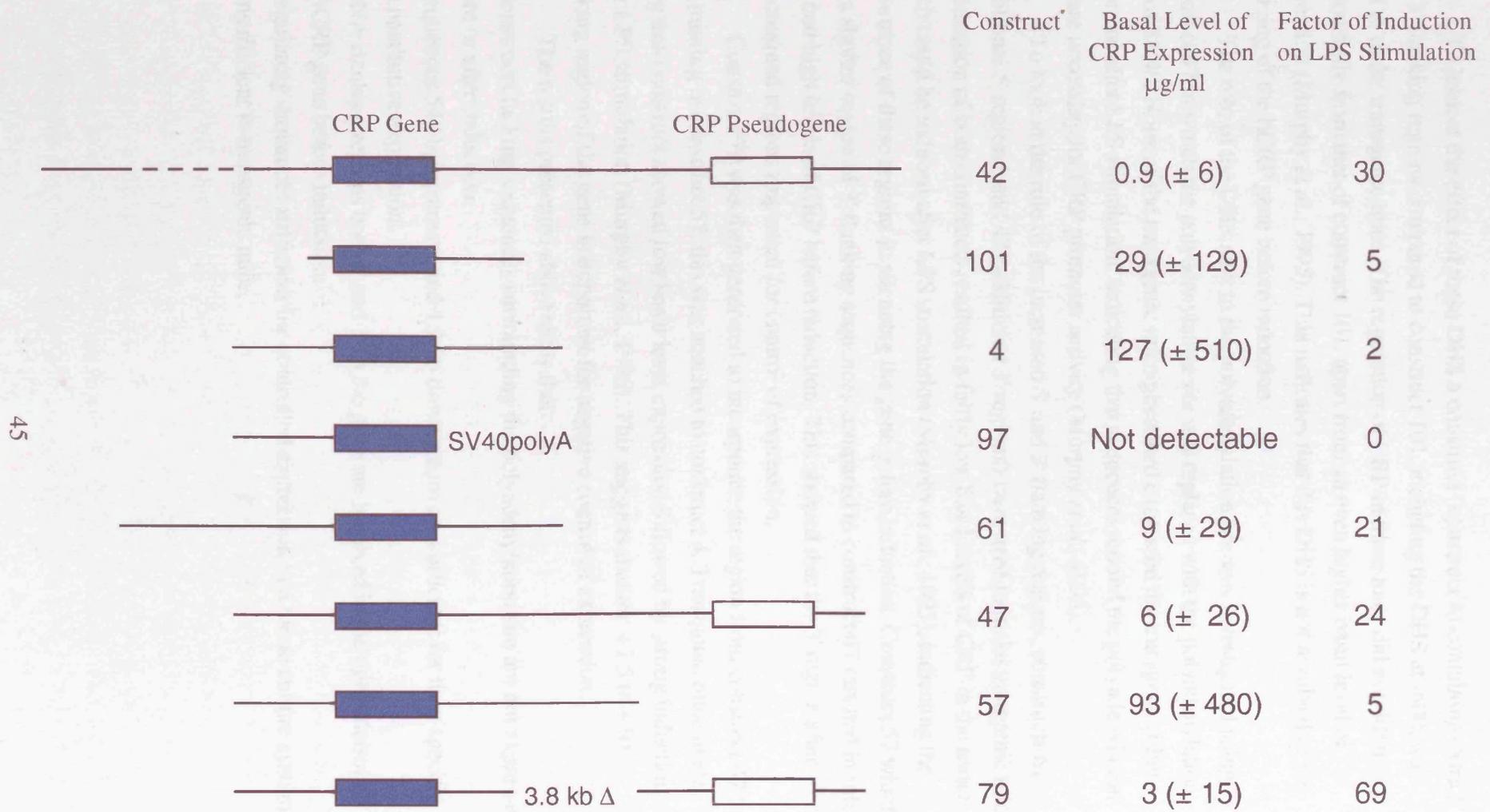
Lines of mice expressing the full length (30 kb) construct, termed construct 42, were made initially. These transgenic mice showed low constitutive levels of CRP expression which was induced 30-fold after LPS injection, and the expression was restricted to the liver (Murphy *et al.*, 1995). These results were therefore consistent with those obtained by Ciliberto (Ciliberto *et al.*, 1987a).

The next construct to be analysed was construct 101; which is only 4 kb in size and contained all the known elements defined for *in vitro* acute phase expression. These transgenic mice showed high basal levels of hCRP expression and this was increased 5-fold following LPS stimulation (Murphy *et al.*, 1995). This suggests that construct 101 lacks the regulatory regions required to silence transgene expression before induction.

It is known that DNA surrounding actively or potentially transcribed genes shows enhanced sensitivity to endonuclease cleavage, and that these DNase hypersensitive sites (DHS) strongly associate with *cis*-acting DNA sequences of relevance for the regulation of gene expression (such as promoters, enhancers and silencers). The DHS around the hCRP transgene in LPS induced and non-induced transgenic mice were mapped (Toniatti *et al.*, 1990a). In non-induced liver this revealed a DHS in the 3' flanking region of the gene immediately downstream from the polyadenylation site. In induced liver there appeared to be three inducible DHS in the 5' flanking region of the gene; one in the cap site region, one at about -250 bp from the start of the gene, and one at about 600 bp upstream from the cap site.

Figure 1.8: Schematic representation of the hCRP constructs and their expression in transgenic mice.

Taken from Murphy *et al*, 1995.



To look at the effect of these DHS a construct (construct 4) containing extra 5' and 3' flanking regions compared to construct 101, including the DHS at -600, was used to make transgenic mice. The regulation of CRP in these mice did not differ substantially from that of construct 101, apart from an even higher basal level of expression (Murphy *et al.*, 1995). This indicates that this DHS is not involved in the silencing of the hCRP gene before induction.

The role of the DHS next to the polyadenylation site was investigated using construct 97 in which the polyadenylation site was replaced with the polyadenylation site of SV40. None of the transgenic mice generated expressed the transgene either before or after LPS stimulation, indicating that sequences around the polyadenylation site are necessary for CRP promoter activity (Murphy *et al.*, 1995).

To look at the role of the increased 5' and 3' flanking regions, constructs 61 (additional 5' regions) and 47 (additional 3' regions) were used to make transgenic mice. Introduction of both constructs resulted in fairly low basal levels of CRP in the serum which could be induced after LPS stimulation (Murphy *et al.*, 1995), indicating the importance of these regions in silencing the gene before induction. Construct 57 which had a shorter region of 3' flanking sequences compared to construct 47 resulted in mice that had high levels of CRP before induction. This showed that the 3' region after +7.51 bp contained regions important for control of expression.

Construct 79 was then generated to incorporate the region from construct 47 that was missing in construct 57, this was attached to construct 4. Transgenic mice made using this construct showed low basal level expression followed by strong induction after LPS stimulation (Murphy *et al.*, 1995). This suggests that the +7.5 to +10 flanking region of the gene is important for negative control of expression.

The results presented above show that:

1. Constructs lacking sequences surrounding the polyadenylation site are not expressed before or after induction.
2. Sequences 540 bp upstream and 1.2 kb downstream are sufficient for liver specific but constitutive expression.
3. DNA flanking regions both 5' and 3' to the gene are involved in the tight control of the hCRP gene before induction.
4. Regulatory sequences sufficient for controlled expression in a tissue culture system are insufficient in transgenic mice.

1.4.7.3 Inducers of CRP Expression.

It was first reported that a protein factor secreted by human peripheral blood monocytes stimulated with endotoxin, or T-cell lines from patients with adult T-cell leukaemia and infected with HTLV1 were capable of stimulating CRP synthesis from human hepatoma cell lines (Goldman and Liu, 1987). This factor was initially called B-cell stimulatory factor type 2, and then subsequently recognised as IL-6. The main inducer of expression contained in MoCM was also shown to be IL-6 by pre-incubation of MoCM with antibodies against IL-6 before using this to stimulate Hep3B cells transfected with 219 bp of the CRP promoter linked to the CAT gene. Depletion of IL-6 from the MoCM reduced CRP promoter induction to an undetectable level (Ganter *et al.*, 1989). Using an expression vector containing the CRP coding region and about 1 kb of 5' flanking sequence transfected into PLC/PRF/5 cells it was shown that IL-6 alone would induce CRP synthesis from the vector (Li *et al.*, 1990). This also indicated the presence of IL-6 responsive elements in this region of the promoter.

Expression of the endogenous CRP gene in cultured human hepatoma cells is under different control in different cell lines. For example in NPLC/PRF/5 cells IL-6 alone is capable of inducing CRP production, whereas in Hep3B cells both IL-6 and IL-1 are required (Ganapathi *et al.*, 1988). The effect of these two cytokines on 355 bp of the CRP promoter linked to a reporter gene (CAT) after introduction into Hep3B cells was therefore studied. These studies showed that IL-6 is much more active than IL-1 β in inducing the CRP promoter (Ganter *et al.*, 1989). The expression of CAT induced by IL-6 alone is 25% of that induced by MoCM, and the expression induced by IL-1 β alone is 4.6%; but a combination of the two cytokines gives expression of CAT comparable to that induced by MoCM (about 90%) (Ganter *et al.*, 1989). No further increase in CAT expression was seen when IL-6 was used in combination with TNF, IL-2 or interferon- γ . This shows that the CRP gene is regulated cooperatively by IL-6 and IL-1. To determine the effect of IL-6 and IL-1 on the acute phase responsive elements previously identified in the CRP promoter (Arcone *et al.*, 1988), a series of 5' and 3' deletions of the promoter were used to transfect Hep3B cells and the effect of MoCM, IL-6, IL-1 or a combination of IL-6 and IL-1 looked at. The constructs with 5' deletions of the promoter to -94 bp were all still induced cooperatively by IL-6 and IL-1 (Ganter *et al.*, 1989). However, with 3' deletion mutants IL-1 dependent inducibility is lost on exchange of the CRP TATA and 15 bp of the 5' untranslated region (UTR) with the SV40 promoter. It was therefore concluded that the two previously identified acute phase responsive elements are the target of IL-6 and that IL-1 control of expression is exerted in the sequences located between position -42 and +15 (Ganter *et al.*, 1989). Looking at the pattern of mRNA production, it was seen that MoCM, IL-6 and IL-6

with IL-1 all induce transcription from the CRP promoter with a similar efficiency. It was therefore concluded that IL-6 acts as a transcriptional inducer of the CRP gene and IL-1 acts post-transcriptionally to increase expression, possibly by enhancing translation of these transcripts (Ganter *et al.*, 1989).

1.4.7.4 Nuclear Factors Involved in CRP Expression.

DNase I footprinting analysis of 250 bp 5' of the CRP gene revealed two protected regions in the presence of Hep3B cell extracts (Majello *et al.*, 1990). These map to the regions previously identified as important for acute phase expression, APRE1 located between nucleotides -87 and -46 and APRE2 located between nucleotides -106 and -137 (Arcone *et al.*, 1988). Because the region upstream of the CRP gene to nucleotide -94 (containing APRE1) is the minimal region capable of supporting inducible expression in tissue culture, the factors that interact with region APRE1 and the sequences involved in this interaction were studied in more detail. Mutants in this area were created and used to determine the effect of the mutation on inducible expression. This revealed two subregions; mutations in the β subregion (from nucleotide -70 to -57) completely abolished the ability to be induced by MoCM, and mutations in the α subregion (from nucleotide -57 to -45) show reduced inducibility (Majello *et al.*, 1990). Oligonucleotide probes to subregions α and β were made and gel retardation experiments, with nuclear extracts from untreated and MoCM treated Hep3B cells, revealed a complex (termed hepatocyte-acute phase factor 1, H-APF-1) that bound to subregion β in both treated and untreated cells. With subregion α , only a factor from MoCM treated cells bound to the probe (termed H-APF-2) (Majello *et al.*, 1990). Mutations in these subregions also prevent binding of the respective factors.

Tissue specific gene expression relies mainly on the presence of cell-type specific *trans*-acting factors that interact with the promoter and enhancer regions of the genes. Many hepatocyte specific or enriched transcription factors have been identified and one of the best characterized is HNF-1. Sequence analysis of the β subregion showed homology between this region and the consensus binding sequence for HNF-1. Gel retardation assays with nuclear extracts from Hep3B cells showed that probes corresponding to subregion β and two typical HNF-1 binding sites all gave the same pattern of gel retardation (Toniatti *et al.*, 1990b), indicating that all three regions bound the same nuclear factor, that is HNF-1. A mutant oligonucleotide probe of subregion β that abolished the consensus HNF-1 binding sequence was then unable to bind to HNF-1 (Toniatti *et al.*, 1990b).

The other region of the CRP promoter (from nucleotide -175 to -133) that exhibited protection in the DNase I footprinting analysis (Majello *et al.*, 1990) also had

a consensus binding sequence for HNF-1 (termed subregion γ). An oligonucleotide probe corresponding to this region was also shown to bind to the same nuclear factor as subregion β and the two typical HNF-1 binding sites (Toniatti *et al.*, 1990b). Mutation of both subregions β and γ abolished CRP expression, indicating the importance of HNF-1 binding in expression of the gene.

HNF-1 is present in hepatocytes both before and after stimulation with MoCM, but CRP expression is only induced fully after stimulation with MoCM. Analysis of the APRE1 showed interaction of the α subregion with the IL-6 inducible nuclear factor H-APF-2 and a binding site for this factor was also located in the APRE2. Both of these acute phase responsive elements are therefore characterized by the presence of binding sites for both HNF-1 and H-APF-2. It was therefore proposed that the role of HNF-1 is to confer liver specific expression, as it is a hepatocyte specific factor and has been shown to be necessary for transcription of the CRP gene; and the role of H-APF-2 is to confer IL-6 inducible expression (Toniatti *et al.*, 1990b). H-APF-2 was shown to correspond to C/EBR family of IL-6 inducible nuclear factors (Ramji *et al.*, 1993).

Deletion mutants of the CRP promoter have shown that the region between nucleotides -123 to -85 is required for optimal CRP expression in response to IL-6. This region was found to contain a sequence homologous to acute response elements in the rat α_2 -macroglobulin gene as well as to the interferon- γ activation site (GAS), and mutation of this site resulted in reduced IL-6 inducibility (Zhang *et al.*, 1996). It is known that members of the family of signal transducers and activators of transcription (STAT) bind to GAS-like sequences and the STAT family is also thought to be involved in mediating IL-6 effects. Overexpression of STAT3, but not STAT1 α , was shown to increase CRP expression in response to IL-6, and mutation of the GAS-like site abolished this effect (Zhang *et al.*, 1996). This indicates that STAT3 also participates in the transcriptional activation of CRP in response to IL-6.

1.5 Cytokines.

This topic has been extensively covered in many books, and reviews (including Thomson, 1998; Callard and Gearing, 1994; Hamblin, 1994; Arai *et al.*, 1997; Meager, 1998; Nicola, 1995), so only a brief overview is given here.

The body needs a wide range of effector molecules to act as intercellular mediators in the regulation of cellular function in order to bring about cell proliferation, integrated control of physiological systems and defence of the whole organism. One group of these intercellular mediators are the cytokines which include interleukins, interferons, colony stimulating factors, tumour necrosis factors, growth factors and chemokines.

Cytokines are inducible, secreted proteins and are produced in response to exogenous stimuli such as chemicals, organisms, injury and radiation. Many cytokines themselves can also interact with cells to induce the production of other cytokines (or positively feedback to induce their own synthesis). They exhibit a wide variety of primary structures, sizes, post-translational modifications and conformations; although despite this diversity, X-ray crystallography studies have revealed that there are relatively few three-dimensional structures.

The cell surface receptors for cytokines have many common structural features, which is not surprising because of the common structures of many cytokines. For example, the basic structure of the cytokine receptor (CKR) family, which mainly interact with the α -helical cytokines, is two extracellular domains of about 100 amino acids with the N-terminal domain containing a number of conserved cysteine residues and a conserved motif in the domain next to the cell membrane. This basic structure can be modified by duplication of the two domains in some cases, addition of immunoglobulin-like or fibronectin-like domains, and addition of other transmembrane glycoproteins to form a receptor complex.

The mechanism by which binding of a cytokine to its receptor generates a signal into the cytoplasm is poorly understood. It is thought that cytokine binding either induces a conformational change of the receptor or that binding induces dimerization or oligomerization of receptor chains which then results in activation of the receptor intracellular domain or associated intracellular enzymes.

Cytokines act as intercellular messengers and, after binding to specific cell surface receptors, induce changes in cellular metabolism and growth. Individual cytokines can have many different activities; that is they are pleiotropic. However, many cytokines produce similar effects; for example GM-CSF, G-CSF and M-CSF all act to stimulate the proliferation and differentiation of haematopoietic progenitors.

Because of the role of GM-CSF in the proliferation and activation of white blood cells, this was the cytokine that was chosen to be used to attempt to make transgenic mice that were resistant to disease. This cytokine is therefore discussed in more detail below.

1.6.1 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF).

Colony stimulating factors were first discovered using the semi-solid agar method of growing haematopoietic cells, and were shown to stimulate the formation of colonies of granulocytes and macrophages (Bradley and Metcalf, 1966). Four types of colony stimulating factor have been identified and these were classified according to the type of colony they produce using this culture method: granulocyte-colony-stimulating factor (G-CSF) stimulates the production of granulocytic colonies; macrophage-CSF (M-CSF) stimulates the formation of monocyte/macrophage colonies; granulocyte-macrophage-CSF (GM-CSF) stimulates the production of both granulocytic and macrophage cells; and multi-CSF (otherwise known as IL-3) stimulates granulocyte and macrophage colony formation and also has a broad range of other proliferative effects on erythroid, megakaryocytic, eosinophil, mast, stem and multipotential cells (Metcalf, 1986).

1.6.2 GM-CSF Secretion.

GM-CSF secretion was first identified in activated CD4⁺ helper T cells, but was subsequently shown that GM-CSF can be produced by a broad range of cell types; see table 1.7.

Table 1.7: Cells expressing GM-CSF. Adapted from Gasson (Gasson, 1991) and Ruef and Coleman (Ruef and Coleman, 1990).

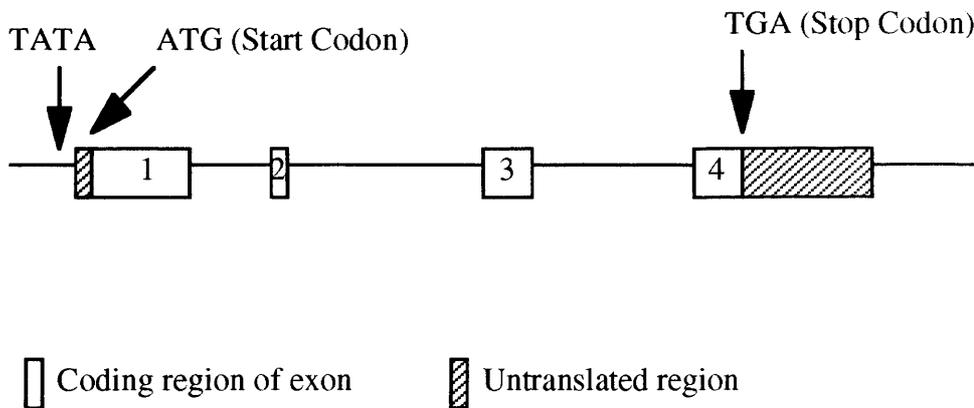
Cell Type	Reference
T lymphocytes	Hermann <i>et al.</i> , 1988
B lymphocytes	Pluznik <i>et al.</i> , 1989
Macrophages	Thorens <i>et al.</i> , 1987
Neutrophils	Kita <i>et al.</i> , 1991
Eosinophils	Broide <i>et al.</i> , 1992
Mast cells	Wodnar-Filipowicz <i>et al.</i> , 1989
Keratinocytes	Chodakewitz <i>et al.</i> , 1988
Osteoblasts	Horowitz <i>et al.</i> , 1989
Fibroblasts	Kaushansky <i>et al.</i> , 1988
Trophoblasts	Ruscetti <i>et al.</i> , 1982
Endothelial cells	Malone <i>et al.</i> , 1988
Smooth muscle cells	Munker <i>et al.</i> , 1986
Mesothelial cells	Demetri <i>et al.</i> , 1989
Various epithelial cells	Waellert <i>et al.</i> , 1995 Jung <i>et al.</i> , 1995

1.6.3 GM-CSF Genetics.

The murine GM-CSF cDNA was isolated from a Con-A activated murine helper T cell library. Sequence analysis of this cDNA revealed a single open reading frame consisting of 141 codons (Miyatake *et al.*, 1985). The sequence suggested a signal sequence of approximately 17 amino acids which would give a mature protein, after processing, of about 120 amino acids. This would give a calculated molecular weight of 12,000 D compared to the reported molecular weight of mGM-CSF of 29,000 D; this difference can be explained by post-translational glycosylation of the molecule.

When the sequences of the murine and human genes for GM-CSF were compared it was revealed that they are highly homologous. The nucleotide sequences share about 70% homology and the amino acid sequences share about 50% homology (Miyatake *et al.*, 1985), indicating that these genes may have evolved from a common ancestor. Both genes are composed of three introns and four exons, which span approximately 2.5 kb of genomic DNA, and are organised in a similar manner (Stanley *et al.*, 1985). See figure 1.9.

Figure 1.9: Schematic representation of the organisation of the GM-CSF gene adapted from Miyatake *et al.*, 1985.



The size of exons 2, 3 and 4 are identical in both species and therefore encode the same number of amino acids; however exon 1 of the human gene is 9 bp longer than the corresponding exon in the mouse. The length of the introns of each gene is nearly the same in both species. There is also conservation of the 5'-flanking regions of the two genes suggesting that these sequences are involved in regulating expression of the genes.

Both the human and mouse genomes contain only one copy of the GM-CSF gene (Miyatake *et al.*, 1985). In the mouse the GM-CSF gene is located on chromosome 11, and the human GM-CSF gene has been mapped to the long arm of chromosome 5 (5q21-q32) (Huebner *et al.*, 1985). This localization of the human GM-CSF gene is interesting as deletion of this region of the chromosome (5q⁻ chromosome disorder) can often be detected in patients suffering from refractory anaemia (resulting in a reduced leukocyte count) [Wisniewski and Hirschhorn, 1983] (Swolin *et al.*, 1981) and acute myelogenous leukaemia (van den Berghe *et al.*, 1976).

1.6.4 GM-CSF Protein Structure.

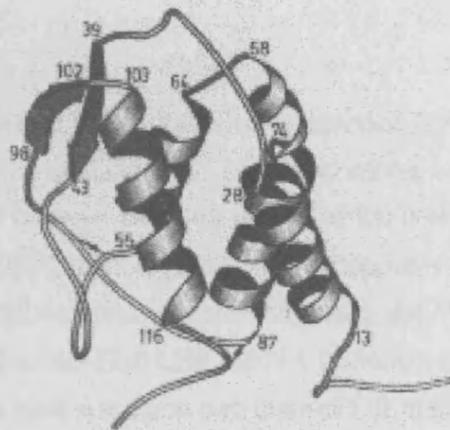
The human cDNA for GM-CSF encodes a protein of 144 amino acids which, after cleavage of the signal peptide results in a mature protein of 127 amino acids. Mature murine GM-CSF is slightly smaller at 124 amino acids, and share approximately 56% homology with the human GM-CSF at the amino acid level. Both are secreted as glycosylated proteins, but glycosylation does not appear to be necessary for biological function as recombinant GM-CSF produced by *E. coli* has the same biological function as the glycosylated form (Kaushansky *et al.*, 1987). GM-CSF from both mouse and human contains four cysteine residues which link the protein with two internal disulphide bridges. These two disulphide bonds are important for biological

activity and are highly conserved between species. Despite the high degree of homology between human and murine GM-CSF at both the nucleotide and amino acid level, GM-CSF is highly species specific.

The structure of human GM-CSF has been determined to 2.4Å resolution by X-ray crystallography and reveals a structure consisting of a two stranded antiparallel β sheet with a bundle of four α helices (Diederichs *et al.*, 1991). The α helices are all antiparallel and form a twisted open barrel, with the open edge filled in by one strand of the β sheet. The two disulphide bonds are at the same end of the molecule and connect helix B to strand 2 of the β sheet and helix C to the carboxyl terminus. See figure 1.10.

A study of mutants in which various tripeptide deletions were made identified four regions in murine GM-CSF that were essential for activity (residues 18-22, 37 to 44, 54 to 64 and 96 to 118). These four regions correspond to helix A, β strand 1, helix B, and a stretch including β strand 2 and helix D. These regions are thought to be important for structural reasons and mutation of these may alter the structure and therefore interfere with binding of GM-CSF to its receptor.

Figure 1.10: The three dimensional structure of GM-CSF taken from Diederichs *et al.*, 1991.



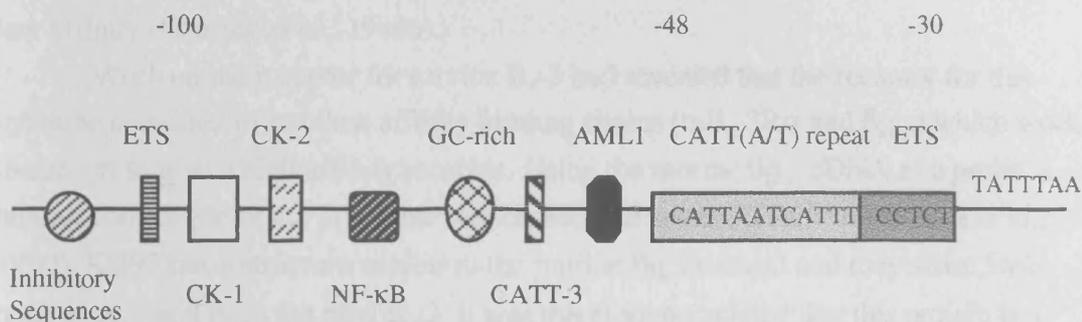
1.6.5 Expression of GM-CSF.

Many agents have been identified that can modulate GM-CSF secretion. Enhancement of GM-CSF production is induced by cytokines such as IL-1, IL-2 and the TNFs; microbes such as *Listeria monocytogenes*, *Leishmania* and *Shistosoma* species and microbial products such as LPS; and drugs such as lithium, retinoic acid

and phorbol esters. Decreased GM-CSF production is also induced by cytokines such as high doses of interferons; and drugs such as vitamin D, ethanol and prostaglandins (reviewed by Ruef and Coleman, 1990).

There is strong conservation of the 5'-flanking regions of the mouse and human genes for GM-CSF, suggesting that this region plays a role in regulating expression of these genes. A variety of transcriptional regulatory elements controlling the expression of GM-CSF have been identified in this 5' region, see fig 1.11 (Nimer and Uchida, 1995).

Figure 1.11: Schematic representation of human GM-CSF 5' flanking sequences indicating the presence of various regulatory regions. Taken from Nimer and Uchida (Nimer and Uchida, 1995).



One study identified a decanucleotide sequence (cytokine consensus 1, CK1) located 78 bp upstream of the GM-CSF TATA promoter consensus sequence, which could also be identified in the 5' flanking region of the multi-CSF, IL-2 and IFN- γ genes (Stanley *et al.*, 1985). This region confers responsiveness to CD28 signalling and is also known as the CD28 response element (Fraser and Weiss, 1992). The region from -48 to -37, relative to the GM-CSF mRNA initiation site, contains a repeated sequence CATT(A/T) which was shown to control full mitogen-inducible promoter activity (Nimer *et al.*, 1988)(Nimer *et al.*, 1990).

The transcription factor NF- κ B, which is a widely utilised mediator of immediate-early gene expression, has also been implicated in binding to the GM-CSF promoter. Purified NF- κ B bound to one site in the promoter (-82 to -91) with an affinity similar to that of other functional NF- κ B binding motifs; and also bound to another site (-98 to -108) with a lower affinity (Schreck and Baeuerle, 1990).

There are also important negative regulatory elements in the region 5' of the GM-CSF gene, which may be important in limiting the expression of GM-CSF in certain cell types (Fraser *et al.*, 1994).

An AT rich region at the 3' end of GM-CSF has also been shown to be important for expression by determining mRNA stability (Shaw and Kamen, 1986).

1.6.6 The Cellular Receptor for GM-CSF.

The cellular receptor for GM-CSF consists of two binding components, the GMR α which binds GM-CSF with a low affinity and the GMR β which exhibits no appreciable binding for GM-CSF.

A cDNA clone encoding a receptor for human GM-CSF was cloned from a placental cDNA library by expression cloning (Gearing *et al.*, 1989b). This cDNA encoded a protein of 400 amino acids with a molecular weight of about 80 kDa, and showed the common motif of the cytokine receptor superfamily in the extracellular domain. The cloned receptor, when expressed in COS cells, bound hGM-CSF with a low affinity (Gearing *et al.*, 1989b).

Work on the receptor for murine IL-3 had revealed that the receptor for this cytokine consisted of two low affinity binding chains (mIL-3R α and β_{IL3}) which work in concert to give a high affinity receptor. Using the murine β_{IL3} cDNA as a probe, a human homologue of the β_{IL3} gene was cloned and termed KH97 (Hayashida *et al.*, 1990). KH97 has a structure similar to the murine β_{IL3} subunit and they share 56% homology, but it does not bind IL-3. It was therefore postulated that this protein is a component of the human IL-3 receptor but it does not play a role in binding IL-3.

The KH97 protein does not bind GM-CSF either, but coexpression of this protein with the low affinity GM-CSF receptor resulted in a high affinity receptor for GM-CSF (Hayashida *et al.*, 1990). Although the KH97 protein has no binding capacity for hGM-CSF by itself, it is cross-linked with GM-CSF when the high affinity receptor is formed. The 80kDa GM-CSF receptor is now referred to as GMR α and the KH97 protein as the common β subunit or β_c (because it also acts as the second chain of the receptors for IL-3 and IL-5).

The sharing of the β_c by the receptors of these three cytokines explains the similar function of these cytokines in stimulating proliferation and development of haemopoietic cells. These three cytokines also cross-compete for receptors (Lopez *et al.*, 1991), and this is mediated by competition for the limiting numbers of β_c subunits by the different α subunits.

The genes for the receptor subunits have been mapped, and the hGMR α gene maps to the pseudoautosomal region of the X and Y chromosomes (Gough *et al.*, 1990) while the β_c subunit maps to chromosome 22 (22q12-22q13) (Shen *et al.*, 1992).

Sequence analysis of the N-terminal α helical regions of the murine GM-CSF, IL-3 and IL-5 genes reveal two conserved amino acids. Mutation of these in mGM-CSF

inhibits binding of the cytokine to the β subunit of the receptor, which therefore implicates these amino acids as the site of binding to the receptor (Shanafelt *et al.*, 1991).

A soluble form of the GMR α subunit has also been identified (Brown *et al.*, 1995)(Murray *et al.*, 1998). This lacks the transmembrane and cytoplasmic domains and competes with the transmembrane-anchored GMR α and the oligomeric receptor complex for binding to GM-CSF (Brown *et al.*, 1995). This soluble GMR α has also been shown to antagonize GM-CSF activity *in vitro* (Murray *et al.*, 1998), suggesting that it may also act as a modulator of GM-CSF activity *in vivo*.

1.6.7 GM-CSF Signal Transduction.

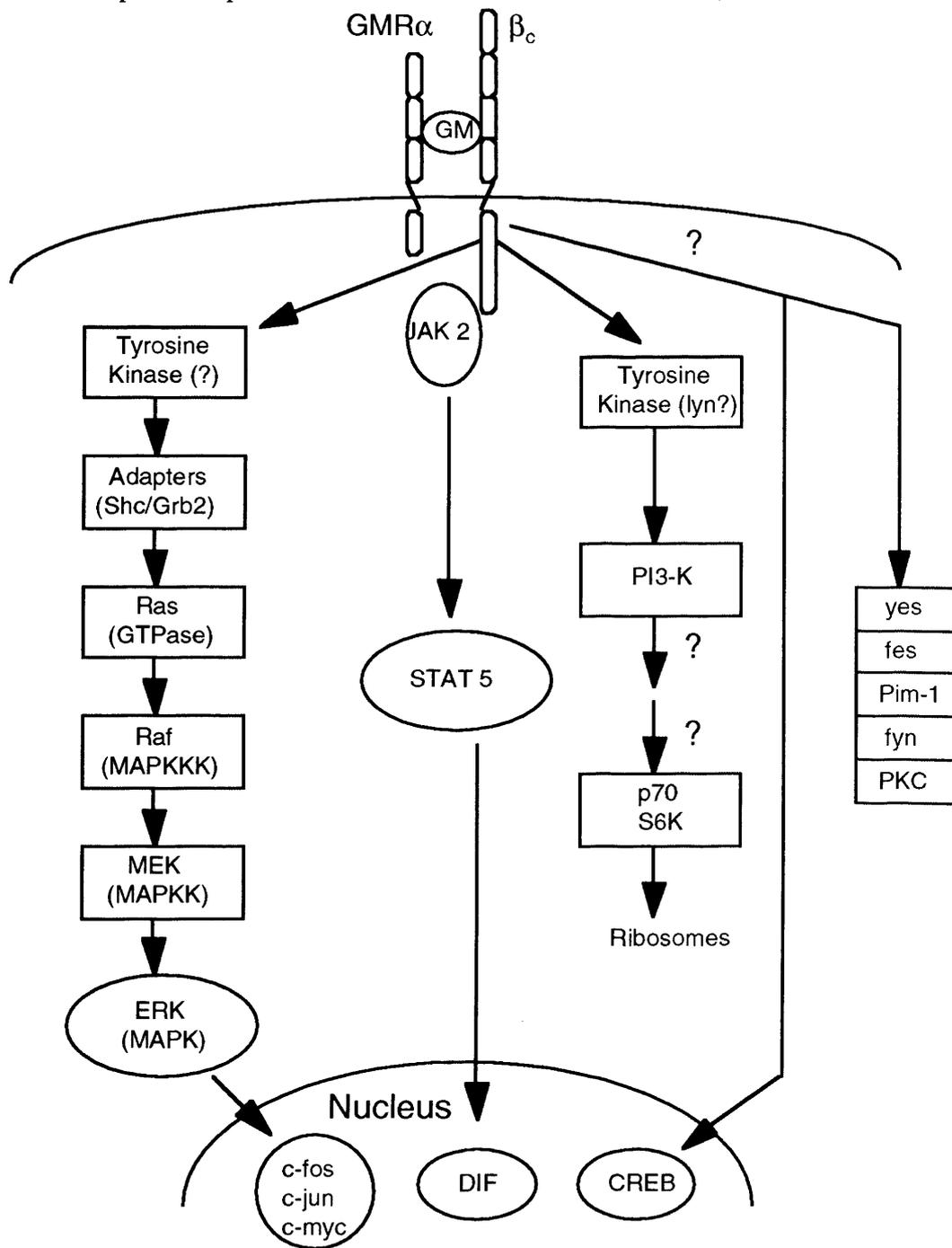
Low affinity binding of GM-CSF to the GMR α is not sufficient to activate signalling in responsive cells, however the cytoplasmic region of the GMR α is necessary for efficient signal transduction. Association between the α and β subunits seems to be the most crucial step for signal transduction.

Although neither of the GM-CSF receptor subunits has an intrinsic tyrosine kinase, evidence links the involvement of tyrosine kinases with the signalling pathways of GM-CSF; including the fact that tyrosine kinase inhibitors block the proliferative response to GM-CSF and IL-3 (Satoh *et al.*, 1992). GM-CSF has been shown to activate tyrosine phosphorylation rapidly, even when the cells are stimulated at 4°C, indicating that the kinase and its initial substrates are closely associated with the receptor before ligand stimulation.

One of the major tyrosine phosphorylated proteins induced by GM-CSF stimulation is the β_c subunit, although the significance of this is not known.

There seem to be four major signalling pathways stimulated by binding of GM-CSF to its receptor, see figure 1.12. One of these involves the Ras-MAP (mitogen activated protein) kinase pathway, in which the initial step involves tyrosine kinase activation of adapter molecules which then activate other protein kinases leading to a sequence of protein activation cumulating in the activation of various transcription factors (Sakamoto *et al.*, 1994).

Figure 1.12: The putative signal transduction pathways initiated by binding of GM-CSF to its receptor. Adapted from Gomez-Cambronero and Veatch, 1996.



Abbreviations: Shc: Src-homology 2-adaptor protein; Grb: growth factor receptor-bound protein; MAPKKK: mitogen-activated protein (MAP) kinase kinase kinase; MEK: MAP kinase/ERK-activating kinase; ERK: extracellular signal-regulated kinase; MAPKK: MAP kinase kinase; MAPK: MAP kinase; JAK 2: Janus kinase-2; STAT5: signal transducer and activator of transcription-5; DIF: differentiation-induction factor; PI3-K: phosphoinositide 3-kinase; PKC: protein kinase C; CREB: cyclic AMP response element-B.

Another major pathway is the "JAK-STAT" pathway. The initial stage is the phosphorylation of the β_c subunit and also the transcription factor activator STAT-5 which then activates the transcription factor DIF (differentiation-induced factor)(Ihle, 1994)(Ihle, 1995)(Karnitz and Abraham, 1995a). A third pathway involves the accumulation of phosphatidylinositol-3,4,5-triphosphate (PI 3) through the action of a tyrosine kinase. PI 3-kinase is one of the chain of activated proteins leading to the activation of the ribosomal kinase p70S6K (Jucker and Feldman, 1995)(Karnitz *et al*, 1995b). The fourth pathway that may be involved in signalling through the GM-CSF receptor has not been fully determined but may involve various molecules that are triggered by GM-CSF and the transcription factor CREB (cyclic AMP response element-B)(Lee *et al*, 1995). It is not yet known what role each pathway has in triggering the cellular effects seen with GM-CSF or to what extent the pathways interact.

1.6.8 Mice Transgenic for GM-CSF.

Mice transgenic for GM-CSF were created using the Moloney murine leukaemia virus (MoMLV) long terminal repeat (LTR), which contains promoter and enhancer elements, fused to the GM-CSF gene (Lang *et al.*, 1987). Two offspring were identified as carrying the transgene; the pattern of transgenic offspring obtained from one of these founders ('female' line) indicated that the insertion site of the transgene was in one of the X chromosomes, the other founder ('male' line) had the transgene inserted into one of the autosomal genes.

The serum levels of GM-CSF in all transgenic mice were consistently elevated to approximately 2000U/ml, at least forty-fold greater than in normal mice (Lang *et al.*, 1987). GM-CSF could also be detected in the intraocular fluid and peritoneal washes of the transgenics and in the urine of male transgenics from the 'male' line.

The mice showed a normal growth pattern until six weeks of age, after which the transgenic mice developed a condition of progressive weight loss and weakness and died prematurely, with most dying by 150 days (Lang *et al.*, 1987).

1.6.8.1 The Macrophages of GM-CSF Transgenic Mice

A marked feature of the transgenic mice was an increase in peritoneal cavity cellularity, with counts of up to 2.5×10^8 cells from transgenic mice compared to between 2 and 6×10^6 cells from littermate controls (Lang *et al.*, 1987). These cells were predominantly macrophages and were enlarged in size compared to controls, and appeared to be in a functionally activated state. An increased number of macrophages

was also seen in the pleural cavity of transgenic mice. In contrast, the peripheral blood white cell count in transgenic mice was not different to that of control littermates.

Bone marrow cell counts were similar in transgenic and controls, although a slightly higher percentage of monocytes and some macrophages were seen in the transgenics. A normal frequency of granulocyte-macrophage progenitors was seen in the bone marrow and when these cells were isolated they exhibited normal responsiveness to both GM-CSF and other growth factors. The spleens of the transgenic mice varied in size from atrophy to slight enlargement and contained some enlarged macrophages.

The elevated numbers of peritoneal macrophages seen in the GM-CSF transgenic mice correlates with the amount of detectable GM-CSF in the peritoneum, and also with the levels of IL-1 (Gearing *et al.*, 1989a). The origin of peritoneal macrophages is controversial with data indicating that the population may be self-sustaining (due to local mitotic activity) rather than originating from infiltration of cells from the blood (Parwaresch and Wacker, 1984)(Volkman, 1976). No abnormalities in the bone marrow or spleen cell numbers, or numbers and types of progenitor cells in these tissues was detected; so it is therefore unlikely that the increased number of peritoneal cells is due to infiltration of monocytes generated in these tissues (Metcalf *et al.*, 1992). The peritoneal macrophages in transgenic mice were shown to exhibit elevated mitotic activity and it therefore seems more likely that the increased number of peritoneal macrophages is due GM-CSF-induced local proliferation of these cells (Metcalf *et al.*, 1992). There is also an increase in the number of binucleate and multinucleate cells in the peritoneum as the mice age which is probably due to the long-term consequences of overstimulation by GM-CSF (Metcalf *et al.*, 1992).

Cells isolated from the peritoneum of GM-CSF transgenic mice were shown to be predominantly macrophages (approximately 85% of the cells compared to about 50% of the cells from littermate controls) (Elliott *et al.*, 1991). Flow cytometric analysis of the cells showed that the transgenic macrophages expressed higher levels of two macrophage activation markers (mac-2 and mac-3), indicating that these cells are activated to a different state than the non-transgenic macrophages (Elliott *et al.*, 1991). Both basal and stimulated (using Zymosan or PMA) superoxide release was significantly higher in transgenic cells than controls (Elliott *et al.*, 1991). Superoxide has also been implicated in macrophage-mediated tissue damage, so elevated superoxide release in transgenic macrophages may also play a role in the development of the lesions seen in these animals (see later). No significant differences were seen in cellular cytotoxicity between transgenic and control macrophages (Elliott *et al.*, 1991). These results indicate that up-regulation of cellular function in transgenic macrophages is

selective rather than uniform. The functional changes are most likely to be achieved by stimulation by GM-CSF, either directly or through other mediators (such as IL-1).

Isolated peritoneal macrophages from transgenic mice were shown to be about twice as efficient at phagocytosis of *Listeria monocytogenes* than macrophages from control animals (Tran *et al.*, 1990). However, bacteriolysis by the cells from transgenic mice was not significantly different to that of control cells, despite the fact that the transgenic cells release more superoxide which would be expected to increase bacteriolysis (Tran *et al.*, 1990). Because the cells are more efficient at phagocytosis of these bacteria, the fact that they do not kill ingested bacteria more efficiently than controls should not matter and it would be expected that these mice are more able to resist infection.

1.6.8.2 Tissue Lesions in GM-CSF Transgenic Mice.

A striking abnormality of all the transgenic mice was opacity of the eyes, and accumulations of large numbers of enlarged macrophages in the eyes (Lang *et al.*, 1987). Accumulation of macrophages were also seen in striated muscle, with the more extensive lesions being associated with destruction of the muscle cells. Similar, but smaller, lesions were also occasionally seen in cardiac muscle. Macrophage accumulation was also seen in the loose connective tissue of the mesentery and surrounding various organs, with fibrous tissue developing in some of the older transgenic mice.

GM-CSF transgene transcripts were detected in the ocular tissue, peritoneal cells and skeletal muscle of mice with macrophage lesions (Lang *et al.*, 1987). No mRNA corresponding to the transgene was detected in the bone marrow. One explanation for this pattern is that the transgene is expressed by macrophages, and that activation of the transgene occurs at a stage of cell differentiation outside the bone marrow.

The ocular development of GM-CSF transgenic mice can be divided into three sections: (1) fertilization to day 5 after birth where the transgenics are indistinguishable from the littermate controls; (2) day 5 to day 7 after birth when additional cells are present in the anterior chamber and vitreous of the eyes of the transgenic mice; (3) day 8 after birth to adulthood when these additional cells invade the other issues of the eye and cause tissue damage (Cuthbertson and Lang, 1989). These additional cells bear macrophage surface markers, and GM-CSF transgene mRNA could be detected in these cells (Cuthbertson and Lang, 1989). Normal ocular development involves removal of the temporary hyaloid vasculature in the vitreous and redundant neurones from the retina by macrophages (known as hyalocytes). Tissue destruction in the transgenic mice begins at the same time during development as the remodelling by hyalocytes in normal mice. The

proposed model for ocular damage in GM-CSF transgenic mice is therefore that the normal developmental cell death in the eye provides a stimulus for the infiltration of macrophages in the transgenic mice. Because these macrophages express the transgene, the GM-CSF produced can autostimulate the macrophages enabling them to enter other parts of the eye and cause tissue damage. The invading macrophages have been shown to express the genes for IL-1 α , TNF α and basic fibroblast growth factor (bFGF) (Cuthbertson *et al.*, 1990). Both TNF α and bFGF are angiogenic *in vitro* and may induce the neovascularization seen in the vitreous and cornea of adult transgenic mice. A common response of neural tissue to damage is the production of glial fibrillary acidic protein (GFAP), and it is thought that IL-1 α and bFGF can simulate the production of GFAP. These macrophage products may therefore mediate the damage and death of the cells of the eye.

Because there is no increase in bone marrow macrophage progenitors, the increased numbers of macrophages accumulating in tissues is not due to an increased number of progenitors. An explanation of this increase in activated macrophages may be that GM-CSF is able to prolong the survival of these cells as well as activate them. These macrophages in the transgenic mice would appear to be responsible for the tissue damage in the eye and skeletal muscle as there are abnormal accumulations of macrophages in these areas, and activation of the cells is likely to be caused by production of GM-CSF by macrophages.

Expression of the cytokines TNF α , IL-1 α and bFGF was also detected in the infiltrating macrophages and granulocytes of skeletal muscle, which suggests that these cytokines also play a role in the wasting condition seen in the GM-CSF transgenic mice prior to death (Lang *et al.*, 1992). Again, production of these cytokines is probably due to autostimulation of the monocytic cells involved in remodelling of the tissue, due to the expression of the GM-CSF transgene by these cells.

GM-CSF transgenic mice were also shown to express higher levels of the plasminogen activator uPA than littermate controls (Elliott *et al.*, 1992). uPA converts the proenzyme plasminogen to the fibrinolytic enzyme plasmin and has been implicated in many processes such as inflammation, cell migration and tumour metastasis. The abnormal production of uPA in GM-CSF transgenic mice may therefore also play a role in the development of ocular, muscle and other pathological lesions such as the occurrence of spontaneous intraperitoneal haemorrhage seen in some transgenic mice.

1.6.9 GM-CSF Knockout Mice.

Mice that are homozygous for a disrupted GM-CSF gene develop normally and show no major perturbation of haematopoiesis, in either bone marrow or blood populations (Stanley *et al.*, 1994). This indicates that the absence of GM-CSF is compensated for in haemopoiesis by other regulators such as G-CSF, M-CSF, IL-3 and IL-5. These mice do however develop abnormal lungs with extensive infiltration of lymphocytes, surfactant accumulation and numerous large intraalveolar macrophages (Stanley *et al.*, 1994). A prominent feature of this lung pathology is infection with a range of opportunistic bacterial and fungal infections. The immune response of the GM-CSF knockout mice is sufficient to prevent death, but the ongoing pathology indicates that the response to infection is defective. The role of GM-CSF in the prevention of lung pathology may be the local activation of macrophages involved in control of infection or surfactant clearance.

1.6.10 Action of GM-CSF.

GM-CSF is a highly active biologically molecule and induces many effects on the immune system. Its roles in haemopoiesis and in the activation of mature white blood cells are discussed below.

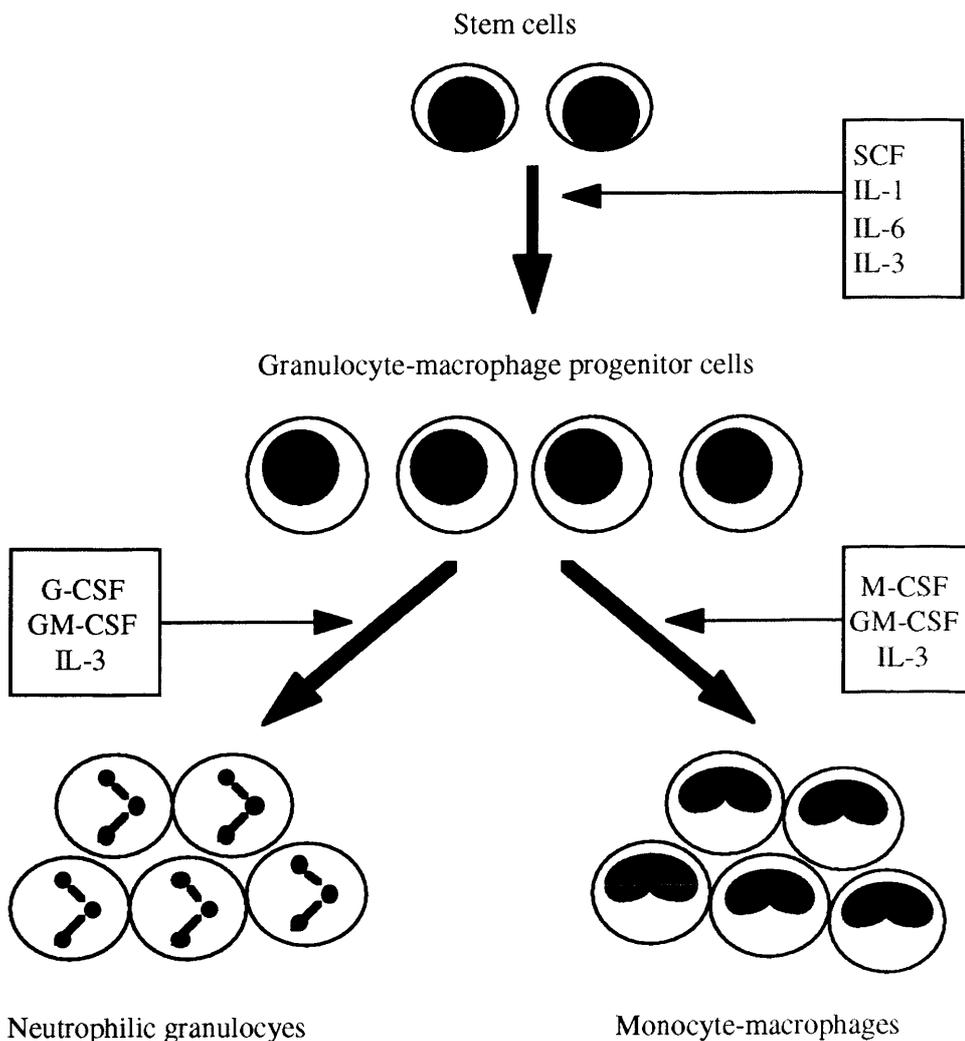
1.6.10.1 Role of GM-CSF in Haemopoiesis.

The turnover of cells of the haemopoietic system of an average person can be estimated at close to 1×10^{12} cells per day, including 2×10^{11} erythrocytes (Erslav, 1983) and 7×10^{10} neutrophilic leukocytes (Dancey *et al.*, 1976). These mature blood cells are derived from a relatively small population of bone marrow stem cells which are laid down during embryonic development. These haemopoietic stem cells are pluripotent and can undergo differentiation to form all types of blood cells, such as neutrophils, basophils, eosinophils, monocytes, lymphocytes, platelets and erythrocytes. The stem cells differentiate to mature blood cells in the bone marrow and are then released into the general circulation. Within the bone marrow the stem cells are found in association with stromal cells and matrix molecules; the stromal cells produce a range of growth factors (either released from the cells or presented as integral membrane components) which influence haemopoiesis. These growth factors can be characterized into three main groups: those which influence B- or T-lymphocytes (such as IL-1 and IL-2), those which have a direct effect on the proliferation and development of multipotent cells

(such as stem cell factor (SCF) and IL-6), and those which influence the growth and development of myeloid progenitor cells (the colony-stimulating factors).

Colony stimulating factors were first discovered using the semi-solid agar method of growing haematopoietic cells, and were shown to stimulate the formation of colonies of granulocytes and macrophages. As mentioned, four types of colony stimulating factor have been identified and these are classified according to the type of colony they produce using this culture method. See figure 1.13. In addition to its effects on cells of the granulocyte-macrophage lineage, GM-CSF has also been shown to stimulate eosinophil and megakaryocyte colony formation (Robinson *et al.*, 1987), and acts synergistically with erythropoietin to stimulate growth of erythroid precursors (Donahue *et al.*, 1985). There have also been reports that GM-CSF can stimulate the proliferation or function of T-cells (Santoli *et al.*, 1988) and B-cells (Haas *et al.*, 1989).

Figure 1.13: Cytokine control of haemopoiesis.



Unique receptors exist for each colony-stimulating factor and stem cells possess receptors for a number of growth factors; then, as the stem cells undergo differentiation, there is a progressive loss of receptors for certain factors (Nicola, 1987). This fits with the idea that as cells become more specialised they lose the ability to react to some growth factors. It would also seem that the growth factors interact with each other in order to induce differentiation of stem cells. For example, exposure of stem cells to single growth factors such as SCF, G-CSF, GM-CSF, M-CSF or IL-6 did not induce the cells to proliferate; however, when the cells were exposed to a combination of growth factors such as SCF and GM-CSF together, a massive proliferative response was seen (Dexter and Heyworth, 1994). Further experiments suggest that, in this system, SCF acts as a survival inducer and that GM-CSF acts as the mitogenic stimulus.

1.6.10.2 GM-CSF Action on Mature Cells.

GM-CSF not only acts to stimulate proliferation of immature progenitor cells, it can also enhance the functions of mature effector cells.

1.6.10.2.1 Neutrophils.

In vitro recombinant human GM-CSF was shown to stimulate the antibody-dependent cell-mediated cytotoxicity (ADCC) of neutrophils, increasing cytotoxicity from 12.5% to 31.5% (Lopez *et al.*, 1986). Addition of GM-CSF to neutrophils resulted in an increase of serum-dependent (complement-mediated) phagocytosis of baker's yeast (Lopez *et al.*, 1986). This was an increase in the number of neutrophils exhibiting phagocytosis and in the number of ingested yeast per neutrophil. Isolated human neutrophils treated with GM-CSF also exhibit enhanced phagocytosis of opsonized *Staphylococcus aureus*, and again this was shown to be due to an increased percentage of the cells exhibiting phagocytosis and an increase in the number of organisms taken up by each cell (Fleischmann *et al.*, 1986).

GM-CSF also primed neutrophils for degranulation and superoxide production after stimulation with FMLP (*N*-formylmethionylleucylphenylalanine) (Lopez *et al.*, 1986). Increased priming, by GM-CSF, of the superoxide respiratory burst was not seen in response to zymosan particles (Leino *et al.*, 1993) and it was hypothesized that this difference is due to selective GM-CSF priming of the different signal-transduction pathways of the FMLP and zymosan receptors. GM-CSF also induced morphological changes, an elongated irregular shape, in neutrophils (Lopez *et al.*, 1986) which has been correlated with superoxide release and activation of protein kinase C. Enhancement of the functional antigens GFA-1, GFA-2 and Mo-1 but not β_2m was induced by GM-CSF, suggesting that GM-CSF can selectively up-regulate the expression of

functionally important molecules (Lopez *et al.*, 1986). GM-CSF was also shown to enhance the survival of neutrophils *in vitro* with approximately 6 hours prolonged survival (Lopez *et al.*, 1986).

In vivo GM-CSF induces a triphasic response after injection (Lord *et al.*, 1989). This consists of an initial neutropenia (5 to 60 min after injection) probably due to activation of the neutrophils resulting in increased adherence to endothelium. Administration of GM-CSF to non-human primates was shown histologically to enhance neutrophil adherence to pulmonary endothelium during this initial neutropenia (Yong *et al.*, 1992). This initial phase is followed by leukocytosis (beginning 3 to 4 hours after injection) due to release of neutrophils from the bone marrow. This is then followed by proliferation and differentiation of neutrophilic precursors in the bone marrow.

Studies *in vivo* also showed that defective phagocytic killing by neutrophils could be corrected in patients infected with HIV treated with GM-CSF (Baldwin *et al.*, 1988). Treatment of patients with aplastic anaemia with GM-CSF resulted in a priming of the respiratory burst activity of neutrophils (Ohsaka *et al.*, 1992). Also, an enhanced neutrophil-mediated ADCC response was seen in patients infected with HIV treated with GM-CSF (Baldwin *et al.*, 1988). These *in vivo* results therefore confirm the results seen *in vitro*.

1.6.10.2.2 Monocytes.

Human peripheral blood mononuclear cells incubated for 24 hours with recombinant GM-CSF were shown to be stimulated to produce IL-1 α , IL-1 β and TNF in a dose dependent manner (Sisson and Dinarello, 1988). The production of each cytokine in response to GM-CSF was complete by 12 hours. Nearly all the measured TNF was secreted by 24 hours, whereas most of the IL-1 was cell associated (in the cytosolic compartment).

Recombinant murine GM-CSF was shown to inhibit the replication of *Trypanosoma cruzi* in murine peritoneal macrophages both when the cells were incubated with GM-CSF before infection and when the cells were stimulated with GM-CSF after infection (Reed *et al.*, 1987). GM-CSF also stimulated the ability of these macrophages to release H₂O₂, indicating a possible mechanism for the increased microbistatic activity. Stimulation of human mononuclear cells and macrophages (allowed to differentiate for 7 days after isolation) with recombinant human GM-CSF also inhibited the intracellular replication of *T. cruzi* (Reed *et al.*, 1987). GM-CSF also stimulated killing of *Leishmania tropica* in infected murine macrophages (Handman and Burgess, 1979). Isolated human mononuclear cells treated with GM-CSF were also shown to be enhanced for phagocytosis of heat-killed yeast, and *Candida albicans*

blastospores; although GM-CSF was not able to prevent germination of the blastospores (Bober *et al.*, 1995). Hyphae of *Aspergillus fumigatus* are also damaged more by monocytes stimulated with GM-CSF than those not stimulated with GM-CSF (Roilides *et al.*, 1996). Addition of steroids (such as dexamethasone) to monocytes prevents the ingestion and killing of bacteria, but this steroid suppression could be prevented by the addition of GM-CSF to phagocytosis and killing assays using *Staphylococcus aureus* (Bober *et al.*, 1995). These results indicate that GM-CSF is effective in enhancing the macrophage phagocytic response to bacteria, fungi and parasites.

The proliferation of viruses in macrophages shows a very different pattern. Peripheral blood monocytes stimulated with GM-CSF were shown to support the replication of HIV and influenza virus better than those not stimulated with GM-CSF (Perno *et al.*, 1989).

Pre-exposure of monocytic cells to GM-CSF for 30 min also enhances the chemotactic response of these cells towards FMLP (a model for bacterial cell wall constituents) and LTB₄ (a chemoattractant produced at inflammatory sites) (Bober *et al.*, 1995). This indicates that GM-CSF can quickly prime monocytes to migrate towards chemoattractants found in bacterial lesions or sites of trauma.

Incubation of murine peritoneal macrophages with GM-CSF also increased the tumouricidal and tumouristatic activities of these cells (Wing *et al.*, 1982).

Looking at isolated monocytes from patients with septic shock, it was shown that these cells exhibit a greater respiratory burst than those from healthy donors, that the response to secondary stimulation was attenuated, and that surface expression of CD14 (which is involved in signal transduction) is decreased. Incubation of monocytes from patients with septic shock with GM-CSF restored the response of these cells to secondary stimulation and also restored the expression of CD14 to normal levels (Williams *et al.*, 1998).

Administration of GM-CSF to patients suffering from small cell lung cancer both before and after chemotherapy induced the expression of MHC class II and CD44 (a mediator of adhesion) on the surface of peripheral blood monocytes (Aman *et al.*, 1996). This treatment also resulted in increased levels of sCD25 (soluble IL-2R) and IL-1Ra in the serum, a small increase in IL-8 was also demonstrated (Aman *et al.*, 1996).

1.6.10.2.3 Eosinophils.

Isolated human eosinophils treated with GM-CSF were shown *in vitro* to have enhanced ADCC for tumour cells, increasing cytotoxicity from 5.8% to 48.6% (Lopez *et al.*, 1986). GM-CSF also activated eosinophil-mediated phagocytosis of opsonized baker's yeast, with an increase in the number of cells showing phagocytosis and an increase in the number of yeast ingested per cell (Lopez *et al.*, 1986). Survival of isolated eosinophils in culture was also enhanced by the addition of GM-CSF; an increase in survival of approximately 9 hours was seen (Lopez *et al.*, 1986).

1.6.10.3 Other Effects of GM-CSF.

The most important cell type for the initiation of primary and secondary (memory) T cell responses is the dendritic cell. Intradermal injection of GM-CSF induces migration of dendritic cells (Langerhans cells) to the skin and also enhances keratinocyte growth (Kaplan *et al.*, 1992). Stimulation of dendritic cells in culture by GM-CSF also induces the expression of high levels of MHC class II molecules which are involved in antigen presentation to T cells (Morrisey *et al.*, 1987).

GM-CSF has also been shown to augment the antibody response; normal volunteers given a single injection of Hepatitis B vaccine and GM-CSF together developed protective antibody titres (Tarr *et al.*, 1996). Also, repeated injections of GM-CSF was shown to increase the efficacy of an anti-idiotypic vaccine to the Lewis Y carbohydrate in primates (Liehl *et al.*, 1995).

GM-CSF is also thought to induce beneficial anti-tumour effects and this was examined in a study of extracted tumour cells from mice which were then engineered to produce mGM-CSF before being transplanted back into the mice. The GM-CSF secreting cells were able to induce an anti-tumour response which was protective (Dranoff *et al.*, 1993).

1.6.11 Clinical Applications of GM-CSF.

Due to its ability to stimulate proliferation and differentiation of white blood cells, there is great potential for GM-CSF to be used as a therapeutic agent. GM-CSF has been used in many clinical trials to enhance neutrophil recovery after cytotoxic chemotherapy (Arnberg *et al.*, 1998)(Deb *et al.*, 1998)(Mustacchi *et al.*, 1997)(Dierdorf *et al.*, 1997). The administration of GM-CSF following autologous bone marrow transplantation accelerated the recovery of neutrophil levels and shortened the duration of antibiotic therapy required and hospital stay (Nemunaitis *et al.*, 1991)(Singhal *et al.*,

1997). Aplastic anaemia is the result of a deficiency of haematopoietic progenitor cells, and GM-CSF treatment has been used to increase the production of mature cells from the small pool available (Vadhan-Raj *et al.*, 1988)(Kojima, 1996). Neutropenia is also one of the complications associated with AIDS. Treatment of these patients with GM-CSF resulted in elevated neutrophil, monocyte and eosinophil counts (Groopman *et al.*, 1987).

GM-CSF also has effects on wound healing. Introduction of GM-CSF intradermally in patients with lepromatous leprosy resulted in enhanced keratinocyte growth, the selective recruitment of Langerhans cells into the dermis and enhanced wound healing (Kaplan *et al.*, 1992). GM-CSF has also been used to promote healing of ulcers (Costa *et al.*, 1994), skin grafts (Pojda and Struzyna, 1994), and in the prevention of sepsis following burns (Molloy *et al.*, 1995).

The use of GM-CSF to treat infections has also been proposed. Infection with *Mycobacterium avium* is a common problem in immunocompromised patients. Patients with disseminated mycobacterial infection were treated with GM-CSF; and macrophages isolated from these patients showed increased superoxide production and increased mycobacteriostatic/mycobacteriocidal activity *in vitro* compared with macrophages isolated from the same patients before GM-CSF treatment (Bermudez, 1994)(Kemper *et al.*, 1998). This suggests that GM-CSF may have the potential to treat mycobacterial infections. Treatment of patients with acute visceral leishmaniasis due to *Leishmania chagasi* were treated with GM-CSF (in combination with pentavalent antimony). The neutrophil, monocyte and eosinophil counts were higher in the treated patients and they suffered fewer secondary bacterial and viral infections than those not given GM-CSF (Badaro *et al.*, 1994). Neutropenic patients with fungal infections often have a poor prognosis, therefore these patients were treated with GM-CSF (in combination with the antifungal agent amphotericin B). Most of the patients responded to GM-CSF with an increased neutrophil count, and half completely recovered as a result of the treatments (Bodey *et al.*, 1994).

GM-CSF may also be useful as a vaccine adjuvant; due its abilities to increase MHC class II expression, enhance dendritic cell migration, induce localized inflammation, and induce systemic effects on the haemopoietic network. Studies with monkeys showed that injection of GM-CSF at the same time, but at a different injection site, as injection with human IL-3 resulted in higher anti-IL-3 antibody titres than in monkeys injected with IL-3 alone (Liehl *et al.*, 1994). This indicates that GM-CSF administered systemically can boost the immune response to an antigen. The efficacy of GM-CSF adjuvant activity when administered with an antigen was examined by giving mice repeated doses of BSA with GM-CSF. The GM-CSF treated mice developed a greater antibody response to BSA than those not treated with GM-CSF (Liehl *et al.*,

1994). An area of current research is the use of antigen/GM-CSF fusion proteins. Immunization of mice with a fusion of GM-CSF and a specific idiotypic expressed on a B-cell lymphoma resulted in antibody production and protection against progression of the lymphoma (Tao and Levy, 1993).

1.7 Project Strategy.

The main aim of this project is to produce transgenic mice that are resistant to infectious disease. Because GM-CSF stimulates the proliferation and differentiation of white blood cells it is thought that an increase in the levels of GM-CSF in the mice would lead to an increase in the numbers and activity of these cells and therefore to an increased ability of the mice to fight infections. However, it has been shown that constitutive expression of GM-CSF in transgenic mice leads to many deleterious side effects (Lang *et al.*, 1987), although isolated macrophages from these mice do show increased activation including increased phagocytosis of organisms (Tran *et al.*, 1990).

It was therefore reasoned that in order to benefit from the action of increased levels of GM-CSF, without the deleterious side effects, it was necessary for the expression of GM-CSF to be controlled. This control of expression should result in GM-CSF being produced rapidly at the onset of an infection or an injury which would then lead to rapid proliferation and activation of white blood cells and therefore to an increased ability to deal with the trauma. In order to prevent the side effects of long term expression of GM-CSF, this production of GM-CSF would have to be stopped once the trauma had passed. The acute phase response is characterised by this pattern of expression (a rapid increase in the levels of certain proteins which returns to low basal levels once the trauma has passed), so it was therefore decided to exploit this pattern of expression for GM-CSF.

The promoter of the acute phase protein CRP was chosen to drive the expression of GM-CSF as this gene has been extensively characterised in transgenic mice (Ciliberto *et al.*, 1987a)(Murphy *et al.*, 1995), and shown to be expressed as a major acute phase protein in transgenic mice. The project strategy was therefore to clone the GM-CSF structural gene into the background of the CRP promoter and then use this to create a transgenic mouse line for further analysis.

1.8 Project Applications.

The use of transgenic technology to create disease resistant animals is an exciting field, and the aim of this project was to see if mice expressing GM-CSF in a controlled manner could be protected from infectious disease. This has commercial applications in the creation of animals with increased immunity, for example for the food industry. It is also useful to identify the role played by GM-CSF in the host response to pathogens and therefore to understand the disease process better in the search for therapeutic agents. Another possible use for these animals would be that they would have an increased immune response and would potentially be useful in the production of antibodies to otherwise poorly immunogenic molecules. Studies have shown that GM-CSF acts as an efficient vaccine adjuvant (Liehl *et al.*, 1994) and these mice would provide a system where the GM-CSF would be produced by the mouse itself on administration of antigen.

A controlled expression system would also be another useful tool in the exploration of the effect of many different proteins (particularly those involved in the immune response). Much useful information about the *in vivo* effects of different proteins has been obtained from studies of transgenic and knockout mice; but perturbation of these proteins often leads to health or developmental problems so controlled expression would enable the study of the role of these proteins in a more natural situation.

Chapter 2: Materials and Methods.

All chemicals from Sigma or Fisons unless otherwise stated.

2.1 Molecular Biology Techniques.

2.1.1 Small Scale Preparation of Plasmid DNA.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

Single colonies were used to inoculate 10 ml of Luria broth (LB) supplemented with antibiotic (where appropriate) and grown overnight at 37°C with shaking (200 rpm).

Luria Broth / Agar -

10 g	Tryptone/Peptone	
5 g	Yeast Extract	
5 g	NaCl	in 1 litre water

(For Luria Agar add 18 g Agar)

A total of 3 ml of this suspension was harvested by centrifugation at 15,000 x g for 3 min in a microfuge. The medium was removed to leave the pellet as dry as possible, and the pellet was resuspended in 100 µl of solution 1 by vortexing.

Solution 1 -

50 mM	Glucose	
25 mM	Tris-Cl pH 8.0	
10 mM	EDTA pH 8.0	Diluted in water

The bacteria were lysed by the addition of 200 µl of freshly prepared solution 2, and the contents of the tube mixed by inversion.

Solution 2 -

0.2 N	NaOH	
1%	SDS	Diluted in water

The cellular proteins and high molecular weight DNA were precipitated by the addition of 150 µl of cold solution 3, and the contents of the tube mixed well by vortexing, inverted, for approximately 10 sec. The tubes were then incubated on ice for 3-5 min.

Solution 3 -

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

The cellular proteins and high molecular weight DNA were pelleted by centrifugation at 15,000 x g for 5 min and the supernatant transferred to a fresh tube. To remove all

remaining cellular proteins the samples were phenol/chloroform extracted by adding an equal volume (450 μ l) of a mixture containing equal volumes of phenol and chloroform, mixing by inversion and centrifuging at 15,000 x g for 2 min. The aqueous phase was transferred to a fresh tube and the plasmid DNA precipitated by the addition of 2 volumes (900 μ l) of ethanol at room temperature. The contents of the tube were mixed by vortexing and allowed to stand for 10 min at room temperature. The DNA was then pelleted by centrifuging at 15,000 x g for 15 min in a microfuge. The supernatant was removed and the pellets washed once with 70% ethanol. The pellets were then allowed to dry (inverted over tissue or under vacuum) before the DNA was resuspended in 50 μ l of TE containing DNase free RNase.

TE with RNase -

10 μ l	Tris-Cl (1M)
2 μ l	EDTA (0.5M)
2 μ l	RNase (10 mg/ml)
986 μ l	water

2.1.2.1 Large Scale Preparation of Plasmid DNA by Equilibration through a Caesium Chloride Gradient.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

The bacteria from a 1l culture were pelleted by centrifugation at 2,000 xg for 15 min at 4°C ; the supernatant was discarded and the pellet resuspended in 200 ml ice cold STE.

STE -

0.1 mM	NaCl
10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

The suspension was pelleted again by centrifugation (2,000 xg for 15 min at 4°C) and the pellet resuspended in 18 ml of solution I.

Solution I -

50 mM	Glucose
25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA (pH 8.0)

To lyse the bacteria, 2 ml of a solution of 10 mg/ml lysozyme in 10 mM Tris-HCl (pH 8.0) was added and incubated at room temperature for a few minutes; then 40 ml of fresh solution II added.

Solution II -

0.2 M	NaOH
1%	SDS

The contents of the tube were mixed thoroughly and incubated at room temperature for 5-10 min; then 20 ml of ice cold solution III added to precipitate the cellular proteins and high molecular weight DNA.

Solution III -

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

(This gives a solution of 3 M Potassium and 5 M Acetate)

The contents of the tube were mixed and the tube stored on ice for 10 min; then the protein and high molecular weight DNA pelleted by centrifugation at 20,000 x g for 20 min at 4°C. The supernatant was filtered through 4 layers of prewetted tissue into a clean tube; then the plasmid DNA precipitated by the addition of 0.6 volumes of isopropanol (24 ml) and incubated at room temperature for 10 min. The nucleic acids were recovered by centrifugation at 12,000 x g for 30 min at room temperature. The supernatant was discarded and the pellet washed with 70% ethanol. The ethanol was removed and the tube left open for 5-10 min at room temperature to allow final traces of ethanol to evaporate. The pellet was dissolved in TE and purified by equilibrium centrifugation in a caesium chloride (CsCl)-ethidium bromide gradient.

For each 1 ml of DNA solution 1 g of solid CsCl was added, and mixed gently until dissolved. A solution of ethidium bromide (10 mg/ml in water) was added to give a final concentration of 600 µg/ml. This solution was transferred to a tube suitable for a Beckman rotor; any space in the tube was topped up with mineral oil. The tubes were centrifuged at 100,000 rpm for about 4.5 hours (or 80,000 rpm overnight).

After centrifugation, two bands were visible; the top being nicked circular or linear DNA and the bottom being plasmid DNA. The bottom band was collected through a hypodermic needle inserted into the side of the tube. The ethidium bromide was removed by adding isopropanol saturated with water and CsCl and mixing the two phases well; this was separated by centrifugation (15,000 x g for 3 min) and the aqueous (lower) phase transferred to a fresh tube. This was repeated until all the pink colour (of the ethidium bromide) disappeared. The aqueous phase was then dialysed against several changes of water, to remove the CsCl.

2.1.2.2 Large Scale Preparation of Plasmid DNA using the Qiagen Maxiprep Kit.

A 250 ml culture of bacteria was grown up in luria broth (with appropriate antibiotics) overnight at 37°C with shaking. The cells were harvested by centrifugation at 2,000 xg for 15 min at 4°C. The supernatant was discarded and the pellet of cells completely resuspended in 10 ml of resuspension buffer P1 (50 mM Tris-HCl (pH 8), 10 mM EDTA, and lyophilised RNase A dissolved in the buffer to a concentration of 100 µg/ml). The cells were lysed by the addition of 10 ml of the lysis buffer P2 (200 mM NaOH, 1% SDS) and incubated at room temperature for 5 min. The cellular proteins were precipitated by the addition of 10 ml of the neutralization buffer P3 (3 M potassium acetate, pH 5.5), the contents of the tubes mixed and incubated on ice for 20 min. The cellular proteins were pelleted by centrifugation at 20,000 x g for 30 min at 4°C, then the supernatant was filtered through a prewetted folded filter.

A Qiagen-tip 500 was equilibrated by applying 10 ml of the equilibration buffer QBT (750 mM NaCl, 50 mM MOPS (pH 7), 15% ethanol) to the top and allowing the column to empty by gravity flow. The supernatant, containing the plasmid DNA, was then applied to the tip and allowed to enter by gravity flow. The resin was washed with 2 volumes of 30 ml of wash buffer QC (1 M NaCl, 50 mM MOPS (pH 7), 15% ethanol) by applying to the top of the column and allowing to empty by gravity flow. The DNA was eluted from the resin with 15 ml elution buffer QF (1.25 M NaCl, 50 mM Tris-Cl (pH 8.5), 15% ethanol), then precipitated by adding 0.7 volumes of isopropanol and pelleted immediately by centrifugation at 15,000 x g for 30 min at 4°C. The supernatant was removed, the pellet of DNA washed with 5 ml of 70% ethanol then allowed to air dry before redissolving in 1 ml of water.

2.1.3 Bacterial Transformation by Electroporation.

2.1.3.1 Preparation of Electrocompetent Cells.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

An overnight culture of bacteria was grown in LB with any appropriate antibiotic supplements. The following day, a 1:100 v/v dilution into LB (with appropriate antibiotics) was made and the cells grown to an OD₆₀₀ of 0.6 to 0.75. The cells were then pelleted by centrifugation, in sterile tubes, at 2,000 xg for 15 min at 4°C. The supernatant was discarded and the cells resuspended in a volume of ice cold water equivalent to the original culture volume. The centrifugation step was repeated and the cells resuspended in 5 ml of ice cold 10% glycerol. This suspension was pelleted by centrifugation at 2,000 xg for 20 min at 4°C, and the supernatant discarded. The cells

were resuspended in ice cold 10% glycerol; 500 µl for each 250 ml of original culture. This was aliquoted into sterile tubes and stored at -70°C.

2.1.3.2 Electroporation.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

The electroporator was turned on and allowed to warm up for a few minutes; then the capacitor was set at 25 mFD, resistance at 200 ohms and voltage at 2.5 kV. The bacterial cells were thawed on ice (about 10 min). 1-2 µl of DNA was aliquoted into chilled cuvettes then 40 µl of cells added; the cuvettes were gently tapped to mix the cells and DNA and ensure they fell to the bottom of the cuvette, then stored on ice for 5 min.

The cuvettes were then dried thoroughly and placed in the sample chamber, making contact with the electrodes, then the buttons pressed until the pulse had been delivered. Immediately after electroporation 1 ml of SOC medium was added, and the transformation mixture transferred to a sterile tube, before being incubated shaking at 37°C for 1 hr.

SOC medium -

0.5%	Yeast Extract (Oxoid)
2%	Peptone (Oxoid)
10 mM	Sodium chloride (NaCl)
2.5 mM	Potassium chloride (KCl)
10 mM	Magnesium sulphate (MgSO ₄)
10 mM	Magnesium chloride (MgCl ₂)
20 mM	Glucose

The transformation mixtures were then plated out with 200 µl of neat or diluted mixtures spread across LA plates, then incubated at 37°C overnight.

2.1.4 Digestion of DNA with Restriction Enzymes.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

For a 20 µl reaction, the DNA was placed in an eppendorf tube and 2 µl of the appropriate 10X enzyme buffer (supplied with the enzyme) added; the volume was adjusted to 20 µl by the addition of water, then the enzyme added. One unit of enzyme is the amount of enzyme required to digest 1 µg of DNA to completion in 1 hour; enzymes are usually supplied at a concentration of 10 units/µl. The reaction mixture was placed at the appropriate temperature and incubated for 2-3 hours (or overnight).

Reactions were typically carried out in a volume of 20 µl however if larger amounts of DNA were to be digested the volumes were scaled up accordingly.

2.1.5 Separation of DNA Bands through Agarose Gels.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

DNA fragments were separated by agarose gel electrophoresis using 0.7 or 1% agarose for smaller fragments and 0.4% agarose for large fragments, the agarose was made up in 1X TAE, with ethidium bromide added to a concentration of 50 ng/ml.

50X TAE -

242 g	Tris	
18.61 g	EDTA	in 1 litre water
pH adjusted to 7.7 with acetic acid		

Electrophoresis was carried out in a tank of 1X TAE. Loading buffer was added to each sample before loading onto the gel.

Loading buffer -

0.25% w/v	Bromophenol Blue	
0.25% w/v	Xylene Cyanol	
30%	Glycerol	in water

The higher percentage agarose gels were run at 80-120 V and the low percentage gels were run at 40-80 V for an appropriate time. Two DNA size markers were used; a 1 kb ladder (Gibco) with a range of 0.075-12.216 kb, and a High Molecular Weight ladder (Gibco) with a range of 8.271-48.502 kb. The DNA fragments were located using UV light.

2.1.6 Purification of DNA from Agarose Gels.

DNA samples were run out through agarose gels, the band to be purified was located under UV light and cut out (as close to the band as possible) using a clean scalpel. The DNA was then extracted using the Sephaglas BandPrep kit (Pharmacia Biotech) following the manufacturers instructions. This involved solubilizing the gel, adsorbing the DNA to the Sephaglas beads (a glass matrix), washing the beads and finally eluting the DNA from the beads using an elution buffer.

2.1.7 Polymerase Chain Reaction (PCR).

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

Regions of DNA were amplified using the polymerase chain reaction, which uses two oligonucleotide primers that hybridise to opposite strands and flank the target DNA region to be amplified. The elongation of the primers is catalysed by the enzyme *Taq* DNA polymerase, which is a heat stable DNA polymerase isolated from the

eubacterium *Thermus aquaticus* . Repetitive cycles of template denaturation, primer annealing and extension of the annealed primers results in the exponential accumulation of specific DNA fragments. The ends of these fragments are defined by the 5' ends of the primers.

Reactions were carried out in 0.5 ml eppendorf tubes, and cycled in a thermocycler. The reactions consisted of the template DNA (genomic or plasmid); the two primers that flank the target sequence (used at a concentration of 100 pmol per 100 µl reaction); a mix of the four nucleotides dATP, dCTP, dGTP and dTTP which are used in the extension of the primers (these were used at a concentration of 1.25 pmol); the DNA polymerase enzyme; a buffer (supplied with the enzyme); and MgCl₂ at various concentrations optimised for each reaction. Typically reaction volumes of 100 µl were used but this was reduced to 20 µl for PCR screening reactions. The reactions were overlaid with a drop of mineral oil to prevent evaporation of the reaction during cycling. The reactions were heated to 94°C to denature the DNA; then cooled to allow annealing of the primers (this temperature was dependant on the nature of the primer and varied between 45°C and 58°C); then heated to 72°C to allow primer extension (the length of time the reactions were held at this temperature was dependant on the size of the target sequence to be amplified, using the rough guide of approximately 1 min for every 1 kb of DNA to be amplified). A typical reaction was cycled at -

94°	for	5 min	1 cycle	
94°	for	1 min)	
58°	for	2 min)	35 cycles
72°	for	4 min)	
72°	for	10 min	1 cycle	

The products of amplification were analysed by electrophoresis through agarose gel.

2.1.8 Dephosphorylation of DNA.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

This technique removes the terminal 5' phosphate groups from DNA, it was used to prevent self-ligation of plasmids digested with only one restriction enzyme. The DNA was first digested to completion with the chosen restriction enzyme, extracted once with phenol/chloroform (1:1 v/v) and precipitated with 0.1 volume of 3 M NaAc and 2 volumes of ethanol.

The DNA was dissolved in a minimum volume of 10 mM Tris-HCl (pH 8.0) then 5 µl of 10X CIAP buffer (giving a final concentration in the reaction mixture of 50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA), H₂O to 48 µl and enzyme (calf

intestinal alkaline phosphatase)(Gibco) added; 0.01 units of calf intestinal alkaline phosphatase (CIAP) are needed to remove the terminal phosphates of 1 pmol of 5' ends of DNA. The reaction was incubated at 37°C for 30 min then a second aliquot of enzyme was added and the incubation continued for a further 30 min, to ensure complete dephosphorylation. The reaction was stopped by the addition of 40 µl of H₂O, 10 µl of 10X STE and 5 µl of 10% SDS and heating to 68°C for 15 min.

10X STE -

100 mM	Tris-HCl (pH 8.0)
1 M	NaCl
10 mM	EDTA

The reaction was extracted twice with phenol/chloroform (1:1 v/v) and twice with chloroform then precipitated with 0.1 volume of 3 M NaAc and 2 volumes of ethanol.

2.1.9 Ligation of Two Fragments of DNA.

Based on the method described in Sambrook et al (Sambrook *et al* , 1989)

The two fragments were purified (for example by BandPrep or ethanol precipitation), then a range of ligation mixtures set up (usually 10:1, 1:10 and 1:1). Each reaction consisted of the DNA fragments to be ligated, 5X ligation buffer (250 mM Tris-Cl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000), H₂O to adjust the volumes and an appropriate amount of DNA ligase enzyme (Gibco). The reactions were incubated at room temperature for 30-60 min then at 4°C overnight. The ligation mixtures were combined and precipitated with 0.1 volume of 3 M NaAc and 2 volumes of ethanol. The ligated DNA was resuspended in a small quantity of H₂O and introduced into electrocompetent *E. coli* (method 2.1.3.2) or packaged into cosmids (method 2.1.11).

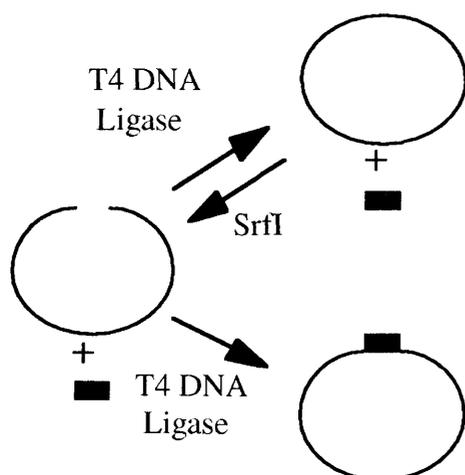
2.1.10 Cloning PCR Products into the pCR-Script™ Vector.

The pCR-Script™ SK(+) cloning kit (Stratagene) permits the efficient cloning of PCR fragments, and the ligation efficiency is increased by the simultaneous, opposite reactions of the *SrfI* restriction enzyme and T4 DNA ligase on non-recombinant, religated vector DNA (see figure 2.1).

Cloning into the vector was carried out according to the manufacturers instructions. Briefly, the PCR product was mixed with the pCR-Script vector, the

reaction buffer, *SrfI* restriction enzyme and T4 DNA ligase then incubated at room temperature to allow ligation of the insert into the vector. The plasmids produced from this step were then transformed into supercompetent *E.coli* XL-1 Blue, using a heat pulse.

Figure 2.1: The pCR-Script cloning method.



2.1.11 Packaging of Cosmids.

Cosmids are modified plasmids that have the DNA sequences (*cos* sequences) that are required for packaging into bacteriophage λ particles. To clone into cosmids, fragments of DNA of approximately 35-45 kb are ligated into linearised vector; if the DNA fragment is flanked by two different vector molecules the two *cos* sites (one from each vector) will cleave during packaging and the DNA between these two sites will be packaged into bacteriophage particles. After infection of *E. coli* by these phage, the cohesive ends of the *cos* sites cause the DNA to circularise, and it can then replicate as a plasmid.

Packaging was carried out using the ready prepared Gigapack II packaging extract system (Stratagene) according to the manufacturers instructions.

2.1.12 DNA Sequencing.

DNA was sequenced using the Sequenase™ Version 2.0 kit from USB(Amersham). This is based on the chain termination method where the synthesis reaction is terminated by the incorporation of dideoxynucleoside triphosphates (ddNTPs) which will not allow continued DNA elongation. Four separate reactions, each with a different ddNTP, ensures that polymerisation is terminated at each possible

base and therefore the sequence can be determined. A radioactively labelled nucleotide is included so the chains can be visualised by autoradiography after separation by electrophoresis.

2.1.12.1 Denaturation of Plasmid DNA for Sequencing.

Prior to sequencing, the plasmid DNA (15 μ l or approximately 2 μ g) was denatured by the addition of 4 μ l of 1M NaOH / 1mM EDTA and incubated at room temperature for 5 min. The reaction was neutralised by the addition of 2 μ l of 3M sodium acetate (pH 5.2) followed quickly by the addition of 60 μ l of cold (-20°C) ethanol to precipitate the DNA, and incubated at -70°C for 10 min. The DNA was pelleted by centrifugation at 13,000 x g for 15 min, washed once with 70% ethanol and dried (under vacuum or air dried) then resuspended in 7 μ l of water. The denatured DNA was then sequenced immediately.

2.1.12.2 Dideoxy Chain-Termination Sequencing.

For each set of sequencing reactions a single primer/template annealing reaction was performed. The reactions were set up in eppendorf tubes as follows:

- 1 μ l primer (50 ng/ μ l)
- 2 μ l sequencing buffer
- 7 μ l DNA (1-2 μ g)

The reactions were heated to 65°C for 2 min then allowed to cool slowly to room temperature. The labelling reactions were carried out by the addition of the following:

- 1 μ l DTT 0.1 M
- 2 μ l labelling mixture (diluted 1:10 to contain
0.75 μ M dGTP, dTTP and dCTP)
- 3.25 μ l Sequenase™ enzyme / label mix (containing 0.375 μ l of enzyme,
2.13 μ l of enzyme dilution buffer and 0.5 μ l of [α -³⁵S]dATP)

The labelling reactions were incubated at room temperature for 3 min, then 3.5 μ l of the reaction was dispensed into each of four wells of a microtitre plate containing 2.5 μ l of the ddNTP termination mixtures. The termination reactions were incubated at 37°C for 10 min, after which 7 μ l of stop solution was added to each tube.

2.1.12.3 Analysis of Sequencing Reactions by Electrophoresis.

The samples were run through a 1X TBE polyacrylamide gel.

For each gel, 60 ml of the TBE/acrylamide/urea mixture was required. Polymerization of the gel was initiated by the addition of 540 µl of 10% ammonium persulphate and 22.5 µl of TEMED. The gel was poured between two plates and allowed to set (for approximately 1 hour).

10X TBE-

109 g Tris
55 g Boric Acid
93 g EDTA Diluted in water to 1l

1X TBE Acrylamide Urea-

86 g Urea
20 ml 10X TBE
30 ml 40% Acrylamide Diluted in water to 200 ml

Immediately before loading the gel, the samples were heated to 80°C for 2 min; 5 µl of sample was loaded into each lane. The gels were run (1.75 kV, 60W, 45mA) for different lengths of time depending on how far away from the primer the region of interest was. After running, the gel was soaked (for approximately 15 min) in a solution of 10% acetic acid, 12% methanol to remove the urea. The gel was then washed with distilled water, laid on Whatmann 3MM paper and dried in a vacuum drier at 80°C. The dried gel was laid on X-ray film at least overnight then the film developed to identify the resulting sequence.

2.1.13 Southern Blotting.

Based on method by Southern (1975).

2.1.13.1 Capillary Blot.

Genomic DNA from tail tip biopsies was prepared as in method 2.3.7. A 10 µg sample of each DNA was digested with the restriction enzyme *EcoRI*; a negative control of genomic DNA isolated from MF1 mice and positive controls of the pC79/GM plasmid and genomic DNA isolated from a known transgenic mouse were also included. The digests were then precipitated by the addition of 0.1 volume of 3 M NaAc and 1 volume of ethanol and the resulting pellets resuspended in 20 µl water. The samples were then mixed with a loading buffer containing xylene cyanol and bromophenol blue and run out on a 0.6% agarose gel at 80V. After electrophoresis, the gel was incubated in 0.25 M HCl at room temperature for approximately 30 min or until the dyes had changed colour. The gel was then rinsed twice with distilled water and the DNA

denatured by incubation in denaturation buffer for 30 min at room temperature with shaking.

Denaturation buffer -

1.5 M	NaCl	
0.5 M	NaOH	diluted in water

The gel was then rinsed twice with distilled water then incubated in neutralization buffer for 15 min at room temperature with shaking. This step was then repeated.

Neutralization buffer -

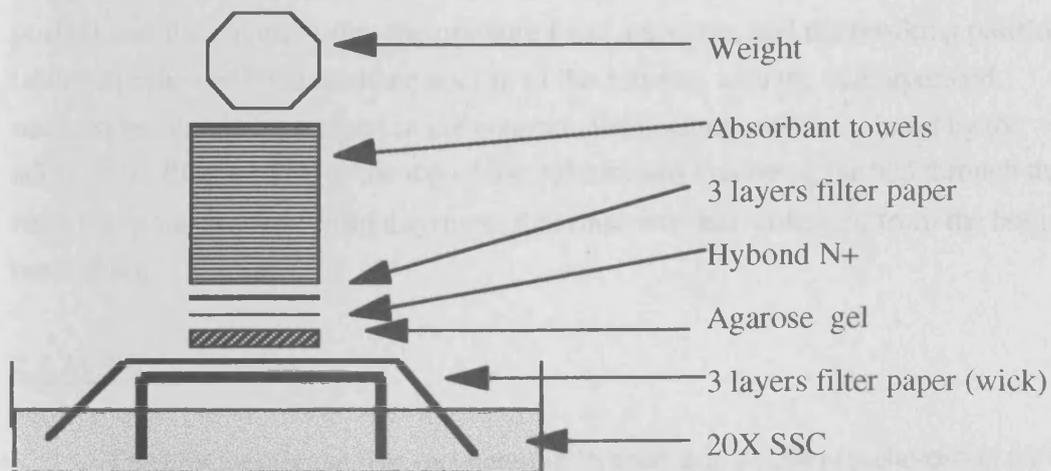
1.5 M	NaCl	
0.5 M	Tris-HCl (pH 7.2)	
0.001 M	EDTA	diluted in water

A capillary blot was then set up to transfer the DNA to the nylon membrane Hybond-N+ (Amersham), see fig 2.2. Transfer of DNA to the membrane was allowed to proceed overnight.

20X SSC -

3 M	NaCl	
0.3 M	Sodium citrate	diluted in water

Fig 2.2: Capillary blot.



After transfer, the blot was dismantled and, before the gel was removed, the position of the wells marked on the membrane with pencil. The membrane was washed with 2X SSC and allowed to air dry before fixing the DNA by UV crosslinking.

2.1.13.2 Preparation of the Probe.

The probe for Southern blotting was radioactively labelled using the Prime-It™ random primer labelling kit (Stratagene). Random hexanucleotide primers were annealed to the DNA template (25 ng) by heating the reactions in a boiling water bath for 5 min then allowing the reactions to cool to room temperature. The DNA probes were then produced by extension of the random primers with the Exo(-) Klenow enzyme; the radioactive label was introduced to the probes by the addition of [α -³²P] dCTP to a nucleotide mix containing dATP, dGTP and dTTP. These reactions were incubated at 37°C for 10 min, then the primer extension reactions stopped by the addition of 2 μ l of stop mix.

The unincorporated nucleotides were removed from the probe using NucTrap™ probe purification columns (Stratagene), these columns contain a resin that binds oligonucleotides from 17 bp in size. The column was prewetted with 70 μ l of STE buffer which was loaded onto the top of the column then pushed through with a syringe.

STE buffer -

100 mM	NaCl	
20 mM	Tris-HCl (pH 7.5)	
10 mM	EDTA	diluted in water

The probe reaction mixture was then loaded onto the top of the column and slowly pushed into the column using the pressure from a syringe, and the resulting purified labelled probe collected from the bottom of the column, with the unincorporated nucleotides remaining trapped in the column. The column was then rinsed by the addition of 70 μ l of STE to the top of the column and this being pushed through the resin using the pressure from a syringe; this rinse was also collected from the bottom of the column.

2.1.13.3 Hybridization.

The blot membrane was incubated in hybridization buffer (preheated to 65°C) for 1 hour at 65°C, in a hybridization oven (Hybaid).

Hybridization buffer -

5 X	SSC	
0.1%	SDS	
5%	Dextran Sulphate	
5%	Liquid Block (Amersham)	Diluted in water

Sheared non-homologous DNA (calf thymus DNA) was also added to the hybridization buffer.

The probe was denatured by heating in a boiling water bath for 5 min before adding this to the hybridization buffer and the blot and incubating at 65°C overnight. Following hybridization, the buffer with the probe was removed from the blot and the blot rinsed several times with wash buffer (prewarmed to 65°C).

Wash buffer -

0.2 X SSC

0.1 % SDS diluted in water

After washing, the blot membrane was wrapped in cling film and exposed to X-ray film, initially overnight, before development of the film to identify bands.

2.1.14 Primers.

Primer	Sequence	Use
FU5PR	AAT AAT TTT <u>GCG GCC</u> GCT CTT CCC <u>GAA GCT</u> CTG ACA CC	Production of the fusion between CRP and mGM-CSF. Underlined region indicates the position of the introduced <i>NotI</i> site
TM7(2)	GAC AGT GAT CGG GCT ACG CGT <u>TGT CTG GCC</u> <u>AAA AGC</u>	Production of the fusion between CRP and mGM-CSF. Underlined region indicates the region of the primer that is complementary to the 3' end of the CRP signal peptide and therefore acts as the priming region; the rest of the oligonucleotide is complementary to the 5' end of the GM-CSF coding sequence
FU3PR	GGA TTA CGA <u>GCG GCC</u> GCA TCC TAT <u>TAT TTT</u> TGG ACT GG	Production of the fusion between CRP and mGM-CSF. Underlined region indicates the position of the introduced <i>NotI</i> site
TM8	GCT TTT GGC CAG ACA ACG CGT AGC CCG ATC <u>ACT GTC</u>	Production of the fusion between CRP and mGM-CSF. Underlined region indicates the region of the primer that is complementary to the 5' end of the GM-CSF coding sequence and therefore acts as the priming region; the rest of the oligonucleotide is complementary to the 3' end of the CRP signal peptide. Also used for screening the C79/GM transgenic mice
CRSEQ	AGC TAC CTC CTC CTG CCT GG	In the 5' region of CRP facing back to sequence across the <i>NotI</i> site
AW1	GAG ACT ACT TCT ACC TCT TC	In the 5' region of the GM-CSF gene facing back to sequence across the region of the fusion between CRP and GM-CSF
GMSEQ	AAC TCC GGA AAC GGA CTG CG	In the 3' region of the GM-CSF gene facing out to sequence across the <i>NotI</i> site
CPA1	GTT AGT CAC AAC TTC TAA AGC	In the 3' region of the CRP promoter facing back into the gene, across the polyadenylation sequence, used for screening the C79/GM transgenic mice
CR1	GTG TGA TCT GAG AAA CCT CTC ACA TTT G	For PCR screening of the CRP transgenic mice
CR2	AGA CGT GGG GCC CAT GCG GGT GTA AAA AAC C	For PCR screening of the CRP transgenic mice
CRPR1	CTG AGG CCA <u>GCG GCC</u> GCT CCT GAA <u>GGT ACC</u> TCC CGG TT	Facing out from 3' end of CRP gene, used to produce acute phase vectors. Underlined region indicates the position of the introduced <i>NotI</i> site
CRPL1	TCG GGA AGA <u>GCG GCC</u> <u>GCA AAA TTA TTA TTT</u> CAG ACC AGA GA	Facing out from 5' end of CRP gene, used to produce acute phase vectors. Underlined region indicates the position of the introduced <i>NotI</i> site

2.1.15 Bacterial Strains.

Strain	Genotype	Reference
<i>E. coli</i> JM101	<i>supE thiΔ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Messing (1979)
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)</i>	Yanisch-Peron <i>et al.</i> (1985)
<i>E. coli</i> JM110	<i>dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Yanisch-Peron <i>et al.</i> (1985)
<i>E. coli</i> XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'[proAB⁺ lacI^q lacZΔM15 Tn10(ter^r)]</i>	Bullock <i>et al.</i> (1987)
<i>S. pneumoniae</i> D39	Serotype 2	NCTC 7466

2.1.16 Plasmids.

Plasmid	Description / Use
pCR-Script	For cloning of PCR products. A 2961 bp phagemid incorporating a <i>SrfI</i> site into the pBluescript II SK(+) phagemid; containing and ampicillin resistance gene and the <i>lacZ</i> gene for blue-white selection of clones containing an insert.
pUC18-GM-CSF	
pFusion	290 bp of the CRP promoter fused to the mGM-CSF coding region, with engineered <i>NotI</i> sites flanking the fusion, cloned into the pCR-Script vector.
pCosCRP1	Isolated from a human genomic library in vector pCos2EMBL using a full-length cDNA for human CRP as the probe, insert length of about 35.2 kb (Ciliberto <i>et al.</i> , 1987b).
pWE15	8.2 kb cosmid cloning vector. Clontech catalogue no. 6340-1, GenBank Accession no. M99569. Contains neomycin resistance (for selection in eukaryotic cells) and ampicillin resistance (for selection in bacteria).
pBNB	Derived from a 5.2 kb BamHI fragment of pCos-CRP1 by PCR using primers engineered to contain <i>NotI</i> sites facing out from the CRP gene, then ligation of these <i>NotI</i> sites to produce a plasmid containing CRP flanking regions but no structural gene.
pBNB/GM	<i>NotI</i> fragment of pFusion ligated into <i>NotI</i> site of pBNB.
pC79	Construct 79 derived from pCos-CRP1 and incorporating the regions -1.7 kb to +3.14 kb and +7.5 kb to +10.7 kb relative to the CRP gene (Murphy <i>et al.</i> , 1995).
pC79-Not	Derived from pC79 by PCR using primers engineered to contain <i>NotI</i> sites facing out from the CRP gene, then ligation of these <i>NotI</i> sites to produce a plasmid containing CRP flanking regions but no structural gene.
pC79/GM	<i>NotI</i> fragment of pFusion ligated into <i>NotI</i> site of pC79-Not.
pCAT-Control	A CAT expression vector containing SV40 promoter and enhancer sequences, resulting in strong expression of CAT in many eukaryotic cells.

2.2 Tissue Culture Methods.

2.2.1 Standard Passage of Hep3B Cell Line.

Hep3B (ECACC 86062703) are a human hepatocyte carcinoma cell line derived from an eight-year-old negro male.

Once the cells were confluent (approximately every 3 days), the growth medium was removed and the cell monolayer washed twice with sterile PBS.

Phosphate buffered saline (PBS) -

8 g	NaCl
0.2 g	KCl
1.44 g	Na ₂ HPO ₄
0.24 g	KH ₂ PO ₄

(Dissolved in water, pH adjusted to 7.4 with HCl then made up to 1l with water)

Enough trypsin/EDTA solution (0.5 g trypsin, 0.2 g EDTA per litre of modified Puck's saline A)(Gibco) to cover the cells was added and any excess removed. The flasks were then incubated at 37°C 5% CO₂ until the cells detached from the surface of the flasks (usually 5-10 min). The cells were then resuspended in a small amount of fresh growth medium (by gentle shaking) and divided between a number of new flasks at a seeding density 1-3 x 10⁵ cells per cm² of flask. More medium was added to these flasks so the medium covered the bottom of the flask. The caps were loosely closed and the flasks incubated at 37°C with 5% CO₂.

Growth medium -

EMEM (Eagles minimal essential medium with Earles balanced salt solution)(Gibco)

10% Foetal bovine serum (Gibco)

1% Non-essential amino acids (Gibco)

(Optional) 1% Penicillin/Streptomycin (5000 U/ml penicillin, 5000 µg/ml streptomycin)(Gibco)

2.2.2 Cryopreservation of Mammalian Cells.

A number of flasks of cells were grown to confluence; the cells were then trypsinised (as described before) and resuspended in growth medium by gently shaking the flasks. The cells were pelleted by centrifugation (200 xg for 10 min), the supernatant discarded, and the cells resuspended in freeze medium to give a final concentration of approximately 4 x 10⁶ cells/ml.

Freeze medium -

- Normal medium with 20% serum and 8-10% cryoprotectant (DMSO or glycerol).
- No antibiotics included.

The cells were aliquoted into 1ml quantities and frozen to -70°C at a rate of $1^{\circ}\text{C}/\text{min}$ by placing them in a box surrounded with isopropanol in a -70°C freezer. The ampoules were then transferred to a liquid nitrogen store.

2.2.3 Recovery of Cells from Liquid Nitrogen Storage.

A vial of cells was taken out of liquid nitrogen storage and defrosted quickly at 37°C . The cells were resuspended in approximately 10 ml growth medium, then pelleted by centrifugation at $200 \times g$ for 10 min. The supernatant was discarded and the pellet resuspended in growth medium, transferred to a flask and incubated at 37°C with 5% CO_2 .

2.2.4 Transfection of DNA into Mammalian Cells.

Introduction of DNA into mammalian cells was facilitated by the calcium phosphate transfection system, using the ProFection™ kit (Promega). This involves mixing DNA with calcium chloride (CaCl_2) and a phosphate buffer to form a fine precipitate which is presented to the cultured cells and may be taken up by them.

The cells were cultured in 60 mm dishes until about 80-90% confluence was achieved. Three hours before the transfection, the culture medium was removed and replaced with fresh medium. The DNA containing transfection mix was prepared by mixing, for each dish, 10 μg of DNA with 37 μl of 2M CaCl_2 and adjusting the volume to 300 μl with water. This was then added dropwise to 300 μl of 2X HEPES-buffered saline (50 mM HEPES (pH 7.1), 280 mM NaCl, 1.5 mM Na_2HPO_4), vortexing continuously during addition of the DNA mix. This was incubated at room temperature for 30 min to allow the precipitate to develop. The solution was then added dropwise to the cells, swirling the plates to distribute the DNA precipitate. The cells were then incubated at 37°C with 5% CO_2 for the required length of time before interleukin stimulation or harvesting.

2.2.5 Interleukin Stimulation of Transfected Cells.

Experiments by Dr Bernard Burke showed that the optimal concentrations of interleukins to use for stimulation of an acute phase type response in Hep3B cells was

500 U/ml Interleukin-6 (R&D Systems) and 200 U/ml Interleukin-1 (R&D Systems) with 1 mM Dexamethasone (see results section 4.5.2).

After 12-16 hours post transfection, the culture medium was removed and washed twice with PBS. The stimulation medium was then prepared to give a final concentration of 500 U/ml Interleukin-6, 200 U/ml Interleukin-1 and 1 mM Dexamethasone, and 3 ml of this added to the cells. For 20 ml this required -

- 4 µl IL-1 (at stock concentration of 1000 U/ml)
- 5 µl IL-6 (at stock concentration of 2000 U/ml)
- 20 µl Dexamethasone (at stock concentration of 1 mM)
- 20 ml Dulbecco's Minimal Essential Medium + 1% Pen/Strep

2.2.6 Harvesting of Transfected Cells.

The cells were harvested 24 hours after interleukin stimulation. The medium was removed from the cells and stored at -70°C for future analysis to look at secretion of products from the cells. The cell layers were then washed twice with PBS making sure all the PBS was removed from the final wash. The cells were then lysed by the addition of 250 µl of diluted lysis buffer (Promega) and incubated at room temperature for 10 min. The cells were scraped off the surface of the dish with a toothpick and the lysate transferred to a microfuge tube. The cell debris was removed by centrifugation at 15,000 x g for 15 sec, and the supernatant (cell lysate) transferred to a fresh tube and stored at -70°C for future analysis.

2.3 Animal Work Techniques.

2.3.1 Viable Counting of *Streptococcus pneumoniae*.

The samples were serially diluted in sterile PBS down to a dilution of 10⁻⁶. For viable counting inocula or larger samples 20 µl of sample was mixed with 180 µl of sterile PBS, then 20 µl of this mixed with 180 µl of PBS and so on to create the serial dilutions. With small samples, such as a mouse tail bleed, 10 µl of the sample was mixed with 90 µl of PBS then 20 µl of this serially diluted as before. Three samples of 20 µl of each dilution were spotted onto sections of plates of Blood Agar Base (BAB) (Oxoid) + 5% Horse Blood (Oxoid); this was done in duplicate. The spots were allowed to dry into the agar then the plates incubated inverted in a CO₂ candle jar at 37°C overnight.

Colonies were counted in the sections that had between 20-100 colonies on each spot for the lowest dilution. To work out the number of colony forming units in 1 ml of the original sample the following equation was used:

$$\text{cfu/ml} = \frac{\text{Total number of colonies in the section}}{3} \times \frac{1000}{20} \times \text{Dilution Factor}$$

2.3.2 Maintenance of Mice.

Mice were housed in standard cages under specific pathogen free conditions, fed and watered ad libitum according to standard procedures at Glasgow University Central Research Facility.

Transgenic mice were bred in house by setting up crosses between transgenic and non-transgenic animals (following screening of these parent animals). The young were weaned from the mothers at approximately 3 weeks of age with the males and females separated at this time.

2.3.3 Tail Bleeding Mice.

The mice to be bled were placed in a heated chamber or under a heat lamp for approximately 10 min before bleeding in order to bring the veins closer to the surface of the tail. The animal was then placed in a ventilated tube held horizontally in a retort stand and the tail fed through a hole in the lid of the tube. A needle (with the bevel uppermost) was directed through the skin, following the course of the vein, and a sample of blood gently drawn into the attached syringe. To prevent clotting of the blood, 10 µl of heparin was first drawn into the needle and the drawn blood was therefore mixed with this.

2.3.4 Cardiac Bleeding Mice.

This was performed under terminal anaesthesia with Halothane. The animal was anaesthetised, using a face mask, with 2.5% v/v fluothane (Halothane)(Zeneca), 1L O₂/min and anaesthesia was confirmed by observing no pinch reflex reaction. The animal was then placed on its back and a needle inserted into the heart, by directing the needle under the rib cage to the right of the sternum, and a sample of blood was then drawn into the attached syringe. After exsanguination, the mice were killed by cervical dislocation.

2.3.5 Collection of Plasma from Blood Samples.

The blood cells were pelleted from the heparinised blood by centrifugation at 6,000 x g for 3 min and the resulting plasma was transferred to another tube.

2.3.6 Animal Passage of Pneumococci and Preparation of Standard Inoculum.

Based on methods described by Canvin *et al.* (1995)

Frozen cultures of *Streptococcus pneumoniae* were streaked to single colonies on BAB + 5% horse blood and incubated in a candle jar at 37°C overnight. Approximately 4-5 colonies was taken from this plate and used to inoculate 10 ml of Brain Heart Infusion (BHI) broth (Oxoid), then the culture incubated statically at 37°C overnight. The overnight culture was centrifuged at 2,000 x g for 15 min to pellet the bacteria. The supernatant was then removed and the bacterial pellet resuspended in 10 ml sterile PBS.

The bacterial suspension was used to inoculate two MF1 mice (Harlan) intraperitoneally, with 200 µl of the suspension given to each mouse. Once the animals started to look sick (method 2.3.8) they were exsanguinated by cardiac puncture; this was usually 24 hours post infection.

The blood (50 µl) was used to inoculate 10 ml of BHI broth and the culture incubated statically overnight at 37°C. The bacteria in this overnight culture were pelleted by centrifugation at 15,000 x g for 15 min. The supernatant was discarded and the pellet resuspended in 1 ml of fresh serum broth (BHI broth + 20% heat inactivated foetal calf serum (Gibco)). This was used to inoculate a 10 ml culture of serum broth to give an OD₅₀₀ of about 0.7, which was then incubated statically at 37°C for about 5 hours or until the OD₅₀₀ of the culture reached 1.6. The standard inoculum was then aliquoted out and frozen at -70°C.

Before use in an experiment, the frozen standard inoculum was viable counted (see method 2.3.1) and checked for optochin sensitivity by streaking out onto a plate of BAB + 5% horse blood and placing an optochin antibiotic disc (Oxoid) on the plate at the site of the first streak. Using the viable count data the standard inoculum was virulence checked by intranasal inoculation of a group of MF1 mice (Harlan) with 1x10⁶ cfu in 50 µl of PBS (see method 2.3.7) and monitoring the animals until they became moribund.

2.3.7 Intranasal Challenge with *Streptococcus pneumoniae* .

Based on methods described by Canvin *et al.* (1995)

Standard inoculum aliquots were thawed out at room temperature. A 0.5 ml sample of this was taken and pelleted at 15,000 x g for 3 min in a microfuge then resuspended in 0.5 ml sterile PBS. From viable count information on that batch of standard inoculum the suspension was diluted (in PBS) to give the required colony forming units in 50 µl.

The animals to be challenged were anaesthetised with 2.5% v/v fluothane, 1L O₂/min and anaesthesia confirmed by observing no pinch reflex reaction. The animals were scruffed, with the nose held upright, and the inoculum introduced intranasally by adding a series of small droplets of the 50 µl inoculum into the nostrils with a pipette; ensuring each drop was inhaled before addition of the next. After inoculation the animals were laid on their backs until recovery. The viable count of the inoculum was then determined (as previously described).

The animals were monitored for symptoms of disease until they became moribund (symptoms were recorded on monitoring sheets) method 2.3.8, when they were killed by dislocation of the neck.

2.3.8 Monitoring sickness of Mice.

Mice were monitored for symptoms of disease and assigned a score according to the following checklist.

1. Normal
2. Hunched
3. Starey Coat
4. Lethargic
5. Moribund

2.3.9 Preparation of DNA for use in the Generation of Transgenic Mice.

The DNA to be used was purified using the Band Prep Kit (Promega). Any particulate matter in the DNA was removed by using a NACS column (BRL), in order to prevent the DNA injection needles becoming blocked. The solutions needed were

Buffer A 0.2 M NaCl in TE
 Buffer B 1.0 M NaCl in TE
 Buffer C 2.0 M NaCl in TE

For 10 ml	1 M Tris	100 ml
	0.5 M EDTA	20 ml
	5 M NaCl	0.4 ml (A)
		2.0 ml (B)
		4.0 ml (C)
	Water	9.5 ml (A)
		7.9 ml (B)
		5.9 ml (C)

The NACS column was hydrated by application of 2 ml of buffer C, then washed with 2 ml buffer A. The DNA was diluted into 1 ml of buffer A then loaded onto the column and allowed to slowly percolate through the matrix. The solution was collected as it came through and reapplied to the column. The column was then washed with 4 ml of buffer A. The DNA was then eluted from the column by addition of 4 x 100 µl aliquots of buffer B and each aliquot collected. The DNA was precipitated by the addition of 300 µl of ethanol and incubation at -20°C overnight. The DNA was pelleted by centrifugation at 15,000 x g for 10 min, washed once with 80% ethanol and redissolved with 20 µl per tube of filter sterilised microinjection TE. The amount of DNA in each tube was determined by measuring OD 260 nm and the samples were diluted to give approximately 300 ng of DNA in 20 µl of buffer.

Microinjection TE -

10 mM Tris-Cl pH 7.4
 0.1 mM EDTA

2.3.10 Production of Transgenic Mice by Microinjection.

Large numbers of embryos are needed for microinjection therefore females were superovulated to induce the production of large numbers of eggs. Superovulation was induced by pregnant mare's serum (PMS) which mimics follicle-stimulating hormone and stimulates the egg containing follicles to mature, and human chorionic gonadotropin (hCG) which mimics luteinizing hormone induces the rupture of the mature follicles to release the eggs. After hCG administration the female mouse was placed in a cage with a stud male then checked in the morning for a copulation plug. Mice that had copulation plugs were killed, the oviducts were removed, and the eggs flushed out of the oviducts.

Microinjection was performed using an inverted microscope, with a fixed stage, with one micromanipulator mounted at each side of the microscope stage; one to control the holding pipette and one to control the injection needle.

To perform the microinjection the eggs were transferred to the injection chamber (a glass depression slide) and picked up with the holding pipette. The injection pipette was filled with DNA and then also moved into the injection chamber. The eggs were manoeuvred using the injection pipette so that the pronuclei were clearly visible, then the injection pipette was pushed through the cell membrane into the pronucleus. A small amount of DNA (1-2 picolitres) was introduced to the pronucleus and successful injections identified by a visible swelling of the pronucleus. The injection pipette was then quickly pulled out of the egg to prevent attachment of nuclear components to the pipette.

After microinjection the healthy eggs were surgically transferred to the oviducts of pseudopregnant recipient females. That is, females in oestrus that had been mated with sterile (vasectomized) males resulting in the female reproductive tract being receptive for the transferred embryos.

2.3.11 Genomic DNA Extraction for Screening Transgenic Mice.

Following the method described by Couse et al (Couse *et al.*, 1994).

Tail tip biopsies were taken from animals anaesthetised with Halothane and the cut tail cauterised. Tail tip samples were either used immediately or frozen at -70°C until use.

A sample of the tail tip biopsy (4-6 mm) was transferred to a SST Vacutainer tube (Becton Dickinson) with 1 ml lysis buffer. These were incubated at 55°C overnight until all the tissue was visibly digested.

Lysis buffer -

50 mM	Tris-HCl pH 8.0
100 mM	EDTA pH 8.0
0.125%	SDS
0.8 mg	Proteinase K

The digests were deproteinised by phenol extraction. The SST tubes contain an inert gel plug which migrates between the organic and aqueous phases upon centrifugation resulting in the organic phase containing the cellular protein being trapped below the gel plug. This was done by adding 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) to the digests, mixing by inversion, then centrifugation of the samples at 2000 x g for 10 min. This step was repeated in the same tube. A final chloroform extraction was

performed by adding 1 ml of chloroform to the tube, mixing by inversion, and centrifugation of the samples at 2000 x g for 10 min.

The aqueous phase was transferred to two 1.5 ml microfuge tubes (0.5 ml into each tube) each containing 50 µl of 3M sodium acetate (pH 6.0). Using sodium acetate at pH 6.0 reduces the possibility of co-precipitation of SDS and EDTA with the DNA therefore making it more suitable for screening by PCR or Southern blot hybridisation. The DNA was then precipitated by adding 1 ml of 100% Ethanol (at room temperature) and immediate centrifugation at 14,000 x g for 7 min. The supernatant was removed and the DNA pellets washed with 70% ethanol by adding the wash then centrifugation at 14,000 x g for 3 min, the wash was then removed and the samples spun briefly (14,000 x g for 1 min) to bring all the remaining wash to the bottom of the tube where it was removed using a pipette. The pellets were allowed to air dry, inverted over tissue, for approximately 30 min. The DNA pellets were then resuspended in 300 µl of water (total 600 µl for each sample).

2.3.12 PCR Screening of Transgenic Mice from a Blood Sample.

Two methods of screening by PCR using a blood sample were used.

2.3.12.1 RapidPrep™ Genomic DNA Isolation Kit.

This method involved the use of ion-exchange chromatography to remove the DNA from the blood. The DNA was prepared using the RapidPrep™ kit (Pharmacia Biotech) and following the manufacturers instructions. Briefly, this involved lysis of the blood cells (both red and white) and removal of the white blood cell nuclei by centrifugation. The cell nuclei were then lysed by an extraction buffer containing guanidinium isothiocyanate (which disrupts cellular material and inhibits nucleases) and the resulting solution was passed through an anion exchange column which bound the DNA. The column was washed to remove contaminants, and then the DNA was eluted from the column using a high salt buffer.

The resulting DNA was then used in PCR screening reactions.

2.3.12.2 GeneReleaser™ Reagent.

This method involved the use of GeneReleaser™ (Bioventures, Inc) which sequesters the cell lysis products that could inhibit polymerases and releases DNA from

whole blood. This therefore avoids excessive manipulations to purify DNA from a blood sample.

A sample of 1 μ l of whole blood was placed in the bottom of a 0.5 ml thermocycling tube and mixed with 19 μ l of resuspended GeneReleaser™ resin. The samples were overlaid with mineral oil and cycled as below to release the DNA from the blood cells.

65°C	30 sec
8°C	30 sec
65°C	90 sec
97°C	180 sec
8°C	60 sec
65°C	180 sec
97°C	60 sec
65°C	60 sec
80°C	Hold

A master mix of amplification reagents containing the two primers, a mix of dNTPs, the *Taq* enzyme (Gibco), 10X enzyme buffer (200 mM Tris-Cl (pH 8.4), 500 mM KCl), an appropriate concentration of magnesium, and water (to adjust the volume) was prepared. Once the first stage of thermocycling was held at 80°C, 19 μ l of the master mix was added to each tube and the reactions cycled at previously described (method 2.1.7).

To screen the CRP transgenic mouse line the primers used were CR1 and CR2. To screen the GM-CSF transgenic line the primers used were CPA1 and TM8 (section 2.1.13).

2.3.13 Screening of Transgenic Mice by PCR from Genomic DNA.

The genomic DNA prepared from tail tip biopsies was diluted 1/10 and 2 μ l of this used in each reaction. A master mix of amplification reagents containing the two primers, a mix of dNTPs, the *Taq* enzyme (Gibco), 10X enzyme buffer (200 mM Tris-Cl (pH 8.4), 500 mM KCl), an appropriate concentration of magnesium, and water (to adjust the volume) was prepared. 18 μ l of the master mix was added to each tube, the reactions overlaid with mineral oil and the reactions cycled as previously described (method 2.1.7).

2.3.14 Mouse Strains.

Mouse Strain	Description	Reference
CRP	Transgenic mouse carrying the human CRP gene. Background strain C57/BL6 x SJL F1 cross.	Ciliberto <i>et al</i> (1987a)
MF1	Outbred strain, developed at Olac, UK from a cross between mice of a Swiss background. Albino coat.	Harlan
CBA/ca	Inbred strain derived from a nucleus colony which originated at Jackson Laboratories, Bar Harbour, Maine. Agouti coat.	Harlan
C57/Black6	Inbred strain derived from a nucleus colony at the National Institutes of Health, Bethesda, Maryland. Intense black coat.	Harlan
C57Black6/ CBA F1 Cross	Cross between C57/Black6 and CBA/ca, used in the production of transgenic mice.	Harlan
C79/GM-B6	Transgenic mouse carrying C79/GM construct, crossed with C57/Black6/CBA F1 strain	Results chapter 5
C79/GM-MF1	Transgenic mouse carrying C79/GM construct, crossed with MF1 strain	Results chapter 5

2.4 Immunological Techniques.

2.4.1 Testing for Presence of CRP by ELISA.

Based on a method obtained from David Samols at Case Western Reserve University School of Medicine, Cleveland, OH (personal communication).

High binding immunoassay plates were coated with primary antibody (Rabbit anti human CRP from Sigma *C-3527) at 1 mg total protein per well in 100 µl coating buffer (5X TBS). 100 µl of antibody was aliquoted into each well, the plate was covered with cling film and the plate incubated at 4°C overnight.

5X TBS -

250 mM Tris
1 M NaCl

Dissolved in water, pH adjusted to 7.4 then made up to 1 L

The wells were emptied by inversion then tapped on absorbent paper to remove all liquid. The wells were then washed three times with TBS/Tween by filling the wells with wash buffer then emptying by inversion and removing all liquid by tapping on absorbent paper.

Wash Buffer (TBS/Tween) -

200 ml Stock (5X) TBS

0.5 ml Tween 20

Made up to 1 L with water

Non-specific binding of other antibodies or serum proteins was blocked with 1% BSA in TBS (1X), 200 µl of this blocking buffer was aliquoted into each well, the plate was covered with cling film and incubated at 4°C overnight.

The wells were emptied and washed as before. The samples and standard curve were prepared using blocking buffer (1% BSA in TBS) as the diluent. The standard curve was prepared using purified human CRP (Sigma *C-4063) diluted to a top standard value of 40 ng/ml then serially diluted 1.5 fold down from this to obtain the standard curve. Mouse serum samples were diluted initially 1/100 and 1/1000 but serum from transgenic animals often required further dilution. 100 µl of standard or sample was aliquoted into wells, the plate covered with cling film and incubated at 37°C for 2-3 hours.

The wells were emptied and washed as before. The detection antibody (sheep anti human CRP, The Binding Site *AU044) was prepared for use at a dilution of 1/1000 in blocking buffer; 100 µl of this was aliquoted into each well, the plate was covered with cling film and incubated at 37°C for 2-3 hours.

The wells were emptied and washed as before. The conjugated antibody (donkey anti sheep IgG/horseradish peroxidase conjugate, The Binding Site *AP360) was prepared for use at a dilution of 1/1000 in blocking buffer; 100 µl of this was aliquoted into each well, the plate was covered with cling film and incubated at 37°C for 2-3 hours.

The assay was developed using TMB as a substrate.

Substrate - (for one plate)

10 ml Substrate buffer (1.36 g sodium acetate in 100 ml water

- pH adjusted to 5.5 with citric acid)

100 µl Tetramethylbenzidine (6 mg TMB in 1 ml DMSO)

3.2 µl 30% Hydrogen peroxide (added just before use)

The wells were emptied and washed as before, then 100 µl of substrate added to each well and the plate incubated in the dark to allow the colour to develop (approximately 15 min). After the colour had developed sufficiently the colour change was stopped by the addition of 50 µl of 10% sulphuric acid.

The plate was then read at OD 450 nm (reference filter 570 nm) using a Dynatech ELISA reader. A standard curve was plotted for each plate, and an equation for the best fit line determined (using the Cricket Graph graph drawing program), the concentration of CRP in the samples was then determined using this equation.

2.4.2 Testing for Presence of mGM-CSF by ELISA.

2.4.2.1 Pharmingen ELISA Pairs.

Based on the method supplied with the antibody pairs.

High binding immunoassay plates were coated with coating antibody (rat anti-mouse GM-CSF, Pharmingen *18091D) at 4 mg/ml in coating buffer (0.1M NaHCO₃ pH 8.2). 50 µl of diluted antibody was aliquoted into each well, the plate was covered in cling film and incubated at room temperature overnight.

The wells were emptied by inversion then tapped on absorbent paper to remove all liquid. The wells were then washed four times with PBS/Tween by filling the wells with wash buffer then emptying by inversion and removing all liquid by tapping on absorbent paper.

20 X PBS -

80 g	NaCl
3.325 g	Potassium dihydrogen orthophosphate (KH ₂ PO ₄)
12.1 g	Dipotassium hydrogen orthophosphate (K ₂ HPO ₄)

Dissolved in water, pH adjusted to 7.0 then made up to 500 ml with water

PBS + 0.05% Tween (Wash buffer) -

50 ml	20 X PBS
0.5 ml	Tween 20
950 ml	Water

Non-specific binding of other antibodies or serum proteins was blocked with 1% BSA in PBS (1X), 200 µl of this blocking buffer was aliquoted into each well, the plate was covered with cling film and incubated at room temperature for approximately 3 hours.

The wells were emptied and washed as before. The samples and standards were prepared using blocking buffer as the diluent. The standard curve was prepared using purified recombinant murine GM-CSF (Pharmingen *19291T) diluted to a top standard of 1000 pg/ml then serially diluted 2 fold down from this to obtain a standard curve.

Mouse serum samples were initially diluted 1/10 or 1/100, although further dilution of the serum from transgenic animals was often required. 100 µl of standard or sample was aliquoted into the wells, the plate covered in cling film and incubated at room temperature overnight.

The wells were emptied and washed as before. The detection antibody (biotinylated rat anti-mouse GM-CSF, Pharmingen *18102D) was prepared for use at

2 mg/ml in blocking buffer; 100 µl of this was aliquoted into each well, the plate covered with cling film and incubated at 37°C for 2-3 hours.

The wells were emptied and washed as before. The streptavidin-HRP conjugate (Amersham) was prepared at a dilution of 1/400 in blocking buffer; 100 µl of this was aliquoted into each well, the plate covered with cling film and incubated at 37°C for 2-3 hours.

The wells were emptied and washed as before. The assay was developed using TMB as a substrate as described (method 2.4.1). Once the colour change had developed sufficiently (approximately 30 min) the colour change was stopped by the addition of 50 µl of 10% sulphuric acid. The plate was then read at OD 450 nm (reference filter 570 nm) using a Dynatech ELISA reader.

2.4.2.2 Quantikine™ Kit from R&D Systems.

Following the protocol supplied with the kit.

The required number of wells were placed in the plate frame and 50 µl of assay diluent RD1W (a buffered protein solution) added to each well. The standard curve was prepared by reconstitution of the mouse GM-CSF standard with 5 ml of calibrator diluent RD5T (a buffered protein solution) to give a concentration of 500 pg/ml. This was used as the top standard and serially diluted 2 fold to obtain the rest of the standard curve. The remainder of the standard was aliquoted into single use aliquots and frozen at -70°C. 50 µl of standard or sample (appropriately diluted) was aliquoted into the wells, the plate covered with an adhesive strip and incubated at room temperature for 2 hours.

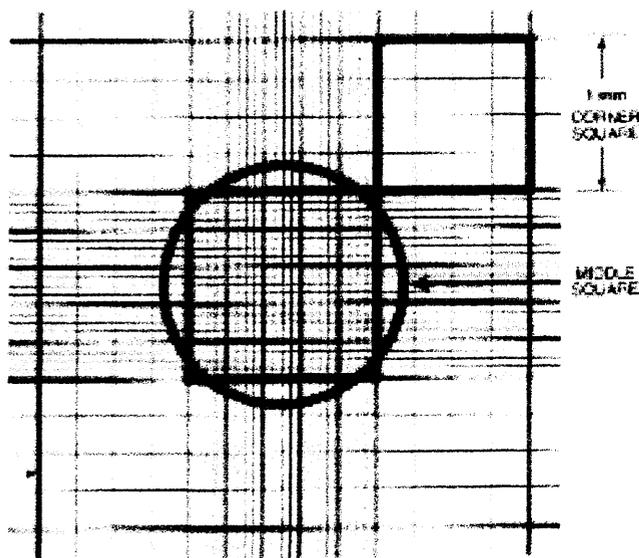
The wells were emptied and washed (as described previously) for a total of five washes using diluted wash buffer. The anti-mouse GM-CSF conjugate was diluted in conjugate diluent and 100 µl added to each well, the plate was covered with an adhesive strip and incubated at room temperature for 2 hours.

The wells were emptied and washed for a total of five washes. The substrate was prepared by mixing equal volumes of colour reagents A (stabilized hydrogen peroxide) and B (stabilized tetramethylbenzidine). 100 µl of the substrate was added to each well and the plate incubated in the dark for 30 min to allow the colour change to occur. The colour change was stopped by the addition of 100 µl of stop solution (diluted hydrochloric acid solution) to each well. The plate was read at OD 450 nm (reference filter 570 nm) using a Dynatech ELISA reader.

2.4.3 Counting of White Blood Cells using a Haemocytometer.

A suspension of the blood sample to be counted was prepared by mixing 4 μl of whole blood with 32 μl of PBS and 4 μl of Zapoglobin® (Coulter Electronics Ltd), to lyse the red blood cells. With the cover slip of the haemocytometer in place, a small amount of the suspension was transferred to the counting chambers by capillary action. The cells in the middle square were then counted (see fig 2.3).

Fig 2.3: Diagram of a standard haemocytometer chamber, circle indicates the approximate area covered at 100 x magnification.



This square of the haemocytometer represents a total volume of 0.1 mm^3 (10^{-4} cm^3); the cell concentration per ml was therefore determined using the following calculation.

$$\text{Cells per ml} = \text{Count in the Square} \times \text{Dilution Factor} \times 10^4$$

2.4.4 Fluorescence Activated Cell Sorting (FACS).

All reactions were performed in 5ml Falcon tubes (Becton Dickinson).

The blood cells were pelleted by centrifugation at $350 \times g$ for 5 min then resuspended in 50 μl FACS buffer.

FACS Buffer -

PBS with 1% v/v Foetal Calf Serum and 0.1% w/v sodium azide

To prevent non-specific binding of antibody to the Fc receptors on the cells,

5 µl of Fc block (anti-CD16/CD32 antibody) was added to the cell suspension and incubated at room temperature for 5 min. The fluorescence labelled antibodies were then added to the cell suspensions and incubated at room temperature, in the dark, for 5 min. To lyse the red blood cells, 2 ml of FACSLYSE (Becton Dickinson) was added to the cell suspension and incubated at room temperature, in the dark, for 10 min. The cells were then washed twice by centrifugation (350 x g for 5 min) followed by resuspension in 2 ml of FACS buffer. The cells were washed again by centrifugation (350 x g for 5 min) and resuspension in 2 ml FACSFLOW (Becton Dickinson). The cells were then prepared for analysis by centrifugation (350 x g for 5 min) and resuspension in 100 µl FACSFLOW, then counted using a FACScan machine (Becton Dickinson). Data analysis was performed using the program PCLysis (Becton Dickinson).

2.5 Statistical Analyses.

Analyses were carried out using Minitab software. All data are presented as means ± standard error of the mean. Data were analysed by Mann-Whitney U-Test, or χ^2 test as indicated in the text relating to each result. Statistical significance was assumed at $P < 0.05$ (5%).

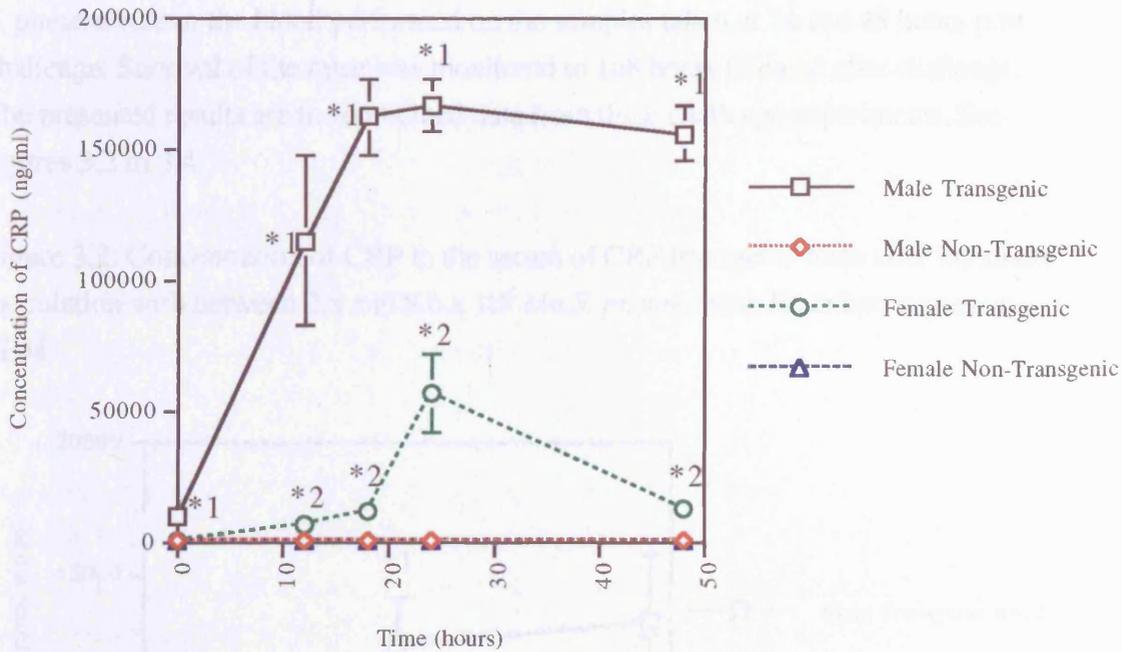
Chapter 3: Results - CRP Transgenic Mice.

3.1 Acute Phase Expression of CRP in CRP Transgenic Mice Following an Inflammatory Stimulus.

To determine the pattern of expression of CRP in the CRP transgenic mice following an inflammatory stimulus, groups of mice were challenged with LPS. Founder animals for the colony were obtained from U. Ruther in Heidelberg (Ciliberto *et al.*, 1987a). The mice were screened for the presence of the transgene by PCR and those possessing the transgene were termed transgenic and those shown not to carry the transgene were termed non-transgenic. Groups of four male transgenic, male non-transgenic, female transgenic and female non-transgenic mice of between 16 and 21 weeks of age were injected intraperitoneum with 100 µg of LPS dissolved in 100 µl of water (that is 100 µl of a 1 mg/ml solution of LPS). A tail bleed was taken from the mice before LPS challenge (time 0 hours) then at times 12, 18, 24 and 48 hours following challenge with LPS. The serum was collected from the blood samples and the concentration of CRP in the serum determined by ELISA. For each group n = 4. See figure 3.1.

At the start of the experiment (that is the basal levels of expression), the concentration of CRP in the serum of the male transgenic mice (9422 ± 2838 ng/ml serum) was higher than the level of CRP in the female transgenic mice (21 ± 13 ng/ml serum) and this was a significant difference ($5\% > P > 1\%$ Mann-Whitney U test). After challenge with LPS the level of CRP expression in both male and female transgenic mice rose with peak levels of expression in both groups at 24 hours after challenge. The difference between the levels of CRP in the male transgenic group compared with the female transgenic group was significant at each timepoint ($5\% > P > 1\%$ at each timepoint Mann-Whitney U test). The difference between the male transgenic and non-transgenic mice was also significant at each timepoint ($5\% > P > 1\%$ at each timepoint Mann-Whitney U test). The difference between the female transgenic and non-transgenic mice was also significant at each timepoint ($5\% > P > 1\%$ at each timepoint Mann-Whitney U test) except at time 0 hours ($P > 5\%$ Mann-Whitney U test). The levels of CRP in both the male and female non-transgenic mice were virtually undetectable and did not alter significantly throughout the course of the experiment.

Figure 3.1: Concentration of CRP in the serum of CRP transgenic mice after intraperitoneal injection with 100 µg LPS. For each group n = 4. Error bars represent SEM.



*1 Significant difference between male and female transgenics, and male transgenic and non-transgenic mice at all timepoints (5% > P > 1% or P < 1% Mann-Whitney U test)

*2 Significant difference between female transgenic and non-transgenic mice at all times except 0 hours (5% > P > 1% or P < 1% Mann-Whitney U test)

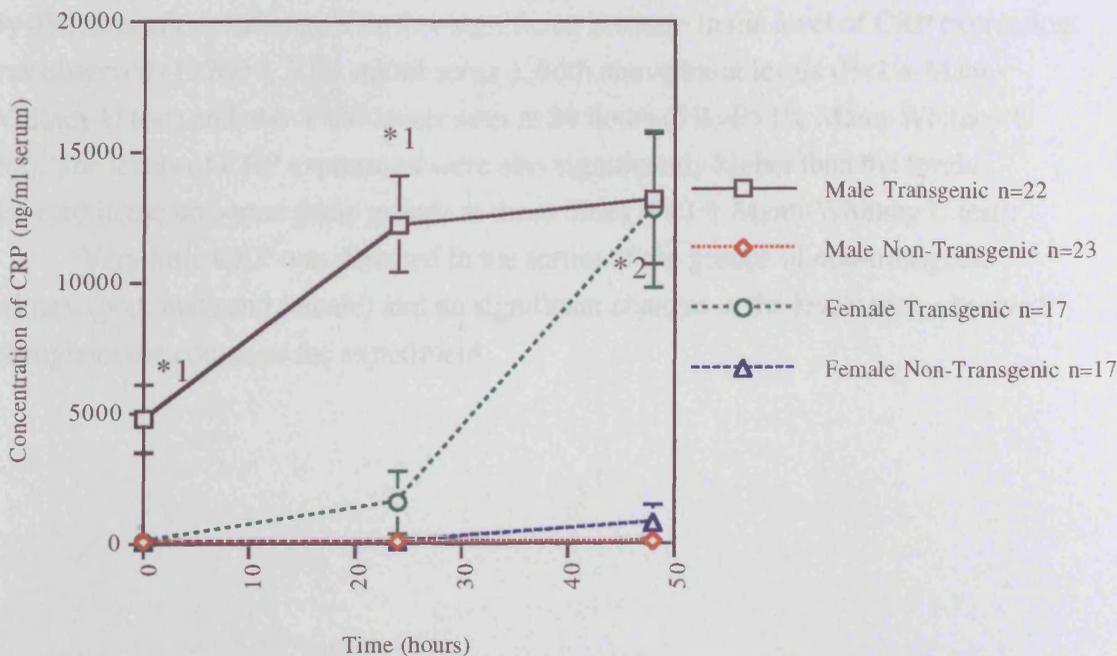
The antibodies used in the ELISA to detect the presence of CRP in murine serum were raised against human CRP and were shown to be specific for human CRP in human serum. Cross species reactivity had not been determined for either antibody, so the very low levels of CRP detected in the serum of the non-transgenic mice may have been due to cross reaction of the antibodies with murine proteins (for example mCRP or mSAP).

3.2 Pneumococcal Challenge of CRP Transgenic Mice.

To determine if challenge with an infectious organism stimulated an acute phase response of CRP expression in the CRP transgenic mice, and if this expression of CRP was protective against infectious challenge, groups of mice were infected intranasally with *S. pneumoniae*. The total number of animals challenged was 79; 22 male transgenics, 23 male non-transgenics, 17 female transgenics, and 17 female non-transgenics. These mice were aged between 9 and 45 weeks of age. The animals were

inoculated intranasally with between 2.8×10^5 and 8.6×10^5 cfu *S. pneumoniae* in 50 μ l of PBS. Blood samples were taken from each mouse, by tail bleeding, before the challenge (time 0 hours) and at 24 and 48 hours after challenge. The concentration of CRP in the serum was determined at each timepoint, and a viable count of the number of *S. pneumoniae* in the blood performed on the samples taken at 24 and 48 hours post challenge. Survival of the mice was monitored to 168 hours (7 days) after challenge. The presented results are the combined data from three challenge experiments. See figures 3.2 to 3.4.

Figure 3.2: Concentration of CRP in the serum of CRP transgenic mice after intranasal inoculation with between 2.8 and 8.6×10^5 cfu *S. pneumoniae*. Error bars represent SEM.



*1 Significant difference between male transgenics and all other groups ($P < 1\%$ Mann-Whitney U test)

*2 Significant increase in the levels of CRP in the female transgenic group by 48 hours ($5\% > P > 1\%$)

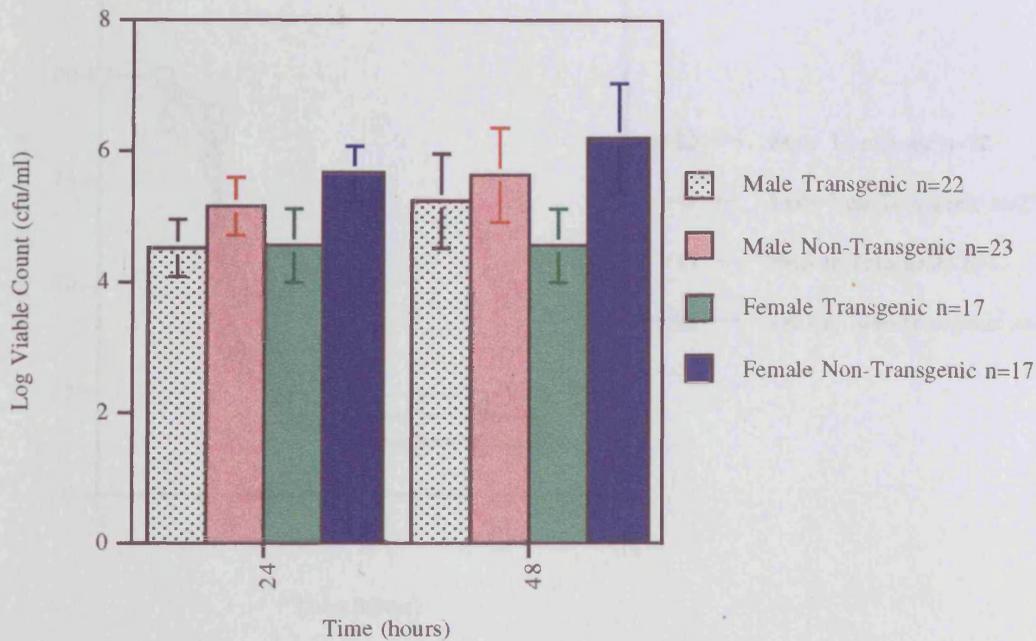
At the start of the experiment (basal levels of expression), the levels of CRP in the male transgenic mice (4755 ± 1282 ng/ml serum) were significantly higher than all the other groups ($P < 1\%$ for each comparison Mann-Whitney U test). This is also comparable to the basal levels of CRP expression detected before administration of LPS (9422 ± 2838 ng/ml serum)(figure 3.1). By 24 hours after challenge with *S. pneumoniae*, the level of CRP expression in the male transgenic mice had increased

(12165 ± 1837 ng/ml serum) and this was significantly different from the basal level of expression (P<1% Mann-Whitney U test). The detectable levels of CRP in the male transgenic group at this time was also significantly higher than the levels in all the other groups (P<1% for all comparisons Mann-Whitney U test). The levels of CRP in the male transgenic mice increased again by 48 hours after challenge (13174 ± 2493 ng/ml serum) and this was significantly higher than the basal level of expression (5%>P>1% Mann-Whitney U test), although not significantly different from the levels detected at 24 hours. At this time the level of CRP expression in the male transgenic group was also significantly higher than the levels detected in the non-transgenic groups (P<1% Mann-Whitney U test).

The level of CRP expression in the female transgenic group also increased significantly by 24 hours after challenge with *S. pneumoniae* (1505 ± 1201 ng/ml serum compared to basal levels of 12 ± 8 ng/ml serum)(P<1% Mann-Whitney U test). By 48 hours after challenge a further significant increase in the level of CRP expression was observed (12760 ± 3031 ng/ml serum), both above basal levels (P<1% Mann-Whitney U test) and above the levels seen at 24 hours (5%>P>1% Mann-Whitney U test). The levels of CRP expression were also significantly higher than the levels detected in the non-transgenic groups at these times (P<1% Mann-Whitney U test).

Very little CRP was detected in the serum of the groups of non-transgenic animals (both male and female) and no significant changes in the levels were observed throughout the course of the experiment.

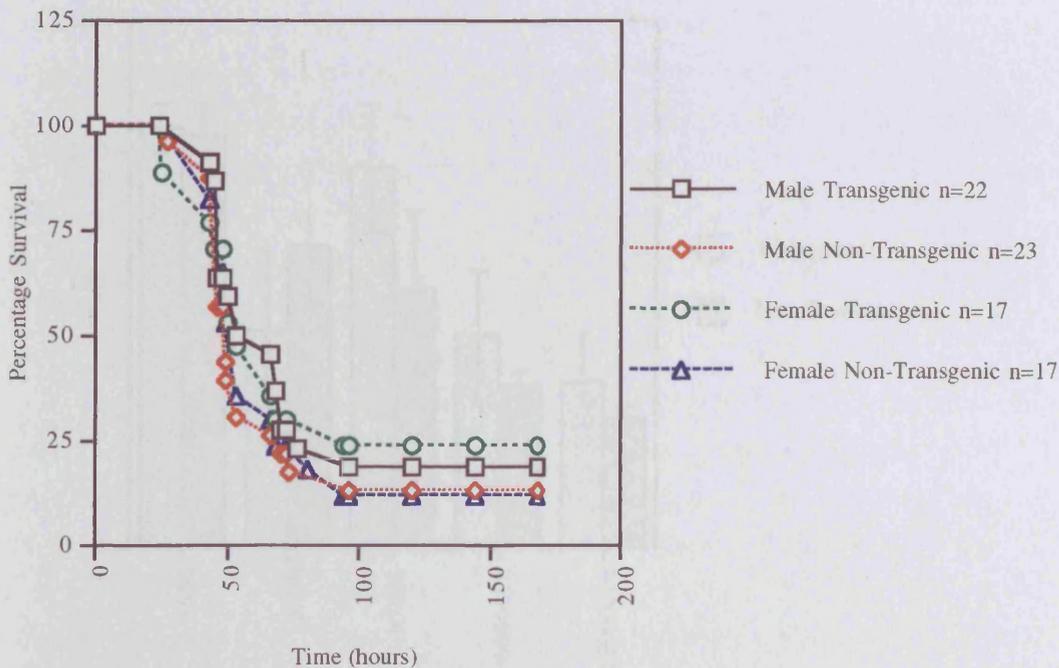
Figure 3.3: Log viable count of *S. pneumoniae* in the blood of CRP transgenic mice after intranasal inoculation with between 2.8 and 8.6×10^5 cfu *S. pneumoniae*. Error bars represent SEM.



The detection limit of the assay was 167 cfu/ml, that is 1 colony in one of the three 20 μ l drops at a dilution of 10^{-1} (ie $1/3 \times 50 \times 10 = 167$ cfu/ml). Any samples that did not have detectable bacteraemia were therefore counted as just below the detection limit of the assay (that is 166 cfu/ml).

Bacteraemia was detected in all the groups by 24 and 48 hours after challenge. By 24 hours after challenge, bacteraemia was detected in 14 of the 22 male transgenic group, 17 of the 23 in the male non-transgenic group, 11 of the 17 in the female transgenic group and 15 of the 17 in the female non-transgenic group. There was no significant difference in the level of bacteraemia detected in each group at either timepoint (Mann-Whitney U test). There were also no significant increases in the level of bacteraemia within each group by 48 hours after challenge (Mann-Whitney U test).

Figure 3.4: Survival of CRP transgenic mice after intranasal inoculation with between 2.8 and 8.6×10^5 cfu *S. pneumoniae*.



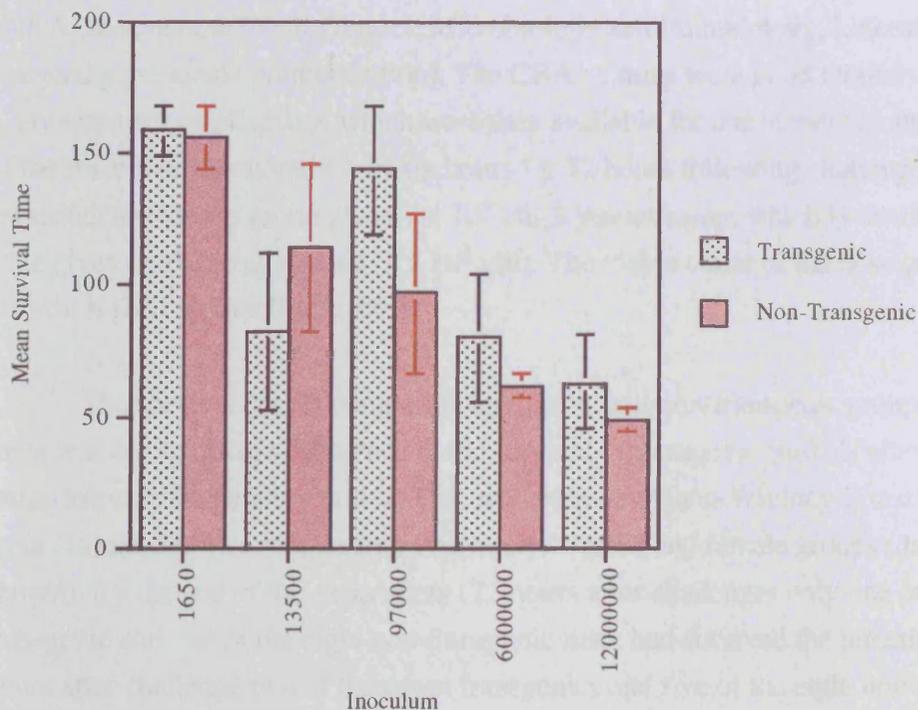
The survival curves for each group of mice show a similar pattern and there is no significant difference in the number of transgenic and non-transgenic animals that survived the experiment, as determined by X^2 test and Mann-Whitney U test.

This was an unexpected result as it had previously been shown that passive immunization with CRP (Mold *et al.*, 1981)(Yother *et al.*, 1982), and other studies with this line of transgenic mice (Szalai *et al.*, 1995), protected mice against fatal infection with *S. pneumoniae*. It was reasoned that the inoculum dose of *S. pneumoniae* given to the mice in these experiments may not have been optimal in order to see protection, so further experiments were performed to determine the optimal dose.

3.3 *S. pneumoniae* Dosage Challenges of CRP Transgenic Mice.

To determine the best inoculum dose of *S. pneumoniae* to give the mice, a dose related challenge was performed, in which groups of four or five transgenic and non-transgenic mice were given different inoculum doses of *S. pneumoniae* intranasally. Both male and female mice were included in each group. Survival of the mice was monitored to 168 hours (7 days) following challenge, and those animals that survived the challenge were therefore determined to have survived for 168 hours. See figure 3.5.

Figure 3.5: Mean survival time of CRP transgenic mice after intranasal challenge with different doses of *S. pneumoniae*. Error bars represent SEM.



There were no significant differences in the mean survival time between the groups of transgenic and non-transgenic mice for any of the doses given (Mann-Whitney U test).

3.4 Intraperitoneal Challenge of CRP Transgenic Mice with *S. pneumoniae*.

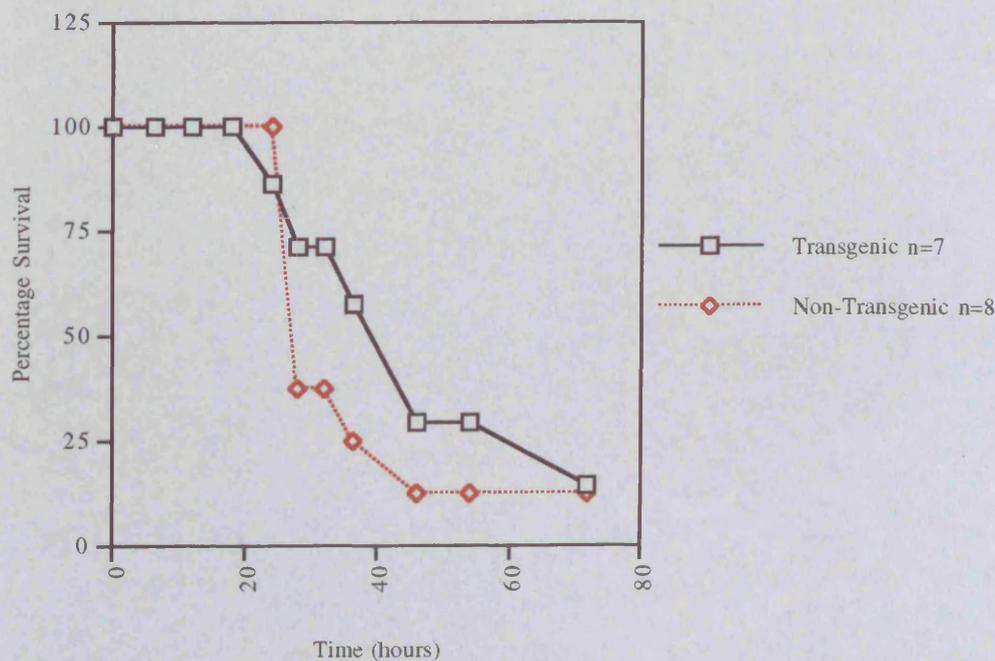
It had previously been shown that mice transgenic for CRP are protected from challenge with *S. pneumoniae* administered intraperitoneally (Szalai *et al.*, 1995). It was therefore surprising that the results of our challenge with *S. pneumoniae* administered intranasally did not show protection of the transgenic mice. It was therefore decided to examine the role of the route of infection using the strain of *S. pneumoniae* that had been used in the intranasal challenges, that is D39 a type 2 encapsulated strain compared to the Szalai group who used WU2 a type 3 strain.

Groups of four male transgenic, male non-transgenic, female non-transgenic and three female transgenic mice were infected intraperitoneally with different doses of *S. pneumoniae* D39 (1×10^3 , 1×10^4 and 1×10^5 cfu in 200 μ l of PBS). These mice were from crosses between male CRP transgenic and female CBA/ca mice, and were aged

between 19 and 32 weeks of age. The CRP transgenic mice were crossed with CBA/ca females as part of a long term programme to breed the transgene onto a CBA/ca background as this strain of mice had been shown to be highly susceptible to infection with *S. pneumoniae* (Neill Gingles, Microbiology and Immunology, Leicester University personal communication). The CBA/ca mice were good mothers so produced a large number of offspring, which were then available for use in these studies. Survival of the mice was monitored every six hours for 72 hours following challenge. The results presented are for the group given 7×10^3 cfu *S. pneumoniae*, which is similar to the dose given by the Szalai group (1×10^4 cfu). The viable count of the dose given was 7×10^3 cfu in 200 μ l. See figure 3.6.

The survival curves comparing transgenic and non-transgenic groups show that there was no significant difference in the survival of transgenic animals after intraperitoneal inoculation with D39 *S. pneumoniae* (Mann-Whitney U test). There were also no significant differences between the male and female groups (data not shown). By the end of the experiment (72 hours after challenge) only one of the seven transgenic and one of the eight non-transgenic mice had survived the infection. By 30 hours after challenge two of the seven transgenics and five of the eight non-transgenics had succumbed to the infection, but this difference was not significant.

Figure 3.6: Survival of CRP transgenic x CBA mice after intraperitoneal inoculation with 7×10^3 cfu *S. pneumoniae*.



This was a surprising result as it did not support previously published work (Szalai *et al.*, 1995). There were a number of differences in this experiment however; including a different strain of *S. pneumoniae* (type 2 not type 3) and the CRP transgene being on a different background (CRP transgenic x CBA/ca crosses rather than crosses within the colony of C57/BL6 x SJL mice).

Summary of Results from the CRP Transgenic Mice.

1. Acute phase expression of CRP was induced by the inflammatory stimulus LPS.
2. Acute phase expression of CRP was induced by intranasal challenge with *S. pneumoniae*.
3. This acute phase expression of CRP was not sufficient to protect the mice from fatal infection induced by intranasal challenge with *S. pneumoniae*.
4. Altering the inoculum dose of *S. pneumoniae* given to the mice did not alter this lack of protection.
5. In our experiments, the CRP transgenic mice were also not protected against intraperitoneal challenge with *S. pneumoniae*.

These results showed that acute phase expression of CRP in these transgenic mice could be induced by an inflammatory stimulus (LPS), and by challenge with an infectious organism (*S. pneumoniae*), but that this acute phase expression of CRP is not protective against infection with *S. pneumoniae*. In order to develop a system that would confer protection against disease, it was therefore reasoned that a broader upregulation of the immune system would be required. The cytokine GM-CSF, which has many effects on the immune system including increasing the production and activation of white blood cells, was therefore chosen to be expressed with acute phase kinetics in transgenic mice.

Chapter 4: Results - Preparation of the Constructs for Acute Phase Expression of GM-CSF.

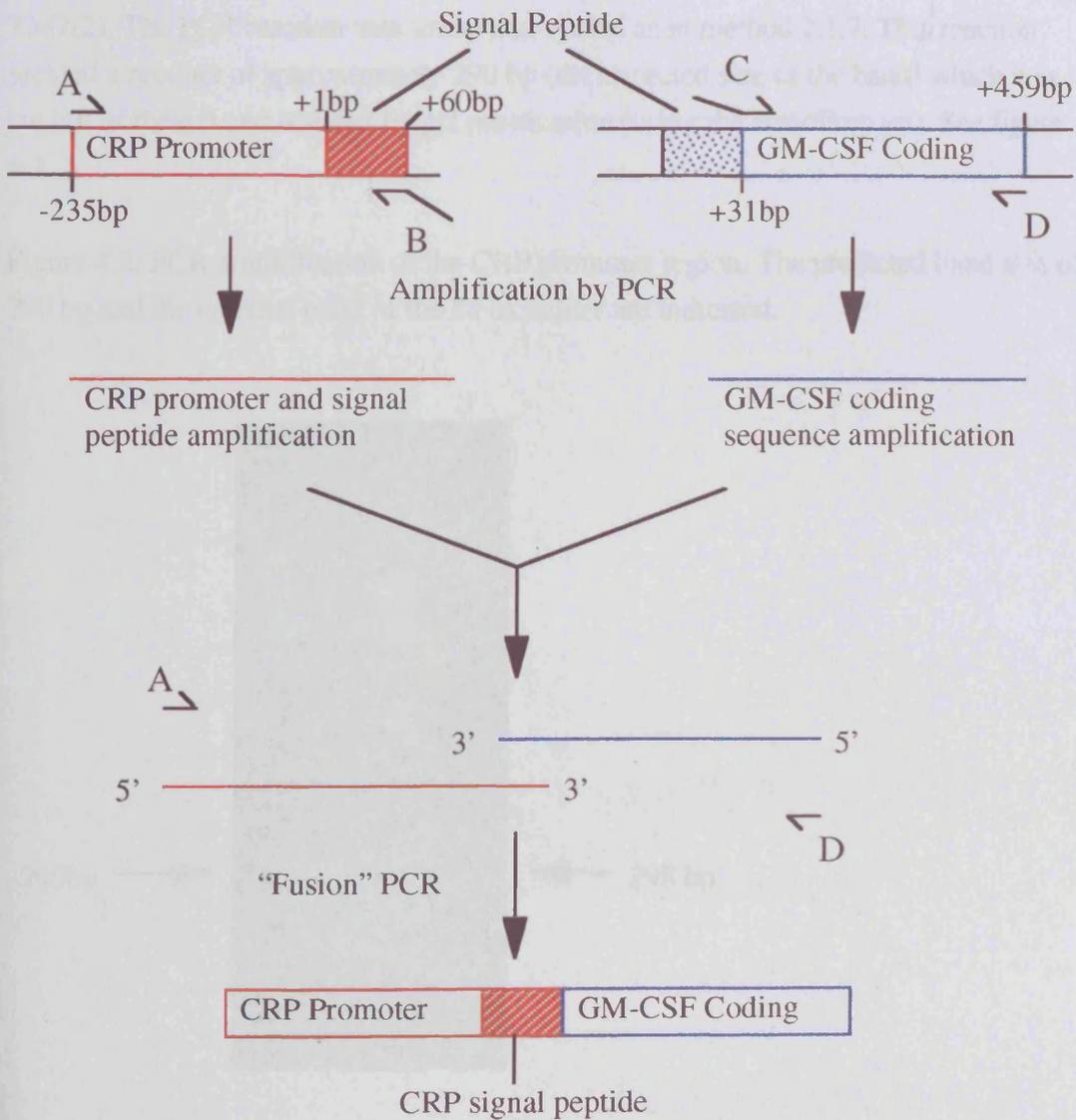
4.1 Construction of the fusion between CRP and GM-CSF.

A fusion (pSH1) between 280 bp of the 5' region of CRP and the coding sequence of GM-CSF was initially made in this laboratory by Shirley Hanley (see figure 4.1). This fusion was sequenced using manual methods and, when all the sequence was aligned, it was seen that there was a base substitution in the coding sequence of GM-CSF which would have altered the amino acid sequence of the protein. A new fusion therefore had to be constructed.

Production of the fusion involved three sets of PCR reactions (see figure 4.1). One reaction amplified the CRP promoter and signal peptide, another amplified the coding region of mGM-CSF, and the third created the fusion product. The primers were designed so that the primer from the 3' region of the CRP signal peptide had a region complementary to this sequence (the region involved in amplification of the product) and a region that was complementary to the 5' region of the GM-CSF coding sequence; conversely the primer from the 5' region of GM-CSF had a region complementary to this sequence and a region complementary to the 3' region of the CRP signal peptide. This resulted in products that had complementary ends which could then be used to construct the fusion.

This region of the CRP promoter (-235 to +1) was chosen to make the initial fusion as this region had previously been shown to control the acute phase expression of CRP in tissue culture systems (Arcone *et al.*, 1988)(Li *et al.*, 1990). Signal peptides are found on proteins destined for secretion or insertion into the cell membrane and consist of a sequence of about 20 amino acids which directs the protein into the endoplasmic reticulum during synthesis. It was decided to use the signal peptide for CRP, rather than that of mGM-CSF, as expression of the protein should be directed to the liver of transgenic animals and CRP is normally produced and secreted from liver cells. It was therefore predicted that the use of the CRP signal peptide would efficiently direct the secretion of mGM-CSF from liver cells. Production of mGM-CSF solely from liver cells would also overcome some of the problems that had been shown to be associated with uncontrolled expression of mGM-CSF in many tissues such as the muscles (Lang *et al.*, 1987) and eyes (Cuthbertson and Lang, 1989).

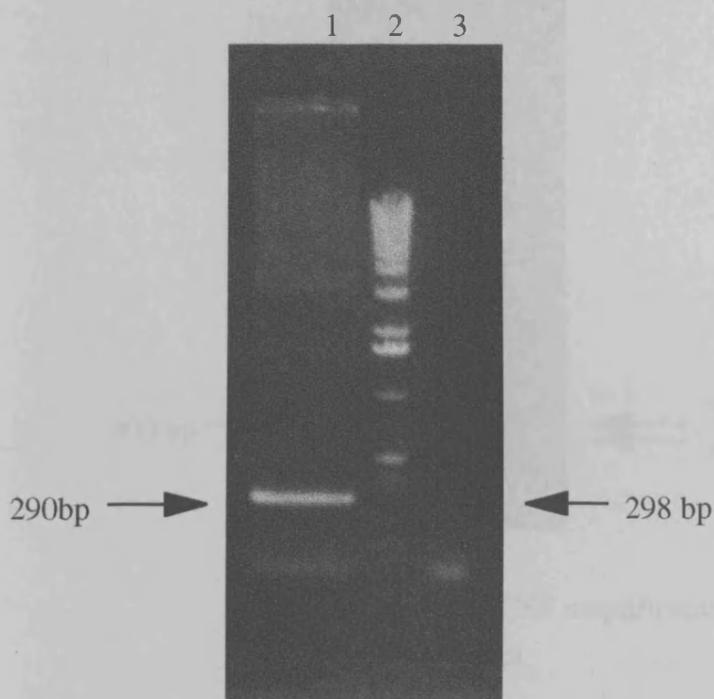
Figure 4.1: Construction of a fusion between the CRP promoter and the GM-CSF coding region by PCR.



Because of the difficulties encountered in working with the 30kb cosmid (see section 4.2), it was also decided to employ a different cloning strategy in order to clone the fusion into the larger background of the CRP promoter that is necessary for acute phase expression in transgenic mice. For this cloning strategy, the fusion was created using primers engineered to contain sites for the rare cutting enzyme *NotI* as the outside primers (primers A and D from figure 4.1). The larger acute phase vectors, containing the regions of the CRP promoter necessary for acute phase expression in transgenic mice, were also engineered to contain *NotI* sites (see section 4.2 and figure 4.6) so that the fusion between 280bp of the CRP promoter and the coding region of GM-CSF could be easily cloned into these vectors.

The 5' region of CRP was amplified from the previous clone (pSH1) as sequence analysis of this region of the construct matched the expected sequence. The amplification was performed using primers FU5PR (with an engineered *NotI* site) and TM7(2). The PCR reaction was set up and cycled as in method 2.1.7. This reaction yielded a product of approximately 290 bp (the expected size of the band) which was cut out of the gel and isolated by gel purification (using the BandPrep kit). See figure 4.2.

Figure 4.2: PCR amplification of the CRP promoter region. The predicted band size of 290 bp and the relevant band of the DNA ladder are indicated.

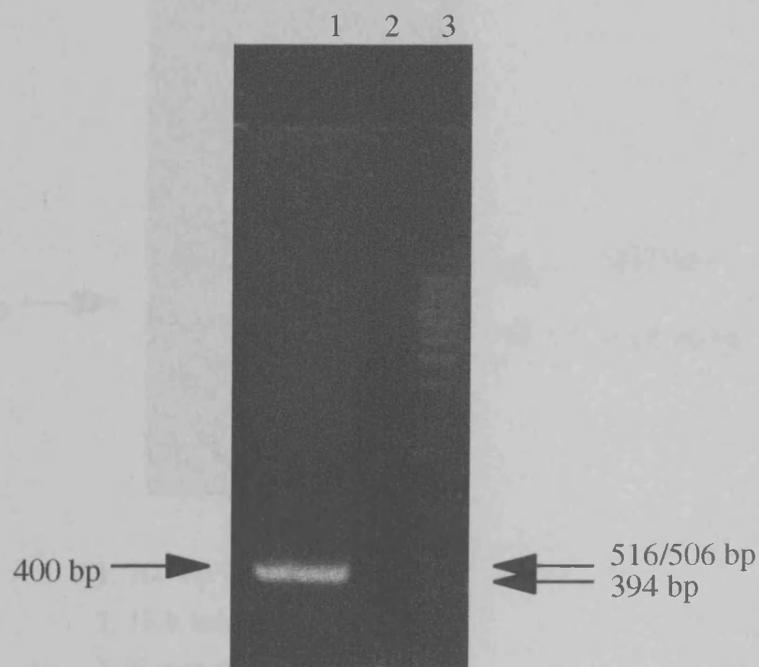


1. 290 bp 5' CRP amplification
2. 1kb ladder
3. Negative control

The mGM-CSF coding region was amplified from a commercially sourced plasmid (R&D Systems) containing the murine GM-CSF gene in the plasmid vector pUC18; this had the mGM-CSF codons modified for more efficient expression in the *E. coli* system, and to contain useful restriction sites. More efficient production of mGM-CSF may have been predicted if a cDNA of murine GM-CSF had been used, or if the coding region of mGM-CSF had been amplified from mouse genomic DNA; however these cDNA clones were not available. The amplification was performed with

the primers FU3PR (containing an engineered *NotI* site) and TM8. The reaction was set up and cycled as in method 2.1.7. This amplification yielded a product of approximately 400 bp (the expected size of this product), which was cut out of the gel and isolated by gel purification (using the BandPrep kit). See figure 4.3.

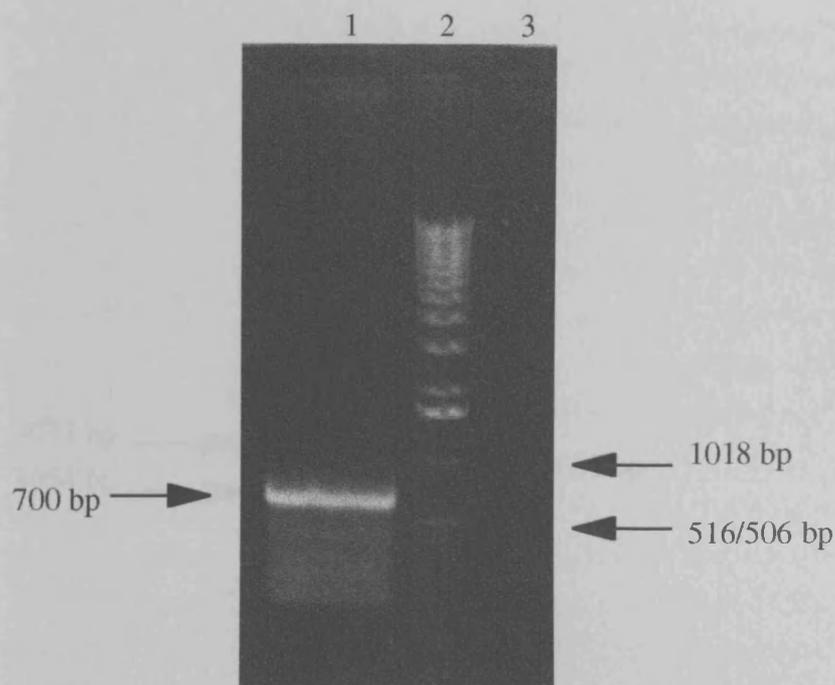
Figure 4.3: PCR amplification of the coding region of mGM-CSF. The predicted product size was 400 bp, and the relevant bands of the DNA ladder are indicated.



1. 400 bp mGM-CSF amplification
2. Negative control
3. 1Kb ladder

These purified products were then used to construct the fusion between this region of the CRP promoter and the coding sequence of mGM-CSF. The amplification was performed using equal amounts of each of the PCR products (0.4 μ g of each), and these were given one cycle of a low annealing temperature to allow the complementary ends to join together before the fusion product was amplified using the primers FU5PR and FU3PR. This amplification yielded a product of approximately 700 bp (the expected size of the fusion) which was cut out of the gel and isolated by gel purification (using the BandPrep kit). See figure 4.4.

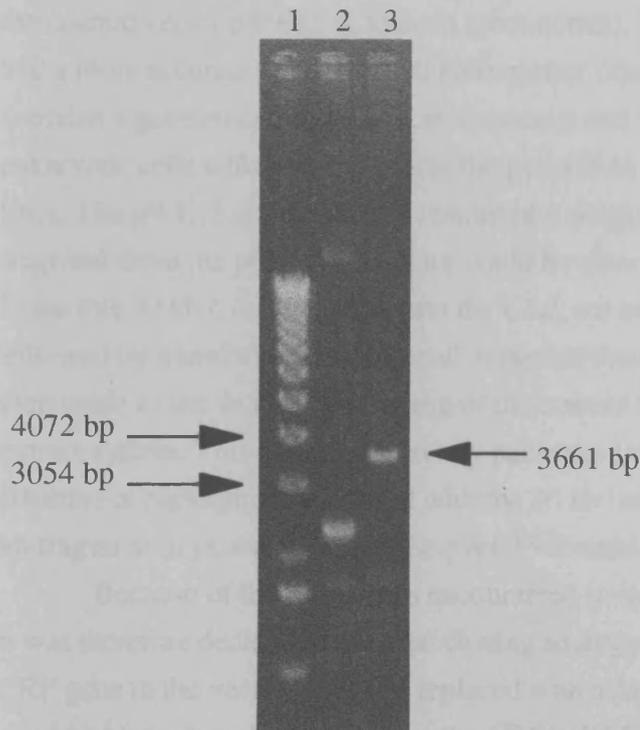
Figure 4.4: PCR amplification of the fusion between the CRP promoter and the coding region of mGM-CSF. The size of the PCR product and the relevant bands of the DNA ladder are indicated.



1. 700 bp fusion PCR amplification
2. 1Kb ladder
3. Negative control

This amplification product was then cloned into the pCRScript vector, following the manufacturers protocol. A number of clones containing an insert of approximately the right size were obtained. One of these was chosen and shown to linearize to the correct band size using the enzyme *StuI* (which was predicted to cut the vector only once). See figure 4.5.

Figure 4.5: Cloning the fusion into pCR-Script. The pCR-Script vector is 2961 bp in size therefore cloning the CRP/mGM-CSF fusion (700 bp) into this should yield a product of 3661 bp. The relevant bands of the DNA ladder are indicated.



1. 1kb ladder
2. Uncut plasmid
3. *StuI* digested clone (linearized plasmid)

The fusion was manually sequenced using the primers CRSEQ, TM7(2), AW1, FU3PR and GMSEQ. The sequence of the fusion was shown to match the predicted sequence, indicating that no mistakes had been introduced during the PCR amplifications and that the fusion was suitable for use in further stages of the cloning strategy.

4.2 Construction of the Acute Phase Vectors.

The initial attempts to make an acute phase vector for expression of mGM-CSF in transgenic mice involved using the 30 kb genomic clone of CRP (pCosCRP1) that had been shown to express CRP with acute phase kinetics in transgenic mice (Ciliberto *et al.*, 1987a). A restriction enzyme site (*BsaBI*) in the region 5' to the CRP gene and a blunt cutting enzyme site at the end of the CRP gene would be used to cut the cosmid to remove the CRP gene. The same restriction site in the 5' region of CRP and another

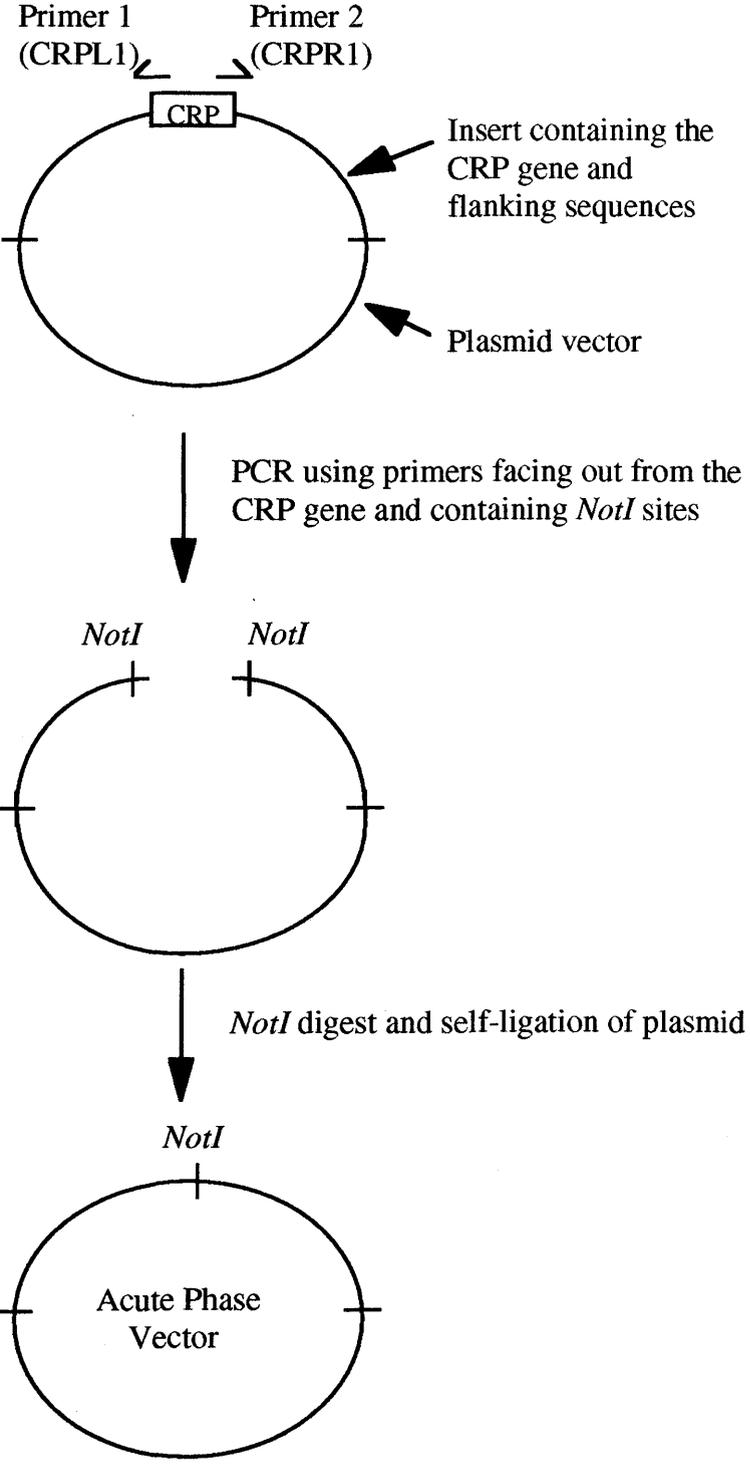
blunt cutting enzyme site at the end of the GM-CSF gene would be used to cut the fusion out of the pCRScript plasmid, and this could then be ligated into the vector created from the 30kb cosmid.

It was decided to clone the 30kb *ClaI* fragment from the pCosCRP1 vector into the cosmid vector pWE15 (Clontech laboratories). The sequence of this vector is known and a more accurate map of the 30 kb fragment could then be generated. Also this vector contains a gene encoding neomycin resistance and an origin of replication for eukaryotic cells which would enable the generation of stably transfected eukaryotic cell lines. The pWE15 cosmid vector contained a single *ClaI* site into which the 30 kb *ClaI* fragment from the pCosCRP1 vector could be cloned. Several attempts were made to ligate this 30 kb *ClaI* fragment into the *ClaI* cut and dephosphorylated pWE15 vector followed by transformation of *E. coli* however these were unsuccessful. Attempts were then made to use *in vitro* packaging of the cosmid using the Gigapack II XL packaging extract system. This system selectively packages large inserts and should therefore be effective at packaging the cosmid with the 30 kb insert. These attempts at cloning the 30 kb fragment of pCosCRP1 into the pWE15 cosmid vector were also unsuccessful.

Because of the difficulties encountered in working with this large piece of DNA it was therefore decided to alter the cloning strategy. For the new cloning strategy, the CRP gene in the vector would be replaced with a *NotI* restriction site, and *NotI* sites would be introduced at the ends of the CRP/mGM-CSF fusion. This would then enable the fusion to be cloned into the vector after digestion of each with *NotI*. Primers facing out from the ends of the CRP gene were engineered to create *NotI* sites within them, and then used to amplify round the plasmid resulting in a PCR product which was then cut with *NotI* and self ligated to give a plasmid consisting of the flanking sequences of CRP without the CRP gene. See figure 4.6.

Two different approaches were followed, one to create a full length acute phase vector (30 kb) and the other to utilise a shorter construct (C79) that had been shown to express CRP with acute phase kinetics in transgenic mice (figure 4.7).

Figure 4.6: Cloning strategy to produce the acute phase expression vectors.



4.3 Production of the pBNB/GM Construct.

See figure 4.7 for the cloning strategy to produce the different GM-CSF acute phase expression vectors.

In order to create the full length construct, a 5 kb fragment obtained from digestion of the pCosCRP1 construct with *Bam*HI was to be used as a 'shuttle' vector for the manipulations to replace the CRP gene with a *Not*I site and subsequently the GM-CSF gene. This 5 kb *Bam*HI fragment of pCosCRP1 corresponded to construct 4 used previously in the generation of transgenic mice which was shown to confer fairly high basal levels of CRP expression (127 ± 510 μ g/ml of serum) and low inducibility (expression increased by a factor of 2)(Murphy *et al.*, 1995). The planned strategy was to clone this fragment (with the CRP gene replaced by GM-CSF) back into the full length flanking DNA. The acute phase vector based on the 5 kb *Bam*HI fragment, with the CRP gene replaced with a *Not*I site was created as in figure 4.6 by Dr Bernard Burke and designated pBNB.

This vector was digested with *Not*I, and the linearized plasmid isolated by gel purification (using a BandPrep kit). The plasmid was then dephosphorylated, to remove the terminal phosphates from the cut ends and prevent self ligation of the plasmid. The plasmid containing the CRP/mGM-CSF fusion was also cut with *Not*I and the 700 bp fragment produced by this digestion isolated by gel purification. The 700 bp fragment was then ligated into the cut vector and transformed into *E. coli*. Plasmid DNA was prepared from the transformants (method 2.1.1) and the plasmids digested with *Bam*HI to identify the clones containing the fusion insert. See figure 4.8.

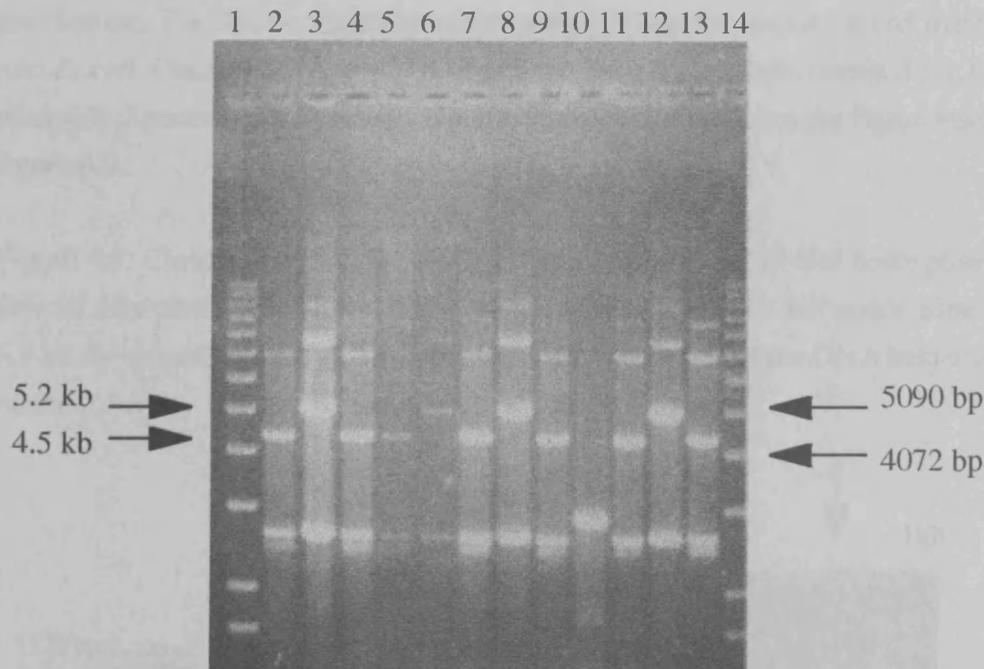
Four clones were identified as carrying the insert, and restriction digest analysis confirmed that two of these contained the insert in the correct orientation, and were designated pBNB/GM (result not shown).

In order to clone this fragment back into the full length flanking sequences, partial digests of the pCosCRP cosmid with *Bam*HI were performed, the appropriate band (approximately 25 kb) extracted from an agarose gel and the 5 kb fragment obtained by *Bam*HI digestion of pBNB/GM ligated into the cut cosmid. Many attempts at this cloning strategy were attempted (by Dr Bernard Burke) but initially proved unsuccessful.

Because this strategy had proved unsuccessful, a second acute phase vector based on a smaller region of the CRP promoter was constructed (section 4.4).

Subsequent work with this system (by Dr Bernard Burke) resulted in a clone that contained the BNB/GM fragment in the background of the 30 kb vector, but this was shown to be incorrectly oriented.

Figure 4.8: Cloning the CRP/mGM-CSF fusion into the pBNB acute phase vector. *Bam*HI digestion of the plasmids cut out a fragment of 4.5 kb for vector alone and 5.2 kb for clones containing the insert. The relevant bands of the DNA ladder are indicated.



1 and 14. 1kb ladder

3, 6, 8 and 12. Clones containing the insert

2, 4, 5, 7, 9, 10, 11 and 13. Vector alone with no insert

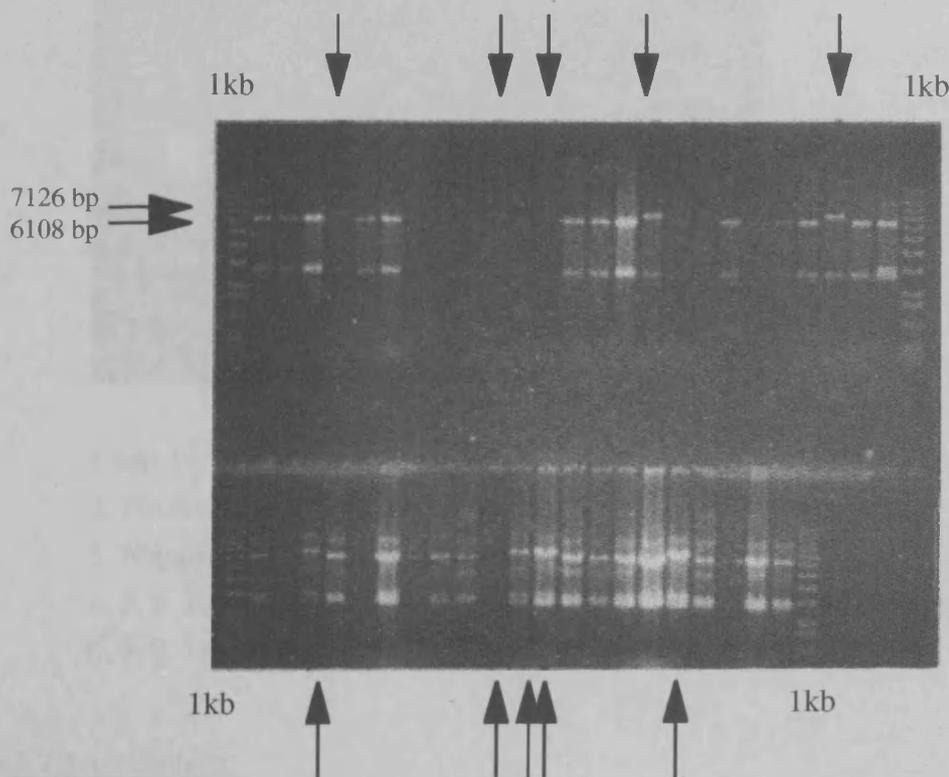
4.4 Production of the pC79/GM Construct.

The other acute phase vector that was generated utilised the C79 construct kindly donated by Ulrich Ruther (European Molecular Biology Laboratory, Heidelberg). This construct contained 1.7 kb of 5' and 3.8 kb of 3' sequence and was created by combining a *Bam*HI -*Hin*DIII fragment from -1.7 kb to +3.14 kb and a *Kpn*I -*Bam*HI fragment from +7.5 kb to +10.5 kb (Murphy *et al.*, 1995). This gave a construct containing the CRP gene and the CRP pseudogene with a 3.8 kb deletion between these regions (see figure 1.8). The construct had been previously shown to be regulated, in transgenic mice, with a low basal level of expression ($3 \pm 15 \mu\text{g/ml}$ of serum) and a high level of induction upon stimulation with LPS (increased by a factor of 69) (Murphy *et al.*, 1995). The acute phase vector based on this construct, with the CRP gene replaced

with a *NotI* site, was created as in figures 4.6 and 4.7 by Dr Bernard Burke and designated pC79-Not.

This plasmid was linearized by digestion with *NotI* and the ends dephosphorylated. The plasmid containing the CRP/mGM-CSF fusion (pFusion) was also cut with *NotI* and the 700 bp fragment produced by this digestion isolated by gel purification. The 700 bp fragment was then ligated into the cut vector and transformed into *E. coli*. Plasmid DNA was prepared from the transformants (method 2.1.1) and the plasmids digested with *BamHI* to identify the clones containing the fusion insert. See figure 4.9.

Figure 4.9: Cloning the CRP/mGM-CSF fusion onto the pC79-Not acute phase vector. *BamHI* digestion of the plasmids cut out a fragment of 6.2 kb for vector alone and 6.9 kb for clones containing the insert. The relevant bands of the DNA ladder are marked. Arrows indicate those clones that contain an insert.

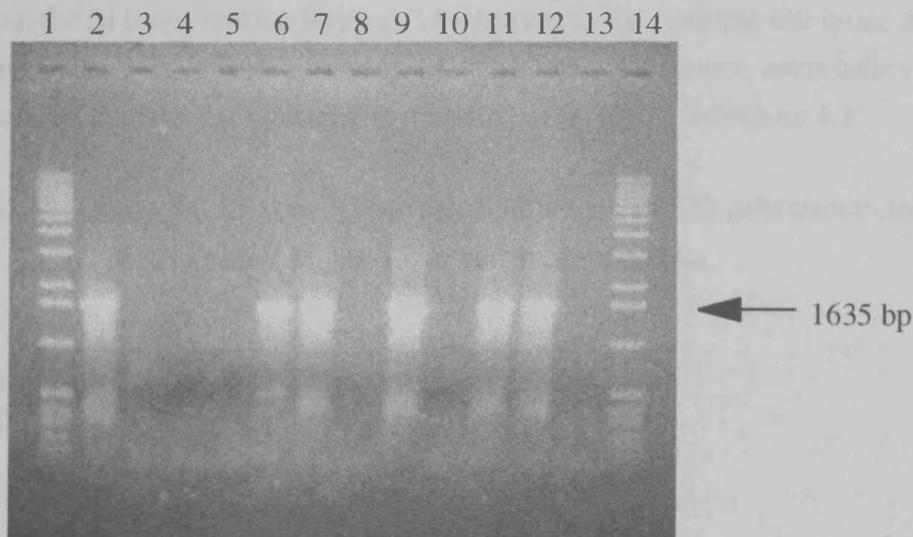


Ten clones were identified as carrying the insert, and the orientation of the insert was confirmed by PCR using the primers TM8 (at the 5' end of the GM-CSF gene) and CPA1 (in the 3' flanking region of CRP, facing back into the CRP gene).

The positive control included in the reactions was the pBNB/GM clone made previously, and the negative control included the pC79-Not clone. The reactions were set

up and cycled as in method 2.1.7. The amplifications revealed bands of the expected size (1.5 kb) from five of the clones, indicating that these contained the fusion insert in the correct orientation. See figure 4.10).

Figure 4.10: Confirmation of the orientation of the CRP/mGM-CSF fusion insert in the pC79-Not acute phase vector by PCR. One primer was complimentary to the sequence of the insert and the other was complimentary to the sequence of the pC79-Not vector; so that a PCR product would only be amplified from clones with the insert in the correct orientation. The expected size of the product was 1.5 kb, and the relevant band of the DNA ladder is indicated.



1 and 14. 1kb ladder

2. Positive control (pBNB/GM construct)

3. Negative control (pC79-Not vector alone)

4, 5, 8, 10 and 13. Clones with the fusion in the incorrect orientation

6, 7, 9, 11 and 12. Clones with the fusion in the correct orientation

4.5 Tissue Culture.

In order to assess the acute phase kinetics of the different mGM-CSF containing constructs that were generated, an *in vitro* tissue culture expression system was studied. The cell line that was used for transfection studies was Hep3B (European Collection of Animal Cell Cultures 86062703) which is a human hepatocyte carcinoma derived from an 8 year old negro male. Acute phase expression of the endogenous CRP gene is only

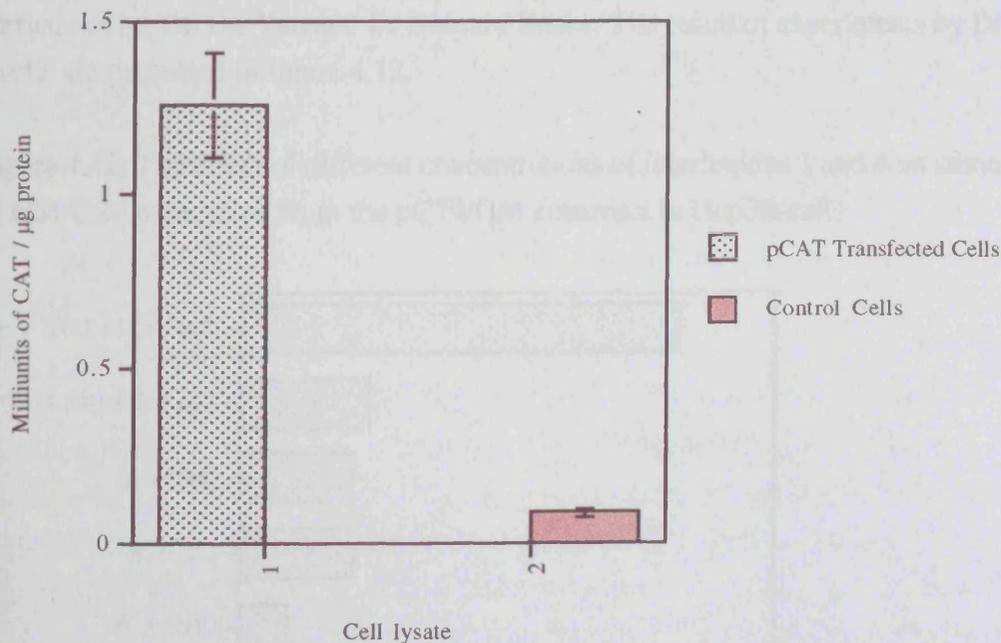
seen from cells of hepatic origin, and Hep3B is one of the cell lines that has been used extensively for studies of CRP expression (Arcone *et al.*, 1988).

4.5.1 Control Transfections.

To establish the tissue culture system, initial experiments examined the production of protein from the constitutive expression vector pCAT-Control which contains SV40 promoter and enhancer sequences that drive the expression of the CAT gene. This enzyme is not produced by mammalian cells so any CAT activity must be the result of expression of the introduced plasmid.

This vector was introduced to Hep3B cells using the ProFection system, incubated for 24 hours and the level of CAT activity in the resulting cell lysate analysed using the Quan-T-CAT™ assay system (Amersham). As a control, some cells were also mock transfected with the transfection mixture but no DNA. See figure 4.11.

Figure 4.11: Assay of CAT activity, per μg of protein, in Hep3B cells transfected with pCAT control vector. Error bars represent SEM.



Expression of CAT was only seen in the cells transfected with the pCAT-control vector and not in the mock transfected cells indicating the specificity of the CAT assay. This result indicates that the transfection system that was used efficiently transfects the vector into the cells and the vector is expressed in this cell line.

4.5.2 Optimization of Interleukin Stimulation of Acute Phase Expression.

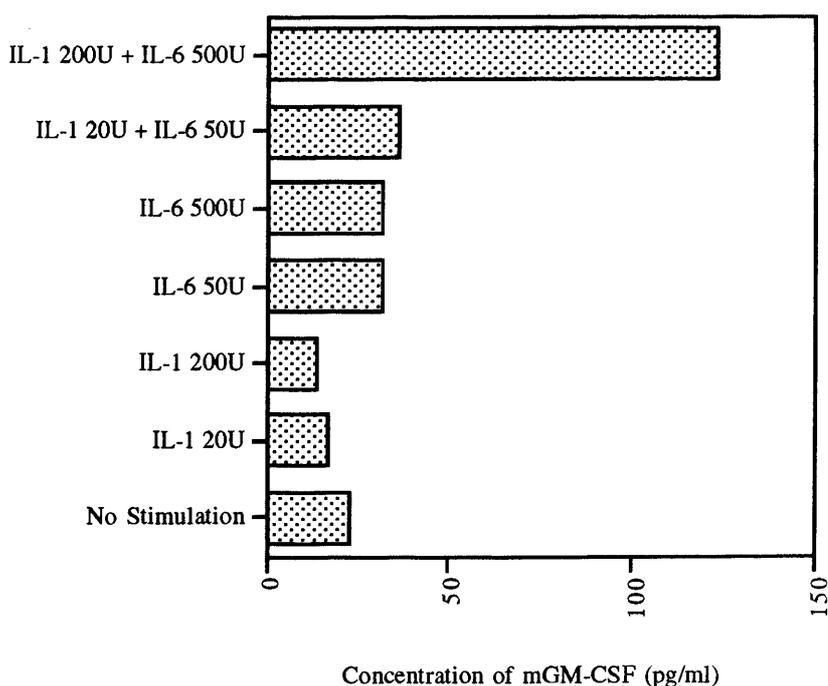
A fusion between the 280 bp 5' of the CRP gene and the reporter gene CAT was made previously (kindly provided by Shirley Hanley), in order to look at the control of expression by this region in a tissue culture system, and the effect of interleukin stimulation. This construct was based on the pCAT-enhancer vector (Promega) which contains SV40 enhancer sequences but no promoter, the cloning of promoter regions into this vector results in the high expression of CAT from the vector.

The pCRP/CAT construct was introduced to Hep3B cells using the ProFection transfection system and the pattern of CAT expression following interleukin stimulation examined. The pCAT-enhancer vector alone was used as a control.

Expression of CAT was only seen in the cells transfected with the pCRP/CAT construct and not the pCAT enhancer vector alone. This result indicates that this region of CRP is functioning as an effective promoter. However, the concentration and combinations of interleukins used in these experiments did not efficiently induce acute phase expression of CAT driven by the CRP promoter (result not shown).

Further work on the inducibility of the CRP promoter with interleukins was carried out by Tai On Yau and Dr Bernard Burke. The result of experiments by Dr Burke are presented in figure 4.12.

Figure 4.12: The effect of different concentrations of interleukins 1 and 6 on stimulation of GM-CSF expression from the pC79/GM construct in Hep3B cells.



This result shows the synergistic effects of IL-1 and IL-6 in this system. The optimal concentration of interleukins for expression from this promoter were therefore determined to be 200U of IL-1 and 500U of IL-6. These concentrations of interleukins were used in subsequent transfection and stimulation experiments.

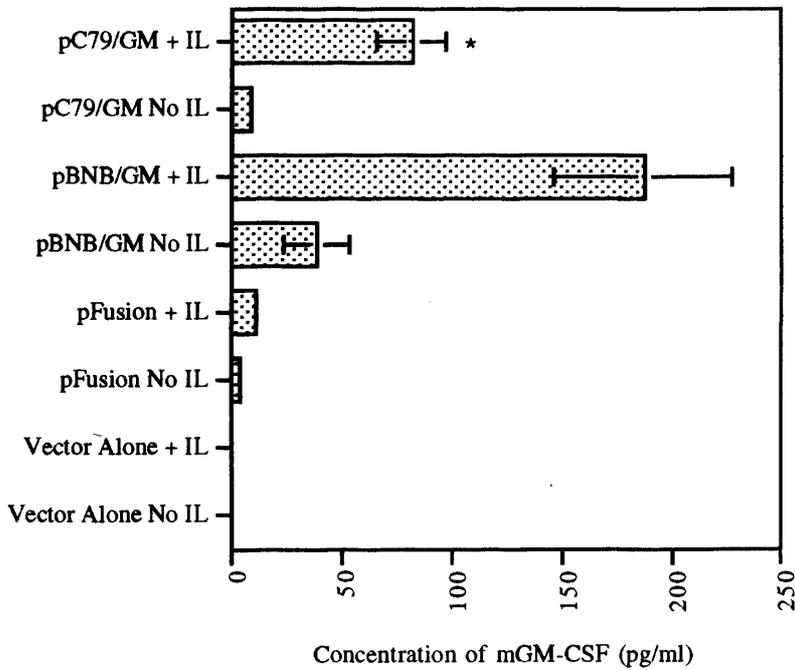
4.5.3 Comparison of the CRP/mGM-CSF Constructs.

In order to analyse the pattern of expression of GM-CSF controlled by the different acute phase vectors, the three constructs that had been produced were analysed using the tissue culture system of expression following interleukin stimulation in Hep3B cells.

The constructs were introduced to Hep3B cells using the ProFection transfection system (Promega) then, 16 hours after transfection, the medium was replaced with medium containing IL-6 at 500 units/ml and IL-1 at 200 units/ml. The cells were then incubated for 24 hours before collection of the samples. The amount of mGM-CSF in the samples was determined by using the Quantikine-M-GM-CSF ELISA kit (R&D Systems). See figures 4.13 and 4.14. The figures show the combined results from either two (pFusion), three (pBNB/GM) or four (pC79/GM) separate transfection experiments.

The basal levels of expression of GM-CSF from the three constructs were not significantly different from each other, nor were the levels induced by interleukin stimulation (Mann-Whitney U test). The fusion alone showed very low levels of GM-CSF expression both before and after interleukin stimulation; although some induction of expression was seen in response to interleukin stimulation (2.7 X induction over basal levels). The pBNB/GM construct showed fairly high basal levels of expression (38.8 ± 25.8 pg/ml), and also high levels of GM-CSF expression after interleukin stimulation (196 ± 69.2 pg/ml). The pC79/GM construct gave lower basal levels of expression (8.9 ± 4.2 pg/ml) than the pBNB/GM construct, but also showed high levels of GM-CSF expression after stimulation with interleukins (87.8 ± 14.3 pg/ml), and this was a significant increase ($5\% > P > 1\%$ Mann-Whitney U test). The level of induction of the pC79/GM construct was higher than that of the pBNB/GM construct (induction above basal levels by a factor of 5.1 for pC79/GM compared to 9.9 for pBNB/GM).

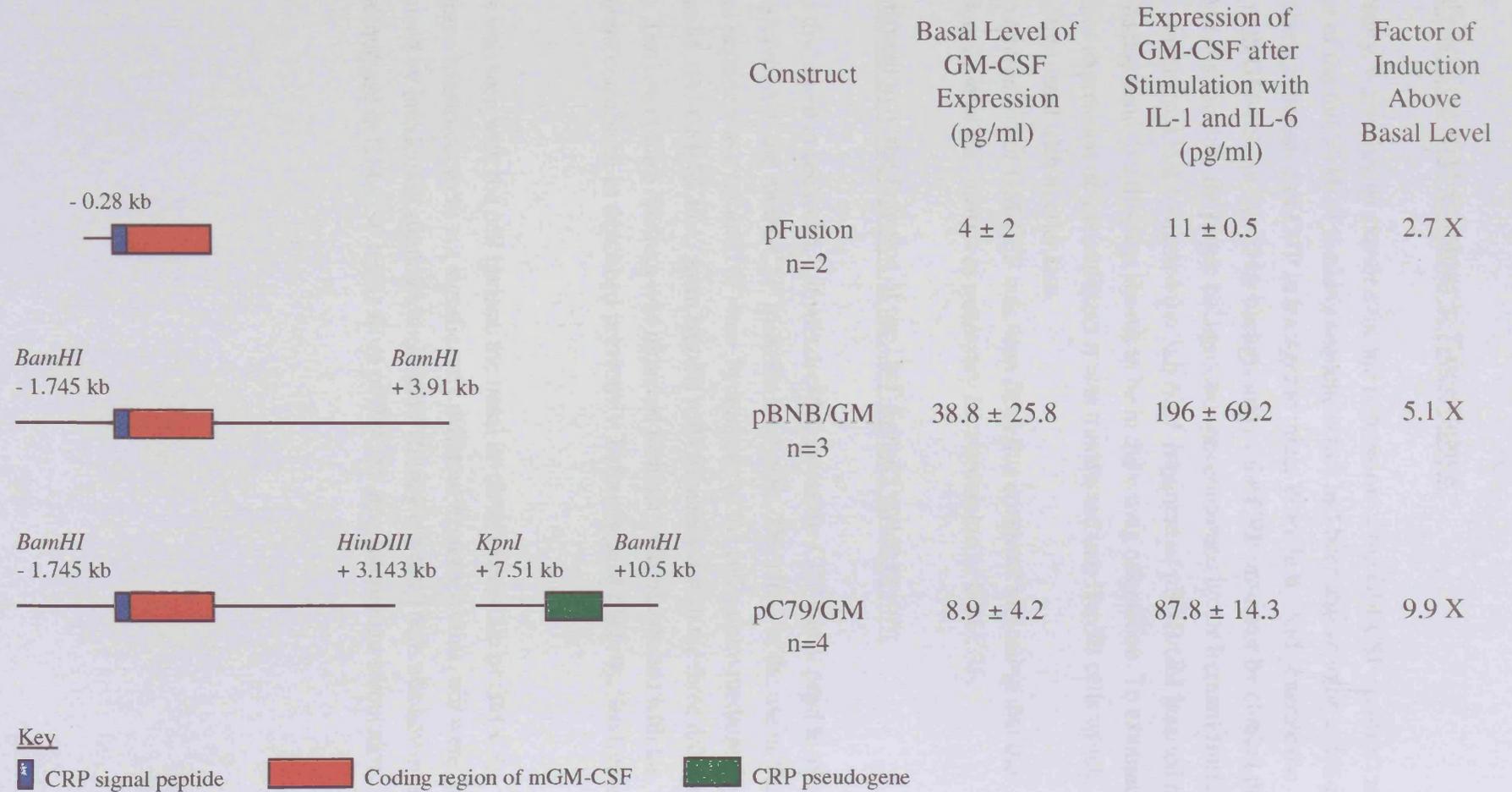
Figure 4.13: Amount of mGM-CSF in cell lysate samples from transfection experiments comparing the expression of the three acute phase constructs 24 hours after stimulation with interleukins. Error bars represent SEM.



* Significant increase in mGM-CSF expression after stimulation with IL-1 and IL-6 (5% > P > 1% Mann-Whitney U test)

The pattern of expression of the pC79/GM construct therefore gave a pattern of expression most closely resembling an acute phase response (that is low basal levels of expression before and high expression following stimulation with interleukins), and this construct was therefore used to make transgenic mice.

Figure 4.14: Schematic representation of the three constructs made and their pattern of expression in the tissue culture system.



4.5.4 Analysis of the 30 kb Construct in Tissue Culture.

Ideally, we wished to examine the the expression of the GM-CSF construct in the context of the full 30 kb of flanking sequences that had been shown to be necessary for acute phase expression of CRP in transgenic mice. Work to try and generate the construct with GM-CSF in the 30 kb background of the CRP promoter by cloning the pBNB/GM construct into the larger background was continued by Dr Bernard Burke. A clone was constructed that contained the 5kb *NotI* fragment of pBNB/GM inserted into the 30 kb background, but this was shown to be in the wrong orientation. To examine the pattern of expression of this construct it was transfected into Hep3B cells which were then stimulated with interleukins.

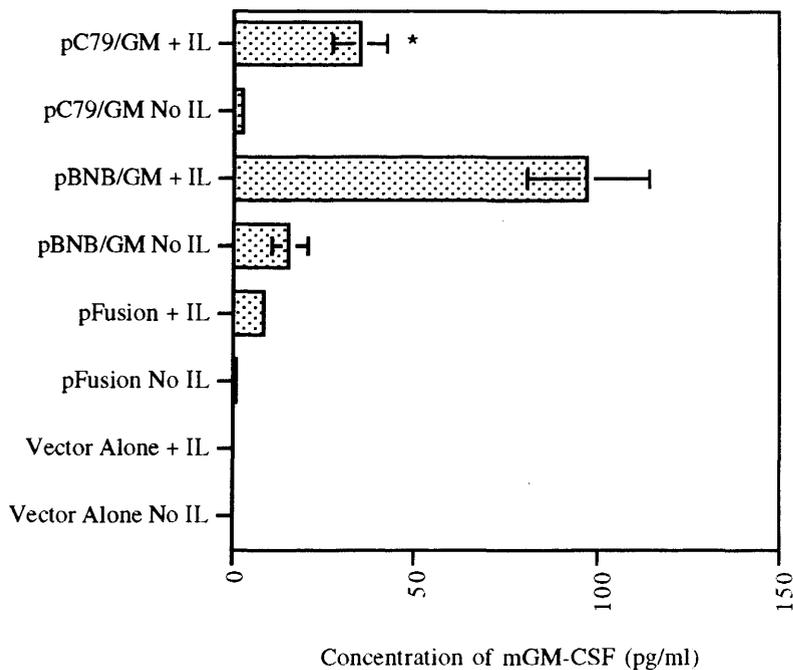
No expression of GM-CSF was seen from this construct indicating that the correct orientation of the insert was necessary for expression of GM-CSF.

4.5.5 Confirmation of the Function of the CRP Signal Peptide *in vitro*.

As discussed in section 4.1, it was decided to use the CRP signal peptide to direct the secretion of the mGM-CSF from the liver cells. The effect of the use of the CRP signal peptide was examined *in vitro* by analysis of the cell culture medium (by Quantikine-M-GM-CSF ELISA) from Hep3B cells transfected with the three different constructs. The cell culture medium was removed from the cells transfected with the three different constructs, as described previously, before lysis of the cells. See figure 4.15.

As was seen with the cell lysates, the basal levels of expression of GM-CSF from the three constructs were not significantly different from each other, nor were the levels induced by interleukin stimulation (Mann-Whitney U test). There was however a significant increase in GM-CSF levels from pC79/GM after interleukin stimulation.

Figure 4.15: Concentration of mGM-CSF in culture medium samples from transfection experiments comparing the expression of the three acute phase constructs 24 hours after stimulation with interleukins 1 and 6. Error bars represent SEM.



* Significant increase in mGM-CSF expression after stimulation with IL-1 and IL-6 (5% > P > 1% Mann-Whitney U test)

It can be seen that GM-CSF was detectable in the culture medium of these cells indicating that the protein is being effectively secreted from the cells. Visual examination of the cells using an inverted microscope before removal of the medium revealed no apparent abnormalities of the cells compared to cells that had not been transfected. This suggests that the cells were healthy and that detection of GM-CSF in the culture medium was not due to lysis of the cells, but that it was being secreted from the cell by the normal processes. Taking into account the volume of the original samples, the total amount of GM-CSF detected in the medium was also greater than the total amount of GM-CSF detected in the lysate (for example, 84 pg in the medium compared to 17.6 pg in the cell lysate for the pC79/GM construct stimulated with interleukins), again indicating the active secretion of GM-CSF from the cells.

It can also be reasoned that the CRP signal is correctly cleaved from the GM-CSF as the protein is found free in the cell culture medium and is not cell associated. This therefore validates the use of the CRP signal peptide in the creation of the fusion between CRP and GM-CSF and it can be predicted that mice transgenic for these constructs would successfully secrete GM-CSF from the liver cells.

Summary of the Results from the Preparation of the Constructs.

1. A fusion between the coding region of mGM-CSF and 290 bp of the CRP promoter was constructed.
2. Two acute phase expression vectors (pBNB and pC79-Not) containing further regions of the CRP promoter were constructed.
3. The CRP/mGM-CSF fusion was cloned into these two acute phase expression vectors.
4. Comparison of the kinetics of GM-CSF expression of these constructs in a tissue culture system showed that the pC79/GM construct gave low basal levels of expression of GM-CSF and expression was strongly induced upon stimulation with interleukins.
5. The CRP signal peptide used in these constructs directed the secretion of GM-CSF from the tissue culture cells.

We were unable to produce the ideal construct for acute phase expression of mGM-CSF in transgenic mice, that is, mGM-CSF in the 30 kb of flanking DNA of the CRP promoter that had been shown to control acute phase expression of CRP in transgenic mice. It was therefore decided to use the pC79/GM construct to produce transgenic mice; as this construct had been shown, in the tissue culture system, to exhibit fairly low levels of basal expression of GM-CSF and was strongly induced after stimulation with interleukins.

Chapter 5: Results - Production and Screening of Transgenics.

5.1 Screening CRP Transgenic Mice.

In order to set up a routine method for screening the colony of CRP mice for presence of the transgene, it was decided to set up a PCR screen from a blood sample. Primers CR1 and CR2 were designed to do this with one primer at each side of the CRP gene in order to amplify a band of approximately 800 bp.

The initial attempt to set up the screening procedure involved the use of whole blood boiled in water (to release the DNA from the white blood cells). These attempts were unsuccessful probably due to the inhibitors of the polymerase enzyme *Taq* which are released from the blood cells. Another approach was to place a drop of blood on a piece of filter paper, allow this to dry then use a small section of the blood spot in the PCR reaction. This approach was also unsuccessful. The next approach was to isolate genomic DNA from blood using the RapidPrep™ genomic DNA isolation kit (Pharmacia). This gave clear differentiation between animals possessing or not possessing the transgene (see figure 5.1) although the preparations were time consuming and a comparatively large sample of blood was required (up to 50 µl) which meant that good bleeds were necessary.

The method that was finally developed and used routinely to screen the colony used the reagent GeneReleaser™ (Bioventures). This releases DNA from whole blood and sequesters the cell lysis products that could inhibit the *Taq* polymerase. Only a small amount of blood (1 µl) was needed for the reaction and this was mixed with the GeneReleaser™ reagent in the amplification tube before a series of heating and cooling steps to lyse the blood. The amplification mix was then added to the tube and cycled to amplify the product. See figure 5.2.

Figure 5.1: Screening of CRP transgenic mice after isolation of genomic DNA from whole blood using the RapidPrep DNA isolation kit. Bands of approximately 800 bp indicate a positive result, the relevant bands of the DNA ladder are also marked.

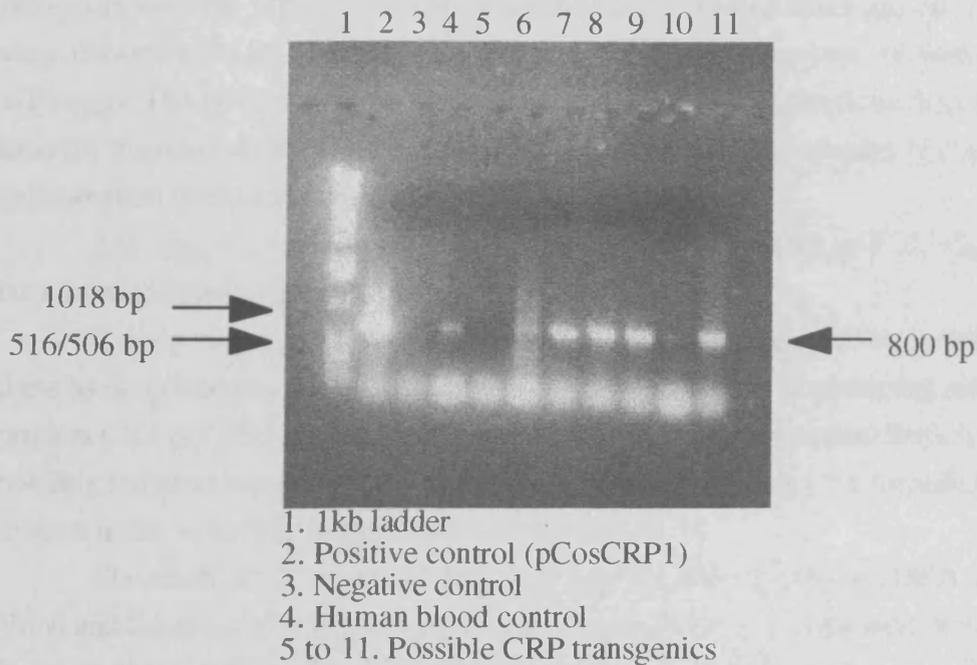
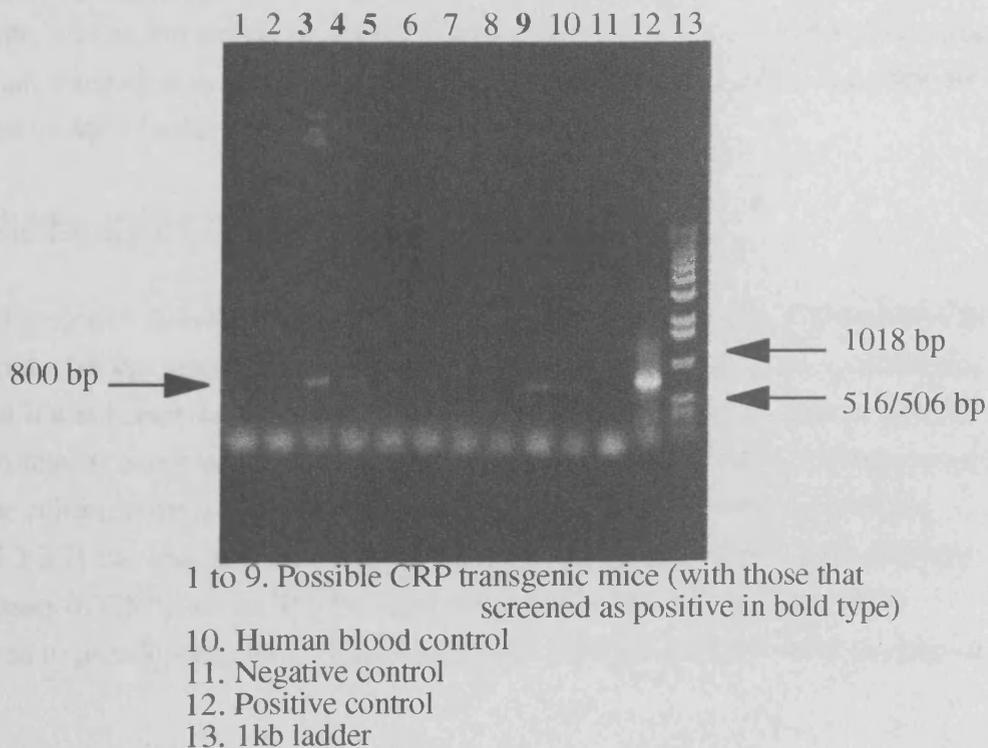


Figure 5.2: Screening of CRP transgenic mice using whole blood and the GeneReleaser™ reagent. Bands of 800 bp indicate a positive result. The relevant bands of the DNA ladder are marked.



5.2 Remaking the CRP Transgenic Line.

Because the murine model of pneumococcal infection developed in this laboratory used the MF1 mouse line it was decided to try to remake this line using the same construct (30 kb *Clal* fragment from the pCosCRP1 clone) microinjected into MF1 eggs. The DNA for microinjection was prepared as described (method 2.3.7) and used for microinjection of MF1 eggs by the transgenic unit at Leicester University (in collaboration with Dr Andrew Collick and Ms Jane Brown).

148 eggs were microinjected and implanted in 8 transfers, and 28 offspring were born from the resulting pregnancies.

Tail tip biopsies of the offspring were taken and genomic DNA prepared from these as described (method 2.3.9). The DNA was used for PCR screening using primers CR1 and CR2 and also for Southern blotting (by Dr Bernard Burke). The possible founders were also screened from a blood sample using the GeneReleaser™ reagent in the same way as the existing CRP line.

The result of all the methods of screening, PCR from genomic DNA, PCR from blood and Southern blotting (results not shown) indicated that there were no transgenic founders obtained from these microinjections.

It was subsequently realised that the production of transgenic mice using MF1 eggs is a difficult procedure as the egg membrane is very elastic. It is therefore difficult to pierce the egg membrane with the injection needle, which results in a low frequency of successfully injected embryos. Because of the problems associated with the use of MF1 eggs, subsequent injections to produce the C79/GM line of transgenic mice was carried out using eggs from the established line of mice. The transgene could then be bred onto an MF1 background by traditional breeding strategies.

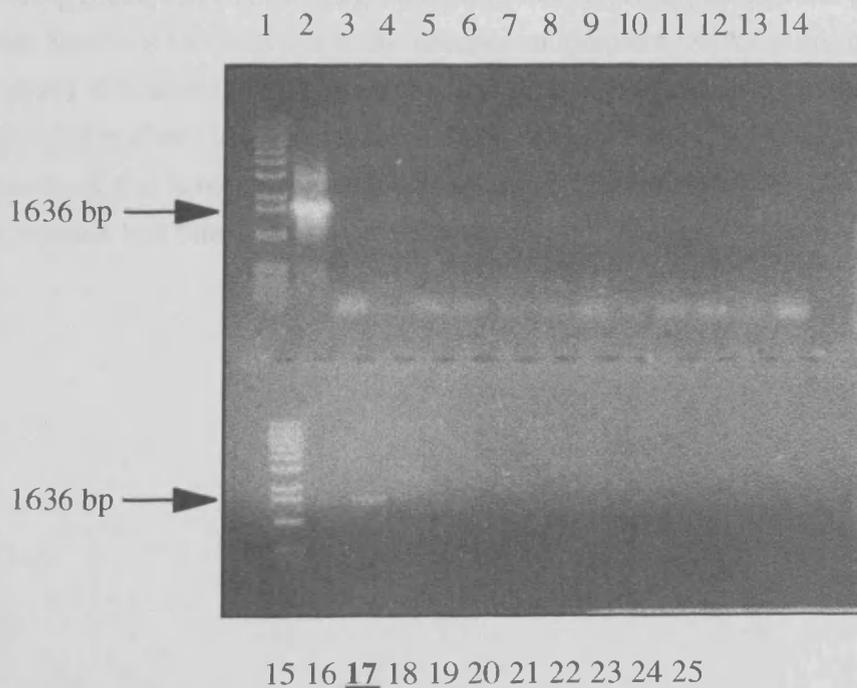
5.3 Production of the C79/GM Line of Transgenic Mice.

The 6.9 kb *BamHI* fragment from pC79/GM (containing the CRP/mGM-CSF fusion in the background of the CRP promoter) was used to make a line of transgenic mice that it was hoped would express mGM-CSF in the regulated manner of an acute phase protein. This construct was selected as it showed the best pattern of expression in the tissue culture system. The DNA for microinjection was prepared as described (method 2.3.7) and was used in microinjections of fertilized F2 mouse eggs obtained from crosses of C57Black6/CBA F1 mice. The successfully injected eggs were transferred to pseudopregnant C57Black6/CBA F1 females and allowed to develop to term.

685 eggs were microinjected and implanted in 40 transfers, and 171 offspring were obtained from the resulting pregnancies.

Tail tip biopsies were taken from these possible founder animals and genomic DNA prepared from these as described (method 2.3.9). The DNA was used for PCR screening using the primers TM8 and CPA1 (those used to check the orientation of the fusion in the acute phase vector), and also FU5PR and FU3PR (those used to create the fusion between CRP and mGM-CSF). The prepared DNA was also screened by Southern blotting (by Dr Bernard Burke). The presented result is the identification of one of the founder mice by PCR analysis of genomic DNA using the primers TM8 and CPA1 (figure 5.3).

Figure 5.3: PCR screening of possible founder C79/GM mice using primers TM8 and CPA1. A band of 1.5 kb indicates a positive result. The relevant band of the DNA ladder is marked.



- 1 and 15. 1kb ladder
- 2. Positive control (pC79/GM plasmid)
- 3. Negative control
- 4 to 14 and 16 to 25. Possible transgenic founders
- 17. Band in this lane indicates positive mouse (designated 3.3)

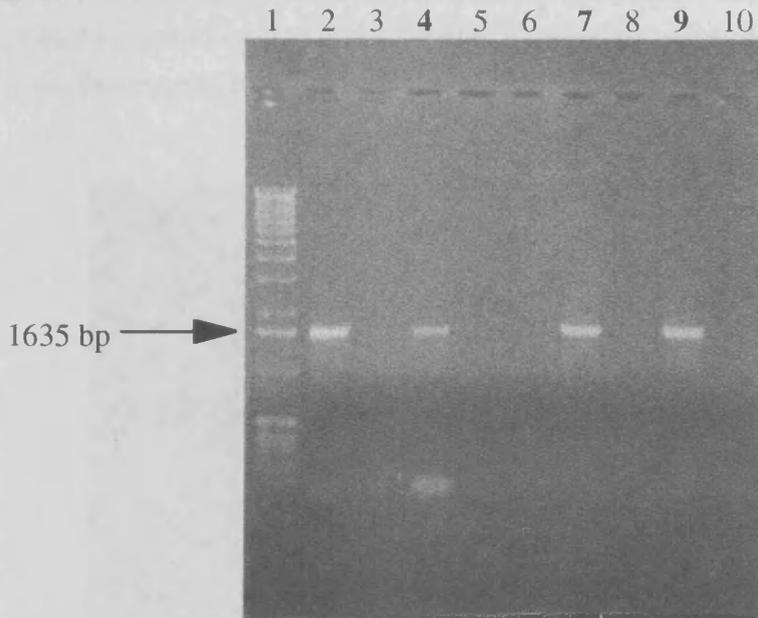
Two of the 171 possible founders screened were seen to be transgenic by both PCR and Southern blotting (designated 3.3 and 180.3). These were both male mice and

were then used in a breeding program to generate a colony of mice. One of the males (3.3) failed to produce any offspring, but the other (180.3) bred normally. It is fairly common for founder animals to be sterile; this may be due to the fact that the insertion site of the transgene can not be controlled so the site of insertion may disrupt a gene that is essential for reproduction.

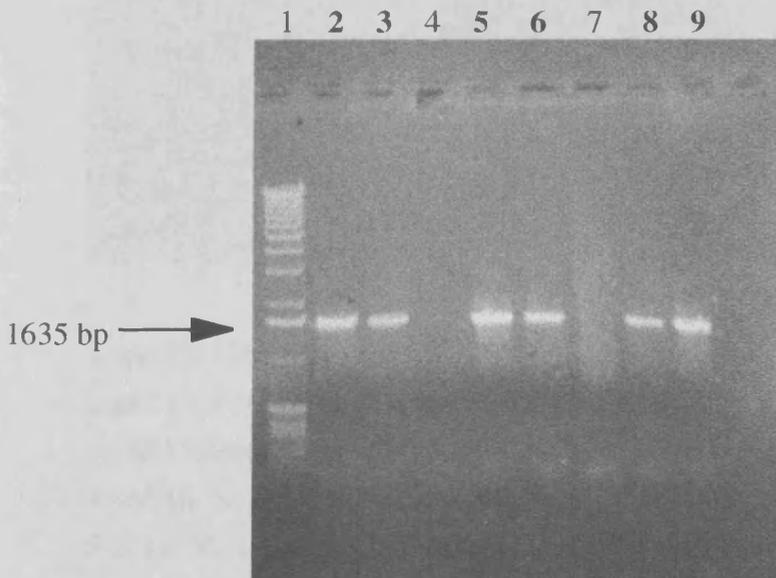
The offspring from the founder male were also screened by PCR and Southern blotting of genomic DNA prepared from tail tip biopsy, and the results of the two different screening methods compared. See figures 5.4 and 5.5.

The same mice that screened as carrying the transgene by PCR also screened as carrying the transgene by Southern blotting, except for mouse C36. Analysis of the enzyme digests on the gel used for Southern blotting showed that there appeared to be no DNA in the digest of the DNA from this mouse (result not shown). This may have been due to contamination of the digest with nucleases or the loss of the DNA pellet during precipitation of the digest. It would therefore appear that the unexpected result of this Southern blot was due to the absence of template DNA for this mouse, and that the results of screening by PCR and by Southern blot do in fact correlate. It was therefore decided to continue the screening of this colony of mice by PCR analysis only as the results of this screening compared to Southern blotting are the same and PCR analysis is a much less time consuming process.

Figure 5.4: PCR screening of the offspring of the C79/GM transgenic mouse line. A band of 1.5 kb indicates a positive result. The relevant band of the DNA ladder is marked.

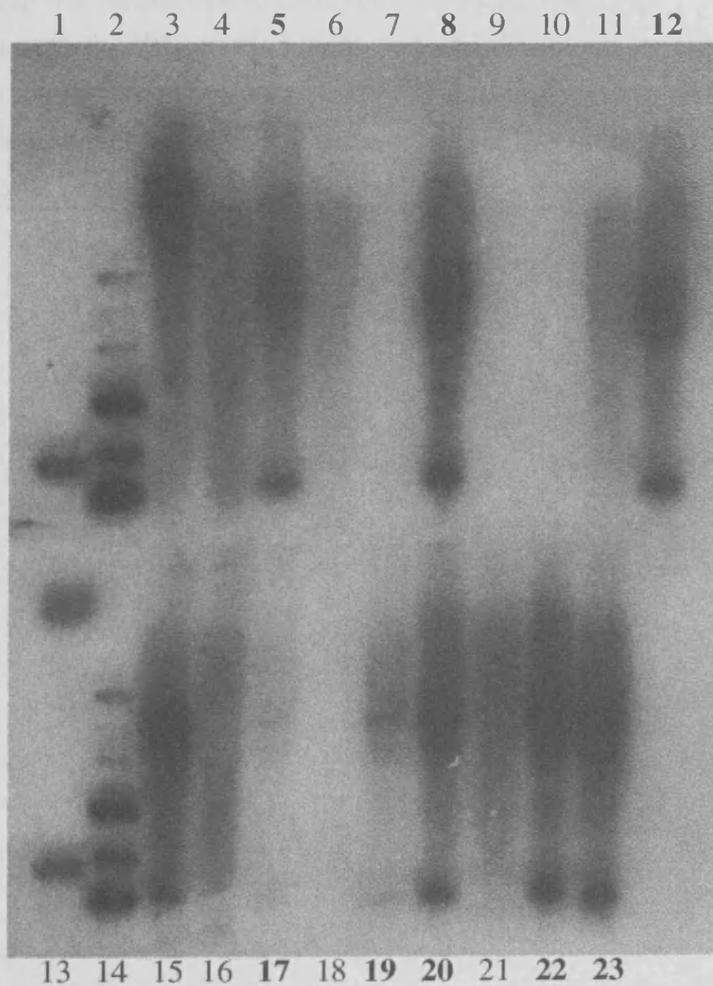


- 1. 1kb ladder
- 2. Positive control
- 3. Negative control
- 4 to 10. Mice C31 to C37. Mice C31, C34 and C36 show up as positives (**bold type**)



- 1. 1kb ladder
- 2 to 9. Mice C38 to C45. Mice C38, C39, C41, C42, C44 and C4 show up as positives (**bold type**)

Figure 5.5: Southern blot screening of the offspring of the C79/GM transgenic mouse line. The DNA was digested with the enzyme *EcoRI*, the bands separated through an agarose gel, and transferred to a nylon filter. The probe was produced from the C79/GM plasmid used to make the line of transgenic mice. A positive result is indicated by the presence of two bands of approximately 9 and 11 kb.



1 and 13. 1kb ladder

2 and 14. Positive control (pC79/GM plasmid)

3 and 15. Positive mouse control

4 and 16. Negative mouse control

5 to 12. Mice C31 to C38. Mice C31, C34 and C38 show up as positive (bold type)

17 to 23. Mice C39 to C45. Mice C39, C41, C42, C44 and C45 show up as positives (bold type)

Summary of the Results of Production and Screening of Transgenics.

1. PCR screening methods using whole blood and DNA from tail tip biopsy were developed.
2. The results of screening by Southern blot and PCR were comparable, so routine screening was subsequently carried out by PCR alone.
3. Remaking the CRP transgenic line on an MF1 mouse background was not achieved.
4. Two founder mice carrying the C79/GM transgene were produced. Only one of these was able to breed and gave rise to the C79/GM line of transgenic mice that were used in subsequent experiments.

Chapter 6: Results - Analysis of the C79/GM Transgenic Mice.

6.1 Expression of GM-CSF in C79/GM Transgenic Mice Following an Inflammatory Stimulus.

To determine whether the CRP promoter was driving the expression of GM-CSF with acute phase kinetics, in the transgenic mouse line that was created using the C79/GM construct; the mice were given an inflammatory stimulus (using LPS) and the pattern of GM-CSF in the serum measured over a period of 48 hours.

In the first experiment female mice from crosses between the founder male (C57Black6/CBA F2 generation) and MF1 females were used (this was the first generation of mice from crosses designed to breed the transgene onto an MF1 background). The mice were injected intraperitoneally with 100 µg of LPS (Sigma) in a volume of 100 µl of water (i.e. 100 µl of a 1 mg/ml solution of LPS). The mice had been screened for presence of the transgene previously and two transgenic and two non-transgenic mice were sacrificed at timepoints 0, 12, 18 and 24 hours post LPS injection and a further two non-transgenic mice were also sampled at 48 hours (due to the available numbers of transgenic mice, it was not possible to sample these at 48 hours). A cardiac bleed was taken from these mice and this blood was used to make a total white cell count (using a haemocytometer) before collecting the serum for analysis of the concentration of GM-CSF in the sample by ELISA. See figures 6.1 and 6.2.

Due to the small number of mice in each group, it was not possible to perform statistical analyses, so the trends are discussed.

The results indicate that, in this line of transgenic mice, GM-CSF is being expressed in the manner of an acute phase protein. The level of GM-CSF in both the transgenic (19.5 ± 19.5 pg/ml serum) and non-transgenic mice (10 ± 10 pg/ml serum) is very low at time 0 hours. In transgenic mice, following administration of LPS, the level of GM-CSF detected in the serum is increased dramatically by 12 hours (to 4580 ± 327 pg/ml serum) compared to basal levels and the levels in non-transgenic mice at this time (7.5 pg/ml serum). The level of GM-CSF in the transgenic mice began to fall by 18 hours after LPS injection (285.5 ± 3.5 pg/ml serum), however this is still higher than the level in non-transgenic mice at this time (11 ± 11 pg/ml serum). By 24 hours after LPS injection, the level of GM-CSF in the transgenic mice had fallen back to basal levels of expression.

Figure 6.1: Concentration of mGM-CSF in serum samples from C79/GM x MF1 female mice after intraperitoneal administration of 100 μ g of LPS. n=2 for each timepoint. Error bars represent SEM.

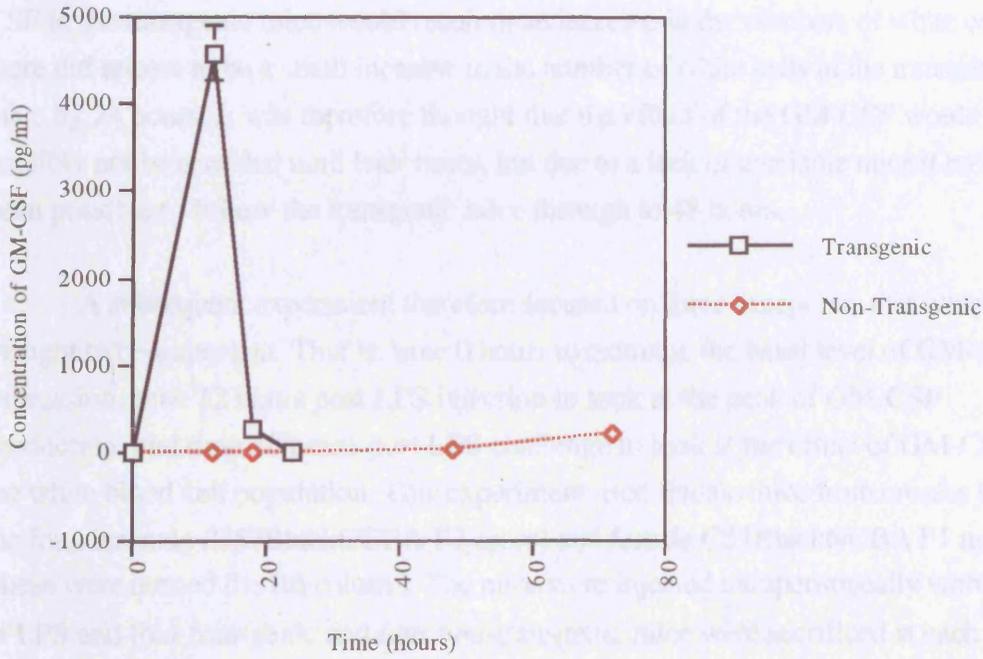
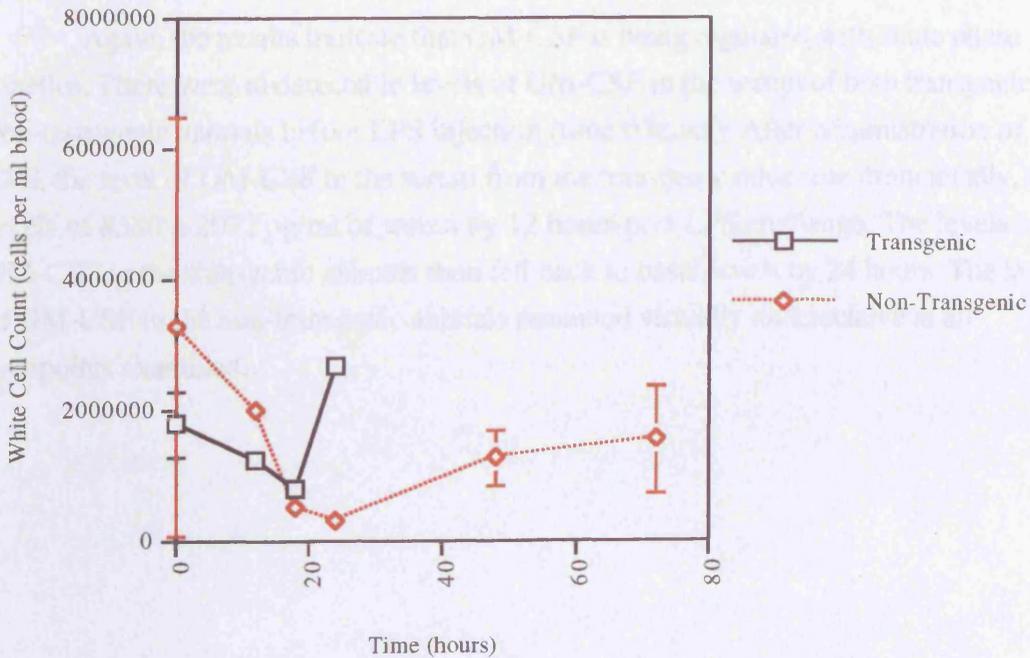


Figure 6.2: Total white blood cell count of blood samples from C79/GM x MF1 female mice after intraperitoneal administration of 100 μ g of LPS. n=2 for each timepoint. Error bars represent SEM..



The total white cell count from the transgenic and non-transgenic mice did not differ greatly throughout the course of the experiment. The white cell counts from these mice also fall within the stated average values of murine white cell counts, $6-15 \times 10^6$ cells/ml (Wolfensohn and Lloyd, 1994). It was thought that the increased levels of GM-CSF in the transgenic mice would result in an increase in the numbers of white cells, and there did appear to be a small increase in the number of white cells in the transgenic mice by 24 hours. It was therefore thought that the effect of the GM-CSF would possibly not be revealed until later times, but due to a lack of available mice it had not been possible to follow the transgenic mice through to 48 hours.

A subsequent experiment therefore focused on three timepoints that were thought to be important. That is, time 0 hours to examine the basal level of GM-CSF expression, time 12 hours post LPS injection to look at the peak of GM-CSF production, and time 48 hours post LPS challenge to look at the effect of GM-CSF on the white blood cell population. This experiment used female mice from crosses between the founder male (C57Black6/CBA F2 cross) and female C57Black6/CBA F1 mice (these were termed the B6 colony). The mice were injected intraperitoneally with 100 μ g of LPS and four transgenic and four non-transgenic mice were sacrificed at each timepoint with a cardiac bleed being taken. This blood was again used to determine the total white cell count of the whole blood, then the serum separated from this and stored at -70°C for analysis of the concentration of GM-CSF by ELISA. See figures 6.3 and 6.4.

Again, the results indicate that GM-CSF is being regulated with acute phase kinetics. There were undetectable levels of GM-CSF in the serum of both transgenic and non-transgenic animals before LPS injection (time 0 hours). After administration of LPS, the level of GM-CSF in the serum from the transgenic mice rose dramatically, with levels of 8580 ± 2072 pg/ml of serum by 12 hours post LPS challenge. The levels of GM-CSF in the transgenic animals then fell back to basal levels by 24 hours. The levels of GM-CSF in the non-transgenic animals remained virtually undetectable at all timepoints examined.

Figure 6.3: Concentration of mGM-CSF in serum samples from C79/GM x B6CBF1 female mice after intraperitoneal administration of 100 μ g of LPS. n=4 for each timepoint. Error bars represent SEM.

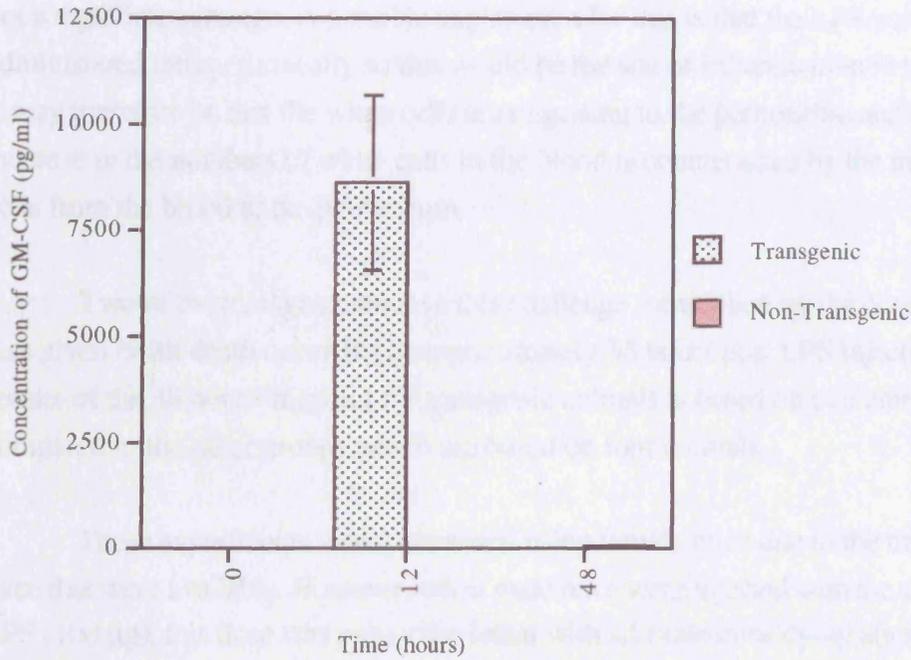
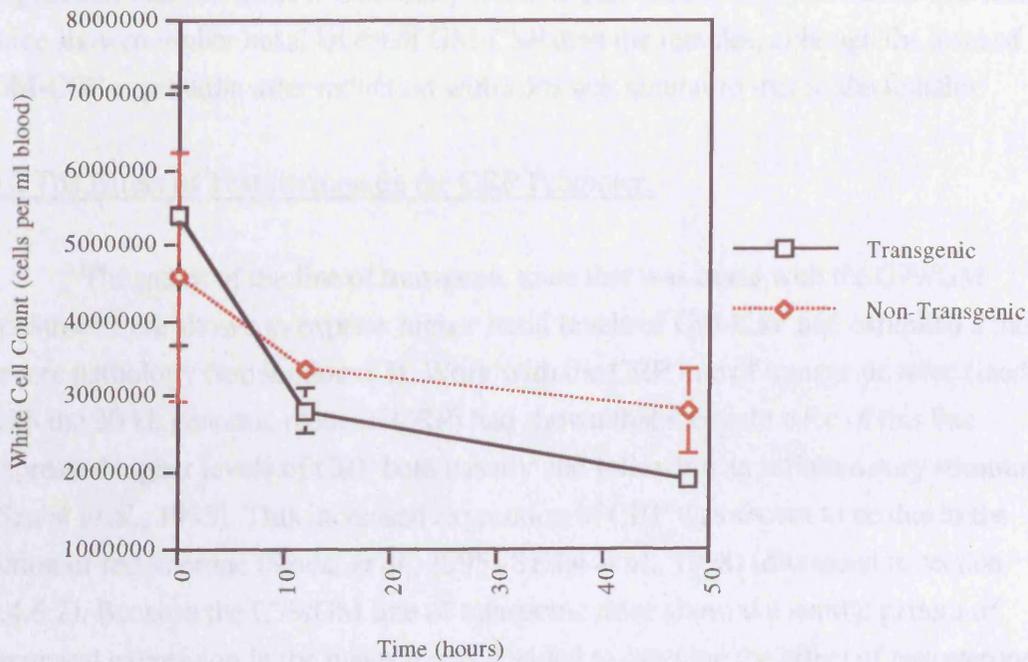


Figure 6.4: Total white blood cell count of blood samples from C79/GM x B6CBF1 female mice after intraperitoneal administration of 100 μ g of LPS. n=4 for each timepoint. Error bars represent SEM.



The white blood cell count again did not alter significantly during the course of the experiment (Mann-Whitney U test). There were no significant changes in the number of white cells in the blood of the transgenic animals by 48 hours which indicates that the small increase in white cell numbers observed in the previous experiment was not a significant change. A possible explanation for this is that the LPS was administered intraperitoneally so this would be the site of inflammation in these animals. It may therefore be that the white cells are migrating to the peritoneum and that any increase in the numbers of white cells in the blood is counteracted by the migration of cells from the blood to the peritoneum.

Two of the transgenic mice in this challenge were killed by the dose of LPS that was given (with death occurring at approximately 36 hours post LPS injection), so the results of the 48 hour timepoint for transgenic animals is based on two animals compared to the other groups which are based on four animals.

These experiments were performed using female mice due to the numbers of mice that were available. However, when male mice were injected with the same dose of LPS (100 µg), this dose was seen to be lethal with all male mice dying after just a few hours. These experiments were repeated by Dr Bernard Burke using both male and female mice in the study and also using a lower dose of LPS (25 µg). A more sensitive, commercially available, ELISA system (Quantikine™ M-GM-CSF) was also employed to determine more accurately the basal level of GM-CSF in these mice. It was shown that the female mice did in fact have very low basal levels of GM-CSF, and that this expression was increased dramatically following an inflammatory stimulus. The male mice showed higher basal levels of GM-CSF than the females, although the level of GM-CSF expression after induction with LPS was similar to that of the females.

6.2 The Effect of Testosterone on the CRP Promoter.

The males of the line of transgenic mice that was made with the C79/GM construct were shown to express higher basal levels of GM-CSF and exhibited a more severe pathology (see section 6.3). Work with the CRP line of transgenic mice (made with the 30 kb genomic clone of CRP) had shown that the male mice of this line expressed higher levels of CRP both basally and following an inflammatory stimulus (Szalai *et al.*, 1995). This increased expression of CRP was shown to be due to the action of testosterone (Szalai *et al.*, 1995)(Szalai *et al.*, 1998) (discussed in section 1.4.6.2). Because the C79/GM line of transgenic mice showed a similar pattern of increased expression in the males it was decided to examine the effect of testosterone on

this construct in a tissue culture system. It was reasoned that if a testosterone mediated effect could be shown to influence the higher basal levels of expression in the male mice then the regulatory elements of the promoter controlling this effect could be identified and subsequently a different acute phase expression vector eliminating these regions constructed.

The two constructs BNB/GM and C79/GM were studied as these were made with different regions of the CRP promoter and flanking DNA, so it was predicted that these might show a different responsiveness to testosterone. The constructs were introduced to Hep3B cells using the ProFection system then, 16 hours after transfection, the culture medium was replaced with stimulation medium containing combinations of interleukins and human testosterone. The cells were incubated in the stimulation medium for 24 hours before samples were collected and assayed for the presence of GM-CSF using the Quantikine-M-GM-CSF ELISA. See figure 6.5 and table 6.1.

Figure 6.5: Concentration of mGM-CSF in cell lysate samples from tissue culture experiment looking at the effect of testosterone on the CRP promoter.

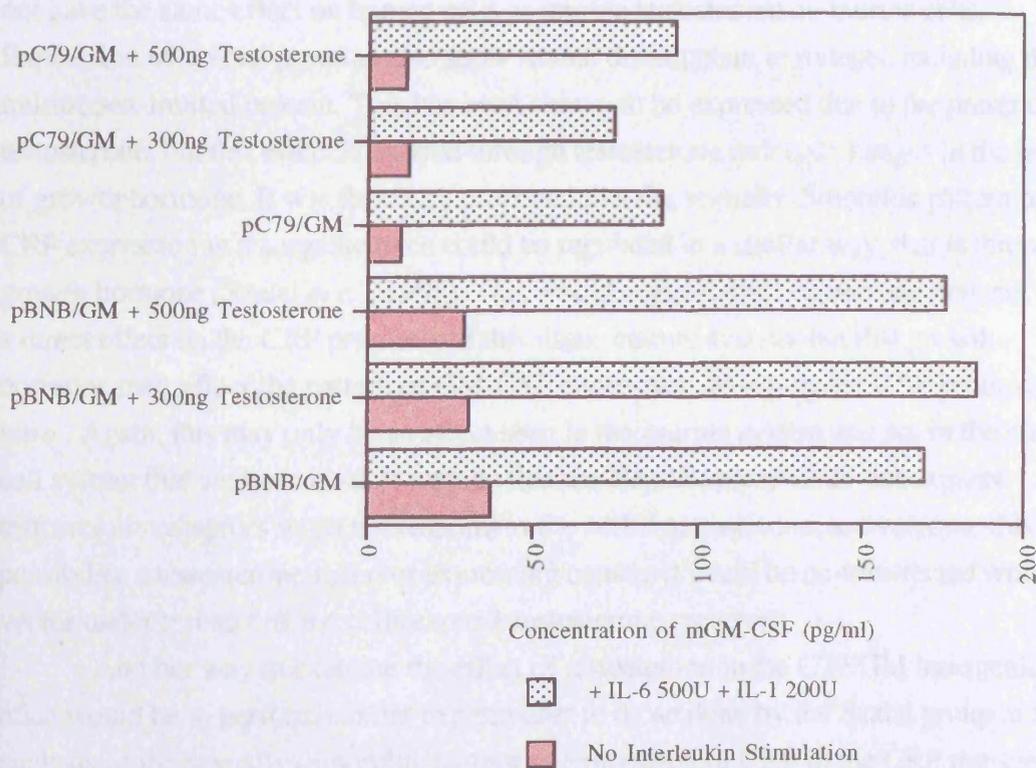


Table 6.1: Induction of GM-CSF expression by testosterone.

Treatment	Induction on Addition of Testosterone (no interleukins)	Induction on Addition of Testosterone (with interleukins)
BNB/GM + 300 ng Testosterone	1.2 X Decrease	1.1 X
BNB/GM + 500 ng Testosterone	1.2 X Decrease	1.04 X
C79/GM + 300 ng Testosterone	1.3 X	1.2 X Decrease
C79/GM + 500 ng Testosterone	1.2 X	1.05 X

This showed that testosterone in this system did not appear to have any effect on either the basal levels of GM-CSF expression (induction with no interleukins) or the level of inducible expression (induction with interleukins). This may be because the tissue culture system used was a human cell line and stimulated with human testosterone. A sexually dimorphic pattern of CRP expression is not seen in the human system and is only seen in the CRP transgenic mice, therefore human testosterone may not have the same effect on human cells as murine testosterone on murine cells. Expression of several proteins also show sexual dimorphism in rodents, including the murine sex-limited protein. This has been shown to be expressed due to the presence of testosterone, but this effect is exerted through testosterone induced changes in the levels of growth hormone. It was therefore proposed that the sexually dimorphic pattern of CRP expression in transgenic mice could be regulated in a similar way, that is through growth hormone (Szalai *et al.*, 1998). This would suggest that testosterone may not have a direct effect on the CRP promoter in this tissue culture system, but that growth hormone may affect the pattern of GM-CSF expression driven by the CRP promoter *in vitro*. Again, this may only be an effect seen in the murine system and not in the human cell system that we have used. It may be that the Hep3B cells used do not express testosterone receptors so do not respond to the added testosterone, to overcome this possibility a testosterone-receptor expressing construct could be co-transfected with the vector under test so that the cells express testosterone receptors.

Another way to examine the effect of testosterone in the C79/GM transgenic mice would be to perform similar experiments to those done by the Szalai group in the analysis of the sexually dimorphic pattern of expression of CRP in the CRP transgenic mice (Szalai *et al.*, 1995). That is to see if the basal levels of expression of GM-CSF in the male mice are reduced following castration and are restored following implantation with testosterone releasing pellets.

6.3 Pathology in the C79/GM Transgenic Mice.

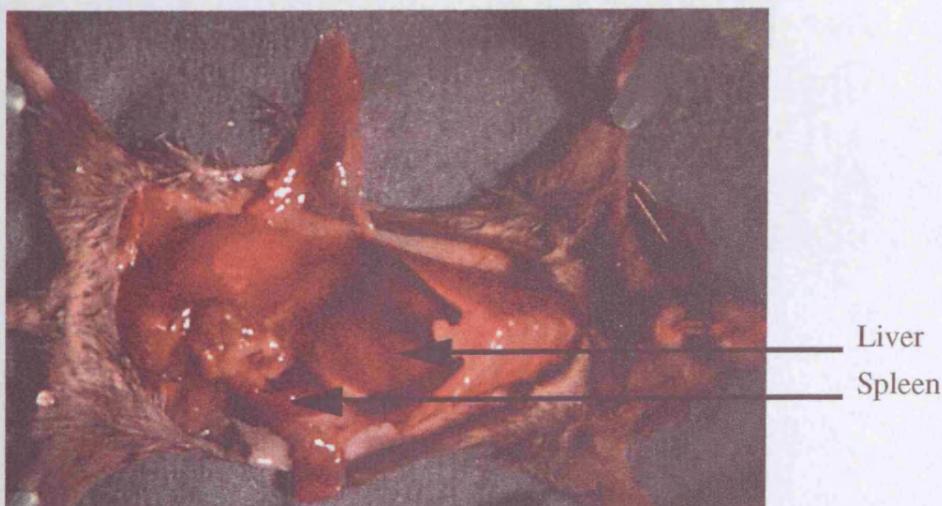
Mice were screened for the level of expression of GM-CSF, and transgenic mice expressing high or low levels of GM-CSF and non-transgenic mice (as controls) were culled and examined post mortem. The non-transgenic mice appeared normal, but the transgenic mice exhibited a number of pathological differences. These were more pronounced in the mice expressing higher levels of GM-CSF and also more pronounced in the male mice. The abdominal muscles appeared to be very thin and transparent, the liver was enlarged and mottled and the spleen was massively enlarged with rounded edges. See figures 6.6.

The transgenic mice also showed a reduced survival time compared with the non-transgenic animals, with some mice dying as young as seven weeks of age. Postmortem analysis of these mice (when possible) revealed the same pathological changes of thin abdominal muscles, enlarged spleen and liver with mottled edges to the liver. These pathologies were often very severe in the mice that died at a young age and it was reasoned that the cause of death in these animals was the noted pathology.

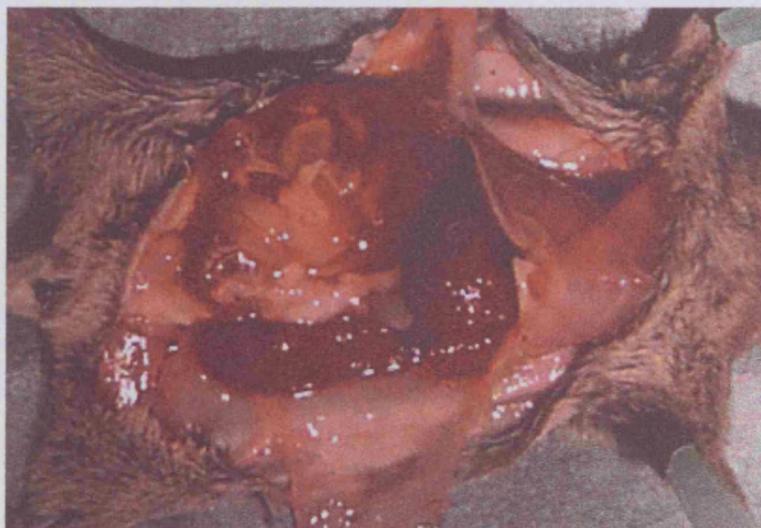
Histological analysis of the spleen revealed a normal white pulp and a massively increased red pulp containing many precursor cells of all of the different white blood cell lineages (see figures 6.7). Similar extramedullary haemopoiesis was also seen in the liver, although to a lesser extent (see figures 6.8). Because the pathological changes were more pronounced in the male mice, all the figures show sections of tissues taken from male mice (transgenic or non-transgenic controls).

Figure 6.6: Gross pathology of the C79/GM-CSF transgenic mice.

6.6A: Non-transgenic mouse showing normal spleen and liver



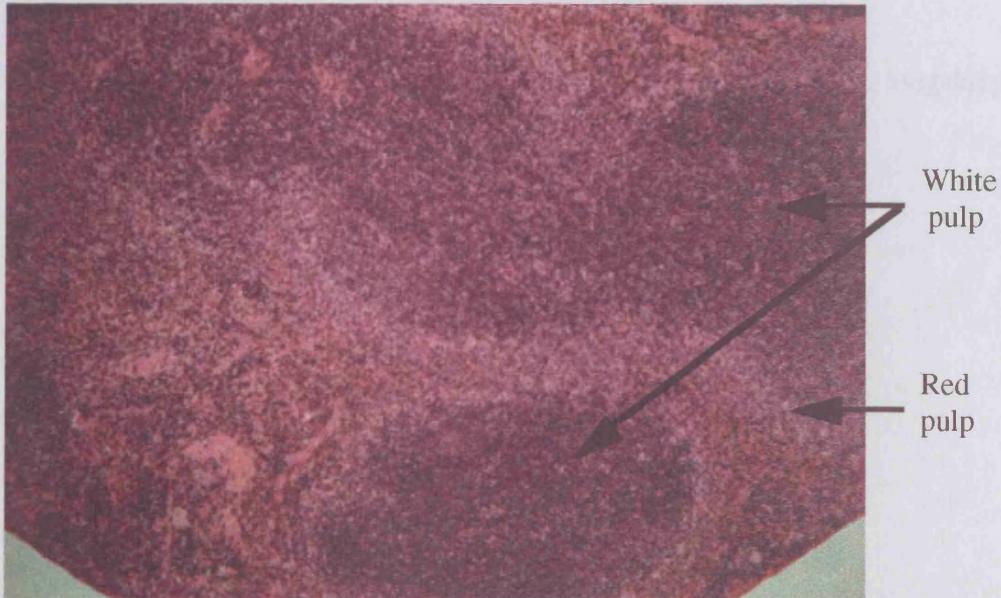
6.6B: Transgenic mouse showing enlarged spleen and liver, and mottled edges of the liver.



6.6C: Transgenic mouse showing massively enlarged spleen and liver, and mottled edges of the liver.



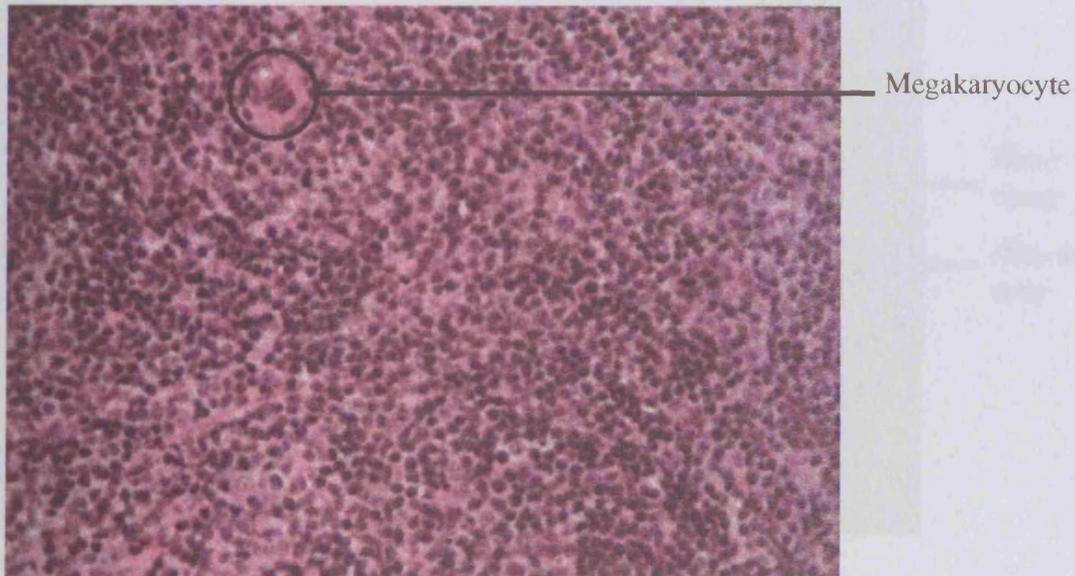
Figure 6.7: Histological analysis of the spleen from C79/GM-CSF transgenic mice.
6.7A: Spleen from non-transgenic male, magnification X100. Normal spleen showing normal white pulp and normal red pulp.



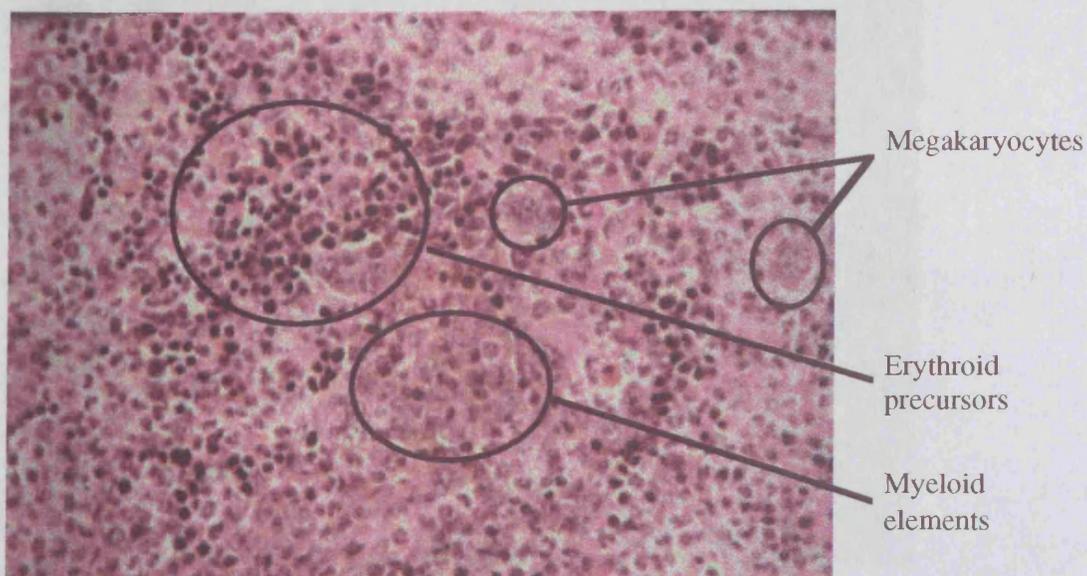
6.7B: Spleen from transgenic male, magnification X100. The red pulp is greatly expanded and has largely replaced the white pulp.



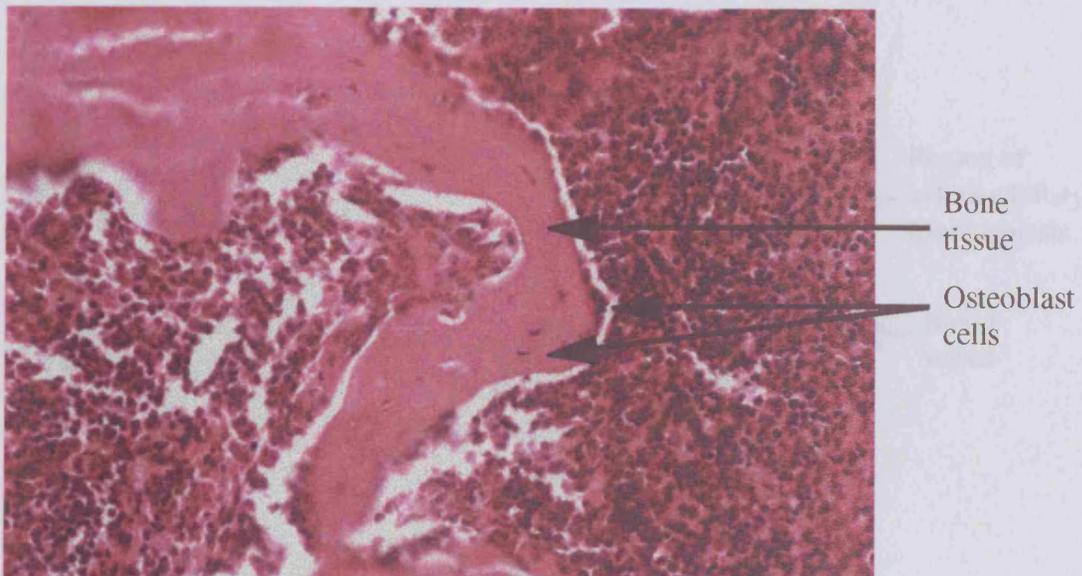
6.7C: Spleen from non-transgenic male, magnification X400. Normal spleen, although occasional megakaryocytes are identified, indicating a low degree of extramedullary haemopoiesis.



6.7D: Spleen from transgenic male, magnification X400. The red pulp contains a very large amount of extramedullary haemopoiesis including erythroid and myeloid elements and megakaryocytes.

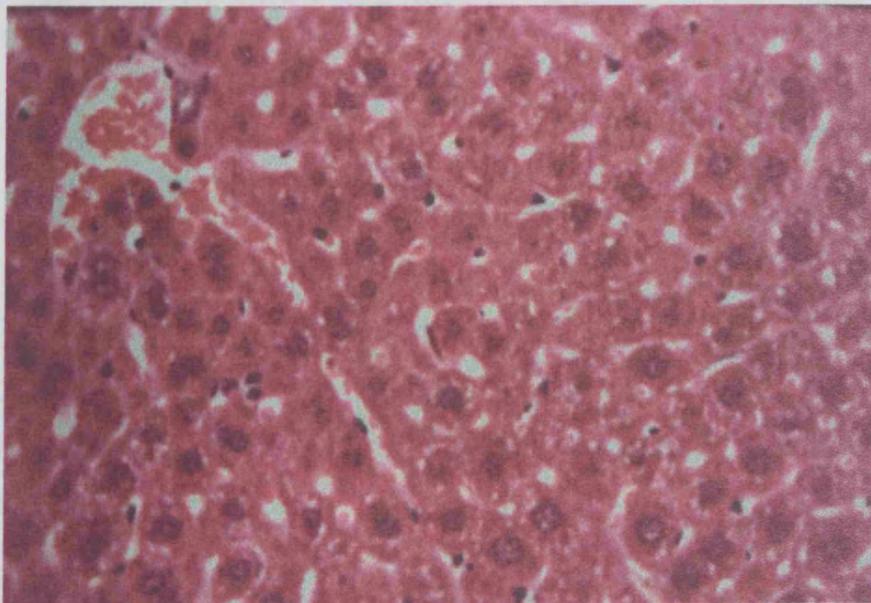


6.7E: Spleen from transgenic male showing the presence of a large trabecula of metaplastic bone.

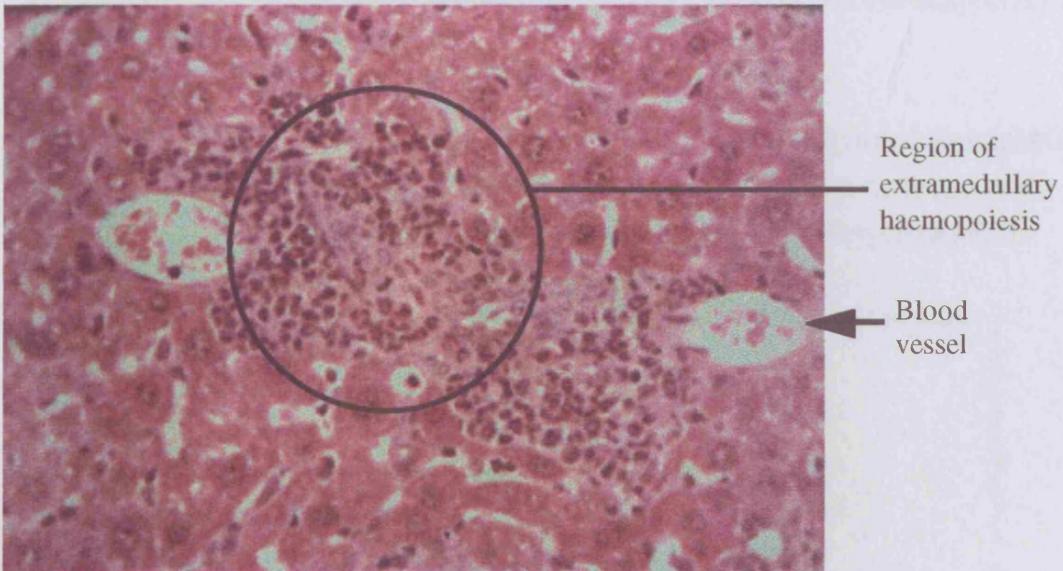


Figures 6.8: Pathology of the liver from C79/GM-CSF transgenic male mice.

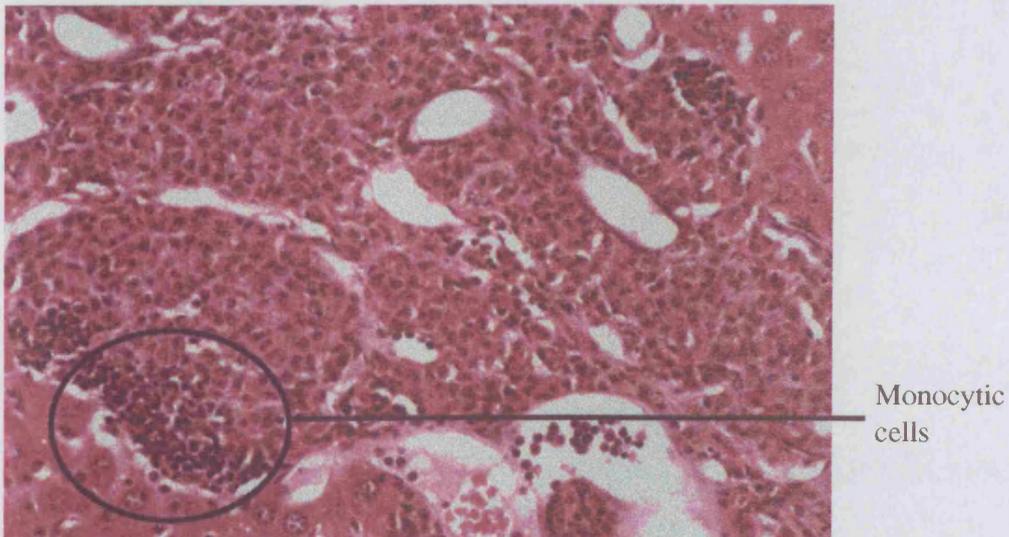
6.8A: Liver from non-transgenic male, a normal liver.



6.8B: Liver from transgenic male, magnification X400. The portal tract contains numerous cells, many of which are mature neutrophils.



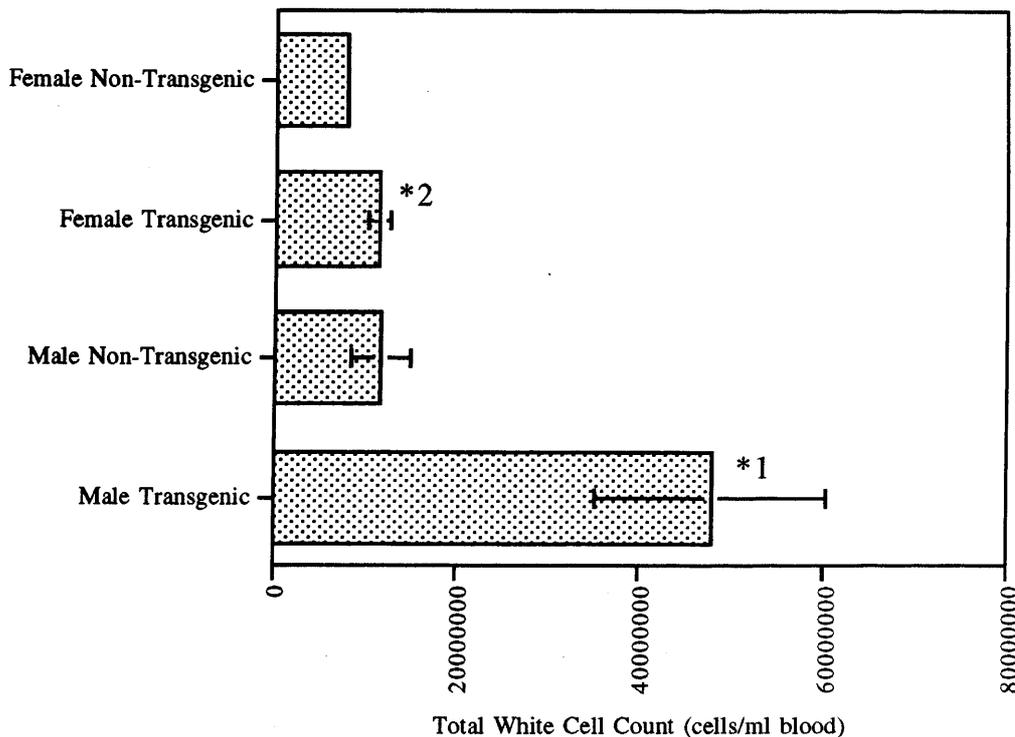
6.8C: Liver from transgenic male, showing collections of monocytic cells.



6.4 White Blood Cell Counts from C79/GM Transgenic Mice.

Total white blood cell counts from the blood of the mice were determined using a haemocytometer. See figure 6.10.

Figure 6.10: Total white cell count in the blood of GM-CSF transgenic mice (combined data from all the animals examined at 0 hours before challenge with *S. pneumoniae*); for each group n=22. Error bars represent SEM.



*1 Significant difference between the counts from the male transgenic mice and all the other groups ($P < 1\%$ for all comparisons Mann-Whitney U test)

*2 Significant difference between the counts from the transgenic females and female non-transgenics ($5\% > P > 1\%$ Mann-Whitney U test)

The cell count from the male transgenic group of mice ($4.8 \times 10^7 \pm 1.3 \times 10^7$ cells/ml blood) was higher than the counts from the other groups ($1.2 \times 10^7 \pm 3.2 \times 10^6$ cells/ml blood for male non-transgenics, $1.1 \times 10^7 \pm 1.1 \times 10^6$ cells/ml blood for female transgenics, and $8 \times 10^6 \pm 8.2 \times 10^5$ cells/ml blood for female non-transgenics). The difference between the counts from the male transgenic group and the other groups was significant in each case ($P < 1\%$ Mann-Whitney U test). There was also a significantly higher count of white cells in the blood of the female transgenic mice compared to the female non-transgenic mice ($5\% > P > 1\%$ Mann-Whitney U test).

Microscopic examination of blood smears taken from the mice also indicated the presence of an increased number of white cells in the blood of male transgenic mice compared to the other mice. See figures 6.11 A and B.

Figure 6.11A: Typical field of view from a smear of blood from a male C79/GM transgenic mouse, stained with Giemsa stain, magnification X400.

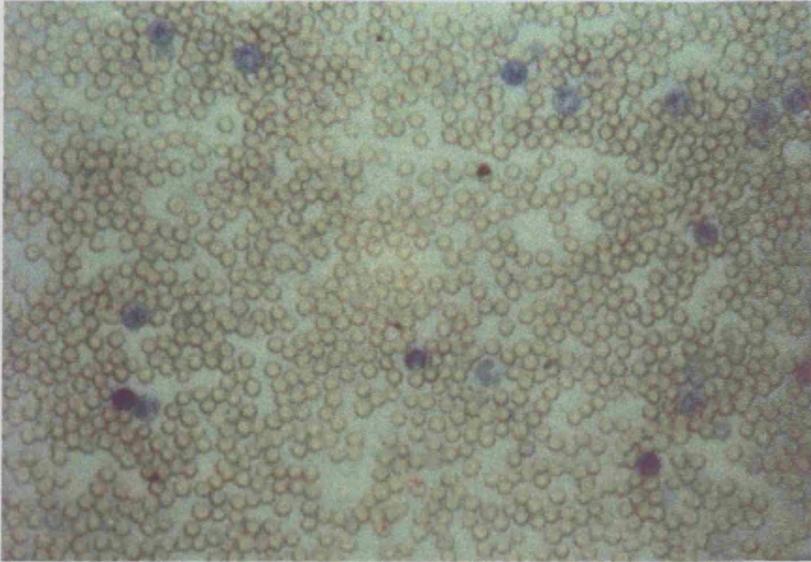
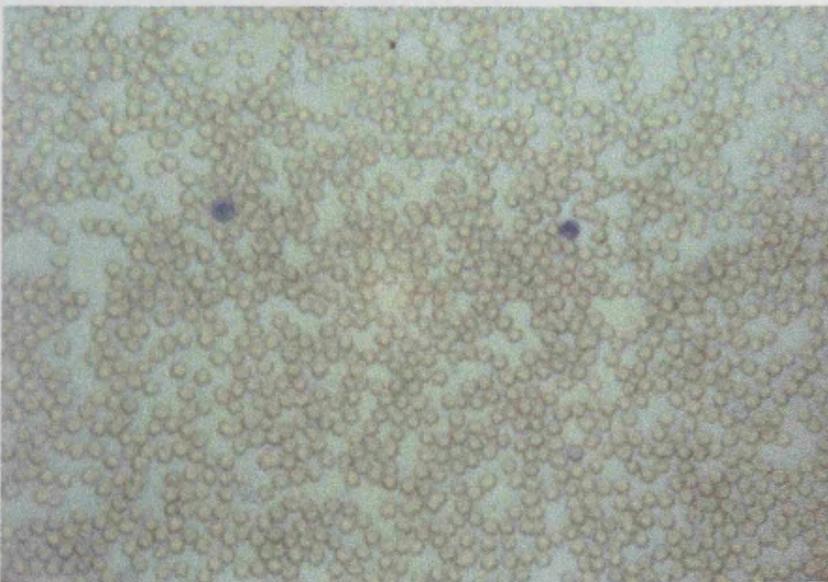


Figure 6.11B: Typical field of view from a smear of blood from a male non-transgenic mouse, stained with Giemsa stain, magnification X400.



FACS analysis of the blood of one each of the male and female transgenic and non-transgenic mice was carried out at each timepoint using three combinations of antibodies. One set used the antibodies F4/80 which labels monocytic cells and mac3

which labels activated macrophages; the second set of antibodies was CD11b which labels granulocytes and monocytes and Ly6g which labels neutrophils; the third antibody used IgG1 which was included as a negative control. The pattern of staining was similar within each group throughout the course of the experiment, and the results of analysis with the antibodies F4/80 and mac3 are presented. See figure 6.12 to 6.15.

For each set of data, the scatter plots (top) indicate the size (y axis labelled SSC) and density (x axis labelled FSC) of the cells and the histograms (bottom) indicate the extent of staining of the cells with the fluorescent antibody. The high staining peaks of cells were gated then transposed back onto the scatter plots to identify the groups of cells that were highly stained by the antibody (black dots on the scatter plot).

Both the male and female transgenic mice had a large group of cells that stained strongly with the monocytic marker F4/80, but the non-transgenic mice had very few cells that were stained with this marker. Both the male and female transgenic mice also had a group of larger and more dense group of cells that were stained with the mac3 antibody indicating that these were activated macrophage cells. These results indicate that the transgenic mice possess more macrophage-like cells than the non-transgenic mice and that the cells from transgenic animals are activated to a different state than the cells from the non-transgenic animals.

Figure 6.12: FACS analysis of the blood of a C79/GM transgenic male mouse.

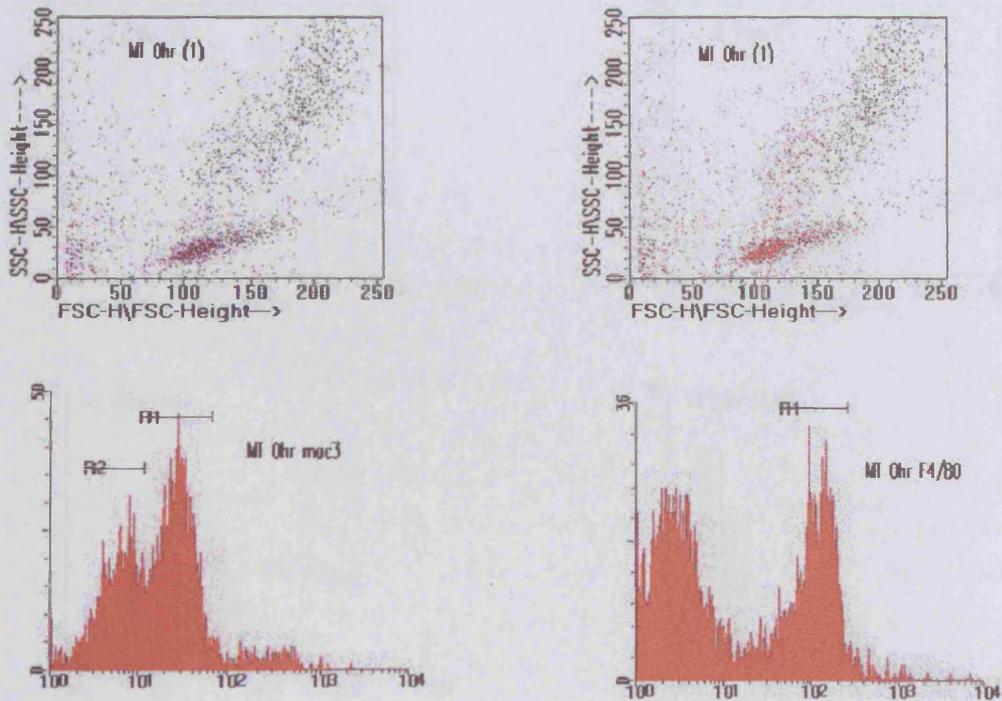


Figure 6.13: FACS analysis of the blood of a C79/GM non-transgenic male mouse.

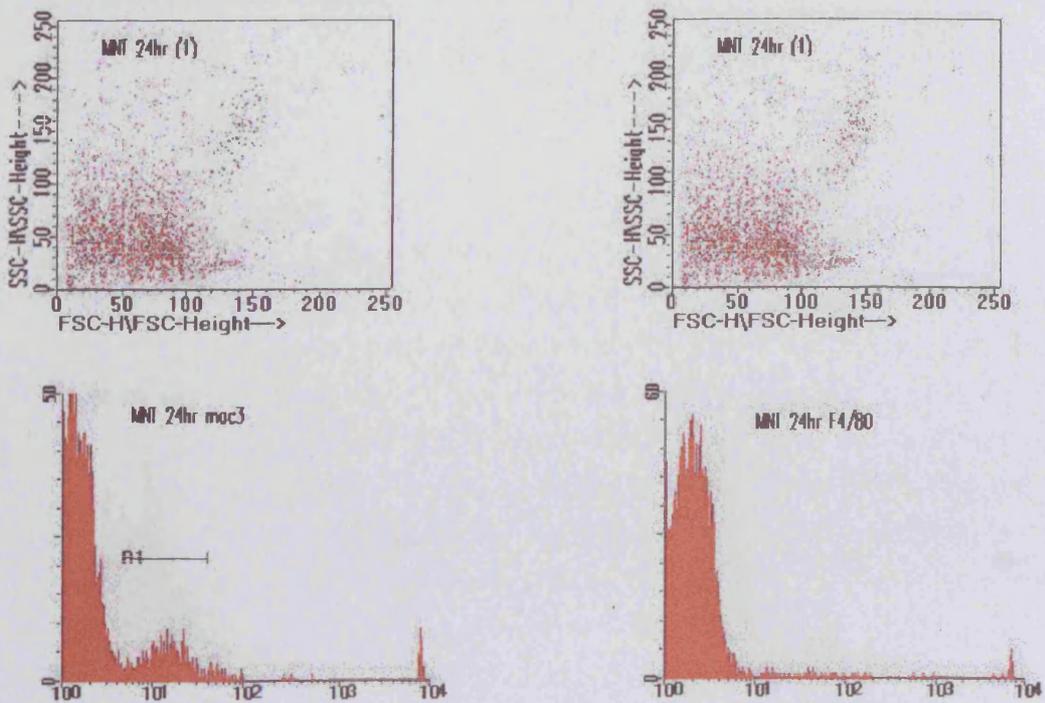


Figure 6.14: FACS analysis of the blood of a C79/GM transgenic female mouse.

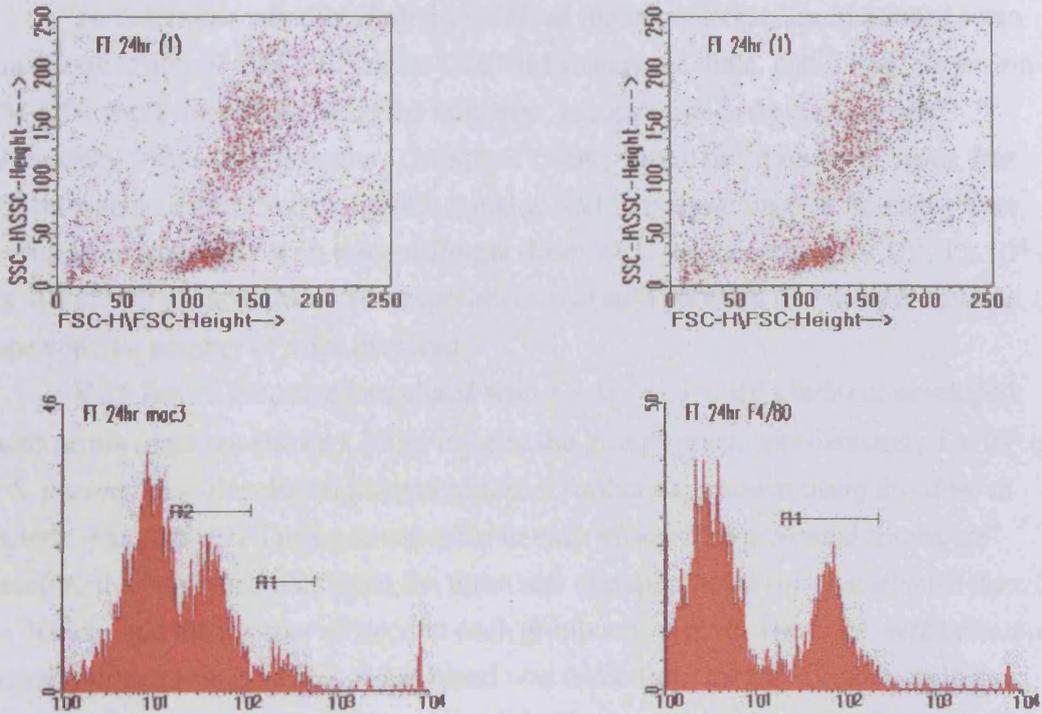
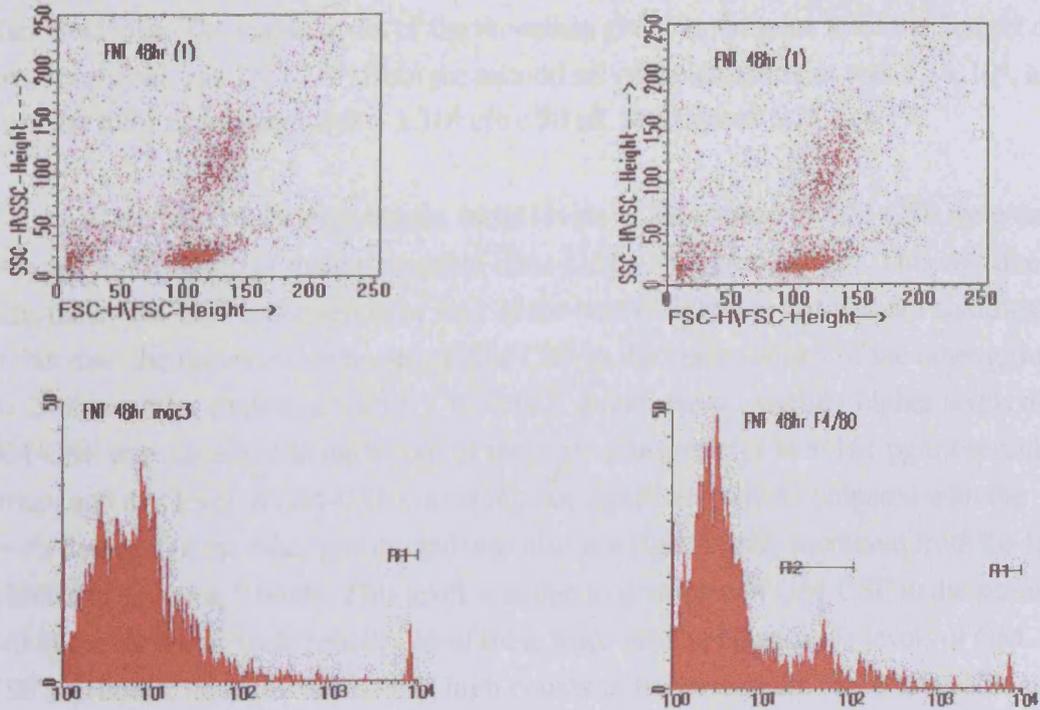


Figure 6.15: FACS analysis of the blood of a C79/GM non-transgenic female mouse.



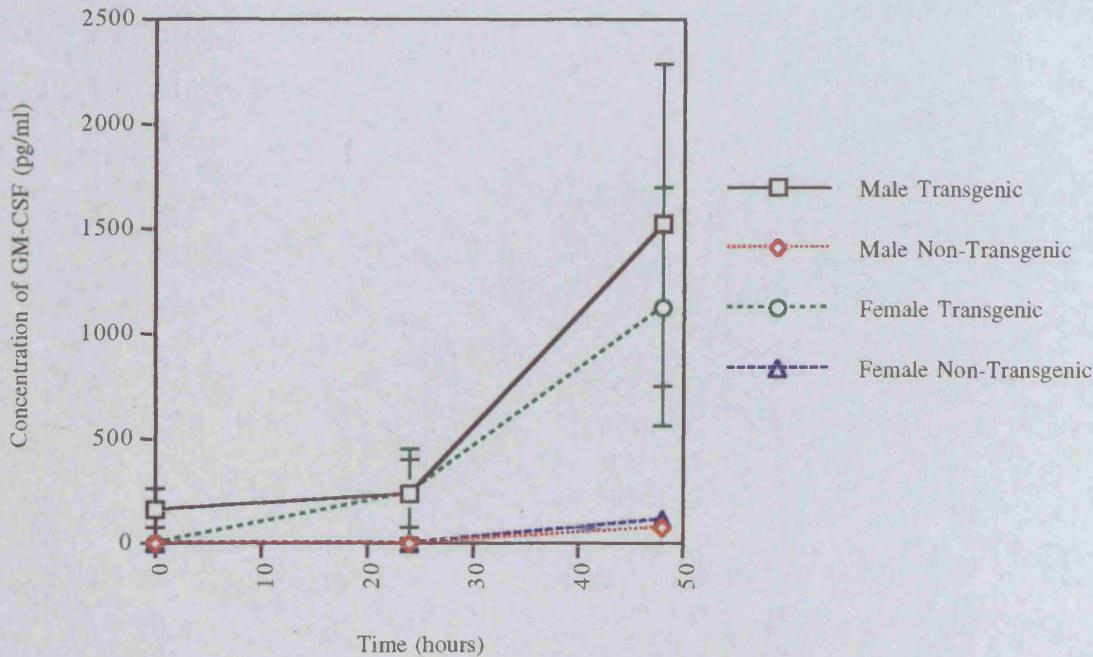
6.5 Intranasal Challenge of C79/GM Transgenic Mice with *S. pneumoniae* .

To determine whether challenge with an infectious organism stimulated acute phase expression of GM-CSF in the C79/GM transgenic mice, and if this expression of GM-CSF was protective against the infection, groups of mice were challenged intranasally with *S. pneumoniae* . Groups of twenty mice (five transgenic males, five non-transgenic males, five transgenic females, and five non-transgenic females) were inoculated intranasally with three different doses of *S. pneumoniae* (1×10^3 , 1×10^4 or 1×10^5 cfu/50 μ l inoculum). The experiment was split between two different groups, to cope with the number of mice involved.

Very few of the mice inoculated with 1×10^3 or 1×10^4 cfu/50 μ l developed bacteraemia (data not shown). More mice in the groups given approximately 1×10^5 cfu of *S. pneumoniae* developed pneumonia, so a further experiment using this dose of bacteria was performed using seven mice in each group. The presented results are therefore the combined data from the three sets of experiments (of mice given a dose of 1×10^5 cfu and the number of mice in each group was twelve. The mice were between ten and thirteen weeks of age. A tail bleed was taken from the mice before challenge (time 0 hours) and at 24 and 48 hours post challenge. At all time points the total white cells count was determined using a haemocytometer and the serum collected for analysis of the concentration of GM-CSF present (by ELISA). At time points 24 and 48 hours after challenge a viable count of the number of *S. pneumoniae* in the blood was also determined. The survival of the mice was also monitored to a time of 144 hours (6 days) after challenge. The viable count of the inoculum given to the mice from the first set of the experiment was 7.9×10^4 , from the second set of the experiment was 3.3×10^4 , and from the third experiment was 5×10^4 cfu / 50 μ l. See figures 6.16 to 6.19.

At the start of the experiment, basal levels of expression of GM-CSF were only detected in the group of male transgenic mice (161 ± 96 pg/ml serum). This was due to detectable GM-CSF in the serum of four of the twelve animals, and was not significantly higher than the undetectable levels of GM-CSF in the serum of any of the other groups. By 24 hours after challenge with 1×10^5 cfu *S. pneumoniae* , slightly higher levels of GM-CSF were detected in the serum of the male transgenic (234 ± 161 pg/ml serum) group, and this level of GM-CSF was again not significant when compared with the levels detected in the other groups and was also not significantly increased from the level within this group at 0 hours. This level was due to detection of GM-CSF in the serum of two of the eleven animals tested; one of these mice also had detectable levels of GM-CSF at 0 hours, the other mouse had high counts of bacteria in the blood at this time and was killed by the infection by 44.25 hours.

Figure 6.16: Concentration of GM-CSF in the serum of C79/GM transgenic mice after intranasal inoculation with between 3.3 and 7.9×10^4 cfu *S. pneumoniae*. n=12 for each group. Error bars represent SEM.



** No significant differences in the levels of GM-CSF either between or within groups (Mann-Whitney U test).

By 48 hours after challenge the level of GM-CSF in the male transgenic group had again increased (1516 ± 769 pg/ml serum), although this was not significantly different from the levels detected in the other groups or from the level detected at 0 or 24 hours within this group. The level of GM-CSF at this timepoint was due to detection of GM-CSF in the serum of six of the eleven animals. Three of these mice had very high levels of GM-CSF (over 5000 pg/ml serum) and were also seen to have high counts of bacteria in the blood at this time; these mice were all subsequently killed by the infection by 53 hours post challenge. The other three mice had much lower levels of GM-CSF (between 20 and 170 pg/ml serum), had no bacteria present in the blood at this time, and subsequently survived to 144 hours after the infection.

In the female transgenic group no basal level of expression of GM-CSF was detected but by 24 hours after challenge GM-CSF was detected (227 ± 214 pg/ml serum). This was due to detectable GM-CSF in the serum of two of the twelve animals and was not a significant increase over the undetectable levels at 0 hours. By 48 hours after challenge the level of GM-CSF in the female transgenic group had increased (1122 ± 571 pg/ml serum) although this level of GM-CSF was not significantly higher than the levels at 0 or 24 hours. The level at 48 hours was due to detectable GM-CSF in the

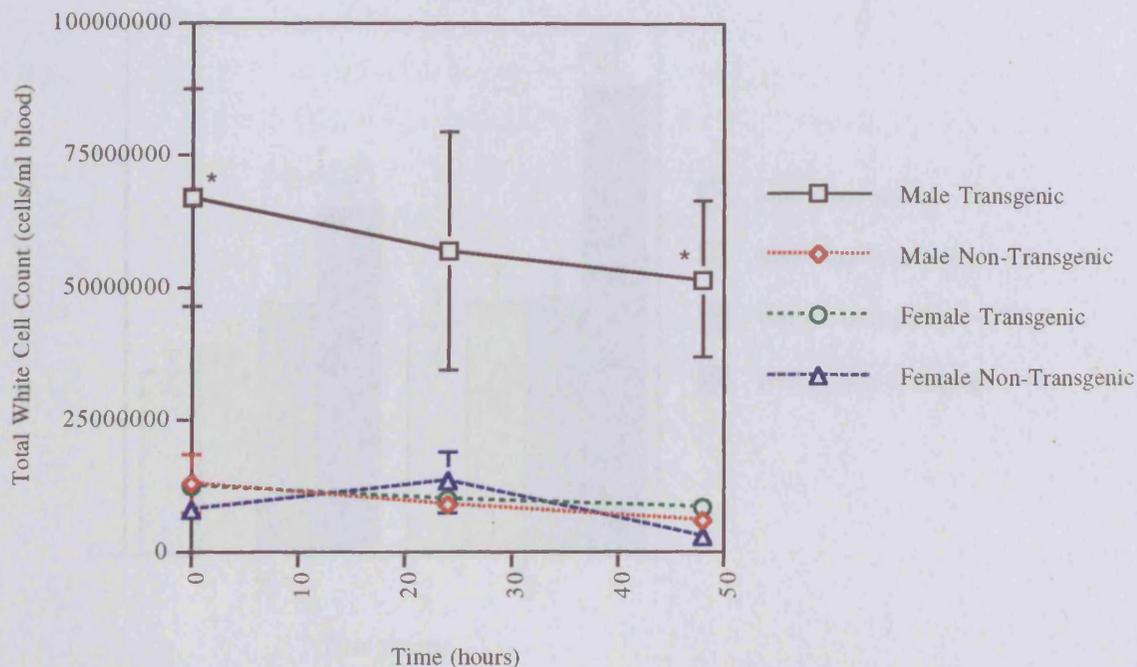
serum of five of the twelve female transgenic mice, with two of these mice being the same as those with detectable GM-CSF at 24 hours after challenge. All the mice with detectable GM-CSF in the serum, at both timepoints, also had bacteria present in the blood at these times and were subsequently killed by the infection by 53 hours post challenge.

In the male and female non-transgenic groups some GM-CSF was detected by 48 hours after challenge. These levels were due to detectable GM-CSF in only one of the twelve male non-transgenic mice, and four of the ten remaining female non-transgenic mice. Three of these female non-transgenic mice also had high bacterial counts in the blood at this time (over 10^9 cfu/ml blood), and were killed by the infection by 44 hours after challenge.

Although the increases in the levels of GM-CSF expression in the transgenic animals are not statistically significant, the trend of the data (figure 6.16) does indicate that GM-CSF is being expressed as an acute phase protein following intranasal challenge with *S. pneumoniae*.

These results may indicate that expression of GM-CSF is stimulated by the presence of bacteria in the blood as high levels of GM-CSF are only detected in most groups during bacteraemia. The male transgenic mice appear to express low basal levels of GM-CSF, but high levels are only seen in the bacteraemic animals. However, the presence of bacteria in the blood did not increase the levels of GM-CSF in all of the transgenic animals. Two male and four female transgenics had bacteria in the blood by 48 hours after challenge but none of these had detectable levels of GM-CSF at this time and all subsequently survived to 144 hours post challenge. This may also indicate that the mice that develop bacteraemia and have high levels of GM-CSF succumbed to the infection whereas those that developed some degree of bacteraemia but did not express high levels of GM-CSF survived the challenge.

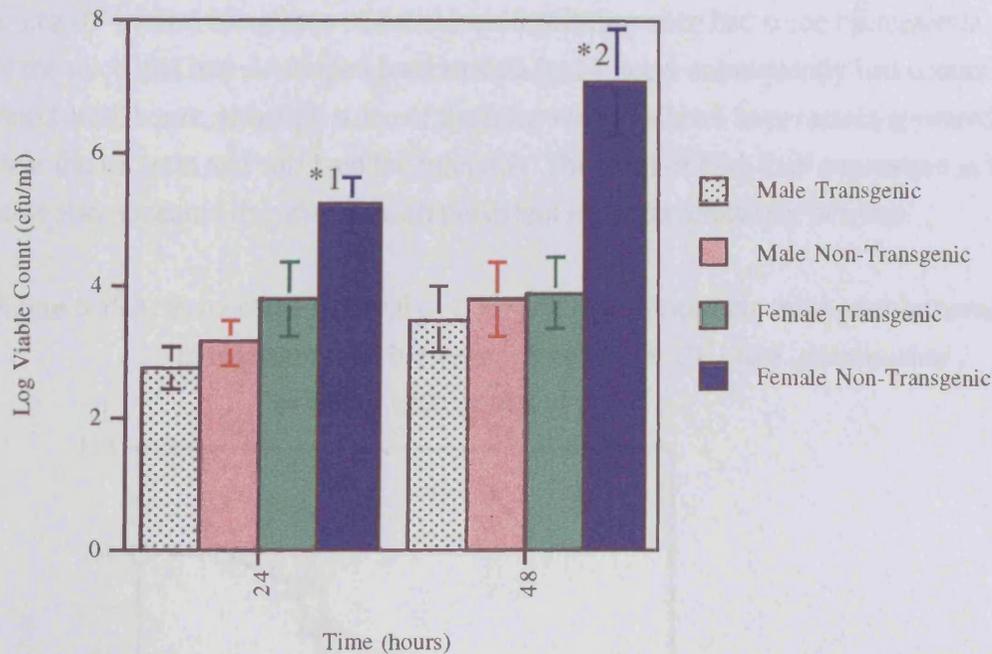
Figure 6.17: Total white blood cell count in the blood of C79/GM transgenic mice after intranasal inoculation with between 3.3 and 7.9×10^4 cfu *S. pneumoniae*. n=12 for each group. Error bars represent SEM.



* Significant difference between the male transgenic group and all the other groups at 0 and 48 hours ($P < 1\%$ Mann-Whitney U test).

The total white cell count in the male transgenic group was higher than all the other groups throughout the course of the experiment. At the start of the experiment the difference between the counts of the male transgenic group ($6.7 \times 10^7 \pm 2.1 \times 10^7$ cells/ml blood) and that of the other groups was significant (male non-transgenic group $1.3 \times 10^7 \pm 5.7 \times 10^6$ cells/ml blood, female transgenic group $1.2 \times 10^7 \pm 1.6 \times 10^6$ cells/ml blood, female non-transgenic group $7.7 \times 10^6 \pm 8.4 \times 10^5$ cells/ml blood; $P < 1\%$ for each comparison Mann-Whitney U test). At 48 hours, the difference between the counts of the male transgenic group were also significantly different from those of all the other groups ($P < 1\%$ Mann-Whitney U test), but at 24 hours after challenge the differences were not significant ($P > 5\%$ Mann-Whitney U test). The counts remained at a similar level in each group throughout the course of the experiment and no significant increase or decrease in the counts within each group was seen (Mann-Whitney U test).

Figure 6.18: Log of the viable count of *S. pneumoniae* in the blood of C79/GM transgenic mice after intranasal inoculation with between 3.3 and 7.9×10^4 cfu *S. pneumoniae*. $n=12$ for each group. Error bars represent SEM.



*1 Significant difference between female non-transgenic mice and male mice (both transgenic and non-transgenic) ($P < 1\%$ Mann-Whitney U test)

*2 Significant difference between female non-transgenic mice and male mice (both transgenic and non-transgenic) ($P < 1\%$ Mann-Whitney U test), and between female non-transgenic and female transgenic mice ($5\% > P > 1\%$ Mann-Whitney U test)

The detection limit of the assay was 167 cfu/ml, that is 1 colony in one of the three $20\mu\text{l}$ drops at a dilution of 10^{-1} (ie $1/3 \times 50 \times 10 = 167$ cfu/ml). Any samples that did not have detectable bacteraemia were therefore counted as just below the detection limit of the assay (that is 166 cfu/ml).

Some members of all the groups of mice had developed bacteraemia by 24 hours after challenge. In the male transgenic group ($3.5 \times 10^4 \pm 3.3 \times 10^4$ cfu/ml blood) this was due to presence of bacteria in the blood of three of the twelve mice, in the male non-transgenic group ($6.2 \times 10^4 \pm 5.7 \times 10^4$ cfu/ml blood) six of the twelve mice had some bacteria in the blood, in the female transgenic group ($6.1 \times 10^5 \pm 3.3 \times 10^5$ cfu/ml blood) five of the twelve mice had bacteria in the blood, and in the female non-transgenic group ($3.4 \times 10^6 \pm 2.8 \times 10^6$ cfu/ml blood) ten of the mice had some bacteraemia. The blood counts of all the groups appeared to rise by 48 hours after challenge although there were no significant differences in the counts within any group (Mann-Whitney U test). In the male transgenic group ($3.5 \times 10^5 \pm 2.7 \times 10^5$ cfu/ml blood) this was due to presence of

bacteria in the blood of five of the eleven mice, in the male non-transgenic group ($6.8 \times 10^6 \pm 6.7 \times 10^6$ cfu/ml blood) seven of the twelve mice had some bacteria in the blood, in the female transgenic group ($4.7 \times 10^7 \pm 4.7 \times 10^7$ cfu/ml blood) nine of the twelve mice had bacteria in the blood, and in the female non-transgenic group ($1.3 \times 10^9 \pm 6.6 \times 10^8$ cfu/ml blood) ten of the eleven remaining mice had some bacteraemia. Most of the mice that had developed bacteraemia by 24 hours subsequently had counts in the blood at 48 hours, although some of the mice with low level bacteraemia appeared to clear the bacteria and survived the infection. The level of GM-CSF expression in the mice also appeared to correlate with the extent of bacteraemia (see before).

Figure 6.19A: Percentage survival of C79/GM male transgenic mice after intranasal inoculation with between 3.3 and 7.9×10^4 cfu *S. pneumoniae* .
n=12 for each group.

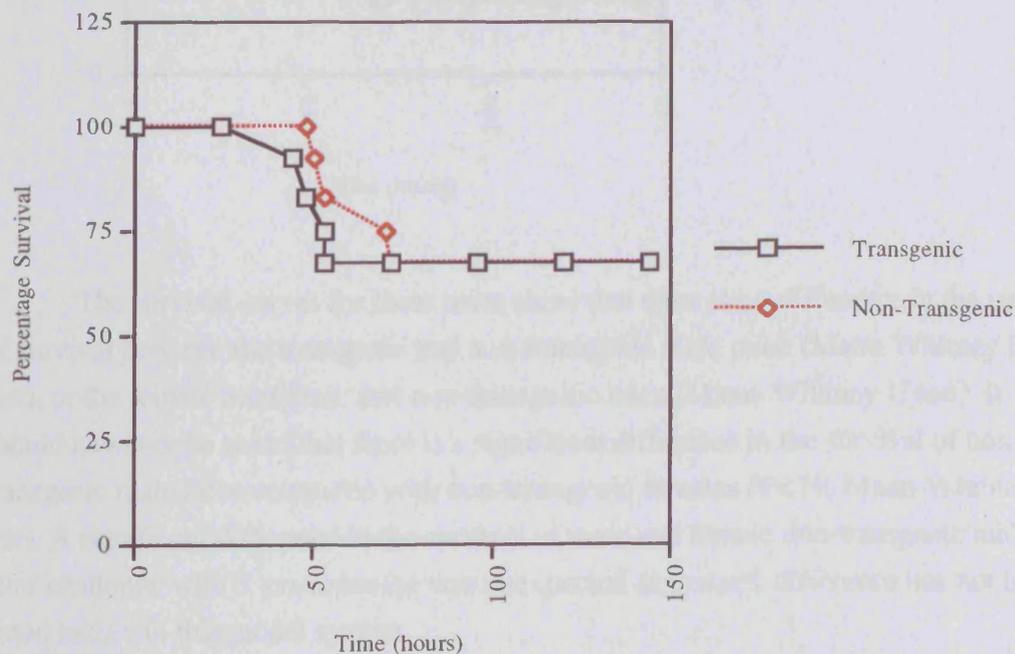
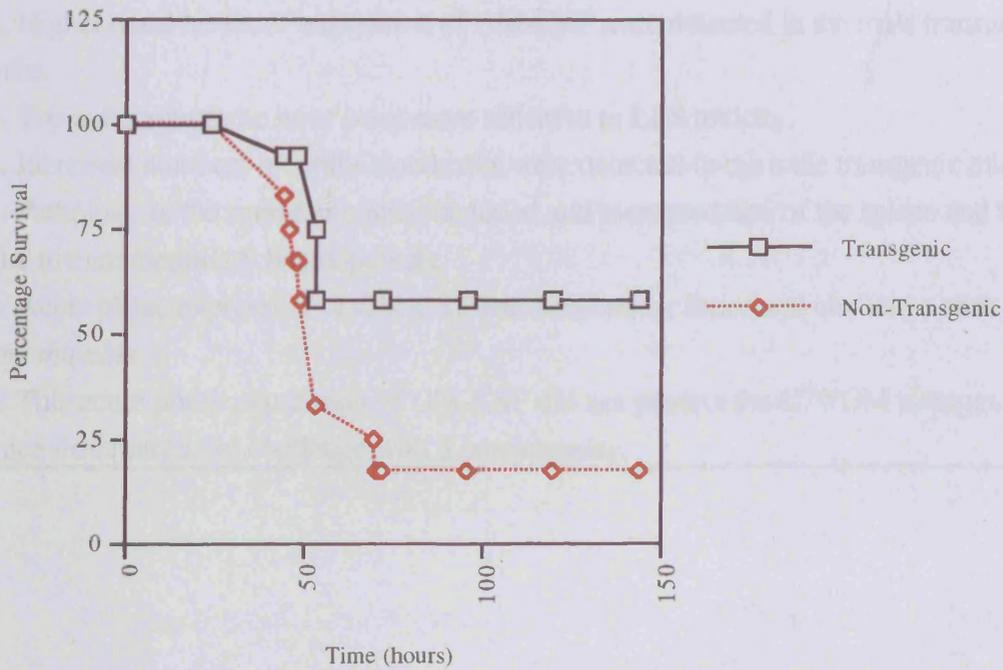


Figure 6.19B: Percentage survival of C79/GM female transgenic mice after intranasal inoculation with between 3.3 and 7.9×10^4 cfu *S. pneumoniae* .
 n=12 for each group.



The survival curves for these mice show that there is no difference in the pattern of survival between the transgenic and non-transgenic male mice (Mann-Whitney U test), or the female transgenic and non-transgenic mice (Mann-Whitney U test). It should however be noted that there is a significant difference in the survival of non-transgenic male mice compared with non-transgenic females ($P < 1\%$ Mann-Whitney U test). A significant difference in the survival of male and female non-transgenic mice after challenge with *S. pneumoniae* was unexpected as this sex difference has not been noted before in this model system.

Summary of the Results from the C79/GM Transgenic Mice.

1. Acute phase expression of GM-CSF was induced by the inflammatory stimulus LPS.
2. Higher basal levels of expression of GM-CSF were detected in the male transgenic mice.
3. The male transgenic mice were more sensitive to LPS toxicity.
4. Increased numbers of white blood cells were detected in the male transgenic mice.
5. Pathology in the transgenic mice included an increased size of the spleen and liver due to extramedullary haemopoiesis.
6. Acute phase expression of GM-CSF was induced by intranasal challenge with *S. pneumoniae* .
7. This acute phase expression of GM-CSF did not protect the C79/GM transgenic mice from intranasal challenge with *S. pneumoniae*.

Chapter 7: Discussion.

The aim of the project was to manipulate the immune response to produce transgenic animals that were resistant to infectious disease. Initially the interaction between CRP and *S. pneumoniae* was examined using a line of CRP transgenic mice. This interaction is related to the ability of CRP to bind to the bacteria and any protection against infection would not be expected to extend to a large number of other organisms, so methods to more generally upregulate the immune response were explored. A system of controlled expression of GM-CSF was developed by placing the mGM-CSF gene under the control of the CRP promoter, and a line of transgenic mice was produced. The kinetics of GM-CSF expression and the response of the animals to challenge with *S. pneumoniae* was then examined.

7.1 Production of Transgenic Mice for Acute Phase Expression of GM-CSF.

A fusion between 280 bp of the CRP promoter, the CRP signal peptide, and the coding region of mGM-CSF was successfully produced using the PCR strategy described (figure 4.1 and section 4.1), and the sequence of the fusion was shown to match the expected sequence. This fusion was then cloned into two different acute phase vectors (pBNB and pC79-Not) that contained more of the flanking sequences of the CRP promoter. Due to time constraints and the difficulties associated with working with large pieces of DNA, it was not possible to complete the original cloning strategy to place the GM-CSF gene under the control of the 30 kb of CRP promoter flanking sequences that had been shown to control liver specific acute phase expression of the CRP gene in transgenic mice. The pattern of expression of the three constructs that were successfully made was examined in a tissue culture system; with the basal levels of expression and the induction of expression in response to interleukins 6 and 1 examined.

The use of the commercially sourced mGM-CSF clone with the codons modified for expression in the *E. coli* system for amplification of the mGM-CSF coding region was justified; as some expression of mGM-CSF was seen from all the constructs examined. This therefore indicated that the *E. coli* modified codon usage is effective in mammalian cells with mGM-CSF, recognisable by ELISA, being produced by all the constructs examined. A bioassay for the activity of the expressed protein was not performed. Bioassays for GM-CSF involve culture of bone marrow cells and are time consuming and technically complicated to set up. As a bioassay for GM-CSF was not routinely used in the lab it was decided that biological activity of GM-CSF would be best determined by analysis of transgenic mice made with the constructs.

The use of the CRP signal peptide in these constructs was also seen to be justified as GM-CSF was detected in the cell culture medium with all the constructs examined (see figure 4.15). This indicated that the CRP signal peptide was directing the secretion of GM-CSF from the tissue culture cells (derived from liver cells) and that correct cleavage of the signal peptide occurred. It was therefore predicted that the CRP signal peptide would direct the secretion of GM-CSF from the liver cells of transgenic mice created with these constructs.

It was seen that the fusion alone expressed very little GM-CSF both before and after interleukin stimulation. Comparing the expression of GM-CSF from constructs pBNB/GM and pC79/GM revealed that both the basal and interleukin stimulated levels of expression of GM-CSF from pBNB/GM were higher than those from pC79/GM. The factor of induction of the pC79/GM construct, however, was higher than the factor of induction of the pBNB/GM construct (9.9 times increase for pC79/GM compared to 5.1 times increase for pBNB/GM).

These *in vitro* results correspond to the results seen when the original (CRP containing) constructs were used to make transgenic mice (Murphy *et al.*, 1995). It was shown that transgenic mice carrying construct 4 (corresponding to acute phase vector pBNB) had higher basal levels of CRP (127 ± 510 μg CRP/ml of serum) compared to mice transgenic for construct 79 (3 ± 15 $\mu\text{g}/\text{ml}$). The construct 4 transgenic mice also showed lower induction of CRP expression (induced by a factor of five) than the construct 79 transgenic mice (induced by a factor of 69).

The pattern of expression of the pC79/GM construct therefore gave a pattern of expression most closely resembling an acute phase response (that is low basal levels of expression before and high expression following stimulation with interleukins). Because the CRP construct this vector was based on had also been shown to be regulated in transgenic mice with a low basal level of expression and strong induction on administration of an inflammatory stimulus, it was reasoned that pC79/GM would be the best construct to use to create transgenic mice. However, it should be noted that there were still detectable basal levels of GM-CSF expressed from this construct so this was still not the ideal construct to use, but it had not been possible to construct the ideal expression vector (GM-CSF in the 30 kb of the CRP promoter flanking regions).

Two transgenic founder mice were obtained from the 171 offspring resulting from the microinjections. This was quite a low frequency of integration of the transgene as it should be expected that at least 25% of the offspring from microinjection experiments carry the transgene (Mann and McMahon, 1993). The low frequency obtained from microinjection of the pC79/GM construct was probably due to inefficient injection of the DNA into the pronucleus of the eggs as this was the first line of transgenic mice to be produced by the technical staff of the transgenic unit. It was

assumed that the transgene would not be lethal to the developing embryos as lines of transgenic mice expressing mGM-CSF under the control of a constitutive promoter had been successfully generated (Lang *et al.*, 1987), so the expression of mGM-CSF in developing mouse embryos is not associated with fetal death.

The potential founder mice were screened for presence of the transgene by PCR analysis of genomic DNA from a tail tip biopsy (using two different sets of primers) and by Southern blot analysis of genomic DNA. Both of these screening methods identified only these two mice as carrying the transgene. Subsequent comparison of the results obtained from screening the mice by both PCR and Southern blot analyses revealed that the results obtained were the same for each type of analysis, therefore the colony of mice were routinely screened by PCR analysis alone.

7.2 Acute Phase Expression in Response to the Inflammatory Stimulus LPS.

7.2.1 CRP Transgenic Mice.

Initial studies of acute phase expression in transgenic mice utilised a line of CRP transgenic mice (Ciliberto *et al.*, 1987a). These mice were created using a 30 kb fragment from a cosmid clone (Ciliberto *et al.*, 1987b) containing the human CRP gene. The mice had previously been shown to express human CRP solely in the liver and with the same acute phase kinetics as in man. That is, CRP was initially undetectable in the serum of the mice, expression was increased dramatically following an inflammatory stimulus then returned to basal levels by 48 hours after induction.

Initial experiments examined the response of the CRP transgenic mice to an inflammatory stimulus (LPS). LPS is the major component of the Gram-negative bacterial cell wall. It mediates the release of many different cytokines and is the component of the bacteria that can induce septic shock. In order to compare the acute phase kinetics of CRP expression in the transgenic mice in response to a general inflammatory stimulus and to infection with Gram-positive bacteria, it was necessary to determine the baseline expression of CRP in response to LPS. Experiments by other groups had also indicated a sex related difference in the expression of CRP from this line of transgenic mice in response to LPS (Szalai *et al.*, 1995) so this difference was also examined.

The CRP transgenic mice, after induction with LPS, did express CRP with the kinetics of an acute phase protein and there were significant differences in the levels of expression between male and female mice (figure 3.1). In both the male and female transgenic groups of mice, CRP expression was rapidly induced to give peak levels of

expression at 24 hours post LPS challenge and the levels fell slightly, but remained much higher than basal levels by 48 hours after challenge. However, in the female transgenic group of mice, CRP levels did not increase as rapidly or to the same extent as the male group. The pattern of acute phase expression seen in our experiments was different to that seen by Ciliberto (Ciliberto *et al.*, 1987a) (figure 1.6), who detected peak levels of expression of CRP by 15 hours after challenge with LPS and the levels of expression had returned to basal levels by 48 hours after challenge. The delay in the peak levels of expression and the extended time of expression of CRP that we saw may have been due to the different sampling techniques employed. In our experiment the same mice were sampled at each timepoint by serial tail bleeds whereas the Ciliberto group sampled different mice for each timepoint. Tail bleeding causes some injury and stress to the animals, and injury and stress are known to contribute to the acute phase response, so the mice may have been stimulated somewhat by each successive bleed. This could therefore explain why the levels of CRP had not returned to basal levels as quickly as would have been expected. This could be examined by sampling at further timepoints than 48 hours (although this was the maximum number of bleeds allowed on our home office licence) or by sacrificing a different group of mice for each timepoint (which was prohibited by the small numbers of mice it was possible to produce from the breeding colony).

The peak levels of CRP expression induced by LPS in our experiments are comparable to those seen by other groups, using the same amount of LPS (100 µg) as in our experiment. The Ciliberto group detected peak levels of 130 µg/ml at 15 hours after challenge with LPS (Ciliberto *et al.*, 1987a), and the Murphy group detected peak levels of 179 µg/ml at 15 hours after challenge (Murphy *et al.*, 1995), compared to our experiment with peak levels of 167 µg/ml at 24 hours after challenge. This indicates that the CRP gene was activated to a similar extent in all these experiments.

The basal level of CRP expression in the male transgenic group of mice was significantly higher than the basal level of expression in the female transgenic group, and the induced level of CRP expression was also higher in the male than the female transgenic group. This confirms the result seen by the Szalai group who also saw a sexually dimorphic pattern of expression of CRP in this line of transgenic mice (Szalai *et al.*, 1995), with significantly higher levels of both basal and induced CRP expression. Further work, including castration and subsequent implantation of testosterone releasing pellets, by this group showed that this difference was mediated by testosterone. The testosterone mediated sexually dimorphic pattern of CRP expression in these mice was subsequently postulated to be the result of testosterone affecting the levels of growth hormone in the male mice, as had been shown for other rodent proteins such as the sex-limited protein of mice (Szalai *et al.*, 1998).

7.2.2 C79/GM Transgenic Mice.

The pC79/GM construct had been shown to express mGM-CSF in the manner of an acute phase protein in a tissue culture system, and a line of transgenic mice was subsequently made using this construct. To determine whether the transgene was being expressed in the mice with the kinetics of an acute phase protein, the mice were challenged with the inflammatory stimulus LPS. Female mice only were used in these experiments as not enough male mice were available.

The basal levels of expression of GM-CSF in the group of transgenic mice were very low (not significantly different from the non-transgenic group). Following induction with LPS the levels of GM-CSF rose rapidly with peak levels of expression at 12 hours, then fell back to basal levels by 24 hours after challenge (see figure 6.1). This is the pattern of expression that would be expected of an acute phase protein, so it can be reasoned that the region of the CRP promoter (C79 construct) used to make these transgenic mice is regulating the expression of the GM-CSF gene in the manner of an acute phase protein in transgenic mice. This also correlates with the results seen when the C79 construct was used previously to make transgenic mice, when the construct expressed low basal levels of CRP and was induced strongly after administration of LPS (Murphy *et al.*, 1995). In fact, from this experiment, the factor of induction of GM-CSF expression seems a lot higher than the induction of CRP driven by the C79 construct (GM-CSF expression was induced by a factor of 458 compared to CRP which was induced by a factor of 69). This may however be due to the nature of the C79 transgene which was shown to be induced to varying levels; that is, in the 11 lines of transgenic mice that were created with the C79 construct the level of induction ranged from a factor of 3.9 to 3123 above varying basal levels of expression (from undetectable levels to 14.9 µg/ml of serum) (U. Ruther personal communication).

This pattern of acute phase expression of GM-CSF, in the C79/GM transgenic mice, following induction with LPS more closely resembles the pattern of CRP expression that was seen by Ciliberto (Ciliberto *et al.*, 1987a) than we saw in our experiments with the CRP transgenic mice. This may have been due to the sampling technique employed in these experiments as groups of mice were sacrificed at each timepoint rather than samples taken by serially bleeding the same mice. As previously mentioned, tail bleeding can cause some injury and stress to the animals which could stimulate the acute phase response to some extent.

Another explanation could be that in this line of transgenic mice a different region of the CRP promoter was used to drive the expression of the protein which could affect the acute phase kinetics. This would be unlikely as acute phase expression in the CRP transgenic mice was controlled by a larger section of the CRP promoter

(30 kb) whereas acute phase expression in the C79/GM mice was controlled by a shorter section of the CRP promoter (7.5 kb in total). It would therefore be assumed that the greater region of the CRP promoter would act to more tightly regulate acute phase expression.

Further analysis of the acute phase expression of GM-CSF in the C79/GM mice showed that the male transgenic mice tended to have higher basal levels of GM-CSF expression than the females, although the peak levels of induction were about the same. (See discussion section 7.5.1)

It was also seen that the male mice were much more sensitive to LPS than the female mice, as challenge with 100 µg of LPS killed almost all of the male mice and substantial mortality was observed after challenge with 25 µg of LPS. This lethality induced by LPS in the male mice was thought to be due to the higher basal levels of GM-CSF that are seen in these male mice. GM-CSF has been shown to prime monocytes for endotoxin-induced TNF production *in vitro* (Cannistra *et al.*, 1988). It has also been shown to potentiate the toxicity of LPS *in vivo* using models of LPS induced septic shock and endotoxin-induced liver injury in GalN-sensitized mice (Tiegs *et al.*, 1994). Administration of GM-CSF to these mice before challenge with LPS resulted in greater mortality from septic shock or greater liver injury, depending on the model used (Tiegs *et al.*, 1994). Levels of TNF α were also significantly increased in the animals pretreated with GM-CSF before administration of LPS. Neutralization of endogenous GM-CSF with antibodies was also shown to reduce LPS mediated lethality or liver damage; however neutralization of GM-CSF did not protect against liver damage induced with TNF (Tiegs *et al.*, 1994). These results therefore demonstrate that GM-CSF is able to prime for LPS-induced TNF production *in vivo*. Another group also showed that GM-CSF rapidly primes mice for enhanced TNF α production following administration of LPS, and that levels of IL-1 β and IL-6 were also enhanced by pretreatment with GM-CSF (Brisette *et al.*, 1995). This group also showed that GM-CSF priming of an enhanced cytokine response to LPS also occurred in leukopenic mice and therefore suggested that the effect of GM-CSF is exerted on macrophages and in particular the resident tissue macrophages (Brisette *et al.*, 1995). Mice made tolerant to LPS also show an attenuated TNF α response to LPS however pretreatment of these mice with GM-CSF can restore the TNF response (Bundschuh *et al.*, 1997). Cultures of cells from these mice also exhibit a reduced response to LPS but treatment with GM-CSF can partially restore the TNF response to LPS (Bundschuh *et al.*, 1997). GM-CSF deficient mice have also been shown to be more tolerant to the effects of LPS than GM-CSF sufficient control (Basu *et al.*, 1997).

These results all indicate a role for GM-CSF in the potentiation of the cytokine response to LPS and in particular to production of TNF α . It can therefore be reasoned that the higher basal levels of GM-CSF in the male mice of the transgenic line that we have made may contribute to a priming effect of the macrophages so that they are more susceptible to LPS due to an enhanced production of TNF α . The detection of cells in the blood carrying macrophage activation markers (by FACS analysis) also indicates that the blood monocytic cells could be primed to some extent, as the cells usually only differentiate to macrophages after migration into tissues and not in the blood.

A similar effect could also be seen in the female transgenic mice as half of these mice challenged with LPS were killed by the dose given compared to the non-transgenic controls that all survived the dose given; although the female transgenic mice tended to survive longer than the male transgenic mice. This may be due to the increased levels of GM-CSF induced by LPS (an increase of about 450 fold) subsequently increasing the production of TNF rather than priming of the response due to the higher basal levels of GM-CSF seen in the male mice.

7.3 Acute Phase Expression in Response to Challenge with Infectious Bacteria.

7.3.1 CRP Transgenic Mice.

The CRP transgenic mice were shown to express CRP with acute phase kinetics following challenge with a Gram-negative bacterial inflammatory stimulus. The next stage was to determine if challenge with Gram-positive bacteria induced the same pattern of acute phase expression of CRP. The model system of intranasal challenge with infectious *S. pneumoniae*, that was routinely used in the laboratory, was employed to examine the expression of CRP in response to infection with Gram-positive bacteria.

Infection with *S. pneumoniae* resulted in an increase in the expression of CRP in both the male and female transgenic groups by 24 hours after challenge (figure 3.2). The basal level of expression of CRP was also significantly higher in the male transgenic group than the female transgenics. The increase in expression of CRP by 24 hours following challenge was also significantly higher in the male transgenic than the female transgenic group. However, by 48 hours after challenge, the level of CRP expression in the female transgenic group was similar to that of the male transgenic group.

The pattern of expression of CRP following challenge with *S. pneumoniae* was therefore different to that seen after induction with LPS. The levels of CRP did not increase as rapidly, with peak levels of expression seen at 48 hours following challenge

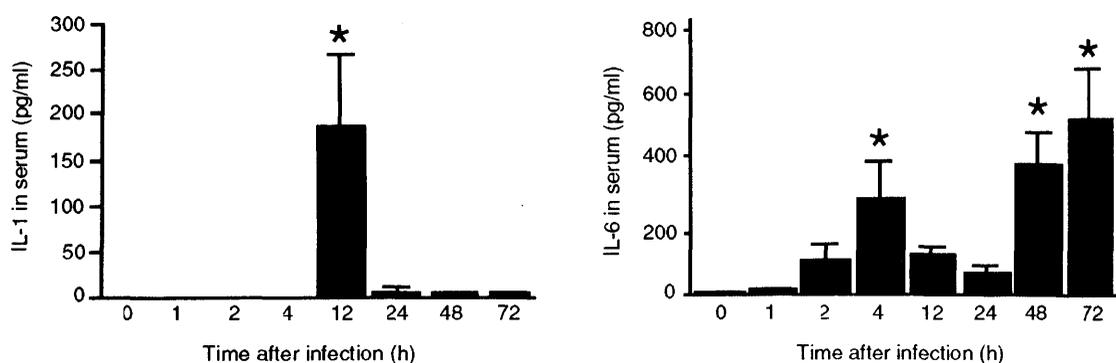
with *S. pneumoniae* compared to 24 hours after induction with LPS. The levels of CRP were also not seen to fall back to basal levels, although again this may have been seen if samples were taken at further timepoints.

The peak levels of CRP expression induced by challenge with *S. pneumoniae* were also lower than the peak levels induced by LPS, with the male transgenics achieving only 8% and the female transgenics only 22% of the levels seen after induction with LPS. This indicates that intranasal challenge with *S. pneumoniae* does not result in as strong activation of the CRP promoter as induction with LPS. A similar result was seen by the Szalai group who examined the effect of intraperitoneal infection with *S. pneumoniae* on the expression of CRP in this line of mice (Szalai *et al.*, 1995). Expression of CRP induced by 25 µg of LPS resulted in detectable levels of CRP of 92 µg/ml which were higher than the peak levels of expression seen after intraperitoneal challenge with *S. pneumoniae* (57 µg/ml), although it is unclear whether this is a significant difference. This suggests that, regardless of the route of infection, the levels of CRP induced by Gram-positive bacteria are lower than the levels of CRP induced by challenge with LPS.

Expression of CRP in tissue culture systems has been shown to be regulated synergistically by the cytokines IL-6 and IL-1 (Ganter *et al.*, 1989)(Li *et al.*, 1990). The pattern of expression of different cytokines, including IL-6 and IL-1, in response to intranasal infection with *S. pneumoniae* has also been studied (Bergeron *et al.*, 1998). This work showed that IL-6 can be detected systemically (in the serum) in a biphasic manner with an initial peak of IL-6 at 4 hours after infection followed by a drop in the levels to 24 hours and a second, higher, peak at 72 hours after infection; IL-1 is only detected in the serum at 12 hours after infection (Bergeron *et al.*, 1998) (see figure 7.1). This suggests that the CRP promoter could be induced by these systemic levels of IL-6 very quickly after intranasal infection with *S. pneumoniae*. However, expression of CRP in tissue culture systems has shown that IL-1 acts synergistically with IL-6; that is the expression induced by both cytokines together is greater than the sum of the expression induced by either cytokine alone (Ganter *et al.*, 1989)(and this thesis). If induction of the CRP promoter is controlled in this way *in vivo* then this may help explain why the CRP promoter is not activated as strongly or as quickly by intranasal infection with *S. pneumoniae*. The IL-1 necessary for strong activation of the CRP promoter is only detected systemically at 12 hours after infection, by which time the levels of IL-6 have dropped after the initial peak (Bergeron *et al.*, 1998). This would suggest that high levels of both cytokines are not present after infection at the same time so, although IL-6 alone can activate the CRP promoter by 2 hours after infection, the synergistic activation of the CRP promoter by IL-1 is not seen.

It would therefore be of interest to determine the levels of both IL-1 and IL-6 in the serum of the mice throughout the course of the experiment following intranasal challenge with *S. pneumoniae*, and to correlate these with the expression of the CRP transgene. Ongoing work within our group (Infection and immunity, Glasgow University) is attempting to characterise the pattern of expression of different cytokines following pneumococcal challenge, and this work may be extended to examine the cytokine response in both the CRP and C79/GM transgenic mice.

Figure 7.1: The levels of IL-6 and IL-1 in the serum of mice after intranasal infection with *S. pneumoniae*. (Bergeron *et al.*, 1998)



7.3.2 C79/GM Transgenic Mice.

The C79 acute phase vector had also been shown to drive the acute phase expression of GM-CSF in the C79/GM transgenic mice after challenge with LPS. The pattern of expression of GM-CSF of these mice in response to infection with Gram-positive bacteria was then examined by intranasal challenge with *S. pneumoniae*.

Basal levels of GM-CSF expression were only detected in the male transgenic mice. By 24 hours after challenge with *S. pneumoniae* the levels of GM-CSF in both male and female groups of transgenic mice had risen slightly, although this was not a significant increase above basal levels for either group. By 48 hours after challenge the levels of GM-CSF expression in both groups of transgenic animals had increased further although again this was not a significant increase above basal levels of expression (figure 6.16). The peak levels of GM-CSF expression were also not significantly different between male and female transgenics. Low levels of GM-CSF were also detected in both the male and female non-transgenic groups by 48 hours after challenge. It was reasoned that this was due to the normal response of the mice to bacteraemia as the mice that had detectable GM-CSF in the serum also had very high bacterial counts at this time.

The kinetics of GM-CSF expression induced by challenge with *S. pneumoniae* was very different to that induced by LPS. A much slower response of GM-CSF expression was induced by challenge with *S. pneumoniae* with peak levels of expression seen at 48 hours after infection (compared to 12 hours after challenge with LPS) and no decrease back to basal levels (although this may have been seen if samples were taken at further timepoints). This pattern of expression was similar to the pattern of CRP expression seen when the CRP transgenic mice were challenged intranasally with *S. pneumoniae*, although the increase in the levels of GM-CSF was not significant whereas the increase in the levels of CRP was significant (compare figure 3.2 and figure 6.16).

One possible explanation of this is that the sensitivity of the ELISA for GM-CSF is different to that of the ELISA for CRP; so that the lowest level of detection of GM-CSF is higher than the actual levels and the full extent of the induction of expression is not therefore detected. Another possible reason for the increase in GM-CSF expression not being significant could be due to the complex interactions of GM-CSF with the immune system, which could result in some feedback of GM-CSF on its own production. For example, the production of anti-inflammatory cytokines such as IL-10 which could damp down the acute phase response.

Also as was seen with CRP expression in the CRP transgenic mice, the peak level of GM-CSF expression induced by challenge with *S. pneumoniae* was lower than the peak levels induced by LPS. Peak GM-CSF levels of 8580 pg/ml were induced by LPS compared to only 227 pg/ml induced by challenge with *S. pneumoniae* (only 2.6%). The C79 construct, derived from the 30 kb clone of the CRP gene used to make the CRP transgenic mice, is therefore driving the expression of GM-CSF in transgenic mice in a similar way to the full length construct. This is supported by the work of the Murphy group who showed that the C79 construct directed the expression of CRP in transgenic mice in a similar way to the full length construct, with low basal levels and strong induction by LPS (Murphy *et al.*, 1995). This construct consists of flanking regions of DNA both 5' and 3' of the CRP gene, and the 3' flanking region includes the CRP pseudogene. Both the 5' and 3' flanking regions were shown to necessary for tight control of the basal levels of expression of the CRP gene in transgenic mice, and are therefore thought to contain regions involved in negative transcriptional regulation.

7.4 The Role of Proteins Expressed with Acute Phase Kinetics in the Progression of Disease Following Challenge with *S. pneumoniae*.

7.4.1 CRP Transgenic Mice.

All of the groups of mice infected intranasally with *S. pneumoniae* developed bacteraemia and there was no significant difference in the levels of bacteraemia detected in each group at either 24 or 48 hours after challenge. This was also reflected in the survival patterns of each group, in the fact that there was also no significant difference in the number of surviving animals in each group or in the mean time of survival.

These were unexpected results as other groups had shown that CRP is protective against infection with *S. pneumoniae* (discussed in section 1.4.3.3). Two groups have shown that intravenous or intraperitoneal administration of CRP to mice prior to infection with *S. pneumoniae* resulted in protection of the animals from the infection (Mold *et al.*, 1981)(Yother *et al.*, 1982). Protection was observed after infection with two different strains of type 3 pneumococci and one strain of type 4 pneumococci which led to the assumption that CRP-mediated protection is effective against many different strains of *S. pneumoniae* (Yother *et al.*, 1982). Experiments with the CRP transgenic mice have also shown protection of the transgenic mice against intraperitoneal infection with *S. pneumoniae* type 3 (Szalai *et al.*, 1995). A significantly lower level of bacteraemia was detected in the transgenic mice (at times 24 and 48 hours after infection); and a significantly higher proportion of the transgenic mice survived the infection. Although this protection is not complete as only four of the twelve transgenic mice survived the infection compared to 100% mortality of the non-transgenic animals.

It was thought that the dose of *S. pneumoniae* used in our infection studies could have been either too high or too low to see increased survival of the CRP transgenic mice; so further experiments were performed using a range of different doses to try to optimise the system. No increased survival of the CRP transgenic mice was seen with any of the different doses used, which suggests that the CRP transgenic mice are not protected from challenge with *S. pneumoniae*, in our system.

One possible reason why we did not see protection of the CRP transgenic mice infected with *S. pneumoniae* could be the route of infection. In our experiments, the mice were infected intranasally compared to other groups where the bacteria were administered intraperitoneally or intravenously. The intranasal route of infection mimics more closely the natural route of infection of the bacteria, so it would be expected that this would be a system that more closely resembles the natural interactions of pathogen and host. Other work with an intranasal model of infection with *S. pneumoniae* has shown that IL-6 can be detected early in the infection, but IL-1 is only detected 12 hours

after infection, which may affect the induction of the transgene, see before (Bergeron *et al.*, 1998). A different pattern of cytokine production may be seen following intraperitoneal or intravenous infection resulting in faster activation of the CRP promoter and therefore production of more CRP in a shorter time.

To examine the effect of the route of infection CRP transgenic mice were infected intraperitoneally with the same strain of *S. pneumoniae* as was used in the intranasal challenges. Protection of the CRP transgenic mice from fatal infection with type 2 pneumococci administered intraperitoneum was also not seen.

Analysis of CRP binding to different serotypes of *S. pneumoniae* has revealed that different strains bind different amounts of CRP. Serotype 27 was shown to bind the most CRP (9×10^6 molecules/cfu) due to the presence of C-polysaccharide in the capsule; serotype 3 bound 0.9×10^6 molecules/cfu and serotype 6 bound 1.1×10^6 molecules/cfu (Mold *et al.*, 1982b). Therefore a possible reason why we did not see protection is that we used a different strain of *S. pneumoniae* than was used by other groups. In our experiments D39, a type 2 encapsulated pneumococcus was used. Whereas the strain of *S. pneumoniae* used in the infection studies with CRP transgenic mice was a type 3 strain (WU2) (Szalai *et al.*, 1995). Other groups also showed that CRP administered prior to infection was protective against type 3 and type 4 pneumococci (Mold *et al.*, 1981)(Yother *et al.*, 1982). No information about the CRP binding capacity of type 2 pneumococci is available, but it may be that this strain has less C-polysaccharide in the cell wall and is therefore less able to bind CRP (and therefore facilitate clearance) than some of the other strains studied. This could be examined by a more thorough analysis of the CRP binding capacities of different strains, or by repeating the infection studies with different strains of *S. pneumoniae* to examine whether protection is seen with different strains. It should be noted that *in vitro* experiments to examine CRP opsonization of type 3 pneumococci did not show increased phagocytosis (Mold *et al.*, 1982b), however a protective effect of administered CRP was seen *in vivo* (Mold *et al.*, 1981)(Yother *et al.*, 1982). This is surprising as the mechanism of protection by CRP *in vivo* was proposed to be due to opsonization of the bacteria resulting in increased phagocytosis.

Protection of mice against fatal infection by either passive immunisation or acute phase expression of CRP therefore appears to be highly specific. Pretreatment of mice with CRP has been shown to confer no protection on infection with *Salmonella typhimurium* (Nakayama *et al.*, 1983). CRP-mediated protection against infection seems to be specific to *S. pneumoniae*, with protection possibly limited to only a few of the many (90) different serotypes.

Because CRP is so specific in its action, the expression of CRP in transgenic animals would be assumed not to confer protection against a wide range of infections.

In order to create animals that would be resistant to a wider range of infectious diseases, it was therefore reasoned (by Professor Tim Mitchell) that it would be better to upregulate other immune mechanisms, for example phagocytosis, which should result in increased clearance of many different infections.

Because the cytokine GM-CSF is involved in activation of phagocytic cells and stimulates the production of new granulocytes and macrophages, this was a good candidate to be used to create animals that would have upregulated phagocytic mechanisms and therefore be more able to clear infections. Constitutive expression of GM-CSF in transgenic mice had been shown to result in many harmful side effects leading to premature death of the animals (Lang *et al.*, 1987); although isolated macrophages from these mice were shown to exhibit enhanced phagocytic activity against *Listeria monocytogenes* (Tran *et al.*, 1990). It was therefore reasoned that expression of GM-CSF in a controlled manner in transgenic mice (that is only during an infection) would result in an upregulation of phagocytic activity, when the GM-CSF was produced, but the harmful side effects would not be seen. To this end, it was decided to utilise the pattern of expression of the acute phase proteins. Because mice transgenic for the human CRP gene had been shown to express human CRP with the kinetics of a major acute phase protein, following an inflammatory stimulus, it was reasoned that if the expression of GM-CSF was controlled by the CRP promoter then GM-CSF would be expressed as an acute phase protein. Because constitutive expression of GM-CSF had been shown to result in these side effects, this was also a useful cytokine to use to evaluate the efficacy of the acute phase expression system used. That is, any leaky expression of the promoter would be indicated by the detection of any alteration of blood cell numbers or other pathological changes.

7.4.2 C79/GM Transgenic Mice.

All of the groups of mice infected with *S. pneumoniae* developed bacteraemia, and there were no significant differences in the blood counts detected in each group at either 24 or 48 hours after challenge (figure 6.18). The detectable levels of GM-CSF in individual mice appeared to correlate with bacteraemia; and this may indicate that expression of GM-CSF is stimulated by the presence of bacteria in the blood as high levels of GM-CSF are only detected in most groups during bacteraemia. For example, the male transgenic mice express low basal levels of GM-CSF, but high levels are only seen in the bacteraemic animals.

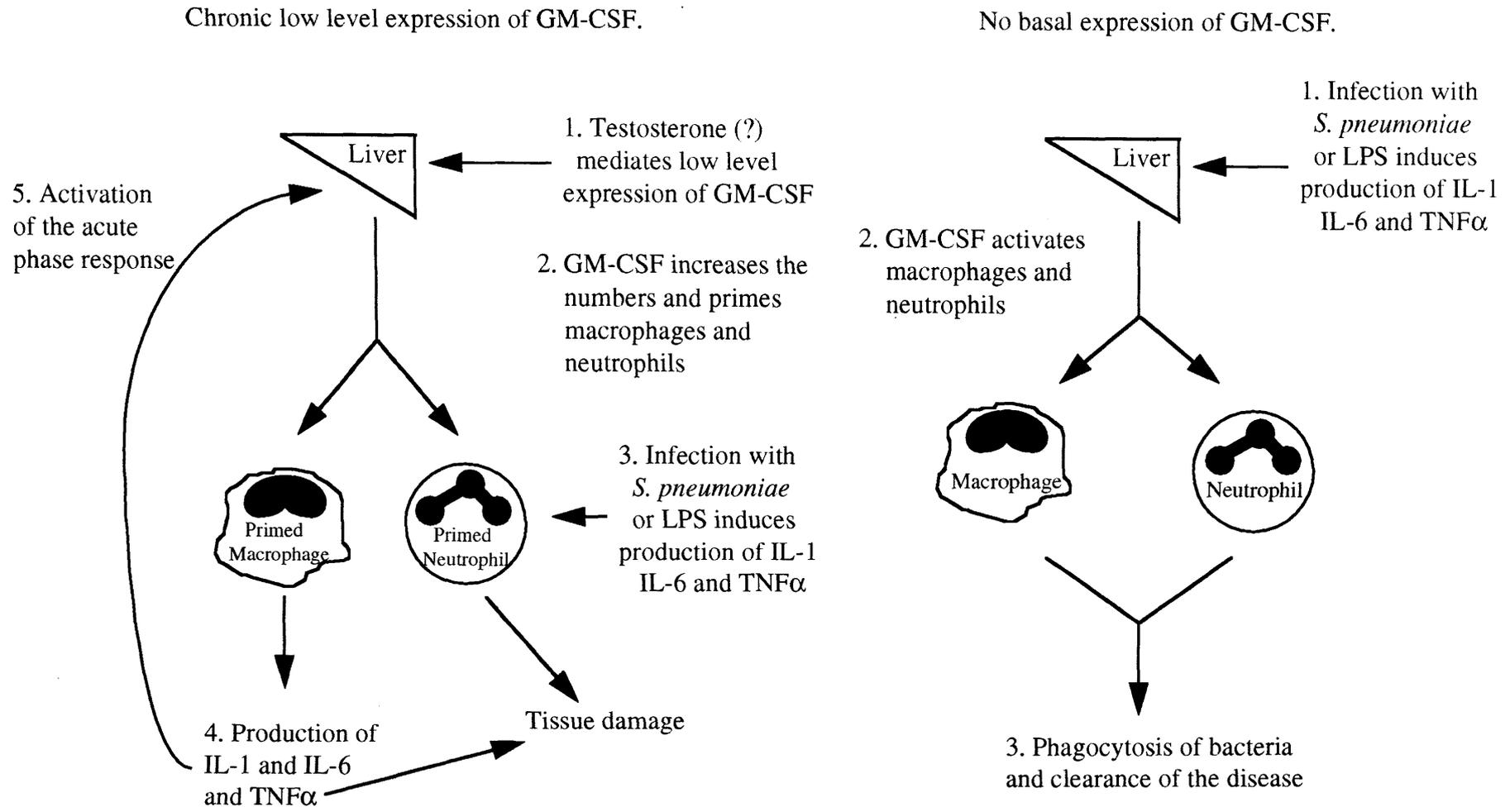
This would suggest that Gram-positive bacteria, such as *S. pneumoniae*, do not stimulate an acute phase response until they enter the blood stream. It has been shown that mice infected intranasally with *S. pneumoniae* have detectable levels of IL-6 in the blood by 2 hours after challenge and IL-1 by 12 hours (Bergeron *et al.*,

1998)(discussed in section 7.3.1). It would therefore be expected that the acute phase response (and therefore the expression of GM-CSF driven by the CPR promoter) would be initiated by these cytokines shortly after infection. However, the levels of IL-6 and IL-1 detected by this group may not reflect the physiological levels necessary to produce a strong acute phase response. Measurement of IL-6 levels in mice injected intraperitoneum with 1 µg of LPS showed serum levels of 14 ng/ml (14,000 pg/ml) by two hours after challenge (Brissette *et al.*, 1995). This is much higher than the serum levels of 280 pg/ml of IL-6 detected by four hours after intranasal challenge with *S. pneumoniae* (the first peak of IL-6 expression) (Bergeron *et al.*, 1998). This could also help explain why the peak levels of GM-CSF or CRP expression in the respective lines of transgenic mice are much lower after intranasal challenge with *S. pneumoniae* compared to challenge with LPS.

The presence of bacteria in the blood did not increase the levels of GM-CSF in all of the transgenic animals. Two male and four female transgenics had bacteria in the blood by 48 hours after challenge but none of these had detectable levels of GM-CSF at this time and all subsequently survived to 144 hours post challenge. This may also indicate that the mice that develop bacteraemia and have high levels of GM-CSF succumbed to the infection whereas those that developed some degree of bacteraemia but did not express high levels of GM-CSF survived the challenge.

Administration of GM-CSF has been shown to prime mice for enhanced cytokine production in response to LPS and to TNF α (Brissette *et al.*, 1995). A possible mechanism to explain the death of the transgenic mice that produce high levels of GM-CSF in response to bacteraemia is that these high levels of GM-CSF prime the white blood cells of the mice to produce large amounts of many different cytokines in response to TNF α . TNF α was shown to be detected in the serum of mice challenged intranasally with *S. pneumoniae* by 12 hours after infection and the levels increased rapidly to 72 hours after challenge (Bergeron *et al.*, 1998). The TNF α produced in response to bacteraemia could then induce the production of a range of cytokines from the GM-CSF primed macrophages; including IL-6, IL-1 and more TNF α . Positive feedback could result in the production of more GM-CSF (induced by IL-6 and IL-1) and TNF α , which could lead to tissue damage and septic shock resulting in death of the animals (see figure 7.2).

Figure 7.2: Possible feedback mechanism by which GM-CSF influences its own production.



No significant difference in the survival of the transgenic and non-transgenic groups of mice was seen (figure 6.19). This would indicate that the presence of the transgene is not protective against intranasal challenge with *S. pneumoniae*.

It should however be noted that there is a significant difference in the survival of non-transgenic male mice compared with non-transgenic females. A significant difference in the survival of male and female non-transgenic mice after challenge with *S. pneumoniae* was unexpected as this sex difference has not been noted before in this model system. However, the model system mainly used female MF1 mice, and when male and female mice of a crosses between CBA/ca and Balb/c mice were compared some difference was seen between the survival of the sexes following challenge with *S. pneumoniae* (Neill Gingles, Leicester University personal communication). There may therefore be some genetic basis of the difference between the two non-transgenic groups in our experiments, and this is enhanced in the genetic background of the mice we were using.

Another explanation for the transgene not being protective against pneumococcal infection could be the effect of the low basal levels of GM-CSF expression resulting in the pathology of the spleen. The spleen is known to play an important role in the host defence against *S. pneumoniae* (Wara, 1981); and patients with asplenia or functional asplenia are predisposed to severe infections with encapsulated bacteria. One effect of low basal levels of expression of GM-CSF in the transgenic mice was splenomegaly which was associated with extensive extramedullary haemopoiesis (discussed in section 7.5.3). This pathology results in disruption of the structure of the spleen and it is likely that this is associated with disrupted function. Clearance of bacteria from the blood by the phagocytes in the spleen is therefore likely to be less efficient. Other work, however, has shown that pretreatment of splenectomized mice with GM-CSF can enhance the clearance of *S. pneumoniae* (and therefore survival of the mice) introduced intravenously (Hebert *et al.*, 1997) or as an aerosol (Hebert and O'Reilly, 1996).

Another possible explanation for the lack of protection against pneumococcal infection seen in the transgenic mice is the potential positive feedback mechanism mentioned previously (figure 7.2). This would involve activation of macrophages by the basal levels of expression of GM-CSF, which would result in production of the cytokines IL-6, IL-1 and TNF α from the macrophages, which could result in the production of more GM-CSF (by IL-6 and IL-1) and TNF α (by TNF α); leading to tissue damage.

The total white blood cell counts in the male transgenic group of mice were significantly higher than all the other groups throughout the course of the experiment (discussed section 7.5.1). There were no significant changes in the cell numbers within each group throughout the experiment. GM-CSF has been shown to stimulate an

increase in the numbers of circulating neutrophils and monocytic cells (Ruef and Coleman, 1990), so it was predicted that acute phase expression of GM-CSF would lead to a rapid increase in the numbers of white blood cells. An increase in the numbers of cells following challenge with *S. pneumoniae* may not have been seen because higher levels of GM-CSF in the transgenic mice were not seen until 48 hours after challenge. An increase in the numbers of white cells in response to increased levels of GM-CSF may therefore not have been detected until later in the experiment; this could be examined by sampling from later timepoints. It would also be interesting to monitor cell activation markers by FACS analysis to determine the effect of the increased levels of GM-CSF on the activation state of the cells.

7.5 Further Characterization of the C79/GM Transgenic Mice.

7.5.1 Higher Basal Levels of Expression of GM-CSF in Male Transgenic Mice.

The male transgenic mice were shown to have higher basal levels of expression of GM-CSF than the female transgenics. This resulted in the male transgenic mice being more sensitive to LPS, having an increased number of white blood cells and exhibiting a more severe pathology than the female transgenic mice.

A similar pattern of higher basal levels of expression of CRP was seen in the CRP transgenic mice and this effect was shown to be dependent on the increased levels of testosterone in the male mice (Szalai *et al.*, 1995)(Szalai *et al.*, 1998). The effect of testosterone on the expression of GM-CSF from constructs C79/GM and BNB/GM was examined in the tissue culture system.

No significant increase in the expression of GM-CSF from either construct was seen after the addition of testosterone. This may be because the tissue culture system used was a human cell line and stimulated with human testosterone. A sexually dimorphic pattern of CRP expression is not seen in the human system and is only seen in the CRP transgenic mice, therefore human testosterone may not have the same effect on human cells as murine testosterone on murine cells. Expression of several proteins also show sexual dimorphism in rodents, including the murine sex-limited protein. This has been shown to be expressed due to the presence of testosterone, but this effect is exerted through testosterone induced changes in the pattern of growth hormone. It was therefore proposed that the sexually dimorphic pattern of CRP expression in transgenic mice could be regulated in a similar way, that is through growth hormone (Szalai *et al.*, 1998). This would suggest that testosterone may not have a direct effect on the CRP promoter in this tissue culture system, but that growth hormone may affect the pattern of GM-CSF expression driven by the CRP promoter *in vitro*. Again, this may only be an

effect seen in the murine system and not in the human cell system that we have used. These possibilities could be examined by using a murine cell line of hepatic origin and the use of murine testosterone and growth hormone to stimulate expression. These experiments were not performed as a suitable murine cell line was not available. The role of testosterone in the sexually dimorphic pattern of expression of GM-CSF in these mice could also be examined *in vivo* using castration and subsequent testosterone replacement experiments as described by Szalai (Szalai *et al.*, 1995).

The detectable basal levels of expression of GM-CSF in the transgenic mice also indicates that the C79 construct is not controlling the acute phase expression of GM-CSF as tightly as was hoped. This may be due to the nature of the C79 construct as the basal level of CRP expression varied in the 11 lines of transgenic mice made using this construct (from undetectable levels to 14.9 $\mu\text{g/ml}$). The mean basal level of CRP expression in these lines of mice was $2.6 \pm 1.6 \mu\text{g/ml}$ compared to mean basal levels of expression of $0.88 \pm 0.85 \mu\text{g/ml}$ (varying from undetectable to 6 $\mu\text{g/ml}$) for the 11 lines of transgenic mice made using the full length (30 kb) construct (U. Ruther personal communication). This also indicates that the site of integration of the transgene affects the expression of the transgene. It is thought that if the transgene integrates at a site close to strong regulatory elements of other genes (either positive or negative regulatory elements) then these could influence the expression of the transgene. The more consistent pattern of expression of the 30 kb construct in different lines of transgenic mice may therefore be a reflection of the size of the construct, in that the regions involved in regulation of the CRP gene are separated from the influence of regulatory regions at the integration site.

In order to achieve tightly controlled acute phase expression of GM-CSF in transgenic mice it may be necessary to use the full length construct as the basis for the acute phase vector as this exhibited a more consistent control of basal levels of expression of CRP. Another possible way to achieve tight control of expression would be to create many lines of C79/GM transgenic mice and screen these for the desired pattern of expression. The work of the Murphy group showed that the different lines of transgenic mice made with each construct could show varying patterns of expression; for example with the C79 construct one of the lines had basal CRP levels of 14.9 $\mu\text{g/ml}$ and was induced by a factor of 3.9 with LPS, whereas another line had basal CRP levels of 0.01 $\mu\text{g/ml}$ and was induced by a factor of 520 with LPS. Another approach, that would solve the problems of the effect of the site of integration, would be to create a targeted construct so that the site of integration could be controlled. Such a construct could be introduced to ES cells, by homologous recombination, then chimeric mice created by the introduction of the ES cells to blastocysts. Possible genes to be replaced would include the murine CRP gene, although this is not expressed in mice which may

be due to the presence of strong negative regulatory elements surrounding the gene; or one the murine acute phase protein genes such as SAP.

None of these approaches would overcome the problem of the higher basal levels of GM-CSF seen in the male transgenic mice, which is thought to be due to the presence of testosterone / growth hormone responsive elements in the CRP promoter. Therefore an approach that is now being followed in our laboratory is to try and identify the regions involved in activation of the CRP promoter, in particular any elements responsive to testosterone or growth hormone, then to design a new acute phase vector based on this analysis.

7.5.2 White Blood Cells.

Counts of the total number of white blood cells revealed that the male transgenic mice had significantly higher numbers of cells in the blood than both the non-transgenic mice and the female transgenics (figure 6.10). One of the main functions of GM-CSF is its role in the stimulation of haemopoiesis as it stimulates the formation of granulocytic and monocytic colonies from cultures of bone marrow cells (Metcalf, 1986) (discussed in section 1.6.10.1). GM-CSF has also been shown to be involved in the formation of eosinophil and megakaryocyte cells (Robinson *et al.*, 1987), and (with erythropoietin) stimulates the growth of erythroid precursors (Donahue *et al.*, 1985).

It should also be noted that the group of female transgenic mice had significantly higher numbers of white blood cells than the group of female non-transgenics (figure 6.10). However, this is compared to the white cell counts from the mice challenged with *S. pneumoniae* (figure 6.17), where the counts do not differ significantly between the female transgenic and non-transgenic groups. This may be due to the larger group sizes associated with figure 6.10 (22 compared to 12) highlighting the smaller differences between these two groups.

It could therefore be suggested that the higher basal levels of expression of GM-CSF in the male transgenic mice are stimulating haemopoiesis with the result of an increased number of white blood cells in these animals. This result also indicates that the mGM-CSF produced by the transgene is in fact biologically active. It had already been shown that GM-CSF produced by the C79/GM construct (both in tissue culture and in transgenic mice) could be detected by ELISA but it was possible that this GM-CSF would not be biologically active. The fact that GM-CSF associated effects (that is increased production of white blood cells) were observed in this line of transgenic mice would therefore suggest that the GM-CSF is functioning *in vivo* as would be expected.

FACS analysis of the cells from the blood of the transgenic mice revealed that a greater proportion of the cells from the transgenic mice (both male and female) stained

with the antibody F4/80 which labels monocytic cells (figures 6.12 to 6.15). Also in the transgenic mice, another group of larger and more dense cells were stained with the antibody mac3 which identifies activated macrophages. This is unusual as circulating monocytic cells do not normally carry markers of macrophage activation as the cells only usually begin to differentiate once they have migrated into the tissues. These results suggest that the transgenic mice contain a greater number of monocytic cells than the non-transgenic animals and that these cells are activated to a different state.

GM-CSF not only influences the progression of haemopoiesis, but has effects on mature cells including the activation of granulocyte and macrophage cells (discussed in section 1.6.10.2)(reviewed by (Ruef and Coleman, 1990)(Gasson, 1991)). The result of FACS analysis of the blood of the C79/GM transgenic mice indicates that the macrophages from these animals are activated compared to the cells from the non-transgenic animals, and it can be reasoned that this is the result of higher levels of GM-CSF in the transgenic mice. The presence of a greater number of monocytic cells and activated cells in the blood of the transgenic female mouse was surprising as the total white cell count data indicated that there was no significant difference in the number of cells in this group of mice. The transgenic female mice also did not have detectable basal levels of expression of GM-CSF so it was surprising that a biological effect could be detected in the mice; in particular no GM-CSF was detected in the serum of the mouse studied by FACS analysis. This pattern of cells in the female transgenic mouse, revealed by FACS analysis, may be due to slightly higher expression of GM-CSF than normal (but still not detectable by ELISA) in this one mouse resulting in an enhanced biological effect. This could be examined by FACS analysis of the blood of a greater number of mice in order to see if this female transgenic mouse was unusual. It would also be useful to correlate the concentration of GM-CSF in the serum of the mice with the FACS analysis of the blood cells, as the concentration of GM-CSF did vary between animals.

Only one marker of macrophage activation was examined, so further studies looking at other activation markers would be useful to further determine the state of maturation and activation of the blood cells from the transgenic animals. Other studies of macrophage activation and function such as respiratory burst activity, cellular cytotoxicity and *in vitro* bacterial killing assays would also be useful to further characterise the effect of low levels of GM-CSF expression on the macrophages of these transgenic mice.

7.5.3 Pathology.

The most striking pathology seen in the C79/GM transgenic mice was the grossly enlarged spleen seen particularly in the male mice but also in some of the older female mice. Histological analysis of the spleen revealed substantial extramedullary haemopoiesis, and this was also observed in the liver of the transgenic animals.

Precursor cells of all the different types of blood cells were identified in the sections of spleen and liver examined. During murine fetal development haemopoiesis occurs in the spleen and liver, but after birth haemopoiesis is normally limited to the bone marrow.

Because of the role of GM-CSF in the stimulation of haemopoiesis, it is not surprising that the higher basal levels of GM-CSF expression seen in the C79/GM transgenic mice (particularly the male mice) is stimulating haemopoiesis; it is however surprising that haemopoiesis is not restricted to the bone marrow. Haemopoiesis is normally a tightly regulated process controlled by the interactions of many different cytokines, so it may be that even a slightly higher level of GM-CSF than normal is enough to 'tip the balance' towards the production of increased numbers of cells. The slightly higher basal levels of expression of GM-CSF in the C79/GM transgenic mice may therefore stimulate the usually dormant stem cells in post-natal murine spleen and liver. The identification of a low level of extramedullary haemopoiesis in the spleens of the non-transgenic animals is not entirely surprising as this has been seen to occur in many different strains of mice. This would also help explain the extensive extramedullary haemopoiesis seen in the transgenic mice, as the low basal levels of GM-CSF may be upregulating the normally low level of extramedullary haemopoiesis in these mice. There have also been reports that clinical treatment of humans with GM-CSF can lead to extramedullary haemopoiesis (Celsing *et al.*, 1992), which supports the idea that the extramedullary haemopoiesis in the spleen and liver of the C79/GM transgenic mice is due to the increased levels of GM-CSF.

The identification of bone formation in the spleen was also unusual, and no other reports of this occurring have been found. GM-CSF has been shown to stimulate osteoblast proliferation (MacDonald and Gowen, 1992) and these are the cells that are involved in the formation of new bone. The bones of the C79/GM transgenic mice appeared to be normal, so it is unusual that GM-CSF mediated bone formation is only occurring in the spleen. No bone abnormalities or formation of bone material in the spleen was observed in the transgenic mice constitutively expressing GM-CSF either (Lang *et al.*, 1987).

CONCLUSIONS.

During this project it has therefore been shown that mice transgenic for the human CRP gene express CRP with acute phase kinetics following challenge with both an inflammatory stimulus and infectious *S. pneumoniae*. The levels of CRP expression induced by the model system of infection with the pneumococcus were not as high as those produced by challenge with LPS, and it is suggested that this is due to the pattern of IL-6 and IL-1 production induced by intranasal challenge with *S. pneumoniae*. The acute phase expression of CRP induced by intranasal infection with *S. pneumoniae* was not protective, with the transgenic mice developing the same levels of bacteraemia as the non-transgenic mice and there was no significant difference in the overall time of survival between the two groups. This contradicts work by other groups who showed that CRP, either administered before infection or with acute phase kinetics in transgenic mice, was protective against infection with *S. pneumoniae*. It was suggested that this is due to the different route of infection or the different strain of pneumococci that were used in our experiments.

Because acute phase expression of CRP had been shown not to be broadly protective against infection (and possibly only protective against some serotypes of *S. pneumoniae*), a system to upregulate other immune mechanisms (such as phagocytosis) thereby leading to protection against a wider range of infections was proposed. It was reasoned that acute phase expression of the cytokine GM-CSF would lead to increased levels of GM-CSF at the onset of an infection, resulting in an increase in the numbers and activation of white blood and an increased ability to combat infection.

Acute phase vectors based on the CRP promoter were created so that the CRP coding region was replaced with the coding region for GM-CSF. Analysis of these constructs in a tissue culture system revealed one construct (pC79/GM) that gave the best pattern of acute phase expression (low basal levels and strong induction with interleukins 6 and 1), and this construct was used to create a line of transgenic mice.

Analysis of the C79/GM mice showed that these mice expressed GM-CSF with acute phase kinetics following challenge with LPS and with *S. pneumoniae*. However, basal levels of GM-CSF were detected in the transgenic mice, with higher levels in the male transgenics. The higher basal levels in the male transgenics were associated with: (a) higher numbers of white blood cells and an increased expression of activation markers on these cells; (b) extramedullary haemopoiesis in the spleen (resulting in splenomegaly) and liver with some mice dying prematurely; (c) increased sensitivity to

LPS with most of the transgenic males killed by low (25 μ g) doses of LPS. Acute phase expression of GM-CSF in the transgenic mice was not protective against fatal infection with *S. pneumoniae*.

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Appendix I: Addresses of Suppliers.

Amersham International,
Amersham Place,
Little Chalfont,
Buckinghamshire HP7 9NA.

BDH Laboratory Supplies,
Poole,
Dorset BH15 1TD.

Becton Dickinson,
Between Towns Road,
Cowley,
Oxford OX4 3LY.

The Binding Site,
PO Box 4073,
Birmingham B29 6AT.

Bio-Rad Laboratories,
Maylands Avenue,
Hemel Hempstead,
Hertfordshire HP2 7TD.

Bioventures Inc,
848 Scott Street,
Murfreesboro,
TN 37129, USA.

Clontech Laboratories Inc,
Unit 2, Intec 2, Wade Road,
Basingstoke,
Hampshire RG24 8NE.

Coulter Electronics Ltd,
Northwell Drive,
Luton,
Bedfordshire LU3 3RH.

Dynatech Laboratories Ltd,
Daux Road,
Billingshurst,
Sussex RH14 9SJ.

ECACC (European Collection of
Animal Cell Cultures),
PHLS Centre for Applied Microbiology
and Research,
Porton Down,
Salisbury SP4 0JG.

Fisons Scientific,
Bishop Meadow Road,
Loughborough,
Leicestershire LE11 0RG.

Harlan,
Shaw's Farm,
Blackthorn
Bicester,
Oxon OX6 0TP

Hybaid Ltd,
111-113 Waldegrave Road,
Teddington,
Middlesex.

Gibco BRL,
3 Fountain Drive,
Inchinnan Business Park,
Paisley PA4 9RF.

Sigma Chemical Company,
Fancy Road,
Poole,
Dorset BH17 7NH.

NCTC (National Collection of Type
Cultures,
61, Colindale Avenue,
London NW9 5HT.

Stratagene Ltd,
Cambridge Innovation Centre,
Cambridge Science Park,
Milton Road,
Cambridge CB4 4GF.

Pharmacia Biotech,
23 Grosvenor Road,
St. Albans,
Hertfordshire AL1 3AW.

(Oxoid) Unipath Ltd,
Basingstoke
Hampshire.

PharMingen,
10975 Torreyana Road,
San Diego,
CA 92121, USA.

Whatman Ltd,
Springfield Mill,
Maidstone,
Kent.

Promega,
Epsilon House,
Enterprise Road,
Chilworth Research Centre,
Southampton S01 7NS.

Zeneca,
Hurdsfield Industrial Park,
Macclesfield.

Qiagen,
Boundary Court,
Gatwick Road,
Crawley,
West Sussex RH10 2AX.

R&D Systems,
4-10 The Quadrant,
Barton Lane,
Abingdon,
Oxon OX14 3YS.