The Role of Cellular Adhesion Molecules in Renal Transplantation.

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by

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ABSTRACT

This thesis investigates the role of endothelial cell adhesion molecules in the process of renal transplantation, both by an in-vitro cell culture system using human umbilical vein endothelial cells (HUVECS) and in-vivo by immunohistological analysis of biopsies taken from renal allografts.

In Chapter 1 the clinical problems associated with renal failure and renal transplantation are reviewed as well an historical perspective of renal transplantation and an overview of the immunological problems which may occur in a renal allograft.

The cellular and molecular mechanisms involved in the inflammatory process are reviewed in Chapter 2, with particular reference to the role of endothelial cell adhesion molecules and inflammatory cytokines. In-vivo studies of cell adhesion molecules are also reviewed. In Chapter 3 the materials and methods employed are recorded.

In Chapter 4 studies of endothelial cell adhesion molecule expression as measured by flow cytometry of HUVECS, with or without cytokine stimulation, are presented, showing that different adhesion molecules are expressed under different conditions and at different times.

Chapter 5 investigates two assays for the measurement of polymorphonuclear leucocyte, (PMN), adhesion to HUVECS. A radioactive chromium release assay is used in preference to one employing Rose Bengal dye. The optimum assay conditions are investigated and used to produce a normal range for adhesion of PMN's from normal subjects.

Adhesion of PMN's from renal transplant and haemodialysis patients is compared to that of normals in Chapter 6, showing that PMN's from transplant and haemodialysis patients demonstrate increased adherence to HUVECS.

Chapter 7 investigates the expression and distribution of endothelial

cell adhesion molecules on biopsies taken from renal allografts both pre and post transplantation. The distribution of adhesion molecules within the kidney is related to clinical events and shows that their expression alters in different pathological states.

Chapter 8 is a general discussion of the results of the experimental work.

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Statement of originality

All the work in this thesis is completely original and has not to the best of my knowledge been previously performed or published.

The work in this thesis was carried out by myself in the Department of Surgery transplant laboratory at Leicester General Hospital. I received help in the isolation and culture of human umbilical vein endothelial cells from Ian Underwood and Natu Mistry. The work on the immunohistological staining and scoring was performed by Angela Lycett. Dr Terry Horsburgh helped me develop the flow cytometry and chromium release assay methods as well as with general scientific advice. Carl Edwards helped with statistical analysis and graphics. Sue Massey helped with the typing of the manuscript.

Ethical committee approval was obtained for the collection of tissue samples used for the studies involved in this thesis.

The work has not been submitted, and is not currently being submitted, for any other degree at this or any other university.

M.D. THESIS

THE ROLE OF CELLULAR ADHESION MOLECULES IN RENAL TRANSPLANTATION

Chapter 1. Introduction

Chapter 2. The role of cell adhesion molecules in the inflammatory process.

Chapter One

Introduction

1.1 Historical Perspectives

Little more than thirty years ago, the fate of the patient who developed irreversible renal failure was universal; they died. The advent of dialysis and renal transplantation as forms of renal replacement therapy have allowed many of these patients to survive. The actual numbers of people who die from renal failure is difficult to calculate because many may die in the community without a diagnosis being made. However, it is estimated that about two hundred patients per million of population in the USA die annually from renal failure although this includes deaths from malignancies of the renal tract (Burton and Hirschuan, 1979). The rate of patients presenting in end stage renal failure who are suitable for renal replacement therapy varies from around one hundred patients per million (Luke, 1983) to fifty two patients per million if patients over sixty five are excluded (Pendreigh et al, 1972). There are geographical variations in presentation and other factors which influence how many patients are on renal replacement therapy programmes in different countries, which include attitudes to diabetics, financial implications and political dogma.

In the United Kingdom, the number of patients per million of population treated have risen in the past twenty years dramatically after it was shown that fewer than forty patients per million were on renal replacement programmes, a number similar within the European Community to Greece and the former Yugoslavia and seventeenth overall in Europe (Wing, 1983). Currently around 200 patients per million of population are treated on renal replacement therapy programmes in the United Kingdom. However,

the UK does transplant a large number of patients compared to the rest of Europe (Rosansky and Eggers, 1987).

1.2 Renal Replacement Therapy

As the glomerular filtration rate falls below 10 ml/min the patient starts to become progressively uraemic. The individual patient's profile, including their clinical state, biochemical parameters and social circumstances dictate when renal replacement therapy should start and which modality is most appropriate. In practice, haemodialysis and renal transplantation maintain the largest numbers of patients with end stage renal failure, but peritoneal dialysis is increasingly favoured and haemofiltration is also an effective treatment.

The dialysis of blood from uraemic dogs was first described by Abel in 1913 (Abel et al, 1914), and in humans by Haas in 1925 (Haas, 1925). The development of the modern dialysis membrane, allowing removal of solutes by diffusion across a semi-permeable membrane, was by Kolff in 1944 using cellophane (Kolff and Berk, 1944). Patients could be dialysed using glass cannulae but the vessels were sacrificed after each dialysis and this could only be a very short term option to allow native kidneys to recover from acute renal failure. Longer term haemodialysis was made possible by the introduction of the Scribner Shunt (Quinton et al, 1960). Unfortunately there is a high rate of vessel infection and thrombosis which leads to the shunts failure and limits its usefulness overall. However, it did allow long term renal replacement therapy to become widespread and contributed significantly to the development of renal transplantation.

The development of the arterio venous fistula by Brescia and Cimino in 1966 provided safe and reliable access for haemodialysis (Bresica et al, 1966). In this operation the cephalic vein is anastomosed to the radial artery at the wrist. The time it takes for a fistula to mature, usually

between four to eight weeks, can be covered by the use of modern types of percutaneous subclavian vein catheter. The development of the wrist fistula owed much to the pioneering work of Carrel in the development of vascular suturing techniques earlier in the century (Carrel, 1902).

The principle of dialysis revolves around diffusion of solutes from areas of high concentration (in the blood) to areas of low concentration (dialysis fluid) across a semi- permeable membrane (the dialysis membrane). Thus, uremic toxins, water and electrolytes such as potassium can be removed from the blood across membranes usually of cuprophane or cellulose acetate arranged in parallel plates, hollow tubes or hollow fibre, through which the blood passes and around which the dialysis fluid flows. Many variations on the theme of the artificial kidney are now available to suit individual patient's needs (Hoenich and Kerr, 1983).

An alternative to the removal of solutes by diffusion, as in haemodialysis, is to remove them by convection. This is called haemofiltration and works by slow continuous ultrafiltration of blood. This avoids rapid osmotic shifts and episodes of hypotension. It is used particularly in haemodynamically unstable patients such as those with multiple organ failure or sepsis. A filtrate of blood is obtained by hydrostatic pressure across a differentially permeable membrane with the filtrate running to waste. The filtered blood is then reconstituted with a sterile fluid resembling the lost extracellular fluid but without the uremic toxins (Miller et al, 1990). The system was developed for removal of higher molecular weight toxins cleared poorly by conventional haemodialysis (Quelhorst et al, 1977) and is very efficient. However, it requires the volume of the replacement fluid to be between 20 and 40 litres and it therefore costs twice as much as dialysis. It is particularly useful as treatment for acute renal failure because it can remove large volumes of fluid easily and has improved cardiovascular stability.

The idea of using the peritoneal membrane for dialysis is not quite as old as haemodialysis but nevertheless was accepted in the 1950s (Odel et al, 1950). However, the technology to put the theory in practice did not really appear until the 1970s when Tenckhoff described the use of a silicon rubber catheter with a Dacron cuff which could be implanted into the peritoneal cavity and safely left in situ for long periods of time (Tenckhoff and Schechter, 1968). In 1976, the concept of leaving fluid within the peritoneal cavity for long periods of time, long dwell time, was originated by Popovich (Popovich et al, 1976) and this, allied with the use of polyvinyl chloride bags for storage of dialysis fluid (Oreopoulos et al, 1976) made peritoneal dialysis a reality for many patients. The most usual form is termed continuous ambulatory peritoneal dialysis or CAPD.

The technique first involves the surgical implantation of a permanent transcutaneous silicon catheter into the peritoneal cavity to provide access for fluid exchange. The dialysis is performed by making between three and five exchanges of approximately 2 litres of dialysis fluid each day. Control of fluid and electrolyte balance is essentially along similar principles to haemodialysis. Uremic toxins move from the blood supply in the peritoneal vessels across the peritoneal membrane into the dialysis fluid. Fluid balance is regulated by using various concentrations of glucose in the dialysis fluid, and increased electrolyte clearance from the blood is achieved by reducing the concentration of the electrolyte in the dialysate. This form of dialysis, CAPD, is the main use of the technique although continuous cyclical peritoneal dialysis (CCPD) is also used. In this method the fluid is cycled in and out of the peritoneal cavity automatically by a mechanical device and has the advantage of being available for use at home at night, leaving the patient free from dialysis during the day.

1.3 Renal Transplantation

For suitable patients, a successful renal transplant is often the most satisfactory form of renal replacement therapy. It improves the quality of life for most recipients, allowing much greater freedom from hospitalisation and hospital care. Despite the expensive medication, immunosuppression in particular, required by transplant patients, transplantation is much less expensive than both CAPD or HD, costing between 20-30% of either after the first year has past.

1.3.1 History of Renal Transplantation

The first attempts at renal transplantation date back to the beginning of the century. Experimental work was pioneered by Ullman and Decastello in Vienna, who transplanted the kidney of a dog from the abdomen to its neck vessels and demonstrated some function. They also performed a dog to goat transplant which functioned for a very short time. They also attempted to anastomose a renal graft to the circulation of a uremic patient but failed due to technical reasons (Ullman, 1914).

Carrell, working in Lyon under Jaboulay, established the modern method of vascular suturing in his famous paper of 1902. He went on to work in America performing autografts of kidneys in dogs and cats and showing that allografts could function, albeit briefly. He was awarded the Nobel Prize for the work in 1912 (Carrel, 1902).

The first attempt of transplantation in humans was probably by Jaboulay, implanting kidneys from a pig and a goat to the arm or thigh of a patient with renal failure (Jaboulay, 1906) but no function ensued. Unger in 1909 transplanted a kidney from a primate to a child dying from renal failure, also unsuccessfully (Unger, 1910). Although the transplant failed to function, the procedure represented an advance in that an intracorporeal location was used for the graft and the desirability of genetic similarity

between donor and recipient was recognised.

Because of the lack of success in this work in terms of function, interest waned, although the technical basis of transplantation had been established. In 1949, a Ukranian surgeon, Vorony, reported transplant of human cadaveric kidney to humans, the first being performed in 1936. Technically the operations were successful but again, no function ensued (Voronoy, 1936).

The next advances came in the 1950s with the growth in knowledge in the field of immunology. The immunological basis of rejection was first described by Gibson and Medawar in the 1940s using a skin graft model (Gibson and Medawar, 1943). They showed that skin grafts between syngeneic mice were accepted permanently, while allogeneic grafts stimulated an immune response in the host and were consequently destroyed (rejected), one to three weeks after grafting. They also showed that mice injected at birth with allogeneic cells would subsequently accept permanently a skin graft from the same donor strain and that this state of unresponsivness, the first experimental demonstration of immunological tolerance, could be abolished, with consequent rejection of the skin graft, by injection of host strain lymphocytes from a normal mouse or from one that had previously rejected a graft from the allogeneic strain. Lymphocytes from the latter mouse induced more rapid and intense graft rejection, showing that, as a result of previously rejecting an allograft, it had developed persistant immunity, manifested by the reactivity of its lymphoid cells. This early work suggested the importance of cell mediated immunity in allograft rejection. Simonsen, experimenting with dog kidney transplants, reported on the mechanism of kidney rejection in this model (Simonsen et al, 1953). Dempster, in 1953, demonstrated that the positioning of a renal allograft in the pelvis was preferable to a superficial site and demonstrated that an immunological mechanism was responsible for failure. He also found that

whole body irradiation of the transplant recipient could delay rejection, but not prevent it altogether (Dempster, 1953).

The modern transplant era began with grafts being performed at the Peter Bent Brigham Hospital, Boston by David Hume and colleagues (Hume et al, 1955), without immunosuppression. Hume used haemodialysis to prepare the patients and concluded that previous blood transfusion might be beneficial and that host bilateral nephrectomy was necessary for control of post-transplant blood pressure. Small doses of steroids were used but not thought to be significant in graft survival. In 1954 the group performed the first transplant between identical twins, one suffering from renal failure. This was the first human transplant to function and more twin to twin grafts were subsequently performed with good results (Murray et al, 1976). Dempsters work led to attempts at immunosuppression in a variety of centres between 1959 and 1962 using whole body irradiation, which led to a limited number of successes but major problems in the recipients due to overwhelming infection. In 1958 doctors transplanting bone marrow as a treatment for leukaemia, unhappy with the success rate of irradiation, reasoned that anti-cancer drugs should be immunosuppressive. 1959 saw the publication of a paper showing depressed immune responses in rabbits treated with 6 mercaptopurine (6-MP), an anti cancer drug (Schwartz and Dameshek, 1959). This work was taken up by Roy Calne at the Royal Free Hospital, London,who showed that 6-MP extended graft function in a dog renal allograft model where irradiation had failed to do so (Calne, 1960) and later the use of a derivative of 6-MP called BW57/322, now known as azathioprine, was shown to be suitable for human use. Though originally azathioprine was used alone, Starzl in Denver reported improved results by combining azathioprine with prednisolone and by using living donors (Starzl et al,1963). Transplantation was seen to be successful and led to the adoption of this regime as a standard therapy and greatly encouraged the

furthur development of transplantation.

Along with the development of azathioprine, methods of tissue typing first came into use in 1962 (Hamburger et al, 1962), improving graft survival. Dausset described an antigen MAC, later known as HL-A2, identified by a number of antisera from multi-transfused patients. This was later shown to be part of the major histocompatibility complex in man (HLA), (Dausset, 1980). In 1966 Kissmeyer-Nielsen demonstrated that the use of a direct crossmatch between donor cell and recipient serum could lead to a marked reduction in the number of cases of antibody mediated hyperacute rejection at the time of transplantation thus improving graft survival (Kissmeyer-Nielson et al 1966). The parallel development of tissue matching and immunosuppressive therapies led to an extension of graft survival times, the wider choice of donor recipient combinations and the safe use of cadaveric kidneys. The 1970s saw the establishment of renal allografts as a acceptably safe and effective form of renal replacement therapy.

The next two major advances came towards the end of the 1970s, first was the successful application of HLA-DR typing to renal transplantation (Ting and Morris, 1978) and second was the use of cyclosporin. Cyclosporin was the first immunosuppressive agent to rival azathioprine for twenty years and its success was proved again by now Prof. Roy Calne showing greatly increased efficacy over azathioprine and prednisolone. Cyclosporin has come to replace the original regime either alone, or in combination with prednisolone and/or azathioprine (Calne et al, 1978).

The results of renal transplantation have improved dramatically in the last thirty years. Initially both patient and graft survival were both near zero. Now a patient survival of 95% or more at one year with graft survival of 90% can be expected in the best centres. These results are despite the fact that patients with high risk concurrent medical conditions

are being transplanted compared to years ago, when they might have been considered unfit for transplantation. Diabetics in particular come into this category. Much of the success is due to better pre operative preparation of the patient and of the donor kidney. However, problems do occur and these fall into three main categories: technical problems, infectious problems and immunological problems.

There are very many technical complications associated with renal transplantation and this is beyond the scope of this discussion to detail them all. They include thrombosis or rupture of the renal artery (Belzer et al, 1972), renal artery stenosis (Rankin et al, 1977), thrombosis of the renal vein (Merion and Calne, 1985), formation of lymphocele (Schweizer et al, 1972), ureteric sloughing and urinary fistula (Lieberman et al, 1982, Glass et al, 1982), ureteric stenosis (Solinger et al, 1986), and bladder leaks,

The success of renal transplantation depends on a compromise between achieving sufficient immunosuppression to avoid rejection of the graft and maintaining a level of immune competence sufficient to protect the recipient from infection. In the early years of transplantation, the instance of severe and lethal infection was high and discouraging, but in recent years a compromise has gradually been reached so that cadaveric renal transplantation now offers equivalent patient survival to haemodialysis. The problem of infection, however, remains of considerable concern and contributes substantially to the mortality and morbidity of renal transplantation. Although most infections in the renal transplant recipient are caused by common pathogens, devastating opportunistic infections occur sufficiently frequently to require a multi-disciplinary approach involving infectious disease experts and sophisticated microbiological backup (Cohen et al, 1978).

Patients are susceptible to infection for many reasons. They are

already uremic and therefore immunosuppressed from that cause. They are often anaemic and wasted due to protein restriction. They undergo a major surgical procedure and are left with many ports of entry for infection such as urinary catheters, wound drains and intravenous catheters. The graft itself may be pre-contaminated during harvesting. The immunosuppressive drugs then broadly suppress immune competence. Other risk factors are age, diabetes, neutropenia and hepatitis (La Quaglia et al, 1981).

It is beyond the scope of this discussion to detail all the specific infections that renal transplant patients can contract. The reader is referred to a textbook of microbiology and virology.

The immunological response to a renal allograft can be seen in terms of three types of rejection:

Hyperacute rejection

This occurs within a short time after the kidney has been reperfused. The kidney may initially become pink and apparently be functioning well but within an hour or so it will become poorly perfused, oedematous and cease to function. This is due to the presence of preformed antibodies and these recipient's serum against antigens on the graft. Antibodies bind to the vascular endothelium, fix complement and cause intravascular thrombosis. Polymorphonuclear leucocytes infiltrate tissue causing necrosis (Morris and Ting, 1982). There is no treatment and the graft should be removed.

Hyperacute rejection is now very unusual in clinical practice because of the routine procedure of pre-transplant crossmatching. Recipient serum and donor cells are combined to detect any preformed antibodies before the graft is implanted. If the crossmatch is positive then antibodies are present and the transplant should not proceed.

Acute rejection

This can occur any time from the first few days post transplant onwards. It is uncommon after 12-24 weeks but can occur at any time if

immunosuppression is stopped. The symptoms and signs of acute rejection have been greatly modified by the use of cyclosporin. Without immunosuppression acute rejection causes the characteristic features of malaise, pyrexia, tachycardia and hypertension. The graft is tender and swollen and there is a reduction in urine output with a worsening of all biochemical parameters of renal function. With the use of cyclosporin rejection episodes may be 'silent' apart from a steady deterioration in biochemical function. The diagnosis should be made by needle biopsy of the graft using ultrasound guidance followed by histological examination. Other investigations may help to exclude the other common causes of graft dysfunction. Ultrasound scanning can establish if any obstruction to outflow is present. Radioisotopes scanning can establish if the graft perfusion has altered suggesting an arterial inflow problem. Arteriography may be indicated if a renal artery stenosis is suspected. However, histological examination of a biopsy from the transplant kidney remains the best way to diagnose acute rejection.

The histological features of acute rejection fall into two main categories: acute cellular rejection and acute vascular rejection. In cellular rejection large numbers of cytotoxic T-lymphocytes invade the interstitium of the graft, infiltrating and destroying renal tubules. In vascular rejection the endothelium becomes activated with adherent lymphocytes seen on the cell surface. There is vascular necrosis and haemorrhage into the kidney substance. Both cellular and vascular rejection may be present together in particularly severe cases. Untreated acute rejection will lead to graft loss quite quickly, but with prompt diagnosis and treatment only minimal damage may occur.

Treatment of acute rejection is by an increase of immunosuppression. Commonly, 0.5 gm of methylprednisolone is given intravenously for three days. A high dose of oral steroids may be used instead. Monoclonal

antibodies (OKT3, Cilag UK) or polyclonal antibody preparations (Anti Thymocyte Globulin [Merieux UK] or Anti Lymphocyte Globulin [Fresenius UK]) are also widely used for the treatment of steroid resistant acute rejection and are very effective although have a higher incidence of side effects notably increased risk of infection and de novo malignancy. Most cases of acute rejection are reversible with accommodation of the available anti rejection drugs. However, large increases in immunosuppression leave the patient open to infections which may be life threatening in their severity.

Chronic rejection

This is a much less well defined process than hyperacute or acute rejection. Clinically there is a gradual deterioration in renal function which may occur some years after transplantation. Biopsy and histology is essential to make the diagnosis although other investigations will often be necessary to rule out other causes of dysfunction. Histologically there is a characteristic obliteration of the vascular tree. There is progressive expansion of the interstitium by fibrous tissue and arterioles have become thickened and obliterated. The glomeruli can be partially or completely obliterated. There is no treatment for chronic rejection. Other causes of gradual renal impairment such as cyclosporin toxicity and other drug interactions should be considered (Matas et al, 1983).

Immune Response to Allografts

In clinical practise the host immunological response to a transplanted organ is still a major problem, despite the use of immunosuppressant drugs such as cyclosporin, azathioprine and prednisolone. The immediate response to an allograft, as established by Medawar in experiments on rabbit skin grafts, is very rapid necrosis of the allograft (Gibson and Medawar, 1943). This is prevented in renal transplantation by accurate pre transplant tissue typing and cross matching to avoid placing a graft into a recipient with preformed circulating antibody against the

organ, and by the use of immunosuppression. However, despite prophylactic immunosuppression, an immunological response causing the clinical picture of acute rejection still often occurs in the first few weeks and months post transplant, and often requires additional immunosuppression. Later, insidious immunologically based damage to the graft leads to the clinical situation of worsening renal function accompanied by histological changes of chronic rejection (Matas et al, 1983).

The immunogenicity of the renal allograft is determined by the major histocompatibility complex antigens (MHC) which are glycoproteins present on the cell surface. The MHC antigens in man are termed the HLA or human leucocyte antigens and subdivided into Class I and II antigens based on function and expression. Class I is divided into three types A, B and C. Class II is divided into three types DR, DP, DQ, with considerable polymorphism between the groups (Klein et al, 1981). Separate minor histocompatibility antigens also exist which may have an effect on eventual graft outcome as shown by animal studies (Fabre and Morris, 1975).

Class I molecules are expressed on almost all nucleated cells and are responsible for the activation of CD8 positive T lymphocytes. Class II molecules are less widely distributed normally but can be expressed by most cell types under suitable cytokine stimulus; they recognise CD4 positive T lymphocytes. For an immune response to be initiated the donor MHC antigens must be presented to the recipient immune system. This presentation is usually performed by macrophages or dendritic cells of the recipient, which present the antigen on their cell surfaces in association with the MHC molecules. Only small fragments of the whole antigen are required to stimulate sensitised T-cells (Grey et al, 1984). Once the T-cell is stimulated, it then produces cytokines such as interleukin 2 which causes maturation of the cytotoxic T-cell and interleukin 4, which is required for growth and differentiation of B lymphocytes into antibody producing cells

(51). Gamma interferon is also produced and this stimulates MHC Class II production along with other functions. The majority of T helper cells have the CD4 phenotype and interact with Class II antigens. CD8 lymphocytes are a similar population which may react with Class I to provide T cell help (Meuer et al, 1982).

The role of specific cell types in the acute or chronic rejection process is the subject of much current research and the role for cytotoxic T cells has probably the strongest body of evidence to support it. The cytotoxic T-cell response was examined in the in-vitro system of the mixed lymphocyte reaction (MLR) where lymphocytes that are MHC mismatched are mixed and found to proliferate in response to one another producing cells that specifically lyse the other cell type (Hayry and Defendi, 1970). This in-vitro model has been questioned with some workers finding that anti-donor specific cytotoxic T-cell transfusions accelerate graft destruction. (Englers et al, 1982) with other workers demonstrating the existence of donor specific cytotoxic T-cells to be present in non-rejecting grafts (Dallman et al, 1987). CD4+ve and CD8+ve cells have been implicated in graft rejection in experiments showing that mouse skin grafts can be prolonged by giving monoclonal antibodies which act against both cell types with an additive effect being demonstrated (Cobbold et al, 1984).

Vascular endothelium may be a primary site for antigen presentation in the process of rejection since it can express Class I and Class II MHC antigens and the vascular changes that occur during rejection are well documented. Parenchymal cells may also be a target for tissue destruction, possibly secondary to the vascular attack. In chronic rejection, there are marked arterial changes leading to obliteration of the vascular lumen and this also suggests that the endothelium is a main target for initiation of this process.

The immune response to a tissue allograft is a very complex

phenomenon both in the manner in which the graft antigens are recognised and in the response to this recognition which results in graft damage. The process is becoming clearer and it is possible to describe the phenomenon of rejection even if the precise relationship of the many factors involved remain to be defined (Dallman and Morris, 1978).

The end point of immune sytem activation in response to allografted organs is tissue damage or destruction. The role of preformed antibodies to foreign MHC molecules in the tissue destruction that occurs after immune system activation in hyperacute rejection is well recognised. The role of antibody in the process of acute or chronic rejection is less well defined but the changes of acute rejection such as arterial wall destruction, thrombosis and interstitial haemorrhage may be antibody led. Immune mediated inflammation the primary histopathological change seen in organs undergoing acute allograft rejection and the inflammatory process will be the subject of discussion in the next chapter.

Chapter Two

The Role of Cell Adhesion Molecules in the Inflammatory Process.

2.1 Historical Perspective.

The changes in the tissues during acute inflammation were first described by Celsus (30BC - AD38). He recognised cardinal signs of calor or heat, rubor or redness, tumor or swelling and dolor or pain. A fifth sign functio laesa or limitation of movement has been added to Celsus' original observations. Historically this fifth sign was attributed to Galen who lived between 130 and 200 AD. However, it has become apparent that Galen never added a fifth sign (Rather, 1971), but it was in fact added by Virchow in Cellular Pathology published in 1858 (Virchow, 1865). Whilst the macroscopic changes associated with the inflammatory process are well established, the microscopic changes are the subject of much recent investigation.

An explanation of the changes during acute inflammation has been provided using microscopic studies. The first to employ such techniques was Cohnheim (1889). Using light microscopy he observed changes in the living transparent tissue of the frog's tongue and foot web during inflammation caused by mechanical injury or chemical irritation. He noted that the inflammatory reaction is composed of a number of phenomenon, all of which involve the small blood vessels in the inflamed tissue. Indeed, the first observable events of the inflammatory process were haemodynamic changes (Muir 1982).

The next major advance in this field came with the invention of the electron microscope. Marchesi and Flory (1960), and later Flory and Grant (1961), described experiments performed on rat mesentery and rabbit ear. Flory and Grant used ultraviolet radiation to inflame the thin tissue over

the central table of a transparent chamber in rabbits' ears and this provoked an acute inflammatory response. They observed the living ear with a light microscope, and having noted that leucocytes were adhering in large numbers to the walls of the vessels in the ear, the tissues were fixed using osmium tetroxide. The tissue was then excised and fixed to be viewed on an electron microscope. They made the following observations:

"With the light microscope polymorphs were seen during life to adhere to the walls of the vessels. At first the cells appeared to be attached to a sticky surface along which they were pushed slowly by the force of the bloodstream. They became deformed and appeared somewhat pear-shaped because the portion in contact with the endothelium did not move freely. In some time they became firmly fixed to the wall and a few emigrated into the surrounding tissues. Using the electron microscope, leucocytes were seen to come into contact with the endothelium and for some reason adhere, becoming flattened on the side in contact with the endothelium. In some instances, leucocytes appear to be moving on the inner surface of the endothelium by amoeboid motion. When close apposition is attained, the leucocyte puts out pseudopodia which can deform the endothelium. The endothelial cytoplasm may be pushed aside over a wide area while the leucocyte penetrates down to the basement membrane of the endothelial cells. The present observations do not make it clear why leucocytes adhere to inflamed endothelium. There is no electron-dense substance in the narrow space between endothelial and adherent leucocyte but occasionally there is a dense area near the endothelium, sometimes in the angle formed by endothelium and leucocyte which could be considered to represent cement. Since the dense material is seen so rarely, it seems unlikely to have anything to do with sticking."

In conclusion, Flory and Grant found no clear morphological evidence

that the surface of the endothelium becomes sticky, although they did occasionally see electron-dense material in relation to the endothelium. They concluded that their observations still left unanswered the question why leucocytes adhere to endothelium in the first instance.

In the 1970s techniques for isolating leucocytes from whole blood were refined and this allowed detailed study of neutrophils, monocytes and lymphocytes. It was discovered that under certain circumstances the adhesivity of these cells in-vitro could be increased, for instance, after stimulation by various chemical mediators. At this time it was thought that the endothelial cells lining blood vessels were entirely passive in the inflammatory process and that leucocytes were solely responsible for the observable changes. This was despite the fact that ultrastructural examination of endothelial cells in areas of contact dermatitis showed that endothelial cells became plump and protruded into the lumen. The cells were shown to display quantities of biosynthetic material such as endoplasmic reticulum and Golgi apparatus, and on the basis of this, appearances were described as activated, implying a functional consequence to the altered morphology (Willms-Kretschler et al, 1967). However, because it was, at that time, extremely difficult to study isolated endothelial cells, the view of the endothelium as a potentially dynamic layer lapsed and in general the endothelium was considered passive in the inflammatory process. In this view, "activation" could be dismissed as a morphologic manifestation of the response to injury (Pober 1988).

A major advance was made in 1973 when Jaffe and co-workers described a method for isolation and long-term in-vitro culture of endothelial cells derived from human umbilical vein (Jaffe, 1973). This was an adaptation of an earlier method by Maruyama, who had cultured endothelial cells from umbilical veins without being able to characterise the cells in culture (Maruyama, 1963). Gaffe demonstrated that cultured endothelial cells could

be grown as monolayers and characterised by the electron microscopy appearance of characteristic cytoplasmic inclusions called Weible-Palade bodies (Weibel and Palade, 1964).

In the early 1980s, as the technique of human umbilical vein endothelial cell culture became accepted and refined, many different groups started to explore the potential of this in-vitro cell line. Other pathological techniques also became available which aided in the study, in particular the production of monoclonal antibodies. This allowed the new techniques of radioimmunoassay, FACs (Fluorescence Activated Cell sorting) analysis, immunoelectron microscopy and immunoprecipitation to be applied. Using these techniques Pober and Gimbrone (1982) showed that endothelial cells cultured under standard conditions expressed Class I major histocompatibility complex (MHC) antigens but not Class II antigens. This led very rapidly to a return to the concept of the endothelium as having a dynamic role to play in the interactions between circulating cells and tissues. Pober (1988) has defined endothelial activation as "quantitative changes in the level of expression of specific gene products (ie proteins) that in turn endow endothelial cells with new capacities that cumulatively allow endothelial cells to perform new functions." Further work soon followed showing that Class II MHC antigens could be expressed on endothelial cells in response to the cytokine gamma interferon (IFN g) whether produced by recombinant gene technology or by peripheral blood lymphocytes. As other cytokines have been produced they too have been shown to be capable of inducing endothelial activation, in particular the interleukin family; Interleukin 1 a, Interleukin 1 b, Interleukin 2, Interleukin 6, Interleukin 8, Interferon a, Interferon b, tumour necrosis factor and Lymphotoxin.

The most recent step forward in the investigation of the cellular events involved in inflammation has been the discovery of specific cell

adhesion molecules. These are now known to be present on both leucocytes and endothelial cells. The first to be identified was endothelial leucocyte adhesion molecule E-Selectin (Bevilacqua et al, 1987). Soon afterwards another endothelial cell adhesion molecule was detected, ICAM-1 (intercellular adhesion molecule) was shown to be the ligand for a similar adhesion molecule on lymphocytes (Marlin and Springer, 1987). In the very short time since these discoveries have been made, numerous other adhesion molecules have been identified, both on leucocytes and endothelial cells. The response of endothelial cells to different cytokine stimuli has now been widely investigated, particularly with regard to the effect on adhesion molecule expression. In-vitro work has helped define the role of adhesion between leucocytes and endothelial cells in the process of inflammation. However, it is clear even from this work, that different types of inflammation may proceed by different pathways ie the inflammatory response to an invading bacterial pathogen is different to that mounted against, for example a transplanted organ. Clearly to look at the process as a whole, in-vitro studies can only have a limited usefulness. Much less in-vivo work has been carried out in this field as yet. However, the amount of in-vivo work is rapidly increasing, not least because of the recognition that the endothelium is a potential commercial target for diagnostic and therapeutic intervention (Pober and Cotran, 1990).

2.2 Overview of the cellular and molecular mechanisms involved in the inflammatory process

The following is an account of the mechanisms by which the inflammatory response is currently thought to be generated and regulated. I have broken these mechanisms down into sections for ease of description, but clearly the process is a dynamic one, and all of these processes occur in an overlapping fashion. It should be stressed that the complete picture

is still far from clear, especially in the areas of cytokine generation and the interactive pathways between leucocytes and endothelial cells. However, much has been learned in the past few years in these areas and further information becomes available as time goes by.

The events that lead to leucocyte migration from the blood into the tissues in response to an extra-vascular stimuli which constitutes the inflammatory response can be simply summarised as follows:

In an area of tissue trauma caused by mechanical damage, bacterial invasion or presence of some other noxious stimuli, cytokine signals are generated by circulating T lymphocytes which activate the adjacent endothelium, primarily on post capillary venules. Circulating phagocytes are also activated. As a consequence of activation, one or both cell types become adhesive (Schulz and Harlan, 1988). The increased adhesiveness of these cell types allied with local vaso-motor changes allows leucocytes to adhere to the endothelium. They are seen to leave the main vascular flow and marginate along the vessel wall. Very rapidly, usually within minutes of the triggering stimuli, leucocytes can be seen rolling along the surface of the endothelial cell (Hogg 1992). The leucocyte then ceases to roll, becomes firmly attached to the endothelium and then diapedeses between the junctions of endothelial cells, finally emigrating through the sub-endothelial matrix to participate in the inflammatory reaction (Alison et al, 1955). Dead, damaged or foreign tissue can subsequently be removed by the action of phagocytic cells which produce degrading enzymes, toxic oxygen products and cytokine stimulators. Although essential for host defence and repair, phagocyte adherence and emigration may sometimes have pathological consequences (Harlan 1985).

At different times during the inflammatory response, the leucocyte sub-population involved will change. This also is determined by the type of stimuli responsible for initiating inflammation. Neutrophils are seen

to be the predominant cell type during the first few hours after the onset of inflammation (Issekutz and Movat, 1980). Later, mononuclear phagocytes become the most common cell type in the affected tissues peaking within 12-24 hours after stimulation (Paz and Spector, 1962) If the inflammatory stimuli is immune in basis, for example, an organ transplant, then lymphocytes will be the predominant cell type. The different kinetics of emigration and subsequent accumulation in tissues of leucocytes may be explained in part by differences in expression or configuration of adhesion molecules on the leucocyte or the endothelium (Carlos and Harlan, 1990).

2.3 Cell Adhesion molecules involved in endothelial cell and leucocyte adhesion

Three main groups of adhesion molecules are recognised which mediate the interaction of leucocytes and endothelial cells:

(1) The selectins, which are dominant in lymphocyte and neutrophil interaction with vascular endothelium (Springer, 1990)

(2) The immunoglobulin super-family, which includes the antigen specific receptors of T and B lymphocytes

(3) The integrin family, which is important in dynamic regulation of adhesion and migration

2.3.1 The Selectins

The selectin family consists of three similar single-chain integral membrane glycoproteins. The three molecules so far identified have a similar structure and have been called (i) E-selectin (ii) L-selectin (iii) P-selectin (Table 1.1, Fig 1.1). The N-terminus of the mature protein consists of a region of approximately 120 residues homologous to a family of calcium-dependent lectins (Drickamer, 1988). This domain is succeeded by an epidermal growth factor (EGF) - like motif similar to those in the EGF precursor and in many other proteins, including coagulation proteases.

There then follows a series of short consensus repeats, each containing about 62 residues that are related to those in complement regulatory proteins such as CR1 and C4B binding protein (Hourcade, 1989). The number of short consensus repeats varies between these molecules which gives them their individuality. L-selectin has two repeats; E-selectin has six repeats; P-selectin has nine repeats. Each molecule ends with a hydrophobic trans-membrane region followed by a short cytoplasmic tail. All three of the molecules in the selectin family are heavily glycosylated, consistent with the presence of numerous potential sites for addition of N-linked oligosacharides in the amino acid sequence (Bevilacqua et al, 1987, Johnson et al, 1989a, Siegelman et al, 1986).

Table 1.1 The Selectin Family of adhesion molecules

SELECTIN	CD ANTIGEN DESIGNATION	EARLIER NAMES	EXPRESSED BY	TARGET CELL
L SELECTIN	-	MEL-14 LAM-1 LEU-8 TQ-1 LECAM-1 LEC.CAM-1 DREG.56	PMN's. Monocytes. Lymphocyte Subsets.	Lymph node high Endothelial venules. Activated Systemic EC's.
E SELECTIN	-	ELAM-1	Cytokine Activated EC's.	PMN's. Monocytes. Memory T--lymphocytes.
P-SELECTIN	CD62	GMP140	Rapidly Activated EC's. Platelets.	PMN's. Monocytes. Lymphocyte Subsets.

PMN - Polymorphonuclear Leucocyte
EC - Endothelial Cell
CD - Cluster Differentiation

The selectins are asymmetric molecules consisting of a number of cysteine-rich, cross-linked modular domains (McEver, 1991). The amino acid sequences of the lectin-like and EGF-like domains of the selectins are over 60% identical and the consensus repeats share over 40% of sequence identity. The sequence relationships and organisation of the conserved domains strongly suggest that the selectins arose as a result of exon duplication and rearrangement. This is supported by analysis of the human genes encoding each protein (Ord et al, 1990, Johnson et al, 1990). The human genes for all three selectins are tightly clustered in a region spanning no more than 30 KB at bands Q21-24 on the long arm of chromosome 1. This tight clustering is also present on the equivalent region of mouse chromosome 1, indicating that, for unknown reasons, the genes have remained physically associated over evolution from at least mouse to man (Watson et al, 1990). Interestingly the human genes of the complement regulatory protein family, which encode consensus repeats related to those in the selectins, are quite closely clustered on the long arm of chromosome 1 at band Q32 (Hourcade et al, 1989).

2.3.2 Selectin Receptors

The receptors which recognise selectins have not yet been fully characterised. However, recent work has provided insights into the nature of these receptors. The selectin group were postulated to recognise carbohydrate ligands because the NH2 terminal domains have a lectin motif (Stoolman,1989, Springer, 1990, Bevilacqua et al 1989). A survey of the glycolipids and N-linked carbohydrate groups of surface glycoproteins of neutrophils revealed uncommon terminal sequences with a (1,3) Fucose residues known generically as Lewis X (Le^x) and sialylated Lewis X(SLe^x) as shown in Figure 1.2 (Laurie-Phillips et al, 1990).

L-selectin is present on leucocytes but its endothelial receptor is

still unknown. One receptor (or possibly several receptors) for L-selectin is expressed on cultured human endothelial cells treated with cytokines (Polley et al, 1991, Hallman et al, 1991, Spertini et al 1991, Smith et al, 1991). The receptor for L-selectin on lymph node high endothelial venules may be the sulphated, fucosylated, sialylated glycoprotein - SLe^x (Zimmerman et al, 1992). L-selectin may interact with E-selectin (Kishimoto et al, 1991), which is expressed on cytokine activated endothelial cells. However, this may not be a unique interaction because temporal expression of a counter-receptor for L-selectin on tumour

FIG 1.1 The Selectin Family.

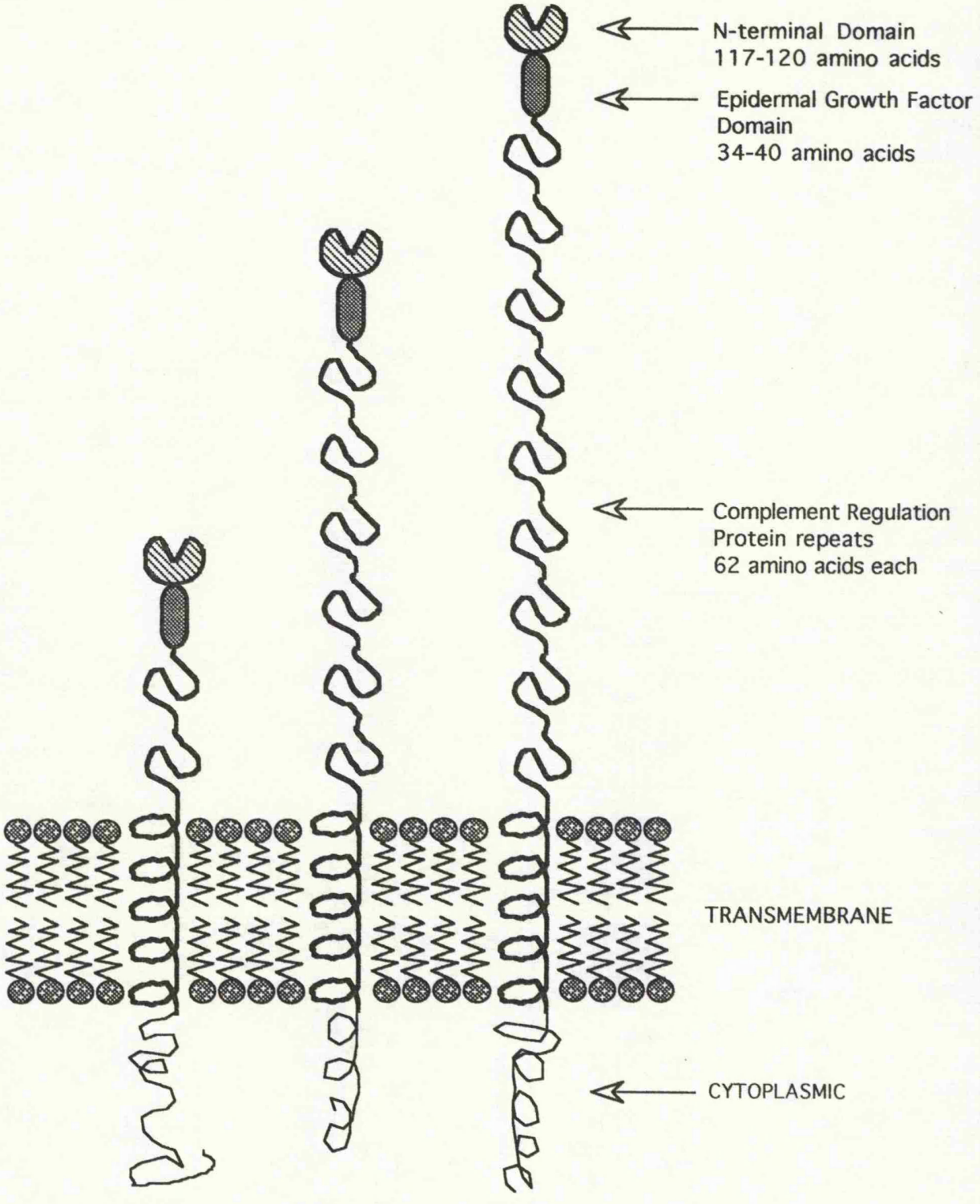
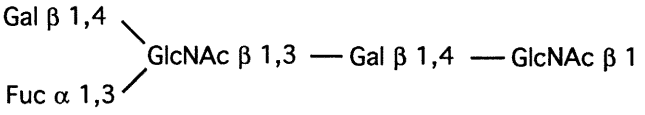
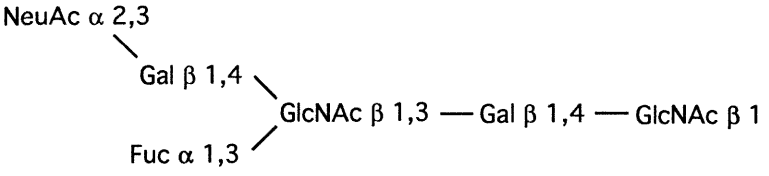


Fig 1.2 Carbohydrate Ligands for Selectins

Lewis x (CD 15)



Sialylated Lewis x



Legend

Gal - Galactose
Fuc - Fucose
GlcNac - N-Aceyl-Glucosamine
NeuAc - Sialic acid

necrosis factor a (TNF a) activated endothelial cells is different from E-selectin expression (Spertini et al, 1991). Under some conditions, the contribution of L-selectin to activated leucocyte binding is apparent only if shear is applied (Hallman et al, 1991). L-selectin has been implicated in neutrophil migration from the circulation into tissues (Smith et al, 1991).

A large amount of in-vitro work exists characterising the properties of E-selectin and P-selectin in cultured human umbilical vein endothelial cells. The properties of L-selectin have also been extensively studied, particularly as a lymphocyte homing receptor in murine models (McEver, 1991).

2.3.3 L-Selectin

L-selectin is expressed by approximately 70% of circulating leucocytes (Tedder et al, 1990), and may function as a leucocyte re-circulation receptor. In fact, it was first recognised as a homing receptor that mediates adhesion of murine leucocytes to high endothelial venules of peripheral lymph nodes using a monocolonal antibody, Mel 14, which blocks this adhesion. The human structure of this molecule is thought to be identical (Tedder et al, 1989, Gallatin et al, 1983). L-selectin is present on neutrophil cell surfaces and ex vivo can provoke adhesion of bone marrow neutrophils to inflamed high endothelial venules (Jutila et al, 1989). L-selectin is also expressed by monocytes but since neither monocytes nor neutrophils re-circulate through peripheral lymph nodes, there may be structural and function differences between the myeloid L-selectin and lymphoid L-selectin molecules. It may also be that accessory molecules not present on myeloid cells are required for emigration into peripheral nodes (McEver, 1991). The amino acid sequence of human L-selectin is identical in all cells (Ord et al, 1990). Recent

in-vivo data suggest that L-selectin may promote adhesion of neutrophils to endothelium at sites of inflammation (Jutila et al, 1989). In-vitro, adherence requires activation of endothelium with cytokines such as IL-1 or tumour necrosis factor (Smith et al, 1991).

Whilst the role of L-selectin in neutrophil migration is not clear (Spertini et al, 1991) it is probable that L-selectin and the b2 integrins (see below) may act together in targeting neutrophils to inflamed tissues. Reagents that block either molecular system inhibit neutrophil adhesion to cytokine activated endothelial cells (Tedder et al, 1990). A soluble L-selectin chimera inhibits neutrophil accumulation at extra vascular sites in-vitro (Watson et al, 1991). Evidence that L-selectin is important in the initial adhesion of neutrophils to endothelial cells comes from work showing that L-selectin mediates rolling of neutrophils on endothelial cells (Ley et al, 1991, Von Adrian 1991). Rolling may be the first adhesive interaction in inflammation, but the endothelial cell counter-receptor that interacts with L-selectin during rolling has not yet been identified. In response to local concentrations of chemotactic factors or signals expressed by activated endothelial cells further changes take place in the neutrophil during the rolling process which enhances the avidity of adhesion, probably via b 2 integrin up-regulation. The initial binding mediated by L-selectin may be critical for the b 2 integrin component to work under flow conditions (Zimmerman et al, 1992).

L-selectin is rapidly shed, probably by proteolytic cleavage, from both lymphocytes and neutrophils after cellular activation (Tedder et al, 1990, Jutila et al, 1989). Rapid shedding of L-selectin occurs after stimulation by phorbol esters or IL-8 from lymphocytes and neutrophils. Soluble L-selectin can influence L-selectin mediated adhesion in lymphocytes, inhibiting adhesion up to 20% (Schleiffenbaum et al, 1992).

In summary L-selectin is present on a wide variety of leucocytes and

mediates the initial adhesive interaction between these cells and endothelial cells. Their exact counter-receptors are still unknown, and the mechanisms of interaction with other adhesion molecules, both before and after shedding from the cell surface, are the subject of further research.

2.3.4 E-Selectin

E-selectin is also known as ELAM or endothelial leucocyte adhesion molecule. It is expressed on the luminal surface of endothelial cells and serves to bind leucocytes via the Sialyl-Lewis X (SLe^x) carbohydrate residue, as described above.

E-selectin has been extensively studied in-vitro using human umbilical vein in endothelial cells, and to a much lesser extent in-vivo.

E-selectin is not present on unstimulated human umbilical vein endothelial cells and its induced expression requires new RNA and protein synthesis (Bevilacqua et al, 1989 and 1987). Induction of mRNA for the protein is preceded by activation of a transcription factor (Montgomery et al, 1991). Cytokines which are known to stimulate endothelial cells in culture to express E-selectin are tumour necrosis factor a, Interleukin-1, or lipopolysaccharide (Bevilacqua et al 1985, Pober and Cotran, 1990). Surface expression of E-selectin requires approximately 1 hour, is maximal at 4-8 hours, and is over by 24-48 hours in-vitro. Polymorph adhesion to endothelial cells parallels this time course with the important exception that there is residual enhanced adhesion after E-selectin disappears from the surface (Springer, 1990). Monoclonal antibodies against E-selectin inhibit neutrophil adhesion to cytokine activated endothelial cells and some leukemic cell lines (Bevilacqua et al, 1995). The role of E-selectin in the adherence of monocytes to cytokine stimulated endothelial cells remains to be determined (Springer, 1990).

Recent studies also show that E-selectin mediates activation independent adhesion of memory CD4+ve T cells (Shimizu et al, 1991a), and the T cell E-selectin ligand may be preferentially, if not exclusively, expressed on memory T cells rather than naive T cells. This adhesion can occur without activation of the memory T cell with agents such as 12-O-tetradecanylphorbol13-acetate (TPA). E-selectin has also been shown to be a tissue selective endothelial cell adhesion molecule involved in the homing of a unique skin associated subset of circulating memory T cells defined by the expression of the cutaneous lymphocyte associated antigen (Picker et al, 1991).

E-selectin has been shown to be present in-vivo at sites of acute inflammation (Cotran et al, 1986). It is particularly found in post capillary venules, the principle sites of leucocyte extravasation during inflammation (Hession et al, 1990). Clearly since E-selectin has been shown to bind neutrophils, monocytes and memory T lymphocytes it must play different roles in different types of inflammation. The induction of adhesion molecules such as E-selectin on human umbilical vein endothelial cells by inflammatory cytokines provides a mechanism for the selective attachment of lymphocytes to endothelium and their subsequent migration into the site of tissue injury. As there are few activated T cells in the circulation, adhesion pathways must exist that allow inflamed endothelium to capture circulating resting T cells. The binding of memory but not naive T cells to E-selectin suggests that the E-selectin pathway not only plays a part in the preferential migration of memory cells into inflammatory sites, but may also contribute to the differential recirculation patterns of naive and memory T cells (Shimizu et al, 1991). In contrast, neutrophil trafficking via E-selectin into areas of tissue inflammation caused by, for example, invasion of a bacterial pathogen, is a case of E-selectin acting in response to a stimulus rather than in the case

of memory T lymphocytes acting as an aid to routine immunological surveillance.

After expression there is evidence that E-selectin is shed from the cell surface, possibly by proteolytic cleavage. A soluble form of E-selectin has been detected in the supernatant of cultured endothelial cells after stimulation with Interleukin 1. It has also been detected in the serum of patients with a variety of chronic inflammatory disease such as polyarteritis nodosa, giant cell arteritis, and scleroderma. However, serum levels of soluble E-selectin were not greater in patients with recent onset or active disease, and only weakly increased in patients with multiple organ system involvement (Carson et al, 1993).

Soluble E-selectin has also been found in the blood of septic patients, probably reflecting a generalised upregulation of E-Selectin in the acute inflammatory state (Newman et al, 1993).

2.3.5 P-selectin

P-selectin is also known as GMP-140 or CD62. It is in an integral membrane glycoprotein of 140 kds. It is stored in the Weible Palade bodies of endothelial cells (Hatton et al, 1989, Bonfarti et al 1989, McEver et al, 1989) and is also present in the alpha granules of platelets (Stenberg et al, 1985, Berner et al, 1986). In-vivo, P-selectin is present in platelets and their precursor cells, megakaryocytes, and in endothelium of post capillary venules (McEver et al 1989). Unlike E-selectin, P-selectin is constitutively expressed in normal non-inflamed tissues (McEver et al, 1989). P-selectin is rapidly mobilised to the surface of endothelial cells after stimulation by products of the clotting cascade such as thrombin or histamine (McEver and Martin, 1984, Hsu-Lin et al 1984). This redistribution is caused by fusion of the granule membranes with the plasma membrane, a process which may take between seconds and minutes. In

cultured endothelium, induced surface expression of P-selectin is transient and returns to basal levels by 20-60 minutes as a result of endocytosis (Hatton et al, 1989).

P-selectin binds to neutrophils and monocytes but not to lymphocytes (Gene et al, 1990, Larsen et al, 1989). Neutrophils bind specifically to purified P-selectin and to cells transfected with DNA encoding P-selectin (Gene et al, 1990). P-selectin supports the adhesion of neutrophils and monocytes to thrombin stimulated platelets (Hamburger and McEver, 1990) and to endothelium stimulated with agonists such as histamine (Gene et al, 1990). If P-selectin is radio-labelled, it can be shown to bind specifically and reversibly to a considerable number of receptors on neutrophils and monocytes (Moore et al, 1991). All of these interactions may be blocked by monoclonal antibodies to P-selectin. The expression of P-selectin on endothelium activated with histamine or thrombin suggests that is it involved in the very rapid adhesion of neutrophils and monocytes to vascular endothelium exposed to these agonists. The fact that activated platelets express P-selectin on their surface suggests that this is a mechanism by which neutrophils and monocytes may be recruited to the inflammatory response in sites of haemorrhage or thrombosis (Henry, 1990). The binding of neutrophils to endothelial cells via P-selectin appears to require the cooperation of platelet activating factor (PAF) (Lorant et al, 1991). The incorporation of P-selectin into model membranes mediates rolling of neutrophils, which is the first step towards firm binding and migration of leucocytes into the sub endothelial tissues (Lawrence and Springer, 1991).

A form of P-selectin lacking the transmembrane domain exists which makes this a soluble protein (Johnston et al, 1989b). Soluble P-selectin is found in the plasma of normal individuals (Dunlop et al, 1992). It is possible that this form is secreted by endothelial cells or platelets under

various conditions and that it binds to leucocyte receptors in the fluid phase. However, experimental work in this area is contradictory with some workers reporting that a solublised P-selectin inhibits leucocyte adhesiveness whilst others report that solublised P-selectin does not inhibit leucocyte adhesiveness to endothelial cells in-vitro (Gamble et al, 1990, Wong et al, 1991).

2.3.6 Immunoglobulins - Introduction

A large number of adhesion molecules known to be involved cell to cell interactions have been characterised, and many have turned out to be members of the immunoglobulin gene super family of proteins (Williams, 1989). The group includes major histocompatibility antigens Class I and II, the T cell receptor designated CD3 as well as other T-cell receptors designated CD4 (T helper lymphocyte) and CD8 (T killer lymphocyte). Lymphocyte functional antigen 2 (CD2) and lymphocyte functional antigen 3 (CD58) are also included in this family. However, only three or possibly four members of the immunoglobulin super-family have shown to be important in terms of endothelial cell leucocyte adhesion. These molecules are designated inter-cellular adhesion molecule 1 and 2 (ICAM-1 and ICAM 2) and vascular cell adhesion molecule 1 (VCAM). A further molecule ICAM 3 has recently been identified (De-Fougerolles and Springer, 1992). ICAM-1 is also designated CD54 (Springer, 1990) (Table 1.2).

2.3.7 Immunoglobulin Superfamily adhesion receptors

Members of the immunoglobulin super-family share common structural features first defined for antibodies. The usual immunoglobulin domain is composed of 90-100 amino acids arranged in a sandwich of 2 sheets of anti parallel b strands, which are usually stabilised by a disulphide bond at its centre (Williams and Barclay, 1988, Alzari et al, 1988). Loops of

amino acids connecting this framework of b strands usually define the specificity of the molecule for its cognate ligand. Within the immunoglobulin super-family domains can have one of three configurations designated C1, C2 or V, depending on the arrangement of particular highly conserved amino acids. Most adhesion molecules of the Ig super-family contain C2 domains (Lobb, 1992). The immunoglobulin cell receptors, which are specialised for antigen recognition, are the only known members of this family with variable regions that undergo somatic diversification (Figure 1.3).

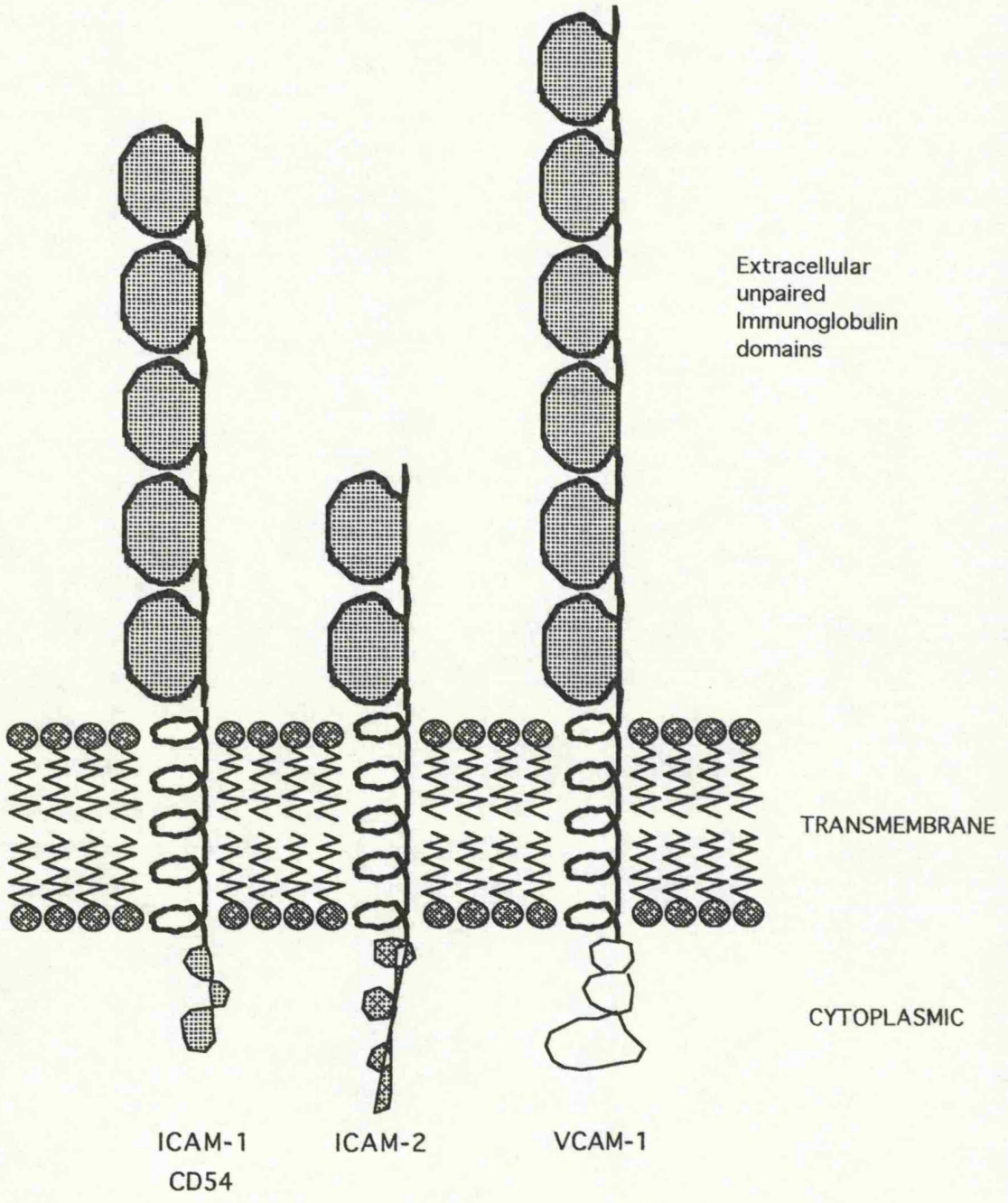
The function of molecules of the immunoglobulin superfamily in adhesion, in evolutionary terms, predates specialisation for antigen recognition, which occurs only in vertebrates. Immunoglobulin super-family members are present in insects as nervous system adhesion molecules involved in adhesion and vesiculation (Shaw and Luce, 1987). The immunoglobulin domain may have diversified and been adapted so widely in evolution because of the stability of the disulphide bonded b strand structure. An interesting feature of the adhesion molecule group within the immunoglobulin family is that in contrast to immunoglobulins and MHC molecules, which have paired immunoglobulin domains, the domains of ICAM and VCAM are unpaired (Staunton et al, 1989).

ICAM-1 is a heavily glycosylated cell surface protein of molecular weight about 90 kds containing 5 tandem extra-cellular Ig-like domains. Only the first two domains are required for function however (Rothlein et al, 1986). Electron micrographs of ICAM-1 show that it is a bent rod 18.7 nm long. This is only compatible with a model in which its five domains are

Table 1.2 Immunoglobulin Family of Adhesion Molecules.

Immunoglobulin	Cell Type	Expression	Ligand	Cell Type
ICAM-1	EC	Constitutive and cytokine induced	CD11a/CD18 CD11b/CD18 CD11c/CD18	All Leucocytes
ICAM-2	EC	Constitutive no cytokine effect	CD11a/CD18	All Leucocytes
ICAM-3	Neutrophils Monocytes Lymphocytes	Cytokine induced	Unknown	Endothelial cell.
VCAM	EC	Cytokine induced	VLA-4	Lymphocytes

Fig 1.3 Immunoglobulin Superfamily



unpaired, and arranged end to end at a slight angle to the b strands (Simmons et al, 1988). Immunoglobulin-like domains are required for function. ICAM-1 interacts with the leucocyte adhesion integrin LFA-1 (see below).

ICAM 2 is an integral membrane protein with two immunoglobulin-like domains in contrast to the five domains of ICAM-1 (Staunton et al, 1989). ICAM 2 is very closely related to the two most N-terminal domains of ICAM-1 (35% identity) and these two molecules bare much greater resemblance to each other than they do to other molecules within the immunoglobulin superfamily, demonstrating the existence of sub-family of immunoglobulin-like ligands that bind the same integrin receptor (Staunton et al, 1989).

VCAM is the third immunoglobulin superfamily adhesion receptor which has a role in endothelial cell leucocyte adhesion. It was identified using an expression cloning technique which confirmed that it belonged to the immunoglobulin family and it was therefore designated Vascular Cell Adhesion Molecule 1 (VCAM) (Osborne et al, 1989). VCAM is a glycosylated cell surface molecule of molecular weight about 110 Kd, containing either six or seven extra-cellular immunoglobulin like domains, generated by alternate splicing. Only the first two domains of VCAM are required for its adhesive function (Osborne et al, 1989)(Figure 1.3).

2.3.8 Functions of immunoglobulin-like adhesion molecules

ICAM-1 is expressed at low levels of human umbilical vein and endothelium in-vitro and on endothelium in-vivo (Dustin et al, 1986). Surface expression of ICAM-1 on cultured endothelial cells is increased by tumour necrosis factor a, Interleukin 1, lymphotoxin and Interferon- gamma (Pober et al, 1986a, Pober et al, 1986b, Pober et al, 1987, Renkonen, 1989); Phorbol Ester (Lane et al,1989) or Lipopolysaccharide (Pober et al,

1986b). In contrast, ICAM2 is constitutively expressed on human umbilical vein endothelial cells, but unlike ICAM-1, its expression is not increased by LPS (Staunton et al, 1989). VCAM is minimally expressed on unstimulated human umbilical vein endothelium but is rapidly induced by recombinant tumour necrosis factor a, recombinant IL-1b and Lipopolysaccharide. In contrast to ICAM-1, VCAM is not induced on dermal fibroblasts or arterial muscle cells after stimulation with recombinant tumour necrosis factor, or on keratinocytes after stimulation with recombinant interferon-gamma (Carlos et al, 1990).

The leucocyte receptors for the immunoglobulin adhesion molecules are members of the integrin family, CD11A/CD18 (LFA1) receptor recognises ICAM-1 and ICAM2. Whilst the CD11B/CD18 (MAC1) receptor only recognises ICAM-1 (Diamond et al, 1990, Diamond et al, 1991). ICAM-1 may also bind the CD11C/CD18 (P150/95) molecule (Stalker and springer, 1991). The ligand for VCAM is the very late antigen 4 molecule (VLA/4) (Shimizu et al, 1991b). Further discussion of these interactions with reference to the cell types involved can be found in the section under Integrin Function (see below).

2.3.9 Integrins

The term integrin was proposed in 1986 to describe a family of integral membrane receptors thought to link or integrate the cytoskeleton of one cell with that of another cell or with the extracellular matrix (Hynes, 1987). The role of integrins in the internal cytoskeleton of the cell is much less clearly defined than the role of the integrins in cell to cell adhesive interaction (Hogg, 1989). The integrin family consists of a series of heterodimers involved in a variety of cell adhesion functions. Almost all cell types express these structures and integrins extend through most of the phylogenic tree. These receptors have several structural and

functional homologies so that it is believed that they differentiated from a common ancestral gene (Rusolatili and Pierschbacher, 1987, Dejana, 1991). Only four of the integrin group have so far been shown to be directly involved in the adhesion of the leucocytes to endothelial cells.

All of the integrins are composed of two non-covalently linked sub-units called the alpha and beta sub-unit, and both of these are transmembrane proteins. The alpha chain is the larger of the two with a molecular weight ranging from 150,000 to 200,000 in non-reduced conditions compared with the smaller beta chain with a molecular weight of 90,000 to 110,000 in non reduced conditions. Both the alpha and beta chains present a small C terminal cytoplasmic domain and a large N terminal extracellular domain with a short transmembrane segment (Hogg, 1991).

There were previously thought to be three main groups of integrins based on the sharing of a common beta sub unit (beta 1, 2 or 3) by various alpha sub units. However, it is now known that at least eight different beta chains have been characterised and genomic analysis suggests that there may be at least fifteen beta sub units. In addition, at least thirteen different alpha chains have been described so far. Since it is known that many different combinations of alpha and beta sub unit can exist, this is potentially a large family of similar molecules. Since new alpha and beta chains are likely to be discovered the original classification using just the beta 1, 2 and 3 chains has clearly become insufficient and any subsequent classification would be necessarily considered provisional. However, from the point of view of adhesion of leucocytes to endothelial cells, the original classification is still of use since it encompasses all the molecules involved (Hogg, 1991). The three main groups under this system are:

1 Beta 1 (CD29) or the VLA proteins

2 Beta 2 (CD11/CD18 family) or the leucocyte integrins

3 Beta 3 (CD61) or the cytoadhesins.

Since the leucocyte adhesion molecules all fall into the beta 1 and beta 2 groups, I will concentrate primarily on these.

The beta 1 family molecules are also known as the VLA (very late activation) antigens because two of them, VLA1 and VLA2 , appear on lymphocytes 2-4 weeks after antigenic stimulation in-vitro (Hemler, 1990). VLA4 (CD49D/CD29) is an unusual beta 1 integrin expressed on resting lymphocytes, monocytes and neural crest derived cells and functions as both a matrix and cell receptor (Hemler, 1990). VLA4 is the only beta 1 integrin involved in cell adhesion.

The beta 2 integrin group comprises three molecules that are all involved in the adhesion of leucocytes to endothelial cells. They are:

1 CD11A/CD18 or LFA1 (lymphocyte function associated antigen 1)

2 CD11B/CD18 also known as MAC1 (macrophage 1)

3 CD11C/CD18 also known as P150-95

Each of the three beta 2 integrins is constitutively expressed on the neutrophil plasma membrane and their absence in certain heritable deficiencies causes severe deficiency of neutrophil adhesion (Zimmerman et al, 1992, Anderson and Springer, 1987), with other fatal consequences in childhood (Table 1.3).

Table 1.3 Integrin family of Adhesion Molecules.

INTEGRIN	CELL TYPE	LIGAND	CELL TYPE
CD11a/CD18	All	ICAM-1	Endothelial cell
LFA-1	Leucocytes	ICAM-2	Endothelial cell
CD11b/CD18	Neutrophils	ICAM-1	Endothelial cell
Mac-1	Monocytes	Fibrinogen	Thrombosis cascade
β_2 Family	(Myeloid cells)	Factor X	Thrombosis cascade
	Large Granular Lymphocytes	iC3b	Complement cascade
CD11c/CD18	Neutrophils	iC3b	Complement cascade
p150-95	Monocytes	ICAM-1	Endothelial cell
β_2 Family	Tissue macrophages		
VLA-4	Lymphocytes	VCAM	Endothelial cell
β_1 Family	Monocytes		
CD29	Eosinophils		
	Basophils		

2.3.10 Integrin structure

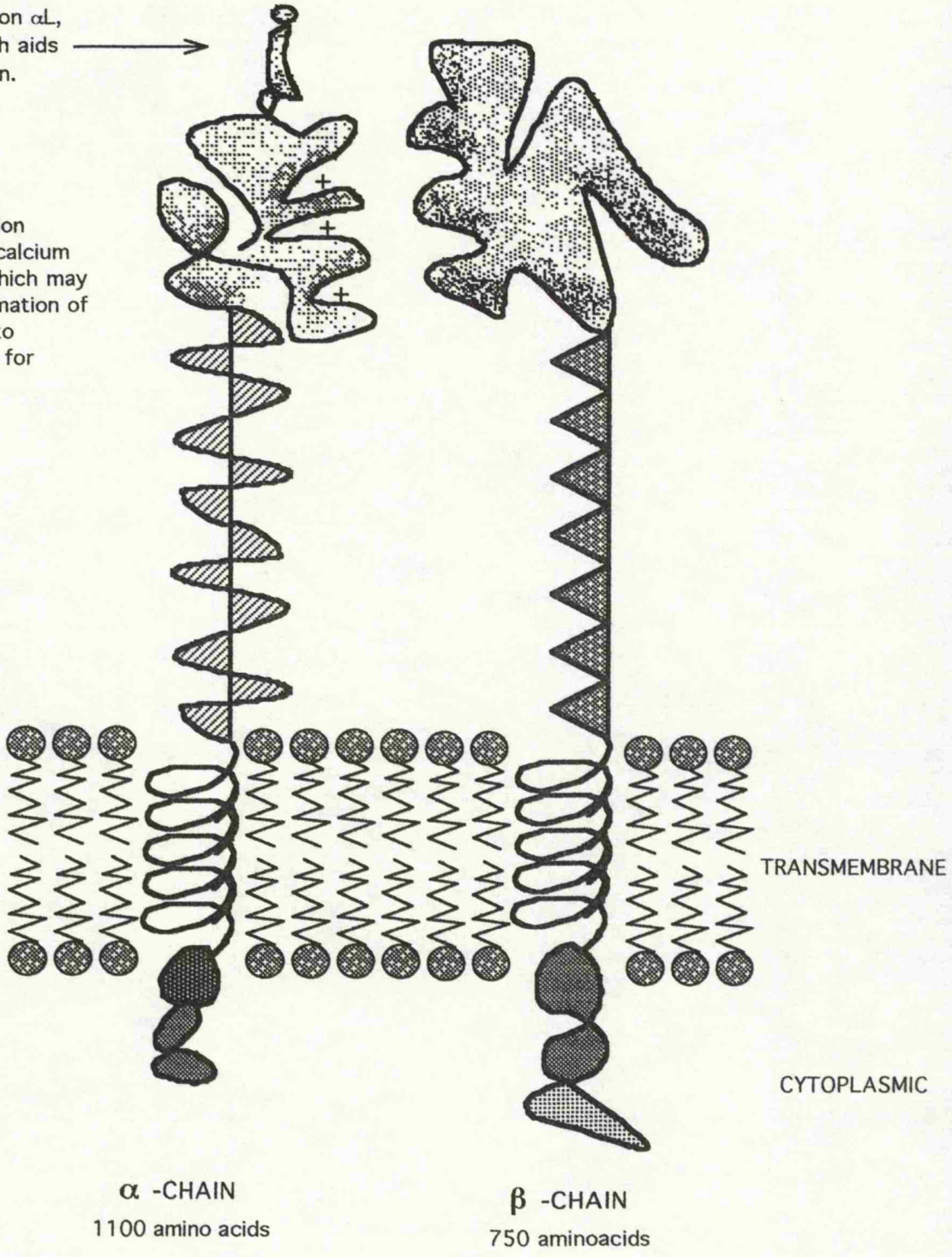
The alpha sub-unit is made up of an amino acid chain which has 25% homology between the various known alpha chains. This identity can increase to between 45 and 63% between sub units of the same group. A key feature of the alpha sub unit is seven homologous tandem repeated sequences (I-VII) of approximately 65 amino acids found at the N terminus. Three (V-VII) or four (IV-VII) of these domains (depending on the particular alpha sub unit) contain putative divalent cation binding sites (Hogg, 1991). As integrin function is either magnesium or calcium dependent, the divalent cations must exert their effects by binding to these sites. Different chains use different cations, for example, CD11A/18 binds magnesium whereas VLA5 binds calcium (Staatz et al, 1989). Some integrins including the beta 2 family and VLA1 and 2 also contain an inserted or I-region of about 200 amino acids located between repeated domains 2 and 3. These domains are similar to those found in other proteins such as Von Willebrand factor, cartilage matrix protein and complement factor (Kishimoto et al, 1989, Larson et al, 1989). The fact that these domains bind to collagen, cartilage proteoglycan or complement component C3b suggests that the I-region may also be involved in similar ligand binding (Hogg, 1991).

The beta chains that have so far been characterised present a relatively high amino acid sequence homology between 37-48%. The best preserved areas appear to be in the cytoplasmic and transmembrane domains. There is a highly cross-linked four domain sequence containing 37-56 cysteines within a stretch of 190 amino acids (Hogg, 1991). Studies of the beta 3 chain have demonstrated that the amino-terminal region is folded into a large disulphide bonded loop formed between cysteines near the amino terminus and those of the first cysteine which repeat (Beer and Coller, 1989). There is also an area of high homology at the end terminus

which is thought to participate in alpha beta heterodimer formation. It is also suggested that an association of the amino terminal globular domains of the alpha and beta sub-units form the extracellular ligand binding regions of the receptor (Albeda and Buck, 1990, D'Souza et al, 1988) (Figure 1.4).

Some integrins, but not all of them, recognise in their ligand a sequence of amino acids which form a tripeptide ARG-GLY-ASP (RGD amino acid code) (Hogg, 1991). This sequence is

Fig 1.4 The Integrin family



αLβ2 = CD 11a/CD 18 or LFA-1

αMβ2 = CD 11b/CD18 or Mac-1

αXβ2 = CD11/CD 18 or p150,95

α5β1 = CD49d or VLA-4

present on many proteins although not all are recognised by an integrin receptor. Proteins involved include fibrinogen, vitronectin, fibronectin, thrombospondin, Von Willebrand factor, and also non adhesive proteins such as thrombin (Oldberg et al, 1988, Bar-Shavit et al, 1991).

Apart from the beta 1, beta 2 and beta 3 chains, additional beta chains have been recently sequenced (Hemler, 1990, Ginsberg et al, 1988). The beta 4 chain (Kennel et al, 1989) the beta 5 chain (Hemler, 1990) and the beta 6 chain (Sheppard et al, 1990) have been sequenced and at least three additional beta chains have been described although not sequenced; Beta p in lymphocytes (Holzmann et al, 1989), the beta chain of the melanoma laminin receptor (Kramer et al, 1989) and the beta 3b in macrophages (Krissansen, 1990).

It can be seen that the potential for diversity within the integrin family is large and therefore members of the group are expected to play a part in widely different cellular adhesive functions.

2.3.11 Integrin function

The cells which express the integrin molecules associated with cellular adhesion can be seen in Table 1.3 along with ligands on endothelial cells. It can be seen that the surface adhesion molecules on endothelial cells that are recognised by the integrins are in fact the immunoglobulin molecules ICAM-1, ICAM2 and VCAM (Springer, 1990). CD11A is found on all leucocytes but CD11B and CD11C are in general confined to the cells of the myeloid lineage. Because CD11C is most highly expressed by tissue macrophages its activities have been more difficult to investigate (Hogg, 1991). The VLA 4 integrin is present on lymphocytes, monocytes, basophils and eosinophils but not neutrophils.

For neutrophils the CD11B sub unit primarily mediates stimulated neutrophil binding to endothelium (Harlan et al, 1985) although the CD11A

subunit also contributes to adherence (Forsyth and Levinsky, 1989). In the case of monocytes all three CD11 molecules have been implicated in binding to endothelium (Arnaout et al, 1988). Monocytes can also bind to VLA4 and this may also be one of the major lymphocyte trafficking pathways (Shimizu et al, 1991).

Differences clearly exist in the mechanisms by which the integrin group of adhesion molecules increase or decrease cellular affinity, in terms of the ways in which cell adhesion can be influenced using their different binding pathways. The functional status of the immunoglobulin ligands ICAM-1, ICAM2 and VCAM also have a part to play in influencing the process. In the case of CD11A (LFA1) changes in cell adhesion due to binding of this molecule have been shown to be due to qualitative rather than quantitative change in the cell surface expression of the molecule. The fact that no new molecules need to be expressed on the surface of the leucocyte in order for increased adhesion to occur is logical because it is known that adhesion by cytotoxic T-cells can be regulated over a very short timescale; they can adhere to target cells, deliver a lethal hit, detach and engage with another target cell, with a cycle time as short as 1-5 minutes (Poenie et al, 1987). Therefore a rapid mechanism for increasing the adhesive potential of the cell is required. We know that increased expression of ICAM-1, the ligand for CD11A, after cytokine induction is detectable in-vitro or in-vivo after 4-6 hours and is maximal by 9-24 hours (Munro et al, 1989). This makes it a candidate for initiating a slower more sustained increase in adhesion rather than the rapid increase that is required in other inflammatory situations. Leucocytes treated in-vitro with phorbol ester show an increased adhesion to endothelial cells with no demonstrable upregulation of either ICAM-1 or CD11A (Rothlein et al, 1986). This rapid upregulation is mediated by CD11A and can be explained by experiments showing that stimulating resting T-lymphocytes with phorbol

esters, or cross-linking the T-cell receptor with monoclonal antibodies converts cellular CD11A from a low to a high affinity state with no change in surface density (Dustin and Springer, 1989). Affinity peaks 5-10 minutes after stimulation of the T-cell receptor (TCR) and returns to resting values by 30 minutes. The transience of the high affinity state provides a mechanism for regulating lymphocyte adhesion. Contact of T-cell receptors with cells bearing specific antigen generates intracellular signals that lead to the conversion of CD11A to a high affinity state and regulates CD11A/ICAM-1 dependent adhesion in an antigen specific manner. CD11A is a flexible adhesion molecule controlled by the TCR (Springer, 1990).

As previously suggested the binding of divalent cations is crucial for integrin activation. It appears that calcium is bound to inactive CD11A and must be displaced as a prelude to induction of an activated integrin. This suggests that calcium and magnesium may have critical and differing effects on the confirmation of the receptor. This mechanism has implications for the rolling of leucocytes on the endothelial surface whilst the selectin group of molecules mediate the rapid attachment and detachment of leucocytes to endothelium. The subsequent strong binding is mediated via the integrin molecules, a mechanism by which binding of a leucocyte to an endothelial cell via a selectin pathway subsequently increases the avidity of the integrin receptor on the leucocyte may exist. It is also likely that the tight binding of the integrin via its ligand in some way loosens the adhesivity of the selectin pathway thus allowing deadhesion of the leucocyte and movement into the sub endothelial tissues (Springer, 1990).

Intracellular storage pools for CD11B and CD11C have been demonstrated in phagocytes (Bainton et al, 1987, Lacal et al, 1988, Miller et al, 1987), but no cytoplasmic storage has been identified for CD11A.

Neutrophil and monocyte expression of CD11B and CD11C increases following the stimulation by a number of factors, but the surface expression of CD11A is not appreciably increased by stimulation. This suggests that increased leucocyte binding mediated by CD11A may occur in a different fashion to binding mediated through CD11B and 11C. Stimulants which have been shown to increase the neutrophil and monocyte expression of CD11B and CD11C include calcium ionophore, phorbol ester, and fMLP (f-met-leu-phe), with the latter two being involved in regulation of phosphorylation of the beta 2 subunit (Freyer et al, 1988, Vedder and Harlan, 1988, Chatila, 1990, Buyon et al, 1990). Other stimulators include GM-CSF (Lopez et al, 1986, Devereux et al, 1989, Solinski et al, 1988), C5a and TNF (Lo et al, 1989a, Gamble et al, 1985), TDGF and LTB4 (Miller et al, 1987) and increases in ambient temperature (Dransfield et al, 1986, Schleiffenbaum et al 1898). However, increased surface expression of CD11b may not actually cause increased adherence. Increased surface expression of CD11b has not necessarily led to increase in the adherence of neutrophils to endothelium monolayers in-vitro (Lo et al, 1989b). It has been shown that treatment of neutrophils with recombinant human GM-CSF produces an increase in surface expression of CD11b but does not augment adherence to endothelium (Lopez et al, 1986). In addition, increased adhesion can occur without increased surface expression of CD11b. If an anion channel blocking agent is used to inhibit surface expression of CD11b neutrophils can still show increased adherence to endothelium (Vedder and Harlan, 1988). While these findings do not exclude a contribution of upregulation to increased neutrophil adhesiveness, they indicate that increased surface expression is neither necessary nor sufficient for increased adherence to endothelium. It seems likely that increased adhesiveness results primarily from alterations in the conformation of CD11b/CD18 (Carlos and Harlan 1990). CD11b has other roles apart from adherence to cytokine activated endothelium via the ICAM-1

receptor (Smith et al, 1989, Diamond et al, 1990). It plays a role if phagocytosis via iC3b-coupled immune complexes (Ross et al, 1989). CD11b also acts directly as a receptor for pathogens. It binds zymosan, the yeast cell wall polysaccharide (Ross 1985), lipopolysaccharide from the bacterial outer membrane (Wright et al, 1989) and the haemagglutinin of bordetella pertussis (Relman et al, 1990).

The CD11c molecule is the least well understood of the beta 2 integrins. Its endothelial cell receptor is as yet unknown. It is known that CD11c present on tissue culture matured monocytes behaves like a receptor for complement products in that it has specificity for IC3b (Myones et al, 1988). It has been shown to play a role in adherence of monocytes to endothelium in-vitro (Stacker and Springer, 1991).

The binding of VLA4, the fourth integrin involved in cell adhesion, to the endothelial cell ligand VCAM has been shown by several groups (Vennegoor et al, 1992, Rice et al, 1990). VLA4 plays a major part in the trafficking of lymphocytes into the tissues but is not present on neutrophils (Rice et al, 1991). This has been demonstrated using a T cell line, cultured in-vitro, which does not carry any of the beta 2 integrins on its surface. Adhesion of this cell line to cytokine stimulated endothelial cells can still occur, thus demonstrating a beta 2 integrin independent pathway (Vennegoor et al, 1992). However, the effect of cytokines on the VLA/VCAM pathway has been less fully investigated and it is not known whether VLA4 adhesivity can be enhanced in a similar way to the beta 2 integrin group.

2.4. Cytokines in the inflammatory response

2.4.1 Introduction

Inflammatory reactions are almost inevitably associated with specific types of leucocyte infiltration. Since the identity of the leucocyte and

degree of the infiltration depend on the nature of the stimulus and temporal state of the lesion, it follows that some cytokines should be specific for a given type of leucocyte (Matsushima et al, 1992). Various types of leucocyte and endothelial derived cytokines have been identified since the early 1970s (Verghese et al, 1989). Advances in biotechnology have allowed purified natural or recombinant cytokines to be available in large quantities, which has allowed many reproducible studies to be carried out, both in-vitro and in-vivo. The one drawback to this work is that comparison of the in-vivo inflammatory response, where all the relevant cytokines are working together, to the in-vitro situation where only one or two cytokines can be studied at one time, may not be strictly accurate. Again, in-vivo experiments involving large non-physiological doses of injectable cytokines may not be relevant to the individual cytokine actions during inflammation. The following is an account of those cytokines which are known to play a role during inflammation, particularly with respect to the adhesive properties of endothelial cells and leucocytes.

Cytokines are regulatory molecules. They are produced by a variety of cell types and exist to coordinate the development and action of multi-cellular organisms. They are probably very basic in evolutionary terms, facilitating the progression from unicellular to complex multi cellular life forms. They have been defined as follows: cytokine is one term for a group of protein cell regulators, variously called leucokines, monokines, interleukins and interferons, which are produced by a wide variety of cells in the body, play an important role in the many physiological responses, are involved in the pathophysiology of a range of diseases and have therapeutic potential (Balliwill and Burke, 1989). In addition, cytokines all affect in some way the functions of the immune system and are important mediators of inflammatory responses.

Cytokines differ from hormones because they usually have an action

over very short distances as autocrine or paracrine intercellular signals, and only occasionally have widespread systemic affects. Hormones, in contrast, are usually produced by well defined endocrine glands and have a role in whole body haemostasis via the circulation. Cytokines are not in constant production, unlike hormones which are constitutively produced, but are synthesised and released in response to emergencies, helping to activate cells, particularly of the immune system.

Cytokines have several common characteristics:

1 They are simple polypeptides or glycoproteins.

2 Constitutive production is low or absent. Production is regulated by various inducing stimuli at the level of transcription or translation.

3 Cytokine production is transient and the action radius is usually short (typical action is autocrine or paracrine, not endocrine).

4 Cytokines produce their actions by binding to specific high affinity cell surface receptors.

5 Most cytokine actions are brought about by gene expression in the target cells. Phenotypically cytokine actions lead to an increase or decrease in the rate of cell proliferation, change in cell differentiation state or a change in the expression of some differentiated function.

6 Although the range of action displayed by individual cytokines can be broad and diverse, at least some actions of each cytokine are targeted at haemopoetic cells (Vilcek and Le, 1991).

The change seen in endothelial cells in response to cytokines have been termed endothelial activation (Pober, 1988). This contrasts with the changes seen in endothelial cells in response to stimuli which do not provoke protein synthesis, such as secretory cell de-granulation in response to histamine or thrombin. This has been termed endothelial stimulation (Pober and Cotran, 1990).

The earliest observable changes during the acute inflammatory

response are haemodynamic changes and these are mediated via the endothelial cell. The agents responsible for these changes are not cytokines because de novo protein synthesis would take too long to be effective, therefore other more rapidly produced molecules are involved and these will be mentioned here for completeness. Vasodilation causes increased blood flow and therefore increased numbers of locally circulating leucocytes. Vasodilation is due to the relaxation of vascular smooth muscle tone. This is accomplished in a two step fashion. Agonists such as histamine, thrombin and leucotriene C4 (LTC4) which are produced very rapidly during the very early stages of acute inflammation, act on endothelial cells and provoke release of secondary mediators which are responsible for smooth muscle relaxation (Brenner et al, 1989). Two well described endothelial cell products are thought to be important in vasodilation and a further product is known to have a vasoconstrictor role. These are prostacyclin (PGI2), nitric oxide (or endothelial derived relaxing factor) and endothelin (endothelial cell contracting factor) (Dinh-Xuan et al, 1989).

PGI2 is an arachidonic acid metabolite which is synthesised and secreted by endothelial cells in response to elevation of cytoplasmic free calcium through an inositol triphosphate dependent mechanism. Elevated calcium, in turn, is believed to activate phospholipase A2 which leads to release of arachidonic acid from membrane phospholipid (Jaffe et al, 1987). The levels of the enzyme which convert arachidonic acid to prostacyclin are subject to control by other cytokines involved in inflammation such as interleukin 1 or tumour necrosis factor, both of which increase the prostacyclin release from endothelial cells after stimulation with thrombin and histamine (Zavidco et al, 1989).

If the endothelium is treated with indomethacin, a cyclo-oxygenase inhibitor, then the tissues are still able to contract and relax pointing

to another mechanism independent of the arachidonic metabolites. Two factors have been identified, although there may be more yet to be discovered. These are nitric oxide (EDRF) and endothelin (EDCF). Nitric oxide is a simple but highly diffusable gas, and endothelin is a more complex structure peptide both produced by endothelial cells. Nitric oxide acts by inducing relaxation of underlying smooth muscle by enhancing intracellular cyclic GMP levels. This action is comparable to that of nitro-vasodilators and nitric oxide can be regarded as an endogenous vasodilator (Dinh-Xuan et al, 1989). The mechanism of action of endothelin is less certain but may depend on an influx of extracellular calcium. Its role as a contracting factor is less clearly defined.

2.4.2 The role of interleukin-1 and tumour necrosis factor

Interleukin 1 (IL-1) and tumour necrosis factor (TNF) elicit a set of pro- inflammatory, pro-thrombotic responses in vascular endothelial cells, which largely overlap with each other (Mantovani et al, 1989). In many functional aspects they can be considered together.

Interleukin 1 (IL-1) is a term encompassing two polypeptides (IL-1a and IL-1b) which share the same cell surface receptors and have very similar biological activity. IL-1 is not constitutively produced by cells of the immune system however, production can be induced under stimulatory conditions in many cell types, most notably by mononuclear phagocytes. It is produced in large amounts in response to infection, inflammatory agents, microbial toxins, products of activated lymphocytes, complement and clotting components (Dinarello, 1992). Interleukin 1 receptor antagonist (IL-1RA) is produced concurrently with IL-1, this molecule is thought to act as an antagonist to the actions of IL-1 and help to limit inflammation and aid resolution (Dinarello and Thompson, 1991).

IL-1, tumour necrosis factor and IL-6 share the ability to stimulate

T and B lymphocytes, augment cell proliferation, and to initiate or suppress gene expression for several proteins (Dinarello, 1992). Many different agents stimulate IL-1 production including endotoxin, exotoxins from staphylococcus and streptococcus bacteria, thrombin, bile salts, androgen metabolites, complement components and other cytokines (Dinarello, 1991). After stimulation by endotoxin, mononuclear cells produce peak IL-1 b mRNA at 3-4 hours and this is sustained for 6-8 hours before decreasing rapidly (Dinarello, 1991).

If IL-1 is administered intravenously to human recipients, such as those undergoing cancer therapy and bone marrow transplantation, it produces fever, sleep, anorexia, generalised myalgia, arthralgia, headache, gastrointestinal disturbance, hypotension and shock (Smith et al, 1990).

Concentrating on the effect at the cellular level, IL-1 can directly inhibit contraction of vascular smooth muscle (Beasley et al, 1989) and can also induce slow (24-48 hours) production of PGI2 by increased levels of the enzyme cyclo-oxygenase, thereby causing vasodilation (Dejana et al, 1984). Tumour necrosis factor acts in a similar fashion (Okusawa et al 1988). Endothelial cells normally provide an anti-coagulant environment but exposure to IL-1 alters this and induces procoagulant activity. Endothelial cells show decreased production of tissue type plasminogen activator and they augment production of an inhibitor of plasminogen activator (TPA) (Bevilacqua et al 1986). IL-1 also increases levels of platelet activating factor (PAF) which is a platelet and leucocyte activator and also a vasoconstrictor (see below) (Bussolino et al, 1986).

Cultured endothelial cells exposed to IL-1 increase the expression of adhesion molecules which leads to the adherence of leucocytes to the endothelial cell surface (Dinarello, 1992). IL-1 itself is not directly chemotactic, but can provoke release of other chemotactic cytokines such as Interleukin 8, monocyte chemotactic protein (MCP1), and colony stimulating

factor (CSF) (Broudy et al, 1987, Sieff et al, 1988, Wang et al, 1989). IL-1 also induces production of cytokines which inhibit leucocyte adhesion and chemotactic action, and this probably represents a negative feedback circuit which may limit the inflammatory response and have a role to play in protecting the endothelial cell from potential damage by adherent leucocytes (Mantovani et al, 1989).

Interleukin 1 inhibits endothelial cell proliferation but promotes the growth of smooth muscle cells and fibroblasts (Raines et al, 1989). It induces platelet derived growth factor (PDGF) release by endothelial cells and smooth muscle cells and this may account for its proliferative effect on smooth muscle cells and fibroblasts. It is possible that IL-1 has a role to play in not only the vascular response to inflammation and thrombosis, but also in the process of atherogenesis (Raines et al, 1989).

IL-1 alters endothelial cell function in many ways and tumour necrosis factor acts in a very similar manner as do bacterial lipopolysaccharides. Most of the effects have delayed onset and are dependent on protein synthesis suggesting an effect at the DNA level (Mantovani et al, 1989).

Tumor necrosis factor (TNF) is in fact made up of two molecules TNFa (cachectin) and TNF b (lymphotoxin). TNFa is produced by many cells including fibroblasts, macrophages and T and B cells, whereas TNFb is a product predominantly of activated lymphocytes (Manogue et al, 1991). The TNFs are peptides of similar amino acid length between 150-250. Both induce necrosis of MET-A sarcoma of mice in-vivo hence the term tumour necrosis factor (Ruddle et al, 1991). For the purposes of this review both can be taken together in terms of their effects on endothelial cell function, especially with regard to the inflammatory response. However, TNFb may be less active than TNFa in inducing CSF production, expression of leucocyte adhesion molecules and the stimulation of interleukin 1 release

(Mantovani et al, 1989). TNF augments major histocompatibility complex class I antigens in endothelial cells whereas IL-1 has little effect on MHC expression (Pober et al 1987). TNF and IL-1 are additive in-vitro at optimum dosage in inducing various endothelial cell functions including procoagulant activity expression of neutrophil adhesion molecules and CSF production (Doukas and Pober, 1990).

2.4.3 Interleukin-8

Interleukin 8 (IL-8) is synthesised by endothelial cells (Shroder et al, 1989) in response to stimulus by IL-1, TNFa and LPS (Streiter et al, 1989). IL-8 is a low molecular weight polypeptide which can stimulate neutrophil movement (chemokinesis) and if presented as a concentration gradient can cause directed chemotaxis (Matsushima et al, 1989). IL-8 has several actions on neutrophils which are apparently contradictory. It up-regulates the expression of the b2 integrins on the neutrophil surface, thus increasing the adhesive potential (Detmers et al, 1990), whilst at the same time it down-regulates the L-selectin molecule by causing it to be shed into the extracellular environment. This latter activity led it to be called leucocyte adhesion inhibitor (LAI) (Gimbrone et al, 1989). However, this is logical when it is realised that L-selectin is important in initial cell binding, particularly in the cell rolling, whereas the b2 integrins are important in later stronger binding followed by cell migration. IL-8 may be working to facilitate this movement from surface binding to penetration into the sub endothelial matrix. In other words it acts to loosen neutrophil attachment rather than cause complete detachment (Pober and Cotran, 1990). T lymphocytes respond in a similar fashion to IL-8 (Matsushima et al, 1989).

In-vivo IL-8 injection into the skin causes predominantly neutrophil

infiltration within one hour and maximally at 3 hours (Carsen et al, 1989). It causes lymphocyte infiltration as lower doses. IL-8 causes an increase in vascular permeability in a neutrophil-dependent manner. Injection of the IL-8 into a rabbit knee-joint causes massive neutrophil extravasation and migration into the joint, followed by lymphocyte infiltration and joint destruction. IL-8 may be an important factor in diseases which are characterised by neutrophilia and/or neutrophil infiltration into organs. Examples are rheumatoid arthritis, psoriasis, glomerulonephritis, adult respiratory distress syndrome, ischaemic bowel disease and ischaemia reperfusion injury. IL-8 probably has pro inflammatory activities in these diseases (Matsushima et al, 1992).

IL-8 also induces neutrophil de-granulation measurable by release of cellular constituents such as b glucuronidase, elastase, myeloperoxidase, gelatinase, and lactoferrin (Willems et al, 1989). IL-8 also elicits a rapid respiratory burst through formation of superoxide and hydrogen peroxide molecules within the neutrophil. Eosinophils and monocytes do not respond to IL-8 which remains selective for neutrophils and lymphocytes.

2.4.4 Platelet activating factor

Platelet activating factor (PAF) is a biologically active phospholipid which is produced by activated rather than resting endothelial cells (Prescott et al, 1984). Synthesis and release is stimulated by agonists such as thrombin, histamine and leucotriene C4. Synthesis is via two enzymes - a calcium dependent phospholipase A2 and a specific acetyl transferase, which are both regulated by phosphorylation (Zimmerman et al, 1992). PAF is not released into the fluid phase by endothelial cells in-vitro, but remains expressed on the cell surface. When a neutrophil comes into contact with the endothelial cell complete with membrane-bound PAF molecule, then the neutrophil becomes activated by upregulation of

CD11a/CD18 and CD11b/CD18, the beta 2 integrins (Zimmerman et al, 1990). Thus, when PAF is expressed by activated endothelial cells, it acts as a signal to induce adhesiveness of neutrophils (Zimmerman et al 1992), This adhesion and activation of a target cell by a membrane bound molecule on another cell has been termed juxtacrine activation (Massague, 1990). This may help to localise the signal and thus the inflammatory response. Overall response is also limited by the fact that PAF is rapidly degraded in activated endothelial cells (Naylor et al, 1984). PAF in the fluid phase in-vitro causes shedding of leucocyte L-selectin but it is not known if PAF bound on the cell surface has the same effect, although this seems likely.

2.4.5 Interferon g

Interferon g (IFNg) was the first cytokine to be produced in large quantities by recombinant gene technology. It was therefore the first cytokine able to be tested against human umbilical vein endothelial cells and therefore much information exists as to its mode of action. There are three different interferons; IFNg, INFa and IFNb. There is however little similarity in gene structure between these three. IFNg is a 166 amino acid protein for which the gene is located on chromosome 12 (Naylor et al, 1984). IFNg production takes place in activated T lymphocytes but not in other cell types (Maeyer et al, 1991).

Initial experiments using IFNg and endothelial cells showed that it was the only cytokine that can induce expression of Class II MHC molecules on the endothelial cell surface (Collins et al, 1984), as well as increasing expression of Class I antigens (Pober et al, 1983). The action of interferon g in inducing Class II expression by HUVECS was important because beforehand it was thought that expression of Class II antigens was the sole property of bone marrow derived antigen presenting cells and traditional elements of the immune system such as lymphocytes (Pober,

1988). This work demonstrated very clearly the dynamic role that endothelial cells play in the immune system.

The ability to express Class II MHC molecules may contribute to allograft rejection, since the vascular endothelial cells of a graft can serve to prime the T lymphocytes of the host (Geppert and Lipsky, 1985).

IFNg also has profound effects on cell adhesion. It causes increased expression of ICAM-1, particularly 24-72 hours post exposure (Smith et al, 1988). It does not however increase expression of E-Selectin or VCAM. It also induced production of JE/MCP-1, another inflammatory cytokine which is a monocyte chemoattractant (Rollins et al, 1990). IFNg also augments the production of IL-1 by lipopolysaccharide stimulated endothelial cells (Miossec and Ziff, 1986). IFNg does not produce the effects on coagulation and fibrinolysis that IL-1 and TNF do. It is an important mediator of immune inflammation.

2.4.5 Monocyte chemoattractant protein

Monocyte chemoattractant protein (MCP-1/JE) is a monocyte chemo-attractant which is produced by human endothelial cells. Expression of mRNA encoding for MCP1 is maximal within 3 hours of stimulation of endothelial cells with IL-1b or TNF. IFNg also stimulates production but only after 24 hours. This protein is released by cultured endothelial cells into the surrounding media and can be detected up to 48 hours after exposure to IL-1b. This protein is a monocyte chemo-attractant that may contribute to the accumulation of monocytes at sites of inflammation (Rollins et al, 1990).

2.4.6 Other cytokines involved in endothelial leucocyte interactions

Interleukin 6 (IL-6) is a protein with 212 amino acids which acts on a wide variety of tissues and has differing effects depending on the

effector cell type (Taga and Kishimoto, 1992). It acts on lymphoid and non lymphoid cell lines and is produced by endothelial cells after stimulation with other cytokines such as IL-1, TNF and LPS (Sironi et al, 1989). IL-6 influences cytotoxic T cell differentiation (Okada et al, 1988), macrophage differentiation (Shabo et al, 1988) and immunoglobulin production by B cells (Muraguchi et al, 1988). IL-6 may therefore play a central role in host defence mechanisms such as immune responses, haematopoesis and acute phase reactions (Taga and Kishimoto, 1992).

Granulocyte and granulocyte-macrophage colony stimulating factors (GM-CSF) induces migration and proliferation of endothelial cells in a similar way to their primary action which is in proliferation of myelomonocytic cell lines (Bussolino et al, 1989). However GM-CSF does not stimulate pro-inflammatory or pro-thrombotic changes in endothelial cells (Mantovani et al, 1989).

Transforming growth factor beta (TGFb) and fibroblast growth factors (FGFs) are both produced by activated mononuclear phagocytes (Nathan, 1987). TGFb inhibits in-vitro endothelial cell growth and inhibits the effect of IL-1 and tumour necrosis factor on neutrophil adhesion to endothelial cells in-vitro (Gamble and Vadas, 1988a). It therefore down-regulates several pro-inflammatory activities. FGFs are mitogenic and induce endothelial cell chemotaxis and protein synthesis. They have a similar role as TGFb but do not inhibit leucocyte adherence to endothelial cells or influence coagulation or thrombolysis (Joseph-Silverstein and Rifkin, 1987).

In conclusion it can be seen than the actions of the various cytokines known to play a part in the inflammatory response are complex. Although it is possible to study cytokine action in vitro, this is often limited to one or two cytokines within a particular experimental system. In vivo, cytokine actions may be far more complex and difficult to

accurately assess.

2.5 Summary of the leucocyte endothelial cell adhesion event

The steps occurring during adhesion of leucocytes to endothelial cells can be seen summarised in Figures 1.5 and 1.6. Essentially a three-step model of cell adhesion has been proposed for these interactions in vivo. In the first step the inactive leucocyte, either a polymorph (Fig 1.5) or lymphocyte (Fig 1.6) comes into contact with the endothelial cell surface and binds initially via the selectin group of adhesion molecules and their counter receptors. These receptors do not support firm adhesion but allow rolling of the leucocyte on the endothelial cell surface. During this rolling procedure the second step of cell adhesion occurs which involves the delivery of an activation signal to the leucocyte from the endothelial cell. This is mediated via cytokines such as platelet activating factor. This triggers the third step of cell adhesion which is a change in the conformation of the receptor site of the integrin adhesion molecule family making it more adhesive. These molecules are then more able to bind to their counter receptors, the immunoglobulin family, and via this mechanism firm adhesion is achieved. During this time the endothelial surface may be made more adhesive by the action of other cytokines serving to recruit more leucocytes to the site of inflammation. Finally, migration of leucocyte occurs from the endothelial cell surface to sub endothelial tissues and this event may again be mediated by cytokine action (Butcher, 1991).

Fig 1.5 Polymorph migration into areas of acute inflammation.

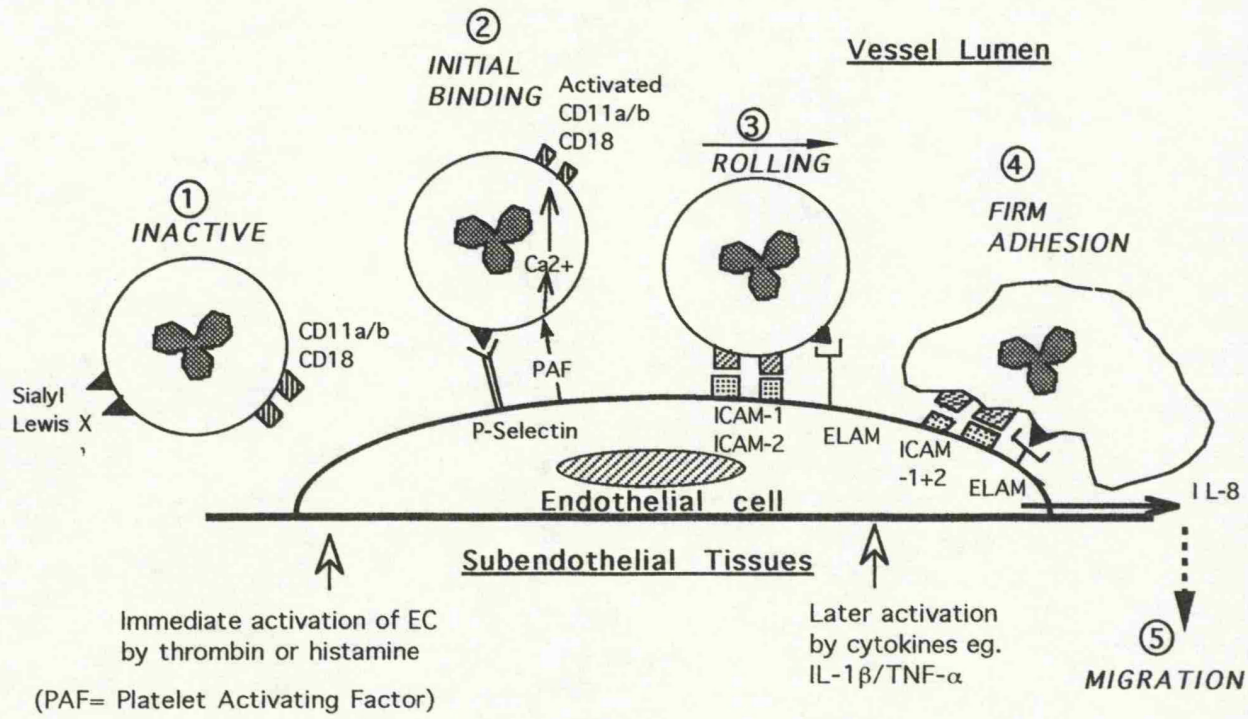
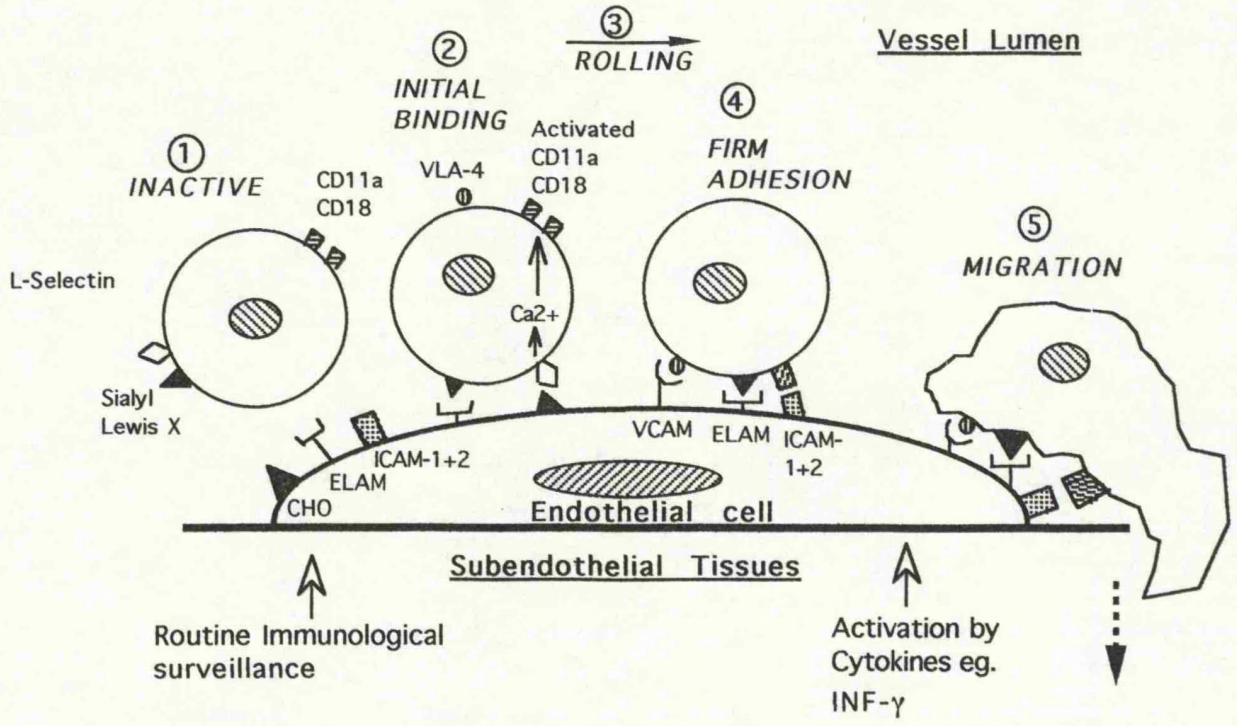


Fig 1.6 Lymphocyte migration into areas of acute inflammation.



2.6 Current understanding of cellular adhesion molecules in-vivo

2.6.1 Introduction

In-vitro studies with isolated phagocytes and cultured endothelial cells have been invaluable in identifying candidate proteins involved in cellular adhesion, elucidating their structure and regulation, and suggesting potential cellular interactions. However, the relative importance of a particular adhesion protein in phagocyte adherence to endothelium and emigration cannot be established until in-vivo studies are performed (Carlos and Harlan, 1990).

The availability of monocolonal antibodies which bind to cellular adhesion molecules have allowed the study of these molecules in-vivo. Broadly speaking these studies fall into four main categories:

(1) Investigation using immunohistological techniques to establish presence or absence of cellular adhesion molecules in various disease states.

(2) Studies inhibiting binding of leucocytes to endothelial cells in in-vivo models of disease states and then exploring the consequences. These are functional studies with potential therapeutic applications.

(3) Studies inhibiting binding of leucocytes to endothelial cells in various disease states to try to influence outcome, ie therapeutic manoeuvres.

(4) Studies to detect soluble forms of the adhesion molecules in various disease states for diagnostic and prognostic purposes.

There are several different groups of disorders in which research is taking place to determine the incidence of adhesion molecules as well as their diagnostic and therapeutic potential. I will consider these under the headings of:

(1) Inflammation and Disorders

(2) Transplantation

(3) Ischaemia Reperfusion Injury

(4) Malignancy.

I will describe these groups of disorders in order mentioning the various ways in which adhesion molecule study is helping to shed light on the disease process.

2.6.2 Cell Adhesion molecules in disease processes.

There are obviously a wide range of disorders which are characterised in one way or another by an inflammatory process. It is therefore not surprising that a good deal of research has already been carried out in several different clinical areas. The various studies which have been carried out will be reviewed in the following section and then a summary will be given of our current knowledge of the adhesion of polymorphs and lymphocytes to endothelial cells, taking into account experimental in vivo data. Before reviewing this work, however, it is appropriate to describe a disorder, leucocyte adhesion deficiency, in which the main problem lies in an absence of inflammation.

Leucocyte adhesion deficiency is a rare congenital condition, is characterised by a complete deficiency of the beta 2 integrins on leucocytes. CD11a, CD11b, CD11c are all absent or reduced (Anderson et al, 1985) and this is caused by mutations in the common beta 2 sub-unit. This leads to a catastrophic failure of neutrophil adherence and emigration in response to an appropriate stimulus (Harian et al, 1985). Surgical biopsies taken from LAD patients demonstrate an absence of pus at sites of bacterial infection (Anderson et al, 1985). These patients suffer recurrent life threatening bacterial infections often fatal in childhood unless treated by bone marrow transplant (Springer, 1990). That other congenital adhesion molecule deficiencies have not been identified may suggest that they are rapidly fatal perhaps in-utero.

Although the leucocyte adhesion deficiency is the only clinical syndrome that has been identified which is known to involve cell adhesion molecule deficiencies, its characterisation has led to a great deal of interest in in-vivo models of phagocyte emigration, since the cellular adhesion molecule deficiency has such a profound effect. Several studies have been carried out using anti-adhesion molecule monoclonal antibodies which in various ways mimic the LAD syndrome, in in-vivo models of inflammation. This has been limited to animal work and has provided confirmatory evidence for the findings of the previous in-vitro work on cultured cell lines. Unfortunately, not all human anti-cell adhesion molecule monoclonal antibodies cross react with the phagocyte or endothelial protein of animal species that are appropriate for various experimental models. This therefore has limited the animal models available for investigation (Carlos and Harlan, 1990).

Harlan's Group have investigated experimental inflammation in the rabbit using the monoclonal antibody 60.3 which is an anti CD18 molecule (Pan integrin). In the rabbit, instillation of E Coli, E Coli Toxin, or PMA into the lung would normally provoke a massive neutrophil influx. However, pre treatment of the rabbit with anti CD18 antibody markedly inhibited accumulation of neutrophils in the lung (Doerschuk et al, 1989). However, if the same experiment were performed using streptococcus pneumoniae instillation in the lung, neutrophil influx was not inhibited by use of the antibody. If Streptococcus pneumoniae was introduced into the peritoneum, neutrophil influx into this site could be reduced by administration of anti CD18. Neutrophils therefore must be able to use a CD18 independent mechanism to migrate into the lungs in response to Streptococcus pneumoniae, but use a CD18 dependent mechanism in response to E coli (Doerschuk et al, 1990).

Arfors and colleagues pre treated rabbits with an anti CD18

monoclonal antibody and then injected intra-dermally chemotactic factors FMCP, leucotriene (LT) B4 and C5a, as well as histamine. Four hours later, plasma leakage of radio-labelled albumin and neutrophil accumulation were determined. Both neutrophil accumulation and plasma leakage were abolished in the inflammatory skin lesions of the rabbits treated with the anti CD18 monoclonal antibody compared to the controls, except for the case of histamine induced plasma leakage which was unaffected. Intravital microscopy of vessels in rabbit muscle revealed that neutrophil adherence to venules and migration into sub-endothelial tissues was abolished in the treated animals, but rolling of neutrophils on the venule wall was unaffected. They concluded from this work that the CD18 complex was of vital importance in neutrophil adherence in response to chemotaxins (Arfors et al, 1987).

In another study, Price and colleagues used the same anti CD18 antibody. They implanted polyvinyl sponges subcutaneously in rabbits and measured the effect of antibody administration on neutrophil migration into the sponges. Intravenous infusion of the anti CD18 antibody resulted in dose dependent inhibition of in-vivo neutrophil migration with almost complete paralysis of neutrophil migration at higher antibody doses. They concluded that anti CD18 antibodies might prove to be potent in-vivo anti inflammatory agents in such disorders as immune complex vasculitis or adult respiratory distress syndrome in which neutrophil activation may be important pathophysiologically (Price et al, 1987).

Tuomanen and colleagues used another anti CD18 monoclonal antibody (IB4) in a rabbit model of bacterial meningitis. Injection of anti CD18 antibody effectively blocked the accumulation of leucocytes in the cerebrospinal fluid of animals challenged intracisternally with living bacteria, bacterial endotoxin or bacterial cell wall. This effect was associated with protection from blood brain barrier injury as measured by

exclusion of serum proteins from CSF in antibody treated animals. The densities of bacteria in CSF and the degress of bacterial killing due to ampicillin were not affected by the antibody. Animals receiving the antibody had a delayed development of bacteremia and a significantly reduced inflammatory response during ampicillin induced bacterial killing. Therapy with the monoclonal antibody prevented development of brain oedema and death in animals challenged with lethal doses of streptococcus pneumoniae. These studies indicate that the major mechanism of leucocyte migration across the blood brain barrier involve the CD11/CD18 receptors and that inflammatory leucocytes recruited by this mechanism are a major cause of blood brain barrier injury and cerebral oedema during meningitis (Tuomanen et al, 1989).

Rosen and Gordon used an antibody against the CD11b receptor in a mouse model. They showed that in-vivo, after IV injection of antibody, the recruitment of myelomonocytic cells in the mouse peritoneal cavity in response to thioglycollate instillation was inhibited compared to controls (Roson and Gordon, 1987). Using the same antibody they also showed that using a model of delayed type hypersensitivity (DTH) in mice, administration of anti CD11b antibody could inhibit inflammatory cell recruitment and oedema in the first 24 hours of the reaction. However, it was not effective in preventing the reaction after 24 hours. They concluded that CD11b plays a quantitatively important role in T cells dependent inflammatory recruitment (Rosen et al, 1989).

Jutila and colleagues used a mouse model of peritonitis through thioglycollate infusion to study monoclonal antibodies against CD11a and b as well as L-selectin (MEL-14 antibody). They showed that in this model MEL-14 mediates early interaction between neutrophils and endothelium during inflammation which was not dependent on the activation of the neutrophil, whilst the CD11a and b pathways were transformed to an active

state on contact of the neutrophil to endothelial cells. The MEL-14 antigen was then found to down regulate. This confirmed the in-vitro work suggesting the L-selectin acted in a different way to the integrin family in neutrophil adhesion (Jutila et al, 1989).

Lewinsohn and colleagues used a similar MEL-14 monoclonal antibody against L-selectin in a mouse model of acute inflammation. They implanted an E coli-soaked gelfoam sponge in the skin and peritoneal cavity of mice. Intravenous injection of MEL-14 antibody inhibited neutrophil extravasation into the site (Lewinsohn et al, 1987).

Watson and colleagues have gone one step further and developed an antibody which recognises the L-selectin/MEL-14 antigen but which is a soluble IgG chimera of the murine homing receptor. It consists of the extracellular domain of the murine homing receptor ligated to the hinge CH2 and CH3 domains of the human IgG-1 heavy chain. This antibody was shown to decrease the number of neutrophils that migrate into the mouse peritoneum in response to the inflammatory irritant thioglycollate. The potential advantages of using a chimeric molecule include its ability to form an immunoglobulin-like dimer with a potential higher avidity and serum half life, as well as improved purification through IgG binding protein A - sepharose chromatography (Watson et al, 1991).

These studies confirm that adhesion molecules, first defined in-vitro, do indeed pay a pivotal role in in-vivo inflammation and that the movement of leucocytes can be manipulated with possible clinical applications.

Inflammatory skin disorders are relatively common and have therefore proved to be useful in the study of cellular adhesion molecules. Biopsies are readily available and some skin lesions can be induced for study purposes, such as delayed type hypersensitivity reactions. The presence of E-selectin in particular has been studied in a variety of skin biopsies

from patients with psoriasis, allergic contact dermatitis, lichen planus, lymphoid hyperplasia, non specific chronic dermatitis, cutaneous drug eruptions, pityriasis lychenoides et variociformis acuta, granuloma annulare and pityriasis rubra piliaris (Picker et al, 1991). In the majority of these inflammatory skin lesions, E-selectin staining was intensely positive using a three stage immunoperoxidase technique. However, tissue from extracutaneous sites such as from myocarditis, inflammatory bowel disease and hepatitis, were generally negative for E-selectin (Picker et al, 1991). The E-selectin present in these essentially chronic inflammatory skin lesions are thought to represent a pathway for migration of memory T-cells into the skin.

Rheumatology is another clinical area in which inflammatory disorders are common. A good example is rheumatoid arthritis which is characterised histologically by infiltration of the synovial membrane of joints by mononuclear cells, mainly T-cells. Sanchez-Madrid's group studied T-lymphocytes isolated from the blood, synovial fluid and synovial membranes of patients with rheumatoid arthritis. They measured the adhesiveness of the T cells to E-selectin and VCAM and found that the synovial T cells showed enhanced binding to E-selectin and VCAM compared to peripheral blood T cells. They concluded that the increased adhesiveness of synovial T cells to their endothelial ligands may reflect pathological phenomena occurring in the inflamed tissue of patients with rheumatoid arthritis, and therefore this may extend current understanding of the pathophysiology of this disease (Postigo et al, 1992).

Inflammatory conditions also predominate in respiratory medicine where asthma in particular has been studied in terms of the contribution of cellular adhesion molecules in the pathogenesis of the disease. Asthma is a condition characterised by airway eosinophilia, epithelial desquamation, and extreme hyper-responsiveness of the bronchi (ten to one thousand times

normal) to inhaled agents. Wegner and colleagues have produced a primate model of airway hypersensitivity. They showed that ICAM-1 was up-regulated on the inflamed bronchial endothelium in-vivo of primates after inhalation of allergen. In-vitro an antibody to ICAM-1 was found to reduce eosinophil adhesion to endothelial cells, and using this antibody in the in-vivo model of asthma, airway eosinophilia and hyper-responsiveness were reduced. They concluded that ICAM-1 may be pivotal in the pathogenesis of airway hyper-responsiveness and asthma and that it may contribute to onset and progression of other diseases characterised by airway inflammation such as chronic bronchitis, emphysema and idiopathic pulmonary fibrosis, or eosinophil infiltration and tissue sensitisation-destruction such as rhinitis, nasal polyposis, chronic urticaria and atopic dermatitis (Wegner et al, 1990).

ICAM-1 has been shown to be the cellular receptor for the major group of rhinoviruses which belong to the picornavirus family and cause about 50% of the common cold. Marlin and colleagues showed that a soluble purified form of the ICAM-1 molecule was effective in inhibiting rhinovirus infection (Marlin et al, 1990). This has profound implications for antiviral therapy. Particularly soluble forms of CD4, the receptor for human immunodeficiency virus (HIV), are now being tested for clinical efficiency in the acquired immune deficiency syndrome (AIDS) (276).

A soluble form of ICAM-1 has been characterised in the serum (277). It is possible to detect soluble forms of adhesion molecules by an ELISA technique. This has been used by Newman to evaluate the levels of soluble E-Selectin (sE-Selectin) in the serum of patients suffering from chronic inflammatory vascular disease, such as giant cell arteritis, polyarteritis nodosa and scleroderma. He found that these individuals had raised soluble E-Selectin levels compared to normal controls. These levels were not affected by corticosteroids or non steroidal anti inflammatory agents. The

diagnostic and prognostic potential of this assay are yet to be evaluated but provide an exciting new field of investigation (Newman, 1991).

2.6.3.1 Cell Adhesion molecule studies in transplantation

Some adhesion molecule studies have been carried out in renal transplantation, cardiac transplantation, bone marrow transplantation and liver transplantation. However, studies have not been widespread or exhaustive in any of these areas. Some limited clinical trials using adhesion molecules have been performed, particularly in bone marrow transplant and renal transplant.

2.6.3.2 Renal Transplantation

Bishop and Hall studied 25 biopsies taken from renal allografts which were proven to have allograft rejection histologically, and compared them to controls. ICAM-1 was found to be expressed strongly by large vessel endothelium, peri-tubular capillaries and by glomeruli in normal kidneys but not by renal tubular cells. With rejection expression of ICAM-1 was markedly increased. Expression of ICAM-1 by glomeruli and by large vessel endothelium was unchanged by rejection. The vascular endothelium of the kidney was identified as having the highest constitutive expression of ICAM-1. They concluded that this increased expression of ICAM-1 by vascular endothelium may be a contributing factor in the preferential binding of activated effector cells and may be a contributing factor in the preferential destruction of capillary endothelial cells observed in rejecting renal allografts (Bishop and Hall, 1989).

Fuggle and colleagues studied the distribution of E-Selectin, ICAM-1 and VCAM in needle core specimens taken from 50 pre transplant renal allografts and 42 post transplant allografts. They also measured the level of CD45 and CD29 positive leucocyte infiltration and the upregulation of

HLA Class II antigens. Pre-transplantation there was considerable variation between biopsies in endothelial expression of E-Selectin and in proximal tubular expression of ICAM-1 and VCAM. After transplant upregulation of ICAM-1 was detected in the proximal tubules of 26% of biopsies and induced expression of E-Selectin and VCAM was detected on the endothelium of 29% and 33% of biopsies respectively. Adhesion molecules were up-regulated at times of graft rejection and were associated with increased levels of leucocyte infiltration. They concluded that upregulation of cellular adhesion molecules in transplant kidneys was evidence of endothelial activation and probably facilitates the entry of leucocytes into the allograft. The induced cellular adhesion molecules, together with HLA Class II antigens may render the graft more susceptible to damage mediated by allo-specific cytotoxic T lymphocytes (Fuggle et al, 1992).

Cosimi and colleagues have performed a trial using a monoclonal antibody against ICAM-1 in a model of renal transplant rejection in cyanomolgus monkeys. They used the anti ICAM-1 monoclonal antibody both for induction of immunosuppression after transplantation and treatment of allograft rejection post transplantation. They found that the anti ICAM monoclonal antibody administered prophylactically as the sole immunosuppressive agent for 12 days significantly prolonged graft survival beyond untreated controls. Biopsies of the transplant kidneys showed a decrease in focal oedema, little or no haemorrhage and an infiltrate of mononuclear cells that was significantly less than controls. The oedema and haemorrhage are believed to result from peri tubular capillary injury (Colvin, 1990). In those animals treated for acute rejection with anti ICAM monoclonal antibody for 10 days, 6 of 8 monkeys demonstrated prompt improvement in renal function with reversal of rejection. Of the remaining two, one died from anaesthetic complications and one of acute pylonephritis

with no evidence of rejection. After injection of antibody, the main site of deposition was found to be the graft vascular endothelium as well as the endothelium of normal organs. Antibody was cleared from the endothelium at 4 days (when biopsies were taken). They hypothesised that the antibody blocks adhesion to graft ICAM-1 molecules on vascular endothelium and that anti ICAM-1 monoclonal antibodies can inhibit T cell mediated injury in-vivo, and that ICAM-1 is a critical molecule in the pathogenesis of acute cellular rejection (Cosimi et al, 1990).

2.6.3.3 Cardiac Transplantation

In cardiac transplantation, Taylor and colleagues have studied the presence of adhesion molecules on biopsies taken from normal donor hearts and from the myocardium of transplant recipients with rejection episodes. They found that ICAM-1, MHC Class I and Class II, and VCAM are expressed on arterioles and venules with E-Selectin expression confined to venules (Taylor et al, 1992). During rejection episodes, expression of all adhesion molecules were increased and this was mirrored by an increase in the endothelial activation markers PAL-E and FV111-Ca, found in rejection shown in an earlier study (Hengstenberg et al, 1990). They concluded that the expression of induced ICAM-1 and E-Selectin on endothelium within cardiac biopsies is an early event in rejection leading to enhanced migration of cells into the allografted heart (Taylor et al, 1992). A similar study by Briscoe and colleagues of 15 cardiac biopsies taken from transplant recipients, showed that in biopsies which were positive for CD3 positive infiltrating cells, ICAM-1 and VCAM were present on post capillary vascular endothelium, although E-Selectin was not found (Briscoe et al, 1991).

2.6.3.4 Liver Transplantation

In liver transplantation, Adams and colleagues studied the expression of ICAM-1 on tissue from donor livers, stable transplants, on rejection complicated transplants and rejecting transplants. There was greater ICAM-1 expression on bile ducts, endothelium and peri venular hepatocytes (structures affected by the rejection process) in patients with acute rejection and in donor livers, patients with stable transplants, all patients with non rejection complications. Expression of ICAM-1 was highest in patients who progressed to chronic irreversible rejection. ICAM-1 expression was reduced after prednisolone treatment and they suggested that this might be an important mode of action of this drug (Adams et al, 1989).

2.6.3.5 Bone Marrow Transplantation

Bone marrow transplantation in situations where there is an MHC mismatch between donor and recipient, requires immunosuppressive treatment on both sides. Children with congenital immune deficiency states who cannot mount an immunological response can be subject to the phenomenon of graft versus host disease if the engrafted cells are not haploidentical. Children who have partial immune deficiency receiving non-haploidentical grafts have a particularly bad prognosis because of rejection of the graft due to residual immune activity of the patient. Diseases which fall into this latter bad prognosis group are the Wiscott-Aldrich Syndrome, Osteopetrosis, Gauchers Disease, Metachromatic leukodystrophy and Fanconi's Anaemia. Fisher and colleagues performed a clinical trial using an anti CD18 monoclonal antibody in seven children with partial immunodeficiency states to facilitate the engraftment of partially matched related donor bone marrow. They also used conventional therapy including busulphan, cyclophosphamide, ALG (antilympocyte globuline) and cyclosporin in these

patients as well as in 7 historical controlled patients. The monoclonal antibody caused transient pyrexia but was generally well tolerated and led to rapid engraftment in all 7 patients although 2 died later of infectious complications. In the control group treated identically except for the anti CD18 monoclonal antibody, only 1 patient partially engrafted (Fisher et al, 1991).

Although this was a small study, it has obviously raised great hopes for future possibilities in this field. However, a further study has not been encouraging using the anti CD18 monoclonal antibody in bone marrow transplantation in leukemic patients.

Norton and Stone studied skin biopsies from allogenic bone marrow transplant recipients and performed immunohistology looking for ICAM-1. However, although ICAM-1 did seem to be increased on epidermal keratinocytes during episodes of graft versus host disease (GVHD) it was not diagnostic and was of little value (Norton and Sloan, 1991). However, in similar work studying E-Selectin and VCAM expression in a comparable group of patients, the same research has showed that both E-Selectin and VCAM were up-regulated in biopsies of cutaneous graft versus host disease. VCAM especially was of potential benefit in diagnosing early GVHD and a prospective study has been undertaken to evaluate antibodies to VCAM in predicting clinical outcome in patients with rashes after transplantation (Norton et al, 1991).

That GVHD may be treated with anti cell adhesion molecule antibodies was investigated by Harning and colleagues in mice. They showed that an anti LFA1 (CD18) monoclonal antibody was useful in significantly reducing GVHD and enhancing survival in mouse bone marrow transplant (Harning et al, 1991).

2.6.4.1 Cell Adhesion molecules in studies of Ischaemia Reperfusion Disorders.

The spectrum of diseases which can be included in the general category of ischaemia reperfusion injury disorders is very wide. Clearly ischaemia which does not resolve leads to irreversible tissue necrosis which has major pathological consequences in some of the most common diseases of the developed world such as cerebro-vascular accident, myocardial infarction and arterial embolic events to any organ. However, if sites of tissue ischaemia which prove to be non fatal to the patient are then reperfused with blood, then a different set of tissue changes occur. In addition, tissue which adjoins necrotic areas is also subject to these changes. For example, the areas of myocardium around infarcted tissue secondary to coronary artery thrombosis show characteristic pathological changes that come in the broad category of ischaemia reperfusion injury. There is evidence that the overall tissue damage in reperfused tissue is often more extensive than in the ischaemic tissue prior to reperfusion and eventual infarct size can be significantly influenced by the extent of the reperfusion injury. A great deal of research has been performed to investigate the cellular mechanisms of damage to cells and possible ways of treating them. Disorders which might benefit from alteration of reperfusion injury are wide ranging from limb ischaemia, organ transplantation, myocardial infarction, stroke and hypovolemic shock.

Evidence suggests that one of the main causes of tissue injury occurring after reperfusion is damage mediated by activated neutrophils. The recognition of cellular adhesion molecules and development of monoclonal antibodies to block adhesion has shed light on mechanisms of reperfusion injury and has led to exciting work attempting to attenuate tissue damage in reperfusion injury. This is a large subject and I will limit this discussion to a brief resumé of the pathophysiology of ischaemia

reperfusion injury and the areas where cellular adhesion molecules have been investigated in this context.

2.6.4.2 Cellular Events

During a period of ischaemia the biochemistry of the cell is altered because of the lack of oxygen. Abnormal metabolites build up which initiate the injury caused during the reperfusion phase. Particularly harmful are reactive oxygen metabolites such as the super oxide anion, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Granger, 1988).

In brief, investigations have shown that during the ischaemic period adenosine triphosphate (ATP) is depleted and broken down to hypoxanthine. Normally, hypoxanthine is converted to xanthine by the enzyme xanthine dehydrogenase, but this enzyme is also depleted during ischaemia and is itself degraded to xanthine oxidase. This latter enzyme can build up quite rapidly in the tissues during ischaemia (Friedl et al, 1990). When reperfusion of the cell occurs and oxygen becomes available, the reaction of hypoxanthine and xanthine oxidase, now present in large amounts, leads to a pathological reaction forming xanthine and the highly reactive super oxide ion $O_2.-$. The superoxide ion is then further metabolised to form hydroxyl radials OH^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Ratych et al, 1987, McCord et al, 1987).

The oxygen free radicals themselves cause part of the cellular injury post reperfusion (Granger et al, 1981). However, it is also thought that oxygen radicals may increase the neutrophil component of the injury by either altering the endothelial cell directly and thereby increasing neutrophil adherence and chemotaxis (Repine et al, 1987), or by leaking out along endothelial cells and converting serum components into chemotactic or adherence promoting mediators for neutrophils (Petrone et al, 1980). Overall, the main pathological effect of oxygen radical release is the

generation of chemotactic activity leading to the directed migration of activated neutrophils into the reperfused tissue with consequent injury (Welbourne et al, 1991).

Arachidonic acid metabolites are also important in cellular damage. These are molecules such as prostaglandins PGE2, PGD2 and PGF2a and 6-keto PGF1a, thromboxanes TXB2 and TXA2 and leucotrienes LTC4, LTD4, LTE4 and LTB4. In the presence of oxygen radicals, intercellular free calcium rises rapidly and activates plasma membrane phospholipase A2 which generates the arachidonic acid metabolites (Ernster, 1988). Inhibition of the oxygen radicals using scavenging enzymes prevents release of these metabolites (Klausner et al, 1989a).

There is evidence that the arachidonic acid metabolites can influence neutrophils in ischaemia reperfusion injury. LTB4 and TXA2 are both chemo attractants for neutrophils (Spgnuolo et al, 1980, Gimbrone et al, 1984) and may cause neutrophil diapedesis (Welbourne et al, 1990). TXA2 and LTB4 also stimulate the neutrophil directly to produce elastase and H_2O_2 (Larfors et al, 1987, Paterson et al, 1989). Leucotrienes and thromboxanes also have a direct action on blood flow. They cause vasoconstriction which can lead to poor capillary flow and exacerbate the ischaemic insult. There is evidence of this effect alone may be of major importance in the pathophysiology of reperfusion injury, particularly in renal reperfusion injury where neutrophils may not play as greater part as in other organs (Thornton et al, 1989).

The evidence that the neutrophil is of major importance in the generation of reperfusion injury comes from several studies where anti adhesion molecules have been used to block neutrophil adhesion via the CD18 (beta 2 integrins system) molecule in models of ischaemia reperfusion. The adhesion event itself is important because under the influence of the pathologically generated cytokines detailed above, the neutrophil binds to

and damages the endothelial cell by creating a micro environment at the cell interface into which proteolytic enzymes and oxygen free radicals are released at a high concentration. This causes lysis of essential structural matrix proteins such as elastin, which leads to increased microvascular permeability (Weiss, 1989, McCord, 1985).

Several antibodies exist to block the CD18 complex on neutrophils and these have been tested models of reperfusion injury. Removing neutrophils from the circulation before reperfusion has also been attempted in similar models.

After myocardial infarction neutrophil sequestration into reperfused tissue is quantitatively related to the severity of the ischaemic insult and it has been shown that drugs which prevent neutrophil adhesion or activation can reduce the extent of tissue damage caused during reperfusion injury. Depleting the model of neutrophils prior to injury has been shown to reduce infarct size in the dog (Romson et al, 1983, Litt et al, 1989, Mullane et al, 1984).

Antibodies against the CD18 complex have been shown to block neutrophil accumulation in the ischaemic reperfused heart and reduce infarct size (Simpson et al, 1988, Winquist et al, 1990). An antibody against ICAM-1, the ligand for CD18, also reduces infarct size in the same dog model (Seewaldt-Becker et al, 1989).

The consequences of circulatory shock followed by resuscitation is, in essence, the development of full body ischaemia reperfusion injury, and it is not surprising an injury to multiple organs is frequently a consequence (Vedder et al, 1988). Clinical studies of the multiple organ failure syndrome in trauma patients has shown a close correlation with prior hypovolaemic shock (Faist et al, 1983). The concept of the neutrophil being responsible for some of the changes leading to multiple organ failure have been investigated in animal models. An anti CD18

monoclonal antibody was used by Vedder and colleagues (Vedder et al, 1988) in a rabbit model of hypovolaemic shock. They showed that if control animals were subject to one hour of shock sufficient to produce a cardiac output 30% of normal, and were then resuscitated, only 29% survived five days and all had gross and histological evidence of injury to lungs, liver and gastrointestinal mucosa. In contrast, animals treated with the anti CD18 monoclonal antibody resuscitation all survived and had absent or markedly attenuated organ injury. Mileski and colleagues performed a similar study in primates (Rhesus monkeys) showed that treated animals had less chance of dying, required less fluids, gained less weight, maintained a higher hematocrit level and did not develop gastritis. They concluded that inhibition of neutrophil adherence or aggregation at times of resuscitation reduces fluid requirements and gastric injury in monkeys after haemorrhagic shock (Mileski et al, 1990).

The same antibody was used by Walsh et al in a model of acute lung injury in septic pigs treated with Pseudomonas aeruginosa. Treated animals had significantly less neutropenia, bronchoalveolar lavage protein content and extravascular lung water collection than untreated animals although systemic hypotension, hypoxemia and cardiac index were not altered in the treatment group (Walsh et al, 1991).

On the basis of these studies, Thornton and colleagues investigated the effect of blocking neutrophil adhesion using the same anti CD18 monoclonal antibody as used previously, in a rabbit model of renal ischaemia. They subjected rabbits to varying times of renal ischaemia by renal artery occlusion and compared control animals to those treated with the antibody in terms of renal function and histological appearance. They found no differences between the two groups. In a further experiment comparing two groups of rats, no difference could be found between controls and rats pre-treated with an anti neutrophil serum administration prior to

renal ischaemia. They concluded that the neutrophil is not a critical participant in the renal ischaemia reperfusion process (Thornton et al, 1989).

Neutrophil depletion by administration of anti neutrophil serum or nitrogen mustard administration has also been studied by other investigators in the rat. Hellberg and colleagues reported that neutrophil depletion during renal ischaemia caused some increase in the immediate reperfusion filtration rate, although longer time periods were not studied (Hellberg et al, 1988). In another similar study, Klauser showed that the blood urea did not rise as high in neutrophil depleted animals as in controls after a renal ischaemia under 24 hours (Klausner et al, 1989b). It is interesting that the kidney is the only organ where anti neutrophil adhesion antibodies have not conferred benefit in mediating ischaemia reperfusion injury. This may be because renal endothelium is in some way different to other types of endothelium or because neutrophils use additional adhesion pathways in renal endothelium and the monoclonal antibody employed was not appropriate. These questions remain to be answered (Paller et al, 1989).

The area of ischaemia reperfusion injury is clearly a large one. Whilst anti adhesion monoclonal antibodies have led to great to advances in the understanding of cellular mechanisms, much is still to be understood before effective human trials can be planned.

2.6.5 Cell Adhesion molecule in studies of Malignancy

A limited amount of work has been done in the field of adhesion molecule expression by malignant tissue. One of the main characteristics of malignant cells is their ability to invade other tissues. The loss of normal cellular adhesion mechanisms or the gaining of new ones, may be important in this role. Another characteristic of malignant cells is the

ability to spread to remote sites and again the expression of adhesion molecules by either the cancer cells or the target organ may be of importance in determining which cancers spread to a particular site.

Liotta found that neoplastic cells of invasive tumours can enzymatically digest some components of basement membranes, and have altered expression of Laminin receptors which are involved in cell anchorage to the basement membrane (Liotta et al, 1986). The relationship between the extracellular matrix and the tumour cells is also important. Poorly differentiated tumours may have few detectable fibronectin and collagen receptors. Neoplastic cells can influence the extracellular matrix by producing degrading enzymes, or by inducing host cells to change the extracellular matrix environment (Plantefabers and Hynes, 1989).

For tumour cells to metastasise the normal cell to cell adhesion amongst the neoplastic cells must be lost or modified. Abnormal expression of adhesion molecules may predispose to cell detachment and then dissemination. This is supported by the fact that 70% of colorectal cancers do not express normal adhesion molecule genes and this may lead to loss of cell to cell adhesion (Pauli and Knudson, 1988). Abnormal expression of Laminin receptors has been demonstrated in hepatocellular carcinomas (Fearon et al, 1990), breast cancers (Grigioni et al, 1991) and colon cancers (Horanhand et al, 1985).

As with leucocyte migration in inflammation, the first step in the metastatic process of blood borne tumour cells is adhesion to endothelial cells in the target organ (Yow et al, 1988). Melanoma cells and colon cancer cells have been shown to bind to E-Selectin and VCAM (Keelan and Haskard, 1982). Expression of the ligand for E-Selectin, Sialyl Lewis X, has been shown to be increased by colon cancer cells (Rice and Bevilacqua, 1989).

The tumour cells themselves may secrete cytokines that cause

endothelial cells to express adhesion molecules thus aiding metastatic spread. Haemopoetic and non haemopoetic tumour cells can secrete interleukin 1 and/or tumour necrosis factor. Interleukin 1 promotes adherence of tumour cells to endothelial cells in-vitro (Matsushita et al, 1990) and augments experimental metastases or human melanoma cells if given to athymic mice (Dejana et al, 1988). It may be that organs with activated blood vessels may be at increased risk of tumour cell adhesion compared to organs with a non inflamed vascular bed.

The expression of ICAM-1 has been investigated with respect to haematological malignancies by Boyd and colleagues (Giavazzi et al, 1990). Tumours which were solid, single masses, showed intensive staining for ICAM-1 whilst tumours which were diffuse, poorly localised and highly metastatic stained very weakly for ICAM-1. ICAM-1 has been shown to be strongly expressed by lymph nodes containing Hodgkins Disease, although reactive lymph nodes also demonstrated high levels. In contrast E-Selectin and VCAM were only expressed on lymph nodes affected by conditions characterised by high level of interleukin 1 and tumour necrosis factor production such as granulomatosis and Hodgkins Disease especially the nodular sclerosing form of Hodgkins Disease (Boyd et al, 1989). ICAM-2 as well as ICAM-1 was shown to be more strongly expressed by lymphomas than by hyperplastic lymph nodes (Ruco et al, 1992).

It is clear, even from the limited amount of work carried out so far, that adhesion molecules are very important in the biology of malignancies. They may well hold the key as to why some tumours, especially lymphoid malignancies, have a tendency to spread whilst some do not (Renkonen et al, 1992).

2.7 Aims of the study

The aim of this study was to investigate the role that cell adhesion molecules play during the process of renal transplantation. This question was firstly addressed by using an *in-vitro* model of endothelial cell function employing cultured human umbilical vein endothelial cells. Using this model the expression of endothelial cell adhesion molecules under inflammatory cytokine stimulus was investigated with the technique of flow cytometry.

This model was then adapted so that the adhesive potential of polymorphonuclear cells from healthy volunteers and from different patient groups could be measured. A method of quantifying this adhesion using release of radioactive chromium was compared with a method utilising Rose Bengal dye.

Finally tissue specimens taken from renal allografts, both pre and post-transplantation, were assessed by immunohistology for the distribution of adhesion molecules within the kidney and for adhesion molecule expression during times of post-transplant allograft dysfunction. In particular E-selectin expression was studied to investigate it's potential use as a diagnostic tool in the diagnosis of renal allograft rejection.

CHAPTER 3

Materials and Methods

3.0 Primary culture of Human umbilical vein endothelial cells.

3.1 Collection of umbilical cords

Arrangements were made for the collection of umbilical cords from the maternity department. (During this project cords were kindly collected by the midwives of the maternity ward, Leicester General Hospital.) Cords were collected into 150 ml plastic tubs and labelled with the date and time collected as shown:

Cord Pot for Dept Surgery		Cord Details
Ext 4607	Date:	
Please complete details on pot lid		Time:

Cords were found to provide viable endothelial cells for culture up to 36 hours after delivery. After this time viability was greatly reduced.

3.2 Isolation of endothelial cells from umiblical cords

This procedure was done in a sterile manner using a class II Microbiological cabinet. Before starting the procedure all equipment and consumables were prepared and placed in the hood to avoid contamination of the surrounding area with blood. Sterile, unpowdered surgical gloves were worn at all times when handling human tissue.

Procedure

1 The umbilical cord was removed from the sample pot and the exterior was swabbed with 70% propan-2-ol. The cord was then placed in a disposable kidney dish for ease of handling.

2 The cord was inspected for any visible blood clots, these were

massaged out of the cord vessels and the external clamp marks were located.

3 Using a sterile scalpel the cord was transected 1-2 cm inside the clamp marks.

4 The umbilical vein was located. The umbilical vein is larger than the two umbilical arteries and is less muscular walled. The end of the vein was gently dilated using a small stock forceps.

5 A pre-sterilised (by immersion in 70% propan-2-ol), metal cannula, was attached to a 10 ml syringe containing Hanks Balanced salt solution, this helped to maintain sterility and also helped in manipulation of the cannula into the vein. This cannula was then inserted into one end of the umbilical vein (end 1).

6 The cannula was tied firmly into the umbilical cord by using a strong surgical suture.

7 This procedure was repeated at the other end of the cord except the 10 ml syringe was left empty (end 2).

8 The vein was flushed with calcium and magnesium free Hanks Balanced Salt Solution (HBSS/CMF) massaging gently along the length of the vein to remove blood from the lumen. 10-20 ml of HBSS/CMF was found to be sufficient to obtain a clear effluent. The flush from the full syringe at end 1 was collected in the empty syringe at end 2 and discarded.

9 Once the effluent was clear any residual HBSS/CMF was aspirated from end 2 with end 1 raised.

10 An empty syringe was inserted into end 2 and a syringe containg 0.1% collaganese solution that had been preheated to 37°C in a water bath was inserted into end 1. The collaganese was injected into the cord, then the cord (with both syringes in place) was placed in a pre warmed beaker containing 0.9% saline, in a 37°C water bath.

11 The cord was then incubated for 20 mins.

12 The cord was removed from the beaker, the empty syringe at end 1 was

replaced with a syringe containing 10 ml HBSS supplemented with 20% fetal calf serum (HBSS/FCS) and the collagenase. The cord was gently massaged to facilitate endothelial cell detachment and then flushed through with the HBSS/FCS into the syringe at end 2. The FCS prevents further collagenase digestion of the detached endothelial cells by providing a gross excess of substrate for the collagenase to act on.

13 The effluent was placed in a sterile universal container and centrifuged at 1000 rpm for 10 minutes.

14 The supernatant was poured off and the pellet was resuspended in 10 ml HBSS/FCS and centrifuged at 1000 rpm for ten minutes.

15 The supernatant was poured off and the pellet was resuspended in 5 ml of endothelial culture medium. This was then transfered to a 25 ml tissue culture flask.

16 The flask was placed in an incubator at 37°C maintained at 5% carbon dioxide, with the cap of the flask loosened to allow gas to diffuse into the flask.

17 At least 24 hours were allowed for the endothelial cells to adhere to the flask before attempting to change the culture medium.

18 When the media had been changed and non adherent debris removed from the flask, the cell layer was visualised using an inverted phase contrast microscope, under which the cells had a characteristic cobblestone appearance.

3.3 Materials for primary culture of human umbilical vein endothelial cells

All chemicals were obtained from Sigma Chemical Co. Ltd, UK. Media and FCS was purchased from Imperial Labs, UK.

1 Stainless steel canulae

2 Strong silk sutures

3 Kidney dish

4 70% Propan-2-ol

5 Scalpel

6 Forceps

7 Calcium and Magnesium Free Hanks Balanced salt solution

8 Hanks Balanced salt solution with calcium and magnesium

9 Fetal calf serum

10 Collagenase solution. This is made up using: 98.5 ml RPMI; 1.5 ml calcium chloride (1 Molar); 100 mg collagenase (Sigma blend H). After thorough mixing 5 ml aliquots are stored at -20°C in 10 ml syringes having been filtered through a .22µm filter to sterilise.

11 Universal containers

12 Endothelial cell culture medium, which is made up using: RPMI 1640 (149 ml); fetal calf serum (40 ml); penicillin/streptomycin (2 ml); endothelial cell growth supplement (1 ml); Heparin (2 ml); L-glutamine 200 mMol (2 ml); sodium pyruvate 100 mMol (0.4 ml).

3.4 Subculture of human umbilical vein endothelial cells

Twenty four hours after isolation of the endothelial cells from a human umbilical vein, the cells which were viable were adherent to the tissue culture flask. The media was then poured off and replaced with a further 5 ml of endothelial cell culture medium. The flask was examined under an inverted phase contrast microscope. The adherent endothelial cells had a characteristic appearance and these could easily be identified. (see plates 3.1 and 3.2). When the confluent endothelial cells had formed a monolayer,it had a cobblestone appearance. When the monolayer had covered the entire flask, the cells were ready for subculture which allowed cell numbers to be expanded for experimentation.

The cells had to first be detached from the culture flask by using a

combination of Trypsin and agitation.

1 All procedures were performed in a Class II microbiological cabinet.

2 The supernatant was poured from the flask, and replaced with 1ml trypsin solution. Trypsin solution was made up in the laboratory and after thorough mixing this solution was stored in 5 ml aliquots at -20°C: Trypsin 2.5% (20 ml), Phosphate buffered saline ph 7.2 (460 ml), EDTA 1% (10 ml), HEPES Buffer 1 M (10 ml).

3 The flask was then incubated at 37°C for 5 minutes, or until the cells were detached.

4 The flasks were checked to ascertain cell detachment by observation under an inverted phase contrast microscope. A sharp tap to the side of the flask often helped detach cells.

5 10 ml HBSS with calcium + 20% foetal calf serum was added to the flask, as soon as the cells were detached to prevent the Trypsin further digesting the endothelial cells and compromising viability.

6 The cell suspension was transfered to a sterile universal container and centrifuged for 5 min. at 150 g.

7 The supernatant was poured off from above pellet and the pellet was resuspended in 10 ml HBSS/FCS. This suspension was then re-centrifuged for 5 min. at 150g.

8 Stage 7 repeated.

9 The pellet was resuspended in 15 ml endothelial cell culture medium and the cells from one confluent 25 ml flask were divided into three 25 ml flasks by adding 5 ml media to each.

10 Cells were incubated at 37°C in a 5% carbon dioxide atmosphere as before.

11 The culture media was changed as required (approximately every 48-72 hrs) until confluence was reached.

3.5 Preparation of human umbilical endothelial cells for experimental work

HUVECS prepared by trypsination (Ch 3 ii) can be cultured in any tissue culture grade experimental flasks or wells, for the purpose of these studies twenty four and ninety six well (NUNC) tissue culture plates were convenient to use. After trypsinisation, HUVECS were resuspended at a concentration of 1 $x10^5$ cells per ml of endothelial cell culture medium. One ml of cells were added per well if a 24 well plate was used and 0.1 ml per well if a 96 well plate was used. Plates were incubated for 24 hours and then visually inspected using a phase contrast microscope. At this stage the cells were usually fully adherent, confluent monolayers and ready for further experimental work.

3.6 Characterisation of endothelial cells

Endothelial cells have a characteristic 'cobble stone' appearance when viewed under a phase contrast microscope. Endothelial cells strongly express factor VIII on the cell surface and this was used to characterise the cells and confirm cell culture purity. At each subculture stage representative aliquots of cells were taken and prepared for factor VIII assessment. This was done using the cytospin method.

3.7 Cytospin Method

1 Cell suspensions were obtained at a concentration of 2 x 10^5 cells per ml. Six slides should were prepared with 100 µL of cells required for each slide.

2 The cells were transferred onto microscope glass slides in the following way: a standard glass microscope slide was placed on a metal cytospin holder.

Plate 3.1 Human umbilical vein endothelial cells viewed under phase contrast microscopy, showing characteristic 'cobble stone' morphology (magnified x400).

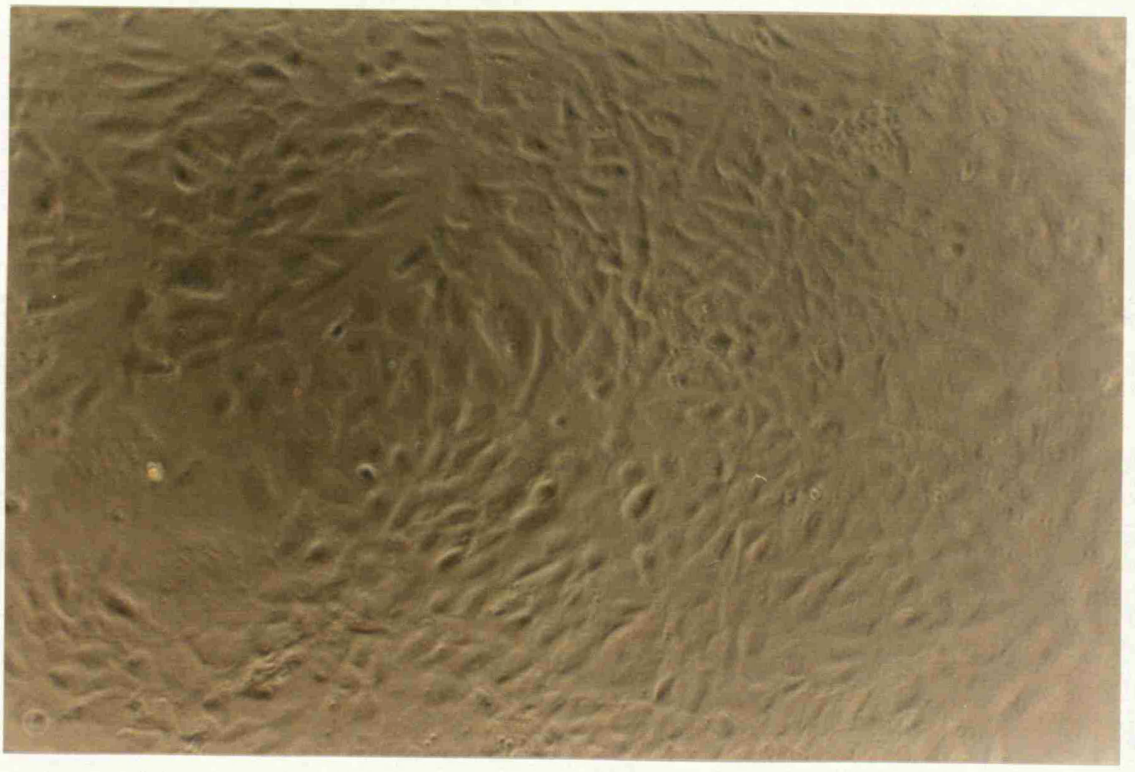


Plate 3.2 Human umbilical vein endothelial cells stained with Rose Bengal dye, showing a confluent monolayer (magnified x100).

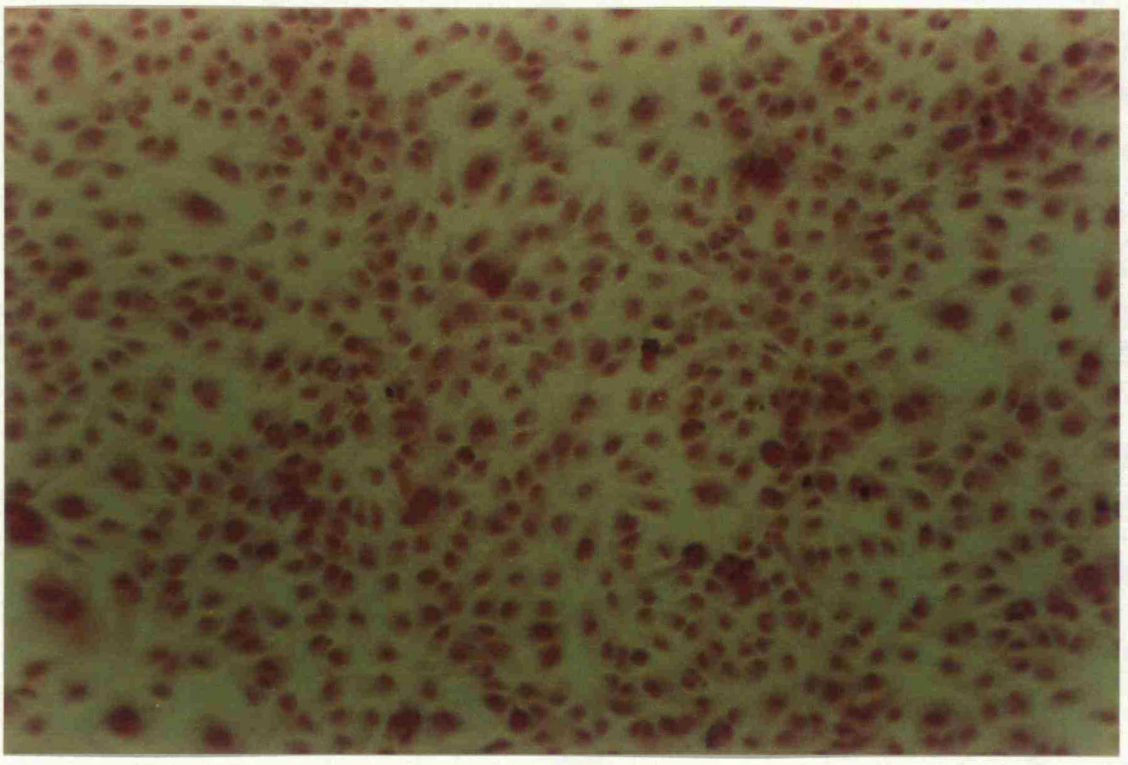


Plate 3.3 Individual parts of the CytoSpin Assembly. Left to Right, Metal holder, CytoSpin card, Microscope Slide, Plastic CytoSpin bucket.

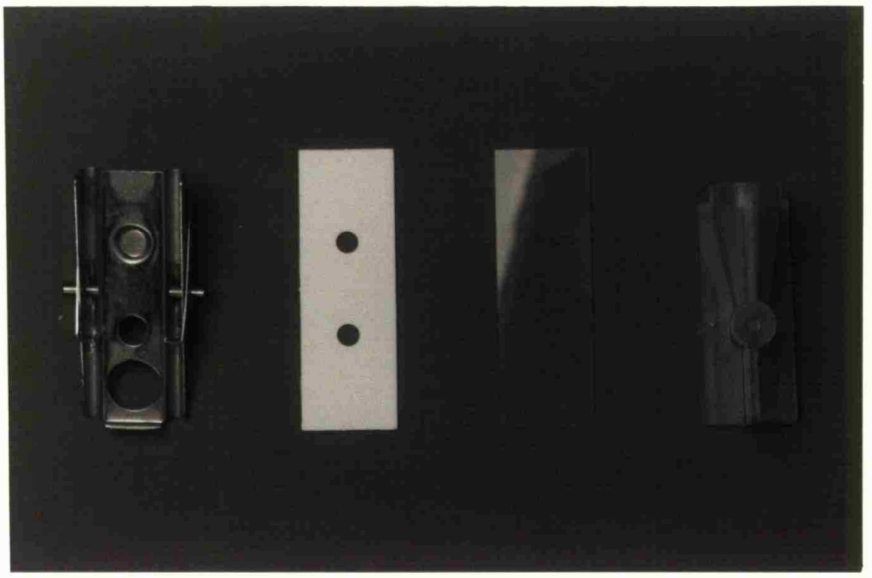
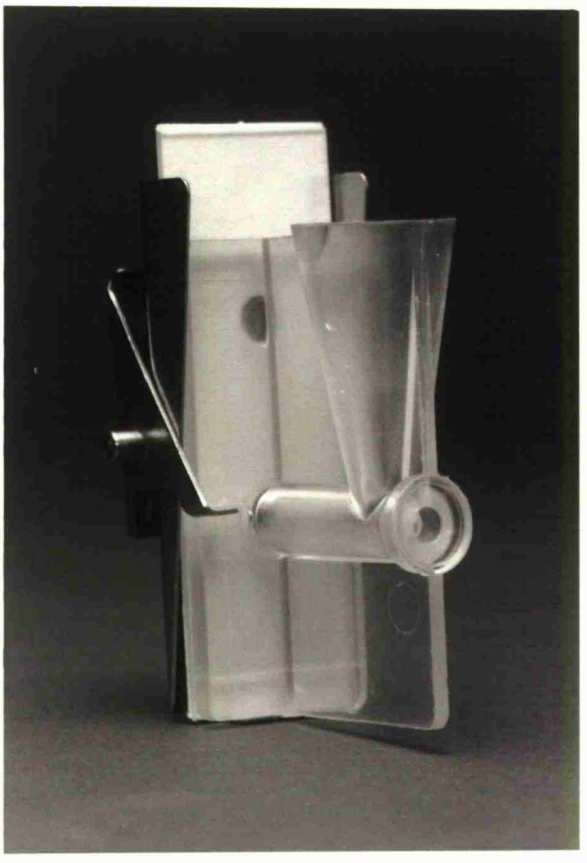


Plate 3.4 Assembled CytoSpin parts.



An absorbent cytospin card with a punched central hole was then placed on the slide and covered with a plastic bucket which had a chamber to insert the cell suspension. This chamber leads to a tube which abuts the glass slide at the punched hole in the card. A locking clamp mechanism ensured that the four different components stayed firmly applied together. See photographs Plates 3.3 and 3.4.

3 The slide holder was placed in the centrifuge and 100 µL cells were then placed in each bucket.

4 The slides were centrifuged at 150g for 7 minutes on the low acceleration setting.

5 The clamp mechanism was released using a special tool and to avoid smearing the central cell preparation on the slide the cytospin card was carefully bent away from the slide before removal.

6 The slide was allowed to air-dry for one hour, after this slides could be stored at -20°C in bags containing silica gel to prevent condensation.

7 For immunocytochemistry the slides were removed from the freezer and allowed to thaw in their bags for one hour at room temperature (see immunocytochemistry method).

8 This method produced a microscope slide with a central portion of human umbilical vein endothelial cells, which were present as a monolayer, which was confirmed using light microscopy before staining.

3.8 Cell counting

The counting of cells was done using a standard technique.

1 50 µL of the cell suspension to be counted was added to 200 µL of counting fluid and thoroughly mixed. Counting fluid was prepared by dissolving 1 mg/L gentian violet in 2.0 % w/v acetic acid in distilled water.

2 A pre-etched cell counting slide (Neubauer) was washed in methanol solution and a cover slip applied to its surface obtaining a good fit between the two.

3 The diluted cell suspension was introduced under the edge of the cover slip of the microscope slide. A minimum suspension to cover the etched counting chambers was used to ensure a monolayer of cells which restricts the counting error.

4 Four sets of 25 grids were counted and an average taken.

5 The cell concentration is worked out using the following formula:

Average number of cells in 4 grids = c

Cells per ml = $c \times 5 \times 10^6$

3.9 Flow cytometry of human umbilical vein endothelial cells

1 Human umbilical vein endothelial cells were subcultured into tissue culture flasks or wells. When the cells were confluent they were removed from the culture vessels by trypsinisation, as previously described. After the post trypsinisation washing sequence they were resuspended in phosphate buffered saline and counted. They were then further diluted using phosphate buffered saline to a final concentration of 1×10^5 cells/ml.

2 100 µL of cells were drawn off and placed in a 5 ml test tube.

3 1 µL of appropriately diluted monoclonal antibody was added to the endothelial cells and thoroughly mixed using a vortex agitator.

4 The tube was placed on ice for 30 minutes in the light to incubate.

5 After incubation excess unbound monoclonal antibody was removed by washing using the following procedure. 3 ml of phosphate buffered saline was added and mixed using the agitator, the cell suspension was then centrifuged at 150 g for 5 minutes. The supernatant was discarded and the cells were resuspended in phosphate buffered saline and centrifuged as before. After discarding the supernatant again the cells were re-suspended

in 50 µL of 0.1% (w/v) human albumin in phosphate buffered saline containing 0.005% (w/v) human AB serum.

6 50 µL FITC conjugated anti mouse IgG polyclonal antibody (all antibodies were resuspended in 100 µL of a mixture of phosphate buffered saline/azide/bovine serum.) was added to this suspension.

7 The tube was then incubated at 4°C in the dark.

8 Excess FITC conjugated anti mouse antibody was removed by washing. 3 ml phosphate buffered saline was added and mixed and the suspension then centrifuged at 150 g for 5 minutes. The supernatant was discarded and the cells resuspended. This washing step should be repeated three times.

9 After the final wash the cell pellet was resuspend in 100 µL of 1% of paraformaldehyde phosphate buffered saline (care is required in the handling of paraformaldehyde which is toxic).

10 The cells were then ready to be analysed using the flow cytometer. Some of the flow cytometer parameters were preset, the Amplitude to 1.00, the Threshhold to 200 and the gates to count 2000 cells.

11 After counting the flow cytometer software was used to obtain both a numerical and graphic representation of cells counted.

3.10 Collection of blood samples

1 All blood samples were regarded as hazardous and treated with care. Gloves were worn by the phlebotomist and all needles were immediately disposed of into sharp safe boxes.

2 A tourniquet was placed around the upper arm of the blood donor.

3 Venous filling was encouraged by asking the donor to squeeze their hand into a fist and by sharply tapping the vein to be used.

4 The skin above the vein was cleaned with an alcohol swab.

5 Two blood collection systems were used in this study, either a needle and syringe or the vacutainer blood collecting system. The needle was

introduced into the vein and the syringe or the blood bottles were then filled as required.

6 When sufficient blood had been taken, the tourniquet was released.

7 The needle was removed from the vein and pressure immediately applied to the puncture site with a cotton wool ball pressing for one to two minutes to ensure haemostasis.

8 The punture site was then covered with a disposable dressing.

9 Blood samples were stored in a 4°C fridge until ready for use.

3.11 Isolation of neutrophils from whole blood by density centrifugation

This method is as described by Eggleton (Eggleton et al, 1989).

The main advantages of this method were that it was simple, relatively rapid to perform, cell morphology was well preserved and the method does not impair the function of the cells. A relatively high yield of cell is possible if blood is collected into potassium EDTA tubes with as many as 4×10^6 cells extracted from 1mL of blood. The cell preparations contained approximately 10% lymphocytes, 5% eosinophils and 2% monocytes. This purity was not as good as that obtained using gradient polymers however Percoll gradients, which are commonly used in this method, can affect polymorph function by being phagocytosed while the cells are layered on the gradient. This may then affect the ability of the polymorph to perform cell adhesion. It was also important that the blood be collected into potassium EDTA because citrate and heparin have been found to stimulate the oxidative burst of polymorphonuclear leucocytes.

1 4 ml venous blood was collected in postassium EDTA to a universal container.

2 The blood was then added to 16 ml cold (4°C) isotonic ammonium chloride. This was mixed well and incubated at 4°C for fifteen minutes.

3 This mixture was then centrifuged at 160 g for 10minutes.

4 The supernatant was discarded.

5 The cell pellet was gently resuspended in 10 ml phosphate buffered saline.

6 This suspension was centrifuged at 55 g for 10 minutes.

7 The supernatant was discarded.

8 The cell pellet was gently resuspended in 5 ml phosphate buffered saline.

9 This suspension was centrifuged at 55 g for 5 minutes.

10 The supernatant was discarded and the cells resuspended in phosphate buffered saline.

11 The cell concentration was determined by the Haemocytometer counting method.

12 The cell suspension was further diluted, if required, to the required concentration and stored at 4°C until use.

3.12 Method of renal transplant biopsy

This method is taken from Nicholson 1990 (Nicholson et al, 1990).

Prior to biopsy blood coagulation as measured by INR (International Normalised Ratio) was within the normal range and the diastolic blood pressure was less than 100 mmHg. The procedure was performed under aseptic conditions. The transplanted kidney was localised and orientated using ultrasound scanning, during this project an Aloka SSD-630 real time machine was used with a fully sterilisable 3.5 MHz linear array probe.

A transverse or longitudinal section of the kidney was demonstrated in a position which was suitable for easy manipulation of the biopsy gun and needle. The ultrasound probe was held steady throughout the procedure by an assistant. The skin and subcutaneous tissues down to the capsule of the transplanted kidney were anaesthetised by the local infiltration of 1-2% lignocaine solution. A scalpel blade (no 11) was used to pierce the

skin over the transplant to facilitate smooth passage of the biopsy needle. A 14 gauge Tru-cut biopsy needle (Travenol Laboratories Incorporated, Deerfield, USA), mounted in the Biopty gun (Radiplast AB, Uppsala, Sweden) was used. The Biopty gun is an automatic firing device which works by a spring loaded mechanism (Lindgren, 1982). The Tru-cut needle was introduced through the subcutaneous tissues and down to the capsule of the kidney under ultrasound control. The needle was carefully passed through the fibrous pseudocapsule of the transplanted kidney. The capsule presents variable resistance, depending on the age of the transplant, and with long-standing organs is often quite thick. The biopsy track and needle tip can be seen very clearly ultrasonically and this allows precise targeting. The biopsy needle tip was positioned just underneath the pseudocapsule and the angle of attack adjusted so that a superficial cortical biopsy was taken.

Following biopsy, firm pressure was applied over the biopsy site for 5 minutes. A pressure dressing was then applied and the patient kept on strict bed rest for 12 hours. Intensive observation of the pulse and blood pressure was made during this time interval. Serial urine samples were displayed at the bedside to judge the presence and progress of macroscopic haematuria.

The use of both the Biopty gun and the ultrasound scanner were essential to the success of the method. The Biopty gun performed a 2-stage cutting action of the Tru-cut needle mechanically and we believe that this improved both the size and quality of the tissue core obtained when compared with the manual Tru-cut technique.

The use of real time ultrasound allowed accurate orientation of the biopsy procedure so that the renal vessels and ureter are avoided. The ultrasound scan also clearly distinguished between renal cortex and medulla and, with experience, selective biopsy of the renal cortex could be

achieved. This ensured a good glomerular count and avoided damage to the blood vessels in the medulla and hilum of the kidney.

3.13 Storing and mounting of tissue for histology

This method is applicable to all tissue specimens collected either using the Tru-cut biopsy technique or by open biopsy.

1 All specimens were collected into universal containers prefilled with minimum essential media (MEM). Specimens were immediately transported to the laboratory and stored at 4°C whilst the further equipment required was prepared.

2 A small volume of liquid nitrogen was poured into a dewar (protective glasses and gloves were worn at all times whilst handling liquid nitrogen).

3 The specimen and media were poured into a petri dish and using a sharp scalpel blade a representative slice of tissue (minimum 3mm depth by 10 mm length) was cut, ensuring that tissue was sliced and not torn. The Tru-cut kidney biopsies were not large enough to require cutting down and were left intact.

4 A few drops of Tissue-Tex OCT embedding medium were poured onto a small piece of cork disc, taking care to avoid the introduction of any air bubbles.

5 The specimen was picked up using a 22 gauge orange needle and mounted in the OCT by drawing it through the fluid to flatten the specimen out.

6 The mounted specimen was plunged into the liquid nitrogen using large metal forceps and held under until bubbling ceased and the specimen was frozen.

7 A small screw top vial was labeled with patient details and the specimen was placed in it.

8 The vial was stored in liquid nitrogen until the specimen was ready for further processing.

3.14 Cutting and fixing frozen sections using frigocut cryostat

a Cabinet was set at -20°C for cutting kidney specimens.

b Defrost timer was set for 12 midnight.

c The clearance angle of the knife block was set at 7.5° (the first mark represents 5°)

d The cutting thickness was set at 5 mm.

Cutting sections

The biopsy was transfered from the main liquid nitrogen tub to the cryostat cabinet using a small dewar flask filled with liquid nitrogen. Protective equipment was used, mask and gloves, because of the danger of vial explosion due to rapid expansion of liquid nitrogen trapped in vial.

1 The vial containing cork mounted biopsy was placed in the cabinet and left for one hour to warm to -20°C.

2 The cork was mounted on the object block with OCT and placed on a freezing stage until the OCT had hardened.

3 The object block was placed on the cutting stage and the cutting stage position was adjuted as required. The biopsy was trimmed until the excess OCT had been cleared and tissue was being cut, the sections were then collected on from the knife edge using an anti-roll plate (position altered as required). The sections were picked up on gelatinised microscope slides (slides dipped in solution of 0.6 gelatin in 100 ml distilled water + 0.05 g chrome alum - added after gelatin is dissolved. Slides were then left to air dry and stored in large storage box). Enough sections were cut for 2 staining runs.

4 The section was allowed to air dry for at least one hour before fixing.

5 The slides were labelled in pencil with the biopsy number and details of patient, date cut, etc. were entered in a biopsy record book which was kept with the biopsy equipment.

Fixing sections

1 The slides with sections on them were placed in a rack and into black dishes containing acetone at room temperature for 10 minutes, then allowed to air dry.

2 Half the slides were stored in racks in labelled bags containing silica gel in a -20°C freezer (or overnight at room temperature for staining next day). The other half were wrapped with tape and dividers, and placed in labelled sample bags containing silica gel and stored in airtight boxes in a separate -20°C freezer. The details were then entered into the cut biopsy record book.

3.15 Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining

This method is applicable to both sections of tissue and cell preparations prepared by cytospin. Sections can be used fresh after cutting and fixing or can be used from frozen. All incubations were carried out at room temperature in humid chambers using a drop of antibody/substrate on section. All biopsies were first ringed with a Dako wax pen.

1 Slides were rehydrated in phospate buffered saline for 15 minutes.

2 Sections (pre fixed in acetone) were incubated with 20% AB serum in PBS (AB/PBS) for 30 minutes.

3 The slides were drained and wiped around the sections of tissue. 100 µL of appropriately diluted (in AB/PBS) monoclonal antibody was added to each section and incubated for 45-60 mintues in humid chambers.

4 The slides were washed three times for 5 minutes each in PBS using racks and black dishes.

5 The slides were drained and wiped, and 100 µL of rabbit anti-mouse 'link' antibody diluted 1:50 (20 µL in 1 ml) in AB/PBS was added to each section. The slides were then incubated for 30 minutes.

6 The slides were washed 3 times for 5 minutes each in PBS.

7 The slides were drained and wiped and 100 µL APAAP complex diluted 1:50 (20µL in 1 ml in AB/PBS) was added to each section. The slides were then incubated for 30 minutes.

8 The slides were washed 3 times for 5 minutes each in PBS. NB the last wash contained 2.5 ml 0.1M levamisole.

9 The sections were then incubated for 20 minutes with freshly made substrate - 10 mg Naphthol AS/BI phosphate + 10 ml buffer (0.2 M Tris-HCL pH9) to this was added to 10 mg Fast red TR salt + 100 µL 0.1M levamisole. Note: Naphthol-AS-BI-phosphate + Fast Red are considered possible carcinogens; gloves were worn at all times.

10 The slides were then rinsed with distilled water.

11 The sections were counterstained with Mayer's Haemalum for 10 minutes.

12 The slides were then left to soak in tap water for 2-3 minutes.

13 The sections were then mounted wet using pre-warmed glycerol gelatin.

3.16 Preparation of Cytokines

3.16.1 Interleukin-1ß

The Interleukin-1ß (IL-1ß) used in these studies was obtained from British Biotechnology and produced by recombinant gene technology. The product was supplied as 2 mg of vials lyophilised powder, with a stated activity of 200 U/vial. The vials were reconstituted with 2 ml of 0.22 mm filter sterilized phosphate buffered saline plus 0.1% (w/v) human serum albumin, and aliquoted in 100 ml lots into sterile 1 ml storage tubes (Sarstedt, 'Cryovials', UK). Aliquots were stored at -20°C until needed, and discarded after 3 months. Before use vials were thawed at room temperature, and the contents of the vial added to 0.9 ml of fresh ECM

giving a stock solution of 20 U/ml.

3.16.2 Interferon-gamma

The Interferon-gamma (IFN-g) used in these studies was obtained from Sigma Chemicals (I-6507) and was supplied as vials of 0.1 ml INF-g solution in phosphate buffered saline plus human serum albumin, with a stated activity of 500,000 U/vial. The vials were diluted with 9.9 ml of 0.22 mm filter sterilized phosphate buffered saline plus 1% (w/v) human serum albumin (Final concentration 50,000 U/ml), and aliquoted in 0.5 ml lots into sterile 1 ml storage tubes (Sarstedt, 'Cryovials', UK). Aliquots were stored at -70°C until needed, and discarded after 3 months. Before use the tubes were thawed at room temperature, and contents diluted as required.

3.17 FACScan examination of cell surface markers: Details of anitbodies used.

Endothelial cell suspensions were examined by flow cytometry (see section 3.9), using a range of monoclonal antibodies the details of which are given in Table 3.1. (p 107). All antibodies used were monoclonal antibodies derived from mouse cell lines or whole animals.

3.18 Statistical analysis of data.

All statistical analyses were performed using an Apple Macintosh Classic 4/40 computer using MINTAB™ 8.0 and Instat™ 1.12 statistical packages. Advice concerning the use of parametric and non-parametric statistics was obtained from Dr T Horsburgh and other members of the Dept. of Surgery.

When appropriate statistical tests for parametric data (Students t test) were applied. However due to the small sample numbers encountered, particularly in Chapter seven, it was felt necessary to use distribution free or non-parametric tests. We used the Mann-Whitney U test and the paired Wilcoxon test where appropriate on the advice of a statician. Results are expressed in the normal way with significance reached when $p<0.05$. This is shown in the text and on graphs and tables.

Table 3.1 Antibodies used in FACScan examination of endothelial cell surface markers.

Target Antigen Number	Specificity	Ig Type	Source	Dilution	Catalogue
Anti-Class I	W6/32+	IgG2A	NS1/1£	1:1000	84112003
Anti-Class I	ABC Common++	IgG2A	Ascites	1:1000	MCA81A
Anti-Class II	DR*	IgG2A	L243@	1:1000	34736A
Anti-Class II	DQ*	IgG1	SK10$	1:1000	347450
Anti-ICAM-1	CD54**	IgG1	BB1G-1L$	1:1000	BBA3
Anti-E-Selectin	-**	IgG1	BB1G-E6$	1:1000	BBA2
Anti-PECAM	CD31**	IgG1	9G11$	1:1000	BBA7
Anti-VCAM	-**	IgG1	BB1G-V1$	1:1000	BBA5

Legend:

£Myeloma cell line
@Derived from Myeloma NS-1/1-Ag4
$Derived from Myeloma NS-1
+European Collection of Animal Cell Cultures, PHLS, Porton Down, Salisbury, UK
++SeroTec, Kidlington, Oxon, UK
*Becton-Dickinson, Oxon, UK
**R&D Systems Europe, Oxon, UK

CHAPTER FOUR

The effect of exogenous stimuli on the expression of endothelial cell surface antigens.

4.1 Introduction

The aim of these experiments was to investigate the effects of the inflammatory cytokines INF-gamma (INF-g) and Interleukin-1 beta (IL-1b) on endothelial cell expression of HLA Class 1 and Class II antigens and on the expression of the endothelial cell adhesion molecules E-Selectin, ICAM-1 and PECAM. The time course over which these molecules were expressed on the cell surface after cytokine stimulation was investigated as were a range of cytokine concentrations, to determine which produced maximum antigen expression.

The renal transplant patient has a number of unique stresses acting on the endothelial lining of their blood vessels, including uraemic toxins and immunosuppressive agents. The potential effect of these agents on HUVEC antigen expression was investigated *in vitro* using HUVEC cultures treated with cyclosporin A and methylprednisolone.

4.2 Methods

4.2.1 Cell Culture Methods

Endothelial cells (EC) were cultured in 24 well tissue culture plates (see Methods). After sub-culture, the cells were allowed to adhere to the plates for 24 hours before experimentation.

The supernatant medium was poured from the plates and replaced with 0.5 ml fresh endothelial cell medium (ECM), into which were added cytokines or immunosuppressive drugs at varying concentrations.

Assays of each stimulant concentration were performed in triplicate. To obtain samples for time course observations identical 24-well plates were prepared simultaneously. All plates were incubated at 37°C in a 5% CO_2/95% O_2 atmosphere.

4.2.2 Effect of INF-gamma on endothelial surface antigen expression

The concentrations of INF-gamma (INF-g), used were obtained by dilution of the stock solution described in the Methods section. The final INF-g concentrations (U/ml) used were:- 0, 10, 50, 100, 500, 1000, 5000 and 10,000.

One plate was removed from the incubator at 24, 48, 72 and 96 hours, the endothelial cells were examined with an inverted phase contrast microscope to confirm that a monolayer of cells was present. Cells were then detached by trypsinisation and washed (see Methods).

4.2.3 Effect of IL-1 beta on endothelial cell surface antigen expression

The concentrations of IL-1 beta (IL-1b), used were obtained by dilution of the stock solution described in the Methods section. The final IL-1b concentrations (U/ml) used were:- 0, 0.1, 1 and 5.

One plate was removed from the incubator at 1, 2, 3 and 4 hours, the endothelial cells were examined with an inverted phase contrast microscope to confirm that a monolayer of uncontaminated cells was present. Cells were then detached by trypsinisation and washed (see Methods).

4.2.4 Effect of Cyclosporin A on endothelial cell surface antigen expression.

Cyclosporin A (CyA) (Sandoz Pharma, Switzerland) was obtained in its intra-venous form at a concentration of 100 mg/ml. Dilutions of this stock were prepared with fresh ECM to produce final concentrations (mg/ml) of 0, 0.1, 1, 10 and 50.

Two different protocols were used to examine the effects of CyA on endothelial cell antigen expression. Protocol 1 examined the effects of pre-treatment of endothelial cells (EC) with CyA before stimulation. EC were pre-incubated in ECM + CyA for 48 hours, this medium was poured off and replaced with 0.5 ml of either fresh ECM or ECM+5 U/ml IL-1b and the cells were incubated for a further 4 hours before surface antigen assay. Protocol 2 examined the effects of simultaneous incubation of CyA and IL-1b. EC were incubated with ECM+CyA for 4 hours either with or without 5 U/ml IL-1b.

The EC suspensions from these assays were examined for increases in E-Selectin expression, since IL-1b only stimulated E-Selectin expression and for HLA Class I expression (with anti Class I ABC common) as a control.

4.2.5 Effects of Methylprednisolone on endothelial cell surface antigen expression.

Methylprednisolone (MPred) was obtained as an intra venous preparation and made up as a stock solution at a concentration of 500 mg/ml in ECM. Dilutions of this stock were prepared with fresh ECM to produce final concentrations (mg/ml) of 0.25, 2.5 and 25.

Two different protocols were used to examine the effects of MPred on endothelial cell antigen expression. Protocol 1 examined the effects of pre-treatment of endothelial cells (EC) with MPred before stimulation. EC were pre-incubated in ECM + MPred for 24 hours, this medium was poured off

and replaced with 0.5 ml of either fresh ECM or ECM+5 U/ml IL-1b and the cells were incubated for a further 4 hours before surface antigen assay. Protocol 2 examined the effects of simultaneous incubation of MPred and IL-1b. EC were incubated with 0.5 ml ECM+MPred for 4 hours, either with or without 5 U/ml IL-1b.

The EC suspensions from these assays were examined for increases in E-Selectin expression, since IL-1b only stimulated E-Selectin expression, and for HLA Class I expression (anti Class I ABC), as a positive control.

4.2.7 FACScan examination of cell surface marker expression

The EC suspensions were processed for flow cytometry, (see Methods 3.9), with cell surface antigens assayed using the following mouse monoclonal antibodies (MoAb). Further details are available from Table 3.1

1 Anti-Class I (W6/32, Dept of Surgery, Leicester, UK)

2 Anti-Class I (ABC Common, SeroTech, Oxon, UK)

3 Anti-Class II (DR, Becton-Dickinson, Oxon, UK)

4 Anti-Class II (DQ, Becton-Dickinson, Oxon, UK)

5 Anti-ICAM-1 (R&D Systems Europe, Oxon, UK)

6 Anti-E-Selectin (R&D Systems Europe, Oxon, UK)

7 Anti-PECAM (R&D Systems Europe, Oxon, UK)

0.5 ml of stock MoAb solution was used in each case. Control cell suspensions, without MoAb but with FITC conjugated anti-mouse MoAb and with MoAb but without FITC conjugated anti-mouse MoAb, were used in these experiments.

4.3 Results

All flowcytometer results are expressed as median fluorescence, but it was important to allow for the fact that endothelial cells show a background fluorescence without labelling when examined in a flowcytometer.

In order to obtain fluorescence above this normal background the median fluorescences of the labelled cells have had the median fluorescence of an unlabelled HUVEC suspension subtracted from them. As an example the data used to plot Fig 4.1 is shown in Table 4.1, (page 117), with annotations to explain the steps involved in calculating the median fluorescence.

4.3.1.1 Effects of INF-gamma on endothelial cell surface antigen expression

The results of the flow cytometric analyses are shown in Figs 4.1 to 4.6, with median fluorescence shown on the y-axis, (pages 118-123).

4.3.1.2 INF-gamma and expression of HLA Class I

Figs 4.1 and 4.2 show HLA Class I expression as detected by two separate antibodies. The W6/32 antibody has a lower affinity for the Class I antigen than ABC common, but still shows the change in Class I expression. This demonstrates that low affinity antibodies are capable of detecting changes in cell surface antigen expression and allowed the use of the other antibodies in these experiments without extensive affinity studies. High initial median fluorescence demonstrated that expression of HLA Class I was constitutive in cultured endothelial cells. HLA Class I expression was up regulated by INF-g, this up regulation was concentration dependent with maximal stimulation seen at 72 hours. The minimum concentration required for stimulation was <10 U/ml, with maximal stimulation seen with 500 U/ml.

4.3.1.3 INF-gamma and expression of HLA Class II

HLA Class II expression is shown in Figs 4.3 and 4.4. Unstimulated HUVEC's showed no HLA Class II DR or DQ expression. HLA Class II expression was rapidly increased after INF-g stimulation, in a

concentration dependent manner. Maximum expression occurred between 24 and 72 hours post stimulation. The minimum concentration required for stimulation was 50 U/ml with maximal stimulation seen with 1000 U/ml. The Class II DQ expression was not stimulated by by INF-g. HLA Class II expression reduced with increasing time in culture in unstimulated cells.

4.3.1.4 INF-gamma and expression of Adhesion molecules

Fig 4.5 shows expression of ICAM-1. ICAM-1 was constitutively expressed by cultured HUVEC and is increased by INF-g stimulation in a concentration dependent manner. Maximum expression occurs between 48 and 72 hours post stimulation. The minimum concentration required for stimulation was <10 U/ml with maximal stimulation was seen at 1000 U/ml.

Fig 4.6 shows expression of PECAM and E-Selectin. PECAM and E-Selectin were expressed constitutively by HUVEC cells, however PECAM is expressed at a higher level then E-Selectin. Neither PECAM or E-Selectin were up regulated by INF-g stimulation.

4.3.2 Effects of IL-1 beta on endothelial cell surface antigen expression

The results of these experiments are shown in Figs 4.7-4.12, (pages 124-127)

4.3.2.1 IL-1 beta and expression of HLA Class I.

Fig 4.7 shows the expression of HLA Class I stimulated with 5 U/ml IL-1b, as detected by the ABC common antibody. HLA Class I was constitutively expressed but there was no effect of IL-1b on expression of HLA Class I over time.

4.3.2.2 IL-1 beta and expression of HLA Class II.

Fig 4.8 shows the expression of HLA Class II stimulated by 5 U/ml

IL-1b, as detected by the anti-HLA Class II DR antibody. There was no effect of IL-1b on endothelial expression of HLA Class II DR or DQ over time.

4.3.2.3 IL-1 beta and expression of adhesion molecules.

Fig. 4.9 shows expression of ICAM-1 on endothelial cells stimulated with 5 U/ml IL-1b. There was no effect of IL-1b on the constitutive expression of ICAM-1.

Fig. 4.10 shows the expression of PECAM on endothelial cells stimulated by 5 U/ml IL-1b . There was no effect of IL-1b on the constitutive expression of PECAM.

Fig 4.11 shows the expression of E-Selectin on endothelial cells stimulated with IL-1b at different concentrations. IL-1b stimulates the expression of E-Selectin in a dose dependent manner over time. These results suggested that the E-Selectin expression was sub-maximal at 4 hours post-stimulation and a further experiment using 5 U/ml IL-1b over a time course of 24 hours was carried out. The results of this experiment are shown in Fig 4.12, E-Selectin expression appears to reach a peak between 4 and 8 hours post stimulation, but had returned to almost unstimulated levels by 24 hours post-stimulation.

4.3.3 Effect of Cyclosporin A on endothelial surface antigen expression.

Figs 4.13 and 4.14, (page 128), show the effects of both CyA treatments on endothelial cell Class I expression. As can be seen CyA had no effects on HLA Class I expression at concentrations 0.01 to 1.0 mg/ml, however cytotoxic effects are seen at 10 mg/ml and above reducing the median fluorescence to 0.

Figs 4.15 and 4.16, (page 129), show the effects of both CyA treatments on endothelial cell E-Selectin expression. Concentrations of

CyA of 10 mg/ml and above were seen to be cytotoxic in that there was complete detachment of the HUVEC molayer from the cell culture plate as viewed under phase contrast microscopy. There appeared to be no effect of lower concentrations of CyA on E-Selectin expression except for a small, non-significant, decrease in expression at a concentration of 1.0 mg/ml.

4.3.4 Effects of Methylprednisolone on endothelial cell surface antigen expression.

Figs 4.17 and 4.18, (page 130), show the effects of both Methylprednisolone (MPred) treatments on endothelial cell Class I expression. As can be seen MPred had no effects on HLA Class I expression at concentrations of 0.25 mg/ml and 2.5 mg/ml, however MPred is seen to be cytotoxic at 25 mg/ml reducing the median fluorescence to 0.

Figs 4.19 and 4.20, (page 131), show the effects of both MPred treatments on endothelial cell E-Selectin expression. A concentration of 25 mg/ml MPred of was cytotoxic similar to that of high concentrations of Cyclosporin, and there was no effect of lower concentrations of MPred on E-Selectin expression.

4.4 Conclusions

1. HLA Class I, Class II DR and ICAM-1 expression were up regulated at the HUVEC surface by INF-g in this *in vitro* model.
2. The optimum concentration of INF-g to produce this maximal expression was similar for these molecules, between 500 and 1000 U/ml, as was the time course over which this increased expression occurred, between 48 and 72 hours.
3. Only E-Selectin expression was up regulated at the HUVEC surface by IL-1b in this *in vitro* model.
4. The optimum concentration of IL-1b was 5 U/ml with maximal expression

at 4 to 8 hours post-stimulation.

5. The immunosuppressive agents tested, Cyclosporin A and Methylprednisolone, did not affect the IL-1b stimulated expression of E-Selectin. However it should be emphasised that these experiments using Cyclosporin and Methylprednisolone were rather basic in their design and only give a limited insight into the effects of these agents on adhesion molecule expression by HUVECS. Since the concentrations of drug used were only just sub-lethal to the HUVECS it may be that cell function was impaired, limiting normally expected cell responses. Furthur studies are indicated using lower drug concentrations and monitoring other cell functions to ensure normal responses before it can be confidently asserted that HUVEC cell adhesion molecule expression is not effected by immunosuppressive agents.

Table 4.1 Data used to derive the graph in Figure 4.1.

Incubation Time (hrs)	Background*	Units of Interferon-γ /ml 10000	5000	1000	500	100	50	10	0
0**	200	294	294	294	294	294	294	294	294
24+	200	394	384	368	336	323	304	302	294
48+	202	441	416	453	318	298	248	208	244
72+	225	448	452	471	456	408	376	325	255
96+	298	410	434	427	405	404	342	327	283

**At time= 0 the binding of antibody to unstimulated cells was assayed to provide a measure of the constitutive expression of the surface molecules. The value represents a median of 2 replicates measured on two separate days (n=4), the same cells were used to seed the treated cultures and thus all treatments share this common value.

* The median fluorescence of endothelial cells passed through the flowcytometer without antibody labelling of any kind. The cells used for this were cultured at the same time as the IFN-γ treated cells and processed in the same way as treated cells apart from the addition of antibody.

+All values are a median of 2 replicates measured on two separate days (n=4), the values for treated cells have had the background fluorescence subtracted.

Fig 4.1 Interferon stimulated endothelial cell expression of HLA Class I (W6/32).

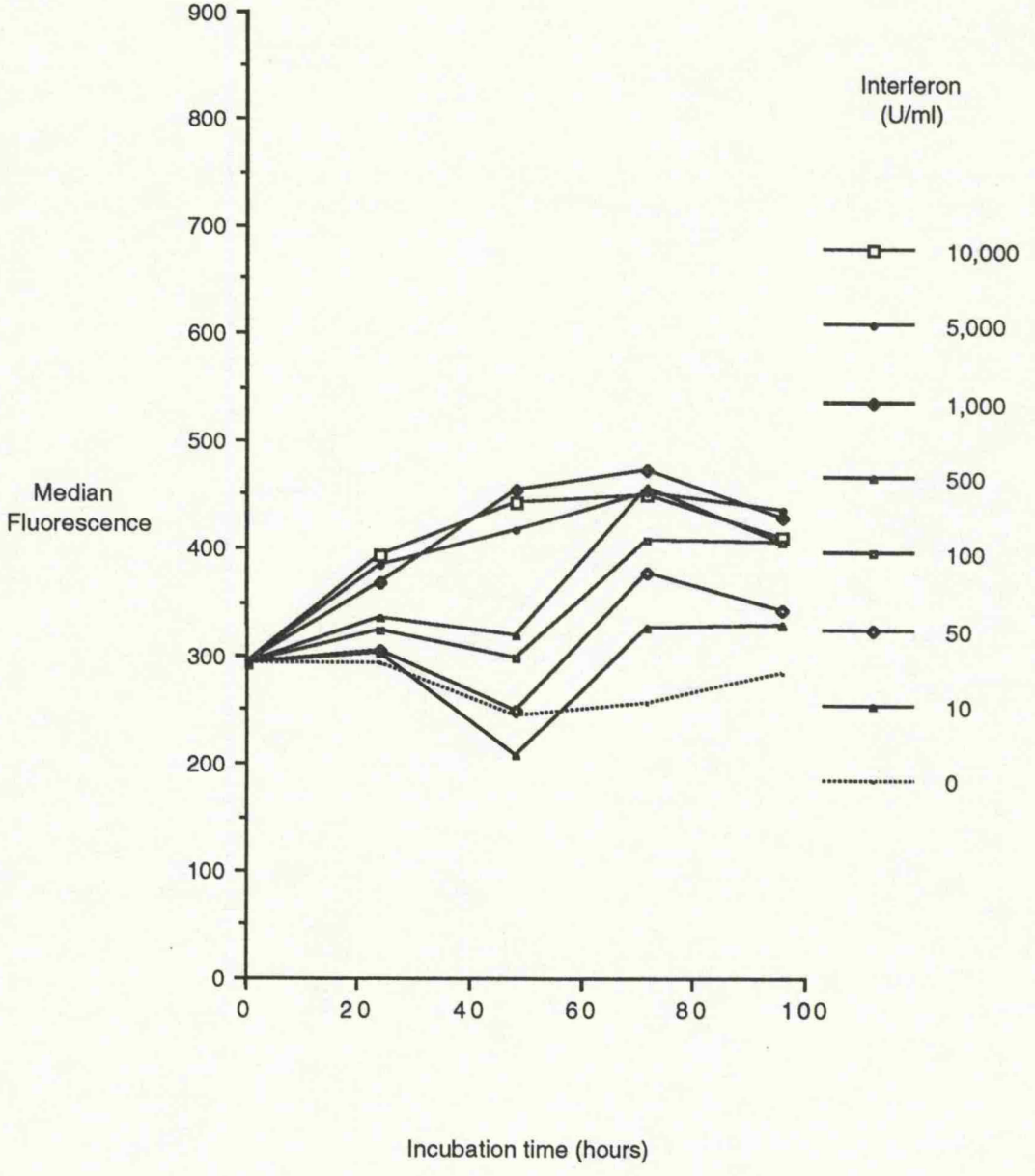


Fig 4.2 Interferon stimulated endothelial cell expression of HLA Class I (ABC Common).

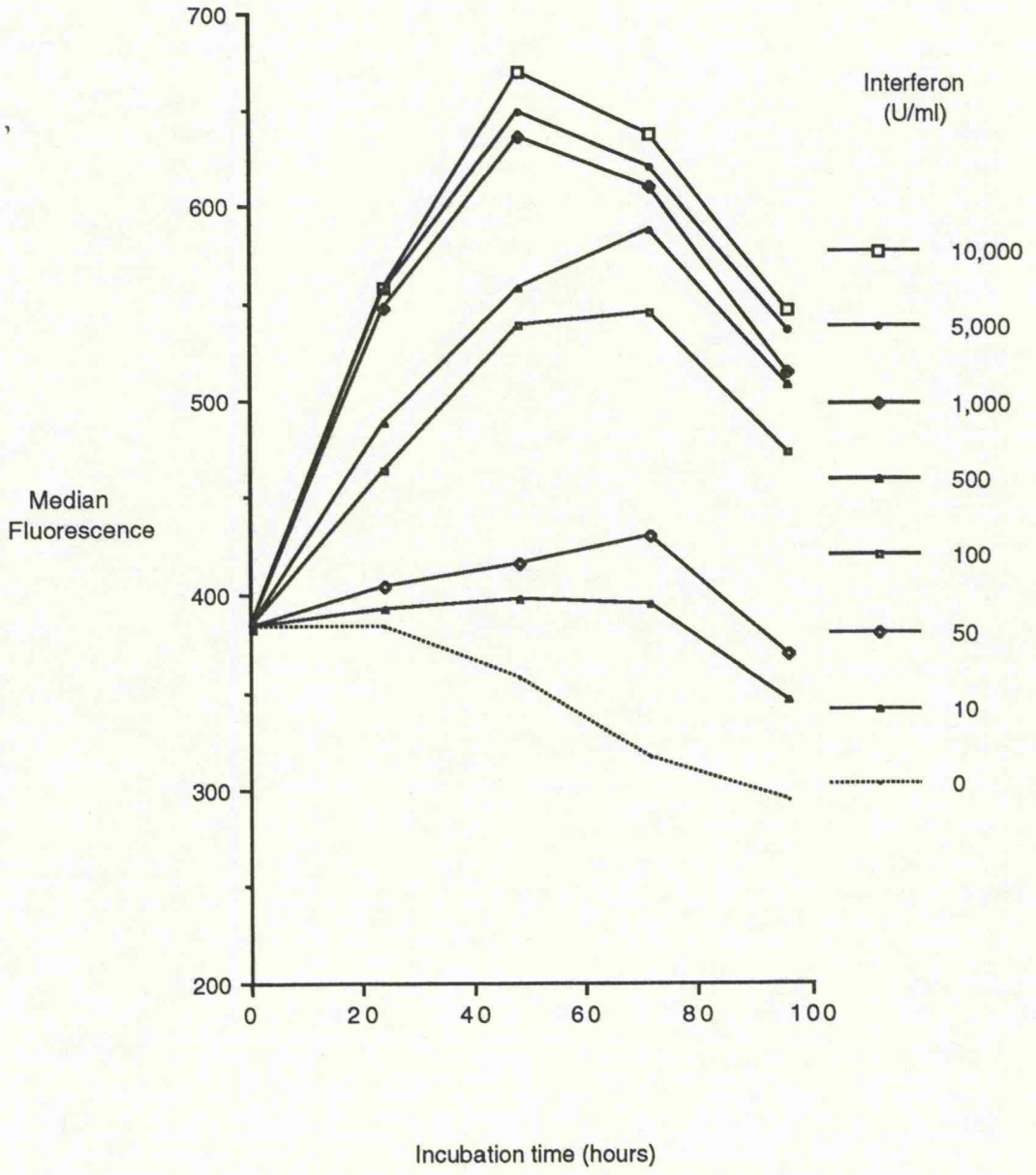


Fig 4.3 Interferon stimulated endothelial cell expression of HLA Class II DR.

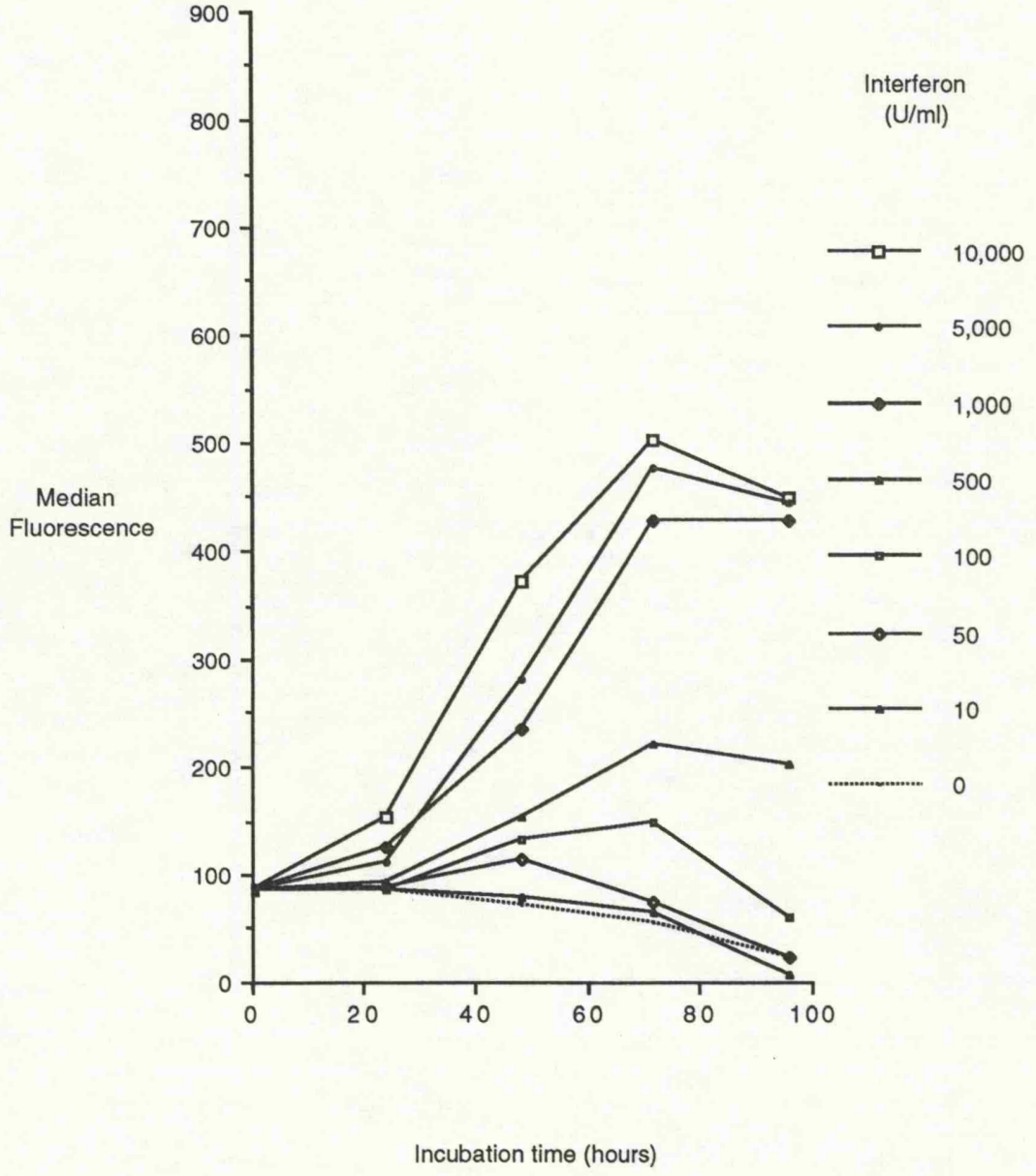


Fig 4.4 Interferon stimulated expression of HLA Class II DQ.

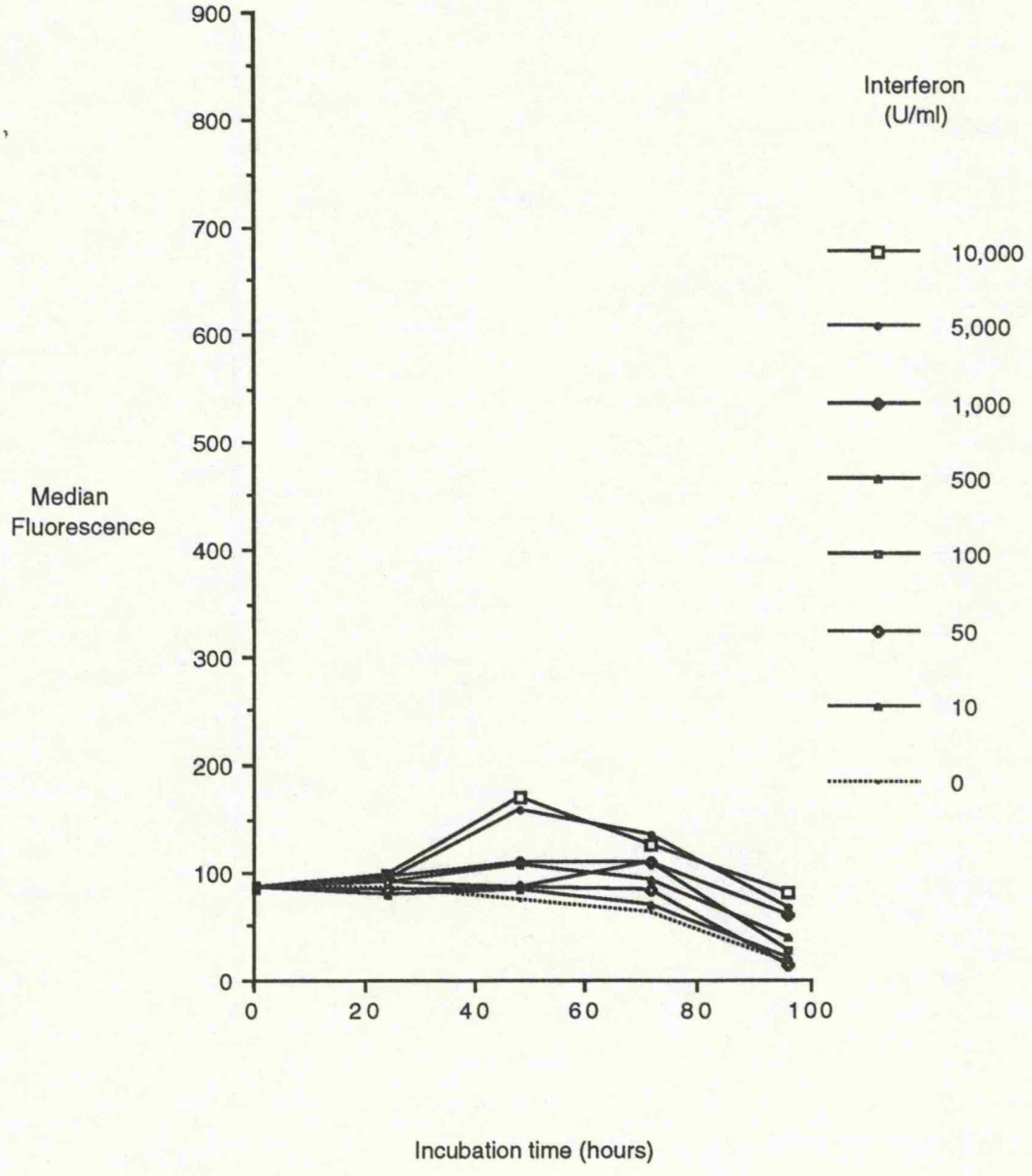


Fig 4.5 Interferon stimulated endothelial cell expression of ICAM-1

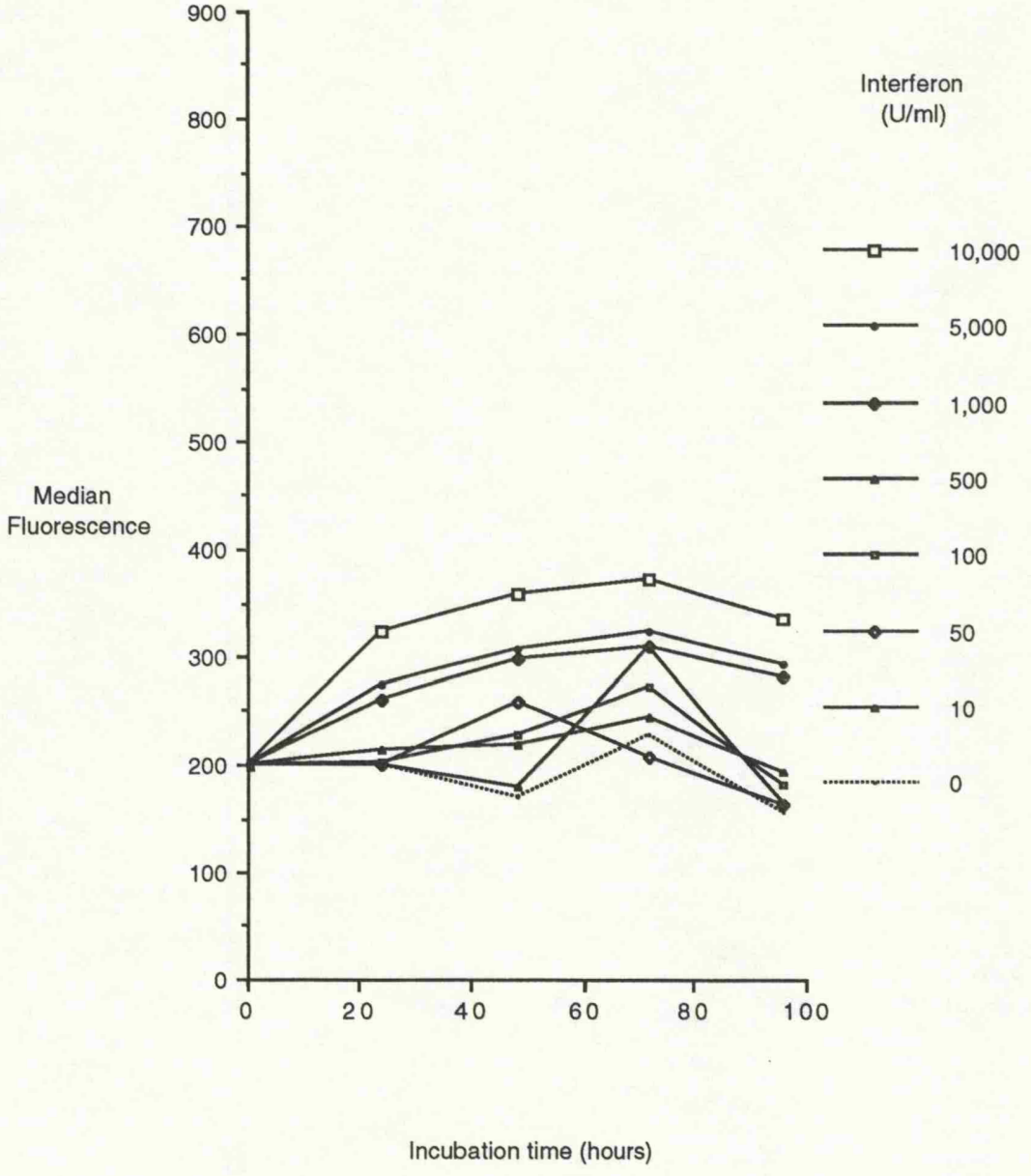


Fig 4.6 Interferon stimulated endothelial cell expression of adhesion molecules E-Selectin and PECAM.

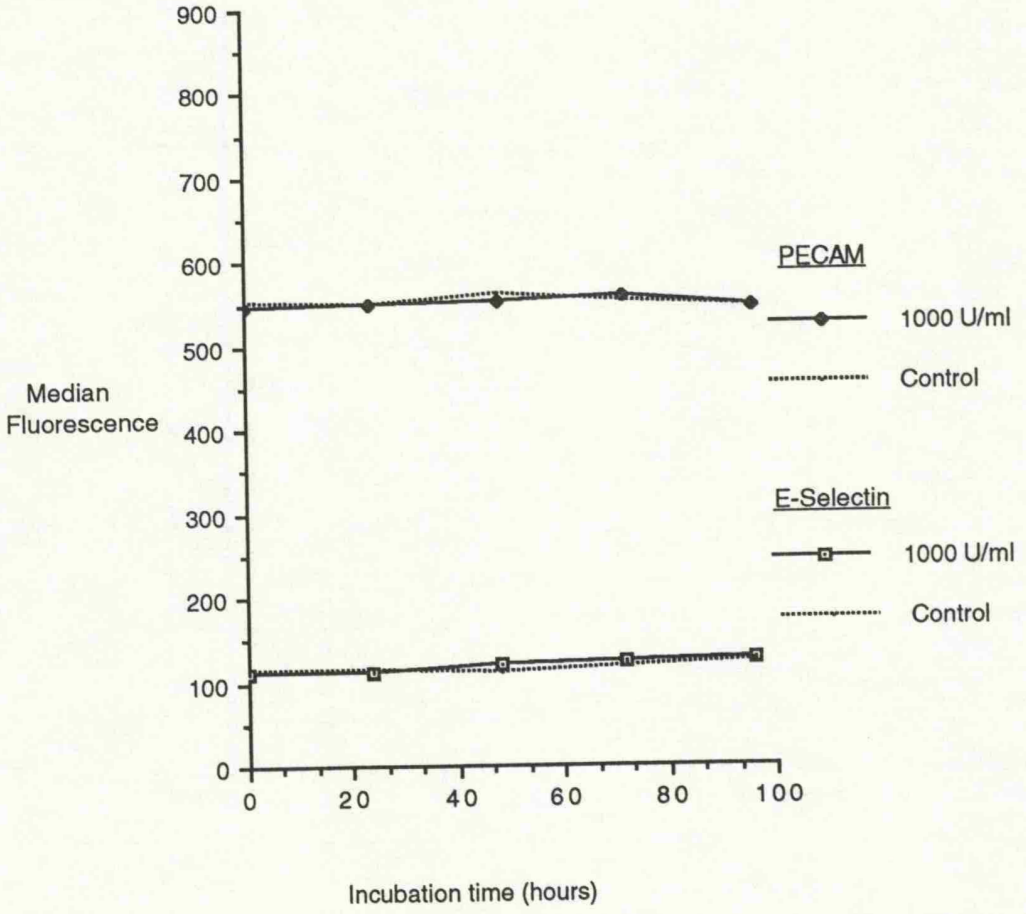


Fig 4.7 IL-1 Stimulated Expression of HLA class 1 (ABC Common)

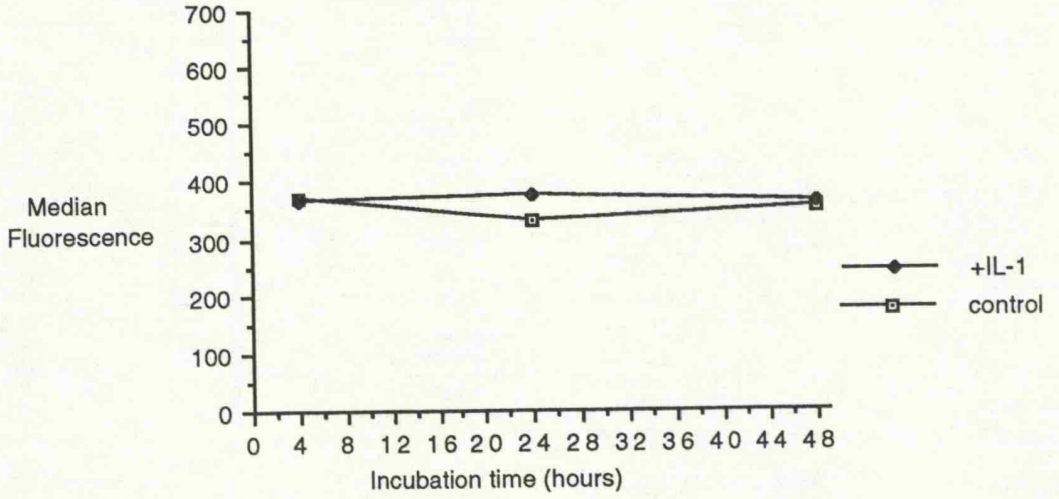


Fig 4.8 IL-1 stimulated expression of HLA class II (HLA-DR).

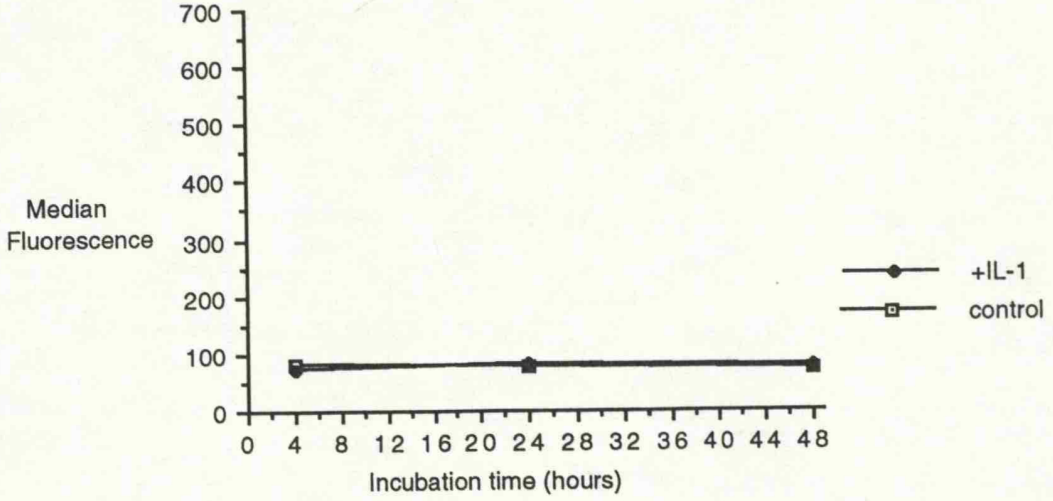


Fig 4.9 IL-1 stimulated expression of ICAM-1

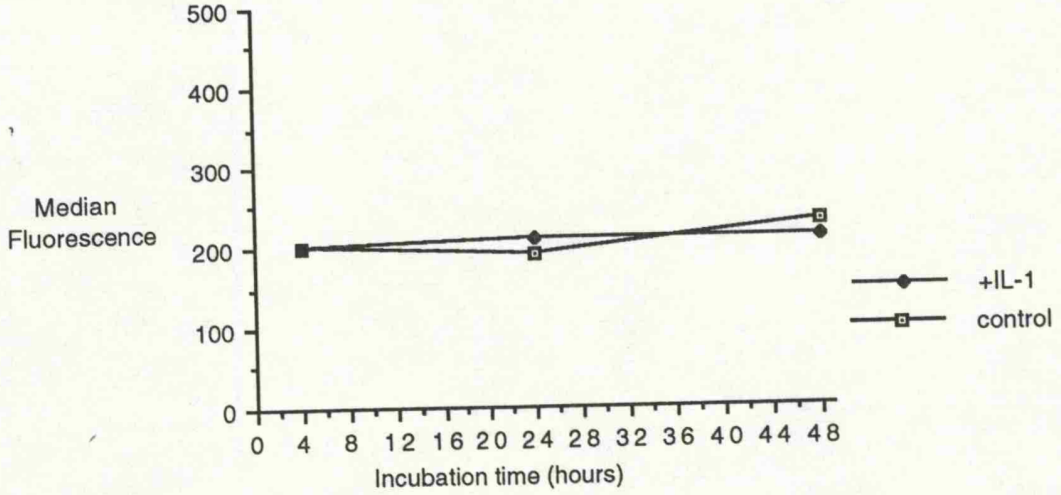


Fig 4.10 IL-1 stimulated expression of PECAM

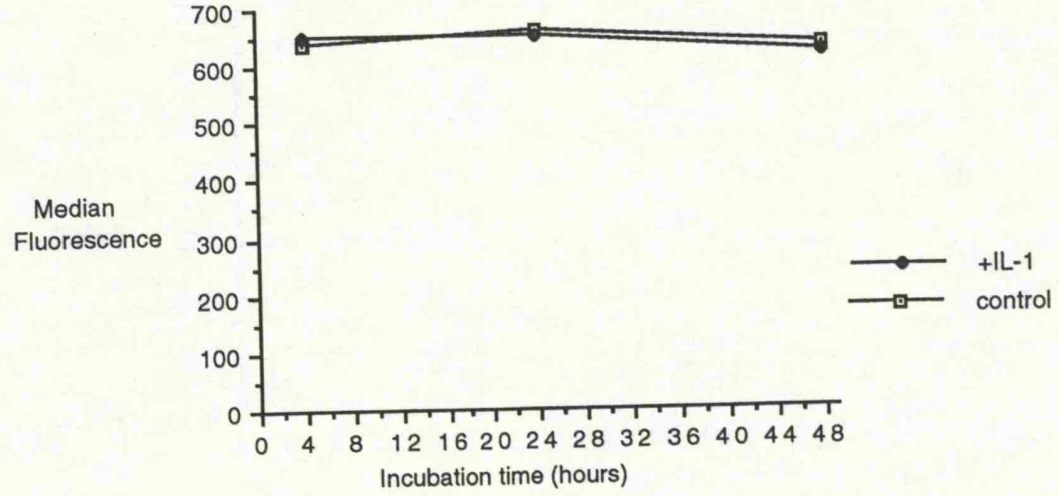


Fig 4.11 Effect of increasing IL-1β concentration on endothelial cell expression of E-Selectin.

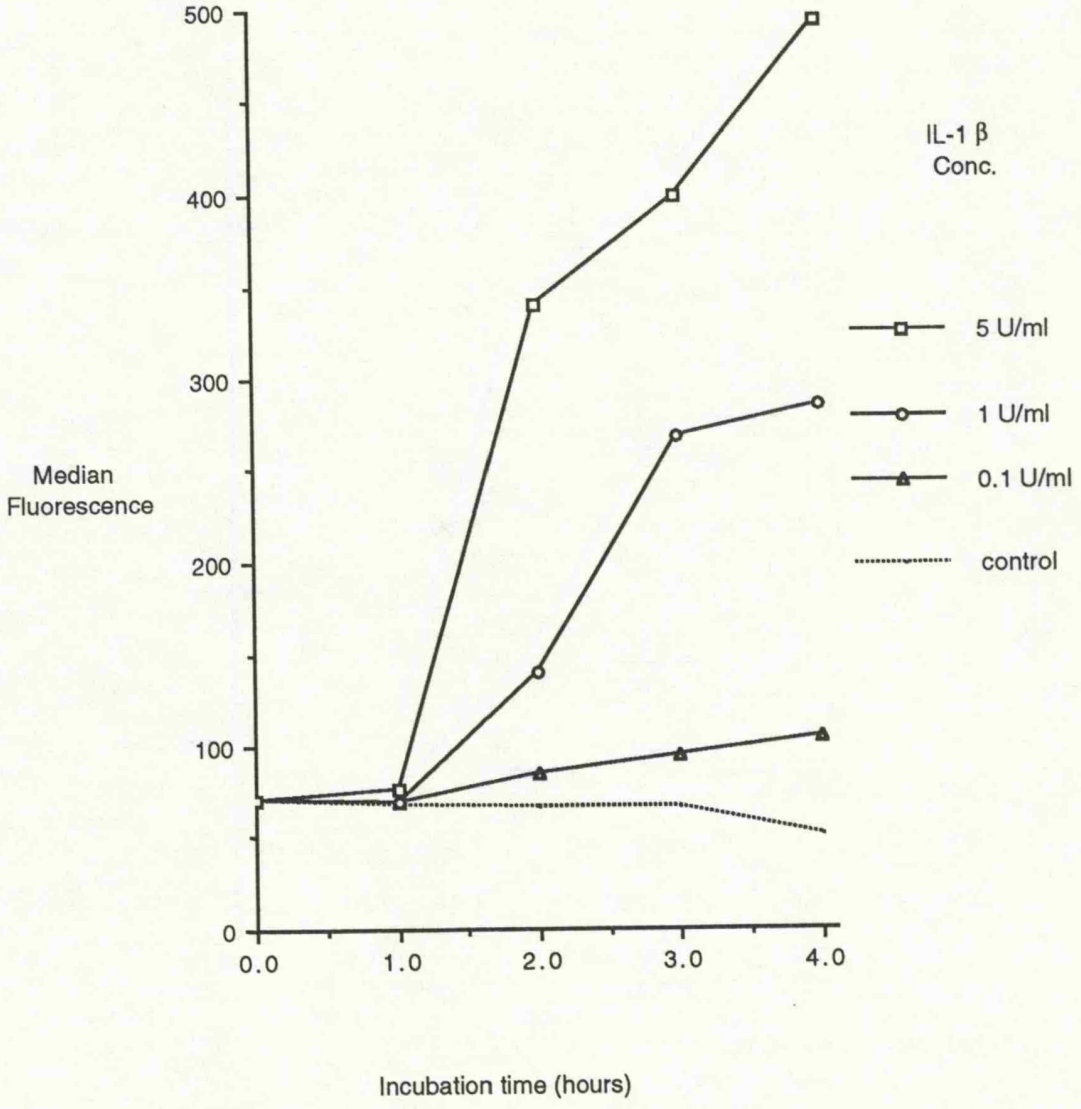


Fig 4.12 IL-1 stimulated endothelial expression of E-Selectin.

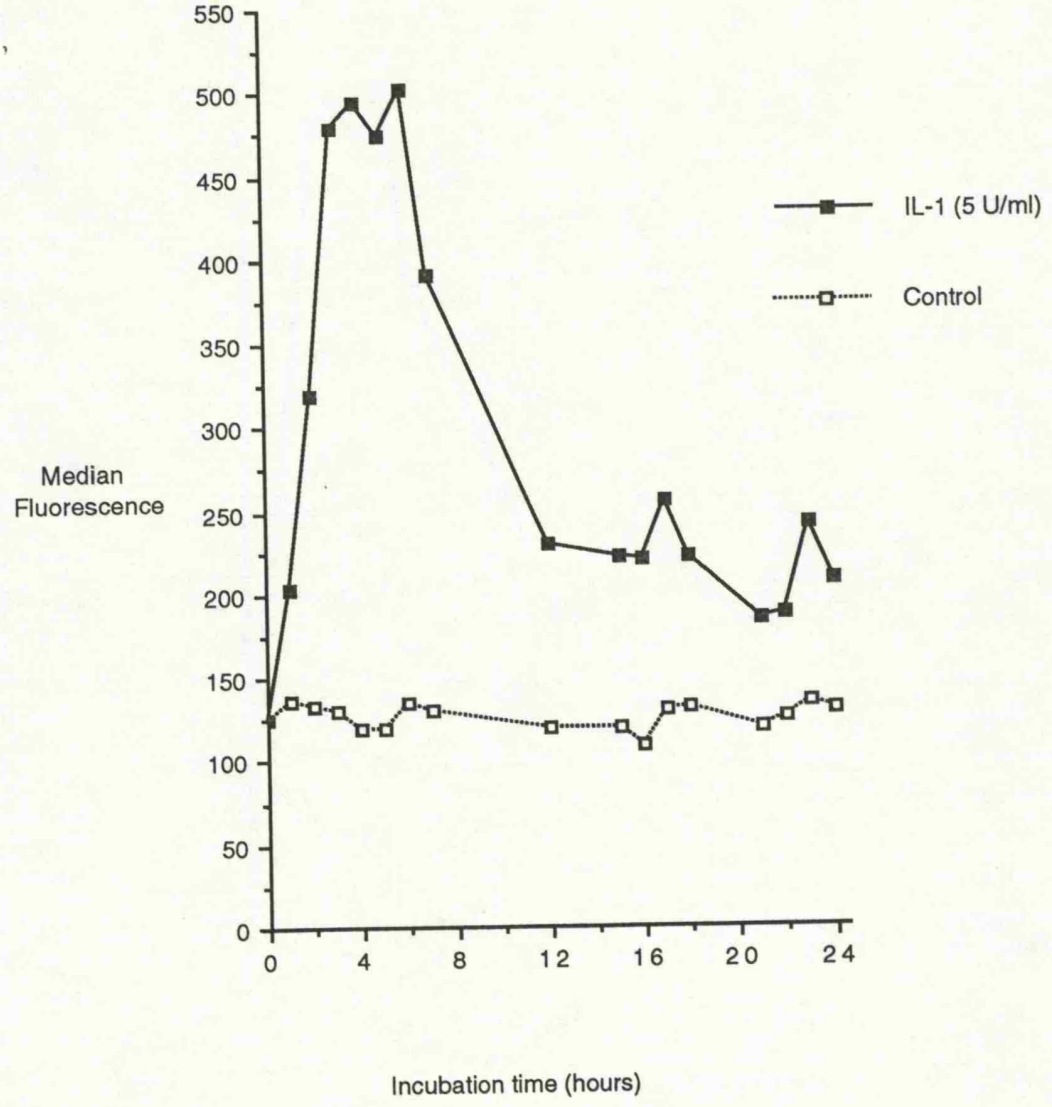


Fig 4.13 IL-1 βstimulated expression of HLA Class 1 after 48 hour preincubation with Cy A.

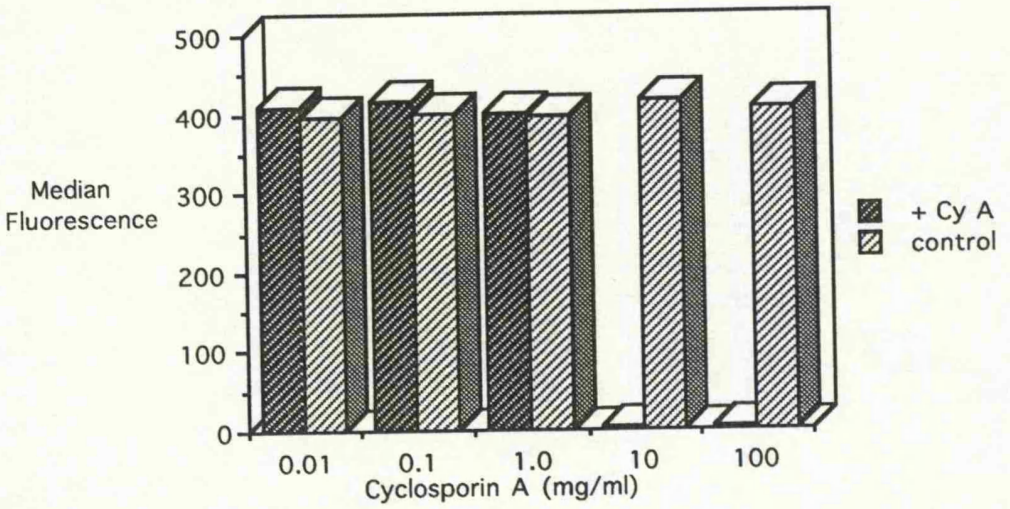


Fig 4.14 IL-1 βStimulated expression of HLA Class 1 after 4 hours incubation with Cy A

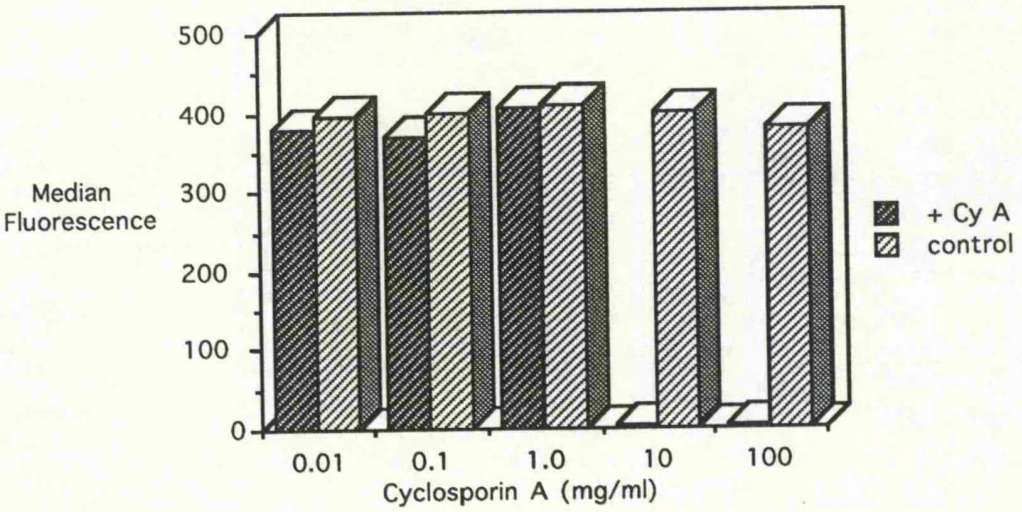


Fig 4.15 IL-1β stimulated expression of E-Selectin after 48 hour pre-incubation with cyclosporin A.

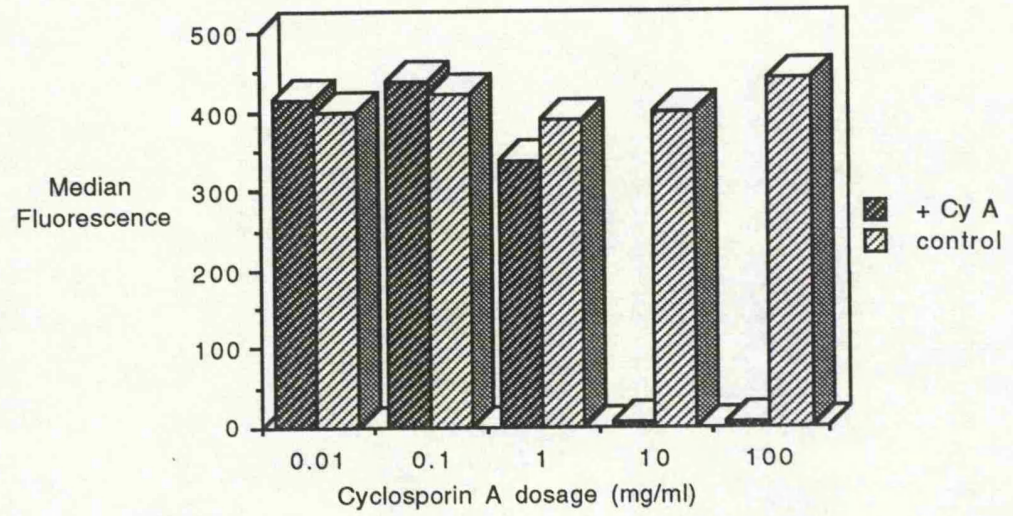


Fig 4.16 IL-1β stimulated expression of E-Selectin after 4 hours incubation with cyclosporin A.

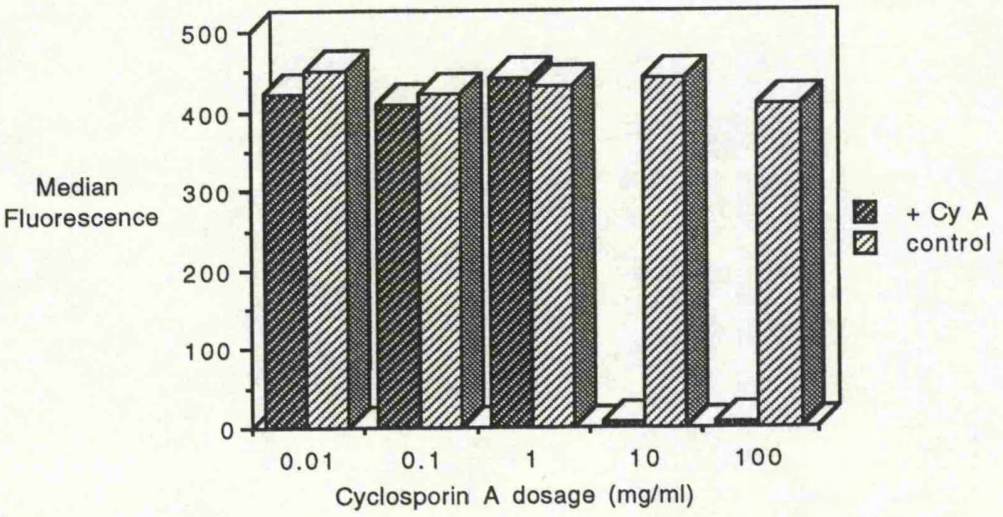


Fig 4.17 Effect of Methylprednisolone on IL-1 stimulated Endothelial expression of HLA Class 1

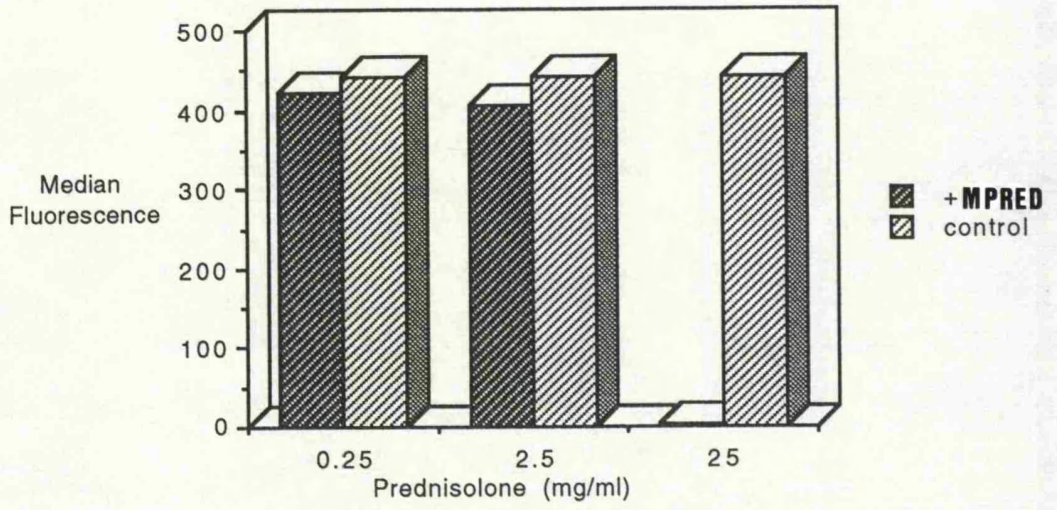


Fig 4.18 IL-1 stimulated expression of E-Selectin after 4 hours pre-incubation with methylprednisolone.

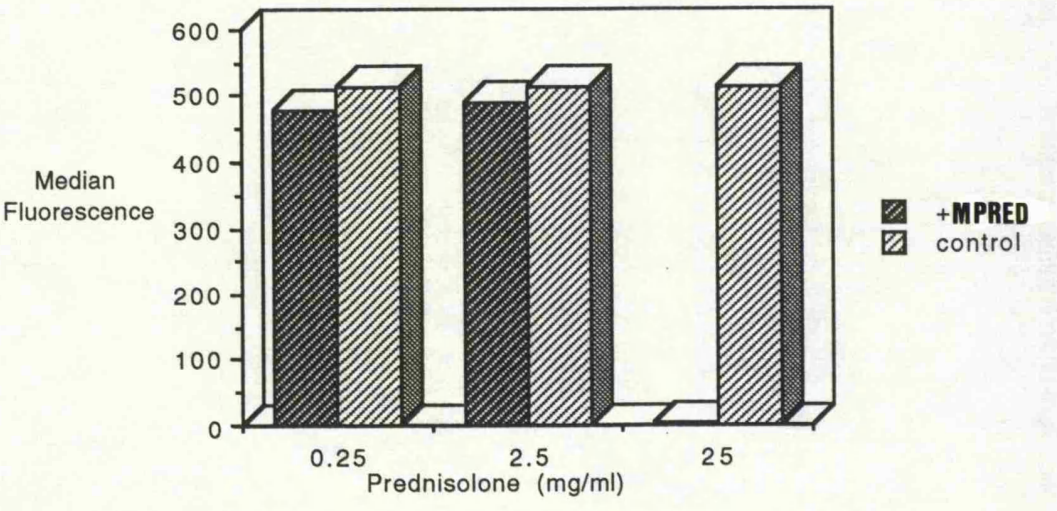


Fig 4.19 IL-1 stimulated expression of HLA Class 1 after 24 hours pre-incubation with methylprednisolone.

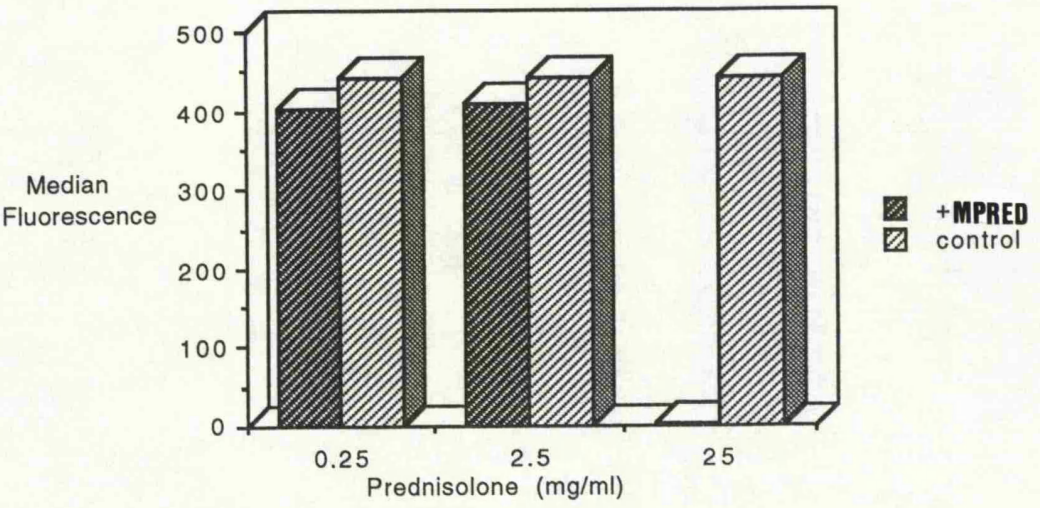
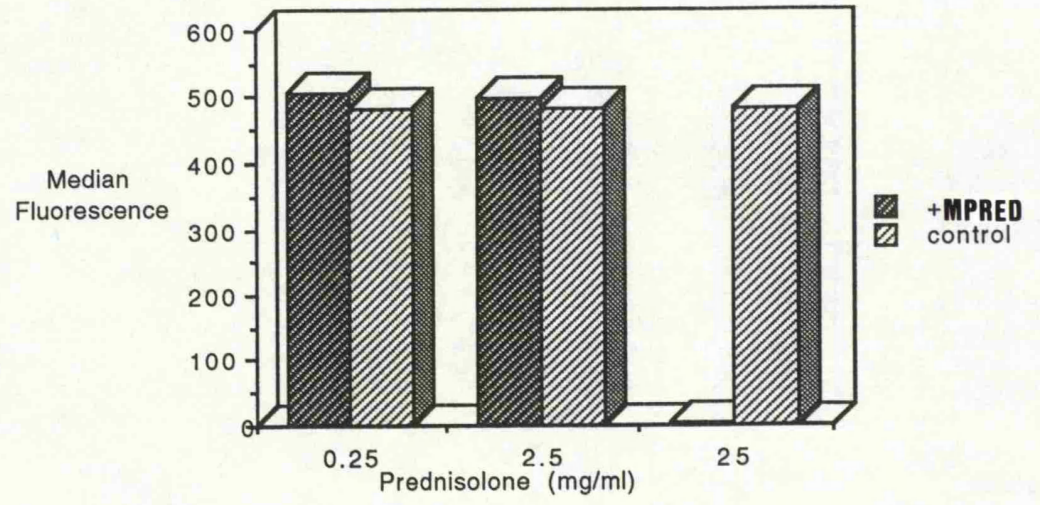


Fig 4.20 IL-1 stimulated expression of E-Selectin after 24 hours pre-incubation with methylprednisolone.



CHAPTER FIVE

In vitro studies of cellular adhesion

5.1 Introduction

We established in the previous chapter that the *in vitro* expression of endothelial cell adhesion molecules can be influenced by stimulation by inflammatory cytokines. However, increased expression of adhesion molecules does not give information as to the functional nature of these molecules or about the adhesive potential of the endothelial cell under *in vivo* conditions.

The aim of these studies was to investigate the effects of altering the endothelial cell adhesive potential under cytokine stimulation by using the measurement of neutrophil adherence to stimulated and unstimulated endothelial cells. The functional assay of neutrophil adherence is a complex subject and for this reason two different adhesion assay methods were used. The methods were standardised and assessed as to their sensitivity and ability to give a quantitative measure of neutrophil adherence. They were a colorimetric assay using Rose Bengal dye and a radiation release assay using Chromium 51 (^{51}Cr). Neutrophils were separated from whole blood using a density centrifugation technique which does not achieve as pure a population of neutrophils as other density gradient techniques, but does avoid the use of Percoll or dextran, which have been shown to have an activating effect on leucocytes (Eggleton et al. 1989).

Using these assays the adhesion of neutrophils, taken from normal healthy controls, to stimulated and unstimulated HUVEC monolayers was measured to establish a normal range.

5.2.1 Rose Bengal Dye method for estimation of neutrophil adhesion to HUVECS.

A stock solution of Rose Bengal powder (Acid red 94: 4,5,6,7,-Tetrachloro-2',4',5',7'-tetraiodofluorescein. Sigma Chemicals Ltd UK) was made up to a 0.25% solution of Rose Bengal in Phosphate Buffered Saline (0.25g Rose Bengal in 100 ml PBS).

Endothelial cell staining was achieved using the method of Gamble JR and Vadas MA (Gamble and Vadas 1988). HUVECs were plated in 96 well plates (Nunc, GIBCO, Paisley, Scotland, UK.) at a concentration of 10^4 cells/well in 200 ml of ECM and allowed to adhere to the plate by incubation at 37°C in 5% CO_2 for 24 hours. Adhesion was confirmed by examination using phase contrast microscopy and wells were usually found to be confluent or almost confluent prior to use. Before assay the endothelial cells were washed once using Endothelial Cell Medium (ECM) this medium was replaced with either fresh ECM alone or Fresh ECM plus Interleukin-1b added at varying final concentrations. Endothelial cells were then incubated for 4 hours at 37°C in 95% air/5% CO_2 atmosphere, after which time E-Selectin expression was maximal (See Chapter 4).

Neutrophils (PMN) were separated by Density Centrifugation (Methods 3.11). After 4 hours incubation endothelial cells were washed once and then incubated with neutrophils of known concentration for 30 min at 37°C in a volume of 200 ml. After this time the non-adherent PMNs were removed by agitation and the wells were washed twice. All media was then removed by aspiration and 100 ml of Rose Bengal stock solution was added and left to stand for 5 minutes. The dye solution was then aspirated from each well and then each well was washed twice. The remaining endothelial cells and adherent PMNs were lysed to release the dye using 200 ml of ethanol+PBS solution (1:1 mix). After 30 minutes cells were judged to be fully lysed by examination under a phase contrast microscope to confirm that no intact

cells were present. The optical density of the well was then determined using a ELISA plate reader (Labsystems Group, Basingstoke UK) at a wavelength of 540 nm.

Plate 5.1 shows the HUVEC monolayer prior to addition of PMNs and plate 5.2 shows the HUVEC monolayer after addition of PMNs demonstrating an even coverage of cells, (p136).

5.2.2 Rose Bengal Dye Adhesion Assay Results

Fig 5.1, (p138), shows the absorbance of a 0.25% w/v solution of Rose Bengal in PBS, as measured on a Phillips PU8065 Spectrophotometer at different wavelengths. This showed that the maximum absorbance of a Rose Bengal solution was 555 nm and all subsequent experiments used the plate reader set to 540 nm which was the closest available filter. This differs from the method of Gamble and Vadas, which used a 590 nm filter.

Fig 5.2, (p138), shows the effect of altering PMN concentration on adhesion to HUVEC monolayers, showing mean Optical Density ± Standard Error (n=12). It shows that PMN binding to unactivated HUVEC monolayers is independent of the concentration of PMNs used in the assay. However, when IL-1b activated HUVEC monolayers were used the adhesion increased in proportion to the concentration of PMNs used.

Fig 5.3, (p139), shows the effect of different washing media on the adhesion of activated HUVEC monolayers to 96-well tissue culture plates. This showed that the washing media required at least 10% FCS to be present to prevent excessive loss of the HUVEC monolayer after repeated washings. On the basis of these results HBSS+20% FCS was used for washing in all further experiments.

Fig 5.4 (p139), shows the effect of increasing IL-1b concentration on the adhesion of PMNs to HUVEC monolayers as mean Optical Densities, the OD obtained using no IL-1b and no PMNs is shown as a separate bar for ease of

plotting. The graph shows the PMN adhesion increases with increasing IL-1b concentration. However, at low concentration of IL-1b the OD obtained was lower than the OD of the HUVEC monolayer alone. The reason for this may be that at low IL-1b concentrations PMN adhesion to the HUVEC monolayer is a weak transient phenomenon, causing the PMNs to detach after washing. The underlying HUVEC monolayer may have had reduced exposure to Rose Bengal because of the overlying PMNs and consequently is unable to take up a maximal amount of dye. However, when the PMNs are not added the HUVEC monolayer is able to take up its maximum capacity for the dye and therefore the OD of the monolayer alone may be higher than that of the monolayer treated with low dose IL-1b and PMNs.

Plate 5.3, (p137), shows HUVECs after the washing phase demonstrating loss of cells from the monolayer. Plate 5.4, (p137), shows the same cells as Plate 5.3 under high power demonstrating that the adherent cells show PMN morphology and that there has been detatchment of cells from the HUVEC monolayer.

5.2.3 Rose Bengal Adhesion Assay Conclusions.

This assay does not give rise to a percentage figure of PMNs bound to the endothelial cells because the non-adherent cells compete with the endothelial cells for uptake of the dye. When these non-adherent cells are removed with the wash, dye which would have normally been taken up by the endothelial cells is lost leading to a downward drift of the baseline OD the lower the PMN adherence is. This partition of dye between the PMN and the endothelial cells leads to the OD being a qualitative and not quantitative measure of adherence, which makes this assay limited in its usefulness and for this reason it was not used further.

Plate 5.1 HUVECs stained with Rose Bengal dye prior to addition of PMNs (magnification x100).

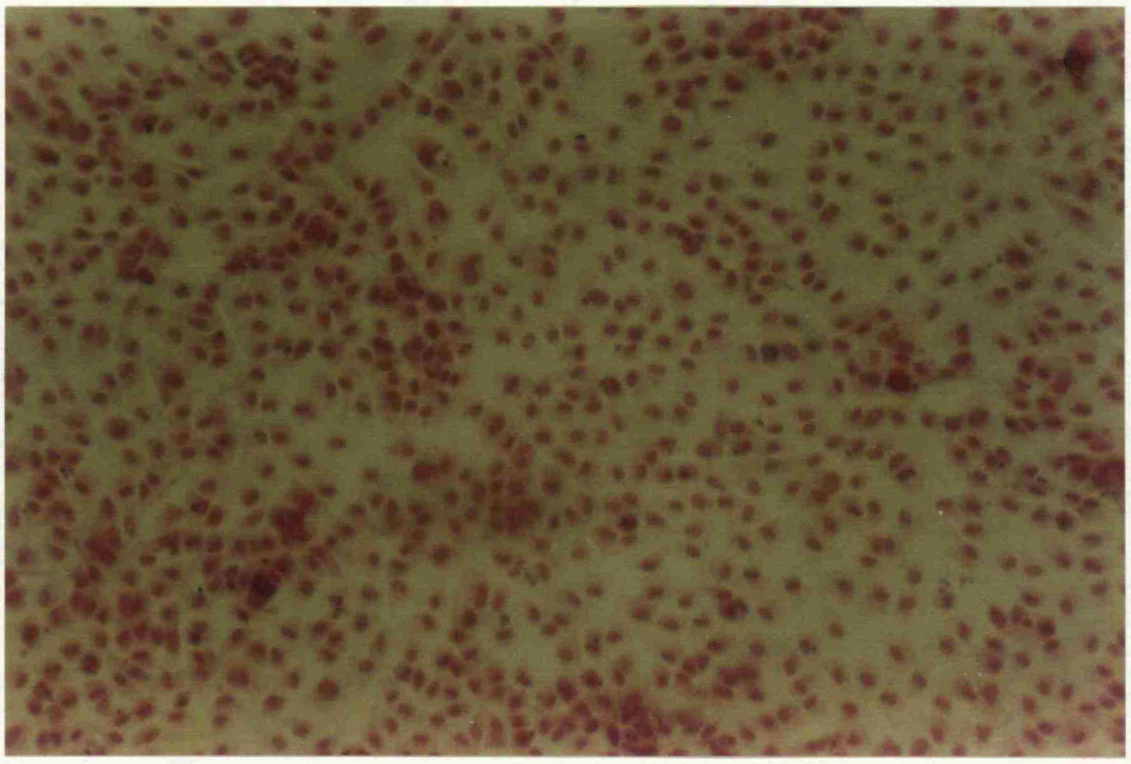


Plate 5.2 HUVECs stained with Rose Bengal dye post-addition of PMNs (magnification x100).

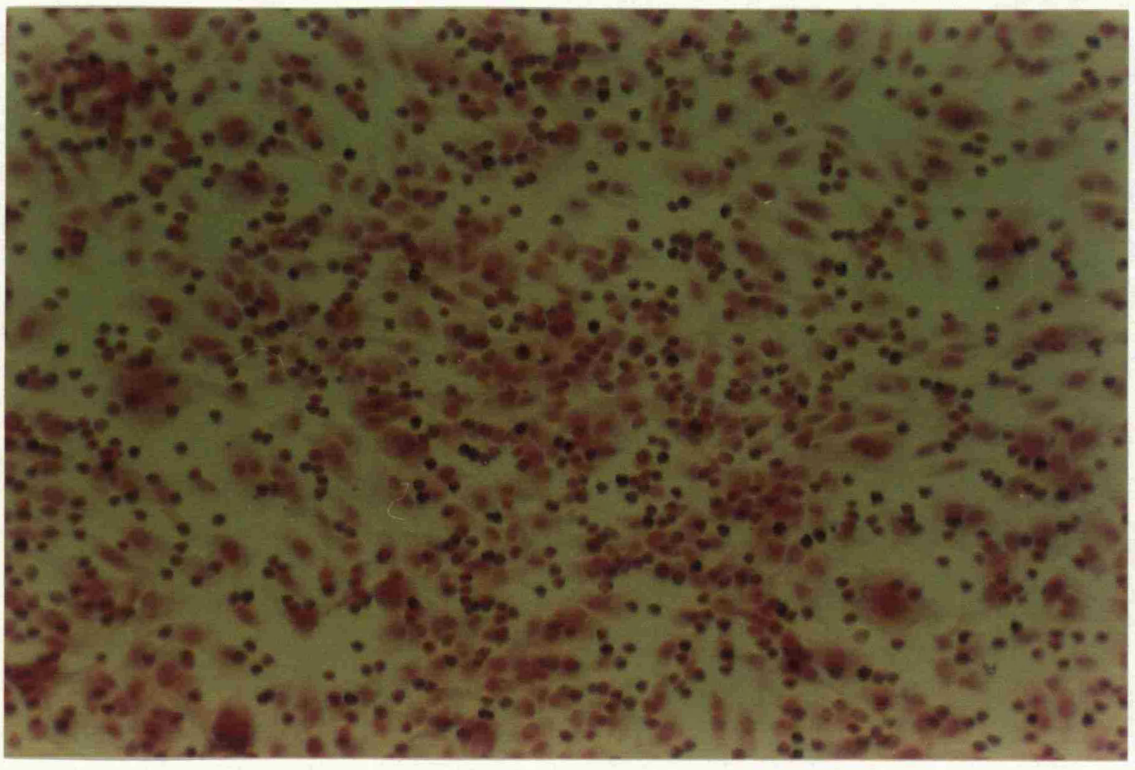


Plate 5.3 HUVECs stained with Rose Bengal dye after washing (magnification x100).

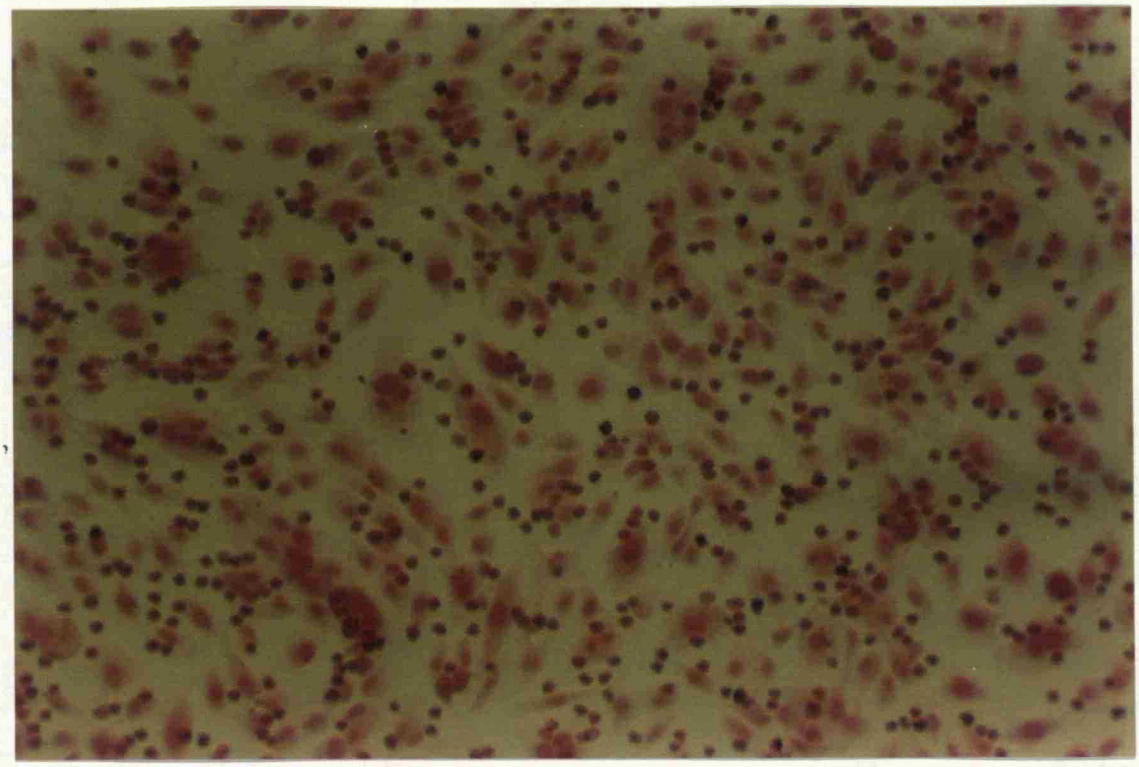


Plate 5.4 HUVECs stained with Rose Bengal dye after washing (magnification x400).

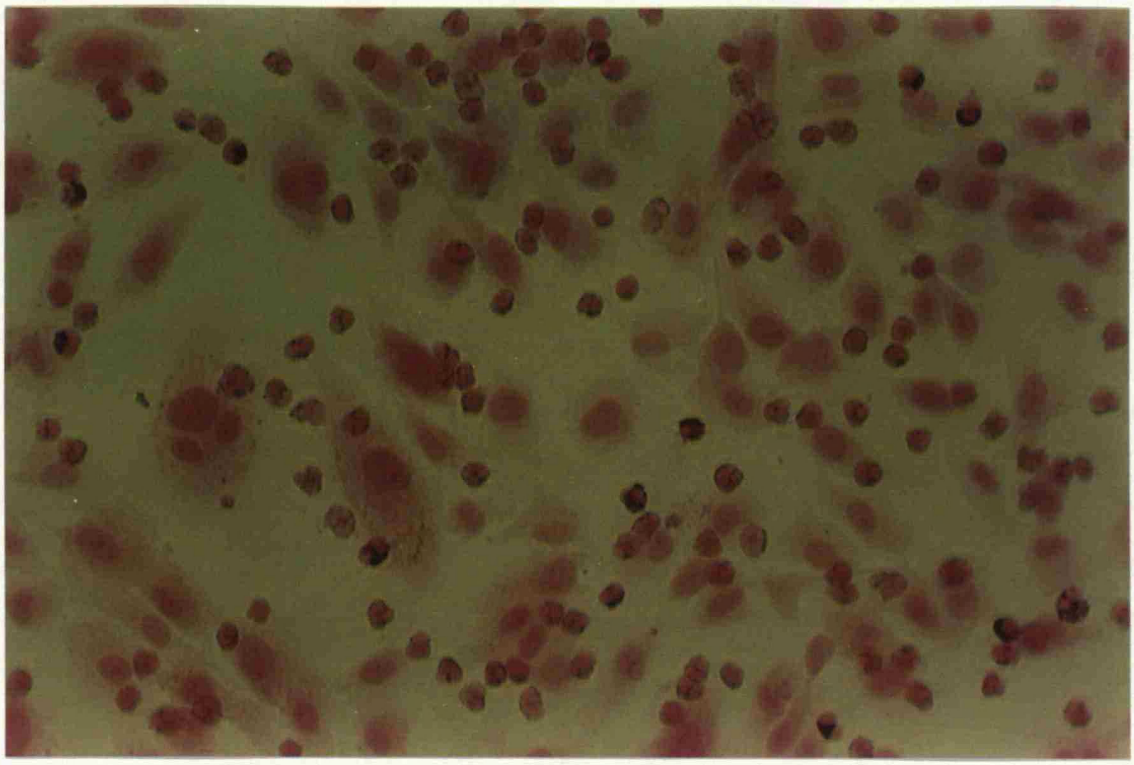


Fig 5.1 Absorbance range of Rose Bengal in PBS to determin the maximal absorbace.

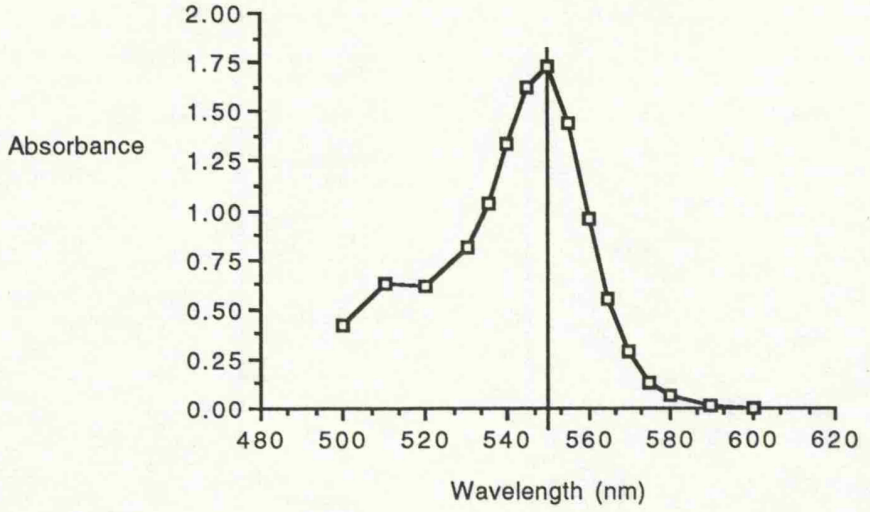


Fig 5.2 Effect of PMN concentration on adhesion to activated HUVEC layers. Rose Bengal assay.

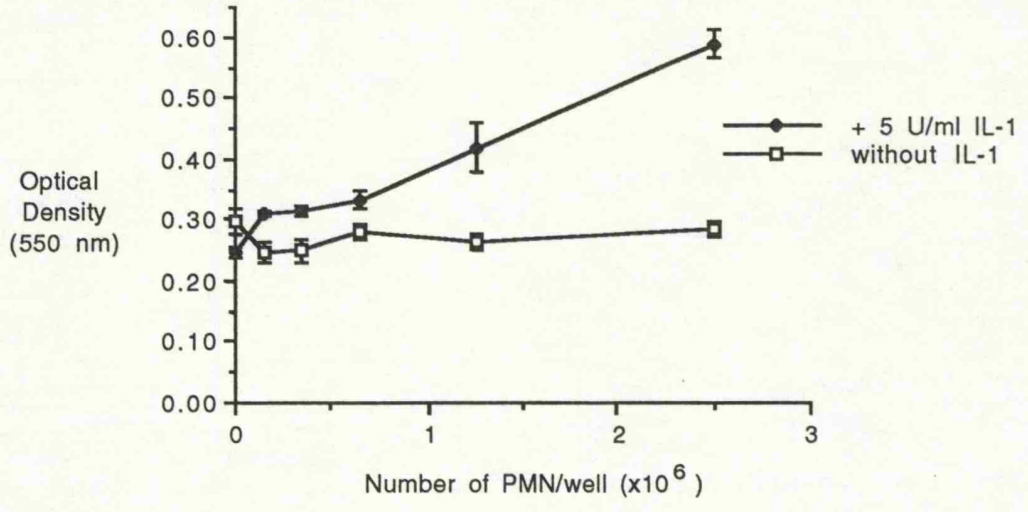


Fig 5.3 Effect of wash medium on the adhesion of activated HUVEC layers to 96-well tissue culture plates.

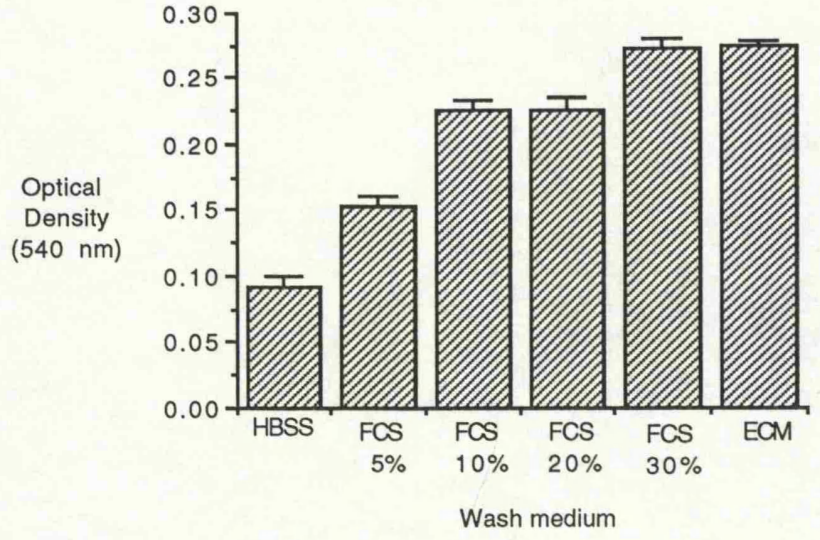
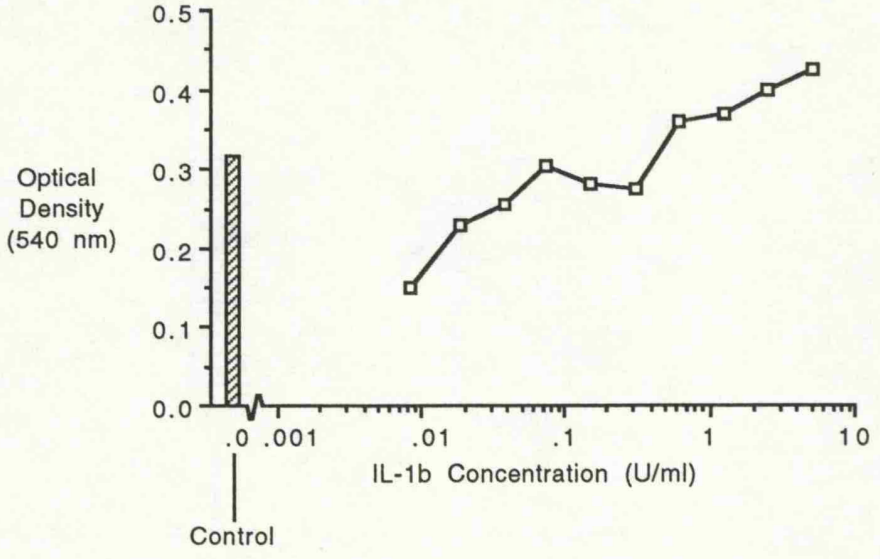


Fig 5.4 Effect of IL-1 βconcentration on PMN binding to HUVEC monolayers



5.3 Assay of neutrophil adhesion to HUVECs using radioactive chromium release.

The method used was based on that described by Gamble and Vadas (Gamble and Vadas 1988). Essentially neutrophils were separated from whole blood, radiolabelled with ^{51}Cr, and allowed to adhere to a layer of HUVECs. This layer was then washed, and the remaining radioactivity measured as an estimate of neutrophil adhesion. It was felt that this procedure could have several sources of inter and intra-assay variation and a range of assay conditions were investigated to obtain a procedure with maximum reproducibility.

5.4 Chromium labelling of neutrophils.

This method was based on the method of Gallin JT, et al.(1973). Neutrophils were separated from whole blood by density centrifugation, and after washing were suspended in 1 ml of HBSS+0.1% BSA (v/v)/1mM Ca^{++}.

Radiolabelled chromium, ^{51}Cr, 50 ml was added as sodium chromate (Amersham, UK) and incubated for one hour at room temperature with regular gentle agitation. At the end of the incubation period, excess free ^{51}Cr was removed by washing 3 times in HBSS/ 10 % FCS.

Neutrophils were then resuspended in ECM and their concentration determined by direct cell counting using a haemocytometer and microscope. Cells were then diluted to a known concentration in ECM.

Further dilutions of cells were prepared and aliquots of each concentration placed in a small plastic test tube (PT0944, LiP Equipment UK) and radioactivity measured in a gamma-counter (LKB, Sweden). The correlation between cell concentration and the number of counts per minute, was then calculated (Fig 5.4a, p156). This showed that the number of counts per minute was proportional to the concentration of cells counted, demonstrating an even uptake of ^{51}Cr into the cells.

For experimental purposes cells were made up to final concentrations of $2x10^6$/ml and then 0.5 ml added per experimental well ($1x10^6$ cells in total). 0.5 ml of cells were also counted directly for each neutrophil preparation to give the total number of counts added per experimental well. This was later used to calculate the percentage of cells binding in the cell adhesion assays.

5.4.1 Effect of the number of endothelial cells on PMN binding to HUVEC monolayers.

The effect of altering the concentration of endothelial cells added per well is shown in Fig 5.5 and as a percentage binding in Fig 5.6 (p157). Different concentrations of endothelial cells were added to 24-well plates as previously described (see Methods), final cell concentrations used were $1x10^5$ cell/well, $1x10^4$ cell/well and $1x10^3$ cell/well. After 24 hours incubation at 37°C in 95% O_2/5% CO_2 atmosphere the monolayer was washed once with 1 ml of fresh ECM. 0.5 ml of PMNs pre-labelled with ^{51}Cr were added to each well at a concentration of $1x10^6$ PMN/well and incubated for 45 minutes at 4°C. The wells were then washed with HBSS/20% FCS, and the remaining adherent cells were then lysed to release ^{51}Cr from the remaining PMNs. The radioactivity in each well was then measured using a gamma-counter and compared to the known total radioactivity contained in the original 0.5 ml of PMNs.

At an endothelial cell concentration of $1x10^5$ cells/well there was a statistically significant difference between the PMN binding to IL-1b stimulated cells and controls (Mann-Whitney, $p<0.05$). There was also a statistically significant increase in binding at an endothelial cell concentration of $1x10^4$ cells/well (Mann-Whitney, $p<0.05$), however this increase was less than that obtained at a concentration of $1x10^5$ cells/well. At a concentration of $1x10^3$ endothelial cells/well there was

no increase in PMN binding after IL-1b stimulation. $1x10^5$ endothelial cells/well had already been demonstrated as being necessary to obtain confluence in a well after 24 hours incubation by light microscopy. Since confluence was an easily measurable end-point to demonstrate full endothelial cell adhesion to the well, $1x10^5$ endothelial cells/well was chosen to be the concentration of choice in this assay.

5.4.2 Effect of IL-1b concentration on PMN binding to HUVEC monolayers

The effect of altering the concentration of IL-1b on PMN adhesion to endothelial cells was measured. Endothelial cell monolayers and radiolabelled PMN suspensions were prepared as previously described (Section 5.4.1). Dilutions of IL-1b were made up from stock solution, as previously described. Endothelial cells were pre-incubated with IL-1b for 4 hours at 37°C in 95% Air/5% CO_2 atmosphere prior to the adhesion assay being performed. Concentrations of IL-1b were made up in ECM to give the final concentrations (U/ml) of 5.0, 0.5, 0.05, 0.005 and 0. The determination of adhesion was then performed as previously described (Section 5.4.1).

The proportion of PMN bound to the endothelial cell monolayers increased following a logarithmic dose response curve as shown in Fig 5.7 (p158). Without IL-1b stimulation 6.9% of the original PMNs added bound to the monolayer. At an IL-1b concentration of 0.005 U/ml 7.44% of cells bound, at 0.05 U/ml 13.1% of cells bound, at 0.5 U/ml 12.9% of cells bound, and at 5.0 U/ml 17.6% of cells bound. On the basis of these results it was decided to use an IL-1b concentration of 1 U/ml in further experiments.

5.4.3 Effect of PMN Concentration on adhesion to HUVEC monolayers

The effect of altering the concentration of PMNs, on adhesion to endothelial cells both unstimulated and stimulated with IL-1b was measured. Endothelial cell monolayers and radiolabelled PMN suspensions were prepared as previously described (Section 5.4.1). Dilutions of PMNs were made up from a solution of known PMN concentration in fresh ECM to final working concentrations of $1.8x10^6$, $0.9x10^6$, $0.45x10^6$, $0.225x10^6$, $0.115x10^6$, $0.0575x10^6$, and $0.029x10^6$. A dilution of IL-1b was made up from stock solution, as previously described, to give a final working concentration of 1U/ml. Endothelial cells were pre-incubated with IL-1b for 4 hours at 37°C in 95% Air/5% CO_2 atmosphere prior to the adhesion assay being performed. The determination of adhesion was then performed as previously described (Section 5.4.1).

The effect of altering the concentration of PMNs added to HUVEC monolayers is shown in Fig 5.8, (p159), expressed as a percentage of the cells originally added to each well. The upper line shows the adhesion of PMNs to IL-1b stimulated cells and the lower line adhesion to unstimulated cells. The graphs shows that over the concentration range $0.45x10^6$ to $1.8x10^6$, there was no significant effect of PMN concentration on the percentage of cells bound to the HUVEC monolayer, either unstimulated or stimulated. Fig 5.9 shows the total counts per minute (cpm) obtained from each PMN dilution. This shows a linear relationship between PMN number added to the monolayer and the cpm obtained over the concentration range of PMNs used. This linear relationship was true for both stimulated and unstimulated HUVEC monolayers. These two graphs show that the proportion of PMNs adherent to a HUVEC monolayer is independent of cell concentration between $0.45x10^6$ and $1.8x10^6$ PMN/well. On the basis of these results it was decided to use $1.0x10^6$ PMNs per well in future experiments.

5.4.4 Effect of incubation time and temperature on PMN adhesion to endothelial cell monolayers.

The effect of altering the time of incubation of PMNs and HUVEC monolayers was investigated along with the temperature at which incubation was performed. Endothelial cell monolayers and radiolabelled PMN suspensions (2×10^6/ml) were prepared as previously described (Section 5.4.1). 3 plates of endothelial cells were prepared simultaneously, to enable the study of 3 different temperatures. A dilution of IL-1b was made up from stock solution to give a final working concentration of 1 U/ml. Endothelial cells were pre-incubated with IL-1b for 4 hours at 37°C in 95% Air/5% CO_2 atmosphere prior to the adhesion assay being performed.

The adhesion assay was performed at either 4°C, 20°C or 37°C. The incubation was performed for 15 min, 30 min, 45 min and 60 min at each temperature. After these times the determination of adhesion was performed as previously described (Section 5.4.1).

Figs 5.10 and 5.11 show the adhesion of radiolabelled PMNs to HUVEC monolayers at 4°C, Figs 5.12 and 5.13 the adhesion at 20°C and Figs 5.14 and 5.15 the adhesion at 37°C (p160-162). These figures express adhesion over time, expressed as either total radioactivity per well or percentage PMN adhesion respectively. The graphs show an increase in adhesion over time, with adhesion increased by pre-incubation with IL-1b at all temperatures. Fig 5.16 (p163), summarises the results of total radioactivity recovered at the various times and temperatures in one plot. Adhesion at 4°C to unstimulated HUVECs is less than at 37°C which is in turn less than that at 20°C, this pattern is reproduced for stimulated HUVECs. When the results of total radioactivity are expressed as a percentage PMN binding in Fig 5.17 (p164), the same pattern of results is seen.

Fig 5.18 (p165), shows the difference in binding of PMNs to stimulated HUVECs when compared to unstimulated HUVECs, at each time and temperature. These results are expressed as the percentage of PMNs binding to the stimulated HUVEC monolayer minus the percentage of PMNs binding to the unstimulated HUVEC monolayer. The greatest difference in adhesion is seen at 4°C when incubated for 45 minutes, and this incubation time and temperature was used for all subsequent experiments.

5.4.5 Effect of washing on PMN adhesion to endothelial cell monolayers.

There were a number of variables that needed to be considered in the standardisation of the washing technique used in the adhesion assay, including the extent of PMN adhesion to the culture wells without HUVECs, the degree of detachment of the HUVEC monolayer after repeated washings, the quantity of detachment of adherent PMNs after washing and the possible effects of pre-coating wells to improve HUVEC adhesion. The aim of these experiments was to determine whether increasing the number of washes would remove only non-adherent PMN and the number of washes required to achieve this, or whether increased washes would also remove previously adherent PMN and, or the HUVEC monolayer itself.

5.4.5.1 Effect of washing on the adhesion of PMNs to culture wells alone.

Tissue culture wells are designed to be adherent surfaces for cultured cells. In this context it was felt important that the amount of PMN adhesion to the wells, independent of HUVECs, be determined and whether this adhesion, if any, could be reduced by washing.

PMNs were isolated from whole blood by differential centrifugation (see Methods) and diluted to a final concentration of 2.0×10^6 PMN/ml in

ECM. 0.5 ml of this PMN stock were then added to unseeded tissue culture wells and allowed to incubate at 4°C for 45 minutes. After that time the wells were washed with HBSS/10% FCS. The procedure for washing was 1 ml of wash solution was added to the well, the well was then gently agitated, the contents poured off into a disposal container containing 5% RBS surfactant (Chemical Concentrates (RBS) London, UK) for later safe disposal. The well was then inverted and blotted onto tissue paper to remove any remaining fluid on the well rim. In this experiment wells were washed either 1, 2, 3 or 4 times using the above method. A control well with no PMNs added was also incorporated into the method to assess any background radioactivity of the well and lysing solution. After washing 1 ml of lysing fluid (1% Triton-X, Sigma Chemicals, UK) was added to the well, incubated for 30 minutes at room temperature, the well was then agitated and aspirated completely and the fluid transfered to a small plastic tube (PT0944) and the radioactivity measured using a gamma-counter.

Fig 5.19 (166), shows the total radioactivity as expressed in cpm obtained from 6 duplicate wells either without PMNs, or with PMNs after 1 to 4 washes. The total radioactivity declined after each wash, the majority of adhering cells being removed after the first wash. The radioactivity reached background levels, as compared to the control wells, after 4 washes. Fig 5.20 (166), shows the radioactivity obtained after each wash as a percentage of the total radioactivity added to the wells in the original PMN suspension. After 1 wash 14.75% of the original radioactivity added remained in the well, demonstrating that PMNs are adherent to tissue culture wells, but the removal of all these non-specifically adherent cells could be achieved after 4 washes.

5.4.5.2 Effect of increasing washings on PMN adhesion to unstimulated and IL-1b stimulated HUVEC monolayers.

Following the previous section the effect of multiple washings on PMN adhesion to unstimulated and IL-1b stimulated HUVEC monolayers was assessed.

Endothelial cell monolayers and radiolabelled PMN suspensions were prepared as previously described (Section 5.4.1). The radiolabelled PMNs were diluted to a working concentration of 2.0×10^6 cells/ml. A dilution of IL-1b was made up from stock solution, as previously described, to give a final working concentration of 1U/ml. Endothelial cells were pre-incubated either with or without IL-1b for 4 hours at 37°C in 95% Air/5% CO_2 atmosphere prior to the adhesion assay being performed. To measure both the inter-plate and inter-day variation of the assay, pairs of 24-well tissue culture plates containing confluent endothelial cell monolayers were assessed on 3 separate days. Each plate was incubated for 4 hours prior to the adhesion assay being performed, 12 wells with fresh ECM and the other 12 wells with fresh ECM containing IL-1b. The wells were then washed once with fresh ECM and 0.5 ml of fresh ECM containing 2.0×10^6 radiolabelled PMN/ml was added to each well. The plates were then incubated at 4°C for 45 minutes to allow PMN adhesion. The wells were then washed either 1, 2, 3, or 4 times following the same procedure as that in Section 5.4.5.1. The determination of adhesion was then performed as previously described (Section 5.4.1).

Fig 5.21 (167), shows the total radioactivity recovered from unstimulated HUVEC monolayers after successive washings. The radioactivity from each pair of plates is represented by using different symbols as detailed in the Figure legend. Overall radioactivity was removed with each successive wash, the total radioactivity recovered showed some intra-plate variation, with an average coefficient of variance of 17.0% after 3 washes (see Table 5.1, p177). Inter-plate variability was also seen with an average coefficient of variance of 14.9% after 3 washes (see

Table 5.1). Variation of radioactivity between days was related to the different amount of radiolabelling of PMNs on different days, and therefore the absolute radioactivity cannot be directly related between days. Therefore to compare results from different days PMN adhesion to HUVEC monolayers was expressed as a percentage of the total PMNs added per well, with reference to the total radioactivity added per well and this is shown in Fig 5.22 (p167).

Fig 5.23 (168), shows the total radioactivity recovered from IL-1b stimulated HUVEC monolayers after successive washings. The radioactivity from each pair of plates is represented by using different symbols as detailed in the Figure legend. The total radioactivity recovered showed some intra-plate variation, with an average coefficient of variance of 13.1% after 3 washes (see Table 5.1, p177). Inter-plate variability was also seen with an average coefficient of variance of 16.1% after 3 washes (see Table 5.1). Overall radioactivity appeared to maintain a steady level over the first 3 washes, only falling significantly after 4 washes. This sharp fall was investigated further by examination with inverted phase contrast microscopy of HUVEC monolayers after each wash. It was found that up to 3 washes the monolayer remained intact, but that after 4 washes the monolayer was inclined to detach from the tissue culture plate, thus introducing a large error. Fig 5.24 (p168), shows that in percentage terms the adhesion of PMNs to HUVEC monolayers is significantly increased after pre-stimulation of the monolayers with IL-1b over control values. This increased adhesion remained constant over 3 washes, only declining after the fourth wash. Fig 5.25 (p169), shows the combined results from Figs 5.22 and 5.24 to enable a direct comparison between the percentage adhesion of PMNs to stimulated and unstimulated HUVEC monolayers emphasising the difference in PMN adhesion to stimulated and unstimulated HUVEC monolayers.

Plate 5.5 shows the HUVEC monolayer after addition of ^{51}Cr labelled

PMNs demonstrating even coverage of the monolayer. Plate 5.6 shows the same HUVEC monolayer after 3 washes demonstrating that whilst PMNs have been removed the HUVEC monolayer remains intact with and even coverage of remaining PMNs (p155).

5.4.5.3 Effect of increasing washings on PMN adhesion to unstimulated and IL-1b stimulated HUVEC monolayers in gelatin coated wells.

Following the previous experiment showing HUVEC detachment from the tissue culture well surface after repeated washings a method of stabilising the HUVEC monolayer was sought. Tissue culture wells were coated with 0.25 ml of warm, sterile 0.2% gelatin solution (Sigma Chemicals Ltd, UK), which was sufficient to just cover the well bottom, and the gelatin allowed to set for 24 hours at 4°C. The wells were then seeded with HUVECs as previously described and the PMN adhesion assay performed as in section 5.4.5.2.

Fig 5.26 (p170), shows the effect of washing on PMN adhesion to unstimulated HUVEC monolayers in gelatin coated wells and Fig 5.27 (p170), shows the same data expressed as a percentage of PMN adhesion. Examination of the wells under phase contrast microscopy after each wash showed minimal detachment of the monolayer even after 4 washes and this is supported by the marked decrease in intra-plate variation seen in Fig 5.26, with an average coefficient of variance 8.26% after 3 washes (see Table 5.2, p178). The average interplate coefficient of variation was 0.4% after 3 washes (see Table 5.2).

Fig 5.28 (p171), shows the effect of washing on PMN adhesion to IL-1b stimulated HUVEC monolayers in gelatin coated wells and Fig 5.29 (p171), shows the same data expressed as a percentage of PMN adhesion. In agreement with the results for unstimulated HUVEC monolayers the IL-1b

stimulated HUVECs showed minimal detachment from the well surface after 4 washes. The sharp fall in measured PMN adhesion seen after 4 washes on uncoated tissue culture wells was not seen in the gelatin coated wells supporting the hypothesis that HUVEC detachment was the reason for this fall. The average coefficient of variation for intra-plate variation was 19.3% after 3 washes (see Table 5.2, p178). The average interplate coefficient of variation was 30.1% after 3 washes (see Table 5.2).

5.4.5.4 Effect of the type of lysing fluid used on the measured PMN adhesion to HUVEC monolayers.

Incomplete lysis of the HUVEC monolayer and adherent PMNs would lead to large errors between wells on a plate and to investigate this effect different lysing fluids were used. The adhesion assay was performed on stimulated and unstimulated HUVEC monolayers as previously described (Section 5.4.1). After adhesion of PMNs to the HUVEC monolayer lysis was performed using 1 ml per well of NaOH 2.0 M, NaOH 4.0 M, HCl 2.0 M or NOP-4 for 30 minutes. The well contents were aspirated and placed in a tube for gamma-counting, the well was then washed once with a further 1 ml of lysing 6fluid which was also added to the same tube.

Fig 5.30 (p172), shows the effect of the different lysis solution on measured PMN adhesion to stimulated and unstimulated HUVEC monolayers expressed as a percentage of bound PMN. There was no significant difference seen between any of the lysis fluids used and on the basis of its lower toxicity and corrosiveness, NOP-4 was used for all further experiments.

5.5 Effect of Pre-stimulation of PMN with Phorbolmystirate acetate

To investigate the maximum adhesive potential of PMNs in this assay

system, phorbolmystirate acetate (PMA) was used to pre-stimulate PMN suspensions before addition to either IL-1b stimulated or unstimulated HUVEC monolayers. PMA is a non-specific mitogen which is an extremly potent activator of the Protein Kinase C (PKC) pathway. Amongst other effects this causes the opening of Calcium channels into the cell and increases the oxygen consumption of the cell. Stimulation of PKC by PMA leads to the activation of many different cell types. PMA (Sigma Chemicals, UK.) was made up from a stock solution to final concentrations of 10 ng/ml and 100 ng/ml. PMA was diluted in dimethylsuphoxide (DMSO, Sigma Chemicals, UK.) and for this reason a control adding DMSO alone was also used in addition to a control without PMA or DMSO.

Endothelial cell monolayers and radiolabelled PMN suspensions were prepared as previously described (Section 5.4.1). The radiolabelled PMNs were diluted to a working concentration of 2.0×10^6 cells/ml.

The PMN suspension was then incubated for 1 hour at room temperature with either 100 ml of fresh ECM, DMSO, DMSO with 10ng/ml PMA, or DMSO with 100 ng/ml PMA. The PMN suspension was then washed three times using fresh ECM to remove DMSO and PMA before re-suspension in fresh ECM at a final concentration of 2.0×10^6 PMN/ml.

A dilution of IL-1b was made up from stock solution, as previously described, to give a final working concentration of 1U/ml. Endothelial cells were pre-incubated either with or without IL-1b for 4 hours at 37°C in 95% Air/5% CO_2 atmosphere prior to the adhesion assay being performed. The wells were then seeded with HUVECs as previously described and the PMN adhesion assay performed as in section 5.4.5.2.

Fig 5.31 (p173), shows the total radioactivity bound to the the wells of HUVECs, and the effect of PMA and IL-1b stimulation on the PMN binding. The percentage binding is shown in Fig 5.32 (p173). The binding of unstimulated PMN to unstimulated and stimulated HUVEC monolayers was

similar to that seen with DMSO treated PMNs.

PMA stimulated PMNs show significantly increased binding to unstimulated and IL-1b stimulated HUVEC monolayers when compared with unstimulated PMNs. This effect was seen at both concentrations of PMA. The maximum adhesion of PMN to HUVECs using this assay technique was 40%.

When unstimulated PMNs were used, IL-1b caused a significant increase in binding to HUVEC monolayers as had been seen before. However when PMA stimulated PMNs were used IL-1b stimulation of the HUVEC monolayer did not increase the PMN adhesion over and above the already increased adhesion to unstimulated HUVECs.

This disproportionate effect of PMN stimulation on the adhesion to HUVEC monolayers indicated that the overall activation state of the PMNs used in this assay has a greater effect on PMN binding HUVECs than the state of activation of IL-1b stimulated endothelial cells.

5.6 Establishment of a normal range for the ^{51}Cr PMN adhesion assay.

Blood was collected from 18 normal, healthy volunteers who had no evidence of infection at the time of sampling (12 male, 6 female, mean age 33.8 years, range 27-50 years). Neutrophils were prepared as previously described and PMN adhesion was assayed on both unactivated and IL-1b activated HUVEC monolayers.

The results of the adhesion assay are shown in Figs 5.33 and 5.34 (p174), the median adhesion to unactivated HUVEC monolayers was 7.75%, with an interquartile range of 4.88%-9.85%. The median adhesion to activated HUVEC monolayers was 20.9%, with an interquartile range of 16.1%-22.8%.

The results were analysed to establish if any relationship existed between sex and PMN adhesion, the results are presented in Figs 5.35 and 5.36 (p175). There was no significant difference between male and female PMN adhesiveness.

Intra-individual variation in PMN adhesiveness between days was also assessed. 3 volunteers were sampled on 4 different days each at weekly intervals, neutrophils were separated as described and their adhesiveness measured. The results of these assays are shown in Fig 5.37 (p176). There was no statistically significant variation in the same individual's PMN adhesiveness between different days (Wilcoxon Signed Rank Test).

5.7 Conclusions

1. The assay of neutrophil adhesion using Rose Bengal dye release, whilst giving an estimate of cell adhesion, was not able to give any clear idea of the magnitude of adhesion. The assay also had sources of error which proved difficult to eradicate. The method was therefore not used further.

2. The assay of neutrophil adhesion using the chromium release method had the advantages of being more reliably reproducible and able to give an estimate of percentage adherence of neutrophils to endothelial cells. This allowed a comparison of neutrophil adhesion between individuals to be made.

3. In the chromium release method the following assay conditions were found to be the most reliable:

a. HUVECS should be seeded onto 24 well tissue culture plates, pre-coated with gelatine solution, at a concentration of $1x10^5$ cells per well, 24 hours prior to assay. The HUVECS should then be incubated for 4 hours in fresh endothelial culture medium with or without interleukin 1b at a concentration of 1 unit\ml

b. 1ml of neutrophils, at a concentration of 2 $x10^6$/ml after pre-incubation with 50ml of Cr^{51} for one hour at room temperature, should be added per well.

c. Neutrophil incubation with the endothelial cells should be at 4^0c

for 45 minute.

d. The wells should be washed at the end of the adhesion period with BSS\10% FCS, 1ml\well, three times.

e. Remaining radioactivity in the wells after the washing phase should be released by 30 minute incubation with 1ml of NOP-4 followed by a further 1ml per well.

4. Using these assay conditions it was found that the addition of Interleukin 1b to the assay increased the adhesive potential of the HUVEC monolayer by 2-3 times. the pre-incubation of neutrophils with PMA increased the adhesion by 4-5 times.

5. Using neutrophils from healthy subjects the median adhesion to unstimulated endothelial cells was 7.75% and to stimulated endothelial cells was 20.9%.

Plate 5.5 HUVEC monolayer post addition of ^{51}Cr labelled PMNs (magnification x100).

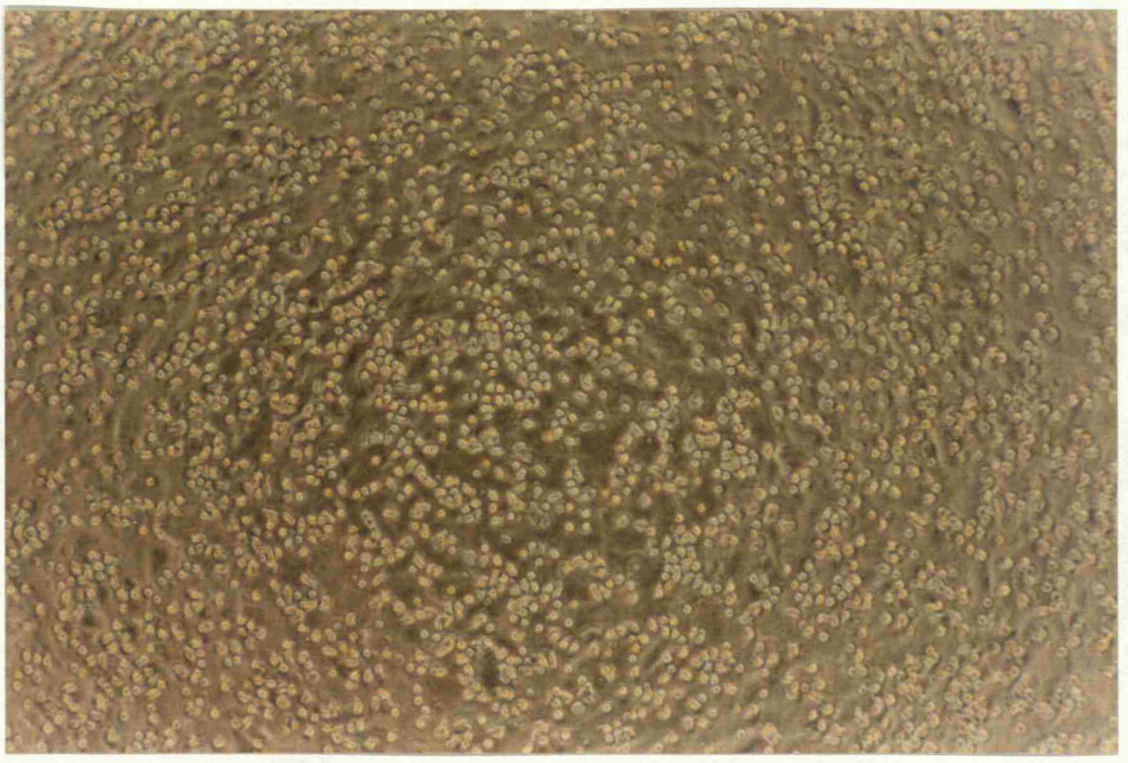


Plate 5.6 HUVEC monolayer post addition of ^{51}Cr labelled PMNs after 3 washes (magnification x100).

Fig 5.4a Correlation between measured radioactivity and labelled neutrophil cell numbers.

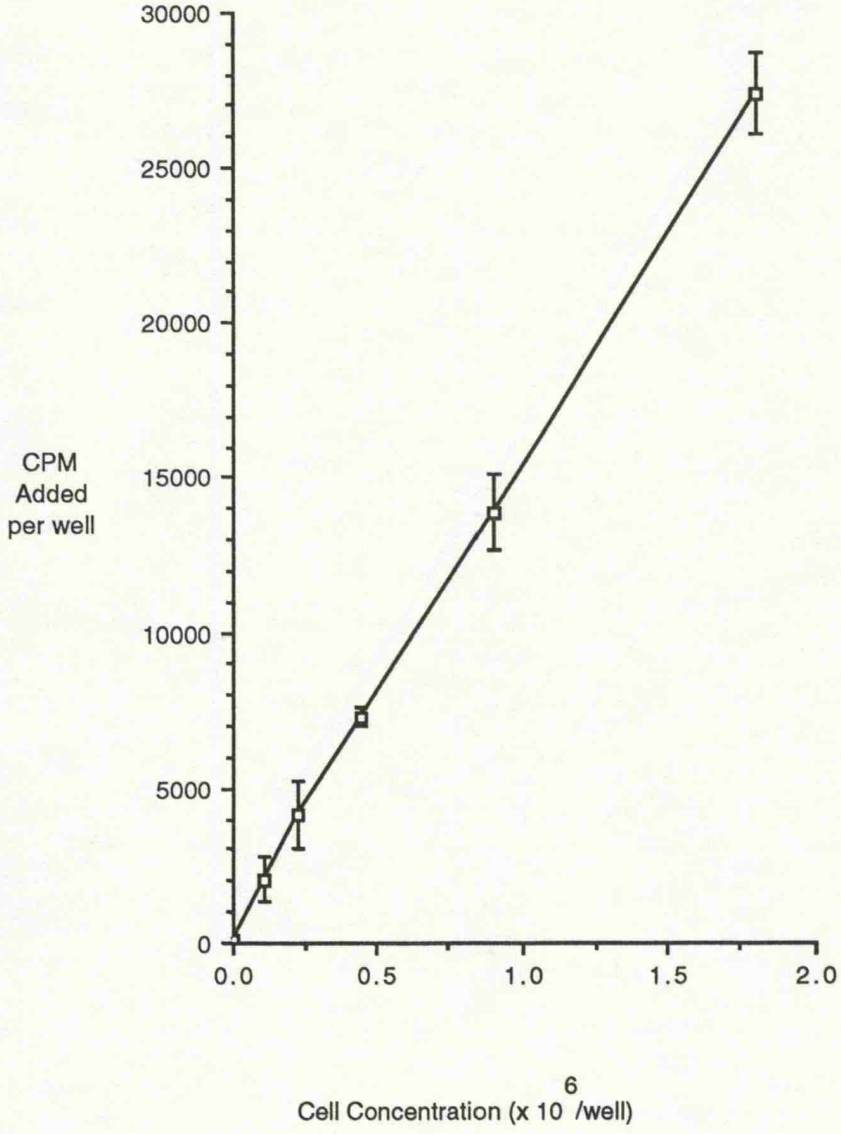


Fig 5.5 Effect of endothelial cell concentration and Interleukin-1β on PMN binding.

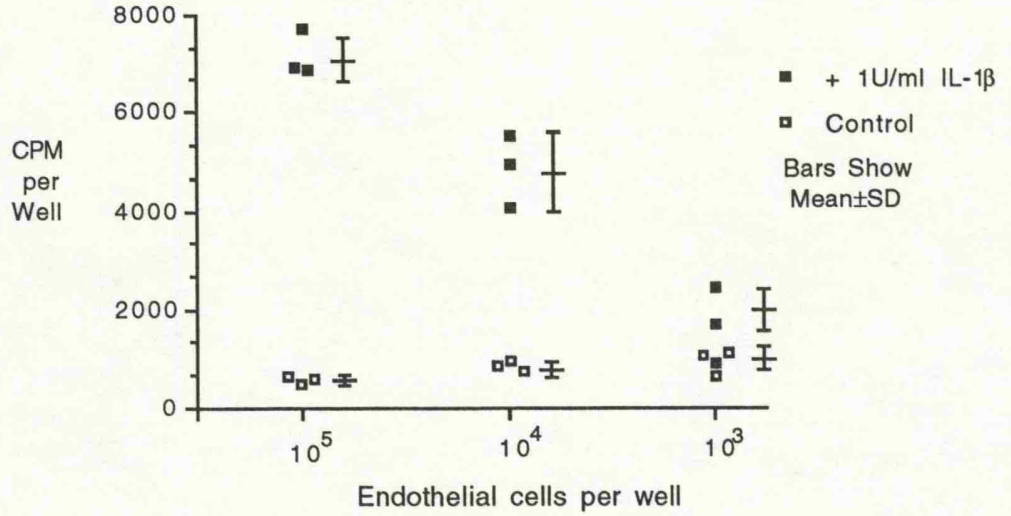


Fig 5.6 Effect of endothelial cell concentration and IL-1β on percentage PMN binding.

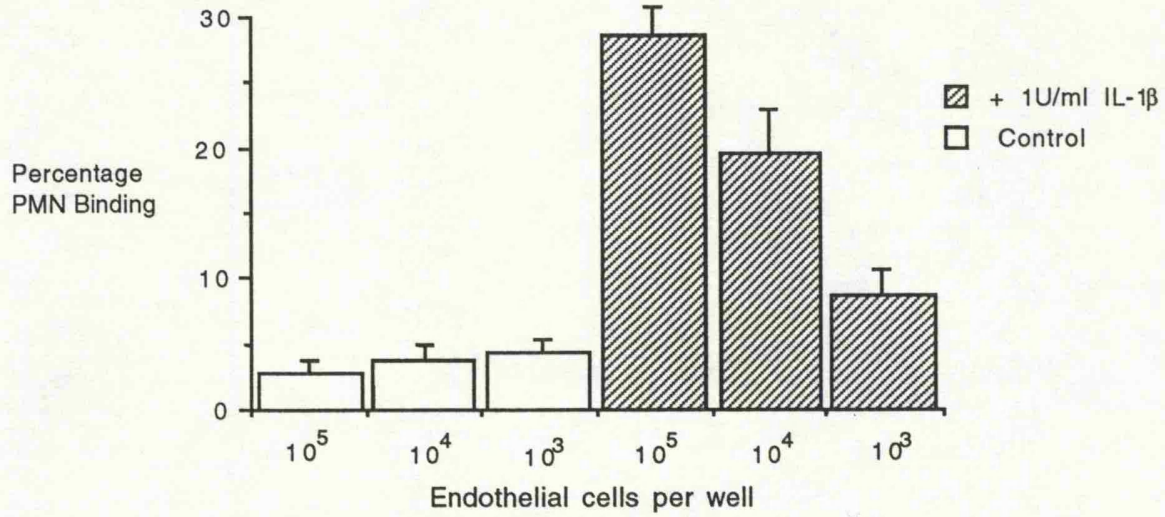


Fig 5.7 Effects of IL-1 β concentration on PMN adhesion to HUVEC monolayers.

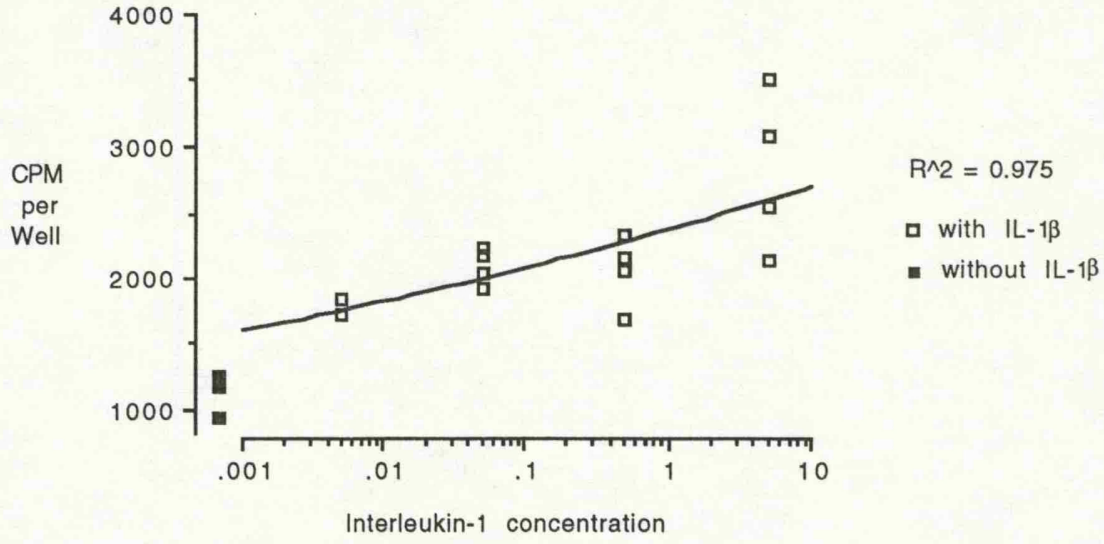


Fig 5.8 Effect of PMN concentration on adhesion to HUVEC monolayers treated with Interleukin-1β .

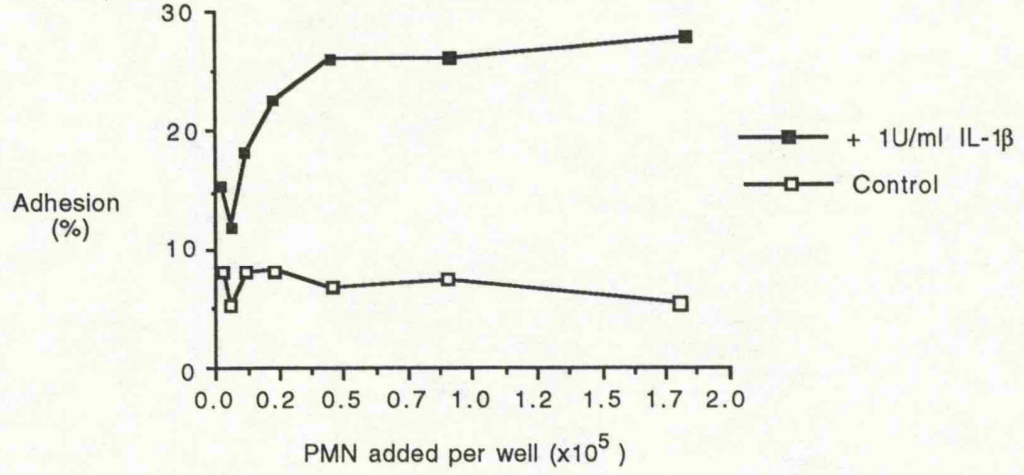


Fig 5.9 Correlation of PMN adhesion with total number of PMN added

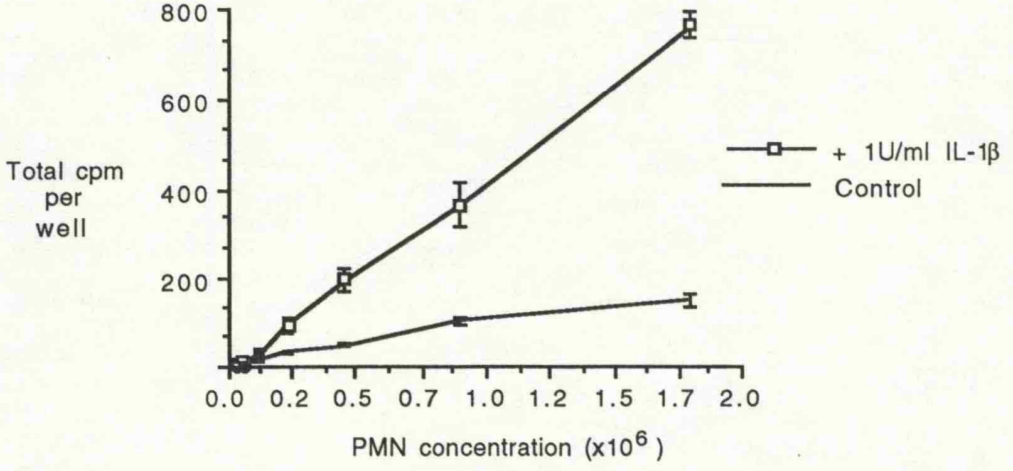


Fig 5.10 Effect of time on the binding of PMN to HUVEC monolayers at 4'C

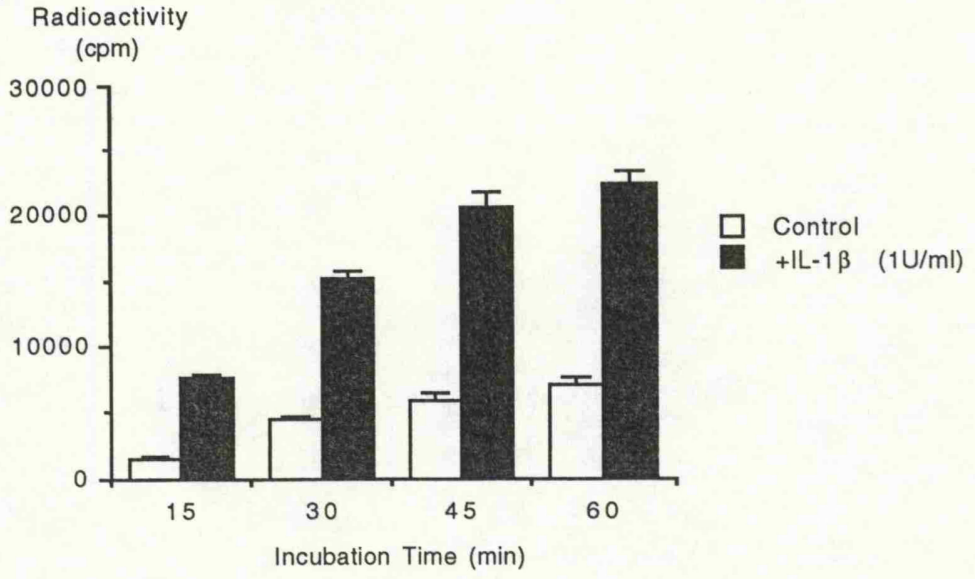


Fig 5.11 Effect of time on the percentage binding of PMNs to HUVEC monolayers at 4'C.

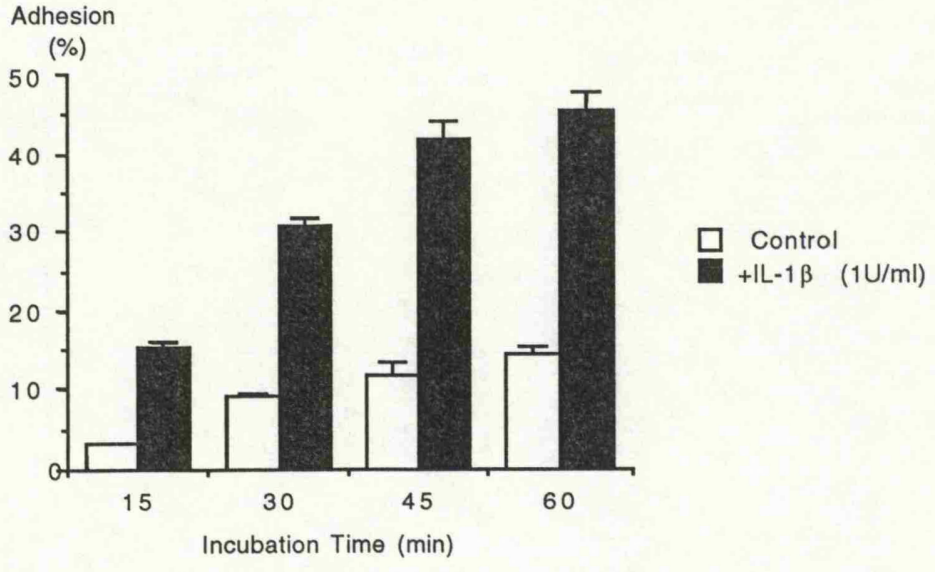


Fig 5.12 Effect of time on the binding of PMN to HUVEC monolayers at 20'C.

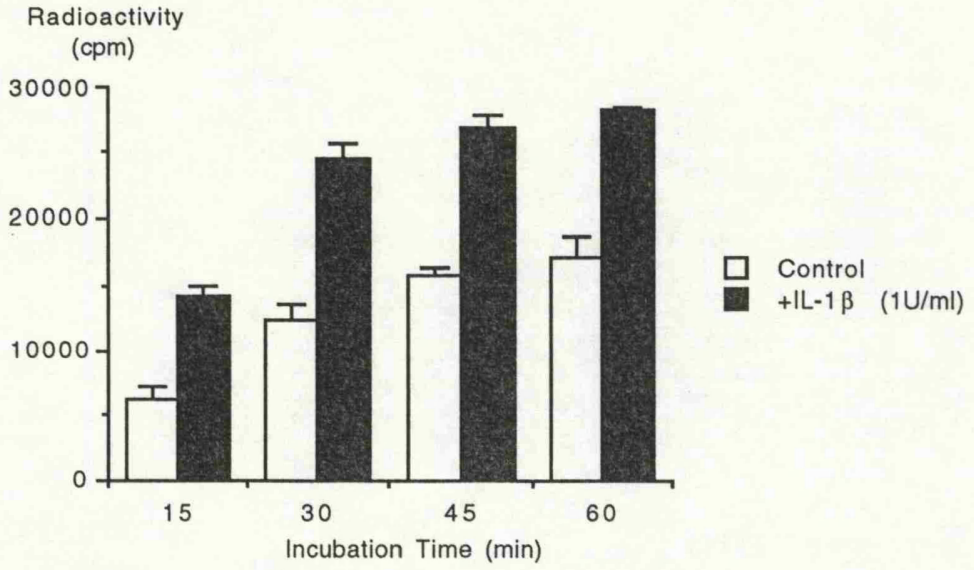


Fig 5.13 Effect of time on the percentage binding of PMN to HUVEC monolayers at 20'C.

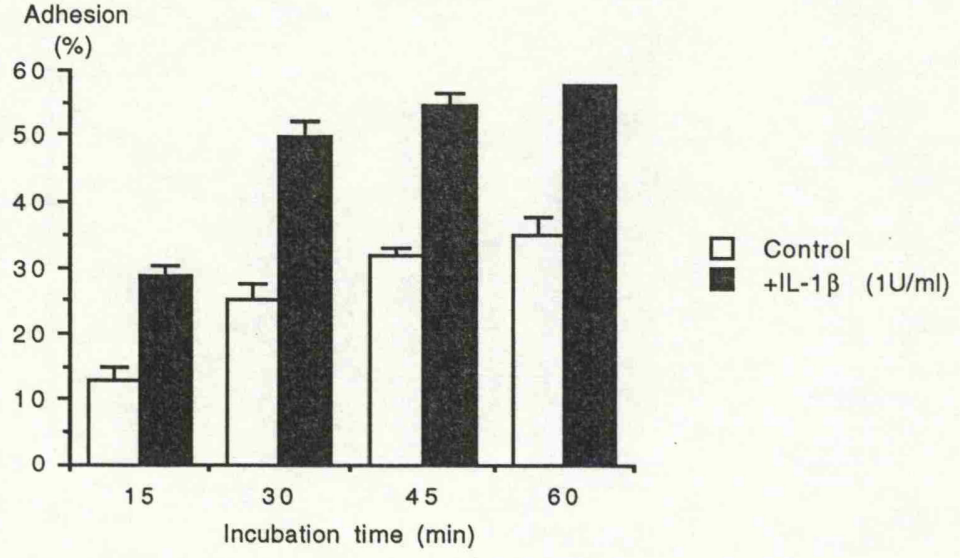


Fig 5.14 Effect of time on the binding of PMNs to HUVEC monolayers at 37'C.

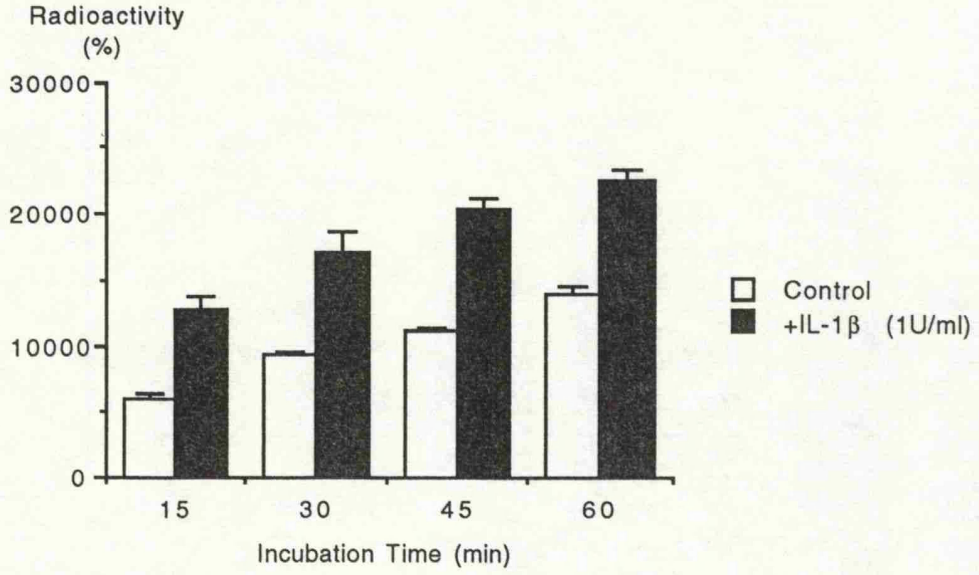


Fig 5.15 Effect of time on the binding of PMNs to HUVEC monolayers at 37'C.

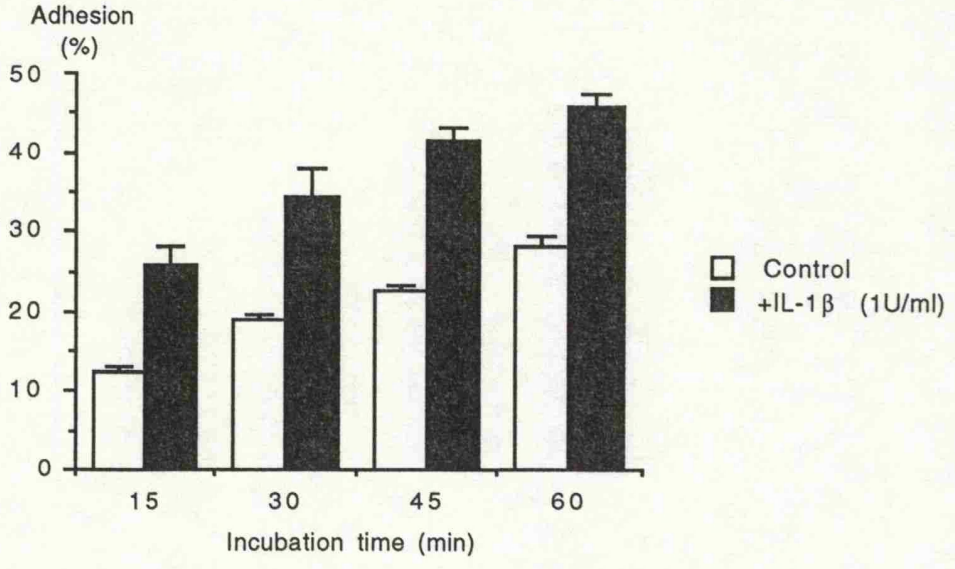


Fig 5.16 Effect of time and temperature on PMN adhesion to HUVEC monolayers with or without IL-1β.

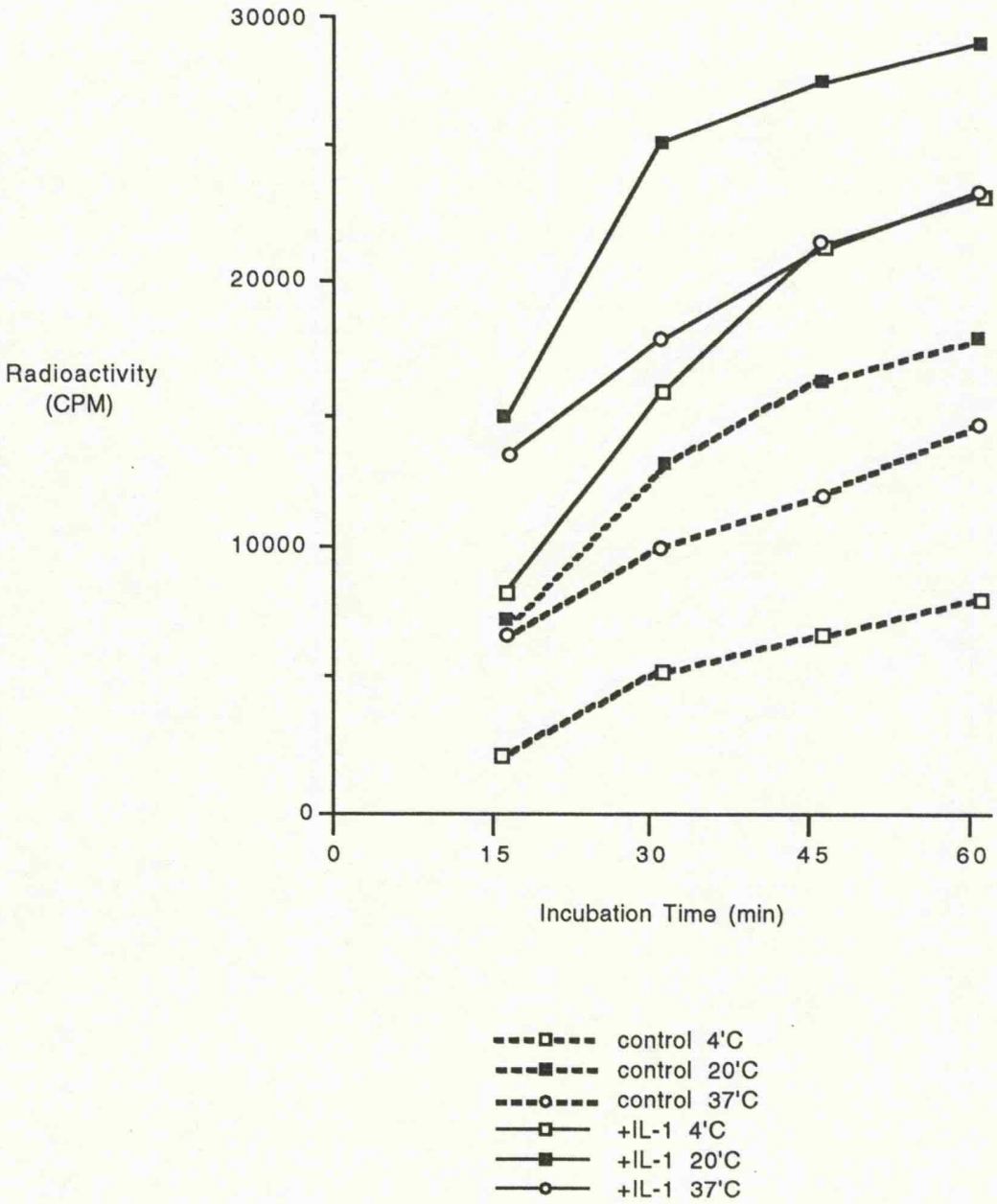


Fig 5.17 Effect of time and temperature on % PMN adhesion to HUVEC monolayers with or without IL-1β.

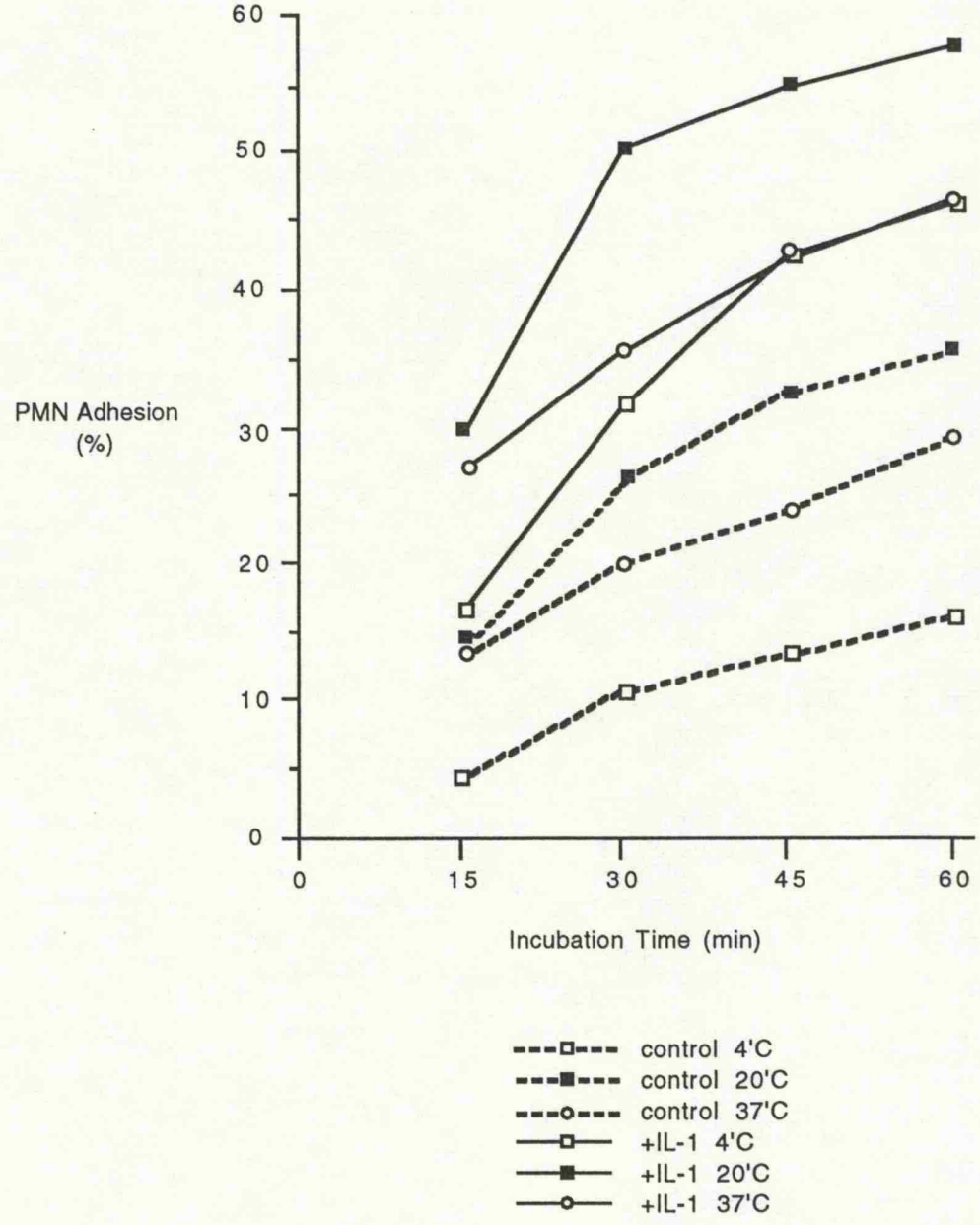


Fig 5.18 The % increase in PMN adhesion with time, comparing unstimulated and IL-1β stimulated HUVEC monolayers.

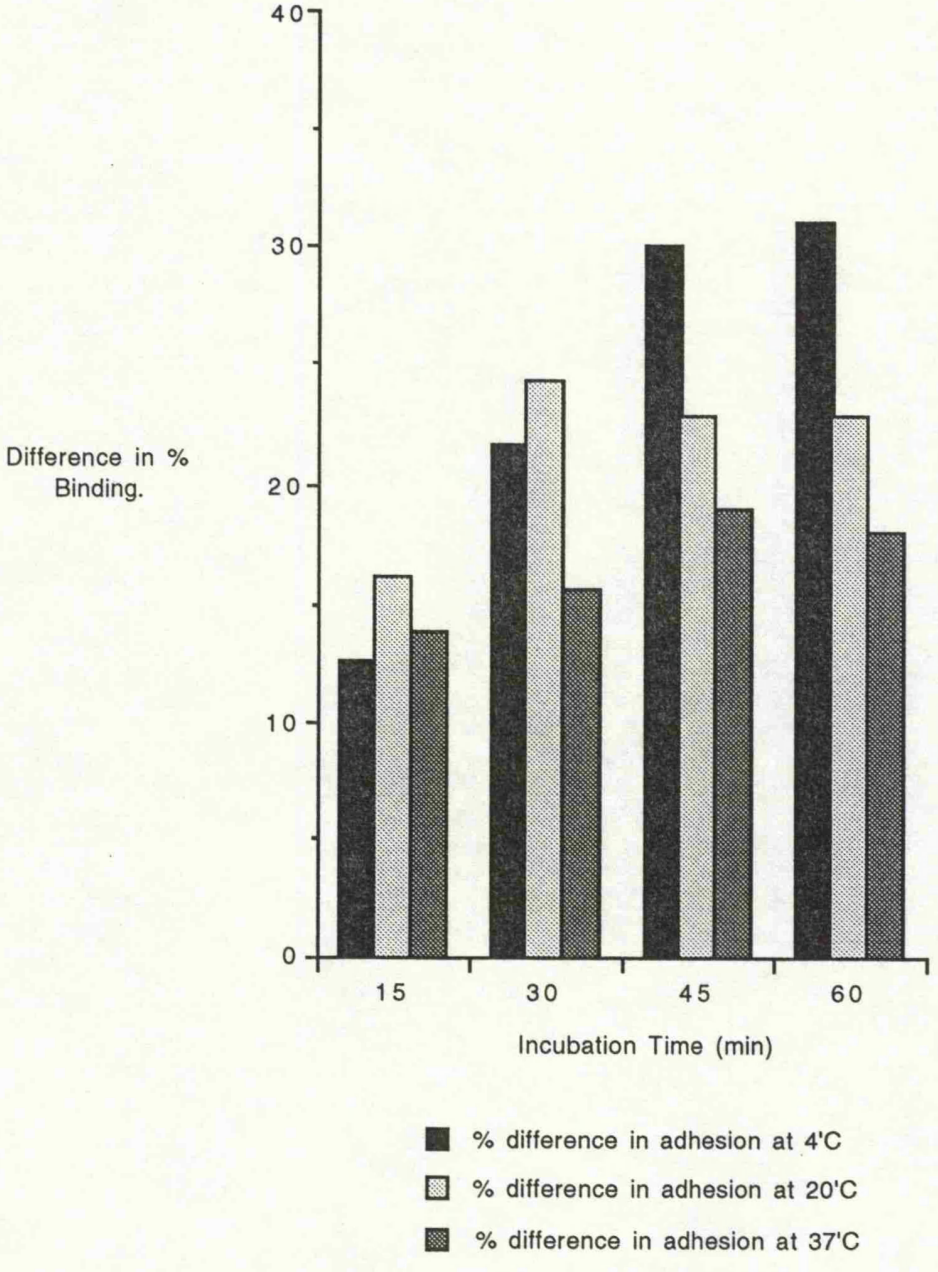


Fig 5.19 Effect of washing on PMN adherence to culture wells.

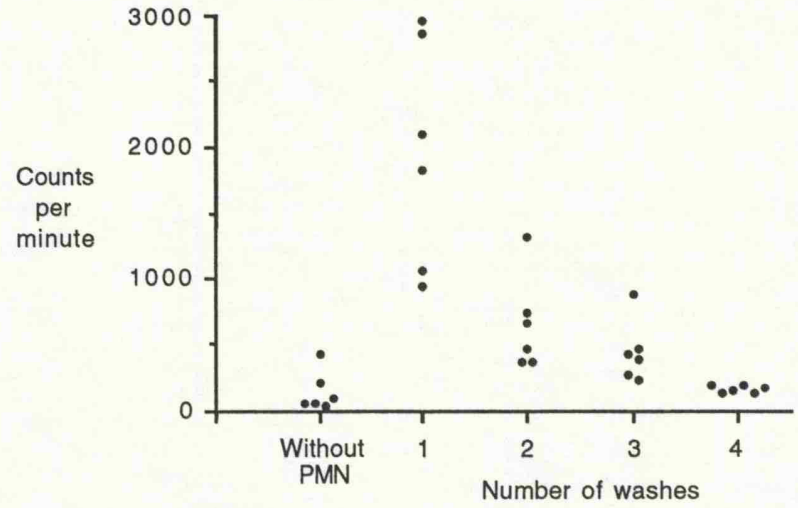


Fig 5.20 Effect of washing on percentage PMN adherence to culture wells.

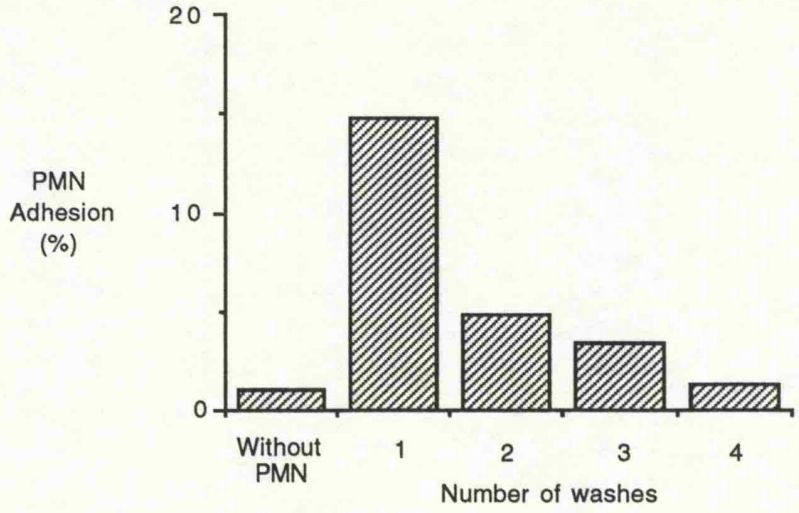


Fig 5.21 Effect wash number on PMN adhesion to HUVEC monolayers.

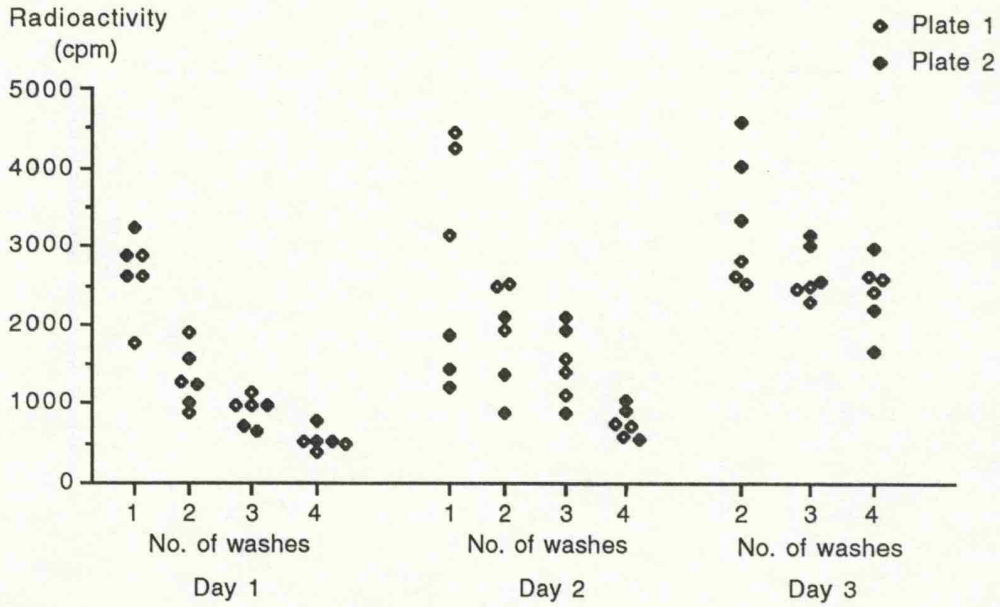


Fig 5.22 Effect of washing on the percentage adhesion of PMN to HUVEC monolayers.

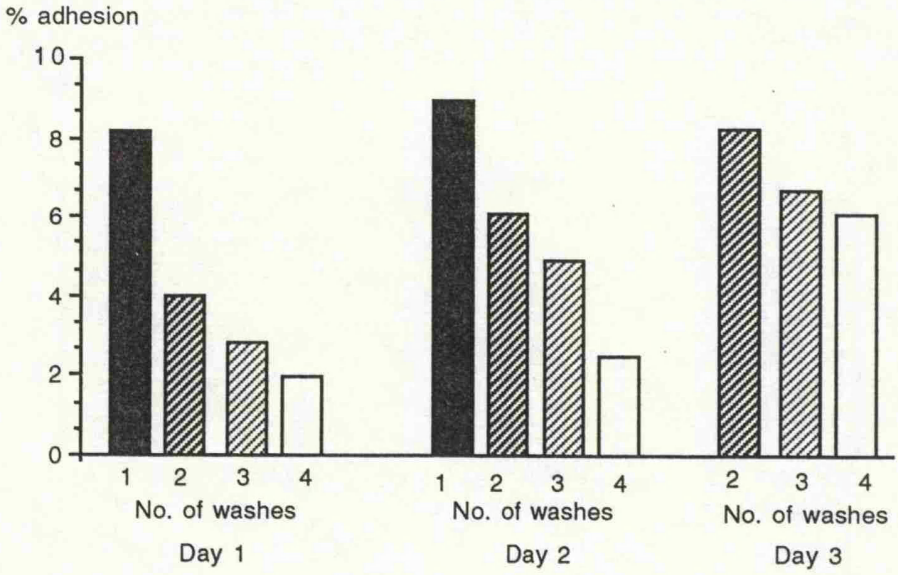


Fig 5.23 Effect of wash number on PMN adhesion to IL-1β stimulated HUVEC monolayers.

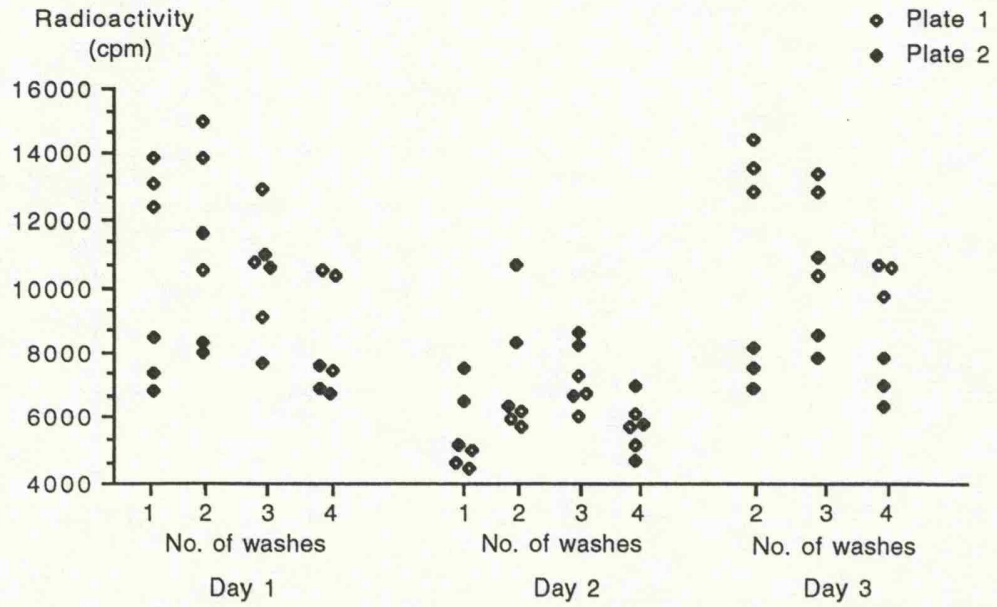


Fig 5.24 Effect of wash number on percentage PMN adhesion to IL-1β stimulated HUVEC monolayers.

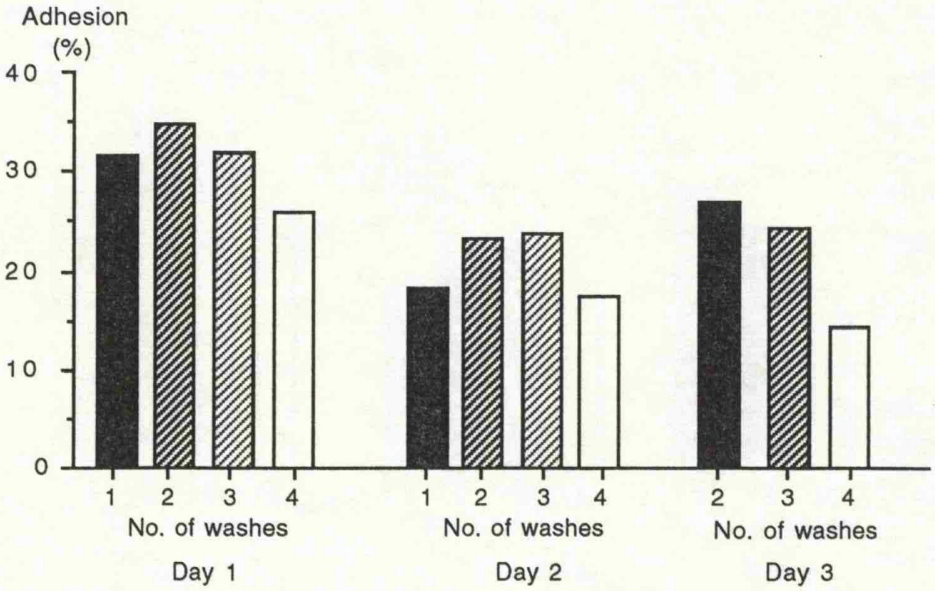


Fig 5.25 Effect of washing on the adhesion of PMNs to stimulated and unstimulated HUVEC monolayers.

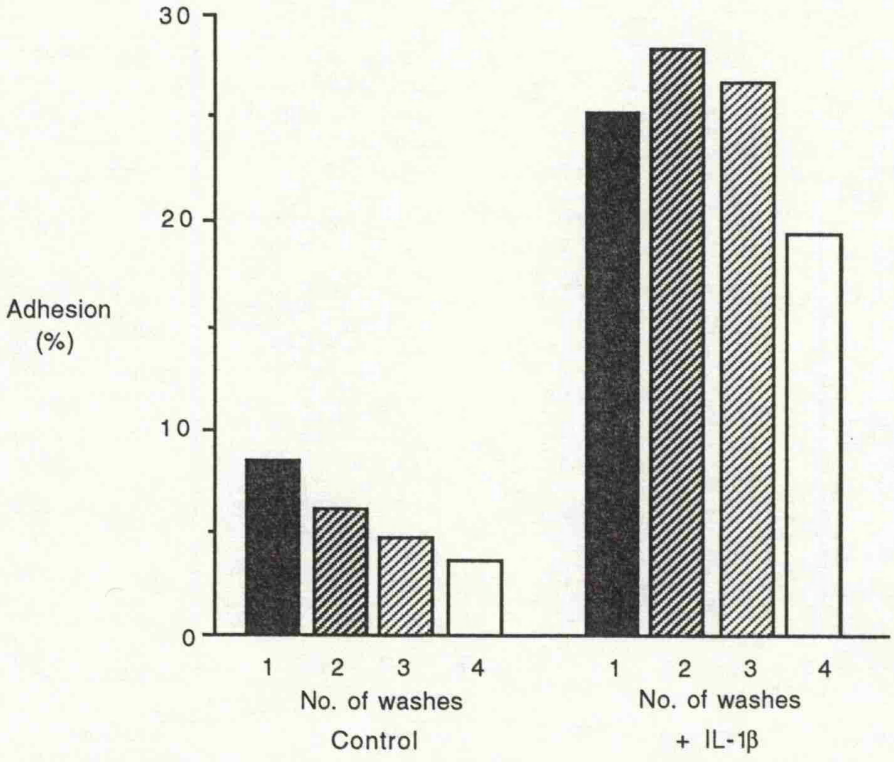


Fig 5.26 Effect of washing on PMN adhesion to unstimulated HUVEC monolayers in gelatin coated wells.

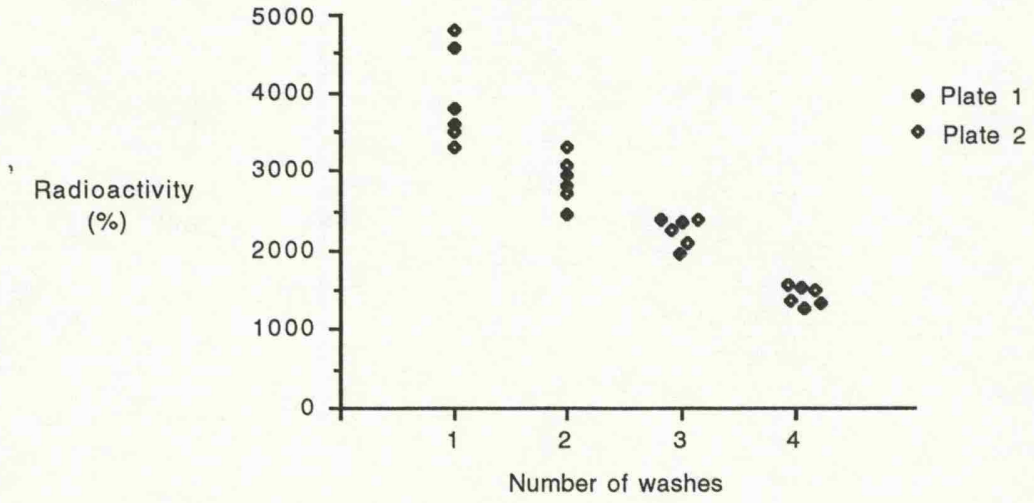


Fig 5.27 Effect of washing on percentage PMN adhesion to unstimulated HUVEC monolayers in gelatin coated wells.

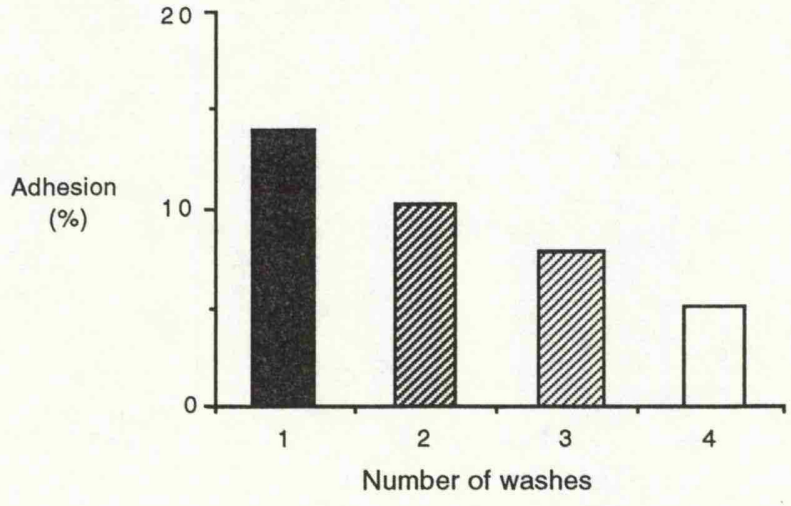


Fig 5.28 Effect of washing on PMN adhesion to IL-1 β stimulated HUVEC monolayers in gelatin coated wells.

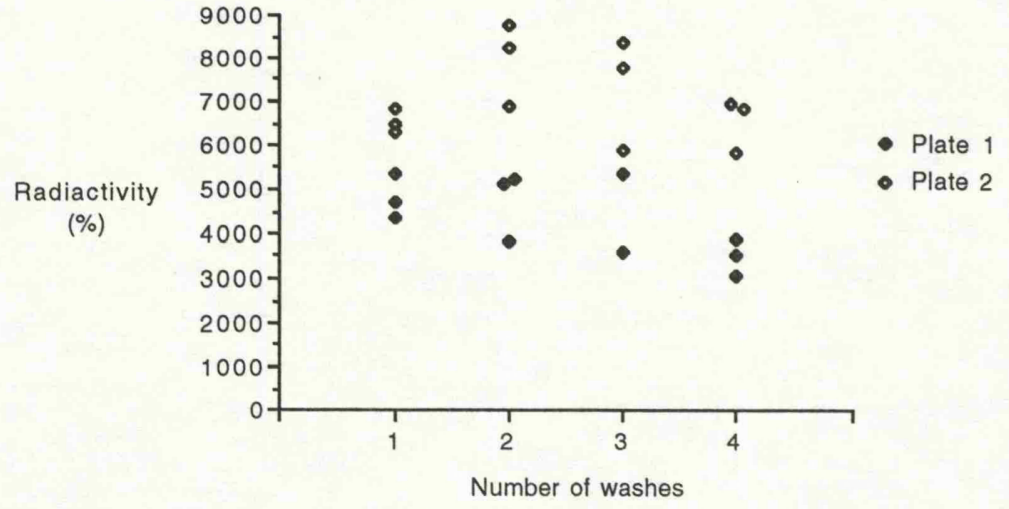


Fig 5.29 Effect of washing on percentage PMN adhesion to IL-1β stimulated HUVEC monolayers in gelatin coated wells.

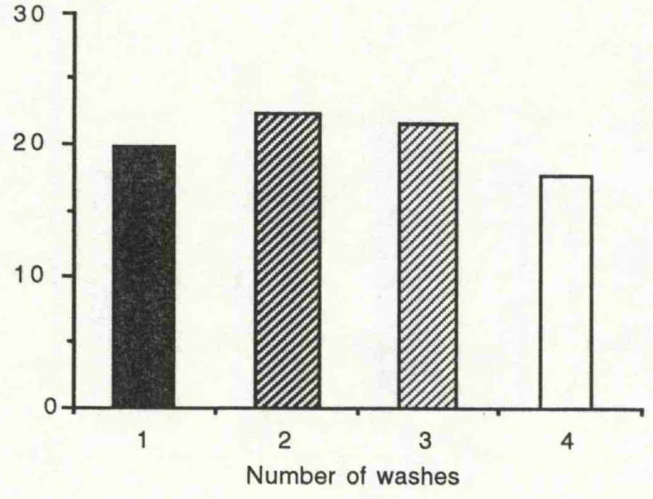


Fig 5.30 Effect of different lysis solutions on measured PMN adhesion to HUVEC monolayers.

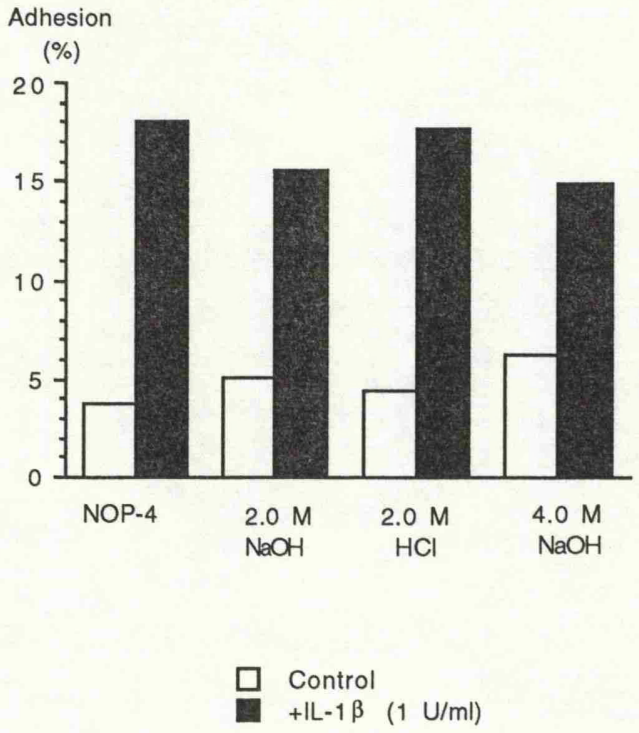


Fig 5.31 Effect of PMA stimulation on PMN adhesion to stimulated and unstimulated HUVEC monolayers.

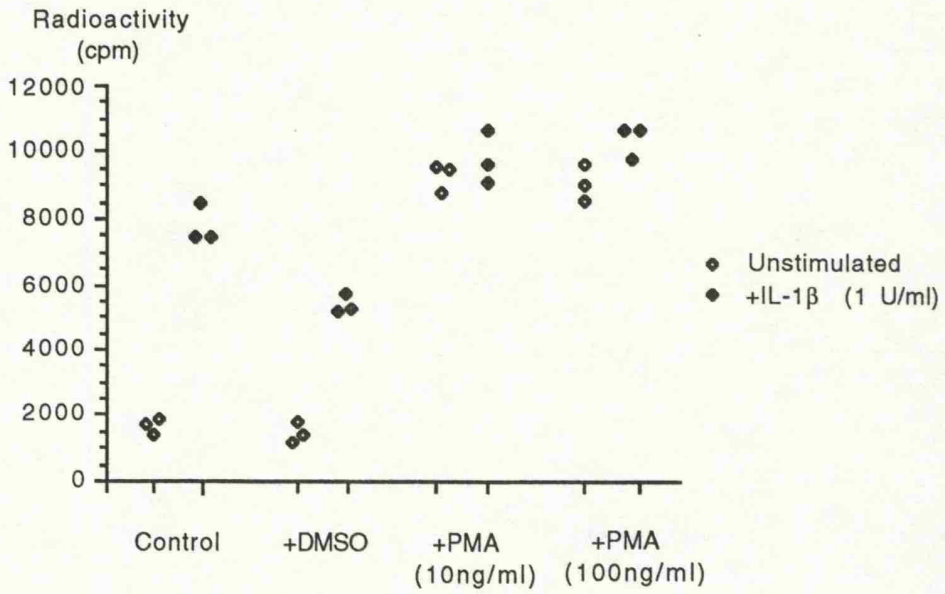


Fig 5.32 Effect of PMA stimulation on percentage PMN adhesion to stimulated and unstimulated HUVEC monolayers.

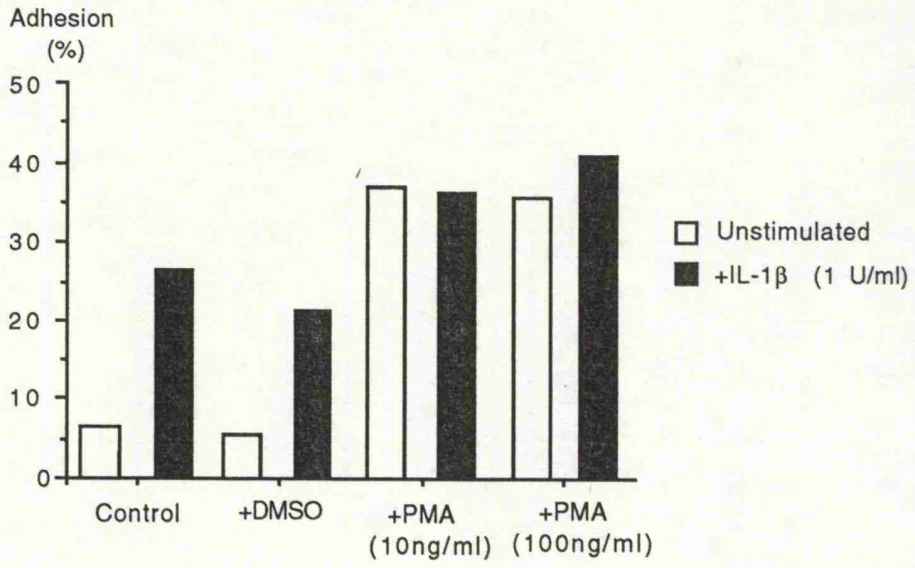


Fig 5.33 Adhesion of PMNs from normal individuals to unstimulated HUVEC monolayers

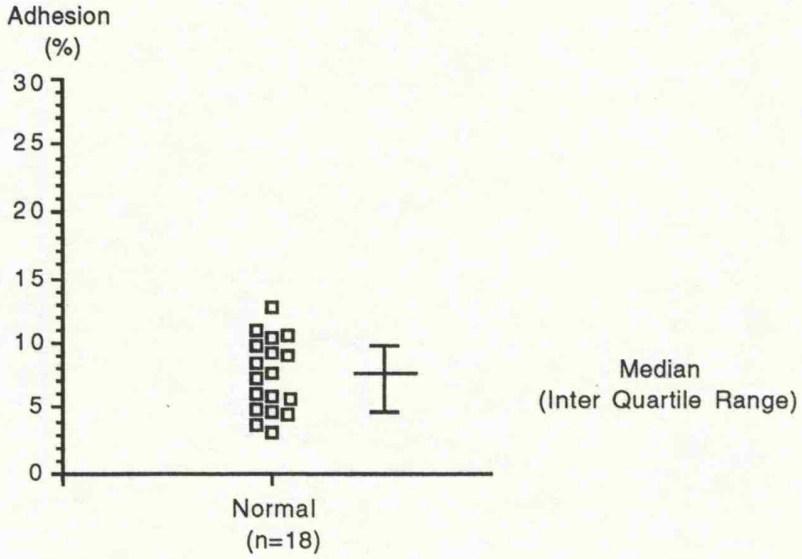


Fig 5.34 Adhesion of PMNs from normal individuals to IL-1β stimulated HUVEC monolayers

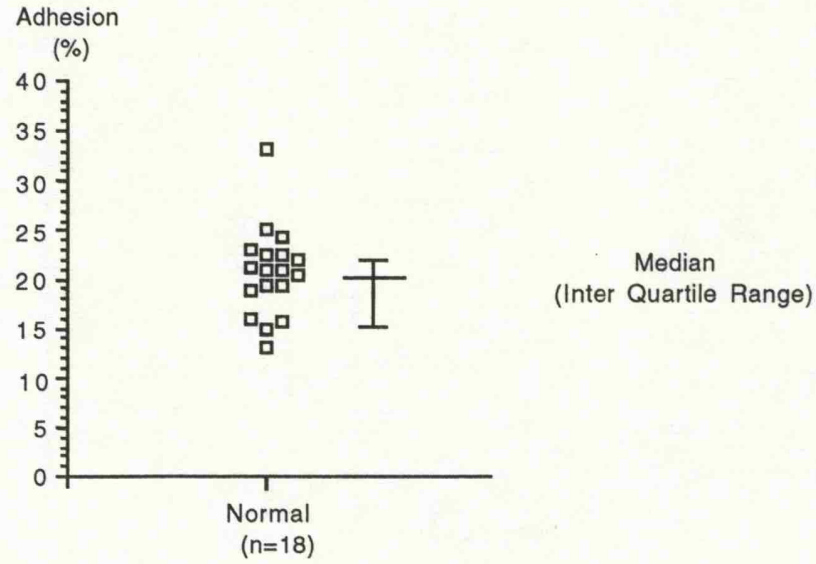


Fig 5.35 Effect of sex on PMN adhesion to unstimulated HUVEC monolayers.

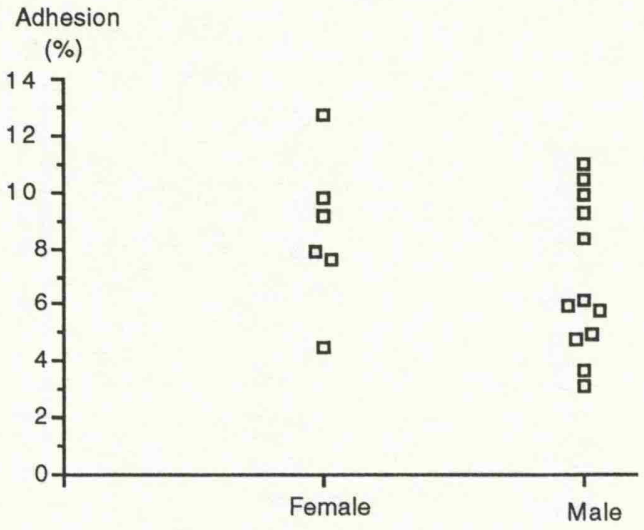


Fig 5.36 Effect of sex on PMN adhesion to stimulated HUVEC monolayers.

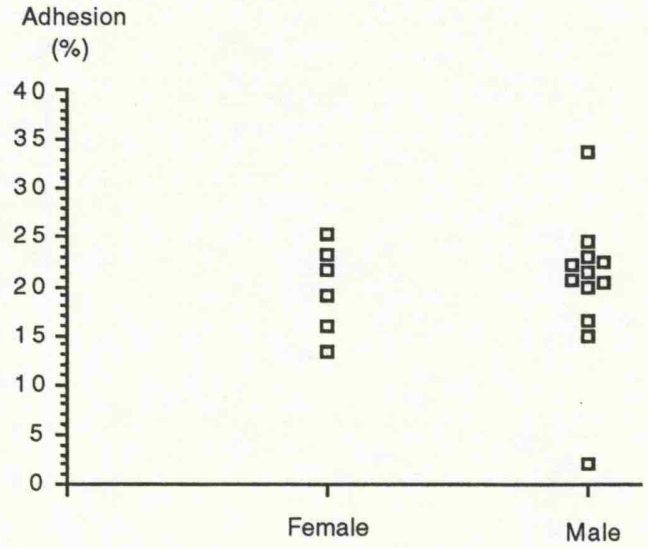


Fig 5.37 Intra-Individual variation in PMN adhesivness.

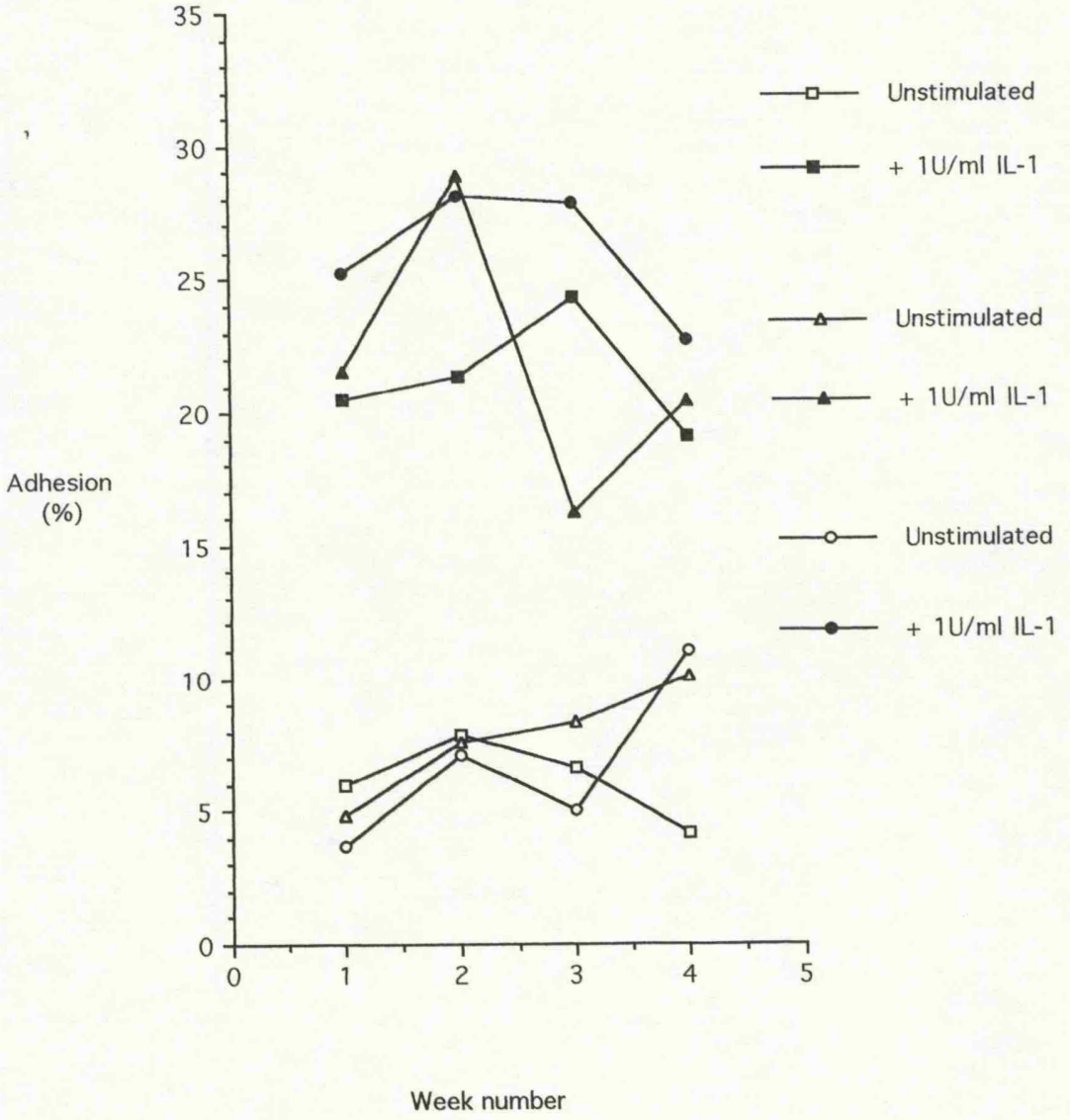


Table 5.2 Coefficient of variation of total radioactivity recovered compared between number of washes on gelatin coated plates.

UNSTIMULATED

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	9.1%	9.8%	7.5%
Wash 3	10.4%	6.12%	0.4%
Wash 4	9.3%	6.9%	3.7%

STIMULATED

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	16.4%	12.1%	48.4%
Wash 3	21.2%	17.5%	30.1%
Wash 4	11.7%	9.7%	42.5%

Table 5.1 Coefficient of variation of total radioactivity recovered compared between number of washes on uncoated plates.

UNSTIMULATED

Day 1

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	21.6%	22.1%	4.6%
Wash 3	9.3%	23.4%	19.3%
Wash 4	6.8%	24.7%	15.2%

Day 2

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	13.8%	41.5%	32.6%
Wash 3	16.5%	39.6%	12.8%
Wash 4	14.3%	29%	14.5%

Day 3

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	5.8%	15.8%	28.5%
Wash3	3.1%	10.3%	12.5%
Wash 4	2.5%	28.7%	8.4%

Average Coefficient of variation over 3 days.

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	13.7%	26.4%	21.9%
Wash3	9.6%	24.4%	14.9%
Wash 4	7.8%	27.5%	12.7%

STIMULATED

Day 1

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	21.3%	17.5%	23.8%
Wash 3	18.3%	17.4%	16.6%
Wash 4	6.5%	18.1%	19.7%

Day 2

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	25.4%	4.3%	24.0%
Wash 3	12.8%	9.2%	10.9%
Wash 4	20.1%	8.4%	1.6%

Day 3

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	8.0%	5.5%	40.1%
Wash 3	7.5%	13.3%	20.7%
Wash 4	13.2%	5.0%	26.4%

Average Coefficient of variation over 3 days.

	Intra-Plate(1)	Intra-Plate(2)	Inter-plate
Wash 2	18.2%	9.1%	30.8%
Wash 3	12.9%	13.3%	16.1%
Wash 4	13.3%	10.5%	15.9%

CHAPTER SIX

Application of the chromium labelled neutrophil assay to measurement of PMN adhesivness in patient groups

6.0 Introduction

In Chapter 5 we explored the sources of variability in the ^{51}Cr labelled PMN adhesion assay. Having optimized the assay conditions, the assay was then used to investigate the adhesive potential of PMNs from a range of normal subjects, patients in end stage renal failure (ESRF) and recipients of renal allografts. Patients in ESRF were all haemodialysis patients whose neutrophils were sampled immediately prior to dialysis, in order to assess the effects of prolonged uremia and to exclude the possible immediate activating effects of haemodialysis. Recipients of renal allografts all had functioning grafts at the time of sampling and were dialysis independent. The renal allograft patients were divided into two groups: those with proven infection at the time of sampling and those who were infection free.

6.1 Effect of Haemodialysis on PMN adhesion to HUVEC monolayers.

10 Haemodialysis patients were sampled over one 72 hour period. 4 ml of blood was collected into EDTA anticoagulant, from either the arteriovenous fistula or their central venous dialysis catheter. The blood was transported to the laboratory immediately and neutrophils were separated using the differential centrifugation technique (see Methods section). The adhesive potential of these neutrophils was then assayed using the standardised ^{51}Cr labelled PMN assay as described in section 5.4.5.2.

The demographic and clinical data from these patients is presented in

Table 6.1 (p184). There was a predominance of females in the patient group, which was due to the cohort of patients undergoing dialysis at the time this study could be carried out. Data from the normal cohort however, shows no relationship between sex and PMN adhesiveness as demonstrated in Figs 5.35 and 5.36 (p175).

Table 6.1 also shows the results of the adhesion assay for each patient. There was no relationship between access device type and the adhesiveness of PMNs, or to the length of time the patients had been in the dialysis programme. The mean whole blood white cell count was 7.72 $x10^9$/dL, which was within the normal range (4-11$x10^9$/dL). Mean serum urea concentration was 25.15 mmol/L, and mean serum creatinine concentration was 935 mmol/L, both of which were significantly raised above normal (urea 2.5-6.5 mmol/L, creatinine 60-120 mmol/L).

Figs 6.1 and 6.2 (p187), show the adhesion to unstimulated and stimulated HUVEC monolayers respectively, of PMNs from haemodialysis patients compared to normals, uninfected transplant patients and infected transplant patients. Examination of the graphs indicated that there are significant differences between the groups and the analysis of the data is shown in Table 6.3 (p188).

Table 6.3 shows that the PMN adhesion of Haemodialysis patients (12.7%) was significantly raised over control values (7.75%) when the assay was performed using unstimulated HUVECs (p=0.0023). This suggests that the adhesive potential of the haemodialysis patient's PMNs was elevated above normal levels. This was not true when stimulated HUVECs were used (Haemodialysis=23.85%, Normal=20.9%).

6.2 Effect of renal transplantation on PMN adhesion to HUVEC monolayers.

24 patients with functioning renal allografts, who were dialysis independent, were sampled at varying periods after transplantation from 5

days to 7 years. 4 ml of blood was collected into EDTA anticoagulant, from clean venepuncture of the cephalic vein. The blood was transported to the laboratory immediately and neutrophils were separated using the differential centrifugation technique (see Methods section). The adhesive potential of these neutrophils was then assayed using the standardised ^{51}Cr labelled PMN assay as described in section 5.4.5.2.

The demographic and clinical data from these patients is presented in Table 6.2.1 and 6.2.2 (p185-186). Of the 24 patients, 5 had infections at the time of sampling and these are indicated, with infection type, in the table.

Table 6.2.1 and 6.2.2 also show the results of the adhesion assay for each patient. The adhesion assay indicated an increased level of adhesion in patients with infection and the analysis of clinical parameters was carried out after splitting the patients in to two groups, uninfected (n=19) and infected (n=5). The mean whole blood white cell count was 8.4 $x10^9$/dL for the uninfected group, which was within the normal range, and 14.2 $x10^9$/dL for the infected group, which is above the normal range. Mean serum urea concentration was 16.7 mmol/L and 16.3 mmol/ for the uninfected and infected groups respectively, both significantly above normal. The mean serum creatinine concentration was 282 mmol/L and 217 mmol/L, for the uninfected and infected groups respectively, both significantly above normal.

The relationship between the length of time the sample was taken post transplantation and neutrophil adhesiveness was investigated. For this the transplant group was divided into those patients less than 6 months post transplant and those who were more than 6 months post-transplant, on the basis that immunosuppressive therapy had decreased to baseline by this time. No correlation was found between the time post transplant and white cell count, urea, creatinine or PMN adhesiveness on either stimulated or

unstimulated HUVEC monolayers.

Figs 6.1 and 6.2 (p187), show the adhesion to unstimulated and stimulated HUVEC monolayers respectively, of PMNs from uninfected transplant patients and infected transplant patients compared to normals and haemodialysis patients. Examination of the graphs indicated that there are significant differences between the groups and the analysis of the data is shown in Table 6.3 (p188).

Table 6.3 shows that the PMN adhesion of uninfected transplant patients (24.2%) was significantly raised over control values (20.9%) when the assay was performed using stimulated HUVECs (p=0.04). This was not true when unstimulated HUVECs were used (Uninfected transplant=7.6%, Normal=7.75%).

Uninfected transplant patients showed a lower PMN adhesiveness (7.6%) to unstimulated HUVECs, when compared to haemodialysis patients (12.7%), (p=0.0027). Uninfected transplanted patients (24.2%) show slightly increased adhesiveness of PMNs to stimulated HUVECs when compared to haemodialysis patients (23.85%), but this was not a significant increase. However, uninfected transplant patients have an elevated PMN adhesiveness to stimulated HUVECs (24.2%), when compared to normal individuals (20.9%) (p=0.04).

Infection appeared to cause a general increase in PMN adhesiveness to both unstimulated and stimulated HUVECs. Table 6.3 shows that the PMN adhesion of infected transplant patients (34.0%) was significantly elevated above the both normal individuals(20.9%) and uninfected transplant patients (24.2%) using stimulated HUVECs (p= 0.0015 and 0.0014 respectively). When unstimulated HUVECs were used elevated adhesion was seen in infected patients (13.8%), however this elevation failed to reach statistical significance above normals (7.75%) or Uninfected transplant patients (7.6%). When PMN adhesion, using stimulated HUVECs, in infected patients

(34.0%) was compared to haemodialysis patients (23.85%) there was significantly increased adhesion (p=0.006).

6.3 Conclusions

1. Using the chromium release assay for measuring neutrophil adhesion to endothelial cells it was found that patients with end stage renal failure undergoing haemodialysis had a significantly higher level of neutrophil adhesion to unstimulated HUVECS than normal controls. Patients with renal transplants who had evidence of infection tended to also to have increased adhesion over normal controls but this was not statistically significant.
2. Using the chromium release assay it was found that patients with end stage renal failure undergoing haemodialysis had a significantly higher level of neutrophil adhesion to unstimulated HUVECS than patients with functioning renal transplants who had no evidence of infection. There was no difference in neutrophil adhesion between haemodialysis patients and patients with renal transplants who had evidence of infection, using unstimulated HUVECS.
3. Using interleukin 1b stimulated HUVECS, neutrophils from normal controls were significantly less adhesive than patients with renal transplants, both infected and uninfected, but not haemodialysis patients.
4. Using interleukin 1b stimulated HUVECS, neutrophils from uninfected renal transplant patients were significantly less adhesive than patients with renal transplants who had evidence of infection, but were not different from those neutrophils from haemodialysis patients.
5. Using interleukin 1b stimulated HUVECS, neutrophils from infected renal transplant patients were significantly more adhesive than neutrophils from haemodialysis patients.
6. Overall the greatest level of neutrophil adhesion was seen amongst the group of patients with renal transplants who had evidence of infection.

Table 6.1 Demographic and Clinical Information of the Haemodialysis patients studied, with neutrophil adhesion data.

Patient No.	Sex	Age at Sampling	Cause of End Stage Renal Failure	Time on Dialysis (years)	White Cell Count ($x10^9/l$)	Urea (mmol/L)	Creatinine (μmol/L)	Neutrophil Adhesion (%) Control	+IL-1β	Dialysis Access Type.
1	F	51	Chronic Pylonephritis	1	9.8	30.4	1399	7.23	16.2	AVF
2	F	48	Unknown	0.25	4.7	33.6	681	12.8	21.3	AVF
3	F	63	Hypertension	6	7.5	16.2	996	16.2	24.6	AVF
4	F	59	Interstitial nephritis	1	6.5	24.4	1233	9.9	28.2	AVF
5	F	58	IDDM	5	6.9	27.3	873	17.7	26.5	AVF
6	F	54	SLE+IDDM	6	5.6	19.9	962	12.6	25.1	Vascath
7	F	65	Vasculitis	0.25	3.5	33.5	892	11.0	34.1	Vascath
8	F	50	Sickle cell nephropathy	3.5	11.6	31.7	1022	13.4	20.9	Vascath
9	F	58	IDDM	4	9.8	17.9	740	7.2	19.3	AVF
10	M	63	Renal artery stenosis	1.9	11.3	16.6	560	17.8	22.8	AVF

AVF = Arteriovenous fistula.
Vascath = Percutaneous Double lumen central venous catheter.
IDDM = Insulin Dependent Diabetes Mellitus

Table 6.2.1 Demographic and Clinical Information of the Renal transplant recipients studied, with neutrophil adhesion data.

Patient No.	Sex	Age at Sampling	Cause of End Stage Renal Failure	Time post Transplant	White Cell Count ($x10^9/l$)	Urea (mmol/L)	Creatinine (μmol/L)	Neutrophil Adhesion (%) Control	+IL-1β	Infection at time of sample?
1	F	39	Glomerulonephritis	5.66 years	8.2	36.9	540	5.5	19.8	no
2	M	71	Hypertension	14 days	9.8	10.5	135	7.1	27.5	no
3	M	38	IDDM	5 weeks	13.0	12.1	164	7.8	16.6	no
4	F	41	Polycystic Kidneys	3 months	6.8	10.1	172	4.9	22.8	no
5	M	63	IDDM	5 years	11.0	23.3	294	7.6	24.2	no
6	M	47	SLE	5 days	6.7	12.4	222	5.8	27.6	no
7	F	64	Chronic Pylonephritis	9 days	12.8	6.4	69	6.3	22.2	no
8	M	45	Single Kidney	10 weeks	10.6	8.7	198	3.4	17.4	no
9	M	67	Unknown	4.33 years	10.4	33.0	633	3.4	15.1	no
10	F	15	Infantile Polycystic Kidneys	3 months	5.5	9.2	142	2.7	19.2	no
11	M	23	Vesicoureteric reflux Pylonephritis	5 years	4.8	8.5	121	5.0	33.7	no
12	M	27	Focal Segmental Glomerulosclerosis	2 months	10.4	28.4	979	8.6	30.7	no

Table 6.2.2 Demographic and Clinical Information of the Renal transplant recipients studied, with neutrophil adhesion data (continued).

Patient No.	Sex	Age at sampling	Cause of End Stage Renal Failure	Time post Transplant	White Cell Count ($x10^9/l$)	Urea (mmol/L)	Creatinine (μmol/L)	Neutrophil Adhesion (%) Control	+IL-1β	Infection at time of sample?
13	F	52	Glomerulonephritis	3 months	5.9	9.0	144	8.04	30.9	no
14	F	56	IDDM/Pylonephritis	4.5 years	7.1	38.3	533	9.8	29.0	no
15	F	53	IDDM	6 years	6.3	8.6	13.1	8.6	29.4	no
16	M	57	Polycystic Kidneys	3 months	10.4	23.5	274	7.9	25.6	no
17	M	51	Unknown	6 years	5.0	16.9	256	12.4	23.08	no
18	F	24	Chronic Pylonephritis	6.66 years	7.5	18.5	236	13.9	20.4	no
19	M	31	Hypertension	2.25 years	6.4	4.3	123	11.9	26.7	no
20	M	23	Chronic Pylonephritis	1.33 years	16.2	11.9	256	21.1	38.9	Septacaemia + Respiratory Infection
21	M	17	Lawrence-Moon -Biedle Syndrome	6 months	5.7	9.6	165	13.79	30.84	Septacaemia *E.coli* culture
22	M	38	Polycystic Kidneys	7 years	9.1	14.1	238	15.4	34.7	Upper Resp. Tract Infection (-ve culture)
23	M	63	IDDM	11 days	27.1	37.9	291	5.4	31.3	UTI *E. coli* culture
24	M	53	IDDM	10 days	12.7	7.8	137	7.2	34.2	*Staph. aureus* wound infection

Table 6.3 Percentage PMN adhesion to HUVEC monolayers of Normals, Transplant patients, and Haemodialysis patients (median and interquartile range (Q1-Q3)).

Patient type	Control HUVECs	IL-1β Stimulated HUVECs
Normals (n=18)	7.75(4.88-9.85)	20.9(16.1-22.8)
Haemodialysis (n=10)	12.7(9.2-16.6)	23.85(20.5-26.9)
All Transplants (n=24)	7.7(5.4-11.4)	27.1(20.85-30.9)
Uninfected Transplant (n=19)	7.6(5.4-8.6)	24.2(19.8-29.0)
Infected Transplant (n=5)	13.8(6.3-18.3)	34.0(31.1-36.8)

Tests of Significance, p values (Mann-Whitney).

Patient type	Control HUVECs	IL-1β Stimulated HUVECs
Normal vs.		
Haemodialysis	**0.0023**	0.088(ns)
All Transplants	0.66(ns)	**0.005**
Uninfected Transplant	0.91(ns)	**0.040**
Infected Transplant	0.11(ns)	**0.0015**
Uninfected Transplant vs.		
Haemodialysis	**0.0027**	0.7655(ns)
Infected Transplant	0.124(ns)	**0.0014**
Infected Transplant vs.		
Haemodialysis	1.0(ns)	**0.006**

Fig 6.1 Effect of End Stage Renal Failure, transplant and infection on the adhesion of PMN to unstimulated HUVEC monolayers.

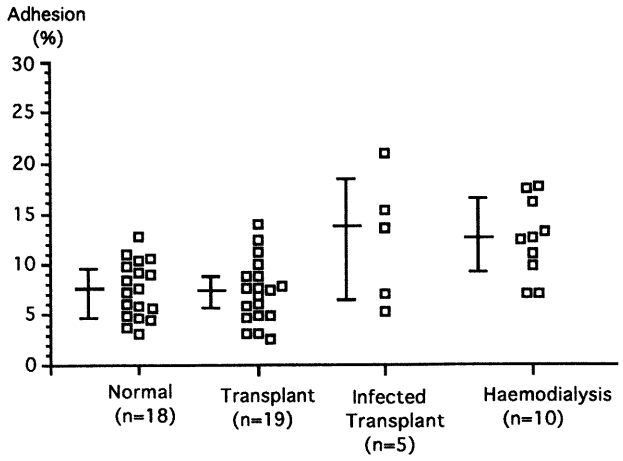
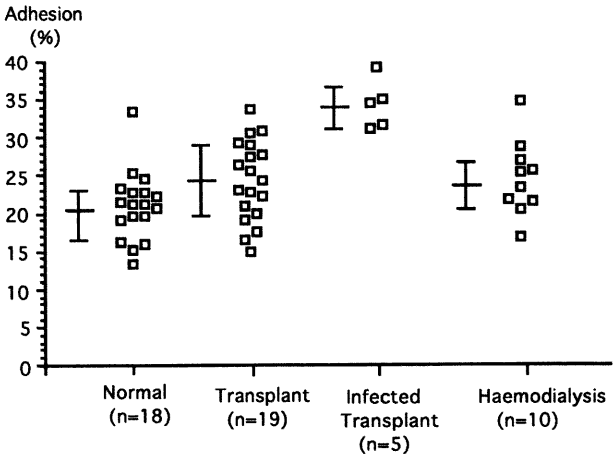


Fig 6.2 Effect of End Stage Renal Failure, transplant and infection on the adhesion of PMN to stimulated HUVEC monolayers.



Bars Indicate
Median ± Inter-Quartile Range

CHAPTER SEVEN

In-vivo expression of Cell adhesion molecules in renal allografts

7.1. Introduction

Inflammation is characterised by the local accumulation in the tissues of blood leucocytes, plasma proteins and fluid as a result of injury, infection or antigenic stimuli. The control of leucocyte passage is, in part, regulated by the expression of adhesion molecules on the surface of both vascular endothelial cells and leucocytes. The pattern of adhesion molecule expression varies with time and the type of stimulus, thus regulating the tempo of the inflammatory response and the leucocyte population entering the tissues. Cytokines, produced both locally and systemically, are important regulators of adhesion molecules, being both stimulatory and inhibitory during the inflammatory process. Whilst localised inflammatory conditions, such as allograft rejection, would be expected to cause increased adhesion molecule expression, more generalised conditions such as hypovolaemic or septic shock may also have a similar effect.

Leucocyte infiltration is an key part of renal allograft rejection with lymphocyte's and macrophages predominant. Lymphocyte sub-populations can be investigated by looking at antigen expression on the cell surface: leucocyte common antigen identifies white cells in general, presence of CD3 identifies lymphocytes, CD4 is present on T-helper and inducer lymphocytes, CD8 is present on cytotoxic and suppressor T lymphocytes.

During acute cellular renal allograft rejection the initial inflammatory response is associated with the release of the cytokines IL-1 and TNF with subsequent T-lymphocyte activation and, if untreated, graft destruction. The de-novo synthesis and expression of the interleukin 2

receptor, (IL-2R), represents the early consequences of antigen or mitogen induced activation of mature resting T lymphocytes. The subsequent interaction of IL-2 with IL-2R promotes rapid expansion of the effector T cell population. As such IL-2R's presence is an established marker for immune activation.

Evaluation of the expression of endothelial adhesion molecules in renal allografts might therefore provide a reliable index of immune activation permitting appropriate anti-rejection therapy to be given. In these studies biopsies were obtained from renal allografts both pre-transplantation and post-transplantation and assessed, using immunohistology, for the presence of adhesion molecules and cellular infiltrate. The findings were then related to the patients clinical course and histopathological diagnosis to determine the usual pattern of adhesion molecule expression that might be expected, and the potential that adhesion molecule expression might have as a diagnostic aid in renal allograft dysfunction.

7.2. Patients and methods

7.2.1 Patient details

Biopsies were obtained from 16 pre-transplant and 119 post-transplant renal allografts.(for the biopsy method see methods chapter). Pre-transplant samples were taken immediately prior to transplantation, before the kidney was reperfused. Post-transplant samples were obtained both at times of graft dysfunction and routinely at intervals of one, six, twelve and twenty four months. Half of the sample was snap-frozen in liquid nitrogen and used for immunohistological analysis, and half preserved in formalin for histopathological examination by an independent histopathologist.

Details of the organ donors from whom the pre-transplant biopsies were taken are shown in appendix A, table A1 (p232). Following transplantation all recipients were immunosuppressed with a combinations of azathioprine, cyclosporin and prednisolone. Episodes of allograft rejection, diagnosed on clinical criteria and confirmed by histopathology were treated with a three day course of intravenous methylprednisolone (0.5 gms/day). Steroid resistant rejection, as diagnosed by histopathology, was treated with a 10-14 day course of the monoclonal antibody OKT-3. A complete record of patient details for each post-transplant biopsy is shown in appendix C (p243-247).

7.2.2. Immunohistological methods

In this study the distribution of the cell adhesion molecules E-selectin, VCAM, ICAM-1 and PECAM in renal transplant biopsies was studied. HLA Class 1 and Class 2 antigen expression was also assessed. The intensity of staining of these antigens on renal tubules, glomeruli, inter-tubular endothelium (ITE) and large vessels within the kidney biopsy was measured. Any cellular infiltrate found on the biopsy was also assessed for the presence of leucocyte common antigen, CD3, CD4, CD8 and IL-2 receptor.

Details of the immunohistology sample preparation and staining are shown in the methods chapter. Staining intensity was scored according to a simple semiquantitative system using a scale of 0-5 where 0=absent, 1= very weak, 2=weak, 3= moderate, 4=strong, 5= very strong. Examples of biopsies which demonstrate the staining of different antigens and different scores of staining intensity are shown in Plates 7.1-7.13 (p205-217).

7.3. The distribution of cell adhesion molecules in renal transplant biopsies

7.3.1. Introduction

Results are shown for each structure within the biopsy, either tubules, glomeruli, ITE or large vessels, as a dot plot. The intensity of antigen staining is shown on the y axis for HLA class 1 and 2, E-selectin, VCAM and ICAM-1. The type of biopsy is shown on the x axis; either pre-transplant, post transplant normal or post transplant acute cellular rejection (post-transplant ACR). The results for PECAM are given separately in the text. The raw data for each dot plot is shown both in real and percentage terms in appendix B, tables B1-B15 (p234-242). The full set of adhesion molecules were assessed in 16 pre-transplant biopsies, 13 post-transplant normal biopsies and 15 post-transplant ACR biopsies.

7.3.2 Renal tubules

Figures 7.1-7.5 (p218-219), show antigen expression on renal tubules. Figure 7.1 shows HLA class 1 expression. In pre-transplant biopsies 6% were positive with a median intensity of 0. In post-transplant normal biopsies 14% were positive with a median expression of 0. In post-transplant ACR biopsies 94% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where p=0.0001, and the pre-transplant group where p=0.0001.

Figure 7.2 shows HLA class 2 expression on renal tubules. In pre-transplant biopsies 38% were positive with a median intensity of 0. In post-transplant normal biopsies 22% were positive with a median expression of 0. In post-transplant ACR biopsies 60% were positive with a median expression of 1. There was no significant difference in antigen expression between the groups.

Figure 7.3 shows E-selectin expression on renal tubules. In pre-transplant biopsies 6% were positive with a median intensity of 0. In post-transplant normal biopsies 0% were positive with a median expression of 0. In post-transplant ACR biopsies 12% were positive with a median expression of 0. There was no significant difference in antigen expression between the groups.

Figure 7.4 shows VCAM expression on renal tubules. In pre-transplant biopsies 75% were positive with a median intensity of 2. In post-transplant normal biopsies 69% were positive with a median expression of 1.5. In post-transplant ACR biopsies 100% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.005$, and the pre-transplant group where $p=0.022$.

Figure 7.5 shows ICAM-1 expression on renal tubules. In pre-transplant biopsies 55% were positive with a median intensity of 2. In post-transplant normal biopsies only 7% were positive with a median expression of 0. In post-transplant ACR biopsies 45% were positive with a median expression of 1.5. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.024$. The pre-transplant group also showed significantly greater expression than the post-transplant normal group where $p=0.0006$. There was no difference between the pre-transplant group and the post-transplant ACR group.

7.3.3. Glomeruli

Figures 7.6-7.10 (p220-221), show antigen expression on renal glomeruli. Figure 7.6 shows HLA class 1 expression. In pre-transplant biopsies 100% were positive with a median intensity of 4. In post-transplant biopsies 100% were positive with a median expression of 4.

In post-transplant ACR biopsies 89% were positive with a median expression of 4. There was no significant difference in expression between the groups.

Figure 7.7 shows HLA class 2 expression on renal glomeruli. In pre-transplant biopsies 94% were positive with a median intensity of 2.5. In post-transplant normal biopsies 100% were positive with a median expression of 2. In post-transplant ACR biopsies 56% were positive with a median expression of 1. The post-transplant ACR group showed significantly less expression than the post-transplant normal group where $p=0.03$, and the pre-transplant group where $p=0.0075$.

Figure 7.8 shows E-selectin expression on renal glomeruli. In pre-transplant biopsies 6% were positive with a median intensity of 0. In post-transplant normal biopsies 0% were positive with a median expression of 0. In post-transplant ACR biopsies 0% were positive with a median expression of 0. There was no significant difference in antigen expression between the groups.

Figure 7.9 shows VCAM expression on renal glomeruli. In pre-transplant biopsies 100% were positive with a median intensity of 3. In post-transplant normal biopsies 90% were positive with a median expression of 2.5. In post-transplant ACR biopsies 100% were positive with a median expression of 3. There was no significant difference in antigen expression between the groups.

Figure 7.10 shows ICAM-1 expression on renal glomeruli. In pre-transplant biopsies 100% were positive with a median intensity of 4. In post-transplant normal biopsies 90% were positive with a median expression of 4. In post-transplant ACR biopsies 100% were positive with a median expression of 3. There was no significant difference in antigen expression between the groups.

7.3.4. Renal intertubular endothelium

Figures 7.11-7.15 (p222-223), show antigen expression on the renal inter-tubular endothelium. Figure 7.11 shows HLA class 1 expression. In pre-transplant biopsies 100% were positive with a median intensity of 4. In post-transplant normal biopsies 100% were positive with a median expression of 3.5. In post-transplant ACR biopsies 100% were positive with a median expression of 3.5. There was no significant difference in antigen expression between the groups.

Figure 7.12 shows HLA class 2 expression on renal inter-tubular endothelium. In pre-transplant biopsies 94% were positive with a median intensity of 2. In post-transplant normal biopsies 92% were positive with a median expression of 2. In post-transplant ACR biopsies 100% were positive with a median expression of 2.5. There was no significant difference in antigen expression between the groups.

Figure 7.13 shows E-selectin expression on renal inter-tubular endothelium. In pre-transplant biopsies 31% were positive with a median intensity of 1.5. In post-transplant normal biopsies 14% were positive with a median expression of 0. In post-transplant ACR biopsies 80% were positive with a median expression of 2. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.0003$, and the pre-transplant group where $p=0.0215$.

Figure 7.14 shows VCAM expression on renal inter-tubular endothelium. In pre-transplant biopsies 38% were positive with a median intensity of 0.5. In post-transplant normal biopsies 14% were positive with a median expression of 0. In post-transplant ACR biopsies 26% were positive with a median expression of 0.5. There was no significant difference in antigen expression between the groups.

Figure 7.15 shows ICAM-1 expression on renal inter-tubular endothelium. In pre-transplant biopsies 100% were positive with a median

intensity of 3.5. In post-transplant normal biopsies 100% were positive with a median expression of 3. In post-transplant ACR biopsies 100% were positive with a median expression of 4. There was no significant difference in antigen expression between the groups.

7.3.5 Large blood vessels

Figures 7.16-7.20 (p224-225), show antigen expression on the large blood vessels within the kidney. Figure 7.16 shows HLA class 1 expression. In pre-transplant biopsies 94% were positive with a median intensity of 3. In post-transplant biopsies 100% were positive with a median expression of 3. In post-transplant ACR biopsies 100% were positive with a median expression of 3.5. There was no significant difference in antigen expression between the groups.

Figure 7.17 shows HLA class 2 expression on large blood vessels. In pre-transplant biopsies 86% were positive with a median intensity of 2. In post-transplant normal biopsies 90% were positive with a median expression of 2. In post-transplant ACR biopsies 71% were positive with a median expression of 2. There was no significant difference in antigen expression between the groups.

Figure 7.18 shows E-selectin expression on large blood vessels. In pre-transplant biopsies 13% were positive with a median intensity of 0. In post-transplant normal biopsies 10% were positive with a median expression of 0. In post-transplant ACR biopsies 50% were positive with a median expression of 1. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.043$, but not the pre-transplant group where there was no difference between the groups.

Figure 7.19 shows VCAM expression on large blood vessels. In pre-transplant biopsies 38% were positive with a median intensity of 0. In post-transplant normal biopsies 20% were positive with a median expression

of 0. In post-transplant ACR biopsies 42% were positive with a median expression of 1. There was no significant difference in antigen expression between the groups.

Figure 7.20 shows ICAM-1 expression on large blood vessels. In pre-transplant biopsies 100% were positive with a median intensity of 2.5. In post-transplant normal biopsies 100% were positive with a median expression of 3. In post-transplant ACR biopsies 100% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where p=0.0323, but not the pre-transplant group where there was no difference between the groups.

7.3.6. Cellular infiltrate

Figures 7.21-7.25 (p226-227), show the intensity of staining of the cellular infiltrate within the kidney biopsy. Figure 7.21 shows intensity of staining of the leucocyte common antigen. In pre-transplant biopsies 79% were positive with a median intensity of 2. In post-transplant normal biopsies 62% were positive with a median expression of 2. In post-transplant ACR biopsies 100% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where p=0.0013, and the pre-transplant group where p=0.001.

Figure 7.22 shows intensity of staining of the CD3 antigen on the cellular infiltrate within the kidney. In pre-transplant biopsies 72% were positive with a median intensity of 2. In post-transplant normal biopsies 62% were positive with a median expression of 2. In post-transplant ACR biopsies 100% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where p<0.0001, and the pre-transplant group

where $p<0.0001$.

Figure 7.23 shows intensity of staining of the CD4 antigen on the cellular infiltrate within the kidney. In pre-transplant biopsies 50% were positive with a median intensity of 1.5. In post-transplant normal biopsies 54% were positive with a median expression of 2. In post-transplant ACR biopsies 100% were positive with a median expression of 3. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.0001$, and the pre-transplant group where $p<0.0001$.

Figure 7.24 shows intensity of staining of the CD8 antigen on the cellular infiltrate within the kidney. In pre-transplant biopsies 43% were positive with a median intensity of 1. In post-transplant normal biopsies 60% were positive with a median expression of 1. In post-transplant ACR biopsies 94% were positive with a median expression of 2. The post-transplant ACR group showed significantly more expression than the post-transplant normal group where $p=0.0023$, and the pre-transplant group where $p=0.0058$.

Figure 7.25 shows intensity of staining of the IL-2 receptor antigen on the cellular infiltrate within the kidney. In pre-transplant biopsies 0% were positive with a median intensity of 0. In post-transplant normal biopsies 23% were positive with a median expression of 0. In post-transplant ACR biopsies 53% were positive with a median expression of 1. The post-transplant ACR group showed significantly more expression than the pre-transplant group where $p=0.007$. There was no difference between the pre-transplant group and the post-transplant normal group ($p=0.091$), or between the post-transplant normal group and the post-transplant ACR group ($p=0.072$).

7.3.7. Expression of PECAM in renal transplant biopsies

The tissue distribution and expression of PECAM in all three types of renal transplant biopsy was identical. No PECAM was found to be present on renal tubules on any biopsy. All biopsies expressed PCAM on intertubular endothelium, glomeruli and large blood vessels, with a median staining intensity of 3-4. There was no difference in staining intensity between pre-transplant, post-transplant normal or post-transplant acute cellular rejection biopsies.

7.4. Expression of E-selectin on post-transplant biopsies taken either electively or for graft dysfunction

7.4.1 Introduction

In the previous section it was shown that E-selectin expression is very low in both pre-transplant and post-transplant normal biopsies but is significantly increased on the intertubular endothelium in post-transplant acute cellular rejection biopsies (fig 7.13, p222). To investigate further the value of E-selectin as a tissue marker for acute rejection, it's expression on the renal intertubular endothelium of 122 biopsies of post-renal transplant renal allografts was studied. These biopsies were taken from 70 patients both as a result of graft dysfunction and as part of a routine graft surveillance programme. The results of the two groups have been analysed separately.

7.4.2. Results of E-selectin expression in biopsies taken for dysfunctional analysis.

The results of E-selectin expression on the intertubular endothelium of biopsies taken for dysfunctional analysis is shown in table 7.1, (p228). Details of those patients biopsied are shown in appendix C (p243-247).

Seventy-seven biopsies were obtained in total. 42% showed histological changes consistent with acute cellular rejection and of these 88% were E-selectin positive. 23% showed acute tubular necrosis and of these 72% were E-selectin positive. 8% of the biopsies showed chronic allograft rejection and of these 4/6 (67%) were positive for E-selectin. Two of the biopsies (3%) were reported as histologically normal and both were E-selectin negative. 25% of the biopsies were classified as other diagnoses and 63% of these were E-selectin positive. The histological diagnoses of this group is shown in appendix A, table A3 (p233), the majority showing varying degrees of chronic interstitial fibrosis.

7.4.3. Results of E-selectin expression in biopsies taken for routine analysis.

As part of a routine graft surveillance programme for monitoring of renal allografts it was possible to examine the expression of E-selectin on 45 biopsies where no clinical evidence of graft dysfunction existed. Details of the patients from whom biopsies were taken are also shown in appendix C (p243-247).

The results of this group are shown in table 7.2 (p228). 58% of the biopsies were reported histologically to be normal and of these 19% were E-selectin positive. One biopsy showed acute tubular necrosis and one chronic allograft rejection, neither of which were E-selectin positive. 20% of the biopsies showed features consistent with acute cellular rejection and 78% were E-selectin positive despite the absence of any clinical suspicion of this diagnosis. As in the dysfunction group there were a number of other diagnoses, 18%, of which most showed chronic interstitial fibrosis (see appendix A, table A2, p233), and amongst which 38% were E-selectin positive.

Overall 74% of biopsies taken for dysfunctional analysis were

E-selectin positive against 33% of routine biopsies.

7.4.4. Biochemical and haematological profile of patient groups.

The mean blood creatinine levels in the post-transplant normal group was 147.9 umol\L (normal range 60-120 umol\L). This was significantly lower than the acute tubular necrosis group (mean 533 umol\L, $p<0.05$), and the acute cellular rejection group (mean 286 umol\L, $p<0.05$), but not the chronic rejection group (mean 353 umol\L) or the other diagnosis group (mean 239.4 umol\L).

The mean blood urea levels in the post-transplant normal group was 10.3 mmol\L (normal range 2.5-6.5 mmol\L). This was significantly lower than the acute tubular necrosis group (mean 26.9 mmol\L, $p<0.05$), and the acute cellular rejection group (mean 17.4 mmol\L, $p<0.05$), but not the chronic rejection group (mean 17.3 mmol\L) or the other diagnosis group (mean 14.5 mmol\L).

The mean blood white cell count in the post-transplant normal group was 8.6x 10^9\L (normal range 4-12 10^9\L). This was significantly lower than the acute tubular necrosis group (mean 11.7x10^9\L, $p<0.05$), and the acute cellular rejection group (mean 11.9x10^9\L, $p<0.05$), but not the chronic rejection group (mean 7.5 mmo10^9\L) or the other diagnosis group (mean 9.7 10^9mmol\L).

7.5. Analysis of E-selectin expression verses CD4 expression in post renal transplant biopsies

7.5.1. Introduction.

In section 7.4 it was shown that the expression of E-selectin, although elevated in episodes diagnosed as acute cellular rejection, was also elevated during other episodes of allograft dysfunction. Cellular

infiltration into the kidney is a major component of acute cellular rejection and so the relationship between E-selectin expression and lymphocyte content within the graft biopsy might be more informative and more helpful in the diagnosis of acute cellular rejection. The post-transplant biopsies in section 7.4 were therefore examined for E-selectin expression and CD4 positive content. Biopsies were grouped by histological diagnosis irrespective of whether they were taken for dysfunction or routine surveillance. The groups were classified as normal, acute cellular rejection, chronic rejection or acute tubular necrosis.

7.5.2. Results

The correlation of E-selectin intensity to the magnitude of the CD4 positive infiltrate is shown in tables 7.3-7.6 (p229-230).

Table 7.3 shows the results for those biopsies reported as histologically normal. eighty-one percent of biopsies were negative although 35% had small numbers of infiltrating CD4 positive T lymphocyte's. Over half (54%) of all normal biopsies had some level of CD4 positive lymphocyte content; however, both E-selectin staining intensity and CD4 content were generally low, with all biopsies having a combined E-selectin/CD4 intensity of 2 or less.

Table 7.4 shows the results for those biopsies reported histologically as acute cellular rejection. Three biopsies could not be stained for CD4 because of technical difficulties leaving 38 for analysis. 80% were positive for both E-selectin and CD4, 3% were negative for both antigens and the remainder (17%) were CD4 positive but E-selectin negative. Over half (52%) of the biopsies showed a score of 2 or more with both CD4 and E-selectin.

Table 7.5 shows the results for those biopsies reported histologically as chronic rejection. Although only a small number were

available, of the biopsies diagnosed as chronic rejection 78% were positive for both E-selectin and CD4, none were negative for both antigens and the remainder (22%) were positive for CD4 alone. Over half of the biopsies scored 2 or more.

Table 7.6 shows the results for those biopsies reported histologically as acute tubular necrosis. 73% were both E-selectin and CD4 positive. 16% were negative for both antigens and the remaining 11% were CD4 positive but E-selectin negative. In the acute tubular necrosis group there were a greater number that had a combined E-selectin/CD4 intensity of greater than 2 (32%) compared to normal transplant biopsies.

Figure 7.26 (p231), shows a correlation of E-selectin and CD4 infiltrate in the post-transplant biopsy group taken as a whole. There is a very strong correlation between intensity of E-selectin expression and CD4 positive cell infiltration with Pearson's correlation coefficient $r=0.728$ and $p<0.001$ (Students t-test).

7.6. Conclusions.

1. In pre-transplant biopsies E-selectin was found only occasionally on the ITE and not on other renal structures. VCAM was expressed moderately on tubules and glomeruli and very weekly on ITE and large vessels. ICAM-1 was expressed strongly on glomeruli and ITE, and moderately on tubules and large vessels. HLA class 1 was strongly expressed on glomeruli, ITE and large vessels but not at all on tubules. HLA class 2 was weekly expressed on glomeruli, ITE and large vessels and very weekly on tubules. There was a week or very week cellular infiltrate within the biopsies.

2. In post-transplant normal biopsies antigen expression was very similar to that seen in pre-transplant biopsies. The only exception was in ICAM-1 expression on tubules which was significantly decreased in the

post-transplant normal biopsies.

3. In post-transplant ACR biopsies E-selectin expression was significantly increased on ITE compared to the other two groups, and on large vessels compared to post-transplant normal biopsies. VCAM and HLA class 1 expression was significantly increased on tubules compared to the other two groups. ICAM-1 expression was significantly increased on tubules and large vessels compared to post transplant normal biopsies. HLA class 2 expression was decreased compared to both other groups. All antigens on the cellular infiltrate were significantly increased over both other groups except IL-2R which was significantly increased over pre-transplant biopsies only.

4 In biopsies taken for assessment of dysfunction, E-selectin was expressed more frequently on ITE than on biopsies taken for routine analysis. Although E-selectin tended to be expressed more strongly on ACR biopsies it was also expressed in other forms of allograft dysfunction.

5 .Analysis of the relationship between E-selectin and CD4 showed that in biopsies showing acute cellular rejection the combined expression tended to be higher than for other diagnoses but was not significantly higher particularly with respect to the acute tubular necrosis group.

6. For the post-transplant group as a whole there was a significant correlation between E-selectin expression and CD4 cellular infiltrate.

Plate 7.1 Pre-transplant renal allograft biopsy showing E-selectin expression. Staining intensity 1.

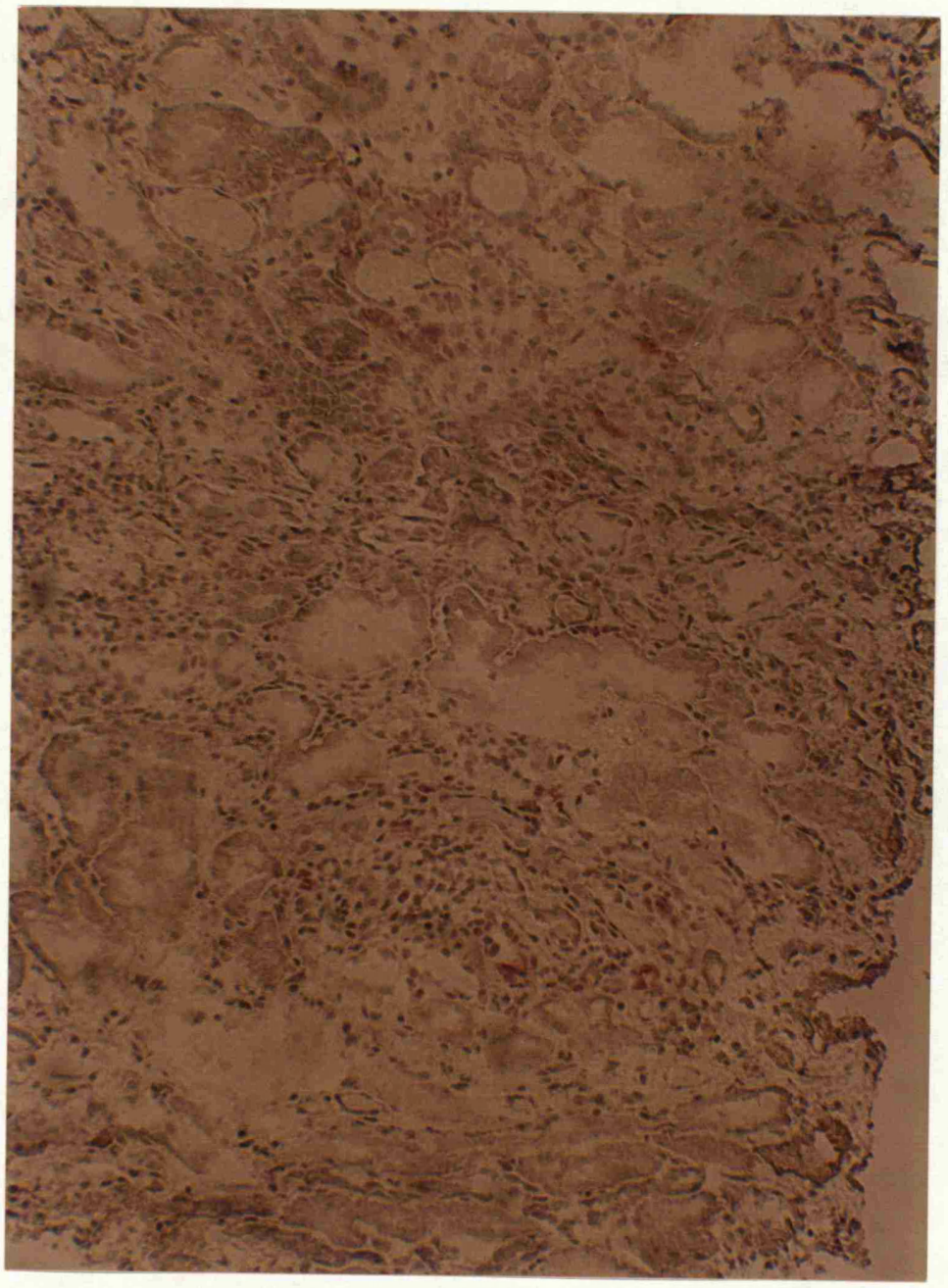


Plate 7.2 Pre-transplant renal allograft biopsy showing T-4 expression.

Staining intensity 3. (same patient as plate 7.1)

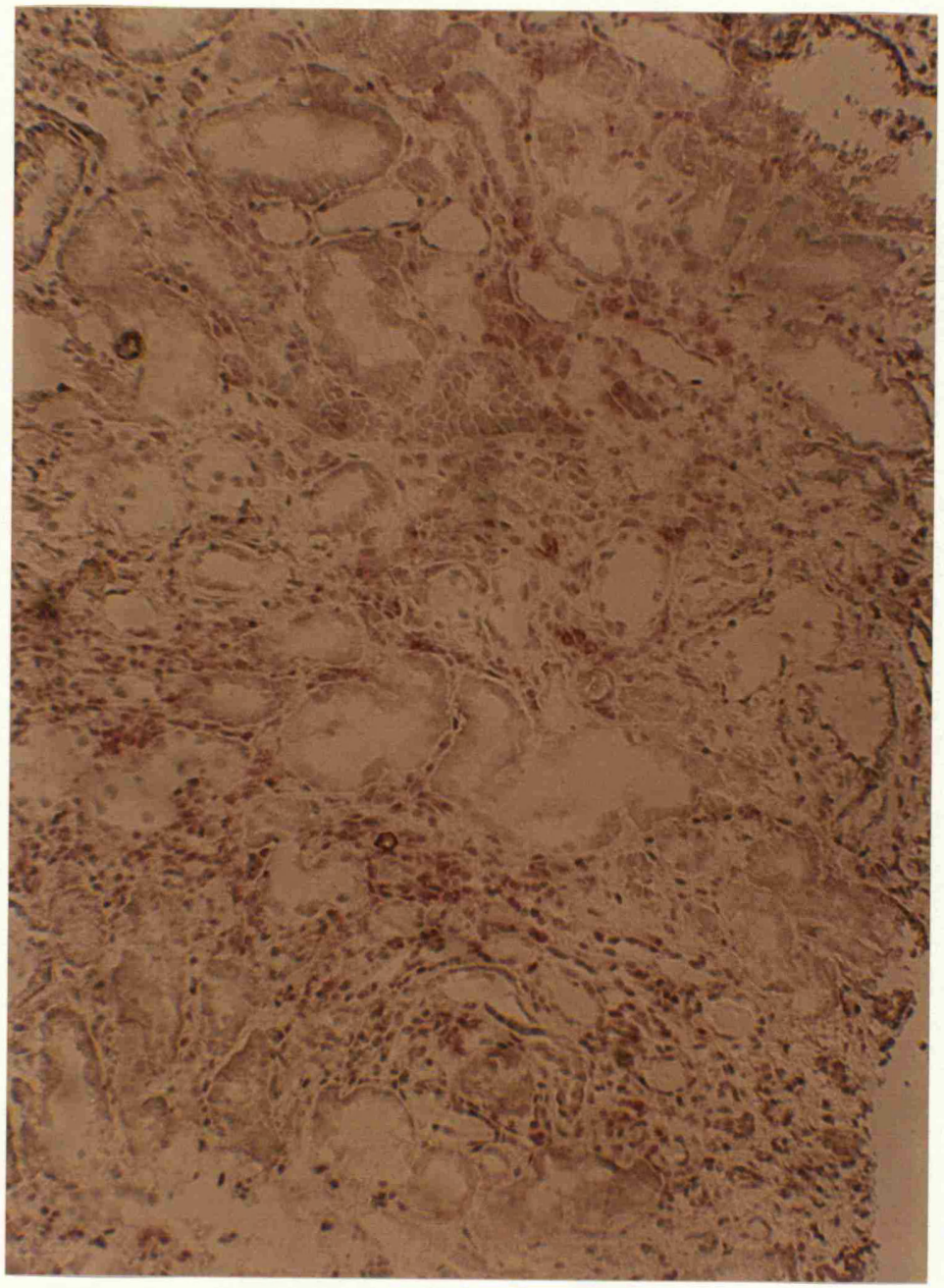


Plate 7.3 Post-transplant renal allograft biopsy showing E-selectin expression in a rejecting allograft. Staining intensity 2

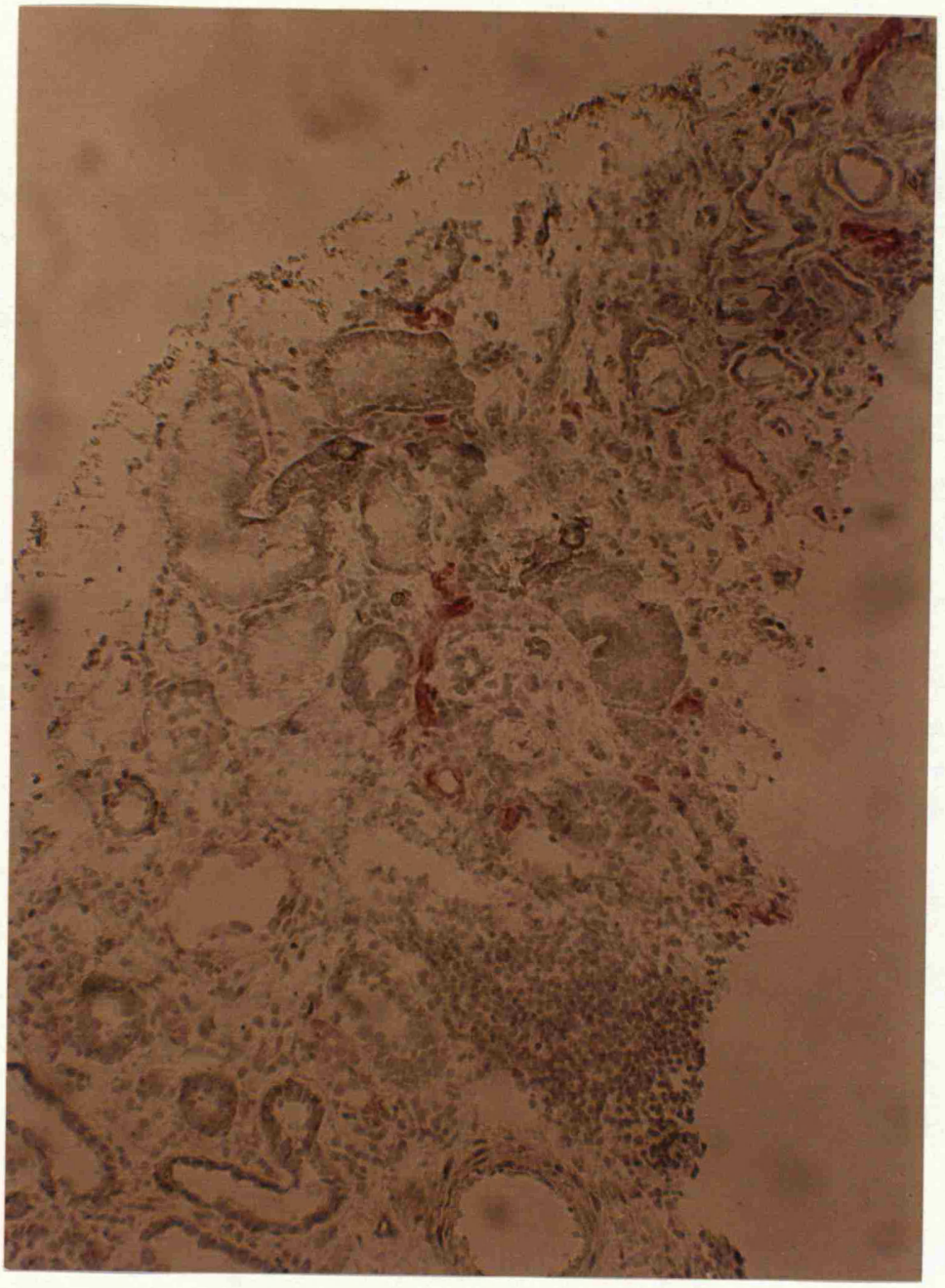


Plate 7.4 Post-transplant renal allograft biopsy showing T-4 expression.

Staining intensity 4. (same patient as plate 7.3)

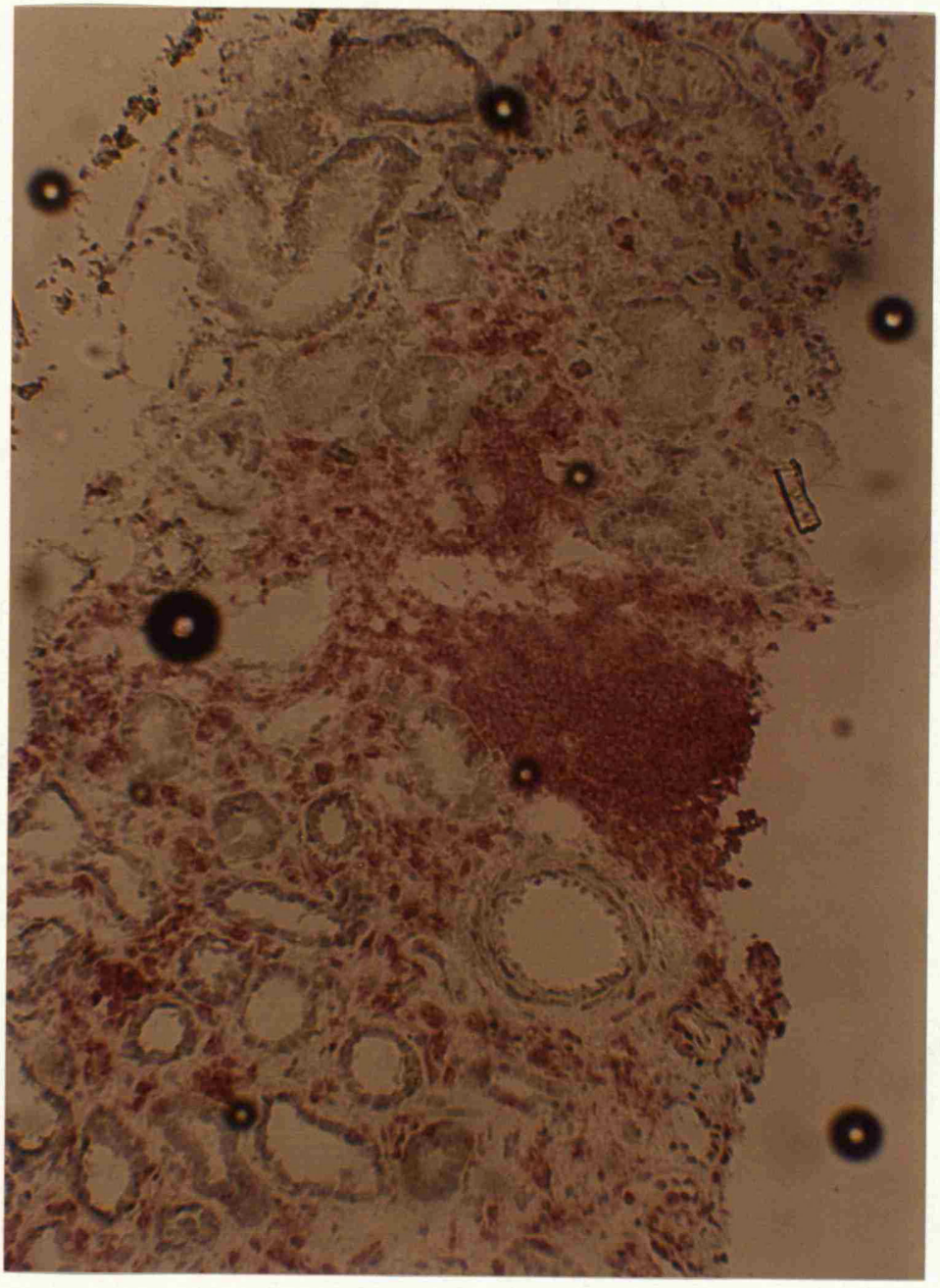


Plate 7.5 Post-transplant renal allograft biopsy showing HLA Class 2 DR expression. Staining intensity 3.

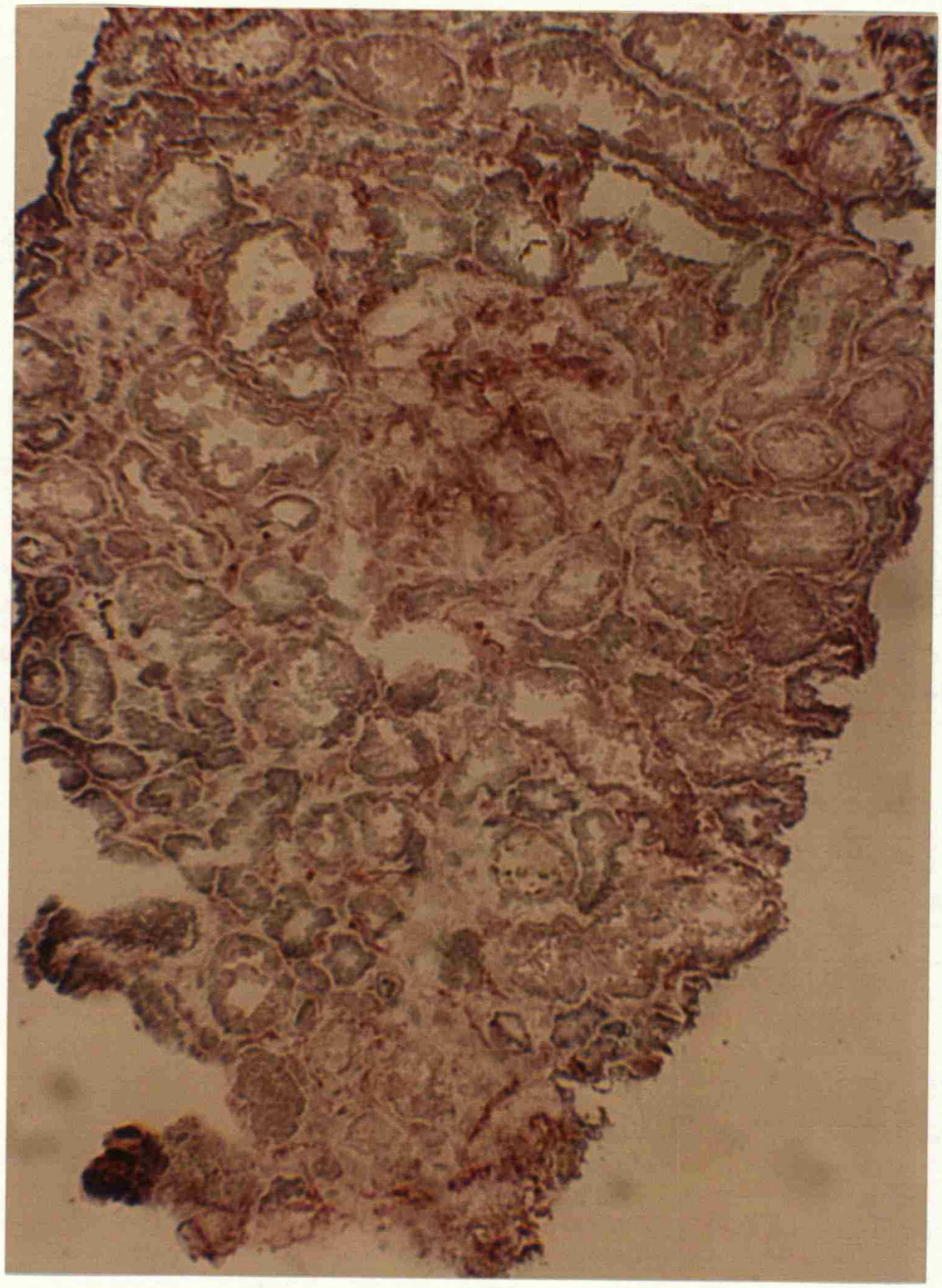


Plate 7.6 Post-transplant renal allograft biopsy showing VCAM expression.

Staining intensity 3.

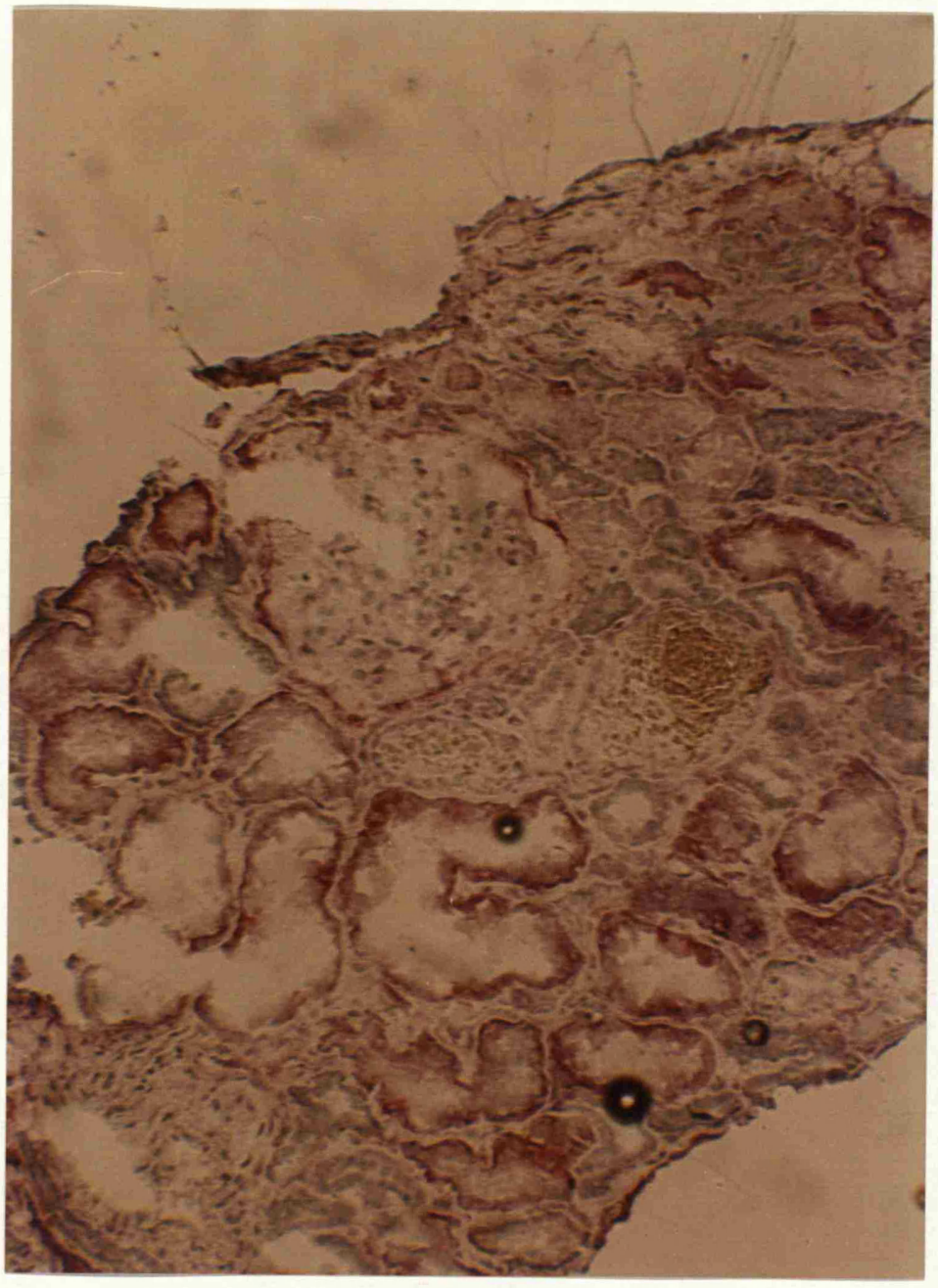


Plate 7.7 Post-transplant renal allograft biopsy showing E-selectin expression in a rejecting allograft. Staining intensity 2.

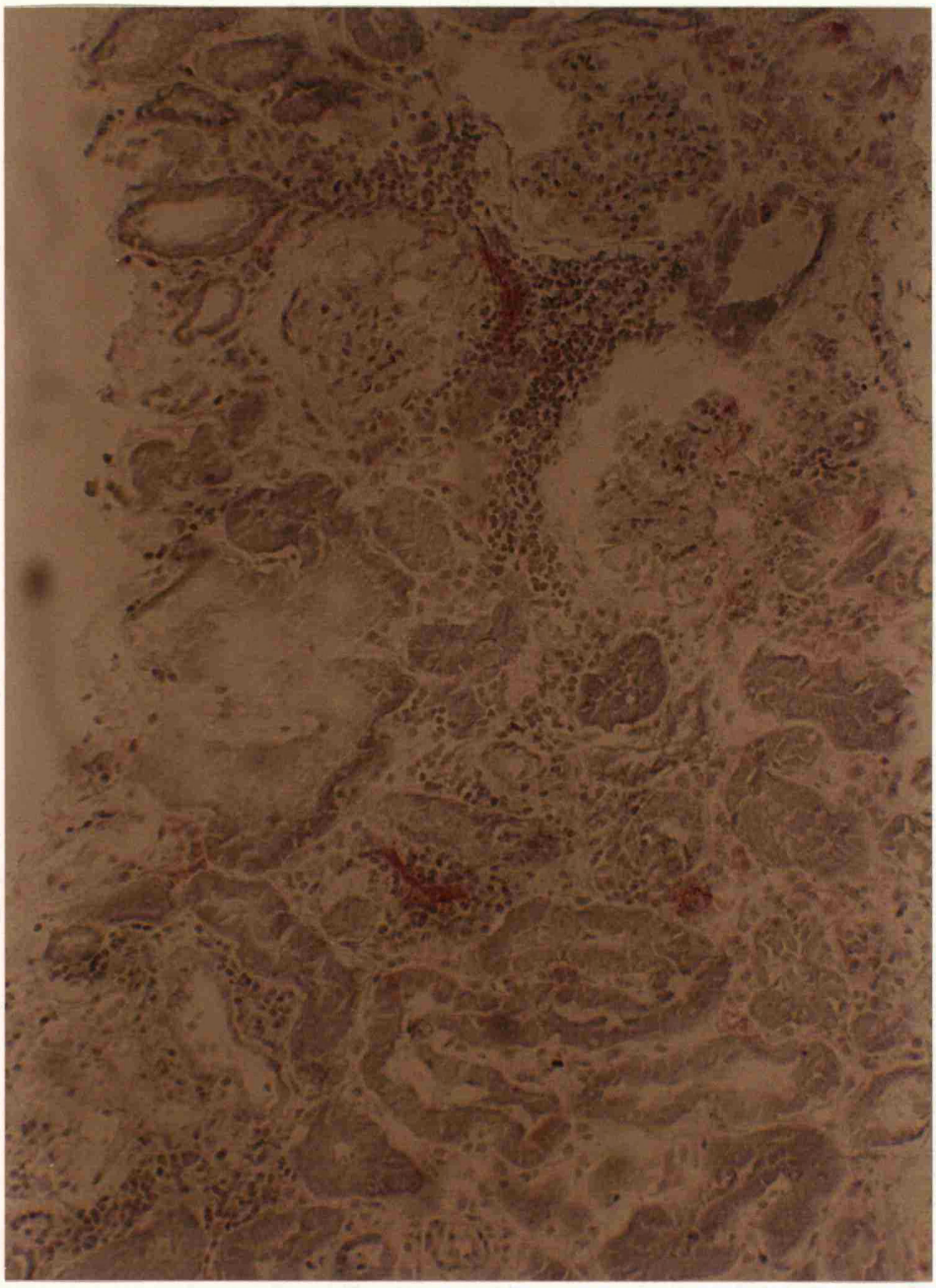


Plate 7.8 Post-transplant renal allograft biopsy showing T-8 expression in a rejecting allograft (same patient as plate 7.7). Staining intensity 3.

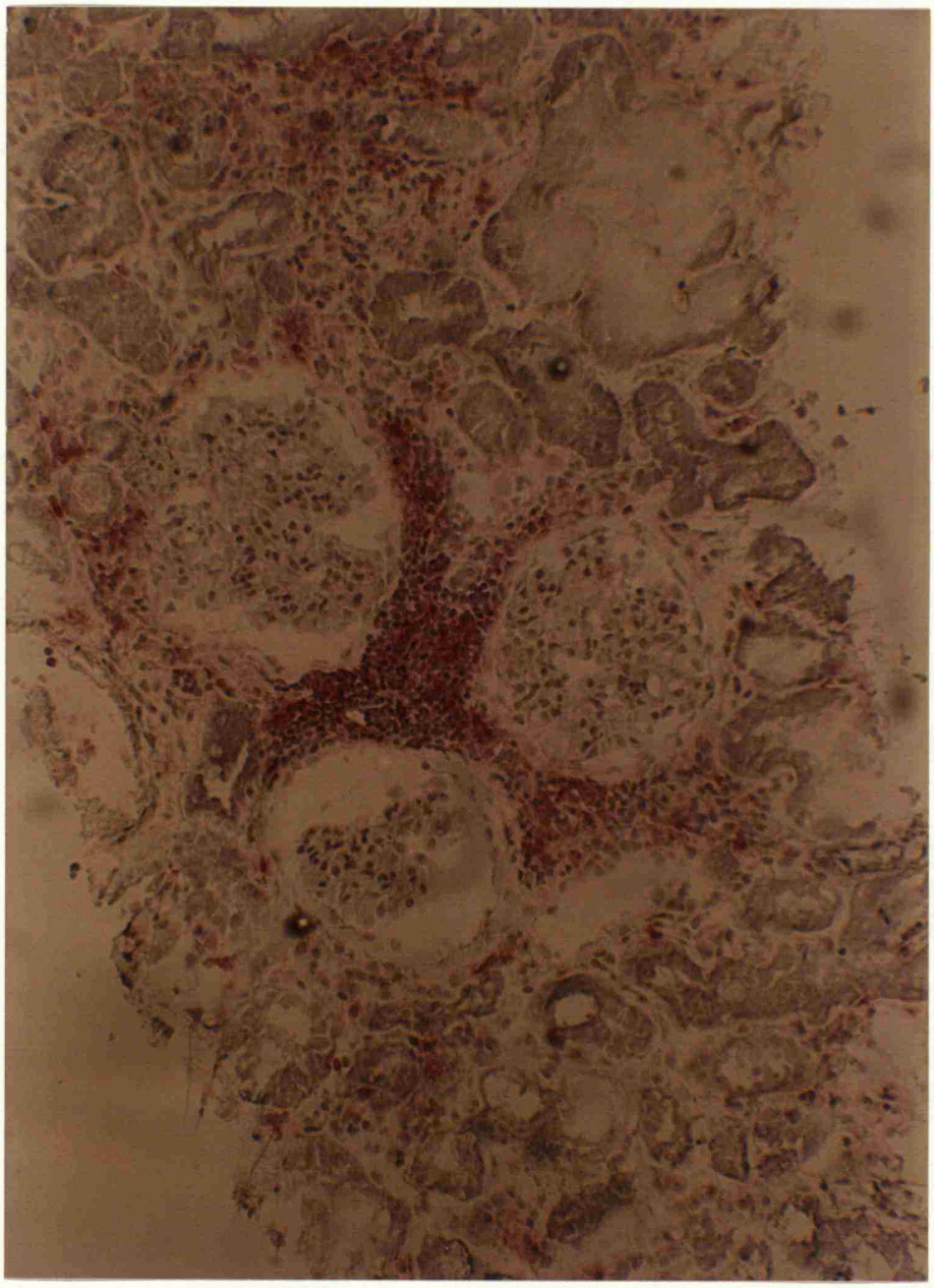


Plate 7.9 Post-transplant renal allograft biopsy showing T-4 expression in a rejecting allograft (same patient as plate 7.7). Staining intensity 4.

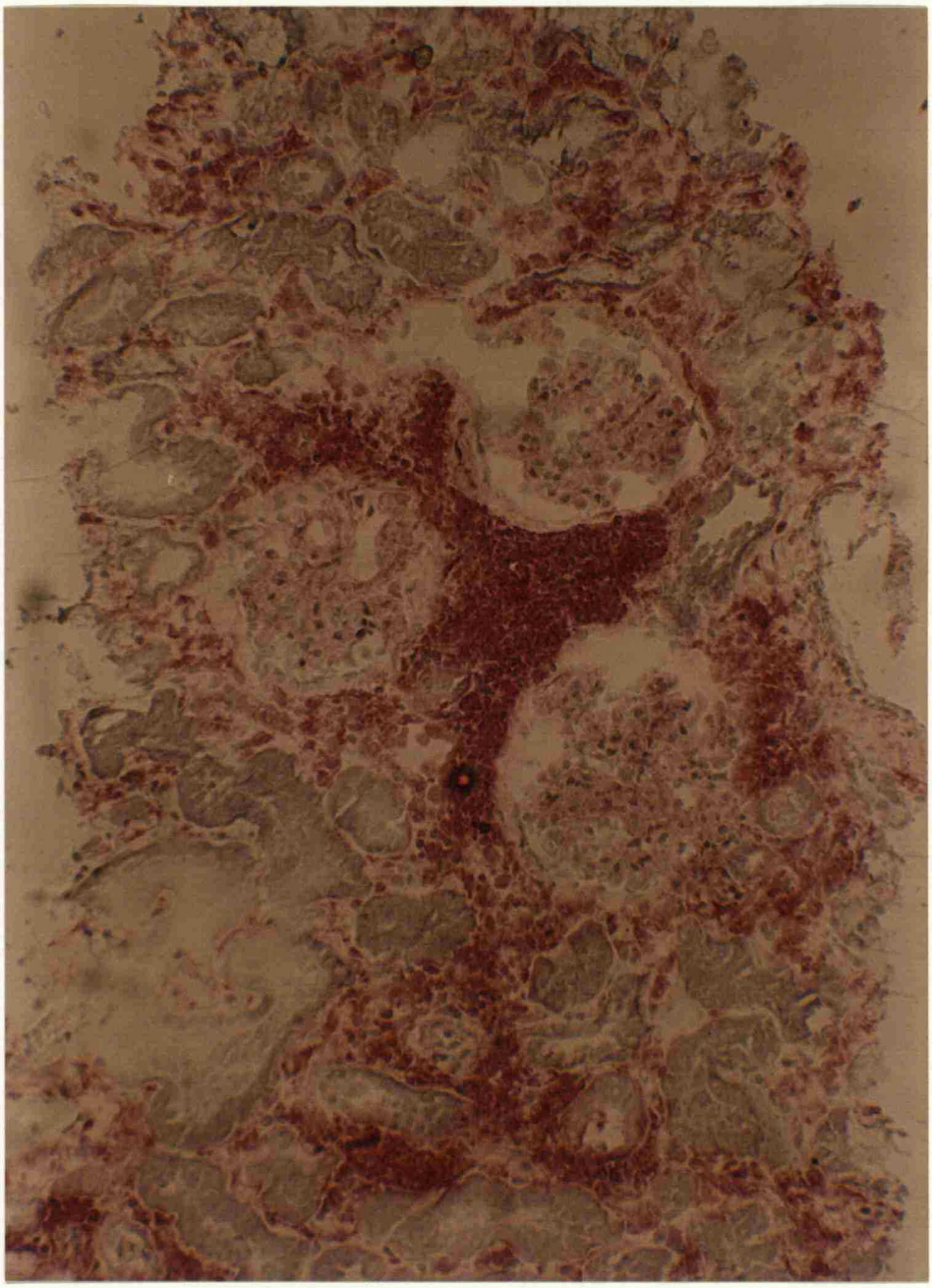


Plate 7.10 Post-transplant renal allograft biopsy showing HLA Class 1 ABC common expression. Staining intensity 4.

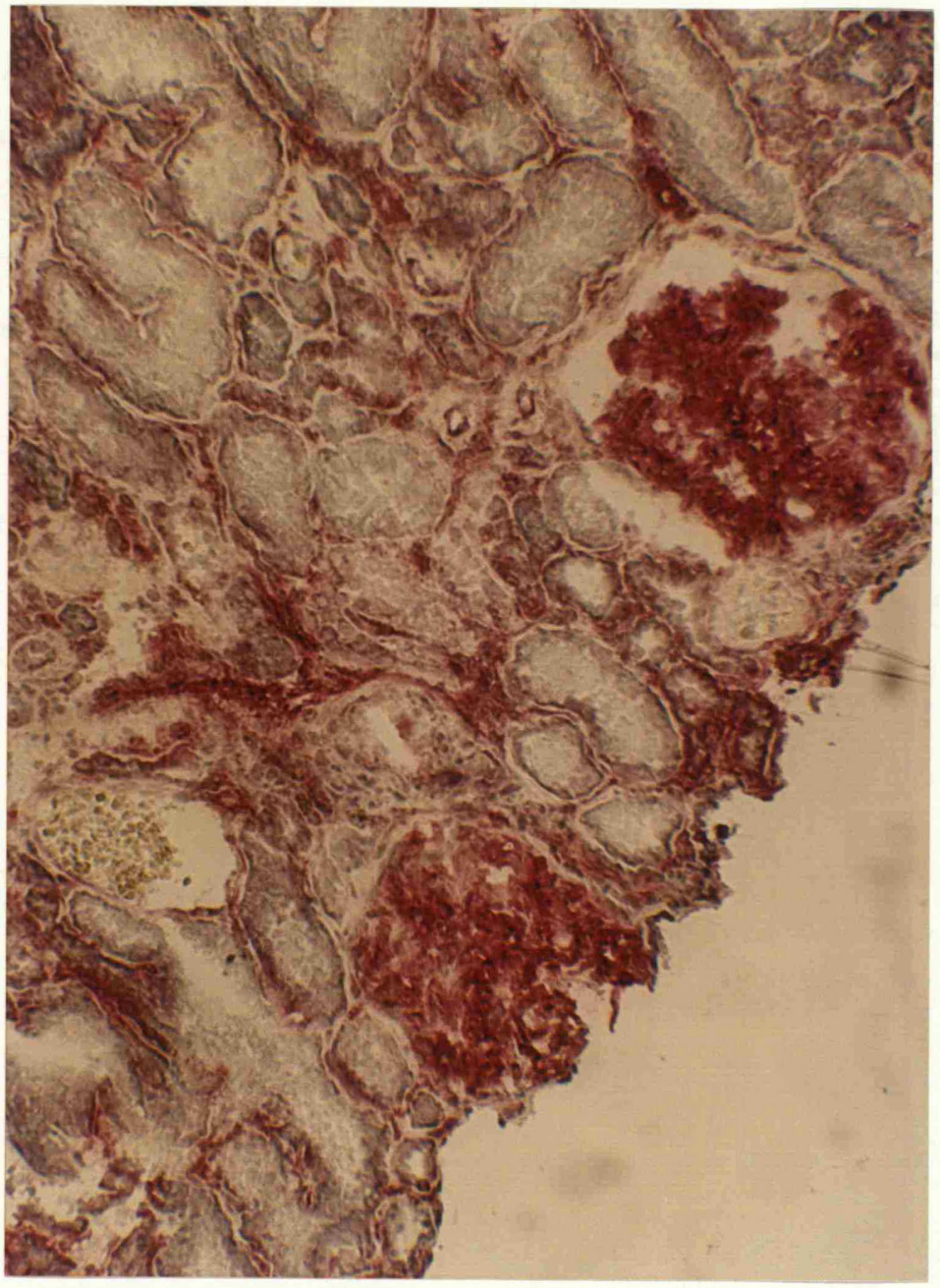


Plate 7.11 Post-transplant renal allograft biopsy showing PECAM expression.

Staining intensity 4.

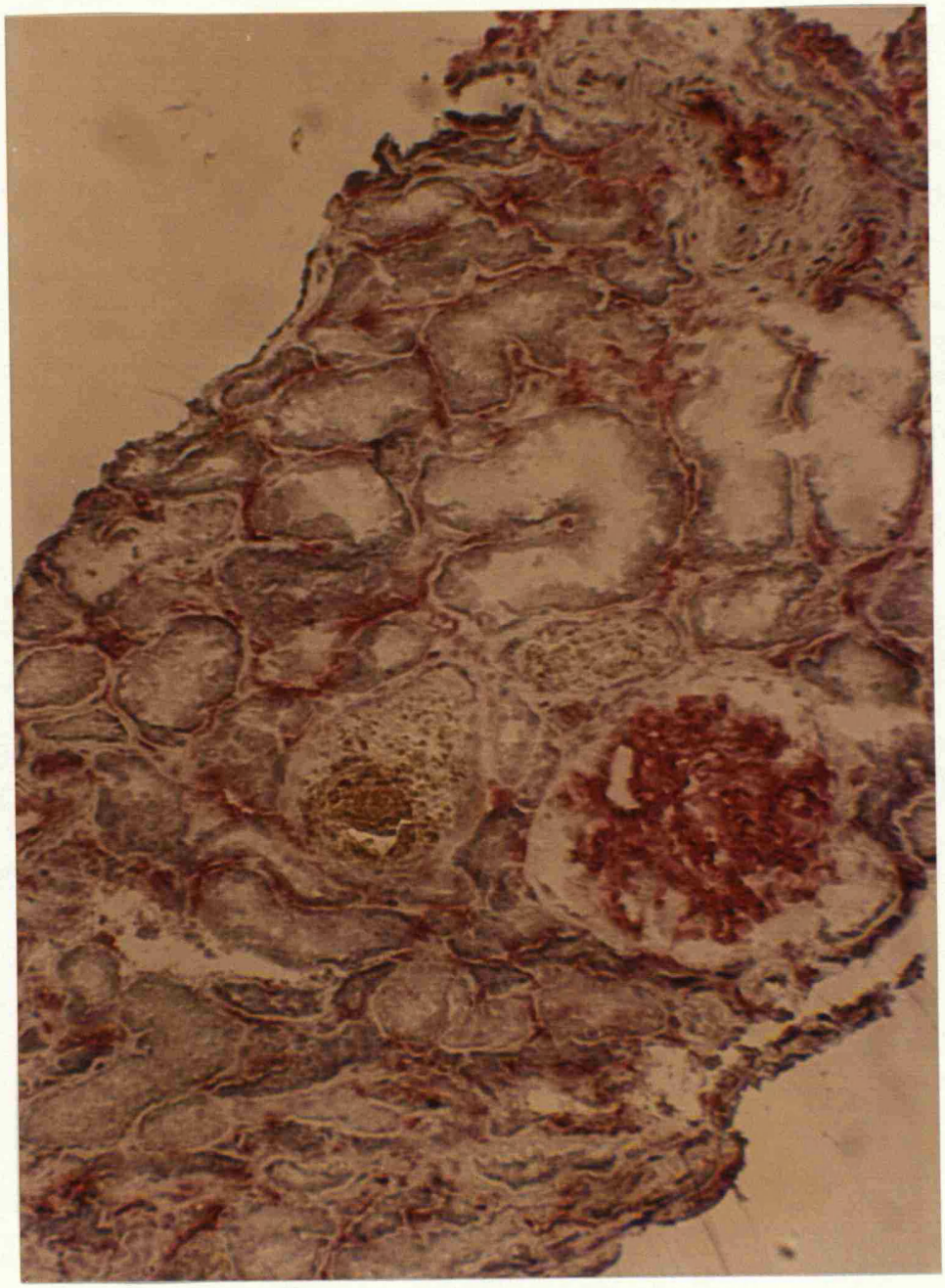


Plate 7.12 Post-transplant renal allograft biopsy showing ICAM-1 expression. Staining intensity 4.

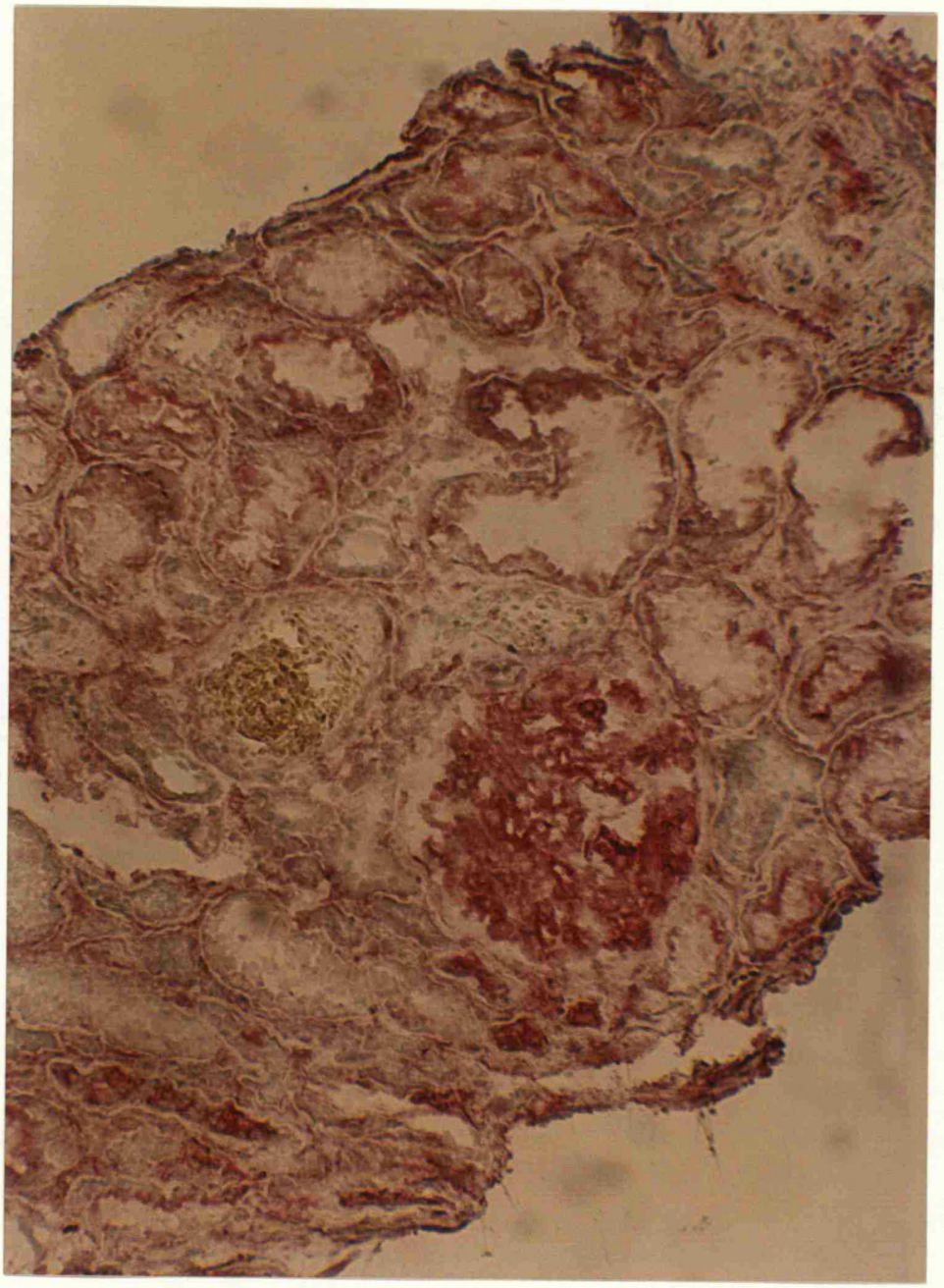


Plate 7.13 Post-transplant renal allograft biopsy showing Cytokeratin staining. Staining intensity 5.

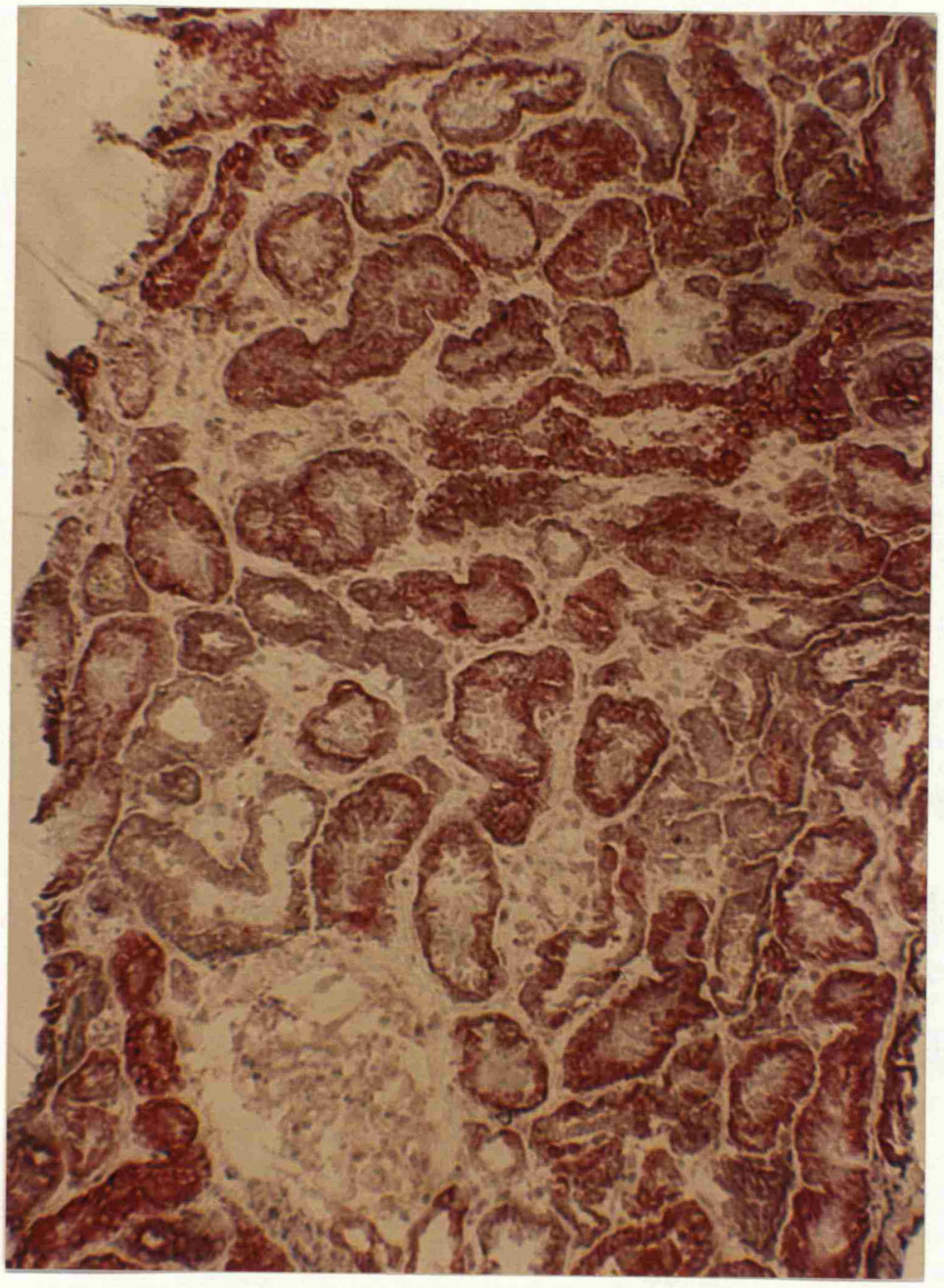


Fig 7.1 Class 1 antigen expression in Transplant biopsies-Tubules

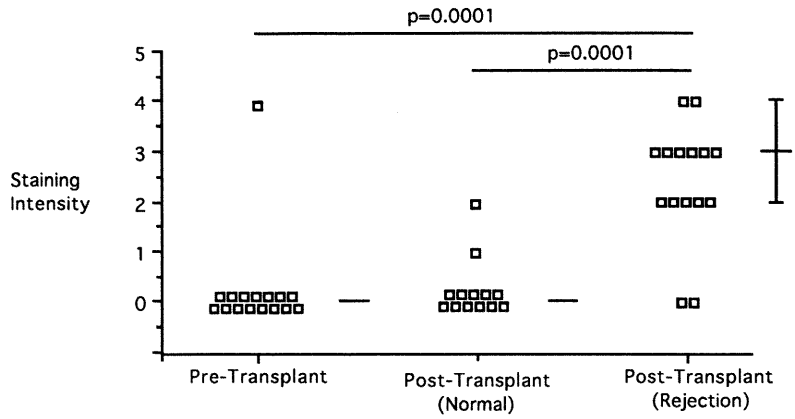


Fig 7.2 Class 2 antigen expression in Transplant biopsies-Tubules

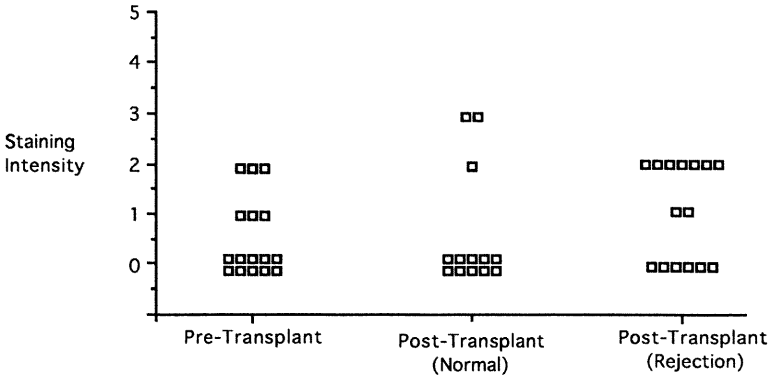


Fig 7.3 E-Selectin antigen expression in Transplant biopsies-Tubules

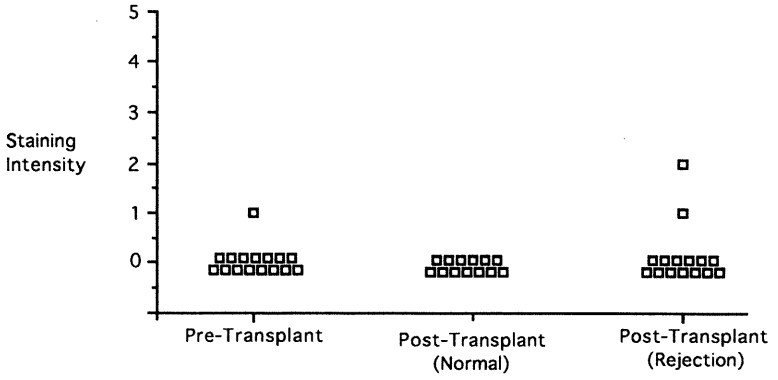


Fig 7.4 VCAM antigen expression in Transplant biopsies-Tubules

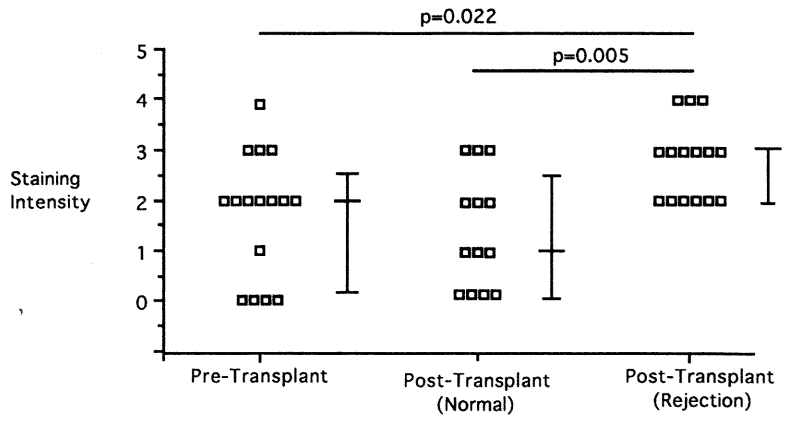


Fig 7.5 ICAM antigen expression in Transplant biopsies-Tubules

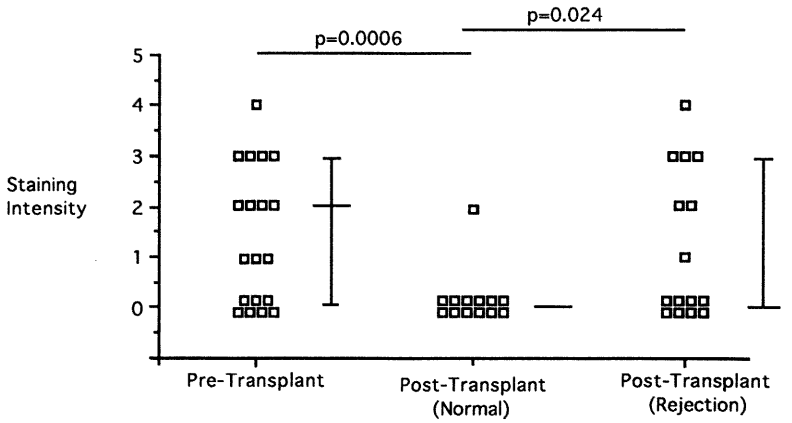


Fig 7.6 Class 1 antigen expression in Transplant biopsies-Glomeruli

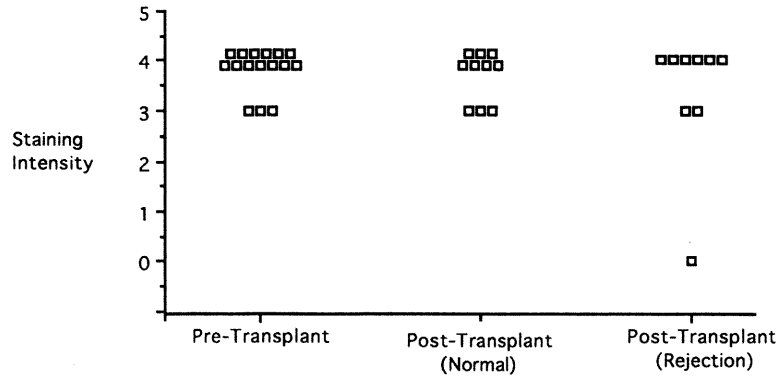


Fig 7.7 Class 2 antigen expression in Transplant biopsies-Glomeruli

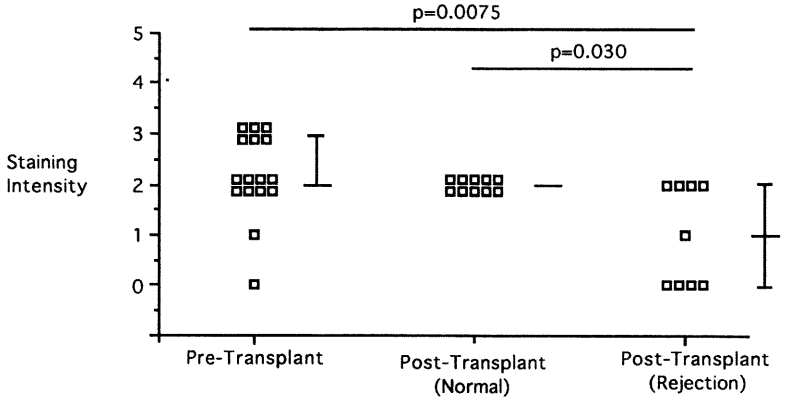


Fig 7.8 E-Selectin antigen expression in Transplant biopsies-Glomeruli

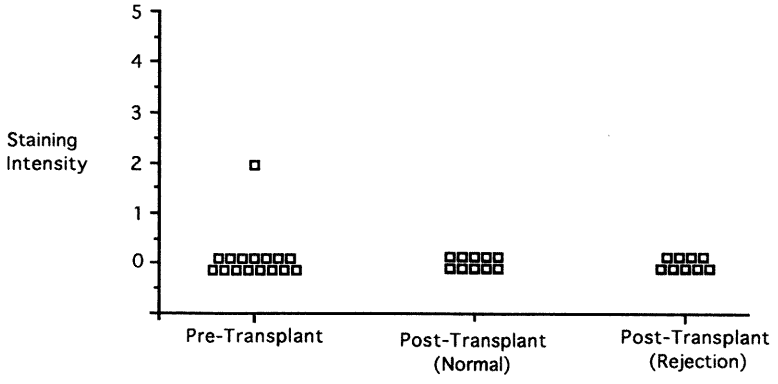


Fig 7.9 VCAM antigen expression in Transplant biopsies-Glomeruli

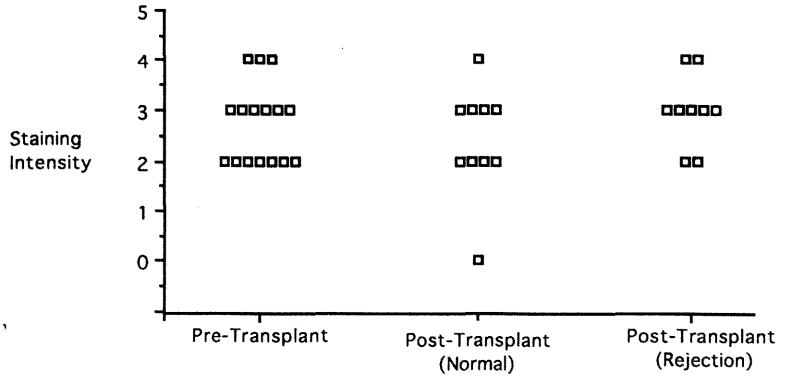


Fig 7.10 ICAM-1 antigen expression in Transplant biopsies-Glomeruli

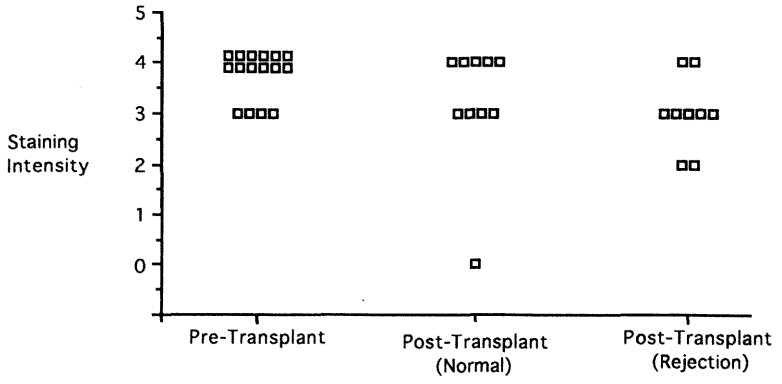


Fig 7.11 Class 1 antigen expression in Transplant biopsies-ITE

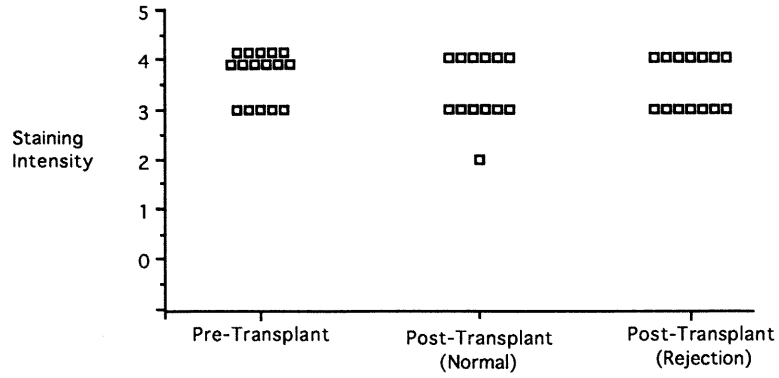


Fig 7.12 Class 2 antigen expression in Transplant biopsies-ITE

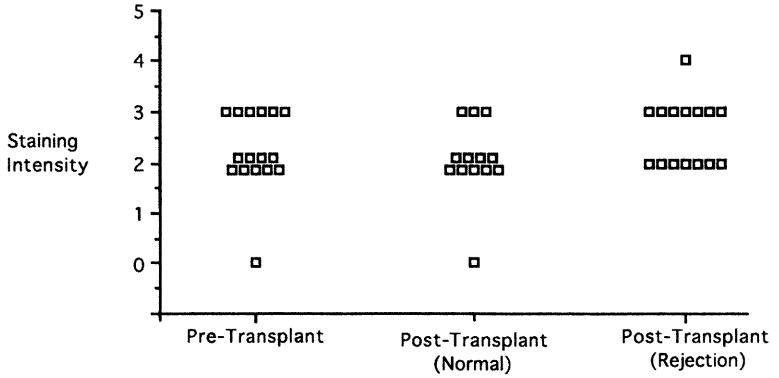


Fig 7.13 E-Selectin antigen expression in Transplant biopsies -ITE

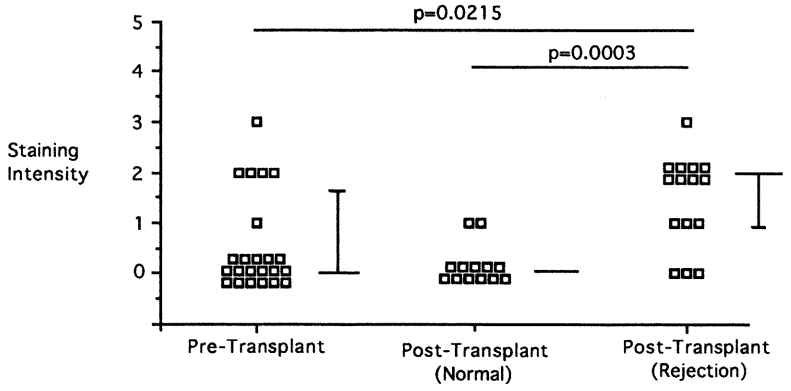


Fig 7.14 VCAM antigen expression in Transplant biopsies-ITE

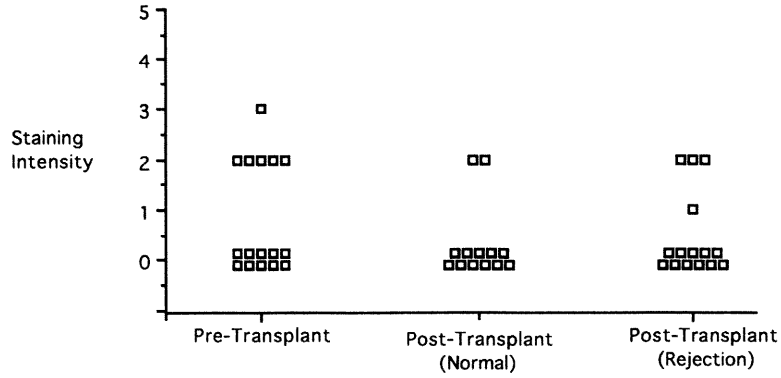


Fig 7.15 ICAM-1 antigen expression in Transplant biopsies-ITE

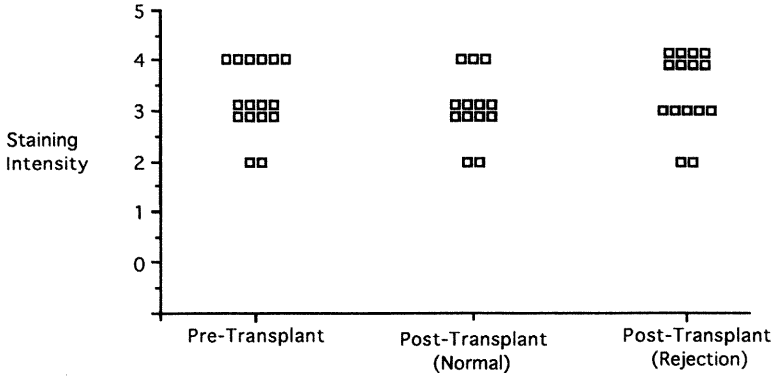


Fig 7.16 Class 1 antigen expression in Transplant biopsies-Large vessels

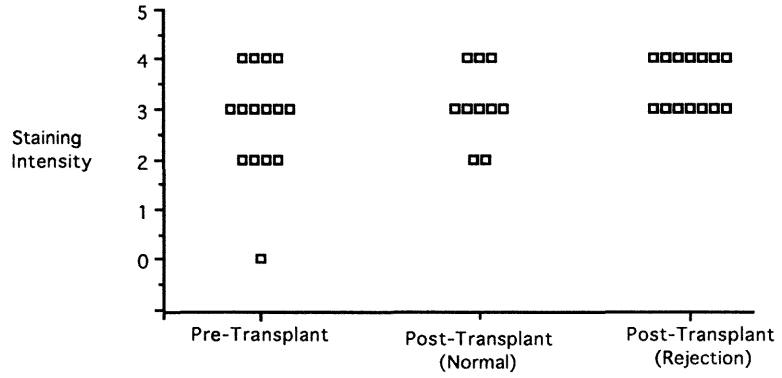


Fig 7.17 Class 2 antigen expression in Transplant biopsies-Large vessels

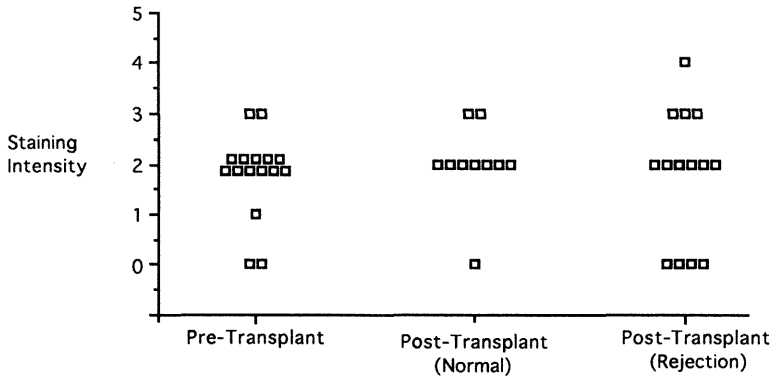


Fig 7.18 E-Selectin antigen expression in Transplant biopsies-Large vessels

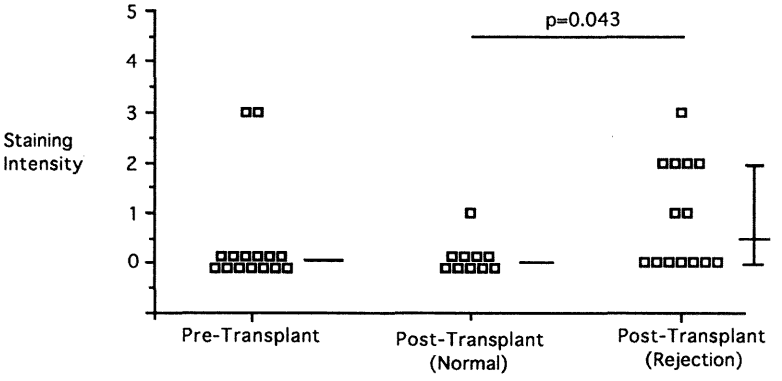


Fig 7.19 VCAM antigen expression in Transplant biopsies-Large vessels

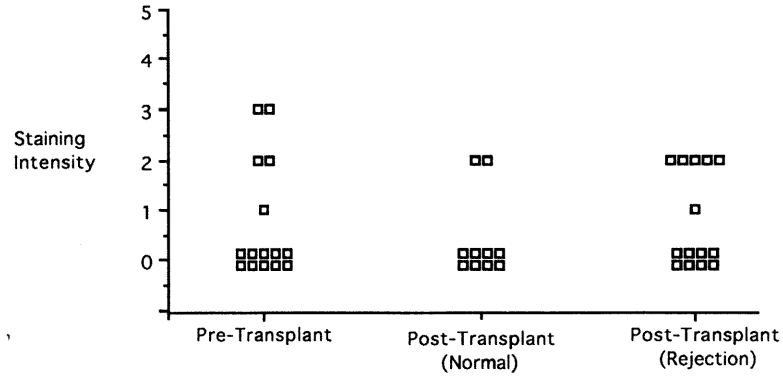


Fig 7.20 ICAM-1 antigen expression in Transplant biopsies-Large vessels

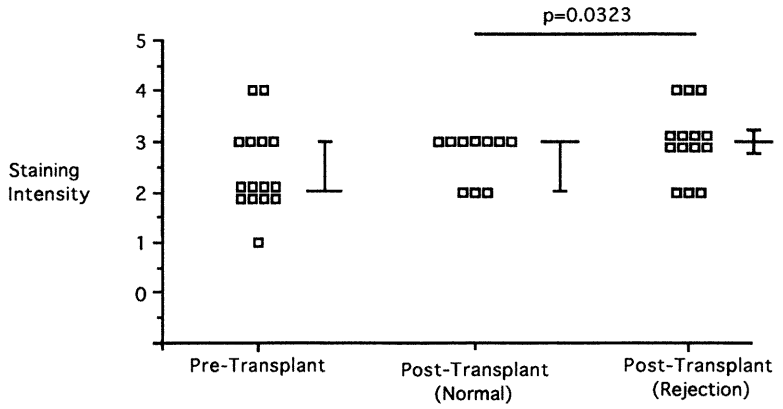


Fig 7.21 Leucocyte Common antigen expression in Transplant biopsies-Cellular Infiltrate

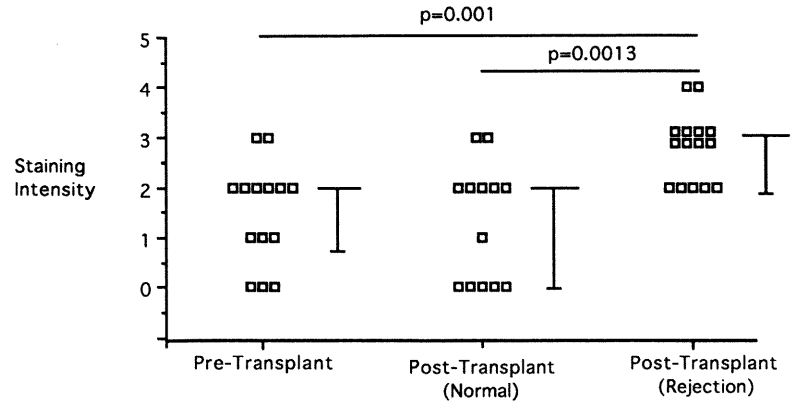


Fig 7.22 CD3 antigen expression in Transplant biopsies-Cellular Infiltrate

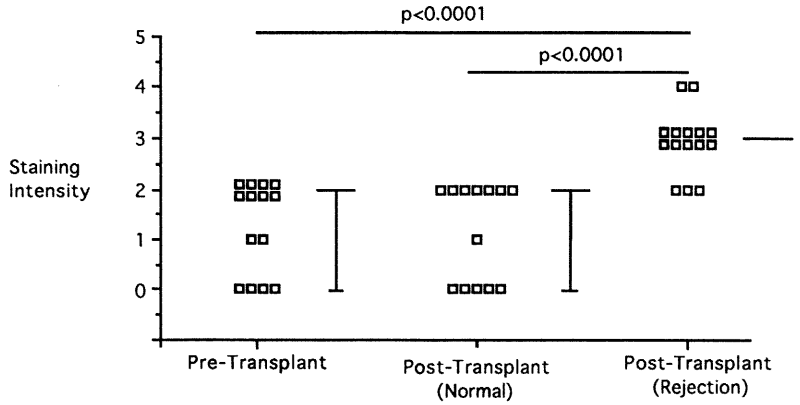


Fig 7.23 CD4 antigen expression in Transplant biopsies-Cellular Infiltrate

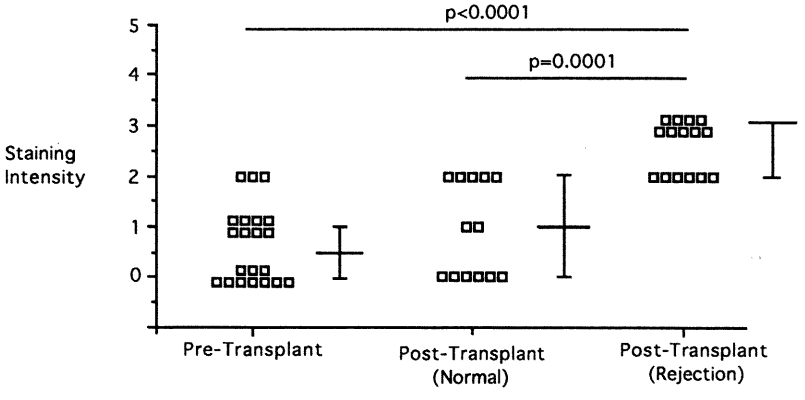


Fig 7.24 CD8 antigen expression in Transplant biopsies-Cellular Infiltrate

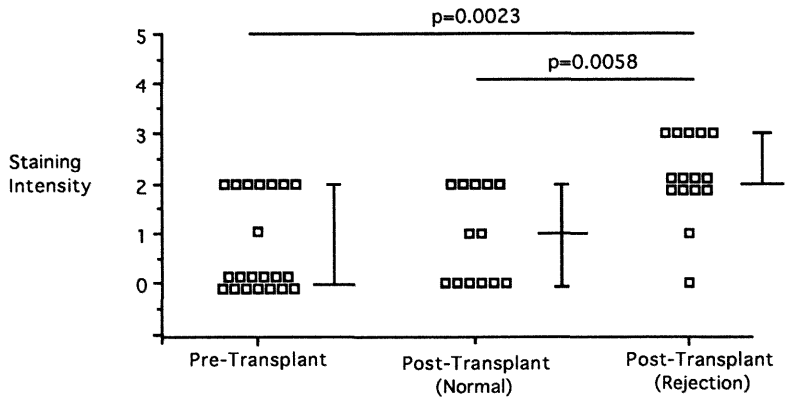


Fig 7.25 IL-2R antigen expression in Transplant biopsies-Cellular Infiltrate

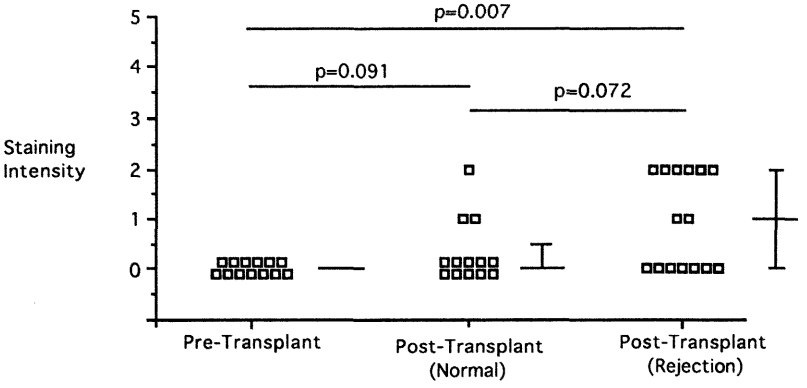


Table 7.1 Presence of E-Selectin on Post-transplant kidney biopsies taken for analysis of dysfunction .

Biopsy Report	Number	E-Selectin positive	Intensity of E-Selectin staining				
			1	2	3	4	5
Normal	2(3%)	0(0%)	-	-	-	-	-
ATN	18(23%)	13(72%)	7	4	2	-	-
ACR	32(42%)	28(88%)	6	13	7	2	-
CR	6(8%)	4(67%)	1	1	2	-	-
Other	19(25%)	12(63%)	8	2	2	-	-
Total	77(100%)	57(74%)	22	20	13	2	-

Table 7.2 Presence of E-Selectin on Post-transplant kidney biopsies taken electively.

Biopsy Report	Number	E-Selectin positive	Intensity of E-Selectin staining				
			1	2	3	4	5
Normal	26(58%)	5(19%)	4	1	-	-	-
ATN	1(2%)	0(0%)	0	-	-	-	-
ACR	9(20%)	7(78%)	2	3	2	-	-
CR	1(2%)	0(0%)	-	-	-	-	-
Other	8(18%)	3(38%)	3	-	-	-	-
Total	45(100%)	15(33%)	9	4	2	-	-

n = number of biopsies in each group

ATN = acute tubular necrosis

ACR = acute cellular rejection

CR = chronic rejection

Table 7.3 Relationship between E-Selectin and CD4 staining intensity in post-transplant kidney biopsies. Biopsy Report Normal n=26

	E-Selectin Intensity (% of biopsies)						
CD4 Intensity	0	1	2	3	4	5	Total
0	46	-	-	-	-	-	46
1	8	-	-	-	-	-	8
2	27	11	4	-	-	-	42
3	-	-	-	-	-	-	0
4	-	4	-	-	-	-	4
5	-	-	-	-	-	-	-
Total	81	15	4	0	0	0	

Table 7.4 Relationship between E-Selectin and CD4 staining intensity in post-transplant kidney biopsies. Biopsy Report Acute Cellular Rejection n=38.

	E-Selectin Intensity (% of biopsies)						
CD4 Intensity	0	1	2	3	4	5	Total
0	3	-	-	-	-	-	3
1	3	3	-	-	-	-	6
2	3	3	13	3	-	-	21
3	5	10	18	5	3	-	41
4	3	3	-	10	-	-	16
5	-	-	5	5	3	-	13
Total	17	19	36	23	6	-	

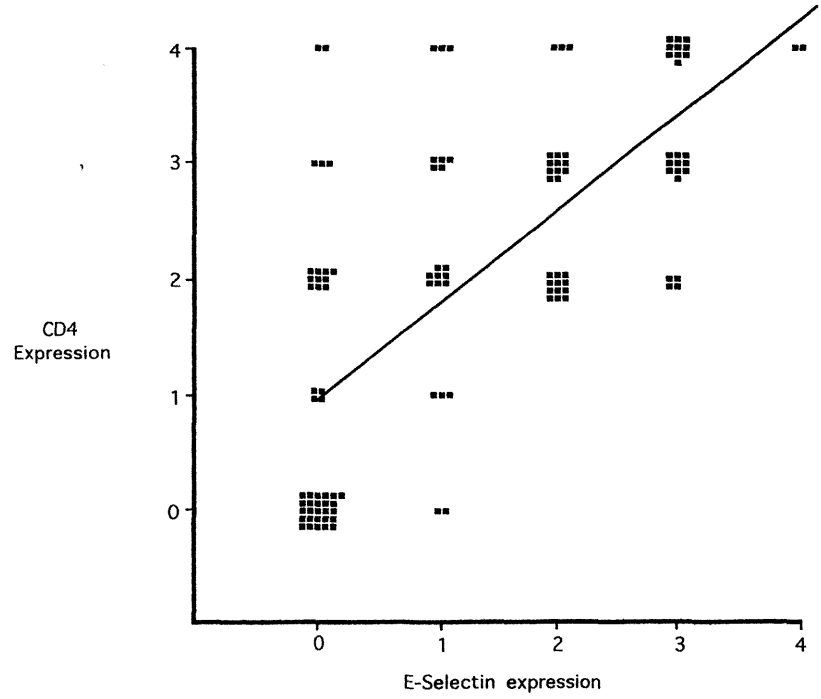
Table 7.5 Relationship between E-Selectin and CD4 staining intensity in post-transplant kidney biopsies. Biopsy Report Chronic Rejection n=9

	E-Selectin Intensity (% of biopsies)						
CD4 Intensity	0	1	2	3	4	5	Total
0	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
2	-	11	-	-	-	-	11
3	22	11	33	-	-	-	66
4	-	-	-	-	-	-	-
5	-	-	-	22	-	-	22
Total	22	22	33	22	-	-	

Table 7.6 Relationship between E-Selectin and CD4 staining intensity in post-transplant kidney biopsies. Biopsy Report Acute Tubular Necrosis n=18.

	E-Selectin Intensity (% of biopsies)						
CD4 Intensity	0	1	2	3	4	5	Total
0	16	-	-	-	-	-	16
1	-	5	-	-	-	-	5
2	-	10	5	-	-	-	15
3	11	16	16	11	-	-	54
4	-	5	-	5	-	-	10
5	-	-	-	-	-	-	-
Total	27	36	21	16	0	0	

Fig 7.26 Scatter plot of E-Selectin and CD4 Expression in post tranplant biopsies.



Regression line of CD4 (y) vs. E-Selectin (x):

$y = 0.942 + 0.837x$

Pearson's Correlation Co-efficient: $r = 0.728$*

*Significance test of correlation because n>60.
Students t-test; t=11.4 : therefore p<0.001

Appendix A

Details of pre-transplant and post-transplant biopsies.

Table A.1 Donors details of kidneys from which pre-transplant kidney biopsies were taken along with transplant recipient details.

Recipient number	Date of Transplant	Donor Type	Donor Age	Cause of Death	Drugs at Retrieval	Infection
1	15.1.92	LRD	43	-	-	-
2	18.6.92	CD	29	Asthma	Dopamine,Dobutamine,DDVAP	-
3	10.6.93	CD	45	SAH	Adrenaline, DDVAP, GTN	-
4	28.7.92	CD	49	RTA-HI	DDVAP	-
5	24.12.91	CD	19	RTA-HI	DDVAP, Prochlorperazine	-
6	31.8.91	CD	27	ICH	Dopamine	-
7	17.11.91	CD	14	RTA-HI	Dopamine,Dobutamine,DDVAP	-
8	27.12.91	CD	55	SAH	Dopamine	-
9	14.1.92	CD	21	ICH	-	-
10	23.11.91	CD	37	ICH	Dopamine,Dobutamine,DDVAP	-
11	4.10.91	CD	37	ICH	Dopamine	-
12	29.8.91	CD	25	ICH	DDVAP	-
13	20.8.92	CD	47	CA	Dobutamine	-
14	31.12.91	CD	30	HI	-	-
15	24.12.91	CD	53	RTA-HI	Dopamine,DDVAP,Labetalol	-
16	13.8.93	CD	67	ICH	Dopamine,Verapamil	-
17	16.2.92	CD	20	RTA-HI	Dopamine	-
18	13.9.91	CD	45	ICH	Dopamine,DDVAP	-
19	9.7.93	CD	29	Asthma	DDVAP,Phenytoin,Insulin	-
20	26.3.92	CD	57	SAH	Dopamine	-
21	24.8.91	CD	53	RTA-HI	Dopamine,Dobutamine	-
22	24.8.91	CD	48	SAH	-	-
23	31.8.91	CD	27	ICH	Dopamine	-

Legend

LRD= Living Related Donor, CD= Cadaver Donor
SAH= Sub-Arachnoid Haemorrhage, RTA= Road Traffic Accident, HI= Head Injury,
ICH= Intra-Cranial Haemorrhage, CA= Cardiac Arrest.
DDAVP= Desmopressin
GTN= Glyceryl Trinitrate

Appendix B

Details of Antigen expression on Pre and Post-Transplant Biopsies.

Table B.1 Antigen Expression on Pre-Transplant Biopsies: Tubules (n=16)

	0	1	2	3	4	5
Class 1	15 94%	- -	- -	- -	1 6%	- -
Class 2	10 62%	3 19%	3 19%	- -	- -	- -
E-Selectin	15 94%	1 6%	- -	- -	- -	- -
PCAM	16 100%	- -	- -	- -	- -	- -
VCAM	4 25%	1 6%	7 44%	3 19%	1 6%	- -
ICAM	7 44%	- -	4 25%	4 25%	1 6%	- -

Table B.2 Antigen Expression on Pre-Transplant Biopsies: Glomeruli (n=16)

	0	1	2	3	4	5
Class 1	- -	- -	- -	3 19%	13 81%	- -
Class 2	1 6%	1 6%	8 50%	6 38%	- -	- -
E-Selectin	15 94%	- -	1 6%	- -	- -	- -
PCAM	- -	- -	- -	4 25%	12 75%	- -
VCAM	- -	- -	7 43%	6 38%	3 19%	- -
ICAM	- -	- -	- -	4 25%	12 75%	- -

Table B.3 Antigen Expression on Pre-Transplant Biopsies: ITE (n=16)

	0	1	2	3	4	5
Class 1	- -	- -	- -	5 31%	11 69%	- -
Class 2	1 6%	- -	9 56%	6 38%	- -	- -
E-Selectin (n=21)	17 74%	1 4%	4 18%	1 4%	- -	- -
PCAM	- -	- -	- -	2 12%	14 88%	- -
VCAM	10 62%	- -	5 32%	1 6%	- -	- -
ICAM	- -	- -	2 12%	8 50%	6 38%	- -

Table B.4 Antigen Expression on Pre-Transplant Biopsies: Large Vessels (n=15)

	0	1	2	3	4	5
Class 1	1 6%	- -	4 27%	6 39%	4 27%	- -
Class 2	2 14%	- -	11 72%	2 14%	- -	- -
E-Selectin	13 87%	- -	- -	2 13%	- -	- -
PCAM	- -	- -	1 7%	4 27%	10 66%	- -
VCAM	10 62%	1 7%	2 14%	2 14%	- -	- -
ICAM	- -	1 7%	8 52%	4 27%	2 14%	- -

Table B.5 Antigen Expression on Pre-Transplant Biopsies: Cellular Infiltrate (n=14)

	0	1	2	3	4	5
Leu Common	3 21%	3 21%	6 43%	2 15%	- -	- -
CD3	4 28%	2 15%	8 57%	- -	- -	- -
CD4 (n=23)	10 48%	8 38%	3 14%	- -	- -	- -
CD8 (n=21)	13 62%	1 5%	7 33%	- -	- -	- -
IL-2R	13 100%	- -	- -	- -	- -	- -

Table B.6 Antigen Expression on Normal Post-Transplant Biopsies: Tubules (n=13)

	0	1	2	3	4	5
Class 1	11 85%	1 7%	1 7%	- -	- -	- -
Class 2	10 78%	- -	1 7%	2 15%	- -	- -
E-Selectin	13 100%	- -	- -	- -	- -	- -
PCAM	13 100%	- -	- -	- -	- -	- -
VCAM	4 31%	3 23%	3 23%	3 23%	- -	- -
ICAM	12 44%	- -	1 7%	- -	- -	- -

Table B.7 Antigen Expression on Normal Post-Transplant Biopsies: Glomeruli (n=10)

	0	1	2	3	4	5
Class 1	- -	- -	- -	3 30%	7 70%	- -
Class 2	- -	- -	10 100%	- -	- -	- -
E-Selectin	10 100%	- -	- -	- -	- -	- -
PCAM	- -	- -	1 10%	3 30%	6 60%	- -
VCAM	1 10%	- -	4 40%	4 40%	1 10%	- -
ICAM	1 10%	- -	- -	4 40%	5 50%	- -

Table B.8 Antigen Expression on Normal Post-Transplant Biopsies: ITE (n=13)

	0	1	2	3	4	5
Class 1	- -	- -	1 8%	6 46%	6 46%	- -
Class 2	1 8%	- -	9 69%	3 23%	- -	- -
E-Selectin	11 86%	2 14%	- -	- -	- -	- -
PCAM	- -	- -	1 8%	6 46%	6 46%	- -
VCAM	11 86%	- -	2 14%	- -	- -	- -
ICAM	- -	- -	2 14%	8 62%	3 23%	- -

Table B.9 Antigen Expression on Normal Post-Transplant Biopsies: Large Vessels (n=10)

	0	1	2	3	4	5
Class 1	- -	- -	2 20%	5 50%	3 30%	- -
Class 2	1 10%	- -	7 70%	2 20%	- -	- -
E-Selectin	9 90%	1 10%	- -	- -	- -	- -
PCAM	- -	- -	1 10%	3 30%	6 60%	- -
VCAM	8 80%	- -	2 20%	- -	- -	- -
ICAM	- -	- -	3 30%	7 70%	- -	- -

Table B.10 Antigen Expression on Normal Post-Transplant Biopsies: Cellular Infiltrate (n=13)

	0	1	2	3	4	5
Leu Common	5 38%	1 8%	5 38%	2 16%	- -	- -
CD3	5 38%	1 8%	7 54%	- -	- -	- -
CD4	6 46%	2 16%	5 38%	- -	- -	- -
CD8	5 38%	2 16%	6 46%	- -	- -	- -
IL-2R	10 77%	2 16%	1 7%	- -	- -	- -

Table B.11 Antigen Expression on Post-Transplant Biopsies (Acute rejection): Tubules (n=15)

	0	1	2	3	4	5
Class 1	1 6%	1 6%	5 33%	6 40%	2 13%	- -
Class 2	6 40%	2 13%	7 47%	- -	- -	- -
E-Selectin	13 88%	1 6%	1 6%	- -	- -	- -
PCAM	15 100%	- -	- -	- -	- -	- -
VCAM	- -	- -	6 40%	6 40%	3 20%	- -
ICAM	8 55%	1 6%	2 13%	3 20%	1 6%	- -

Table B.12 Antigen Expression on Post-Transplant Biopsies (Acute rejection): Glomeruli (n=9)

	0	1	2	3	4	5
Class 1	1 11%	- -	- -	2 22%	6 66%	- -
Class 2	4 44%	1 12%	4 44%	- -	- -	- -
E-Selectin	9 100%	- -	- -	- -	- -	- -
PCAM	- -	- -	- -	3 33%	6 67%	- -
VCAM	1 11%	- -	4 44%	4 44%	- -	- -
ICAM	- -	- -	2 22%	5 56%	2 22%	- -

Table B.13 Antigen Expression on Post-Transplant Biopsies (Acute rejection): ITE (n=15)

	0	1	2	3	4	5
Class 1	- -	- -	- -	7 47%	8 53%	- -
Class 2	- -	- -	7 47%	7 47%	1 6%	- -
E-Selectin	3 20%	3 20%	8 54%	1 6%	- -	- -
PCAM	- -	- -	- -	5 33%	10 67%	- -
VCAM	11 74%	1 6%	3 20%	- -	- -	- -
ICAM	- -	- -	2 13%	5 33%	8 54%	- -

Table B.14 Antigen Expression on Post-Transplant Biopsies (Acute rejection): Large vessels (n=14)

	0	1	2	3	4	5
Class 1	- -	- -	- -	7 50%	7 50%	- -
Class 2	4 29%	- -	6 43%	3 21%	1 7%	- -
E-Selectin	7 50%	2 14%	4 29%	1 7%	- -	- -
PCAM	- -	- -	- -	4 29%	10 71%	- -
VCAM	8 58%	1 7%	5 35%	- -	- -	- -
ICAM	- -	- -	3 21%	8 58%	3 21%	- -

Table B.15 Antigen Expression on Post-Transplant Biopsies (Acute rejection): Cellular Infiltrate (n=15)

	0	1	2	3	4	5
Leu Common	- -	- -	5 33%	8 55%	2 12%	- -
CD3	- -	- -	3 20%	10 67%	2 13%	- -
CD4	- -	- -	6 40%	9 60%	- -	- -
CD8	1 6%	1 6%	8 55%	5 33%	- -	- -
IL-2R	7 47%	2 13%	6 40%	- -	- -	- -

Biopsy Number	Age	Diagnosis	Post Tx Weeks	Immuno-Suppression	Creatinine (umol/L)	Urea (mmol/L)	White Cells (x109)	Biopsy Type	HistoPathological Diagnosis
1	22	Vesico Ureteric Reflux	4	Aza/Pred/CyA	107	4.6	5.6	Routine	Normal
2	22	Vesico Ureteric Reflux	8	Aza/Pred/CyA	112	5.7	13.1	Diagnostic	Normal
3	22	Vesico Ureteric Reflux	24	Aza/Pred/CyA	153	9.6	5.3	Routine	Normal
4	56	Polycystic Kidneys	104	Pred/CyA	125	7.6	9	Routine	Normal
5	42	Chronic Pylonephritis	104	Pred/CyA	169	10.5	13.2	Routine	Normal
6	47	Chronic Pylonephritis	104	Pred/CyA	148	9.3	9	Routine	Acute Tubular Necrosis
7	66	Amyloidosis	1	Pred/CyA	907	26.6	6.9	Diagnostic	Acute Tubular Necrosis
8	51	Congenital Renal Hypoplasia	1	Pred/CyA	221	14.2	16.1	Diagnostic	Acute Cellular Rejection
9	67	Polycystic Kidneys	416	CyA	348	26.9	5.5	Diagnostic	Other
10	40	Hypertension	1	Pred/CyA	341	27.4	26.1	Diagnostic	Acute Cellular Rejection
11	40	Hypertension	3	Aza/Pred/CyA	618	21.5	11.7	Diagnostic	Acute Tubular Necrosis
12	55	Diabetes	1	Pred/CyA	209	20.2	6.4	Diagnostic	Acute Cellular Rejection
13	55	Diabetes	1	Pred/CyA	217	11.8	7.6	Routine	Acute Cellular Rejection
14	55	Diabetes	4	Pred/CyA	141	9.5	7.8	Diagnostic	Acute Cellular Rejection
15	32	Vesico Ureteric Reflux	4	Pred/CyA	249	17	11.3	Routine	Other
16	32	Vesico Ureteric Reflux	64	Pred/CyA	258	11	11.4	Diagnostic	Chronic Rejection
17	62	Diabetes	104	Pred/CyA	167	12.5	9.9	Routine	Normal
18	31	Glomerulonephritis	104	Aza/Pred/CyA	166	8.8	6.9	Routine	Normal
19	55	Chronic Pylonephritis	90	Pred/CyA	201	16.2	10.1	Diagnostic	Acute Tubular Necrosis
20	21	Vesico Ureteric Reflux	148	Aza/Pred/CyA	331	13.3	5	Diagnostic	Other
21	49	Diabetes	2	Pred/CyA	205	20.3	11.5	Diagnostic	Acute Cellular Rejection
22	49	Diabetes	2	Pred/CyA	323	38	18.8	Diagnostic	Acute Cellular Rejection
23	49	Diabetes	4	Aza/Pred/CyA	327	30.1	9.5	Diagnostic	Acute Cellular Rejection
24	21	Vesico Ureteric Reflux	24	Pred/CyA	142	7.2	8.1	Routine	Normal
25	21	Vesico Ureteric Reflux	52	Pred/CyA	141	11.8	7	Routine	Normal
26	21	Vesico Ureteric Reflux	104	Pred/CyA	156	11.6	12.5	Routine	Normal
27	51	Chronic Pylonephritis	10	Pred/CyA	307	28	15.5	Diagnostic	Acute Tubular Necrosis
28	51	Chronic Pylonephritis	15	Pred/CyA	236	40	10.5	Diagnostic	Acute Tubular Necrosis (ACR)

Appendix C: Patient Details for Biopsy specimens.

Biopsy Number	Age	Diagnosis	Post Tx Weeks	Immuno-Suppression	Creatinine (umol/L)	Urea (mmol/L)	White Cells (x109)	Biopsy Type	HistoPathological Diagnosis
29	58	Chronic Pylonephritis	10	Pred/CyA	238	12.7	10.7	Diagnostic	Acute Cellular Rejection
30	58	Chronic Pylonephritis	15	Pred/CyA	211	4.7	6.6	Diagnostic	Acute Cellular Rejection
31	58	Chronic Pylonephritis	18	Pred/CyA	264	7.3	8.1	Diagnostic	Other
32	63	Diabetes	2	Pred/CyA	306	37	15.7	Diagnostic	Acute Tubular Necrosis
33	63	Diabetes	6	Pred/CyA	519	35.7	13.5	Diagnostic	Other
34	37	Chronic Pylonephritis	4	Pred/CyA	87	4	8.2	Routine	Normal
35	37	Chronic Pylonephritis	24	Pred/CyA	130	6.7	8	Routine	Normal
36	37	Chronic Pylonephritis	52	Pred/CyA	124	6.8	12.2	Routine	Other
37	37	Chronic Pylonephritis	104	Pred/CyA	115	7.3	6.7	Diagnostic	Other
38	25	Glomerulonephritis	2	Aza/Pred/CyA	177	10.5	13.4	Diagnostic	Other
39	25	Glomerulonephritis	9	Aza/Pred/CyA	168	13	32.8	Diagnostic	Acute Cellular Rejection
40	25	Glomerulonephritis	14	Aza/Pred/CyA	233	11.6	11.1	Diagnostic	Other
41	51	Unknown	24	Pred/CyA	203	9.5	6.1	Routine	Normal
42	51	Unknown	52	Pred/CyA	199	13	5.2	Routine	Normal
43	36	Diabetes	1	Pred/CyA	785	36.2	12	Diagnostic	Acute Cellular Rejection
44	36	Diabetes	2	Pred/CyA	430	36.4	21.4	Diagnostic	Acute Tubular Necrosis (ACR)
45	36	Diabetes	3	Pred/CyA	307	27.8	15.1	Diagnostic	Acute Tubular Necrosis
46	36	Diabetes	56	Pred/CyA	222	16.6	13.6	Diagnostic	Other
47	56	Amyloidosis	1	Pred/CyA	955	37	9.4	Diagnostic	Acute Tubular Necrosis (ACR)
48	56	Amyloidosis	4	Pred/CyA	146	10.9	7.5	Routine	Normal
49	56	Amyloidosis	24	Pred/CyA	236	17	9.9	Routine	Acute Cellular Rejection
50	56	Amyloidosis	128	Pred/CyA	548	36	16.3	Diagnostic	Other
51	56	Glomerulonephritis	5	Aza/Pred/CyA	159	11.7	8.5	Routine	Normal
52	56	Glomerulonephritis	24	Aza/Pred/CyA	177	7.4	8.9	Routine	Normal
53	56	Glomerulonephritis	92	Aza/Pred/CyA	225	16.4	6	Diagnostic	Other
54	40	Diabetes	92	Aza/Pred	208	8.8	7.1	Diagnostic	Acute Cellular Rejection
55	40	Diabetes	118	Aza/Pred/CyA	395	22	6.3	Diagnostic	Chronic Rejection
56	58	Polycystic Kidneys	4	Pred/CyA	197	12.9	9.4	Diagnostic	Other

Appendix C: Patient Details for Biopsy specimens.

Biopsy Number	Age	Diagnosis	Post Tx Weeks	Immuno-Suppression	Creatinine (umol/L)	Urea (mmol/L)	White Cells (x109)	Biopsy Type	HistoPathological Diagnosis
57	45	Systemic Lupus Erythematosus	3	Aza/Pred/CyA	94	9.3	14.8	Routine	Acute Cellular Rejection
58	56	Unknown	24	Pred/CyA	217	14.7	8.7	Diagnostic	Other
59	39	Polyarteritis nodosa	1	Pred/CyA	234	13.8	13.2	Diagnostic	Acute Cellular Rejection
60	43	Chronic Pylonephritis	24	Pred/CyA	115	8.4	16.3	Routine	Normal
61	43	Chronic Pylonephritis	104	Pred/CyA	144	12.2	10.3	Routine	Acute Cellular Rejection
62	27	Glomerulonephritis	2	Aza/Pred/CyA	605	27.1	14	Diagnostic	Acute Cellular Rejection
63	59	Unknown	1	Pred/CyA	1144	27.6	15.2	Diagnostic	Acute Tubular Necrosis
64	51	Glomerulonephritis	1	Pred/CyA	1008	27	9.5	Diagnostic	Acute Tubular Necrosis
65	51	Glomerulonephritis	3	Aza/Pred/CyA	929	28	5.6	Diagnostic	Acute Tubular Necrosis
66	30	Congenital Obstructive Uropathy	4	Aza/Pred/CyA	150	11.9	12.6	Diagnostic	Acute Cellular Rejection
67	30	Congenital Obstructive Uropathy	24	Aza/Pred/CyA	142	10.4	10.5	Routine	Normal
68	30	Congenital Obstructive Uropathy	52	Aza/Pred/CyA	129	8.7	8.2	Routine	Normal
69	39	Glomerulonephritis	4	Pred/CyA	130	7.5	11.1	Routine	Normal
70	39	Glomerulonephritis	68	Pred/CyA	182	10.4	10.4	Diagnostic	Other
71	57	Unknown	5	Aza/Pred/CyA	76	5.5	6.7	Routine	Normal
72	23	Glomerulonephritis	52	Pred/CyA	154	11.5	4.1	Routine	Normal
73	23	Glomerulonephritis	104	Pred/CyA	224	21.6	6.4	Diagnostic	Normal
74	56	Unknown	2	Pred/CyA	198	17.2	22.1	Diagnostic	Acute Cellular Rejection
75	50	Chronic Pylonephritis	24	Pred/CyA	137	13	2.8	Diagnostic	Other
76	44	Glomerulonephritis	260	Aza/Pred/CyA	393	27.5	5.4	Diagnostic	Chronic Rejection
77	65	Small Kidneys	4	Pred/CyA	161	17.5	7.4	Diagnostic	Normal
78	65	Small Kidneys	12	Pred/CyA	168	13.3	14.4	Diagnostic	Acute Cellular Rejection
79	39	Chronic Pylonephritis	156	Aza/Pred/CyA	369	17.8	9.7	Diagnostic	Acute Cellular Rejection
80	48	Polycystic Kidneys	26	Aza/Pred/CyA	385	16.4	8.1	Diagnostic	Other
81	30	Small Kidneys	104	Aza/Pred/CyA	154	6.9	10.8	Routine	Chronic Rejection
82	17	Laurence-Moon-Biedl Syndrome	8	Aza/Pred	153	9.3	3	Routine	Acute Cellular Rejection
83	17	Laurence-Moon-Biedl Syndrome	9	Aza/Pred/CyA	134	9.9	4.4	Diagnostic	Acute Cellular Rejection
84	54	Diabetes	2	Pred/CyA	121	9.4	9.2	Diagnostic	Acute Tubular Necrosis

Appendix C: Patient Details for Biopsy specimens.

Biopsy Number	Age	Diagnosis	Post Tx Weeks	Immuno-Suppression	Creatinine (umol/L)	Urea (mmol/L)	White Cells (x109)	Biopsy Type	HistoPathological Diagnosis
85	55	Small Kidneys	1	Pred/CyA	514	22.4	10	Diagnostic	Acute Cellular Rejection
86	55	Small Kidneys	2	Pred/CyA	524	24.2	16.1	Diagnostic	Acute Tubular Necrosis
87	57	Glomerulonephritis	5	Pred/CyA	99	11	10.1	Routine	Normal
88	49	Glomerulonephritis	2	Pred/CyA	418	19.6	13.9	Diagnostic	Acute Tubular Necrosis
89	49	Glomerulonephritis	3	Pred/CyA	424	14.8	7.4	Routine	Acute Cellular Rejection
90	49	Glomerulonephritis	4	Pred/CyA	343	15.8	11.4	Diagnostic	Acute Cellular Rejection
91	53	Unknown	1	Pred/CyA	162	9.4	10.5	Diagnostic	Acute Cellular Rejection
92	53	Unknown	2	Pred/CyA	191	14.5	14.3	Diagnostic	Acute Cellular Rejection
93	53	Unknown	2	Pred/CyA	251	18.9	11.2	Diagnostic	Acute Cellular Rejection
94	76	Unknown	104	Pred/CyA	167	11.6	8.4	Routine	Normal
95	31	Alport's Syndrome	1	Pred/CyA	174	13.5	19.7	Diagnostic	Acute Cellular Rejection
96	31	Alport's Syndrome	7	Aza/Pred/CyA	290	12.3	22.7	Diagnostic	Other
97	53	Diabetes	24	Pred/CyA	246	19	10.5	Routine	Other
98	25	Renal Artery Stenosis	2	Pred/CyA	601	41.7	12.5	Diagnostic	Acute Cellular Rejection
99	25	Renal Artery Stenosis	2	Pred/CyA	457	30.9	13.3	Diagnostic	Acute Cellular Rejection
100	59	Unknown	416	Pred/CyA	207	11.6	7.7	Diagnostic	Other
101	17	Vesico Ureteric Reflux	112	Aza/Pred/CyA	237	14.5	6.5	Diagnostic	Chronic Rejection
102	43	Sarcoidosis	112	Pred/CyA	69	4.6	8.1	Routine	Normal
103	23	Chronic Pylonephritis	24	Pred/CyA	149	8.3	8.2	Routine	Other
104	63	Unknown	2	Pred/CyA	271	22.1	7.5	Diagnostic	Acute Tubular Necrosis
105	28	Chronic Pylonephritis	24	Aza/Pred/CyA	151	9.2	7.1	Routine	Acute Cellular Rejection
106	28	Chronic Pylonephritis	28	Aza/Pred/CyA	162	8.6	5.3	Diagnostic	Acute Cellular Rejection
107	28	Chronic Pylonephritis	104	Aza/Pred/CyA	211	17.6	6.5	Routine	Acute Cellular Rejection
108	34	Chronic Pylonephritis	884	Aza/Pred	786	21.2	7.6	Diagnostic	Chronic Rejection
109	19	Glomerulonephritis	4	Pred/CyA	115	12.4	13.2	Routine	Acute Cellular Rejection
110	19	Glomerulonephritis	24	Pred/CyA	120	11.1	9.4	Routine	Other
111	19	Glomerulonephritis	52	Pred/CyA	170	10.6	8.1	Routine	Other
112	54	Glomerulonephritis	4	Pred/CyA	472	34.5	7.8	Diagnostic	Acute Tubular Necrosis

Appendix C: Patient Details for Biopsy specimens.

Biopsy Number	Age	Diagnosis	Post Tx Weeks	Immuno-Suppression	Creatinine (umol/L)	Urea (mmol/L)	White Cells (x109)	Biopsy Type	HistoPathological Diagnosis
113	54	Glomerulonephritis	48	Pred/CyA	315	31.1	11.9	Diagnostic	Normal
114	54	Unknown	2	Pred/CyA	826	38.4	12.1	Diagnostic	Acute Tubular Necrosis
115	37	Chronic Pylonephritis	364	Pred/CyA	1042	24.7	8.6	Diagnostic	Acute Cellular Rejection
116	35	Henoch-Schonlein purpura	104	Aza/Pred/CyA	205	10.9	4.9	Routine	Other
117	57	Glomerulonephritis	312	Aza/Pred	250	17.8	4.2	Diagnostic	Chronic Rejection
118	67	Obstructive Uropathy	104	Aza/Pred/CyA	118	7.2	7.4	Routine	Normal
119	49	Hypertension	104	Pred/CyA	126	6.8	9.2	Routine	Other

Appendix C: Patient Details for Biopsy specimens.

Table A.2 Other Diagnoses in Routine Biopsies

Biopsy Number	Diagnosis	Staining Intensity E-Selectin	CD4
15	Mild Interstitial Fibrosis	0	3
37	Mild chronic Fibrosis	0	0
97	Cyclosporin Toxicity	0	2
103	Mild Interstitial Fibrosis	0	0
110	Mild Interstitial Oedema	1	1
111	Mild Interstitial Fibrosis	0	0
116	Mild Interstitial Fibrosis	1	2
119	Moderate Interstitial Fibrosis	1	3

Table A.3 Other Diagnoses in Diagnostic Biopsies

Biopsy Number	Diagnosis	E-Selectin	CD4
9	Mild Interstitial Fibrosis	1	1
20	Chronic Interstitial Fibrosis	1	3
31	Mild Interstitial Fibrosis	1	4
33	Acute Pyelonephritis	0	0
36	Mild Chronic Fibrosis	1	3
38	Cyclosporin Toxicity	0	2
40	Cyclosporin Toxicity	1	2
46	Severe Chronic Fibrosis	2	4
50	Severe Chronic Fibrosis	2	2
53	Moderate Interstitial Fibrosis	0	1
56	Mild Chronic Fibrosis	1	3
58	Interstitial Fibrosis	1	3
70	Chronic Fibrosis	0	1
75	Interstitial Fibrosis	1	3
80	Severe Interstitial Fibrosis	0	0
96	Cyclosporin Toxicity	0	0
100	Severe Interstitial Fibrosis	3	4
112	Mild Interstitial Fibrosis	3	3

CHAPTER 8

DISCUSSION

8.0 Introduction

The concept of the endothelial cell as a dynamic regulator of its own interaction with leucocytes is a recent one. As little as 20 years ago the endothelium was seen as passive in the generation of the inflammatory response, acting solely as a semi-permeable membrane. The development of the technique of human umbilical vein endothelial cell culture in the early 1980s allowed the functional capacity of endothelial cells to be studied. Initially endothelial cells were found to express MHC Class I antigens and later to express MHC Class II antigens under stimulation by the cytokine Interferon-g. The discovery of endothelial cell adhesion molecules such as E-Selectin and ICAM-1, allied with the previous discovery of adhesion molecules expressed by leucocytes, allowed the definition of a role for endothelial cells in the generation of the inflammatory response.

Despite the characterisation of adhesion molecules and their structures much is unknown about the precise interaction of the different adhesion molecule families in the modulation of the inflammatory response. The important role of cytokines in the regulation of adhesion molecule expression during different types of inflammation has been shown *in vitro*, but the complexities of inflammation *in vivo* make elucidation of cell-cell adhesion molecule interactions very difficult. Whilst *in vivo* work using animal models can help establish links between adhesion molecule expression and inflammation the differences in immune mechanisms between species makes transfer of this knowledge to a clinical situation difficult. *In vivo* work in humans is problematic and therefore establishment of reliable *in vitro* models is necessary to study interactions of human adhesion molecule

systems effectively .

The aim of this study was first to establish a reliable method of obtaining HUVECs for *in vitro* quantification of adhesion molecule expression, secondly to establish a method for quantitative measurement of PMN-HUVEC adhesion, and thirdly examine in vivo adhesion molecule expression in renal transplant biopsies. These methods were combined in an examination of adhesion molecule expression and PMN activation in normal individuals, haemodialysis patients and renal transplant patients.

8.1 Establishment of Primary cultures of human endothelial cells

The primary culture of endothelial cells is preferred to the long term culture of endothelial cell lines because of the potential loss of function of cells after repeated sub-culture. Sources of endothelial cells used in previous work include bovine aorta (excluded because of its non-human nature), human umbilical veins, human omentum and sections of human veins obtained after vascular surgery. It was decided to use human umbilical veins as the source of endothelial cells because of their ease of availability compared with other human sources which rely on surgical procedures and culture techniques which have been previously well characterised.

Human umbilical cords were collected from the Maternity Unit at the Leicester General Hospital as previously described (Chapter 3), this was made easier by the fact that the maternity unit is located adjacent to the Dept of Surgery laboratories. Following the protocol described in Chapter 3 successful primary isolation of endothelial cells was achieved from approximately 75% of cords processed. These cells were grown to confluence and when characterised (Section 3.4) were shown to be pure endothelial cells without contaminating fibroblasts.

8.2 *In vitro* assay of endothelial cell response to stimuli.

The range of stimuli used on HUVECs in these experiments was influenced by the need to simulate conditions found in renal failure and renal transplant patients. A range of stimulatory cytokines was used, as well as immunosuppressive drugs and urea to determine the relative effects of these factors on adhesion molecule expression in these patient groups. There were two available methods to determine adhesion molecule expression in the HUVEC lines either immunocytochemistry using microscopic examination of stained slides or, flow cytometry using cell suspensions labelled with fluorescent antibodies. It was decided to use a flow cytometer based method because of its speed and the improved quantitative nature of results obtained when compared to the relatively slow and less objective analysis of stained slides.

Examination of the results show that different cytokines exert a stimulatory influence on different endothelial cell adhesion molecules. Interferon-g was shown to increase expression of HLA Class I, Class II DR and ICAM-1 but not E-Selectin or P-Selectin. This suggests that INF-g is important in regulation of immune inflammation since ICAM-1 is a ligand for the leucocyte adhesion molecule LFA-1 present on lymphocytes and HLA Class I and II are both responsible for antigen presentation to lymphocytes. The optimum concentration of INF-g to produce this maximal expression was similar for these molecules, between 500 and 1000 U/ml, suggesting that they would be up-regulated together *in vivo*. However, the dose response of cells in vitro is not an accurate guide to *in vivo* responses and the actual levels of INF-g which induce up-regulation may be different *in vivo*. The time course over which this increased expression occurred was between 48 and 72 hours. This is a relatively long period and is also co-incidental with the anticipated time course one would expect of immune mediated inflammation.

In contrast to the results from INF-g only HUVEC expression of

E-Selectin was induced by IL-1b in this *in vitro* model. E-Selectin is a ligand for receptors on neutrophils, monocytes and memory T-lymphocytes. The optimum concentration of IL-1b was 5 U/ml with maximal expression at 4 to 8 hours post-stimulation. Again the precise dose of cytokine may not reflect the *in vivo* environment but the short time course of the response would imply that E-Selectin is involved in acute inflammation. The rapid recruitment of neutrophils into areas of acute inflammation is well recognised and it is likely that E-Selectin is one of the main mediators of this response. The fact that memory T-lymphocytes also use E-Selectin as an adhesion receptor suggests that this may be an important early step in the activation of the immune inflammatory response.

The immunosuppressive agents tested, Cyclosporin A and Methylprednisolone, did not affect the Interleukin 1 stimulated expression of E-selectin, using this rather limited assay system. This possibly suggests that these most commonly used immunosuppressive agents may not alter cell adhesion molecule expression and therefore may not be useful for treating the inflammatory process during allograft rejection at the adhesion molecule level. Methylprednisolone and Cyclosporin A both work by altering lymphocyte function and as such would not play a role in altering cell adhesion molecule function. However, the targeting of other agents, such as anti-cell adhesion molecule antibodies, during allograft rejection might be expected to have a beneficial effect on the course of the immune inflammatory process. It should be remembered, as noted in Chapter 4, that the experiments using Cyclosporin and Methylprednisolone were rather limited in their scope and design and caution should be exercised in over interpreting this data.

8.3 The in-vitro assay of polymorphonuclear cells to endothelial cells

In Chapter 4 the increased endothelial cell expression of surface

adhesion molecules after cytokine stimulation was demonstrated. However this does not give information as to the functional adhesive potential of these molecules. To investigate this we assayed the adhesion of polymorphonuclear cells (PMN) to the endothelial cell monolayer. Since both cell types may alter their adhesiveness for one another it was possible to investigate the conditions under which adhesion might occur.

It was established in Chapter 4 that E-selectin expression is induced by interleukin-1. E-selectin is a ligand used for binding by PMN, monocytes, and memory T lymphocytes. PMN were used to investigate the function of E-selectin in the in vitro culture system because of the ease of isolation from small volumes of blood. To isolate monocytes and lymphocytes requires larger volumes of blood and because they can not be cultured fresh cells must be isolated for each assay. Isolation of PMN from blood can be performed by the use of dextran or density gradient polymers. This gives a low yield of high purity but has the disadvantage of altering PMN cell function and surface properties. The differential centrifugation method was used for PMN isolation because absolute cell purity was not a requirement and because avoidance of cell activation was important. It requires only 4 mls of blood, takes less than 30 mins and avoids cell activation. PMN from several individuals can be isolated at the same time.

The assay of PMN adhesion to HUVEC's can be performed using radioactive chromium labelling or Rose Bengal dye. The Rose Bengal dye method has the advantage of being relatively non-toxic and rapid to perform. The PMN require no pre-treatment and can be isolated from blood and adhesion assayed in less than one hour minimising the cell damage due to storage and processing. The Rose Bengal assay showed that HUVEC's, pre-treated with cytokines, were functionally more adhesive than non pre-treated cells. However the assay gives a qualitative estimate of PMN adhesion rather than a quantitative measure of the numbers of PMN adhering

to the HUVEC monolayer and this limits it's usefulness.

Radio labelled chromium proved more useful than Rose Bengal dye in the assessment of PMN adhesion to HUVEC's. The advantages of the assay are that it gives a percentage adhesion of PMN's to HUVEC's which can be reproduced under varying assay conditions, on different days and between different individuals. The disadvantages of the assay are the use of potentially hazardous radioactive material and the significantly increased assay time, compared to Rose Bengal, leading to the possibility of cell damage due to storage time.

The chromium release assay demonstrated that interleukin-1 stimulated HUVEC's bind 2-3 times as many PMN's as unstimulated HUVEC's. The assay conditions investigated showed that varying conditions could be employed. The final assay temperature of 4^0C was chosen because the metabolic functions of both cell types are low at this level which would allow the assay to measure existing adhesion molecules rather than any potential new adhesion molecule expression that might occur at physiological temperatures. At physiological temperatures the possibility of the HUVEC's phagocytosing the PMN's exists and this would clearly be a major source of error in the assay. *De novo* adhesion molecule expression and phagocytosis may be the reason why there was apparently more adhesion of PMN's at 20^0C and 37^0C than at 4^0C.

The use of PMA to activate PMN's demonstrates that any increased adhesive potential of PMN's can also be investigated using this assay system. This suggests that a dynamic situation exists in-vivo in which the endothelial cell and the PMN are variably adhesive depending on their activation state. Using the final ^{51}Cr assay conditions it was possible to produce a normal range of PMN adhesion to HUVEC's which showed that cytokine stimulated HUVEC's bind almost three times as many PMN's from normal subjects as unstimulated HUVEC's.

8.4 Effect of renal failure and transplantation on expression and function of adhesion molecules

Using the ^{51}Cr release assay the adhesion of PMN's from normal controls, haemodialysis patients and renal transplant patients was compared. The renal transplant patients were further divided into those with no evidence of infection at the time of sampling and those with evidence of infection on the day of sampling. Details of the control group, as presented in Chapter 5, show that these were normal healthy volunteers and it should be emphasised that whilst this provides a reference group for normal PMN adhesion it does not constitute a control group for dialysis or renal transplant patients. A proper control group for these groups would include individuals on the same medication as renal failure patients, without being on dialysis, and those on immunosuppressive agents but not in renal failure. Such patients are difficult to recruit in sufficient numbers to make proper control groups and because of this the 'normal controls' were restricted to healthy volunteers. As such it is impossible to say that the medication that the patient groups were taking did not contribute to the differences seen in PMN adhesion using this assay rather than the pathological condition suffered by the patient.

Using unstimulated HUVEC's PMN's from haemodialysis patients were found to be more adhesive than normal controls. This may imply a degree of chronic activation of PMN's from haemodialysis patients since samples were collected just prior to dialysis (which is known to activate PMN's). The adhesiveness of PMN's immediately post dialysis would be interesting to measure and would be expected to be higher than pre-dialysis because of the activating effect that increased cytokine production during the haemodialysis process has. However, because dialysis causes a neutropenia, the amount of blood required to isolate enough PMN's to perform the assay would be large and could not be justified ethically.

Haemodialysis patients also had PMNs which were more adhesive to unstimulated HUVEC's than transplant patients, who had no evidence of infection, suggesting that renal transplantation may reverse the activated PMN state seen in the dialysis patient. However, transplant patients with evidence of infection had PMN's which showed similar adhesion to unstimulated HUVEC's as PMN's from dialysis patients suggesting that immunosuppressive agents do not to prevent increased neutrophil adhesion during infective episodes.

PMN's from transplant patients (but not haemodialysis patients) were more adherent to Interleukin-1 stimulated HUVEC's than PMN's from normal controls. This may suggest that the PMN's from transplant patients have increased expression of surface adhesion molecules that recognise E-selectin and therefore are more adherent to endothelial cells which have increased E-selectin expression. PMN's from infected transplant patients were more adhesive than PMN's from both non-infected transplant patients and haemodialysis patients using Interleukin-1 stimulated HUVEC's. This probably reflects a general increase in the cytokine environment during an episode of infection. The greatest level of PMN adhesion was seen in PMN's from infected renal transplant patients to stimulated HUVEC's, however the percentage adhesion was not as high as seen in PMN's that had been pre-stimulated with PMA. This demonstrates that there is a large reserve of PMN adhesive potential and that in-vivo there is probably a wide spectrum of the PMN adhesive state.

The aim of this work was to study the distribution of cell adhesion molecules and E-Selectin in particular, in renal allograft biopsies taken pre-transplant, at times of allograft dysfunction and at times of normal allograft function. Previous work has found E-Selectin to be present in both pre-transplant and post-transplant renal biopsies (Fuggle et al, 1993) and rejecting cardiac allograft biopsies (Taylor et al, 1992). However,

other workers have not found E-Selectin to be present in normal kidney biopsies (Brockmeyer et al, 1993) or on biopsies from cardiac allografts (Taylor et al, 1992). We believe that these apparent discrepancies may be explained by differences in the sensitivity of the immunohistological techniques used. In this study we used an APAAP method which, on the basis of our in vitro work, we have demonstrated to be more sensitive in detection of E-Selectin than indirect immunoperoxidase staining. Using APAAP staining we have shown that some kidneys, pre-transplantation, may demonstrate enhanced E-Selectin expression on the intertubular endothelium as well as on the endothelium of larger vessels. E-Selectin was not found on glomeruli or renal tubules in these cases. The presence of ELAM-1 in some pre-transplant kidney biopsies may reflect a general increase in cytokine levels in a patient, who by definition as an organ donor, is critically ill in an intensive care setting.

The distribution of the other adhesion molecules differed from that of E-selectin in pre-transplant biopsies. VCAM was mainly seen on renal tubules and glomeruli. ICAM-1 was strongly expressed on glomeruli and inter tubular endothelium and moderately on tubules and large vessels. PCAM was found strongly on all structures except on renal tubules where it was absent. The CD4+ and CD8+ infiltrate within the graft was found to be minimal except in a few cases where cellular infiltrate was more pronounced again probably reflecting an increase in cytokine environment in a critically ill patient. It is interesting to note the different distribution of adhesion molecules within the renal allograft reflecting the varying pathways of leucocyte migration that exist.

Amongst the post transplant biopsies taken during episodes of graft dysfunction E-Selectin was detected in 74% of cases overall and in 88% of cases with acute cellular rejection. The distribution within the kidney was found to be the same as in the pre-transplant biopsies with the

majority of E-Selectin expression present on the intertubular endothelium. The intensity of staining tended to be greater in the acute rejection group than in biopsies showing acute tubular necrosis or another diagnosis. Other workers have found E-Selectin in 50% of rejecting renal allograft biopsies (342) and 29% overall of post renal transplant biopsies (341). However, in the latter study the timing of the biopsy did not necessarily coincide with a clinical event. Some groups have not found E-Selectin to be present in rejecting renal allografts (Cotran et al, 1986) or in rejecting cardiac allografts (Ferran et al, 1993). The increased E-Selectin expression in our study may again reflect a more sensitive immunohistological technique.

The other cell adhesion molecules studied, ICAM-1 and VCAM, were also more strongly expressed during episodes of acute cellular rejection than on pre and normal post-transplant biopsies.

When the E-Selectin expression was compared with the CD4 + cellular infiltrate there was a significant association between increased leucocyte presence and adhesion molecule induction in the allograft biopsies. This was particularly so in biopsies showing acute rejection. But the difference between these biopsies and ones showing acute tubular necrosis is not strong enough to suggest that the presence of E-Selectin and CD4+ cellular infiltrate could be used as a sole test in the diagnosis of acute renal allograft rejection.

In those patients who had their biopsies reported as histologically normal the E-Selectin expression was weak although CD4+ infiltration was slightly stronger. However, taken together with the pre-transplant biopsies we have shown that E-Selectin may be expressed in apparently histologically normal biopsies and that this is associated with a CD4+ cellular infiltrate. This finding raises questions about the expression of E-Selectin in vivo. In vitro work in Chapter 4 has shown that E-Selectin

expression is maximal 4-6 hours after cytokine stimulation by interleukin-1b and this expression declines over 24 hours. However, this in vitro work does not take into account the ongoing antigenic stimulation that occurs during acute allograft rejection. It seems likely that E-Selectin expression in vivo is a much longer term event than in vitro. In vivo E-Selectin expression may persist some time after an acute immune stimulus has apparently declined. To investigate this fully a biopsy would need to be taken daily post-transplantation to include episodes of dysfunction. Unfortunately this is not practical or ethical in the post-transplant patient.

With the development of monoclonal antibody technology cellular adhesion molecules are now a potential target for therapeutic intervention. Anti ICAM-1 (CD54) monoclonal antibodies have been used with some success in the treatment of renal allograft rejection in cynomolgus monkeys (Cosimi et al, 1990) and in a phase 1 trial in rejecting human renal allografts (Haug et al, 1993). The demonstration of E-Selectin in rejecting renal allografts suggests that this molecule might also be considered as a target for monoclonal antibody therapy. However, since it is not specifically up-regulated in any one condition, this may mitigate against its use as a diagnostic tool but it may be useful as a treatment during the rejection process.

The finding of E-Selectin expression in association with CD4+ cellular infiltration in renal allograft biopsies suggests that this adhesion molecule has an important role in leucocyte recruitment during episodes of graft dysfunction.

However despite the fact that the adhesion molecules have distinct roles to play in terms of the trafficking of different leucocyte populations into the tissues it is interesting that during episodes of allograft rejection, E-selectin, VCAM and ICAM-1 were all found to be

increased in intensity of expression. This suggests that the targeting of individual cell adhesion molecules with an anti-adhesion molecule antibody might not reverse allograft rejection unless all the adhesion molecules could be blocked at the same time.

There has been a lot of recent interest in defining the role of cell adhesion molecules in the process of allograft rejection. Fuggle et al (1993), studied the expression of E-selectin, ICAM-1 and VCAM-1 in needle core biopsies from 20 pre-transplant kidneys and 42 post transplant kidneys. They found variation in expression in the pre-transplant biopsies. In the post-transplant biopsies they found enhanced expression of E-selectin and VCAM-1 on endothelium and of ICAM-1 and VCAM-1 on tubules during episodes of rejection, associated with increased expression of CD45 and CD25. Brockmeyer et al (1993), studied biopsies from 10 normal kidneys and 52 renal allografts with cell mediated rejection. Expression of VCAM-1 and E-selectin was increased in rejecting kidneys and this was shown to be secondary to increased cellular mRNA synthesis. Von Willebrand et al (1995), found a strong upregulation of ICAM-1, E-selectin and VCAM-1 in rejecting renal allografts and this was associated with increased intragraft cellular infiltration. Gaber et al (1995), studied E-selectin and ICAM-1 expression in biopsies from renal allografts taken 30-45 minuits after revascularisation and found an increased amount of both in kidneys with evidence of ischaemic injury. They hypothesised that donor-recipient sensitisation might occur secondary to leucocyte margination in peritubular capillaries and suggested that anti-adhesion molecule treatments may be designed to prevent this early sensitisation.

Thervet et al (1995), studied adhesion molecule expression in pre and post transplant renal allografts. They showed that adhesion molecule expression was increased in post transplant biopsies but could not find a correlation between clinical rejection and adhesion molecule expression.

Mampaso et al (1993) studied 30 renal allograft biopsies taken at times of graft dysfunction. They found that in rejection, the interstitial cell infiltrate was facilitated by the contribution of both LFA-1/ICAM-1 and VLA-4/VCAM-1 cell adhesion mechanisms. They also found that VLA-4/VCAM-1 does not play a role in Cyclosporin nephrotoxicity. This differential expression pattern of VLA-4/VCAM-1 in rejection and Cyclosporin toxicity might provide a valuable immunohistological guide to the diagnosis of allograft dysfunction. Turunen et al (1994), found that ICAM-1 and VCAM-1 were expressed in rejecting renal allografts and also found increased receptors for L-selectin during rejection episodes.

Lautenschlager and Hockerstedt (1993), investigated ICAM-1 expression in liver allografts undergoing acute rejection using fine needle biopsy. ICAM-1 was always seen during rejection episodes and was also found to be expressed during episodes of Cytomegalovirus (CMV) infection. They concluded that ICAM-1 could be considered as an early though unspecific marker for acute allograft rejection. Sedmak et al (1994), also found that CMV could alter adhesion molecule expression. Experimenting on HUVECs infected with CMV they showed that ICAM-1 was induced 24 hours post infection but that VCAM-1 and E-selectin were not. They concluded that ICAM-1 induction on endothelial cells might represent a mechanism by which CMV infection exacerbates the recipient immune response to an allograft.

The measurement of soluble forms of adhesion molecules has been made possible by an ELISA assay now available commercially. Lebranchu et al (1995), found significantly raised levels of VCAM-1 in the serum of renal transplant patients with CMV disease. Soluble VCAM-1 levels fell during treatment of CMV with the anti-viral agant Gancyclovir but increased again in some cases after treatment ended. Kanagawa et al (1994), showed that soluble ICAM-1 levels in serum were raised during episodes of acute renal allograft rejection, but could not find a correlation between allograft

rejection and levels of sICAM-1 in serum. Lebranchu et al (1994), demonstrated an increase in sE-selectin but not, sICAM-1 or sVCAM-1, during episodes of acute renal allograft rejection. Soluble E-selectin levels normalised after treatment with OKT-3 which is a monoclonal antibody used to treat severe allograft rejection. They suggested that serial measurements of soluble E-selectin could be valuable in the diagnosis of renal allograft rejection. In contrast, Behctel et al (1994), found no sE-selectin to be present in the urine of patients with renal allograft rejection. However they did find that urinary sICAM-1 and sVCAM-1 were useful in providing information with regard to the severity and type of allograft rejection.

In liver transplantation, Adams et al (1993), showed that sICAM-1 was released within the liver during graft rejection, probably from activated lymphocytes, and was most usefully measured in the bile rather than in the serum. This was supported by Lang et al (1995), who also found that sICAM-1 was significantly excreted in the bile during episodes of liver rejection. Serum sICAM-1 and VCAM-1 increased during rejection episodes and fell with successful treatment, suggesting a useful prognostic role in the measurement of these molecules.

Finally in heart transplantation, Briscoe et al (1995), examined adhesion molecule expression in serial endomyocardial biopsies. ICAM-1 and E-selectin were most often detected just prior to a rejection epiosode. VCAM-1 and ICAM-1 were both seen during rejection episodes and declined afterwards. There was an association between CD3+ infiltrate and adhesion molecule expression in rejecting biopsies. They suggested that cell adhesion molecules might be used as an adjunct in the diagnosis of rejection.

There has clearly been increasing interest in the role that adhesion molecules play in organ transplantation, particularly with respect to

rejection. In this work it has been shown that endothelial cell adhesion molecules have an important role to play in the cellular response to a renal allograft. Cellular adhesion molecules have been investigated in-vitro and been found to be induced by cytokines and unaffected by immunosuppressive agents. PMN adhesion to endothelial cells has confirmed the functional competence of the adhesion molecules and that PMN's from transplanted patients with intercurrent infections are more adhesive than those from controls. The distribution of adhesion molecules within renal allograft biopsies has been investigated. E-selectin has been shown to be expressed during rejection episodes and that there is a strong relationship to leucocyte infiltration at times of allograft dysfunction. However it has also been shown that E-selectin is expressed during other causes of allograft dysfunction such as acute tubular necrosis and chronic rejection. This finding means that E-selectin cannot be used alone as a marker for allograft rejection and that over-interpretation of it's presence in a renal allograft biopsy must be avoided.

8.5 Future prospects

There are several areas which might be developed further.

1. In-vitro, using the flow cytometry method, other pharmacological agents could be tested to evaluate their effect on adhesion molecule expression. The effect of such agents on adhesion molecule function could then be assessed using the PMN adhesion assay
2. In-vitro the adhesion of lymphocytes to endothelial cells could be examined by adapting the PMN adhesion assay. Work investigating the lymphocyte endothelial cell adhesion pathway would have benefits in developing novel therapies for potential prevention and treatment of allograft rejection
3. Further work is required on the adhesion of PMN's from transplant patients to establish a time course of PMN activation around an infective episode. Similar studies of lymphocyte adhesion at times of acute allograft rejection would be potentially of benefit in the prediction of rejection episodes.
4. Immunohistological studies of a larger series of pre-transplant biopsies may identify grafts which are potentially activated in terms of adhesion molecule expression prior to transplantation. These grafts may be more susceptible to early immune damage and might require the use of a modified immunosuppressive regimen.
5. Pre-treatment of renal allografts with anti-adhesion molecule antibodies may help protect the graft from early leucocyte infiltration and this could be assessed using immunohistology of biopsies taken from pre-treated grafts.
6. The measurement of soluble adhesion molecules in serum, bile, or urine may prove to be highly valuable in the prediction of graft dysfunction. It has obvious advantages in that no tissue specimin is required. However furthur work is required because at present only a

generalised diagnosis of graft disorder can be made from the finding of increased soluble adhesion molecules in serum. To be really useful a specific diagnosis of rejection that would negate the need for a biopsy is required. At present the "Gold Standard" remains a biopsy producing tissue for conventional histology.

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