Leucocytes, Transfusion Related Immunomodulation And Colorectal Cancer - Clinical And Laboratory Aspects

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

Timothy John WHEATLEY

University of Leicester

May 1998

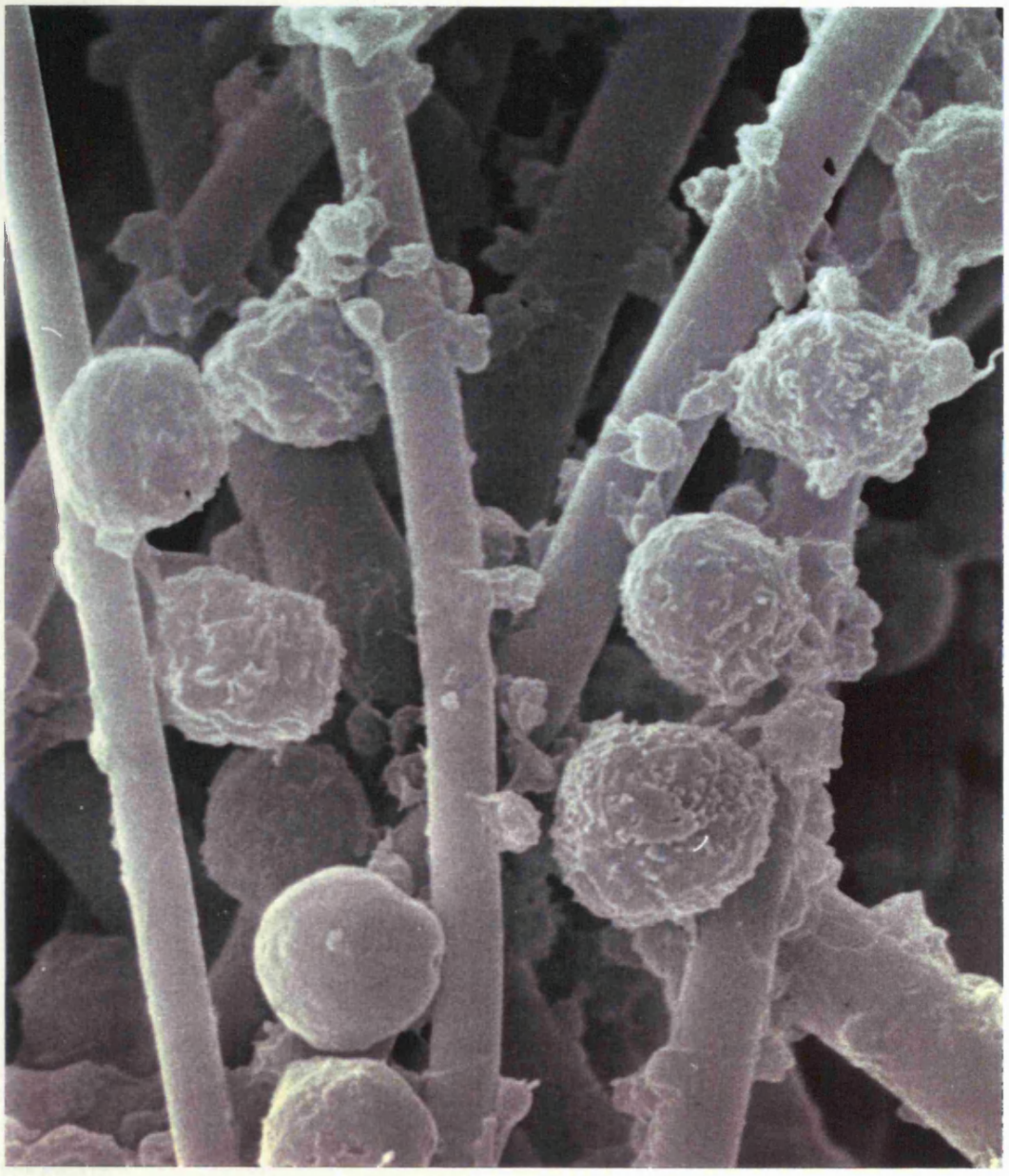
ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

"Real knowledge is to know the extent of one's ignorance."

Confucious 551-479 BC

For my sister.

Plate 1



Leucocytes trapped by the fibres of a high efficiency blood filter (x1,000)

(Scanning electron microscopy, courtesy of Pall)

CONTENTS

Acknowledgements

The work for this thesis was performed at the University Department of Surgery, Leicester General Hospital from 1994 to 1996. I am grateful to all the patients who consented to be studied in the trial, and to their respective consultants who kindly gave permission for their patients to be involved.

Efficient filtration of blood for transfusion was essential to the study and would have been difficult to achieve without the help of Geoff Lloyd and Karen Berrill from Pall Biomedical, and the staff at the Leicester blood transfusion laboratory, in particular John Revill, Deborah James, Nick Nolan and Mandy Gardner.

Collection of blood samples and patient details from the two other hospitals in Leicester was performed by junior medical staff (Dr J McCulloch and Dr M Clark), and Carol Barr proved indispensable at identifying patients and transporting samples.

Readjusting to laboratory work wasn't easy and I must thank the staff in the Department of Surgery for their guidance and tolerance, especially Natu Mistry for maintaining the cell cultures. Terry Horsburgh warrants special thanks for his help in teaching me the assays, criticising my scientific methods and providing immunological advice. Statistical help was provided by Dr J Thomas of Leicester University, and Dr B Higgins of Portsmouth University.

All of the laboratory work other than that mentioned here was performed personally by myself. This manuscript was typed and edited by myself on an Apple Macintosh laptop computer using Microsoft Word.

Mr PS Veitch, Consultant Surgeon, supervised me during the course of the project and I am extremely grateful for his guidance, encouragement and provision of an opportunity to carry out this work. Professor ML Nicholson was a constant support with persistent enthusiasm and good humoured tolerance of my efforts, and I will always appreciate that.

Finally I must thank Professor PRF Bell of the University Department of Surgery for his constant surveillance of my work and express my gratitude at being able to benefit from his experience.

CHAPTER 1

INTRODUCTION

1. OVERVIEW.

The purpose of this first chapter is to review the literature relevant to the subject matter of this thesis, thereby introducing the reader to the scientific work done prior to this date, and outlining the reasons why this piece of research was performed. To achieve this aim the area of transfusion medicine is briefly described along with a detailed analysis of transfusion related immunomodulation and its significance to malignant disease. Then colorectal cancer, one of the most prevalent malignant diseases of mankind, is discussed, including a limited description of its interaction with the immune system and the therapeutic implications thereof. A discussion of cellular cytotoxicity and its relevance to both cancer and transfusion related immunomodulation forms the last section of the chapter.

2. BLOOD TRANSFUSION.

Historical.

The history of blood transfusion dates back to the mid-17th century at a time when medical advances were being spurred on by intense scientific competition between England and France. In 1656 Christopher Wren perfected a technique of injecting liquids into the veins of dogs which he subsequently taught to members of the medical profession, who, within 10 years, had used it to transfer small quantities of blood (2 fluid ounces) from one dog to another (Myhre 1990). At the same time in France Jean Baptiste Denys was experimenting with transfusion of calves blood into dogs, and in 1667 he transfused 2 humans with lambs blood with no recorded ill effect (Denys 1667). His results were published in Philosophical Transactions, the journal of the English Royal Society, and this stimulated English surgeons to perform their own animal to man transfusion later that same year, again without incident (Lower 1667).

The French experiments continued until 1668 when a 34 year old man died following repeated transfusion of calves blood. After the first transfusion he complained of severe pain in the vein receiving the blood, and after the second, 2 days after the first, he vomited, had a nosebleed, complained of loin and chest pain, and passed a large quantity of black urine. Two months later he received a third transfusion during the course of which he suffered from convulsions, became comatose and subsequently died (Myhre 1990). Denys was brought to trial for murder (perhaps the first transfusion related malpractice suit) and although he was subsequently acquitted the Paris Faculty of Medicine declared blood transfusion to be scientifically unsound and dangerous, and the French Parliament made blood transfusion illegal in 1670. Shortly after this a proclamation by the Pope effectively banned transfusion in most of Europe and further studies were abandoned for the next 150 years (Ficarra 1942).

The first recorded transfusion of blood from one human being to another appears to have taken place in Philadelphia in 1795. The medical practitioner accredited with this event was a surgeon, Dr Philip Physick, however there is no traceable case report of this transfusion, merely a mention some thirty years later in a scientific paper about further transfusions (Blundell 1825; Schmidt 1995). It is perhaps fair to assume that the transfusion was unsuccessful! The man who is more widely acknowledged as "The Father of Blood Transfusion" was an Englishman, James Blundell. His experiments in the early nineteenth century updated the animal-to-human experiences of the seventeenth century, and showed that human-to-human transfusion was possible. Over the ten year period from 1819 to 1829 he performed 11 transfusions of human blood, designed a device called the "gravitator" (which allowed venesected blood from a donor to be collected in a funnel and then fed by gravity through a rubber tube into the patient's vein, controlled by a valve), reported that the less the exposure of transfused blood to "air and inanimate objects" the better, and speculated that cooling of blood prior to transfusion may serve to prolong its active life (Myhre 1995). Students of Blundell's techniques continued the application of human-to-human transfusion, albeit in limited numbers, and by 1849 it was viewed to be a successful procedure with a mortality rate of 1 in 3 (Walton 1849).

After this rather courageous start to the science of blood transfusion the stage was set for the development of modern transfusion medicine, beginning in the early twentieth century when Carl Landsteiner made his historic discovery of the ABO blood grouping system (Ficarra 1942). The application of this knowledge along with anticoagulant technology and sterile techniques allowed for the establishment of blood transfusion as an essential part of modern medical practice, and many advances in the treatment of patients, especially in the field of surgery, would have been difficult, if not impossible, without the availability of human blood products.

Along with the acceptance of blood transfusion as a beneficial form of therapy has come the realisation that the collection, storage and transfer of blood from one human being to another is not without risk. Side effects of transfusion range from mild allergic reactions, through bacterial and viral infection, on to more serious immune-related reactions such as acute haemolysis (Table 1:1, p1:7). Febrile, non-haemolytic reactions complicate 1% of transfusions; the risks of acquiring viral hepatitis or the human immunodeficiency virus (HIV) are estimated at 1 in 5,000 and 1 in 400,000 respectively (in the United States); and the risk of a fatal haemolytic reaction is approximately 1 in 600,000 (Klein 1992; Nicholls 1993; Jeter 1995).

One side effect of blood transfusion that has received relatively little publicity over recent years, partly overshadowed by public and professional concern over the risk of HIV transmission, and partly because of continuing controversy over its clinical significance, is immunomodulation.

Immunological:	febrile reactions urticaria alloimmunisation hypersensitivity reactions haemolytic reactions (immediate/delayed).
Infective:	viral. bacterial. protozoal.
Volume related:	cardiogenic (e.g. pulmonary oedema). metabolic (e.g. hyperkalaemia, hypocalcaemia). clotting disorders.

Table 1:1. Detrimental side effects of blood transfusion.

History of transfusion related immunomodulation.

Medawar was one of the first to study the immunomodulatory effect of blood transfusion in 1945 when he demonstrated a commonality of antigens between blood and other tissues (Medawar 1945; Medawar 1946). In a series of animal experiments he noted that a mouse grafted with skin from a suitable homologous donor would rapidly reject a second skin graft from the same donor when grafted several days after rejection of the original. This rapid rejection would also occur if the recipient mouse was transfused with blood from the donor prior to receiving a skin graft, implying that blood sensitised the recipient in the same way as an initial skin graft. Medawar deduced that this "second set" phenomenon could only occur if blood and skin shared at least some common donor antigens.

Medawar's work was undertaken at a time when transplantation surgery was in its infancy, and clinicians were working hard to understand the complexities of graft rejection and how to prevent it. As a direct consequence of his findings the early transplant surgeons adopted a policy of deliberately avoiding blood transfusions, as far as was possible, in potential transplant recipients, believing that if a patient was exposed to foreign antigens from a transfusion there was a very real chance that similar antigens may later be presented to him in his transplant, leading to rejection of the graft by the second set phenomenon.

Over the next 30 years studies on both animals and humans looking into this effect of blood transfusion started to suggest a somewhat different story from that originally told by Medawar. Halasz et al (Halasz 1964) published results of their work with dogs receiving homologous renal transplants, showing that recipients that had been transfused with blood from the donor prior to transplantation had a much more favourable graft survival rate than those that had not. 1973 saw the publication of a large retrospective study of patients who had undergone renal transplantation,

demonstrating that graft survival was increased by up to 20% in those who had received a transfusion prior to transplantation (Opelz 1973). The greatest benefit was seen in patients receiving 5 or more transfusions, completely contradicting the assumptions drawn from Medawar's original work. Over the next 10 years the attitude of transplant surgeons changed dramatically from one of deliberate avoidance to that of deliberate transfusion. Units developed protocols for both donor-specific and random donor transfusion, resulting in a rise in the one-year graft survival rate from 40% to 60% (Opelz 1985).

The 1980's saw the widespread introduction of cyclosporine A as an immunosuppressive agent in transplantation, and this powerful drug had as big an effect on graft survival as pre-transplant transfusion had done in the late 1960's/70's. One year renal graft survival rates rose to the level of 80%, and data from the Collaborative Transplant Study (CTS) suggested that the transfusion effect now only conferred a 5% improvement in one year graft survival, and by 3 years post transplant there was no difference in graft survival between transfused and non-transfused recipients (Opelz 1989). It is unlikely that cyclosporine alone was responsible for the vast improvement in renal transplant outcome that occurred during this time. Concurrent with improvements in immunosuppressive therapy were beneficial changes in patient care (both pre- and post-operatively), patient selection, HLA matching, rejection therapy, and graft perfusion/ transportation techniques (Yuichiiwaki 1988); and the combination of these many factors contributed to the high one year survival rate.

Mechanisms of transfusion related immunomodulation.

Inspite of all the progress in our understanding of immunology there is, as yet, no clear explanation as to how a transfusion of allogeneic blood causes suppression of the recipient's immune function. Some authors believe that transfusion itself is not responsible for a so-called "transfusion effect", rather that it is a surrogate marker for some other event that is responsible; in the case of transplant surgery that transfusion pre-selects patients who will not become sensitised to donor antigens, and in the case of other disease processes that transfusion is a marker for severity of disease and/or treatment which would automatically place the subject in a worse prognostic group (Mickey 1983; Brunson 1990). On the other hand many non-specific changes in immune function have been described following transfusion and the likelihood that a complex, general inhibition of the immune system is responsible for the effect is favoured by many authors (Blumberg1990; Brunson 1990; Mincheff 1993; Blumberg 1994; Jensen 1994).

Some of the effects of transfusion on immune function are as follows:

- decreased cytokine production
- increased suppressor cell number/function
- decreased helper cell number/function
- decreased natural killer cell activity
- decreased monocyte function
- depressed delayed hypersensitivity reactions (Kaplan 1984; Schot 1986; Wood 1988l; Jensen 1992; Jensen 1996).

The alteration of interleukin-2 (IL-2) and prostaglandin E_2 (PGE_2) metabolism has been postulated as being at the heart of these changes (Brunson 1990). IL-2 is a powerful up-regulator of immune responses which is produced by T helper cells and is necessary for the generation of cytotoxic T cells. PGE_2 down-regulates immune function by suppressing macrophage antigen expression and presentation, and

inhibiting Il-2 production and target cell response to IL-2. There are currently three theories to explain these effects.

Clonal deletion.

Clonal deletion is proposed as an antigen specific event whereby a blood transfusion shares one or more antigens with the recipient, something which occurs deliberately in the field of transplantation when donor-specific transfusions are used prior to transplantation. In such a transplant scenario the donor-specific transfusion would prime the recipients immune system to the "foreign" antigens, then when the same antigens are presented again in the form of a solid organ transplant a population of T and B lymphocyte clones are produced (Takiff 1987). The deletion of these clones then occurs by nature of the powerful immunosuppressive agents used at the time of transplantation. Obviously in the straightforward transfusion setting there are no immunosuppressive drugs to delete the clones, and a more natural form of clonal deletion takes place. As foreign MHC antigens are introduced into the thymus evidence is emerging that T cell clones whose receptors are directed against such MHC are deleted (Dzik 1996). As well as occurring in the thymus gland (so called central or thymic deletion) the "dropout" of specific T cell receptors has been demonstrated in peripheral blood, providing that the transfusion shares HLA-DR antigens with the recipient (Munson 1995). This is referred to as peripheral or nonthymic deletion.

Tolerance.

Tolerance, or host anergy, is a non-reaction by the recipient of a transfusion, a failure of the immune system to mount a response. A description of tolerance by Jerne in 1974 put forward the idea of "immune networks", whereby T cell receptors and antibodies that are generated by the immune system in response to foreign antigen presentation are then themselves perceived as new, or foreign, antigenic material (Jerne 1974). Production of T cells and antibodies specific for these antigens would

obviously down-regulate the response to the initial foreign antigen, and the interaction between these elements of the immune system begins the immune network. Repeated presentation of antigens increases the size of such a network where antigen and antibody interactions are extensively interlinked, and serves to lower the overall immune response.

Another way to look at the development of tolerance is to consider anti-idiotypic antibodies. The idiotype of a T cell receptor is the site which actually combines with the antigen, and the antibody that blocks this is known as an anti-idiotypic antibody (Brunson 1990). These antibodies possibly compete for binding sites with foreign antigen, with which they show certain similarities, and even after a single transfusion of blood such antibodies have been detected for both T cell and immunoglobulin receptors (Burlingham 1988).

Current research is focusing on a slightly different aspect of the T cell - antigen interaction, namely that of co-stimulation. Part of the mechanism of T cell activation is co-stimulation by molecules not directly involved in the immune process. In the absence of such co-stimulation T cells may become anergic and unable to respond adequately to antigenic challenge (Boussiotis 1994). This has been shown to occur after exposure to interleukin-10 (secreted by certain T helper cells) *in vitro* , preventing expression of the CD80 co-stimulatory molecules on macrophages (Dind 1993). It also happens following exposure of a recipient to donor MHC molecules (Bell 1993).

Active suppression.

The presence of a suppressive element to the immune response has been recognised for a long time, and forms part of the natural regulatory process of the immune system. Suppressor T cells are produced over a period of days by a multistep process initiated when macrophages present antigen to an inducer T cell (Ts1). The Ts1 cells then transfer details of the antigenic configuration to Ts2 cells, which are an intermediate in the process and not directly suppressive themselves. The Ts2 cells

transfer on the antigenic information to the final cells in the pathway, Ts3 lymphocytes. These have surface receptors for the original antigen as presented to the Ts1 cells and produce non-specific suppressor factors which decrease interleukin-2 receptor expression and decrease macrophage activity (Leivestad 1984; Loertscher 1988; Brunson 1990).

As well as the well established role of T suppressor cells in down-regulating immune responses, there has recently been interest in the activities of T helper cells (Dzik 1996). Two subsets of T helper cells are recognised dependant on the cytokines that each release. Th1 cells release interleukin-2, tumour necrosis factor α, and interferon γ; Th2 cells release interleukins -4, -5, -6 and -10. Th1 cells stimulate cellular immune responses, Th2 cells stimulate humoral immune response, and there appears to be an inhibitory effect between the two groups such that Th1 cells when activated will suppress Th2 activity. Transfusion of allogeneic blood in humans results mainly in a Th2 response, which would in turn suppress Th1 stimulation of cellular immune responses (Kirkley 1995).

Leucocytes and transfusion related immunomodulation.

A blood transfusion contains a cocktail of constituents; erythrocytes, leucocytes, platelets, plasma and anticoagulant drugs. Any one, or any combination of these constituents could be responsible for causing the immunomodulatory effects described. For several years now the commonly held belief has been that the donor leucocytes are responsible, both from work involving leucodepleted blood products, and from experimental investigation of the mechanism of the transfusion effect (Klein 1992; Thomson 1993; Bordin 1994; Jensen 1994). In some of the original studies into the transfusion effect in transplant patients the longest graft survival rates were from those patients who received whole blood transfusions rather than those who received red cell concentrates, or washed red cells (Opelz 1982). Along similar lines those renal transplant patients who received leucodepleted blood transfusions prior to transplantation had identical graft survival to those patients who were never transfused (Persijn 1979). Depression of the delayed hypersensitivity reaction occurs following blood transfusion, but this depression is more marked if the transfusion is of whole blood rather than a red cell concentrate (Schot 1986; Nielsen 1991). Several parameters of immune function (natural killer cell activity, CD4/CD8 lymphocyte ratio, lymphocyte proliferation and interleukin-2 production) in patients undergoing elective colorectal surgery are suppressed more in patients who receive transfusion of whole blood during the peri-operative period than those who receive a leucodepleted transfusion (Jensen 1992; Jensen 1996). Current theories explaining the mechanism of the transfusion effect rely on the presentation of foreign MHC antigens to the recipients immune system by donor cells i.e. lymphocytes (Bordin 1994; Dzik 1996).

Clinical Implications of the Transfusion Effect.

As described previously the initial realisation of the immunomodulatory effect of blood transfusion arose in the field of transplantation, and more recently the advent of powerful immunosuppressive agents (cyclosporine A in particular), and improvements in patient care (both pre- and post-operatively), patient selection, HLA matching, rejection therapy, and graft perfusion/ transportation techniques, have reduced the "transfusion effect" in this field to negligible levels (Yuichiiwaki 1988). In recent years clinicians have considered whether the immunosuppressive effects of transfusion may have influence on other aspects of patient care, and for those treating patients at or around the time of surgery (a time when the need for transfusion often arises) the concern has been whether transfusion may affect the original disease process, and whether it may contribute to patient morbidity during the recovery period. As far as post-operative immunocompetence is concerned the three main areas of interest have been *(i)* tumour recurrence rates, *(ii)* post-operative infection rates, and *(iii)* clinical course of inflammatory disorders such as Crohn's disease.

Transfusion and cancer recurrence.

Once the transfusion effect had been recognised and utilised in the clinical setting of transplantation (albeit erroneously at first) it was inevitable that its influence on other areas of medical practice would be investigated. However, it wasn't until the early 1980's that Burrows and Tartter reported the results of their retrospective review of colorectal cancer patients, demonstrating a positive association between perioperative blood transfusion and cancer recurrence (Burrows 1982). They reviewed 123 patients who had undergone resection of a colorectal cancer in their hospital and had been followed up for at least 5 years. Using multivariate analysis to control for covariate factors they concluded that blood transfusion was the second most important

prognostic variable, after pathological staging. This first report of such a link between blood transfusion and cancer prognosis stimulated interest around the world, and numerous studies on this matter have now been published, some of which support and others which dispute the presence of such an association.

The majority of the work done so far has looked at colorectal cancer as it is a condition which is both common and often requires the sufferer to receive a blood transfusion, either because of disease related anaemia (particularly gastrointestinal tract bleeding), or to replace blood lost as a result of surgical intervention. Thirty three studies so far (Burrows 1982; Agarwal 1983; Burrows 1983; Bickel 1985; Blair 1985; Blumberg 1985; Foster 1985; Francis 1985; Frankish 1985; Nathanson 1985; Ota 1985; Blumberg 1986; Corman 1986; Parrott 1986; Creasy 1987; Francis 1987; Kiff 1987; Ross 1987; Voogt 1987; Weiden 1987; Corman 1988; vanLawick 1988; Beynon 1989; Crowson 1989; Mecklin 1989; Vente 1989; Wobbes 1989; Bentzen 1990; Cheslyn-Curtis 1990; Liewald 1990; Marsh 1990; Jahnson 1992; Busch 1993; Houbiers 1994) have investigated the link between recurrence of this form of cancer and blood transfusion, while yet further studies have concentrated on cancer of the breast (Foster 1983; Tartter 1983; Foster 1984; Nowak 1984; Bickel 1985; Miller 1985; Tartter 1985; Voogt 1987; Crowe 1989; Kieckbusch 1989), lung (Tartter 1984; Bickel 1985; Hyman 1985; Pastorino 1986; Foster 1988; Kelly 1988; Moores 1989), kidney (Manyonda 1986; Mikulin 1986; Moffat 1987), prostate (Heal 1988; McClinton 1990; Davies 1991), stomach (Kaneda 1987; Sugezawa 1989), cervix (Blumberg 1985), vulva (Dalrymple 1986), head and neck (Johnson 1987; Jackson 1989; Jones 1990), larynx (Jackson 1989), bone, (Chesi 1989) and soft tissue sarcoma (Rosenberg 1985). The two most striking features of these various studies are firstly that they are almost all retrospective in nature, and secondly that they reach completely different conclusions - on the one hand that transfusion worsens the prognosis of cancer patients, and on the other that it makes no difference.

There are two questions that need to be answered by any study looking at the hypothesised association between perioperative blood transfusion and poor prognosis. Most important is whether or not such an association exists to any degree of statistical significance, and if so then is it a causal relationship or just casual? The fact that the majority of studies so far have been retrospective makes it very hard to answer either of these questions with any degree of certainty. Without randomisation the decision to transfuse patients or not is dependant on clinical criteria which relate to the nature of the disease and the type and extent of surgery involved in its treatment, and this inevitably leads to significant differences between the two populations of patients. The studies referred to earlier illustrate well between them that the population of cancer patients receiving perioperative blood transfusions are associated with a variety of adverse prognostic factors compared to the non-transfused group, such as age (Nowak 1984; Blumberg 1985; Foster 1985; Mikulin 1986), tumour site and size (Blumberg 1985; Francis 1985; Frankish 1985; Nathanson 1985; Mikulin 1986; Parrott 1986; Francis 1987; Liewald 1990), metastases (Mikulin1986), extent and duration of surgery (Blumberg 1985; Parrott 1986; Pastorino 1986; Heal 1988; Jones 1990), and operative blood loss (Tartter 1983; Parrott 1986). Death due to non-cancer related causes has been shown to be more common and to occur sooner after operation in transfused patients (Hyman 1985; Voogt 1987; vanLawick 1988), and when all these factors are considered together it is easy to appreciate the argument that these patients are already in a "high risk" group and that transfusion is simply a marker of this.

In order to make allowance for these variable factors authors have employed statistical tools including multivariate analysis, and recently three separate groups have combined the results of several studies looking at colorectal cancer and the transfusion effect (Chung 1993; Vamvakas 1993; McAlister 1998). These groups have performed meta-analysis on the pooled results of up to twenty different studies, the intention being to increase analytical power by increasing the sample size, and

thereby provide an estimate of the magnitude of any transfusion effect. Chung et al reviewed 20 studies covering a period from 1951 to 1986 and incorporating 5236 patients in total, and concluded that transfused patients were 69% more likely to suffer a negative outcome (disease recurrence, cancer death, death) than non-transfused - a cumulative odds ratio of 1.69. In contrast, Vamvakas et al failed to show a significant adverse effect from their combined total of 2915 patients, and were of the opinion that the 37% increase in relative risk of cancer recurrence that they observed could be confusing due to residual confounding factors relating to the retrospective, uncontrolled nature of the studies. They felt that any radical alteration of transfusion practices on the basis of existing data could not be justified and encouraged the setting up of randomised, controlled trials. McAlister's group identified 8 studies that fulfilled their inclusion criteria for analysis - namely randomised controlled trials and prospective cohort studies with suitable control groups - and looked at death from any cause, cancer recurrence, and post-operative infections. The relative risk ratios for these end-points were respectively 0.95, 1.05, and 1.00. They concluded that current evidence did not support the theory that allogeneic blood transfusion imparted a worse prognosis to cancer patients, and again encouraged further studies.

Transfusion and postoperative infection rates.

Renal allograft survival and cancer recurrence rates are both long term markers of clinical outcome, whereas postoperative infection usually manifests within 7 to 14 days of surgery and would be expected to be more prevalent in patients who are immunosuppressed to a significant degree. The same workers in America who first reported on cancer recurrence rates published their results of postoperative infection rates amongst transfused and non-transfused patients a few years later (Tartter 1988). They found an increase in infection rate from 4% to 25% which was independently

associated with a perioperative blood transfusion. Increased infection rates of similar magnitude have been reported for abdominal surgery (Jensen 1992; Ford 1993; Edna 1998), orthopaedic surgery (Murphy 1991; Triulzi 1992), cardiac surgery (Miholic 1985; van de Watering 1998), and trauma patients (Edna 1992; Agarwal 1993). Overall these studies appear more supportive of the idea that perioperative blood transfusion has a deleterious effect on patient outcome, even when combined and subjected to meta-analysis (Blumberg 1990; Blumberg 1994). This acceptance is still not universal though, and there remains a body of opinion that this association is due to other variables not adequately controlled for (Vamvakas 1996), although van de Watering's more recent study of cardiac surgery patients had good controls with large sub-groups, and showed a convincing deleterious effect of allogeneic blood transfusion on infection rates, and interestingly on mortality rates.

Transfusion and inflammatory disorders.

Far less well reported is the effect that perioperative transfusion has on the clinical course of Crohn's disease, a condition which may be auto-immune in nature. Two studies have shown a 2 - 3 fold increase in disease recurrence in patients who were not transfused at the time of surgery, compared to those who were (Peters 1989; Williams 1989). The numbers of patients in both studies were small but if the effect was real it may represent a beneficial aspect of transfusion related immunomodulation similar to that described for transplant patients.

3. COLORECTAL CANCER.

Tumours of the large bowel account for 14 and 16% of cancer deaths amongst western men and women respectively, and in Britain 22,000 people per year die from this disease, making it second only to lung cancer as the commonest cause of cancer death in this country (Begent 1992; Deans 1992). It appears to be a disease predominantly of the Western world, being 20 times more likely to affect the average European than black African, and even within countries there are geographical differences, Scotland, for example, having a higher incidence than England. As mentioned already there is a slightly higher incidence amongst women compared to men, and within the broad heading of colorectal cancer men are twice as likely to have rectal tumours, while lesions of the right colon are more common in women.

Numerous factors have been put forward as important in the aetiology of colorectal cancer, and diet is one of the most significant of these. Diets rich in fat, cholesterol and protein but low in fibre (a good description of a Western diet) have significant associations with a higher incidence of large bowel tumours. It is believed that most, if not all, colorectal cancers develop from adenomatous polyps, the polyps representing a pre-malignant stage of the disease, and inherited conditions that give rise to polyps, such as familial adenomatous polyposis and Gardner's syndrome, are associated with a high rate of cancer. Other aetiological factors include inflammatory bowel disease, surgical procedures and radiation.

Staging of colonic cancers is done most commonly according to Dukes' classification (Table 1:2, p1:22). Dukes' original classification described stages A, B, and C (Dukes 1932), and stage D has entered popular use since then. Despite other staging systems and modifications this simple and easy to use classification remains the standard

Tumour stage	Description
A	Limited to bowel wall
B	Involving or penetrating serosa
C	Lymph node involvement
(D)	*(Distant metastases)*

Table 1:2. Dukes' classification of colorectal cancer *(with popular modification)*.

Tumour stage	Frequency (%)	5 year survival (%)
A	10	70
B	35	55
C	29	30
D	26	<10

Table 1:3. Dukes' stage, frequency and survival rates (Chapuis 1985).

throughout the UK, showing a strong correlation with patient survival (Deans 1992). Table 1:3 (p 1:22) shows the relative frequency of the different stages and their respective 5 year survival rates.

By far the majority of theses tumours are adenocarcinomas, rarely they may be lymphomas, squamous cell carcinomas or melanomas. The tumours display a variation of differentiation and can be graded histologically into three groups: well differentiated, moderately differentiated and poorly differentiated. Moderately differentiated tumours account for just over 60%, unfortunately only 9% are well differentiated which is associated with a better prognosis (Chapuis 1985). Overall 5 year survival for this common disease is poor, currently less than 30% (Begent 1992).

The mainstay of treatment is surgical resection, aiming to cure the patient by removing all of the cancer. The problem with this approach if used alone is that a large number of patients have local or distant spread of the tumour which defeats the possibility of a cure (see Tables 1 & 2). Refinements of surgical technique, which should of course be encouraged (Jeekel 1995), will not deal with the problem of metastatic disease. Prevention of metastases can be attempted in two ways; either by preventing the disease itself, which would require a degree of certainty as to the aetiology and pathophysiology; or by detecting tumours early, ideally at the adenomatous polyp or even the Dukes' A stage, and offering early excision (Hardcastle 1986). Adjuvant therapy for metastatic and residual disease consists of chemotherapy, radiotherapy, and immunotherapy, and may be used either pre- or post-operatively.

Cancer, the immune system and immunotherapy.

Virtually all human cancers, when studied under the microscope, show a varying degree of cellular infiltration by lymphocytes, plasma cells and macrophages. The significance of this infiltrate has caused much debate; the negative view being that its presence is merely a reflection of the lymphatic environment of the tumour, while on a more positive note it may represent an immune reaction to "foreign" tumour tissue. The degree of infiltration and quantity of lymphocytes in lymphatics draining affected areas has shown positive correlation with prognosis (Fisher 1971; Ioachim 1976; Lauder 1977).

The beginnings of an understanding of the relationship between tumours and the immune system date back some 100 years when the very concept of an immune system was only just emerging. William Coley, an American surgeon, recognised an association between acute infection and remission of cancer in a patient of his who was cured of a sarcoma after developing the streptococcal skin infection, erysipelas (Coley 1893). He used this observation to treat other cancer patients by deliberate infection with erysipelas, and went on to develop mixed cultures of streptococcus and bacillus prodigiosus, otherwise known as "Coley's mixed toxins". These toxins, which are now recognised as immunostimulants, were used for 40 years with dramatic cures in 25% of patients treated, but also with unfortunately inconsistent effects. At much the same time Paul Ehrlich proposed (in 1909) that the immune system may protect the body against cancer by constant surveillance against transforming cells, and this concept of "immunosurveillance" has since been refined into a theory which is important to the field of tumour immunology and will be referred to again later (Burnet 1970). The basic theory states that tumour cells must present antigens that allow them to be differentiated from normal cells, and in doing so they elicit an immune response that protects the host against tumour growth.

Tumour specific antigens (TSA) have been characterised within the last 10 years, and appear to differ from normal host antigens in two ways; *(i)* single point mutations in otherwise normal proteins, *(ii)* aberrant expression of an otherwise normal protein (Boon 1992; Smith 1994).

If the immune system can recognise tumour cells as foreign why is it that tumours are able to grow at all in people who have functioning immune systems? The answer is unclear but several theories exist. If the immune system is functioning normally then the quantity of foreign cells produced may lead to a state of persistent antigen overload, particularly important at the tumour - host interface (Smith 1994). Also it has been shown that tumours can lose their major histocompatibility complex (MHC) representation, thus limiting the possibility of adequate TSA presentation (Chauvenet and Smith 1978). Alternatively the immune system may not be functioning properly, either with a local microenvironment causing an immunosuppressive effect on normal lymphocytes (Somers 1996), or as a progressive, systemic immunodeficiency (Twomey 1974).

The principle behind immunotherapy for cancer is to boost the natural response to the tumour, and 5 basic strategies are used, illustrated here for colorectal cancer.

1. Nonspecific immune adjuvant agents - these agents are mainly toxins from bacterial cell walls, such as bacillus Calmette-Guerin (BCG). The most successful example has been levamisole (in conjunction with 5-fluorouracil) (Moertel 1990).

2. Active specific immunotherapy - here a non-specific immune adjuvant agent is combined with a TSA to produce tumour specific immunity in the host (Gray 1989).

3. Adoptive immunotherapy - where a population of tumouricidal cells are generated and expanded by cytokines, e.g. lymphokine-activated killer (LAK) cells treated with interleukin-2 (IL-2).

4. Passive specific immunotherapy - monoclonal antibodies can be developed for TSAs and last for 1 - 2 days in vivo.

5. Biologic response modifiers - cytokines such as IL-2 and interferon-γ (IF-γ).

In spite of this wide variety of possible treatments, immunotherapy has not made a great impact on the management of colorectal cancer. Studies of the immune system of patients with colorectal cancer have shown that their responses are already suppressed (Monson 1986) and that the added insult of major surgery suppresses them further (Lennard 1985; Sedman 1988). Tumour cells are released into the circulation at the time of surgery (Salisbury 1965; Leather 1991; Juhl 1994; Wong 1995) and animal work has clearly shown that there is a risk of dissemination (Weese 1986). It would certainly seem sensible to suggest that any impairment of patient immunity at the time of surgery may cause tumour cells to be seeded on so-called "fertile soil", and therefore measures should be taken to prevent this happening. Treatment of these patients in the peri-operative period with low dose cytokines abrogates certain aspects of the immunosuppression (Nichols 1993) although there is as yet no information on the long term outcome of this type of intervention. IL-2 increases cellular cytotoxicity in patients with malignant disease by increasing both numbers and activity of natural killer cells and lymphokine-activated killer cells (Deehan 1995; Atzpodien 1993; Nichols 1992). Histamine receptor antagonists have also been shown to exhibit similar properties (Nielsen 1995), and there is limited evidence that there may be some long term benefits of peri-operative treatment with such drugs (Adams 1994). It appears that one of the histamine receptor antagonists, ranitidine, can limit the transfusion induced immunosuppression described earlier, and

in doing so may reduce the transfusion effect seen in patients undergoing surgery for malignant disease (Nielsen 1989; Browell 1993).

4. CELLULAR CYTOTOXICITY.

Natural killer (NK) cells.

NK cells are large, granular lymphocytes that are capable of killing target cells irrespective of class I or II major histocompatibility antigen expression (non-MHC restricted cytotoxicity) (Lanier 1986; Trinchieri 1989). They comprise, on average, 10 - 15% of human peripheral blood lymphocytes, and have been isolated from spleen, bone marrow, liver, lung and intestine as well as from the tissues of other vertebrates such as mice, monkeys, rats, hamsters, dogs, cats, birds and fish (Robertson 1990). In attempts to accurately identify human NK cells the search for a single surface antigen has so far been unsuccessful. The vast majority are CD3 -ve (>95%) and virtually all non-MHC restricted cytotoxic cells express CD56. Of these CD56 +ve cells approximately 90% are also CD16 +ve, and the 10% or so that are not are possibly inactive precursors of active NK cells (Robertson 1990). For the purpose of laboratory identification it is currently accepted that the majority of human NK cell activity is mediated by CD3 -ve/CD16 +ve, CD56 +ve lymphocytes (Lewis 1992).

NK cells have a variety of roles in normal human physiology. These include:

- tumour cell lysis
- control of microbial infections (viral, bacterial, fungal and parasitic)
- regulation of haematopoiesis
- involvement in graft versus host disease

For the purpose of this thesis the main interest is in their tumouricidal behaviour, and other roles will be only briefly outlined.

Microbial infections

NK cells are recognised to be active against many microbial pathogens, with the majority of work to date focusing on viral infections. Virally infected cells cause activation of NK cells by the production of cytokines and/or the presence of viral glycoproteins (Storkus 1991; Ramsay 1993), these NK cells then proliferate, accumulate at the site of infection and exert control by a combination of lysis of virally infected cells, and antiviral cytokine production (Welsh 1992; Brutkiewicz 1995). The role of NK cells against other pathogens remains less clear. Although *in vitro* studies have demonstrated "natural killing" of gram-positive and gram-negative bacteria (Garcia-Penarrubia 1989), fungi such as *Cryptococcus neoformans* (Lipscomb 1987; Hidore 1989), and parasites (Hatcher 1982; Hauser 1986), it is not obvious how this is mediated, though direct lysis and cytokine release are postulated again (Garcia-Penarrubia 1989; Robertson 1990; Garcia-Penarrubia 1992).

Haematopoiesis

NK cells have both an inhibitory and a stimulatory effect on haematopoiesis, an effect which can alter depending on prevailing experimental or pathological conditions (Trinchieri 1992). Though NK cells are capable of directly lysing progenitor cells with which they come into contact there is more evidence to support the theory that their role in regulation is mediated by the secretion of soluble cytokines, but there remain many unanswered questions regarding this role (Trinchieri 1989; Robertson 1990).

Graft versus host disease (GVHD)

Acute and chronic GVHD remain important causes of morbidity and mortality after bone marrow transplantation, classically involving cytotoxic donor T-cells which react to host tissue in the acute form, and high levels of suppressor cell activity in the chronic form. This manifests clinically with opportunistic infections and autoimmunity respectively. High NK cell activity soon after bone marrow transplantation, presence of NK cells in classic GVHD lesions, and evidence of decreased incidence of GVHD following NK cell depletion all point to an important involvement of NK cells in this phenomenon (Trinchieri 1989; Hogan 1992).

The role of NK cells in tumour biology has been investigated ever since the first reports of infiltrating immune cells in human malignancies (Hamlin 1968) which were later dubbed "natural killers". Their function in the immune response to cancer can be considered under three headings; 1, immunosurveillance; 2, regulation of primary growth; 3, metastasis.

The theory that the human body possesses a surveillance system that constantly polices against the development of cancerous cells is not new. Paul Ehrlich in 1909 published one of the first scientific papers relating to such an idea, proposing that "natural immunity" was responsible for the inactivation of abnormal cells that must be produced during the processes of development and differentiation, and that without such a system carcinomas would be expected to appear with enormous frequency. The theory was developed further into that of "immune surveillance" by Burnet some 60 years later who put forward the idea of an immune system that was capable of recognising and destroying *in situ* tumours before they became established.
This theory is based on the premise that tumours are antigenic by nature, and once recognised they initiate a thymus dependent immune response which results in their destruction. One of the main implications from that hypothesis is that one would expect to find a relatively high incidence of tumours in subjects who suffered from immunodeficiency states. In spite of careful investigation this did not prove to be the case, except with certain lymphoproliferative and cutaneous tumours, and the theory has since been expanded by other workers. This includes looking at all types of cellular cytotoxicity, and also blurring the distinction between immune surveillance and response to primary growth with the consideration that so-called failure of such a system may reflect the fact that true surveillance does not exist as originally described (Stutman 1975; Patek 1988; Robertson 1990).
The evidence that NK cells have a role in tumour surveillance comes mainly from human studies. In keeping with the original theory of immune surveillance certain subjects with low NK cell activity have an increased incidence of malignancies: those

with the Chediak-Higashi syndrome of suppressed NK activity but otherwise normal cellular immunity have a pre-disposition to lymphoproliferative disorders (Roder 1980), patients with familial malignant melanoma have low NK cell activity (Hershey 1979), and subjects with a strong family history of cancer have a lower NK cell activity than those without (Strayer 1984).

Host reaction to primary tumours has been commented on since Ehrlich first wrote about "natural immunity". In 1968 a group from the Royal Marsden Hospital sought to describe and grade the cellular infiltrate in and around primary breast carcinomas, and relate this to the patient's prognosis (Hamlin 1968). They produced a grading system based upon histological grade and intensity of "host defence reaction" (lymphocytic infiltrate), and subsequently found this to correlate closely with prognosis, suggesting that host reaction to the tumour, as well as histological grade, was important prognostically. Interest in the immune response to, and cellular infiltration of, tumours has increased dramatically, and NK cells have been demonstrated infiltrating tumours of the gastrointestinal tract, breast, bladder, lung and skin (Viac 1977; Moy 1985; Tsujihashi 1988; Markey 1989; Kernohan, Sewell et al. 1990; LeFever 1991; Gudmundsdottir 1992; Matsuda 1995).

Animal studies have explored the role of NK cells against tumours in a variety of manners. Animals that are deficient in NK cells, such as homozygous beige mice, show a far more rapid rate of growth of both experimentally induced and transplanted tumours (Karre 1980; Talmadge 1980). Tumour susceptibility of mice which have been deliberately rendered immunodeficient by immunosuppressive drugs can be reversed by NK cell clones (Warner 1982). Specific suppression of NK cell activity by anti-asialo-GM1 serum in nude mice results in accelerated growth of tumours that are NK sensitive, but not of tumours that are NK resistant (Habu 1981). All of this work tends to support the theory that NK cells are both attracted to areas of primary tumour growth, and are instrumental in providing resistance to such growth.

If tumours are to metastasise successfully they must seed themselves by some route to a site distant from their primary site, and then survive and multiply to form a secondary growth. Animal work has shown that inhibition of NK cell activity by treatment with anti-asialo-GM1 serum decreases the ability to eliminate tumour cells from the blood stream after deliberate innoculation (Gorelik 1982; Barlozzari 1985). Such a decrease in tumour cell elimination is associated with increased frequency of development of pulmonary metastases. Similarly in mice a decrease in NK cell activity induced by transfusion of allogeneic blood results in a greater incidence of pulmonary metastases after intra-venous injection of syngeneic tumour cells (Clarke 1993). The evidence for NK cells being active against metastatic tumours in man is limited, but the development of metastatic head and neck cancer correlates with low NK cell activity at initial presentation (Schantz 1987), and patients with metastatic liver disease have lower NK cell activity within the liver than patients with benign liver disease (Winnock 1993).

Summary

Allogeneic blood transfusion causes a non-specific down -regulation of immune function by a complicated mechanism which so far remains unclear. What also remains unclear is whether this transfusion effect is of any prognostic relevance to cancer patients. If indeed it has an adverse effect upon long term survival then ways to abrogate such an effect must be found for cancer patients in whom transfusion of blood is unavoidable. Leucocytes appear to be strongly implicated as the mediator of this transfusion effect, as such it would be logical to hypothesise that removal of leucocytes from blood for transfusion would prevent such an effect occurring. While the ultimate measure of prognosis for cancer patients is survival time (often expressed as 5 year survival rates) the follow-up of patients for several years after surgical treatment for cancer involves a long wait for results, and a more immediate indicator of the success, or otherwise, of such an intervention would be ideal as a prelude to survival figures. Measuring parameters of immune function in cancer patients would be suitable as immunosuppression is the basis of the theory that links blood transfusion to a worsened prognosis. Natural Killer cells are accepted as being important in host resistance to cancer and measurement of their activity provides a means of comparison between patients receiving blood transfusions with and without leucocytes.

Objectives of this thesis

The hypothesis put forward at the beginning of this work was that leucocytes present in transfused blood were responsible for the immunosuppression induced in a recipient by receiving such a blood transfusion, and therefore that immunosuppression could be prevented, or reduced, by removal of leucocytes from transfused blood before it is transfused into the recipient. As the transfusion effect is postulated to be important in cancer patients, and particularly so during the time of surgery, a group of colorectal cancer patients were studied during the peri-operative period. The filtration of blood to remove leucocytes prior to transfusion allowed for comparison between patients receiving standard and leucocyte depleted blood transfusions. Several aspects of cellular immunity were measured in these patients, in particular Natural Killer cell activity was used as an indication of anti-tumour cytotoxicity.

Supplementary to this main question further subsidiary experiments were performed to investigate the nature of the transfusion induced immunosuppression.

CHAPTER 2

METHODS

1. Leucodepleted Blood Transfusion In Colorectal Cancer Patients - Clinical Trial.

1:1 Introduction.

A prospective, randomised, controlled trial involving patients undergoing elective resection of colorectal cancers in the Leicester region was set up to compare the immunosuppressive effect of standard blood transfusion with that of leucodepleted blood transfusion. Local ethical committee approval was obtained prior to the trial commencing, and the consultant surgical staff involved kindly agreed to the inclusion of their patients.

1:2 Design.

In the Leicester area there are between 100 and 150 colorectal cancer resections performed electively each year, and it was predicted that, after exclusion of unsuitable patients, it would be practical to recruit 100 patients within a 12 to 18 month time span. Previous work in Leicester had shown that approximately 40% of these patients receive a blood transfusion in the peri-operative period. By randomising patients pre-operatively to receive either standard or leucodepleted blood should a transfusion be required, it was calculated that 60 patients would receive no transfusion, 20 would receive standard blood, and 20 would receive leucodepleted blood (60%,20% and 20% respectively). *See also* Appendix V - Pilot Study and Statistical Planning, p App:12.

1:3 Setting.

Leicester General Hospital, Glenfield General Hospital and Leicester Royal Infirmary.

1:4 Inclusion criteria.

All patients undergoing elective resection of a colorectal carcinoma at one of the above hospitals were considered for inclusion, providing they were able and willing to provide written, informed consent.

1:5 Exclusion criteria.

1:5:1 - Transfusion of blood within the preceding 6 months (to ensure there was no residual immunomodulation from previous transfusions).

1:5:2 - Concurrent immunosuppressive medication, including oral steroids (thus excluding any confounding activity of drugs known to cause immunomodulation).

1:5:3 - Transfusion reaction requiring cessation of transfusion (as many of these reactions are immunological in nature and would therefore affect the assay results).

1:6 Patient consent.

Once identified as eligible for entry into the trial the nature and purpose of the study was explained personally to each patient and an information sheet provided for future reference. Written consent was then obtained on a separate consent form and filed in the hospital notes.

1:7 Randomisation.

Computer generated random numbers (Microsoft Excel) were used to produce 100 slips of paper indicating that either "standard" or "filtered" blood should be prepared (50 of each - see fig.2:1). These were sealed in consecutively numbered envelopes and opened in turn for each patient recruited to the trial. Blood bank was informed immediately if leucodepleted blood needed to be prepared and the number on the envelope was used as the patient's trial number for identification. The

STANDARD BLOOD

Trial number: **14**

This patient has been allocated to receive standard blood transfusions only. Please record the trial number on the patient detail sheet and take no further action.

FILTERED BLOOD

Trial number: **36**

This patient has been allocated to receive leucodepleted blood transfusions. Please record the trial number on the patient detail sheet and inform your blood bank immediately that this patient requires filtered blood.

Figure 2:1. *Examples of patient randomisation slips.*

randomisation was performed by the main investigator (TW) but he was "blind" to the patient's allocation at the time the assays were performed.

1:8 Leucodepletion.

This was performed in the central blood bank at the Leicester Royal Infirmary under sterile, reproducible conditions according to a previously validated standard operating procedure. The process is described in detail later in this chapter (*see sect. 2*).

1:9 Transfusion.

1:9:1 The usual procedure for patients undergoing surgery when blood products may be required is either to have a "group and save" performed on a sample of their blood, whereby their blood group is determined and serum saved ready for crossmatching, or to have a specified number of units crossmatched ready for use. For patients randomised to the standard transfusion arm there was no change from this routine and blood was made available as requested by the caring team. For those randomised to the leucodepleted arm the blood bank was informed and any units of blood actually crossmatched for that patient were filtered by the blood bank staff (as described later). If for any reason blood was required urgently for a patient in the leucodepleted arm and there was insufficient filtered blood available then standard blood was provided, thus avoiding any potentially harmful delay. Any patients in this situation were withdrawn from the trial.

1:9:2 Patients were not transfused for study purposes. Transfusion was only performed if the caring team felt that it was clinically indicated (operative blood loss >500mls, haemoglobin level <10g/dl), and they were kept "blind" as to whether the patient received standard or leucodepleted blood.

1:10 Blood samples.

20 mls of peripheral venous blood was collected from each patient on at least 3 occasions:

1 - within 24 hours prior to surgery.

2 - day 5 after surgery.

3 - 4 weeks after surgery.

Blood samples were taken using standard venesection technique with a 20 ml syringe and 21 gauge needle from a vein in the antecubital fossa, distended by prior application of a tourniquet to a pressure between systolic and diastolic blood pressure. The blood was then transferred to a sterile universal container containing 200 units of sterile, preservative free heparin, and mixed thoroughly. This was then used for the natural killer (NK) cell activity assay and flow cytometric analysis as described in detail in sections 5 and 6.

1:11 Patient data.

The following information was collected prospectively on each patient:

- name, age, sex and hospital identification.
- previous transfusion history.
- pre-operative haemoglobin, white cell count and haematocrit.
- date and length of operation.
- curative or palliative operation (and the reason why, if palliative).
- post-operative haemoglobin, white cell count and haematocrit.
- major complications.
- tumour stage and grade.
- length of hospital stay.

2. Leucodepletion.

2:1 Introduction.

Modern high - efficiency leucodepleting filters act by passing blood through a complex membrane which selectively removes the white blood cells. This process relies more upon the white cells adherent properties than a size related filtration effect, and the end result is removal of up to 99.9% of leucocytes. For the purposes of this study the Pall BPF4BBS filter system was used in the Leicester blood bank to produce sterile, leucodepleted units of blood with consistently low leucocyte counts.

2:2 Pall BPF4BBS filter system.

The system consists of a filtration disc, a sterile receiving bag for collection of the filtered blood, and 3 lengths of plastic tubing (fig.2:2). The filtration disc and the receiving bag are connected by one length of tubing, another length on the inflow side allows connection to the donor blood bag while the third length provides a bypass to the filtration disc for removal of air as described below.

2:3 Sterile connection.

When a unit of blood was to be filtered it was connected to the filter system using a Sterile Connecting Device (SCD). This machine used a disposable copper blade, heated by passage of an electric current, to slice through the outflow tubing of the blood bag and the inflow tubing of the filter system and join the two together.

2:3:1 The two lengths of tubing to be joined were positioned side by side in the SCD in the correct orientation for joining (fig.2:3a).

2:3:2 A single use, sterile copper blade was heated by an electric current and slid across to divide the two tubes (fig.2:3b).

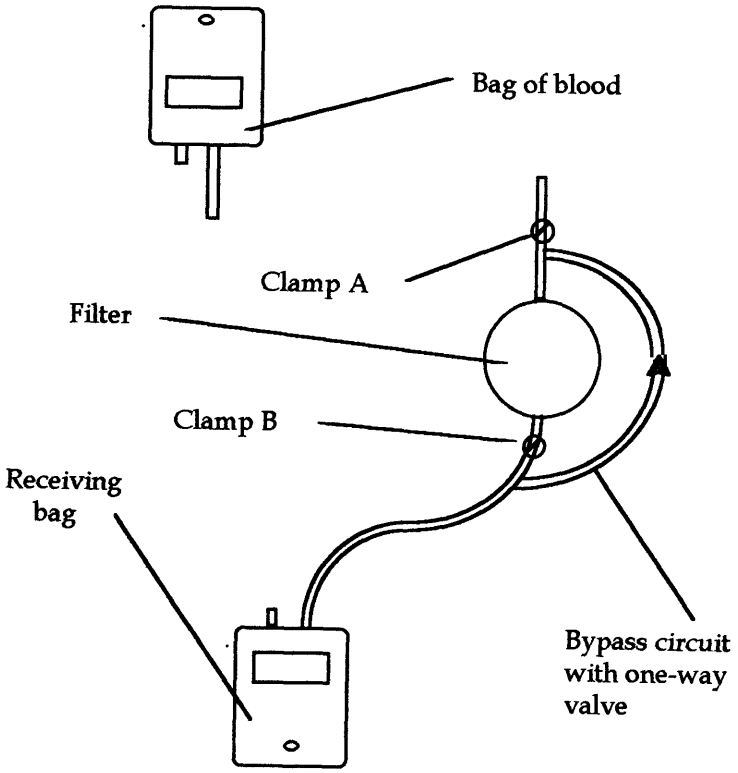


Figure 2:2. *Diagram of the BPF4BBS filtration system before connection to the transfusion bag.*

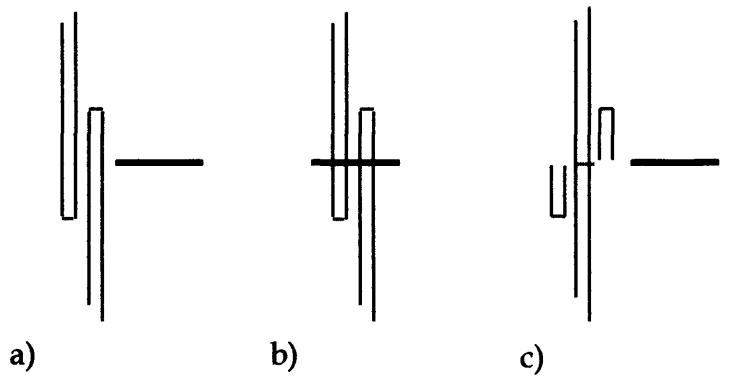


Figure 2:3. *Schematic diagram of the SCD in action:*

a) two tubes to be joined are laid side by side next to the copper blade.
b) the heated blade slides across cutting the tubes at the same level.
c) the blade is withdrawn and the cut ends apposed to form a secure join.

2:3:3 Immediately after this the two cut ends were slid together so that as the plastic cooled and solidified a sterile, water-tight join was formed between the blood bag and filter system (fig.2:3c).

2:4 Filtration.

2:4:1 Clamp A (fig.2:2) was closed, clamp B opened, and the bag of blood was suspended on a hook 1.5 - 2 metres off the ground to allow the system to drain under gravity.

2:4:2 Firm pressure was applied to the bag of blood by manually squeezing it and then clamp A was opened. The filter disc was filled quickly with blood, aided by the pressure on the blood bag, ensuring even distribution over the filtration surface.

2:4:3 Once blood was exiting from the filter disc the pressure was stopped and filtration continued by force of gravity alone, taking 10-15 minutes to complete.

2:5 Air removal.

After filtration there was always a small quantity of air in the receiving bag which had been present in the original system and had to be removed before the filtered blood could be used.

2:5:1 With clamp B closed pressure applied to the receiving bag forced the air back up the tubing and around the filter disc in the bypass circuit through the one way valve.

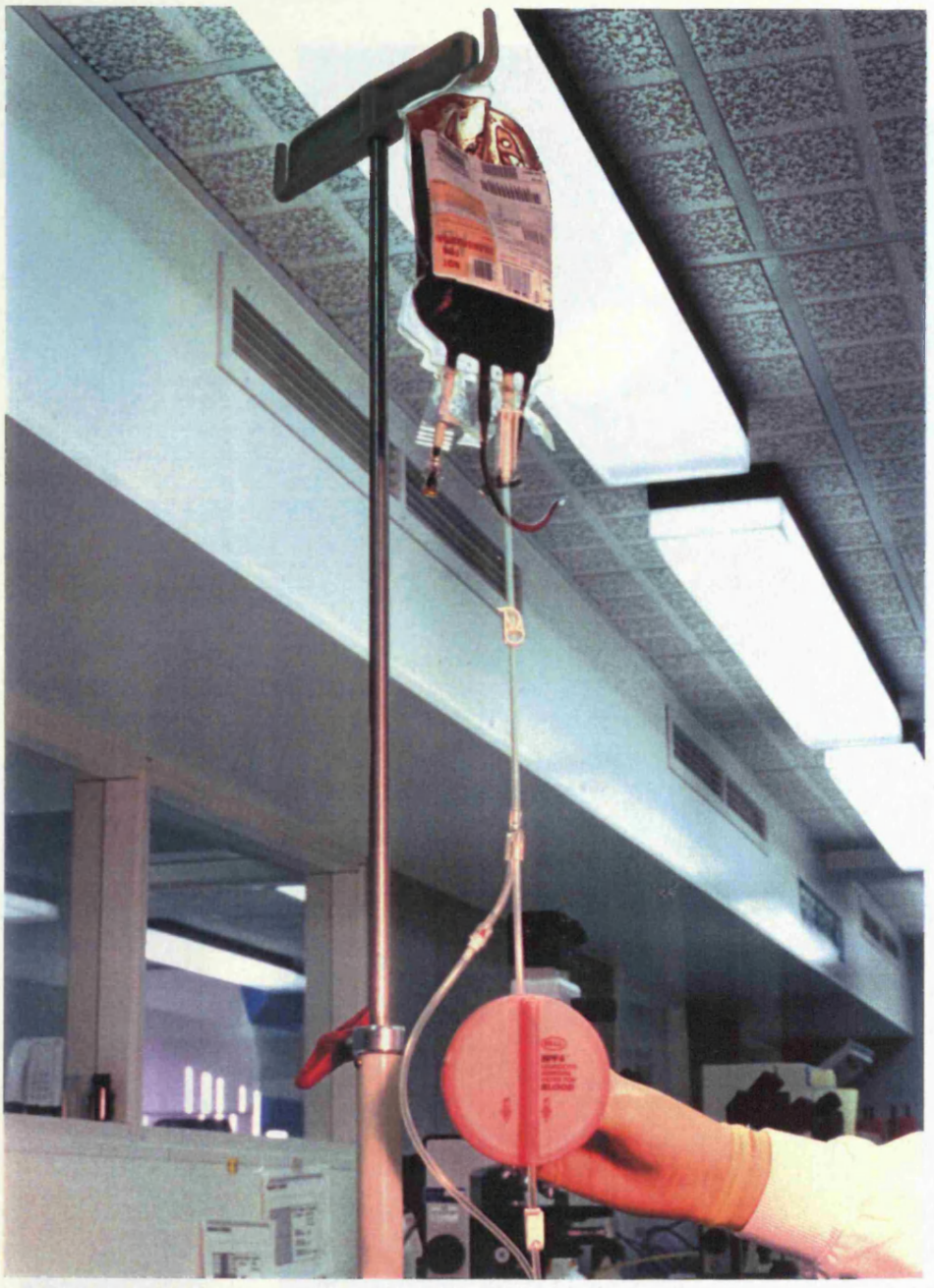
2:5:2 Once all the air had passed back up, clamp B was released and any further blood allowed to drain through the filter disc, stopping when air reached the filter.

2:6 Final preparation of leucodepleted blood bag.

2:6:1 All of the blood / patient details were carefully transferred from the original bag to the receiving bag.

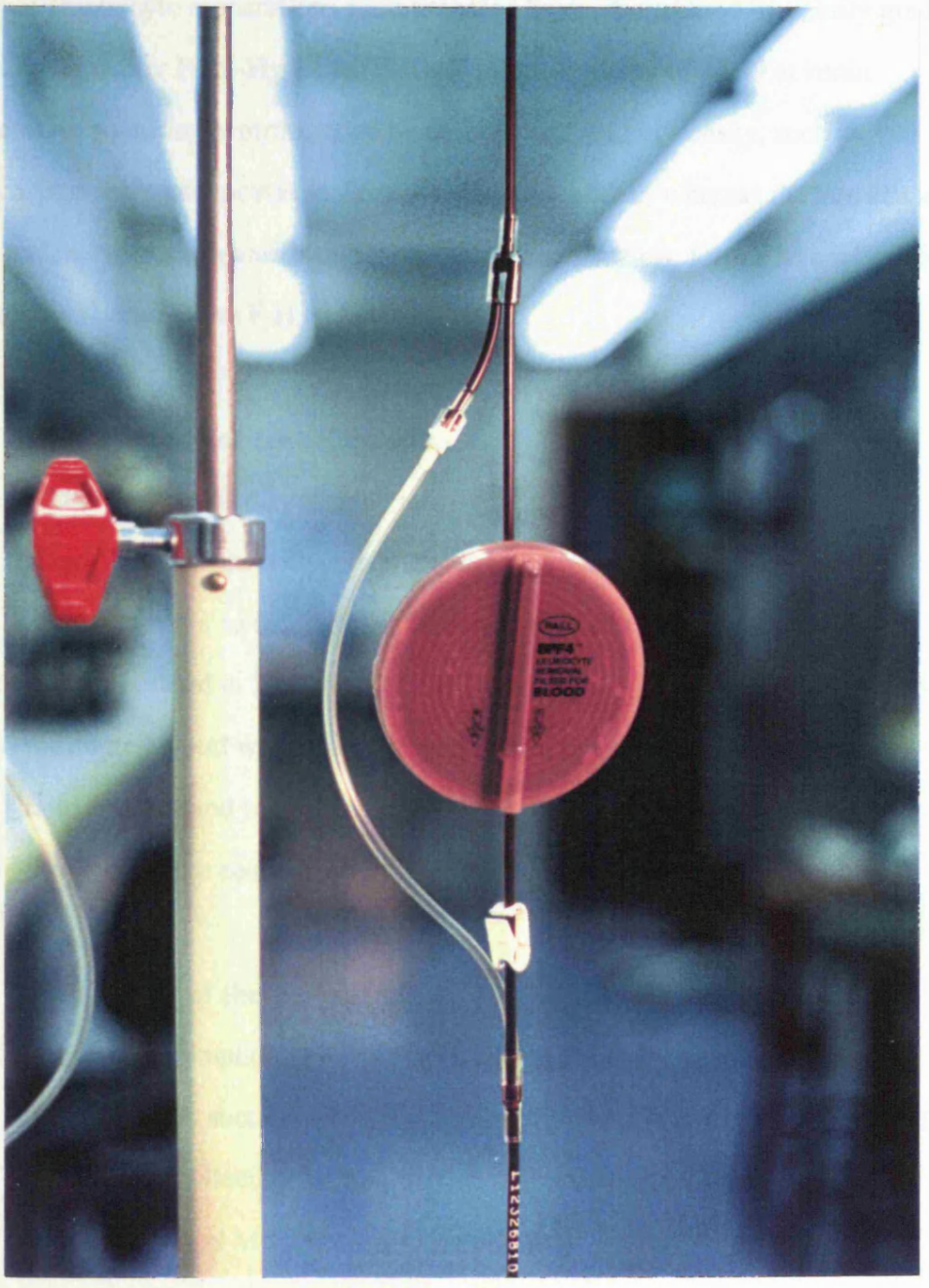
2:6:2 The tubing between the filter disc and the receiving bag was clamped with metal rings and divided to provide a sterile unit of leucodepleted blood.

Plate 2



High efficiency leucocyte depleting filter being primed

Plate 3



High efficiency leucocyte depleting filter in use

3. Lymphocyte Separation.

3:1 Introduction.

Lymphocyte preparations were prepared from whole blood by density gradient centrifugation over Ficol-Hypaque (F-H). F-H has a density of 1.099 at room temperature so during centrifugation blood cells of a greater density, such as erythrocytes and granulocytes, collect as a pellet beneath it, whereas the less dense cells, mainly lymphocytes with some monocytes and platelets, form a layer above it at the interface between the F-H and plasma.

3:2 Density gradient centrifugation.

3:2:1 20ml of whole blood collected into heparin (as described in section 1:10 above) was diluted with an equal volume of balanced salt solution (BSS, Sigma) in order to facilitate separation of the cell types.

3:2:2 F-H was placed in 30ml plastic tubes (Corning Costar) in 10ml aliquots, and the diluted blood was carefully layered onto the surface at an approximate ratio of 1.5 blood to 1.0 F-H (fig.2:4a).

3:2:3 The tubes were centrifuged at 700g for 20 minutes at room temperature.

3:3 Preparation of the Peripheral Blood Mononuclear Cell solution.

3:3:1 The layer of lymphocytes/monocytes between the plasma and F-H was "harvested" by suction with a disposable plastic pipette (Corning Costar) (fig 2:4b,c). The collection of cells, as found in this layer, is referred to as Peripheral Blood Mononuclear Cells (PBMC).

3:3:2 The PBMC layer was washed three times with BSS, centrifuged at 400g for 10 minutes.

3:3:3 After the final wash the pellet of cells was resuspended in 2 ml of Roswell Park Memorial Institute (RPMI, Sigma) medium, to which was added foetal calf serum (FCS, Sigma) as a nutrient in a final concentration of 10%.

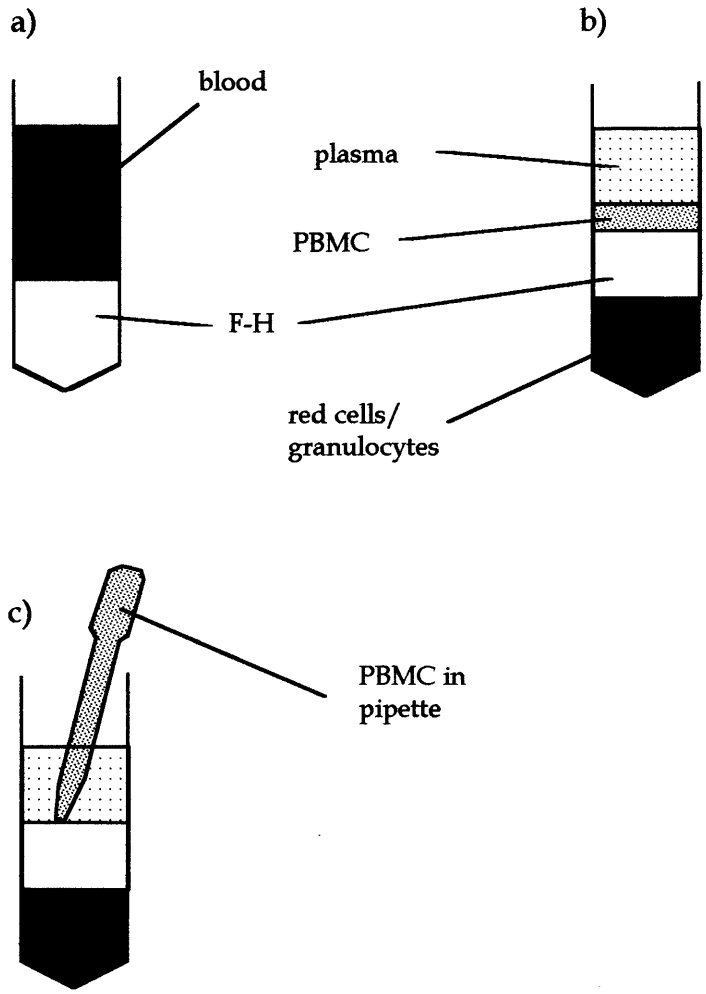


Figure 2:4. *Ficoll-Hypaque separation of PBMC from whole blood:*

a) *before centrifugation.*
b) *after centrifugation.*
c) *harvesting of PBMC.*

3:3:4 An aliquot of 50µl was taken for cell counting as described below. The concentration of the solution was then adjusted by addition of further RPMI/10%FCS medium to bring the cell count to 4 x 10^6 per ml.

4. Cell Counting.

4:1 Introduction.

The determination of the number of white blood cells present in a given solution was an important procedure in more than one part of this study, and at all times accuracy was essential. Cell counting was performed in 4 different areas: *i)* subjects whole blood sample - although routinely used as a useful clinical parameter this measurement was used in the study, along with the results of flow cytometry and chromium release assays, to calculate absolute numbers of NK cells in subjects blood, and to extrapolate the cytotoxic capability of that blood in terms of lytic units per ml (see later); *ii)* lymphocyte separation - the final white cell concentration of the PBMC solutions was adjusted to 4 x 10^6 per ml on the results of the count performed after the final washing (see above); *iii)* chromium release assay - the concentration of the K562 target cells was adjusted to 1 x 10^5 per ml prior to setting up of the assay plates (see later); *iv)* validation of the leucodepletion process - post-filtration white cell counting of samples from the leucodepleted blood bags was performed to ensure consistent and accurate filtration.

4:2 Haemocytometer design.

Two different haemocytometers were used in this study, the standard Neubauer for whole blood and white cell preparations, and the Nageotte for post-filtration leucodepleted specimens. They are both similar in design in that they consist of a rectangular block of glass containing a central section that has been accurately ground down below the level of the surface of the rest of the block, and when this central section is covered over with a thin glass cover slip resting on the adjacent block the space thus formed becomes the counting chamber (fig.2:5). The volume of this space is precisely determined and is accurately reproducible so that a constant volume of cell solution is always counted. The first main difference between the two haemocytometers is in the size of their counting chambers, the Nageotte model having

a much larger chamber, each one holding 50µl of fluid, compared to the Neubauer which only holds 1.8µl.

The surface of the glass block which forms the floor of the counting chamber is etched with lines to form a grid of very small squares which facilitate counting under the microscope. The second difference between the Nageotte and Neubauer is found here in that the Nageotte has a simple grid dividing the area into 40 rectangles whereas the Neubauer has a more complicated system of large squares which are further subdivided into smaller squares (fig.2:6).

4:3 Counting technique.

4:3:1 The haemocytometers were cleaned and dried to exclude contamination by previously used cell solutions.

4:3:2 A glass cover slip was secured over the counting chamber by lightly moistening the glass block and pressing the cover slip in place until it was held firmly over the centre of the chamber.

4:3:3 50µl of cell solution was mixed with 450µl of white cell counting solution (a mixture of gentian violet to stain the white cell nuclei, and acetic acid to lyse superfluous cells) to form a 1:10 dilution.

4:3:4 This mixture was left to stand for at least 5 minutes to allow for adequate staining and lysis, and then mixed thoroughly again prior to counting.

4:3:5 Aliquots of solution were transferred to the counting chambers using a pipette, the tip of which was placed against the edge of the cover slip where it rested over the counting chamber.

4:3:6 The Neubauer haemocytometer would now be ready for counting, however the Nageotte haemocytometer with it's deeper chamber had to be left standing for 15 minutes to allow the cells to settle onto the lower surface of the chamber.

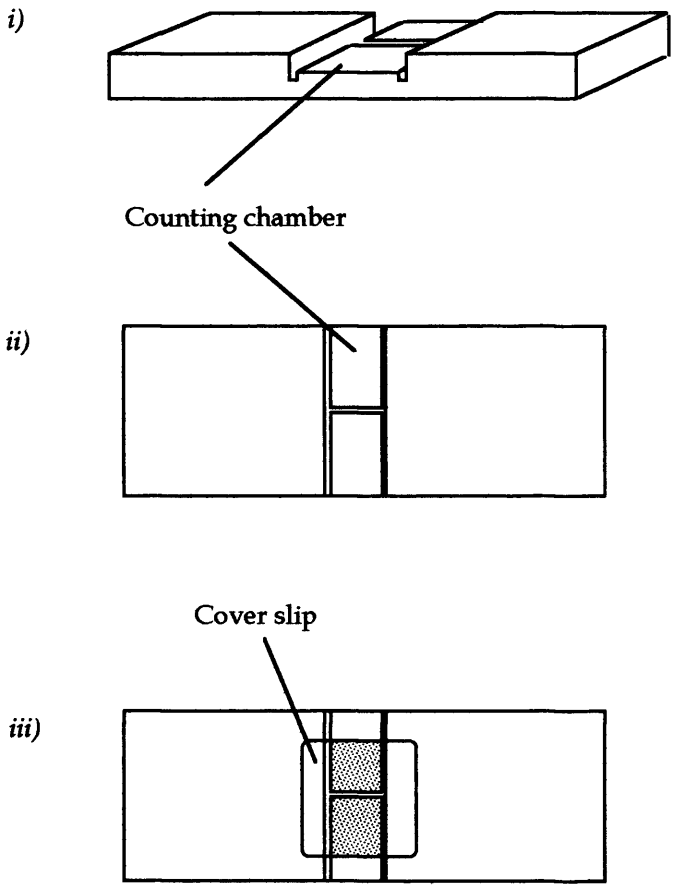
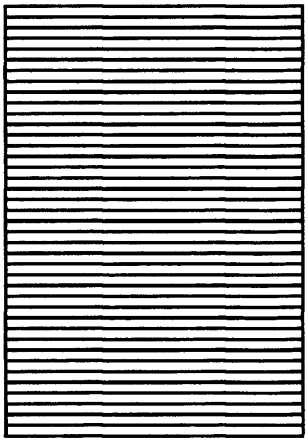
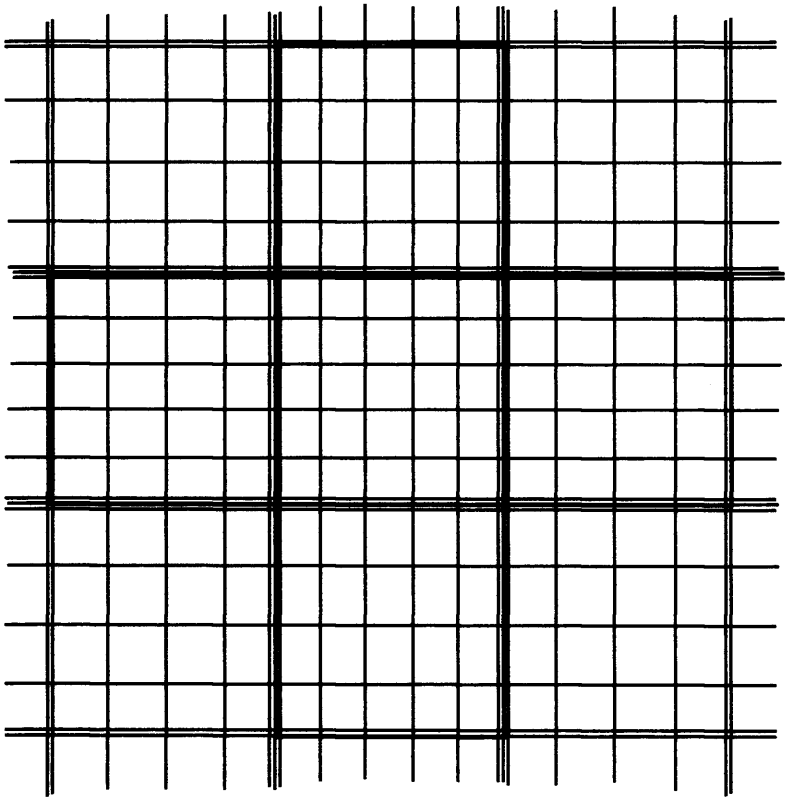


Figure 2:5. *Diagrammatic view of standard haemocytometer showing i) oblique view, ii) aerial view, iii) aerial view with glass cover slip in place and cell solution in counting chamber.*



i) Nageotte counting grid



ii) Neubauer counting grid

Figure 2:6. *Diagrammatic views showing the different counting grids of the Nageotte and Neubauer haemocytometers as seen down the microscope.*

4:3:7 Counting was performed using a standard laboratory light microscope with a 10x objective lens. With the Nageotte chamber counting was done by scanning alternately from left to right then right to left along each rectangle in turn until all 40 were counted. Both chambers were counted giving a volume of 100µl, and the cell concentration calculated with the formula:

$$\frac{\text{cells counted x dilution}}{\text{volume counted}} = \text{conc. (cells/µl)}$$

where : dilution = 10
: volume counted = 100

4:3:8 With the Neubauer chamber the number of cells within one large square was counted, usually the central square bordered by triple scored lines (fig.2:6). Each large square is 1mm x 1mm and the depth of the counting chamber is 0.1mm, thus the volume of solution counted over one large square is:

$1 \times 1 \times 0.1 = 0.1\text{mm}^3$

$= 0.1\mu\text{l}$

Using the same formula for cell concentration:

$$\frac{\text{cells counted x dilution}}{\text{volume counted}} = \text{conc. (cells/µl)}$$

where : dilution = 10
: volume = 0.1

4:3:9 White cell concentrations were expressed as $x10^6$ per ml.

5. Natural Killer Cell Assay.

5:1 Introduction.

Over the years several different methods of measuring NK cell activity have been described, and of these the most commonly used and widely accepted technique is the so-called chromium release assay. In brief this involves labelling target cells with radioactive chromium (^{51}Cr, Amersham), combining these cells with the NK cells under study, incubating the mixture for 4 hours and then measuring the amount of released ^{51}Cr as a direct indicator of the number of target cells lysed by the NK cells.

5:2 Target cells.

Erythroleukaemic K562 cells derived from the pleural effusion of a patient with chronic myeloid leukaemia in blast crisis were used as targets.

5:3 K562 cell culture.

The K562 cells were maintained in culture throughout the course of the study, samples being removed at regular intervals for the purposes of the NK cell assay.

5:3:1 The K562 cells were cultured in medium consisting of RPMI 1640 + 2mM glutamine (Sigma) + 10% FCS.

5:3:2 The culture was kept in a sterile incubator at 37°C with an atmosphere of 5% CO_2.

5:3:3 At regular intervals (approximately every 48 hours) the culture was "cut", removing sufficient cells to maintain the concentration at 2 - 9 x 10^5 per ml, and supplemented with fresh medium.

5:3:4 Cells removed at the "cutting" stage were used for the NK cell assay, and additional cells were removed at any time for the same purpose.

5:4 Cell labelling with ^{51}Cr.

Radioactive sodium chromate (Amersham) was used to label the K562 cells with ^{51}Cr. This was supplied on a monthly basis and made up to a stock solution of 1 MBq per 10µl by dilution with 0.9% saline. 1.8 x 10^5 labelled target cells were required for each NK cytotoxicity assay, and multiples of this figure if several assays were performed at once. Usually between 1 - 2 x 10^6 K562 cells were prepared so that sufficient targets were available.

5:4:1 The cells to be labelled were placed in a single well of a sterile plastic 24-well plate (Corning Costar) in 1 - 2 ml of RPMI/10% FCS culture medium.

5:4:2 10MBq of stock solution of ^{51}Cr was added - 100µl initially but as time progressed from delivery of the stock solution and the activity decayed, the volume of solution used was increased accordingly. Table 2:1. shows the decrease in activity bearing in mind that the half life of ^{51}Cr is 27.7 days.

5:4:3 This plate was placed in a humidified incubator at 37°C with a 5% CO_2 atmosphere for at least 2 hours, though usually overnight.

5:4:4 After incubation the cells were aspirated into a plastic pipette along with the culture medium, taking care to wash all of the cells from the floor of the well, and transferred to a 30ml plastic tube.

5:4:5 25ml of sterile BSS was added, the solution agitated, centrifuged at 1000 rpm for 5 minutes, the supernatant discarded and the pellet of cells resuspended.

5:4:6 This wash was performed three times and after the final wash the cells were resuspended in 2ml RPMI/10% FCS.

5:4:7 An aliquot was removed for cell counting and the concentration adjusted to 1x10^5 per ml.

Days from reference	0	7	14	21	28
Relative activity	1.000	0.839	0.704	0.591	0.496

Table 2:1. *Decrease in relative activity of ^{51}Cr per week from initial reference point.*

5:5 Cytotoxicity assay.

5:5:1 A PBMC solution was prepared from the blood of the subject(s) to be studied as described in section 3, and the concentration adjusted to $4x10^6$ cells per ml.

5:5:2 For each assay 3 columns and 6 rows of a 96-well U-bottom microtitre plate (Gibco) were used, providing results in triplicate and allowing up to 4 experiments to be performed on a single plate (fig.2:7).

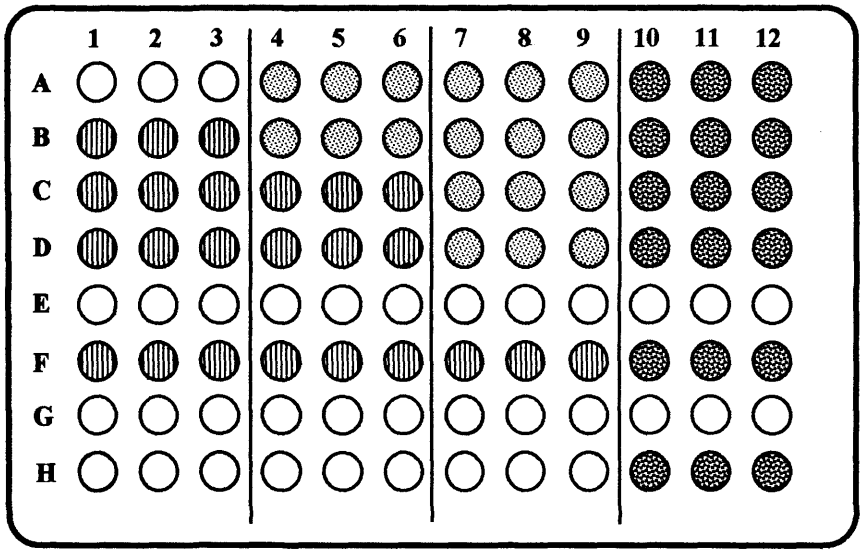
5:5:3 100µl of RPMI culture medium was added to rows B, C, D and F (fig.2:7:a).

5:5:4 100µl of PBMC solution was added to rows A and B (fig.2:7: b).

5:5:5 Using a multi-channel pipette rows B to D were serially diluted by sequentially withdrawing 100µl of solution from the cells in one row, mixing this with the culture medium in the next row and repeating the process again with the following row. The final 100µl removed from row D was discarded, leaving 100µl aliquots of PBMC in rows A to D of cell concentration 4, 2, 1 and 0.5 x 10^6 per ml respectively (fig.2:7: c).

5:5:6 100µl of radio-labelled K562 cells were added to each cell in rows A, B, C, D, F and H giving effector to target ratios of 40:1, 20:1, 10:1 and 5:1 in rows A to D (fig.2:7: d).

5:5:7 100µl of detergent (1%NP40) was added to the cells in row H to lyse the target cells completely (representing a maximum kill), the plate covered and placed in an incubator at 37^0C, 5%CO_2 for 4 hours.



a) *b)* *c)* *d)*

e)

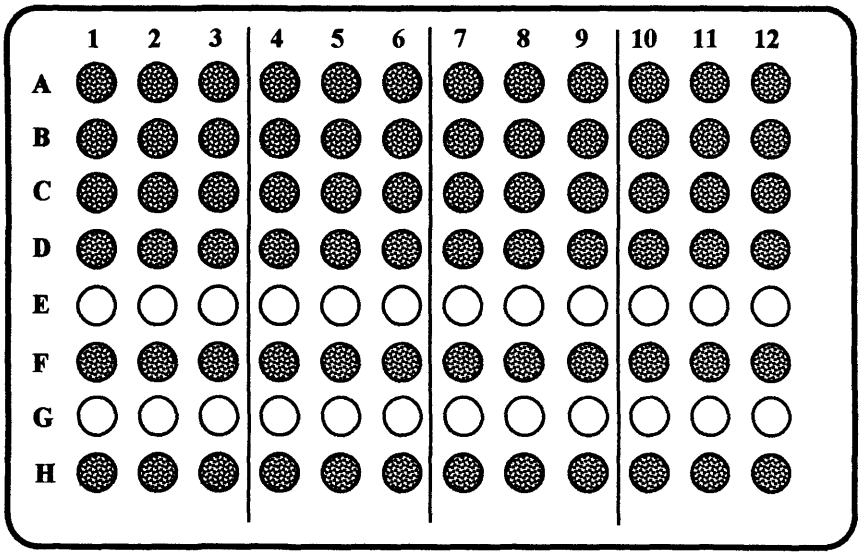


Figure 2:7. *Setting up the 96 well plate for the NK cell cytotoxicity assay:*

a) 100µl RPMI + 10%FCS in rows B,C,D and F: -

b) 100µl PBMC solution added to rows A and B: -

c) serial dilution of rows B to D.

d) 100µl radio labelled K562 added to rows A,B,C,D,F and H: -

e) final view of plate containing 4 separate assays in columns 1-3, 4-6, 7-9, and 10-12. Each well in row H has had 100µl of 1%NP40 detergent added.

5:6 Radioactivity counting.

5:6:1 After incubation the plate was placed in the plate spinning attachment of a centrifuge and spun at 1000 rpm for 5 minutes to sediment the cells.

5:6:2 100µl aliquots of supernatant were removed from each well and placed in 400µl Eppendorf plastic tubes (Gibco).

5:6:3 In addition 3 tubes were filled with 100µl aliquots of the original K562 solution.

5:6:4 The tubes were capped and placed in the counting rack of a gamma-counter and counted for radioactivity over a 60 second period.

5:7 Data analysis.

5:7:1 The formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

was used to calculate the percentage cytotoxicity for each assay.

5:7:2 The counts per minute (cpm) of radioactivity from rows A-D provided the "experimental release" values over 4 effector : target ratios (40:1, 20:1, 10:1, 5:1).

5:7:3 The cpm from row F provided the "spontaneous release" values.

5:7:4 The cpm from row H provided the "maximum release" values.

5:7:5 4 values were obtained from each assay representing the percentage cytotoxicity for each of the 4 effector : target ratios.

5:7:6 These values produced a straight line graph when plotted as percent cytotoxicity against effector : target ratio (on a logarithmic scale), and by regressional analysis the effector : target ratio required to produce 30% cytotoxicty was calculated (fig.2:8).

5:7:7 This value (E:T ratio for 30% cytotoxicity) represented 1 "Lytic Unit" and the results were finally expressed as Lytic Units per 10^6 cells.

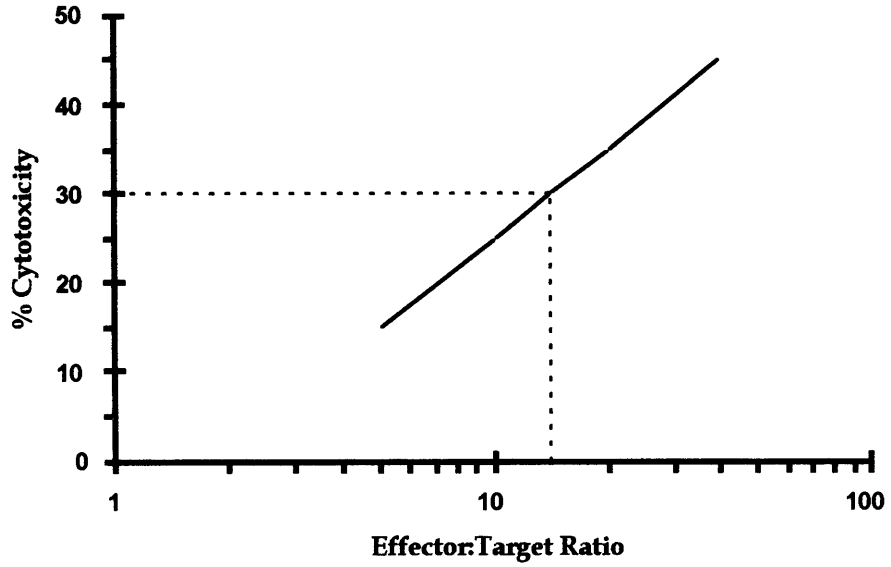


Figure 2:8. *Logarithmic plot of typical cytotoxicity assay results. Dotted line indicates 30% cytotoxicity level.*

6. Flow Cytometry.

6:1 Introduction.

The development of flow cytometry has provided the ability to rapidly, and accurately, study the phenotypic characteristics of different cell populations. Using this technique leucocytes were differentiated by their varying size and granularity, and by their expression of cell surface antigens identified by fluorochrome labelled monoclonal antibodies.

6:2 Principles of flow cytometry.

A solution of cells to be studied are prepared for analysis according to the manufacturers instructions (Sect. 6:4). When introduced to the FACScan™ machine (FACS® = Fluorescence Activated Cell Sorter, Becton Dickinson) the cells are forced under pressure to the optical sensor, through which they pass in a laminar flow of single cells surrounded by a concentric sheath of buffer fluid (Fig.2:9). As they pass through the sensor they are transilluminated by a beam of light from a 15 milliwatt argon-ion laser at a wavelength of 488nm. Some of this light will pass through the cells and is described as being scattered in a forward direction (forward scatter or FSC). Some will be deflected to the side and is referred to as side scatter (SSC. Fig.2:10). FSC light provides a measure that correlates well with cell size, whereas SSC light provides a measure of cell granularity. The cells may also be stained with fluorochrome labelled monoclonal antibodies and the fluorescent characteristics of such cells are detected as part of the SSC light (Fig.2:10). The two commonly used fluorochromes are fluorescein isothiocyanate (FITC) and phycoerythrin (PE). When excited at 488nm by the laser beam FITC emits yellow-green light at a wavelength of approximately 515nm, and PE emits red-orange light at approximately 580nm. These are detected as Fluorescence 1 (FL1) for FITC, and Fluorescence 2 (FL2) for PE.

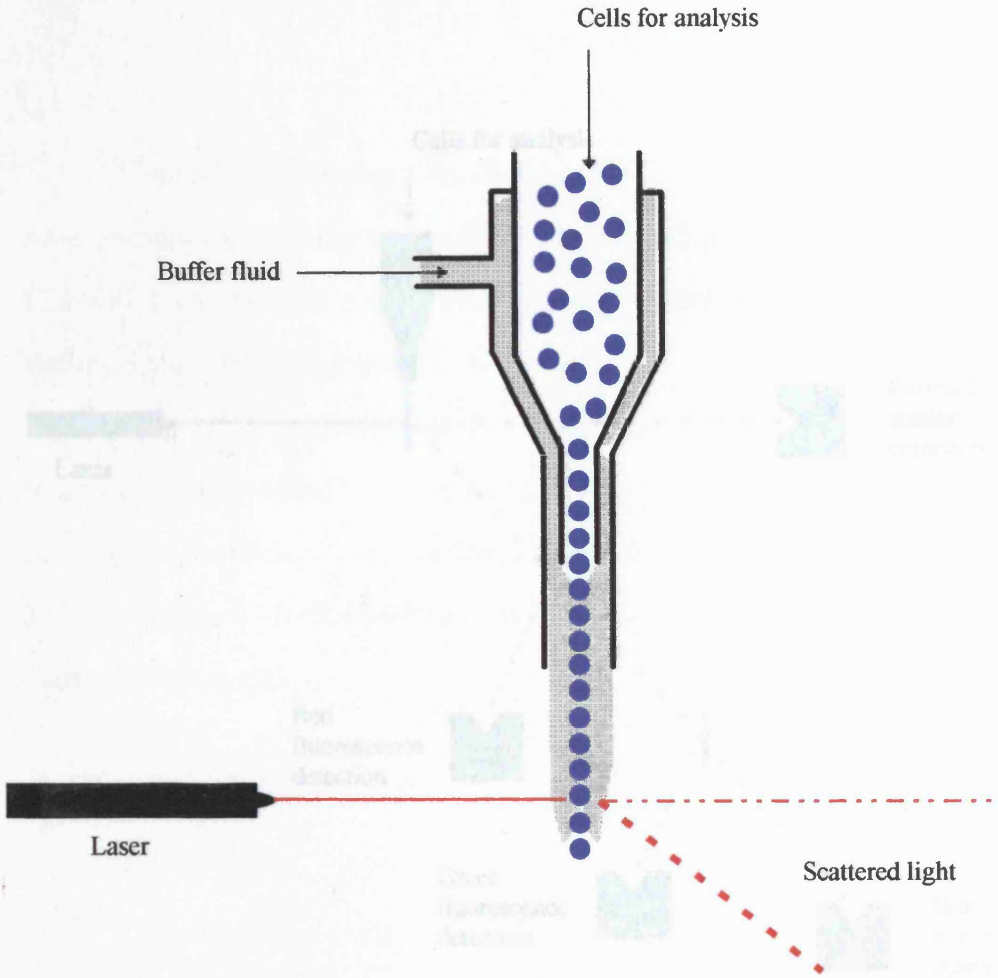


Figure 2:9 *Laser trans-illumination of cells in flow cytometer.*

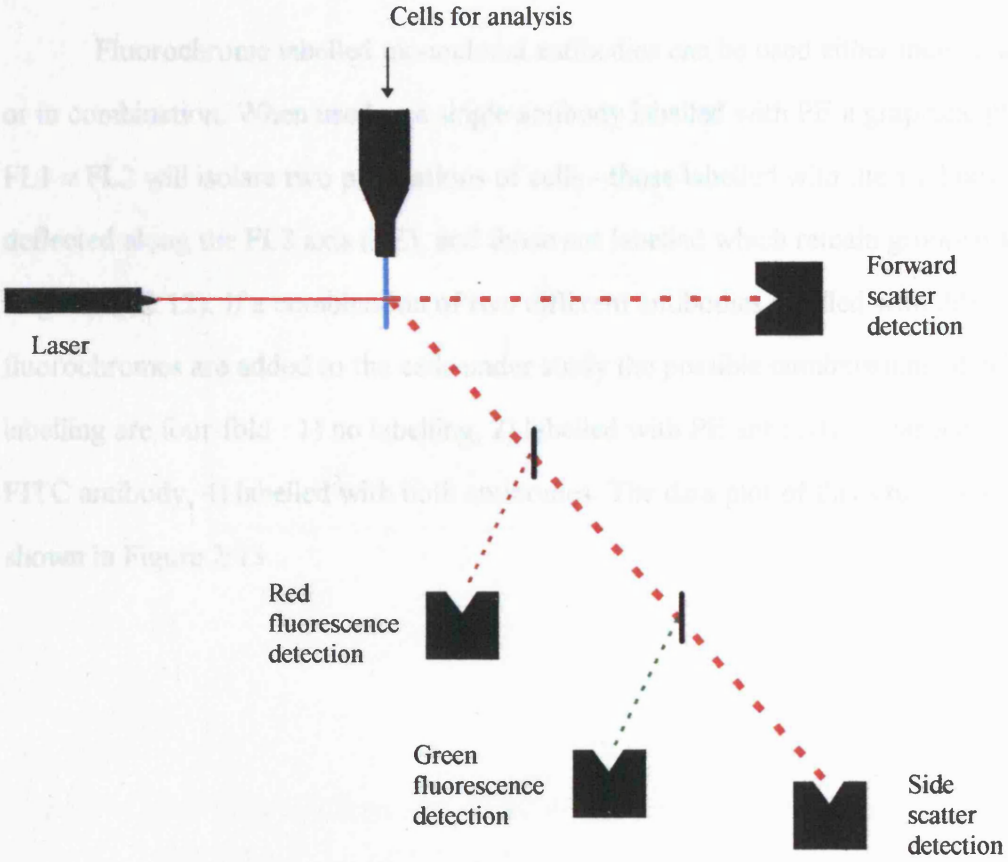


Figure 2:10 *Schematic view of laser light detection during flow-cytometry.*

Using these four characteristics cells can be separated into different types. A graphical plot of FSC v SSC distinguishes between populations of white blood cells, and usually it is possible to see distinct groups representing lymphocytes, monocytes and granulocytes (Fig.2:11).

Fluorochrome labelled monoclonal antibodies can be used either individually or in combination. When used as a single antibody labelled with PE a graphical plot of FL1 v FL2 will isolate two populations of cells - those labelled with the antibody deflected along the FL2 axis (PE), and those not labelled which remain grouped at the origin (Fig.2:12). If a combination of two different antibodies labelled with different fluorochromes are added to the cells under study the possible combinations of cell labelling are four-fold : 1) no labelling, 2) labelled with PE antibody, 3) labelled with FITC antibody, 4) labelled with both antibodies. The data plot of this situation is shown in Figure 2:13.

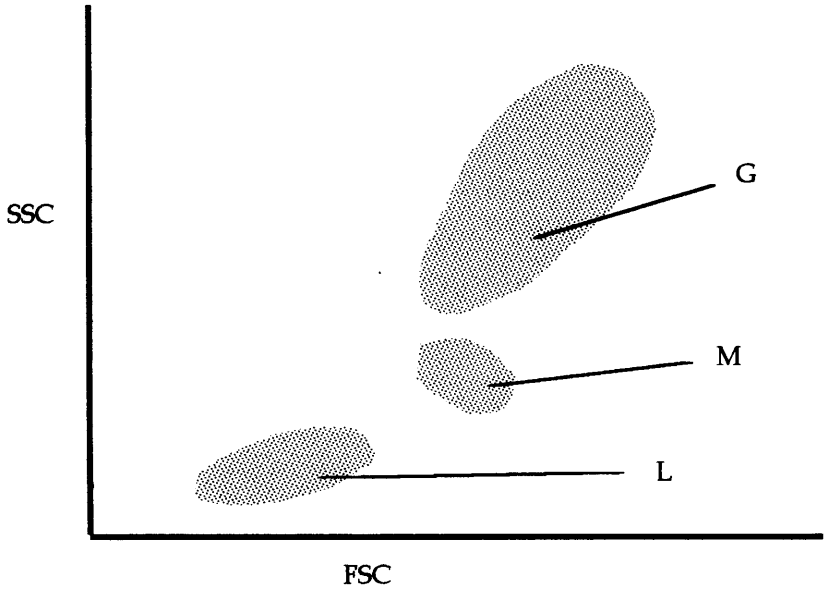


Figure 2:11. *Schematic FACScan plot of FSC v SSC showing populations of lymphocytes (L), monocytes (M), and granulocytes (G).*

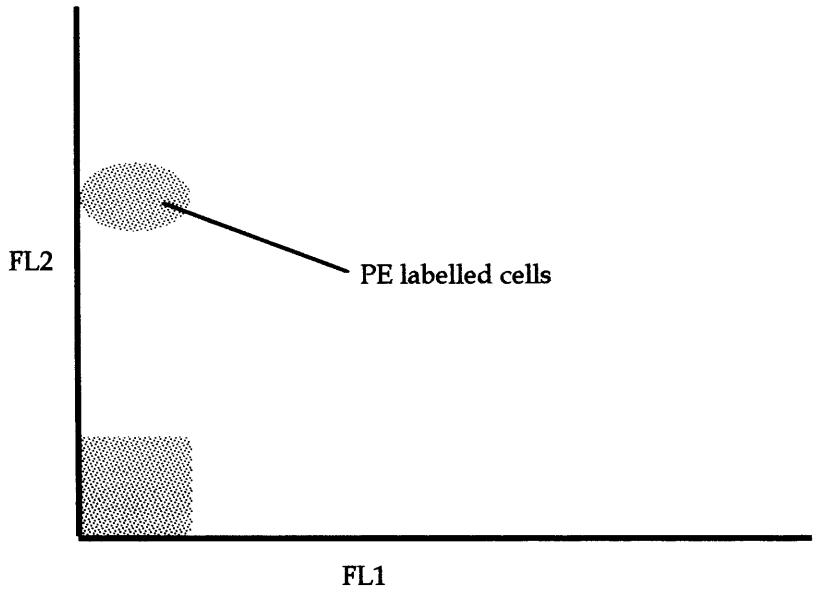


Figure 2:12. *Schematic FACScan plot of FL1 v FL2 for a group of PE monoclonal antibody labelled lymphocytes.*

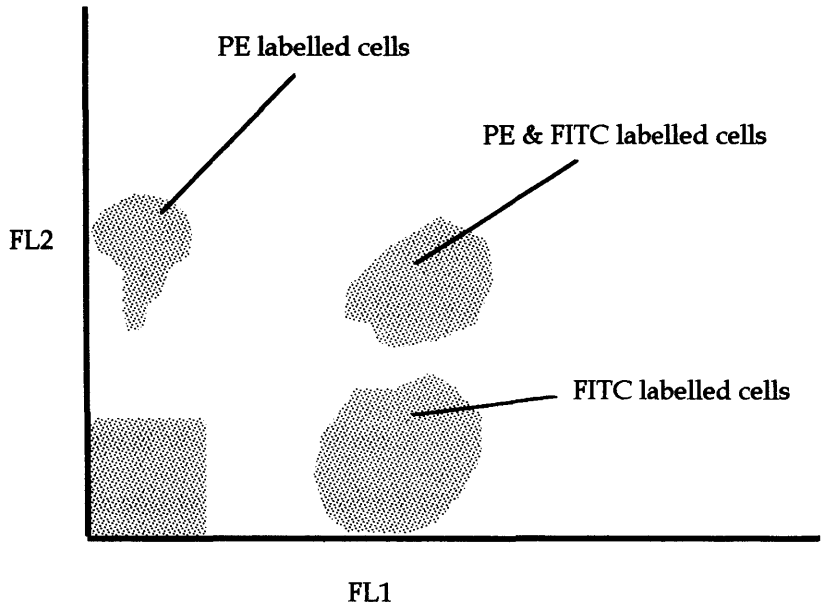


Figure 2:13. *Schematic FACScan plot of lymphocytes labelled with two fluorochrome monoclonal antibodies - one PE, one FITC.*

6:3 Fluorochrome labelled monoclonal antibodies.

Monoclonal antibodies labelled with 2 different fluorochromes were used : fluorescein isothiocyanate (FITC - yellow-green fluorescence), and phycoerythrin (PE - red-orange fluorescence). Some were dual labelled with both FITC and PE.

The antibodies used were as follows (Becton Dickinson):

6:3:1 CD45/CD14 (LeucoGATE™). This was used to set a "gate" on the FACScan machine for lymphocyte acquisition.

6:3:2 CD3/CD16+CD56. Used to identify NK cells.

6:3:3 CD3. T cell marker absent from NK cells.

6:3:4 CD2. pan - T cell marker.

6:3:5 CD4. T helper cell marker.

6:3:6 CD8. T suppressor cell marker.

6:4 Cell preparation and labelling.

During the course of these experiments flow cytometry was used to analyse lymphocyte subsets from subjects peripheral blood samples. The lymphocytes were labelled both with a whole blood method and using PBL solutions. Whole blood was taken as aliquots from the heparinised samples acquired by venepuncture from subjects, and PBL solutions were prepared as described previously. The method for labelling the cells is essentially the same irrespective of source and is described here.

6:4:1 A series of FACScan tubes (plastic U-bottomed sample tubes dedicated for use with the FACScan machine) were labelled with subject details and the type of antibody to be added to each tube, including the first tube labelled as control.

6:4:2 50µl aliquots of cell solution (either whole blood or PBL solution) were pipetted into each tube, and to this was added a 10µl aliquot of fluorochrome

labelled monoclonal antibody according to the label on the tube. No antibody was added to the control tube.

6:4:3 The tube contents were mixed and incubated at room temperature for 30 minutes in the dark.

6:4:4 2ml of FACSLyse solution was added to each tube and a further 10 minutes incubation allowed, again at room temperature in the dark.

6:4:5 The tubes were centrifuged at 2,000 rpm for 5 minutes and the supernatant discarded.

6:4:6 The cells were washed with 3ml phosphate buffered saline (PBS)/azide solution and centrifuged for 5 minutes at 2,000 rpm.

6:4:7 The supernatant was discarded and the cells resuspended in 0.25 ml of 1% paraformaldehyde solution.

6:4:8 The cells were stored in the dark at 4^0C until analysis, which was performed within 24 hours.

6:5 Analysis.

The FACScan machine was set up as standard for analysis of human lymphocytes, and all tubes were thoroughly mixed prior to analysis to ensure cells were resuspended.

For each series of samples the control tube and LeucoGATE tube were analysed first. 10,000 ungated events were measured for each of these tubes and the results used to set a lymphocyte gate. Subsequent tubes from the same series were analysed with this lymphocyte gate set, and 2,000 events measured on each occasion. Quadrant statistics were then applied to the FL1 v FL2 data plots for each tube to determine the percentage of lymphocytes expressing each CD antigen.

7. In Vitro Regulation Of Natural Killer Cell Activity.

7:1 Introduction.

Several drugs have been advocated to upregulate immune function and/or abrogate the transfusion effect in the perioperative period *(see Chapter 1)*. Four of those drugs were evaluated here *in vitro* on natural killer cell assays from the colorectal cancer patients.

7:2 Drugs added to the NK assays.

- Interleukin-2 (IL-2).
- Interferon-γ (IF-γ).
- Cimetidine.
- Ranitidine.

7:3 Modification of NK assay.

In order to perform the *in vitro* assessment of these four drugs the NK assay had to be modified slightly to accommodate the extra reagents while keeping the volumes and concentrations of the cellular solutions the same.

7:3:1 K562 cells were labelled with ^{51}Cr and adjusted to a concentration of $1x10^5$ cells per ml, as described in section 5:4.

7:3:2 A PBMC solution was prepared from the blood of the subject(s) to be studied as described in section 3, but rather than adjusting the concentration to $4x10^6$ cells per ml *(section 5:5:1)* it was adjusted to $8x10^6$ instead, i.e. twice the concentration.

7:3:3 The NK cytotoxicity assay was set up in the 96-well U-bottom microtitre plates as described in sections 5:5:2 - 5:5:7, <u>except</u> that instead of adding 100µl of PBMC to rows A and B *(section 5:5:4)*, 50µl of double strength

PBMC ($8x10^6$ per ml) plus 50µl of drug solution was added. Thus the total volume remained the same, as did the concentration of PBMC, and the drug being studied was incorporated into the assay.

7:3:4 The rest of the assay and analysis was carried out exactly as described in sections 5:6 and 5:7.

7:4 Drug dosage - IL-2 and IFγ.

To determine the optimum dose of IL-2 and IF-γ a series of dose-response assays were performed on blood from 10 healthy volunteers. The IL-2 concentration was varied from 10 International Unit (IU) per ml down to 0.0001 IU per ml over 10x dilutions. The IF-γ dose was varied from $1x10^6$ IU per ml down to $1x10^2$ IU per ml over 10x dilutions.

Based on the results of the dose-response experiments (Appendix IV - Dose-Response Curves, p App:9) the doses used were as follows: IL-2 was used at a concentration of 0.1 IU per ml, giving a dose of $5x10^{-3}$ IU in each 50µl aliquot, and IFγ was used at a concentration of $1x10^4$ IU per ml, giving a dose of 50 IU in each 50µl aliquot.

7:5 Drug dosage - cimetidine and ranitidine.

These H_2 receptor blocking drugs were used in vitro at concentrations similar to therapeutic levels used in patients. For cimetidine this therapeutic level is a plasma concentration of 0.5 - 1.0 mg/l (Abate, Hyneck et al. 1982), for ranitidine it is a plasma concentration of at least 0.15 mg/l (Doltery 1991).

The PBMC solution was used at a concentration of $4x10^6$ cells/ml, which is double the approximate plasma lymphocyte concentration of $2x10^6$ cells/ml.

Cimetidine was therefore used at a concentration of 2 µg/ml, equivalent to a plasma concentration of 1.0 mg/l, and ranitidine at a concentration of 0.5 µg/ml, equivalent to a plasma concentration of 0.25 mg/l.

CHAPTER 3

Transfusion In Colorectal Cancer Patients - Patient Details and Clinical Course.

Introduction.

Presented here are the details of the patients recruited into the blood transfusion trial. As well as their characteristics there are also details of their operations, pathology, haematological parameters and post-operative course. All the patients discussed here were recruited into the trial as described in Chapter 2.

Patient Characteristics.

A consecutive series of 93 patients were entered into the trial between October 1994 and April 1996. Two of these were withdrawn from the study after the development of medical problems which prevented their operations; one patient suffered a myocardial infarction after admission to hospital, and one patient was found to have pneumonia. Five patients proved to have pathology other than colorectal carcinoma once the results of histological examination were available; two had tubulo-villous adenoma, and three had diverticular disease. This left a total of 86 patients who were suitable for final analysis.

Nineteen (22%) of these patients required transfusion; 10 received filtered blood and 9 received standard blood. The remaining 67 were not transfused (Table 3:1).

Age

The median age of the 86 patients was 70 years (range 37 - 86). There was no significant difference in age between those who were transfused and those who were not (71 years v. 69 years: p=0.53 Mann-Whitney). The range of ages was also very similar between the groups (Table 3:2, Graph 3:1).

Sex

Of the 67 patients who were not transfused, 38 (57%) were male. This ratio was very similar in the group of patients who were transfused where 11 (58%) of the 19 patients were male.

Type of Operation.

The most common colorectal resection performed in this study was anterior resection, accounting for 41 of the 86 procedures, and most of these did not require perioperative transfusion (32/41). Right and left hemicolectomy and sigmoid colectomy comprised 34 of the total number of resections and 32 of these were completed without the need for blood transfusion. Ten abdomino-perineal resections were performed, and the majority of these did require transfusion (8/10).

Patients who were transfused mainly underwent abdomino-perineal or anterior resection (17/19). Those who were not transfused showed a fairly equal mix of colonic (n=32) and rectal (n=34) resections (Table 3:3).

Length of Operation. Table 3:4, Graph 3:2

The quickest operation lasted 40 minutes for a right hemicolectomy, and a difficult sub-total colectomy took 5 hours to complete . Within this range the median time for a colorectal resection was 2 hours in the non-transfused group and 2.5 hours in the transfused group, making those operations in which the patients required transfusion significantly longer (p=0.017). There was no significant difference in operating time between the two transfused groups (those receiving filtered blood and those receiving standard).

Blood Loss. Table 3:5, Graph 3:3

This ranged from 0 to 2.5 litres with the heaviest losses being in the transfused group; median values were 200 ml. for the patients who received no transfusion, and 650 ml. for those who did. All of the patients who received a transfusion lost at least

250 ml. of blood, and one of the patients lost 1200 ml. without transfusion being deemed necessary.

The difference between the transfused and non-transfused groups was highly significant (650 ml v. 200 ml; $p<0.0001$), while there was no significant difference within the transfused group between the filtered and standard patients (700 ml v. 650 ml).

Haemoglobin. Table 3:6, Graph 3:4

All of the patient groups showed a significant fall in haemoglobin concentration in the peri-operative period; a median drop compared with pre-operative values of 1.7 g/dl in the non-transfused group ($p<0.001$), and 2.0 g/dl in the transfused group ($p=0.004$). Pre-operative values were not different between the groups, but the post-operative value for those patients who required transfusion was significantly lower than for those who were not transfused ($p=0.036$). Within the group who were transfused there was no difference in either pre- or post-operative values between those who received filtered blood and those who received standard.

Haematocrit. Table 3:7, Graph 3:5

As with haemoglobin there was, within the groups, a significant fall in the haematocrit over the peri-operative period; a median decrease of 5% for the non-transfused group ($p>0.0001$), and 7% for the transfused group ($p=0.0023$). However, there was no significant variation between the groups for either pre-operative or post-operative values.

Length of Hospital Stay. Table 3:8, Graph 3:6

The median number of days in hospital for the patients who were not transfused was 10, up to a maximum of 45 for one patient who developed severe respiratory problems after his anterior resection, required ventilation on the intensive care unit, and died while still in hospital. For those patients who were transfused the median stay was 14 days, up to a maximum of 34 in a patient who was returned to theatre for adhesional obstruction after his abdomino-perineal resection.
The difference in stay between the transfused and non-transfused groups was significant ($p=0.0013$), and within the transfused group those who received standard blood stayed significantly longer than those who received filtered blood (median 17 and 12.5 respectively, $p=0.027$). There was no significant difference in length of stay between the non-transfused group and those who received filtered blood (median 10 and 12.5, $p=0.081$), but those who received standard blood again stayed longer when compared to the non-transfused (median 17 and 10, $p=0.0018$).

Post-operative Complications Table 3:9

The overall wound infection rate for all patients irrespective of transfusion status was 16%, but 44% in the group of patients who received standard blood transfusions. This was significantly greater than the number of wound infections seen in the non-transfused patients. Other complications were infrequent and showed no statistical differences between the groups.

Tumour Stage and Grade Table 3:10

The spread of disease stage and grade between the three groups was quite equal with the majority of tumours being Dukes' stage B or C. No patients with stage D disease were included in the study as surgery in such patients is considered palliative. The majority of tumours were moderately well differentiated, and poorly differentiated tumours were rare

Summary.

There was an equal age and sex distribution between the groups. Although the majority of transfused patients had undergone rectal resections, it was by no means true that the non-transfused, control, group was composed of patients who had undergone "less major" colonic resections, in fact the majority of anterior rectal resections were within this latter group. The operative time reflected the distribution of type of resection and resulted in a prolonged theatre time for those who received a transfusion. Patients in this study were transfused according to clinical need so it was not surprising to see that operative blood loss was higher in the transfused group, and that the fall in haemoglobin was also greater. Of all the variables described in this chapter the only difference within the transfused group of patients was the length of hospital stay, where patients receiving standard transfusions stayed longer than those who had filtered blood, who in turn stayed a similar length of time to those who were not transfused. The wound infection rate was higher amongst the standard transfusion patients, but other complications were uncommon.

Tables and Graphs

Type of transfusion	Number of patients
None	67
Filtered blood	10
Standard blood	9

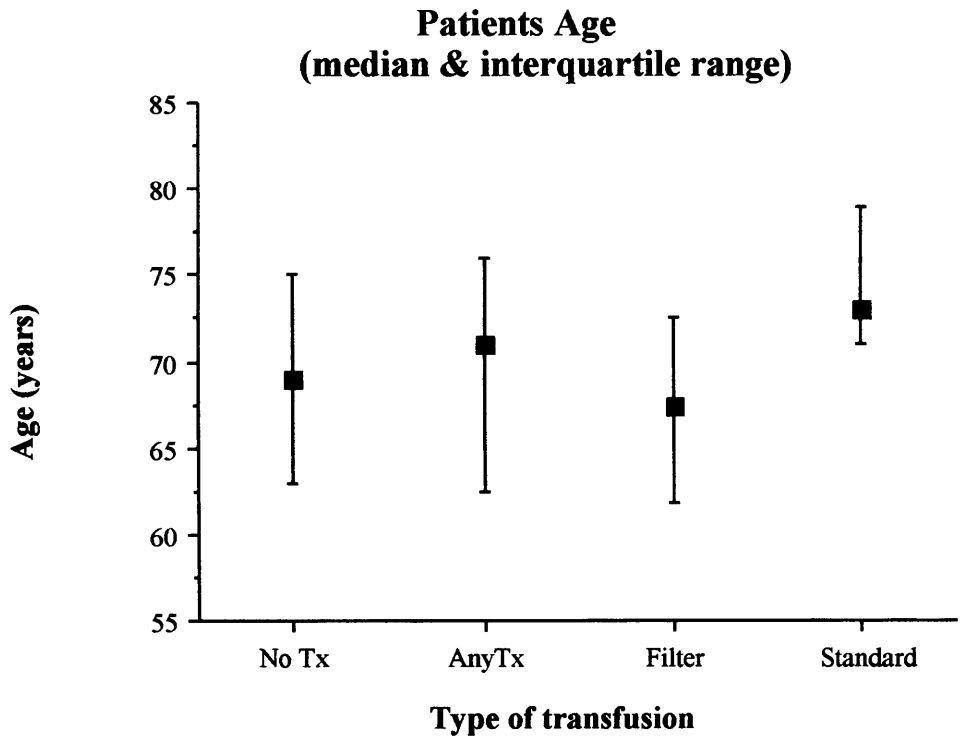
Table 3:1. *Transfusion status of patients included for analysis.*

	All patients	Not transfused	Transfused	Filtered blood	Standard blood
Number	86	67	19	10	9
Median	**70**	**69**	**71**	**67.5**	**73**
Minimum	37	37	41	41	58
Maximum	86	85	86	82	86

Statistical Analysis: (Mann - Whitney)

Not transfused v. Transfused	p = 0.53
Not transfused v. Filtered	p = 0.60
Not transfused v. Standard	p = 0.13
Filtered v. Standard	p = 0.15

Table 3:2. *Patient age in years (median values and ranges).*



Graph 3:1 *Age of patients undergoing colorectal cancer resections - median and interquartile range.*

	All patients	Not transfused	Transfused	Filtered blood	Standard blood
Right hemicolectomy	13	12	1	0	1
Left hemicolectomy	11	10	1	1	0
Sigmoid colectomy	10	10	0	0	0
Anterior resection	41	32	9	4	5
Abdomino-perineal resection	10	2	8	5	3
Subtotal colectomy	1	1	0	0	0
TOTAL	86	67	19	10	9

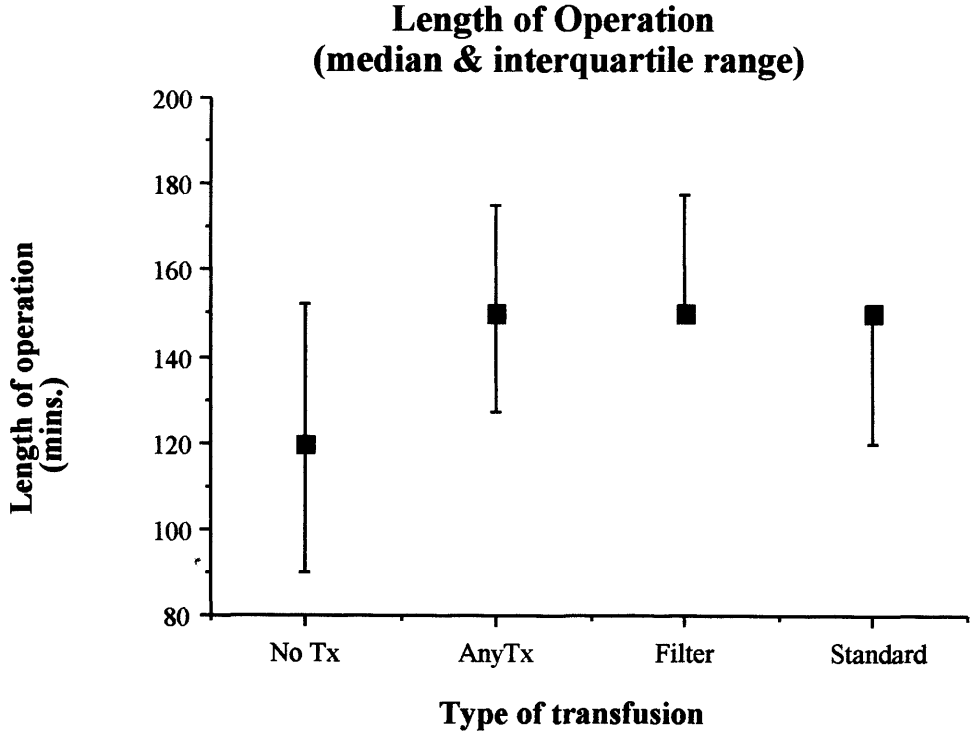
Table 3:3. *Type of operation.*

	Not transfused	Transfused	Filtered blood	Standard blood
Number	67	19	10	9
Median	**120**	**150**	**150**	**150**
Minimum	40	100	120	100
Maximum	300	260	260	180

Statistical Analysis: (Mann - Whitney)

Not transfused v. Transfused	**p = 0.017**
Not transfused v. Filtered	**p = 0.017**
Not transfused v. Standard	p = 0.25
Filtered v. Standard	p = 0.22

Table 3:4. *Length of operation in minutes (median values and ranges).*



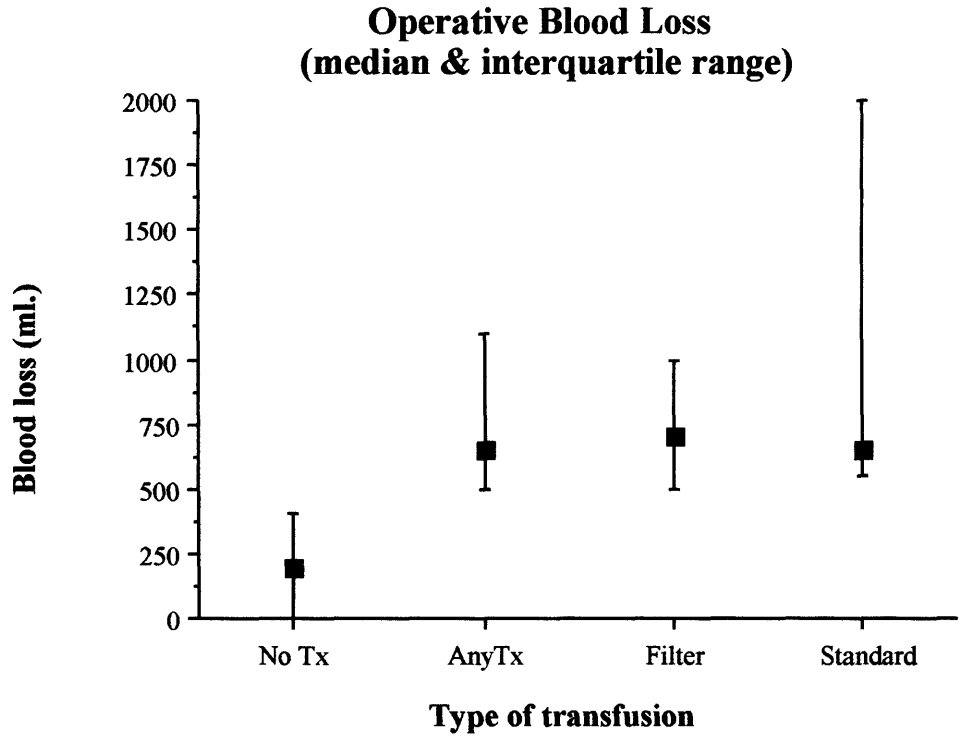
Graph 3:2 *Duration of surgery for colorectal cancer patients - median and interquartile range.*

	Not transfused	Transfused	Filtered blood	Standard blood
Number	67	19	10	9
Median	**200**	**650**	**700**	**650**
Minimum	0	250	400	250
Maximum	1200	2500	2000	2500

Statistical Analysis: (Mann - Whitney)

Not transfused v. Transfused	**$p < 0.0001$**
Not transfused v. Filtered	**$p < 0.0001$**
Not transfused v. Standard	**$p = 0.0002$**
Filtered v. Standard	$p = 0.74$

Table 3:5. *Operative blood loss in millilitres (median values and ranges).*



Graph 3:3 *Volume of blood lost during colorectal cancer resection - median and interquartile range.*

	Not transfused	Transfused	Filtered blood	Standard blood
Pre-operative	**13.4** (9.1-16.5)	**12.7** (9.7-15.8)	**13.0** (9.7-15.8)	**11.3** (10.1-15.0)
Post-operative	**11.7** (9.0-15.6)	**10.1** (7.5-14.9)	**10.0** (7.7-14.9)	**11.0** (7.5-12.7)
Change	1.7	2.0	2.6	1.6

Statistical Analysis:

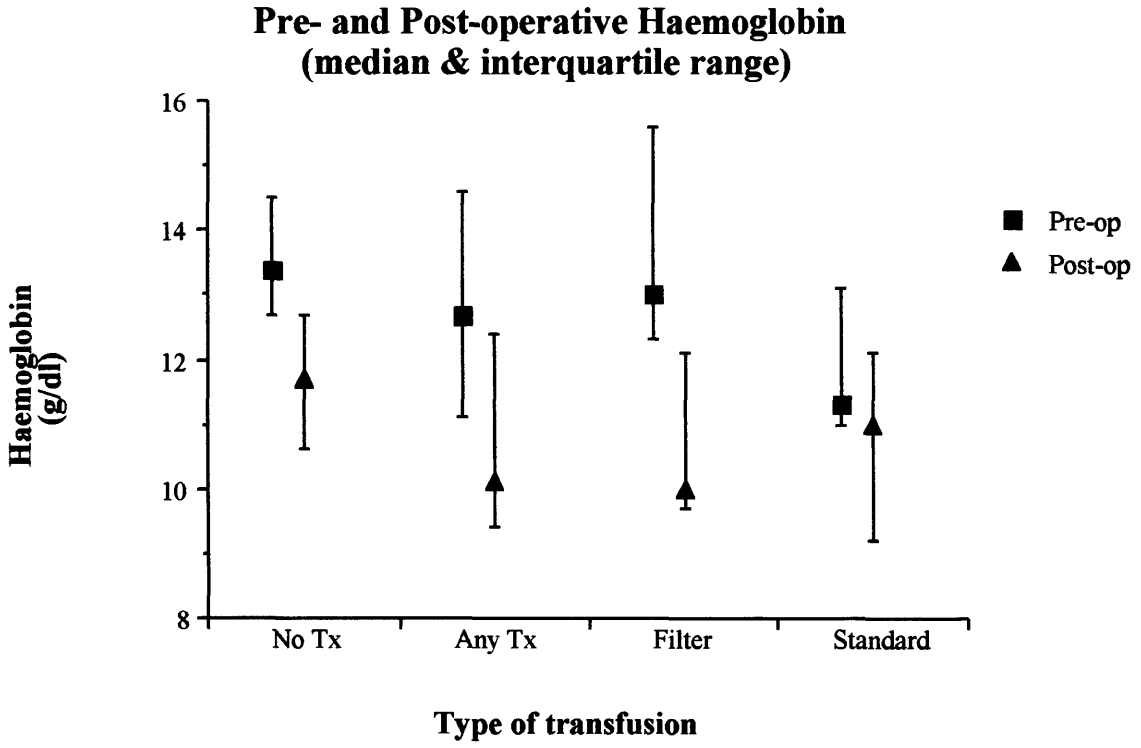
a) Intra-group variation (Wilcoxon)

Not transfused; pre- v. post-operative	**$p < 0.0001$**
Transfused; pre- v. post-operative	**$p = 0.0005$**
Filtered; pre- v. post-operative	**$p = 0.0039$**
Standard; pre- v. post-operative	**$p = 0.020$**

b) Inter-group variation (Mann-Whitney)

Not transfused v. Transfused	Pre-operative	$p = 0.14$
	Post-operative	**$p = 0.036$**
Not transfused v. Filtered	Pre-operative	$p = 0.96$
	Post-operative	$p = 0.09$
Not transfused v. Standard	Pre-operative	**$P = 0.026$**
	Post-operative	$p = 0.16$
Filtered v. Standard	Pre-operative	$p = 0.17$
	Post-operative	$p = 0.74$

Table 3:6. *Median haemoglobin values; g/dl (range).*



Graph 3:4 *Change in haemoglobin value following colorectal cancer resection - median and interquartile range.*

	Not transfused	Transfused	Filtered blood	Standard blood
Pre-operative	**39** (30-49)	**39** (28-46)	**40** (28-46)	**37** (33-44)
Post-operative	**34** (25-43)	**32** (22-44)	**31** (23-44)	**33** (22-37)
Change	5	7	6.5	7

Statistical Analysis:

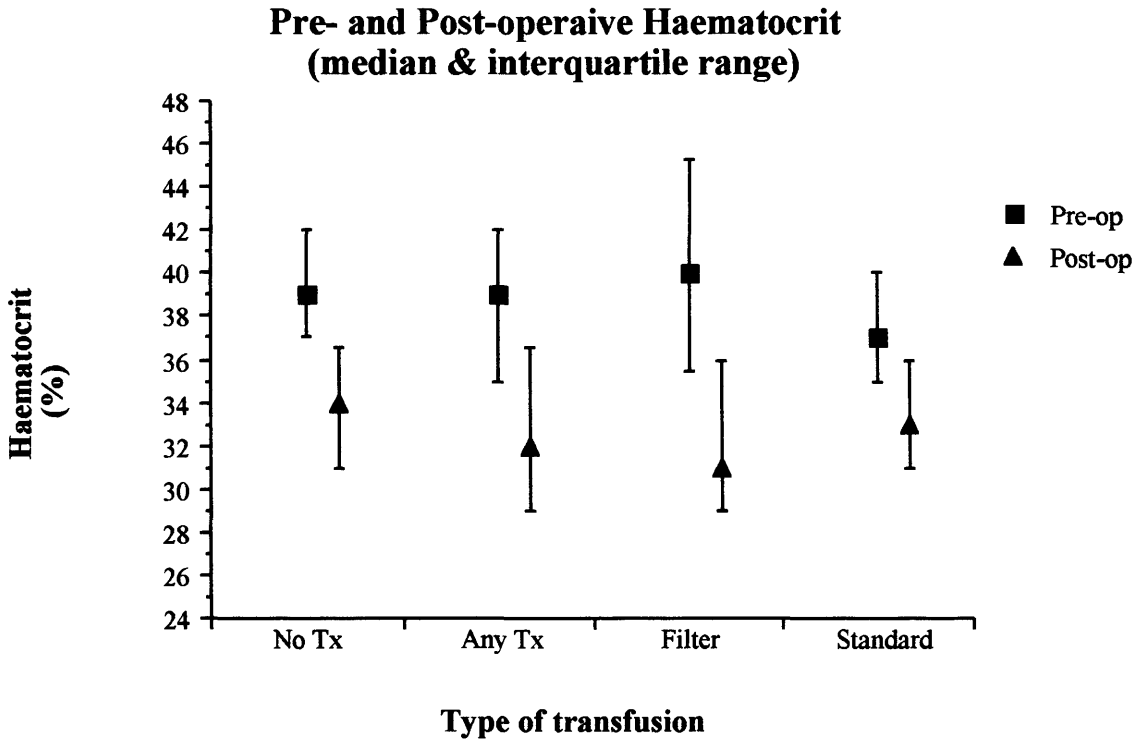
a) Intra-group variation (Wilcoxon)

Not transfused; pre- v. post-operative	**$p < 0.0001$**
Transfused; pre- v. post-operative	**$p = 0.0019$**
Filtered; pre- v. post-operative	**$p = 0.027$**
Standard; pre- v. post-operative	**$p = 0.020$**

b) Inter-group variation (Mann-Whitney)

Not transfused v. Transfused	Pre-operative	$p = 0.49$
	Post-operative	$p = 0.36$
Not transfused v. Filtered	Pre-operative	$p = 0.81$
	Post-operative	$p = 0.43$
Not transfused v. Standard	Pre-operative	$P = 0.19$
	Post-operative	$p = 0.57$
Filtered v. Standard	Pre-operative	$p = 0.35$
	Post-operative	$p = 0.94$

Table 3:7. *Median haematocrit values; % (range).*



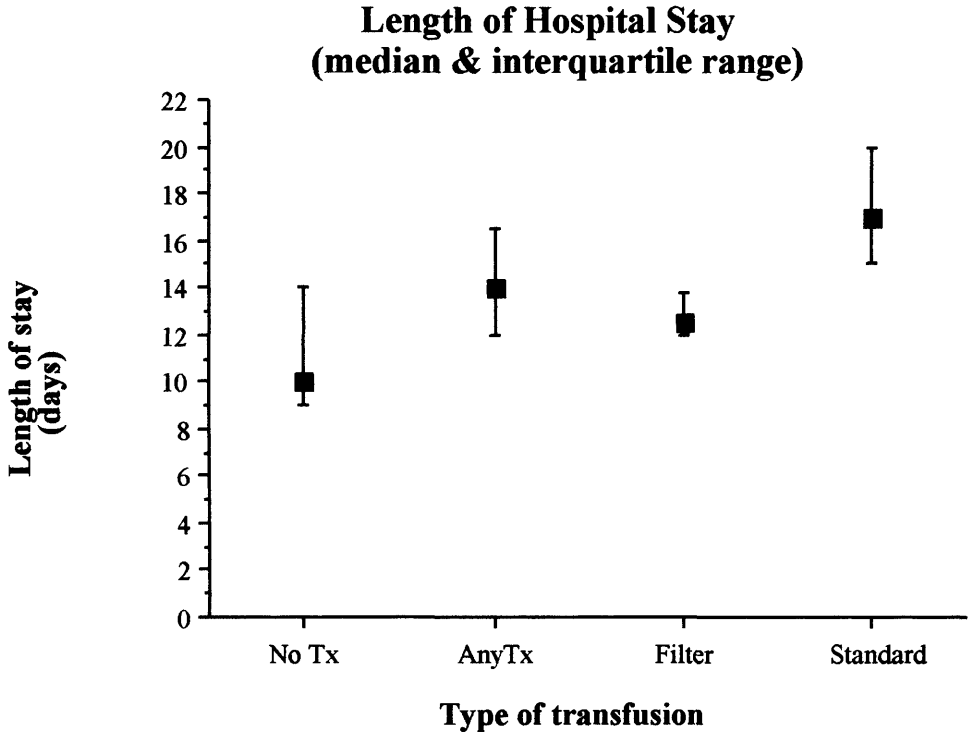
Graph 3:5 *Change in haematocrit value following colorectal cancer resection - median and interquartile range.*

	Not transfused	Transfused	Filtered blood	Standard blood
Number	67	19	10	9
Median	**10**	**14**	**12.5**	**17**
Minimum	5	10	11	10
Maximum	45	34	15	34

Statistical Analysis: (Mann - Whitney)

Not transfused v. Transfused	**p = 0.0013**
Not transfused v. Filtered	p = 0.081
Not transfused v. Standard	**p = 0.0018**
Filtered v. Standard	**p = 0.027**

Table 3:8. *Length of hospital stay in days; median and range.*



Graph 3:6 *Length of hospital stay for patients undergoing colorectal cancer resection - median and interquartile range.*

	Not transfused (n=67)	Filtered blood (n=10)	Standard blood (n=9)
Wound infection	9	1	4
Chest infection	4	1	1
Urinary tract infection	5	0	1
Anastomotic leak	3	0	0
Wound dehiscence	1	0	0

Statistical Analysis: (Fisher's exact)

Wound infections:

Not transfused v. Filtered	p = 1.0
Not transfused v. Standard	**p = 0.04**
Filtered v. Standard	p = 0.14

Other complications:

All comparisons	p>0.05

Table 3:9. *Post-operative complications.*

		Not transfused (n=67)	Filtered blood (n=10)	Standard blood (n=9)
Tumour stage:	A	16 (24)	1 (10)	1 (11)
	B	31 (46)	6 (60)	5 (56)
	C	20 (30)	3 (30)	3 (33)
Histological grade:	Well	12 (18)	3 (30)	2 (22)
	Moderate	50 (75)	6 (60)	7 (78)
	Poor	5 (7)	1 (10)	0 (0)

Statistical Analysis: (Chi-squared)

Tumour stage:

All groups $p = 0.78$

Histological grade:

All groups $p = 0.66$

Table 3:10. *Tumour stage (Dukes' classification) and grade of differentiation for resected cancers - percentage values shown in parentheses.*

CHAPTER 4

Transfusion In Colorectal Cancer Patients - The Immune Response.

Introduction.

The 86 patients described in Chapter 3 all had their natural killer cell activity and lymphocyte subsets measured during the perioperative period, as detailed in Chapter 2 (Methods). That data is presented here comparing the 3 subgroups of patients: *(i)* those who were not transfused (as the control group, n = 67), *(ii)* those who received a filtered blood transfusion (n = 10), and *(iii)* those who received a standard blood transfusion (n = 9).

Natural Killer Cell Activity.

To summarise from Chapter 2: the raw data collected from the ^{51}chromium release assay was expressed in terms of the number of "lytic units" present in 10^6 peripheral blood lymphocytes (LU/10^6 PBL), where one lytic unit was the number of peripheral blood lymphocytes required to lyse 30% of the target cells. Flow cytometry was used to determine the number of natural killer (NK) cells present in each PBL preparation, and from these two data sets the number of lytic units per 10^6 NK cells (LU/10^6 NK) was calculated. Flow cytometry of the patients' whole blood samples determined the number of NK cells per millilitre of whole blood (NKx10^6 /ml. blood), and from that data and the LU/10^6 NK data the number of lytic units per millilitre of whole blood was calculated.

Thus, under the heading of Natural Killer Cell Activity, there are 4 data sets presented for each of the 3 patient subgroups:

(i) cellular cytotoxicity of peripheral blood lymphocyte preparations.

(ii) absolute numbers of Natural Killer cells.

(iii) cytotoxicity of Natural Killer cells.

(iv) cellular cytotoxicity of whole blood samples.

Lymphocyte subsets.

Flow cytometry on whole blood samples was used to determine the percentage of certain lymphocyte subsets present in the trial patients, these being

CD2, CD3, CD4, CD8, and CD16 as described in the Methods section. From these percentages and the absolute lymphocyte count the absolute value of the subsets was calculated and expressed as the number of cells $x10^3$ per millilitre of peripheral blood. The CD4/CD8 ratio was also calculated and expressed as a simple ratio.

Statistical Analysis

The four parameters of Natural Killer Cell Activity, and the absolute values of lymphocyte subsets, were analysed for differences both within and between the three patient subgroups. The Wilcoxon test was used for comparison of paired data sets within the groups (changes from baseline), and the Mann-Whitney test was used to analyse variations between the groups.

Natural Killer Cell Activity.

Characteristics of the raw data.

A general trend was observed in all four data sets, namely that by the time of the first post-operative measurement (Day 5) there had been a noticeable decrease in the measured parameter, be it cytotoxicity or cell numbers, and this was more noticeable in the patient group which had received standard blood transfusions. By Day 28 the four parameters showed varying levels of recovery toward pre-operative values, and in general the patient group which had received standard blood transfusions showed less evidence of recovery than either the control group or those that received leucodepleted transfusions. These characteristics are described in more detail by considering both intra- and inter-group variations over the next few pages. For each data set there is one table and two graphs: the table contains median values (with interquartile ranges) which are expressed graphically in the first of the two graphs; the second graph is a representation of the change from baseline for each parameter where the two post-operative values (Day 5 and Day 28) are plotted as a percentage of the pre-operative value. Statistical analysis of the data is shown below each table and relates only to the absolute data, no analysis was performed on the second graph of each data set - this is shown solely as a visual aid to highlight the degree of change from baseline.

(i) Lytic units per 10^6 peripheral blood lymphocytes. Table 4:1

Graphs 4:1 & 4:2

Intra-group variations.

Cellular cytotoxicity was decreased in all three groups at Day 5, significantly so in both the control group and those who received standard blood transfusions. By Day 28 the control group and those who had received leucodepleted blood transfusions showed a recovery in cytotoxicity to levels which were very similar to their pre-operative values, while the standard transfusion group showed a persistent suppression of cytotoxicity with no significant change between Day 5 and Day 28.

Inter-group variations.

All three groups had no statistically significant differences in peripheral blood lymphocyte cytotoxicity between them both pre-operatively and at Day 5. At Day 28 the control group and those who received leucodepleted transfusions still did not differ significantly, but those who received standard blood transfusions had significantly impaired cytotoxicity when compared with the leucodepleted group.

(ii) Natural killer cells ($x10^6$) per millilitre of whole blood. Table 4:2

Graphs 4:3 & 4:4

Intra-group variations.

The absolute number of Natural Killer cells decreased in all three groups by Day 5, reaching significantly lower levels compared with pre-operative values in both the control group and those who received standard transfusions. At Day 28 the number of Natural Killer cells in the control group's blood had nearly returned to pre-operative levels (although analysis of the paired data showed a statistically significant lower number of cells), and patients from the leucodepleted group had slightly more

Natural Killer cells than pre-operatively. Those patients who received standard blood transfusions showed a small but significant increase in Natural Killer cell numbers from Day 5 to Day 28, but overall the cell concentration was still much lower than pre-operatively.

Inter-group variations.

Pre-operatively the standard transfusion group of patients had significantly more Natural Killer cells than either of the two other groups, and, although this group showed a greater decrease in cell numbers over the time of the study, the absolute numbers of Natural Killer cells showed no significant variation between all three groups at both Day 5 and Day 28.

(iii) Lytic units per 10^6 natural killer cells. Table 4:3

Graphs 4:5 & 4:6

Intra-group variations.

The cytotoxicity of Natural Killer cells decreased in all three groups over the first five days following surgery. From Day 5 to Day 28 the control group and those who received leucodepleted blood showed an increase in Natural Killer cell activity to levels that were significantly higher than at Day 5, but did not quite match pre-operative levels. The patients who had received standard blood transfusion showed little evidence of a recovery of Natural Killer cell cytotoxicity.

Inter-group variations.

There was no significant difference in Natural Killer cell cytotoxicity between all three groups at the pre-operative stage, and also at Day 5 after surgery. By Day 28 this situation was the same when comparing the control group with those who received leucodepleted transfusions, whereas the patients who had been transfused with

standard blood showed a significantly lower cytotoxicity than the leucodepleted group.

(iv) Lytic units per millilitre of whole blood. Table 4:4

Graphs 4:7 & 4:8

Intra-group variations.

The control group shows a dramatic and significant fall in overall cytotoxicity by Day 5, a pattern that is repeated in both of the other groups. By Day 28 both the control group and those who received leucodepleted transfusions have nearly regained their pre-operative levels of cytotoxicity with significant increases in activity from Day 5, but those patients who were transfused with standard blood have not returned to anywhere near their pre-operative cytotoxic activity in spite of also showing a significant increase from Day 5.

Inter-group variations.

Graphs 7 and 8 show a clear trend in these extrapolated values of whole blood cytotoxicity, suggesting that all three groups show a similar degree of lytic ability both pre-operatively and at Day 5 post-operatively, but by Day 28 the patients who were transfused with standard blood show an impairment of cytotoxicity when compared to the other two groups. These changes do not however show any degree of statistical significance.

Lymphocyte Subsets.

Characteristics of the raw data.

The differing subsets all showed a degree of decrease in numbers at the initial post-operative assessment followed by varying amounts of recovery over the 28 day period. The CD4/CD8 ratio was a little different as described below. All data are presented as median values with interquartile ranges, and is shown in tabular and graphical form for each subset. A single graph of absolute values is shown for each subset as there was little inter-group variation of pre-operative results.

(i) **CD2.** Table 4:5
Graph 4:9

Intra-group variations.

Patients who were not transfused showed a significant fall in T-cell numbers at Day 5 which returned to pre-operative levels by Day 28. Those who received leucodepleted transfusions showed a similar fall and recovery pattern, but the decrease in numbers at Day 5 was not statistically significant. Patients who were transfused with standard blood also showed the same response but with a significant fall in numbers at Day 5.

Inter-group variations.

There was no significant difference between the three groups of patients pre- or post-operatively.

(ii) CD3. Table 4:6

Graph 4:10

Intra-group variations.

The CD3 subset ("non-NK" T-cells) showed an almost identical response as the CD2 subset, principally a fall in numbers at Day 5 which recovered by Day 28. This was again significant in those patients who were not transfused as well as those who received standard blood, but not in those who were transfused with leucodepleted blood.

Inter-group variations.

There was no significant difference between the three groups of patients pre- or post-operatively.

(iii) CD4. Table 4:7

Graph 4:11

Intra-group variations.

Patients without transfusion had a marked fall in numbers of T-helper cells at Day 5 that recovered to near pre-operative levels by Day 28. Those who had been transfused with leucodepleted blood showed a similar trend with a significant increase in cell numbers by Day 28 compared with Day 5, whereas those who had received standard blood had significantly lower numbers at both Day 5 and Day 28 compared to pre-operative values.

Inter-group variations.

Again there was no significant difference between the three groups of patients pre- or post-operatively.

(iv) CD8. Table 4:8

Graph 4:12

Intra-group variations.

Those who were not transfused suffered a significant fall in numbers of T-suppressor cells at Day 5 which was followed by a recovery to a level just below pre-operative values by Day 28. The group of patients who received filtered blood transfusions showed a similar but non-significant trend, also repeated in those who were transfused with standard blood but this time with a very significant fall in numbers at Day 5.

Inter-group variations.

Although the standard transfusion group of patients appeared to maintain a slightly higher number of T-suppressor cells than the other two groups during the course of the study this was not significant at any point. Otherwise, as before, there was no difference between the groups.

(v) CD4/CD8 ratio. Table 4:9

Graph 4:13

Intra-group variations.

The ratio of T-helper to suppressor cells increased slightly in the early post-operative period in both the group of patients who were not transfused and those who received leucodepleted blood. This increased ratio was maintained in both groups during the course of the study. The patients who were transfused with standard blood behaved completely differently with a significant fall in ratio at Day 5 which was maintained through to Day 28.

Inter-group variations.

The group who received no blood transfusion did not differ from those who received leucodepleted blood from pre-operative measurements through to Day 28. Those who were transfused with standard blood showed a similar ratio to the other two groups at the pre-operative stage, a significantly lower ratio than the non-transfused group at Day 5, and a lower ratio than either of the other two groups by Day 28.

(vi) CD16. Table 4:10

Graph 4:14

Intra-group variations.

The patients who were not transfused showed a decrease in CD16 +ve lymphocytes at Day 5 which then returned to pre-operative levels by Day 28. Those who received leucodepleted transfusions had a very similar reduction and recovery in cell numbers, but not to a significant degree. The patients who had standard blood transfusions had a very significant decrease in cell numbers at Day 5, and although this increased significantly by Day 28 the value was still much lower than pre-operatively.

Inter-group variations.

Even though the pre-operative number of cells appeared higher for the standard transfusion group than the other two this was not significant, and there was no significant variation between the three groups over the whole study.

Summary

Cellular cytotoxicity measured as lytic activity of peripheral blood lymphocytes showed a clear trend of prolonged immunosuppression for the patients who received standard blood transfusions, and this was mirrored in the derived activity of NK cells alone. NK cell numbers fell dramatically in all 3 groups after surgery though the standard transfusion patients started with higher numbers of NK cells than the other groups. This fall in numbers was prolonged only in the standard transfusion group. The combination of these results to give an overall cytotoxicity of peripheral blood confirmed the impression that patients transfused with standard blood have a prolonged post-operative suppression of immune response compared with the other patients.

The overall number of T lymphocytes, and in particular the non-NK lymphocytes, was decreased in the early post-operative period, but there was no discernible difference due to blood transfusion. T-helper cell numbers appeared particularly badly affected by the combination of surgery and standard blood transfusion, while T-suppressor cell numbers seemed to be boosted by the presence of a standard blood transfusion. The result of these two changes was to cause a significant and prolonged fall in the CD4/CD8 ratio for the 4 weeks following surgery in the standard transfusion group.

Tables and Graphs

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	4.5 (2.5 - 7.0)	4.5 (3.4 - 7.7)	4.0 (3.7 - 4.4)
Day 5	1.5 (0.9 - 3.4)	2.8 (1.2 - 3.8)	1.7 (0.8 - 3.0)
Day 28	4.1 (2.3 - 7.3)	4.5 (3.0 - 7.7)	1.9 (1.7 - 3.2)

Statistical Analysis

Intra-group variations: (Wilcoxon test)

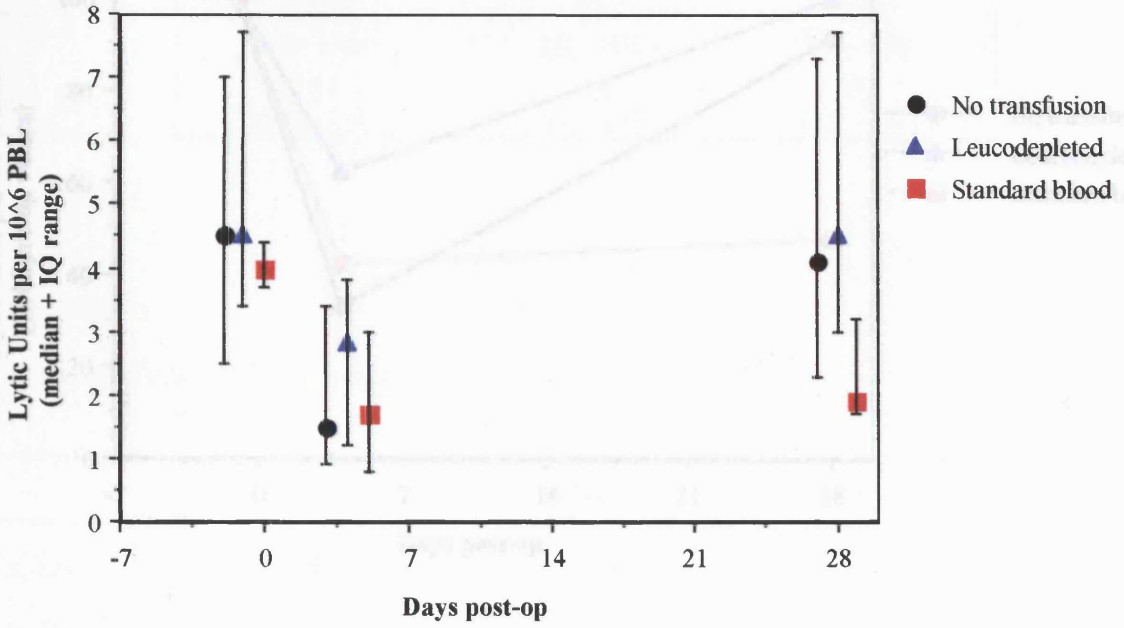
	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.08	**p=0.05**
Pre-op v. Day 28	**p<0.0001**	p=0.63	**p=0.02**
Day 5 v. Day 28	**p<0.0001**	**p=0.01**	p=0.36

Inter-group variations: (Mann-Whitney test)

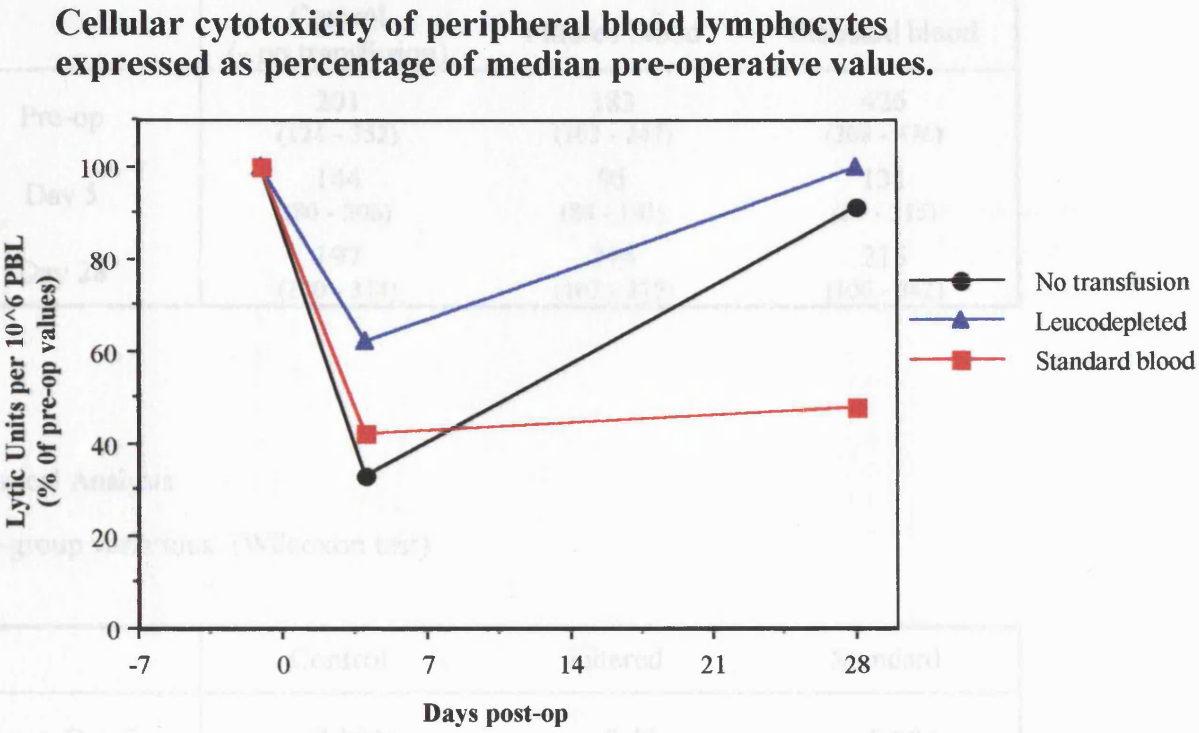
	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.62	p=0.46	p=0.42
Control v. Standard	p=0.99	p=0.97	p=0.12
Filtered v. Standard	p=0.9	p=0.78	p=0.05

Table 4:1. *Lytic units / 10^6 peripheral blood lymphocytes; median (interquartile range).*

Cellular cytotoxicity of peripheral blood lymphocyte preparations from patients receiving standard, leucodepleted and no transfusion.



Graph 4:1


Cellular cytotoxicity of peripheral blood lymphocytes expressed as percentage of median pre-operative values.
Lytic Units per 10^6 PBL (% 0f pre-op values)
100
80
60
40
20
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:2

	Control (- no transfusion)	Filtered blood	Standard blood
Pre-op	201 (124 - 352)	183 (103 - 247)	426 (208 - 476)
Day 5	144 (80 - 206)	95 (88 - 181)	131 (57 - 215)
Day 28	197 (140 - 314)	214 (107 - 275)	215 (106 - 342)

Statistical Analysis

Intra-group variations: (Wilcoxon test)

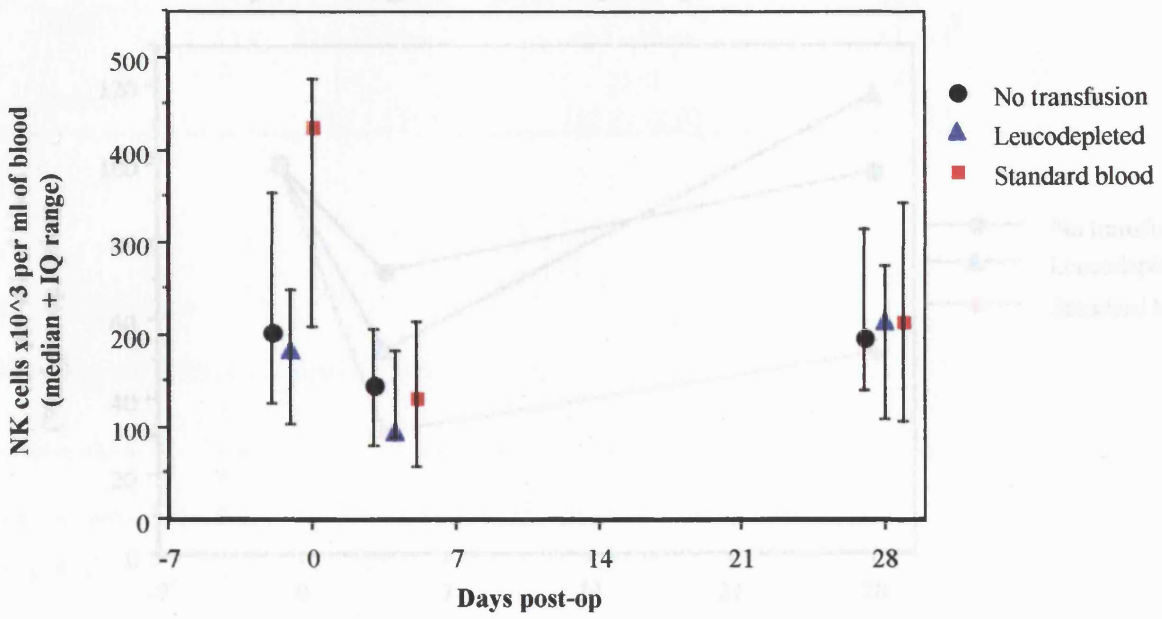
	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.43	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.63	**p=0.004**
Day 5 v. Day 28	**p<0.0001**	p=0.23	**p=0.004**

Inter-group variations: (Mann-Whitney test)

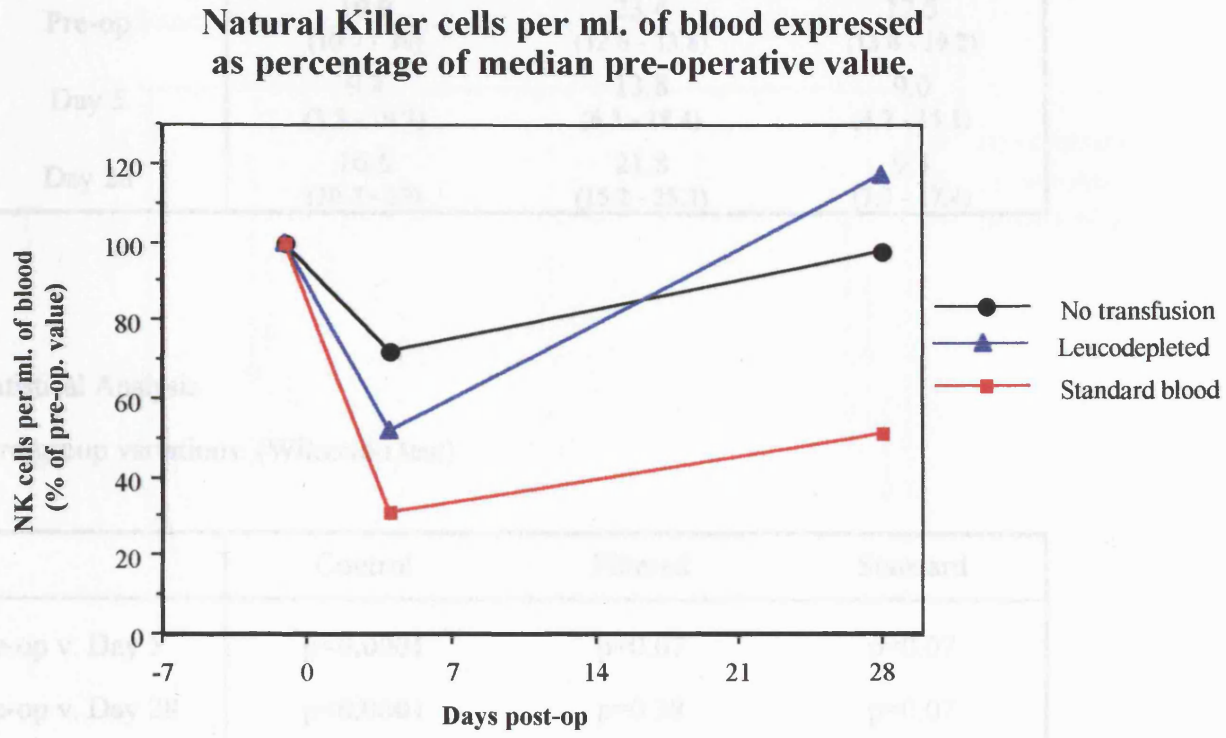
	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.29	p=0.95	p=0.56
Control v. Standard	**p=0.03**	p=0.81	p=0.82
Filtered v. Standard	**p=0.02**	p=0.97	p=0.6

Table 4:2. *Number of natural killer cells $x10^3$ per ml. of blood - median (interquartile range).*

Absolute numbers of Natural Killer cells for patients receiving standard, leucodepleted, and no transfusion.



Graph 4:3.


Natural Killer cells per ml. of blood expressed as percentage of median pre-operative value.
NK cells per ml. of blood (% of pre-op. value)
120
100
80
60
40
20
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:4.

	Control (- no transfusion)	Filtered blood	Standard blood
Pre-op	19.9 (10.7 - 39)	23.4 (12.6 - 33.8)	17.5 (13.8 - 19.2)
Day 5	9.3 (3.5 - 19.2)	13.8 (6.3 - 18.4)	9.0 (4.7 - 15.1)
Day 28	16.6 (10.7 - 33)	21.8 (15.2 - 28.2)	9.5 (7.3 - 17.4)

Statistical Analysis

Intra-group variations: (Wilcoxon test)

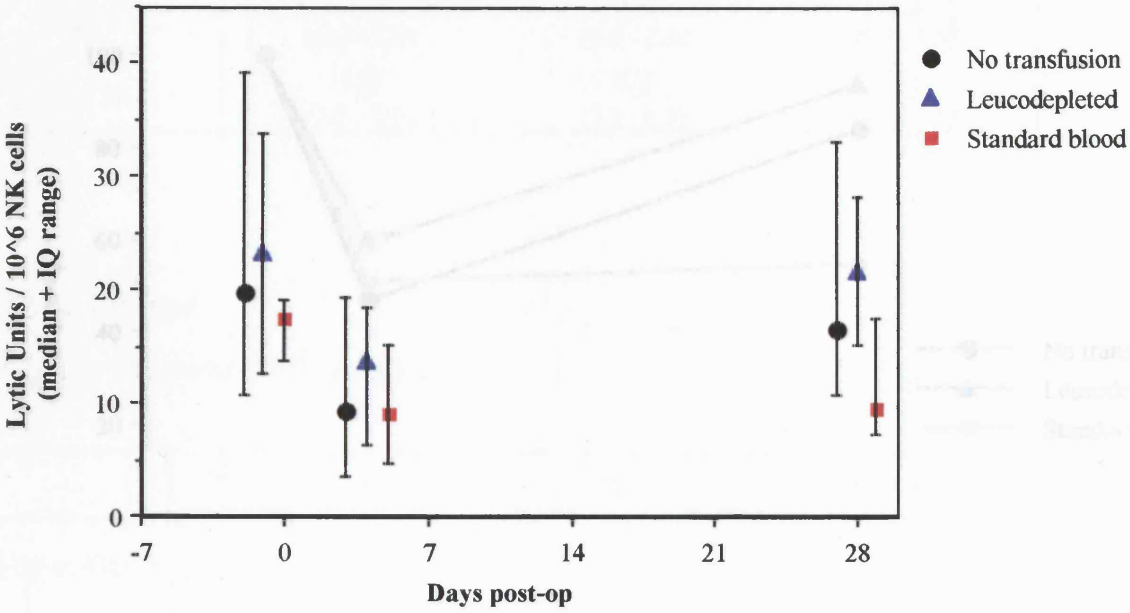
	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.07	p=0.07
Pre-op v. Day 28	**p<0.0001**	p=0.38	p=0.07
Day 5 v. Day 28	**p<0.0001**	**p=0.01**	p=0.43

Inter-group variations: (Mann-Whitney test)

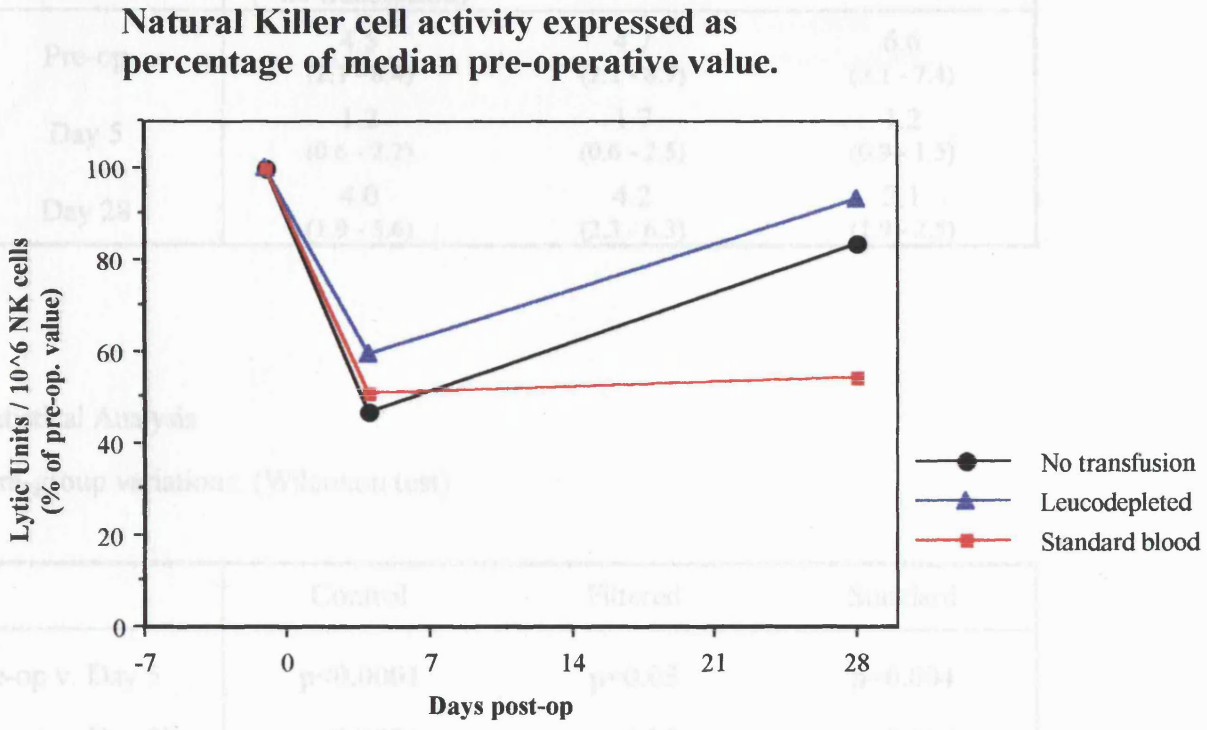
	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.69	p=0.7	p=0.32
Control v. Standard	p=0.35	p=0.94	p=0.1
Filtered v. Standard	p=0.24	p=0.71	p=0.03

Table 4:3. *Lytic units per 10^6 NK cells - median (interquartile range).*

Natural Killer cell activity for patients receiving leucodepleted, standard, and no transfusion.



Graph 4:5


Natural Killer cell activity expressed as percentage of median pre-operative value.
Lytic Units / 10^6 NK cells (% of pre-op. value)
100
80
60
40
20
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:6

	Control (- no transfusion)	Filtered blood	Standard blood
Pre-op	4.5 (2.1 - 8.4)	4.7 (2.2 - 8.7)	6.6 (3.1 - 7.4)
Day 5	1.2 (0.6 - 2.2)	1.7 (0.6 - 2.5)	1.2 (0.9 - 1.5)
Day 28	4.0 (1.9 - 5.6)	4.2 (2.3 - 6.3)	2.1 (1.9 - 2.5)

Statistical Analysis

Intra-group variations: (Wilcoxon test)

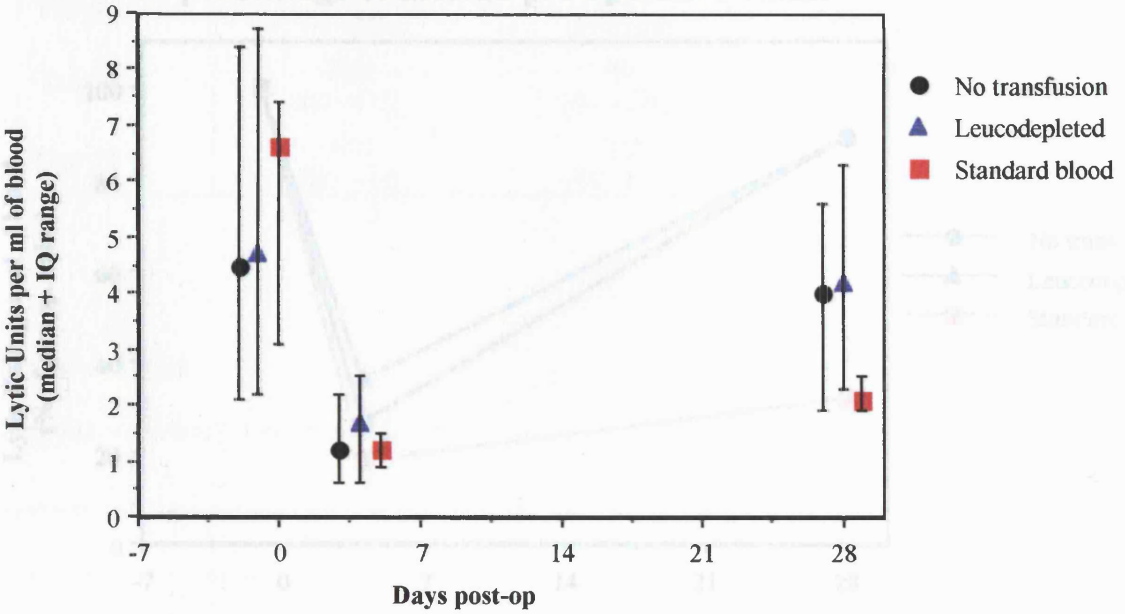
	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	**p=0.05**	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.19	**p=0.004**
Day 5 v. Day 28	**p<0.0001**	**p=0.02**	**p=0.004**

Inter-group variations: (Mann-Whitney test)

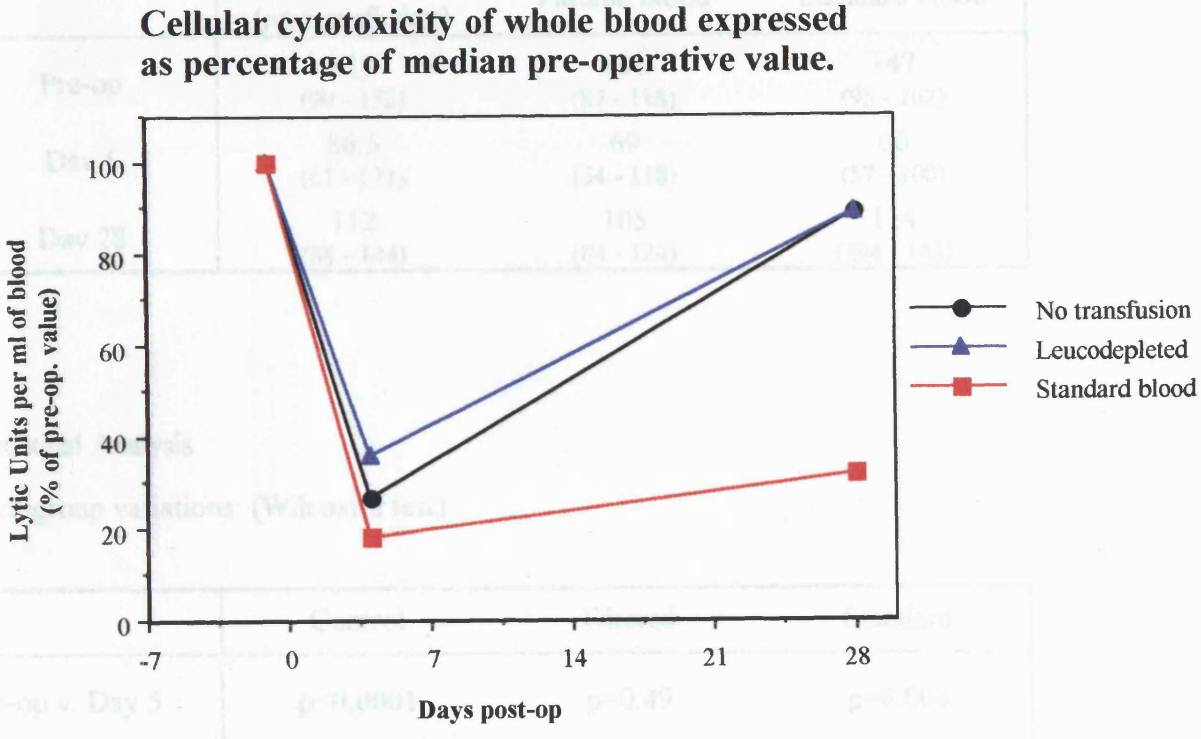
	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.99	p=0.7	p=0.73
Control v. Standard	p=0.42	p=0.86	p=0.12
Filtered v. Standard	p=0.6	p=0.65	p=0.21

Table 4:4. *Lytic units per ml. of whole blood - median (interquartile range).*

Cellular cytotoxicity of whole blood from patients receiving standard, leucodepleted and no transfusion.



Graph 4:7


Cellular cytotoxicity of whole blood expressed as percentage of median pre-operative value.
Lytic Units per ml of blood (% of pre-op. value)
100
80
60
40
20
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:8

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	119 (90 - 152)	101 (87 - 118)	147 (95 - 162)
Day 5	86.5 (61 - 121)	69 (54 - 118)	66 (57 - 100)
Day 28	112 (88 - 144)	105 (84 - 124)	114 (104 - 142)

Statistical Analysis

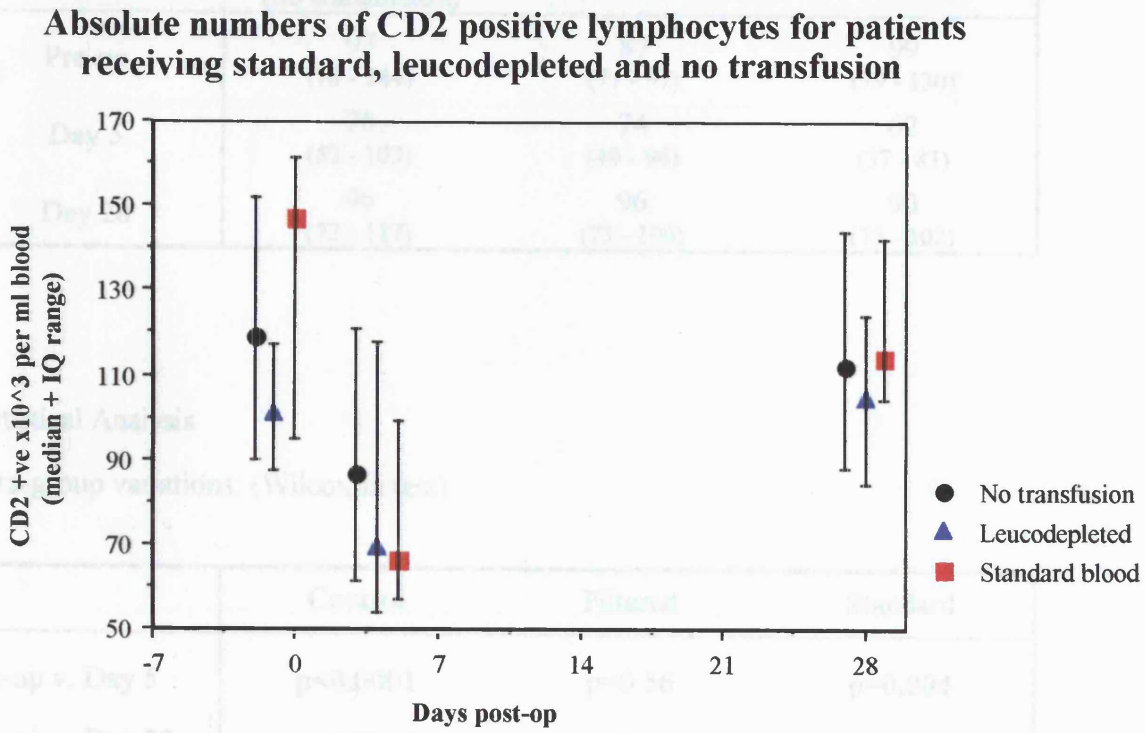
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.49	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.63	p=0.15
Day 5 v. Day 28	**p<0.0001**	p=0.19	**p=0.027**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.19	p=0.5	p=0.36
Control v. Standard	p=0.65	p=0.4	p=0.8
Filtered v. Standard	p=0.13	p=0.97	p=0.35

Table 4:5. *Number of CD2 positive lymphocytes x10^3 per ml. of blood; median (interquartile range).*


Absolute numbers of CD2 positive lymphocytes for patients receiving standard, leucodepleted and no transfusion
CD2 +ve x10^3 per ml blood (median + IQ range)
170
150
130
110
90
70
50
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:9

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	97 (78 - 144)	87 (77 - 97)	99 (79 - 130)
Day 5	76 (52 - 103)	74 (49 - 96)	62 (37 - 83)
Day 28	96 (72 - 127)	96 (73 - 106)	90 (75 - 102)

Statistical Analysis

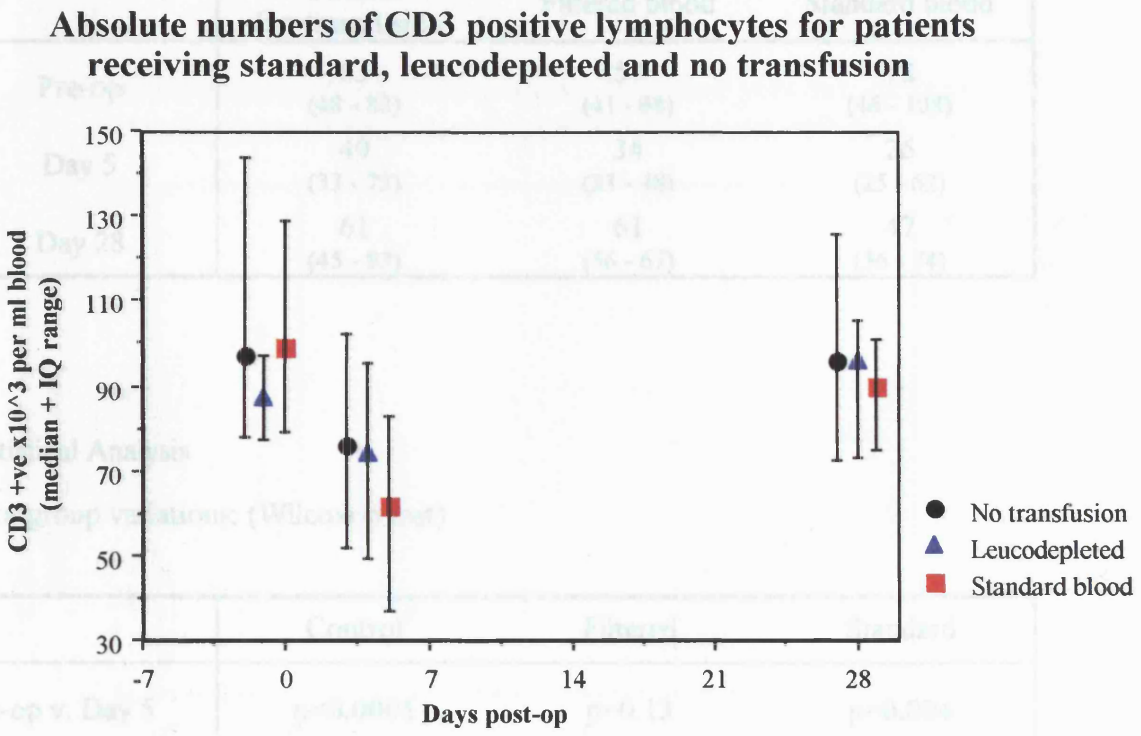
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.56	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.92	p=0.11
Day 5 v. Day 28	**p<0.0001**	p=0.13	**p=0.02**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.17	p=0.83	p=0.45
Control v. Standard	p=0.98	p=0.4	p=0.61
Filtered v. Standard	p=0.44	p=0.71	p=0.87

Table 4:6. *Number of CD3 positive lymphocytes $x10^3$ per ml. of blood; median (interquartile range).*


Absolute numbers of CD3 positive lymphocytes for patients receiving standard, leucodepleted and no transfusion
CD3 +ve x10^3 per ml blood (median + IQ range)
150
130
110
90
70
50
30
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:10

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	63 (48 - 83)	57 (41 - 68)	72 (46 - 108)
Day 5	49 (33 - 72)	34 (23 - 48)	26 (25 - 62)
Day 28	61 (45 - 83)	61 (56 - 67)	47 (36 - 74)

Statistical Analysis

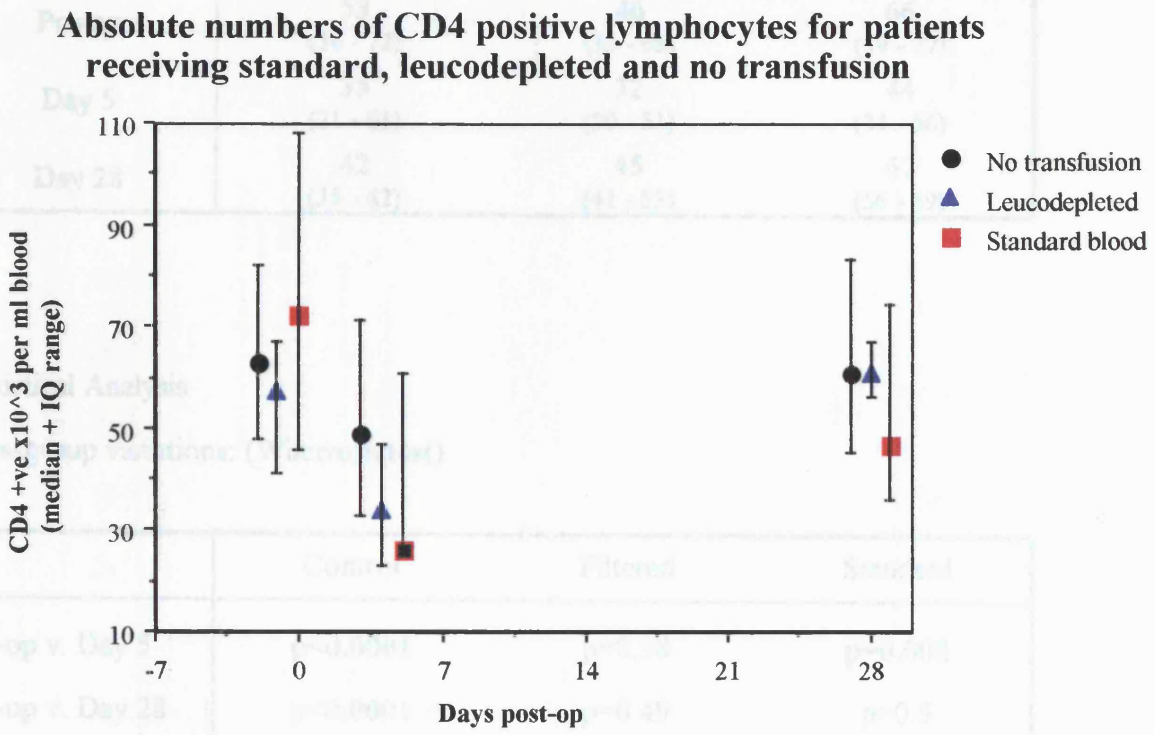
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.13	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.43	**p=0.02**
Day 5 v. Day 28	**p<0.0001**	**p=0.01**	**p=0.02**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.15	p=0.1	p=0.86
Control v. Standard	p=0.83	p=0.24	p=0.39
Filtered v. Standard	p=0.24	p=0.97	p=0.57

Table 4:7. *Number of CD4 positive lymphocytes x10^3 per ml. of blood; median (interquartile range).*


Absolute numbers of CD4 positive lymphocytes for patients receiving standard, leucodepleted and no transfusion
CD4 +ve x10^3 per ml blood (median + IQ range)
110
90
70
50
30
10
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:11

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	53 (36 - 72)	46 (33 - 68)	66 (39 - 77)
Day 5	35 (21 - 61)	32 (20 - 51)	44 (34 - 56)
Day 28	42 (35 - 62)	45 (41 - 63)	67 (56 - 69)

Statistical Analysis

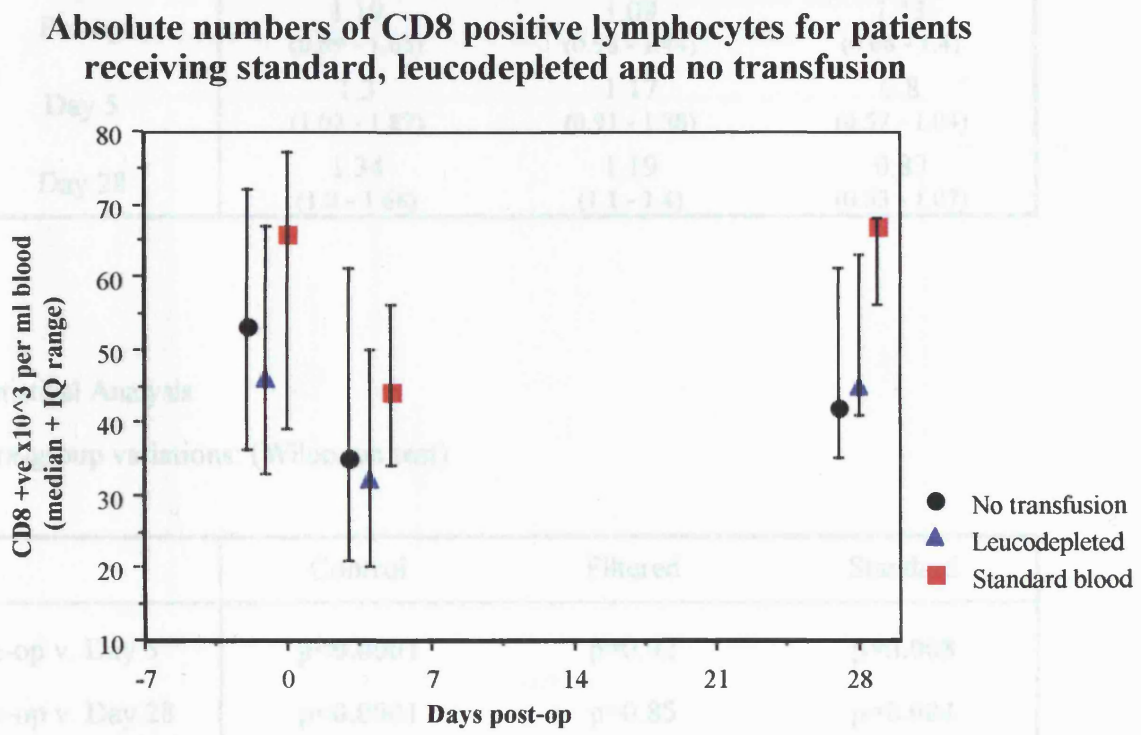
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.38	**p=0.008**
Pre-op v. Day 28	**p<0.0001**	p=0.49	p=0.5
Day 5 v. Day 28	**p<0.0001**	p=0.08	**p=0.023**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.56	p=0.77	p=0.41
Control v. Standard	p=0.49	p=0.33	p=0.09
Filtered v. Standard	p=0.27	p=0.39	p=0.29

Table 4:8. *Number of CD8 positive lymphocytes $x10^3$ per ml. of blood; median (interquartile range).*


Absolute numbers of CD8 positive lymphocytes for patients receiving standard, leucodepleted and no transfusion
CD8 +ve x10^3 per ml blood (median + IQ range)
80
70
60
50
40
30
20
10
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:12

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	1.19 (0.89 - 1.65)	1.09 (0.98 - 1.44)	1.13 (0.68 - 1.4)
Day 5	1.3 (1.02 - 1.87)	1.17 (0.91 - 1.38)	0.8 (0.57 - 1.04)
Day 28	1.34 (1.0 - 1.68)	1.19 (1.1 - 1.4)	0.87 (0.53 - 1.07)

Statistical Analysis

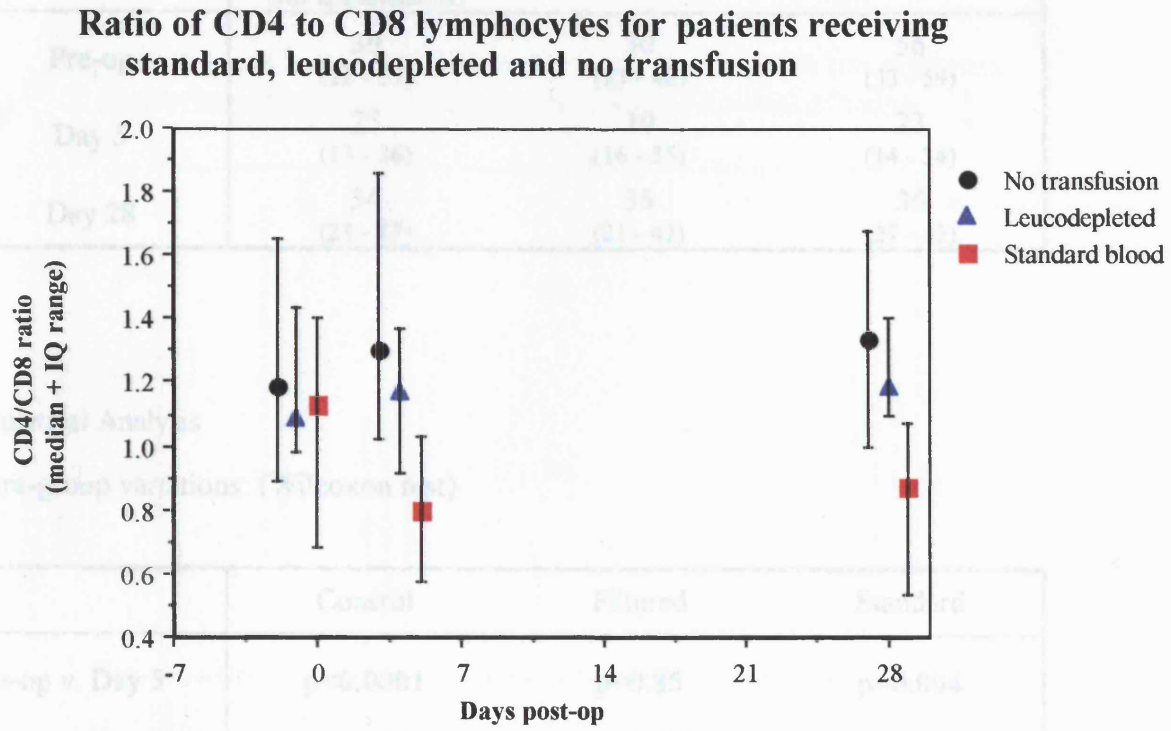
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.92	**p=0.008**
Pre-op v. Day 28	**p<0.0001**	p=0.85	**p=0.004**
Day 5 v. Day 28	p=0.14	p=0.56	p=0.91

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.71	p=0.2	p=0.39
Control v. Standard	p=0.52	**p=0.013**	**p=0.009**
Filtered v. Standard	p=0.87	p=0.18	**p=0.05**

Table 4:9. *Ratio of CD4 to CD8 positive lymphocytes; median (interquartile range).*


Ratio of CD4 to CD8 lymphocytes for patients receiving standard, leucodepleted and no transfusion
CD4/CD8 ratio (median + IQ range)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 4:13

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	39 (22 - 57)	30 (23 - 48)	56 (33 - 59)
Day 5	25 (13 - 36)	19 (16 - 35)	23 (14 - 34)
Day 28	34 (23 - 47)	35 (21 - 42)	36 (27 - 42)

Statistical Analysis

Intra-group variations: (Wilcoxon test)

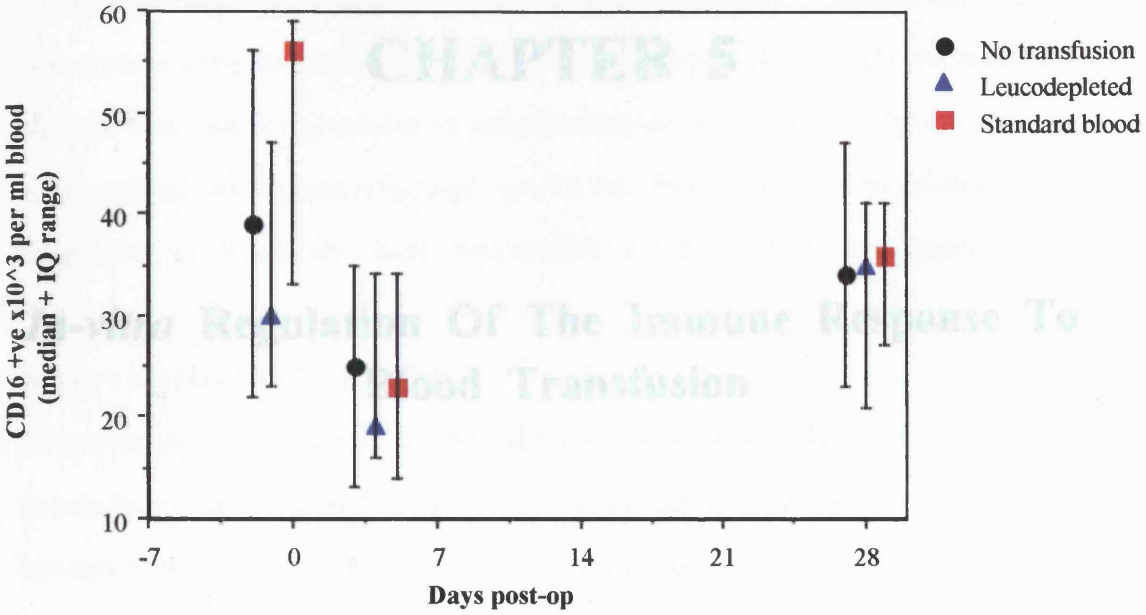
	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.85	**p=0.004**
Pre-op v. Day 28	**p<0.0001**	p=0.38	**p=0.012**
Day 5 v. Day 28	**p<0.0001**	p=0.32	**p=0.004**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.37	p=0.91	p=0.76
Control v. Standard	p=0.26	p=0.52	p=0.98
Filtered v. Standard	p=0.09	p=0.9	p=0.74

Table 4:10. *Number of CD16 positive lymphocytes $x10^3$ per ml. of blood; median (interquartile range).*

Absolute numbers of CD16 positive lymphocytes for patients receiving standard, leucodepleted and no transfusion



Graph 4:14

CHAPTER 5

In-vitro Regulation Of The Immune Response To Blood Transfusion

Introduction.

The effect of four different drugs on the cytotoxicity of patients peripheral blood lymphocytes was assessed as detailed in the Methods section (Chapter 2). These *in-vitro* assays were all variations of the standard ^{51}Cr release assay and as such the results were expressed as Lytic Units per 10^6 peripheral blood lymphocytes. The same patient sub-groups were considered, but as this part of the study commenced after the initial work the number of patients analysed was lower: *(i)* those who were not transfused (as the control group), n = 41, *(ii)* those who received a filtered blood transfusion, n = 5, and *(iii)* those who received a standard blood transfusion, n = 7.

In vitro regulation of cytotoxicity.

To summarise from Chapter 2: a peripheral blood lymphocyte solution was prepared in exactly the same way as for the standard assay, but by doubling the cellular concentration it was possible to use only 50µl per well in the assay plate, rather than 100µl, to give the same number of cells. This left space for addition of 50µl of drug solution which, when combined with 100µl of ^{51}Cr labelled K562 cell solution, provided the same effector to target cell ratio in the same overall volume as the standard assay.

Data presentation.

The data from these four experiments are shown in table form as median values with the interquartile ranges (tables 5:1 - 5:4). This same data is then presented graphically in two forms: initially (graphs 5:1 - 5:4) all three patient groups are plotted together for each of the four experiments, allowing comparison within and between the groups with *in vitro* additives; then (graphs 5:5 - 5:16) individual graphs for each patient group showing cytotoxicity with and without *in vitro* additives for each experiment.

Statistical analysis.

The results of these drug-modified assays were analysed in the same manner as in Chapter 4 using paired data analysis for intra-group variations (Wilcoxon signed rank test), and unpaired analysis for inter-group variations (Mann--Whitney test). Comparisons with the standard assay results were performed as paired data sets for each patient, therefore the Wilcoxon test was used for these analyses.

Effects of the addition of *in vitro* additives on the differences in cytotoxicity within and between the three sub-groups of patients.

The results of the addition of the four drugs to the cytotoxicity assay are described here noting the different responses of the three groups of patients.

(i) Addition of interleukin 2 (IL2). Table 5:1

Graph 5:1

Intra-group variations

The control group showed significant changes in cytotoxicity at each time of measurement with a dip at Day 5 followed by recovery as already demonstrated in Chapter 4. The patients who received leucodepleted blood showed little in the way of suppressed activity at Day 5, and were boosted quite strongly at Day 28 to double the pre-operative value, although not quite to a statistically significant level. Those who received standard transfusions showed the usual Day 5 decrease in activity, remaining significantly suppressed by Day 28.

Inter-group variations

Pre-operatively, and at Day 5 post-operatively, the three groups of patients showed similar cytotoxic activity. By Day 28 this activity was lower in those who had received standard blood compared to both of the other groups, and although those who had been transfused with leucodepleted blood had a median activity higher than the control group this represented a wide range of values which did not differ significantly.

(ii) Addition of interferon gamma (IFNγ). Table 5:2

Graph 5:2

Intra-group variations

The control group showed significant change at each of the three points of measurement. Those who had been transfused with leucodepleted blood showed a non-significant fall in cytotoxicity at Day 5 with activity returning to just greater than pre-operative levels by Day 28. The standard transfusion group suffered a significant decrease in cytotoxicity at Day 5 with restoration of pre-operative values by Day 28.

Inter-group variations

There were no significant differences between the three groups at any of the three measurement days, even though the leucodepleted transfusion group showed a tendency towards more active cytotoxicity.

(iii) Addition of cimetidine. Table 5:3

Graph 5:3

Intra-group variations

The control group again differed significantly on all three days of measurement. Those who received leucodepleted blood had a familiar decrease in cytotoxic activity at Day 5 though not to any significant degree, while the standard transfusion group showed little variation in cytotoxic activity through the course of the experiment.

Inter-group variations

The three patient groups started off with similar cytotoxicity, and by Day 5 the control group and the leucodepleted transfusion group remained similar while the standard transfusion group showed a slightly higher level of activity. By Day 28 all three groups were similar once more.

(iv) Addition of ranitidine. Table 5:4

Graph 5:4

Intra-group variations

Again the control group showed significantly different levels of cytotoxicity throughout the experiment. The leucodepleted transfusion patients showed a good recovery of cytotoxic function to greater than pre-operative levels by Day 28, and the group who received standard blood transfusions had only minimal changes in activity during the experiment.

Inter-group variations

As seen in Graph 4 the only apparent difference between the groups was at Day 28 when the leucodepleted transfusion group appeared to have a greater degree of activity than those who had received standard blood, but from a statistical point of view there were no significant differences at any time.

Comparison of cytotoxicity with and without the addition of *in vitro* additives.

The cytotoxicity of peripheral blood lymphocytes as presented in Chapter 4 is compared here with the results of the same assay performed with *in vitro* additives. As described in the Methods section PBL samples from individual patients were assayed in the standard manner and with the different additives simultaneously, thus providing paired data for direct comparison. As mentioned earlier only 53 patients were assayed with *in vitro* additives (control n=41, leucodepleted transfusion n=5, standard transfusion n=7) and therefore only those 53 sets of results from Chapter 4 are used for comparison.

(i) Interleukin 2 (IL2) Graphs 5:5 - 5:7.

All three groups had their cellular cytotoxicity increased by a factor of 3-5x by the addition of IL2. The pattern of cytotoxicity over the time course of the experiment was not altered in any way, and the degree of statistical significance between the plain assays and those with additives was identical within the three groups.

(ii) Interferon gamma (IFNγ) Graphs 5:8 - 5:10.

For the control group the addition of IFNγ resulted in a minimal, but significant, increase in cytotoxicity over the course of the study. A similar trend was seen with those patients who received leucodepleted blood transfusions, and although the increase was not statistically significant it was more marked at Day 5 and Day 28. Those who had standard transfusions showed small, insignificant increases in cytotoxic activity pre-operatively and at Day 5, but at Day 28 there was a significant boost in activity compared to the plain assay with no IFNγ .

(iii) Cimetidine Graphs 5:11 - 5:13.

The addition of cimetidine to the control group assays caused statistically significant changes in the cytotoxicity values which realistically showed as a very minimal increase pre-operatively and decrease at Days 5 and 28. In the group which had leucodepleted blood the cimetidine appeared to cause a suppression of cytotoxicity, most evident at Day 5 and Day 28. The group which received standard transfusions showed a significant boost in activity in response to cimetidine at Days 5 and 28.

(iv) Ranitidine Graphs 5:14 - 5:16.

Ranitidine added to the control group assays caused a similar effect as cimetidine, a significant but very minimal alteration in cytotoxicity at each point of measurement. For those who received leucodepleted blood the addition of ranitidine made no difference to cytotoxicity, but for those from the standard transfusion group it resulted in a significant boost in activity on Days 5 and 28.

Summary.

The addition of IL2 *in vitro* to the natural killer cell assay resulted in a general increase in NK cell activity for all patients without altering the pattern of cytotoxicity during the post-operative course. IFNγ was responsible for a very minor increase in cytotoxicity generally, but specifically increased activity seen in the standard blood transfusion patients seen at Day 28. Cimetidine and ranitidine both caused an increase in natural killer cell activity in the standard transfusion patients at Days 5 and 28.

Tables and Graphs

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	15.2 (11.2 - 24.7)	14.6 (13.3 - 29.3)	16.3 (11 - 20.9)
Day 5	11.0 (5.9 - 13.9)	14.5 (7.9 - 22.7)	7.1 (3.2 - 12.2)
Day 28	16.2 (10.6 - 27.1)	32.1 (12.1 - 36.1)	9.9 (8 - 15.6)

Statistical Analysis

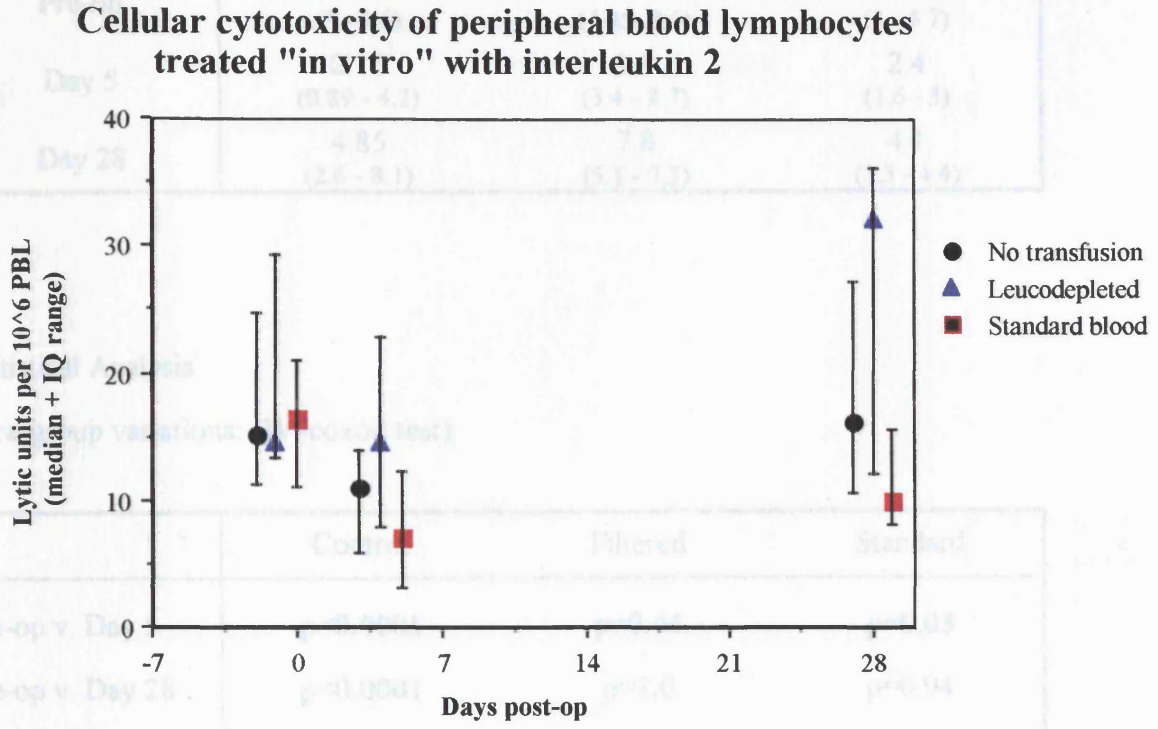
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.44	p=0.38
Pre-op v. Day 28	**p<0.0001**	p=1.0	**p=0.03**
Day 5 v. Day 28	**p<0.0001**	p=0.06	p=0.47

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.7	p=0.4	p=0.46
Control v. Standard	p=0.74	p=0.39	p=0.08
Filtered v. Standard	p=0.53	p=0.27	p=0.07

Table 5:1 *Lytic units / 10^6 peripheral blood lymphocytes treated* in vitro *with interleukin 2; median (interquartile range).*


Cellular cytotoxicity of peripheral blood lymphocytes treated "in vitro" with interleukin 2
Lytic units per 10^6 PBL (median + IQ range)
40
30
20
10
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 5:1

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	5.4 (3 - 7.6)	6.2 (4.25- 8.6)	4.5 (3 - 4.7)
Day 5	2.15 (0.89 - 4.2)	3.9 (3.4 - 2.7)	2.4 (1.6 - 3)
Day 28	4.85 (2.6 - 8.1)	7.8 (5.1 - 9.3)	4.1 (3.3 - 4.4)

Statistical Analysis

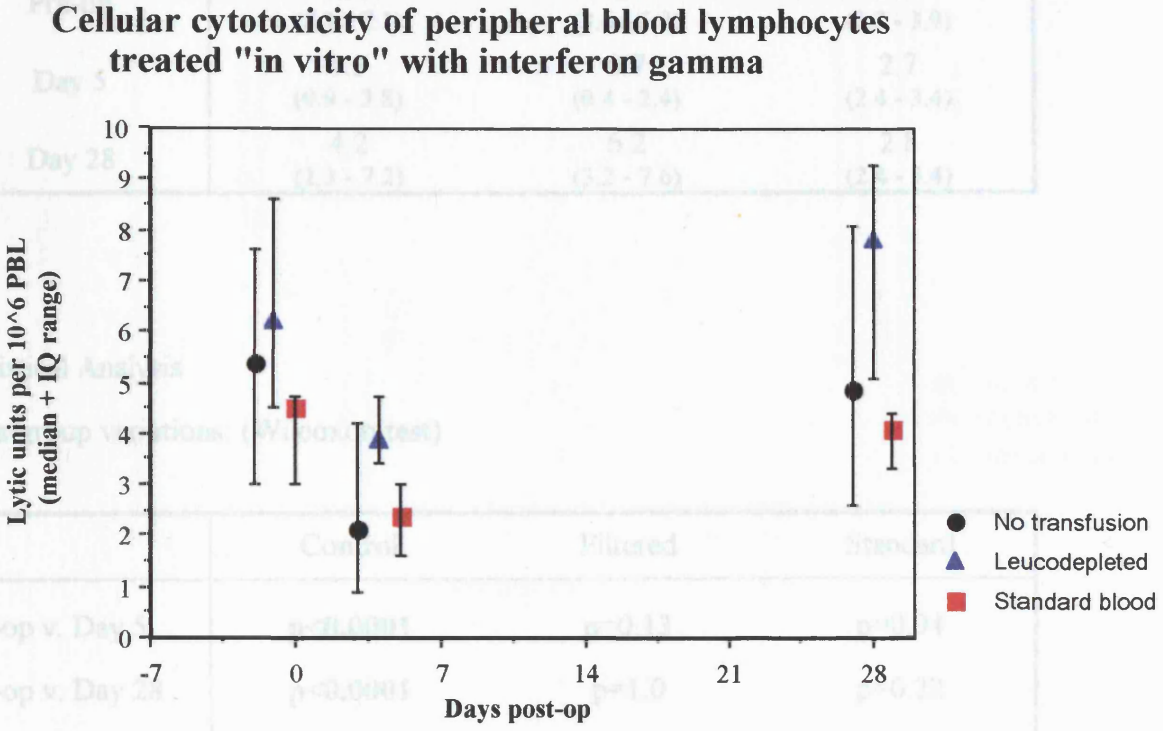
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.44	**p=0.03**
Pre-op v. Day 28	**p<0.0001**	p=1.0	p=0.94
Day 5 v. Day 28	**p<0.0001**	p=0.06	**p=0.03**

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.69	p=0.34	p=0.24
Control v. Standard	p=0.47	p=0.98	p=0.53
Filtered v. Standard	p=0.43	p=0.27	p=0.15

Table 5:2 *Lytic units / 10^6 peripheral blood lymphocytes treated* in vitro *with interferon γ; median (interquartile range).*


Cellular cytotoxicity of peripheral blood lymphocytes treated "in vitro" with interferon gamma
Lytic units per 10^6 PBL (median + IQ range)
10
9
8
7
6
5
4
3
2
1
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 5:2

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	5.2 (2.1 - 7.1)	4.6 (3.6 - 6.9)	3.5 (3.3 - 3.9)
Day 5	1.3 (0.9 - 3.8)	0.7 (0.4 - 2.4)	2.7 (2.4 - 3.4)
Day 28	4.2 (2.3 - 7.2)	6.2 (3.2 - 7.6)	2.8 (2.4 - 3.4)

Statistical Analysis

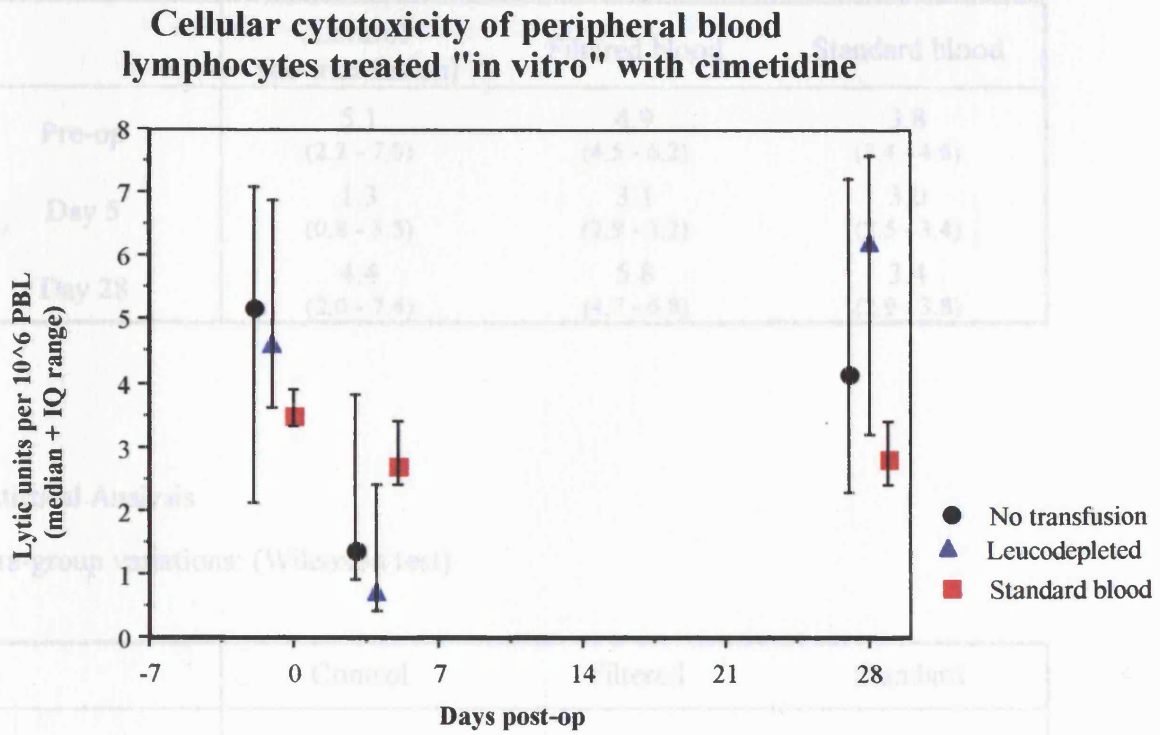
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.13	p=0.94
Pre-op v. Day 28	**p<0.0001**	p=1.0	p=0.22
Day 5 v. Day 28	**p<0.0001**	p=0.06	p=0.81

Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0..91	p=0.47	p=0.7
Control v. Standard	p=0.7	p=0.08	p=0.38
Filtered v. Standard	p=0.34	p=0.15	p=0.43

Table 5:3 *Lytic units / 10^6 peripheral blood lymphocytes treated* in vitro *with cimetidine; median (interquartile range).*


Cellular cytotoxicity of peripheral blood lymphocytes treated "in vitro" with cimetidine
Lytic units per 10^6 PBL (median + IQ range)
0
1
2
3
4
5
6
7
8
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 5:3

	Control (no transfusion)	Filtered blood	Standard blood
Pre-op	5.1 (2.2 - 7.0)	4.9 (4.5 - 6.2)	3.8 (3.4 - 4.6)
Day 5	1.3 (0.8 - 3.5)	3.1 (2.9 - 3.2)	3.0 (2.5 - 3.4)
Day 28	4.4 (2.0 - 7.4)	5.8 (4.7 - 6.8)	3.4 (2.9 - 3.8)

Statistical Analysis

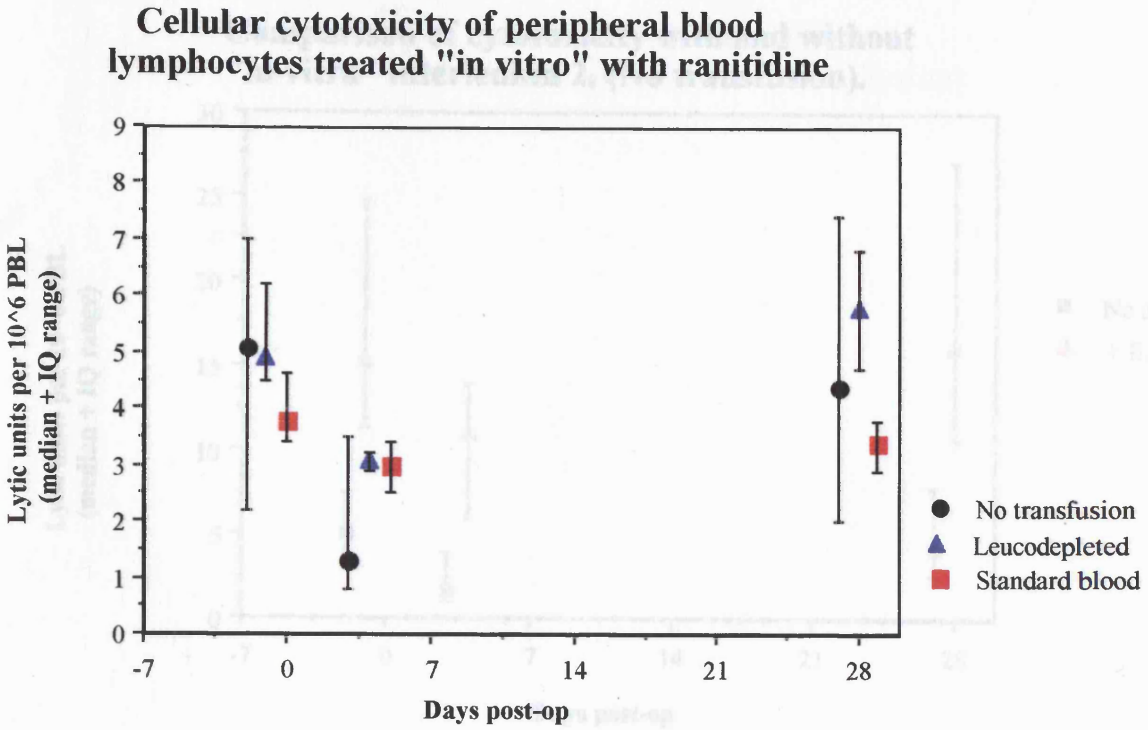
Intra-group variations: (Wilcoxon test)

	Control	Filtered	Standard
Pre-op v. Day 5	**p<0.0001**	p=0.31	p=0..69
Pre-op v. Day 28	**p<0.0001**	p=1.0	p=0.37
Day 5 v. Day 28	**p<0.0001**	p=0.06	p=094

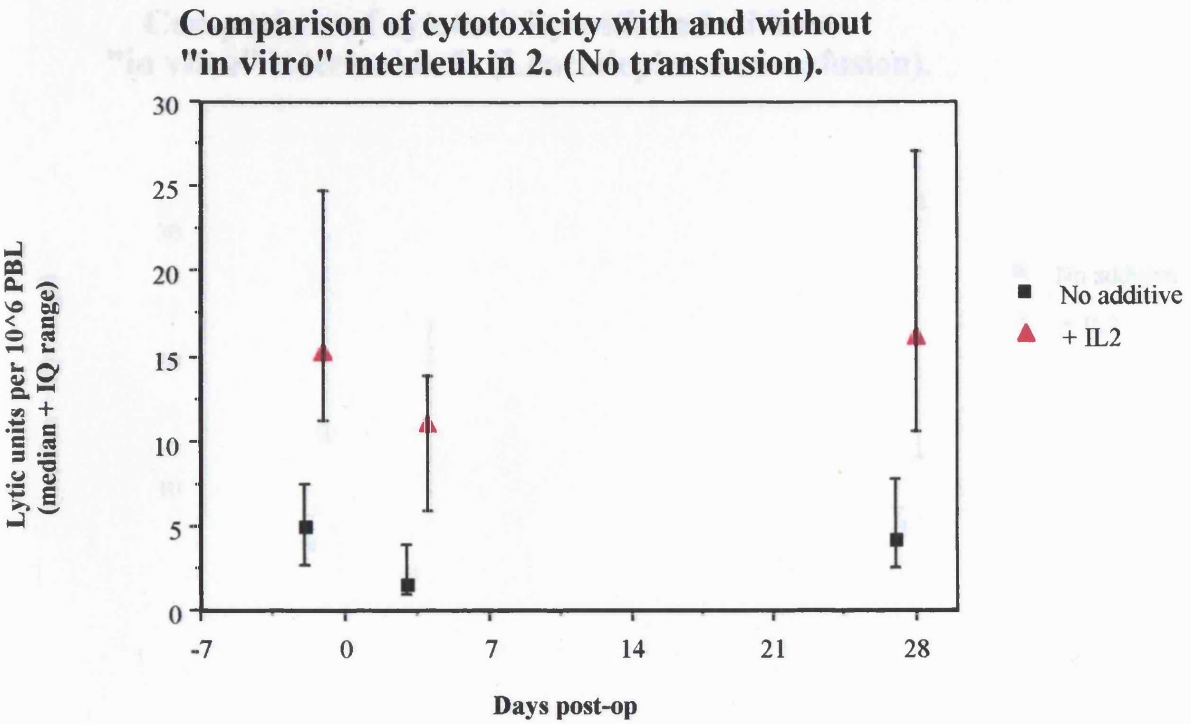
Inter-group variations: (Mann-Whitney test)

	Pre-op	Day 5	Day 28
Control v. Filtered	p=0.93	p=0.49	p=0.38
Control v. Standard	p=0.73	p=0.12	p=0.61
Filtered v. Standard	p=0.43	p=1.0	p=0.2

Table 5:4 *Lytic units / 10^6 peripheral blood lymphocytes treated* in vitro *with ranitidine; median (interquartile range).*


Cellular cytotoxicity of peripheral blood lymphocytes treated "in vitro" with ranitidine
Lytic units per 10^6 PBL (median + IQ range)
9
8
7
6
5
4
3
2
1
0
-7
0
7
14
21
28
Days post-op
No transfusion
Leucodepleted
Standard blood

Graph 5:4



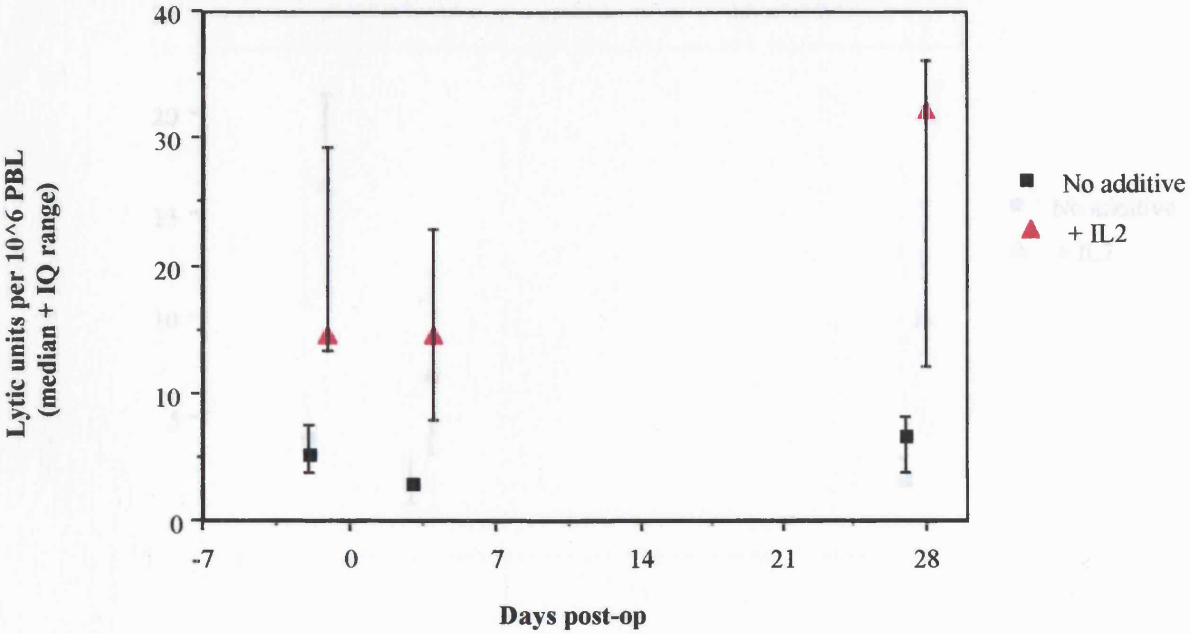
Statistical Analysis (Wilcoxon)

No additive v. plus IL2.

Pre-op.	**p<0.0001**
Day 5	**p<0.0001**
Day 28	**p<0.0001**

Graph 5:5

Comparison of cytotoxicity with and without "in vitro" interleukin 2. (Leucodepleted transfusion).



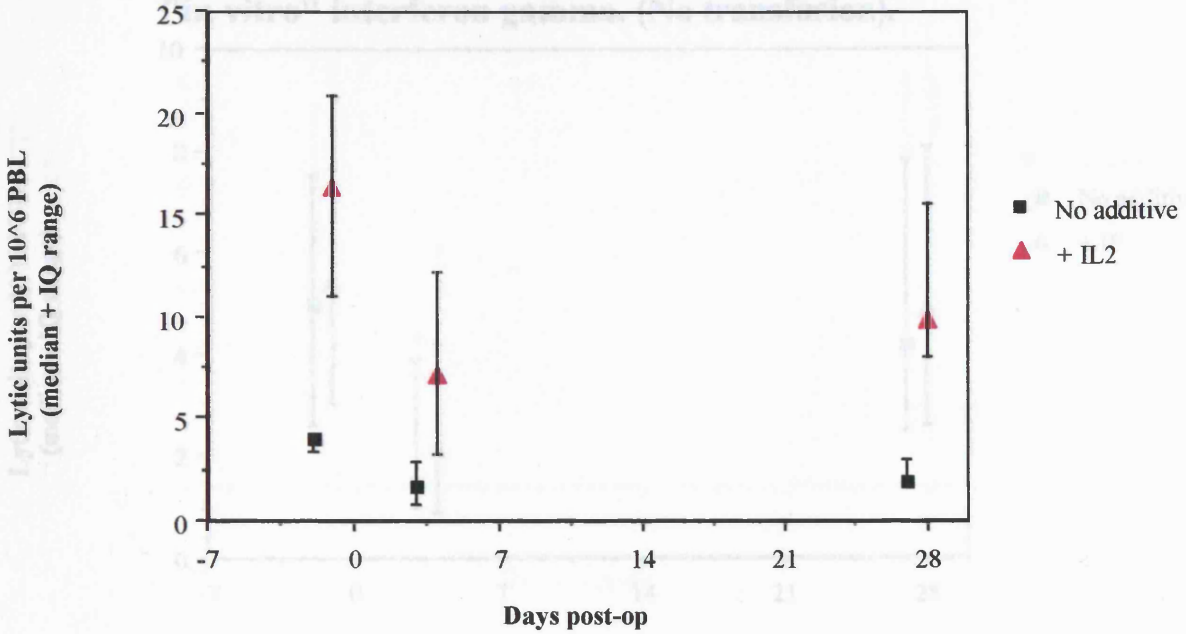
Statistical Analysis (Wilcoxon)

No additive v. plus IL2.

Pre-op.	p=0.06
Day 5	p=0.06
Day 28	p=0.06

Graph 5:6

Comparison of cytotoxicity with and without "in vitro" interleukin 2. (Standard transfusion).



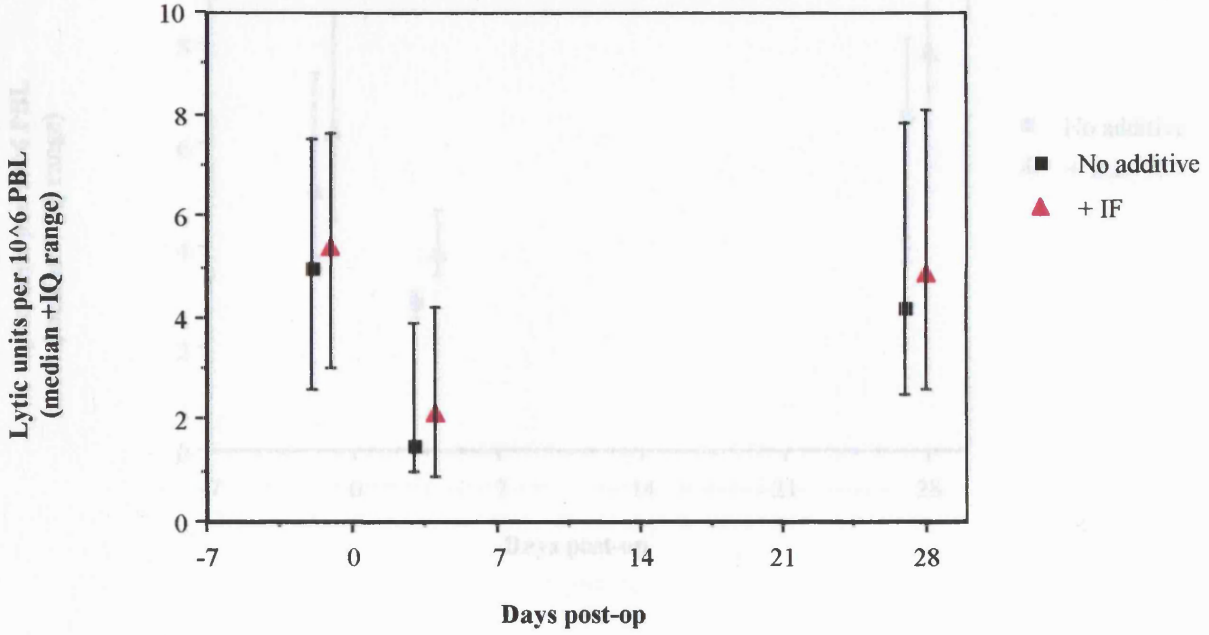
Statistical Analysis (Wilcoxon)

No additive v. plus IL2.

Pre-op.	**p=0.02**
Day 5	**p=0.02**
Day 28	**p=0.02**

Graph 5:7

Comparison of cytotoxicity with and without "in vitro" interferon gamma. (No transfusion).



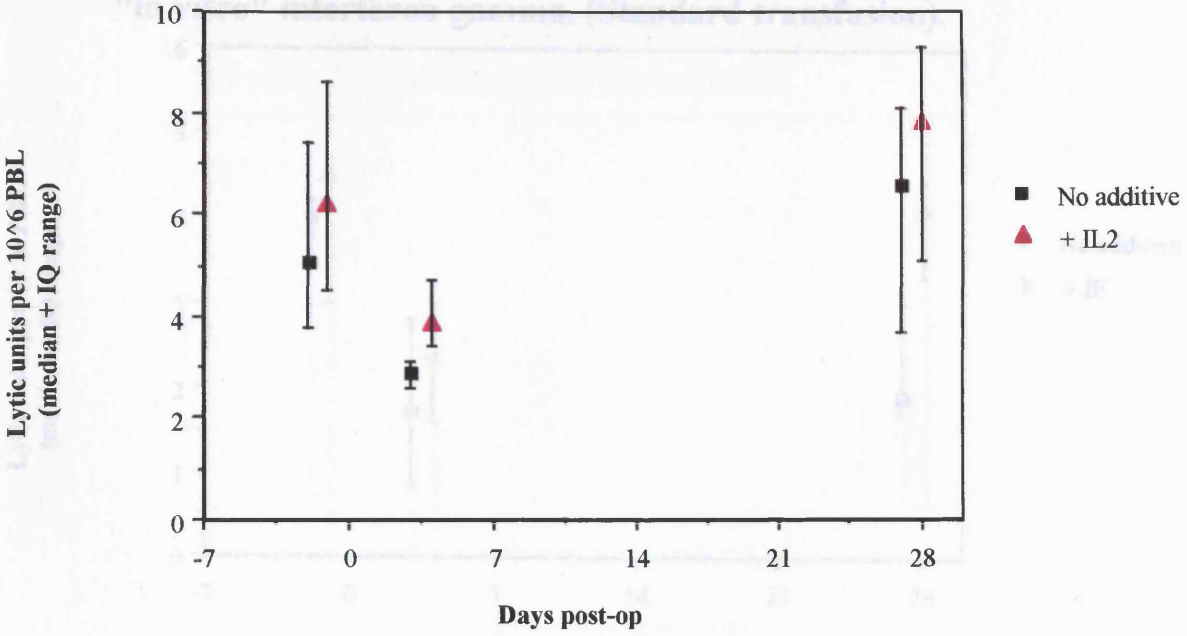
Statistical Analysis (Wilcoxon)

No additive v. plus IFNγ.

Pre-op.	**p<0.0001**
Day 5	**p<0.0001**
Day 28	**p<0.0001**

Graph 5:8

Comparison of cytotoxicity with and without "in vitro" interferon gamma. (Leucodepleted transfusion).



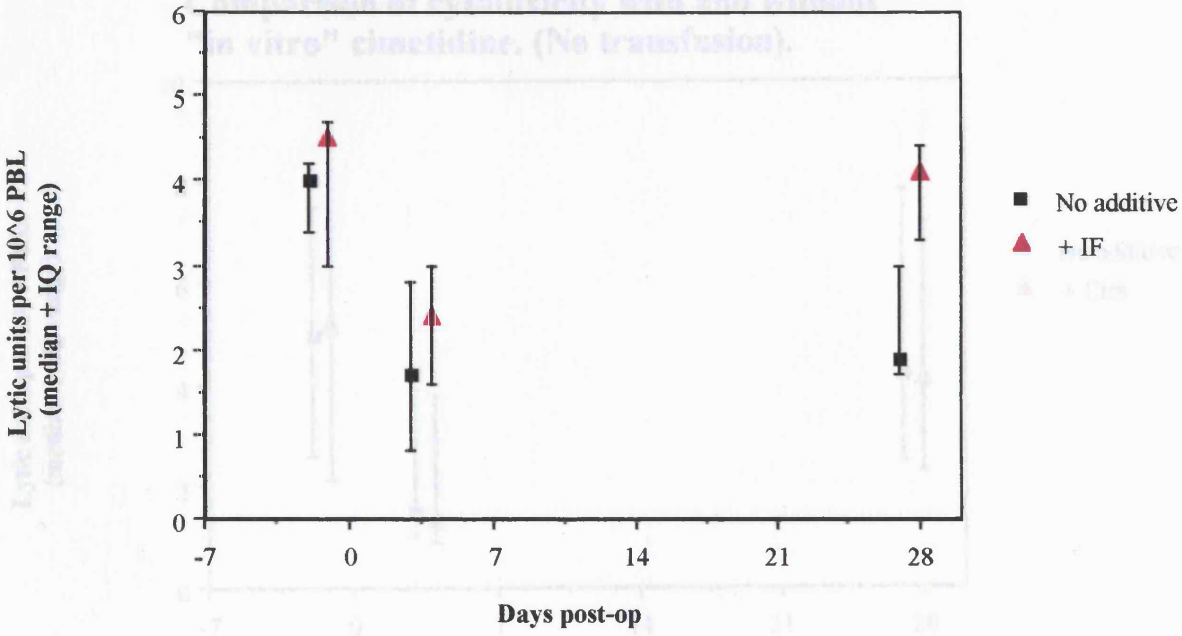
Statistical Analysis (Wilcoxon)

No additive v. plus IFNγ.

Pre-op.	p=0.13
Day 5	p=0.06
Day 28	p=0.06

Graph 5:9

Comparison of cytotoxicity with and without "in vitro" interferon gamma. (Standard transfusion).

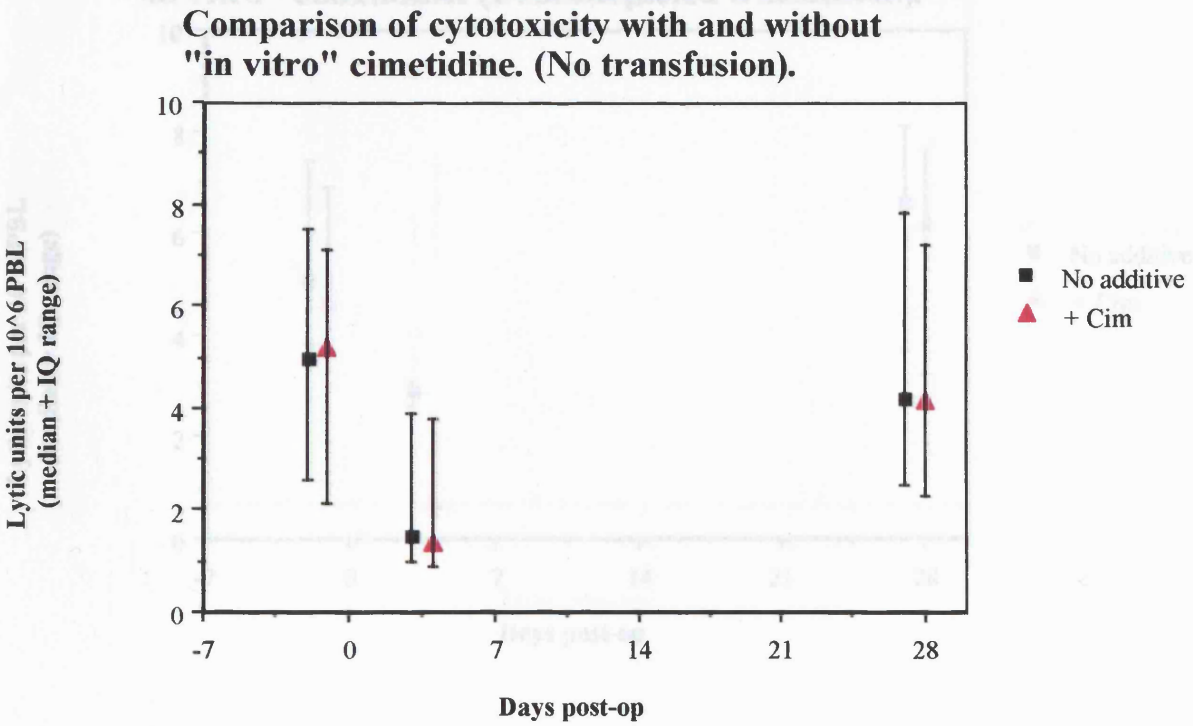


Statistical Analysis (Wilcoxon)

No additive v. plus IFNγ.

Pre-op.	p=0.30
Day 5	p=0.47
Day 28	**p=0.02**

Graph 5:10

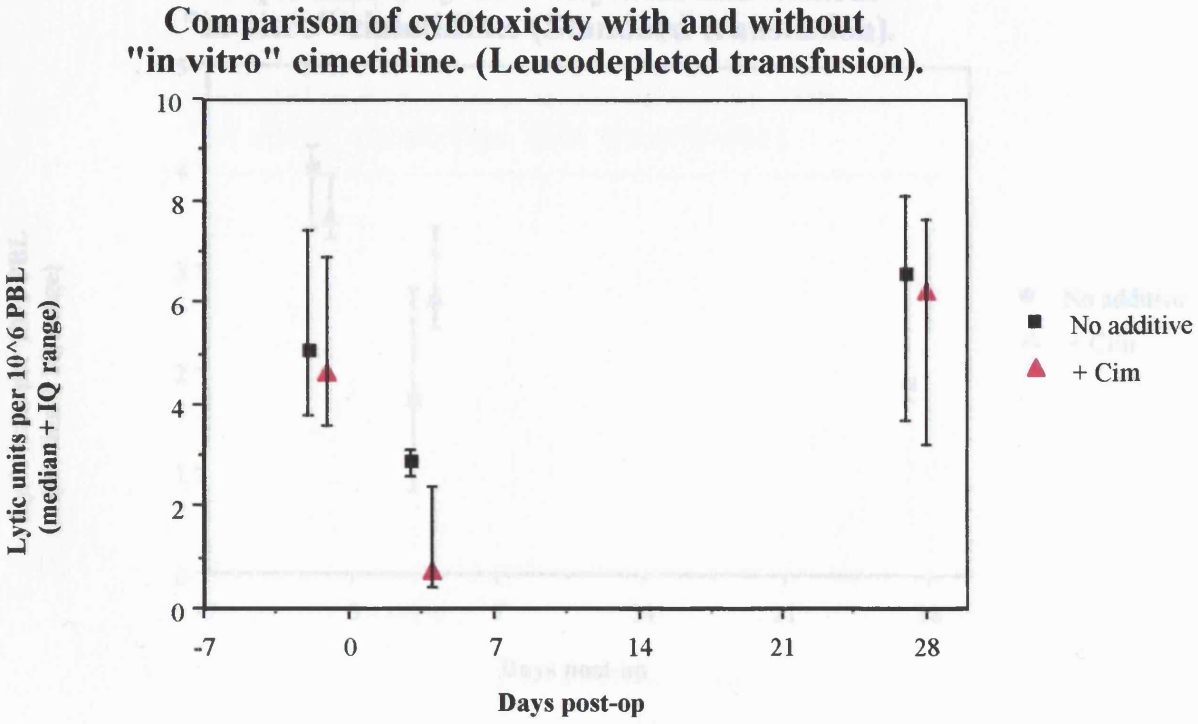


Statistical Analysis (Wilcoxon)

No additive v. plus cimetidine.

Pre-op.	**p<0.0001**
Day 5	**p<0.0001**
Day 28	**p<0.0001**

Graph 5:11



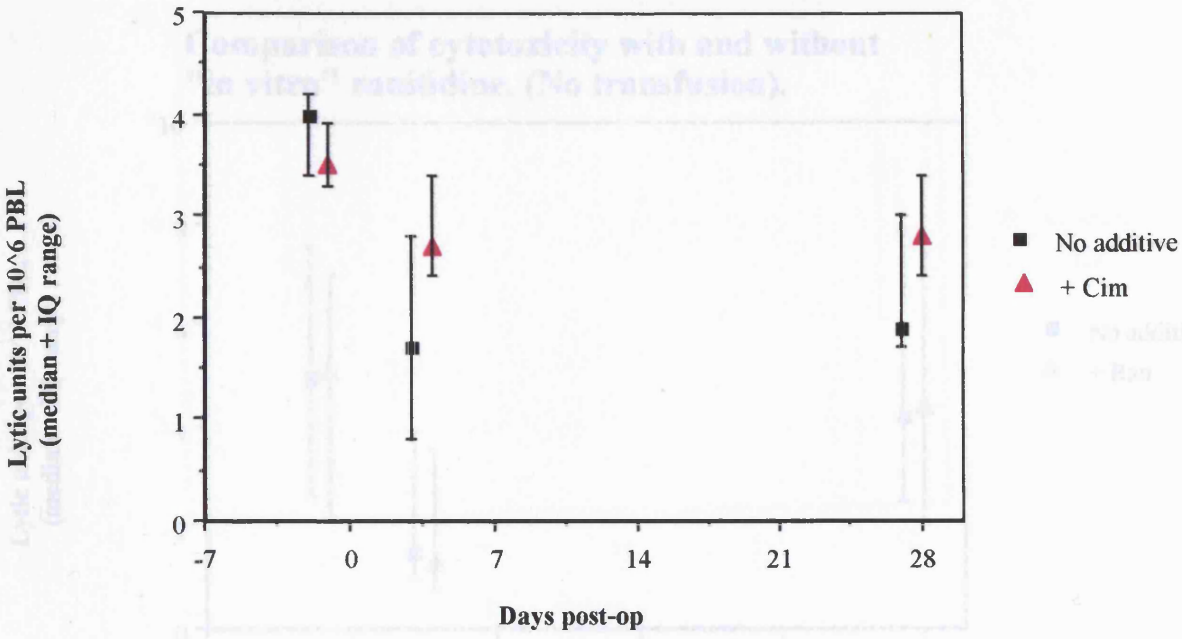
Statistical Analysis (Wilcoxon)

No additive v. plus cimetidine.

Pre-op.	p=0.81
Day 5	p=0.13
Day 28	p=0.06

Graph 5:12

Comparison of cytotoxicity with and without "in vitro" cimetidine. (Standard transfusion).

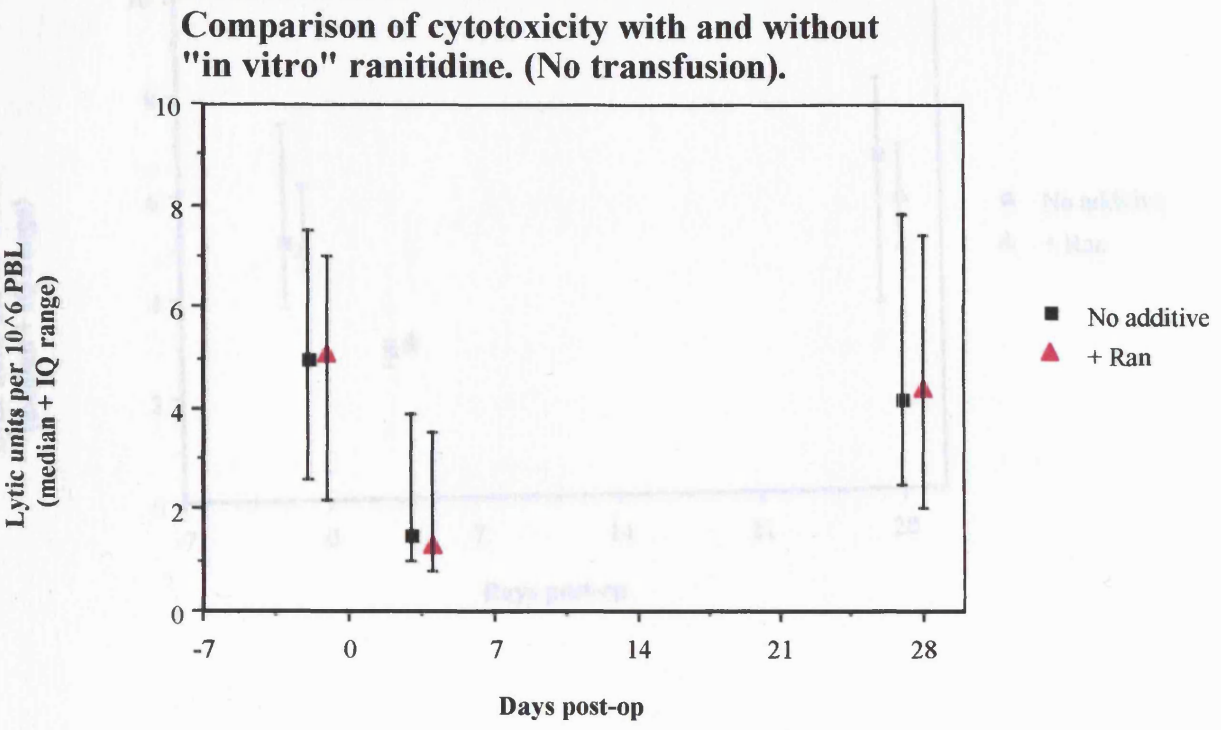


Statistical Analysis (Wilcoxon)

No additive v. plus cimetidine.

Pre-op.	p=0.11
Day 5	**p=0.02**
Day 28	**p=0.02**

Graph 5:13



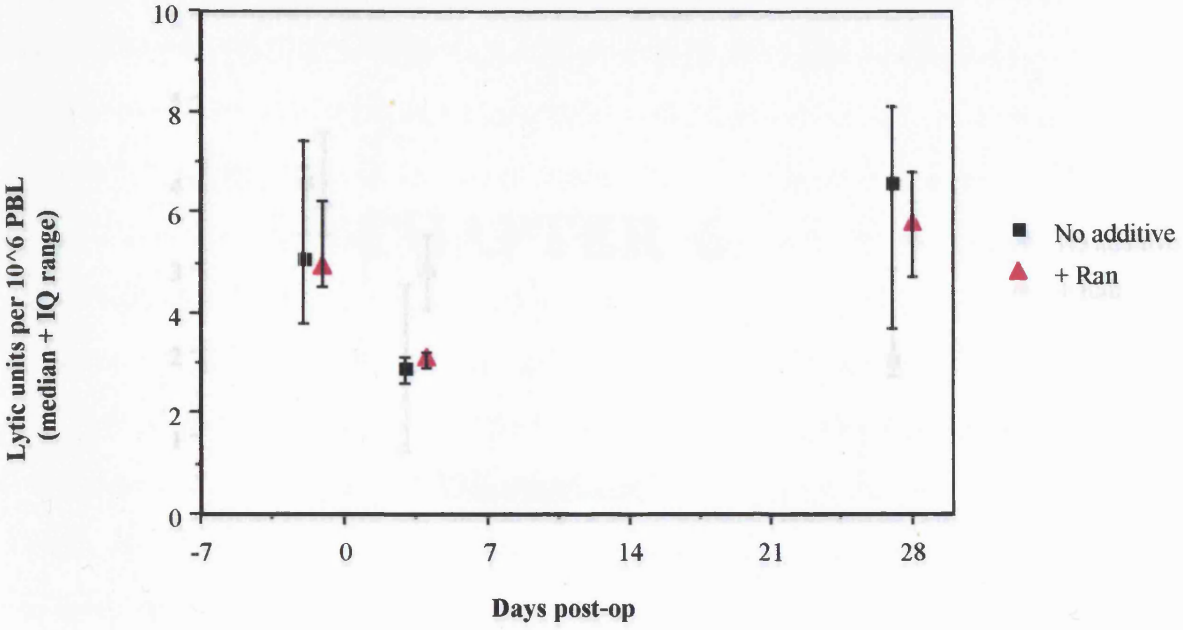
Statistical Analysis (Wilcoxon)

No additive v. plus ranitidine.

Pre-op.	**p<0.0001**
Day 5	**p<0.0001**
Day 28	**p<0.0001**

Graph 5:14

Comparison of cytotoxicity with and without "in vitro" ranitidine. (Leucodepleted transfusion).

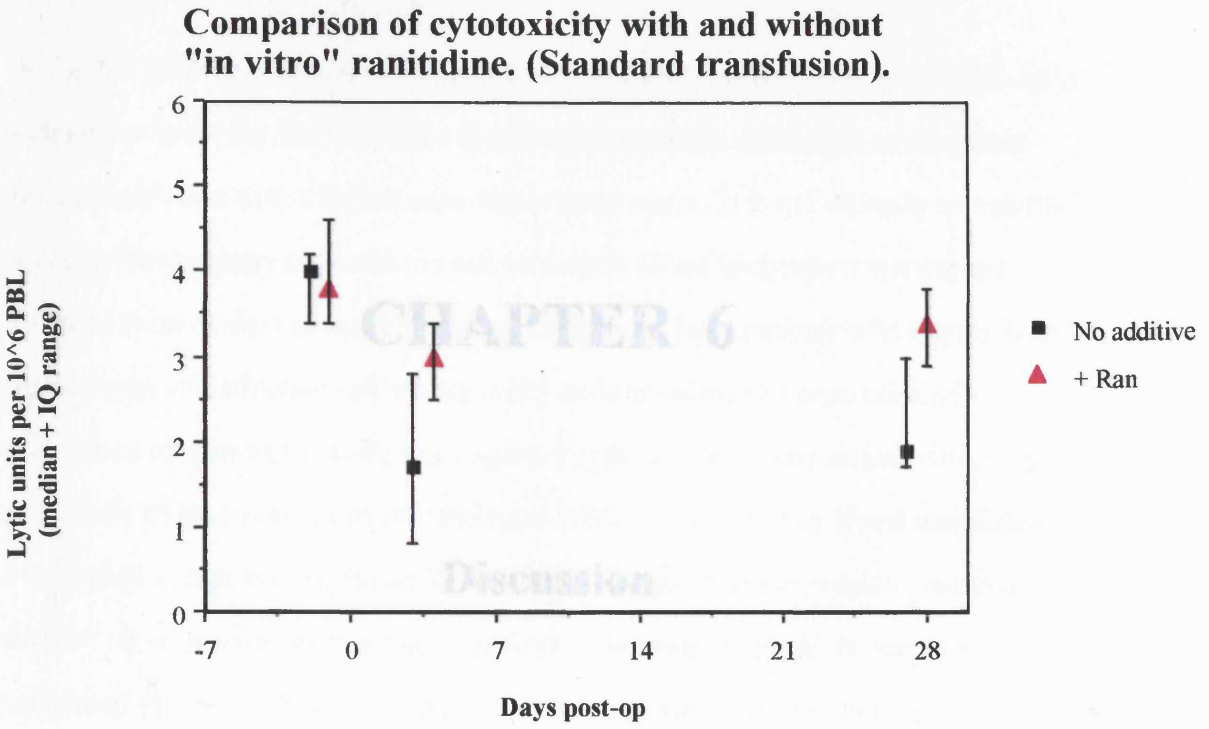


Statistical Analysis (Wilcoxon)

No additive v. plus ranitidine.

Pre-op.	p=0.44
Day 5	p=0.63
Day 28	p=1.0

Graph 5:15



Statistical Analysis (Wilcoxon)

No additive v. plus ranitidine.

Pre-op.	p=0.69
Day 5	**p=0.02**
Day 28	**p=0.02**

Graph 5:16

CHAPTER 6

Discussion

Overview.

One of the problems that has challenged workers studying the transfusion effect in humans has been the fact that there is currently no viable alternative to allogeneic blood transfusion with which comparison can be made . It is not ethically acceptable to randomise patients to receive or not to receive blood transfusion in a manner divorced from clinical need; withholding transfusion from patients who require blood replacement will adversely affect morbidity and mortality, and imposition of transfusion on patients who do not require it exposes them unnecessarily to a range of potentially serious complications (Harrison 1992) Accepting that blood transfusion is a necessary component of medical and surgical management of patients, currently without an acceptable alternative, it has only been possible to either compare transfused patients with non-transfused patients, or compare the transfusion of different types of blood product. The comparison of transfused with non-transfused patients introduces a difference between patient groups and leaves open the possibility that blood transfusion may be a 'surrogate marker' for confounding factors which influence both immune function and prognosis for cancer patients - such as extent of disease, degree of difficulty of surgery, experience of both surgeon and anaesthetist (Vamvakas 1996). The comparison of different types of blood product has not been explored in great depth, but the theory that it may be isolated components of transfused blood that are responsible for the transfusion effect allows for this type of comparison to be usefully investigated (Houbiers 1994; Dzik 1996; Jensen 1996).

The central question that this study was designed to answer was whether it was the presence of leucocytes in transfused blood that was responsible for the immunosuppressive effect induced by transfusion, and in particular for patients undergoing surgery for colorectal cancer. As further investigation of the transfusion

effect and the role of donor leucocytes therein the action of known immunomodulating agents on the three patient sub-groups was studied.

The conclusions that can be drawn from the results of these experiments are discussed here.

Patient Characteristics.

In order to make meaningful comparisons between groups of patients within a study it is important that the groups are similar in terms of their epidemiological characteristics; and as far as this study was concerned it was also important that management and pathology variables were similar too. Criticism of retrospective studies that have compared transfused with non-transfused patients has included the observation that such groups will have important differences in terms of length and difficulty of surgery, amount of blood loss (correlating with induction of hypovolaemic induced stress responses), type of operation and extent of disease (Chung 1993; Vamvakas 1993). It was therefore expected that the non-transfused patients in this study would have some differences to the transfused patients, but the anticipation was also that the standard transfusion patients would be very similar to the leucodepleted transfusion group.

The age and sex distribution (*p 3:2*) simply confirmed that colorectal cancer predominantly affects elderly people, with a slightly higher incidence in males compared to females, without there being significant differences between any of the groups studied. The fact that the majority of colonic resections (right hemicolectomy, left hemicolectomy and sigmoid colectomy) were performed without transfusion (*p3:3*) is at odds with the results stated in several of the retrospective studies into colorectal cancer prognosis and blood transfusion. The transfusion rate for patients with right sided cancers was historically quite high, in the region of 60-70%, compared with 30-40% for patients with left sided colonic cancers (Ross 1987; Tartter 1992; Sene 1993). Patients with rectal tumours were very likely in historical series to be transfused (60-90%), again a much higher rate than that reported here. Transfusion practice has changed over the years and transfusion rates have fallen dramatically over the last 10-20 years. The overall transfusion rates in the retrospective studies referred to in the Introduction ranged from 60-95%, whereas the equivalent figure for this study was just 22%. While the majority of right sided

cancers still present with anaemia current management would avoid blood transfusion unless absolutely necessary (ie. haemoglobin level less than 10g/dl), while 10-20 years ago transfusion was used far more liberally to "top up" patients prior to surgery (Hallissey 1992; Carson 1993). Greater awareness of the potential complications of transfusion are mainly responsible for this change in practice, however patients who lose significant volumes of blood during surgery still require blood transfusion which probably explains why the transfusion rate for rectal cancer operations has not fallen so dramatically.

It is recognised that rectal cancers have a worse prognosis than colonic cancers though the reasons for this remain unclear (Heald 1986). This has led authors to try to correct for possible bias by considering colonic cancers separately to those of the rectum when analysing their data (Blumberg 1985; Ota 1985; Creasy 1987), such a practice however has always been applied to retrospective studies of transfused *versus* non-transfused patients, and the relevance of separating these two tumour groups whilst not making allowance for other confounding variables has been questioned (Vamvakas 1993). In this study the transfused patients (both standard and leucodepleted) mainly had rectal cancers so comparisons between the two transfusion groups was not a problem, and the control group had an almost equal mix of rectal and colonic disease rather than a preponderance of colonic cases.

As predicted the control (non-transfused) group showed certain differences from the transfused patients in terms of shorter operations, less blood loss, and smaller fall in haemoglobin concentration (*pp 3:3-4*); but these variables have been shown in other analyses not to be linked with adverse prognosis other than a correlation with transfusion itself (vanLawick 1988; Jakobson 1990; Tartter 1992).

The fact that those patients who received filtered blood transfusions stayed in hospital for similar lengths of time as those who were not transfused is made interesting because those who had standard transfusions stayed in so much longer than either of the other groups (*p 3:5*). The slightly lower median value for the non-transfused group over the filtered group is probably explained by the number of

colonic resections in the former group who would ordinarily be expected to recover quicker than those undergoing rectal excision. No such explanation can account for the longer stay of the transfused group of patients, not even the contribution of the two "long-stay" patients who were equally distributed between the control group (45 days) and the standard transfusion group (34 days). In fact the only variable recorded in Chapter 3 that was different between the two groups of transfused patients was the type of transfusion administered, though it is impossible to give a hard reason from the available data as to why this should have made such a difference.

Immune Responses.

Cytotoxicity

The direct measurement of cellular cytotoxicity in this study was the ^{51}Cr release assay performed on peripheral blood lymphocyte solutions (*p4:5*), and it was reassuring to see that these results were very similar to those of NK cell cytotoxicity, which was derived from a combination of PBL cytotoxicity results and direct measurements of NK cell numbers present in the PBL solutions (*pp 4:5-6*). By implication the values of cytotoxicity from the PBL solution assays can be taken as an accurate reflection of isolated NK cell cytotoxicity.

The initial post-operative fall in cytotoxicity that was observed in patients here has been previously described and appears to be part of the general immunosuppressive response to surgical stress, appearing as soon as 18 hours after surgery (Pollock 1984; Jensen 1992). Work from Denmark on patients undergoing colorectal resections (not necessarily for malignant disease) had suggested that NK cell activity in the patients would be affected less by surgery alone than by the combination of surgery and transfusion together (Jensen 1992) . The results described in Chapter 4 of this thesis clearly show that surgery by itself is a potent suppressor of NK cell cytotoxicity, and the immunosuppressive effect of blood transfusion (standard or leucodepleted) would seem to be no more potent than, and certainly not cumulative with, that of surgery. However, the suppression of cytotoxicity induced by surgery alone had essentially resolved by 28 days after the operation, whereas the suppressive effect was still evident at this stage in those patients transfused with standard blood. The removal of leucocytes from blood for transfusion appeared in this study to remove the effect on NK cell activity that would otherwise have resulted from receiving a transfusion.

The possibility that the stimulus for transfusion related immunosuppression may be the occurrence of hypovolaemia resulting from haemorrhage produced by trauma has been postulated (Cue 1992) This theory supports the idea of transfusion

being a surrogate marker for another process that would explain the "transfusion effect" as such a haemorrhage would invariably provoke transfusion of blood from involved clinicians, but the evidence of this being true is weak. Transfusion of blood in patients who have not suffered from haemorrhagic hypovolaemia causes immunosuppression in its own right (Blumberg 1994) and those patients transfused with leucodepleted blood in this study, who lost the same amount of blood as those who received standard transfusions, showed little difference in immune function to those patients who lost significantly less blood and consequently were not transfused at all. Haemorrhage, hypovolaemia, and shock may well institute an immunosuppressive mechanism in the same manner that surgical "stress" does, but so does blood transfusion by itself, and the former is not the explanation for the latter.

The suppressive effect on NK cell activity of surgical stress has been studied in animals and humans and most workers have shown a decrease in activity within 24 hours of surgery, returning to pre-operative levels over the following 7 - 10 days (Lukomska 1983; Miyazaki 1983; Pollock 1984; Pollock 1992). The isolated effect of transfusion on NK cell activity has been less well documented in humans but in animals a suppressive effect can be shown (Clarke 1993. The duration of NK cell suppression after transfusion has also not been studied extensively, though patients with a history of blood transfusion during their lifetime have decreased levels of activity compared to those who have never been transfused (Gascon 1984; Kaplan 1984; Mathiasen 1994), and transfusion of whole blood in combination with surgery has resulted in suppressed activity over a similar time scale as reported here (Jensen 1992).

The number of NK cells present in the body is another factor which obviously affects the cytotoxic potential of an individual. Most studies into NK cell activity measure activity in standard concentration lymphocyte preparations, as described in Chapter 2, and although this extrapolates to individual NK cell activity it makes no allowance for overall NK cell numbers. Methods to measure NK activity in whole blood samples and thus allow for variations in cell numbers have been described

(Nielsen 1989; Jensen 1992), but have not been accepted in preference to lymphocyte solution assays.

The difference in NK cell numbers pre-operatively between the standard transfusion group and the other two groups of patients (*pp 4:5-6*) is difficult to explain, especially as there was so little difference between the patients' characteristics. In particular the only pre-operative difference between the two groups of patients who were transfused was their random allocation to receive either standard or leucodepleted transfusions. It is highly unlikely that a problem with collection or counting of the cells could have resulted in such a difference as the individual patients were spaced throughout the time span of the study, and consequently were not associated with any particular batch of reagents, or with any particular time period that might question the reliability of data collection. What seems more likely is that with the small numbers involved a sampling error resulted in the 9 patients in question having NK cell numbers from the upper end of the range of values of the study population.

Such a difference in pre-operative NK cell numbers rendered direct comparison between the groups meaningless, but the sustained decrease in numbers seen in the standard transfusion patients was an important trend which mirrored the changes in cytotoxic activity. Major surgery is known to result in a fall in NK cell numbers (Lennard 1985), but most studies into NK cell function in relation to surgery and/or blood transfusion concentrate on cytotoxic activity alone without reference to cell numbers (Lukomska 1983; Miyazaki 1983; Gascon 1984; Kaplan 1984; Pollock 1984; Pollock 1992; Clarke 1993). NK cells are produced from precursor cells in the bone marrow and then differentiate in the peripheral blood to a resting state of maturity, distributed between the blood and most bodily organs (Trinchieri 1989; Lewis 1992). Although they are sequested from the blood to areas where they may be of use (Moy 1985; Tsujihashi 1988; Kernohan 1990; Gudmundsdottir 1992; Winnock 1993) the numbers present in peripheral blood are an important monitor of availability as: (a) the elimination of potentially metastatic, circulating tumour cells will rely on

both blood and tissue NK cells, (b) NK cells are relatively short-lived and low peripheral blood concentrations will imply low tissue concentrations too.

As with cytotoxic function it would appear that both surgery and transfusion of standard blood have separate effects on the number of NK cells, but the long term decrease is due to transfusion rather than surgery.

It must be remembered that the data for whole blood cellular cytotoxicity presented from this study was derived from the measurements of NK cell numbers and NK cell cytotoxicity rather than being a direct measure of *in vivo* cytotoxicity. This type of data presentation has not previously been described and though it theoretically provides an interesting estimation of the available "killing power" within a patient's blood it's interpretation must be cautious. In particular with the results shown in Chapter 4 the fact that NK cell numbers pre-operatively were so disparate must mean that the contribution of this data set to overall understanding of the results is limited. It is probably more useful to consider the directly measured *in vitro* results separately as described above.

Lymphocyte subsets

The initial post-operative fall in numbers of T-lymphocytes (CD2, and CD3 subsets) generally was mirrored in all the other lymphocyte subsets tested for here, and though the results for CD4 and CD8 subsets were not remarkable by themselves the prolonged suppression of the CD4/CD8 ratio in the standard transfusion group was. The effects of surgery and blood transfusion on lymphocyte numbers have been investigated previously, both in isolation and combined. Surgery alone evokes a response the degree of which is dependant on the severity of surgical stress imparted to the subject (Lennard 1985). Minor surgery results in an overall decrease in lymphocyte numbers, particularly noticeable amongst the T-helper cells (CD4), while major surgery produces a more marked overall lowering of numbers, affecting T-lymphocytes in particular as well as NK cells.

The response of T-helper (CD4) and T-suppressor (CD8) cells to surgery alone appears to be an initial fall in T-helper numbers recovering over the first month, combined with relatively little change in T-suppressor numbers over the same time period (Lennard 1985; Fernandez 1992). If surgery is combined with perioperative transfusion of blood differing responses have been noted amongst these two cell types: either a more marked fall in T-helper numbers with little difference in T-suppressor cells (Fernandez 1992) or a relative increase in T-suppressor numbers without any marked change in T-helper response (Jensen 1996). Though there is some discrepancy between these reported results the derived CD4/CD8 ratio is similar in both cases, showing an initial decrease post-operatively which is sustained in comparison to subjects who only underwent surgery.

The isolated effect of blood transfusion on lymphocyte populations has been studied in patients who have previously received multiple transfusions as part of the management of a haematological disorder, and in patients presenting with malignant disease who have been transfused at some time in their life for a variety of reasons (Gascon 1984; Kaplan 1984; Mathiasen 1994). The studies into multiply transfused haematological patients highlighted changes in both CD4/CD8 ratio and NK cell activity as markers of immune function that have been used in monitoring patients with acquired immunodeficiency syndrome. The CD4/CD8 ratio was decreased in sickle cell patients receiving multiple transfusions in one study where transfusion was occurring every month, but not significantly lowered in another where transfusion was occurring less frequently. Interestingly the NK cell activity in both cases was significantly lowered in transfused patients. The study looking at patients with a transfusion history who presented with colorectal cancer found no differences in a wide range of T-lymphocyte subsets between those who had never been transfused, those transfused greater than 30 days prior to sampling, and those transfused within that 30 day period. In all these studies there was no opportunity to consider pre-transfusion lymphocyte numbers and though absolute numbers are important,

individuals obviously express a range of normal values, and change from such normality may be as important as consideration of a single measurement.

The literature is not clear as to how much of an influence blood transfusion has on lymphocyte populations, in contrast to the recognised effect of surgery. Certainly the results presented in Chapter 4 (*4:8-11*) would suggest that the main factor affecting the lymphocyte subsets in this study was surgery with little or no influence from blood transfusion (standard or leucodepleted), except in the case of the CD4/CD8 ratio. This appeared to respond to standard blood transfusion in a similar manner to NK cell numbers and activity, a manner that was not apparent if the blood transfusion had been leucodepleted.

Manipulation of the Immune Response.

The *in-vitro* addition of IL2, IFNγ, cimetidine, and ranitidine did little to alter the overall pattern of post-operative/post-transfusion immunomodulation observed amongst the three patient groups (*pp6:4-6*). They did, however, cause changes in NK cell activity compared to the unadulterated assays which were of interest when considering the underlying mechanisms involved.

IL2 is a powerful upregulator of many immune functions, not least NK cell activity. The stimulation of cellular cytotoxicity that results from exposure to IL2 includes a direct increase in NK cell cytotoxicity that begins within minutes of interaction with IL2, as well as induction of so-called Lymphokine Activated Killer (LAK) cells (Trinchieri 1989; Lewis 1992; Naume 1994). LAK cells are mononuclear cells derived from IL2 stimulation of resting NK cells in peripheral blood, and are capable of antibody dependant cellular cytotoxicity against tumour cells resistant to NK cell cytotoxicity. Their function is not considered part of NK cell activity, it takes between 6 to 18 hours of IL2 exposure *in-vitro* to induce them, and the K562 cell line is not especially sensitive to their cytotoxic activity; they are therefore not of relevance to this particular study.

Surgical stress results in decreased production of IL2 which persists for up to 9 days post-operatively, and blood transfusion has a similar effect which is still active 16 days after transfusion in mice (Akiyoshi 1985; Wood 1988; Baxevanis 1994). Theoretically then replacement of depleted IL2 could help to counter such an effect. IL2 has been used both therapeutically and experimentally to try and increase host immune response peri-operatively and as adjunctive treatment in patients with malignant disease. Peri-operative treatment with IL2 of patients undergoing surgery for colorectal cancer causes variable increases in cellular cytotoxicity dependant on dosage. Low-dose regimes abolish the post-operative dip in NK cell activity (Nichols 1992; Nichols 1993), while higher doses boost activity to above the base-line (Deehan 1995).

By adding IL2 *in-vitro* , as presented in Chapter 6, it was possible to boost the NK cytotoxicity some 3 - 5 times above the normal level of activity, but the fact that the degree of post-operative/post-transfusion suppression was unaltered by this technique indicates that the underlying mechanism is more complex than deficiency of IL2 during the cytotoxic process. The *in-vivo* techniques referred to above suggest that higher levels of IL2 present at the time of an insult to the immune system (as produced by an operation) protect the host from immunosuppressive effects, presumably by alteration and upregulation of lymphocyte subsets. Unfortunately the effect of this type of therapy has not been investigated with respect to blood transfusion.

IFNγ causes enhanced NK cell activity, as do all interferons, but there has been some suggestion that IFNγ may also act to protect certain tumour cells from NK cytotoxicity by altering Class I MHC expression, and this may explain why IFNγ is a less active stimulator of NK cells than IFNα and IFNβ (Trinchieri 1989; Ferrat 1990; Lewis 1992). IFNγ has been tried as a prophylactic agent to protect trauma and burns patients from sepsis (Polk 1992), and it seemed reasonable to see if it should protect against the transfusion effect. As the results of this study have shown IFNγ had an effect on NK activity not dissimilar to IL2, though the degree of upregulation was much smaller and hardly significant. It was interesting though to see that the prolonged immunosuppressive effect of standard blood transfusion was effectively abolished by *in-vitro* IFNγ treatment, suggesting (a) a different mechanism to surgery-induced down-regulation, and (b) the possibility that cell receptor up-regulation plays a part in the mechanism. The theory of active suppression described in Chapter 1 refers to subgroups of T-helper cells which are mutually inhibitory, the Th1 cells being stimulated by IFNγ (amongst others) and causing suppression of Th2 cells which in turn appear to play a part in the transfusion effect (Brunson 1990; Dzik 1996). Active suppression would seem to require several days to develop to full effect which would explain why IFNγ only had a noticeable result at Day 28 post-operatively.

Histamine can act as an immune stimulant via H_1 (histamine type-1) receptors, but can also suppress immune function via H_2 receptors at concentrations higher than the physiological norm (Beer 1987). Powerful H_2 receptor antagonists have been available for many years now, developed for use in peptic ulcer disease to combat the gastric acid stimulatory effect of H_2 receptors. Cimetidine and ranitidine are the two best known examples of such agents, and both have been investigated regarding possible immunostimulatory effects in relation to cancer. Cimetidine has been reported to increase survival in colonic and gastric cancer patients, and to inhibit colonic cancer growth both *in-vitro* and *in-vivo* (Tonnesen 1988; Adams 1994; Adams 1994). Ranitidine has shown less encouraging results with gastric cancer survival (Wotherspoon 1997), and only minimal stimulation of NK activity in patients with metastatic colorectal cancer (Nielsen 1995). However it does appear to prevent the reduction of cell-mediated immunity seen post-operatively in patients who have received blood transfusions (Nielsen 1989), and has been reported to abrogate the *in-vitro* stimulation of malignant cell growth induced by blood transfusion (Browell 1993).

The results presented in Chapter 5 show both cimetidine and ranitidine producing significant improvement in post-operative NK cell activity for those patients who were transfused with standard blood, supporting the role of the H_2 receptor in the transfusion effect. Both of these agents had no effect on NK cells from the other two groups of patients, suggesting that the H2 receptor blockade is only of value in transfusion induced rather than surgery induced immunosuppression.

Problems with the study.

This project suffered from several problems, most of which related in one way or another to patient numbers. The original plan for the trial made two important assumptions (based on other studies) which were to be highly relevant to the final results; firstly that a transfusion rate of 40% was to be expected within the Leicester region for this type of surgery, and secondly that the main differences in cytotoxicity would be observed at Day 5 post-operatively and would be of the order of 40%. The planning of the study is described in more detail in Appendix V (*pp App:11-14*). The observed transfusion rate over the course of the trial was only 22%, which obviously resulted in a dramatic lowering of transfused patients to study. The reason for this was almost certainly changes in transfusion practices by local medical staff, which in turn has probably been brought about by the relatively high profile given to complications of blood transfusion over the last 5 - 10 years. It is highly unlikely that the trial itself was responsible for the low transfusion rate as the clinical staff involved agreed in principle to the randomisation of their patients several months prior to the trial commencing, and were never informed specifically that certain patients were included. If blood for transfusion was requested for patients who had been randomised to receive leucodepleted blood the bags would arrive in the normal manner from the blood bank, and would be used in the normal manner. The only distinguishing feature about the leucodepleted blood was a slightly different label on the bag.

It did not prove possible to repeat the results of the study from Denmark which had been used to help plan the trial (Jensen 1992), in particular the results during the first post-operative week did not show the large differences between the leucodepleted and standard transfusion groups observed by Jensen. It is possible that such a discrepancy may relate to the different blood products used; the Danish work compared whole blood transfusions with blood filtered through bedside filtration devices, the study presented here utilised SAGM buffy-coat depleted blood compared

with laboratory filtered SAGM units of blood, with consequent differences in the leucocyte load presented to the recipient. A later study published by the same main workers from Denmark still used whole blood rather than buffy-coat depleted products, and returned a transfusion rate of 57% (Jensen 1996).

Data collected from the assays was far more variable than initially anticipated and though the difference between the study groups were of the magnitude anticipated for the original plan the standard deviation of the data points made the likelihood of statistically significant results less likely. Recalculation of the minimum sample size as described in Appendix V after 18 months required a difference in means of 3.0 and a Standard Deviation of 2.7 to be used as the data for the Instat calculator. These figures produced a minimum sample size of 23 if a power of 95% ($\beta = 0.05$) was to be expected; however if a lower power of 80% ($\beta = 0.2$) was accepted as being reasonable for a clinical trial then a sample size of 14 would have been sufficient. Although this figure compared with the original estimate, when combined with the lower transfusion rate the overall number of patients required increased to at least 130. However, with the much larger than predicted standard deviation of the data it was doubtful that increasing the size of the study would have produced more meaningful results, so the trial was completed as originally planned and analysed as presented.

Summary

Overall this study has demonstrated a difference in post-operative immune function between patients who were transfused peri-operatively with either standard, SAGM buffy-coat depleted blood, or blood filtered through high-efficiency leucodepletion filters. The only real clinical difference between these two groups of patients was the number of allogeneic leucocytes infused into them as part of their blood transfusions, otherwise they were well matched. Although the differences in immune function did not always reach convincing levels of statistical significance there were clear trends in the data sets which, particularly if repeated with more patients, should serve to implicate the donor leucocyte as a major factor in the production of the so-called "transfusion effect". To emphasise that point it should be noted that the patients transfused with leucodepleted blood behaved in a similar manner to those who were not transfused at all, though comparison between these two groups is slightly more difficult.

Attempts to ameliorate the transfusion effect with known immunomodulators has shown that while IL2 is a powerful up-regulator of the immune system its effects are not specific for the defects caused by blood transfusion. On the contrary IFNγ would appear to be active against the longer term (> 1 week) immunosuppressive effect of transfusion, supporting one of the proposed theories behind the effect. The H_2 receptor antagonists cimetidine and ranitidine both appear equally effective at reversing the immunosuppressive effect, and seem specific for transfusion immunomodulation rather than that produced by surgery.

Implications for the future.

Though the results of this study are interesting from a laboratory point of view the important question is whether there is any clinical benefit to be gained from it. Certainly the techniques described here provide a method to try to answer the question of whether or not blood transfusion is of poor prognostic significance to cancer patients. A prospective, controlled, randomised, multi-centre trial of standard transfusion versus laboratory leucodepleted transfusion conducted over several years should be able to tell if transfused patients die sooner than those given leucodepleted blood or not transfused at all.

Whether such a trial will ever take place is another matter. Already the transfusion rate is falling (as witnessed here) increasing the workload necessary for a significant result, and transfusion practices are starting to change, investigating pre-deposit autologous transfusion and the use of recombinant erythropoietin to avoid allogeneic transfusion completely. Until such time as a synthetic, or even recombinant, alternative to blood transfusion is available there will still be requirement for the provision of allogeneic blood, but it is interesting that the evidence accumulating in favour of leucodepletion is prompting much wider use of blood filter technology in Western Europe and the USA. The only real disadvantage to providing leucodepleted blood as a routine is the extra cost involved, both in materials and man-power, but greater demand would reduce these to an affordable minimum, and probably prevent the question posed above ever being answered.

APPENDIX

Appendix

APPENDIX I - LEUCODEPLETION VALIDATION

An essential part of the trial was to be certain that when leucodepleted blood was transfused into a patient it had in fact been adequately leucodepleted. For this study leucodepletion was performed under sterile conditions in the main blood bank by one of three trained personnel (Miss Mandy Gardner, Miss Deborah James and Mr Nick Nolan) to ensure reproducible results. Prior to starting the study representatives from the Pall Scientific and Laboratory Services visited the blood bank to validate the leucodepletion process, and a further validation was performed halfway through the study. The results of the validation are presented here.

Validation 1 : 25th August 1994.

Eight units of SAG-M (Saline, Adenine, Glucose - Mannitol) red cell concentrate were filtered during this validation. The filtration was performed as described in Chapter 2 Section 2, samples of blood were taken from the units before and after filtration and the units were weighed after filtration to calculate final volume. Manual white cell counts were performed on the blood samples using Neubauer counting chambers for the pre-filtration samples and Nageotte chambers for the post-filtration samples (see Chapter 2 Section 4). The results are shown in Table (i).

Validation 2 : 24th May 1995.

10 units of SAG-M red cell concentrate were filtered on this occasion. The procedure was as on 25/8/94 except no pre-filtration samples were taken. The results are shown in Table (ii).

Unit No.	Pre-filter WCC $x10^9/l$	Pre-filter WCC per unit	Post-filter WCC per µl	Post-filter unit volume (ml)	Post-filter WCC per unit
380898DX	4.72	-	<0.5	259	$<1.29 \times 10^5$
380913D7	7.88	-	<0.5	253	$<1.26 \times 10^5$
384508D7	5.92	1.93×10^9	0.5	296	1.48×10^5
376959D3	9.47	2.94×10^9	<0.5	280	$<1.40 \times 10^5$
382431D4	3.14	0.94×10^9	<0.5	268	$<1.34 \times 10^5$
945805D0	3.69	1.24×10^9	39.6	296	1.17×10^7
375205D4	1.79	0.58×10^9	1.1	301	3.31×10^5
380565D4	7.41	2.22×10^9	<0.5	272	$<1.36 \times 10^5$

Table (i) : Validation data from 25/8/94

Unit No.	Post-filter WCC per µl	Post-filter unit volume (ml)	Post-filter WCC per unit
969216D9	<0.5	291	$<1.45 \times 10^5$
620649D2	2.0	292	5.84×10^5
618377D8	<0.5	292	$<1.46 \times 10^5$
967153D6	<0.5	282	$<1.41 \times 10^5$
620376D0	<0.5	310	$<1.55 \times 10^5$
619586D5	0.7	263	1.84×10^5
968462DX	<0.5	307	$<1.53 \times 10^5$
967520D5	<0.5	313	$<1.56 \times 10^5$
627354D8	0.8	284	2.27×10^5
623489D5	<0.5	310	$<1.55 \times 10^5$

Table (ii) : Validation data from 24/5/95.

When less than 5 cells were counted in the Nageotte chamber this was considered below the sensitivity of the counting method which is 0.5 leucocytes/µl. For each unit the residual leucocyte load after filtration was calculated from the post-filtration unit volume and the post-filtration white cell count. At each validation the average leucocyte load per unit was calculated as an indication as to the reliable level of leucodepletion that could be expected using that particular filtration method.

The initial validation on 25/8/94 produced an average final leucocyte content of:

- less than or equal to 1.63×10^5 per unit.

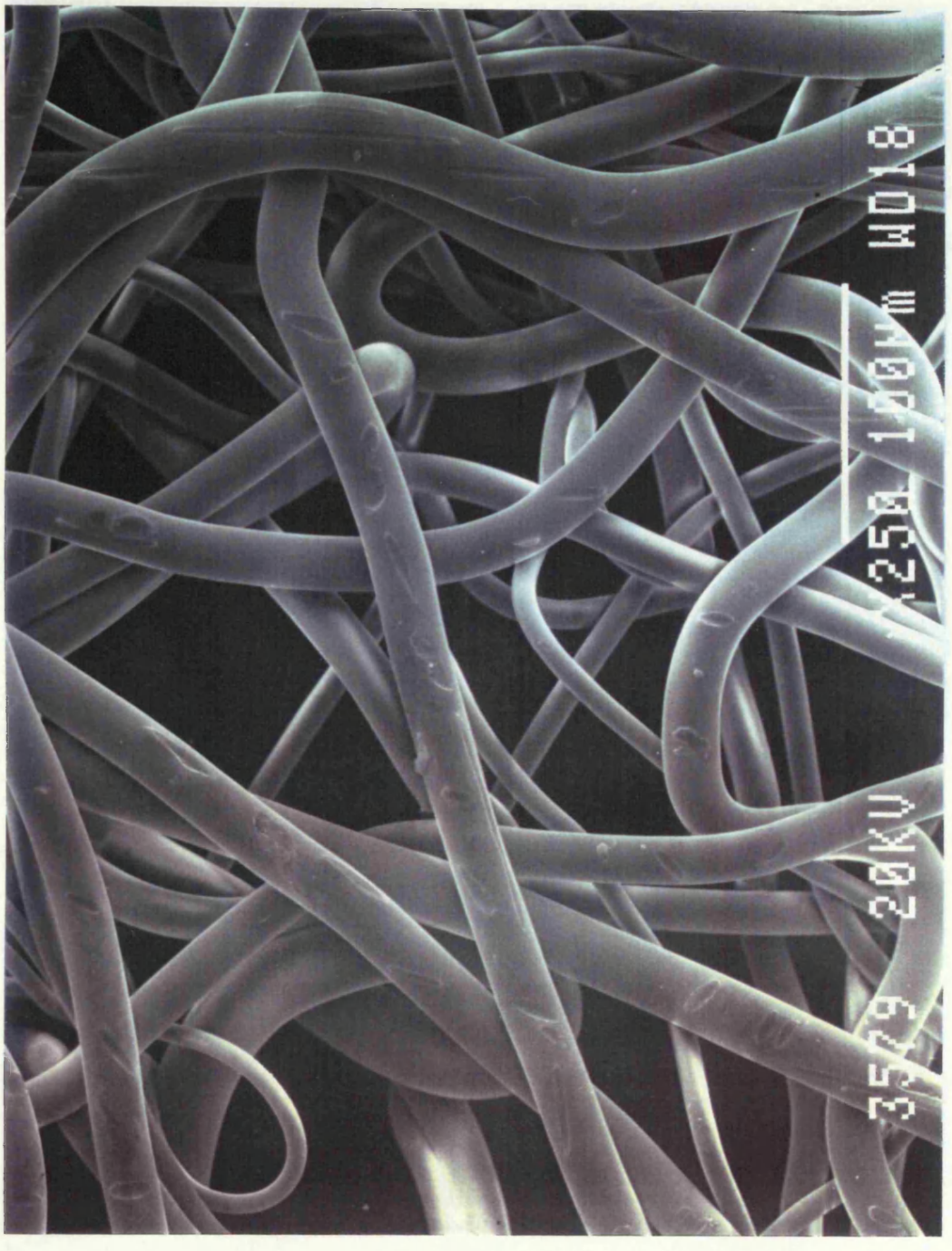
The second validation on 24/5/95 produced an average final leucocyte content of:

- less than or equal to 2.05×10^5 per unit.

These validation results showed that the filtration system was producing a reproducible log4 decrease in leucocyte content (log4 = 10,000 x).

(Of the 8 units validated on 24/8/94 one unit was difficult to filter (unit no. 945805D0), being hard to prime, slow to filter (27 minutes total, three times the average), and ceasing filtration before the whole unit had passed through. This could have been a problem either with the filter or with the blood, however it was an unsuccessful filtration and as such would have been discarded if for clinical use. The filter performed a log2 reduction in leucocytes (1.24×10^9 to 1.17×10^7 per unit), but these results were been excluded from further analysis.)

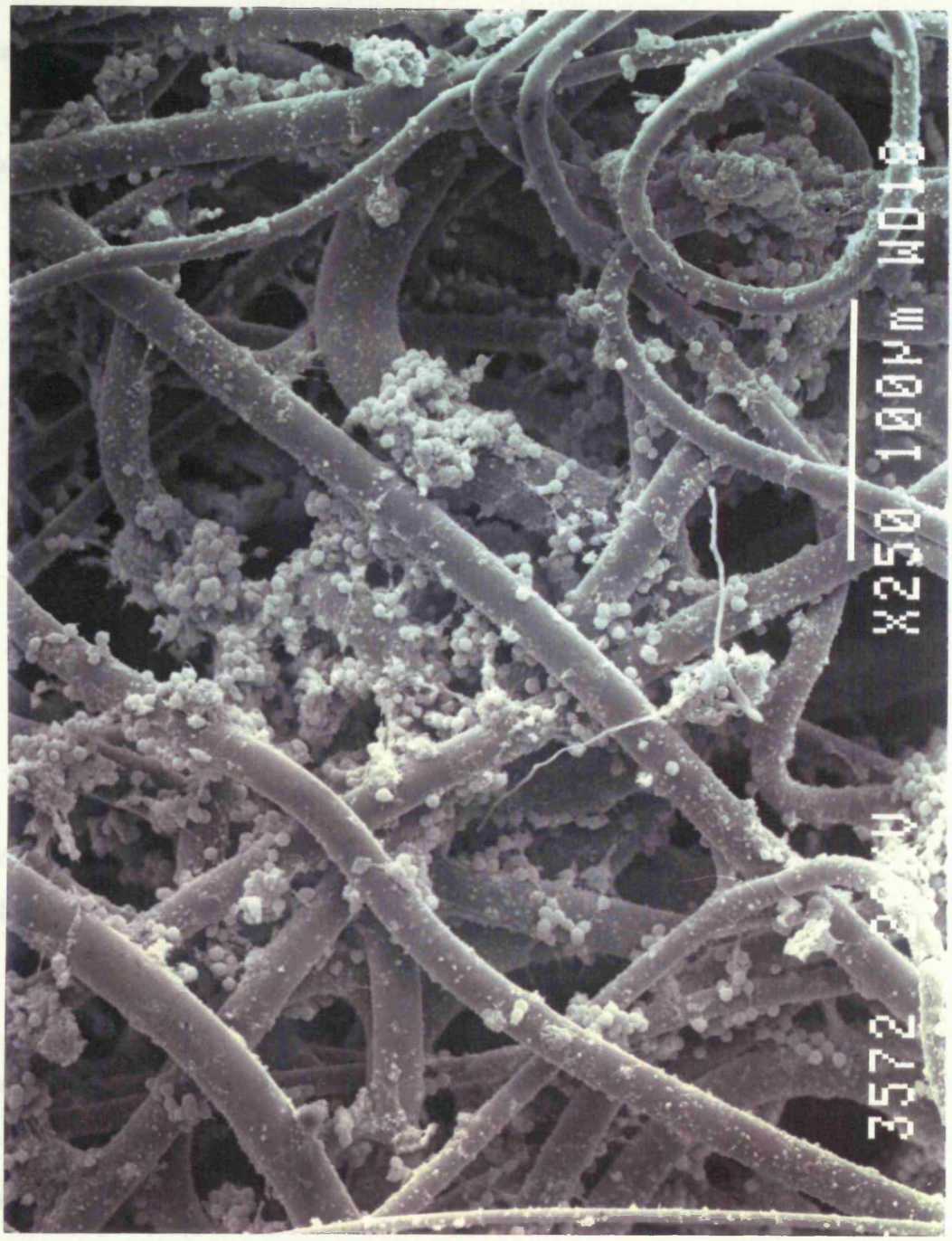
Plate 4



Fibres of a high efficiency leucocyte depleting filter : pre-filtration (x250)

(Scanning electron microscopy, courtesy of Pall)

Plate 5



Fibres of a high efficiency leucocyte depleting filter : post-filtration (x250)

(Scanning electron microscopy, courtesy of Pall)

APPENDIX II - SOLUTIONS

Hanks Buffered Salt Solution (HBSS).

Each 1000 ml contains:

	grams/litre
Calcium chloride	0.185
Magnesium sulphate	0.09767
Potassium chloride	0.4
Potassium phosphate	0.06
Sodium chloride	8.0
Sodium phosphate	0.04788
D-glucose	1.0
Phenol red sodium salt	0.01

(Sigma Chemical Company Ltd.)

In addition, each 1000ml contains 10ml of 1M HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pKa=7.55 at 25°C (Sigma Chemical Company Ltd.).

Roswell Park Memorial Institute - 1640 medium (RPMI).

Each 1000ml contains:

	grams/litre
Calcium nitrate-$4H_20$	0.1
Magnesium sulphate	0.04884
Potassium chloride	0.4
Sodium bicarbonate	2.0

Sodium chloride	6.0
Sodium phosphate dibasic	0.8
L-Arginine	0.2
L-Asparagine	0.05
L-Aspartic acid	0.02
L-Cystine.2HCl	0.0652
L-Glutamic acid	0.02
L-Glutamine	0.3
Glycine	0.01
L-Histidine	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine.HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine.2Na.2H_20	0.02883
L-Valine	0.02
D-Biotin	0.0002
Choline chloride	0.003
Folic acid	0.001
Myo-inositol	0.035
Niacinamide	0.001
p-Amino benzoic acid	0.001
D-Pantothenic acid	0.00025

Pyridoxine.HCl	0.001
Riboflavin	0.0002
Thiamine.HCl	0.001
Vitamin B12	0.000005
D-Glucose	2.0
Glutathione	0.001
Phenol red sodium salt	0.0053

(Sigma Chemical Company Ltd.)

In addition, each 1000ml contains 10ml of 1M HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pKa=7.55 at 25°C (Sigma Chemical Company Ltd.).

APPENDIX III - SUPPLIERS

Amersham International plc.

Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, ENGLAND.

Becton Dickinson.

2350 Qume Drive, San Jose, California, 95131-1812, USA.

Corning Costar Corporation.

One Alewife Center, Cambridge, ENGLAND.

Gibco (Life Technologies Ltd).

PO Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF, SCOTLAND.

Imperial Laboratories Ltd.

West Portway, Andover, Hampshire, SP10 3LF, ENGLAND.

Pall Biomedical.

Europa House, Havant Street, Portsmouth, Hampshire, PO1 3PD,

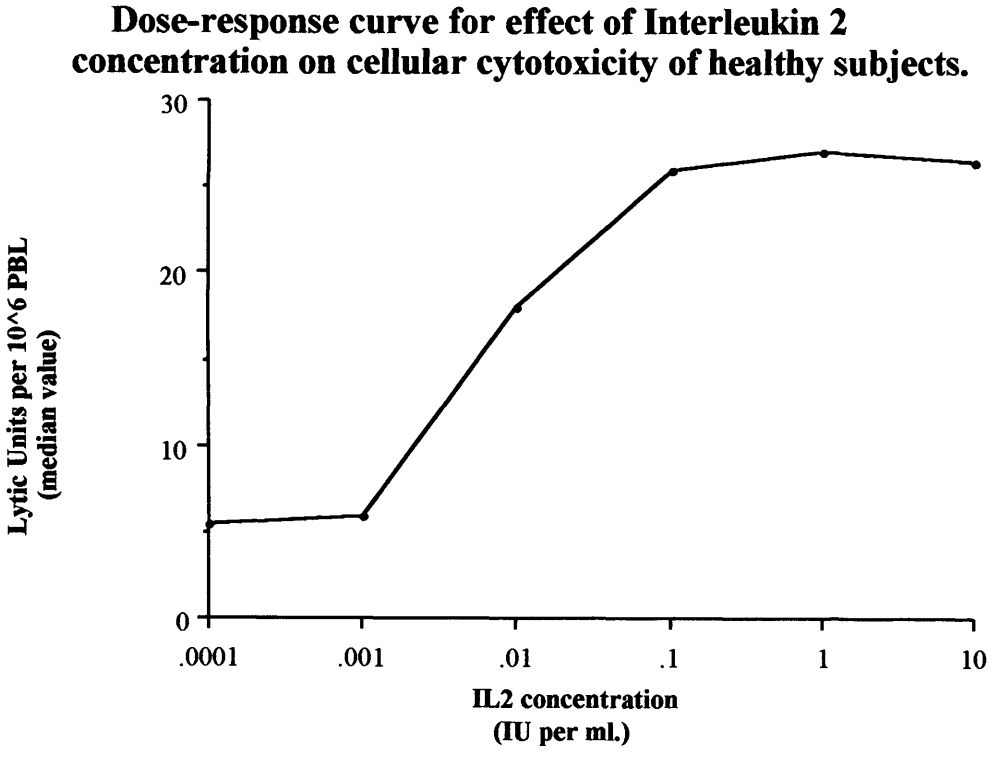
ENGLAND.

Sigma Chemical Company Ltd.

Fancy Road, Poole, Dorset, BH12 4GH, ENGLAND.

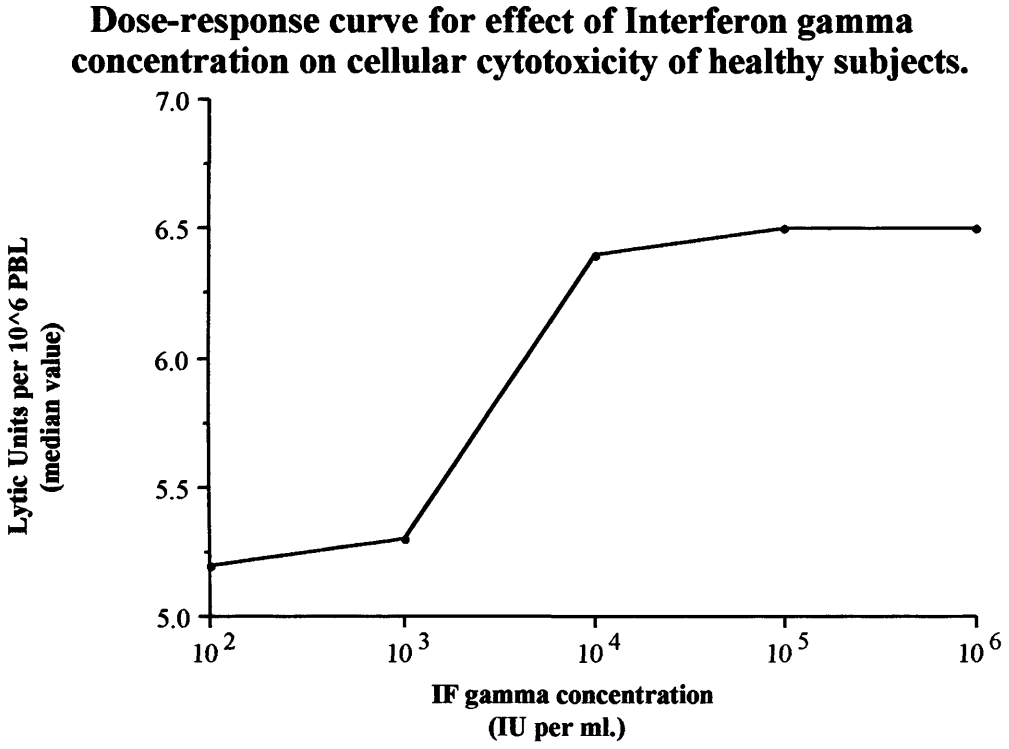
APPENDIX IV - DOSE-RESPONSE CURVES

(i) Interleukin 2



Graph A:1 *Effect of increasing Interleukin-2 concentration on the cellular cytotoxicity of healthy subjects' peripheral blood lymphocytes.*

(ii) Interferon gamma



Graph A:1 *Effect of increasing Interferon gamma concentration on the cellular cytotoxicity of healthy subjects' peripheral blood lymphocytes.*

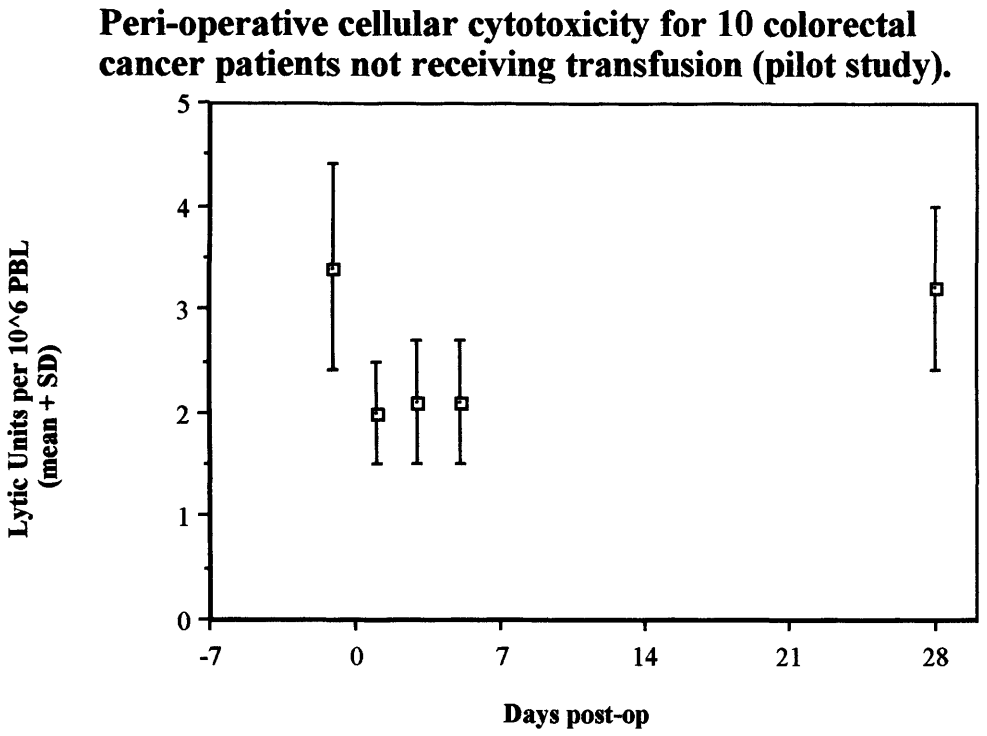
APPENDIX V - PILOT STUDY and STATISTICAL PLANNING

Pilot Study.

Prior to starting the main study a short pilot study was conducted both to test the assays and data collection procedures, and to provide preliminary data for statistical planning. Ten patients who underwent elective resection of colorectal cancers and who did not receive blood transfusion either before or during the peri-operative period (up to four weeks from surgery) were studied. Venous blood samples were taken pre-operatively, and at Days 1,3,5, and 28 after surgery. Peripheral blood lymphocyte preparations from these samples were analysed using the ^{51}Cr release assay as described in the Methods section to provide measures of cellular cytotoxicity. These results, displayed in Table A:1 and Graph A:3, showed very little difference in cytotoxicity between Days 1,3 and 5. As such it was elected to collect the initial post-operative sample for the main study on Day 5 alone, for the simple reason that any peri-operative blood transfusion would be given between Day 0 (i.e. during surgery) and Day 5, and with that regimen any patients who received transfusions would have had both surgery and transfusion completed by the time of first post-operative sampling.

Days post-op	**-1**	**1**	**3**	**5**	**28**
Lytic Units per 10^6 PBL (mean)	3.4	2.0	2.1	2.1	3.2
SD	1.0	0.5	0.6	0.6	0.8

Table A:1 *Cellular cytotoxicity of peripheral blood lymphocyte preparations from 10 patients undergoing colorectal cancer resections without blood transfusion - pilot study.*



Graph A:3 *Cellular cytotoxicity of peripheral blood lymphocyte preparations from 10 patients undergoing colorectal cancer resections without blood transfusion - pilot study (mean values with SD).*

Statistical Planning

To provide an estimate of how many patients would need to be recruited for this study a computer based statistics package was used (Instat for Macintosh, Version 1.12, GraphPad Software). For this program to estimate minimum sample size the Standard Deviation of the proposed sample and the degree of precision required are entered, the latter being the smallest difference between two means that would be considered significant.

Work performed in Denmark (Jensen 1994) had studied NK cell function in 60 patients undergoing colorectal surgery, 26 of whom had no transfusion, 15 received leucodepleted blood transfusions, and 19 received standard transfusions. The patients transfused with standard blood in that study had NK cell function approximately 50% lower than the other two groups throughout the post-operative period, which was statistically significant using Students t-test ($p<0.001$). Considering this magnitude of difference it appeared reasonable to expect at least a 40% difference between the means at Day 5, which from the pilot study data (Table A:1) equated to a value of 0.8 (Lytic Units per 10^6 PBL). The SD for that data point was 0.6, and by entering these two values to the Instat calculator a table of minimum sample sizes for varying α and β values was produced (Table A:2)

α =	**0.10**	**0.05**	**0.02**	**0.01**
β =				
0.20	9	10	13	15
0.10	11	13	16	18
0.05	14	16	19	22

Table A:2 *Minimum sample sizes for two-tailed tests with unpaired data; SD=0.6, difference between means = 0.8.*

Using this table with an α value of 0.05 and a β value of 0.05 - 0.10 the minimum sample size recommended was 13 - 16 patients per group. By over-estimating slightly

to allow for errors a minimum sample size of 20 was decided upon. Although the study from Denmark had an overall transfusion rate of 53%, an earlier study into transfusion practices in the Leicester area had shown a transfusion rate for colorectal cancer surgery of 40% (Veitch unpublished). Thus by aiming to recruit a total of 100 patients the intention was to have 40 patients who required transfusion, of which 20 would receive leucodepleted blood, and 20 would receive standard blood, leaving 60 patients as a control group with surgery but no transfusion.

BIBLIOGRAPHY

Abate, M. A., M. L. Hyneck, et al. (1982). "Cimetidine pharmacokinetics." Clinical Pharmacy **1**: 225-233.

Adams, W. J., J. A. Lawson, et al. (1994). "Cimetidine inhibits in-vivo growth of human colon cancer and reverses histamine stimulated in-vitro and in-vivo growth." Gut **35**: 1632-1636.

Adams, W. J. and D. L. Morris (1994). "Short course cimetidine and survival with colorectal cancer." Lancet **344**: 1768-1769.

Agarwal, M. and N. Blumberg (1983). "Colon cancer patients transfused perioperatively have an increased incidence of recurrence." Transfusion **23**: 421.

Agarwal, N., J. G. Murphy, et al. (1993). "Blood transfusion increases the risk of infection after trauma." Archives of Surgery **128**: 171-177.

Agostino, G. J., B. D. Kahan, et al. (1982). "Suppression of mixed leucocyte culture using leucocytes from normal individuals, uraemic patients, and allograft recipients." Transplantation **34**(6): 367-371.

Akiyoshi, T., F. Koba, et al. (1985). "Impaired production of interleukin 2 after surgery." Clinical and Experimental Immunology **59**: 745-749.

Alexander, J. W., G. F. Babcock, et al. (1992). "The induction of immunological hyporesponsiveness by pre-operative donor-specific transfusions and cyclosporine in human cadaveric transplants." Transplantation **53**: 423-427.

Atzpodien, J., H. Kirchner, et al. (1993). "Expansion of peripheral blood natural killer cells correlates with clinical outcome in cancer patients receiving recombinant subcutaneous interleukin-2 and interferon-α-2." Tumor Biology **14**: 354-359.

Barlozzari, T., J. Leonhardt, et al. (1985). "Direct evidence for the role of LGL in the inhibition of experimental tumour metastases." Journal of Immunology **134**(4): 2783-2789.

Baxevanis, C. N., K. Papilas, et al. (1994). "Abnormal cytokine serum levels correlate with impaired cellular immune responses after surgery." Clinical Immunology and Immunopathology **71**(1): 82-88.

Beatty, J. D. (1992). "Immunotherapy of colorectal cancer." Cancer **70**(5): 1425-1433.

Beer, D. J. and R. E. Rocklin (1987). "Histamine modulation of lymphocyte biology: membrane receptors, signal transduction, and functions." Critical Review of Immunology **7**: 55-91.

Begent, R. H. J. (1992). "Colorectal cancer." British Medical Journal **305**: 246-249.

Bell, Y., D. Shoskes, et al. (1993). "Induction of specific unresponsiveness using transfected cells expressing donor major histocompatibility complex molecules: evidence for indirect presentation of allopeptides in vivo." Transplant Proceedings **25**: 359.

Bentzen, S. M., I. B. Balslev, et al. (1990). "Blood transfusion and prognosis in Dukes' B and C colorectal cancer." European Journal of Cancer **26**: 457-463.

Besouska, K., C. T. Yuen, et al. (1994). "Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity." Nature **372**: 150-157.

Beynon, J., P. W. Davies, et al. (1989). "Perioperative blood transfusion increases the risk of recurrence in colorectal cancer." Dis Colon Rectum **29**: 975-979.

Bickel, K. D., P. I. Terasaki, et al. (1985). "Effect of blood transfusions on survival after cancer surgery." Netherlands Journal of Medicine **28**: 257-258.

Blair, S. D. and S. B. Janvrin (1985). "Relation between cancer of the colon and blood transfusion." British Medical Journal **290**: 1516-1517.

Blajchman, M. A., l. Bardossy, et al. (1993). "Allogeneic blood transfusion induced enhancement of tumour growth: two animal models showing amelioration by leucodepletion and passive transfer using spleen cells." Blood **81**(7): 1880-1882.

Blajchman, M. A. and J. O. Bordin (1995). "The tumour growth-promoting effect of allogeneic blood transfusions." Immunological Investigations **24**(1&2): 311-317.

Blomberg, J., T. Moller, et al. (1993). "Cancer morbidity in blood recipients - results of a cohort study." British Journal of Cancer **29A**(15): 2101-2105.

Blottiere, H. M., C. Burg, et al. (1992). "Involvement of histo-blood-group antigens in the susceptibility of colon carcinoma cells to natural killer mediated cytotoxicity." International Journal of Cancer **52**: 609-618.

Blumberg, N., M. Agarwal, et al. (1985). "A possible association between survival time and transfusion in patients with cervical cancer." Blood **66**(1): 274A.

Blumberg, N., M. M. Agarwal, et al. (1985). "Relation between recurrence of cancer of the colon and blood transfusion." British Medical Journal **290**: 1037-1039.

Blumberg, N. and J. M. Heal (1994). "Effects of transfusion on immune function." Arch Pathol Lab Med **118**: 371-379.

Blumberg, N. and J. M. Heal (1995). "Transfusion and the immune system: a paradigm shift in progress?" Transfusion **35**: 879-883.

Blumberg, N., J. M. Heal, et al. (1986). "Association between transfusion of whole blood and recurrence of cancer." British Medical Journal **293**: 539-543.

Blumberg, N., D. J. Triulzi, et al. (1990). "Transfusion induced immunomodulation and its clinical consequences." Transfusion Medicine Reviews **4**(4(1)): 24-35.

Blundell, J. (1825). "Transfusion of blood (case abstract)." Philadelphia Journal of Medical and Physical Sciences. **9**: 207.

Boon, T. (1992). "Toward a genetic analysis of tumour rejection antigens." Advances in Cancer Research **58**: 179-210.

Borden, E. C. and P. M. Sondel (1990). "Lymphokines and cytokines as cancer treatment: immunotherapy realised." Cancer **65**: 800-814.

Bordin, J. O. and M. A. Blajchman (1995). "Immunosuppressive effects of allogeneic blood transfusions: implications for the patient with a malignancy." Haematology/Oncology Clinics of North America **9**(1): 205-218.

Bordin, J. O., N. M. Heddle, et al. (1994). "Biologic effects of leucocytes present in transfused cellular blood products." Blood **84**(6): 1703-1721.

Boussiotis, V. A., J. G. Gribben, et al. (1994). "Blockade of the CD28 co-stimulatory pathway: a means to induce tolerance." Current Opinion in Immunology **6**: 797-807.

Brivio, F., P. Lissoni, et al. (1993). "Influence of blood transfusions on surgery-induced immune changes in colorectal carcinoma." Minerva Chirurgica **48**: 331-335.

Browell, D. A., J. A. Kirby, et al. (1993). "Ranitidine abrogates the transfusion effect in cancer surgery." Surgical Research Society, oral presentation.

Browell, D. A. and T. W. J. Lennard (1993). "Immunological status of the cancer patient and the effects of blood transfusion on antitumour responses." Current Opinion in General Surgery : 325-333.

Bruil, A., T. Beugeling, et al. (1995). "The mechanisms of leucocyte removal by filtration." Transfusion Medicine Reviews **9**(2): 145-166.

Brunner, K. T., J. Manuel, et al. (1968). "Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr labeled allogeneic target cells in-vitro." Immunology **14**: 181.

Brunson, M. E. and J. W. Alexander (1990). "Mechanisms of transfusion induced immunosuppression." Transfusion **30**(7): 651-658.

Brutkiewicz, R. R. and R. M. Welsh (1995). "Major histocompatibility complex class I antigens and the control of viral infections by natural killer cells." Journal of Virology **69**(7): 3967-3971.

Burlingham, W. J., M. H. Pan, et al. (1988). "Induction of anti-idiotypic antibodies to donor HLA-A2 following blood transfusion in a highly sensitised HLA-A2+ recipient." Transplantation **45**: 1066-71.

Burnet, F. M. (1970). Immunological Surveillance. New York, Pergamon Press.

Burrows, L. and P. Tartter (1982). "Effect of blood transfusion on colonic malignancy recurrence rate." Lancet **2**: 662.

Burrows, L. and P. Tartter (1983). "Blood transfusions and colorectal cancer recurrence: a possible relationship." Transfusion **23**: 419.

Busch, O. R. C., W. C. J. Hop, et al. (1994). "Blood transfusions and local tumor recurrence in colorectal cancer." Annals of Surgery **220**(6): 791-797.

Busch, O. R. C., W. C. J. Hop, et al. (1995). "The effect of blood transfusions on survival after surgery for colorectal cancer." European Journal of Cancer **31A**(7/8): 1226-1228.

Busch, O. R. C., W. C. J. Hop, et al. (1993). "Blood transfusion and prognosis in colorectal cancer." New England Journal of Medicine **328**: 1372-76.

Bykowsky, M. and O. Stutman (1986). "The cells responsible for murine natural cytotoxic activity: a multi-lineage system." Journal of Immunology **137**(4): 1120-1126.

Callaerts, A. J., M. L. Gielis, et al. (1993). "The mechanism of white cell reduction by synthetic filtration." Transfusion **33**: 134-138.

Carson, J. L. and L. R. Willett (1993). "Is a hemaglobin of 10g/dl required for surgery?" Medical Clinics of North America **77**(2): 335-347.

Cerhan, J. R., R. B. Wallace, et al. (1993). "Transfusion history and cancer risk in older women." Annals of Internal Medicine **119**: 8-15.

Chang, L., G. A. Gusewitch, et al. (1993). "Rapid flow cytometric assay for the assessment of natural killer cell activity." Journal of Immunological Methods **166**: 45-54.

Chapuis, P. H., O. F. Dent, et al. (1985). "A multivariate analysis ofclinical and pathological variaties on prognosis after resection of large bowel cancer." British Journal of Surgery **72**: 698-702.

Chauvenet, P. H. and R. T. Smith (1978). "Relation of tumour specific antigens and the histocompatibility complex." International Journal of Cancer **22**: 79-93.

Chesi, R., A. Cazzola, et al. (1989). "Effect of perioperative transfusion on survival in osteosarcoma treated by multimodal therapy." Cancer **64**: 1727-1737.

Cheslyn-Curtis, S., L. P. Fielding, et al. (1990). "Large bowel cancer: the effect of perioperative blood transfusion on outcome." Annals of the Royal College of Surgeons of England **72**: 53-59.

Christou, N. V., J. L. Meakins, et al. (1995). "The delayed hypersensitivity response and host resistance in surgical patients." Annals of Surgery **222**(4): 534-548.

Chung, M., O. K. Steinmetz, et al. (1993). "Perioperative blood transfusion and outcome after resection for colorectal carcinoma." British Journal of Surgery **80**: 427-432.

Clarke, P. J., R. C. Burton, et al. (1993). "Allogeneic blood transfusion reduces murine pulmonary natural killer (NK) activity and enhances lung metastasis of a syngeneic tumour." International Journal of Cancer **55**: 996-1002.

Clarke, P. J. and D. Tarin (1987). "Effect of pre-operative blood transfusion on tumour metastases." British Journal of Surgery **74**: 520-522.

Clarke, P. J., K. J. Wood, et al. (1989). "Increased tumour growth after blood transfusion." Transplantation Proceedings **21**(1): 584-585.

Coley, W. (1893). "The treatment of malignant tumours by repeated innoculation of erysipelas: a report of ten original cases." American Journal of Medical Sciences **105**: 487-511.

Corman, J., R. Arnoux, et al. (1988). "Perioperative blood transfusion and colorectal cancer outcome." Transplant Proceedings **20**: 1128-1129.

Corman, J., R. Arnoux, et al. (1986). "Blood transfusion and survival after colectomy for colorectal cancer." Canadian Journal of Surgery **29**: 325-329.

Creasy, T. S., P. S. Veitch, et al. (1987). "A relationship between perioperative blood transfusion and recurrence of carcinoma of the sigmoid colon following potentially curative surgery." Annals of the Royal College of Surgeons of England **69**: 100-103.

Crosby, E. T. (1992). "Perioperative haemotherapy: II. Risks and complications of blood transfusion." Canadian Journal of Anaesthesia **39**(8): 822-837.

Crowe, J. P., N. H. Gordon, et al. (1989). "Breast cancer survival and perioperative blood transfusion." Surgery **106**: 836-841.

Crowson, M. C., M. T. Hallisey, et al. (1989). "Blood transfusion in colorectal cancer." British Journal of Surgery **76**: 522-523.

Cue, J. I., J. C. Peyton, et al. (1992). "Does blood transfusion or haemorrhagic shock induce immunosuppression?" The Journal of Trauma **32**(5): 613-617.

d'Ambra, M. N. and D. K. Kaplan (1995). "Alternatives to allogeneic blood use in surgery: acute normovolaemic hemodilution and preoperative autologous donation." The American Journal of Surgery **170**(6A): 49-52.

Dalrymple, J. C. and J. M. Monoghan (1986). "Recurrent carcinoma of the vulva: relationship with operative blood transfusion." Journal of Obstetrics and Gynaecology **7**: 65-67.

Davies, A. H., D. Cranston, et al. (1991). "Effect of blood transfusion on survival after radiotherapy as treatment for carcinoma of the prostate." Annals of the Royal College of Surgeons of England **73**: 116-118.

Deans, G. T., T. G. Parks, et al. (1992). "Prognostic factors in colorectal cancer." British Journal of Surgery **79**: 608-613.

Deehan, D. J., S. D. Heys, et al. (1995). "Interleukin-2 augments host cellular immune reactivity in the perioperative period in patients with malignant disease." European Journal of Surgical Oncology **21**: 16-22.

Denys, J. B. (1667). "A letter concerning a new way of curing sundry diseases by transfusion of blood." Philosophical Transactions **2**(27A): 489-504.

Dind, L., P. S. Linsley, et al. (1993). "IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression." Journal of Immunology **151**: 1224--1234.

Djeu, J. Y., J. A. Heinbaugh, et al. (1979). "Role of macrophages in the augmentation of mouse natural killer cell activity by poly I:C and interfon." Journal of Immunology **122**(1): 182-187.

Doci, R., L. Gennari, et al. (1995). "Morbidity and mortality after hepatic resection of metastases from colorectal cancer." British Journal of Surgery **82**: 377-381.

Doltery, C. (1991). Ranitidine. Therapeutic Drugs Ed. C. Doltery. London, Churchill Livingstone. R5-R8.

Dukes, C. E. (1932). "The classification of cancer of the rectum." Journal of Pathology and Bacteriology **35**: 323-332.

Dunlop, M. G. (1997). "Colorectal cancer." British Medical Journal **314**: 1882-1885.

Dzik, S. (1993). "Leucodepletion blood filters: filter design and mechanism of white cell removal." Transfusion Medicine Reviews **7**(2): 65-77.

Dzik, S., M. A. Blajchman, et al. (1996). "Current research on the immunomodulatory effect of allogeneic blood transfusion." Vox Sanguinis **70**: 187-194.

Edna, T. H. and T. Bjerkeset (1992). "Association between blood transfusion and infection in injured patients." The Journal of Trauma **33**(5): 659-661.

Edna, T. H., K. Vada, et al. (1992). "Blood transfusion and survival following surgery for renal carcinoma." British Journal of Urology **70**: 135-138.

Edna, T.H., Bjerkeset, T. (1998). "Association between transfusion of stored blood and infective complications after resection for colorectal cancer." European Journal of Surgery **164**(6): 449-456.

Fernandez, L. A., J. M. MacSween, et al. (1992). "Immunologic changes afer blood transfusion in patients undergoing vascular surgery." The American Journal of Surgery **163**: 263-269.

Ferrat, N. and et.al. (1990). "Recombinant interferon gamma provokes resistance of human breast cancer cells to spontaneous and IL2 activated non-MHC restricted cytotoxicity." British Journal of Cancer **61**: 558-562.

Ficarra, B. J. (1942). "The evolution of blood transfusion." Annals of Medical History **4**: 302-323.

Fisher, B. and E. R. Fisher (1971). "Studies concerning regional lymph nodes. Initiation of immunity." Cancer **27**: 1001-1004.

Ford, C. D., J. W. Gillette, et al. (1994). "Transfusion and second malignancy." Transfusion **34**: 63-65.

Ford, C. D., G. VanMoorleghem, et al. (1993). "Blood transfusions and postoperative wound infections." Surgery **113**(6): 603-607.

Foster, R. S., M. Costanza, et al. (1988). "Blood transfusions and survival after resection of cancers of the breast,colon and lung; the need for prospective randomised trials." Transplant Proceedings **20**: 1125-1127.

Foster, R. S., M. C. Costanza, et al. (1985). "Adverse relationship between blood transfusions and survival after colectomy for colon cancer." Cancer **55**: 1195-1201.

Foster, R. S., J. C. Foster, et al. (1983). "Do blood transfusions have an adverse effect on survival after mastectomy for breast cancer?" Proceedings of the American Society of Clinical Oncology **2**: 60.

Foster, R. S., J. C. Foster, et al. (1984). "Blood transfusions and survival after surgery for breast cancer." Archives of Surgery **119**: 1138-1140.

Francis, D. and R. Judson (1985). "Relation between recurrence of cancer of the colon and blood transfusion." British Medical Journal **291**: 544.

Francis, D. M. A. (1991). "Relationship between blood transfusion and tumour behaviour." British Journal of Surgery **78**: 1420-1428.

Francis, D. M. A. and R. T. Judson (1987). "Blood transfusion and recurrence of cancer of the colon and rectum." British Journal of Surgery **74**: 26-30.

Frankish, P. D., R. K. McNee, et al. (1985). "Relation between cancer of the colon and blood transfusion." British Medical Journal **290**: 1827.

Freidberg, R. C. (1994). "Issues in transfusion therapy in the patient with malignancy." Haematology/Oncology Clinics of North America **8**(6): 1223-1253.

Fry, R. D., J. W. Fleshman, et al. (1989). "Effect of perioperative blood transfusion on recurrence of Crohn's disease." The Lancet : 1048.

Garcia-Penarrubia, p. (1992). Natural killer cells in bacterial infection. The Natural Killer Cell Eds. C. E. Lewis and J. O. McGee. Oxford, Oxford University Press. 67-105.

Garcia-Penarrubia, P., F. T. Koster, et al. (1989). "Antibacterial activity of human natural killer cells." Journal of Experimental Medicine **169**: 99-113.

Gascon, P., N. C. Zoumbos, et al. (1984). "Immunological abnormalities in patients receiving multiple blood transfusions." Annals of Internal Medicine **100**(2): 173-177.

George, C. D. and P. J. Morello (1986). "Immunological effects of blood transfusion upon renal transplantation, tumour operations, and bacterial infections." The American Journal of Surgery **152**: 329-337.

Goodnough, L. T. and G. J. Despotis (1995). "Establishing practice guidelines for surgical blood management." The American Journal of Surgery **170**(6A): 16-20.

Gorelik, E., R. Wiltrout, et al. (1982). "Role of NK cells in the control of metastatic spread and growth oftumour cells in mice." International Journal of Cancer **30**: 107-114.

Gray, B. N., C. Walker, et al. (1989). "Controlled clinical trial of adjuvant immunotherapy with BCG and neuraminidase-treated autologous tumour cells in large bowel cancer." Journal of Surgical Oncology **40**: 34-37.

Greenburg, A. G. (1995). "A physiologic basis for red blood cell transfusion decisions." The American Journal of Surgery **170**(6A): 44-48.

Gudmundsdottir, I. and H. M. Ogmundsdottir (1992). "Variable natural killer function of tumour infiltrating lymphocytes from breast carcinomas." APMIS **100**: 737-746.

Habu, S. and e. al (1981). "In-vivo effects of anti-asialo-GM1. Reduction of NK activity enhancement of transplanted tumour growth in nude mice." Journal of Immunology **127**: 34-38.

Halasz, N. A., M. J. Orloff, et al. (1964). "Increased survival of renal homografts in dogs after injection of graft donor blood." Transplantation **2**: 453-458.

Hallissey, M. T., M. C. Crowson, et al. (1992). "Blood transfusion: an overused resource in colorectal cancer surgery." Annals of the Royal College of Surgeons of England **74**: 59-62.

Hamilton, S. R. (1992). "Molecular genetics of colorectal carcinoma." Cancer **70**(5): 1216-1221.

Hamlin, I. M. E. (1968). "Possible host resistance in carcinoma of the breast, a histological study." British Journal of Cancer **2**: 383-401.

Hardcastle, J. D., N. C. Armitage, et al. (1986). "Faecal occult blood screening for colorectal cancer in the general population." Cancer **58**: 183-184.

Harford, F. J., L. F. Williams, et al. (1988). "Recurrence of colon carcinoma after curative resection and blood transfusion: proposal for a prospective study." Transplantation Proceedings **20**(6): 1130-1134.

Harris, R. and E. A. Ukaejiofo (1970). "Tissue typing using a routine one-step lymphocyte separation technique." British Journal of Haematology **18**: 229-235.

Harrison, S., R. J. C. Steele, et al. (1992). "Predeposit autologous blood transfusion in patients with colorectal cancer: a feasibility study." British Journal of Surgery **79**: 355-357.

Hatcher, F. M. and R. E. Kuhn (1982). "Destruction of Trypanosoma cruzi by natural killer cells." Science **218**: 295.

Hauser, W. E. and V. Tsai (1986). "Acute toxoplasma infection of mice induces spleen cells that are cytotoxic for T. gondii in vitro." Journal of Immunology **136**: 313.

Heal, J. M., C. Chuang, et al. (1988). "Perioperative blood transfusion and prostatic cancer recurrence and survival." American Journal of Surgery **156**: 374-380.

Heald, R. J. and R. D. H. Ryall (1986). "Recurrence and survival afetr total mesorectal excision for colorectal cancer." Lancet **1**: 479-480.

Hershey, P., A. Edwards, et al. (1979). "Low natural killer activity in familial melanoma patients and their relatives." British Journal of Cancer **40**: 113-119.

Hidore, M. R. and J. W. Murphy (1989). "Murine natural killer cell interactions with with a fungal target, Cryptococcus neoformans." Infection and Immunology **57**: 1990.

Hogan, P. G. (1992). Natural killer cells in transplantation. The Natural Killer Cell Eds. C. E. Lewis and J. O. McGee. Oxford, Oxford University Press. 151-174.

Houbiers, J. G. A., A. Brand, et al. (1994). "Randomised controlled trial comparing transfusion of leucocyte-depleted or buffy coat depleted blood in surgery for colorectal cancer." The Lancet **344**: 573-578.

Hyman, N. H., R. S. Foster, et al. (1985). "Blood transfusion and survival after lung cancer resection." American Journal of Surgery **149**: 502-507.

Ioachim, H. L. (1976). "The stromal reaction of tumours: an expression of immune surveillance." Journal of the National Cancer Institute **57**: 465-475.

Irving, G. A. (1992). "Perioperative blood and blood component therapy." Canadian Journal of Anaesthesia **39**(10): 1105-1115.

Jackson, R. M. and D. H. Rice (1989). "Blood transfusion and recurrence in head and neck cancer." Ann Otol Rhinol Laryngol **98**: 171-173.

Jahnson, S. and M. Anderson (1992). "Adverse effects of perioperative blood transfusion in patients with colorectal cancer." European Journal of Surgery **158**: 419-425.

Jakobson, E. B., J. H. Eickhoff, et al. (1990). "Perioperative blood transfusion and recurrence and death afetr resection for cancer of the colon and rectum." : 435-442.

Jeekel, J. (1995). "Developments in colorectal cancer surgery." European Journal of Cancer **31A**(7/8): 1379-1381.

Jensen, L. S. (1994). "Immunosuppression and leucocytes." Curr Stud Haematol Blood Transf. **60**: 64-74.

Jensen, L. S., A. J. Andersen, et al. (1992). "Postoperative infection and natural killer cell function following blood transfusion in patients undergoing elective colorectal surgery." British Journal of Surgery **79**: 513-516.

Jensen, L. S., N. Grunnet, et al. (1995). "Cost effectiveness of blood transfusion and white cell reduction in elective colorectal surgery." Transfusion **35**: 719-722.

Jensen, L. S., M. Hokland, et al. (1996). "A randomised controlled study of the effect of bedside leucocyte depletion on the immunosuppressive effect of whole blood transfusion in patients undergoing elective colorectal surgery." British Journal of Surgery **83**: 973-977.

Jensen, L. S., P. Kissmeyer-Nielsen, et al. (1996). "Randomised comparison of leucocyte depleted versus buffy-coat poor blood transfusion and complications after colorectal surgery." Lancet **348**: 841-845.

Jerne, N. K. (1974). "Towards a network theory of the immune system." Annals of Immunology **125C**: 373-389.

Jeter, E. K. and M. A. Spivey (1995). "Noninfectious complications of blood transfusion." Haematology/Oncology Clinics of North America **9**(1): 187-204.

Johnson, J. T., F. H. Taylor, et al. (1987). "Blood transfusion and outcome in stage 3 Head and neck cancer." Ann Otolaryngol Head Neck Surg **113**: 307-310.

Jones, K. R. and M. C. Weissler (1990). "Blood transfusion and other risk factors for recurrence of cancer of the head and neck." Arch Otolaryngol Head Neck Surg **116**: 304-309.

Juhl, H., M. Stritzel, et al. (1994). "Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow

of gastric, colorectal and pancreatic cancer patients." International Journal of Cancer **57**: 330-335.

Kaneda, M., T. Horimi, et al. (1987). "Adverse effect of blood transfusion on survival of patients with gastric cancer." Transfusion **27**: 375-377.

Kaplan, J., S. Sarnaik, et al. (1984). "Diminished helper/suppressor lymphocyte ratios and natural killer cell activity in recipients of repeated blood transfusions." Blood **64**(1): 308-310.

Karre, K., G. Klein, et al. (1980). "Low natural in-vivo resistance to syngeneic leukaemias in natural killer cell deficient mice." Nature **284**: 624-625.

Kelly, S. M., S. Groshen, et al. (1988). "Blood transfusion and lung cancer recurrence." Cancer **62**: 606-610.

Kernohan, N. M., H. F. Sewell, et al. (1990). "Natural killer cells in cutaneous malignant melanoma." Journal of Pathology **161**: 35-40.

Kieckbusch, M. E., J. R. O'Fallon, et al. (1989). "Blood transfusion exposure does not influence survival in patients with carcinoma of the breast." Transfusion **29**: 500-504.

Kiff, R. S. and R. D. Kingston (1987). "A relationship between perioperative blood transfusion and recurrence of carcinoma of the sigmoid colon following potentially curative surgery." Annals of the Royal College of Surgeons of England **69**: 303.

Kirkley, S. A., J. Cowles, et al. (1995). "Cytokine secretion after allogeneic or autologous blood transfusion." Lancet **345**: 527.

Kitchens, C. S. (1993). "Are transfusions overrated? Surgical outcome of Jehovah's Witnesses." The American Journal of Medicine **94**: 117-119.

Klava, A., A. Windsor, et al. (1997). "Monocyte activation after open and laparoscopic surgery." British Journal of Surgery **84**: 1152-1156.

Klein, E. and A. Mantovani (1993). "Action of Natural Killer cells and macrophages in cancer." Current Opinion in Immunology **5**: 714-718.

Klein, G. and T. Boon (1993). "Tumour immunology: present perspectives." Current Opinion in Immunology **5**: 687-692.

Klein, H. G. (1992). "Wolf in wolf's clothing: is it time to raise the bounty on the passenger leucocyte?" Blood **80**(8): 1865-1868.

Klein, H. G. (1994). "Immunologic aspects of blood transfusion." Seminars In Oncology **21**(2): 16-20.

Klein, H. G. (1995). "Allogeneic transfusion risks in the surgical patient." The American Journal of Surgery **170**(6A): 21-26.

Knuth, A., T. Wolfel, et al. (1992). "T cell response to human malignant tumours." Cancer Surveys **13**: 39-52.

Krishnaraj, R. and G. Blandford (1988). "Age associated alterations in human natural killer cells." Cellular Immunology **114**: 137-148.

Lanier, L. L., J. H. Phillips, et al. (1986). "Natural killer cells: definition of a cell type rather than a function." Journal of Immunology **137**(9): 2735-2739.

Lasek, W., M. Jakobisiak, et al. (1994). "Decreased natural killer cell activity in whole-blood donors does not seem to result in increased cancer incidence." Transfusion **34**(4): 359-360.

Lauder, I., W. Aherne, et al. (1977). "Macrophage infiltration of breast tumours: a prospective study." Journal of Clinical Pathology **30**: 563-568.

Leather, A. J. M., N. Gallegos, et al. (1991). "Value of immunocytochemistry in the detection of circulating malignant cells in colorectal cancer." Gut **32**: 559.

Leather, A. J. M., N. C. Gallegos, et al. (1993). "Detection and enumeration of circulating tumour cells in colorectal cancer." British Journal of Surgery **80**: 777-780.

LeFever, A. V. and A. Funahashi (1991). "Phenotype and function of natural killer cells in patients with bronchogenic carcinoma." Cancer Research **51**: 5596-5601.

Leivestad, T. and E. Thorsby (1984). "Effects of HLA-haploidentical blood transfusions on donor specific immune responsiveness." Transplantation **37**: 175-181.

Lenhard, V., P. Scholler, et al. (1989). "Transfusion induced T suppressor cell activity and experimental tumor growth." Transplantation Proceedings **21**(1): 580-583.

Lennard, T. W. J., B. K. Shenton, et al. (1985). "The influence of surgical operations on components of the human immune system." British Journal of Surgery **72**: 771-776.

Levi, F. A., C. Canon, et al. (1988). "Seasonal modulation of the circadian time stucture of circulating T and natural killer lymphocyte subsets from healthy subjects." Journal of Clinical Investigation **81**: 407-413.

Lewis, C. E. and J. O. McGee, Eds. (1992). The Natural Killer Cell. The Natural Immune System. Oxford University Press.

Liewald, F., R. P. Wirsching, et al. (1990). "Influence of blood transfusions on tumour recurrence and survival rate in colorectal carcinoma." European Journal of Cancer **26**: 327-335.

Lipscomb, M. F., T. Alvarellos, et al. (1987). "Role of natural killer cells in resistance to Cryptococcus neoformans infection in mice." American Journal of Pathology **128**: 354.

Little, A. G., H. S. Wu, et al. (1990). "Perioperative blood transfusion adversely affects prognosis of patients with stage 1 non-small cell lung cancer." American Journal of Surgery **160**(630-633):

Loertscher, R., A. B. Leichman, et al. (1988). "Blocking of T cell activation at the G1A/G1B interface of the cell cycle and prevention of expression of IL-2 receptors by alloactivated suppressor T lymphocytes." Transplantation **45**: 194-201.

Look, K. Y., M. Reisinger, et al. (1993). "Blood transfusion and the risk of recurrence in squamous carcinoma of the vulva." American Journal of Obstetrics and Gynaecology **168**: 1718-1723.

Lower, R. and E. King (1667). "An account of the experiment of transfusion, practised on a man in London." Philosophical Transactions **2**: 557-564.

Lukomska, B., W. L. Olszewski, et al. (1983). "The effect of surgery and chemotherapy on blood NK cell activity in patients with ovarian cancer." Cancer **51**: 465-469.

Maetani, S., T. Nishikawa, et al. (1986). "Role of blood transfusion in organ system failure following major abdominal surgery." Annals of Surgery **203**(3): 275-281.

Manyonda, I. T., D. E. Shaw, et al. (1986). "Renal cell carcinoma: blood transfusion and survival." British Medical Journal **293**: 537-538.

Markey, A. C. and D. M. Macdonald (1989). "Identification of CD16/NKH-I+ natural killer cells and their relevance to cutaneous tumour immunity." British Journal of Immunology **121**: 563-570.

Marquet, R. L., M. A. Papendrecht, et al. (1993). "Blood donation leads to a decrease in natural killer cell activity: a study in normal blood donors and cancer patients." Transfusion **33**(5): 368-373.

Marquet, R. L., S. K. Singh, et al. (1989). "Effect of blood transfusions and surgery on metastatic growth and immune parameters in a rat model." Transplantation Proceedings **21**(1): 586-587.

Marsh, J., P. T. Donnan, et al. (1990). "Association between transfusion with plasma and the recurrence of colorectal carcinoma." British Journal of Surgery 77: 623-626.

Mathiasen, O., L. Lund, et al. (1994). "The effect of previous blood transfusion on lymphocyte subsets and natural killer cell function in patients with colorectal cancer." Vox Sang **67**: 36-41.

Matsuda, M., M. Petersson, et al. (1995). "Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumour-infiltrating, gut mucosal and

peripheral lymphocytes: correlation with the stage of the disease." International Journal of Cancer **61**: 765-772.

McAlister, F. A., Clark, H. D., et al. (1998). "Perioperative allogeneic blood transfusion does not cause adverse sequelae in patients with cancer - a meta-analysis of unconfounded studies." British Journal of Surgery **85**(2): 171-178.

McClinton, S., L. E. F. Moffat, et al. (1990). "Blood transfusion and survival following surgery for prostatic cancer." British Journal of Surgery **77**: 140-142.

Mecklin, J. P., H. J. Jarvinen, et al. (1989). "Blood transfusion and prognosis in colorectal carcinoma." Scandinavian Journal of Gastroenterology **24**: 33-39.

Medawar, P. B. (1945). "Immunity to homologous grafted skin; relationship between antigens of blood and skin." British Journal of Experimental Pathology **127**: 15-24.

Medawar, P. B. (1946). "Relationship between antigens of blood and skin." Nature **157**: 161-162.

Mickey, M. R., S. Cats, et al. (1983). "Transfusion and selection in cadaveric donor kidney grafts." Transplant Procedings **15**: 965-968.

Miholic, J., M. Hudec, et al. (1985). "Risk factors for severe bacterial infections after valve replacement and aortocoronary bypass operations." Annals of Thoracic Surgery **40**: 224-228.

Mikulin, T., C. S. Powell, et al. (1986). "Relation between blood transfusion and survival in renal adenocarcinoma." British Journal of Surgery **73**: 1036-1037.

Miller, B., U. Kock, et al. (1985). "Do perioperative blood transfusions have an influence on the recurrence rate in patients with breast cancer?" Fortschr Med **103**: 995-999.

Mincheff, M. S. (1993). "Blood transfusion and immunomodulation: a possible mechanism." Vox Sang **65**: 18-24.

Mincheff, M. S. and H. T. Meryman (1995). "Blood transfusion, blood storage and immunomodulation." Imunological Investigations **24**(1&2): 303-309.

Miyazaki, S., T. Akiyoshi, et al. (1983). "Depression of the generation of cell-mediated cytotoxicity by suppressor cells after surgery." Clinical and Experimental Immunology **54**: 573-579.

Moertel, C. G., T. R. Flemming, et al. (1990). "Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma." New England Journal of Medicine **322**: 352-358.

Moffat, L. E., G. T. Sunderland, et al. (1987). "Blood transfusion and survival following nephrectomy for carcinoma of the kidney." British Journal of Urology **60**: 316-319.

Mohandas, K. and L. Aledort (1995). "Transfusion requirements, risks, and costs for patients with malignancy." Transfusion **35**: 427-430.

Monson, J. R., C. W. Ramsden, et al. (1987). "Lymphokine activated killer cells in patients with gastrointestinal cancer." Gut **28**: 1420-1425.

Monson, J. R. T., C. Ramsden, et al. (1986). "Decreased interleukin-2 production in patients with gastrointesinal cancer." British Journal of Surgery **73**: 483-486.

Moores, D. W. O., S. Piantadosi, et al. (1989). "Effect of perioperative blood transfusion on outcome in patients with surgically resected lung cancer." Annals of Thoracic Surgery **47**: 346-351.

Moretta, L., E. Ciccone, et al. (1994). "Human Natural Killer Cells: origin, clonality, specificity, and receptors." Advances in Immunology **55**: 341-379.

Moretta, L., E. Ciccone, et al. (1994). "Origin and functions of human natural killer cells." International Journal of Clinical and Laboratory Research **24**: 181-186.

Moy, P. M., E. C. Holmes, et al. (1985). "Depression of natural killer cytotoxic activity in lymphocytes infiltrating human pulmonary tumours." Cancer Research **45**: 57-60.

Munson, J. L., E. vanTwuyver, et al. (1995). "Missing T cell receptor Vb families following blood transfusion: the role of HLA in development of immunisation and tolerance." Human Immunology **42**: 43-53.

Murphy, P., J. M. Heal, et al. (1991). "Infection or suspected infection after hip replacement surgery with autologous or homologous blood." Transfusion **31**(3): 212-217.

Murphy, W. G., P. Phillips, et al. (1995). "Blood use for surgical patients: a study of Scottish hospital transfusion practices." Journal of the Royal College of Surgeons of Edinburgh **40**: 10-13.

Myhre, B. A. (1990). "The first recorded blood transfusions: 1656-1668." Transfusion **30**(4): 358-362.

Myhre, B. A. (1995). "James Blundell - pioneer transfusionist." Transfusion **35**: 74-78.

Nathanson, S. D., B. C. Tilley, et al. (1985). "Perioperative allogeneic blood transfusion." Archives of Surgery **120**: 734-738.

Naume, B. and T. Espevik (1994). "Immunoregulatory effects of cytokines on natural killer cells." Scandanavian Journal of Immunology **40**: 128-134.

Nicholls, M. D. (1993). "Transfusion: morbidity and mortality." Anaesthesia and Intensive Care **21**(1): 15-19.

Nichols, P. H., C. W. Ramsden, et al. (1992). "Perioperative immunotherapy with recombinant interleukin 2 in patients undergoing surgery for colorectal cancer." Cancer Research **52**: 5765-5769.

Nichols, P. H., C. W. Ramsden, et al. (1993). "Peri-operative modulation of cellular immunity in patients with colorectal cancer." Clin Exp Immunol **94**: 4-10.

Nichols, P. H., U. Ward, et al. (1994). "The effect of 5 fluorouracil and alpha interferon and 5 fluorouracil and leucovorin on cellular anti-tumour immune responses in patients with advanced colorectal cancer." : 946-949.

Nielsen, B., P. Hokland, et al. (1989). "Whole blood assay for NK activity in splenectomised and non-splenectomised hairy cell leukaemia patients during IFN-α–2β treatment." Leukaemia Research **13**: 451-456.

Nielsen, H. J. (1995). "Detrimental effects of perioperative blood transfusion." British Journal of Surgery **82**: 582-587.

Nielsen, H. J., J. H. Hammer, et al. (1989). "Ranitidine prevents postoperative transfusion-induced depression of delayed hypersensitivity." Surgery **105**(6): 711-717.

Nielsen, H. J., J. H. Hammer, et al. (1991). "Comparison of the effects of SAG-M and whole blood transfusion on post-operative suppression of delayed hypersensitivity." Canadian Journal of Surgery **34**: 146-150.

Nielsen, H. J., F. Moesgaard, et al. (1995). "Effect of ranitidine and low-dose interleukin-2 in-vitro on NK cell activity in peripheral blood from patients with liver metastases from colorectal cancer." European Journal of Surgical Oncology **21**: 526-530.

Norfolk, D. R. and L. M. Williamson (1995). "Leucodepletion of blood products by filtration." Blood Reviews **9**: 7-14.

North, R. J., M. Awwad, et al. (1989). "The immune response to tumours." Transplantation Proceedings **21**(1): 575-577.

Nowak, M. M. and J. L. Ponsky (1984). "Blood transfusion and disease free survival in carcinoma of the breast." Journal of Surgical Oncology **27**: 124-130.

Opelz, G. (1985). "Current relevance of the transfusion effect in renal transplantation." Transplant Proceedings **17**: 1015-1021.

Opelz, G. (1989). "The role of HLA matching and blood transfusions in the cyclosporine era." Transplant Proceedings **21**: 609-612.

Opelz, G., D. P. S. Sengar, et al. (1973). "Effect of blood transfusions on subsequent kidney transplants." Transplant Proceedings **5**: 253-259.

Opelz, G. and P. I. Terasaki (1978). "Improvement of kidney-graft survival with increased numbers of blood transfusions." New England Journal of Medicine **299**: 799-803.

Opelz, G. and P. I. Terasaki (1982). "International study of histocompatibility in renal transplantation." Transplantation **33**: 87-95.

Ota, D., L. Alvarez, et al. (1985). "Perioperative blood transfusion in patients with colon carcinoma." Transfusion **25**: 392-394.

Parks, K. G. M., S. D. Heys, et al. (1992). "Recombinant interleukin 2 treatment in patients with metastatic colorectal cancer: effect on natural cytotoxicity." Cancer Immunology Immunotherapy **35**: 53-58.

Parrott, N. R., T. W. J. Lennard, et al. (1990). "Blood transfusion and surgery: effect on growth of a syngeneic sarcoma." Annals of the Royal College of Surgeons of England **72**: 77-81.

Parrott, N. R., T. W. J. Lennard, et al. (1986). "Effect of perioperative blood transfusion on recurrence of colorectal cancer." British Journal of Surgery **73**: 970-973.

Parrott, N. R., R. M. R. Taylor, et al. (1987). "The blood transfusion effect and colorectal cancer recurrence." Transplantation Proceedings **19**: 1475.

Pastorino, U., M. Valente, et al. (1986). "Perioperative blood transfusion and prognosis of resected stage in lung cancer." European Journal of Cancer and Clinical Oncology **22**: 1375-1376.

Patek, P. Q. and J. L. Collins (1988). "Tumor surveillance revisited: natural cytotoxic activity deters tumorigenesis." Cellular Immunology **116**: 240-249.

Peller, S., J. Sayfan, et al. (1994). "Immunological profile changes following perioperative autologous vs. homologous blood transfusion in oncologic patients." Journal of Surgical Oncology **56**: 98-101.

Perkins, H. (1995). "Transfusion reactions: the changing priorities." Immunological Investigations **24**(1&2): 289-302.

Persijn, G. G., B. Cohen, et al. (1979). "Retrospective and prospective studies on the effect of blood transfusions in renal transplantation in the Netherlands." Transplantation **28**(5): 396-401.

Perussia, B., S. Starr, et al. (1983). "Human natural killer cells analysed by B73.1, a monoclonal antibody blocking Fc receptor functions." Journal of Immunology **130**(5): 2133-2141.

Peters, W. R., R. D. Fry, et al. (1989). "Multiple blood transfusions reduce the recurrence rate of Crohns disease." Diseases of the Colon and Rectum **32**: 749-753.

Pinto, V., R. Baldonedo, et al. (1991). "Relationship of transfusion and infectious complications after gastric carcinoma operations." Transfusion **31**: 114-118.

Polk, H., W. Cheadle, et al. (1992). "A randomised prospective trial to determine the efficacy of interferon gamma in severely injured patients." American Journal of Surgery **163**: 191-196.

Pollock, R. E., G. F. Babcock, et al. (1984). "Surgical stress-mediated suppression of murine natural killer cell cytotoxicity." Cancer Research **44**: 3888-3891.

Pollock, R. E., E. Lotzova, et al. (1992). "Surgical stress impairs Natural Killer cell programming of tumor for lysis in patients with sarcomas and other solid tumors." Cancer **70**(8): 2192-2202.

Prado, I. B., A. A. Laudanna, et al. (1995). "Susceptibility of colorectal carcinoma cells to natural killer mediated lysis: relationship to CEA expression and degree of differentiation." International Journal of Cancer **61**: 854-860.

Pross, H. F. and M. G. Baines (1982). "Studies of human natural killer cells. 1. In vivo parameters affecting normal cytotoxic function." J Cancer **29**: 383-390.

Ramsay, A. J., J. Ruby, et al. (1993). "A case for cytokines as effector molecules in the resolution of virus infection." Immunology Today **14**: 155-157.

Robertson, M. J. and J. Ritz (1990). "Biology and clinical relevance of human natural killer cells." Blood **76**(12): 2421-2438.

Roder, J. C., T. Haliotis, et al. (1980). "A new immunodeficiency disorder in humans involving NK cells." Nature **284**: 553-555.

Rosenberg, S. A., C. A. Seipp, et al. (1985). "Perioperative blood transfusions are associated with increased rates of recurrence and decreased survival in patients with high-grade soft-tissue sarcomas of the extremities." Journal of Clinical Oncology **3**: 698-709.

Ross, W. B. (1987). "Blood transfusion and colorectal cancer." Journal of the Royal College of Surgeons of Edinburgh **32**: 197-201.

Salcedo, M., P. Hoglund, et al. (1995). "Natural killer cell interaction with murine allogeneic MHC class I molecules." Transplantation **60**: 281-286.

Salisbury, A. J., J. A. Kinna, et al. (1965). "Circulating cancer cells during excision of carcinomas of the rectum and colon with high ligation of the inferior mesenteric vein." Surgery, Gynaecology and Obstetrics **120**: 1266-1270.

Schantz, S. P., B. W. Brown, et al. (1987). "Evidence for the role of natural immunity in the control of metastatic spread of head and neck cancer." Cancer Immunology and Immunotherapy **25**: 141-148.

Schantz, S. P., B. H. Campbell, et al. (1986). "Pharyngeal carcinoma and natural killer cell activity." The American Journal of Surgery **152**: 467-474.

Schmidt, P. J. (1995). "The bicentennial of transfusion in America." Transfusion **35**: 4.

Schot, J. D. L. and R. K. B. Schuurman (1986). "Blood transfusion suppresses cutaneous cell-mediated immunity." Clinical and Experimental Immunology **65**: 336-344.

Scott, N. and P. Quirke (1993). "Molecular biology of colorectal neoplasia." Gut **34**: 289-292.

Sedman, P. C., C. W. Ramsden, et al. (1988). "Effects of low dose perioperative interferon on surgically induced suppression of anti-tumour immune responses." British Journal of Surgery **75**: 976-981.

Sene, A., S. Walsh, et al. (1993). "Blood transfusion does not have an adverse effect on survival after operation for colorectal cancer." Annals of the Royal College of Surgeons of England **75**: 261-267.

Singh, S. K., R. L. Marquet, et al. (1987). "Promotion of tumour growth by blood transfusion." Transplant Proceedings **19**(1): 1473-1474.

Smith, R. T. (1994). "Cancer and the immune system." Paediatric Clinics of North America **41**(4): 841-850.

Somers, S. (1996). "Surgical implications of tumour immunology." Annals of the Royal College of Surgeons of England **78**: 103-109.

Spence, R. K. (1995). "Surgical red blood cell transfusion practice policies." The American Journal of Surgery **170**(6A): 3-15.

Spits, H., L. L. Lanier, et al. (1995). "Development of human T and natural killer cells." Blood **85**(10): 2654-2670.

Steneker, I., H. K. Prins, et al. (1992). "Mechanisms of white cell reduction in red cell concentrates by filtration: the effect of the cellular composition of the red cell concentrates." Transfusion **32**: 42-50.

Steneker, I., M. J. A. vanLuyn, et al. (1992). "Electonmicroscopic examination of white cell reduction by four white cell reduction filters." Transfusion **32**: 450-457.

Storkus, W. J. and J. R. Dawson (1991). "Target structures involved in natural killing: characteristics, distribution and candidate molecules." Critical Reviews in Immunology **10**: 393-416.

Strayer, D. R., W. A. Carter, et al. (1984). "Low natural cytotoxicity of peripheral blood mononuclear cells in individuals with high familial incidences of cancer." Cancer Research **44**: 370-376.

Stutman, O. (1975). "Immunodepression and malignancy." Advances in Cancer Research **22**: 261-422.

Stutman, O. (1989). Cancer. Natural Immunity Ed. D. S. Nelson. London, Academic Press. 749-794.

Sugezawa, A., N. Kaibara, et al. (1989). "Blood transfusion and the prognosis of patients with gastric cancer." Journal of Surgical Oncology **42**: 113-116.

Takiff, H., M. Novak, et al. (1987). "Examination of the clonal deletion hypothesis folowing transfusions in rat cardiac transplants." Transplantation **43**: 145-151.

Talmadge, J. E., K. M. Meyers, et al. (1980). "Role of NK cells in tumour growth and metastasis in beige mice." Nature **284**: 622-624.

Tartter, P., A. Papatestas, et al. (1983). "Breast cancer recurrence is associated with perioperative blood transfusion." Proceedings of the American Society of Clinical Oncology **2**: 51.

Tartter, P. I. (1988). "Blood transfusion and infectious complications following colorectal cancer surgery." British Journal of Surgery **75**: 789-792.

Tartter, P. I. (1992). "The association of perioperative blood transfusion with colorectal cancer recurrence." Ann. Surg. **216**(6): 633-638.

Tartter, P. I. (1995). "Immunological effects of blood transfusion." Immunological Investigations **24**(1&2): 277-288.

Tartter, P. I. and D. M. Barron (1985). "Unnecessary blood transfusions in elective colorectal cancer surgery." Transfusion **25**: 113-115.

Tartter, P. I., L. Burrows, et al. (1984). "Perioperative blood transfusion adversely affects prognosis after resection of stage 1 non-oat cell lung cancer." Journal of Thoracic and Cardiovascular Surgery **88**: 659-662.

Tartter, P. I., L. Burrows, et al. (1985). "Perioperative blood transfusion has prognostic significance for breast surgery." Surgery **97**: 225-229.

Tartter, P. I., S. Quintero, et al. (1986). "Perioperative blood transfusion associated with infectious complications after colorectal cancer operations." American Journal of Surgery **152**: 479-482.

Thomas, M. J. G. (1996). "Royal College of Physicians, Edinburgh: Final consensus statement. Consensus conference on autologous transfusion." Vox Sanguinis **70**: 183-184.

Thomson, A. R. (1993). "Leucodepleted blood components." Anaesthesia and Intensive Care **21**(1): 39-43.

Tonnesen, H., U. Knigge, et al. (1988). "Effect of cimetidine on survival after gastric cancer." Lancet **2**: 990-992.

Trinchieri, G. (1989). "Biology of natural killer cells." Advances in Immunology **47**: 187-303.

Trinchieri, G. (1992). Natural killer cells in haematopoiesis. The Natural Killer Cell Eds. C. E. Lewis and J. O. McGee. Oxford, Oxford University Press. 41-65.

Trinchieri, G. and P. Scott (1994). "The role of interleukin 12 in the immune response, disease and therapy." Immunology Today **15**(10): 460-463.

Triulzi, D. J., K. Vanek, et al. (1992). "A clinical and immunological study of blood transfusion and postoperative bacterial infection in spinal surgery." Transfusion **32**(6): 517-524.

Tsujihashi, H., H. Matsuda, et al. (1988). "Immunocompetence of tissue infiltrating lymphocytes in bladder tumors." Journal of Urology **140**: 890-894.

Twomey, P. L., W. J. Catalona, et al. (1974). "Cellular immunity in cured cancer patients." Cancer **33**: 435-440.

Vamvakas, E. and S. B. Moore (1993). "Perioperative blood transfusion and colorectal cancer recurrence: a qualitative statistical overview and meta-analysis." Transfusion **33**(9): 754-765.

Vamvakas, E. C. (1995). "Perioperative blood transfusion and cancer recurrence: meta-analysis for explanation." Transfusion **35**: 760-768.

Vamvakas, E. C. (1996). "Transfusion-associated cancer recurrence and postoperative infection: meta-analysis of randomised, controlled clinical trials." Transfusion **36**: 175-186.

Vamvakas, E. C. and S. B. Moore (1994). "Blood transfusion and postoperative septic complications." Transfusion **34**(8): 714-727.

Vamvakas, E. C. and H. F. Taswell (1994). "Epidemiology of blood transfusion." Transfusion **34**: 464-470.

Vamvakas, E. C. and H. F. Taswell (1994). "Long-term survival after blood transfusion." Transfusion **34**: 471-477.

van de Watering, L. M., Hermans, J., et al. (1998). "Beneficial effects of leucocyte depletion of transfused blood on post-operative complications in patients undergoing cardiac surgery: a randomised clinical trial." Circulation **97**(6): 562-568.

vanLawick, W. P., B. L. A. M. Langenhorst, et al. (1988). "Effect of perioperative blood loss and perioperative blood transfusion on colorectal cancer survival." European Journal of Cancer and Clinical Oncology **24**: 741-747.

Vente, J. P., T. Wiggers, et al. (1989). "Perioperative blood transfusions in colorectal cancer." European Journal of Surgical Oncology **15**: 371-374.

Viac, J., R. Bustamante, et al. (1977). "Characterization of mononuclear cells in the inflammatory infiltrates of cutaneous tumours." British Journal of Dermatology **97**(1): 1-10.

Vignali, A., M. Braga, et al. (1996). "A single unit of transfused allogeneic blood increases postoperative infections." Vox Sanguinis **71**(3): 170-175.

Voogt, P. J., J. H. Cornelis, et al. (1987). "Perioperative blood transfusion and cancer prognosis." Cancer **59**: 836-843.

Walton, H. H. (1849). "Remarks, statistical and general on transfusion of blood." The Medical Times : 114-117.

Warner, J. F. and G. Dennert (1982). "Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development and metastasis in-vivo." Nature **300**: 31-36.

Watson, M. A., A. A. Diamandopoulos, et al. (1979). "Endogenous cell mediated immunity, blood transfusion and outcome of renal transplantation." The Lancet : 1323-1326.

Waymack, J. P., K. Gugliuzza, et al. (1991). "Effect of blood transfusion on immune function." Journal of Surgical Research **55**: 269-272.

Weese, J. L., F. D. Ottery, et al. (1986). "Do operations facilitate tumour growth? An experimental model in rats." Surgery **100**: 273-277.

Weiden, P. L., M. A. Bean, et al. (1987). "Perioperative blood transfusion does not increase the risk of colorectal cancer recurrence." Cancer **60**: 870-874.

Welsh, R. M. and M. Vargas-Cortes (1992). Natural killer cells in viral infections. The Natural Killer Cell Eds. C. E. Lewis and J. O. McGee. Oxford, Oxford University Press. 107-150.

Williams, J. G. and L. E. Hughes (1989). "Effect of perioperative blood transfusion on recurrence of Crohns disease." The Lancet **2**: 131-133.

Williamson, L. (1994). "Homologous blood transfusion: the risks and alternatives." British Journal of Haematology **88**: 451-458.

Winnock, M., M. G. Barcina, et al. (1993). "Functional characterisation of liver associated lymphocytes in patients with liver metastasis." Gastroenterology **105**(1152-1158):

Wobbes, T., K. H. G. Joosen, et al. (1989). "The effect of packed cells and whole blood transfusions on survival after curative resection for colorectal carcinoma." Diseases of Colon and Rectum **32**: 743-748.

Wong, L. S., W. J. Bateman, et al. (1995). "Detection of circulating tumour cells with the magnetic activated cell sorter." British Journal of Surgery **82**: 1333-1337.

Wood, M. L., R. Gottschalk, et al. (1988). "Effect of blood transfusion on IL2 production." Transplantation **45**: 930-935.

Wotherspoon, H. A., J. R. Anderson, et al. (1997). "Randomised controlled trial of an H2 receptor antagonist in gastric cancer." British Journal of Surgery **84**: 1168-1169.

Yokoyama, W. M. (1995). "Natural killer cell receptors." Current Opinion in Immunology **7**: 110-120.

Yuichiiwaki, J., M. Cecka, et al. (1988). The transfusion effect. Clinical Kidney Transpl Ed. P. I. Terasaki. Los Angeles, UCLA Tissue Typing Laboratory. 283-292.