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ENDOGENOUS IMMUNOSUPPRESSION AND THE EFFECT OF BLOOD TRANSFUSION ON CELL MEDIATED IMMUNITY IN HAEMODIALYSIS PATIENTS

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Submitted for PhD Degree
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DECLARATION

The experimental design of this thesis was completed by the author. The preparation of the patches for the DNCB skin test, the clinical testing and scoring of the skin reactions were performed by the author.

The work on the IgG production utilising the plaque assay was carried out by Dr D Degiannis.

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SUMMARY

In order to clarify the effect of blood transfusion in uraemic patients, 48 dialysis patients who had not received previous blood transfusion were randomly allocated to receive 5 or 10 units of third-party packed cells at two week intervals. Twelve dialysis patients and 12 healthy subjects who had not received previous blood transfusion were also studied as control groups.

The cell-mediated immunity (CMI) of the transfused patients was measured by skin testing with DNCB and four recall antigens before the first transfusion and 14 days after the last unit of blood. In addition, prior to the first and 14 days after each transfusion patients were screened for a) haematological and biochemistry results, b) cytotoxic antibodies, c) complement levels, d) titres of IgG secreting cells (PFC) in the peripheral blood both spontaneously and after stimulation with PWM and SAC, using a protein A plaque assay, and e) prostaglandin E (PGE) production in supernatants from both Con A-stimulated PBMC cultures and unstimulated ones. The two control groups were screened for these parameters on 3-4 occasions over a period of 3 months. The methods and results from this randomised controlled study are described in detail in the first 8 chapters of this thesis. In Chapter 9, I describe results from a large number of patients with regard to factors determining the response to DNCB and its value as a means of predicting renal allograft survival. This retrospective analysis was done from an angle and evidence provided by the prospective study.

The aim of the study was, a) to assess the effect of blood transfusion in uraemic patients, b) to see whether patients with strong or weak reaction to DNCB differ in their response to transfusion, and try to define the optimum number of transfusions for each group to achieve a beneficial effect, c) to see whether blood transfusion-induced alterations in immune responses could be monitored with simple skin tests and d) to define possible factors determining the response to DNCB and assess the predictive value of the skin test regarding graft survival.

In the group of the previously non-transfused dialysis patients more than half were strong DNCB responders. This proportion was two fold higher compared to that of the overall dialysis population.

There was a progressive decline in strong responders as patients received more blood, and in multi-transfused patients CMI was profoundly suppressed. Multivariate analysis revealed that the most significant factor associated with the response to DNCB was blood transfusion. A number of factors did not show any association, namely age, sex, primary renal disease, type of dialysis, parity, ABO and rhesus blood groups, interval between last transfusion and test, cytotoxic antibodies and a number of haematological and biochemical parameters.

HLA-DRW6 positive patients were more likely to be strong responders when tested prior to any blood transfusion, but the DRW6 effect was not observed in patients sensitised after one or more transfusions. This suggests that HLA-DRW6 is a marker of strong CMI, but this strong response can be modified by transfusion.

Sequential skin testing with DNCB and recall antigens did not reflect changes induced by transfusion, as repeated immunisation resulted in stronger anamnestic responses, regardless of the amount of blood or whether they were transfused at all. Follow-up DNCB skin testing showed that blood transfusion at the time of sensitisation to DNCB was a crucial event for secondary responses to DNCB. Patients eliciting a strong primary response retained and gave even stronger responses on subsequent tests regardless of transfusion status at the time of sensitisation to DNCB. Contrary to that, weak responders who were sensitised after transfusion showed a further depression in their secondary responses, while weak responders sensitised prior to any transfusion were more likely to show increased secondary responses. As a result of this, approximately one third of the latter patients changed to strong responders in follow-up tests.

The predictive value of the DNCB test with regard to graft survival in patients treated with azathioprine and prednisolone was best expressed when the last test prior to transplantation was used. Weak DNCB responders had significantly better graft survival compared to strong responders over a five year follow-up. This effect on graft survival was independent of the blood transfusion effect and was most clearly seen in patients receiving 1-5 units of blood. In transplant recipients treated with cyclosporin the DNCB test did not have any predictive value.

The risk of sensitisation to HLA antigens was negligible with 3-4 units of blood, but increased with more transfusions, particularly in women. Strong DNCB responders developed cytotoxic antibodies earlier, but after 5 units both rate and level of panel reactive antibodies was comparable between weak and strong DNCB responders.

Transfusion of 1-10 units of blood did not induce any measurable changes in serum complement levels and the difference between weak and strong DNCB responders was not significant. Serum C3, C4, factor B and C1 inhibitor levels did not correlate with IgG production either spontaneously or after stimulation with SAC or PWM prior to and after transfusion.

Blood transfusion had a suppressive effect on IgG production in the PWM-driven system, but not after stimulation with SAC. As the former system is dependent on suppressor cell activity, whereas the latter is not, it is likely that the blood tranfusion-induced suppression was due to activation of a non-specific suppressor cell system. This activation was evident after the second transfusion and was equally induced in weak and strong DNCB responders, whose response did not change significantly with more than 5 units of blood. Subgroups of patients with a high or low rate of IgG production, both spontaneously and after stimulation with PWM or SAC were observed, but the DNCB skin test failed to identify these patients.

Blood transfusion induced a significant increase of PGE synthesis in Con A-stimulated PBMC supernatants which was more pronounced after the 3rd unit of blood. The difference in PGE concentration between weak and strong DNCB responders prior to and after each of 1-10 transfusions was not significant. A further 5 units of blood did not have any significant effect on PGE release in comparison with the initial five transfusions. PGE concentrations did not correlate with IgG production in the peripheral blood either spontaneously or after stimulation with PWM or SAC. This finding suggests that the blood transfusion-induced suppression of PWM-IgG production was not mediated by PGE. However, patients who were successfully transplanted showed a significant increase in PGE release after the last elective pre-transplant transfusion, as

opposed to a small number of patients with irreversible rejection in whom such an increase was not observed. These findings suggest that multiple mechanisms might be responsible for the blood transfusion effect on graft survival, one of which might be mediated by PGE.

ABBREVIATIONS

ADCC	Antibody dependent cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ATG	Antithymocyte globulin
AZ	Azathioprine
BT	Blood transfusion
cAMP	Cyclic 3.5 adenosine monophosphate
CMC	Cell mediated cytotoxicity
CMI	Cell mediated immunity
CML	Cell mediate lympholysis
Con A	Concanavalin A
CPM	Counts per minute
CR	Complement receptors
CTS	Collaborative transplant study
CYA	Cyclosporin A
DNCB	2,4-dinitrochlorobenzene
DST	Donor specific transfusion
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
ERFC	E-rosette forming cells
FcR	Fc receptor
GVHR	Graft versus host reaction
H-2	Histocompatibility 2
HLA	Human leukocyte antigen system A
IL-1	Interleukin 1
IL-2	Interleukin 2
Ir genes	Immune response genes
ISC	Immunoglobulin secreting cells
KLH	Keyhole limpet haemocynain
LC	Langerhans cell
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MHC	Major histocompatibility complex
MIF (T)	Migration inhibition factors (test)
MLC (R)	Mixed lymphcoyte culture (reaction)
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cell
PFC	Plaque forming cells
PG	Prostaglandin
PGE	Prostaglandin E series
PHA	Phytohaemagglutinin
PITS	PG-induced T cell derived suppressor
PPD	Purified protein derivative
PRA	Panel reactive antibodies
PWM	Pokeweed mitogen
RES	Reticuloendothelial system
SAC	Staphylcoccus aureus, Cowan I
SRBC	Sheep red blood cell
TPT	Third party transfusion
UCLA	University of California, Los Angeles

INTRODUCTION

I THE CELLULAR BASIS OF IMMUNITY

Overview

Immunity is concerned not only with resistance to infectious agents but also to other foreign agents including toxins, living cells and host cancer cells. The word itself derived from the Latin 'Immunitas' which originally meant 'freedom from public service' and later came to mean freedom from disease. The most striking thing about immunity is the specific nature of its enhancement after the recognition and response to what is regarded as foreign. It soon appeared, however, that such specific immune responses might confer unpleasant and harmful effects on subsequent exposure to the provoking antigen. Von Pirquet in 1906 pointed out that what develops after exposure to antigen is an altered reactivity recognised as immunity or hypersensitivity. This changed reactivity he called allergy (1). Von Pirquet's concept was fundamental because behind all human responses he established a common process of specific sensitisation regardless of clinical outcome. To accomplish this, mammalian organisms possess a self-regulating system, involving organs, cells, proteins and numerous low-molecular weight signals.

Immune responses may be classified into two categories, namely, specific and non-specific. Specific immune responses depend upon exposure to a foreign configuration and the subsequent recognition and reaction to it takes place in a highly discriminatory fashion. Non-specific responses, on the other hand, are not dependent upon specific recognition and merely repeat the same general response to the substance. Thus, specific responses are characterised by specificity, heterogeneity and memory which include the induction and interaction of a variety of cells specific for the inducing antigen, while the non-specific immune responses include a limited number of pre-existent cells which are not characterised by the property of memory.

When antigen enters the body two different types of adaptive immunological reactions may occur, namely those dependent on antibody (humoral responses) and those dependent on sensitised lymphocytes

which are themselves the effectors of cell mediated immunity (CMI). These two different types of responses are indicative of the basic division of lymphocytes into B and T cells. This two-compartment concept of immunological responses is interwined in a complex manner, but the distinction has been retained to simplify description. B and T cells interact with each other and with the antigen presenting cells (APC) and are found throughout the body in the blood and lymphoid tissues (2).

The organs that comprise the lymphoid system are classified as primary (central) or secondary (peripheral). The primary organs are the bone marrow, the foetal liver and the thymus, representing the sites where cells derive from a common pluripotent stem cell and give rise to two distinct progenitors for the lymphoid and myeloid cells (3,4). The lymphocytes generated in the central organs differentiate into mature B and T cells in an antigen-independent fashion and then migrate to the secondary organs, namely, the spleen, lymph nodes and the non-encapsulated accumulations of lymphoid tissue thoughout the body, where the specific immune reactions take place. The myeloid cells differentiate into phagocytes which include the granulocytes (neutrophils, eosinophils and basophils) and the mononuclear phagocyte system (monocytes and macrophages).

Lymphocytes

Glick et al in 1956 showed that chickens whose bursa of Fabricius was removed in the neonatal period had a depressed antibody response to salmonella antigens, but rejected foreign grafts, indicating that CMI was intact (5). The converse was achieved in thymectomised neonatal chickens (6) and mice (7). These experiments provided the first evidence of the two-compartment concept of immune mechanisms. Similarities between experimental models and patients with selective immune deficiencies emphasised further this concept (8) and led eventually to the T- (thymus derived) and B- (Bursa-equivalent derived) cell nomenclature (9).

Tests to characterise T and B lymphocytes by their surface markers rather than their functional properties have been extensively developed over the past 10 years. The differences are expressed in cell surface structures which can be identified by suitably labelled

antisera or other reagents (10-12). There are also relative physical and histochemical differences between T and B cells which do not completely distinguish them, but together with the better defined membrane binding sites or receptors provide a means of separating lymphocytes into varying degress of homogeneity (13).

B lymphocytes

B lmphocytes represent about 5-15% of the circulating lymphoid cells. They express a number of surface markers which are characteristic of both animal and human B cells but not resting T cells (14-16). B cells were identified as cells bearing surface immunoglobulin (sIg) namely IgM, IgD, or both IgM and IgD as their surface receptors (17). Nearly all B cells have Fc receptors (FcR) for IgG (18), and two types of surface receptors for complement (CR), CR1 which is the receptor for C4b and C3b, and CR2 which is the receptor for C3d (19). The majority of B cells can also be characterised by the presence of class II MHC Ia (mouse) or HLA-DR (human) antigens (20), receptors for Epstein-Barr Virus (EBV) (21), and for mouse erythrocytes (22).

The classification of B cells based on their stage of differentiation has been reviewed in detail (23). The first recognisable human B lymphocytes, called pre-B cells, appear in the foetal liver during the seventh week of gestation. They contain small amounts of cytoplasmic but not surface IgM, and bear specific B cell antigens and Ia-like determinants, but lack FcR and CR. Immature B cells appear at week 8 of gestation in the liver and bone marrow and they possess surface IgM but few FcR or CR. These cells by the onset of the third trimester reach adult proportions and develop into 'virgin' B lymphocytes bearing sIg, Ia, FcR and CR. Stimulation by antigen, together with some signal from T cells, induces proliferation and differentiation into immunoglobulin secreting cells (ISC) which are called plasma cells.

T lymphocytes

T lymphocytes have a variety of functions in the host defence system and consequently there are different subpopulations, all of which are characterised by specific cell surface antigens consisting

of membrane glycoproteins. Thus, Thy-1 is expressed by all mouse T cells, whereas most human T cells express three surface markers, namely, CD2 (synonyms, T11, Leu5, E rosette receptor), CD3 (T3, Leu 4), and CD5 (T1, Leu1) (24-26). Human T cells can also be identified by their ability to bind and form rosettes with sheep erythrocytes, called E rosetting (27). Other specific cell surface antigens are acquired during differentiation and allow further distinction of T cell subsets responsible for helper (Th), cytotoxic (Tc), and suppressor (Ts) functions, and also regulatory functions (25,26,28-30).

Mouse Th cells express the Qa1, and the Lyt-1, L3T4 antigens which are equivalent to the CD5 (T1, Leu1) and CD4 (T4, Leu3) markers in the human. Th cells are essential for normal activation of B, Tc and Ts cells. By means of the clonally restricted CD4 receptor, they recognise foreign antigen in association with HLA class II antigen on accessory cells.

Ts and Tc cells bear the Lyt-2,3 in mouse or the CD8 (T8, Leu2) antigen in man. Ts cells regulate the T-dependent antibody production or the generation of Tc cells either by cell to cell contact or by production of specific and non-specific soluble mediators. Tc cells are the effector killers against nucleated cells and parasites.

Murine Ia and human HLA-DR antigens are expressed only on T cells when they are activated by antigens or mitogens. All activated human T cells express the interleukin 2 (IL-2) receptor (CD25, TAC). Human T cell subsets can also be separated functionally on the basis of the presence or absence of FcR for either IgG or IgM (31). In addition to these primary markers, one per cent of E-rosette forming cells (ERFC) also express receptors for C4b and C3b (32,33), and there are a number of other surface receptors and antigens the functional significance of which is unknown.

'Null' cells

A third population of human PBMC, the 'null' cells are both E rosette negative and sIg negative (34). Null cells are subdivided on the basis of the presence or absence of either FcR and/or CR (35).

IgG FcR-positive null cells functionally are the predominant antibody dependent cellular cytotoxic cells (ADCC) (36), and may also manifest natural killer (NK) activity against tumour cells (37).

Mononuclear phagocyte system

Mononuclear phagocytes represent a distinct class among PBMC, arising from monoblasts and promonocytes in the bone marrow. Promonocytes develop into monocytes, which after 24-48 hours leave the circulation and migrate to tissues where they become macrophages (38). The term 'macrophages' was given by Metchnikoff in 1892 because they were able to ingest large particles. In 1924 Aschoff introduced the term reticuloendothelial system (RES) to describe the whole spectrum of wandering and fixed mononuclear phagocytes. Apart from the well established role of these cells in defence mechanisms through phagocytosis and killing of micro-organisms, the excitement about their immunological function was triggered by Fishman and Adler in 1963 (39). Later Unanue and Askonas, and Mitchison took the concept further when they showed that antigen associated with live macrophages was capable of producing a strong immune response (40,41). Since then an extensive literature with excellent reviews has defined the role of macrophages in the antigen presentation which leads to antibody production and T cell stimulation, regulation of T cell differentiation, and secretion of the molecules that can regulate lymphocyte function (42-44).

Approximatley 90% of monocytes and macrophages have Fc receptors for IgG (45) while CR for C4b and C3b (CR1) have been described on both monocytes and tissue macrophages and receptors for C3d (CR2) have been found only on monocytes (46). In addition, monocytes and macrophages also have receptors for lymphokines, such as γ-interferon and migration inhibition factor (26).

The diversity of functions assigned to macrophages in the development and expression of immune responses led to the concept that they, like lymphocytes, consist of a heterogeneous cell population. For example only some human macrophages possess receptors for IgE (26). Also since Rosenthal and Shevach showed that the interaction between Th cells and antigen presented by macrophages takes place in an MHC-restricted fashion (47), it has been

established that only a small proportion of activated macrophages bear Ia and HLA-DR surface molecules (26,48). This subpopulation of macrophages is referred to as antigen presenting cells (APC).

Antigen presenting cells

In their classical work in guinea pigs, Rosenthal and Shevach examined the function of immune response (Ir) genes at the level of antigen presentation, using synthetic co-polymers composed of L-glutamic acid plus L-lysine (GL) and L-glutamic acid plus L-tyrosine (GT) as antigens in the activation of Th cells (47). They found that although T cells were capable of responding to either GT or GL, they did so only when the appropriate Ia bearing macrophage presented the antigen. Subsequently, further studies in the mouse and in humans established that T cells, unlike B cells, are not activated by direct interaction of their receptors with soluble antigens, and also the interaction between APC and the responding T cells is Ia restricted, that is, the clones of T cells and APC must share the same allelic form of their class II molecules (44,49).

In the past ten years knowledge of the role of APC in the induction of immune responses has greatly increased. Thus it has become clear that the capacity of APC to synthesise and express Ia antigen on their surface is not constitutive, but varies depending on the state of maturation, on the duration of stimulation and on the balance of stimulatory and inhibitory conditions. Therefore it seems that the Ia marker is not a stable surface molecule, but represents a specific stage of cell activation (44). For example, in murine tissue cultures, Ia positive macrophages synthesised Ia briefly and the period of Ia expression was within the range of 12-48 hours. Subsequently, these cells rapidly become Ia-negative and lost both their antigen presenting function and their capacity to stimulate in a mixed lymphocyte reaction (MLR) (50,51). In humans, the expression of Ia molecules by monocytes also decays in culture, and may be increased by γ-interferon and inhibited by endotoxin and zymosan (52-54). Also, prostaglandins of the E series (PGE), alpha-fetoprotein and glucocorticoids have been found to inhibit Ia expression (44, 55, 56).

Another interesting point which has emerged from recent studies is that each tissue has a characteristic ratio of Ia-positive to Ia-negative macrophages, probably reflecting microenvironmental influences of positive and negative regulatory signals. The production of Ia-positive macrophages in various tissues has been reviewed recently (44).

The stimuli for Ia induction can either be T-independent or T-dependent, the latter consisting of lymphokines secreted by activated T cells. The major lymphokine responsible for Ia induction is γ-interferon (57), which seems to behave in a non-specific manner in that it is not restricted by the MHC haplotype of the responding cell. The increase in Ia-positive cells takes place under all conditions that result in T cell activation, particularly in infections with intracellular pathogens like Listeria monocytogenes (58), Mycobacterium tuberculosis (59), and Trypanosoma cruzi (60). The findings from these and other studies (61,62) show the excellent correlation between the number of Ia-positive macrophages and the degree of antigen-dependent T cell stimulation, which in turn, through an amplification loop of cellular interactions, calls for more Ia-positive macrophages promoting the presentation of antigen.

During the last few years, increasing interest has been focused on the intracellular events of transport and degradation of antigens by APC. This function has been termed antigen processing (reviewed in 44,49,63,64). The initial antigen processing studies were carried out in Unanue's laboratory using the bacterial agent Listeria monocytogenes. It was shown that endocytosis of the antigen by APC was followed by an active metabolic step involving trafficking of the antigen to lysosomes where proteolytic cleavage takes place. The processed antigen is then recycled on the surface membrane after a lag period that takes approximately one hour to complete (44,49,63). Treatment of APC with ammonium chloride or chloroquine completely abrogates the processing and presenting capacity of the treated cells indicating that during processing, the antigen must encounter an acidic intracellular compartment. These results have been confirmed in many antigen systems, and it is now accepted that bacterial antigens and small globular proteins need to be processed (49,63). However, there are antigens which do not require processing including

in humans the large protein fibrinogen (63). This finding suggests that it could be the nature of the antigen that dictates the requirement for processing. The determinant recognised by fibrinogen-specific T cells has been located on a portion of the peptide chain which has enough conformational freedom to allow the segregation of the binding sites of the interacting cells. Thus, it seems that the requirement for antigen processing by APC might be dependent on the configuration of T-cell determinants. Those which require proteolytic cleavage might be those which need conformational flexibility to form the correct Ia and T cell contact structures (63).

As there are a variety of cells other than macrophages that express Ia molecules, the question is whether all these Ia-positive cells can function as APC. B cells constitutively express Ia on their surface (20,44) and can present some antigen to T cells in a MHC restricted fashion (65). Other Ia-positive cells with proven APC function include the Langerhans cells, the Kupffer cells in the liver, the dendritic cells in the spleen, the veiled cells and interdigitating cells in the skin and lymph nodes, and the skin keratinocytes. This last type of cell is normally Ia-negative, but following activation is capable of expressing Ia and is shown to produce a soluble cytokine similar to interleukin-1 (IL-1) (reviewed in 49).

The Langerhans cells have an important role in delayed-type hypersensitivity (DTH) reactions and will be discussed in detail later.

Lymphokines

Lymphokines have been defined as non-immunoglobulin secretory products of activated lymphocytes and macrophages with a wide range of potent physiological effects in inflammation and immunity. There is enough evidence now to suggest that they represent hormone-like intercellular agents that both positively and negatively regulate interactions between T and B cells and macropahges, and non-lymphoid tissues.

The initial demonstration that soluble mediators could be liberated by activated lymphocytes was a by-product of research into the mechanisms of DTH. In 1928 Rich and Lewis showed that migration in culture of tuberculin-sensitised cells from spleen and lymph nodes was inhibited when tuberculin was present in the culture (66). Until the late 60's it was not known how the interaction of sensitised lymphocytes with antigen triggered the local inflammation seen in DTH reactions. Only after the discovery of the first lymphocyte-derived product, the macropahge migration inhibition factor (MIF), by Bennett and Bloom and David (67,68), was it realised that DTH responses are mediated by soluble agents released locally by antigen-activated lymphocytes. In the following years, a number of additional lymphocyte-derived factors were described which were collectively termed 'lymphokines'. The first macrophage-derived soluble helper factor was described by Bach et al in 1970 and subsequently a variety of both helper and suppressor agents were described in culture supernatants (69). By 1977 Waksman in a comprehensive review had listed 98 named lymphokines and monokines (70). As most studies on lymphokines utilised supernatants from antigen or mitogen-stimulated cultures which contained a crude mixture of molecules rather than biochemically pure entities, it could be safely assumed that some of the identified lymphokines represented different forms of the same molecular substances which express different activities depending on the nature of the assay. For example, IL-1 has been variously described as lymphocyte activating factor (LAF), endogenous mediator of fever, the co-mitogen for thymocytes, the stimulant for cartilage resorption, and the stimulant for muscle wasting. Only recently have all these mediators been attributed to a single factor ie IL-1 (71).

Although lymphokines are defined in terms of their immunological properties, the production of some of these factors is not limited to lymphoid cells, but extends to other cell types such as fibroblasts, kidney cells, and other cell lines, and they may regulate growth and division in non-lymphoid tissues (72). With regard to lymphokine production by lymphoid cells, it seems that both T and B cells are capable of producing most lymphokines and those from both sources appear to have similar functional properties while macrophages may also produce lymphokines such as interferon.

Although non-lymphoid cells can produce some lymphokines, the synthesis of others is restricted to T cells, B cells, or macrophages (70,72).

As lymphokine production is the result of cell activation, it is essential to understand the nature of the activating event, since this is the major determinant of whether T or B cells will be the source of a specific lymphokine. In other words the process of activation of lymphokine production is similar to the activation that results in cell proliferation or antibody production. Thus, T-cell polyclonal activators, such as phytohaemagglutinin (PHA) and Concanavalin A (Con A), preferentially induce T-cell lymphokine production, whereas stimulation with B cell polyclonal activators, such as pokeweed mitogen (PWM) and bacterial lipopolysaccharide (LPS), induce B-cell lymphokine production (72).

Lymphokines affect a variety of cells including lymphocytes, macrophages, neutrophils, basophils, eosinophils, thymocytes, bone marrow cells, platelets, fibroblasts, osteoclasts and endothelial cells (69). Thus their precise classification is difficult since a single factor may have different effects on different target cells. Nonetheless, attempts have been made to group lymphokines according to their cellular source (72), by the nature of their target cells (73), and by their immunological properties, ie whether they are specific or non-specific mediators, restricted or unrestricted by MHC, and whether they are helper or suppressor factors (70).

In the following section I shall discuss in detail one of the many lymphokines, the prostaglandins.

Prostaglandins

In 1930 two American gynaecologists, Kurzork and Lieb, made the original observation that uterus strips in contact with human sperm either contract or relax (74). These results were confirmed a few years later by Goldblatt (75) and by von Euler, who showed that the responsible substance was a lipid which he called 'prostaglandin' (76). After thirty years of research, during which some hundred kilograms of vesicular glands from young Icelandic sheep were used to

provide the PG standard, Bergstrom and Samuelsson, and van Dorp et al identified the chemical structure of prostaglandins (PG) and elucidated their biosynthesis from arachidonic acid (77,78).

Chemistry and metabolism

All PG molecules are 20-carbon unsaturated fatty acids including a cyclopentane ring and are synthesised within or close to the cell membrane by cyclo-oxygenase activity. They are synthesised in nearly every tissue of the body and all types of cells can respond to them.

The most widely known fatty acid precursor is arachidonic acid which is stored in the phospholipid portion of cell membranes. Following the activation of endogenous phospholipases, arachidonic acid is released from phospholipids and through the cyclo-oxygenase enzymatic pathway, PGG_2 and PGH_2 are generated; the subscript denotes the number of double bonds in the molecule, ie PGG_2 has two double bonds. PGG_2 and PGH_2 are called endoperoxidases, are short lived and are metabolised to form the six primary prostanoids, namely PGE_2, PGF_{2a}, PGD_2, PGI_2 or prostacyclin, TXA_2 or thromboxane, and a 17-carbon cleavage product, hydroxyheptadecatrienoic acid (HHT). PGE and PGF are rapidly converted to inactive 15-keto-, and 15-keto-13, 14-dihydrometabolites by a dehydrogenase which was first identified in the lung but subsequently has been found in several tissues. These sequential enzymatic conversions occur with such great efficiency that in a single passage of serum-borne PG through the lungs, virtually all PGE is quickly removed from the circulation. This accounts for the short biological half-life of PGs, which, because of this, have been considered as 'local hormones' having essentially no systemic action (reviewed in 79-82).

The generation of PGG_2 from arachidonic acid can be blocked irreversibly by aspirin and reversibly by non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, which blocks the action of cyclo-oxygenase. This important finding was made by Vane et al in 1971 and since then numerous publications have confirmed the anti-inflammatory action of NSAIDs resulting from inhibition of PG synthesis (reviewed in 83).

PGE can be converted to PGA and PGB through isomerases which are normally present in serum (81). The metabolism of PGF_{2a} has been studied in man with tritium-labelled material and it was found that 90% of the administered substance can be recovered in the urine within about five hours. Of the 14 or so urine excretion products known to man, all are dinor or tetranor analogues which are generated by further metabolism through side chain oxidation of the 15-keto, 13,14 dihydro metabolite compounds (82).

Isolation and measurement

Because some intermediate PG metabolies do not remain chemically intact in aqueous solvents, there are restrictions on which compounds can be isolated and measured. The relatively stable end-products of PG synthetase adequately reflect the synthesis of their respective biologicaly active parent compounds, such as PGE_2, PGF_{2a}, PGI_2, or TXA_2 (81).

Sample material is best stored at -15°C to -20°C following extraction from aqueous solvents. The extraction step from aqueous media is facilitated by the lipid solubility of PGs and favoured by acidification of the aqueous media, ie with citric acid. For samples with significant contamination by neutral lipids, initial extraction with petroleum ether is carried out, and following this, separation of the classes of PG compounds serves to lessen the requirement for highly specific anti-PG-antiserum (81).

The most frequent method used for chemical separation has been column chromatography, utilising silicic paper (81,84). For analytical purposes, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas-liquid chromatography with mass spectroscopy (GLC-MS), radioimmunoassay (RIA), coupled enzyme analysis, receptor binding, spectrofluorometric and isotope dilution assays have all been employed to measure PG compounds with varying success regarding accuracy and reproducibility (reviewed in 81, 84). However, quantitative determination of PGs has to date relied mainly on two bioassays, namely GLC-MS and RIA. The adaptability of RIA to most laboratories and the high degree of

sensitivity and reproducibility due to available specific anti-PG antisera made RIA the favoured method for measuring PG levels. This procedure is described in detail in Chapter 7.

Cellular sources of PG production

Most leukocytes can produce PGs, including macrophages, T lymphocytes, granulocytes - in particular neutrophils and to a much lesser degree eosinophils, platelets and possibly B lymphocytes (85, 86). However, leukocytes in various locations of the body produce different quantities of PGs and the significance of PG synthesis by each cell type is not clear. Thus, PGE production by granulocytes is usually much lower than that of macrophages and does not persist for periods longer than 6-8 hours in vitro (85). It is conceivable that PGE release from neutrophils may be primarily responsible for the rapid pro-inflammatory effects, such as vasodilatation and potentiation of vasopermeability, oedema formation and pain, which occur during acute inflammation. By contrast, PGE release from macrophages may be involved predominantly in the longer lasting immunoregulatory circuits which, as will be discussed in the next section, may represent the anti-inflammatory effects of PGs (83, 85).

In comparison to other immune cells, the monocyte/macrophage cells are the major source of PGs, particularly PGE_2 in human peripheral blood (81,83,85,86). The macrophage membrane is rich in precursor fatty acids and phospholipase A1 and A2 which can be activated by several physical and chemical stimuli. To date zymosan, antibody-coated red blood cells, phorbol myristrate acetate, Con A, PHA, LPS, colchicine, silica, carrageenan, dextran sulphate, Fc fragments of IgG, immune complexes, endotoxin and colony stimulating factor have been shown to trigger macrophage PG synthesis (81,85,86). The available evidence clearly indicates that PGs are not stored within cells, but following stimulation are freshly synthesised and instantly released into the extracellular space (80,81,85,86). In that sense PG release and PG synthesis are interchangeable terms.

Measurements of PG synthesis have shown that PGE_2 and TXA_2 are the major products in guinea pigs, mouse and man (81). Isotopic labelling with tritiated arachidonic acid showed that the label is rapidly incorporated into the macrophage cell membrane, is equally

rapidly converted in the cell and is then recovered as tritiated PGE_2, 6-ketoPGF_{1a}, PGF_{2a} and TxB_2. Less than one per cent of the label can be identified as intracellular PG, confirming that virtually all the cyclo-oxygenase products are rapidly secreted (81).

Although there is a basal level of PG synthesis by monocyte/macrophage cells, production increases markedly upon stimulation. Phagocytosis of latex particles does not induce PG synthesis above basal levels but bacteria coated with IgG or complement or both are more efficient stimuli (85). These findings suggest that the phagocytic process itself is not responsible for initiation of PG production, but some alteration on the membrane surface or cell damage is required to potentiate PGE release. Similarly, PGE synthesis by lymphocytes is minimal unless the cells are stimulated by antigens or mitogens to undergo blastogenesis (87).

Human peripheral blood lymphocytes and rat thymocytes have specific membrane receptors for one or more PG types, but restricted distribution of these receptors helps to maintain local specificity of PG actions (88,89). Goodwin et al found specific reversible binding for ^3H-PGE_1 and PGE_2 (but not for ^3H-PGA, F_{1a} or F_{2a}) which could not be inhibited by the addition of 10- to 1000-fold greater amounts of unlabelled PGA, F_{1a} or F_{2a} (88).

Binding of PGs to membrane receptors has long been associated with activation of adenylate cyclase and elevation of intracellular cyclic 3,5-adenosine monophosphate (cAMP) (86,90). As the increase in cAMP levels have been implicated in the regulation of leukocyte activation (91), in vitro experiments using PGE have been widely used to establish the link between the functional properties of PGE and cAMP. However, in addition to cell surface receptors for PGE, there exist active mechanisms for the uptake of PGE into many animal tissues both in vivo and in vitro. This uptake can be inhibited by metabolic poisons, inhibitors of biotransport such as probenecid and anti-inflammatory drugs (92). Thus, it does not necessarily follow that all effects of PGs on immune function are accompanied by changes in cAMP levels. In fact with such evidence of functional heterogeneity in the lymphocytes which are involved in the expression of immunity, it would hardly be surprising for these cells to exhibit functional differences in terms of control mechanisms for their

secretory actions. Adenylate cyclase activation can act as a bidirectional control system with both activation and suppression of lymphocyte secretory processes (93), and this can explain some of the functional differences seen in lymphocytes.

Regulation of immune responses by PGs

In the early 1970s, PGs were linked with inflammation in studies which showed that the mechanical irritation of rabbit eyes released a substance called 'irin' into the anterior chamber of the eye. This substance was later identified as a mixture of PGE_2 and PGF_{2a} (94). However, the role of PGs in the development of inflammatory responses was not generally accepted because they did not reproduce two important cardinal signs of inflammation, namely, increased vascular permeability and pain. The answer to this came a little later when it was shown that PGs enhanced the oedema and pain producing effects of other mediators of inflammation such as histamine, bradykinin and C5 (83). On the other hand, Vane and his colleagues had already established the anti-inflammatory role of NSAIDs by their inhibition of PG synthetase. Thus in the mid 1970's there was abundant evidence showing that PGs were potent mediators of inflammation. In particular, PGs of the E series were found to produce vasodilatation in most systems, to cause fever when injected into the central nervous system of experimental animals and to potentiate the inflammatory effects of certain other mediators. Also they were found to be increased in inflammatory exudates, and their biosynthesis was strongly inhibited by NSAIDs (95).

In spite of the evidence that PGs have pro-inflammatory properties, many reports suggested that PGs may act as anti-inflammatory agents as well. PGs of the E series were found to be potent activators in leucocytes of membrane adenylate cyclase leading to an increase in intracellular cAMP (86,90). Bourne et al measured the release of histamine as an indicator of basophil function and showed that β-adrenergic catecholamines, PGEs and histamine itself stimulated the accumulation of cAMP by activating adenylate cyclase and reducing histamine release (90). At the same time a large number of reports supported the concept that an increase in cAMP in leukocytes served as an intracellular signal for

inhibition (reviewed in 91). Functions such as stimulation of lymphocytes, cytotoxicity, antibody and lymphokine production, release of lysosomal enzymes, killing activity against candida and enzyme induction could consistently be suppressed by raising intracellular cAMP levels (83,85). These findings led to the suggestion that cAMP may be a potent intracellular modulator of leukocyte activities and that agents such as PGE which increase intracellular cAMP may thereby regulate the character and intensity of inflammatory and immune responses (90). This may seem contradictory to the observations supporting the pro-inflammatory role of PGs. However, it seems that PGs have opposing effects on inflammation under different circumstances or at different times during an inflammatory response. The rapidly occuring pro-inflammatory effects may not primarily affect lymphocytes but rather other cell types that are active in inflammed tissue. It has been suggested that the puzzling role of PGE as a pro- and anti-inflammatory mediator may be temporally related to acute and chronic states of inflammation respectively and in both, PGE may act as a homeostatic agent to secure a well balanced immune system (83,85).

In 1971 Smith et al showed that PGE_1 and PGE_2, when added to human PHA-stimulated PBMC could suppress DNA synthesis (96). Thereafter, many investigators using PGs in various experimental systems demonstrated their inhibitory role on the immune system. The list of these inhibitory actions include:-

1) suppression of in vitro humoral immune responses to SRBC

2) inhibition of mitogen stimulation

3) inhibition of antibody-dependent cytotoxicity

4) inhibition of cellular cytotoxicity

5) inhibition of MLR

6) inhibition of lymphokine and monokine secretion

7) inhibition of antibody secretion (97).

In addition to this list, glomerulonephritis in NZB/W hybrid mice, a model for human SLE, was effectively suppressed in animals treated with PGE_1 resulting in prolonged survival of the mice (98). In another report, allografted kidneys in rats treated with a stable analogue of PGE-inhibited splenic T cells resulted in increased survival of graft recipients and improved graft function which was coincident with the suppression of splenic T cell cytolytic activity (99).

In view of the inhibitory actions of PGE it was not surprising that addition of inhibitors of PG synthesis often produced a marked increase in lymphocyte proliferation. Studies using specific PG synthetase inhibitors in different models have resulted in enhancement in a variety of ways including the in vivo primary responses to SRBC, DNA synthesis in response to T cell mitogens, DNA synthesis in mixed lymphocyte responses, in vivo secondary antibody responses and an enhanced DTH reaction (86,92,97). However, it should be noted at this point that the use of indomethacin to block in vitro and in vivo PGE synthesis has certain limitations. Firstly, it has sometimes been used in too high a concentration (more than 5ug/ml) which is toxic to lymphocytes (92). Secondly, PGE synthetase inhibitors also have other actions such as inhibition of cAMP-dependent-protein kinase and phosphodiesterase. Finally, indomethacin, aspirin and the other similar agents inhibit the entire cycloxygenase pathway including the synthesis of thromboxane and prostacyclin, the immunological relevance of which is less well defined (85,92). Thus, any effect ascribed to PGE synthesis through indirect experiments with PGE synthetase inhibitors should be confirmed by abrogation of these effects when exogenous PGE is added in amounts found in control cultures.

T lymphocytes are probably heterogeneous with regard to their sensitivity to PGE. Stobo et al showed that PGE-induced suppression of blastogenic response was 60% in a T cell population of high density, while populations of intermediate density cells were inhibited by only 20%, and populations of low density cells even showed an enhanced blastogenic response (100). This last finding suggests that PGE should not be regarded as a simple, overall inhibitory agent as this inhibition represents a net result of different T cell subsets, the sensitivity of which to PGE largely depends upon cell cycle events. Suppressive effects of PGE were usually noticed during the early phase of lymphocyte activation and addition of PGE after 12-16 hours of culture no longer caused significant suppression of DNA synthesis (85,86,92). The available evidence indicates that the lymphocyte response to PGE may be determined by the lymphocyte subclass tested, the time of addition of PGE, and the amount and type of mitogen employed. Mitogens stimulating T cells such as Con A or PHA appear to be more easily suppressed by PGE than mitogens like PWM which stimulate B cells (92).

With regard to PG-induced regulation of B cell activation it appears that there are species differences. In both the rat and mouse, antibody responses to SRBC measured by plaque forming cells (PFC) are inhibited by PGE, whereas in rabbits and humans the effect of PGE on IgG and IgM synthesis appears to be stimulatory (101).

Despite the well established immunosuppressive effect of PGE under in vitro conditions, there are few diseases which have been influenced by the effects of PGE in vivo. PGs are known to be released by tumour cells and this may be responsible for tumour-induced suppression of the host immune response as suggested by Plescia et al (102). The best example of this principle in disease states is Hodgkin's lymphoma which is characterised by profound defects in CMI. Macrophages from patients with Hodgkin's lymphoma produce PGE at 4 times the normal rate and the reduced response of lymphocytes to mitogens can be entirely reversed in vitro by indomethacin or removal of glass adherent cells from lymphocyte cultures (92). Other diseases which have been associated with either excessive PGE production or altered PGE sensitivity include

rheumatoid arthritis, sarcoidosis, chronic fungal infections, tuberculosis, multiple sclerosis, juvenile periodonitis, Kaposi's sarcoma, extensive burns, graft-versus-host disease, allergic encephalomyelitis and diseases associated with autoimmunity and old age (85,92.94).

The highly specific cell interactions during an immune response are governed by gene-coded surface structures (Ia antigens), IL-1, IL-2 and the activities of a spectrum of lymphocyte subsets with identifiable surface characteristics. The question arises whether an immune system which is responding specifically also requires additional modulation by non-specific factors such as PGE. Convincing experimental evidence that PGE is an essential modulator of normal macrophage-lymphocyte interactions is still lacking. PGE seems to act as only one of many regulatory factors because the inhibition of its production, while leading to enhanced lymphocyte responses does not result in a major change in immune reactivity. Thus PGE may be a modulator of an auxiliary or even reserve regulatory system. The higher release of PGE from activated macrophages led Morley and his colleagues to suggest that macropahge-derived PGE could provide a negative feedback signal that inhibits lymphokine production from stimulated lymphocytes (103). The basic point of this hypothesis is that upon antigenic stimulation, macrophages release IL-2 which stimulates T cells to release lymphokines, including IL-2. One of the lymphokines in turn activates macrophages to produce PGE which causes inhibition of lymphocyte function (83,85,86). The aim of such a feedback regulation could be to maintain a balance between lymphokine-activated macrophages and PGE-inhibited lymphocytes and to adjust the intensity of immune response in a homeostatic manner to the amount and type of antigen introduced into the system. An interesting feature of the PGE-mediated suppression is its rapid induction within 3 to 5 days and its cessation after 2 to 3 weeks with either a restoration to the previous state or a switch to another, more permanent suppressor mechanism (85). The latter finding led Webb and his co-workers to suggest that PGE may induce suppressor activities in T cells. They have characterised two PGE-induced-suppressor factors produced by mouse T cells which they

termed prostaglandin-induced T cell derived suppressor (PITS) (104). PITS has a wide spectrum of suppressive actions, including PHA- and LPS-induced blastogenesis, one-way and two-way MLR, and both T-cell dependent and T-cell independent antibody responses (105,106).

It now seems likely that PGE-induced immunosuppression occurs through at least two mechansisms, one based on macrophages and PGE and the other on PGE-induced T suppressor cells and the synthesis or the release of protein intermediates, the PITS factors which in turn modulate the immune response. In any case, the relative contribution of these mechanisms toward overall regulation of the immune response in vivo remain to be determined.

The complement system

In 1884 Grohmann showed that plasma could kill bacteria in a reaction which did not require leukocytes, and in 1889 Buchner showed that cell free serum possessed the ability to destroy typhoid bacilli. This bactericidal activity which at that time was termed alexin and later complement, was found to be sensitive to heat and did not increase during immunisation. In contrast a second bactericidal factor, now known to be antibody was noted to be heat-stable, increased by immunisation and to react specifically with the immunising organism (107).

The entire complement system, as currently understood, consists of 20 proteins which collectively are a major fraction of the β1 and β2 globulins. These proteins are synthesised in the liver, but several are also produced at extrahepatic sites, such as in monocytes, macrophages, fibroblasts and epithelial cells of the gastrointestinal and genito-urinary systems (108). Two pathways are known to activate the complement cascade, the classical and the alternative pathways. The classical pathway is triggered by interaction of the first component of complement (C1) with antibodies of the IgG and IgM classes in complex with antigen. The proteins of this pathway are numbered C1-C9, though the correct sequence of activation of the components (C1,4,2,3,5,6,7,8,9) does not follow exactly the original discovery and numbering of the components. The alternative pathway is triggered by endotoxins, zymosan, cobra venom factor and IgA without requirement for IgG or IgM and components C1,

C4, and C2. The alternative pathway components are termed factors and each is represented by a letter eg factor B, factor D, properdin (P). The alternative pathway enables complement assisted phagocytosis and inflammation to proceed before the influx of antibody.

Activation of the complement system results in a cascade with interactions of these proteins, leading to the generation of products that have important biological activities and constitute important humoral mediators in inflammation and immunity. Thus, a) C3a and C5a induce increased vascular permeability due to their action on smooth muscle and the release of histamine by mast cells, b) C3b facilitates the localisation and degradation of immune complexes in germinal centres and the opsonisation and phagocytosis of bacteria, c) neutrophils, eosinophils and monocytes are attracted by C5a to the area of inflammation to phagocytose the opsonised micro-organisms ie chemotaxis, d) the membrane attack complex C5-C9, is responsible for the lysis of bacteria and other cells recognised as foreign, and e) the production of T-dependent antibody production in mice is delayed by prior depletion of C3 which also appears to be important to the development of primary B memory cells (107).

Major histocompatibility complex

Landsteiner in his Nobel lecture in 1931 in which he emphasised the parallel between the problems of matching for blood transfusion and transplantation triggered a search for leukocyte blood groups that could form the basis of matching donors and recipients for transplantation. In the middle and late 1930s Gorer, with his pioneering work in mice, showed that a single locus situated in linkage group IX (now known as the 17th chromosome) controlled a cell surface antigen (antigen II) that elicited rapid allograft rejection and could be detected by alloantibody (109). The discovery that genetically controlled antigens present in grafted tissue and absent in the host could result in graft destruction was a milestone in our understanding of graft rejection. The antigens relevant for tissue matching were called histocompatibility antigens and the locus controlling antigen II in mice was coined histocompatibility 2, or H-2 by Snell (110). Subsequent studies using inbred strains of mice

showed that transplants between H-2 incompatible strains were rapidly rejected, whereas transplants between strains differing for antigens not linked to H-2 were more slowly rejected. As the H-2 locus in mice was later shown to be polymorphic and composed of a cluster of antigens, the H-2 complex was designated the major histocompatibility complex (MHC) (reviewed in 111).

Within a short time from the discovery of the H-2 system in mice, studies in man were initiated. In 1954 Dausset et al described the presence of antibodies against human white cells in the sera of leucopenic and multitransfused patients, multiparous women and normal volunteers immunised with leukocytes (112). Soon many different antigenic specificities were detected by such antisera, and genetic analysis of families and populations established the presence of a series of closely linked genes in man. Furthermore, as in the mouse, genetic studies demonstrated a relationship between the tissue alloantigens defined by serological methods and those detected by tissue transplantation. This resulted in the identification of the MHC in man, the HLA system (Human Leukocyte antigen system A) which is a cluster of genes located on the short arm of chromosome 6 (113).

The mouse H-2 complex

The H-2 complex contains at least nine discrete genetic regions in 5 major groups K, I, S, G and D. The I region has been further subdivided in 5 subregions, namely, I-A, I-B, I-J, I-E and I-C (reviewed in 111, 114).

The K and D regions contain the H-2k and H-2d loci which determine cell membrane antigens in graft rejection, cell-mediated lympholysis (CML), mixed lymphocyte reaction (MLR) and graft-versus-host-reaction (GVHR). The S region contains the Ss locus and controls components of the complement system. The G region contains the H-2G locus and controls the resistance of F_1 hybrids to parental transplants of bone marrow or tumours of lymphoid origin. The I region or immune response region was originally identified as a region where specific immune response (Ir) genes are located. The antigens controlled by the I region stimulate MLR and control the immune response to synthetic polypeptides and to naturally occurring protein antigens (111,114,115). They also control CML, GVHR and in

addition essential cell interactions occurring during immune responses such as macrophage-T cell and T cell-B cell interactions (111,114,116). The ability to suppress the immune response to some antigens is also regulated by the I-A or I-B subregion (111,114,115) and several soluble factors that potentiate immune responses have been shown to bear determinants controlled by the I-A subregion (111,114,117).

The H-2 system is one of the most thoroughly studied segments of a chromosome in the mammalian system. Several new techniques, such as DNA hybridisation, gene cloning and monoclonal antibodies have facilitated genetic and functional studies of the H-2 gene complex and permitted identification and isolation of single gene products and cells bearing these products.

The human HLA complex

More than one hundred HLA antigens have already been identified belonging to at least 3 regions, namely class I, class II and class III. The class I genes (HLA-A, HLA-B and HLA-C) encode cell surface antigens that are expressed on most nucleated cells and are recognised by serological techniques. The class II genes are arranged into at least 3 subregions, HLA-DP, HLA-DQ and HLA-DR. The DQ and DR products can be detected serologically whereas the DP products are determined by analysis of T cell reactivity and specifically by the measurement of thymidine uptake during blast transformation in the MLC test. The products of the HLA-D region are expressed on certain cell types, primarily B lymphocytes, monocytes, activated T cells, endothelial and epidermal cells (118). The class III region comprises the genes for the serum complement components C2 and factor B, the 2 genes for the cytochrome P-450 21-hydroxylase and the 2 genes for complement component C4 (C4A and C4B).

HLA antigens are identified by numbers with the letter of the locus as a prefix. Antigens for which good reagents are not generally available have the extra prefix w (from workshop) (113). The various specific combinations of alleles at all loci within the MHC are referred to as haplotypes, and each HLA haplotype is transmitted as a single Mendelian codominant trait. The HLA-A, -B, and -C antigens are membrane glycoproteins with a molecular weight of

44,000 daltons which are associated with a 12,000 molecular weight polypeptide, the $\beta 2$ microglobulin. The gene for $\beta 2$ microglobulin lies on a separate chromosome to that containing the MHC loci and although this peptide does not form part of the antigenic site of the HLA molecule, it is necessary for the expression of the class I antigens. HLA-DR determinants have also a 2 polypeptide chain structure (a and b chain with a molecular weight of 34,000 and 28,000 respectively) (119).

The extreme polymorphism of the MHC and the fact that homologous systems exist in all mammalian species studied suggest that the MHC has an important biological role. Direct proof for this in the mouse was reported by Lilly et al who found that susceptibility to and mortality from Gross leukaemia virus infection were controlled by the H-2 system (120). Also, studies by Benacerraf and McDevitt established that the immune response to various pathogens and antigens is controlled by the Ir genes in the MHC in the mouse, guinea pig and rhesus monkey (115, 121, 122). These findings suggest that the major role of the MHC might be not only its role of histocompatibility matching in organ transplantation, but also its influence on resistance to infectious diseases.

Genetic control of immune responses

Any gene that can modify immune reactivity may be classified as an immune response (Ir) gene. To ensure that the species maintain the ability to resist a variety of micro-organisms at least some of these genes need to be polymorphic. Biozzi et al analysed the immune response in inbred mice with high and low antibody responses to heterologous erythrocytes and identified 10 independent polymorphic Ir loci (123). These loci were non-antigen specific as they controlled the response to many antigens unrelated to the antigen used for selective breeding. The study of antigen-specific Ir genes began with the discovery by Benacerraf's team in 1963 that the ability to respond to selected antigens was controlled by autosomal genes inherited in a simple Mendelian fashion (115,124,125). Using random-bred guinea pigs which were immunised with dinitrophenyl conjugate of the synthetic polymer L-lysine (DNP-PLL), some guinea pigs developed both a specific DTH response and high levels of serum

antibody to DNP-PLL (high responder animals), whereas others failed to develop such a response (non-responder animals). Thus by presenting the immune system with a highly specific antigenic stimulus it was possible to detect specific Ir genes. Later, McDevitt et al studied the genetic control of the immune response in mice to the synthetic polypeptides tyrosine, glutamic acid, alanine and lysine (T,G-A-L) and noted that the Ir gene was linked to the genes of the H-2 complex. Thus they established that the MHC linked Ir genes map to a new region of the H-2 complex, the I region (122,126,127). Since these initial observations, several reports have confirmed that both humoral and cellular immune responses to many types of antigens are under MHC-linked Ir gene control which are the main antigen-specific Ir genes (reviewed in 111,115,128). Studies of the activities controlled by these genes have also shown that they control MLR, cell surface alloantigens (Ia antigens) and cellular interactions in several different species such as mice, guinea pig, rat, chicken, dog and rhesus monkey (44,47,111,115,128,129).

Studies associating HLA and immune responses in man were more difficult because of the problems regarding ethical issues of immunisation, standardisation of exposure to antigens and measurement of immune response in an outbred population. In 1972 Levine et al provided the first evidence for an aetiological link between Ir genes and a specific disease suggesting that the clinical symptoms of hay fever and IgE-mediated skin sensitivity to ragweed antigen were both controlled by HLA (130). Thereafter a number of studies indicated the possible presence of Ir genes in man describing associations between different immune responses to microbial and non-microbial antigens and HLA antigens. Thus, the response to the pollen antigen E was associated with HLA-B7 (131), the occurrence of hepatitis B antigenaemia in Australian aborigines with HLA-Bw35 (132,133) and a high in vitro lymphocyte proliferation stimulated by streptococcal antigens (streptokinase and streptodornase) with HLA-B5 (134). Also a significant association was found between low response to tetanus toxoid and a Japanese HLA-D determinant HO (135), between low response to vaccination with a smallpox-like virus and HLA-Cw3 (136), low response to influenza A virus and HLA-Bw16 (137) and low response

to rubella virus and HLA-A28, -B14 and Bw22 (138,139). These studies were carried out in the late 1970's when HLA-D typing was not performed routinely. Because of the presumed analogy between HLA-D and the murine Ia region, stronger associations are expected to be found in the near future as most associations reported are probably due to linkage disequilibrium for DR (140).

Humans constitute a better study population than mice in the search for possible associations between HLA antigens and susceptibility to diseases. After Lilly's report on leukaemiagenesis in mice (120), Amiel was the first to show an association between HLA and Hodgkin's lymphoma (141). Since then numerous reports have suggested an increased frequency of one or more HLA antigens in various diseases (reviewed in 142, 143). Todate a large number of human diseases have been found to have HLA associations and the majority appear to have a strong relationship with HLA-D antigens. However, haemochromatosis and congenital adrenal hyperplasia, which are both entirely non-immunological diseases have also been found to be associated with HLA. Thus, HLA genes not only influence the immune response but also play a role in other physiological functions such as iron metabolism and steroid hormone formation. It appears, therefore, that mechanisms for disease susceptibility based on genes controlling hormones, Fc receptors and complement components might be possible. Further studies on HLA-associated diseases will enable us to understand the relationships and classification of certain clinical syndromes and may even offer means for therapeutic interventions.

HLA and renal transplantation

Today organ transplantation is an important form of treatment for diseases involving an irreversible loss of organ function. In particular kidney transplantation, which is the commonest form of organ transplantation, constitutes the best treatment for end-stage renal failure. By the end of 1987, approximatley 75,000 renal transplants had been performed in Europe of which approximately 10,000 were performed in 1987 (144).

Evidence that HLA antigens are important in allotransplantation is derived from tissue matching and subsequent graft survival rates. In 1978 an International Forum entitled 'Fifteen years of HL-A: What is the importance of HL-A compatibility for clinical outcome of renal transplantation?' was organised by J Dausset, F Kissmeyer-Nilsen, PJ Morris, JJ van Rood, PI Terasaki and G Opelz. The general view in that Forum was that matching for HLA haplotypes in living related transplants had a clear and significant effect on graft survival. Thus, graft survival at one year in HLA identical siblings was 90%, compared to 70% in one-haplotype matched transplants and 50% in sibling transplants sharing no HLA haplotype (145). These results reflected the experience then and later of most transplant centres (reviewed in 146, 147, 148). The beneficial effect of HLA matching in living related transplants is such that nowadays most centres do not perform transplants between totally mismatched siblings. However, the fact that nearly 10% of the HLA identical transplants are rejected within a year despite immunosuppression implies that other histocompatibility antigens and non-HLA linked genes may play a role in graft rejection (146,148). On the other hand, more than 50% of totally mismatched living related grafts are still functioning after one year, suggesting that host factors other than HLA matching are crucial in graft survival.

In view of the superior graft survival with HLA identical sibling donors one would expect general agreement on the beneficial role of HLA matching in cadaver renal transplantation. However, this has been a controversial issue. The first reports showing that matching for HLA-A, B improved graft survival rates appeared soon after the discovery of these loci in 1968 (149,150). In a recent review of renal transplantation and HLA which has summarised 99 reports from 1969 to 1984, 23 studies described a statistically better graft survival in well-HLA-A,B matched grafts compared to poorly-matched grafts, while in 31 studies the difference between the two groups was not significant and in the remaining studies graft survival rates were given without p values (147). The reasons for the apparent discrepancy are threefold. Firstly the number of cases from single-centre reports is small, secondly graft outcome is influenced by factors other than HLA matching, such as blood

transfusion, immunosuppression and pre-sensitisation and thirdly, the polymorphism of the HLA antigen system makes it virtually impossible to find HLA identical donor-recipient pairs in cadaver transplantation. However results based on large numbers of cases from national and regional organisations (eg Eurotransplant, UK Transplant Service, Collaborative Transplant Study [CTS]) show that the difference in graft survival at one year between 0-1 mismatched HLA-A,B grafts has been in the range of 10-15% (reviewed in 146,147,148). For example, Opelz and Terasaki reported that in the North American Registry with more than 7,000 first cadaveric transplants the one-year graft survival was 53% in the zero HLA-A,B mismatch grafts compared to 42% in the 4 HLA-A,B mismatch group (147). In a Eurotransplant report, the long-term graft survival in 2,522 transplants at 5 years was also better in the zero HLA-A,B mismatch group (51%) compared to that of the 4 mismatch grafts (32%) (151). d'Apice in a controlled study found that matching for HLA-B antigens gave better graft survival, while this did not apply to the HLA-A locus (152). The view that HLA-B antigens might be stronger histocompatibility antigens than HLA-A antigens was supported by another study the following year (153), whereas HLA-C antigens seemed to have no effect on graft survival (154).

Soon after the identification of the first 7 HLA-DR antigens (DR1-DR7) in 1977, transplant centres began to focus their attention on the effects of matching for these antigens, as in theory the expression of these surface markers like Ia antigens in mice trigger the induction of the immune response against allografts. The first report which showed the beneficial role of HLA-DR matching in cadaveric transplantation came from Oxford in 1978 (155), and thereafter was confirmed by both single and multicentre studies (146,147,148,152,154,156). In 385 first cadaver grafts in Oxford the survival at one and 5 years of grafts with no HLA-DR mismatches was 82% and 65% respectively, whereas those with one and 2 DR mismatches were 72% and 49%, and 65% and 49% respectively (148).

Mickey analysed the one year graft survival of 9,345 first cadaver transplants and reported an 82% survival in 98 grafts with no mismatches at any of the HLA-A,B and DR loci (156). Of the 5,574 grafts analysed in the CTS report, 163 had no HLA-A,B,DR mismatches

and their one year survival was similar at 80% which was more than 20% better than grafts mismatched for 5 or 6 antigens (148). Also, the Eurotransplant study showed that in grafts with zero DR mismatches, additional zero A,B mismatches improved the 2 year graft survival to 82%, whereas zero DR mismatches with one, 2 and 3-4 A,B mismatches had 76%, 72% and 57% graft survival respectively (157). The UK Transplant Service has reported that first cadaver grafts with no HLA-A,B,DR mismatches had a striking 93% one year survival (148). It appears, therefore, from these studies that matching for HLA-A,B and DR has a cumulative effect, although other reports found that matching for HLA-A,B had an additional effect only in the DR incompatible grafts, but no effect in grafts with zero DR mismatches (146,154).

After the introduction of cyclosporin in renal transplantation, a Scandinavian multicentre study reported that HLA-DR matching no longer had any effect on graft survival and therefore was unnecessary, as one year graft survival rates for zero, one and 2 DR mismatches were 74%, 78% and 79% respectively (158). Similar results were obtained from Kahan et al (159), Taylor et al (160) and Ferguson and Sommer (161). However, other multicentre and single-centre studies have shown that HLA matching still has a beneficial effect on graft survival in patients treated with cyclosporin. The Canadian multicentre trial showed a clear trend towards improved graft survival with both HLA-A,B and DR matching from 73% to 89% when two-antigen identity was found in either locus (162). A similar trend was reported in 522 transplants by Terasaki's group who found a 6 month graft survival of 79% and 70% in donor-recipient pairs with the best and worst HLA-B and DR matches respectively (163). Perhaps the strongest evidence that HLA-A,B and DR matching continue to be important in renal transplantation comes from Opelz in his CTS report (164) and in a single-centre report from Oxford (148). In the former study the actuarial one year graft survival was 76% in 2,198 transplants treated with cyclosporin compared to 64% of 6,392 transplants treated with azathioprine and prednisolone. This difference was highly significant. The difference in outcome between the best and worst HLA-A and B matched grafts was 10% in both cyclosporin and azathioprine treated patients. Similarly, grafts

well matched at the DR locus had 10% better survival compared to those poorly matched in both groups treated with cyclosporin and prednisolone/azathioprine. Furthermore, matching for HLA-A,B and DR had an additive effect on improving graft survival in both groups, and the best survival of 86% was obtained in 161 first cadaver transplants treated with cyclosporin where there were no mismatches at the HLA-B and DR loci. This result was significantly better than the 67% graft survival of 181 cyclosporin treated recipients with 4 HLA-B and DR mismatched grafts. The results from Oxford were based on 135 cyclosporin treated first cadaver grafts and they showed an 84% graft survival at one and 5 years for the zero DR mismatched grafts compared to 75% and 59% for those with one DR mismatch and 61% and 57% for those with 2 DR mismatches respectively.

Despite the controversies which surround HLA matching in the cyclosporin era, most centres continue to use tissue typing as a means of improving graft survival. As HLA-A and B alleles are highly polymorphic, most attention has been focused on DR matching followed by HLA-B and then HLA-A locus matching.

II DELAYED TYPE HYPERSENSITIVITY

The activation of T cell dependent immune responses results in a state of specific immunity called cell-mediated immunity (CMI). CMI reactions are found a) during infection by certain micro-organisms, b) as a component of immune reactions against tumours and transplanted tissues, c) in some autoimmune diseases, and d) in delayed type hypersensitivity (DTH) reactions.

DTH (type IV hypersensitivity) is a T cell dependent phenomenon manifested as an inflammatory reaction at the site of antigen deposition, usually the skin, which takes more than 12 hours to develop and with the exception of the granulomatous type of DTH, reaches its peak within 24-72 hours of antigen challenge. It is, therefore, distinguished from allergy or immediate hypersensitivity (Type I), antibody-dependent hypersensitivity (Type II), and immune complex mediated hypersensitivity (Type III) (165). There are 4 types of DTH, namely, the Jones-Mote reaction, the tuberculin-type reaction, contact sensitivity and the granulomatous DTH. The Jones-Mote reaction is characterised by numerous basophils in the cellular infiltrate, its maximal reaction time is at 24 hours and it is transferable by immune serum, as well as by cells (166). Tuberculin-like and contact sensitivity both peak at 48-72 hours after antigen challenge and are characterised by intense induration containing mainly mononuclear cells. The site of induration in the tuberculin-like reaction is the dermis, whereas in contact DTH it is the epidermis (165). The reaction on both occasions is transferable by purified T cells, but not B cells or immune serum from immunised to non-sensitised animals (165, 167). In granulomatous DTH the lesion develops over a period of weeks and is the result of a cellular reaction around discrete loci of antigen in high concentration.

DTH is an in vivo phenomenon which provides information on all the factors involved in CMI. This includes the individual capacity to respond to an antigen with the induction of activation of the relevant T cell subsets, the capacity of these cells together with APC and recruited cells to react with the specific antigen, and the capacity of the tissues to respond to the

inflammatory agents generated from these reactions. Several in vitro tests which have been devised to correlate with DTH provide useful information which, however, cannot replace DTH as an in vivo source of evidence concerning the function of interacting cells in CMI.

History

The first description of DTH possibly dates back to 1798, when Jenner observed a papular erythematous lesion which peaked in 24-72 hours after vaccination. In 1890 Koch reported the tuberculin-like reaction and the following year Epstein discussed its diagnostic value in tuberculosis (168, 169). These initial observations were not recognised as DTH reactions, and it was at the beginning of the 20th century that the concept of hypersensitivity was established as an immunological response which can lead to tissue damage. Portier and Richet in 1902 observed the immediate type hypersensitivity (170) which von Pirquet later called allergy (1). At that time the tuberculin reaction was interpreted as a local anaphylactic reaction similar to that described by Arthus in 1903 following the daily subcutaneous injections of horse serum in rabbits.

The tuberculin reaction was separated from anaphylactic reactions by Zinsser who showed that DTH to tuberculin could not be passively transferred in guinea pigs by highly specific immune sera, as opposed to the Arthus reaction which was transferable in this way (171, 172). The first histological confirmation of the two different forms of reaction came from Dienes who described the tuberculin reaction as being characterised by the slow development of an infiltrate with mononuclear cells, whereas in the anaphylactic reaction there was a rapid development of oedema followed by an intense polymorphonuclear infiltration (173,174).

The first contact sensitivity reaction was produced by Jadassohn in 1896 using mercury (175) and several years later Nestler and Cash sensitised themselves to Primula obconica and to East Indian Satinwood respectively (176). The systematic study of the contact sensitivity reaction was carried out by Landsteiner and his school (177). They worked with chloro and nitro-substitution products of benzene such as 2,4-dinitrochlorobenzene (DNCB) and

picryl chloride (2,4,6 trinitrochlorobenzene) and suggested that simple chemicals like these could become antigenic substances upon conjugation with proteins in the skin of sensitised animals. Landsteiner concluded that DNCB is a typical compound of contact dermatitis in humans and he observed that such sensitivity could not be transferred passively with serum in the same way as anaphylaxis. The passive transfer by Landsteiner and Chase in 1942 of contact sensitivity in guinea pigs to picryl chloride by peritoneal exudate cells was a milestone in our understanding of the DTH reaction (178). In 1945 Chase succeeded in passively transfering the tuberculin reaction using the same cell type (179). Also, Chase in 1941 on the basis of previous evidence of different degrees of contact sensitivity within groups of guinea pigs, proved by breeding experiments that the susceptibility to skin sensitisation with DNCB was influenced by heredity (180).

By means of these experiments Landsteiner and Chase established that specific cellular immunity was mediated by sensitised lymphocytes and not by serum antibody, this at a time when T cells were not known to be the specific mediators of these reactions. These investigators found that when they used syngeneic guinea pigs to transfer DTH reactions, all the recipients developed strong and long lasting responses. By contrast, when an outbred population of guinea pigs was used, the DTH reactions did not develop in all recipients and were not as strong and long lasting as those in the inbred population (178). The explanation of these results was not clear at that stage and they were interpreted as rejection-type phenomena. The framework for understanding these findings came later with the description of the MHC-restriction phenomenon by Shevach and Rosenthal (47) which has been discussed in the previous section of this thesis. Thus, the successful transfer of DTH in the outbred population probably occurred because recipients and donors were MHC compatible, whereas in the unsuccessful transfer, the donor-recipient pairs most likely were incompatible.

Medawar and his school studied the relation between DTH and allograft rejection. They showed that a tolerated skin graft could be rejected when the host was injected with lymphoid cells from a syngeneic animal which had been previously injected with donor skin.

This event seemed to have similarities with the passive transfer of DTH. Later, they produced a tuberculin-like reaction by injecting cells from regional lymph nodes of an allograft recipient back to the skin of the donor (181). The finding that a homografted animal expressed a DTH response which was transferable by lymphoid cells to its donor strengthened the relation between skin allograft rejection and DTH then, and until the present day (182, 183).

In 1961 Miller provided the first clear evidence that DTH reactions form a distinct group of immune responses dependent on thymus function by showing that skin graft rejection could be prevented in neonatally thymectomised animals (7,184). Soon after the subdivision of immune responses into cellular and humoral (5-16), Huber et al (185) and Vadas et al (186) showed that the T cells responsible for DTH carry the same surface markers as T helper cells, and therefore are different from T cytotoxic and T suppressor cells which were also known by then to mediate effector cell functions (187-191). The lysis of allogeneic cells by sensitised T cytotoxic cells was documented in tissue cultures (187-189) and the role of T suppressor cells was demonstrated by Asherson and Zembala who produced suppression of contact sensitivity to trinitrochlorobenzene (picryl chloride) in mice by intravenous injection of trinitrobenzene sulfonic acid and subsequently transferred this suppression to non-sensitised recipients (190,191). This sequence of events had been described in guinea pigs as the Sulzberger-Chase phenomenon and represented one of the earlier examples of tolerance to contact sensitivity (192,194). Guinea pigs fed or injected with picryl chloride were no longer able to develop contact sensitivity upon exposure to the chemical on the skin, presumably due to suppressor T cell activity induced by the systematic exposure to the antigen.

The activation of macrophages in the process of DTH was observed in 1928 in the original studies by Rich and Lewis (66). After the discovery of MIF in 1966 it was realised that soluble agents released locally by antigen-sensitised lymphocytes could not only inhibit the migration of non-sensitised lymphocytes from capillary tubes (66-68) but also resulted, when injected intradermally, in skin reactions with DTH characteristics (195).

From this historical review it is interesting to note that the study of DTH started in relation to defence mechanisms in tuberculosis, was then followed by critical experiments on contact sensitivity with fascinating results and later attracted the interest of transplant researchers. The role of DTH has now been defined both in the defence mechanisms of intracellular micro-organisms and in local tissue damage resulting from cell-mediated immunity which involves interactions between sensitised lymphocytes, activated macrophages and lymphokines.

Cellular interactions in DTH

DTH is primarily associated with two types of cells, T helper cells and macrophages which are the APC (167). As DTH is an in vivo phenomenon, the requirement for APC in the induction of DTH is not apparent. However, it was shown that doses of antigen that are not immunogenic by themselves become highly immunogenic when associated with macrophage rich cell populations (40-44, 196).

The mode of antigen presentation to T helper cells in association with class II MHC antigens on the surface of interacting cells has been discussed in the previous chapter (44,47-65). The role of MHC in DTH was shown in the early experiments of Landsteiner and Chase from the successful transfer of DTH reactions using inbred guinea pigs (178), although its significance was not understood then. The explanation came later from the work of Zinkernagel and Doherty (197-199) and Vadas and Miller (196,200,201). The former investigators found that T cytotoxic cells will recognise and lyse virus infected cells only in the context of the same MHC antigen to which sensitisation took place. This finding, together with the observation of Vadas and Miller that identity at the H-2 complex was essential to successfully transfer DTH in mice, suggested that the MHC could form part of the antigen specificity in CMI reactions.

Following this information, two theories developed to explain antigen recognition by immune cells depending on whether the T cell antigen receptor recognises both MHC and antigen together or whether the receptor sees antigen and another surface marker sees the MHC. These theories are referred to as 'associated recognition' and 'dual recognition' respectively (reviewed in 202). With the very recent

characterisation of the T cell antigen receptor, it is now known that both antigen and MHC molecule are recognised together by the receptor (202,203). T helper cells recognise antigen on the surface of APCs in association with class II molecules and T cytotoxic cells in association with class I molecules. T suppressor cells appear to recognise both free antigen and the MHC but it is not clear whether they use the same type of antigen as other T cell subsets (203-205).

The influence of Ir genes on DTH has been reviewed in the previous chapter (111,114-117,121-129). The evidence which showed that DTH reactions are under the control of the Ir genes was provided by experiments using inbred strains of non-responder and responder animals which differed only at the I region of the MHC. These animals were tested for their immune responses to synthetic polypeptides either by quantitating an anti-hapten response using the antigens as carriers or by direct measurement of DTH to the antigens. As DTH reactions are T cell dependent phenomena, the Ir gene control is expresssed at T cell level.

The Langerhans cell

Paul Langerhans described these cells in 1868 as an intraepidermal sensory receptor for extracutaneous signals to the nervous system (206). Having been considered as such for more than a century, evidence obtained in the early 1970's rekindled interest in the Langerhans cells (LC). The possible role of LC as an immunologically active cell was first suggested by the observation that they were morphologically indistinguishable from the histiocytic cell in histiocytosis X (207) and by the finding of mononuclear cells in apposition to LC in DTH lesions induced by DNCB in guinea pigs (208). This information was followed by a search for surface markers which have fully characterised the LC. We now know that LCs are bone marrow-derived, non-phagocystic APC, representing 2-4% of the total epidermal cell population (209, 210). They express Ia (DR), CD1 (T6) and CD4 (T4) antigens, carry Fc receptors for IgG, receptors for C3 and produce IL-1 (reviewed in 211, 212). Their distinctive, racket-like cytoplasmic organelles, the so-called Birbeck granules are the only reliable morphological marker and are CD1 (T6) positive (212). The function of the Birbeck granules is still unknown. Katz

provided evidence that LCs are not a stable resident population of the epidermis, but rather derive from precursor circulating cells that migrate into the skin (210) at a daily repopulation rate of about 45 million LCs for the entire skin (212). The largest number of LCs occur within the epidermis in close proximity to the superficial vascular plexus, the postcapillary venules of which are the sites of inflammatory cell immigration, histamine-induced vascular permeability and immune complex deposition under different pathological conditions. Skin lymphatics start with open ends within the papillary dermis and drain lymph to regional lymph nodes. Lymph nodes, with LCs and the rest of the dendritic APCs, namely the intermediate cells, the interdigitating cells and the veiled cells constitute the skin immune system which plays an important role in immunisation and tolerance mechanisms.

Stingl et al showed that LCs in guinea pigs not only share common surface markers with macrophages but also behave similarly at a functional level in their capacity to present antigen to T lymphocytes in an MHC-restricted fashion (209). Epidermal cell suspensions are also potent stimulators of allogeneic T cell proliferative responses (213) and can induce generation of T cell cytotoxicity against alloantigens and hapten-modified syngeneic cells in mice (214). Small numbers of dendritic cells taken from draining lymph nodes of mice one day after sensitisation with picryl chloride were sufficient to induce primary stimulation of lymphocytes in vitro and to transfer DTH reactions in vivo (215). Similar results were obtained in humans utilising human LCs in autologous and allogeneic MLC (216,217). The response of allogeneic lymphocytes to human LCs depended on the degree of HLA-DR mismatch and the stimulation was blocked by both monoclonal and alloanti-DR sera (217).

There is therefore, abundant evidence of the importance of LCs in the induction of contact hypersensitivity and allogeneic cytotoxicity and their role in graft rejection, tumour immunology and defence mechanisms to viral and other intracellular pathogens.

The list of antigens that can initiate DTH reactions is large and they have been reviewed recently (218). They include:

1) Intracellular bacteria:
   - Mycobacterium tuberculosis
   - Salmonella typhosa
   - Brucella abortus
   - Listeria monocytogenes

2) Fungi:
   - Candida albicans
   - Cryptococcus neoformans
   - Histoplasma capsulatum

3) All viruses and chlamydia

4) Insect bites

5) Serum proteins and other simple protein antigens

6) Simple chemical compounds:
   - Oxalone
   - Beryllium fluoride
   - Potassium dichromate
   - Mercuric chloride
   - Benzene compounds

The DNCB skin test

Since Landsteiner and Jacobs in 1935 reviewed contact sensitivity with simple chemical compounds, sensitisation and challenge skin tests with DNCB have been extensively used as one parameter of CMI (219, 220). DNCB was selected then because it was shown to be a suitable compound and it has been used as such until now, because a) it is a simple in vivo test, b) a primary response is obtained as previous exposure to DNCB is rare (221) and c) 85-95% of normal people can be sensitised to this antigen (222-225).

Following the demonstration in 1930 by Bloch and Steiner-Wourlish that guinea pigs could be sensitised to an extract of the leaves of the plant Primula obconica, which belongs to the

genus of Rhus (226), Landsteiner and Jacobs, while studying plant extracts from this genus, found that the common factor which caused conduct sensitivity in guinea pigs was a chloro or nitro-substituted benzene. Out of 20 compounds which they tested, 10 produced DTH and of these 10 they favoured DNCB and picryl chloride as the most suitable test agents.

The sensitising properties of DNCB are related to its ability to act as a hapten forming covalent bonds with the ε-amino groups of lysine residues of epidermal proteins (227). Using radioactive techniques it was shown that less than 5% of the applied DNCB binds to epidermis and about 85% of the dinitrobenzene groups were bound to lysine residues (228).

The technique of DNCB sensitisation has varied considerably and inevitably there has been a subjective element in the interpretation and quantitation of the test. The most widely used method has consisted of measurement of redness and induration of the skin in response to DNCB. Other methods such as employing engineering calipers for measurement of the induration or radioisotopic measurement of the intensity of the cellular infiltration may be more accurate and objective but are too complex for routine use (167,218).

The pathology of contact sensitivity lesions has been extensively studied in guinea pigs rabbits, mice, rats and humans and the findings have been similar in all species, resembling those of the tuberculin-like reaction (218,229-235). In the latter reaction the findings were confined to the dermis and in contact sensitivity to the epidermis.

On application of DNCB to the skin in the guinea pig, a diffuse mononuclear infiltrate is formed in the upper dermis at 4-6 hours, the peak reaction is reached at 12-15 hours and the same intensity is then maintained for 18-48 hours (232). Intracellular oedema is evident after 6 hours and although Flax and Ganlfield did not describe microscopic vesicle formation (232), Fisher and Cooke noted vacualation of epidermal cells 9 hours after skin testing (235). The majority of infiltrating cells are T lymphocytes, and macrophages form 20-30% of the mononuclear cells with a relative paucity of polymorphonuclear leucocytes at all stages of the reaction. In the tuberculin-like DTH, macrophages form 59% of the mononuclear cells

but there are many polymorphonuclear cells at an early stage of the reaction (218). Over several days, with the constant growth of new cells from the basal layer of epidermis, the antigen containing cells are pushed outwards and ultimately lost by desquamation.

In humans the pattern of cell infiltration is similar to that in guinea pigs except that the cell infiltrate is not as diffuse in the upper part of the dermis but is perivascular in position. Turk et al using the acid phosphatase technique to distinguish macrophages from lymphocytes found that the former constituted 20% and the latter 80% of perivascular cell infiltrates 24 hours after skin testing (218). These histological changes started to appear in skin biopsies performed 48 hours after the test when a minimum of 30ug DNCB was used for primary sensitisation with 3ug being used for challenge tests (234). The nature of changes during the primary sensitisation and challenge reactions were identical (234).

Removal of the skin where the DNCB was applied 16 hours after its application did not prevent the subsequent development of sensitivity, yet the draining lymph nodes if removed at 48-72 hours, were not capable of transfering sensitisation to histocompatible guinea pigs. Successful transfer, however, occurred using lymph nodes removed after the fourth day, and this capacity appeared to correlate with the presence of large pyroninophilic cells in the nodes (230). These cells which were then called 'immunoblasts' (236), are now known as T lymphocytes (212,237,238). Using immunohistochemical double staining techniques in frozen sections of human skin after PPD injections, it was found using T cell specific monoclonal antibodies that the majority of infiltrating cells were T lymphocytes. Most of them had the helper/inducer phenotype and few the suppressor/cytotoxic phenotype (237). Apposition of both T cell subsets to HLA-DR expressing cells occurred in the dermis as well as in the epidermis suggesting that interactions occur in the skin between T cells and APCs. Antigen presenting Langerhans cells increased rapidly in numbers a few hours after antigen sensitisation (208, 238) and it appeared that the majority of them were leaving the epidermis presumably emigrating to regional lymph nodes, spleen and thymus (212,238). B cells and plasma cells were not found in the DTH lesions (238).

With the above knowledge in mind (212,239), one could envisage the movement of the cellular components in human DTH reactions to DNCB as follows. Upon skin sensitisation, DNCB binds to a 'carrier', which is an epidermal protein, and thus the hapten-carrier complex acts as an immunogen and initiates the induction of a specific immune response. Specialised non-phagocytic, HLA-DR positive cells, the Langerhans cells (LC), function as APCs continually leaving the epidermis through afferent lymphatics. The exact localisation of primary sensitisation is still debatable and it remains unclear whether it is the hapten, the sensitised T cell already within the skin, or the LC-hapten complex that is carried to the regional lymph node. Recently it was shown that hapten-carrying dendritic cells can sensitise unprimed syngeneic mice (215). Nevertheless, LCs on their way to the lymph node change morphology and are referred to as 'veiled' cells, which again, on reaching the paracortical area (T cell area) of the lymph node, change morphology to form the interdigitating cells. These cells present the antigen to paracortical T cells which in turn become activated and are induced to proliferate and differentiate. Effector T precursor cells proliferate and some of them differentiate into memory cells, whereas, others become effector cells. Suppressor cell precursors are simultaneously induced to proliferate and they stop the further production of effector cells.

During sensitisation, release of lymphokines plays an important role in DTH reactions. Thus, antigen-binding T cell factors are released into the circulation and sensitise skin mast cells which release serotonin. Through receptors on post-capillary venules serotonin induces vasodilatation which facilitates the entry of antigen-specific T cells into the site of sensitisation. It is only then that the DTH reaction develops in a MHC restricted fashion, characterised by the development of the mononuclear cell infiltrate, in which non-specific tissue monocytes co-operate with specific recirculating T cells to clear the hapten-carrier complex, the antigen.

In Vitro Assays of CMI

Several in vivo functions can take place in cell or tissue cultures with reproducible efficiency and precision. It is beyond the scope of this thesis to describe in detail and review the literature on in vitro assays correlating with CMI. Thus, in this section I shall mention briefly some common in vitro tests used as correlates of CMI and particularly DTH.

<u>Measurement of T lymphocyte surface markers</u>: The most simple, direct and commonly used in vitro assay of CMI is the determination of T cell surface markers. This can be done either by utilising the ability of T cells to non-specifically bind SRBC to their surface CD2 receptors to form E rosettes (ERFC) (27) or using monoclonal antibodies which further detect T cell subsets responsible for helper, suppressor/cytotoxic and other regulatory functions (24-30). Low or absent ERFC have been found in different immunodeficiency states such as various malignancies and viral diseases (240), chronic active hepatitis (241), sarcoidosis (242) and autoimmune diseases (243-245), suggesting that T cells are either selectively removed from the circulation or are unable to form E rosettes.

Characterisation of FcR on T cell subsets is another interesting research topic, as T cells defined by the presence of the IgG FcR (T_G cells) mediate suppressor function in in vitro systems of Ig production and allogeneic MLR, whereas T cells bearing FcR for IgM (T_M cells) mediate helper function (31).

<u>Lymphocyte transformation test (LTT)</u>: The majority of lymphocytes are resting cells which upon antigenic stimulation become activated with synthesis of both DNA and RNA. This burst of metabolic activity can be measured in vitro by the incorporation of tritiated thymidine into the cell during DNA synthesis and it is believed that transformation reflects in vivo events (246-248).

The LTT is basically the same regardless of the antigenic stimulus used in the in vitro system. Mononuclear cell populations are cultured in vitro in the presence or absence of various agents. Depending on the stimulus, variable proportions of lymphocytes will undergo blast transformation and after a predetermined interval the

responses of stimulated cells are compared to those of unstimulated cells by measuring the radioactivity of the activated cells in an automatic scintillation counter (246).

Cell activation in in vitro systems can be produced by mitogens, antigens or lymphoid cells (autologous or allogeneic). The most commonly used mitogens in man are the plant lectins PWM, Con A and PHA and they are considered as polyclonal activators in the sense that they non-specifically trigger multiple cell clones. Thus, unlike antigens, mitogen-induced lymphocyte transformation is not dependent upon prior sensitisation to the particular stimulant (247). Con A and PHA have been considered the classical 'pure' T cell polyclonal activators, although stimulation of B cells through T cell factors has been shown to be possible (247,248). PWM is the standard T cell dependent polyclonal activator of B cells, whereas protein A of staphylococcus aureus, Cowan I (SAC) and Epstein-Barr virus (EBV) are not dependent on T cell presence to induce B cell proliferation and differentiation (247-250). Thus in vitro measurement of Ig production in T-dependent or T-independent systems are useful means of studying cellular interactions.

Antigen-induced blast transformation appears to be dependent on previous in vivo exposure to the particular antigen. Commonly used antigens include PPD, keyhole limpet haemocyanin (KLH), Candida species, tetanus toxoid and DNCB (251-255). These antigens appear to stimulate mainly T cells in fractionated T and B cell populations (254,255), but in the presence of T cells or antigen-stimulated T cell supernatants it was shown that B cell stimulation is also possible (254,255). Furthermore, it was observed that PPD and KLH may act as polyclonal B cell activators of non-sensitised lymphocytes (247). A good correlation was found between in vivo DTH to tuberculin and in vitro PPD-induced blast transformation (252). DNCB when coupled to autologous or allogeneic PBMC induced lymphocyte transformation specifically in leukocyte cultures from men sensitised to DNCB (253). Sequential studies showed that specifically reactive lymphocytes were first detected at 10 days after the in vivo primary sensitisation to DNCB and reached a peak at about 14-21 days (253).

Mixed lymphocyte reaction (MLR): The in vitro LTT using as stimulators allogeneic or autologous lymphocytes constitutes the MLR, which has been considered as the in vitro model of transplantation, and represents T cell blast transformation triggered by allogeneic or autologous non-T cells (146, 256). In the allogeneic MLR, transformation depends on the degree of histoincompatibility between responder T cells and stimulator non-T cells, whereas in the autologous MLR, T cells are activated only by histoincompatible antigens on non-T cells (256).

Both in guinea pigs and humans, lymphocyte transformation in MLC correlated with the presence of DTH in the donor in vivo (257-261), and the intensity of blast transformation to tuberculin in humans showed a good correlation with the size of tuberculin-like DTH in the donor (252,258-260) and not with antibody levels (261).

Because the test takes up to 7 days to perform, it has been used prospectively only in transplants from related donors as several studies showed a good correlation between MLR reactivity and graft survival in one haplotype identical sibling transplants (reviewed in 146). In cadaver transplantation retrospective studies reported conflicting results (146). Today MLR is particularly useful in selecting the best donor-recipient pair between two potential one haplotype identical donors on the grounds of lower MLR reactivity (146).

Also the test has been used to study mechanisms of altered immunoregulation in patients with SLE where it was found that in the autologous MLR non-T cells were defective stimulators (262,263).

Assays of inhibition of cell migration by antigens: In 1932 Rich and Lewis showed that tuberculin inhibited the migration of cells taken from animals with DTH to tuberculin and described cytotoxic changes in these cells (66). The explanation of this phenomenon came later with the discovery of the first lymphokine, the migration inhibition factor (MIF) which inhibited the migration of macrophages from sensitised donors in the presence of the sensitising antigen (67,68). In 1962 George and Vaughan first described a test utilising capillary tubes to demonstrate inhibition of migration of peritoneal exudate cells from guinea pigs sensitised to PPD in the presence of PPD

(264). Since then various modifications of this test have provided the means to establish that this in vitro phenomenon is related both in animals and humans to in vivo DTH to PPD (66-68, 264-267), histoplasmin (266), diphtheria toxoid and ovalbumin (265), candida albicans (268), brucella abortus (269) and DNCB (270).

In clinical practice the migration inhibition test (MIT) has been used to detect cellular hypersensitivity in Hashimoto's thyroidits (271, 272) ulcerative colitis (272), glomerulonephritis (272), Sjogren's syndrome (272), and hay fever (273).

Cytotoxicity assays: In vitro cytotoxicity of effector lymphoid cells against different targets reflects several in vivo CMI mechanisms. Tissue damage is important in the pathogenesis of allograft rejection, tumour immunity, DTH and autoimmune disease (34-37, 274). This tissue damage can be measured in vitro using any of 3 primary methods, namely the entry of dye into damaged cells, loss of the ability to adhere and the release of bound radioisotopes from damaged cells (275). The last method using chromium-51 has proved the most sensitive and reproducible.

Natural cytotoxicity or NK activity does not depend on previous exposure to target cell or in vitro stimulation, and appears to occur primarily through the FcR without need for opsonisation of the target cell with complement or antibody (37,274,276,277). Defects in NK activity have been described in bladder carcinoma (278), in breast carcinoma (279) and in severe combined immunodeficiency disease (280).

Antibody-dependent cytotoxicity (ADCC) involves the coating of target cells by specific anti-target cell antibody and destruction by effector cells through the FcR (36,281-283). ADCC has been expressed for a variety of lymphoid and non-lymphoid cells (282), but the major ADCC effector cell appears to be the K cell (36,283). The role of ADCC in vivo in autoimmunity and in tumour and allograft rejection remains uncertain (182,183).

Cell-mediated cytotoxicity (CMC) has been classified as direct and indirect. The former refers to in vivo sensitisation of effector cells by allogeneic cells and the classical study model has been the GVHR (284). The indirect CMC has been studied in MLR where it

appears to be generated as cell-mediated lympholysis (CML) following blast transformation by allogeneic stimulation (282,285). In both CMC and CML the effector cells appear to be T cells (284,285) and they have been implicated in the mechanisms of graft rejection (reviewed in 182, 183).

DTH skin testing in clinical practice

DTH skin testing using recall antigens or DNCB have been useful means of detecting immunodeficiency states and helping to give an accurate prognosis for many conditions in clinical practice. The list of conditions in which anergy or impairment of DTH reactions occur in disease states is long and includes congenital and acquired immunodeficiency disorders (286), sarcoidosis (286-288), lymphomas and leukaemias (286, 289-291), multiple myeloma (291), various malignancies (292,293), alcoholic cirrhosis (294) and hepatitis B carriage (295). A large number of infections have been associated with anergy (reviewed in 296) and they include measles, rubella, mumps, varicella, infectious mononucleosis, yellow fever, tuberculosis, lepromatous leprosy, syphilis, brucellosis, scarlet fever, streptococcal infections, histoplasmosis, blastomycosis, coccidioidomycosis, toxoplasmosis, schistosomiasis (296) and influenza (297).

Meakins et al produced a number of papers showing a strong correlation between sepsis and mortality following surgery and DTH to recall antigens (298-301). These findings were confirmed by Bradley et al (302).

Also advanced age (286,303,304), debilitating diseases (305), malnutrition (286,306,307) and immunosuppressive agents such as steroids, x-rays and ultraviolet radiation (286,308,309) have been found to depress DTH.

In the following chapter I shall discuss in detail the immune responses in uraemic patients.

III IMMUNE RESPONSES IN URAEMIA

Immunologic alterations in uraemia have been the subject of study for many years. Among the many immune defects which have been attributed to uraemia lymphocytopenia has been recognised since the 1930s (310). Although uraemia has been termed 'nature's immunosuppressive device' (311) and the profound suppression of CMI has been reviewed by several authors (311-317), there are still conflicting findings in the literature with regard to the effect of uraemia on CMI and humoral immune responses. These immunologic alterations are not fully understood and there is uncertainty about the relationship between the susceptibility to infection which occurs in uraemia and the various immunologic defects both in humans (316,318) and experimental animals (319). This is due to the fact that most of the studies have not concurrently investigated both immune and host defence mechanisms in relation to infection susceptibility.

Granulocyte function

In 1956 Balch and Evans showed that uraemic rabbits which were inoculated intradermally with bacteria exhibited smaller inflammatory lesions compared to normal animals (320). This was the first indirect evidence of impaired granulocyte locomotion in uraemia. This finding was confirmed using microcrystalline monosodium urate intradermally in normal and uraemic humans (321). However, reports on in vitro measurement of granulocyte locomotion as studied by granulocyte adherence and chemotaxis gave conflicting results.

Granulocyte adherence in patients with renal failure was found to be impaired (322) or normal (323,324). In addition during haemodialysis it was reported to be enhanced in the first 15 minutes but returning to normal within 60 minutes (325), or impaired 2 hours after the start of haemodialysis, returning to predialysis values by the end of the procedure (324). The discrepancy in the findings with regard to timing of haemodialysis could be explained by the profound granulocytopenia observed at the early stages of haemodialysis (326,327), but the question of whether or not granulocyte adherence is abnormal in chronic renal failure is still unresolved.

Studies on chemotaxis have produced more consistent results. Most investigators have reported depression of both cellular and serum chemotactic activity in both ESRF and haemodialysis patients (323,328-332) and in acute renal failure in rabbits (333). Baum et al found depressed chemotaxis in 40% of 15 undialysed uraemic patients, whereas the remainder and also 28 haemodialysis patients had normal results (334). In the former patients, chemotaxis returned to normal between 3 weeks and 3 months after starting haemodialysis. By contrast, other investigators demonstrated that defective chemotaxis in ESRF and in haemodialysis patients was associated with increased serum chemotactic inhibitory activity (328,331,332) which was mediated by a serum inhibitor of C_5 fragment (332). This defect, once acquired, could not be reversed by haemodialysis and neither could it be correlated with infection or mortality (332).

Opsonisation of S. aureus and Candida albicans has been reported to be normal in chronic renal failure (323,329,335) and the metabolic functions of neutrophils, such as oxidative metabolism, O_2 consumption and quantitative protein iodination were also found to be normal (323,335). Following transplantation, an unexplained defect in Candida opsonisation was observed, but neutrophil metabolic function was normal (335). Opsonins are either heat-labile and these are thought to be mainly complement components or heat-stable which are thought to be antibodies. Serum haemolytic complement activity, C_3 and C_4, were found to be normal in non-dialysed uraemic and haemodialysis patients (331, 334,335). Others reported normal C_{1q} and C_1 esterase inactivator and normal or high C_4 but low C_3 and C_3 proactivator levels in both uraemic and haemodialysis patients when compared to controls (314, 336,337). It seems that although there might be a quantitative reduction in some complement components in renal failure, this does not result in a defective opsonic capacity (323,329). Also it is unlikely that the defective opsonic capacity could be associated with the depressed chemotaxis which seems rather to be due to the generation of complement inhibitory activity and not the low complement levels (328,331,332,334).

Variable results have been reported from the numerous studies of phagocytosis by granulocytes from non-dialysed patients with ESRF and those treated by haemodialysis (316,317,323,324,329,335, 337-340). The majority of these studies reported normal granulocyte phagocytosis by both these groups of patients in the presence of normal or autologous uraemic serum (317,323,324,335). However, a number of studies showed impaired phagocytosis (317,337-339), depending upon the particular micro-organism or particle studied (317). Two investigators reported reversal of the phagocytic defect by haemodialysis (338, 339), and de Gast et al showed that decreased carbonyl iron phagocytosis was found only in blood from the venous side of the dialyser, but not from the arterial side (341). This last finding confirms a previous report suggesting that mechanical trauma during extracorporeal circulation through haemodialysers may cause neutrophil damage and dysfunction (342).

Intracellular killing of Escherichia coli was found to be impaired (340) in patients treated by haemodialysis, whereas killing of Staph. aureus was also depressed in haemodialysis patients (340), but normal in those with chronic renal failure (323).

With regard to the clearing of organisms and antigens from the circulation by the reticulo-endothelial system (RES), it was found that severely uraemic rabbits failed to clear bovine serum albumin following primary sensitisation but clearance was very effective when rabbits were sensitised before uraemia was established (343). In another experimental study of acute renal failure, plasma clearance of coagulase-negative staphylococci was equally effective in normal, sham operated and nephrectomised rabbits (320). In haemodialysis patients the clearance of radiolabelled human serum albumin has been found to occur at a normal rate (344). However, a defect in monocyte Fc-receptor-mediated uptake was described in some patients with glomerulonephritis and chronic renal failure from non-immune causes (345). Reticuloendothelial and mesangial dysfunction was also observed in murine models of immune complex glomerulonephritis (346).

Humoral immune responses

Studies of the effect of uraemia on humoral immunity have produced conflicting findings. Lymphopenia was an early observation (310) and was confirmed by many authors in patients with acute (347) and chronic renal failure (348-350) and in haemodialysis patients (335,348,351,352). These studies described an absolute decrease in both T and particularly B cells while the latter was partially corrected by haemodialysis (349,350). However, despite this well established lymphopenia the evidence for impaired humoral immune responses in uraemia is far from convincing.

Serum IgG, IgA and IgM levels were reported to be decreased (314) or normal (335,340) and the antibody response of the uraemic host to different specific antigens has been variable. In 1956 Dammin et al described normal IgM synthesis in response to immunisation with blood group specific substances (353) and two years later Stoloff et al showed that the anamnestic response to diphtheria toxoid (which is an IgG response) was also normal (354). These initial observations were confirmed later when antibody titres against E. coli and Staph. aureus (312), and Brucella immunisation (355) were all similar to those in normal subjects. In contrast, Wilson et al described poor anti-O (IgM) and anti-H (IgG) responses to typhoid vaccination in uraemic patients (356) and subnormal responses were also observed after immunisation against influenza (357), KLH (348) and bovine serum albumin in uraemic rabbits (343). It is interesting that in the latter study Gowaland et al was able to show that an abnormal antibody response to bovine serum albumin occurred only when rabbits were immunised with the antigen while uraemic. Those animals which were immunised prior to the operation gave normal antibody responses when subsequently challenged with the antigen while uraemic. This would partly explain the discrepancies in the findings by different investigators. It is also likely that factors other than uraemia such as patient selection, drugs, haemodialysis, blood transfusion and malnutrition might play a role in the conflicting results. This was emphasised by a carefully designed study in rats in which animals with moderate to severe

uraemia were found to have comparable antibody responses to sheep red blood cells, E. coli, OX174 bacteriophage and KLH to that found in sham-operated and control animals (358).

From these studies it is apparent that uraemic patients are usually capable of normal antibody production against a variety of antigenic challenges, but a proportion of patients are non-responders to these antigens. Studies on hepatitis B vaccination from our own and other centres showed that the proportion of the non-responders could be as high as 50% (359,360) and so far it is not established whether the defect is due to B cell dysfunction or to a deficiency in the help required from T cells or both.

Cell-mediated immunity

Contrary to the small number of studies on B cell function and antibody production in uraemia, numerous reports have been published on CMI. The abnormalities relating to depressed CMI which involve primarily T cell subsets, their interactions with antigen presenting cells (APC) and B cells, the lymphokines they produce and the effector cells they activate have been more clearly characterised in the uraemic state.

In 1955 Hume et al reported better than expected renal graft survival in nine uraemic humans (361). This observation was confirmed by subsequent studies which showed that uraemia prolonged survival of skin grafts in humans (353), rabbits (362) and rats (363), renal grafts in dogs (364) and cardiac grafts in rats (365). The evidence for immunosuppression induced by uraemia was further strengthened when skin from uraemic animal donors was promptly rejected by normal recipients suggesting that uraemic tissues are not altered per se (362).

The series of studies which followed these reports was directed towards investigation of the mechanisms involved in the depression of CMI in uraemia. Suppressed DTH responses (which will be discussed in the next section) did not appear to correlate with the lymphopenia (366) which has been described in uraemia (335,430,347-352,356). The pathogenesis of this lymphopenia is not clear. Impaired production of lymphocytes is unlikely as a rapid decrease in absolute number occurred within 1-3 days of induction of renal failure in guinea pigs

(367). Structural alterations of the RES such as thymic atrophy associated with thymosin deficiency, poor development of secondary follicles of lymph nodes (317,348,356), hypersplenism (368), shortened half-life of lymphocytes (369), presence of antileukocyte antibodies and vitamin B6 coenzyme deficiency (317) have all been described as features of the uraemic syndrome and might contribute to both lymphopenia and functional abnormalities of lymphocytes.

Recent studies utilising monoclonal antibodies showed decreased per cent and absolute numbers of peripheral blood total T cells as measured by OKT3 and OKT11 (351,352,370). The decreased representation of OKT3 positive cells was mainly due to a decrease in the percentage (351,352) and absolute numbers of OKT4 cell subsets (inducer/helper T-cells) (351,352,370), whereas OKT8 subsets (suppressor/cytotoxic T-cells) were found to be either slightly increased (351), decreased (352) or normal (370) resulting in decreased (351,370) or normal OKT4/OKT8 ratios (352). One of these studies showed that functional assays of suppressor activity did not correlate with the numerical assays suggesting a discordance between marker and functional assays of suppressor cells (370). NK cells as measured by the monoclonal Leu-7 were decreased and NK activity, which is exhibited by cytolysis without prior sensitisation, was impaired in haemodialysis patients (351).

One of the first questions investigators dealt with was whether endogenous immunosuppression in uraemia was due to a cellular defect or to suppression by uraemic serum or plasma. Lymphocytes from uraemic patients have been reported to show impaired ability to stimulate an inflammatory response in the skin of normal recipients compared to cells from normal subjects (371). This lymphocyte transfer test is the in vivo equivalent of the in vitro two-way MLR. In both one-way and two-way MLR, uraemic lymphocytes have been shown to possess defective stimulatory activity in humans (352,372-374) and animals (375,376). One report, however, found that lymphocytes from haemodialysis patients had a normal ability to respond to and stimulate allogeneic lymphocytes in a one-way MLR (377). The authors of this study suggested that differences in cell culture techniques might explain some of the contradictory results. Further evidence of impaired reactivity of uraemic cells comes from reports suggesting

that the graft versus-host reaction is suppressed in uraemic rats (375,378,379) and is mediated by a population of adherent suppressor cells (375,378).

In vitro blast transformation of lymphocytes from uraemic patients after stimulation with PHA has been found to be either depressed (335,340,349,352,373), normal (348,374,377), or even increased (369). Variable results utilising the lymphocyte transformation test (LTT) in uraemia were also reported using other polyclonal activators. Stimulation with PWM was impaired (352) or normal (377) and with PPD was either suppressed (380) or increased (366). Blastogenesis induced by Con-A (340,349,352), LPS (373), KLH and streptokinase-streptodornase (348) was reported to be decreased. From these studies it is apparent that conflicting LTT results were obtained using both T-cell (PHA, Con-A) and B-cell (PWM, LPS) polyclonal activators. Furthermore discrepancies were not limited to studies with uraemic humans, but extended to animal models. In uraemic rats, Alevy et al reported depressed blast transformation in response to PHA, Con-A and LPS (381,382), whereas Miller and Stewart found normal responses to stimulation with Con-A (383). Various reasons have been put forward to explain these discrepancies such as differences in blood samples (341), separation of cells, contamination of or duration of cell culture (348,377), viability of cells, counting efficiency and expression of results (369), concentration of activating substances (383) and suspension of the culture in autologous uraemic serum, normal human serum or fetal calf serum (369,374,377).

From this controversial data it is not possible to say whether the impairment in lymphocyte function is due to a primary defect in cellular function or whether the defect is in the host milieu. Most of the studies described an inhibitory effect of uraemic serum on blastogenesis of uraemic lymphocytes (335,372,374,377,383,384). When lymphocytes were washed and resuspended in normal serum the suppression was abolished (372,374,384). Numerous studies attempted to characterise the metabolites responsible for the inhibitory effect of uraemic sera on lymphocyte transformation. The list of the postulated mechanisms include increased levels of middle molecules such as methylguanidine and guanidinosuccinic acid, cAMP,

corticosteroids, antileukocyte antibodies and lymphocytotoxic antibodies, and decreased levels of thymosin and vitamin B6 coenzyme (317). With regard to whether haemodialysis has any effect on the already compromised CMI, the evidence is inconclusive. Several studies did not find any difference in CMI parameters such as DTH, LTT, MIF and antibody production between non-dialysed and dialysed patients or before and after haemodialysis (348,369,372,374). In contrast, Newberry and Sanford reported improvement in CMI due to a dialysable suppressive factor (384), whereas Ludwig et al described further reduction in PWM-, Con A-, and PWM-induced blastogenesis during the early phase of dialysis followed by a marked increase in the response after 4 hours of haemodialysis (385). It has been claimed that CAPD restored CMI responses more than does haemodialysis due to better clearance of middle molecules (386-388), but this has not been confirmed by others (373,389,390).

Gouillou et al using the Con-A enhancement technique, which is a test that measured Con-A-induced antigen non-specific suppression, reported that diminished CMI responses in haemodialysis patients were partly associated with retention of or increased T-suppressor cell activity (391). This finding was in agreement with results obtained in uraemic rats using MLR and GVH assays (375,378,379). Subsequent studies, in humans however, which investigated Con A-induced suppression found lower or normal suppressor cell activity in haemodialysis patients compared to healthy controls (370,392-394) suggesting that T-suppressor cells were not involved in the immunodeficiency of uraemia. Other factors such as blood transfusion significantly increased the function of suppressor T cells although their number did not change (394).

Other in vitro correlates of CMI which have been reported to be suppressed in uraemia are antibody-dependent cell cytotoxicity and cell-mediated cytotoxicity (395) and production of lymphokines including MIF (270, 348,355), interferon (396) and interleukin-2 (352).

Delayed hypersensitivity

DTH has been traditionally thought to reflect in vivo CMI responses which involve primarily two cell types, T helper cells and APC, as discussed in the previous chapter.

Numerous studies have decribed impaired DTH to skin test preparations in both non-dialysed (348,356,366,397) and dialysed uraemic patients (334,340,348,356,366,387,398-411) and in uraemic animals (412). The substances that have been utilised in skin testing to assess CMI in these reports have included either common recall antigens such as PPD, histoplasmin, candida, monilia, mumps, trichophyton, coccidioidin, streptokinase-streptodornase and proteus (334,340,348,356,366,387,397,398,403-405,409,410) or de novo antigens such as DNCB (398-403,406-408,411), KLH (348) and oxazolone (412). There is general agreement that suppression of DTH is a manifestation of the uraemic syndrome. The proportion of non-responder patients, however, varied widely from 28-87% using recall antigens (356,404) but was less variable (49-84%) using the DNCB skin test (398,406). In control groups the proportion of non-responders to skin tests with recall antigens and DNCB has been reported to be less than 2% and 5-15% respectively (222-225, 399,409). These variable percentages are due to differences in protocols of sensitisation and the timing of sensitisation or other factors that might interfere with expression of CMI by skin testing. For example, Valderrabano et al noticed an increase in non-responders from 46% to 63% within 3 years of introducing a policy of deliberate blood transfusion in their unit (410).

The interest in monitoring CMI in uraemic patients using simple skin tests was promoted by Rolley's initial observation that non-responders to both recall antigens and DNCB had better renal graft survival (398). This finding was supported by a series of publications from our unit (399-402) and elsewhere (403,408-410) while other reports were unable to confirm an association between the response to DNCB and transplant outcome (406,411).

Another area where skin tests found clinical application in uraemic patients, was the association between DTH responses and the susceptibility to both infections and cancer. A number of reports from Canada described a positive correlation between cutaneous anergy

to recall antigens prior to transplantation and development of post-transplant infections and cancer (404,405). These results, however were not confirmed by other studies (413, 414).

Despite these numerous studies it is still not clear at what stage uraemia affects the induction of DTH. From the evidence reviewed in this chapter it appears that uraemia-induced alterations in both morphological and functional properties of T helper cells and APC might be involved in the suppression, suggesting a defect in the afferent limb of the DTH response. On the other hand the fact that a variable proportion of uraemic patients have normal responses to skin tests suggests that uraemic lymphocytes are not intrinsically defective but a suppressive host milieu might alter their reactivity. This concept is supported by the failure of lymph node cells to transfer PPD sensitivity from non-uraemic guinea pigs to uraemic recipients, while transfer has been successful from uraemic donors to non-uraemic recipients (415). This suggests that sensitised lymphocytes which have the capacity to express DTH in a normal recipient are inhibited in the uraemic environment. Furthermore, there is evidence that factors other than uraemia such as malnutrition (286,306,307), infections (296,297), surgery (379), immunosuppressive agents (286,308,309), and blood transfusions (393,394,410) might interfere with the expression of CMI and DTH.

IV THE EFFECT OF BLOOD TRANSFUSION ON GRAFT SURVIVAL

History

Blood has held a mysterious fascination for man from ancient times and the history of blood transfusion is full of mysticism, pseudo-science and fantasy, leading to bold experiments and a multitude of scientific investigations.

One of the earliest references to blood transfusion is in Metamorphoses by Ovid in Medea's effort to restore the youth of Jason's father. She drained his old blood by cutting his throat and then filled his veins with a rich elixir. There are many other ancient references to the use of blood (416). The refusal of Jehovah's witnesses to receive blood transfusion is based on passages from the Old Testament forbiding the eating of blood 'for the blood is the life; and thou shalt not eat the life with the flesh'. Ancient Egyptians bathed in blood to cure elephantiasis and ancient Norwegians drank the blood of seals and whales as therapy for epilepsy and scurvy. Galen recommended the drinking of blood for the cure of rabies and Pliny the Elder and Celsus described the custom of spectators rushing into the arena to drink the blood of dying gladiators. Later in 1492 the blood from three robust young boys was given to Pope Innocent VIII who was suffering from chronic renal failure. This attempt had a devastating outcome as both the Pope and the three young blood donors died shortly after the procedure.

All these early references cannot be considered blood transfusion as we regard it today because they involved transfer of blood per os or through the skin. The first person to advocate blood transfusion in the modern sense was Andreas Libavius in 1615 and his description of the blood transfusion procedure is an example of man's ingenuity. The idea that blood had a certain critical volume within the body below which life was in danger was not appreciated until William Harvey published his monograph De Motu Cordis in 1628 which is the cornerstone of the blood circulation theory. In the 17th century, many scientists claimed the honour of being the first to transfuse blood. Francis Potter in 1652 attempted blood transfusion between chickens and in 1660 Francesco Foli described techniques for blood transfusion using silver tubes connecting a vein of the

recipient with an artery of an animal. The credit for the introduction of intravenous therapy goes to Sir Christopher Wren who in 1656 proposed the administration of drugs into the veins of dogs. Ten years later Richard Lower performed a direct dog-to-dog blood transfusion cannulating the cervical artery of the donor and the jugular vein of the recipient. Lower was the first to demonstrate that a dog could be bled almost to death and then be completely restored by blood transfusion, establishing the role of blood replacement in severe haemorrhage. Jean Denis in 1667 was the first to perform a blood transfusion in a human being. He transfused blood from a lamb into a 15 year old boy suffering from epilepsy and he was also the first to describe a classical haemolytic transfusion reaction. Denis continued his experiments, transfusing blood from lambs to five individuals in total, the last of whom died. This patient's wife charged Denis with murder, but he was exonerated and the widow was subsequently shown to have poisoned her husband with arsenic. Following this incident, the Faculty of Medicine in Paris and the British Parliament prohibited blood transfusion which therefore fell into oblivion for nearly 150 years.

James Blundell, an outstanding obstetrician at Guys Hospital, revived interest in blood transfusion as he felt helpless in the face of massive haemorrhage during delivery. His approach to transfusion was based on a new concept free from ancient ideas of mysticism and the desire to use blood as a tonic or to rid patients of evil spirits. He showed the shocking effects of blood loss which could be reversed by blood transfusion. He also showed that such transfusion was not harmful when the donor and recipient belonged to the same species. In 1818 he performed 10 transfusions in humans of which four were successful and he was rightly credited with being the father of modern blood transfusion. Attempts to circumvent the problem of blood clotting started in 1835 using stirring processes and were brought to a successful conclusion after more than a century with the introduction of the acid-citrate-dextrose solution (ADP) and the citrate-phosphate-dextrose solution (CPD). These attempts in conjunction with the discovery of the ABO blood groups by Landsteiner in 1901 provided practical solutions to some problems faced by early physicians. Other problems relating to the side effects of blood

transfusion such as red cell destruction due to immunologic mechanisms were not understood until after 1900 and generated a great deal of interest among immunologists.

The Effect of Blood Transfusion in Transplantation

In transplant immunology, investigators were aiming to achieve graft acceptance by means of either treating allograft rejection or by inducing immunologic unresponsiveness in the recipient. The conditioning of skin graft recipients by pre-treatment with aliquots of blood from the donors was first demonstrated in rabbits by Medawar in 1946. He suggested that pretreatment with erythrocytes produced prolonged survival of subsequent skin grafts (417). This observation was confirmed by several authors in the late 1950's by experimental transplantation of skin grafts in mice and rabbits (418-420) and in man (421). At the same time Egdahl and Hume (422) and Monroe et al (423) reported hyperacute rejection of renal and musculofascial grafts in dogs and rabbits respectively, when the animals were pretreated with donor blood. These early studies set the scene for a debate on the beneficial or harmful effects of blood transfusion which lasted for over 30 years. The balance between immunological unresponsiveness and the risk of sensitisation induced by blood transfusion has been an exciting and recurring issue in the field of renal transplantation.

Kissmeyer-Nielsen et al in 1966 showed that patients with preformed lymphocytotoxic antibodies exhibited hyperacute rejection of renal grafts (424). This evidence was backed by Terasaki et al who showed poorer graft survival in sensitised transplant patients (425), and these reports deterred transplant surgeons from transfusing patients who were awaiting a kidney transplant.

In 1973 Opelz et al in a retrospective multicentre study of 91 cadaveric renal allograft recipients produced the first clinical evidence of the beneficial role of blood transfusion in graft survival. At one year graft survival was 29% in those who had previously received no blood compared to 47% in those receiving 1-10 units of blood (426). This initial report produced considerable scepticism, but eventually clinicians started to adopt deliberate blood transfusion protocols. This attitude was justified from

reports showing that as the proportion of the non-transfused recipients decreased, graft survival improved. For example, in 1975, the fraction of the non-transfused transplants in the Collaborative Transplant Survey (CTS) was 35% and the graft survival at one year was 45% whereas in 1982-83 those not transfused had dropped to 4% and the graft survival improved to 67% (425). So by the early 1980's only a few sceptics doubted the beneficial effect of blood transfusion on graft survival. The evidence for this effect had accumulated from prospective randomised animal studies and data from one-centre and multi-centre studies in humans.

In rats, Marquet et al reported specific inhibition of organ allograft rejection by donor blood (427). They observed that a single intravenous infusion of as little as 0.05ml fresh donor blood given between one week and several months prior to transplantation increased kidney survival from 12 to 100 days. These results were confirmed and extended by Fabre and Morris (428).

Halasz had described earlier the enhancing effect of donor and third party blood on skin graft survival in dogs (429). These results were confirmed later in experiments with canine renal allografts (430), and were extended by Abouna et al who demonstrated that recipient treatment with whole blood transfusions from multiple donors was associated with significant prolongation of renal graft survival (431). In this study, accelerated rejection did not occur even in the presence of cytotoxic antibodies, whereas Fabre et al, studying three protocols of blood transfusion in a canine model, demonstrated that all transplants which were performed against a positive crossmatch failed to function (432). However, where the crossmatch was negative, the trend of the results was that the transfused dogs had better graft survival than non-transfused animals given the same protocol of azathioprine and prednisolone.

In the rhesus monkey, van Es et al described improved graft survival following one 20ml blood transfusion with further improvement following five 20ml whole blood transfusions (433). Myburgh and Smit studied the effect of multiple blood transfusions on renal graft survival in an outbred population of baboons and found no prolongation of graft survival although donor-recipient MLR was significantly reduced in approximately one half of the animals (434).

In these experimental studies, blood transfusion prolonged graft survival in rats, dogs and rhesus monkeys, although not in the baboons. In all but one study in the dog (432), the animals did not reject grafts in a hyperacute or accelerated manner when transplanted with a kidney from a crossmatch positive donor. Furthermore in the rat model, cytotoxic antibodies reactive with a transplanted kidney appeared to have a strong enhancing effect on graft survival (428).

Following the initial report by Opelz et al (426), a large number of reports agreed on the fact that cadaveric kidney graft survival was increased in transfused patients compared to the non-transfused. Tiwari has reviewed data extracted from 70 reports published between 1972 and 1984, and in all but two there was an increase in one year graft survival in the transfused patients, this increase varying widely from 9% to 70% with a median value between 10% and 20% (435). The variation in the results was ascribed to the small sample size in some reports and also to the so-called centre effect (436). Sengar et al did not find any improvement in the transfused group (437), while Jeffery et al (438) reported a decrease in graft survival. The main criticism of the majority of these studies is that they were retrospective and the groups of patients were often not comparable. Since the transfusion effect seemed abundantly clear, there was reluctance on ethical grounds to embark on prospective, randomised studies. Bucin et al in a prospective study of a small group of 10 transfused and 10 non-transfused patients showed a striking difference namely 90% and 48% graft survival respectively (439). Thus by the early 1980's the finding that transfusion was effective in increasing graft survival was widely accepted. The concern about the risk of sensitisation was outweighed by the 15%-20% improvement in graft survival, particularly as graft survival in the transfused patients with cytotoxic antibodies was not inferior to that of the non-transfused without antibodies (440). Although most agreed that pre-transplant blood transfusion was beneficial rather than harmful, it has never been clear which factors were responsible for the prolongation of graft survival and there was no unanimity on how to use blood transfusion as a means of immunomodulation of the transplant recipient. This was indeed the case, irrespective of whether the blood came from random

donors (not selected on the basis of HLA typing) or from donors identical with the recipient for class I HLA antigens or class I and II antigens, the so-called donor-specific transfusion (DST).

Blood Component Mediating The Transfusion Effect

The active component of blood which is responsible for the preconditioning effect in transplant patients is uncertain. Different types of blood have been used and the results have been inconsistent. Red blood cells may be separated from leukocytes in a variety of ways and the same applies to platelets. Leukocyte-poor blood is that which has been separated from the white cells by centrifugation and removal of the buffy coat or filtering through nylon fibre columns. Washed red cells, providing that the buffy coat is removed at each wash, is an alternative means of producing leukocyte-poor blood. Frozen, thawed red cells, which are deglycerolised by agglomeration (Huggins technique) or by centrifugation, (Meryman technique) contain even fewer leukocytes because most of these cells are killed by the freezing and the thawed cells are then washed to free them from the cryoprotective glycerol and the leukocytes (441). Thus depending on the method used, the number of white cells contaminating the final red cell product can vary widely from nearly 5 billion in whole blood and in concentrated red cells to less than 2 billion in filtered or washed cells and to less than 10 million in frozen cells (441). This varying degree of white cell or platelet contamination of the differing blood products is one likely reason for some of the discrepancies reported in the literature regarding the variation in transfusion effect on graft survival. For example, Polesky et al reported superior graft survival using frozen cells compared to packed cells or whole blood (442), whereas Horimi et al described the opposite (443). A number of authors found no difference between the different blood products (444-446). Briggs et al from our own centre showed that transplant recipients receiving whole blood or washed cells had a graft survival of 77% at one year which was not significantly different from that of patients receiving frozen cells (71%). In contrast, non-transfused patients had a strikingly worse graft survival of 28% at one year (447). Opelz and Terasaki found no benefit from one unit of frozen

cells while multiple transfusions had a positive effect on graft survival (440). Finally Persijn et al in a prospective study described 80% graft survival at 200 days in 40 patients receiving one leukocyte-poor blood transfusion (90% of white cells removed) compared to 33% survival in the remaining 12 patients who received one to three units of leukocyte free blood (98% of white cells removed) (448). From these studies it would appear that leukocytes are the main mediators of the transfusion effect and the main antigenic stimulus for the sensitisation risk as well. Attempts to minimise sensitisation without altering unresponsiveness have focussed on less immunogenic blood products. Borleffs et al described a strong effect on graft survival in rhesus monkeys pretreated with random donor platelets (449), and this finding was confirmed by Oh and McClure (450). Both authors found a reduced rate of sensitisation possibly because platelets bear no class II antigens. However, these results have not been confirmed in dogs (451) and man (452). Chapman et al in a prospective protocol in humans receiving platelets found no development of cytotoxic antibodies but the rejection rate was higher than expected and the survival rate of 44% was worse than in non-transfused patients (452). A poor graft survival of 55% at six months in 36 patients transfused with platelets was also seen in Opelz's multicentre data (453) and this did not further encourage platelet administration as a means of producing the transfusion effect.

In experimental studies in rats and rhesus monkeys the use of plasma only or immunoglobulin did not have any effect on graft survival (449, 454) and this was also the case when ultrasonicated (454) or irradiated blood was given (455). Purified erythrocytes produced enhancement of skin homografts in rabbits (419) but such animal models may not predict the outcome in man since most animal species express HLA antigens on their erythrocytes, while human red blood cells do not.

Amount of Blood

Opelz et al in their initial and subsequent studies found that the beneficial effect of blood transfusion increased as the number of transfusions increased (425, 426, 440). These results were confirmed

by Horimi et al (443) and Ferhman et al (456). The former author, analysing the UCLA data, reported an improvement in graft survival at one year, from 41% in the non-transfused to 52% with one unit of blood. The highest survival rate of 75% was found in those receiving 14 units of blood, whereas subsequent transfusions did not appear to have any further effect (443). Fehrman et al found the best graft survival to be in patients receiving 4 to 19 units of blood or 20 units or more, compared to those receiving zero or 1-3 units. Patients receiving 1-3 units did not have significantly better graft survival compared to those who were not transfused. Kerman et al belong also to the school advocating at least 5 units of pre-transplant blood as they described a difference in graft survival of 41% between 51 transplant recipients receiving more than 5 units and 53 patients receiving less than 5 transfusions (457). In contrast to this school, Persjin et al, analysing the Dutch data, described a striking 87% graft survival at one year in 30 patients receiving only one unit of blood compared to 32% in 74 patients who had not been transfused before transplantation (448). Thus the message from Leiden was that one unit was enough and the effect was long lasting (458). Williams et al from Oxford found that the transfusion effect was achieved with 1-3 units of blood (459) and the same conclusion was reached by Huncicker et al (446), whereas Feduska et al showed that the transfusion effect achieved with 1-5 units of blood was as good as that resulting from 6-10 units or more than 10 units (460). Briggs et al from our centre also reported that the beneficial effect of transfusion was no greater in patients transfused with more than 5 units compared to those given less than 5 units (447).

Ferrara et al in a prospective study used repeated small aliquots of 15ml blood from a single donor to induce HLA unresponsiveness in volunteer recipients (461). He showed that the anti-HLA cytotoxic response decreased progressively with time to a state of complete unresponsiveness despite continuation of immunisation. This idea was used by Wood et al who tried to induce the transfusion effect without provoking sensitisation in skin grafted mice which were transfused repeatedly with small aliquots of 0.2-5ul of donor blood (462). This policy found its clinical

application in a recent paper by Burrows et al who gave three aliquots of third party HLA-defined blood to cadaveric renal transplant recipients treated with azathioprine or CyA (463). The overall graft survival was 83% at one year, with CyA-treated patients having a 95% survival rate compared to 73% for the azathioprine-treated patients. However, Sirchia et al in an earlier prospective study found that small aliquots were not as effective as three standard units of blood in that they achieved 60% graft survival compared with 78% at five months in 16 and 18 patients respectively (464).

Timing of Transfusions

Controversy is limited not only to the number of transfusions required to achieve the maximum effect, but also with respect to the optimum interval between the last transfusion and transplantation and the efficacy as well of per-operative transfusions.

Werner-Favre et al reported that a delay of more than three months between the last transfusion and transplantation significantly decreased six month graft survival from 84% to 58% (464). This view was supported by Hourmant et al who found that patients transfused within three months prior to transplantation had better graft survival when compared to those transfused more than six months before (466). These findings were supported by a study in mice in which maximum graft survival was observed when grafting took place within 2 days of the last of three transfusions and less effect was evident when grafting was delayed for 60 days (467). In contrast, the Leiden group took the opposite view namely that the effect of a single transfusion is long lasting (448, 458). Opelz analysed the CTS data and found an advantage if the interval between transfusion and transplantation was 30 to 90 days as compared to shorter or longer intervals only in patients who had received a single transfusion prior to transplantation (453). This advantage was diminished in recipients who were given 2-5 transfusions and disappeared altogether with more than five transfusions.

Stiller et al claimed that per-operative transfusions improved cadaveric renal allograft survival from 44% to 79% both in those who had never been transfused and in those previously given blood (468).

Williams et al reported improved graft survival with per-operative transfusions only in previously non-transfused recipients (469), whereas Corry et al showed that graft survival of the per-operatively transfused patients was intermediate between the non-transfused and the prior transfused patients (470). Several reports, however, including some from multi-centre studies with large numbers, failed to find any effect from per-operative transfusion (448,453,460). Glass et al argued against the use of per-operative transfusion as they found that it was without benefit in previously non-transfused patients and resulted in poorer graft survival in previously transfused recipients (471).

Storage Time of Blood

As preservatives can protect red cells but not white cells for several weeks, the question arises as to how fresh blood should be and there is very little information about this. One study showed that blood stored for less than three days induced a significantly better graft survival compared to blood stored for more than five days (425). Differences between centres which were attributed to the amount of blood transfused might have been due in part to differences in the storage time of blood.

Effect of HLA Matching in Transfused and Non-transfused Recipients

Early studies suggested that the beneficial effect of transfusion was confined only to well matched transplant recipients (472, 473). While Fehrman et al reported that a beneficial effect of transfusion was found only in patients receiving kidneys with two or less HLA-A and B incompatibilities (456), Moen et al described increased graft survival only in patients receiving DR mismatched grafts (474).

However, most of the studies suggest that HLA matching and transfusion have a synergistic effect on graft survival (453, 457, 459). Kerman et al found that transfusion enhanced the benefit of HLA,B and DR compatibility on graft survival in recipients with less than 2 A,B and 0-1 DR mismatches. These patients had a one-year graft survival rate of 94% when pretreated with more than five transfusions compared to a survival of 38% in those receiving less

than five units of blood (457). Opelz showed that whereas the effect of HLA-DR matching was stronger in non-transfused patients than in transfused ones, even well-matched non-transfused patients did not do as well as mismatched transfused recipients and the highest graft survival rate was observed in those who were both well matched and transfused (453). Schulak et al described a combined effect of HLA-A and B matching and blood transfusion (475). He found a one year graft survival of 87% in patients who were matched for one DR and more than two A and B antigens. This compared with a 42% survival in recipients with a one DR match but a poorer A and B match. The potentiating effect of HLA-A and B matching and transfusion in this study was observed only when blood was given prior to transplantation and not peroperatively.

HLA Matched Transfusions

In order to minimise the sensitisation risk, Nube et al gave HLA-A and B matched blood to 15 patients who had never been transfused before and compared their graft survival both with that of a historical non-transfused control group and a group who received multiple random third-party transfusions (476). The one year graft survival of 87% in those who received the HLA-matched blood was not significantly different from the 76% in those given random blood but was much better than the 76% of the non-transfused group. The sensitisation rate in patients receiving HLA-matched blood was 27% compared to 43% in those given random transfusions (476). In contrast to this report, Albert et al found that HLA-A and B matched transfusions were associated with poor graft survival (477), and Vanrenterghem et al also did not observe any prolongation of graft survival with HLA-A and B matched transfusions (478).

Blood Transfusion and Re-transplantation

Recipients of regrafts have a higher sensitisation rate (479), and transfusion prior to a regraft may further increase cytotoxic antibody levels. Persjin et al found that blood given between a first and second transplant had no effect on regraft survival, but patients who had been transfused prior to the failed first transplant had a 58% one year survival of a second graft compared to 38% in

those who had not received any blood before their first transplant (480). In contrast, the results from the UCLA Transplant Registry did not demonstrate any beneficial effect of transfusion on regraft survival, particularly in those whose first graft had functioned for less than six months (480).

Third Party Transfusions in Living Related Transplants

A number of studies have been carried out with conflicting results. Oei et al found that third-party transfusions (TPT) had no effect on the graft survival of living related transplants (481), whereas Polesky et al reported minimal benefit (442). In another series of studies, Solheim et al (482), Brynger et al (483), and Richie et al (484) reported that graft survival improved in one haplotype matched grafts but not in HLA identical transplants. Opelz in an analysis of the CTS found one year graft survival of 70% in 67 patients with no transfusion, 75% in 150 patients with one to three units of TPT and 90% in 226 patients with more than three TPT (453). Similarly, a beneficial effect of TPT in transplants from related donors was reported both by Fuller et al (445) and from the UCLA Registry (485). In the latter study, graft survival was better with TPT in transplants from parents to offspring, one haplotype-matched siblings and HLA-identical siblings. The optimum transfusion effect was achieved with one to four transfusions and more blood did not increase survival rate.

The findings from studies in dogs and monkeys has not clarified the controversy. A TPT effect in dogs was evident in DLA-mismatched, but not DLA-matched renal allografts (486). In rhesus monkeys, van Es and Balner did not find any synergistic or additive effect of DR matching and transfusions (433), whereas Borleffs et al did (487).

Effect of Donor Transfusion

Frisk et al reported that TPT given to donors led to a significant improvement in graft survival in transfused recipients (488). This observation was supported by Jeekel et al both in humans and rats (489). In the rat model, TPT given to the donor markedly influenced graft survival provided the recipient was transfused as well. The transfusion of recipient-type blood to the donor was not

effective. In humans there was a one year graft survival of 84% in patients receiving their grafts from donors who were transfused in the period immediately preceding death compared with 53% in those whose donors were not transfused (489). Opelz found that the donor's transfusion status had no effect on graft outcome either in the complete series or in the subgroup of recipients who were not transfused (453).

Long-term Effect of Transfusion

The results from a single centre analysis in Scandinavia showed that transfused renal allograft recipients had a 20% better early graft survival rate than non-transfused patients but that this had disappeared by six years after transplantation (490). This confirmed an earlier report from the UCLA Registry which showed that the difference between transfused and non-transfused transplant recipients was established within three months of the transplant and beyond one year, pre-transplant transfusions had little or no effect on graft survival (485).

The Risk of Sensitisation After Third-party Transfusion

Does the risk of sensitisation offset the potential benefit of transfusion? Although sensitisation of some patients is unavoidable, identyfing patients at high risk and learning more about the factors that trigger broad antibody responses has been one of the main themes of research in an attempt to formulate guidelines that may decrease the incidence of sensitisation.

Most evidence suggests that blood transfusion on its own is a poor inducer of a primary immune response but could strongly amplify a response that has occured previously (420, 440, 443, 456, 479). Opelz and Terasaki reported that only 58 of 1852 patients (3%) who received solely packed cell transfusions had antibody reactivity against more than 50% of a random panel (440). Only 1.8% of non-transfused patients had broadly reactive antibodies, compared with 5.6% of patients who received 1-5 transfusions, 4.4% of patients given 6-10 transfusions and 13% of patients who had had more than 10 transfusions. In another study from the same centre, fewer than 2%

of the male patients formed antibodies after 10 transfusions against more than 90% of the panel compared with 14% of the females (491). The difference in reactivity between males and females was associated with the number of previous pregnancies. Almost one in three females with more than three previous pregnancies became broadly reactive (PRA>90% of the panel) after 10 transfusions. Thus the results from UCLA showed that pregnancy is a potent stimulus to the formation of lymphocytotoxic antibodies following transfusion which on its own results in a surprisingly low rate of sensitisation. In accord with this view were the findings from one centre studies by Werner-Favre et al (465), and Fehrman et al (456,479). Also a previously rejected graft was shown to be another strong co-stimulus with blood transfusion in the induction of broad sensitisation. Sanfilippo et al found that only 3% of non-transfused primary graft recipients had panel reactive antibodies (PRA) over 60% compared to 4.5% of the transfused primary transplants, 14% of the non-transfused recipients with one failed graft, and almost 50% of those transfused with two previous failed grafts (492). This group of authors felt that the benefit of blood transfusion out weighed the sensitisation risk as graft survival was better in transfused recipients with PRA compared to non-transfused patients without PRA (440, 443).

In contrast, Betuel et al showed that 62% of 77 prospectively transfused patients formed PRA and 11% of those with titres of more than 76% of a random panel became untransplantable (493). High responders producing antibodies before their fifth transfusion were especially at risk and had graft survival of 50% at two years compared to 73% for those who developed antibodies after the fifth unit. A similar view was taken by D'Apice and Tait who removed patients from the transplant list if PRA developed to more than 10% of the panel after the first two transfusions (494). Solheim et al suggested that if patients who died while waiting for a transplant because of broad sensitisation are designated as 'graft losses,' then the beneficial effect of transfusion was almost lost (495). This view is rather extreme as all deaths could hardly be attributed to sensitisation and the delay of receiving a transplant. It reflects,

however, the disturbing prospect of a delay in transplantation which, in patients with 3-20% PRA was only three months, while in those with more than 80% PRA it was almost two years (496).

The type of sensitisation after transfusion is relevant to its beneficial effect. Antibodies with differing specificities may give rise to a positive crossmatch between recipient serum and donor lymphocytes. These antibodies may be directed against HLA-A and B antigens, B cell autoantigens, B and T cell antigens, T cell alloantigens and HLA-DR and Lewis antigens (497). Antibodies against B cells were found more frequently than antibodies against T cells after transfusion (479, 491). Most investigators consider that IgM-B cell cold antibodies are enhancing antibodies and prolong graft survival, whereas there is controversy about B cell warm antibodies (reviewed in 497, 498). There is evidence that IgM cold-reactive T and B cell autoantibodies are not directed against HLA-A, B, DR specificities and are not damaging to the graft (498). Most centres however, would be reluctant to transplant patients who had positive B-warm or T-warm crossmatches but it seems that even the presence of these antibodies was preferable to no transfusion, since grafts in these patients lasted longer (499).

Donor Specific Transfusion (DST)

In 1949 successful skin transplantation from parent to son was reported after unintentional conditioning by donor blood transfusion (500). The first clinical application of DST in renal transplantation was reported by Eschbach et al in 1965 (501). The transplant outcome in this case report was unsuccessful due to renal vein thrombosis. Two years later Dossetor et al reported the first successful renal transplant after DST (502). Batchelor et al extracted cytotoxic antibodies from the father of a uraemic child after immunisation with his wife's leukocytes. This preparation was then administered intravenously as enhancing antibody when the child received a kidney from his mother, immunosuppression consisting of small doses of azathioprine and prednisolone (503). Newton and Anderson reported four successful transplants, two of whom received donor blood from non-identical relatives and the other two from unrelated live donors (504).

Cochrum et al (505) described a protocol for DST in one-haplotype matched pairs with high reactivity in MLC and it was subsequently widely established in clinical transplantation by Salvatierra et al (506). Utilising one 200ml unit of donor blood given in three aliquots at two week intervals, Salvatierra, reported 95% and 93% graft survival at one and two years respectively in 86 recipients who did not develop cytotoxic antibodies after the DST (506). These results were significantly better than the 56% and 53% graft survival at one and two years respectively in a historical control group of one haplotype pairs with highly reactive MLC who had not received DST. However, a high sensitisation rate of 30% was observed in the series and these patients could not therefore receive a kidney from that live donor. The DST effect was confirmed both in recipients who had not previously received third-party blood (507), and in those who had received such blood (508).

A major problem of DST protocols was the development of a positive crossmatch by recipients against their potential kidney donors. Efforts to minimise the risk of sensitisation included the use of stored blood instead of fresh (509, 510) and the giving of the DST as a single 200ml transfusion rather than 3 aliquots (511). Glass et al found that the simultaneous administration of azathioprine with DST reduced the sensitisation rate from 31% to 14%, yet the one year graft survival remained high at 90% (512). Hillis et al gave Cyclosporin from one week before the first DST in 26 transplant recipients and observed only a transient anti-B cell sensitisation which disappeared over 8-12 weeks (513). These methods seemed to reduce the immunogenicity of blood in terms of lymphocytotoxin production without adversely affecting graft survival.

The beneficial effect of DST in one haplotype matched related donor transplants was confirmed by Opelz in the CTS data analysis (453). However, comparison of patients receiving three units of DST with patients receiving more than three units of random third party blood without DST showed virtually identical graft survival (453).

The few reports so far available regarding DST from unrelated live donors (504, 514, 515) do not allow conclusive comments. In a recent study of 41 such patients, 10 became sensitised (24%) and were excluded from transplantation (515). In the remaining 31 recipients graft survival was 90% at one year.

Mechanism of the Blood Transfusion Effect

The mechanism of the blood transfusion effect has remained uncertain and several attractive hypotheses have attempted to elucidate the phenomenon, sometimes contradicting each other. To date, despite lengthy debate of the pros and cons of various proposed mechanisms, it is impossible to completely resolve the confusion surrounding the transfusion effect in renal transplantation.

Selection theory: In clinical studies, a selection factor has been described in the sense that patients with broadly reactive lymphocytotoxic antibodies after transfusion, labelled as 'high responders', are either excluded from transplantation or receive better matched kidneys (456, 479, 495). According to this theory, blood transfusions eliminate transplant candidates whose outcome is predestined to be poor or act to select better donor-recipient combinations by crossmatching. The observation by Opelz et al, however, that transfusion improves graft survival among multiply transfused transplants who do not produce anti-HLA antibodies provides evidence against this theory, at least in cadaveric transplantation where the number of highly sensitised patients after transfusion is too low to explain the entire transfusion effect (425). This selection theory could be more relevant in DST protocols where the sensitisation rate is higher and the improvement in graft survival would be compatible with selection by crossmatching (425, 516). Findings from experimental models argue against selection being the main cause of the transfusion effect (433, 486), but the significance of cytotoxic antibodies in precipitating hyperacute or accelerated rejection in animals seems to differ from that in man (427-431, 433).

Prolongation of Uraemia: Guttmann found a statistically significant correlation between the number of pre-transplant transfusions and the time of dialysis-dependent uraemia (517). He suggested that this association could further promote a selection factor leaving sensitised patients waiting for longer periods of time for a transplant, thus prolonging the duration of uraemia which is itself immunosuppressive. In a study in our own centre relating graft survival to the DNCB skin test response, we found that the duration of dialysis was associated with the results of this test (402).

Immunisation to CMV: Andrus et al suggested that transfusion induces resistance to CMV infection during the post-transplant course by exposure to the virus in the transfused blood. Subsequent avoidance of the need to discontinue immunosuppression contributes to better graft survival (518). This argument does not apply to DST protocols where few donors have evidence of overt CMV or residual antibodies from prior infection. It is unlikely that CMV is related to the blood transfusion effect.

Transfusion-associated acquired immunodeficiency: Reports of acquired immunodeficiency syndrome (AIDS) among haemophiliacs show that repeated administration of factor VIII with or without other blood products may cause severe immunodeficiency (519, 520). Curran et al reported 18 cases of AIDS in adults who were not in a high-risk group but who had received blood transfusions within five years before diagnosis (521). It is interesting that no blood donor with clinical AIDS was identified although such a case has been reported elsewhere (522). It is not clear, therefore, whether transfusion-associated immunodeficiency is due to the blood product itself or a contaminating virus or other agent (523-525). As the latent period after acquisition is of several years duration, it is difficult to determine whether transfusion acquired immunodeficiency and the beneficial effect on graft survival are related phenomena. However, the former phenomenon has a very low incidence for it to be closely associated with the transfusion effect in transplantation which has been described in numerous studies both clinical and experimental.

Iron Overload: De Sousa has postulated that the transfusion effect could be related to accumulation of iron and products of red cell breakdown which have been shown to exert a non-specific suppression (526). In vitro ferritin suppresses T cell mitogen responses and the MLR (527), and serum ferritin levels were found to inversely correlate with the OKT4/OKT8 ratio in the blood of transfused dialysis patients (528). Patients with iron overload have been reported to be susceptible to infection (529), and to have a worse prognosis in certain forms of malignancy (530). Keown and Descamps applied the significance of these findings to clinical transplantation. They suggested that rapid endocytosis of transfused erythrocytes in vivo may impair antigen presentation and lymphocyte activation resulting in a non-specific erythrocyte-mediated unresponsiveness (531). Later the same authors attributed this effect to an inhibitory action of haemoglobin, ferritin and iron radicals on the proliferation of cytotoxic effector cells (532).

In monkey experiments, graft prolongation was also obtained if erythrocyte-free transfusions were given to the recipients (533). Also in humans, only one unit of blood, which could not cause iron-overload, was sufficient to produce the transfusion effect, a response not seen when leukocyte-free blood was used (448, 458). The results of these studies argue against any important role of iron in the blood transfusion effect.

Induction of Unresponsiveness: There is evidence that blood transfusion induces both non-specific immunosuppression mediated by suppressor cells and specific unresponsiveness mediated by anti-idiotypic antibodies (394, 425, 458, 495, 516, 531-537). Classical enhancement and acquired immunologic tolerance have been considered as possible explanations for the transfusion effect. However, both seem unlikely to be causative principles as by definition they represent donor-specific events and the beneficial effect has been described using a single random blood transfusion (537). Furthermore, if the transfusion effect is mediated by erythrocytes which do not express HLA antigens, then the effect cannot be attributed to any hypotheses involving enhancement or tolerance in their classical sense (531, 532, 537).

In an attempt to reconcile these contradictory findings, van Rood suggested that non-specific and specific mechanisms act sequentially in a two-step or multi-step process (458). He postulated that in an initial stage, suppressor cells are induced which suppress the formation of antibodies to class I HLA antigens. This is followed by a second stage in which anti-idiotype antibodies are formed, preventing activation of T cell clones which could react with the class I antigens of the blood donor. Also these antibodies, if they differ from those of the blood donor, can react too with the class I antigens of the kidney donor. The second stage is thus blood-donor-specific and may also be kidney-donor-specific even when the blood and kidney donors are distinct and do not have antigens in common. However, the inactivation of the responding T cell clones is optimal in DST protocols because in these the blood transfusion donor and kidney donor share antigens.

The central point in this hypothesis is that blood transfusion induces a state of unresponsiveness because T helper cells cannot be activated, and therefore if a kidney is transplanted during this time it will not be rejected. If this is the case, the effect of pre-transplant blood transfusion might be replaced or its importance diminished by drugs which are more effective in suppressing T helper cells such as antithymocyte globulin (ATG) or Cyclosporin (458). However, pre-transplant transfusions, apart from inducing suppressor cell activity may induce T helper cells. This could lead to activation of K cells with their strong destructive capacity of allogeneic cells in monkeys and mice (533). Thus the balance between helper and suppressor activity might be switched either way depending on the disparity or matching for certain histocompatibility antigens between the host and donor.

The suppressor cell mechanism has been the most attractive explanation for the transfusion effect and the supporting evidence has resulted from research efforts utilising various suppressor cell models mostly in animals and to a lesser extent in humans. This has inherent limitations as no consensus exists with regard to the most appropriate model for suppressor cell studies which might be relevant

to man. It has been difficult to establish which of the in vitro and in vivo experimental findings were relevant to the transfusion effect observed in human transplants (516).

MacLeod et al, in a series of papers, suggested that non-cytotoxic, Fc receptor blocking antibodies can develop in response to random and donor specific blood transfusion and their presence in pretransplant recipient sera was associated with improved renal allograft survival (538-541). They found that these blocking antibodies were IgG alloantibodies, not autoantibodies, that they were HLA linked and that they could be detected in the sera of women with successful pregnancies but not in the sera of those with repeated spontaneous abortions (542). Forwell et al from our centre confirmed that indeed the presence of blood-transfusion-induced Fc receptor blocking antibodies revealed a striking correlation with subsequent renal allograft survival (543-545). However, in contrast to the studies of MacLeod, the effect seemed to be mediated by immune complexes rather than monomeric IgG. Serum fractionation studies showed that although the IgG fraction produced significant Fc receptor blocking, this activity did not distinguish transplant acceptors from rejectors. By contrast, the serum fraction of molecular weight more than 19S was present more often in the transplant acceptor group (544). Of interest too, the level of blocking activity produced by this high molecular weight serum factor in vitro was found to correlate with depressed CMI as assessed in vivo by the DNCB skin test, the response to which has been reported elsewhere to correlate with graft survival (399-402).

Donnely et al measured plasma-suppressive activity using an electrophoretic test of normal lymphocyte function to define pretransplant patients with high and low activity, which they attributed to a qualitative but not quantitative change in α_2-macroglobulin (546). They found that plasma suppressive activity was greater in transfused patients compared with controls and correlated with renal graft survival.

From these studies it is apparent that sera from transfused pre-transplant patients exhibit immunosuppressive activity mediated by either specific anti-idiotypic antibodies or non-specific Fc receptor blocking antibodies or immune complexes. However the

relationship between these types of antibody and prolongation of graft survival in clinical transplantation seemed to be limited by differences in the methods used to detect antibody/immune complex-mediated immunosuppression (544).

Clonal deletion theory: Terasaki proposed this hypothesis which states that the primary function of blood transfusion is to immunise patients and not to induce tolerance or enhancement or to serve as a donor selection mechanism (547).

According to this theory the transplant acts as a secondary stimulus to transfusion, and if the patient is then treated with immunosuppressive drugs in high dosage the proliferating reactive cells are killed or inactivated. Hence, the critical differences between a transfused and non-transfused patient is in the timing of the rejection in relation to maximal immunosuppression. To support his theory, Terasaki pointed out that multiple transfusions are more effective than a single transfusion and in experimental transplantation, blood transfusion not followed by immunosuppression has been found to be less effective (547). Woodruff in the early 1960s suggested the possibility of promoting graft survival by clonal elimination (548). He tested this hypothesis experimentally by injecting mice intravenously with allogeneic spleen cells, treating them with whole-body irradiation or immunosuppressive drugs, and challenging them with a skin graft from a mouse of the strain that provided the spleen cells. Clonal elimination was not demonstrated in those experiments, but the use of a free skin graft as a challenge test of non-reactivity is different from experimental models of vascularised whole organ transplants (549). However, recent findings from studies of DST protocols are not in accord with this theory and support the view that transfusion induces inhibition of anti-donor immune responses rather than stimulation (550, 551). These findings suggest that alloreactive T cell blasts induced by allografting following DST may be eliminated, not simply by immunosuppressive therapy, but also by autoregulatory mechanisms, such as anti-receptor antibodies or suppressor cells. This view helps to explain instances in which DST alone, without immunosuppression, enhanced cardiac and renal allograft survival in rats (428, 552).

In conclusion the explanation of the transfusion effect in renal transplantation is unclear. It seems likely that apart from the possible relevance of a selection factor, both non-specific and specific mechanisms are implicated. These involve suppressor cell activity with antibody or immune complex-mediated suppression leading to inactivation or decloning of proliferating reactive host cells and the induction of a state of unresponsiveness.

Blood Transfusion In the Cyclosporin Era

The European multi-centre study on the effect of Cyclosporin in renal graft survival did not provide evidence for a beneficial effect of pre-transplant blood transfusion (553). This was contrary to initial findings from the CTS report (425, 453) and results from Oxford where in two randomised studies, graft survival was significantly better in transfused Cyclosporin treated patients (87%) than in those non-transfused (58%) (554, 555). Results from UCLA also supported this view (556). By 1986 the debate had developed as a large multi-centre study from Scandinavia (557) and other smaller single-centre studies reported the absence of the transfusion effect in Cyclosporin-treated transplant recipients (558, 559). At the 11th International Congress of the Transplantation Society in Helsinki, Opelz, reporting for the CTS, surprised the audience by showing that in his database too, the transfusion effect had disappeared (560). In the years 1982 and 1983, graft survival at one year was 60% in non-transfused and 80% in transfused Cyclosporin-treated recipients. Since then there has been a steady improvement in graft survival among non-transfused transplant recipients, so that by 1985 the benefit of transfusion was reduced to about a 5% difference in graft survival regardless of whether patients were treated with Cyclosporin or not (560). Thus by 1985, graft survival at one year in non-transfused Cyclosporin-treated recipients had improved from 60% to 80% and in non-transfused-azathioprine treated patients, from 50% to 70%. Similar results were observed in recipients of one HLA-haplotype-matched related transplants receiving DST (560). The explanation for these surprising results is not clear. A recent editorial in the Lancet suggested that more accurate diagnosis of rejection utilising renal biopsies and advances in its treatment

could be a contributory factor to the improved results (561). At the beginning, the impact of these findings in changing existing blood transfusion protocols was rather slow and cautious as many units were achieving excellent results with one-year graft survival of 80-85% (561). At the last International Congress of the Transplantation Society in Melbourne, Opelz suggested that the minor beneficial effect of transfusion on graft survival does not justify a deliberate transfusion policy of potential transplant recipients (562).

The Effect of Blood Transfusion Outwith Transplantation

As interest in the blood transfusion effect in the field of clinical transplantation was fading in the last few years, an editorial assembled an impressive series of unexpected consequences of blood transfusion outwith transplantation (563). The list included a) remissions of rheumatoid arthritis by blood transfusions, b) the effect of a husband's blood transfusion in cases of spontaneous abortion, c) enhancement of AIDS by an immunosuppressive factor in the blood, and d) the effect of blood transfusions in the prognosis of neoplasia. There are numerous reports suggesting that non-transfused cancer patients survived longer compared to patients who have received blood. The reports relate to carcinoma of the colon and rectum, breast, lung, kidney, uterine cervix, tissue sarcomas, and prostate (563). Experimental studies in rats have supported these clinical findings by showing that inoculated sarcoma had a faster rate of growth if the animals had been transfused previously with compatible allogeneic blood (564).

The possible implications of blood transfusion in areas other than transplantation was raised some years ago by Woodruff and van Rood (565). They suggested that since the transfusion effect seemed to be partly non-specific, transfusions might modify immune responses to a wide range of antigens of other than transplanted tissue. They also reviewed the literature on the effect of blood transfusion in relation to a) enhancing AIDS; 2) benefiting autoimmune disease; 3) contributing to an increased incidence of lymphomas and skin cancer in transplant recipients and 4) facilitating the growth, spread or recurrence of tumours in other transfused patients. They

concluded that evidence of the relationship between these phenomena was still inconclusive and encouraged the collection of more data (565).

More recently Singh et al revived interest in blood transfusion as a promoting factor of tumour growth by demonstrating a link between sarcoma and blood transfusion in rats (566). Another recent study from Newcastle described an association between recurrence of colorectal cancer and perioperative blood transfusion (567). These reports suggest that it is worth pursuing the transfusion effect outwith transplantation by further prospective studies.

EXPERIMENTAL DESIGN AND AIM OF THE STUDY

The experimental design of the study is shown schematically in Figure 1. Forty eight dialysis patients who had not received previous blood transfusion were randomly allocated to receive 5 or 10 units of third party blood in the form of packed cells at two week intervals prior to admission to the transplant pool. The blood was matched for ABO and Rhesus blood groups but not for HLA antigens. Twelve dialysis patients and 12 healthy subjects who had not received previous blood transfusion were also studied as control groups. The characteristics of the 48 transfused patients and the 12 uraemic controls are shown in Table 1. In the group of the normal controls there were 7 males and 5 females with a mean age of 36 years (range, 24-59 years).

The CMI of the transfused patients was measured by skin testing with DNCB and four recall antigens before the first blood transfusion and 14 days after the last blood transfusion. Haematology and biochemistry results were recorded at these times. In addition, prior to the first and 14 days after each transfusion patients were screened for: a) complement levels, b) cytotoxic antibodies, c) titres of IgG secreting cells (plaque forming cells, PFC) in the peripheral blood, both spontaneously and after stimulation with PWM and SAC using a protein A assay, and d) PGE production in supernatants from both Con-A stimulated cultures and unstimulated ones of PBMC.

The uraemic control patients were skin tested with DNCB and the four recall antigens and these tests were repeated 3 months later, (a time interval equivalent to that of the transfused patients). Haematology and biochemistry results were recorded and complement levels and cytotoxic antibodies were measured and assays of PFC, PGE production were performed on three or four occasions over the period of 3 months. The group of normal subjects was used as a control group for the measurement of the complement levels, PGE production, PFC titres. The methods and results from this randomised controlled study are described in the first 8 chapters. In chapter 9 I describe results from a large

number of patients with regard to factors determining the response to the DNCB skin test and its value as a means of predicting renal allograft survival.

The data were stored in the current Glasgow University Mainframe Computer which is an ICL 2988, operating under the ICL VME system. All computations were done with the statistical computing packages Minitab (568) and SPSS-X (569). The tests of statistics which have been applied using these packages will be discussed in the respective chapters. Probability values were considered significant when $p < 0.05$.

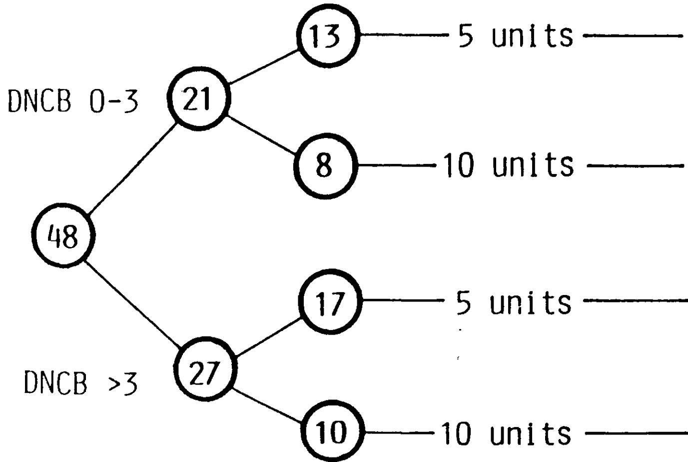
The aim of this thesis was:

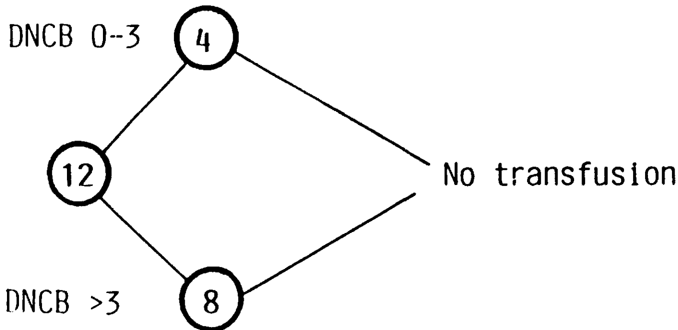
a) to assess the effect of blood transfusion in uraemic patients,

b) to see whether individuals with strong and weak reactions to DNCB and recall antigens differ in their response to blood transfusion, and try to define the optimum number of transfusions for each group to achieve a beneficial effect,

c) to see whether blood transfusion induced changes in the immune response could be monitored with simple skin tests

d) to define possible factors determining the response to DNCB and assess the predictive value of this skin test regarding renal graft survival.





III Normal Controls

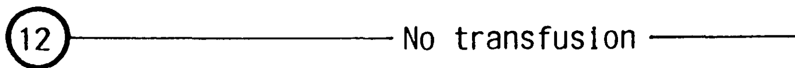


Figure 1. Experimental design of the blood transfusion trial; (numbers in circles represent numbers of subjects allocated within each group).

TABLE 1. CHARACTERISTICS OF THE PATIENTS IN THE BLOOD TRANSFUSION TRIAL

	Transfusion group (n=48)	Uraemic control group (n=12)
Sex:		
Males	37 (77%)	8 (67%)
Females	11 (23%)	4 (33%)
Age:		
Mean ± SD	41 ± 12	44 ± 15
Range	18 - 59	18 - 63
Original renal disease:		
Glomerulonephritis	14 (29%)	5 (42%)
Pyelonephritis	12 (25%)	0
Polycystic kidneys	6 (13%)	0
Hypertensive nephrosclerosis	4 (8%)	3 (25%)
Diabetes	7 (15%)	2 (17%)
Alport's syndrome	1 (2%)	0
Analgesic nephropathy	1 (2%)	0
Hypoplastic kidneys	0	1 (8%)
Renal artery stenosis	0	1 (8%)
Unknown	3 (6%)	0
Type of dialysis:		
Haemodialysis	36 (75%)	8 (67%)
CAPD	12 (25%)	4 (33%)
Parity (n=15):		
0	2	3
1	1	1
2	1	0
3	5	0
4	1	0
5	1	0

CHAPTER 1. SKIN TESTING WITH DNCB

1.1 Introduction

The DNCB skin test is a simple and reproducible in vivo test of CMI. Several reports have been published with regards to the applications of the test both in experimental work (218-220,227,228, 230,232,234,235) and in clinical trials (221-225,398-403, 406-408,411). Previous studies from this unit have shown that only one third of long-term dialysis patients display the strong reaction to DNCB which is characteristic of that seen in normal controls (359,399-402,570). These strong responders, who are presumed to have intact CMI, are more likely to reject a renal allograft (399-402), respond better to vaccination against hepatitis B (359), and are less liable to develop infections (570). One of the main questions during these studies has been what factors determine the response to the DNCB test. It was found that sex, the type of kidney disease, duration of dialysis and blood transfusion status may influence the response to the test (399,402). However, the correlation was not strong, and there was overlap between blood transfusion and the other three variables. Having described the association between blood transfusion, DNCB test and graft survival (399-402), the next questions addressed were, firstly, whether the transfusion-induced immunosuppression could be monitored by means of sequential skin tests, and secondly, whether the poor graft survival in the strong responders could be improved with more transfusions (571). This was the background against which the present randomised controlled study was initiated, as far as the DNCB test is concerned.

1.2 Patients and Methods

Patients

Table I showed the details of the sixty previously dialysis patients who had not received prior blood transfusion and who were tested with DNCB. Forty eight of these patients were subsequently given blood transfusions ('transfused') and the remaining 12 comprised the uraemic controls. In the transfused group there were 37 males and 11 females with a mean age of 41 years (range 18-59 years). All patients had recently been established on dialysis

between one and three months prior to entering the study. Thirty six patients were on haemodialysis (4 hours thrice weekly) and 12 on CAPD. The control group of the 12 uraemic patients was comparable to the 'transfused' patients in terms of sex (8 males and 4 females), age (mean 44.3, range 18-63 years) and type of dialysis (haemodialysis 8 and CAPD 4). Although the original renal diseases were not fully comparable between the two groups, the difference was not statistically significant. After allocation by random numbers, 30 of the 60 patients were transfused with 5 and 18 with 10 units respectively of third party packed red cells at two week intervals. The remaining 12 patients acted as controls. The randomisation was done after the DNCB skin test was carried out in order to achieve comparable numbers of weak and strong reactors in the groups of patients receiving 5 or 10 units of blood, as shown in Figure 1.

Methods

The DNCB skin test was carried out as previously described (359,399,402,571) prior to the first and two weeks after the last transfusion in the transfused patients, and in the uraemic controls it was repeated after 3 months. The method is shown in Figure 1.1 and was carried out as follows:

a) Primary sensitisation: The sensitising solution was prepared by disolving crystals of 1-chloro-2,4-dinitrobenzene (DNCB, BDH Chemicals Limited) in acetone to give a 2% solution (20mg of DNCB per 1ml of acetone). This solution was stored in a glass bottle covered with foil at $4^{\circ}C$, and was discarded after 2-3 days if unused. Using a micropipette, 0.1ml of this solution, ie 2000ug of DNCB, was applied to a 2cm diameter area of the volar aspect of the dominant forearm (or the forearm with the vascular access in haemodialysis patients). The sensitised skin area was cleaned with a medi-swab and was encircled by the ring of a scissors handle. After the application the solution was allowed to dry and was covered with a dressing. Patients were asked to keep it dry for 24 hours.

b) <u>Challenge sensitisation</u>: Fourteen days after the initial sensitisation, patients were tested with five different concentrations of DNCB (2ug, 4ug, 8ug, 16ug and 32ug) which were dried onto 1cm diameter felt pads of aluminium test strips (Al-test, Astra Chemicals, Herts). These five concentrations were prepared by dissolving crystals of DNCB in acetone to give an initial concentration of 3.2mg/ml, which subsequently was serially diluted to give the 1.6mg/ml, 0.8mg/ml, 0.4mg/ml and 0.2mg/ml solutions; 10ul of each of these dilutions were then added onto the corresponding pads of the patches, the lower concentration being dried to the bottom pad. The patches were applied with micropore to the volar area of the opposite forearm for 48 hours. After 48 hours the reaction was scored thus: 0 - no reaction or erythema only; 1 - erythema and induration confined to the patch; 2 - erythema and induration extending beyond the patch; 3 - as for 2 plus blistering. The DNCB score recorded was the sum of the scores for all five patches and therefore gave a range of 0 to 15. The cut off point between strong and weak responders was arbitarily derived from the lowest DNCB score recorded in a group of 15 healthy controls, which was 4. Hence, patients scoring 0-3 were classed as weak responders (Figure 1.2), and patients scoring 4-15 were classed as strong responders (Figure 1.3).

<u>Statistical analysis</u>

Comparison of the DNCB scores was carried out using the paired t test and the unpaired t test. When the numbers were small the significances were double checked with the non-parametric Wilcoxon's rank sum tests (the signed rank test, and the unsigned rank test or the Mann-Whitney-Wilcoxon test). Differences in the distribution of a variable in the groups were estimated using the chi-square test, with Yates' correction when one of the expected values was less than five.

1.3 Results

The results of the DNCB skin testing are shown in Table 1.1. Prior to blood transfusion 27 of the 48 patients were strong responders (56%), and the remaining 21 patients were weak responders

(44%). Among the latter group 10% (5 of 48) of the patients were anergic, ie DNCB score 0. The first test in the control group showed that 67% (8 of 12 patients) were strong and 33% (4 of 12) were weak responders respectively. On repeating the test the majority of both transfused and control patients showed higher DNCB scores (Figure 1.4). In the transfused patients the mean DNCB score increased from 4.8 to 6.8 ($p < 0.001$), and in the controls from 5.8 to 6.9 ($p < 0.05$); the latter difference failed to reach statistical significance using the signed rank test (Table 1.1). Overall, 6 of the 60 patients (12%) changed from weak to strong responders and one vice versa. Anergic patients tended to remain anergic in both transfused and control groups (7 of 8 patients). Six weak responders became strong responders after blood transfusion, and one strong responder changed to a weak responder. In the controls such change was not observed because 3 of the 4 weak responders were anergic (Figure 1.4). As a result of these changes after blood transfusion the proportion of the strong and weak responders in the transfused patients changed to 67% (32 of 48) and 33% (16 of 48) respectively, which was not significantly different from the repeat tests in the controls (Table 1.1).

Table 1.2 and Figure 1.5 show the changes in the DNCB skin test scores in the weak and strong responders for both the transfused patients and the controls. The mean score of the weak responders in the transfused patients changed from 1.5 to 2.4 (NS), whereas in the controls it remained unchanged. By contrast, the mean score of the strong responders increased from 7.3 to 10.1 in the transfused patients ($p < 0.001$), and from 8.5 to 10.1 in the controls (paired t test, $p < 0.025$; signed rank test, NS).

Table 1.3 shows the results of the DNCB test in relation to the number of transfusions. Prior to the transfusion, of those who received 5 units of blood 57%, (17 of 30) and 43% (13 of 30) respectively were strong and weak responders. The comparable figures for those who received 10 units of blood were 56% (10 of 18) and 44% (8 of 18). After blood transfusion the difference in the number of weak and strong responders between the two transfusion groups was not significant. Neither was there a significant difference between these two groups and the controls. Figure 1.6 shows the individual

DNCB scores in patients receiving 5 and 10 units of blood and in the controls. Both transfusion groups showed an increase in the mean DNCB score, although the difference reached statistical significance only among patients receiving 5 transfusions.

Finally, from Table 1.4 and Figure 1.7 it is evident that overall, only strong responders had significant changes in their response to DNCB on repeat testing, regardless of the amount of blood transfused (5 or 10 units) or whether or not they were given transfusion at all.

1.4 Conclusions

1) Sequential skin testing with DNCB does not reflect changes induced by blood transfusion.

2) Repeated immunisation with DNCB in previously non-transfused dialysis patients has as a result stronger anamnestic reactions, providing a primary strong response has been elicited, regardless of the amount of transfusions (5 or 10 units) or whether they were transfused at all.

3) Anergic patients tend to remain anergic, and patients with a strong primary response have stronger responses on repeating the test when primary immunisation has taken place prior to any blood transfusion.

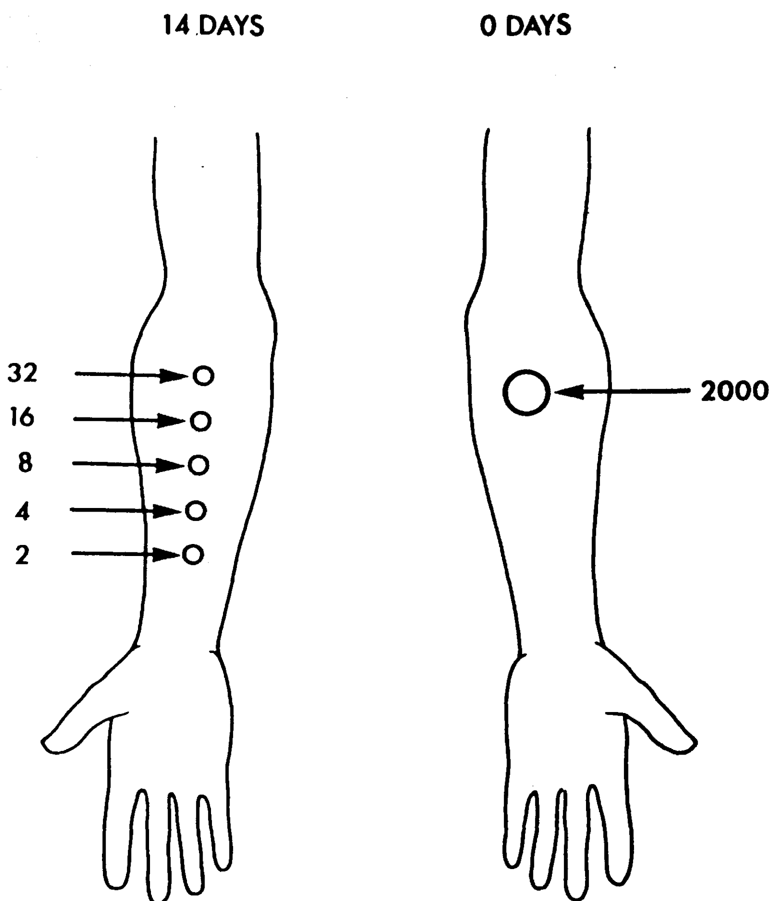


Figure 1.1. Schematic representation of the DNCB skin test; numbers represent the sensitising solution (0 days) and the challenge doses (14 days) of DNCB.

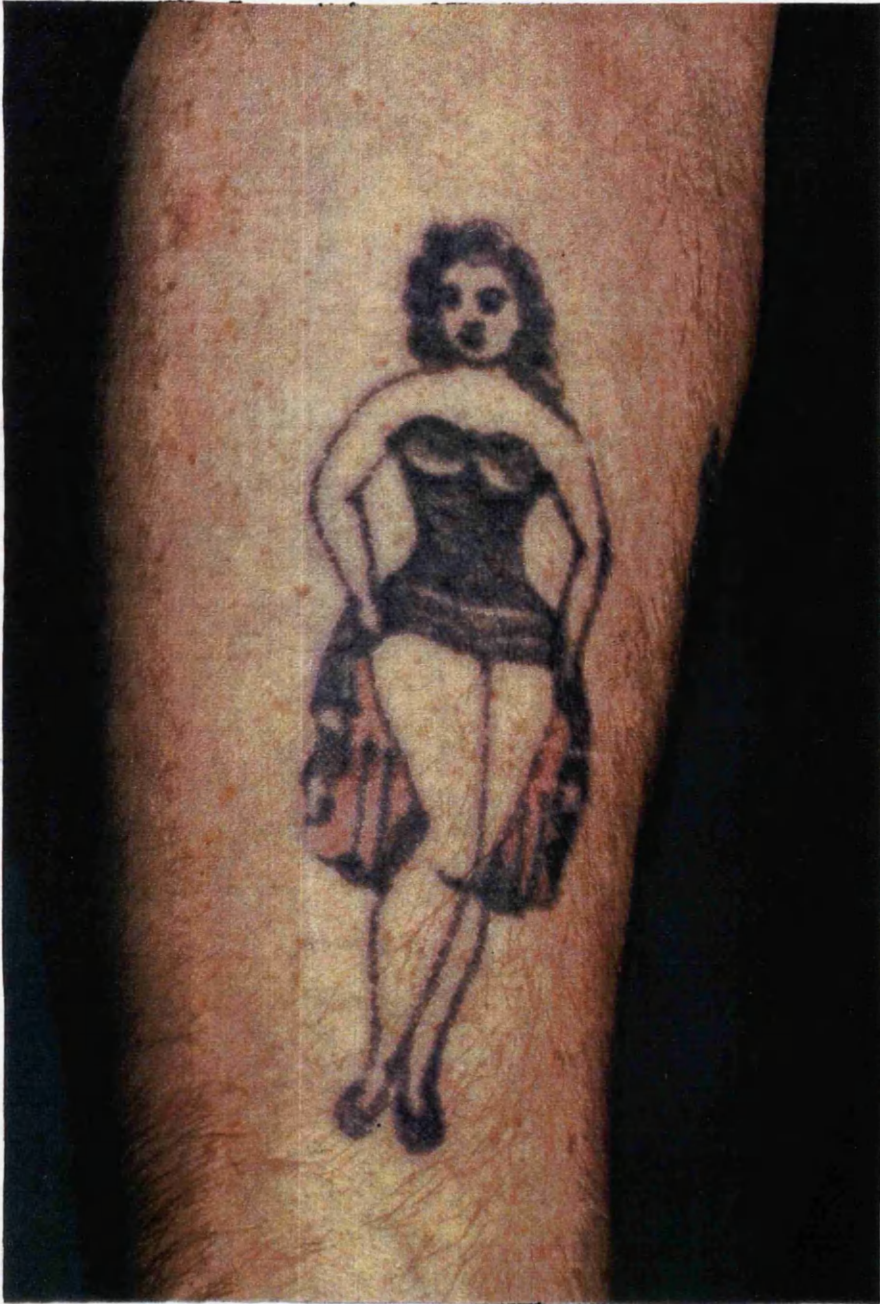


Figure 1.2. Example of a weak DNCB responder; this patient's reaction was only erythema to the top patch and nothing to the remaining four lower patches (DNCB score = 0).

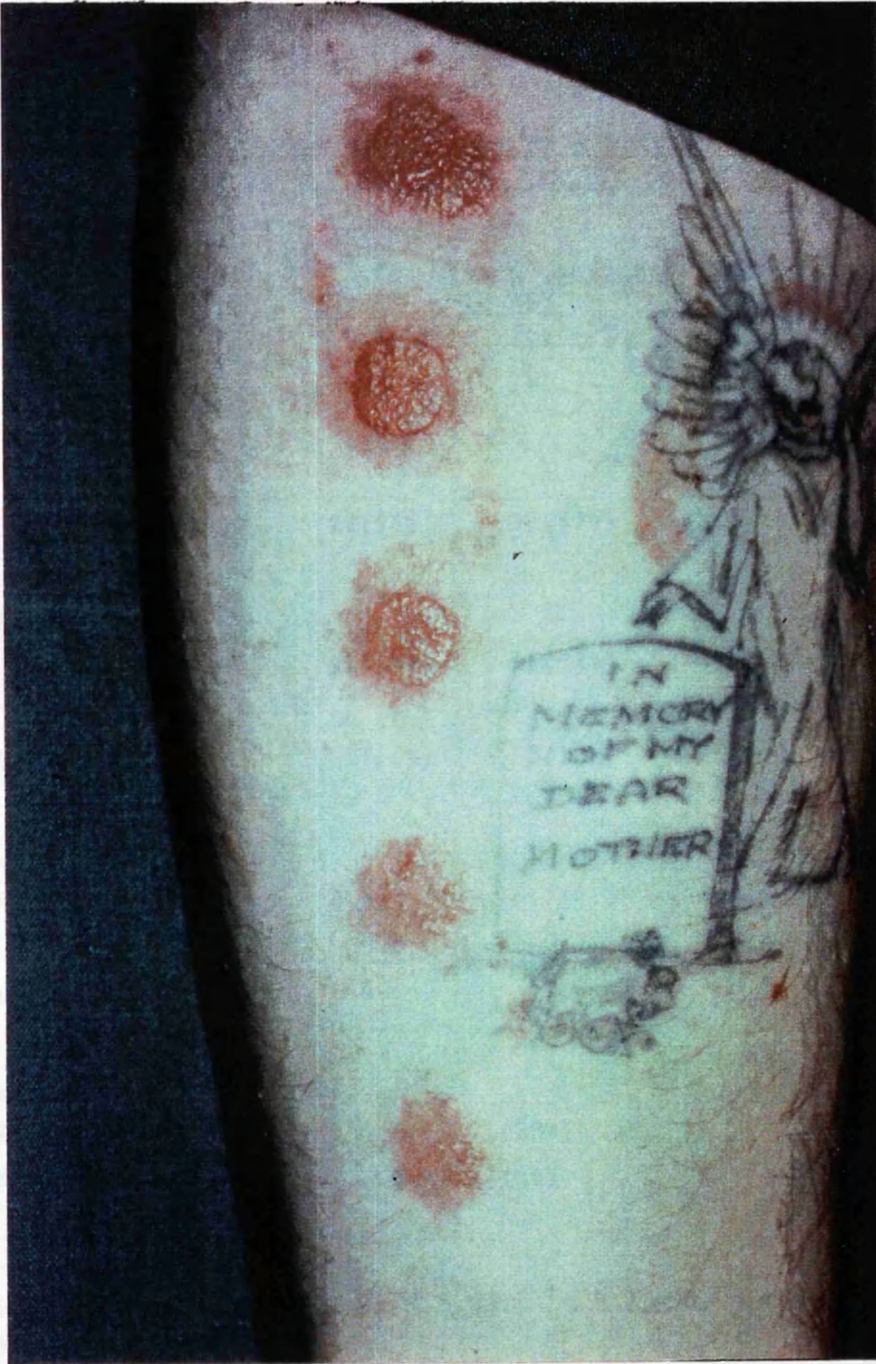


Figure 1.3. Example of a strong DNCB responder; this patient's reaction was blistering to all 5 patches (DNCB score = 15).

TABLE 1.1. RESULTS OF THE DNCB SKIN TEST IN THE TRANSFUSED PATIENTS AND IN THE URAEMIC CONTROLS

	Transfused patients (n=48)		Uraemic controls (n=12)	
	Pre-BT	Post-BT	At 0	At 3 months
Patients with score:				
>3 (strong responders)	27(56%)	32(67%)	8(67%)	8(67%)
0-3 (weak responders)	21(44%)	16(33%)	4(33%)	4(33%)
0 (anergic)	5(10%)	5(10%)	3(25%)	3(25%)
DNCB score:				
Mean ± SD	4.8±3.6*	6.7±4.8*	5.8±5.0^{+}	6.9±5.5^{+}
SEM	0.5	0.7	1.4	1.6
Median	4.0	7.0	5.5$^{\{}$	6.5$^{\{}$
Change of score:				
From 0-3 to >3	6 of 21(29%)		None	
From > 3 to 0-3	1 of 27(4%)		None	

Paired t test: *t = 4.139, $p < 0.001$; $^{+}$t = 2.493, $p < 0.05$.
Signed rank test: $^{\{}$NS

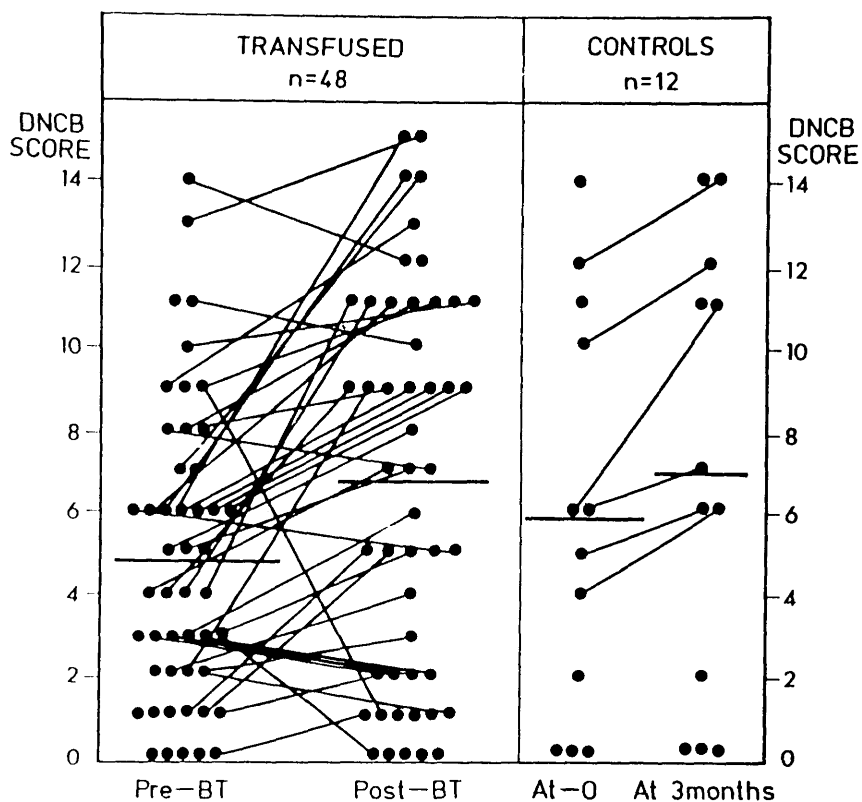


Figure 1.4. DNCB skin test scores in the 48 transfused patients and in the 12 uraemic controls; BT = blood transfusion.

TABLE 1.2. RESULTS OF THE DNCB SKIN TEST IN THE WEAK AND STRONG RESPONDERS

	Transfused patients (n=48)		Uraemic control (n=12)	
	Pre-BT	Post-BT	At 0	At 3 months
DNCB score of weak responders	n=21		n=4	
Mean ± SD	1.5±1.2	2.4±2.4	0.5±1.0	0.5±1.0
SEM	0.3	0.5	0.5	0.5
Median	1.0	2.0	0.0	0.0
DNCB score of strong responders	n=27		n=8	
Mean ± SD	7.3±2.8*	10.1±3.1*	8.5±3.7^{+}	10.1±3.4^{+}
SEM	0.5	0.6	1.3	1.2
Median	6.0	11.0	8.0$^{(}$	8.0$^{(}$

Paired t test: *t = 3.919, $p < 0.001$; $^{+}$t = 2.876, $p < 0.025$.
Signed rank test: $^{(}$NS

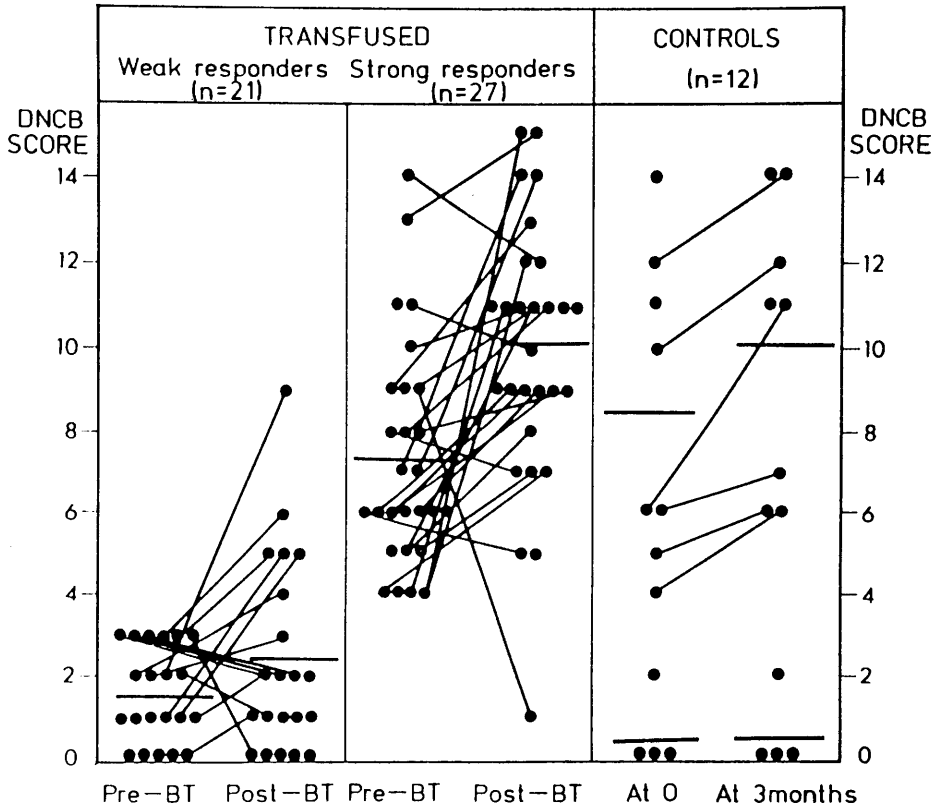


Figure 1.5. DNCB skin test scores in the transfused weak and strong responders and in the uraemic controls.

TABLE 1.3. RESULTS OF THE DNCB SKIN TEST IN THE PATIENTS RECEIVING 5 AND 10 UNITS OF BLOOD AND IN THE URAEMIC CONTROLS

	Transfused patients				Uraemic controls	
	5 units (n=30)		10 units (n=18)		(n=12)	
	Pre-BT	Post-BT	Pre-BT	Post-BT	At 0	At 3 months
strong responders	17(57%)	23(77%)	10(56%)	9(50%)	8(67%)	8(67%)
weak responders	13(43%)	7(23%)	8(44%)	9(50%)	4(33%)	4(33%)
anergic	3(10%)	3(10%)	2(11%)	2(11%)	3(25%)	3(25%)
DNCB score:						
Mean±SD	4.7±3.7*	7.0±4.5*	4.9±3.6[+]	6.1±5.2[+]	5.8±5.0[$]	6.9±5.5[$]
SEM	0.7	0.8	0.9	1.2	1.4	1.6
Median	4.0**	7.5**	4.5[++]	5.0[++]	5.0[((]	6.5[((]
Change of score:						
From 0-3 to >3	6 of 13 (46%)		None		None	
From >3 to 0-3	None		1 of 0 (10%)		None	

Paired t test: *t = 4.330, $p<0.001$; [+]t = 1.451, NS; [$]t = 2493, $p<0.05$.
Signed rank test: **$p<0.04$; [++]NS; [((]NS

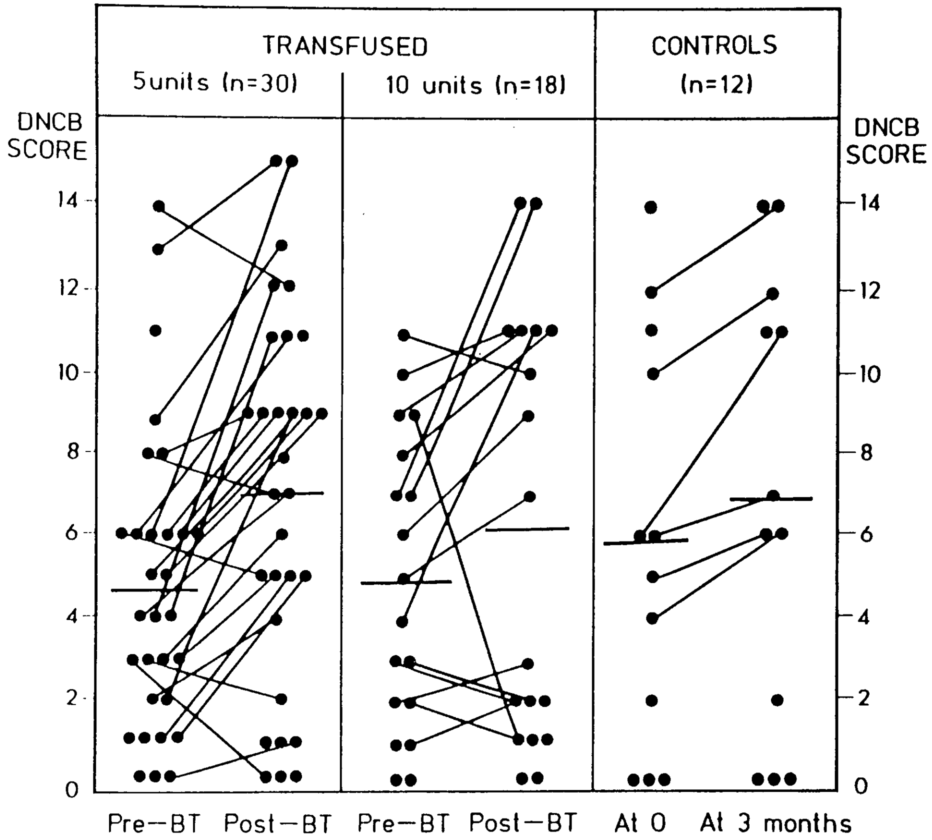


Figure 1.6. DNCB skin test scores in the patients receiving 5 and 10 units of blood and in the uraemic controls.

TABLE 1.4. RESULTS OF THE DNCB SKIN TEST IN THE WEAK AND STRONG RESPONDERS RECEIVING 5 AND 10 UNITS OF BLOOD AND IN THE CONTROLS

	Transfused patients				Uraemic controls (n=12)	
	5 units (n=30)		10 units (n=18)			
	Pre-BT	Post-BT	Pre-BT	Post-BT	At 0	At 3 months
DNCB score of weak responders	n=13		n=8		n=4	
Mean±SD	1.5±1.2	3.0±2.9	1.5±1.2	1.4±1.1	0.5±1.0	0.5±1.0
SEM	0.3	0.8	0.4	0.4	0.5	0.5
Median	1.0	2.0	1.5	1.5	0.0	0.0
DNCB score of strong responders	n=17		n=10		n=8	
Mean±SD	7.1±3.0*	10.1±2.8*	7.6±2.2^{+}	9.9±3.8^{+}	8.5±3.7$^{\{}$	10.1±3.4$^{\{}$
SEM	0.7	0.7	0.7	1.2	1.3	1.2
Median	6.0**	9.0**	7.5^{++}	11.0^{++}	8.0$^{\{\{}$	8.0$^{\{\{}$

Paired t test: *t=3.987, $p<0.005$; $^{+}$t=1.744 NS; $^{\{}$t=2.876, $p<0.025$.

Signed rank test: **$p<0.01$; $^{++}p<0.01$; $^{\{\{}$NS

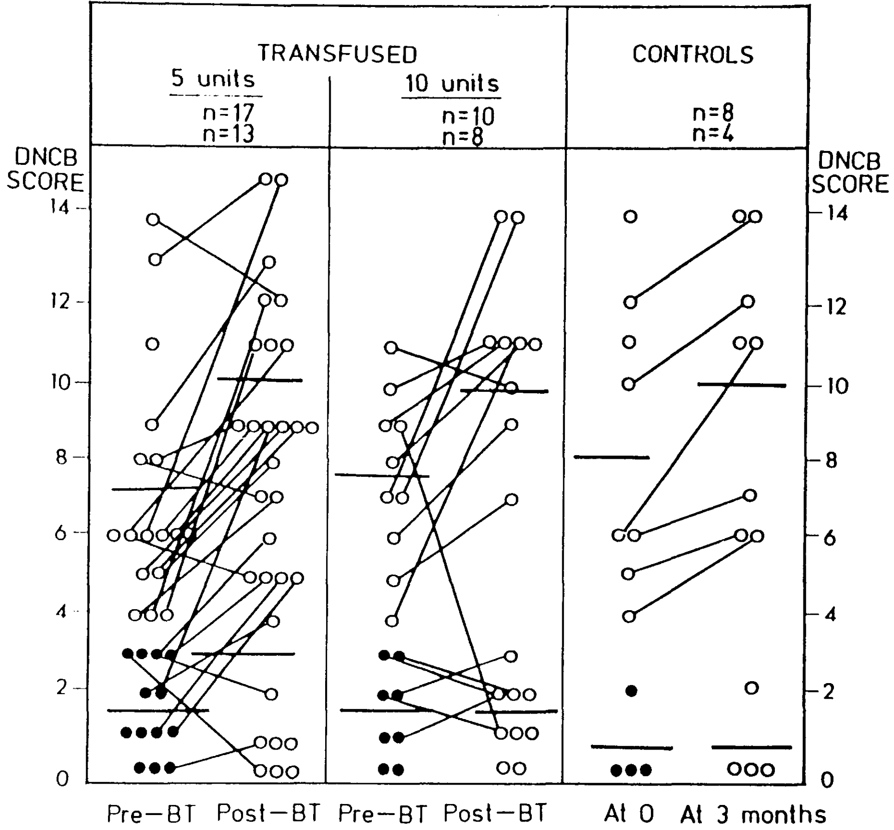


Figure 1.7. DNCB skin test scores in the weak and strong responders receiving 5 and 10 units of blood and in the uraemic controls (● = weak responders, O = strong responders).

CHAPTER 2. SKIN TESTING WITH RECALL ANTIGENS

2.1 Introduction

A possible advantage of the DNCB test over DTH skin tests using recall antigens is that it tests the response to a new antigen and does not therefore require or test for immunological memory (399). However, many reports have shown that anergy to recall antigens may also have predictive value and may be associated with increased morbidity and mortality (298-302,404,405,417), and with improved graft survival (398,410). In a recent study it was suggested that the beneficial effect of blood transfusion on graft survival could be monitored by skin testing with a battery of several recall antigens (410). This chapter describes the use of skin testing in the assessment of the transfusion effect.

2.2 Patients and Methods

Patients

In addition to the DNCB test, 23 of the 60 patients described in the previous chapter were also skin tested with four recall antigens. Seventeen of the patients were in the transfusion group and 6 were uraemic controls. Nine of the 17 patients received 5 units of blood and the other 8 had 10 units. Among the transfused patients there were 14 males and 3 females with a mean age of 42 years (range 25-59 years). The original diseases were glomerulonephritis (6), pyelonephritis (4), polycystic kidneys (3), hypertensive nephrosclerosis (1), diabetic nephropathy (2), and unknown (1). Fifteen patients were on haemodialysis and 2 patients were on CAPD. In the group of the uraemic controls there were 4 males and 2 females with a mean age of 48 years (range 31-63 years). The original diseases were glomerulonephritis (1), hypertensive nephrosclerosis (2), diabetic nephropathy (2), and renal artery stenosis (1). Three patients were on haemodialysis and 3 patients were on CAPD.

Methods

Patients were skin tested as described by Bradley et al (302) with 0.1ml of each of four recall antigens, namely, purified protein derivative (PPD) 100 units per ml (Evans Medical Limited, Liverpool), Candida albicans 0.5% (2003) M2646 (Bencard, Brentford, Middlesex), Streptokinase 10 units plus Streptodornase 2.5 units (Varidase, Lederle Laboratories, Hampshire), and mumps skin test antigen, USP (Eli Lilly and Co., Indianapolis, USA). The antigens were injected intradermaly several centimetres apart on the volar aspect of the forearm and the response was measured as the diameter of induration at 24 and 48 hours. Patients were defined as 'responders' if one or more of the antigens produced 5mm or more induration, and 'non-responders' (anergic) if none of the antigens caused a reaction of 5mm or greater. To quantitate further the response to each antigen the following scoring system was used for each antigen: 0 - induration less than 5mm, 1 - induration 5-9mm, 2 - induration 10-19mm, 3 - induration more than 19mm. The score for the four antigens, therefore, ranged from 0 to 12. The test was carried out at the same time as the DNCB test, ie prior to the first and two weeks after the last blood transfusion in the transfused patients, and twice in the uraemic controls with an interval of approximately 3 months.

Statistical analysis

The comparison of the scores was done using both Students' t tests (paired and unpaired), and the non-parametric rank sum tests (the signed rank test, and the rank test for unpaired samples or the Mann-Whitney-Wilcoxon test). The correlation between the DNCB and the recall antigen scores was done using linear regression analysis.

2.3 Results

The results of skin testing with the recall antigens are shown in Figure 2.1. In the 17 patients who were transfused, the mean score rose from 3.8 prior to blood transfusion to 5.0 after the last transfusion. Similarly, the 6 uraemic controls showed an increase in their mean score from 3.3 to 4.8. The statistical significance of these differences is shown in Table 2.1; the increase in skin test

score of the controls failed to achieve significance using the signed rank test possibly because of the small sample number. Overall, there was only one non-responder (anergic, total score 0) amongst the 6 controls, and the remaining 5 controls together with all the transfusion group were responders (96%). The anergic patient remained anergic on repeat testing. Thus despite the increase in the mean score, by definition there was no change of non-responders to responders and vice versa, unlike the DNCB test.

Figure 2.2 shows the results of skin testing with recall antigens, with regards to the number of transfusions. Both groups of patients receiving 5 and 10 units of blood showed an increase in the mean skin test score from 3.6 to 5.0 and from 4.1 to 5.0 respectively as did the controls whose score increased from 3.3 to 4.8. The statistical analysis of these changes is shown in Table 2.2; only the increase in skin test score seen in patients receiving 5 transfusions was significant using both parametric and non-parametric tests.

Figures 2.3 and 2.4 represent the plots of the recall antigen scores against the DNCB scores, on the first and second occasions in the 23 patients in whom both tests were performed. On neither occasion was there a significant correlation. Furthermore, of the four patients who were anergic to DNCB only one was also anergic to the recall antigens as well. This finding suggests that the DNCB test and the recall antigen test provide a measure of different aspects of the cell-mediated immune response and therefore define different subpopulations of responders and non-responders.

2.4 Conclusions

1) The response to skin testing with recall antigens is not altered by blood transfusion in previously non-transfused patients who have recently been established on dialysis.

2) The response to repeat skin testing with recall antigens tends to be increased in these patients, irrespective of whether they receive blood transfusion, or of the amount of blood they are given.

3) There is no correlation between the response to the DNCB test and the response to skin testing with recall antigens which suggests that the two tests define different sub-populations of responders and non-responders.

TABLE 2.1. RESULTS OF THE SKIN TEST WITH RECALL ANTIGENS IN THE TRANSFUSED PATIENTS AND IN THE URAEMIC CONTROLS

	Transfused patients (n=17)		Uraemic controls (n=6)	
	Pre-BT	Post-BT	At 0	At 3 months
Recall antigen score:				
Mean ± SD	3.8±1.7*	5.0±1.5*	3.3±2.6^{+}	4.8±2.7^{+}
SEM	0.4	0.4	1.1	1.1
Median	4.0**	5.5**	3.5^{++}	5.0^{++}

Paired t test: *t = 4.515, $p < 0.001$; $^{+}$t = 3.503, $p < 0.02$.

Signed rank test: **$p < 0.01$; $^{++}$NS.

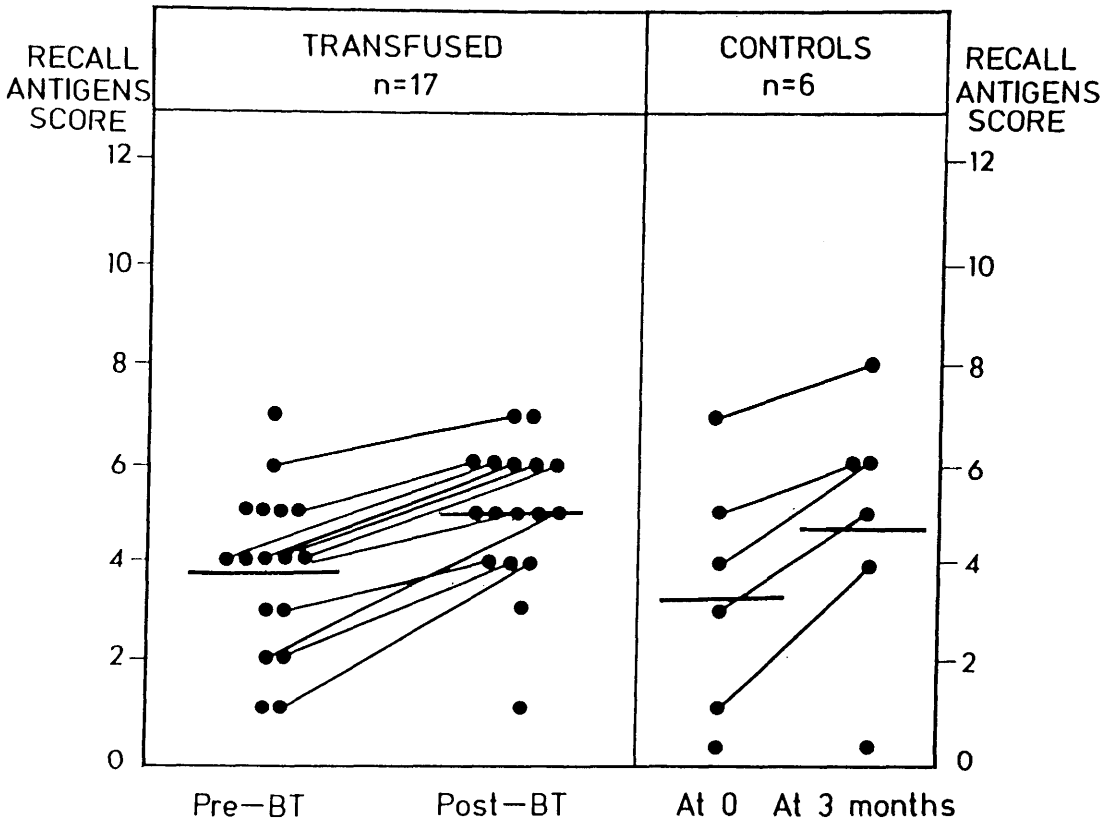


Figure 2.1. Scores of the skin test with recall antigens in the transfused patients and in the uraemic controls.

TABLE 2.2. RESULTS OF THE SKIN TEST WITH RECALL ANTIGENS IN THE PATIENTS RECEIVING 5 AND 10 UNITS OF BLOOD AND IN THE URAEMIC PATIENTS

	Transfused patients				Uraemic controls	
	5 units (n=9)		10 units (n=8)		(n=6)	
	Pre-BT	Post-BT	Pre-BT	Post-BT	At 0	At 3 months
Recall antigen score:						
Mean±SD	3.6±1.7*	5.0±1.2*	$4.1\pm1.7^{+}$	$5.0\pm1.9^{+}$	$3.3\pm2.6^{\$}$	$4.8\pm2.7^{\$}$
SEM	0.6	0.4	0.6	0.7	1.1	1.1
Median	4.0**	5.0**	4.0^{++}	5.5^{++}	$3.5^{((}$	$5.0^{((}$

Paired t test: *t = 3.833, p<0.01; $^{+}$t = 2.497, p<0.05;
$^{\$}$t = 3.503, p<0.02.

Signed rank test: **p < 0.05; $^{++}$NS; $^{((}$NS.

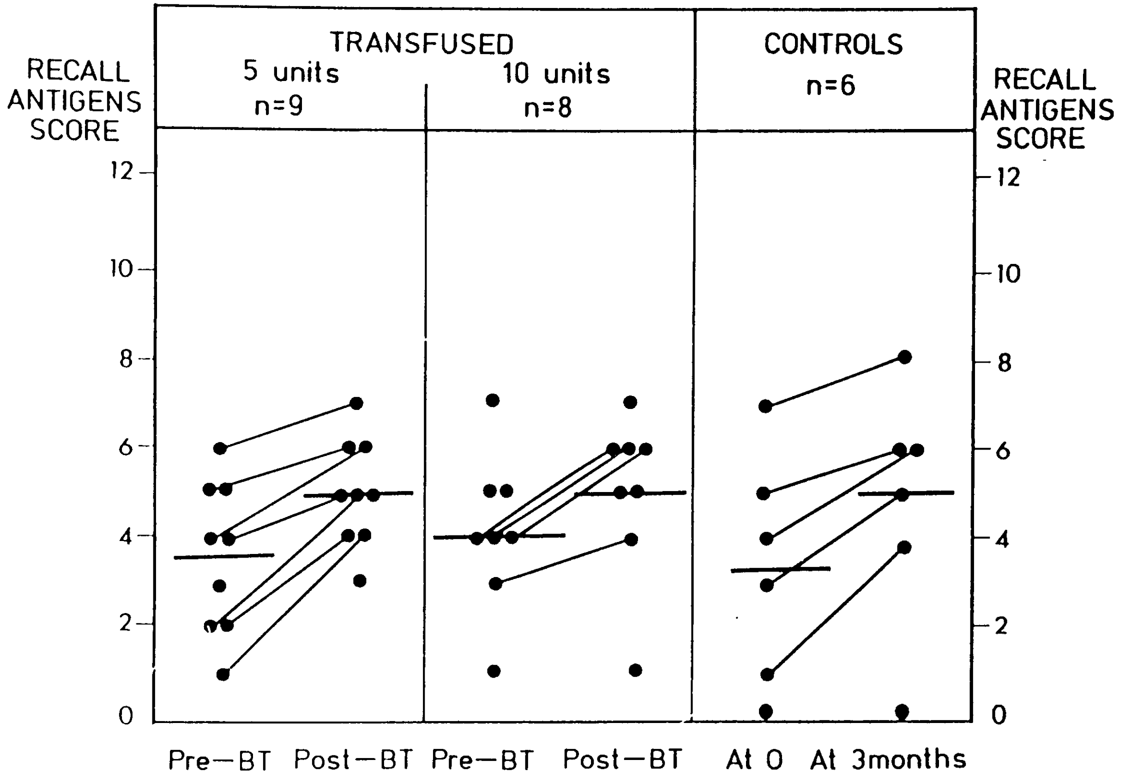


Figure 2.2. Scores of the skin test with recall antigens in the patients receiving 5 and 10 units of blood and in the uraemic controls.

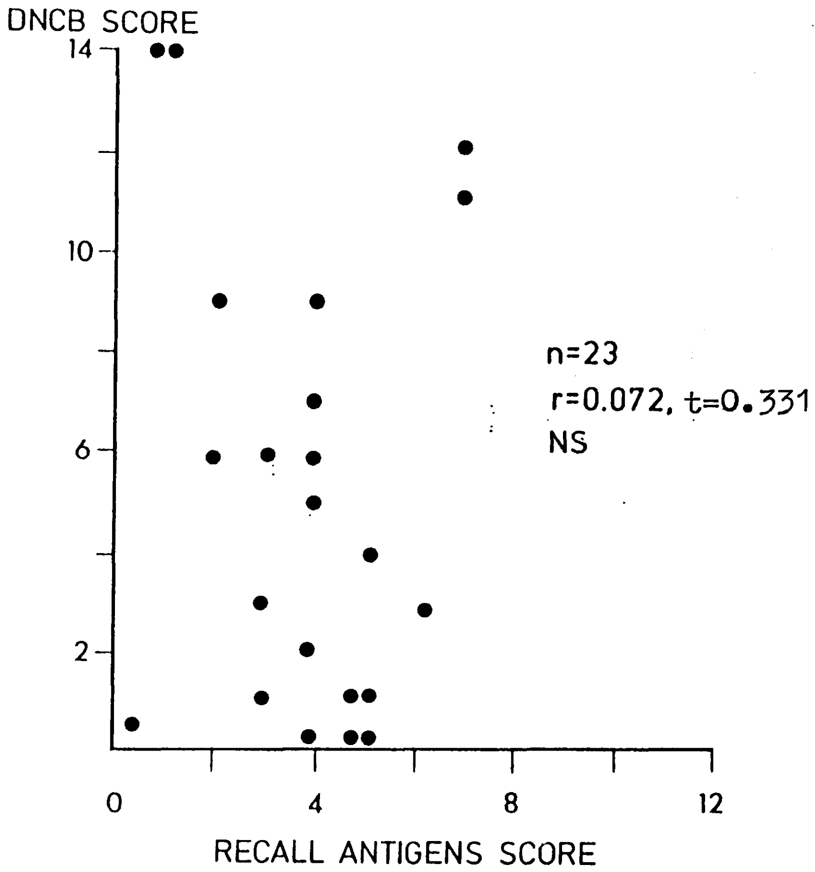


Figure 2.3. Correlation between the DNCB scores and recall antigen scores on the first tests.

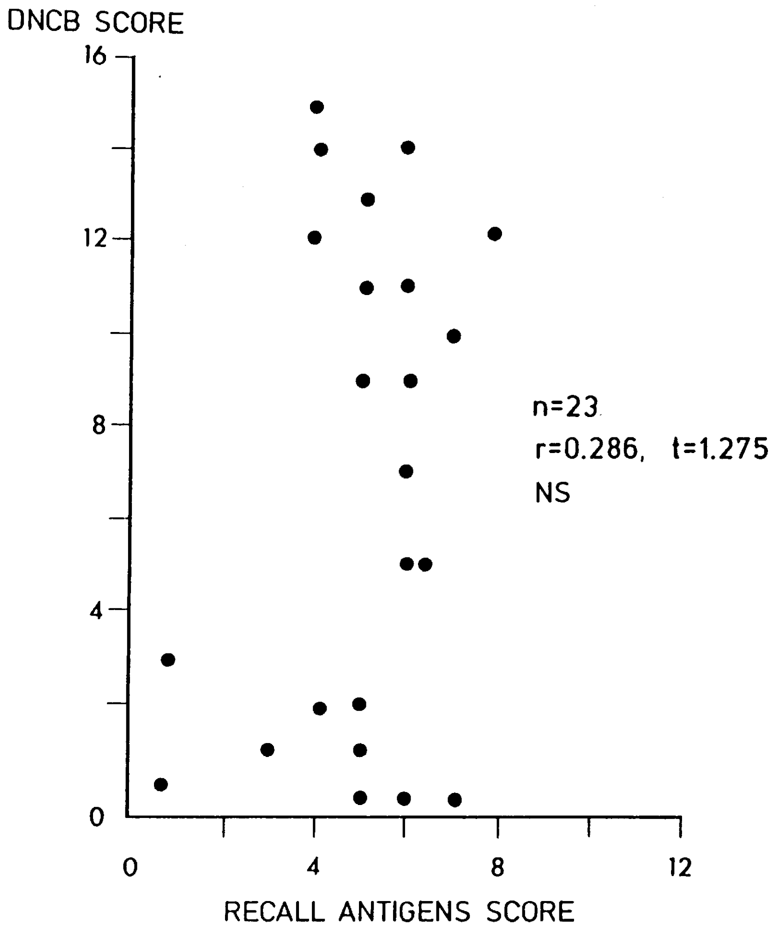


Figure 2.4. Correlation between the DNCB scores and the recall antigen scores on the repeat tests.

CHAPTER 3. FACTORS DETERMINING THE RESPONSE TO SKIN TESTING WITH DNCB AND RECALL ANTIGENS

3.1 Introduction

Although it is generally accepted that the presence of uraemia depresses the immune response there is some experimental evidence which apparently contradicts this well established view (381-383,412). The controversy arises mainly from differences between experimental uraemic models (383) and between in vitro methods used to measure the immune response (341,348,369,374, 377,383). It is possible that the endogenous depression of immune response which results from uraemia may have been overemphasised, and that exogenous factors such as blood transfusion, surgical operations, infections and administration of drugs may all contribute to the depressed immunity seen in uraemic patients (286,296,297, 306-309,379,393,394,399,402,410,571). For this reason, the selected group of patients described in this thesis, who have never received a blood transfusion or renal allograft and who have recently been established on dialysis, constitutes a useful population in which to study the different factors which may determine the immune response.

3.2 Patients and Methods

All 48 patients in the transfusion group and the 12 uraemic controls (Table 1) were studied to see whether there was any association between a number of variables and the DTH skin test results. The variables examined were patient sex, age, original disease, the type of dialysis, parity, and standard haematology and biochemistry results. Blood samples were taken on the morning of the day during which the skin tests were carried out, either before the commencement of haemodialysis or prior to the first bag change in the CAPD patients. On the test day urine was collected for 24 hours to assess the residual urine volume. In order to see whether the response to the DNCB skin test was an HLA-associated phenomenon, the HLA-DR phenotypes and response to the skin test were studied in 57 of the 60 patients who had been tissue typed. Tissue typing was carried out in the regional Tissue Typing Laboratory using the standard NIH-Terasaki method (572).

As the DTH skin test results sometimes changed on repeat testing (see Chapter 1 and 2), the association between the patient variables and the skin test results were examined for both first and repeat tests. However, as the DNCB score increased to a similar degree in both the transfused and control groups on repeat testing, the two groups have been considered together for the analysis of these variables. Furthermore, none of the variables which changed significantly after transfusion showed any correlation with the DTH skin test results as will be discussed in the results section.

Statistical analysis

The significance of differences between means was determined by a univariate analysis using the paired and unpaired t tests. Multivariate analysis (proportional hazards Cox analysis) was not applied because the numbers were small. However, since it is possible in a study with many variables for one of them to come up as signficant by chance, the strength of the statistical significance was checked by correction of the p value by multiplying it by the number of the variables studied (573). Differences in the distribution of a variable in the groups were estimated using the chi-square test with Yates' correction; in the analysis of the association between HLA-DR antigens and the response to the DNCB test the p value was further corrected by multiplying it by the number of antigens studied. The correlation between the haematology and biochemistry results and the skin test scores was done using linear regression analysis.

3.3 Results

Table 3.1 shows that none of the patient details namely, sex, age, original disease, type of dialysis and parity showed a significant effect on the response to the DNCB test. Table 3.2 shows that of 19 haematology and biochemistry variables only 2 changed significantly after blood transfusion in the transfusion group, namely, haemoglobin and serum iron. The former increased from 8.3 ± 1.2 g/dl to 10.9 ± 1.6 g/dl ($p<0.0000$), and the latter from 12 ± 4 umol/l to 20 ± 4 umol/l ($p<0.0000$). These two values after transfusion were also significantly higher compared to the values of

the controls at 3 months ($p<0.001$, and $p<0.0000$ respectively). Serum creatinine and serum albumin also increased after transfusion, but the difference was not sufficient to remain significant after correction of the p value. By contrast, all the variables in the controls remained unchanged. Although in the transfused group there was a rise in haemoglobin and serum iron between the two tests which did not occur in the control group, the increase in the DTH skin test results did not differ between the two groups. Table 3.3 illustrates that none of the patient variables showed a significant correlation with the DNCB scores at the first test. Neither was there any correlation at the second test with regard to any variable and particularly with regard to haemoglobin and iron which increased significantly after blood transfusion. Serum chloride and serum calcium showed a weak correlation on the repeat test which was not significant after correction of the p value. Figures 3.1 and 3.2 represent the plots of the serum chloride and calcium respectively against the DNCB scores, and show an absence of correlation. Also Table 3.4 shows that the haematology and biochemistry values did not differ significantly between the weak and strong DNCB responders on the occasion of both tests.

When skin testing was carried out with recall antigens, there was a weak correlation between serum bicarbonate and serum aluminium and the recall antigen scores at the first test, but this was lost after correction of the p value. None of the haematological or biochemical variables correlated significantly with the scores on repeating the test (Table 3.5). Figures 3.3 and 3.4 represent the plots of the serum bicarbonate and serum aluminium respectively against the recall antigen scores and there appears to be a significant correlation in these graphs. However, this is likely to be accidental, since it has been detected only at the first test and not on repeat testing, whereas both the first and second tests are thought to represent anamnestic reactions.

The question of whether the response to the DNCB skin test is a genetically controlled event was investigated by looking into the possible association between HLA-DR antigens and percentage of strong and weak responders. Figure 3.5 shows the distribution of the DR antigens in the 57 patients. The commonest specificities were DR3

(42%), DR4 (40%) and DR2 (35%), and the least common were DR5 (7%), DR1 (14%) and DR7 (14%), while DRW6 was found in 25%. Undefined specificities and homozygotes were grouped together under blank (23%). From Figure 3.6 it can be seen that at the first test DRW6 positive patients were significantly more likely to be strong responders compared to DRW6 negative patients (93% vs 47%, $p<0.04$; the p value was corrected for the number of DR antigens studied). This association exists even in this small selected group of patients and supports our finding in an earlier larger series (574,575). Finally, the DRW6 effect was not found on repeating the test (86% vs 60%), because of the increase in strong responders among the DRW6 negative patients at that time (Figure 3.7).

3.4 Conclusions

1) In a group of patients with chronic renal failure who were recently started on dialysis and who had not received previous blood transfusion, the response to the DNCB test was not related to the sex, age, original renal disease, type of dialysis or parity of the patients.

2) A number of haematological and biochemical parameters do not correlate with the response to DNCB and recall antigens, and this is the case even for haemoglobin and serum iron which change considerably after transfusion.

3) HLA-DRW6 positive patients are significantly more likely to be strong DNCB responders, but on repeat immunisation this is not observed.

TABLE 3.1. ASSOCIATION BETWEEN PATIENT DETAILS AND RESPONSE TO THE DNCB SKIN TEST (ALL PATIENTS n = 60)

Patient details	First test		Second test	
	Weak responders	Strong responders	Weak responders	Strong responders
Sex:				
Males (n=45)	17(38%)	28(62%)	13(29%)	32(71%)
Females (n=15)	8(53%)	7(47%)	7(47%)	8(53%)
Age (mean ± SD)	40.5±10.6	42.3±13.3	42.4±9.8	41.4±13.3
Original disease:				
Glomerulonephritis (n = 19)	10(53%)	9(47%)	10(53%)	9(47%)
Pyelonephritis (n = 12)	4(29%)	8(71%)	3 (25%)	9(75%)
Polycystic (n = 6)	1(17%)	5(83%)	1(17%)	5(83%)
Hypertension (n = 7)	1(14%)	6(86%)	1(14%)	6(86%)
Diabetes (n = 9)	6(67%)	3(23%)	2(22%)	7(78%)
Miscellaneous (n = 7)	3(43%)	4(57%)	3(43%)	4(57%)
Type of dialysis:				
Haemodialysis (n = 44)	17(39%)	27(61%)	17(39%)	27(61%)
CAPD (n = 16)	8(50%)	8(50%)	3(19%)	13(81%)
Parity:				
Nulliparous (n = 5)	3(60%)	2(40%)	3(60%)	2(40%)
Parous, 1-5 (n = 10)	5(50%)	5(50%)	4(40%)	6(60%)

TABLE 3.2. HAEMATOLOGY AND BIOCHEMISTRY DATA IN THE TRANSFUSED PATIENTS AND IN THE CONTROLS

Variable	Transfusion group (n = 48) Pre-BT	Transfusion group (n = 48) Post-BT	Controls (n = 12) At 0	Controls (n = 12) At 3 months
Hb (g/dl)	8.3±1.2*	10.9±1.6*,**	8.2±1.0	9.0±1.4**
WBC ($x10^9$/l)	6.5±1.4	6.9±1.7	7.3±1.2	7.4±1.3
Na (mmol/l)	140±3	139±3	139±2	140±3
K (mmol/l)	4.6±0.6	4.8±0.7	4.5±0.9	4.5±0.7
Cl (mmol/l)	102±4	101±3	100±5	100±4
CO_2 (mmol/l)	23±2	23±3	23±3	23±3
Urea (umol/l)	21±5	22±5	20±5	20±6
Creatinine (umol/l)	901±183$^{(}$	1001±203$^{(}$	890±269	910±272
Alk phos (U/l)	96±33	91±33	105±48	101±60
Ca (mmol/l)	2.3±0.2	2.4±0.2	2.3±0.2	2.4±0.2
PO_4 (mmol/l)	1.7±0.3	1.8±0.4	1.6±0.5	1.7±0.5
SGOT (U/l)	17±6	17±6	17±6	17±6
SGPT (U/l)	19±8	22±10	22±7	20±8
Albumin (g/l)	39±4$^{\$}$	41±4$^{\$}$	39±5	41±3
Globulin (g/l)	25±4	25±3	27±3	26±3
Iron (umol/l)	12±4^{+}	20±4$^{+,++}$	10±3	10±4^{++}
PTH (ng/l)	1490±1310	1410±1360	1780±1660	1910±2000
Aluminium (umol/l)	1.7±1.0	1.4±0.8	1.9±1.3	1.1±0.5
Urine volume (l)	1.0±0.4	0.8±0.4	0.7±0.3	0.6±0.3

Paired t test: *t = 9.026, p<0.0000; $^{(}$t = 2.522, p_{cor}<0.25, NS

$^{\$}$t = 2.634, p_{cor}<0.19, NS; $^{+}$t = 9.473, p<0.0000

Unpaired t test: **t = 4.133, p_{cor}<0.01; $^{++}$t = 6.873, p<0.0000

TABLE 3.3. CORRELATION COEFFICIENT (r) BETWEEN HAEMATOLOGY AND BIOCHEMISTRY DATA AND THE DNCB SCORES (ALL PATIENTS n = 60)

Variable	DNCB scores First test	DNCB scores Second test
	r	r
Hb (g/dl)	-0.056	0.177
WBC ($x10^9$/l)	-0.177	0.096
Na (mmol/l)	-0.234	-0.002
K (mmol/l)	0.151	0.095
Cl (mmol/l)	0.008	0.263*
CO_2 (mmol/l)	-0.081	-0.248
Urea (mmol/l)	0.064	0.089
Creatinine (umol/l)	-0.046	0.025
Alk phos (U/l)	-0.052	0.025
Ca (mmol/l)	-0.206	-0.318+
PO_4 (nmol/l)	0.019	0.055
SGOT (U/l)	0.100	-0.028
SGPT (U/l)	0.058	-0.057
Albumin (g/l)	0.000	-0.041
Globulin (g/l)	0.081	-0.052
Iron (umol/l)	0.077	0.036
PTH (ng/l)	0.094	-0.119
Aluminium (umol/l)	0.004	-0.149
Urine volume (l)	0.070	0.210

*$t = 2.076$, $p < 0.05$, $p_{cor} < 0.95$, NS

+$t = 2.554$, $p < 0.02$, $p_{cor} < 0.38$, NS

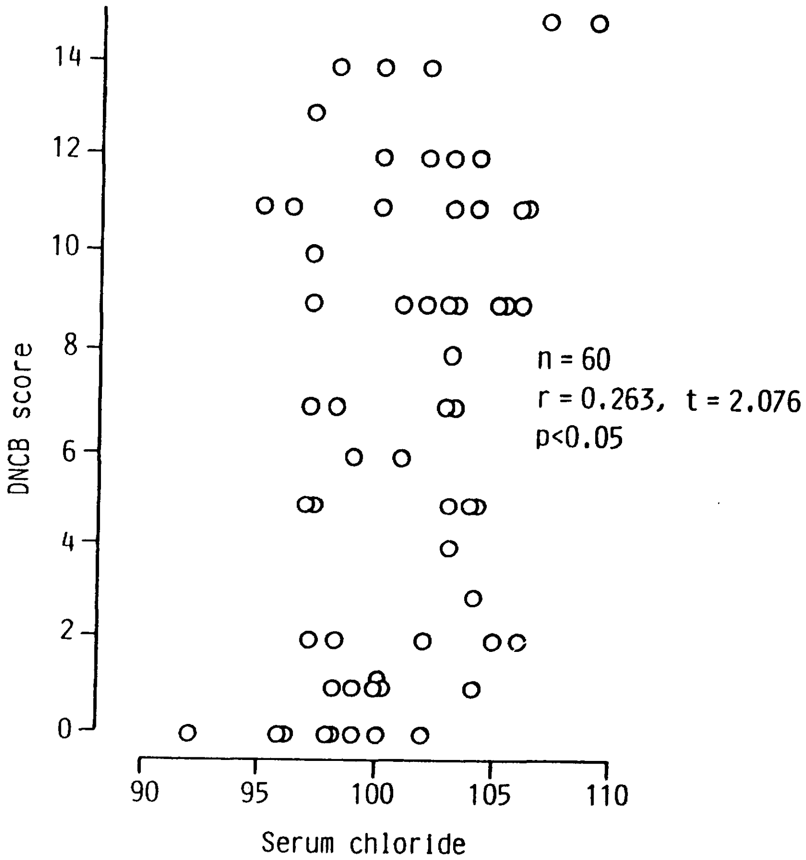


Figure 3.1. Correlation between serum chloride and DNCB scores on repeating the test.

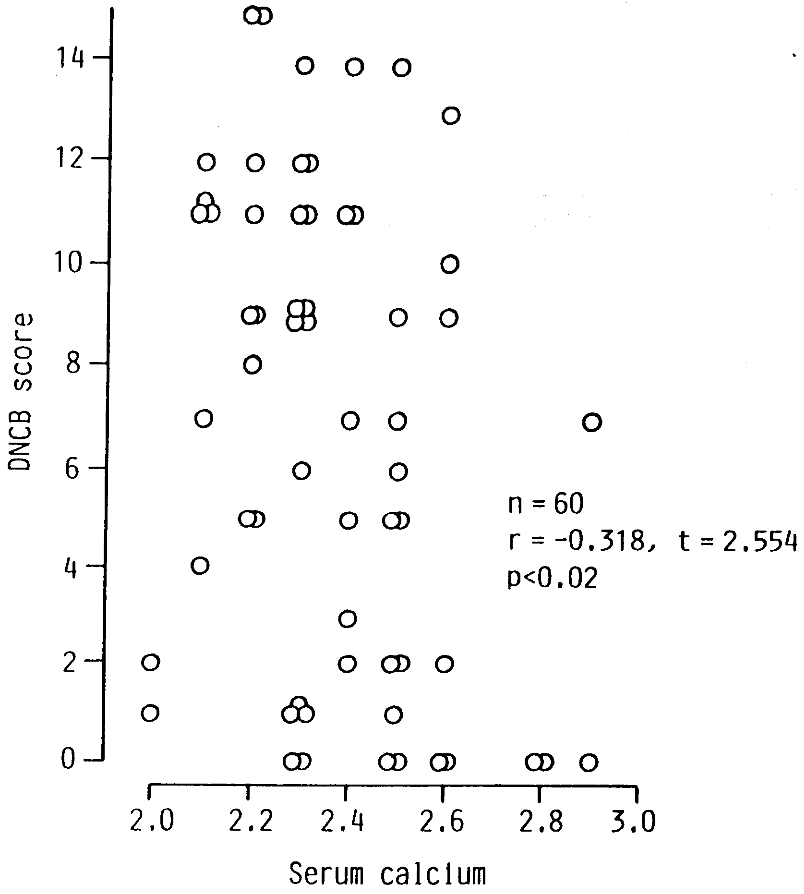


Figure 3.2. Correlation between serum calcium and DNCB scores on repeating the test.

TABLE 3.4. ASSOCIATION BETWEEN HAEMATOLOGY AND BIOCHEMISTRY DATA AND RESPONSE TO THE DNCB SKIN TEST (ALL PATIENTS, n = 60)

Variable	First test: Weak responders (n=25)	First test: Strong responders (n=35)	Second test: Weak responders (n=20)	Second test: Strong responders (n=40)
Hb (g/dl)	8.4±1.2	8.2±1.1	10.0±1.6	10.8±1.8
WBC ($x10^9$/l)	7.1±1.3	6.4±1.4	6.7±1.6	7.1±1.6
Na (mmol/l)	140±2	139±2	140±3	139±3
K (mmol/l)	4.5±0.7	4.6±0.7	4.8±0.6	4.7±0.7
Cl (mmol/l)	102±4	102±4	100±4	101±3
CO_2 (mmol/l)	23±2	23±3	23±3	23±3
Urea (mmol/l)	21±5	21±5	21±5	21±5
Creatinine (umol)	891±220	904±189	984±254	982±203
Alk phos (U/l)	106±32	92±38	93±27	93±45
Ca (mmol/l)	2.3±0.2	2.3±0.2	2.5±0.2*	2.3±0.2*
PO_4 (mmol/l)	1.7±0.3	1.7±0.4	1.8±0.4	1.7±0.4
SGOT (U/l)	17±7	18±5	17±6	17±6
SGPT (U/l)	21±9	19±6	20±9	22±8
Albumin (g/l)	38±5	39±4	42±3	41±4
Globulin (g/l)	25±3	26±4	26±3	25±3
Iron (umol/l)	11±3	11±4	18±6	18±6
PTH (ng/l)	1627±1419	1492±1362	1972±1802	1279±1292
Aluminium (umol/l)	1.8±1.2	1.8±1.0	1.5±0.8	1.3±0.8
Urine volume (l)	0.9±0.4	1.0±0.4	0.7±0.3	0.9±0.4

Unpaired t test: *t = 2.354, $p < 0.02$, $p_{cor} < 0.38$, NS

TABLE 3.5. CORRELATION COEFFICIENT (r) BETWEEN HAEMATOLOGY AND BIOCHEMISTRY DATA AND THE RECALL ANTIGEN SCORES (ALL PATIENTS, n = 20)

Variable	Recall antigen scores First test r	Recall antigen scores Second test r
Hb (g/dl)	0.142	0.437
WBC ($x10^9$/l)	0.044	-0.178
Na (mmol/l)	0.245	0.051
K (mmol/l)	-0.220	0.003
Cl (mmol/l)	0.150	-0.338
CO_2 (mmol/l)	0.537*	-0.265
Urea (mmol/l)	-0.295	-0.257
Creatinine (umol/l)	-0.288	-0.026
Alk phos (U/l)	-0.145	-0.092
Ca (mmol/l)	0.203	-0.269
PO_4 (mmol/l)	-0.428	0.137
SGOT (U/l)	0.225	0.282
SGPT (U/l)	0.275	0.179
Albumin (g/l)	0.267	0.282
Globulin (g/l)	0.232	0.187
Iron (umol/l)	-0.034	0.193
PTH (ng/l)	-0.151	0.068
Aluminium (umol/l)	-0.520^+	0.326
Urine volume (l)	0.153	0.241

*$t = 2.700$, $p < 0.02$, $p_{cor} < 0.38$, NS

$^+t = 2.582$, $p < 0.02$, $p_{cor} < 0.38$, NS

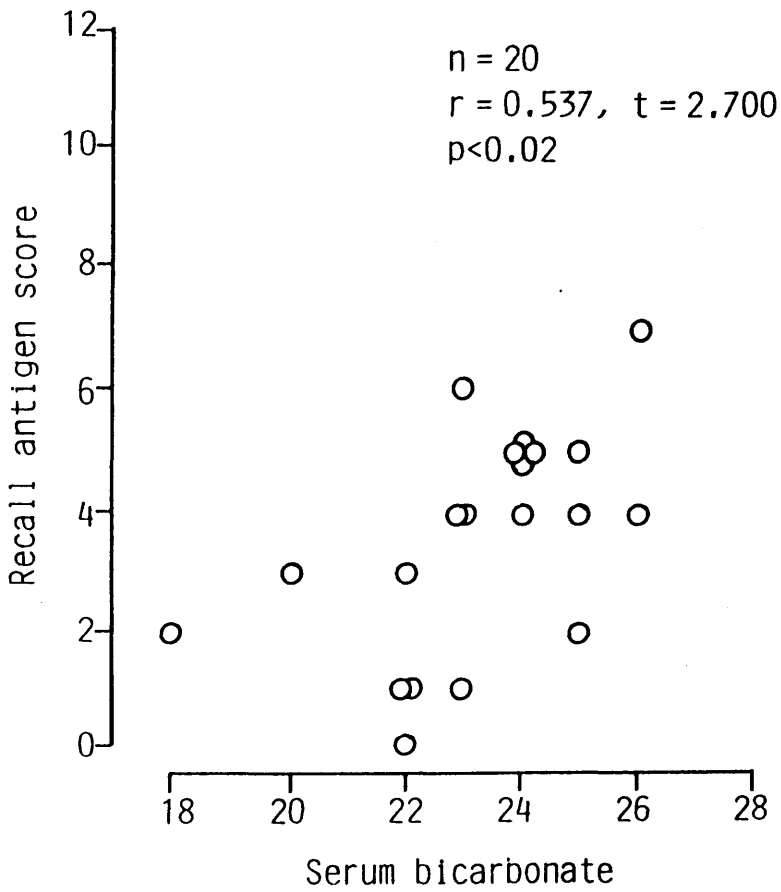


Figure 3.3. Correlation between serum bicarbonate and recall antigen scores at the first test.

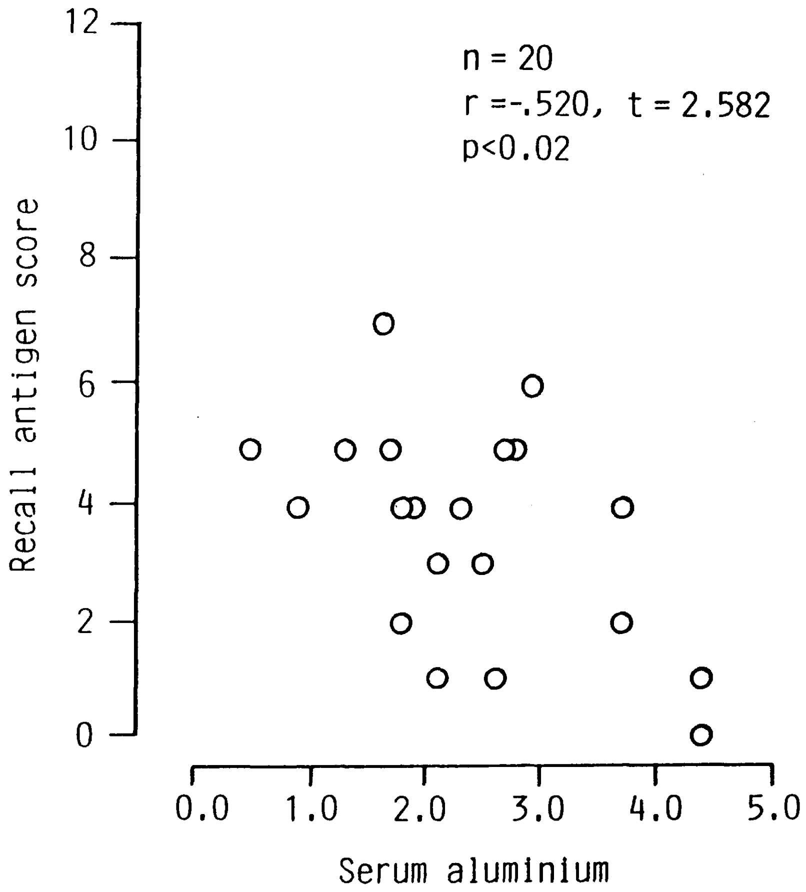


Figure 3.4. Correlation between serum aluminium and recall antigen scores at the first test.

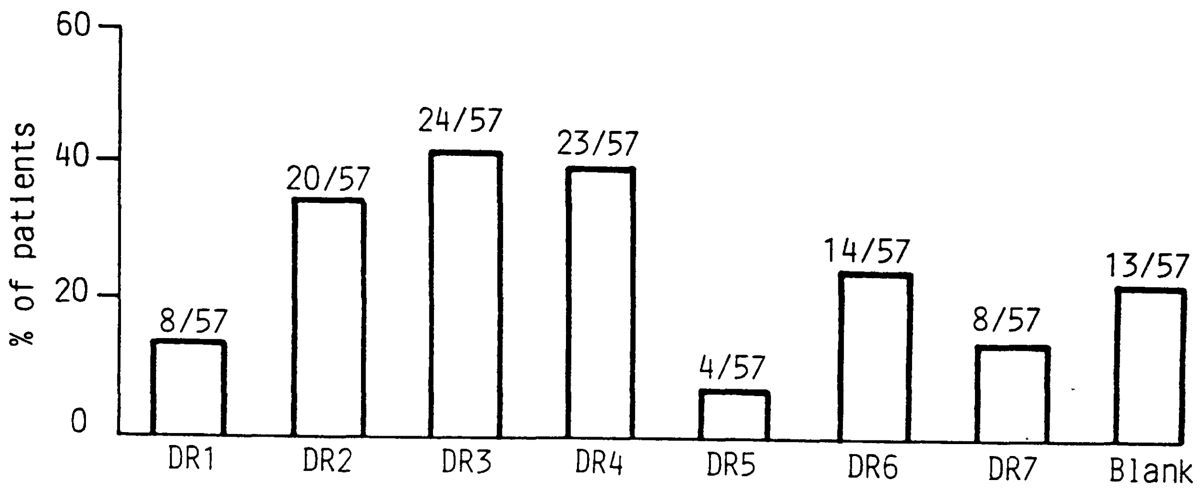


Figure 3.5. Distribution of HLA-DR specificities among the 57 patients.

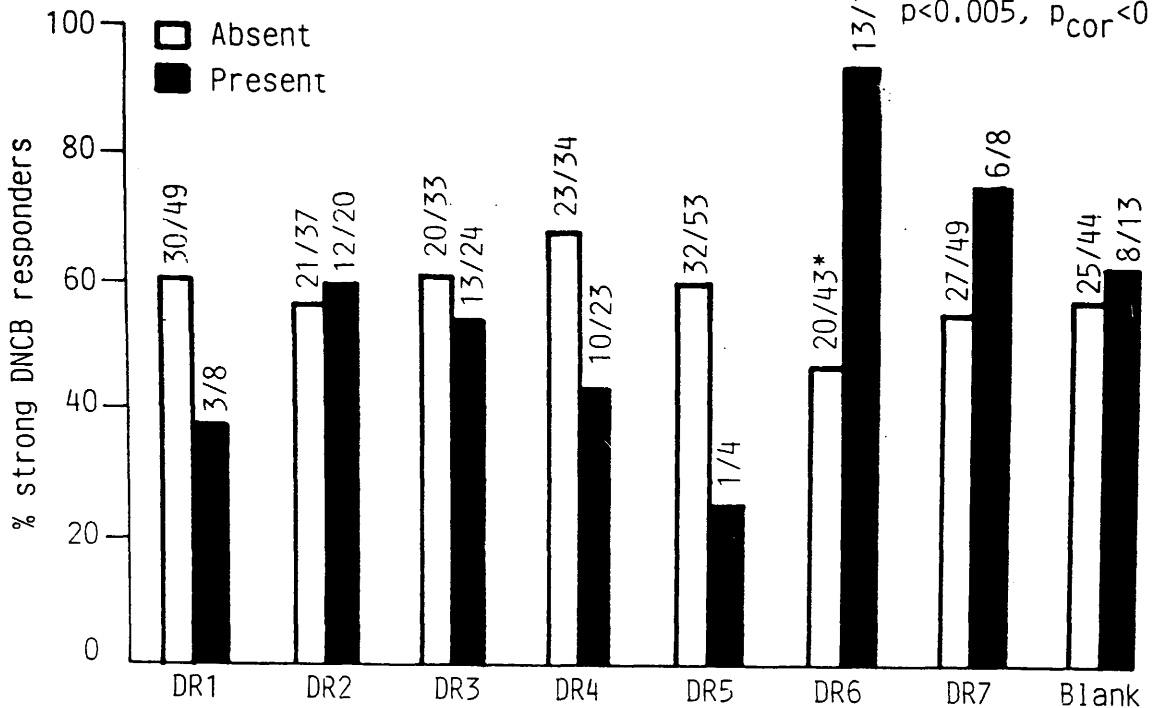


Figure 3.6. Percentage of strong DNCB responders in patients with DR antigens absent and present at the first test.

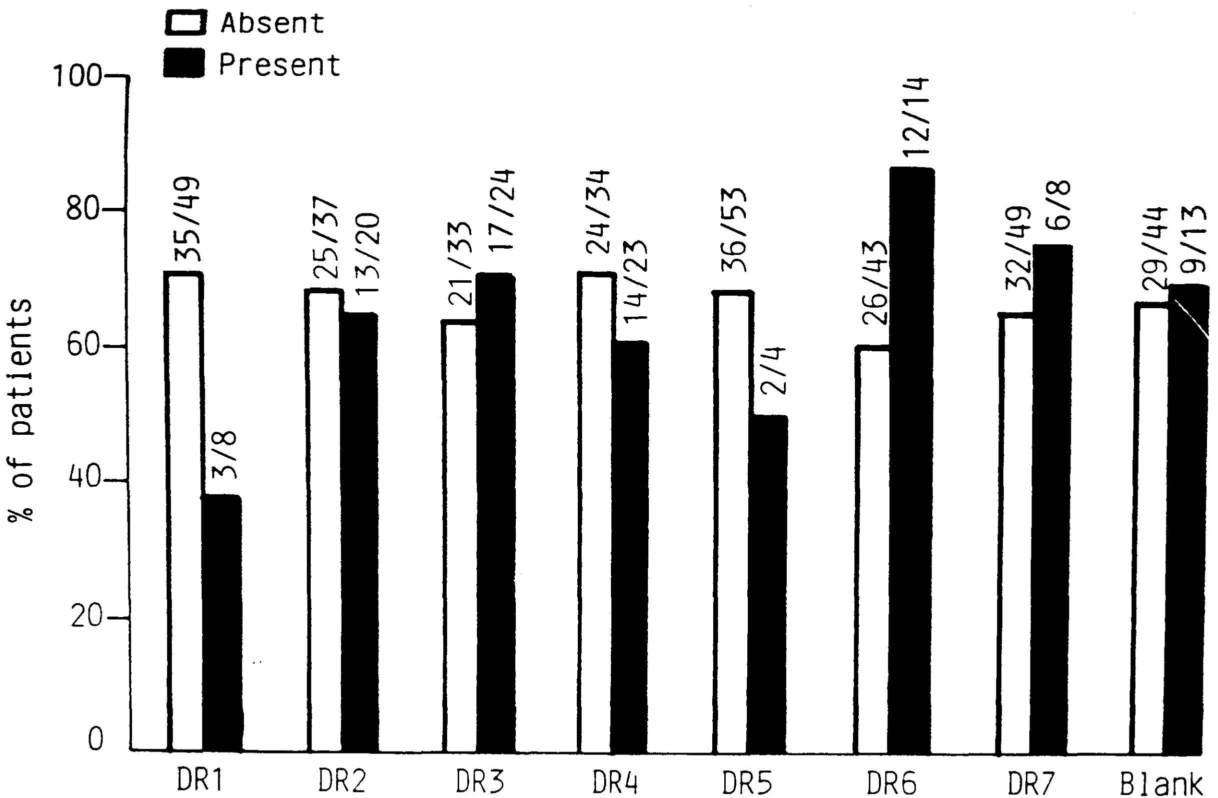


Figure 3.7. Percentage of strong DNCB responders in patients with DR antigens absent and present on repeating the test.

CHAPTER 4. RENAL ALLOGRAFT SURVIVAL IN PATIENTS UNDERGOING TRANSPLANTATION

4.1 Introduction

The aim in the management of the transplant patient is to achieve optimal patient and graft survival while minimising the side effects of immunosuppression. In recent years elective blood transfusion has occupied a large part of this effort both in clinical practice and research. One of the objectives of this study was to see whether transfusion of 5 or 10 units of blood in dialysis patients with presumed impaired (weak DNCB responders) or intact CMI (strong DNCB responders) had any different effect on subsequent renal allograft survival. This chapter will describe details of the transplant outcome in weak and strong DNCB responders who received 5 or 10 units of blood.

4.2 Patients and Methods

Thirty two of the 48 patients in the transfusion group were transplanted with a cadaveric renal graft by the end of 1985. In all cases this was their first transplant. There were 25 males and 7 females, and their mean age was 40 years (range, 20-59 years). The first patient was transplanted at the beginning of May 1983 and the last in mid-July 1985. Up to the end of 1983 azathioprine was used as the main immunosuppressant (along with steroids), and thereafter cyclosporin was introduced. This change in the immunosuppression protocol was not anticipated when this study was designed in August 1982, but it was thought that it would not be ethical to deprive the patients who entered the study from receiving cyclosporin because of its apparent superiority. Therefore, 11 of the 32 transplants received azathioprine and prednisolone, 18 patients received cyclosporine and prednisolone, 2 patients were commenced on cyclosporine and eventually were changed to azathioprine because of delayed graft function, and one patient was treated with cyclophosphamide and prednisolone because she developed persistent azathioprine-induced leucopenia. Azathioprine was given in an average dose of 2mg/kg/day aiming to keep the WBC count more than 4000/mm^3. The starting dose of cyclosporin in the first half of 1984 was 17.5mg/kg/day reducing

to 15mg at one week, and to 10mg at 3 weeks; thereafter the dosage was adjusted to achieve optimal serum creatinine levels and trough whole blood cyclosporin levels ranging between 200-400ng/l. From mid-1984 a lower cyclosporin dose regimen was adopted starting with 15mg/kg/day for one week reducing to 10mg for two weeks, with subsequent further reductions as before. The initial prednisolone dose in both patients receiving azathioprine and cyclosporine was 20mg/day reducing to 15mg at 3 months, 12.5mg at 6 months, and 20mg on alternate days at one year after transplantation. Rejection episodes were treated either by 500mg intravenous methylprednisolone pulses for two or three successive days or by increasing the oral prednisolone dose according to the Belfast regimen (436). The diagnosis of rejection was based on clinical grounds and ultrasound findings, and in most occasions was confirmed by biopsy. Selection of recipients was done on a best match basis giving priority to HLA-DR matching. Transplantation was carried out across a negative T cell crossmatch on both current and historical sera. The time of graft failure was defined either as the time of transplant nephrectomy or of recommencing dialysis. The end point of the follow-up was 31.5.86, giving a follow-up time of between 8 and 45 months for the entire group.

Statistical analysis

The introduction of cyclosporin in this study made the interpretation of the transplantation results more complicated. The already small numbers of patients in the groups of weak and strong responders receiving 5 and 10 units of blood were inevitably affected by the two different immunosuppressive regimens. Therefore, actuarial graft survival was not considered in the statistical analysis. Graft survival has been expressed as the proportion of functioning grafts at the end of the study. Comparison of percentages was done using the chi-square test. Fisher's exact test was computed when there were fewer than 20 cases in a 2x2 table; Yates' corrected chi-square was computed for all other 2x2 tables. Comparison of the number of HLA mismatches and rejection episodes was carried out using the Mann-Whitney-Wilcoxon test.

4.3 Results

Twenty two of the 32 transplants (69%) had a functioning renal graft at the conclusion of the study. Seven of the remaining 10 patients lost their graft because of irreversible rejection, and the other three failures were non-immunological; of these three, two patients died with functioning grafts (one from septicaemia and the other from a myocardial infarct), and one transplant was an early technical failure due to renal artery thrombosis.

Figure 4.1 shows the graft survival in weak and strong DNCB responders receiving 5 or 10 units of blood; in the first part of the figure (a) patients have been allocated on the basis of their response to DNCB at the first test, and in the second part of the figure (b) according to their DNCB status on repeating the test. The repeat test was carried out after the last elective transfusion. Although the graft survival in weak DNCB responders was better on both occasions compared to that of the strong responders the differences were not significant. Twelve of the 16 grafts in the weak responders were functioning by the end of the study (75%), three of the remaining transplants underwent irreversible rejection, and one patient died with a functioning graft 14 months after the operation. In the strong responders 10 of the 16 grafts (63%) were functioning at the end of the study, four grafts had rejected, one patient with ongoing chronic rejection died with a functioning graft at 16 months, and there was one technical failure (Figure 4.1a). The rationale in analysing the graft survival of taking into account the DNCB status on repeating the test (Figure 4.1b) is justified by the fact that 6 of the weak responders who underwent transplantation were found to be strong responders on the repeat test. This implies that there is a proportion of uraemic patients who had delayed strong reaction at the first test, which has not been detected at the reading of the scores 48 hours after the challenge immunisation. The reaction of these patients became clearly strong in the anamnestic response on repeating the test. Two of the 6 weak responders who had changed to strong responders rejected their grafts. This slightly increased the difference in graft survival between weak and strong responders (80% vs 64%), although it was still not significant. This is an interesting point which may affect analyses of larger numbers

of transplants, and will be discussed later in Chapter 9. With regard to the number of transfusions (5 or 10 units) and the graft survival in the weak and strong responders the numbers are too small to draw out any firm conclusion; nevertheless, the difference did not appear to be significant (Figure 4.1).

Table 4.1 shows that the number of patients treated with azathioprine or cyclosporine was comparable in the weak and strong responders (31% and 63% vs 38% and 50% respectively). The proportion of well matched grafts was also not significantly different, and the mean number of mismatches per patient were identical in the two groups. In contrast, there was a significant difference in the number of patients presenting with rejection episodes between weak and strong DNCB responders ($p < 0.025$). Eleven of the 16 weak responders had no rejection episodes (69%),4 patients had one episode (25%), and one patient had three rejections (6%). In the 16 strong responders 5 patients had no rejection episodes (31%), 5 patients had one episode (31%), 2 patients had 2 episodes (13%), and 4 patients had three episodes (25%). Hence, in the former group there was a total of 7 rejections among 5 patients, and in the latter group 21 rejections among 11 patients which gives a mean number of rejection episodes per patient 0.44 in the weak responders compared to 1.31 in the strong responders ($p < 0.025$). There was not any significant difference in the timing of the first rejection episode between the two groups. Four of the 5 weak responders (80%) had their first episode within the first week from the operation compared to 7 of the 11 strong responders (64%).

In Table 4.2 is shown the same information as in the previous Table taking into account the DNCB status on the repeat test. The change in the six patients from weak to strong responders did not upset the comparability of the two groups in terms of immunosuppression and tissue matching. However, the difference in the prevalance of rejection episodes in the two groups did not reach statistical significance because of the smaller numbers in the weak responder group which was reduced from 16 to 10 patients.

4.4 Conclusions

The small number of transplants in the weak and strong DNCB responder group were further affected by the two different immunosuppressive regimens. Thus, the results in this chapter can only suggest trends and do not justify firm conclusions. Weak DNCB responders appeared to have better graft survival and less rejection episodes compared to strong DNCB responders, and whether the patients received 5 or 10 units of blood did not seem to associate significantly with the transplant outcome. These points will be further discussed in Chapter 9 of this thesis based on the findings from a retrospective analysis of a larger number of transplants.

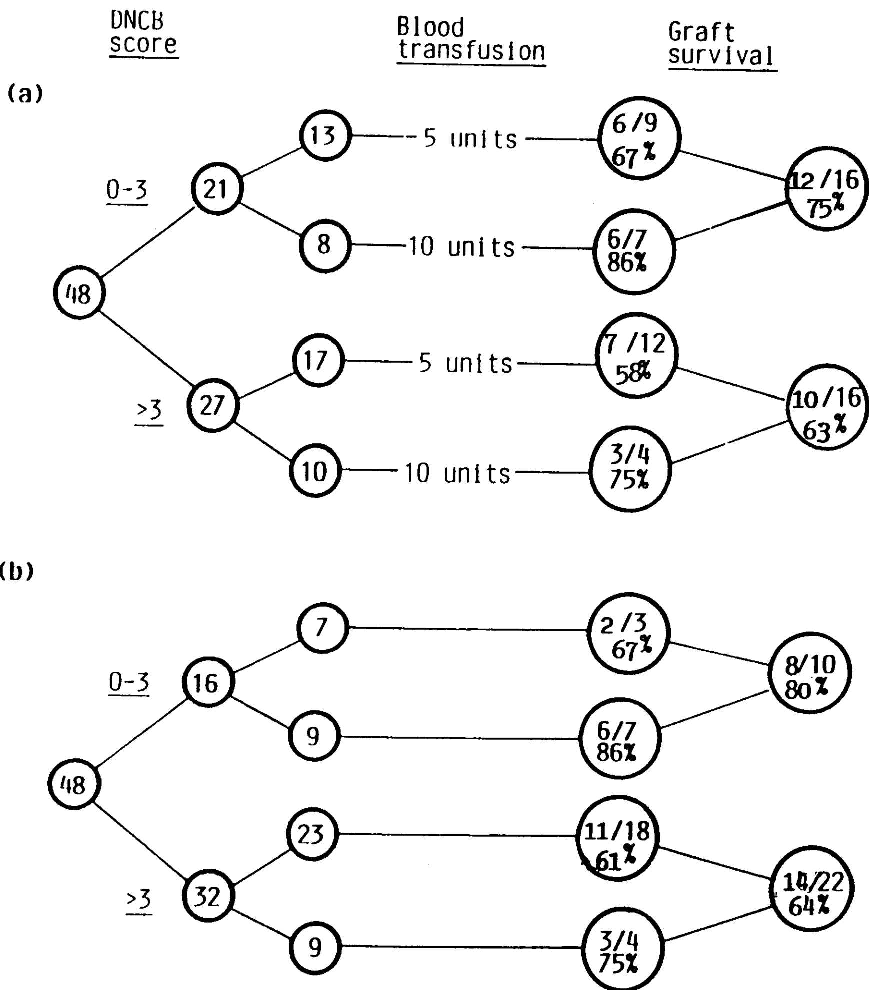


Figure 4.1. Graft survival in weak and strong DNCB responders receiving 5 or 10 units of blood; a = DNCB score at first test, b = DNCB score on repeating the test.

TABLE 4.1. TRANSPLANTATION DATA OF WEAK AND STRONG DNCB RESPONDERS AT THE FIRST TEST

	Weak Responders n=16			Strong responders n=16		
Immunosuppression:						
Azathioprine	5 (31%)			6 (38%)		
Cyclosporin	10 (63%)			8 (50%)		
Combination	1 (6%)			2 (12%)		
Number of patients with HLA mismatches:	**A**	**B**	**DR**	**A**	**B**	**DR**
0	4(25%)	5(31%)	8(50%)	2(12%)	3(19%)	6(38%)
1	4(25%)	7(44%)	7(44%)	8(50%)	12(75%)	8(50%)
2	8(50%)	4(25%)	1(6%)	6(38%)	1(6%)	2(12%)
Mean mismatches/ patient	1.3	0.9	0.6	1.3	0.9	0.6
Number of patients with rejection episodes:*						
0	11(69%)			5(31%)		
1	4(25%)			5(31%)		
2	0			2(13%)		
3	1(6%)			4(25%)		
Total number of rejections	7			21		
Mean rejections/patient:	0.44**			1.31**		
Duration of graft function Mean + SD (months)	17.4+9.5			15.6+11.2		

*x^2 = 9.363, DF = 3, $p < 0.025$; **t = 2.420, $p < 0.025$

TABLE 4.2. TRANSPLANTATION DATA OF WEAK AND STRONG DNCB RESPONDERS ON THE REPEAT TEST

	Weak responders n=10			Strong responders n=22		
Immunosuppression						
Azathioprine	4 (40%)			7 (32%)		
Cyclosporine	5 (50%)			13 (59%)		
Combination	1 (10%)			2 (9%)		
Number of patients with HLA mismatches:						
	A	B	DR	A	B	DR
0	3(30%)	4(40%)	5(50%)	3(14%)	4(18%)	9(41%)
1	3(30%)	4(40%)	4(40%)	9(41%)	15(68%)	11(50%)
2	4(40%)	2(20%)	1(10%)	10(45%)	3(14%)	2(9%)
Mean mismatches/ patient:	1.1	0.8	0.6	1.3	1.0	0.7
Number of patients with rejection episodes:*						
0	8(80%)			8(36.5%)		
1	1(10%)			8(36.5%)		
2	0			2(9%)		
3	1(10%)			4(18%)		
Total number of rejections	4			24		
Mean rejections per patient:	0.40**			1.10**		
Duration of graft function Mean + SD (months):	19.4 + 10.1			15.2 + 10.2		

*x^2 - 6.158, DF - 3, NS; **t - 1.696, NS

CHAPTER 5. DEVELOPMENT OF PANEL REACTIVE ANTIBODIES FOLLOWING TRANSFUSION AND TRANSPLANTATION

5.1 Introduction

The main concern with regards to a deliberate transfusion policy prior to renal transplantation is the risk of producing sensitisation against the HLA antigens of future kidney donors (420,440,443,456,465,479,491-496,509-513). In addition to blood transfusion, sensitisation may also result from a previous failed transplant or from a previous pregnancy (456,465,479,491,492). There is a considerable variation in titre, specificity and persistence of cytotoxic HLA antibodies in patients awaiting transplantation, and this fact may to some extent be due to individual differences in the patient's immune response (457,493,494). The aim of this chapter is to see whether weak or strong DNCB responders differ in the development of lymphocytotoxic antibodies ie panel reactive antibodies (PRA) after elective blood transfusion, and to define the optimum number of transfusions in order to achieve a beneficial effect on graft survival, while keeping the risk of sensitisation to a minimum.

5.2 Patients and Methods

Patients

All 48 patients in the transfusion group and the 12 uraemic controls (Table 1) were followed up to determine the development of PRA (Figure 5.1). Sera from the patients in the transfusion group were harvested prior to the first and 2 weeks after each transfusion, and from the controls at monthly intervals. Thereafter all patients were followed up every month for a mean period of 18.5 months (range 8-36 months) after the last elective blood transfusion. Thirty two of the 48 patients underwent transplantation (see Chapter 4); the remaining 16 patients were not transplanted during the study. The mean follow up in the former group was 16.6 months (range 8-36 months) and in the latter group was 22.3 months (range 8-28 months); during this period 9 of the 16 patients who were not transplanted were given packed cells when transfusion was indicated on clinical grounds (range 1-18 units).

Methods

Sera were screened for PRA using the standard Terasaki-NIH microcytotoxicity test (572) using a long incubation time (576). Purified T lymphocytes prepared by rosetting with neuraminidase were recovered from a selected panel of 30 healthy volunteers covering the range of HLA-A, B and C specificities. In summary, 1ul of T lymphocytes was added to 1ul of antiserum in Greiner microtest plates, and incubated for 60 minutes. Using a multiple needle dispenser 5ul of rabbit complement was added and plates were incubated for a further 120 minutes. Both incubations were carried out at room temperature (22°C). Then, 3ul of 5% eosin, and a few minutes later 8ul of formaldehyde were added. All sera were judged to be positive when more than 20% of the target cells were killed. Positive and negative controls were included in each test. The results were not known until the end of the study, so exclusions with regards to sensitisation status of the patients were not made. This reflects our policy in clinical practice, since patients awaiting transplantation after they have completed the elective 5 units of packed cells, receive further packed cells regardless of the level of PRA should they require transfusion on clinical grounds.

Statistical analysis

Comparisons of differences of sensitisation rates between groups were done using the chi-square test with Yates' correction. Differences of means of PRA were not analysed statistically because the numbers of sensitised patients within subgroups were small. Therefore, findings in this chapter should be viewed as indicative of trends rather than leading to firm concluding remarks.

5.3 Results

None of the 12 controls developed PRA. In contrast, the rate of sensitisation after 5 and 10 units of blood is shown in Table 5.1. Six of the 30 patients receiving 5 units (20%), and 7 of the 18 patients receiving 10 units of blood (39%) had PRA in their latest sera. Looking at the entire range of sera, the peak sensitisation rates were 27% amd 44% respectively. The incidence of sensitisation was higher among the strong DNCB responders compared to the weak

responders, but the numbers were too small to reach statistical significance (Table 5.1). However, the importance of the individual patient's immune response, as measured by the DNCB test, in response to blood-transfusion induced PRA is better demonstrated in Table 5.2, which shows the rate of senisitisation prior to the first and after each transfusion in the weak and strong DNCB responders. Strong DNCB responders developed cytotoxic antibodies earlier compared to weak responders. After the third unit of blood none of the 21 weak DNCB responders had PRA compared to 22% of the strong responders. This difference just failed to reach statistical significance with Yates' correction. After the fifth transfusion, however, the numbers of sensitised patients in the two groups become more comparable. Figure 5.2 shows in logarithmic scale the level of PRA in weak and strong DNCB responders receiving 5 units of blood. After the third transfusion none of the 13 weak responders had PRA compared to an overall mean of 4% of the 17 strong DNCB responders. After the fifth transfusion, however, both weak and strong responders had identical mean PRA at 10%. Figure 5.3 shows the 8 patients who initially had no antibodies and in whom these developed in the course of the 5 transfusions. None of the 6 sensitised patients of both strong and weak DNCB responders had broad reactivity after 4 transfusions (all had less than 35%), and it was only after the fifth unit of blood that patients tended to become more broadly reactive against the panel (32-76%). It is also interesting that the 2 sensitised weak DNCB responders had negligible reactivity prior to the fifth transfusion, but after that they responded in a comparable way to the strong DNCB responders. The same pattern of response was found in the patients receiving 10 transfusions (Figure 5.4). Again, after the fifth transfusion the level of PRA in the 2 groups was almost identical, ranging from 10% after the fifth transfusion to 16% after the tenth unit of blood. During the first 4 transfusions the level of PRA was zero in the weak DNCB responders compared to 8-9% in the strong responders. In Figure 5.5, which shows the patients sensitised in the course of 10 transfusions, it can be seen that, with the exception of one patient, the reactivity prior to the fifth unit is low and becomes stronger thereafter. The exception was a woman with a history of five pregnancies and with a positive (20%)

background reactivity. This patient developed broad sensitisation (more than 80% PRA) after the second transfusion and remained highly sensitised thereafter. The fact that females are of higher risk to becoming sensitised because of previous immunisation from pregnancies (Table 1), is shown in Table 5.3. Although the differences were not significant, a larger proportion of women became sensitised, particularly after 10 units of blood, and they tended to retain the antibodies for longer compared to men, judging from the peak and the latest PRA figures in the 2 groups. Table 5.4 shows the development of cytotoxic antibodies before and after each elective transfusion and the follow-up thereafter in the patients who had not received an allograft by the end of the study. Of the patients who became sensitised during the elective transfusions and did not require further blood on clinical grounds (numbers 1, 3 and 7 in Table 5.4), all 3 eventually lost their antibodies within a year from the last elective transfusion. Patients requiring an occasional further transfusion (numbers 2, 4, 8, 9 and 10) had fluctuating PRA titres, the blood always having a booster effect on their reactivity, but which again was eventually lost or declined. In contrast, 2 patients (numbers 5 and 6) who required a further 18 and 13 units of blood respectively after the elective transfusions, developed broadly reactive antibodies, including those with the more common anti-HLA specificities, namely, HLA-B8, HLA-B12 and HLA-A2. Table 5.5 summarises the data of the patients who underwent transplantation and classifies them according to the development of peak and pre-transplant PRA and also peak titres after the transplant. Patients transplanted with zero PRA in the complete series of pretransplant samples, and with zero (group 1) or very low post-transplant titres (range 4-19%) (group 2), all had successful outcome (mean graft survival 19 months, range 8-31 months). In the third group, 5 patients had a transplant across positive (range 13-61%), but declining PRA titres (range 0-26%), and the post-transplant response was also low (range 0-26%); all 5 patients had a successful outcome (mean graft survival 12 months, range 8-16 months). By contrast all 5 patients transplanted with zero pretransplant PRA, but with high post-transplant titres (range 56-87%) lost their grafts within 5-14 months (group 4). Finally, all

3 patients with high (range 32-100%), non-declining pretransplant titres (range 65-96%), and with equally high post-transplant titres (range 52-100%) also lost their grafts within a month (group 5).

5.4 Conclusions

1) Strong DNCB responders develop cytotoxic antibodies earlier after blood transfusion in comparison with weak DNCB responders, but after 5 units of blood both rate and level of PRA are comparable in the two groups.

2) The risk of broad sensitisation of patients receiving 3 or 4 units of blood is negligible, but may increase with more transfusions.

3) Elective pretransplant transfusion of packed cells should be limited to 3 or 4 units, and thereafter patients should receive less immunogenic blood products such as frozen or filtered red cells should they require transfusion on clinical grounds.

4) Women are at higher risk of developing antibodies, and those with preformed PRA should be given less immunogenic transfusion protocols.

5) Negative or declining PRA titres in the pretransplant sera predict high graft survival, while high titres after transplantation are commonly associated with graft failure.

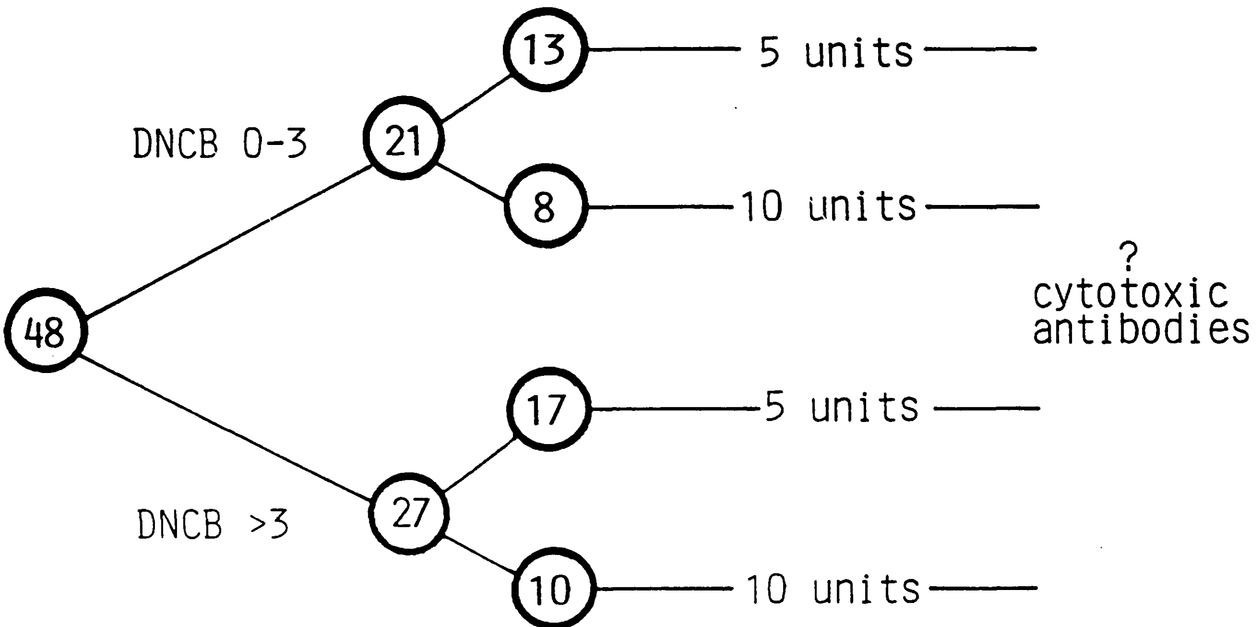


Figure 5.1. Numbers of weak (0-3) and strong (greater than 3) DNCB responders allocated to receive 5 or 10 units of blood.

TABLE 5.1. SENSITISATION RATES AFTER 5 AND 10 UNITS OF ELECTIVE BLOOD TRANSFUSION

	Number of sensitised patients	
	5 units	10 units
Latest PRA:		
All patients	6 of 30 (20%)	7 of 18 (39%)
Weak DNCB responders	2 of 13 (15%)	2 of 8 (25%)
Strong DNCB responders	4 of 17 (24%)	5 of 10 (50%)
Peak PRA:		
All patients	8 of 30 (27%)	8 of 18 (44%)
Weak DNCB responders	2 of 13 (15%)	3 of 8 (38%)
Strong DNCB responders	6 of 17 (35%)	5 of 10 (50%)

TABLE 5.2. SENSITISATION RATE IN WEAK AND STRONG DNCB RESPONDERS AFTER BLOOD TRANSFUSION

	Number of sensitised patients										
Number of transfusion	0	1	2	3	4	5	6	7	8	9	10
Weak DNCB responders	0/21	0/21	1/21	0/21*	1/21	3/21	2/8	1/8	2/8	3/8	2/8
	-	-	5%	-	5%	14%	25%	13%	25%	38%	25%
Strong DNCB responders	1/27	4/27	5/27	6/27*	5/27	5/27	1/10	2/10	2/10	5/10	5/10
	4%	15%	19%	22%	19%	19%	10%	20%	20%	50%	50%

*x^2 = 5.353, $p < 0.025$, Yates' x^2 = 3.495, NS

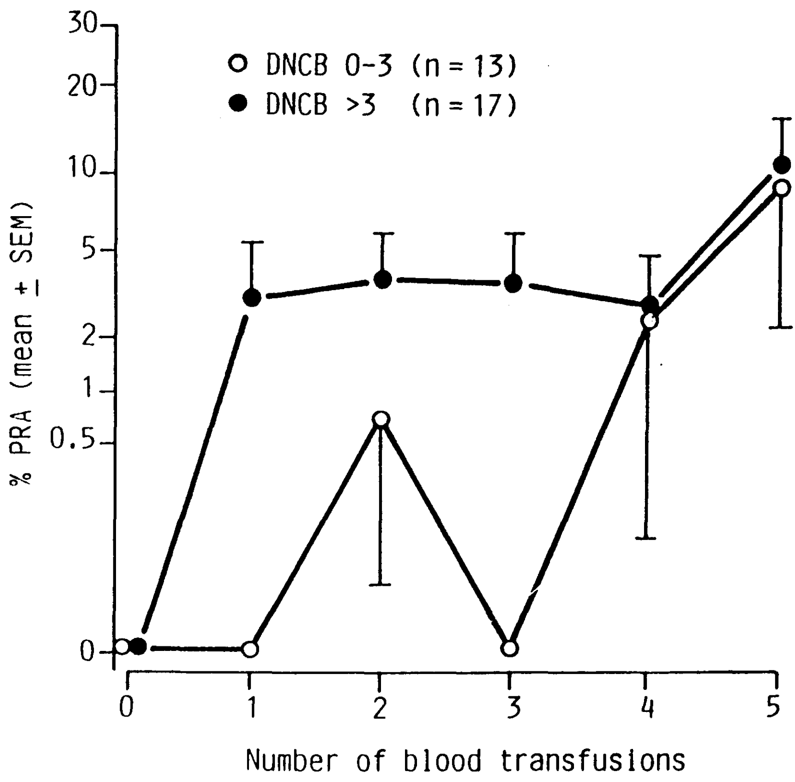


Figure 5.2. Mean ± SEM percent PRA titres in the weak (O) and strong DNCB responders (●) receiving 5 units of packed cells.

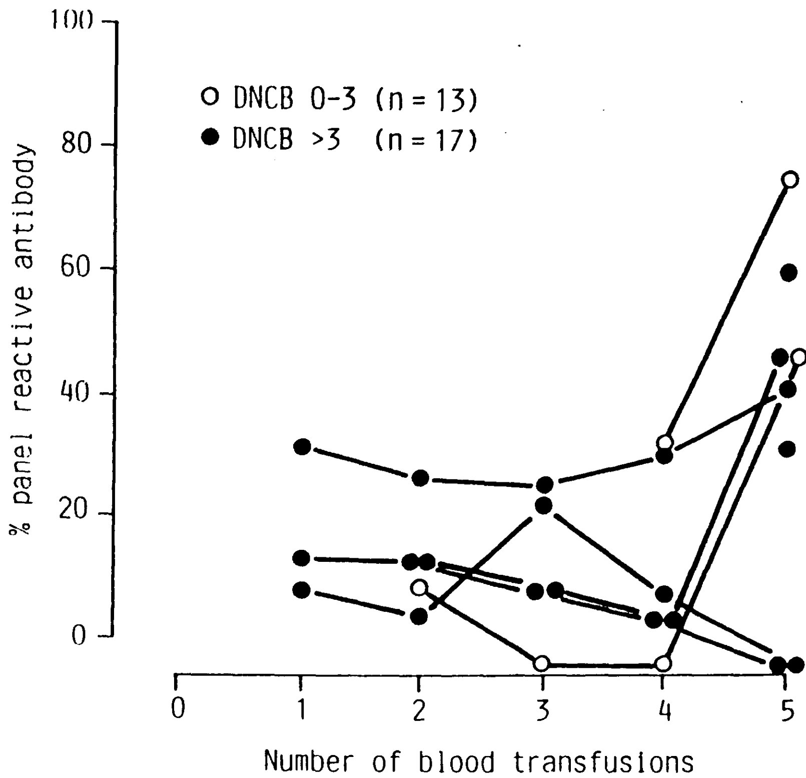


Figure 5.3. Percent PRA titres in the 8 patients who became sensitised in the course of 5 transfusions; O = weak DNCB responders, ● = strong DNCB responders.

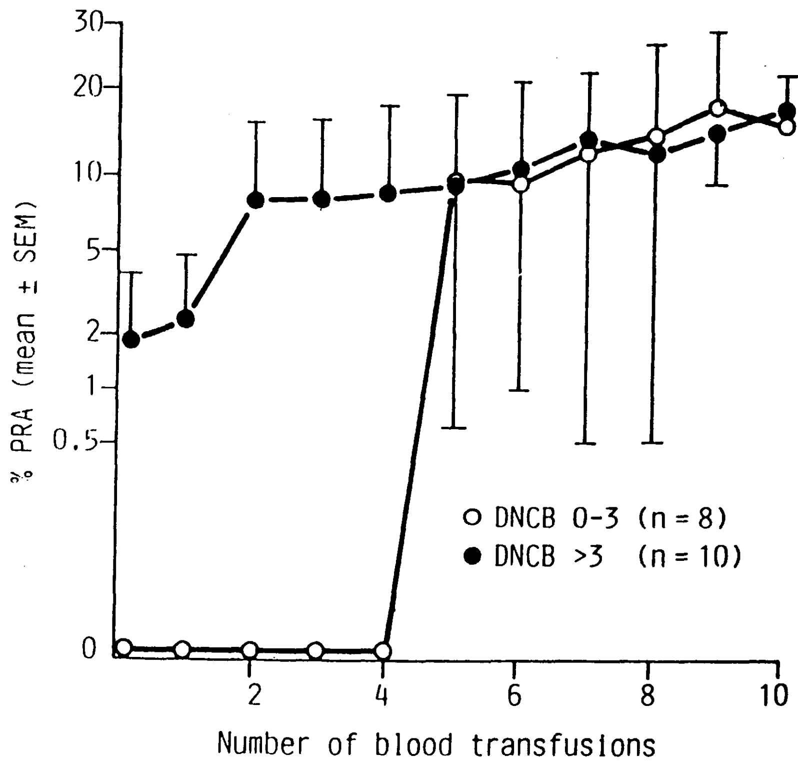


Figure 5.4. Mean ± SEM percent PRA titres in the weak (O) and strong DNCB responders (●) receiving 10 units of packed cells.

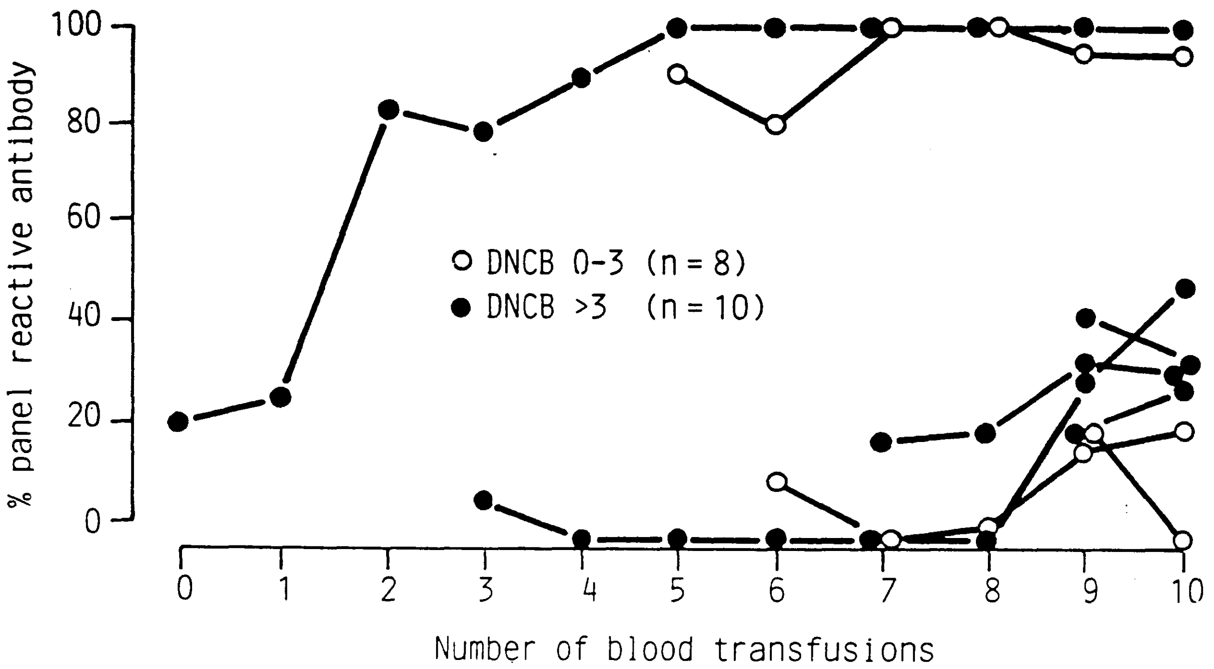


Figure 5.5. Percent PRA titres in the 8 patients who became sensitised in the course of 10 transfusions (O = weak DNCB responders, ● = strong DNCB responders).

TABLE 5.3. SENSITISATION RATES AFTER 5 AND 10 TRANSFUSIONS IN MALES AND FEMALES

	Number of sensitised patients	
	5 units (n=30)	10 units (n=18)
Latest PRA:		
Males	4 of 23 (17%)	4 of 14 (29%)
Females	2 of 7 (29%)	3 of 4 (75%)
Peak PRA:		
Males	6 of 23 (26%)	5 of 14 (36%)
Females	2 of 7 (29%)	3 of 4 (75%)

TABLE 5.4. PANEL REACTIVE ANTIBODIES (PRA) IN PATIENTS NOT UNDERGOING TRANSPLANTATION

% PRA in patients not undergoing transplantation

Patients	Post-BT						Months post last BT						
5BT	0	1	2	3	4	5	4	8	12	16	20	24	28
J.B.	0	0	0	0	0	0	0	•0	•0	0	•0		
W.D.	0	0	0	0	0	0	0	•0	•0	0	•0	0	
K.M.	0	0	0	0	0	0	0	0	0	0	0	0	0
D.E.	0	0	0	0	0	0	0	0					
J.McL. [1]	0	0	13	9	4	48	50	25	0	0	0	0	0
L.A. [2]	0	0	0	0	0	0	18	0	0	•42	•30	4	0
L.McL. [3]	0	0	9	0	0	48	39	11	0				
J.H. [4]	0	9	4	23	8	0	0	0	8	•54	4	10	
C.B. [5]	0	32	27	26	32	43	•46	::65	::91	::90	∴:56		
N.McP. [6]	0	0	0	0	33	76	73	54	::91	•77	•61	∴76	∴80

10BT	0	1	2	3	4	5	6	7	8	9	10	4	8	12	16	20	24	28
P.F.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
J.L.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
A.K. [7]	0	0	0	0	0	0	0	0	0	18	27	35	19	0	0	0		
J.P. [8]	0	0	0	0	0	0	0	0	0	41	32	0	0	•24	4	0	0	
B.J. [9]	0	0	0	4	0	0	0	0	0	29	48	29	•4	0	•24	•41	0	
M.M. [10]	0	0	0	0	0	0	0	0	0	18	0	0	•26	•73	4	•4	•46	7

1. B7, B27, B40; 2. A1, B8; 3. A2; 4. Undefined;
5. Multispecific, CW7, B8, B12(44); 6. Multispecific, A2, B8.
7. B7, B27. 8. Undefined. 9. A1, A11. 10. Undefined.

TABLE 5.5. SUMMARY OF PATIENTS UNDERGOING TRANSPLANTATION

Patients (n=32)	%PRA Peak	%PRA Pre-Tx	%PRA Post-Tx	Graft Failure n	HLA Mismatches A,B	HLA Mismatches DR	CYA n	AZ n	CYA/AZ n
Group 1 (n=14)	0	0	0	0	2.1	0.5	7	6	1
Group 2 (n=5)	0	0	9±5	0	2.4	0.8	3	1	1
Group 3 (n=5)	30±19	5±10	5±10	0	1.6	0.6	5	0	0
Group 4 (n=5)	0	0	70±11	5	2.4	0.6	1	3	1*
Group 5 (n=3)	78±39	82±16	72±25	3	2.3	1.3	2	1	0

CYA = cyclosporine, Az = azathioprine,

*Azathioprine/cyclophosphamide

CHAPTER 6. THE EFFECT OF PRE-TRANSPLANT TRANSFUSION ON IgG-SECRETING CELLS

6.1 Introduction

Previous studies have shown that blood transfusion may alter lymphocyte reactivity of uraemic patients due to an increase in non-specific suppressor activity mediated by either suppressor T cells or monocytes (393,394,531-535,537). Activation of B cells by polyclonal B cell activators is regulated by suppressor T cells. One means of assessing the role of suppressor cells at the cellular level is to examine the production of immunoglobulin by peripheral blood lymphocytes in response to two polyclonal B cell activators that differ in their dependence on regulation by suppressor cells. Pokeweed mitogen (PWM) induced immunoglobulin synthesis is influenced by T suppressor cells (249,250), whereas the response to Staphylococcus Aureus, Cowan I (SAC) is only partially T cell dependent; the proliferation of B cells in response to SAC is not dependent on T cells, while the differentiation into immunoglobulin secreting cells is T cell dependent (577). The aim of this chapter is to describe the effect of third party blood transfusion on IgG-secreting cells both spontaneously and after stimulation with PWM and SAC, using a reverse haemolytic protein A plaque forming cell (PFC) assay.

6.2 Patients and Methods

Patients

Twenty six of the 48 patients in the transfusion group were studied of whom 20 were males (77%) and 6 females (23%) with a mean age of 39.2 years (range 20-59 years). Their original renal diseases were glomerulonephritis 9 (35%), pyelonephritis 7 (27%), polycystic kidneys 3 (11%), hypertensive nephrosclerosis 3 (11%), diabetes 2 (8%), and of unknown origin 2 (8%). Twenty patients were on haemodialysis (77%) and the remaining 6 patients were on CAPD (23%). With regard to their clinical characteristics, these 26 patients were representative of the entire group of 48 transfused patients (Table 1). The breakdown of the 26 patients according to their response to the DNCB skin test and the number of transfusions they received is

shown in Figure 6.1. Ten patients were weak DNCB responders and 16 were strong responders. Fourteen patients (6 weak and 8 strong responders) received 5 units of blood, and the other 12 patients (4 weak and 8 strong responders) had 10 transfusions. Twelve non-transfused uraemic controls (Table 1) and 12 healthy subjects were also studied. In the group of healthy controls there were 7 males and 5 females with a mean age of 36 years (range, 24-59 years).

Methods

The 26 transfused patients were studied prior to and at 14-day intervals after each transfusion. The two control groups were also studied sequentially on 3 to 4 occasions over a period of three months. Peripheral blood mononuclear cells (PBMC) were prepared from heparinised blood by separation on Ficoll Hypaque (Nyegaard, Birmingham, UK), and were cryopreserved in liquid nitrogen. All PBMC samples from each subject were assayed in one experiment to avoid test to test variation. Initial studies had shown that cells could be cryopreserved without loss of plaque forming cell activity.

The PFC assay: The principle of the assay is schematically shown in Figure 6.2. Sheep red blood cells (SRBC) coated with staphylococcal protein A which had been mixed with anti-Ig antisera, PBMC, and complement are incubated in a Petri dish containing agar. Immunoglobulin released by plasma cells from within the population of PBMC combines with the antisera forming complexes which in turn are bound on the surface of the SRBC through the Fc portion of the antisera. Complement activation causes a zone of lysis of SRBC surrounding a central plasma cell which is visible to the naked eye as a plaque on the agar layer.

The PFC assay was performed as described by Gronowicz et al (578) and modified by Bird and Britton (579). SRBC (Flow Laboratories, UK) were washed six times in normal saline and then coated with protein A (Pharmacia, Uppsala, Sweden). The coating was carried out by incubating one volume of SRBC with one volume of protein A (0.5mg/ml) and ten volumes (2.5 x 10M) of chromic chloride (Analar, BDH Chemicals, Poole, England) for 45 minutes at 37°C with continuous rotation. After incubation the protein-A coated SRBC were washed three times with Hank's balanced salt solution (HBSS, Gibco,

Paisley, Scotland) and were used for up to 5 days after preparation. The spontaneous PFC assay was performed by adding the reagents listed below to 750ul of agar, which was supplemented with 2mg/ml of DEAE-dextran (Pharmacia, Uppsala, Sweden) and 1% polyethelene glycol 6000 (PEG 6000, Sigma, St Louis, USA) and was kept in 46°C water bath:

a) 25ul protein-A-coated SRBC

b) 100ul of PBMC suspension

c) 25ul (1:30) rabbit antisera specific for heavy chains of human IgG (Dako Immunoglobulins, Copenhagen, Denmark)

d) 25ul (1:4) of SRBC absorbed guinea pig complement (Sera-Lab England) diluted in HBSS.

After mixing in a vortex mixer, three 200ul drops were pipetted into a plastic 90mm Petri dish (Sterilin, Teddington, England) and a 24mm x 32mm coverslip immediately placed on top of each drop. The plates were then incubated for 8 hours at 37°C in 5% CO_2 in air in a humified incubator. Potential PFC were identified by the naked eye as holes in the agar (Figure 6.3), and the presence of a lymphoid cell at the centre of the haemolytic spots was confirmed by microscopy (Figure 6.4). The plaques were counted and the results were expressed as the mean PFC count (of triplicate determinations per million plated cells). To perform the PFC assay on lymphocyte cultures stimulated with PWM and SAC, 0.5 x 10^6 PBMC in one ml of medium (RPMI 1640 supplemented with 10% foetal calf serum, penicillin/streptomycin and L-glutamine) were cultured for six days in 12x75cm plastic tubes (Sterilin, Teddington, England) in the presence of optimal dilutions of PWM (1:100, Gibco, Paisley, Scotland) or SAC (1:1000) which was prepared following the procedure described by Forsgren et al (580). At the end of the culture period, the cells were washed twice in ice-cold HBSS, and then counted in a haemocytometer. Their viability was assessed and they were then assayed for IgG-secreting cells using the PFC assay as described above.

Statistical analysis

There was a large intersubject variation in the IgG-PFC counts and in order to show this, mean, standard deviation, standard error of mean, range and median values have been estimated. Because of this variation, graphic representation of data was carried out using a semi-logarithmic scale. The distribution of PFC counts was not normal and there was a substantial departure from linearity in the examination of the scattergrams, so the results have been analysed using non-parametric tests. Since the direction of the results could not be predicted, two-tailed significance values (p values) have been applied. Comparison of mean values between unpaired populations was done using the Mann-Whitney-Wilcoxon test which although based on ranks utilises most of the quantitative information inherent in the data. Comparison of mean values in paired samples was carried out using the Wilcoxon matched-pairs signed rank test which utilises both direction and magnitude in the ranking of differences. The correlation between variables was done using two non-parametric counterparts of the Pearson correlation coefficients, namely, the Spearman and the Kendall correlation coefficients. Also, these two tests were used in multiple regression analysis in the examination of inter-relations between more than two variables. Comparison of values between groups was also done using one-way analysis of variance. Although this is a parametric test, it is an extremely useful one, since the F ratio reflects significance of differences both between and within the groups, the latter expressing the degree of individual variation and functioning as a sort of 'standard error of the variance'.

6.3 Results

Table 6.1 and 6.2 show the IgG-PFC counts in the 2 control groups and in the transfusion group prior to and after each transfusion respectively. Judging from the range and standard deviations it is clear that there was a large intersubject variation in both spontaneous IgG-PFC counts and those after stimulation with SAC and PWM. This was more pronounced in the transfusion group particularly after the fifth unit of blood. As a result, the standard error after the tenth unit of blood was approximately

twofold compared to that prior to transfusion. Another point of caution comes from the comparison between mean and median values, the former being higher on all occasions. This suggests that the distribution of the values is positively skewed and mean counts are drawn in the direction of the extreme values.

6.3.1 Comparison of IgG production between controls and transfused patients

From Table 6.1 it is apparent that the IgG-PFC counts in the 2 control groups remained unchanged over a period of 3 months. In contrast, there were significant changes in the transfusion group with regard both to the spontaneous counts and those after PWM stimulation. Before describing the statistical details of these changes, it is worth looking at the comparison of IgG-PFC counts between healthy subjects and non-transfused uraemic patients (Figure 6.5). The difference between the two groups in the spontaneous and SAC-induced IgG-PFC counts was highly significant (mean $\pm$ SEM, 1426 $\pm$ 340 vs 300 $\pm$ 157, $p < 0.0001$, and 15480 $\pm$ 1480 vs 3743 $\pm$ 1220, $p < 0.0000$, respectively), whereas the difference in the PWM-induced counts was not significant (mean $\pm$ SEM, 28707 $\pm$ 2862 vs 26066 $\pm$ 5184). The latter finding suggests that B cell function in non-transfused uraemic patients is comparable to that of normal subjects. Thus, the defect in Ig secretion which was detected in the spontaneous and SAC-driven systems may be due to altered activity of other mediators involved in B cell activation which can be defined by these systems.

Figure 6.6 shows the comparison of IgG-PFC counts between the transfusion group prior to and after the 5th and 10th unit of blood and the uraemic controls at 0 and 3 months. The difference between the 2 groups in the spontaneous IgG-PFC assay at the start of the study (Figure 6.6a) is significant (mean $\pm$ SEM, 214 $\pm$ 24 vs 385 $\pm$ 46, $p < 0.02$) but this is presumed to be a chance finding arising from patient selection. However the difference after the 5th and 10th transfusion was highly significant and was due to an increase in the spontaneous IgG-PFC after transfusion (after 5 BT: mean $\pm$ SEM, 215 $\pm$ 25 vs 460 $\pm$ 44, $p < 0.0002$, after 10 BT: 215 $\pm$ 25 vs 655 $\pm$ 140, $p < 0.008$). Despite this increase, Tables 6.1 and 6.2 show that the

IgG-PFC counts in the transfused group after the 5th or 10th unit of blood, were still significantly lower than those of the normal controls at 3 months (after 5BT: mean ± SEM, 460 ± 44 vs 1579 ± 383, $p<0.001$, after 10 BT: 655 ± 140 vs 1579 ± 383, $p<0.006$). The difference in the IgG-PFC counts after stimulation with SAC between the transfused and non-transfused uraemic groups (Figure 6.6b) was not significant both prior to and after the 5th or 10th transfusion (mean ± SEM, 2935 ± 1414 vs 4551 ± 1032, 2916 ± 1286 vs 3721 ± 709, and 2916 ± 1286 vs 4973 ± 1789, respectively). The difference in SAC-IgG-PFC between uraemic and normal controls at 3 months was highly significant (mean ± SEM, 2916 ± 1286 vs 15642 ± 1545, $p<0.001$) as was the difference between transfused patients after the 5th or 10th unit of blood and the normal controls (3721 ± 709 vs 15642 ± 1545, $p<0.0002$, 4973 ± 1789 vs 15642 ± 1545, $p<0.0008$). The difference between the uraemic control and transfused groups in the IgG-PFC counts after stimulation with PWM (Figure 6.6c) was not significant prior to transfusion (mean ± SEM, 30968 ± 5851 vs 21145 ± 4517), but became significant after the 5th unit of blood due to a decrease in the IgG-PFC counts in the transfused group (mean ± SEM, 32080 ± 5669 vs 13433 ± 2796, $p < 0.002$). However, after the 10th unit of blood the counts had risen again so that the difference had disappeared (mean ± SEM, 32080 ± 5669 vs 21156 ± 9361). In the PWM-driven system IgG production in the transfused group after the 5th unit of blood was significantly depressed in comparison with the normal controls at 3 months (mean ± SEM, 13433 ± 2796 vs 28176 ± 2933, $p<0.004$). In contrast, the difference between uraemic and normal controls at 3 months was not significant (mean ± SEM, 32080 ± 5669 vs 28176 ± 2923). The difference in the mean PWM-IgG-PFC counts between the transfused group after the 10th unit of blood and the normal controls at 3 months did not reach statistical significance (mean ± SEM, 21156 ± 9361 vs 28176 ± 2923).

6.3.2 IgG production in the transfusion group

Despite the large intersubject variation in the IgG-PFC counts the kinetics of their appearance following transfusion was fairly reproducible resulting in significant changes. This is shown in Figure 6.7 which is the graphic version of Table 6.2. In the

spontaneous assay the mean IgG-PFC counts showed an overall progressive increase after each transfusion; the differences between background counts and those after the 1st, 2nd, 3rd, 5th, 8th, 9th and 10th unit of blood were significant (Figure 6.7a). Although the mean values progressively increased up to the 10th transfusion, this did not lead to greater statistical significance, firstly, because the numbers of patients decreased from 26 to 12 after the 5th transfusion, and secondly, because the difference in the median values was less pronounced (Table 6.2). Within the patients receiving 10 units of blood there were three with much higher values, and as a result of this the mean counts were drawn in that direction. In the SAC-driven system there was a mild degree of suppression of the mean IgG-PFC counts by the 5th transfusion and thereafter the mean counts were increased by the 10th unit of blood, but the difference compared to the background counts was not significant at any time (Figure 6.7b and Table 6.2). In contrast, after stimulation with PWM there was a progressive suppression of mean IgG-PFC counts which became significant after the second transfusion and was greater after the 5th unit of blood. However, further transfusion in the 12 patients receiving 10 units of blood had an enhancing effect on the mean IgG-PFC counts and the difference between post-6th to post-10th BT IgG-PFC counts compared to the background ones was not significant (Figure 6.7c and Table 6.2). This finding raises the question as to whether this rise in IgG-PFC counts after the 6th BT is a genuine result which may reflect feedback or contrasuppressor activity after the 6th transfusion. To examine this I looked into the individual changes in the PFC counts. Figures 6.8, 6.9, and 6.10 show the individual spontaneous, SAC-, and PWM-induced IgG-PFC counts respectively, before and after 5 or 10 units of blood. To coincide with the 0.05 level of statistical significance, changes were defined as significant when they fell outwith the range of the mean change plus 2 standard deviations of the 12 uraemic controls over a period of 3 months (Table 6.3). Thus, Table 6.4 shows the proportional changes in PFC counts after 5 and 10 units of blood in the transfused patients. Figure 6.11 illustrates these changes, and Figure 6.12 shows the proportion of patients with unchanged, enhanced or suppressed IgG production.

In the spontaneous system 12 of the 26 patients (46%) had a significant increase in the IgG-PFC counts after the 5th transfusion (mean ± SEM, 112 ± 26%, range, 31% to 339%), in 2 patients (8%) the counts were depressed, and in the remaining 12 patients (46%) there was no significant change (Figure 6.11a and Figure 6.12). In the 12 patients who received 10 units of blood the proportion of patients with increased (7/12), unchanged (4/14) and depressed counts (1/12) after the 5th transfusion remained similar after the 10th transfusion (8/12, 3/12, and 1/12 respectively) (Figure 6.11a); however, the overall mean ± SEM change in these 12 patients increased from 65 ± 30% (range, -68% to 339%) to 120 ± 55% (range, -59% to 621). Although this difference was not statistically significant (Figure 6.8), it suggests that further transfusion in these patients had a further enhancing effect on spontaneous IgG production mainly from the patients who had already responded with an increase after 5 units of blood.

In the SAC-driven system the IgG-PFC counts remained unchanged in most of the patients (19/26, 73%), in 2 patients there was a significant increase (8%), and in the remaining 5 patients (19%) IgG production was significantly depressed after 5 units of blood (mean ± SEM, 53 ± 4%, range 44% to 65%) (Figure 6.11b and 6.12). Further 5 units of blood had again an enhancing effect in IgG production in those patients who had already responded with an increase after 5 transfusions (patients no. 9, 14 and 20) (Figure 6.11). None of these changes after stimulation with SAC was significant.

In contrast, after stimulation with PWM 18 of the 26 patients (69%) had a significant suppression of IgG production after 5 units of blood (mean ± SEM, 54 ± 4%, range 22% to 80%), in 3 patients there was no change (12%), and in the remaining 5 patients (19%) there was a significant increase (mean ± SEM, 131 ± 51%, range, 41% to 332%) (Figure 6.11c and 6.12). In the 12 patients receiving a further 5 units of blood the proportion of patients with increased (3/12), unchanged (1/12) and suppressed counts (8/12) was identical after the 10th transfusion. However, in the 3 patients with increased counts, (number 9, 14 and 20) there was a further enhancement in IgG production (from 332% to 721%, from 84% to 233% and from 98% to 161%, respectively) and in the those with suppression of counts there was

further suppression from a mean $\pm$ SEM, 52 $\pm$ 6% (range, 22% to 80%) to 67 $\pm$ 6% (range, 32% to 87%) (Figure 6.11c). Although these differences were not signficant (Figure 6.10), the very large enhancement in IgG production in the 3 patients outbalanced the suppression observed in 8 of the 12 patients receiving 10 transfusions. Consequently, the difference in IgG-PFC counts after 10 units of blood compared to background values was not significant (Figure 6.10). Therefore, the progressive increase in IgG production after the 5th transfusion in the PWM-driven system described earlier in Figure 6.7c was due to the very high increase induced in 3 patients (No. 9, 14 and 20), rather than to an overall feedback or contrasuppressor activity. Interestingly, these 3 patients had a very marked increase in IgG production after transfusion both spontaneously and after sitmulation with SAC and PWM.

6.3.3 The effect of blood transfusion on IgG production in weak and strong DNCB responders.

The 26 patients in the transfusion group were divided according to their response to DNCB, as it was shown in Figure 6.1, in order to see whether weak and strong DNCB responders differ in IgG production following transfusion. Table 6.5 shows the mean IgG-PFC counts in the 12 uraemic controls divided into the 4 weak and 8 strong DNCB responders. There were no differences between the weak and strong responders over the period of 3 months. Tables 6.6, 6.7 and 6.8 show the mean spontaneous, SAC- and PWM-induced IgG-PFC counts respectively following transfusion in the 10 weak and 16 strong DNCB responders before and after each unit of blood. Statistical analysis of these Tables is shown in Figures 6.13, 6.14 and 6.15 respectively.

The difference in mean spontaneous IgG production between weak and strong DNCB responders was not significant following 1-5 units of blood (Figure 13a,b). However, from the 6th transfusion onwards in the patients receiving 10 units the weak DNCB responders had significantly higher mean IgG-PFC counts compared with the strong responders (Figure 6.13c). This difference was due to the fact that all 4 weak DNCB responders (No. 3, 4, 9 and 20) had a significant increase in spontaneous IgG production by the 10th transfusion compared to 4 of the 8 strong DNCB responders (Figure 6.8 and 6.11a).

Although the numbers are small, this finding suggests that after intensive exposure to alloantigens at least a proportion of patients, who have been classed as weak responders by the DNCB test, will respond with a high rate of IgG production. A similar finding in this group of patients with regards to production of cytotoxic antibodies following transfusion was described in the previous chapter.

After stimulation with SAC the difference between weak and strong DNCB responders in mean IgG-PFC counts following transfusion showed a similar pattern to that of the spontaneous counts (Figure 6.14). However, the statistical significance with respect to the difference between the 2 groups after the 6th-10th unit of blood was weak, and again was due to the very high IgG response in 2 of the 4 weak DNCB responders (No 9 and 20) (Figure 6.9 and 6.11b).

In the PWM-driven system the difference in the mean IgG-PFC counts between the 10 weak and 16 strong DNCB responders became significant after the 2nd, 3rd, 4th and 5th unit of blood ($p<0.03$, 0.03, 0.02 and 0.05 respectively) (Figure 6.15a). This difference was due to suppression of IgG production in the strong DNCB responders in whom the mean IgG-PFC after the 2nd, 3rd, 4th and 5th unit of blood were significantly lower compared to the background counts ($p<0.02$, on all occasions). In contrast, the decrease in the mean IgG-PFC counts in the weak DNCB responders did not reach statistical significance (Figure 6.15a). The difference between weak and strong responders was partly due to an increase in IgG production among the 4 weak responders who received 10 blood transfusions (Figure 6.15c). In this particular group by the 5th transfusion, weak DNCB responders had unchanged mean IgG-PFC compared to background counts, whereas by then strong DNCB responders had a significant suppression ($p<0.04$); after the 7th transfusion there was an increase in the mean IgG production in the former group, which in combination with the further suppression observed in the strong DNCB responders resulted in the significant difference between the 2 groups after the 10th unit of blood ($p<0.03$). However, in the other split group of the 14 patients receiving 5 units of blood (Figure 6.15b) weak DNCB responders had a significant suppression in mean IgG-PFC counts soon after the 2nd unit of blood ($p<0.03$), which

remained so until after the 5th transfusion; strong DNCB responders in this group had also a progressive suppression which did not reach statistical significance compared to pre-transfusion counts, and the difference in mean IgG production between the 2 groups was not significant at any time.

Furthermore, the level of the individual PWM-IgG-PFC counts between weak and strong DNCB responders prior to and after the 5th transfusion was indistinguishable (Figure 6.10). Also, the difference in the proportion of patients in the 2 groups with increased, unchanged or suppressed IgG production did not appear to be significant (Figure 6.11c). After the 5th unit of blood in the 10 weak DNCB responders, 2 patients had enhanced (20%), one patient unchanged (10%) and the remaining 7 patients (70%) suppressed IgG-PFC counts (mean ± SEM, 52±7%); in the 16 strong DNCB responders 3 patients had increased (19%), 2 patients unchanged (13%) and the remaining 11 patients (69%) suppressed IgG-PFC counts (mean ± SEM, 55±4%). Interestingly however, after the 10th transfusion there appeared to be a further enhancement or suppression of IgG production in both weak and strong DNCB responders who already had increased or suppressed IgG-PFC counts respectively by the 5th transfusion. In the former patients (No. 9 and 20) IgG production increased from 332% and 98% after the 5th unit to 721% and 161% after the 10th unit respectively, and in the latter patient (No. 14) from 84% to 233% (Figure 6.11c). In the patients whose IgG-PFC counts showed suppression, in the weak DNCB responders (No. 3 and 4) IgG-PFC counts were further suppressed from 70% and 54% after the 5th transfusion to 87% and 61% after the 10th unit of blood respectively. In the 6 strong DNCB responders (No. 5, 6, 8, 10, 17 and 18) the mean ± SEM suppression after the 5th transfusion was further suppressed from 49±7% to 65±8% after the 10th unit of blood. Although these individual differences are not statistically significant, they may be of biological significance in the sense of individual variation in the BT-induced immunosuppression and the amount of blood required to achieve this suppression. Yet, in all of these, the DNCB skin test did not prove to be a satisfactory means of predicting the

spontaneous, SAC- and PWM- induced IgG response following third party transfusion. It provided, however, a very useful measurement in assessing the complexity of these responses.

6.3.4 Inter-relations between IgG-PFC counts following transfusion, cytotoxic antibodies, rejection episodes and transplant outcome.

So far, it has become evident that there is a different pattern of spontaneous and PWM- induced IgG production following transfusion. It has been shown that spontaneous IgG-PFC counts increased significantly in about half of the patients after 5 units of blood and this increase became more pronounced with a further 5 transfusions. On the other hand, more than two thirds of the patients showed a significant suppression of IgG production in the PWM-driven system, whereas in a similar proportion of patients after stimulation with SAC there was no significant change. It was also shown that few patients had a high rate of IgG production both spontaneously and after stimulation with SAC or PWM following blood transfusion. This raised the question whether the kinetics of IgG-PFC counts in response to blood transfusion in the spontaneous, PWM-, SAC- driven system correlated with each other. In other words did the patients we studied respond with enhancement, suppression or no change in IgG production in all three systems? And if so, how does this response correlate with other variables of clinical importance, such as, cytotoxic antibodies, rejection and transplant outcome?

Table 6.9 shows that the correlation between spontaneous and PWM-IgG-PFC counts was not significant at any time, namely, neither before nor after each of the 1-10 transfusions. In contrast IgG production in the PWM- and SAC-driven system showed a good correlation, and this was also the case plotting SAC-induced against spontaneous counts. Figure 6.16 illustrates the absence of any linearity between spontaneous and PWM-IgG-PFC prior to and after the fifth transfusion, whereas after the tenth unit of blood the correlation was linear because of a simultaneous increase of counts in 2 patients (No 9, 20) against both axes; this correlation was significant using the parametric Pearson test $r=0.737$, $p<0.006$), but the non-parametric Spearman test failed to reach significance because

of the small numbers. Although PWM and SAC, as polyclonal B cell activators, differ in their dependence on T cells, IgG production in these two systems showed a positive correlation before and more convincingly, after transfusion (Figure 6.17). There was also a positive correlation between spontaneous and SAC- induced IgG production following blood transfusion which again was more pronounced after the tenth transfusion (Figure 6.18). This finding suggests that there is a disassociation between spontaneous and PWM-induced IgG production, which tends to disappear after more than 5 units of blood and this seemed to be due to a clearer distinction between patients with enhanced, suppressed or unchanged PFC counts following that amount of transfusion.

Fifteen of the 26 patients studied in this chapter underwent transplantation following blood transfusion (Table 6.10). Nine of the 15 patients had received 5 units and the remaining 6 patients 10 units of blood. The mean waiting time from the last elective transfusion to transplantation was 4.9 months (range, 1-15 months). Two patients were treated with azathioprine and prednisolone, one patient had azathioprine followed by cyclosporin and the remaining 13 patients received cyclosporin and prednisolone. Nine patients did not have any rejection episodes while of the other 6 patients, 4 had one episode, one had 2 episodes and the final patient had 3 episodes. Thirteen of the 15 patients had a functioning graft at the end of the study, one patient's kidney underwent irreversible rejection at 7 months, and one patient died with a functioning graft 16 months after transplantation.

Tables 6.11 and 6.12 show the relation between rejection episodes, IgG-PFC counts and cytotoxic antibodies after the fifth and tenth transfusion respectively, using both parametric and non-parametric correlation coefficients judged from the appearance of linearity in the scattergrams. The correlation between spontaneous SAC- and PWM-IgG-PFC counts has already been discussed (Table 6.9), and it is obvious that using Pearson's correlation coefficients in Table 6.11 the statistical significance is stronger compared to that of Spearman's in Table 6.9. IgG production in the PWM- and SAC-driven system does not correlate with either PRA or rejection episodes. Spontaneous IgG-PFC counts after the fifth unit of blood

correlated with PRA at that time (Pearson's r=0.497, $p<0.01$), but this was not the case after the tenth transfusion (Table 6.12); however, the significance of the correlation after 5 units of blood was based only on three positive values of PRA (Figure 6.19a) and application of the non-parametric Spearman test showed that the correlation with this test was not significant on either occasion. Similar limitations applied in correlating spontaneous IgG-PFC counts with the rejection episodes which proved to be not significant after five units of blood and showed a weak correlation after the tenth transfusion ($p<0.05$). This again was not significant using non-parametric tests (Figure 6.19b). The correlation between rejection episodes and PRA was not significant (Figure 6.19c). Finally, the transplant outcome has not been included in this multiple regression analysis, because there was only one patient with irreversible rejection.

6.3.5 Summary of results

Polyclonal B cell activation studies, which were carried out to assess the response to elective transfusion, in previously non-transfused uraemic patients, showed a large intersubject variation. Despite this, uraemic patients prior to transfusion had significantly lower spontaneous and SAC- induced counts of IgG secreting cells compared to normal controls, whereas the difference in PWM- induced IgG-PFC counts was not significant. After blood transfusion, IgG production in the PWM-driven system was suppressed in more than two thirds of the patients compared to that in normal controls and non-transfused uraemic controls. On the other hand, spontaneous IgG-PFC showed a significant increase in response to transfusion, whereas SAC-IgG-PFC remained overall unchanged. Within the main groups there were subgroups with enhanced, unchanged or suppressed IgG production, both spontaneously and after stimulation with SAC or PWM. The DNCB skin test did not separate patients with a high or low rate of IgG production in response to transfusion. Although not conclusive, there was some indication that blood transfusion-induced immunosuppression may be more easily produced in strong DNCB responder whereas weak responders were capable of very high IgG production, particularly after more than 5 units of blood.

Whatever effect blood transfusion had in the individual patients (enhancement, suppression or unresponsiveness) it was demonstrable by the fifth transfusion. A further 5 units of blood did not have a significant effect in the same direction, ie enhancement or suppression, and the overall proportion of patients with these responses did not change. There was a clear disassociation between spontaneous and PWM- induced IgG production before and after one to 5 units of blood and the former showed a weak correlation with PRA and rejection episodes. However because of the small numbers, a correlation between PRA and rejection episodes was not established.

6.4. Conclusions

1) Spontaneous and SAC- induced IgG production in previously non-transfused uraemic patients was significantly lower compared with normal subjects. In contrast, the counts of IgG secreting cells after stimulation with PWM was normal.

2) Blood transfusion had a suppressive effect on IgG production in the PWM-, but not in the SAC- driven system, resulting in significantly lower IgG-PFC counts compared to those of uraemic and normal controls.

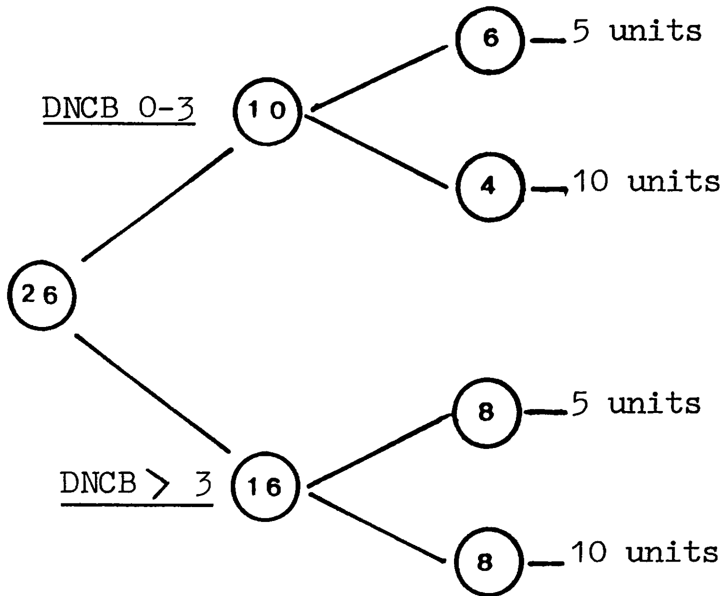
3) Spontaneous IgG production following transfusion was increased and there was some evidence, though inconclusive, that this may correlate with the development of cytotoxic antibodies and subsequent rejection episodes.

4) Subgroups of patients with a high or low response of IgG production, both spontaneously and after stimulation with SAC and PWM were observed, but the DNCB skin test failed to identify these patients.

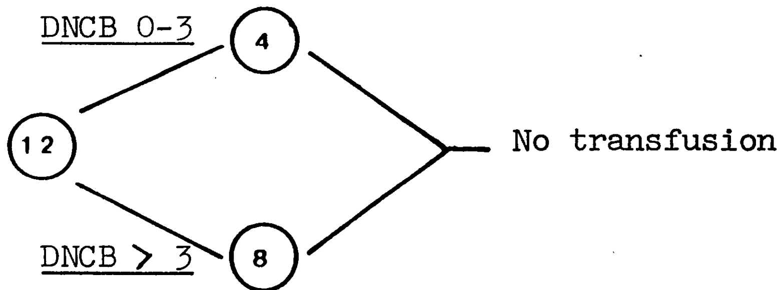
5) The blood transfusion-induced suppression of IgG-PFC counts detected in the PWM- driven system appeared to be at least equally induced in weak and strong DNCB responders. Some of the former patients were capable of responding with a very high rate of IgG production, particularly after more than 5 transfusions.

6) More than 5 units of packed cells had a further, though non-significant enhancing or suppressive effect in spontaneous, SAC- and PWM-induced IgG production, but the overall proportion of patients with this varying response did not change.

I. Transfused



II. Uraemic controls



III. Normal controls

Figure 6.1. Breakdown of the subjects according to their response to the DNCB skin test and the number of blood transfusions; 0-3 = weak responders, > 3 = strong responders.

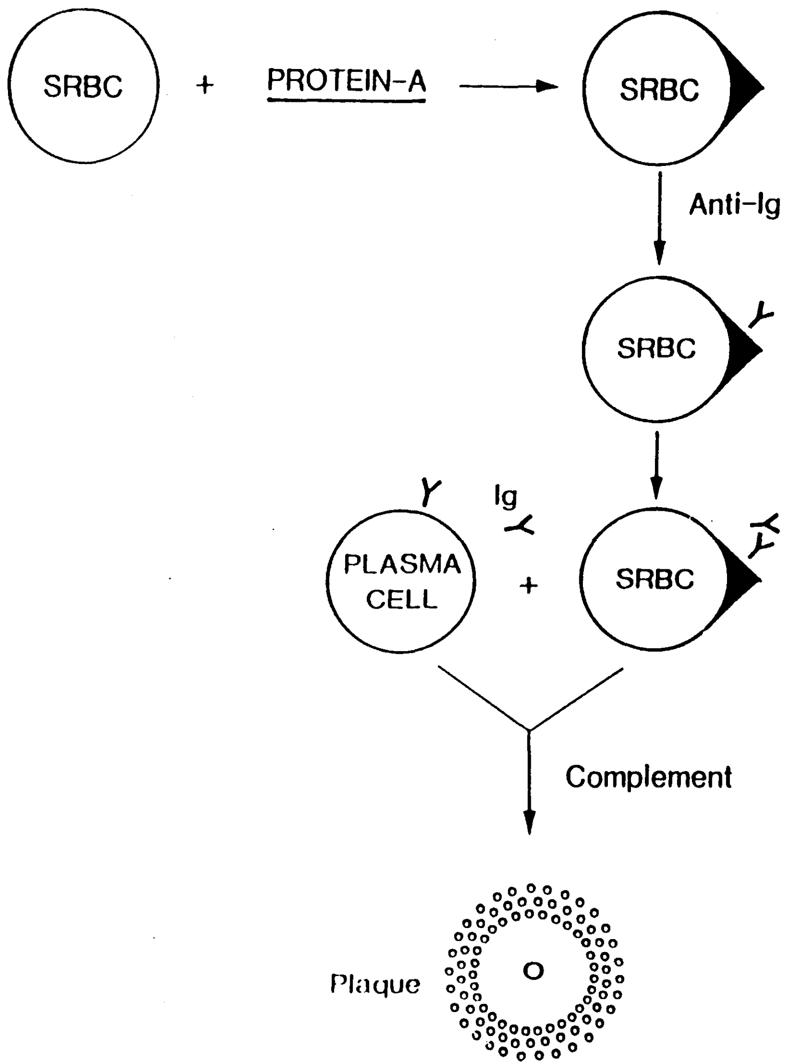


Figure 6.2. Schematic representation of the reverse haemolytic protein-A plaque assay.

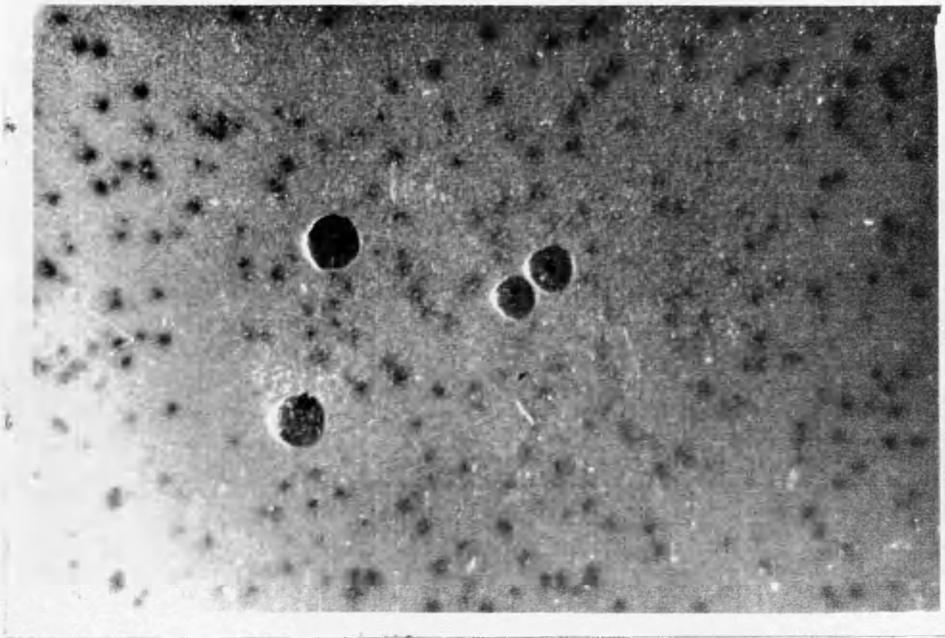


Figure 6.3. Macroscopic appearance of haemolytic plaques in a Petri dish.

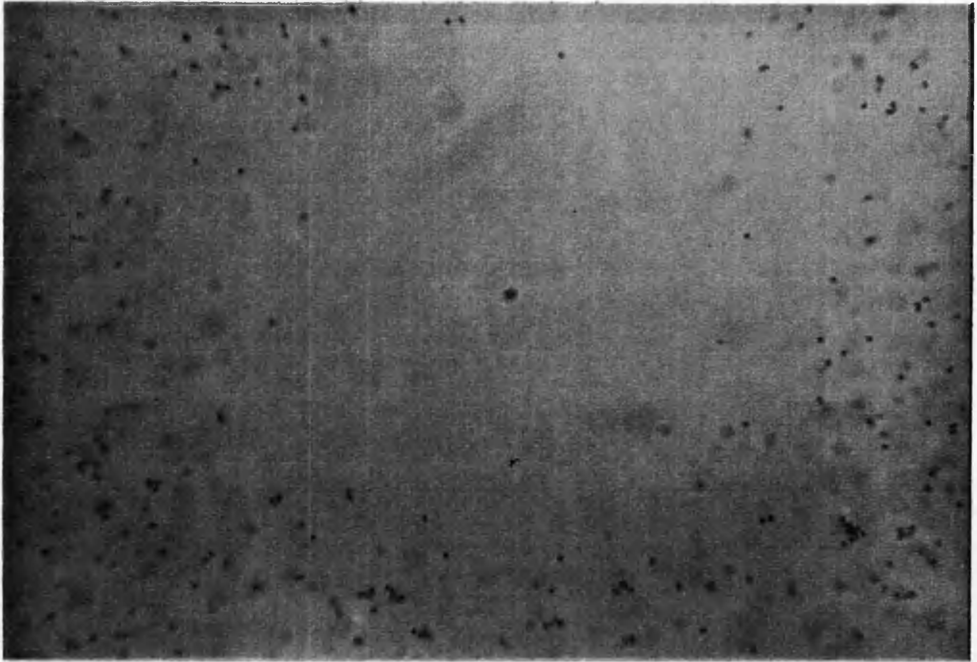


Figure 6.4. Microscopic appearance of a haemolytic plaque with a central lymphoid cell and the zone of SRBC lysis surrounding it.

TABLE 6.1. SPONTANEOUS, SAC-, AND PWM-INDUCED IgG-PFC COUNTS IN THE TWO CONTROL GROUPS

	IgG-PFC counts/10^6 cells			
	Uraemic controls (n=12)		Normal controls (n=12)	
	At 0	At 3 months	At 0	At 3 months
Spontaneous:				
Mean ± SD	214±82	215±87	1426±1178	1579±1326
SEM	24	25	340	383
Median	208	194	943	963
Range	84-353	95-386	416-3654	462-4532
SAC stimulation:				
Mean ± SD	2935±4897	2916±4454	15480±5128	15642±5353
SEM	1414	1286	1480	1545
Median	1370	1421	13540	13190
Range	1120-18432	956-19958	11892-28705	10942-27200
PWM stimulation:				
Mean ± SD	30968±20269	32080±19638	28707±9913	28176±10125
SEM	5851	5669	2862	2923
Median	25254	24909	28767	27126
Range	9750-72500	12410-67502	11498-43504	13673-45370

TABLE 6.2 SPONTANEOUS, SAC-, AND PWM-INDUCED IgG-PFC COUNTS IN THE TRANSFUSED PATIENTS PRIOR TO AND AFTER EACH TRANSFUSION OF 5-10 UNITS OF PACKED CELLS

	IgG-PFC counts/10^6 cells					
No. of transfusion	0 n=26	1 n=26	2 n=26	3 n=26	4 n=26	5 n=26
Spontaneous:						
Mean ± SD	385±232	422±200	445±186	455±177	414±196	460±222
SEM	46	39	37	35	38	44
Median	287	335	428	446	363	428
Range	105-975	185-844	202-925	196-833	178-925	192-1110
SAC stimulation:						
Mean ± SD	4551±5260	4273±4633	3971±3803	3929±3673	3783±3701	3721±3617
SEM	1032	909	746	720	726	709
Median	2434	2463	2405	2539	2377	2472
Range	452-20278	410-18730	560-13132	440-11530	395-12005	460-13709
PWM stimulation:						
Mean ± SD	21145±23031	19721±22251	17730±20013	16810±20066	15932±17439	13433±14258
SEM	4517	4364	3925	3935	3420	2796
Median	11939	10757	8651	7876	6873	6906
Range	2081-95470	2360-94350	2490-87125	2219-76688	2312-66250	1541+60003

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	IgG-PFC counts/10^6 cells				
No. of transfusions	**6** n=12	**7** n=12	**8** n=12	**9** n=12	**10** n=12
Spontaneous:					
Mean ± SD	512±222	522±253	638±329	642±442	655±485
SEM	64	73	95	128	140
Median	463	527	627	479	421
Range	197-875	185-954	200-1315	215-1747	190-1572
SAC stimulation:					
Mean ± SD	4569±5272	4860±5862	5071±6483	5033±6333	4973±6197
SEM	1522	1692	1872	1828	1789
Median	3054	2954	3093	3025	2982
Range	510-17986	540-19405	350-22351	415-21775	430-21177
PWM Stimulation:					
Mean ± SD	17406±21694	17746±23573	21389±38806	21012±34177	21156±32429
SEM	6262	6805	11202	9866	9361
Median	8467	8228	8340	9030	9211
Range	2154-78625	1953-82532	1810-138749	1954-120544	1650-113940

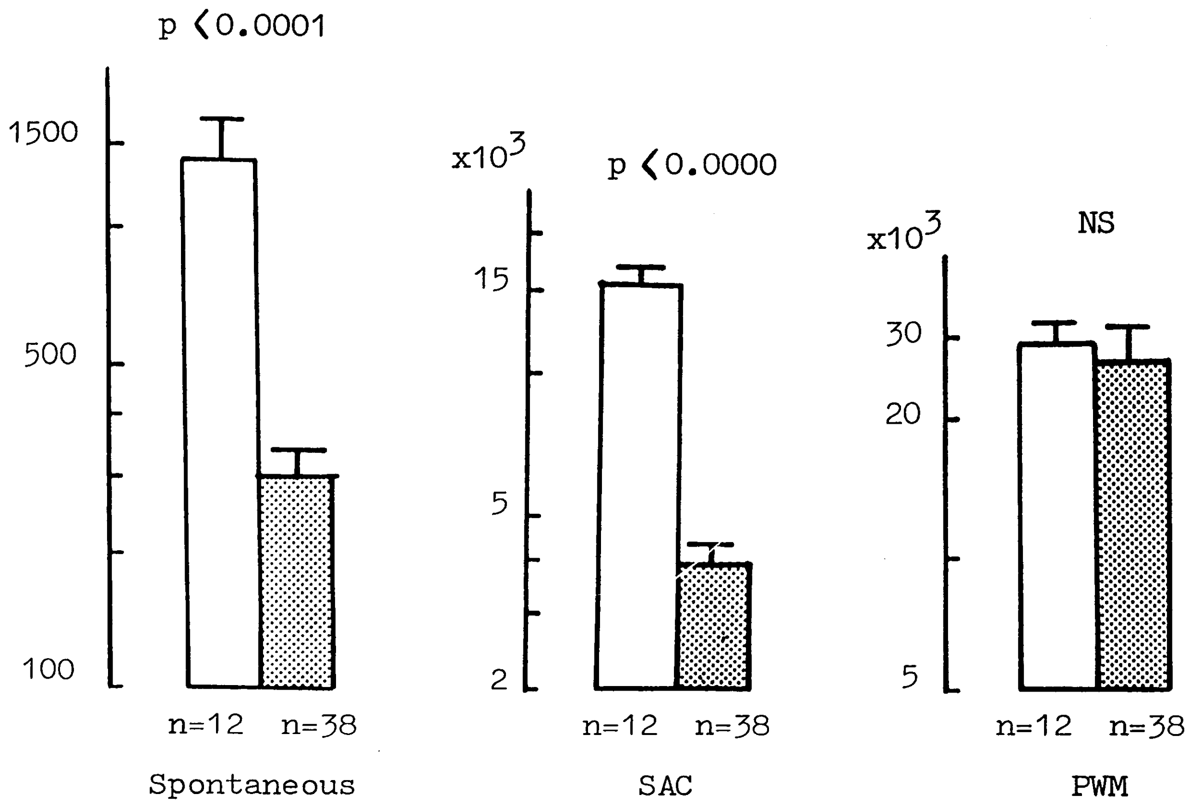


Figure 6.5. Comparison of IgG-PFC counts (mean ± SEM) between normal controls and previously non-transfused uraemic patients (pre-transfusion counts of the patients in the transfusion group and counts at 0 of the uraemic controls); open bars = control, shaded bars = uraemic patients.

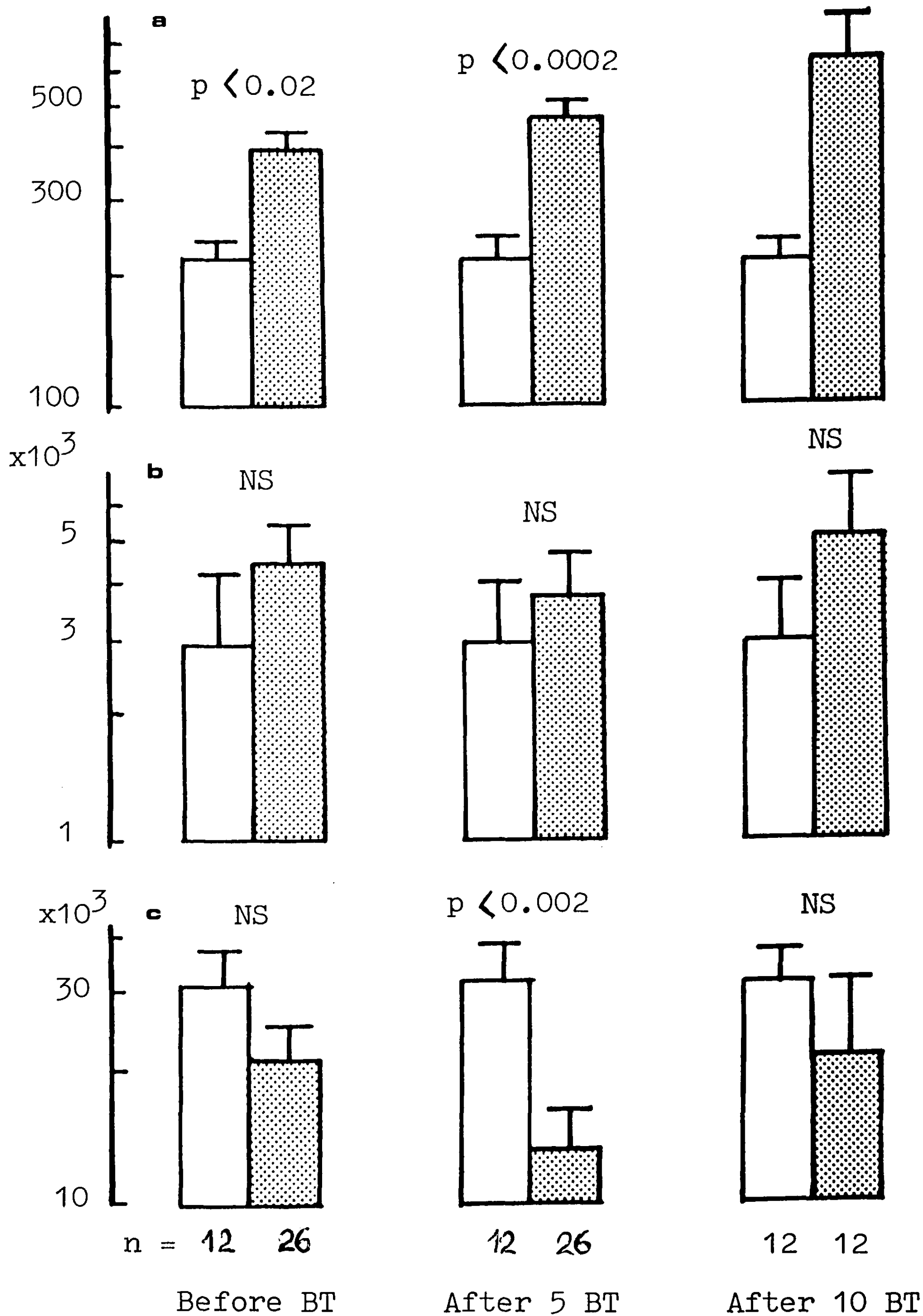


Figure 6.6. Comparison of IgG-PFC counts (mean ± SEM) between the transfusion group prior to and after the 5th or 10th unit of blood and the uraemic controls at 0 and at 3 months; a = spontaneous, b = SAC stimulation, c = PWM stimulation; open bars = control, shaded bars = transfusion group.

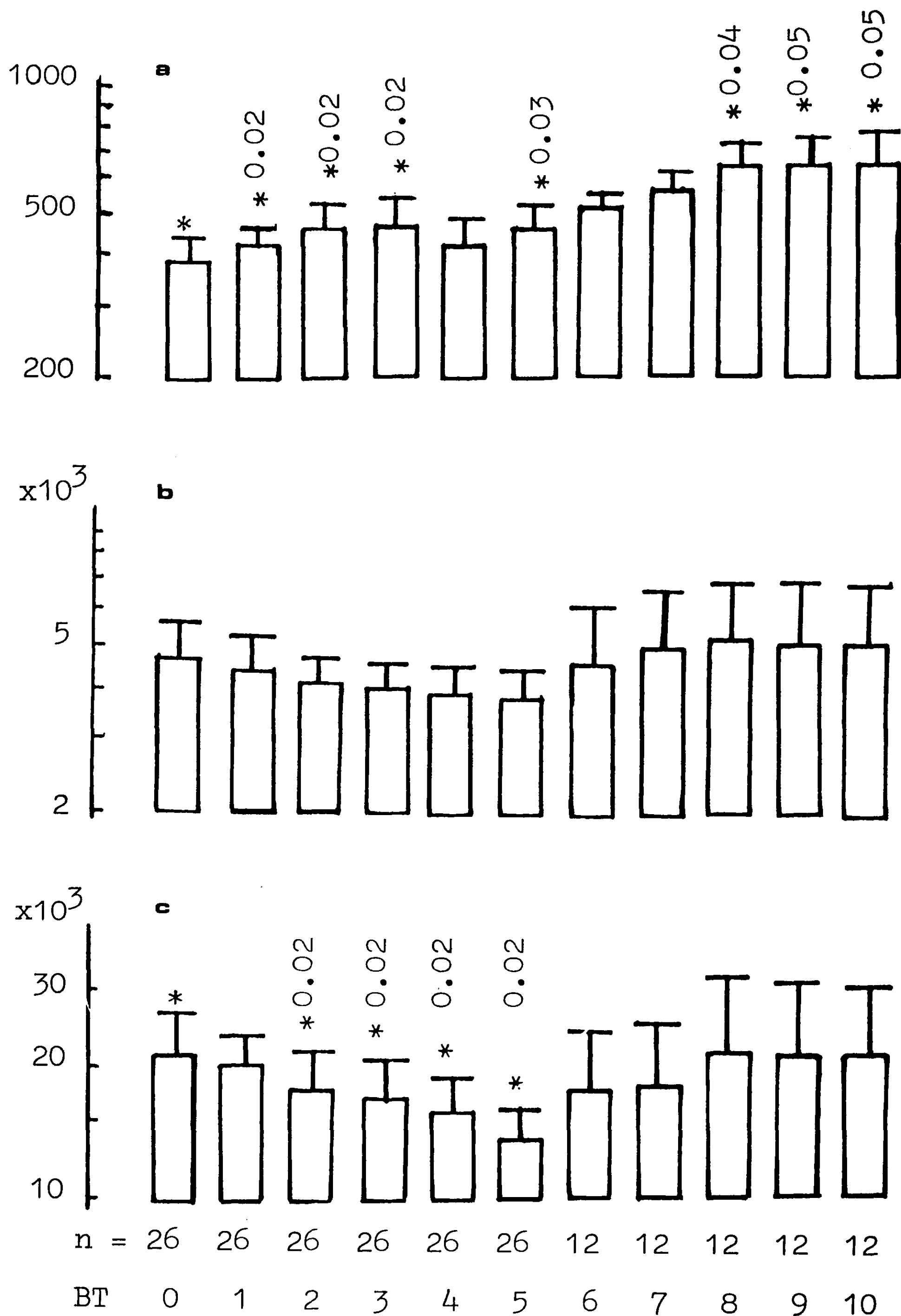


Figure 6.7. IgG-PFC counts (mean ± SEM) in the transfused patients prior to and after each of 5-10 units of blood; a = spontaneous, b = SAC stimulation, c = PWM stimulation; p values against background counts.

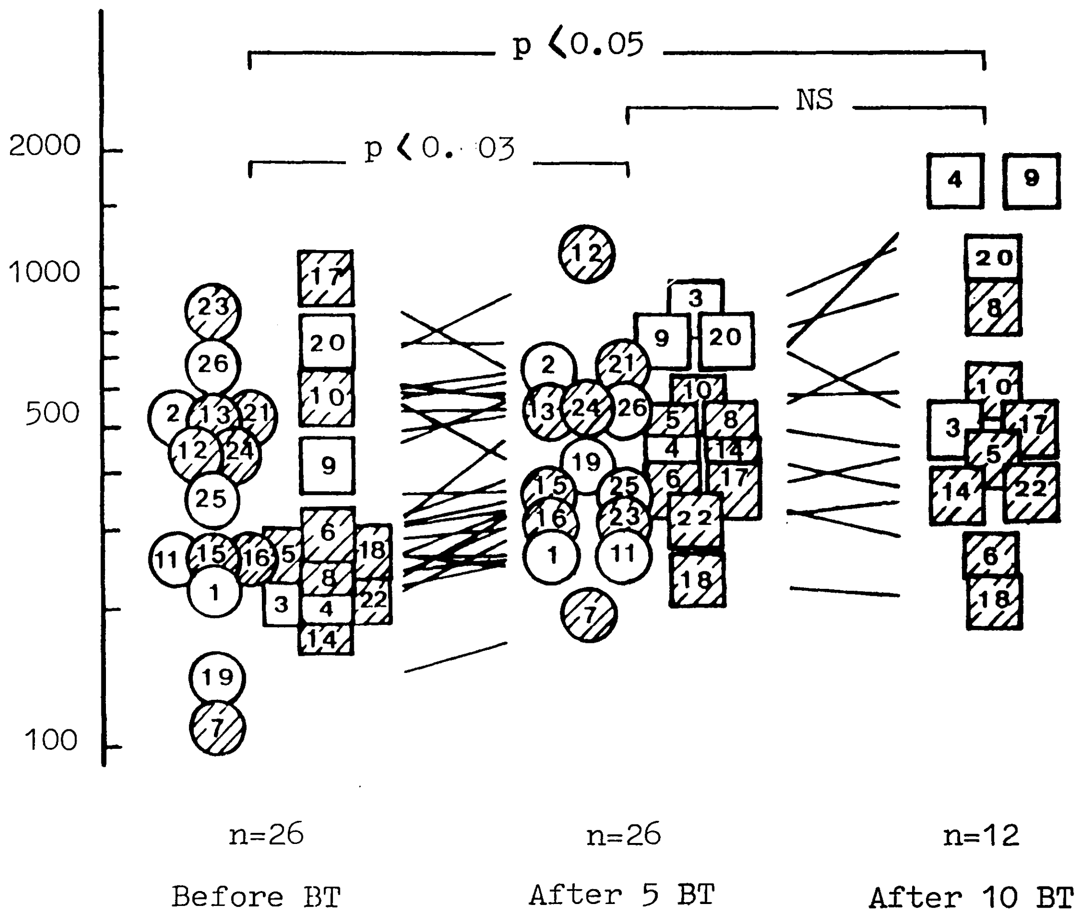


Figure 6.8. Spontaneous IgG-PFC counts in the transfusion group before and after the 5th or 10th unit of blood; ○= patients receiving 5BT, □ = patients receiving 10BT; open symbols = weak DNCB responders, shaded symbols = strong DNCB responders; numbers inside symbols correspond to numbers of patients in Table 6.4.

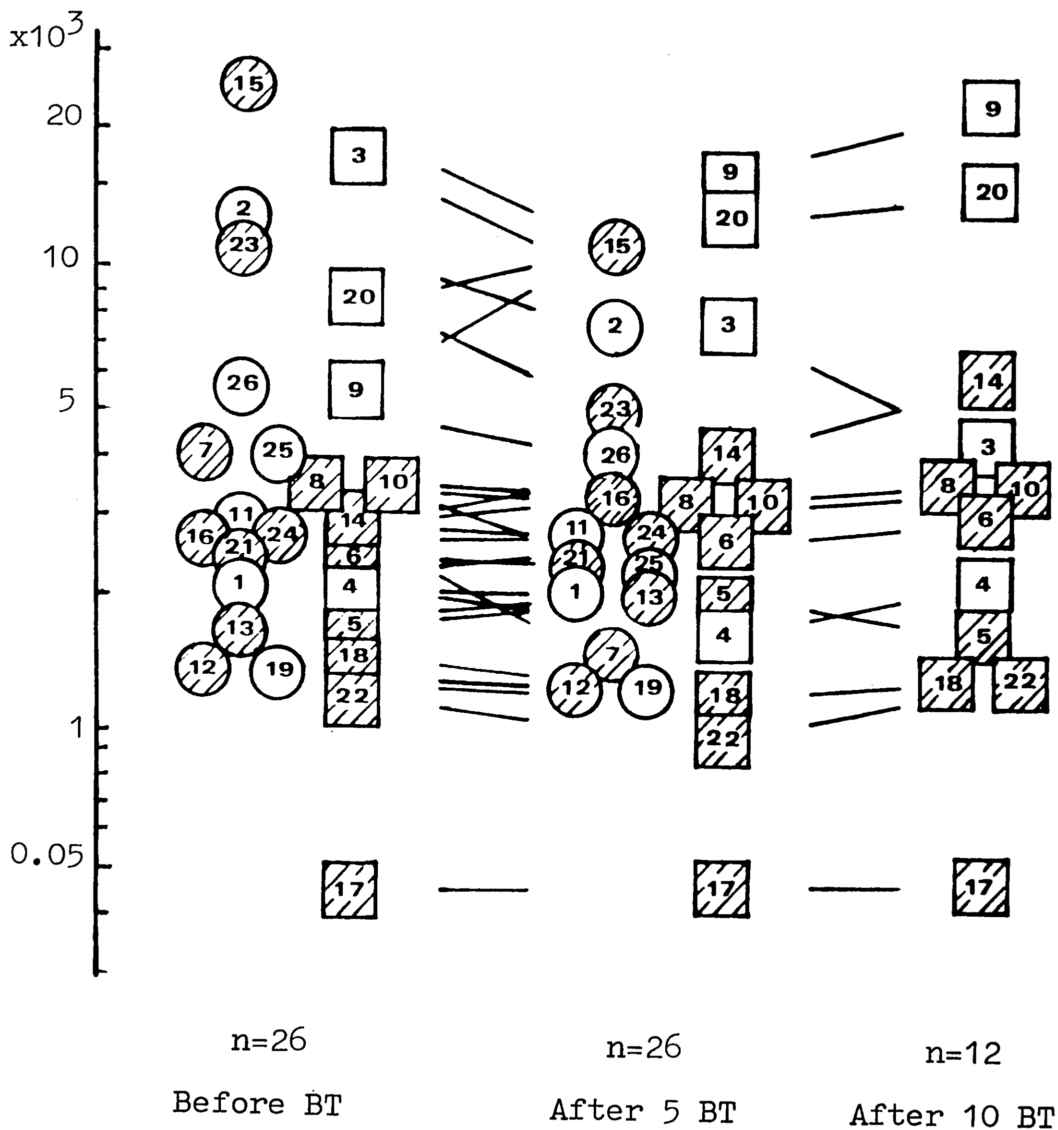


Figure 6.9. IgG-PFC counts after stimulation with SAC in the transfusion group before and after the 5th or 10th unit of blood; ○ = patients receiving 5BT, □ = patients receiving 10BT; open symbols = weak DNCB responders, shaded symbols = strong DNCB responders; numbers inside symbols correspond to numbers of patients in Table 6.4.

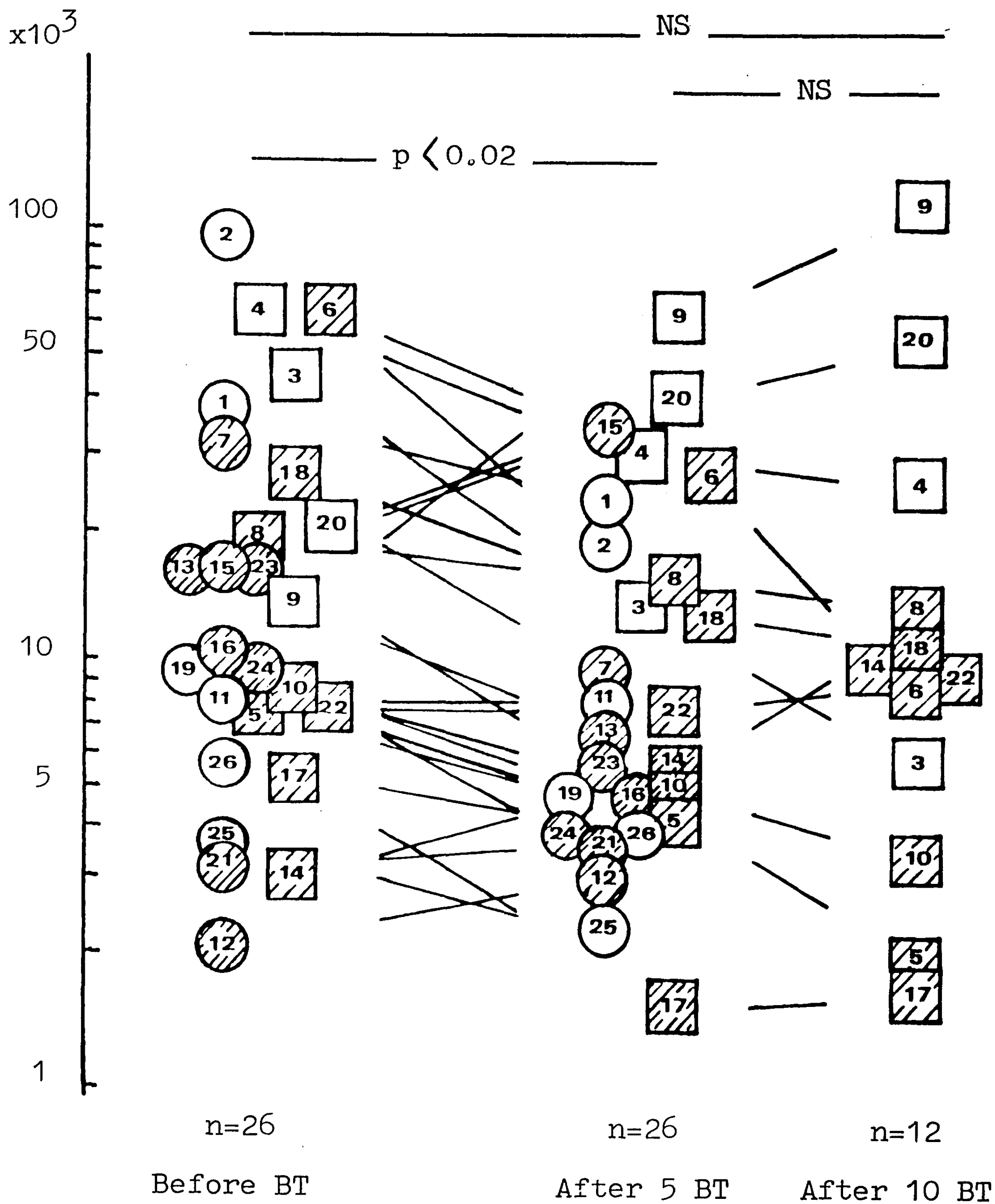


Figure 6.10. IgG-PFC counts after PWM stimulation in the transfusion group before and after the 5th or 10th unit of blood; ◯ = patients receiving 5BT, □ = patients receiving 10BT; open symbols = weak DNCB responders, shaded symbols = strong DNCB responders; numbers inside symbols correspond to numbers of patients in Table 6.4.

TABLE 6.3. PERCENT CHANGE OF IgG PFC COUNTS IN THE 12 URAEMIC CONTROLS OVER A PERIOD OF 3 MONTHS

	% change of IgG-PFC		
Controls	Spontaneous	SAC	PWM
1. SW	-24	-8	-2
2. MS	+3	-5	+3
3. MMcG	+0.5	+33	+4
4. JB	-4	+13	+6
5. CMcD	-2	+16	+7
6. AK	+11	+4	+7
7. WY	+20	+34	+7
8. JH	+17	-12	+8
9. WB	+16	-1	+9
10. DH	+13	-3	+15
11. CB	-15	+5	+17
12. JMcI	-10	-15	+27
Mean ± SD	+2 ± 14	+5 ± 16	+9 ± 8

TABLE 6.4. PERCENT CHANGE OF IgG-PFC COUNTS FROM BACKGROUND VALUES AFTER 5 OR 10 UNITS OF BLOOD IN THE TRANSFUSED PATIENTS

	% Change of IgG-PFC					
	Spontaneous		SAC		PWM	
Patients	Post 5BT	Post 10BT	Post 5BT	Post 10BT	Post 5BT	Post 10BT
1. JB	+15		-9		-38	
2. TS	+13		-44		-80	
3. SW	+339	+135	-57	-78	-70	-87
4. MM	+102	+621	-24	-3	-54	-61
5. WH	+92	+49	+13	-2	-45	-76
6. AK	+4	-26	+5	+7	-58	-87
7. KM	+45		-65		-71	
8. JP	+83	+272	-4	-7	-22	-32
9. JMcG	+88	+286	+151	+288	+332	+721
10. JD	-11	-5	-8	-3	-41	-61
11. WY	-13		+11		-0.3	
12. PM	+169		-6		+41	
13. TC	+6		+17		-61	
14. JL	+127	+93	+33	+87	+84	+233
15. LA	+34		-46		+100	
16. JH	+7		+14		-53	
17. BJ	-68	-59	-2	-5	-70	-68
18. MS	-10	-21	-8	-3	-56	-63
19. WG	+197		-5		-53	
20. CS	+4	+37	+42	+62	+98	+161
21. RF	+22		+7		+3	
22. PF	+31	+58	+15	+13	+2	+20
23. RH	-70		-55		-67	
24. RB	+38		+5		-62	
25. JG	-10		-3		-40	
26. LMcL	-23		-19		-30	

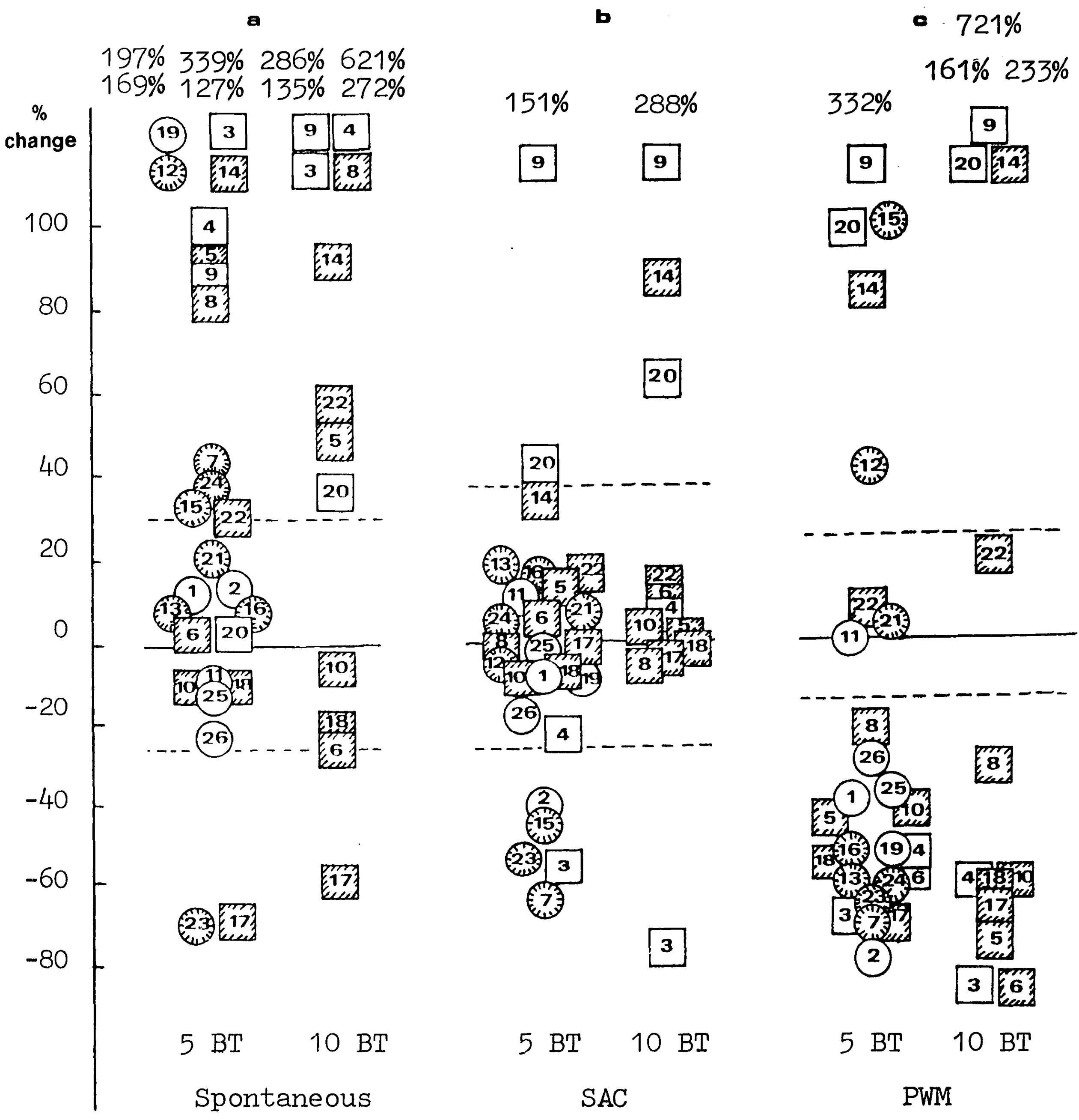


Figure 6.11. Percent change of IgG-PFC counts after 5 or 10 units of blood in the transfusion group; area between lines represent the mean ± 2SD range of change in the uraemic controls; ◯ = patients receiving 5 BT, □ = patients receiving 10 BT, open symbols = weak DNCB responders, shaded symbols = strong DNCB responders.

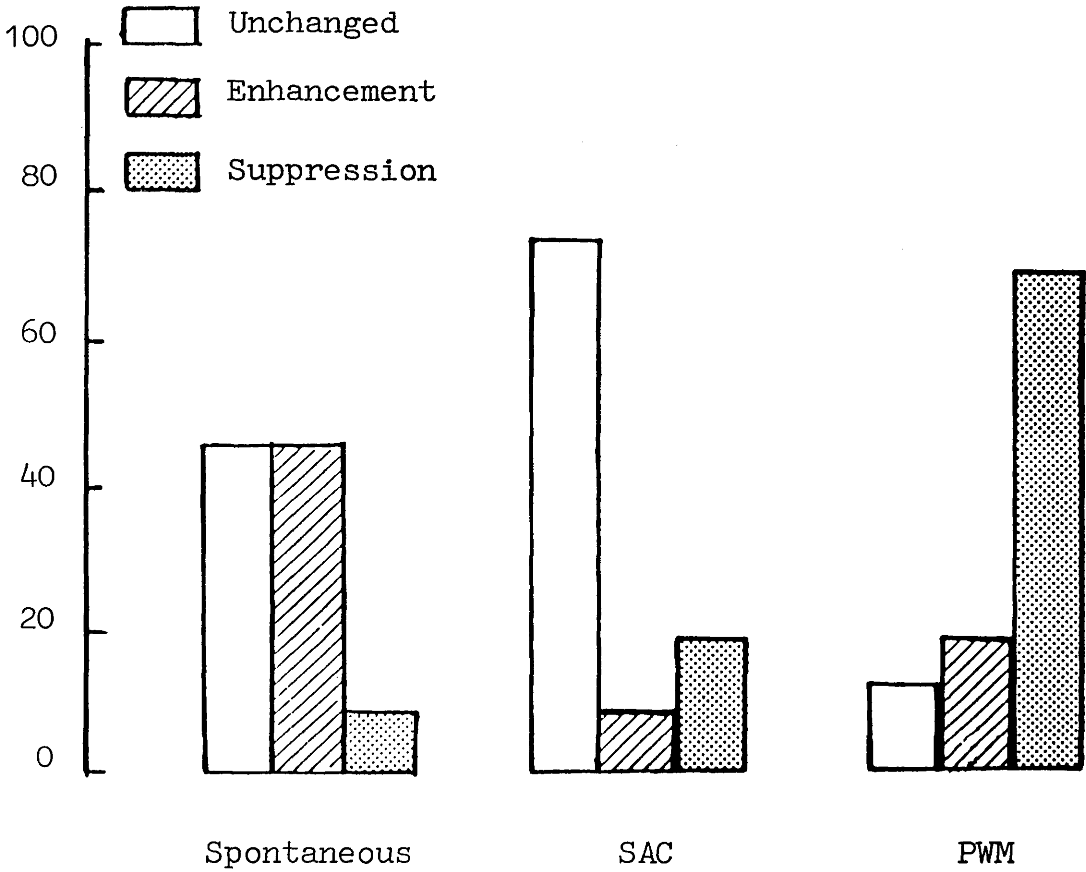


Figure 6.12. Proportion of patients with unchanged, enhanced or suppressed IgG production following 5 units of transfusion both spontaneously and after stimulation with SAC and PWM.

TABLE 6.5 IgG-PFC COUNTS IN THE WEAK AND STRONG DNCB RESPONDERS OF THE URAEMIC CONTROLS

IgG-PFC (mean ± SD counts/10^6 cells)

	Weak responders (n=4)		Strong responders (n=8)	
	At 0	At 3 month	At 0	At 3 month
Spontaneous	224±41	229±59	209±99	208±101
SAC-stimulation	1801±596	2061±519	3501±6035	3343±5516
PWM-stimulation	34130±21432	36332±23111	29414±20982	29954±19015

TABLE 6.6 SPONTANEOUS IgG-PFC COUNTS IN THE WEAK AND STRONG DNCB RESPONDERS PRIOR TO AND AFTER EACH TRANSFUSION OF 5-10 UNITS OF PACKED CELLS

No. of BT	Spontaneous IgG-PFC (mean ± SD counts/10^6 cells)										
	0	1	2	3	4	5	6	7	8	9	10
Weak responders:											
All (n=10)	365±205	456±241	482±232	501±200	481±271	505±221					
5 BT (n=6)	352±194	386±239	384±178	442±186	316±165	380±150					
10 BT (n=4)	384±250	563±231	629±245	588±213	728±198	694±173	735±216	774±131	962±253	1001±564	1138±530
Strong responders:											
All (n=16)	397±253	400±174	422±156	426±161	372±122	431±225					
5 BT (n=8)	424±249	464±175	485±156	436±142	393±147	493±299					
10 BT (n=8)	371±272	367±159	360±137	416±188	351±98	370±103	400±120	441±225	476±229	462±247	413±221

TABLE 6.7. IgG-PFC COUNTS AFTER STIMULATION WITH SAC IN THE WEAK AND STRONG DNCB RESPONDERS PRIOR TO AND AFTER EACH TRANSFUSION OF 5-10 UNITS OF PACKED CELLS

	SAC-induced IgG-PFC (mean ± SD counts/10^6 cells)										
No. of BT	0	1	2	3	4	5	6	7	8	9	10
Weak responders:											
All (n=10)	5632±5790	5370±4783	5588±4927	5502±4761	5211±4837	5131±4788					
5 BT(n=6)	3746±4799	3702±4500	3757±4628	3376±4038	2865±2458	2703±2458					
10 BT(n=4)	8461±6686	7873±4576	8335±4512	8692±4269	8730±4940	8772±5407	8982±7703	9893±8461	10427±9549	1035±9226	10125±9056
Strong responders:											
All (n=16)	3876±4973	3588±4554	2961±2591	2946±2491	2891±2566	2840±2429					
5 BT(n=8)	5650±6675	5156±6155	3805±3353	3713±3236	3654±3390	3500±3184					
10 BT(n=8)	2101±1115	2020±970	2117±1230	2180±1216	2128±1137	2181±1230	2363±1248	2344±1268	2393±1543	2374±1516	2397±1564

TABLE 6.8. IgG-PFC COUNTS AFTER STIMULATION WITH PWM IN THE WEAK AND STRONG DNCB RESPONDERS PRIOR TO AND AFTER EACH TRANSFUSION OF 5-10 UNITS PACKED CELLS

No. of BT	PWM-induced IgG-PFC (mean ± SD counts/10^6 cells										
	0	1	2	3	4	5	6	7	8	9	10
Weak responders:											
All (n=10)	30188±30197	28668±28980	28452±26672	27268±27175	25699±22138	20349±18602					
5 BT (n=6)	26839±36143	26280±35501	23357±33152	19767±28850	18141±24833	10262±9191					
10 BT (n=4)	35212±22413	32249±19761	36094±13140	38519±23389	37038±12521	35480±19713	34787±32164	35836±35614	49867±62114	48721±51989	49218±4730?
Strong responders:											
All (n=16)	15494±15757	14130±15315	11029±10778	10274±10464	9828±10462	9111±8909					
5 BT (n=8)	13090±9575	10547±4603	10157±8929	9154±9485	9063±10617	8440±9941					
10 BT (n=8)	18897±20668	17712±21262	11901±12941	11393±11909	10593±10976	9782±8381	8716±6105	8701±7010	7150±4222	7158±4370	7125±4317

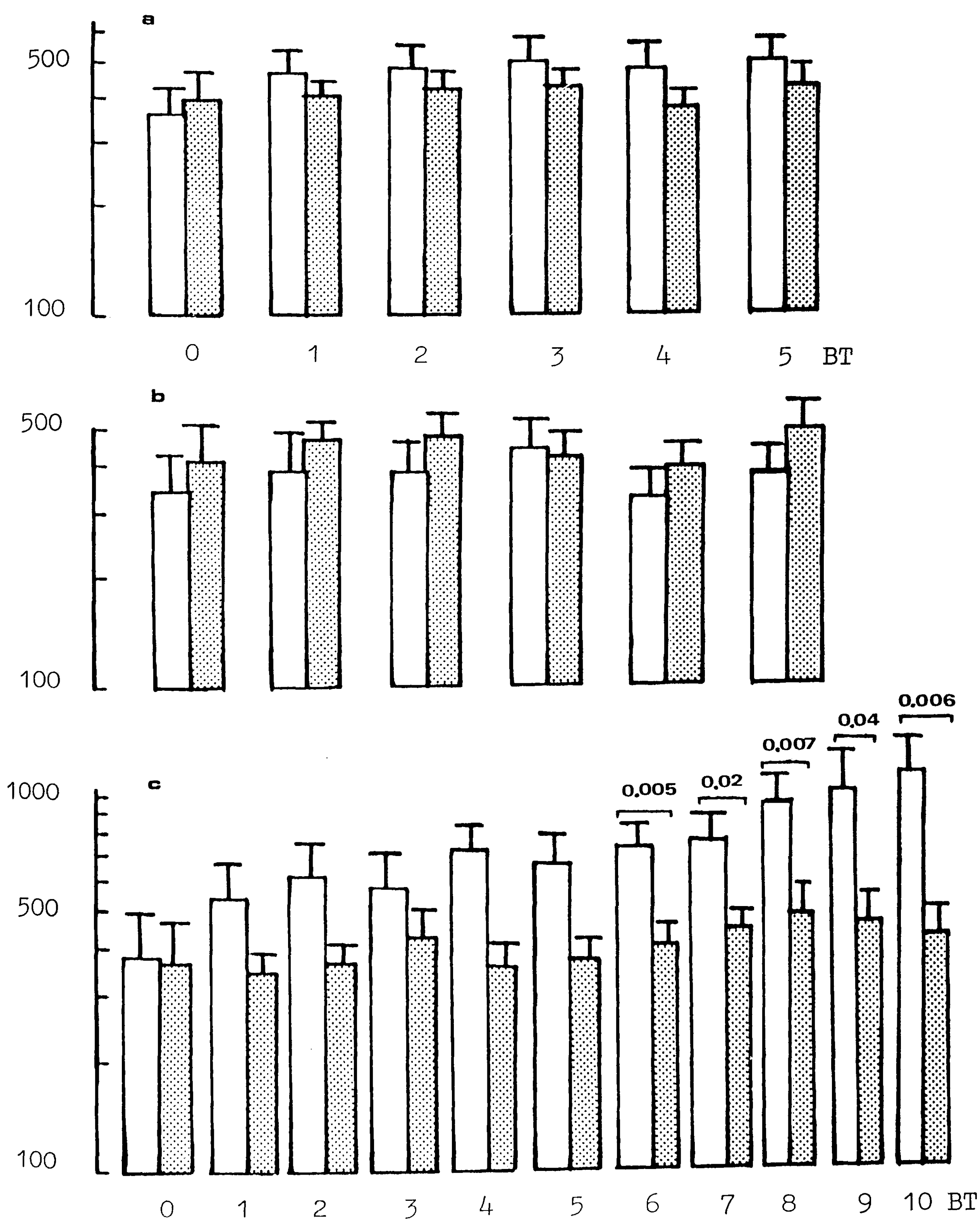


Figure 6.13. Spontaneous IgG-PFC counts (mean ± SEM) in the weak (open bars) and strong DNCB responders (shaded bars) before and after each transfusion; a = all 26 transfused patients (weak responders = 10, strong responders = 16); b = patients receiving 5 units (weak responders = 6, strong responders = 8); c = patients receiving 10 units (weak responders = 4, strong responders = 8).

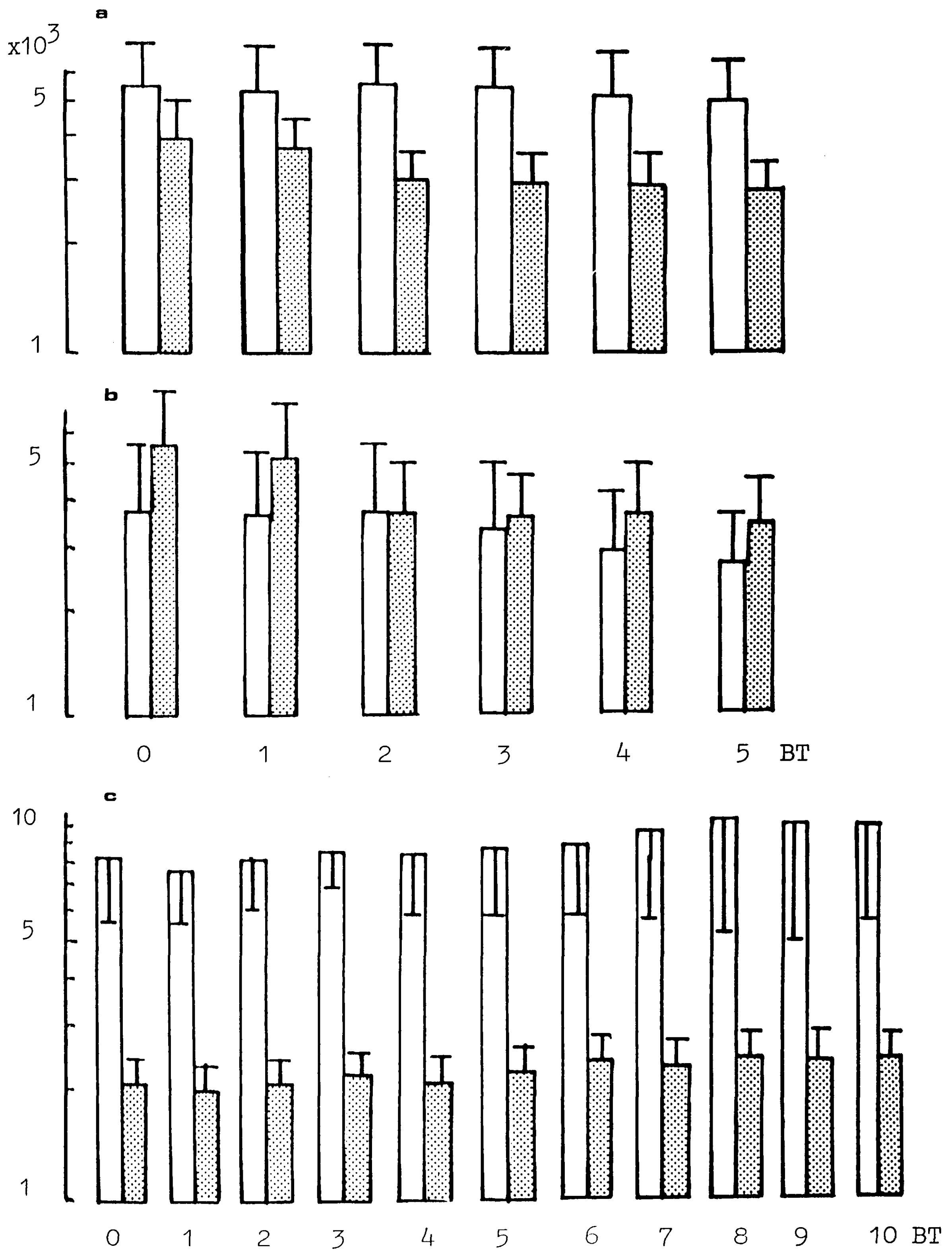


Figure 6.14. IgG-PFC counts (mean ± SEM) after stimulation with SAC in the weak (open bars) and strong DNCB responders (shaded bars) before and after each transfusion; a = all 26 transfused patients (weak responders = 10, strong responders = 16); b = patients receiving 5 units (weak responders = 6, strong responders = 8); c = patients receiving 10 units (weak responders = 4, strong responders = 8).

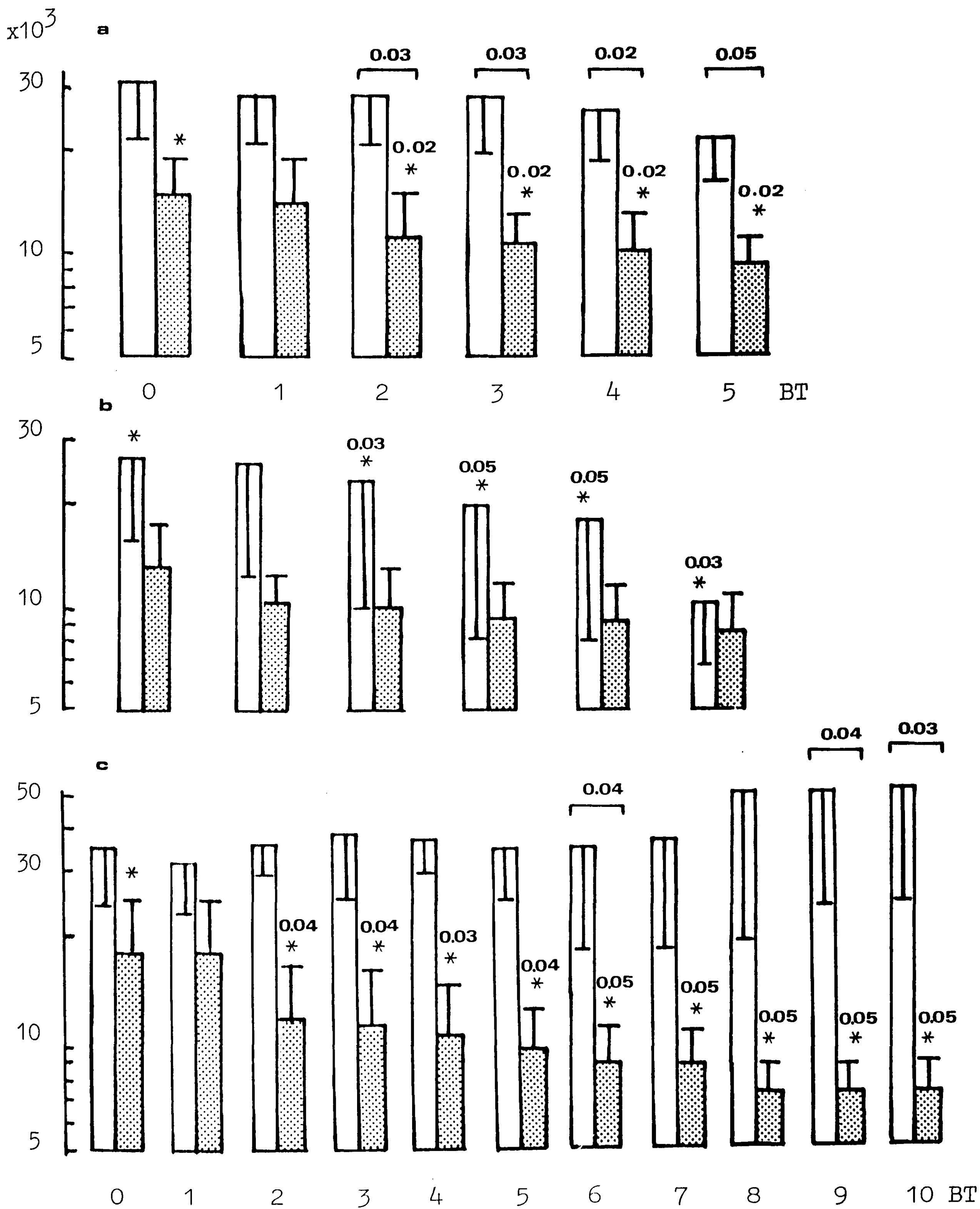
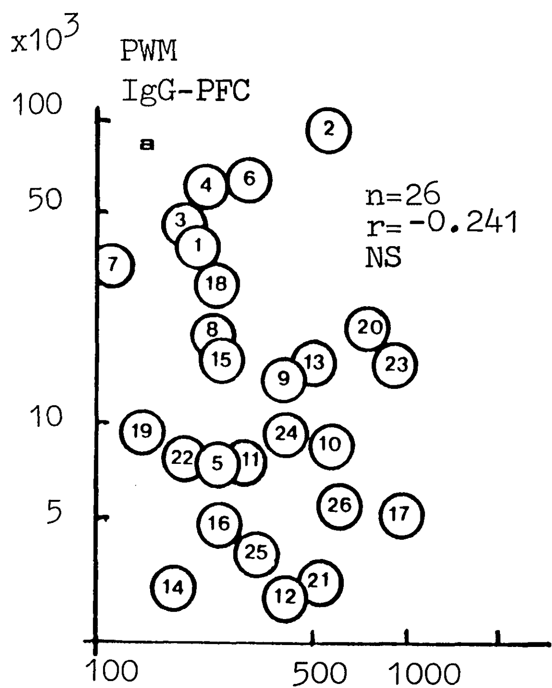
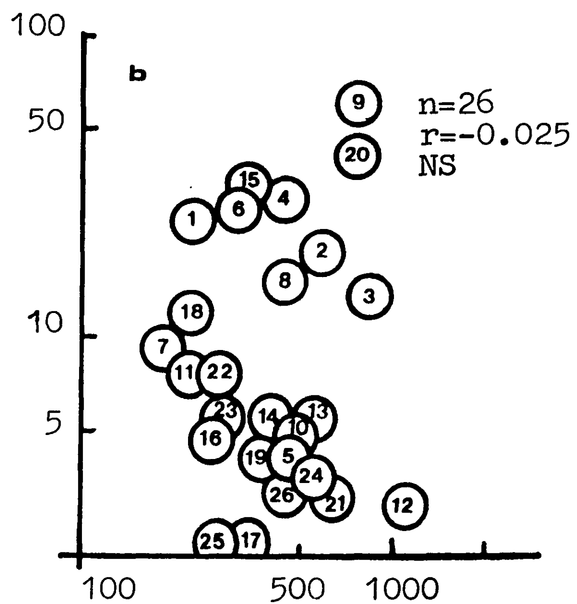


Figure 6.15. IgG-PFC counts (mean ± SEM) after stimulation with PWM in the weak (open bars) and strong DNCB responders (shaded bars) before and after each transfusion; a = all 26 transfused patients (weak responders = 10, strong responders = 16); b = patients receiving 5 units (weak responders = 6, strong responders = 8); c = patients receiving 10 units (weak responders = 4, strong responders = 8).

TABLE 6.9. SPEARMAN CORRELATION COEFFICIENTS AND TWO-TAILED SIGNIFICANCE BETWEEN IgG-PFC COUNTS BEFORE AND AFTER EACH BLOOD TRANSFUSION

Number of transfusion	PWM-IgG-PFC vs Spontaneous IgG-PFC	PWM-IgG-PFC vs SAC-IgG-PFC	SAC-IgG-PFC vs Spontaneous IgG-PFC
0 (n=26)	-0.241 NS	0.497 0.010	0.046 NS
1 (n=26)	0.060 NS	0.442 0.024	0.569 0.002
2 (n=26)	-0.105 NS	0.485 0.012	0.474 0.015
3 (n=26)	-0.049 NS	0.561 0.003	0.488 0.011
4 (n=26)	0.241 NS	0.540 0.004	0.523 0.006
5 (n=26)	-0.025 NS	0.566 0.003	0.350 NS
6 (n=12)	-0.028 NS	0.539 NS	0.594 0.042
7 (n=12)	0.315 NS	0.469 NS	0.713 0.009
8 (n=12)	0.392 NS	0.546 NS	0.762 0.004
9 (n=12)	0.539 NS	0.462 NS	0.769 0.003
10(n=12)	0.476 NS	0.559 NS	0.525 NS





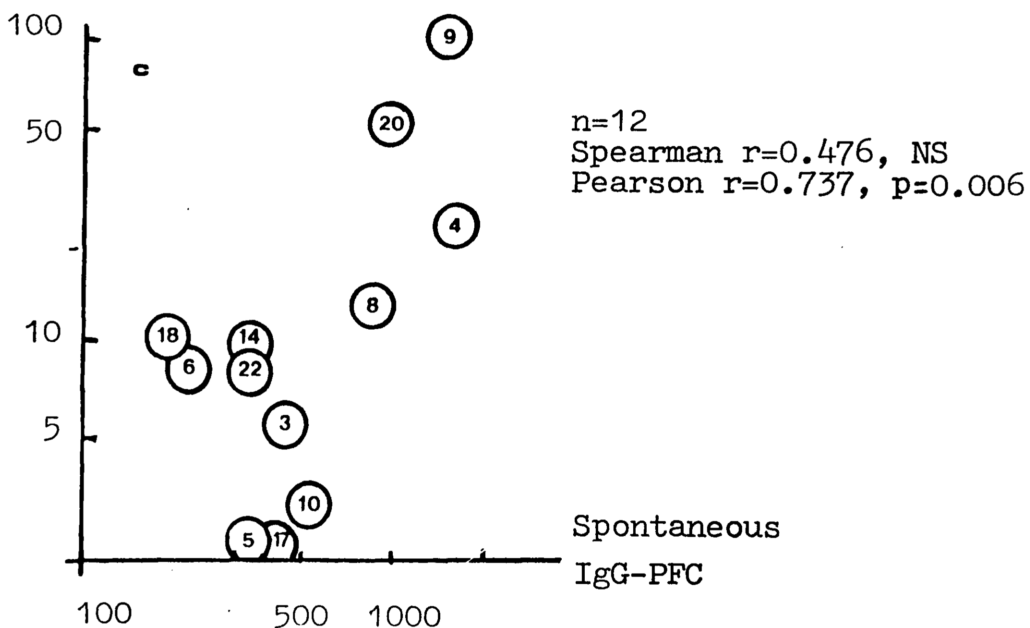


Figure 6.16. Correlation between spontaneous and PWM-induced IgG-PFC counts in the transfusion group; a = before transfusion, b = after the 5th transfusion, and c = after the 10th transfusion.

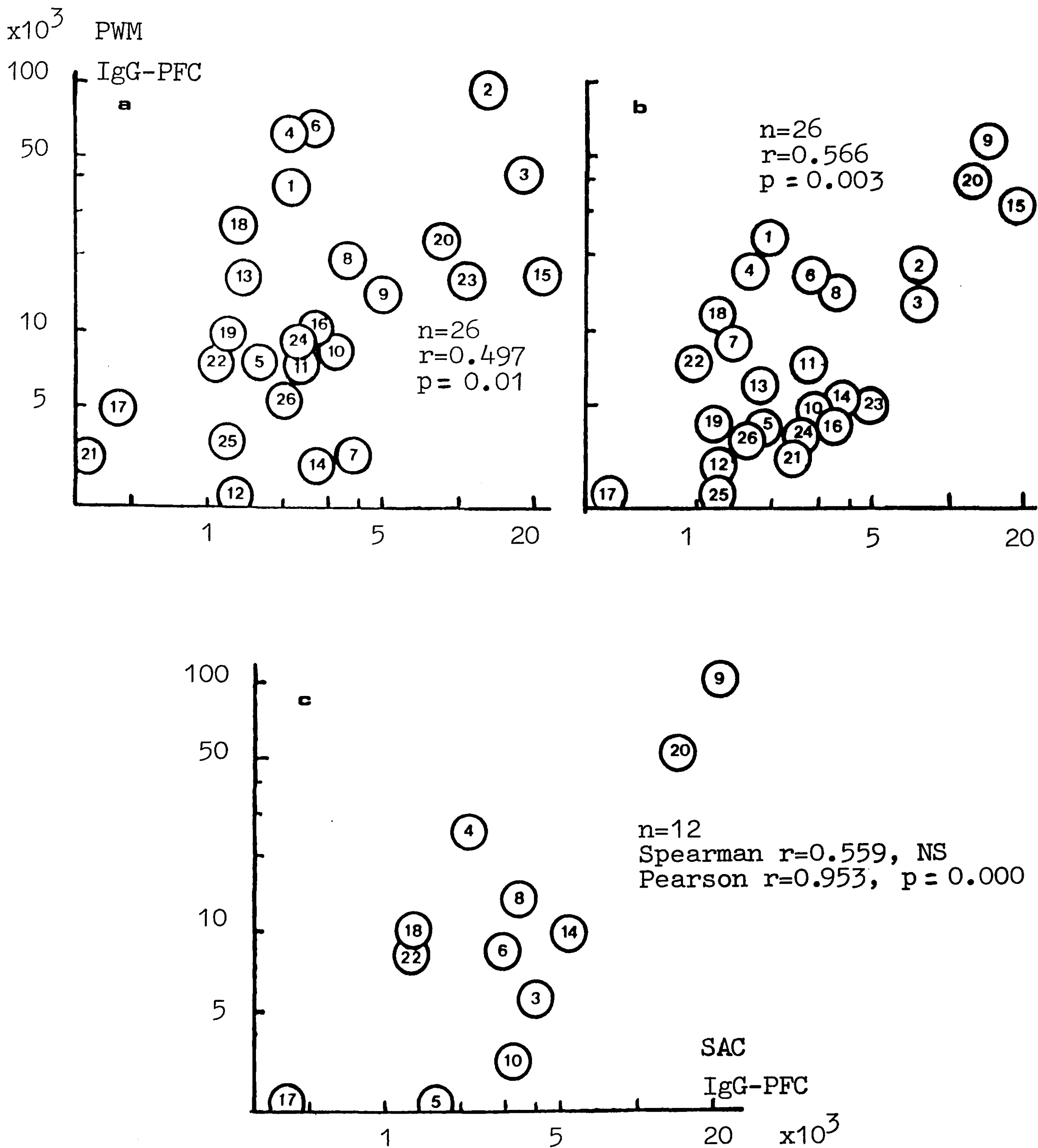


Figure 6.17. Correlation between PWM- and SAC- induced IgG-PFC counts in the transfusion group; a = before transfusion, b = after the 5th transfusion, and c = after the 10th transfusion.

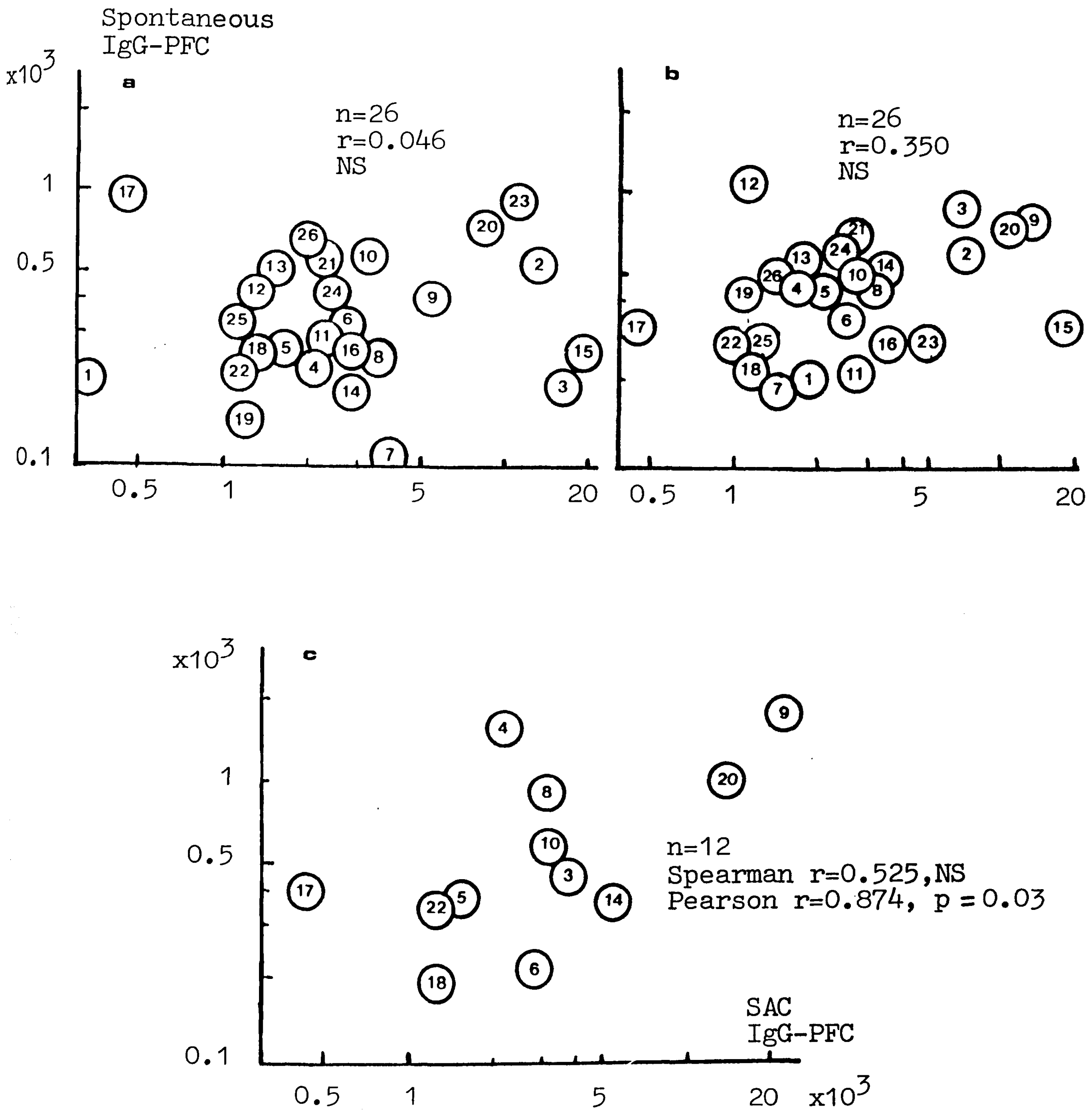


Figure 6.18. Correlation between spontaneous and SAC-induced IgG-PFC counts in the transfusion group; a = before transfusion, b = after the 5th transfusion, and c = after the 10th transfusion.

TABLE 6.10. PATIENTS UNDERGOING TRANSPLANTATION WITH POLYCLONAL B CELL ACTIVATION STUDIES FOLLOWING BLOOD TRANSFUSION

Patients* n=15	Number of transfusions	Months last BT-Tx	Immuno-suppression	Rejection episodes	Transplant outcome
2. TS	5	1	Azathioprine	0	Functioning
3. SW	10	3	Azathioprine	0	Functioning
5. WH	10	3	Cyclosporin	0	Functioning
9. JMcG	10	8	Cyclosporin	3	Functioning
10. JD	10	9	Cyclosporin	1	Functioning
11. WY	5	15	Cyclosporin	1	Functioning
12. PM	5	8	Cyclosporin	0	Functioning
13. TC	5	4	Cyclosporin	1	Died Functioning
18. MS	10	3	Cyclosporin	0	Functioning
19. WG	5	1	Cyclosporin	1	Rejection
20. CS	10	1	Cyclosporin	0	Functioning
21. RF	5	2	Cyclosp/Azath	2	Functioning
23. RH	5	1	Cyclosporin	0	Functioning
24. RB	5	10	Cyclosporin	0	Functioning
25. JG	5	4	Cyclosporin	0	Functioning

*Numbers of patients presented to correspond numbers of patients in Table 6.4 and Figures

TABLE 6.11. INTERRELATIONS BETWEEN REJECTION EPISODES, IgG-PFC COUNTS AND CYTOTOXIC ANTIBODIES (PRA) AFTER THE 5TH TRANSFUSION

	Pearson correlation coefficients and 2-tailed significance			
Variables	PWM-IgG-PFC	SAC-IgG-PFC	Spontaneous IgG-PFC	%PRA
SAC IgG-PFC	0.784 n=26 p=0.000			
Spontaneous IgG-PFC	0.188 n=26 NS	0.384 n=26 NS		
%PRA	-0.209 n=26 NS	0.092 n=26 NS	0.497* n=26 p=0.01	
Rejection episodes	0.047 n=9 NS	0.300 n=9 NS	0.012 n=9 NS	0.287 n=9 NS

*Spearman r=0.314, NS; Kendall r=0.262, NS

TABLE 6.12. INTERRELATIONS BETWEEN REJECTION EPISODES, IgG-PFC COUNTS AND CYTOTOXIC ANTIBODIES PRA AFTER THE 10TH TRANSFUSION

	Pearson correlation coefficients and 2-tailed significance			
Variables	PWM IgG-PFC	SAC IgG-PFC	Spontaneous IgG-PFC	%PRA
SAC IgG-PFC	0.953 n=12 p=0.000			
Spontaneous IgG-PFC	0.737 n=12 p=0.006	0.624 n=12 p=0.03		
%PRA	-0.031 n=12 NS	-0.127 n=12 NS	-0.158 n=12 NS	
Rejection episodes	0.810* n=6 p=0.05	0.746 n=6 NS	0.801** n=6 p=0.05	0.272 n=6 NS

Spearman: * r=0.338, NS, **r=0.676, NS
Kendall: * r=0.258, NS, **r=0.603, NS

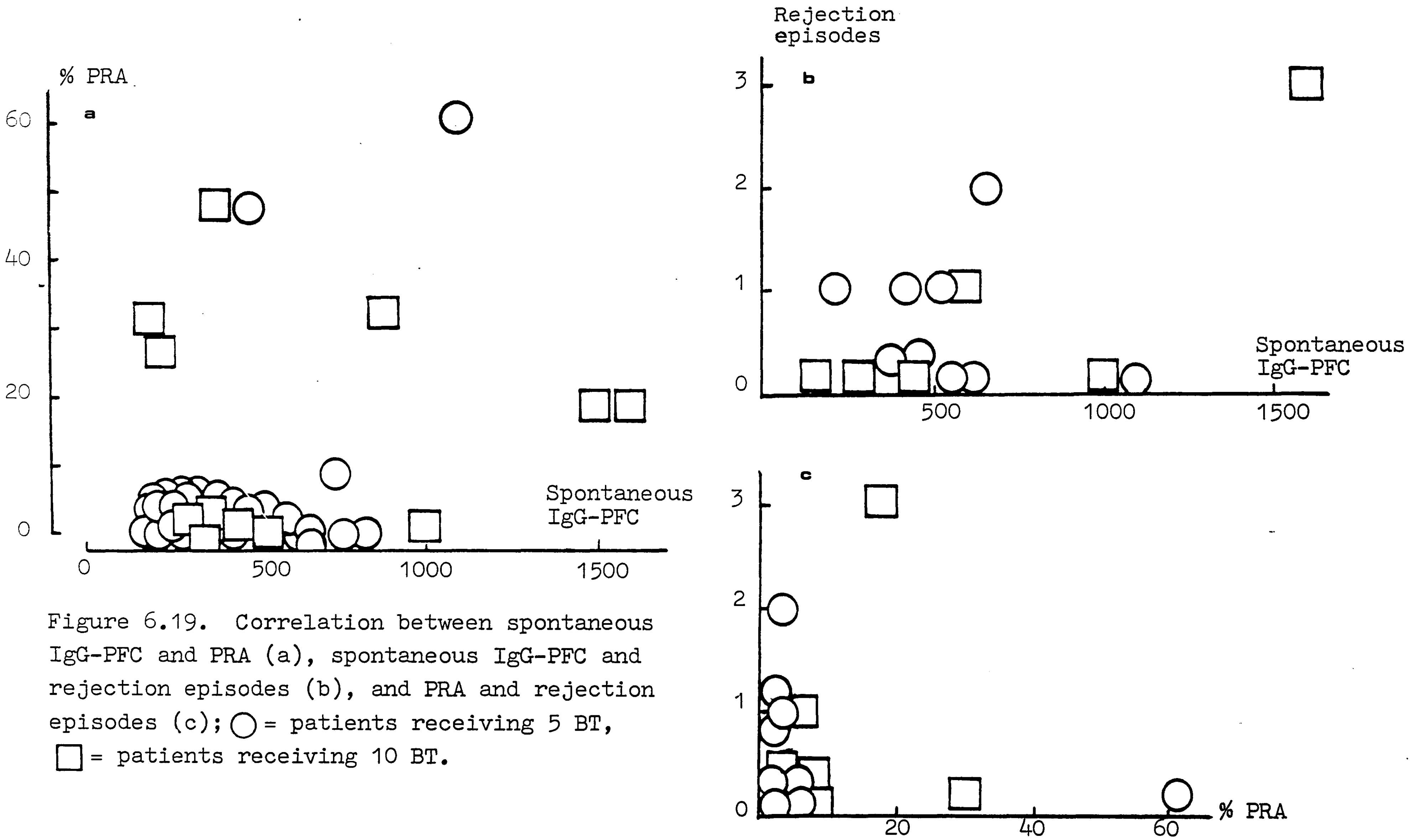


Figure 6.19. Correlation between spontaneous IgG-PFC and PRA (a), spontaneous IgG-PFC and rejection episodes (b), and PRA and rejection episodes (c); ○ = patients receiving 5 BT, □ = patients receiving 10 BT.

CHAPTER 7. EFFECT OF BLOOD TRANSFUSION ON IN VITRO PROSTAGLANDIN E RELEASE IN UNSTIMULATED AND Con-A STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELL CULTURE SUPERNATANTS

7.1 Introduction

The concept that prostaglandins of the E series (PGE) mediate inflammatory responses and function as regulators of immune responses has been widely established (79-83,85-106). Prostaglandins have been shown to directly inhibit the lymphocyte blastogenic responses to both antigenic and mitogenic stimulation (97), as well as to alter lymphocyte responsiveness indirectly through an effect on other mediators. It has been reported that they inhibit the production of interleukin 2 and down-regulate the expression of Class II MHC antigens by antigen presenting cells thereby indirectly inhibiting T cell activation and proliferation (103,581-585). However, the role of PGE on B cell proliferation and differentiation has not been clearly defined. There is now convincing evidence to suggest that there are species differences with regards to PGE participation in B cell activation (Reviewed in 101). For example in both the rat and mouse, antibody responses, as measured by haemolytic plaque formation, are inhibited by PGE (101), whereas in rabbits and humans, PGE stimulates IgG and to a lesser extent IgM production (101). Reports on the effect of PGE on human primary and secondary antibody responses to vaccination give conflicting results (101). Most of the studies which have attempted to define the immunoregulatory role of PGE have been carried out in experimental models or healthy humans. PGE-mediated immunoregulation is abnormal in certain diseases (85,92,94,101,102) but little is known about it in the uraemic state. The complexity of such potential regulatory mechanisms in uraemia may be further influenced by exogenous events, such as blood transfusion. In a preliminary report from this study it was suggested that blood transfusion may induce changes in PGE secretion which differed in weak and strong DNCB responders (586).

This chapter aims to examine to what extent blood transfusion may interfere with PGE synthesis and host cellular and humoral responses in previously non-transfused uraemic patients.

Patients and Methods

Patients

In all 48 patients from the transfusion group (Table 1) with the exception of one female, PGE concentrations were carried out in PBMC culture supernatants before and after each of the 5 or 10 blood transfusions. Hence, among the 47 transfused patients described in this chapter, there were 37 males and 10 females and their mean age was 41 years (range 18-59 years). The woman who was not studied in this chapter was treated by haemodialysis and her original renal disease was hypertensive nephrosclerosis. She was a strong DNCB responder and she had been allocated to receive 10 units of blood. Therefore, the overall characteristics of the original transfusion group (Table 1) were only slightly affected and the random allocation of the patients with regards to their response to the DNCB skin test and the number of transfusions they received was altered by one accordingly (Figure 7.1). In addition to the 47 patients in the transfusion group the 12 uraemic controls and the 12 healthy subjects were also studied.

Methods

Specimen collection and handling: Blood samples were taken in a preservative free heparin bottle before and 14 days after each transfusion in the transfusion group and on 3 - 4 occasions in the controls over a period of three months. The mononuclear cells were separated on a Ficoll-Hypaque gradient and 4 x 10^6 cells were cultured in 2ml of medium 199, which contained glutamine, penicillin-streptomycin and 10% pooled plasma (Gibco-Bioccult, Paisley, Scotland) without and with 10ug/ml of concanavalin A (con A, Sigma Chemicals Co., St Louis, USA) for 48 hours at 37°C in 5% CO_2. After 48 hours the culture supernatants were harvested and were stored at -20°C. Supernatants from each subject collected serially in this way were tested in the same experiment along with specimens from one uraemic and healthy control to avoid test to test variation. In a few patients blood samples were not taken on every occasion after blood transfusion and in a few others there was insufficient cells to carry out the cultures. Thus, different numbers of the 48

patients appeared prior to and following transfusion. To compensate for this, statistical analysis was carried out using both matched, paired and unpaired tests.

PGE Radioimmunoassay

Principles of the method: The PGE radioimmunoassay (Clinical Assays Travenol - Genentech, Diagnostics, Cambridge, Mass., USA) offers a sensitive method of measuring PGE content in serum, plasma, culture supernatants and tissues by measuring the competitive binding of tritium-labelled PG and unlabelled PG antibody to PG. The principle of the assay is based on the competitive binding principles of radioimmunoassays as developed by Yalow and Berson (587), and the technique described here is a modification of procedures published by Levine et al (588) and Gutierre-Cernosek et al (589). Alkaline treatment of extracted samples converts PGE to prostaglandin B (PGB). The known-radioactive PGB from sample extracts or PGB standards compete with a constant amount of tritium-labelled PGB_1 ($[^3H]$ PGB_1)tracer for binding sides on the PGB_1 antibody which is held at a limiting concentration. The amount of $[^3H]$ PGB_1 which will bind the antibody is inversely proportional to the amount of non-radioactive PG in the assay tube. A standard curve is prepared from 6 standards ranging from 8.2 to 2000pg/tube and the PG concentrations of the samples are interpolated from the standard curve. Separation of antibody bound PGB from free PGB is achieved by precipitating the anti-PG/PG complex with the second antibody. After centrifugation the pellets are resuspended and the bound radioactivity is measured in a liquid scintillation counter.

Test procedure: The reagents supplied with the kit were.

1) Tritium PGB_1 tracer,
2) Rapid anti-PGB_1 serum,
3) Tris buffer concentrate,
4) Normal rabbit serum,
5) Goat anti-rabbit serum,
6) PGB_1 standard calibrated at 40ng/ml,
7) Gelatine 250A.

Preparation of the standards: Five standards were prepared from reconstituted PGB_1 standard at 40ng/ml. Into 5 polypropylene tubes (12 x 75mm) marked 1/3, 1/9, 1/27, 1/81, 1/243, 0.6ml of gel tris buffered was pipetted using a 1.0ml semi-automatic adjustable pipette. The reconstituted PGB_1 standard was called the 1/1 standard, 0.3ml of which was added to the 1/3 tube and mixed thoroughly. Subsequently 0.3ml of the 1/3 tube was added in the tube marked 1/9 and after thorough mixing serial dilution was repeated in the same fashion to the last tube. Thus the standard PGB_1 concentrations in the 1/1, 1/3, 1/9, 1/27, 1/81, and 1/243 tubes were 2000, 667, 222, 74, 25, and 8.2pg/0.05ml respectively.

Preparation of the samples: In order to convert PGE into PGB 50ul of the samples were diluted with 950ul gel tris buffer in calibrated tubes with screw caps and 100ul of 1N sodium hydroxide was added. The caps were screwed tightly and after being well mixed the tubes were placed in a boiling bath for five minutes. The samples were left to cool in room temperature and the pH was adjusted to 7.4 using 1N acetic acid.

Assay procedure: The assay procedure included the preparation of the standard curve from which the unknown PGE content in the sample was determined.

1) All reagents reached room temperatures and were mixed before use.

2) The polypropylene tubes were labelled in duplicate as follows:

Tube no.	Contents of tubes	PGB_1 added (pg/0.05ml)
T1, T2	Total counts (tracer)	-
1, 2	Non-specific binding (NSB)	-
3, 4	PGB_1 blank (B_o)	0
5, 6	PGB_1 standard, 1/243	8.2
7, 8	PGB_1 standard, 1/81	25
9, 10	PGB_1 standard, 1/27	74
11, 12	PGB_1 standard, 1/9	222
13, 14	PGB_1 standard, 1/3	667
15, 16	PGB_1 standard, 1/1	2000
17, 18	Patient sample extract	unknown

3) Gel tris buffer was added in the following volumes, 1.1ml to tubes 1 and 2, 1.0ml to tubes 3 and 4, and standard tubes 3-16, and 0.6ml to sample extract tubes 17 to end.

4) 50ul of each PGB_1 standard and 400ul of each patients sample extract were added into the appropriate tube in duplicate.

5) 50ul of reconstituted $[^3H]$ PGB_1 tracer was added to all tubes and the tubes T1, T2 were capped.

6) 50ul of rabbit anti-PGB_1 serum was added to all the tubes except T1, T2 and NSB and was mixed thoroughly on a vortex mixer.

7) Tubes were incubated for 60 minutes in a 37°C incubator from the time the antiserum was added.

8) 100ul of reconstituted normal rabbit serum was added to all tubes except T1, T2, followed by addition of 100ul of goat anti-rabbit serum to all tubes except T1, T2 with thorough mixing on a vortex mixer.

9) Tubes were covered and incubated for at least 18 to 20 hours at 2-8°C.

10) All tubes but T1, T2 were centrifuged in a refrigerated centrifuge at 2-8°C for 30 minutes at a relative centrifugal force of 1600 g.

11) Each tube was decanted except T1, T2 into a waste beaker and the rim of each tube was vigorously tapped on to absorbent paper to remove any residual supernatant.

12) 100ul of 0.1N sodium hydroxide was added to all tubes including T1, T2 and they were vortexed to mix and dissolve the precipitate.

13) The solution of all tubes was decanted into correspondingly numbered scintillation vials ensuring maximum transfer and the tubes were discarded.

14) 10ml of scintillation fluid was added to all vials and after vortexing, all vials were counted in a beta scintillation counter for at least 5 minutes.

The results were calculated from the standard curve. Counts per minute (CPM) for each tube were recorded and the percent bound (Bn) for each standard was calculated from the formula:

$$Bn = \frac{CPM - NSB}{Bc} \times 100$$

where NSB = non-specific binding (average CPM in vials 1,2), and Bc = corrected maximum binding, calculated from the average CPM in the blank vials 3,4 minus the NSB, that is, Bc = B0 - NSB. The percent bound for the PGB_1 standards (vertical axis) were plotted against the PGB_1 concentration (horizontal axis) on 4-cycle semi-logarithmic graph paper and the best fitting curve was drawn. Table 7.1 and Figure 7.2 show typical data and a typical standard curve respectively. The percent bound corresponding to each control and patient sample extract were calculated from the same formula and were located on the vertical axis. The point at which a horizontal line intersected the standard curve was read as the PGB_1 concentration in pg/tube from the horizontal axis. Correction of the PGB_1 concentration for each extracted sample to the amount present in the original sample was carried out by multiplying with a correction factor, namely 60, which was calculated based on the original amount of the sample used in the extraction procedure, an extraction efficiency factor, and a dilution factor for the fraction of extract used as inhibitor in the assay. The final PGE concentration in the original sample was expressed in ng/ml/$4x10^6$ cells.

Statistical analysis

There was a large intersubject variation in the individual PGE concentrations and examination of histograms showed that the distribution of values was not normal. Therefore, comparison of mean values between and within groups was done using the Mann-Whitney test. For paired matched data the Wilcoxon rank test was applied. Correlation between sets of individual parameters was carried out using Pearson (parametric) and Spearman (non-parametric) correlation coefficients depending on the appearance in the scattergrams.

7.3 Results

7.3.1 Effect of blood transfusion on in vitro PGE synthesis

Results of mean ± SD, SEM, median and range of PGE release in supernatants before and after each unit of blood in the transfusion group, and in the two control groups over a period of three months are shown in Table 7.2. In vitro synthesis of PGE in unstimulated and Con-A stimulated culture supernatants from PBMC showed a large intersubject variation both in the transfusion group and in the two control groups. The distribution of the values was positively skewed, but despite the wide range the SEM was not high due to the large number of patients who were included in this study. The statistical analysis of Table 7.2 is shown in Figure 7.3.

PGE synthesis both in unstimulated and stimulated cultures in the two control groups did not change significantly over the period of three months. In contrast, there were highly significant changes induced by blood transfusion in the transfusion group. PGE synthesis was significantly higher in Con-A stimulated culture supernatants after the 3rd, 4th, 5th and 10th unit of blood compared to background values (mean ± SEM, 19 ± 3 vs 46 ± 6, $p<0.0002$; 19 ± 3 vs 37 ± 6, $p<0.006$; 19 ± 3 vs 36 ± 5, $p<0.007$ and 19 ± 3 vs 30 ± 6, $P<0.04$ respectively). This increase in PGE release peaked after the third blood transfusion and remained elevated until after the 6th unit of blood. Thereafter PGE synthesis fell to background levels but rose again after the 10th unit of blood. In vitro PGE synthesis following the 3rd transfusion was significantly higher compared to that of the uraemic controls at 3 months (mean ± SEM, 46 ± 6 vs 19 ± 5, $p<0.04$). An unexpected finding from the analysis of these results was that in

unstimulated culture supernatants PGE concentrations after the 3rd unit of blood showed a significant increase compared to background values (mean $\pm$ SEM, 18 $\pm$ 3 vs 38 $\pm$ 6, $p<0.007$). This could be due to mitogenic activity in the culture medium (ie pooled plasma) which was maximal after the 3rd transfusion.

Figure 7.4 shows the individual PGE concentrations of Con-A stimulated cultures in the transfused patients prior to and after the 3rd, 5th and 10th unit of blood and in the 2 control groups. PGE values were widely scattered and covered the full scale of measurable concentrations using the estimated standard curves. Most of the values in the transfused patients before transfusion were under 20ng/ml (25 of 39, 64%), which was the average concentration in the uraemic controls over the period of 3 months. After the 3rd, 5th and 10th unit of blood the respective figures in the transfused patients were 24% (8 of 34), 43% (16 of 37) and 31% (4 of 13). This indicates that an increase in PGE synthesis after transfusion occurred in most of the patients. This is illustrated in Figure 7.5 which shows the individual changes in PGE synthesis in stimulated cultures after the 3rd, 5th and 10th unit of blood in the transfused patients and in the two control groups. Twenty one of the 26 patients had increased PGE synthesis after the 3rd unit of blood (81%), while the remaining 5 patients (19%) had unchanged or decreased synthesis. The overall increase was highly significant (Wilcoxon paired rank test: mean $\pm$ SEM, 15 $\pm$ 3 vs 48 $\pm$ 7, $p<0.0004$). After the 5th and 10th unit of blood 72% (23 of 32 patients) and 77% (10 of 13 patients) respectively had increased PGE concentrations and the remaining patients had decreased values. The overall increase after the 5th transfusion was also highly significant (mean $\pm$ SEM, 17 $\pm$ 2 vs 38 $\pm$ 6, $p<0.007$), but after the 10th unit of blood the significance was weak (mean $\pm$ SEM, 12 $\pm$ 3 vs 30 $\pm$ 8, $p<0.04$). In contrast, in the two control groups the changes were not significant.

7.3.2 Effect of blood transfusion on in vitro PGE synthesis in weak and strong DNCB responders

Results of PGE concentrations in unstimulated and Con-A stimulated culture supernatants in the weak and strong DNCB responders before and after transfusion are shown in Table 7.3 and

Figure 7.6. At the start of the study the difference between weak and strong DNCB responders in PGE concentrations was not significant in either unstimulated or stimulated cultures. Also, the difference between the two groups was not significant at any time after each transfusion. However, strong DNCB responders showed a significant increase of PGE synthesis in the stimulated cultures following the 3rd, 4th and 5th transfusion compared to their own background values ($p<0.001$, $p<0.03$, $p<0.009$ respectively), whereas weak DNCB responders did not. After the 3rd unit of blood the former group had significantly higher PGE release in the unstimulated cultures as well ($p<0.01$). Figure 7.7 shows the individual changes of PGE concentrations in stimulated cultures in the weak and strong DNCB responders after the 3rd and 5th transfusion. It is obvious that in strong responders the increase in PGE synthesis was highly significant on both occasions ($p<0.006$ and $p<0.009$), while weak responders showed an increase of weak significance only after the third unit of blood ($p<0.03$).

With regard to the number of transfusions, the difference of PGE values in patients receiving 5 or 10 units was not significant (Table 7.4 and Figure 7.8), and this was also the case when the patients were divided into weak and strong DNCB responders. (Table 7.5).

7.3.3 Correlation between PGE synthesis, IgG production, cytotoxic antibodies and transplant outcome.

The polyclonal B cell activation studies which were described in the previous chapter showed that there was a significant increase in spontaneous IgG-PFC counts after transfusion while in the PWM driven system IgG production was suppressed. These studies were carried out on aliquots of blood samples with the PBMC of which the in vitro PGE concentrations in cultures stimulated with Con-A were also measured. Table 7.6 shows the Spearman correlation coefficients between these PGE concentrations before and after each blood transfusion and the respective spontaneous, SAC- and PWM- induced IgG- PFC counts. There was no correlation between in vitro PGE synthesis and IgG production spontaneously or after stimulation with PWM at any time prior to or after transfusion. In contrast in the

SAC- driven system there was a strong positive correlation before transfusion (Spearman r=0.504, p<0.017, Pearson r=0.556, p<0.007) (Figure 7.9) which disappeared after transfusion. These findings suggest that, firstly, PWM- induced IgG production is not sensitive to an immunoregulatory role of PGE in B cell activation, and secondly, SAC- induced IgG production appears to be PGE- sensitive in the non-transfused uraemic patients but this effect disappears after transfusion.

Plotting of PGE concentrations against cytotoxic antibodies after 5 and 10 units of blood (described in Chapter 5) did not show any correlation.

Twenty six of the 47 patients studied in this Chapter were subsequently transplanted and had PGE radioimmunoassays performed before and after the last elective blood transfusion (5 or 10 units). Nineteen grafts were functioning at the end of the study, 4 had irreversibly rejected, 2 patients had died with functioning grafts and in one case the transplant was a technical failure. Of the 19 successful transplants, 12 were treated with Cyclosporin (63%), 6 had Azathioprine (32%) and one had a combination of the two regimens (5%). Of the 4 patients who rejected their grafts, 3 received Azathioprine and one Cyclosporin. Although the number of patients with graft rejection was small and the difference in immnosuppressive treatment was in favour of the graft acceptors, an interesting difference emerged from comparison of PGE synthesis in the 2 groups (Figure 7.10). Rejectors had higher PGE concentrations in Con-A stimulated culture supernatants compared to those of acceptors before pre-transplant transfusion (mean $\pm$ SEM, 45 $\pm$ 10 vs 17 $\pm$ 5, $p<0.05$). After the last elective transfusion (5th or 10th unit) the increase of PGE synthesis was highly significant in the latter group (mean $\pm$ SEM, 17 $\pm$ 5 vs 46 $\pm$ 9, $p<0.005$), but in the former 4 patients the change was not significant (mean $\pm$ SEM, 45 $\pm$ 4 vs 48 $\pm$ 26). This observation, although inconclusive, suggests that differences in PGE kinetics before and after transfusion exist in pre-transplant patients and may play a role in the mechanisms involved in graft rejection.

7.4 Conclusions

1) In vitro PGE synthesis in Con-A stimulated PBMC culture supernatants in previously non-transfused uraemic patients was comparable to that of healthy subjects.

2) Blood transfusion in these patients induced a significant increase of PGE synthesis which was more pronounced after the 3rd, 5th and 10th units of blood transfused.

3) The difference in PGE concentrations in stimulated cultures between weak and strong DNCB responders before any transfusion was not significant, but the latter group showed a more vigorous PGE synthesis in response to subsequent blood transfusion compared to that of the weak DNCB responders.

4) Transfusion of a further five units of blood did not have any significant effect in PGE concentrations in comparison with the initial five units, the maximum effect of which was observed after the 3rd transfusion in patients receiving both 5 and 10 units.

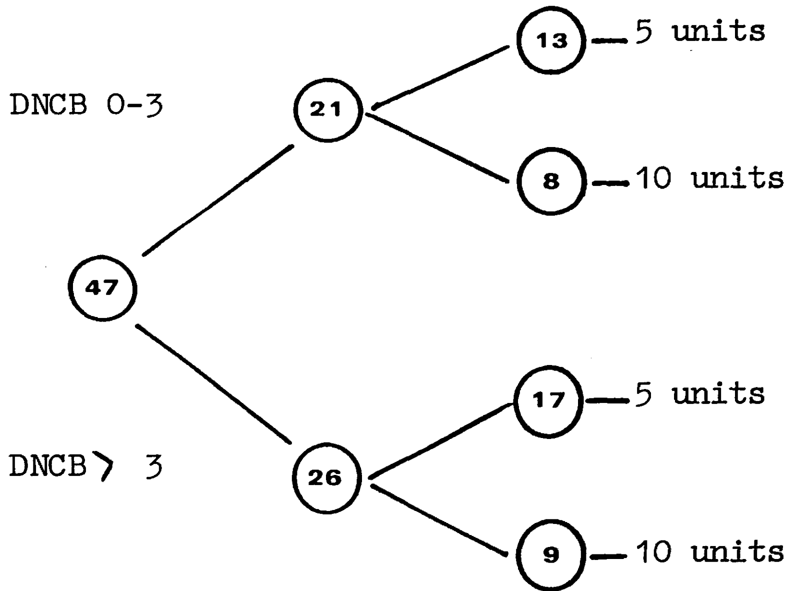
5) PGE concentrations did not correlate with IgG production in the peripheral blood either spontaneously or after stimulation with PWM before and after one to ten transfusions.

6) In contrast, IgG production in the SAC-driven system showed a good correlation with PGE synthesis before transfusion which disappeared after the first unit of blood.

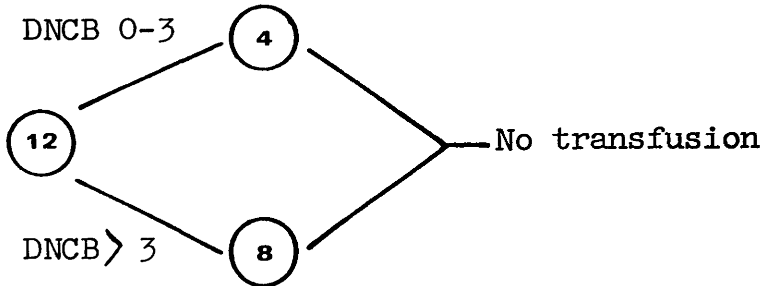
7) In vitro PGE release in stimulated PBMC cultures was not associated with the development of cytotoxic antibodies following transfusion.

8) Patients who were successfully transplanted showed a significant increase of PGE concentrations in stimulated cultures after the last elective pre-transplant transfusion. In contrast, in a small number of patients with irreversible graft rejection such an increase was not observed.

I. Transfused Patients



II. Uraemic controls



III. Normal controls

Figure 7.1. Breakdown of the subjects according to their response to the DNCB skin test and the number of blood transfusions; 0-3 = weak responders, > 3 = strong responders.

TABLE 7.1 RECORDING OF TYPICAL DATA IN A PGE RADIOIMMUNOASSAY

Tube no.	Contents of tubes	CP bound	% bound (average)
T1	Total counts (tracer)	11353	-
T2	Total counts (tracer)	11680	-
1	Non-specific binding (NSB)	320	-
2	Non-specific binding (NSB)	337	-
3	PGB1 blank (Bo), 0pg/tube	5064	-
4	PGB1 blank (Bo), 0pg/tube	5371	-
5	PGB1 standard, 8.2pg/tube	5227	96%
6	PGB1 standard, 8.2pg/tube	4719	
7	PGB1 standard, 25pg/tube	4508	90%
8	PGB1 standard, 25pg/tube	4944	
9	PGB1 standard, 74pg/tube	3998	73%
10	PGB1 standard, 74pg/tube	3839	
11	PGB1 standard, 222pg/tube	2752	51%
12	PGB1 standard, 222pg/tube	2912	
13	PGB1 standard, 667pg/tube	1566	24%
14	PGB1 standard, 667pg/tube	1465	
15	PGB1 standard, 2000pg/tube	1091	12%
16	PGB1 standard, 2000pg/tube	977	
17	Patient sample (AM)	1575	26%
18	Patient sample (AM)	1612	

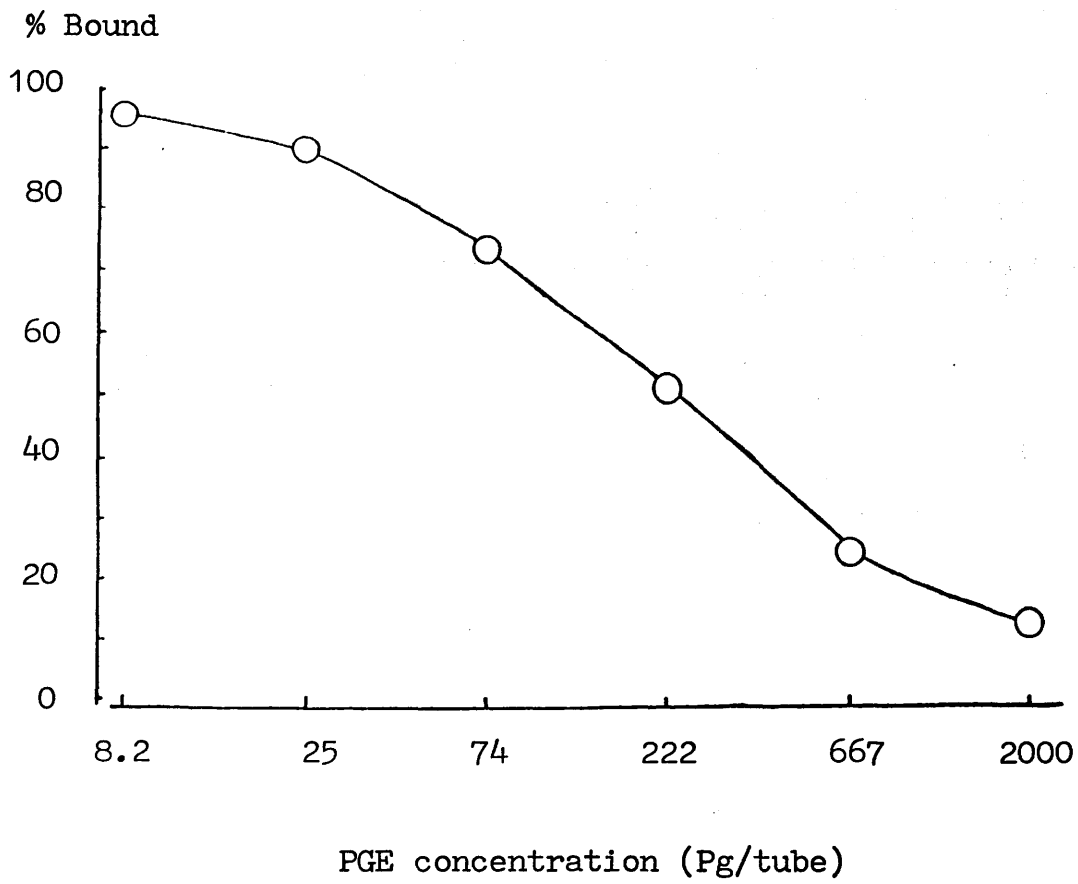


Figure 7.2. Typical standard curve in a PGE radioimmunoassay from the data in Table 7.1.

TABLE 7.2. PROSTAGLANDIN E CONCENTRATIONS IN UNSTIMULATED AND CON-A STIMULATED PBMC CULTURE SUPERNATANTS IN THE TRANSFUSION GROUP BEFORE AND AFTER EACH OF 1-10 UNITS OF PACKED CELLS AND IN THE TWO CONTROL GROUPS

	PGE concentrations (ng/ml)										
No. BT	0	1	2	3	4	5	6	7	8	9	10
Unstimulated:	n=36	n=30	n=32	n=31	n=31	n=36	n=11	n=12	n=12	n=10	n=12
Mean ± SD	18±16	22±23	19±17	38±31	25±28	23±24	25±17	13±7	13±13	15±18	13±8
SEM	3	4	3	6	5	4	5	2	4	6	2
Median	11	11	16	29	16	13	22	13	9	10	14
Range	1-71	1-78	0-65	0-110	2-120	2-120	7-59	2-25	3-48	3-62	3-31
Stimulated:	n=39	n=30	n=32	n=34	n=33	n=37	n=13	n=13	n=12	n=10	n=13
Mean ±SD	19±19	30±31	25±21	46±35	37±32	36±33	38±31	21±19	23±20	19±17	30±21
SEM	3	6	4	6	6	5	9	5	6	5	6
Median	14	16	17	35	26	24	31	13	13	14	28
Range	1-102	0-120	1-74	0-120	2-120	3-120	10-120	7-79	5-66	5-62	4-66

	Uraemic controls		Normal controls	
	At 0	At 3 months	At 0	At 3 months
Unstimulated:	n=12	n=10	n=10	n=11
Mean ± SD	23±24	17±17	17±13	16±31
SEM	10	5	4	9
Median	9	12	11	6
Range	2-111	3-60	6-43	2-107
Stimulated	n=12	n=11	n=12	n=12
Mean ±SD	20±21	19±15	27±24	26±33
SEM	6	5	7	10
Median	12	14	21	9
Range	2-78	5-52	4-81	1-107

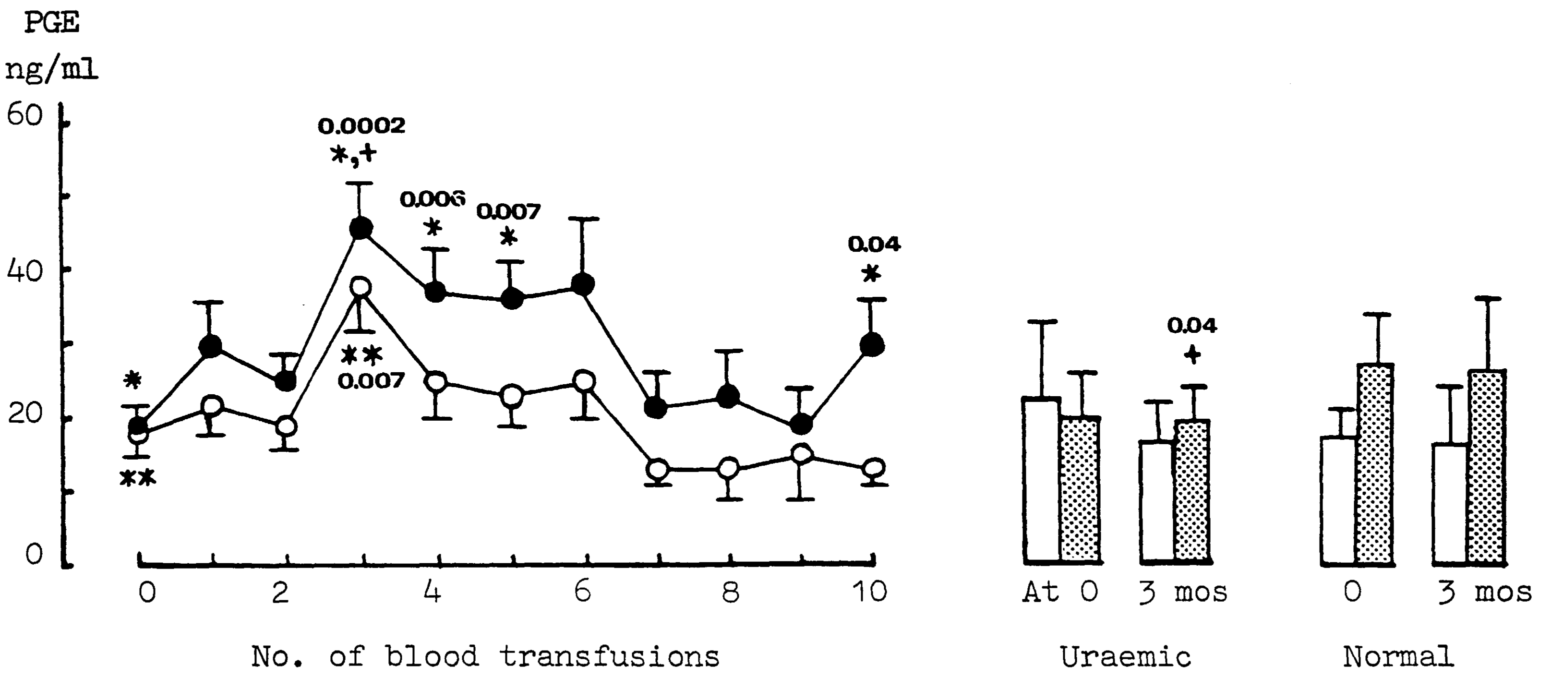


Figure 7.3. Mean $\pm$ SEM PGE concentrations in unstimulated (open circles/bars) and Con A stimulated PBMC culture supernatants (closed circles/bars) in the transfusion group before and after transfusion, and in the 2 control groups at 0 and 3 months; the number of patients tested on each occasion are shown in Table 7.2.

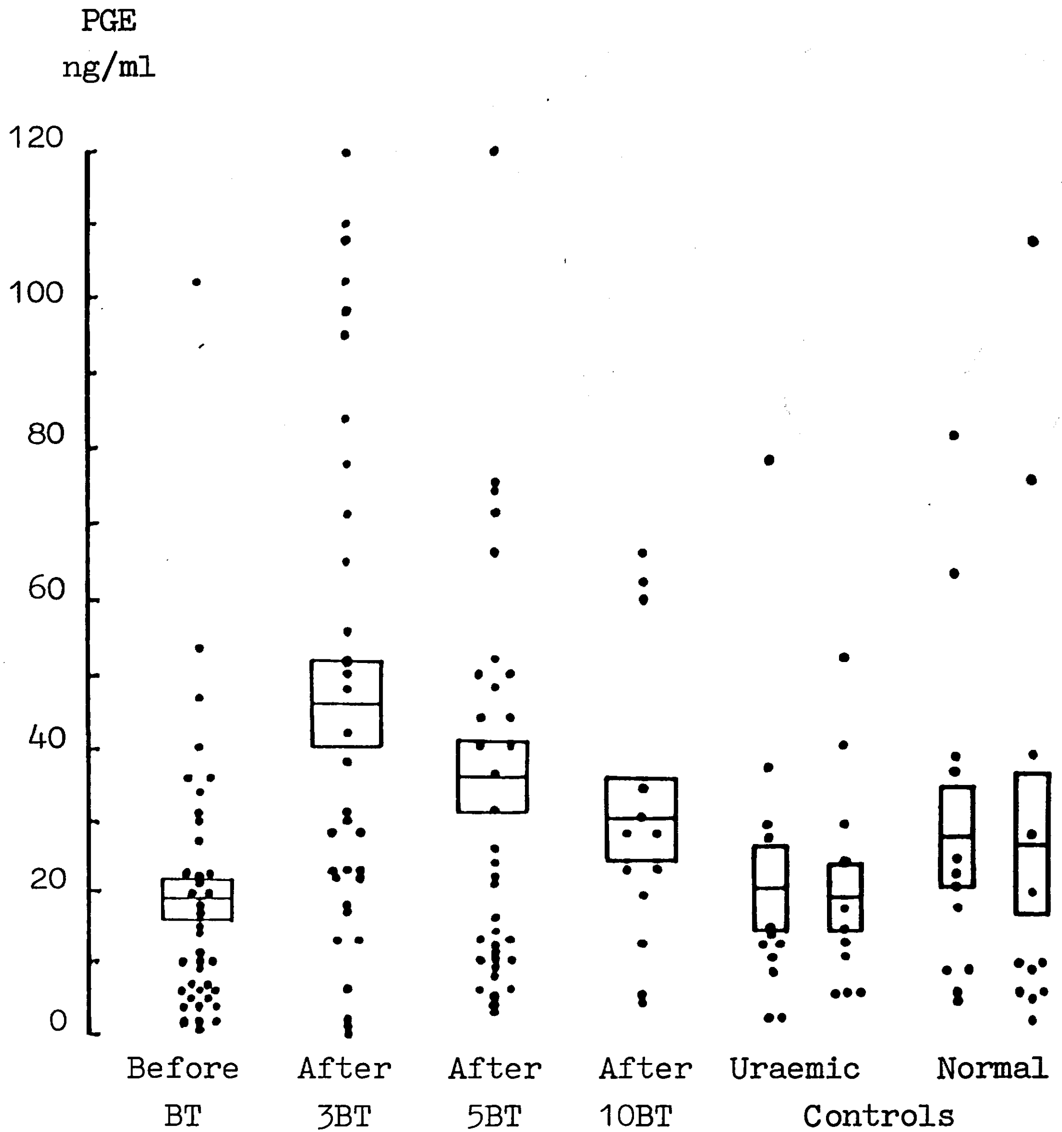


Figure 7.4. PGE concentrations released in PBMC culture supernatants stimulated with Con A in previously non-transfused dialysis patients before and after 3,5 and 10 units of packed cells, and in the uraemic and normal controls at 0 and 3 months; square areas represent mean ± SEM; statistics are the same as in Figure 7.3.

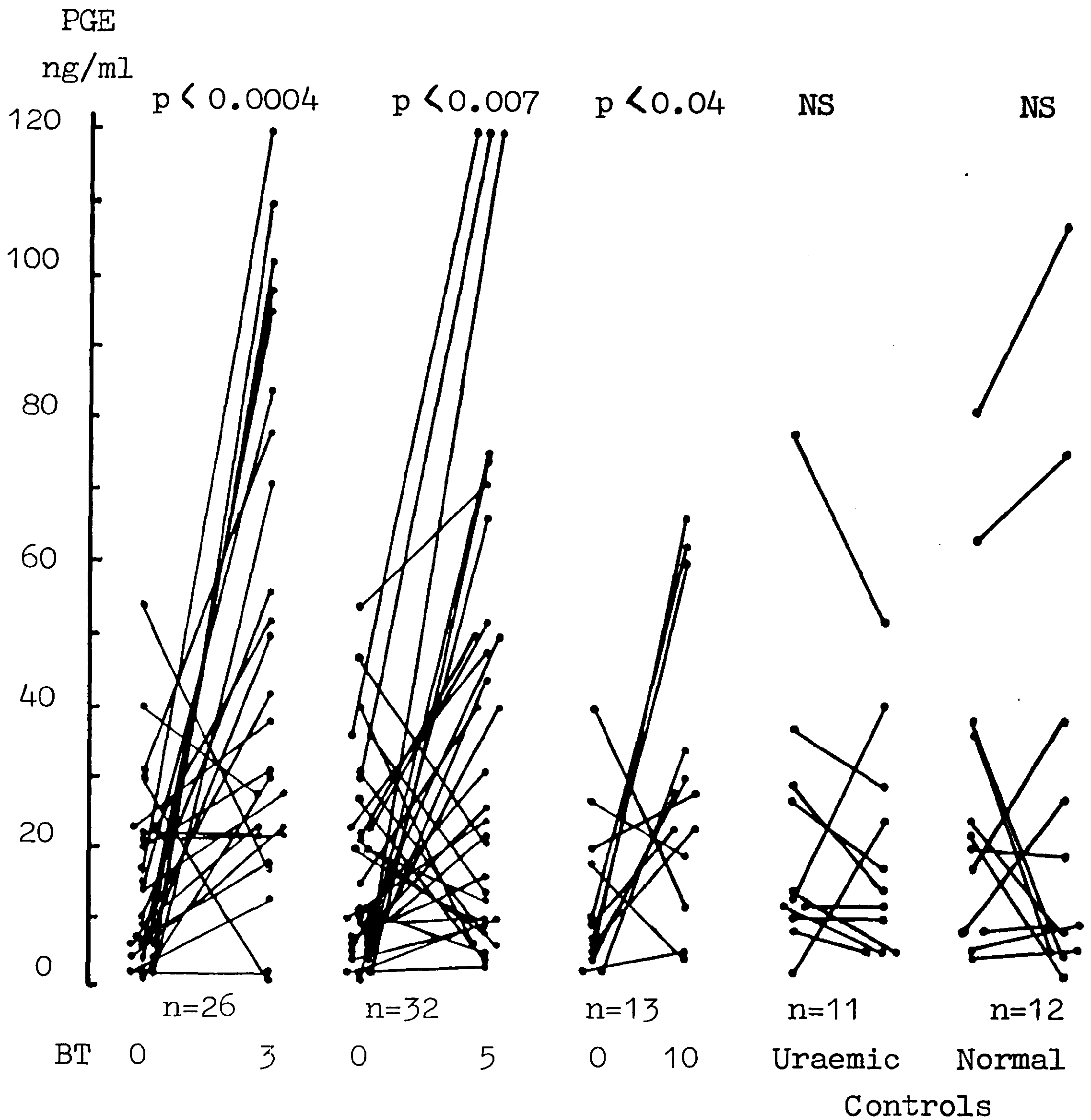


Figure 7.5. PGE concentrations released in Con A stimulated PBMC culture supernatants in the transfusion group before and after 3,5 and 10 units of packed cells, and in the uraemic and normal controls at 0 and 3 months.

TABLE 7.3. PROSTAGLANDIN E CONCENTRATIONS IN UNSTIMULATED AND CON-A STIMULATED PBMC CULTURE SUPERNATANTS IN THE WEAK AND STRONG DNCB RESPONDERS IN THE TRANSFUSION GROUP BEFORE AND AFTER EACH OF 1-10 UNITS OF PACKED CELLS

Mean ± SEM PGE concentrations (ng/ml)

No BT	0	1	2	3	4	5	6	7	8	9	10
DNCB 0-3:	n=15	n=12	n=13	n=12	n=14	n=15	n=6	n=5	n=6	n=6	n=4
Unstimulated	21±4	21±8	15±3	34±8	32±10	16±3	31±8	13±2	18±7	10±2	18±5
	n=17	n=12	n=13	n=14	n=14	n=15	n=7	n=6	n=6	n=6	n=5
Stimulated	23±6	25±7	25±6	41±9	46±11	26±5	30±7	26±11	30±10	13±2	29±9
DNCB >3:	n=21	n=18	n=19	n=19	n=17	n=21	n=5	n=7	n=6	n=4	n=8
Unstimulated	16±3	23±5	22±4	40±7	19±4	28±7	18±5	13±3	8±2	22±14	11±2
	n=22	n=18	n=19	n=20	n=19	n=22	n=6	n=7	n=6	n=4	n=8
Stimulated	16±3	33±8	25±5	50±8	30±5	43±8	47±17	17±4	16±4	29±13	31±8

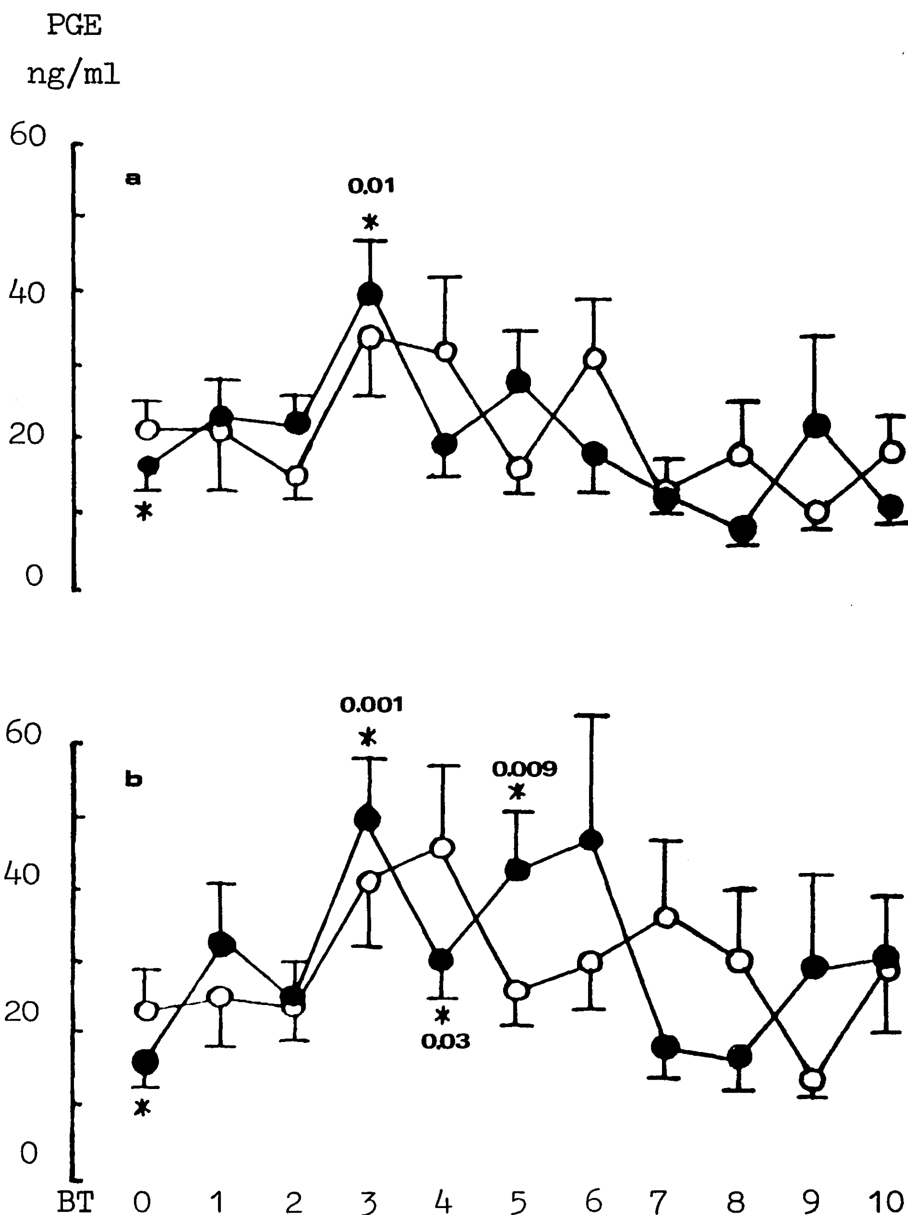


Figure 7.6. Mean ± SEM concentrations in unstimulated (a) and Con A stimulated (b) PBMC culture supernatants in the transfusion group before and after each of 1-10 units of packed cells; open circles = weak DNCB responders, closed circles = strong DNCB responders; number of patients tested on each occasion shown in Table 7.3.

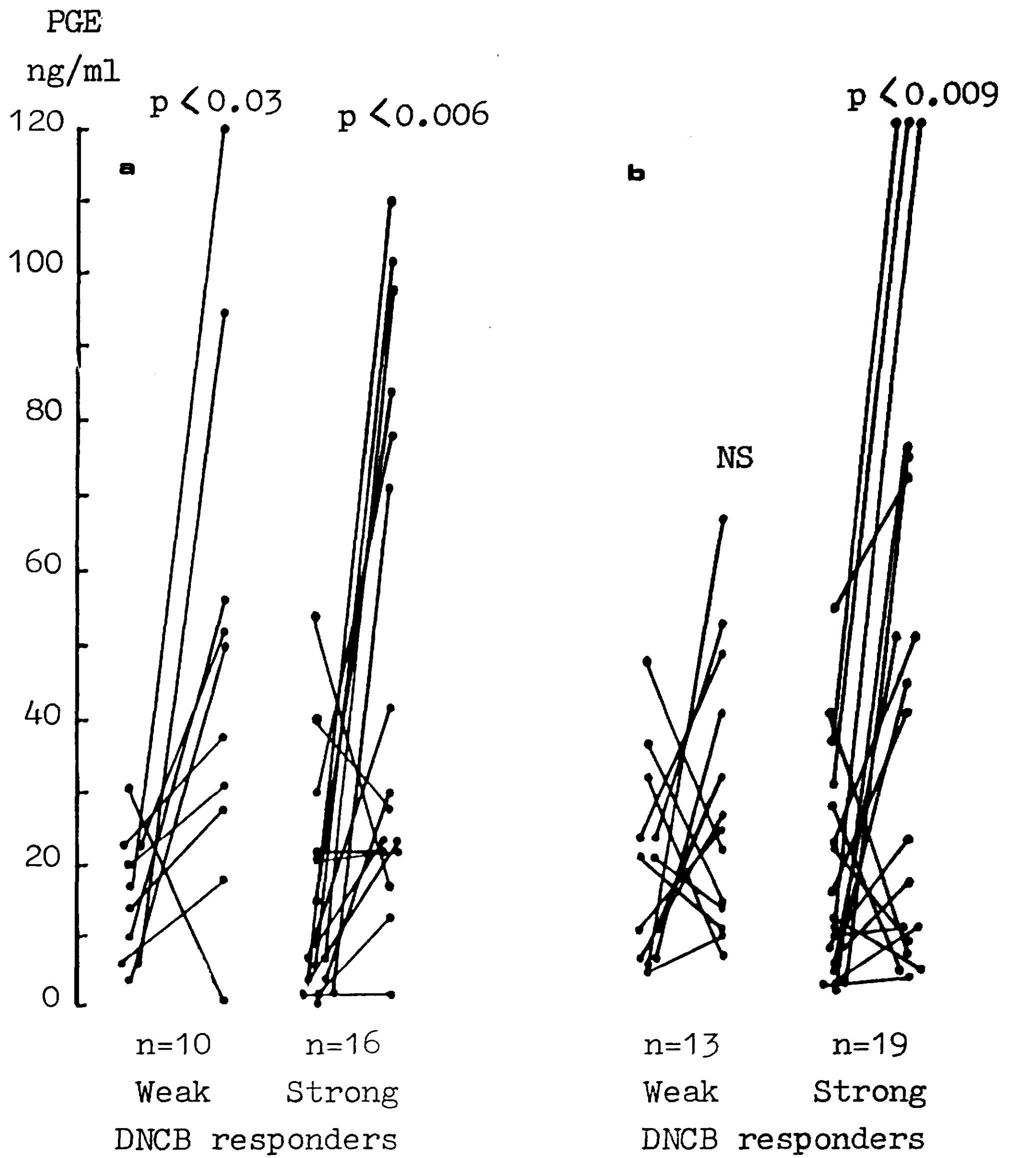


Figure 7.7. PGE concentrations in PBMC culture supernatants stimulated with Con A in the weak and strong DNCB responders before and after the 3rd transfusion (a), and before and after the 5th transfusion (b).

TABLE 7.4. PROSTAGLANDIN E CONCENTRATIONS IN UNSTIMULATED AND CON-A STIMULATED PBMC CULTURE SUPERNATANTS IN PATIENTS RECEIVING 5 OR 10 UNITS OF PACKED CELLS

Mean ± SEM PGE concentrations (ng/ml)

No. BT:	0	1	2	3	4	5	6	7	8	9	10
5 BT:	n=23	n=19	n=20	n=20	n=23	n=25					
Unstimulated	21±4	26±6	22±4	41±8	19±3	26±6					
	n=25	n=19	n=20	n=23	n=24	n=25					
Stimulated	19±3	33±7	28±5	50±8	31±5	42±7					
10 BT:	n=13	n=11	n=12	n=11	n=8	n=11	n=11	n=12	n=12	n=10	n=12
Unstimulated	12±3	15±5	14±3	32±7	41±3	16±3	25±5	13±2	13±4	15±6	13±2
	n=14	n=11	n=12	n=11	n=9	n=12	n=13	n=13	n=12	n=10	n=13
Stimulated	18±7	24±9	20±6	37±9	52±16	21±6	38±9	21±5	23±6	19±5	30±6

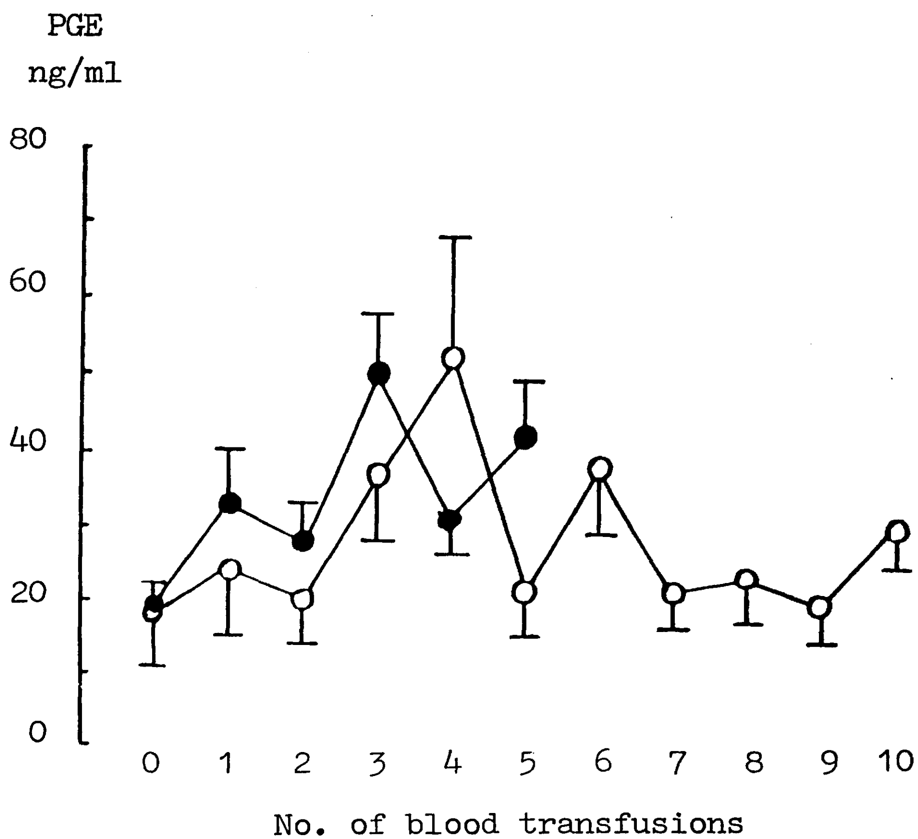


Figure 7.8. Mean ± SEM PGE concentrations in Con A stimulated PBMC culture supernatants before and after each transfusion in patients receiving 5 and 10 units of packed cells; number of patients tested on each occasion are shown in Table 7.4.

TABLE 7.5. PROSTAGLANDIN E CONCENTRATIONS IN UNSTIMULATED AND CON-A STIMULATED PBMC CULTURE SUPERNATANTS IN THE WEAK AND STRONG DNCB RESPONDERS RECEIVING 5 OR 10 UNITS OF PACKED CELLS

Mean ± SEM PGE concentrations (ng/ml)

	1	2	3	4	5	6	7	8	9	10	
DNCB 0-3:											
5 BT	n=10	n=9	n=8	n=7	n=10	n=10					
Unstimulated	25±6	26±10	15±1	32±13	18±5	15+4					
	n=11	n=9	n=8	n=9	n=10	n=10					
Stimulated	21±4	28±10	25±7	41±14	29±7	29±7					
10BT	n=5	n=3	n=5	n=5	n=4	n=5	n=6	n=5	n=6	n=6	n=4
Unstimulated	12±3	5±1	18±7	37±9	65±29	17±4	31±8	13±2	18±7	10±2	18±5
	n=6	n=3	n=5	n=5	n=4	n=5	n=7	n=6	n=6	n=6	n=5
Stimulated	27±15	16±4	25±13	41±10	88±26	18±4	30±7	26±11	30±10	13±2	29±10
DNCB >3:											
5 BT	n=13	n=10	n=12	n=13	n=13	n=15					
Unstimulated	18±4	26±7	27±6	45±10	20±5	32±9					
	n=14	n=10	n=12	n=14	n=14	n=15					
Stimulated	18±4	38±11	31±7	56±9	32±7	51±11					
10 BT	n=8	n=8	n=7	n=6	n=4	n=6	n=5	n=7	n=6	n=4	n=8
Unstimulated	13±5	18±7	12±2	28±10	18±6	16±5	18±5	13±3	8±2	22±14	11±2
	n=8	n=8	n=7	n=6	n=5	n=7	n=6	n=7	n=6	n=4	n=8
Stimulated	12±5	27±12	16±5	35±15	23±6	24±10	47±17	17±4	16±5	29±13	31±8

TABLE 7.6. CORRELATION BETWEEN SPONTANEOUS SAC-AND PWM-INDUCED IgG-PFC COUNTS IN THE PERIPHERAL BLOOD AND PGE CONCENTRATIONS IN PBMC CULTURE SUPERNATANTS STIMULATED WITH CON-A BEFORE AND AFTER 1-10 UNITS OF BLOOD

	Spearman correlation coefficients		
Variable	Spontaneous IgG-PFC	SAC- IgG-PFC	PWM IgG-PFC
Before BT (n=22)	-0.234 NS	0.504 $p<0.017$	0.385 NS
After 1BT (n=20)	0.357 NS	0.253 NS	0.233 NS
After 2 BT (n=19)	-0.185 NS	-0.058 NS	0.112 NS
After 3 BT (n=21)	-0.121 NS	0.147 NS	0.109 NS
After 4 BT (n=19)	-0.226 NS	0.081 NS	0.033 NS
After 5 BT (n=21)	0.082 NS	-0.287 NS	-0.329 NS
After 6 BT (n=9)	0.200 NS	0.000 NS	0.050 NS
After 7 BT (n=10)	0.207 NS	0.000 NS	0.499 NS
After 8 BT (n=10)	0.236 NS	0.018 NS	0.406 NS
After 9 BT (n=8)	0.119 NS	-0.381 NS	0.000 NS
After 10 BT (n=11)	0.100 NS	-0.082 NS	0.100 NS

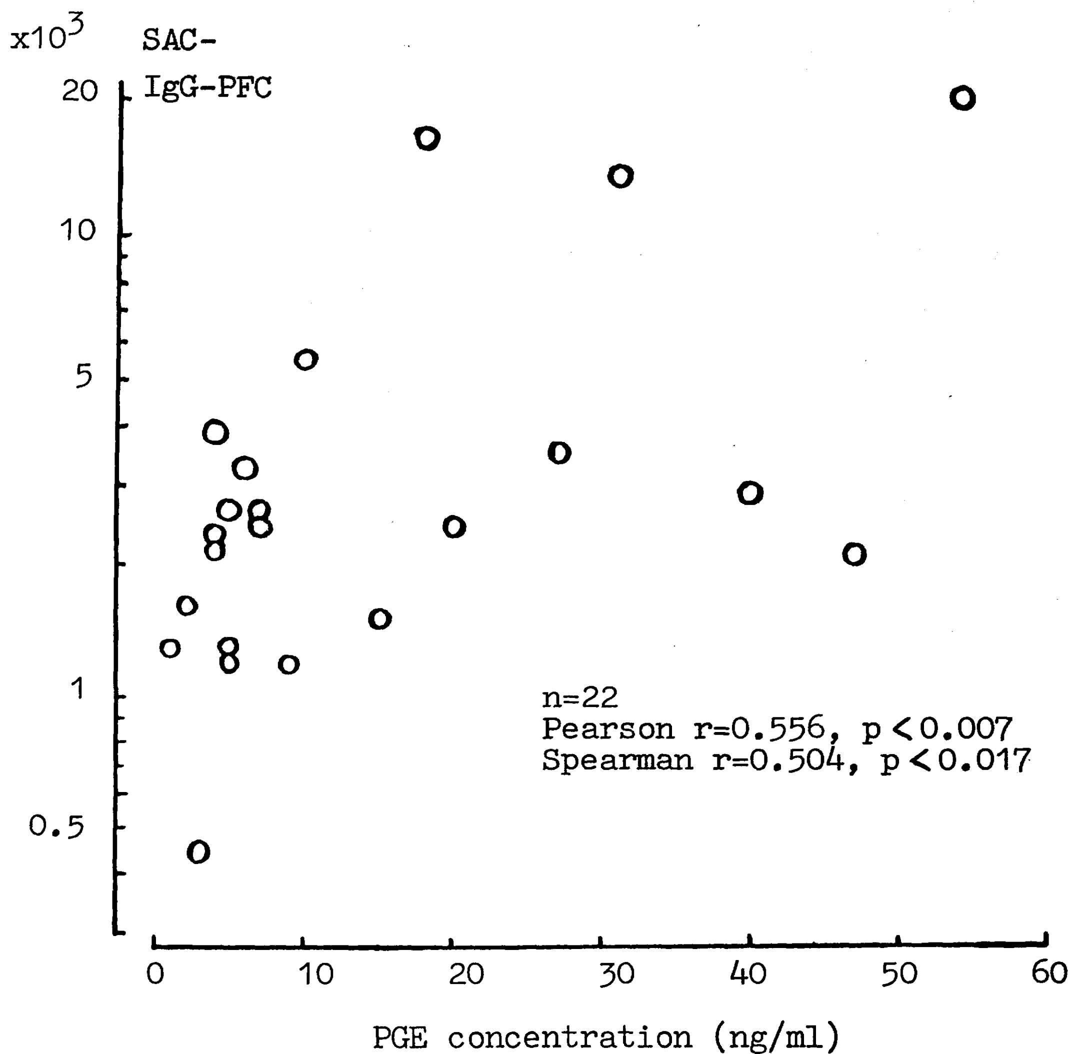


Figure 7.9. Correlation between PGE concentrations in PBMC culture supernatants stimulated with Con A and SAC-induced IgG-PFC counts in the peripheral blood; plotted values represent those prior to any blood transfusion in 22 of the 48 patients in the transfusion group.

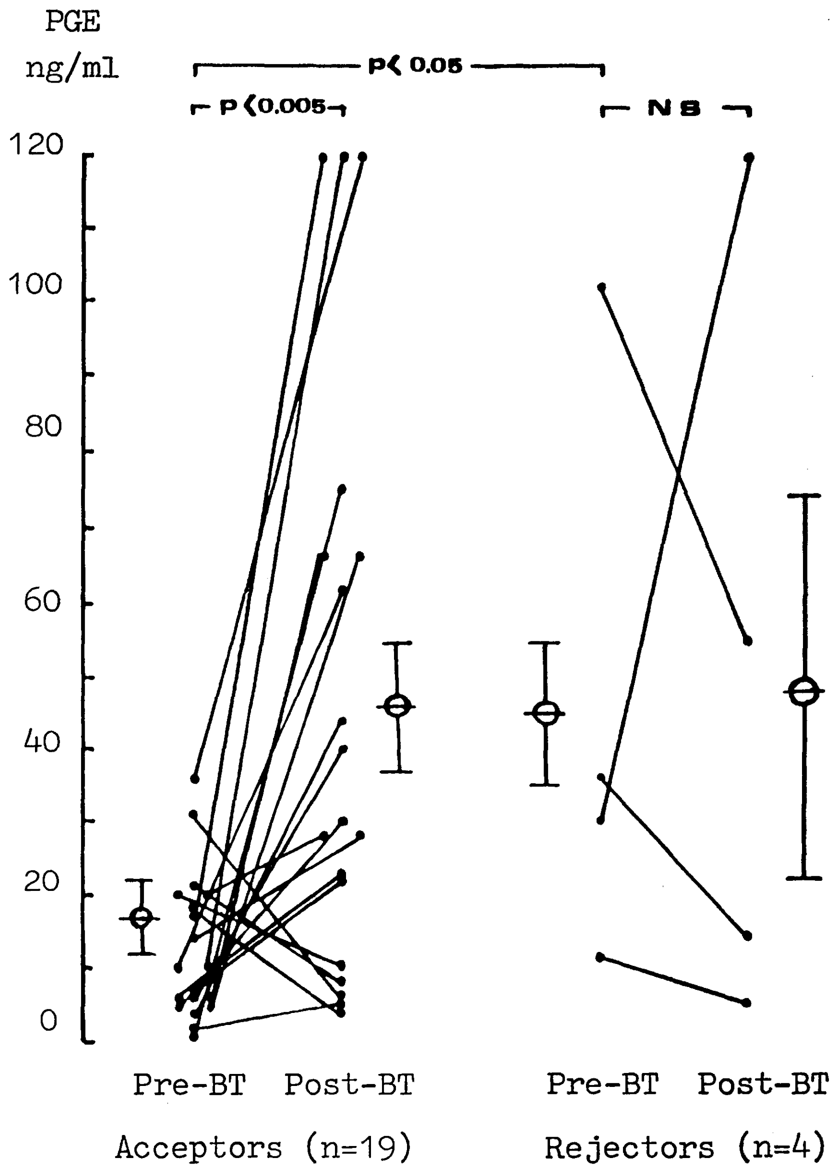


Figure 7.10. PGE concentrations in Con A stimulated PBMC culture supernatants before and after 5 or 10 units of packed cells in patients who subsequently had a transplant and accepted or rejected the graft.

TABLE 8.1 SERUM COMPLEMENT LEVELS IN THE TRANSFUSION GROUP BEFORE AND AFTER EACH TRANSFUSION, AND IN THE URAEMIC AND NORMAL CONTROLS

Mean ± SEM (ug/ml)

No. of BT	0 n=12	1 n=28	2 n=23	3 n=24	4 n=27	5 n=37	6 n=14	7 n=13	8 n=11	9 n=12	10 n=12
C3	1164±51	1137±49	1138±55	1095±69	1074±57	1085±37	1014±62	1043±88	1057±74	964±60	1115±77
C4	475±35	473±33	490±37	472±42	478±35	478±30	514±51	538±63	495±52	474±52	526±57
Factor B	185±10	180±11	188±13	173±11	170±9	173±7	172±13	177±20	167±11	157±12	168±14
C1 Inhibitor	280±14	263±11	281±11	256±12	268±12	283±9	267±15	259±21	250±10	256±13	281±12

Uraemic controls	At 0 n=12	At 3 months n=12
C3	1259±96	1208±49
C4	489±65	476±26
Factor B	208±72	212±26
C1 Inhibitor	324±41	319±22

Normal Controls	At 0 n=12	At 3 months n=12
C3	1251±53	1221±76
C4	350±26	327±30
Factor B	226±21	210±17
C1 Inhibitor	253±17	215±12

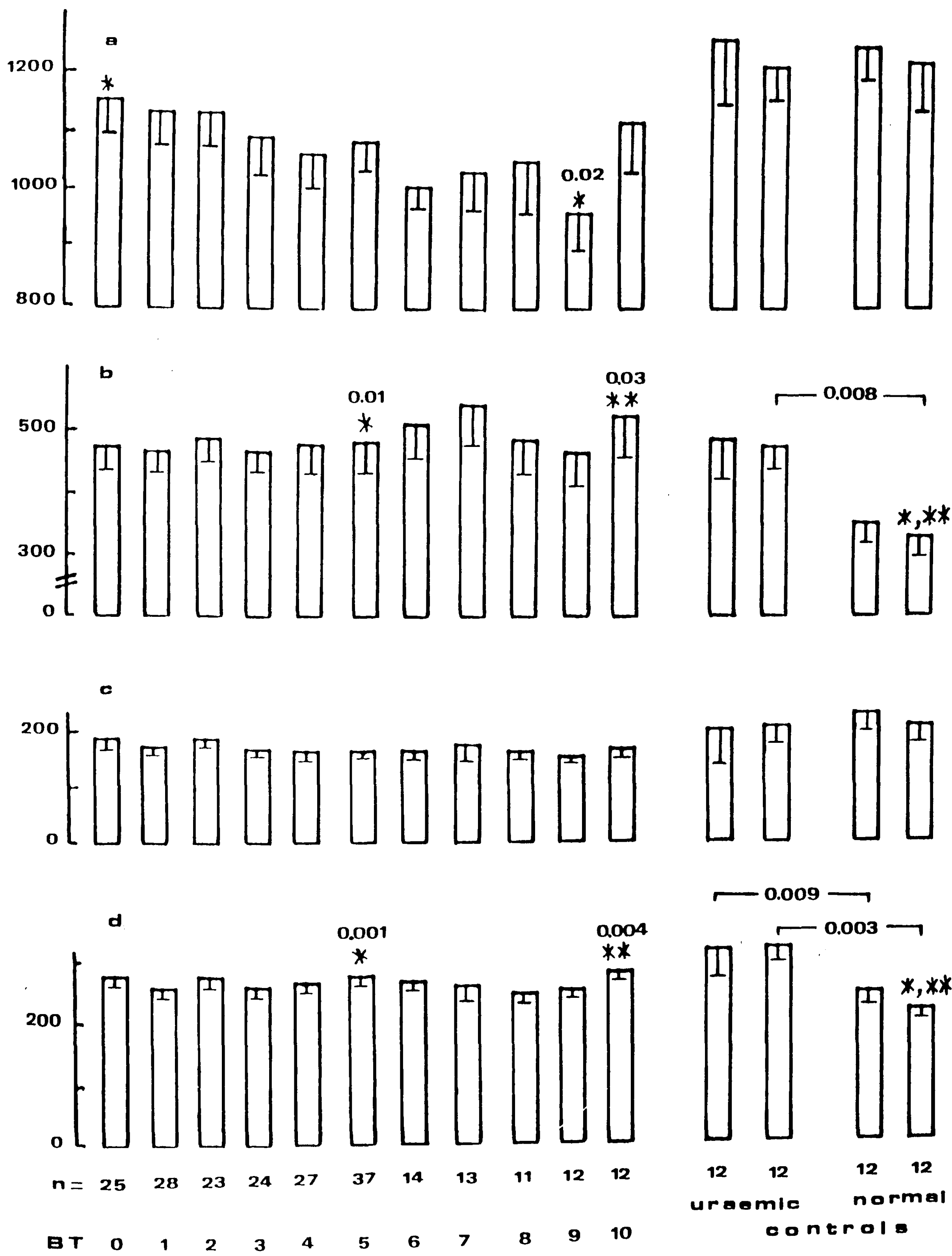


Figure 8.1. Serum complement levels in the transfusion group before and after each blood transfusion (BT), and in the uraemic and normal controls at 0 and at 3 months; a = C_3, b = C_4, c = factor B and d = C_1 inhibitor.

TABLE 8.2. SERUM COMPLEMENT LEVELS IN THE WEAK AND STRONG DNCB RESPONDERS BEFORE AND AFTER EACH BLOOD TRANSFUSION

Mean ± SEM (ug/ml)

No. of BT	0	1	2	3	4	5	6	7	8	9	10
DNCB 0-3:	n=10	n=12	n=12	n=11	n=11	n=17	n=8	n=6	n=6	n=6	n=5
C3	1308±77 [a]	1222±81	1271±54 [b]	1236±94	1236±81 [c]	1150±61	1058±76	1109±117	1146±100	985±90	1096±138
C4	475±64	451±56	477±42	470±64	494±69	429±40	411±33	450±56	432±50	379±56	422±62
Factor B	198±16	188±19	211±12	184±17	185±11	173±8	174±18	194±34	176±11	158±19	168±22
C1 Inhibitor	274±29	273±17	286±14	263±17	279±20	275±15	255±12	232±16	241±11	247±18	266±11
DNCB > 3:	n=15	n=16	n=11	n=13	n=16	n=20	n=6	n=7	n=5	n=6	n=7
C3	1068±56 [a]	1074±58	994±80 [b]	976±90	962±67 [c]	1030±43	955±104	987±134	951±98	944±87	1198±59
C4	475±42	490±40	503±65	474±57	468±37	520±41	651±83	614±103	570±91	569±73	600±77
Factor B	176±13	174±13	150±19	165±15	160±13	172±10	168±20	163±25	156±21	156±16	161±20
C1 Inhibitor	283±14	256±15	275±19	250±18	261±15	291±13	283±33	281±35	261±19	266±20	292±20

a, $p<0.02$; b, $p<0.008$; c, $p<0.02$

TABLE 8.3. ASSOCIATION BETWEEN THE DNCB SKIN TEST AND COMPLEMENT

Variable	Spearman correlation coefficients				
	C3	C4	B Factor	C1 Inhibitor	
DNCB 1st test	-0.221 n=25 NS	0.107 n=25 NS	0.007 n=25 NS	0.263 n=25 NS	Before BT
	-0.050 n=37 NS	0.203 n=37 NS	-0.011 n=37 NS	0.126 n=37 NS	After 5BT
	0.031 n=12 NS	0.308 n=12 NS	0.082 n=12 NS	0.313 n=12 NS	After 10BT
DNCB repeat test	-0.006 n=37 NS	0.161 n=37 NS	0.067 n=37 NS	0.180 n=37 NS	After 5BT
	0.219 n=12 NS	0.125 n=12 NS	-0.125 n=12 NS	0.318 n=12 NS	After 10BT

Spontaneous IgG-PFC

n=26
Pearson r=-0.394, p 0.02
Spearman r= -0.341,NS

Serum C_1 Inhibitor

SAC-IgG-PFC

x10^3

n=26
Pearson r=0.357, p 0.04
Spearman r= -0.356, NS

Serum C_1 Inhibitor

Figure 8.2. Correlation between spontaneous IgG-PFC and SAC-induced IgG-PFC counts with serum C_1 Inhibitor levels.

CHAPTER 8. THE EFFECT OF BLOOD TRANSFUSION ON SERUM COMPLEMENT LEVELS

8.1 Introduction

Activation of the complement system leading to hypocomplementaemia and complement mediated tissue injury may occur in certain types of glomerulonephritis such as post-streptococcal and mesangiocapillary glomerulonephritis (reviewed in 590). In chronic renal failure, chemotaxis, opsonisation, phagocytosis and bacteriolysis, in all of which complement has a role, are impaired (314,323,328,329,331-337). Thus abnormalities of the complement system may exist in chronic renal failure yet only a few reports have been published and these contain conflicting results (331-337). As blood transfusion is associated with suppression of the immune response and altered lymphocyte reactivity (393,394,531-535,537), and complement has an immunoregulatory role in antibody induction and in the generation and maintenance of memory B cells (reviewed in 107), it seemed worth looking at complement levels before and after planned blood transfusion in renal failure. Thus in this chapter the association between complement levels and IgG production occurring spontaneously and after stimulation with SAC and PWM is described both in weak and strong DNCB responders and before and after blood transfusion.

Patients and Methods

Patients

In 40 of the 48 transfused patients (Table 1) a complement profile was carried out prior to and following blood transfusion. Among these 40 patients there were 31 males and 9 females and their mean age was 40.4 years (range 18-59 years). The original renal diseases were glomerulonephritis 14 (35%), chronic pyelonephritis 10 (25%), polycystic kidneys 4 (10%), hypertension 3 (7.5%), diabetes 5 (12.5%), Alport's syndrome 1 (2.25%) and of unknown origin 3 (7.5%). Thirty one of these patients were on haemodialysis and the remaining 9 patients were on CAPD. As a result of the randomisation at the begining of the study these 40 patients were allocated according to their response to the DNCB test and the number of transfusions as

follows. Of the 19 weak DNCB responders, 13 patients received 5 units of packed cells and 6 patients had 10 units; the remaining 21 patients were strong DNCB responders of whom 11 patients received 5 units of blood and the remaining 10 patients had 10 units. In addition to the patients in the transfusion group the 12 uraemic and the 12 normal controls were also studied.

Methods

Serum samples in the transfusion group were harvested before and 14 days after each transfusion from the same blood specimens taken to perform the other tests described in previous chapters. There was not sufficient sera to carry out complement profiles on all occasions, so the number of patients differs prior to and following each transfusion. To compensate for this, statistical analysis was done using both matched paired and unpaired tests. The two control groups were tested 3 to 4 times over a period of 3 months.

Estimation of C3, C4, factor B and C1 inhibitor serum levels were carried out using the single radial immunodiffusion technique (107) which is the standard method used in this hospital. Functional tests of complement activation were not done because sera has to be stored at -70°C and not at -20°C, as was the case in this study.

Statistical Analysis

The individual variation in complement levels was not large and the standard error was relatively small and consistent. However, the distribution of the values was not normal, so comparison of mean complement levels between and within groups was done using the Mann-Whitney test for unpaired samples and the matched paired Wilcoxon test for paired samples. Regression analysis was done using both Pearson (parametric) and Spearman (non-parametric) correlation coefficients depending on the appearance in the scattergrams.

8.3 Results

Table 8.1 shows the mean serum complement levels in the transfusion group prior to and after each transfusion, and in the two control groups over a period of three months. The statistical analysis is shown in Figure 8.1.

The difference in mean C3 levels following each blood transfusion in comparison with the background mean value was not significant at any time except following the ninth unit of blood (Figure 8.1a). This significance was a weak one and very likely to be accidental since it is not consistent with the overall pattern. The difference in C3 levels in the two control groups was not significant over a period of three months. Also, C3 concentrations prior to and after the fifth or tenth unit of blood were not significant in comparison with both uraemic and normal controls at 0 and at 3 months, respectively.

Comparison between C4 levels prior to and following each transfusion showed no significant difference and this was the case also when mean values were compared between the transfusion group before and after the fifth or tenth transfusion and those of the uraemic controls at 0 and at 3 months. However, mean C4 concentrations of patients both in the transfusion group and in the uraemic controls appeared to be consistently higher compared to those of the normal controls. Mean C4 levels in the transfusion group after the fifth and tenth unit of blood were significantly higher compared to levels in normal controls at 3 months (mean $\pm$ SEM, 478 ± 30 v 327 ± 30, $p<0.01$, and 526 ± 57 v 327 ± 30, $p<0.03$ respectively). Also the difference in C4 concentration between uraemic and normal controls at 3 months was significant (mean $\pm$ SEM, 476 ± 26 v 327 ± 30, $p<0.008$).

A similar finding was observed in C1 inhibitor concentrations. Blood transfusion did not induce any changes in serum levels but mean values in both transfused and uraemic controls were consistently higher compared to those of the normal controls. Levels after the fifth or tenth transfusion were significantly higher than those of normal controls at 3 months (mean $\pm$ SEM, 283 ± 9 v 215 ± 12, $p<0.001$ and 281 ± 12 v 215 ± 12, $p<0.004$ respectively). Also the C1 inhibitor concentration was significantly higher in the uraemic controls than in the normal controls at 0 and at 3 months (mean $\pm$ SEM, 324 ± 41 v 253 ± 17, $p<0.009$ and 319 ± 22 v 215 ± 12, $p<0.003$ respectively) (Figure 8.1d). This finding shows that uraemic patients regardless of blood transfusion status tend to have higher C4 and C1 inhibitor serum levels than healthy subjects.

There were no differences in factor B levels between the transfusion group, the uraemic controls and the normal controls in any combination of comparisons (Figure 8.1c).

Table 8.2 shows the serum complement levels in the transfusion group divided into weak and strong DNCB responders. Blood transfusion did not have any effect on C3, C4 factor B and C1 inhibitor concentrations in either group. The difference in mean C3 levels between weak and strong DNCB responders prior to and after the second and fourth transfusion was significant ($p<0.02$, $p<0.008$ and $p<0.02$ respectively), but this was an inconsistent finding since the difference in C3 levels between normal controls, who by definition are strong DNCB responders, and the overall group of uraemic patients was not significant (Figure 8.1a). To further check this, regression analysis of DNCB scores at the first and on the repeat test against the concentration of complement components was performed and did not show any correlation before and after the fifth or tenth transfusion (Table 8.3).

In order to see whether there was any association between complement components and IgG production, the individual serum C3, C4, factor B and C1 inhibitor levels before and after each transfusion were plotted against the respective spontaneous, SAC- and PWM- induced IgG- PFC counts. There was no correlation between serum C3 concentrations and IgG production in any system and this was the case also with regard to C4 and factor B levels. There was a weak correlation between serum C1 inhibitor concentration after the fifth transfusion and the respective spontaneous and SAC-IgG-PFC counts (Figure 8.2). The significance of this correlation, although interesting in theory, is rather uncertain and inconsistent, since it was not confirmed by the non-parametric Spearman test and was not observed on any occasion other than after the fifth transfusion.

8.4 Conclusions

1) Transfusion of 1-10 units of blood does not induce any measurable changes in C3, C4, factor B and C1 inhibitor levels in previously non-transfused uraemic patients.

2) C3 and factor B concentrations in uraemic patients are comparable to those of normal controls, whereas C4 and C1 inhibitor levels are significantly higher.

3) There is no difference in C3, C4, factor B and C1 inhibitor levels between weak and strong DNCB responders and blood transfusion has no effect on complement component concentrations in either group.

4) There is no correlation between serum C3, C4, factor B and C1 inhibitor levels and IgG production both spontaneously and after SAC or PWM stimulation before or after transfusion.

5) It should be emphasised that the above statements are made with regard to measureable serum complement levels and do not exclude possible activation of the complement cascade or its components in cellular interactions in response to blood transfusion.

CHAPTER 9. THE RESPONSE TO THE DNCB SKIN TESTING IN URAEMIC PATIENTS AND ITS PREDICTIVE VALUE IN RENAL TRANSPLANTATION

9.1 Introduction

We have shown in previous studies that strong DNCB responders are more likely to reject a renal allograft (399-402,571). This association between the DNCB response and graft survival was found irrespective of whether the patient had received previous blood transfusion or the type of blood product given frozen red cells, whole blood or packed cells. These results were obtained from the analysis of data during a period when deliberate blood transfusion prior to transplantation was not being practised. Since 1979, all patients have been deliberately given a minimum of 5 units of packed cells prior to the transplant. With the adoption of this deliberate transfusion policy, we observed that the DNCB skin test had lost its predictive value because the improvement in graft survival had occurred almost entirely among the strong DNCB responders (571). We looked into factors determining the response to the DNCB and found that sex, polycystic kidney disease, duration of dialysis and blood transfusion might influence the response to the test (402). As there is an overlap between the effect of blood transfusion and the other three factors, we needed to clarify the precise effect of transfusion on the DNCB response. This was done by setting up the randomised control study which has been discussed in the previous chapters. This study confirmed that a strong response to DNCB is more likely in previously non-transfused uraemic patients than in those already transfused and is maintained after transfusion. This suggests that primary immunisation to DNCB is a crucial event and blood transfusion may interfere with the response to it.

The aim of this chapter is to analyse retrospectively our experience with DNCB skin testing from that point of view and try to explain the apparent discrepancies in our previous studies.

Patients and Methods

Patients

From 1975 to 1984 the DNCB skin test was carried out in 535 patients. There were 321 males (60%) and 214 females (40%) with a mean age of 38.7 years (range, 10-69 years). The original renal diseases were glomerulonephritis 194 (36%), pyelonephritis 133 (25%), polycystic kidneys 54 (10%), hypertension 39 (7%), diabetes 24 (5%), Alport's syndrome 13 (2%), analgesic nephropathy 8 (1.5%), hypoplastic kidneys 8 (1.5%), SLE 6 (1%), nephronophthisis 5 (1%), amyloidosis 4 (1%), Henoch Schonlein purpura 4 (1%), renal artery stenosis 3 (.5%), interstitial nephritis 3 (.5%), tuberculosis 3 (.5%), renal vascular disease 3 (.5%), Wegener's granulomatosis 2 (.5%), 7 patients (1.5%) had miscellaneous diseases and in 22 (4%) the disease was of unknown origin. Forty one of these patients were in end-stage renal failure and tested prior to commencement of dialysis, 464 patients were tested while on dialysis (372 on haemodialysis and 92 on CAPD) in 4 different hospitals in Glasgow, and the remaining 30 patients were tested after transplantation while receiving immunosuppression.

The DNCB skin tests were carried out as described in Chapter 1 by three doctors over a period of 10 years. Twenty percent of the patients (105 of 535) were sensitised by Dr A Diamandopoulos during 1975 - 1978, 55% (296 of 535) by Dr M A Watson during 1978 - 1981 and the remaining 25% (134 of 535) by the author during 1981 - 1984. From 1975 - 1981 the timing of sensitisation to DNCB was random, but from 1981 onwards was within 3 to 6 months from commencement of dialysis. Follow-up tests were performed after variable intervals of 3, 6, 12, 24 or more months from each other. Prior to May 1979 there was no deliberate blood transfusion policy and patients were given whole blood, fresh concentrated red cells or frozen red cells, as required for symptomatic anaemia. From May 1979 onwards, all patients were deliberately transfused with 5 units of fresh concentrated red cells, one unit at two week intervals, before their names were added to the transplant waiting list. After the elective blood transfusion more blood (whole blood or packed cells) was given

to patients if clinically indicated. In addition to this, patients on the transplantation list from one of the 3 hospitals, received one unit of elective transfusion every 6 months after the last elective transfusion.

Three hundred and forty four of the 505 patients who had the DNCB skin test prior to or after commencement of dialysis were subsequently transplanted. Two hundred and eighty four of these patients were immunosuppressed with azathioprine and prednisolone, 57 received cyclosporin A and prednisolone and the remaining 3 had a combination of the two regimens. From 1975 to May 1979 a high initial dose of prednisolone was used and thereafter a low prednisolone regimen was adopted. Cyclosporin A was introduced at the beginning of 1984. The immunosuppression protocols have been described in previous studies (390,399,447,571) and in Chapter 4. The diagnosis of rejection episodes was based on clinical grounds, combined with the use of ultrasound and biopsy, and they were treated either by iv methylprednisolone pulses or by a 10 day course of high dose oral prednisolone. Selection of recipients was done on a best match basis and transplantation was carried out only when the T cell crossmatch was negative using both current and historical sera. HLA matching, and the crossmatch and PRA techniques have been described in Chapter 5. The time of graft failure was defined as the time of transplant nephrectomy or recommencing dialysis. For the estimation of patient survival, patients were considered at risk from the date of transplantation to 6 months after graft failure. The follow-up of these patients was extended to the end of 1985.

Statistical Analysis

Data regarding the following variables were recorded and stored in the computer. Sex, age, original renal disease, nephrectomy prior to transplantation, the hospital where patients were treated, ABO and rhesus blood group, parity, type of dialysis, duration of dialysis, number and type of blood transfusion, the DNCB scores on each occasion, the time interval and number of blood transfusions between tests and transplantation, HLA-DR phenotypes, transplant outcome,

type and number of transplant, HLA mismatches, type of immunosuppression, onset of graft function, number of rejection episodes, timing of first rejection episode, and PRA at the time of the DNCB test, and prior to and after transplantation. Statistical analysis in this chapter was carried out using both univariate and multivariate analysis. Crosstabulation of distribution of two or more categorical variables was computed using the chi-square test. Fisher's exact test was applied when there were fewer than 20 cases in a 2 x 2 table and Yates' correction was computed for all other 2 x 2 tables. In the analysis of possible association between HLA DR antigens and response to DNCB, p values were corrected multiplying them by the number of DR antigens included in the analysis. Comparison of mean values between groups was carried out using one way analysis of variance, the Mann-Whitney and the Wilcoxon tests. Regression analysis was done using the Pearson and Spearman tests. Multiple regression analysis was carried out to check correlation and possible overlapping effect between variables. Stepwise discriminant analysis was also used to see which were the most significant variables in the model, and multiple regression was repeated controlling for these variables in order to confirm and exclude the overlapping effect. Graft survival was estimated using life table analysis and comparisons between subgroups was done using a statistic D, which was calculated from the survival scores using the algorithm of Lee and Desu (569). D is distributed as chi-square with g-1 degress of freedom, where g = the number of groups under the null hypothesis that the subgroups are samples from the same survival distribution. The larger the D statistic, the more likely that the subgroups come from different survival distributions.

Results

9.3.1 Response to DNCB in the pre-dialysis, dialysis and transplant patients

Results of the DNCB scores in the pre-dialysis, dialysis and transplant patients are shown in Figure 9.1. The scores of a historical group of 15 normal controls were also included in this

figure to illustrate the cut off point between weak and strong DNCB responders. The difference in the mean score between pre-dialysis and dialysis patients was not significant (3.5 ± 4.0 v 3.2 ± 3.5), but transplant patients, as expected, had significantly lower reponse compared to both groups (1.0 ± 1.7 v 3.5 ± 4.0, $p<0.005$, and 1.0 ± 1.7 v 3.2 ± 3.5, $p<0.001$ respectively). The proportion of strong responders was also significantly lower among the transplant patients (3 of 30, 10%) compared to that of pre-dialysis and dialysis patients (16 of 41, 39%, $p<0.01$ and 160 of 464, 34%, $p<0.01$ respectively) (Figure 9.2). Two thirds of the transplant patients were anergic (20 of 30, 67%) compared to approximately one third of the two other groups (17 of 41, 41% and 148 of 464, 32%).

All 30 transplants were sensitised to DNCB under immunosuppression with azathioprine and prednisolone within the first year from the operation. The idea of doing this was to test the effectiveness of the pharmacological immunosuppression in relation to graft survival. This idea was eventually abandoned because of the small proportion of strong responders among transplants. Of the 3 strong responders, however, all had functioning grafts at the end of the study after 11, 11 and 10 years from the time of the test, and so did 23 of the 27 weak responders (85%) with an average of a 10 year graft survival. Of the remaining 4 transplants in the latter group, 2 died with a a functioning graft after 6 months and 3 years from left ventricular failure and empyema of the gall bladder respectively, one rejected the graft after 5 years and one transplant was a technical failure. Judging from these findings, it is unlikely that the DNCB test would have been of any predictive importance in this group of patients. Therefore, these patients will not be further discussed in the following sections.

The analysis which will be described from here on is based on the pre-dialysis and dialysis patients. These two groups were comparable in terms of sex, age and original renal disease. Fifty two percent of the latter patients were tested within three months from commencement of dialysis, 80% within one year and 11% (50 of 464) after 3 or more years of dialysis (Table 9.1).

9.3.2 Factors determining the response to DNCB in end-stage renal failure.

Among the 505 patients (41 predialysis and 464 dialysis patients) who were considered for analysis in this section, there were 329 weak DNCB responders (65%), and the remaining 176 were strong responders (35%). The results of the DNCB scores of these patients are shown in Table 9.2 and Figure 9.3 illustrates the cummulative percentages. Thirty three percent of the patients (165 of 505) were anergic to DNCB and only 5% (34 of 505) had a score >10. Their mean DNCB score was significantly lower compared to that of the historical group of normal controls (3.2 ± 3.6 v 9.1 ± 2.6, $p<0.001$). The CMI, therefore, in these patients as assessed by the DNCB skin test was profoundly depressed. To examine what factors might be associated with the response to DNCB, we looked into the possible association with the following variables, firstly, in a univariate fashion analysis.

Sex: Among the 505 patients there were 303 males (60%) and 202 females (40%). The males appeared to have significantly more strong responders compared to females (118 of 303, 39% v 58 of 202, 28%, $p<0.01$) (Figure 9.4).

Age: The mean age in the weak and strong responders was 38.4 ± 13.4 years (range, 10-69 years) and 40.2 ± 12.5 years (range, 12-67 years), respectively. The difference between the two groups is not significant. Also, the prevalence of strong responders in the different age groups was not significantly different (Figure 9.5).

Original renal disease: Figure 9.6 shows that the frequency of strong responders patients with polycystic kidneys, although statistically not significant, was the highest among the various other diseases.

Nephrectomy: Twenty two of the 505 patients had had bilateral nephrectomy prior to the test. Only two of them were strong responders (9%), while in the remaining 483 non-nephrectomised patients there were 174 (36%), $p<0.02$ (Figure 9.7).

Hospital: The hospital where patients had been treated at the time of testing was not a significant factor (Figure 9.8).

Underlined ABO and rhesus blood groups: In 444 of the 505 patients information with regard to ABO and rhesus blood groups was available. The incidence of strong responders was not significantly different in the four ABO blood groups nor in the rhesus positive or negative patients (Figure 9.9).

Parity: Data on parity was available in 186 of the 202 females (92%). In 75 of them there was no history of pregnancy, miscarriage, stillbirth or termination and in the remaining 103 there was such a history. The difference in strong responders among nulliparous and parous women was not significant (Figure 9.10).

Type of dialysis: Of the 464 dialysis patients 372 were treated with haemodialysis and the remaining 92 with CAPD. The difference in the response to DNCB between the two groups was not significant (Figure 9.11).

Duration of dialysis: The mean duration of dialysis prior to the test in the weak DNCB responders was 12.2 $\pm$ 19.5 months (range, 1-168 months), while that of the strong responders was 5.9 $\pm$ 11.7 months (range, 1-94 months). The difference between the two groups is highly significant ($p<0.0002$). Figure 9.12 shows that the proportion of strong responders progressively decreased from 48% (53 of 111) among patients sensitised within a month from commencement of dialysis to 12% (6 of 49) in those sensitised after two years of dialysis ($x^2 = 23.400$, DF = 5, $p<0.0003$).

Blood transfusion: The variable with the strongest association, among all significant variables, with the response to DNCB was the number of blood transfusions prior to the test. The mean number of transfusions in the 329 weak responders was 5.5 $\pm$ 8.4 units (range, 0-92 units), while that of the 176 strong responders was 1.9 $\pm$ 3.4 units (range, 0-24 units), $p<0.0000$. Figure 9.13 shows the progressive decline in strong response to DNCB with the number of transfusions prior to the test. Among the 171 patients who were sensitised to DNCB prior to any transfusion there were 97 strong responders (57%). In patients sensitised after one unit of blood there were 55% strong responders (12 of 22), but after two

transfusions there was a marked fall to 28% (21 of 74) and this decline progressed to only 6% (1 of 16) in the patients sensitised after more than 20 units of blood (x^2 = 68.467, DF = 7, p<0.0000).

Interval between last transfusion and test: Among the 334 patients who were sensitised to DNCB after one or more units of blood the time interval between the last transfusion and the test was known in 292 patients (87%). The difference between the 212 weak responders whose mean interval was 16.4 ± 40.8 weeks (range, 1-468 weeks), and that of the 80 strong responders whose interval was 17.8 ± 38.9 weeks (range, 1-240 weeks) was not significant. Also, the proportion of strong responders was not significantly different among patients sensitised after variable time from the last transfusion (Figure 9.14).

Panel reactive antibodies (PRA): Information on PRA prior to the test was available in 351 patients. Two hundred and twenty two of them were weak responders and the remaining 129 were strong responders. The difference between the two groups in PRA in both current and historical sera was not significant. Weak responders in current sera had a mean ± standard deviation of 4.2 ± 15% PRA (range, 0-91%) and strong responders had 4.2 ± 16% (range, 0-95%). In historical sera the respective values for weak and strong responders were 6.2 ± 18.6% (range, 0-100%) and 4.9 ± 17.4% (range, 0-95%). Figure 9.15 shows that the proportion of strong responders was not significantly different in the split groups of patients with 0, 1-20, 21-80 and 81-100% PRA prior to the test. These findings could be explained on the basis of observations from Chapters 5 and 6, where weak DNCB responders were capable in eliciting comparable antibody production to that of strong responders when given more than 3 units of blood. Thus, pre-existence of PRA in multi-transfused patients does not seem to preclude, interfere or predict subsequent response to DNCB.

Period when the test had been performed: It has already been mentioned that the 505 patients were tested over a period of 10 years by 3 different investigators. There were differences in the timing of the test with regard to blood transfusion and commencement of dialysis between the three periods. In the early period patients

were sensitised to DNCB irrespective of their transfusion status and the duration of dialysis. After 1981, however, we attempted to sensitise patients soon after commencement of dialysis and there was a deliberate policy to sensitise more patients prior to any blood transfusion and see what happened with the follow-up testing. Figure 9.16 shows that there were progressively more strong responders during the three periods (25%, 32% and 45% respectively, $x^2 = 10.826$, DF = 2, $p<0.005$). This raised the question of reproducibility of the test and possible research bias or subjective error, but multivariate analysis, as will be discussed later, showed that this was not the case.

HLA-DR phenotypes: Table 9.3 shows the frequency of HLA-DR antigens in 362 of the 505 patients (72%) in whom tissue typing had been carried out. Table 9.4 shows the frequency of weak and strong DNCB responders in relation to their HLA-DR phenotypes. There were more strong responders among DRW6 positive patients (52%) but the difference was not significant after correction of the p value for the number of antigens.

So far, with regard to factors which might be associated with the response to DNCB, 5 variables were found by univariate analysis to be significant. These were sex, nephrectomy, duration of dialysis, blood transfusion and the period during which patients were sensitised to DNCB. There is overlap between these variables, and this is clearly demonstrated in Table 9.5, which shows the correlation coefficients between these variables. All four variables which correlated with the response to DNCB showed a strong correlation with blood transfusion as well (bottom line in Table 9.5). These correlations were confirmed with the Spearman rank coefficients. Thus, patients sensitised in the earlier period had received more blood, were on dialysis for longer and included more nephrectomised patients among them. The same conclusion applies vice versa for the duration of dialysis and nephrectomised patients. With regard to sex, females had received more blood ($p<0.002$), but there was no correlation between sex and the other three variables, namely,

time period, duration of dialysis and nephrectomy. Therefore, it seemed logical to see what happens when the overlapping effect of blood transfusion is taken into consideration.

The partial correlation procedure in the SPSSX statistical package computes correlation coefficients controlling for one or more other variables. Using this procedure, correlation between the DNCB scores and the other four significant variables, while controlling for the variable blood transfusion, gave the results shown in Table 9.6. The variables, nephrectomy and time period were not significant any more and sex showed only a weak correlation ($p<0.05$). The duration of dialyis, however, showed a strong inverse correlation with the DNCB scores ($p<0.007$).

Stratification of patients in non-transfused and transfused prior to the test.: To double check these results, cross-tabulation of categorical variables and frequencies of continuous variables were carried out after patients had been stratified into those who were sensitised to DNCB prior to any transfusion and those sensitised after one or more transfusions. Figure 9.17 shows the proportion of weak and strong responders according to their sex in the 171 and 334 patients who were sensitised to DNCB prior to any and after one or more units of blood respectively. The difference was not significant in either group. From the number of males and females in this figure it is apparent that there were more females in the transfused group compared to males (150/202, 74% v 184 of 303, 61%, x^2 = 9.909, $p<0.005$). Also, females in the transfused group had received more units of blood compared to males (mean $\pm$ SD, 7.0 $\pm$ 7.7 v 5.9 $\pm$ 8.4, $p<0.05$). From these findings it appears that there is a contradiction with the findings from the partial correlation analysis, where sex was found to be weakly significant after excluding the transfusion effect. This is due to the fact that in the latter analysis, the response to DNCB was used as a continuous variable (ie, DNCB scores, 0-15), while here this variable has been transformed to a dichotomous variable (ie, weak or strong responders). This contradiction implies that the significance of sex in the response to DNCB, if any, could be due not only to the fact

that females were more likely to have been transfused prior to sensitisation, but also they were likely to have received more blood as well. With regard to the nephrectomised patients, all 22 of them had been transfused prior to the test and had received more transfusions (mean ± SD, 12.0 ± 12.6 v 6.1 ± 7.6, $p<0.03$) compared to non-nephrectomised patients. Figure 9.18 shows the proportion of weak and strong responders in the 334 transfused patients in relation to nephrectomy. The difference in strong responders between patients who had and had not had nephrectomy is not significant (10% v 24%). The proportion of strong responders during the three time periods in the 171 non-transfused patients was not significantly different (68%, 54% and 56% respectively) (Figure 9.19). However, in the transfused patients the difference was significant (13% v 24% v 34%, $x^2 = 8.155$, DF = 2, $p<0.02$) due to a difference in the number of transfusions prior to the test. Patients in the first period had received a mean ± SD of 8.8 ± 13.0 units of blood compared to 5.6 ± 6.3 and 6.8 ± 6.6 in the second and third period ($p< 0.008$ and $p<0.012$ respectively).

Table 9.7 shows the statistics for the duration of dialysis and the number of blood transfusion in the subgroups of the non-transfused and transfused weak and strong DNCB responders. Patients sensitised to DNCB after one or more units of blood had been on dialysis for longer compared to those who were sensitised prior to any transfusion (mean ± SD, 12.8 ± 19.8 v 3.9 ± 7.5, $p<0.0000$). The difference in the duration of dialysis between weak and strong responders in the non-transfused patients, however, was not significant (4.1 ± 7.2 v 3.7 ± 7.8). It should be said, though, that the range of dialysis duration in 90% of these patients (129 of 144) was 1-6 months. Thus one can hardly comment on the significance of the duration of dialysis from findings in this particular subgroup. In contrast, in the 319 transfused patients weak responders had been on dialysis for longer compared to strong responders (mean ± SD, 14.2 ± 21.0 v 8.3 ± 14.4, $p<0.02$) and had also received significantly more units of blood (7.1 ± 9.0 v 4.3 ± 3.8, $p<0.002$). Hence, as the duration of dialysis is closely related to the number of transfusions ($r=0.237$, $p<0.000$, Table 9.5) and since it failed to come up as a

significant variable in the stepwise discriminant analysis which will be described further, I would tend to attribute the significance of this variable described here to the amount of blood rather than to the duration of dialysis per se.

Association between HLA DRW6 and response to DNCB: All the other variables, but one, which were not significantly associated with the response to DNCB in the original analysis, were also not significant after the stratification for blood transfusion status. The exception was found in the HLA-DR phenotypes, in that the presence of the HLA-DRW6 was significantly associated with a stronger response to DNCB. Table 9.8 shows the proportion of strong responders in patients sensitised prior to any and after one or more units of blood according to their HLA-DR phenotypes and Figure 9.20 shows this information graphically. HLA-DRW6 positive patients were significantly more likely to be strong responders (17 of 19, 90%) when sensitised prior to any blood transfusion compared to HLA-DRW6 negative patients (58 of 150, 50%), $p_{cor}<0.01$. In patients tested after transfusion, however, the DRW6 effect was not found, as strong responders in this group did not significantly differ among DRW6 positive and negative patients (6 of 25, 24% v 59 of 203, 29%).

Stepwise discriminant analysis: The stepwise discriminant analysis is a statistical technique that defines the set of variables which maximises discriminating power in the model of the analysis, or in other words defines the variables that best discriminate between, or separate groups. Using this technique to define the variables that best correlate with the response to DNCB from the 505 cases which were processed, only 73 cases with no missing values in any of the discriminating variables were used in this analysis. Two variables, namely, sex and the number of transfusions prior to the test came up as significant factors when the tolerance level was set at $p<0.05$. After five steps of selection, none of the remaining variables passed the tolerance criterion, including the DRW6, apparently because of the small numbers in this model of analysis.

9.3.3 Follow-up DNCB skin testing

Table 9.9 summarises the results of mean DNCB scores in the entire group of 505 patients and the breakdown groups during follow-up skin testing. Seventy five percent (380 of 505) of the patients had a second test performed, 51% (259 of 505) were followed up with a further third test and only 7% (35 of 505) had a fourth test done. The mean DNCB score in the entire population increased significantly from 3.2±3.6 to 3.9±4.4, 4.1±4.9 and 5.5±6.1 on the second, third and fourth test ($p<0.0001$, $p<0.0001$, $p<0.02$ respectively) (Figure 9.21). This increase reflected an increase of similar significance in the subgroup of patients who were sensitised to DNCB prior to any blood transfusion whose score increase from 4.8±3.8 to 6.1±4.3, 6.7±4.9 and 7.1±6.4 after the second, third and fourth test respectively. In contrast, in patients who were sensitised to DNCB after transfusion, the mean score did not show any significant change. However in the breakdown groups of weak and strong responders an interesting pattern emerged with regard to timing of sensitisation to DNCB. Figure 9.22 shows that strong responders in the groups of patients sensitised both before and after transfusion had a significant increase in their mean DNCB scores. In the former group the score increased from 7.4±2.9 to 8.7±3.3, 10.0±3.3 and 12.3±3.6, and in the latter group from 7.2±2.9 to 7.8±3.5, 8.7±3.2 and 8.2±5.7 after the second, third and fourth tests respectively. In contrast, weak responders showed an opposing change. Patients sensitised prior to transfusion had a significant increase in the mean score from 1.4±1.2 to 2.6±2.8 and 2.7±3.2 after the second and third test as opposed to those sensitised after transfusion who showed a significant decrease from 0.9±1.1 to 0.6±1.3 and 0.6±1.9 at the respective times.

These findings imply that strong responders tend to retain and even give stronger response on subsequent tests regardless of blood transfusion status at the time of sensitisation to DNCB. Contrary to that, transfusion status seems to be crucial at sensitisation for weak responders, in the sense that those who were sensitised prior to any blood are likely to increase their response in follow-up tests

and at least a proportion of them change to strong responders. This view is demonstrated in Figures 9.23 - 9.26, which show cummulative percentages of weak and strong responders on the first, second and third test with respect to blood transfusion status at sensitisation to DNCB. From Figure 9.23 is apparent that 33% (21 of 63) of the weak responders sensitised prior to transfusion changed to strong responders on the second test, and a further 30% (13 of 44) changed on the third test. In contrast there was only a 6% (5 of 82) and 4% (2 of 53) change from strong to weak responders after the second and third test respectively in those who were sensitised before transfusion (Figure 9.24). Using the McNemar chi-square test, which fits best for before and after treatment changes, these differences between changes from weak to strong responders and vice versa, ie, 33% v 6% and 30% v 4%, were both significant at $p<0.004$. Figure 9.25 shows that the proportional change from weak to strong responders was negligible in patients sensitised after one or more units of blood (3 of 172, 0.2% on the second test, and a further 3 of 123, 0.2% on the third test). Also the change from strong to weak responders in this group of patients was not significant (7 of 63, 11% after the second test and one of 39, 3% after the third test) (Figure 9.26). Within strong responders of patients sensitised prior to and after transfusion, the proportion of patients with higher scores increased markedly. In the former group at the first test, 19 of the 97 strong responders (20%) had a score of more than 9 compared to 29 of 53 at the second test (55%) (x^2=19.437, $p<0.0001$) (Figure 9.24). Similarly, in the strong responders who were sensitised after transfusion there was an increase from 19% (15 of 79) to 38% (15 of 39), (x^2=5.213, $p<0.02$).

Table 9.10 shows the time intervals between serial tests in the entire population and the various groups and subgroups. Although the difference in months between the second and third tests was significant between patients sensitised prior to and after transfusion (11.1 ± 12.3 v 16.5 ± 15.0, $p<0.003$), this was not the case for the interval between the first and second test and between the

third and fourth test. Therefore, one could not explain the pattern of response to DNCB observed in Figure 9.21 on the basis of shorter or longer intervals between the tests in the two groups.

Table 9.11 shows the number of transfusions given between the tests. Patients sensitised prior to any transfusion had less blood in the period between the first and second test compared to those sensitised after transfusion (3.7 ± 4.9 v 6.7 ± 11.6, $p<0.004$), but thereafter both groups received a comparable number of transfusions. Furthermore although strong responders in the latter group received significantly more blood compared to strong responders in the former group (6.1 ± 8.1 v 3.6 ± 5.1, $p<0.02$), both groups displayed a comparably significant increase in their mean DNCB score on the second test (Figure 9.22). In contrast, weak responders who were sensitised after transfusion, although they did not receive significantly more blood between tests compared to weak responders sensitised before transfusion, showed a further descrease in their mean scores on the second and third test as opposed to their comparison group which showed a significant increase.

From these results, it is obvious that the DNCB test after the primary response, when carried out serially gives an anamnestic reaction, which to a certain extent depends on the blood transfusion status at the time of sensitisation. Subsequent transfusions did not seem to interfere with secondary responses to DNCB in strong responders, while in weak responders they may have a further suppressive role.

9.3.4 The predictive value of the DNCB skin test in renal transplantation.

Three hundred and forty four of the 505 predialysis and dialysis patients whose CMI was assessed using the DNCB test underwent transplantation in the years between 1975-1985. These transplants represented 64% (344 of 535) of the total number of transplants carried out successively during this period. Patients treated with azathioprine represented 75% of the consecutive transplants from 1975-1984 (284 of 334), but the 57 transplants

treated with cyclosporin accounted for only 36% of the total transplants receiving cyclosporine between 1984-85 (57 of 157 transplants). Overall, there were 213 males (62%) and 131 females (38%) and their mean age was 37.6 years (range, 14-65 years). Eighty six percent (295 of 334) of the patients were treated with haemodialysis and the remaining 14% (49 of 334) with CAPD prior to transplantation. The overall duration of dialysis prior to the operation was 25.4 $\pm$ 25.0 months (range, 1-177 months). Ninety three percent of the patients received cadaveric grafts (320 of 334), and the remaining 7% (24 of 334) received grafts from live related donors. Three hundred and thirteen were first transplants (91%), 25 second (7%) and 6 third transplants (2%). There were only 17 patients (5%) who did not receive any blood prior to transplantation in the early period, 120 patients had 1-5 units (40%), 112 patients had 6-10 units (28%) and the remaining 95 patients (27%) were multitransfused (range 11-101 units of blood). Sixty seven percent of the total amount of blood given prior to transplantation was whole blood or packed cells and the remaining 33% was frozen or filtered cells. The mean interval between the last transfusion and the operation was 7.4 months (range, 1-144 months). Sixty six percent of the patients were transplanted within six months from the last transfusion, 20% within a year and the remaining 14% after more than a year had elapsed. At the end of the study, irrespective of follow-up length, 184 patients had a functioning graft (54%), 128 had rejected irreversibly (37%), 22 died with functioning grafts (6%), 5 transplants were technical failures (1.5%), 4 died later on dialysis after they had rejected the graft (1.5%) and one patient died on dialysis after his kidney was lost due to technical failure.

Among all the transplants there were 227 weak DNCB responders (66%) and the remaining 117 were strong responders (34%). These percentages were similar in the breakdown groups of patients treated with azathioprine and cyclosporin (66%, 34%, 65%, and 35% respectively). Table 9.12 summarises the characteristics of weak and strong responders in the patients treated with azathioprine. There were more females among the weak DNCB responders, and patients in

this group had been on dialysis for longer and had received more transfusions prior to transplantation compared to strong responders. The difference, however in age, type and number of transplant and in HLA-A,-B and DR mismatches between the two groups was not significant.

In the life table analysis of graft survival all graft losses were considered as failures. Only the three patients who had combined immunosuppression with azathioprine and cyclosporine were excluded from the analysis in the breakdown groups by immunosuppressive regimen. As there was a considerable change from weak to strong DNCB responders from the first to last test, particularly in patients sensitised to DNCB prior to any blood transfusion, life table analysis of patient and graft survival in weak and strong DNCB responders was carried out using both the first and the last test prior to transplantation. In patients who only had one DNCB test, the results were used both in the analyses of the first test and those involving the last test. Figure 9.27 gives the overall actuarial patient and graft survival in the 344 transplants. Patient survival was 91%, 90%, 89%, 87% and 87% from one to five years respectively after transplantation. In the breakdown groups of transplants treated with azathioprine patient survival was almost identical to that of the entire population at these times (90%, 89%, 88% and 86%) and in the cyclosporin group with shorter follow-up, patient survival at 12 and 18 months was 96%. The overall graft survival, including all losses, was 52%, 40%, 30%, 22% and 16% from one to five years after transplantation respectively. Graft survival in patients treated with azathioprine was 58%, 47%, 35%, 26% and 20% at these times while in patients treated with cyclosporin it was 79% at 6 months and 65% at one year. However it should be said that due to the small number of patients and the short follow-up time the standard error of the cummulative survival at one year in the cyclosporin group was 7% as opposed to that of the azathioprine group which was one percent. Neither the first nor the last DNCB test had

any predictive value with regards to patient survival in the weak and strong responders which in both groups was identical to that of the entire population.

Figure 9.28 shows the actuarial graft survival in the 189 weak and 95 strong responders, as classed by the first DNCB test in the group treated with azathioprine and prednisolone. Graft survival in the weak responders was 62%, 50%, 38%, 29% and 23% from one to five years after transplantation respectively which was significantly better compared to 49%, 41%, 29%, 21% and 14% in the strong responders at these times ($p<0.02$). The difference in graft survival between the two groups was of greater significance when the last DNCB test was used to form the breakdown groups (Figure 9.29). On this occasion, weak responders had 64%, 52%, 41%, 32% and 25% graft survival from one to five years respectively as opposed to 48%, 38%, 26%, 17% and 10% in the strong responders ($p<0.0003$).

Table 9.13 summarises graft survival data in the weak and strong responders treated with cyclosporin using both the first and the last DNCB test. The difference between the two groups was not significant at any time during the nine months of follow-up. Thus, from this small group of transplants which as stated represents only 36% of the overall cyclosporin treated transplants, it appears that the DNCB test has no predictive value in the cyclosporin era.

From the description of the characteristics of weak and strong DNCB responders in Table 9.12, it is apparent that weak DNCB responders received more blood transfusion prior to transplantation compared to strong responders. Is the predictive value of the DNCB test, therefore, in the azathioprine group affected by the amount of transfusion? Table 9.14 gives the cumulative graft survival in weak and strong responders of that group, stratifying the patients according to the number of transfusions they received prior to transplantation. Using the first DNCB test, all breakdown groups of weak responders, namely, without any blood transfusion, 1-5 units, 6-10 and more than 10 units of blood, had better graft survival compared to that of the respective breakdown groups of strong responders. The difference in graft survival between the two

breakdown groups receiving 1-5 units just failed to reach statistical significance. However, using the last DNCB test, the difference in graft survival between weak and strong responders was more pronounced in all subgroups and was highly significant in those receiving 1-5 units of blood (Figure 9.30). As the difference in HLA matching was comparable in the two subgroups, it is reasonable to assume that the predictive value of the DNCB test with regard to graft survival in patients treated with azathioprine is genuine. From table 9.15, by contrast, one can see that in patients treated with cyclosporin the DNCB test does not have any value in predicting a difference in graft survival between weak and strong responders receiving 5, 6-10 or more than 10 units of blood prior to transplantation.

Table 9.16 compares the graft survival rates between the 17 patients who were transplanted without any transfusion and those receiving 1-5, 6-10 and more than 10 units of blood, regardless of their response to DNCB. Graft survival was 19% and 21% better respectively at one year in patients receiving 1-5 and 6-10 units of blood, compared to patients without transfusion. This difference at one year was significant ($p<0.02$), but the difference between the overall curves of cumulative graft survival was not significant. In the cyclosporin group, all patients had been transfused and comparison of graft survival rates was made between breakdown groups of patients receiving 1-5, 6-10 and more than 10 units of blood (Table 9.16). During the short follow-up the number of transfusions did not appear to affect graft survival at 3, 6 and 9 months in the cyclosporin group.

It is beyond the scope of this thesis to look at all factors that might be associated with renal allograft survival. Taking into account that the transplants studied in this section represent 64% of all consecutive transplants which were carried out between 1975 and 1985, I attempted to look at some of these factors. In a univariate fashion analysis the variables that showed a significant correlation with the transplant outcome are shown in Table 9.17. The most significant variables, when all transplants were considered, were the type of immunosuppression, (the cyclosporin group had better graft

survival, p-0.0001), the number of rejection episodes (p-0.0001) and the development of cytotoxic antibodies after transplantation (p-0.0001). Other significant variables were the number of HLA DR mismatches (p-0.008), the response to the last DNCB test (weak responders had better graft survival, p-0.025), and the donor source (live related donors were associated with better graft survival than cadaveric ones (p-0.03). In the azathioprine group, in addition to these variables, two more came up as significant, namely the time of the first rejection episode and the hospital where patients were treated prior to transplantation. The earlier the first rejection episode occurred the worse was the outcome (p=0.01), and patients treated in Stobhill Hospital and the Royal Infirmary appeared to have slightly better graft survival (p-0.03). However, the definition of the hospital variable is rather loose, as there was a considerable interchange of patients from one hospital to the other. Interestingly, in the small group of patients treated with cyclosporin the only variables which showed significant correlation with the transplant outcome were the number of HLA DR mismatches and the development of PRA after transplantation (p-0.005 and p-0.001 respectively). Finally, the variables that came up as significant in Table 9.17 were analysed in a stepwise discriminant analysis, using only 101 of the 334 transplants because the remaining 233 had at least one missing discriminating variable. The set of variables which was separated as having, by order of significance, the most discriminating power with regard to transplant outcome were the development of cytotoxic antibodies after transplantation, the last DNCB test and the type of immunosuppressive regimen (chi-square-27.803, DF-3, p<0.00001).

9.4 Conclusions

1) In a large population sample of predialysis and dialysis patients, regardless of any stratification for any parameter, CMI as assessed by the DNCB test was profoundly suppressed. Two-thirds of the patients had a weak response to DNCB and half of them were anergic. The remaining one-third of patients had a strong response comparable to that of normal subjects.

2) Among transplant patients with functioning grafts who had the DNCB test while under immunosuppression, two-thirds were anergic to DNCB, and only one in ten of those patients was a strong responder. The DNCB test did not appear to have any predictive value with regard to graft survival when carried out under immunosuppression.

3) In a univariate fashion analysis the factors which showed a significiant association with the response to DNCB were sex, nephrectomy prior to transplantation, time period when patients were tested, duration of dialysis and number of blood transfusions prior to the test. Blood transfusion proved to be the most significant variable in a multivariate analysis which excluded the interdependence and the overlapping effect between these variables.
Also, when patients were stratified in terms of whether they had been transfused or not at the time of sensitisation to DNCB the significance of the other variables was lost.

4) In a group of patients who were tested prior to any transfusion, the proportion of strong responders was two-fold higher compared to that of the overall unstratified population. There was a progressive decline in strong responders as patients received more blood.

5) DRW6 positive patients were more likely to be strong responders when tested prior to any blood transfusion. However, in patients sensitised after one or more transfusions, the DRW6 effect was not observed.

6) A number of factors did not show any association with the response to DNCB, namely, age, original renal disease, hospital of dialysis treatment, ABO and rhesus blood groups, parity, type of dialysis (CAPD or haemodialysis), interval between last blood transfusion and test and pre-existence of panel reactive antibodies prior to the test.

7) Follow-up DNCB skin testing showed that blood transfusion at the time of sensitisation to DNCB was of crucial importance for secondary responses to DNCB. Patients eliciting a strong primary response retained and gave even stronger responses on subsequent tests regardless of blood transfusion status at the time of sensitisation to DNCB. Contrary to that, weak responders who were sensitised after transfusion showed a further depression in their secondary responses, while weak responders sensitised prior to any transfusion were more likely to increase these secondary responses. As a result of this, approximately one-third of the latter patients changed to strong responders in follow-up tests.

8) The predictive value of the DNCB test with regard to graft survival in patients treated with azathioprine and prednisolone, was best expressed when the last test prior to transplantation was used. Weak DNCB responders had signficantly better graft survival compared to strong responders over a five year follow-up. This effect on graft survival was independent of the blood transfusion effect and was most clearly seen in patients receiving 1-5 units of blood.

9) In transplant recipients treated with cyclosporin, the DNCB test did not have any predictive value.

10) In this series of transplants (which are 64% of all consecutive transplants in the Unit over that period), the set of factors which correlated significantly with the transplant outcome in a stepwise discriminant analysis were the development of panel reactive antibodies after transplantation, the last DNCB skin test and the immunosuppressive regimen. Patients treated with cyclosporin had better graft survival and interestingly most of the factors that were found to correlate significantly with the transplant outcome in patients treated with azathioprine did not correlate in the cyclosporin group.

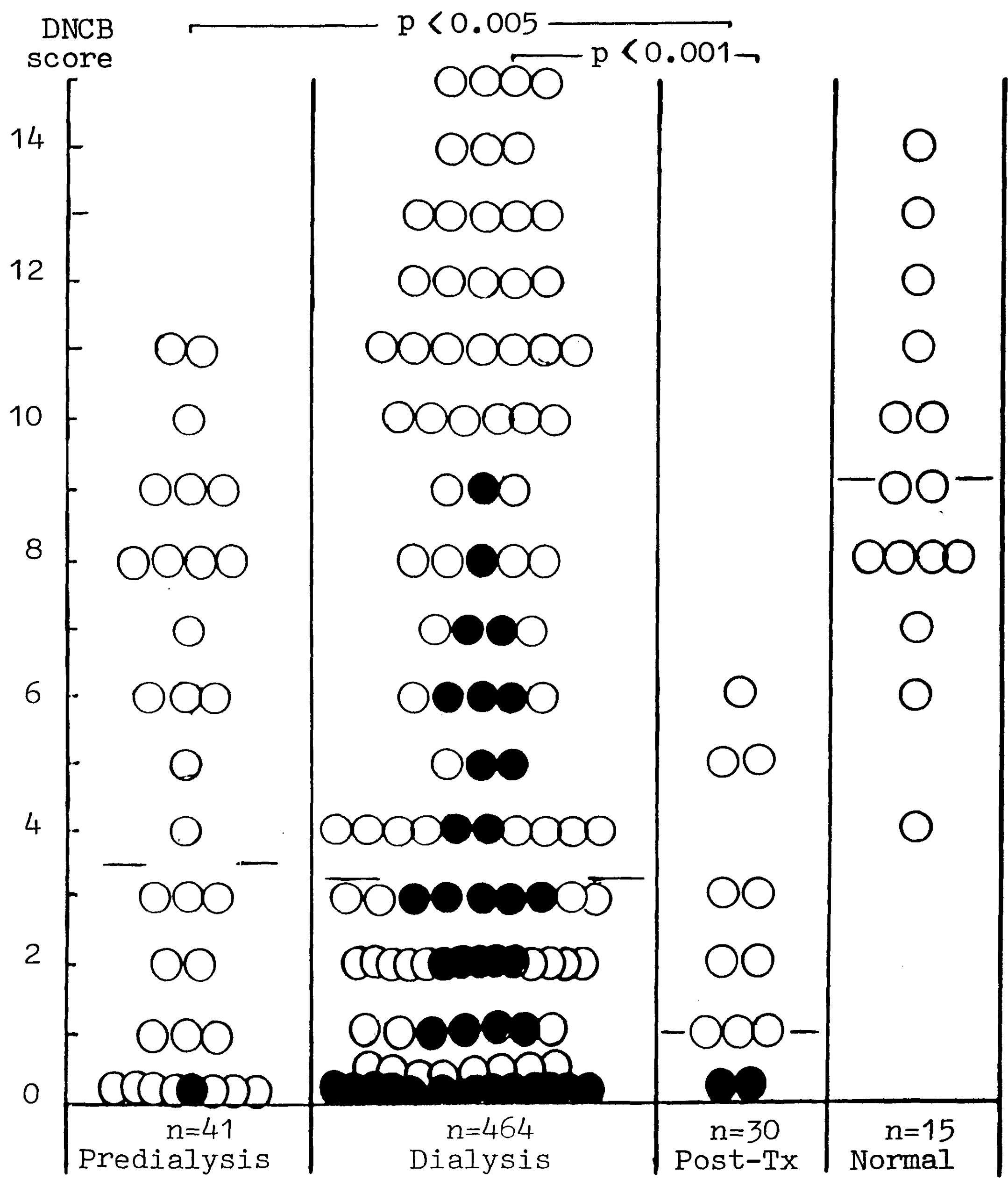


Figure 9.1. DNCB scores at the first test in predialysis, dialysis and transplant patients compared to normal controls; O = one patient, ● = 10 patients.

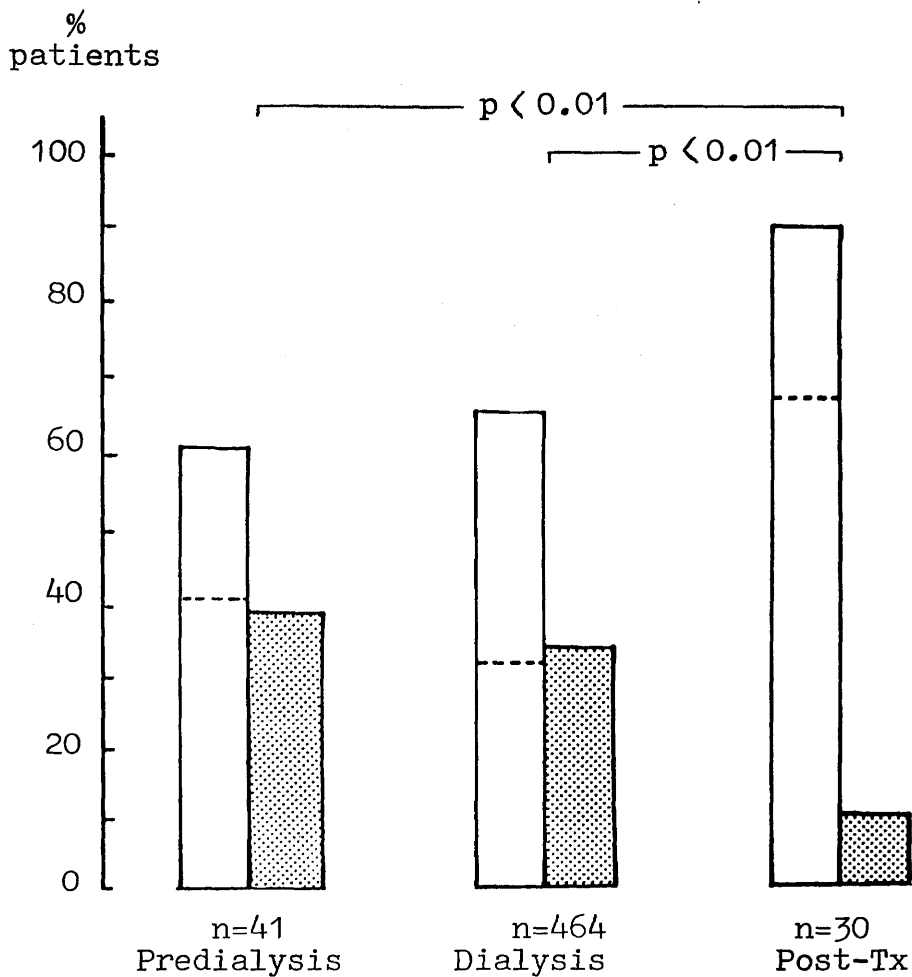


Figure 9.2. Percent of weak (open bars) and strong DNCB responders (shaded bars) in predialysis, dialysis and transplant patients; areas under dotted lines represent anergic patients (DNCB score = 0).

TABLE 9.1. CHARACTERISTICS OF PRE-DIALYSIS AND DIALYSIS PATIENTS

	Pre-dialysis n=41	Dialysis n=464
Sex:		
Male	24 (59%)	279 (60%)
Female	17 (41%)	185 (40%)
Age (Mean ± SD)	39 ± 13	41 ± 12
Original disease:		
Glomerulonephritis	8 (20%)	172 (37%)
Pyelonephritis	12 (29%)	111 (24%)
Polycystic	6 (15%)	46 (10%)
Miscellaneous	10 (24%)	119 (26%)
Unknown	5 (12%)	16 (3%)
Duration of dialysis:		
3 months	0	241 (52%)
6 months	0	81 (18%)
1 year	0	47 (10%)
2 years	0	45 (19%)
>3 years	0	50 (11%)

TABLE 9.2. RESULTS OF DNCB SCORES IN 505 PATIENTS WITH END-STAGE RENAL FAILURE

DNCB score	No. of patients	%	Cumulative %
0	165	32.7	32.7
1	46	9.1	41.9
2	61	12.1	54.0
3	57	11.3	65.3
4	29	5.7	71.0
5	22	4.4	75.4
6	35	6.9	82.1
7	23	4.6	86.7
8	18	3.6	90.3
9	15	3.0	93.3
10	7	1.4	94.6
11	7	1.4	96.0
12	7	1.4	97.4
13	5	1.0	98.4
14	3	0.6	99.0
15	5	1.0	100.0

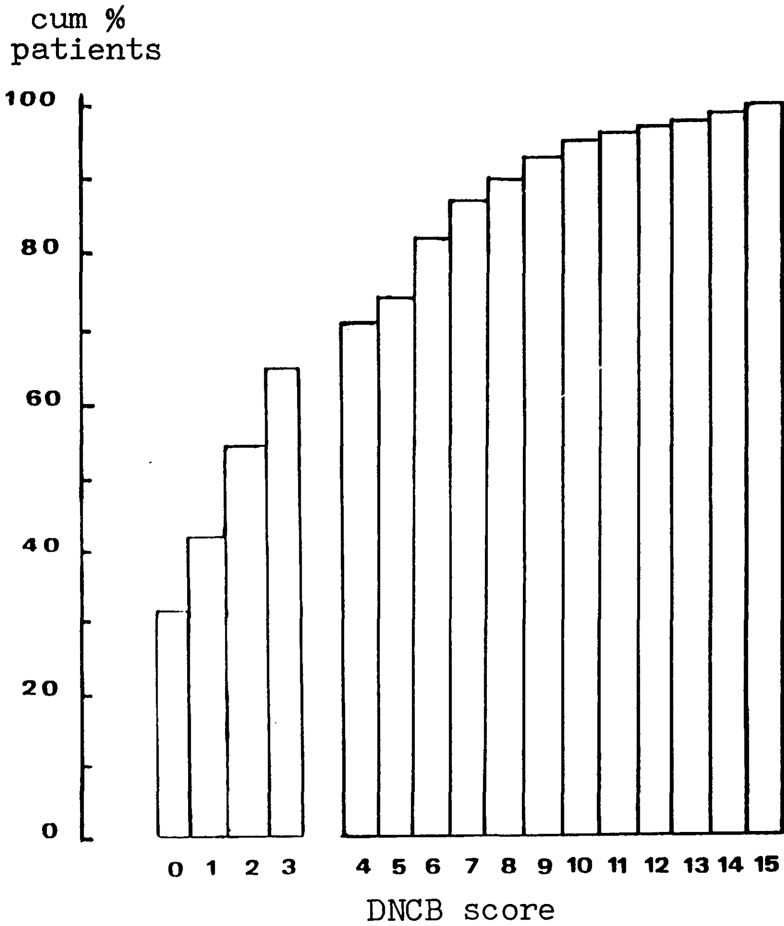


Figure 9.3. Cumulative percentages of DNCB scores in the 505 predialysis and dialysis patients.

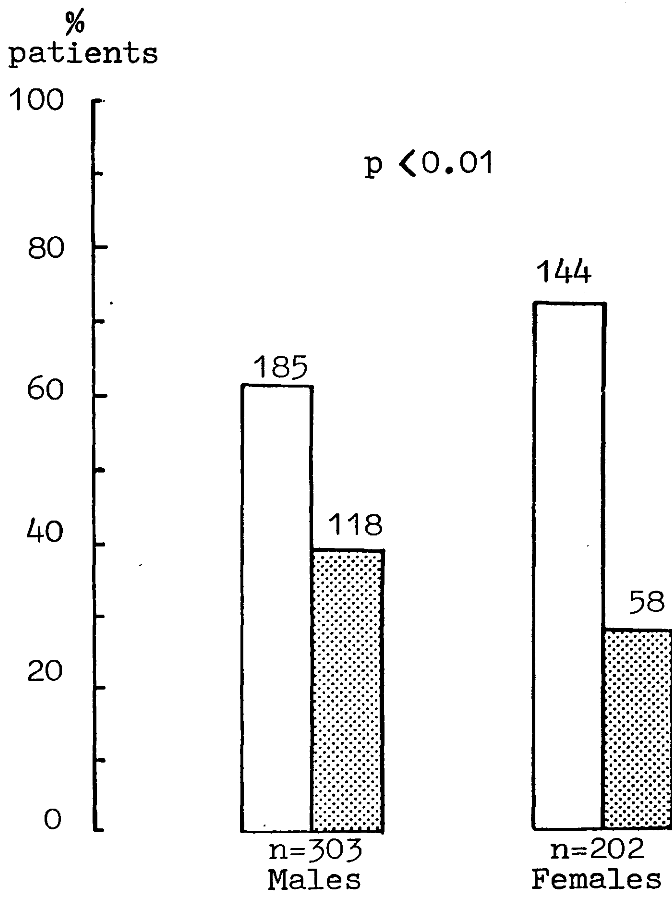


Figure 9.4. Weak (open bars) and strong DNCB responders (shaded bars) in males and females; numbers on top of the bars represent number of patients.

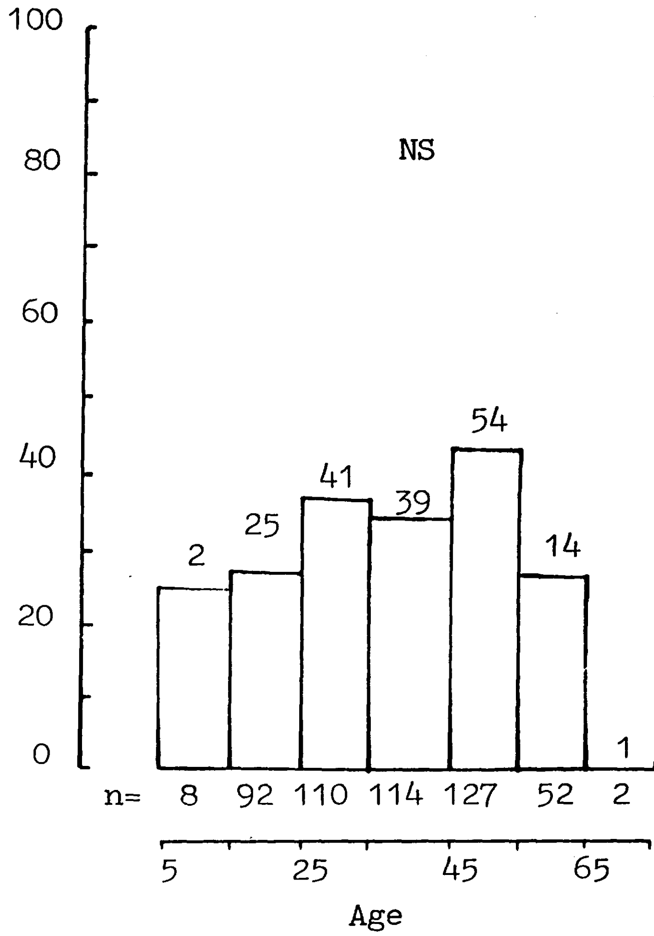


Figure 9.5. Percent of strong DNCB responders in different age groups in 505 predialysis and dialysis patients.

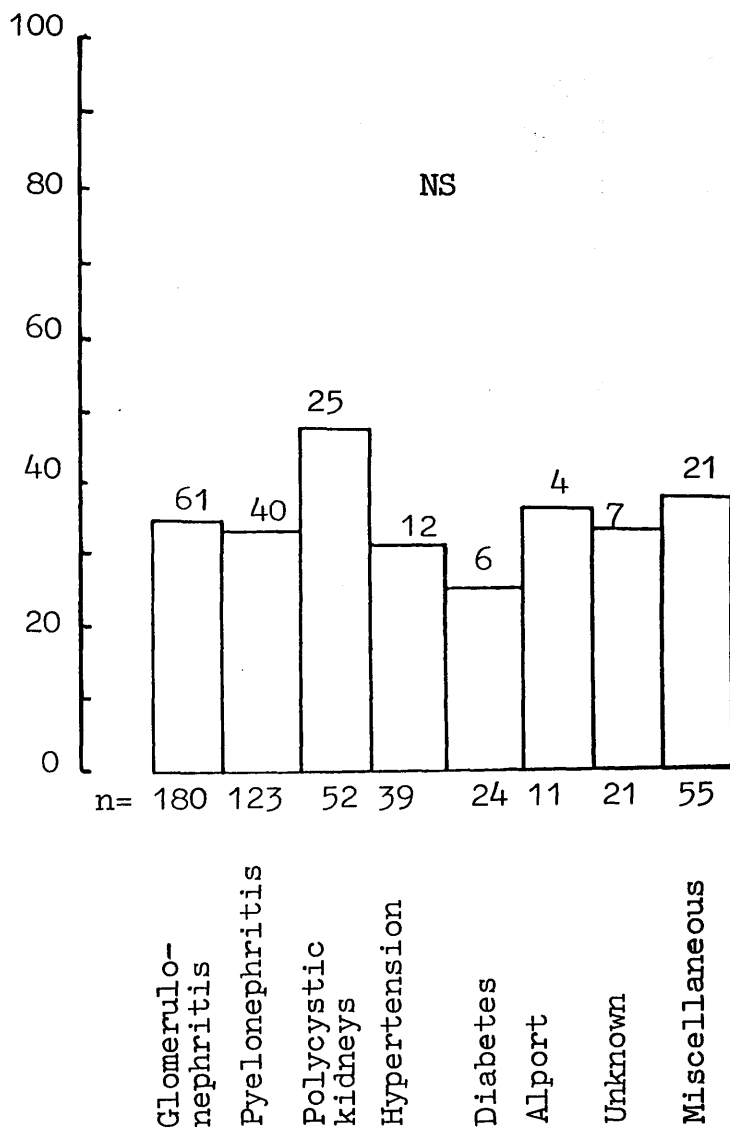


Figure 9.6. Percent of strong DNCB responders in the 505 patients in relation to their original renal diseases.

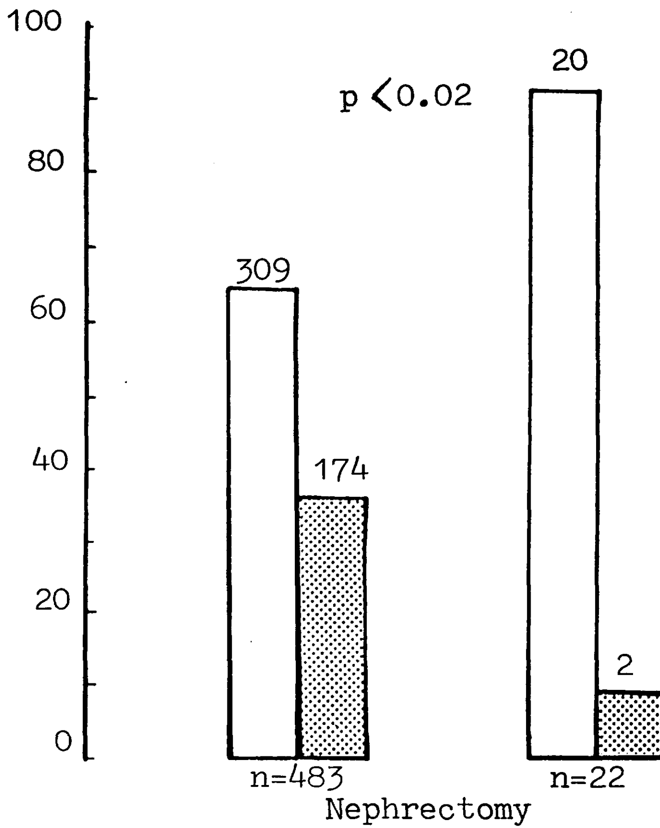


Figure 9.7. Weak (open bars) and strong DNCB responders (shaded bars) in the 505 patients without and with nephrectomy of their own kidneys prior to the test.

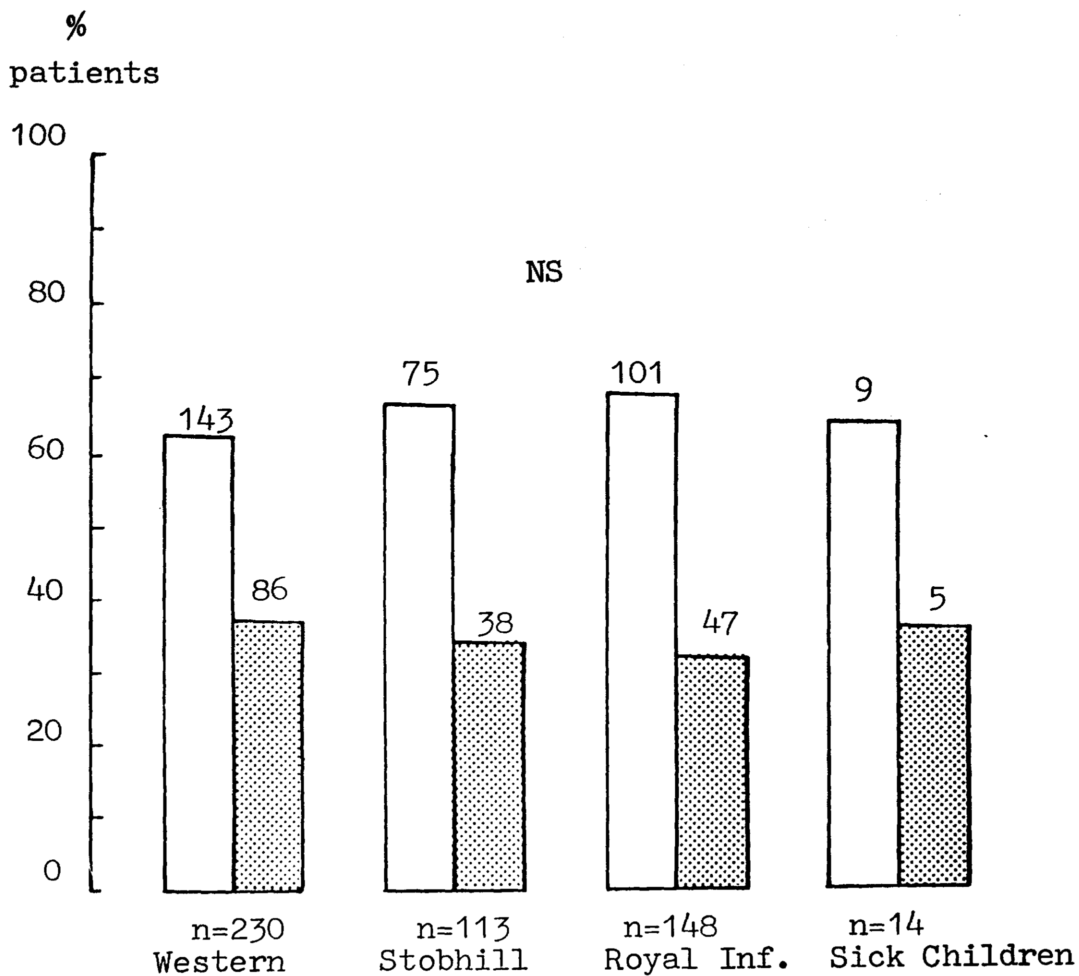


Figure 9.8. Percent of weak (open bars) and strong DNCB responders (shaded bars) in the 505 patients who were treated in four different hospitals in Glasgow.

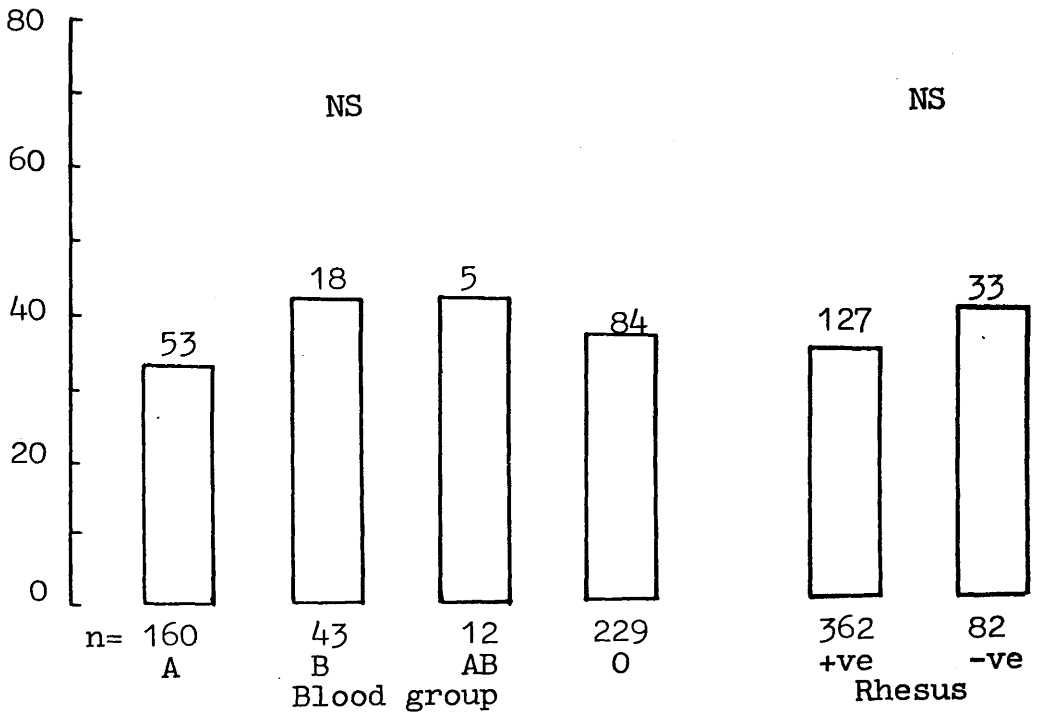


Figure 9.9. Percent of strong DNCB responders in 444 patients in relation to their blood group and rhesus.

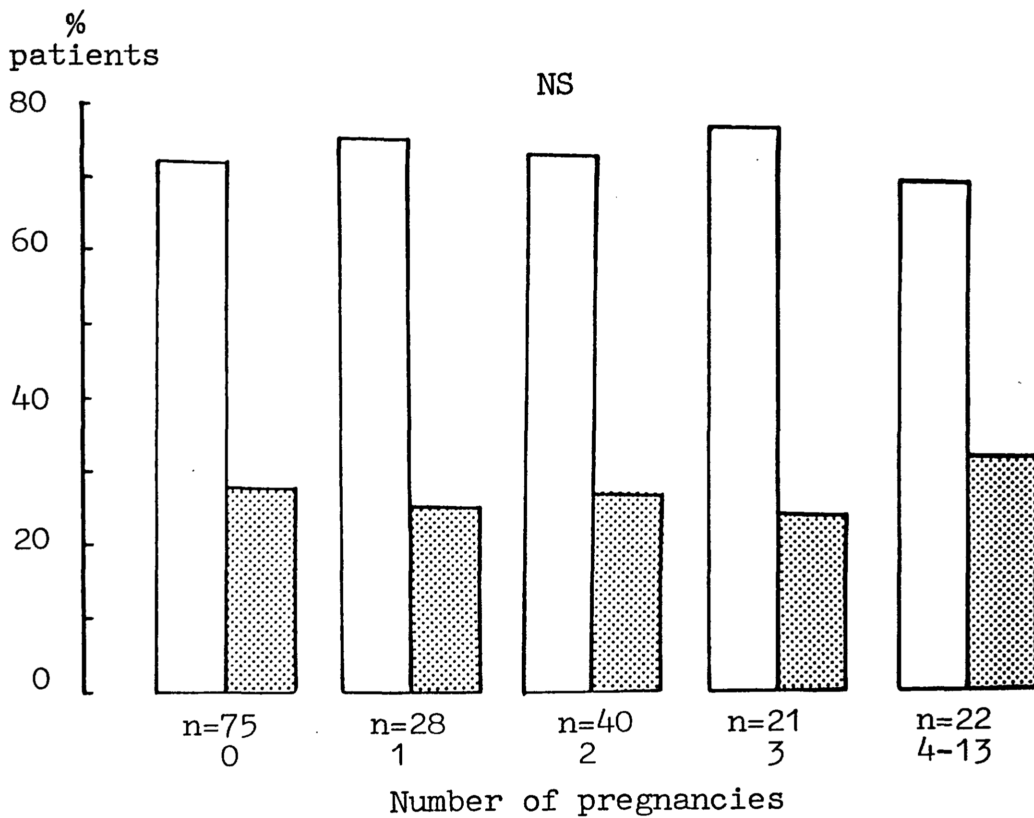


Figure 9.10. Percent of weak (open bars) and strong responders (shaded bars) in 186 dialysis women in relation to parity.

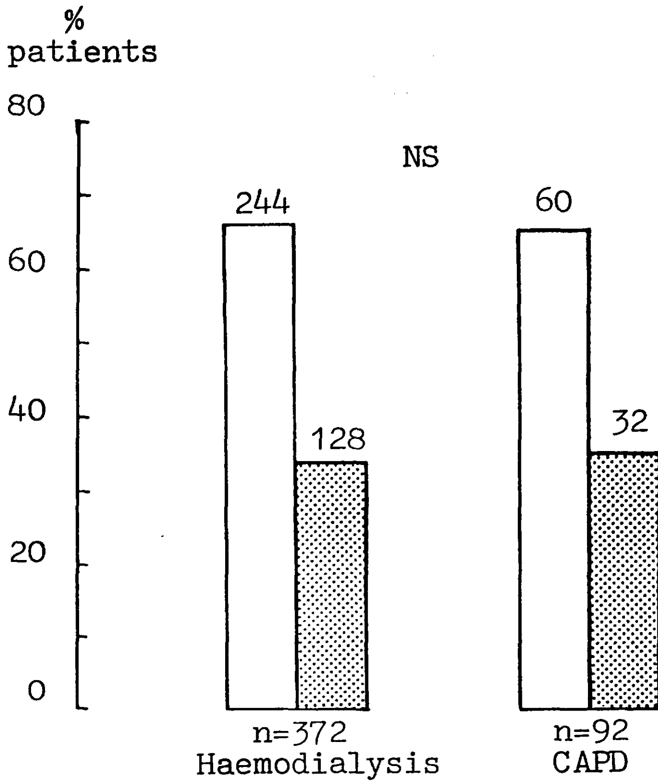


Figure 9.11. Percent of weak (open bars) and strong DNCB responders (shaded bars) in 372 haemodialysis and 92 CAPD patients.

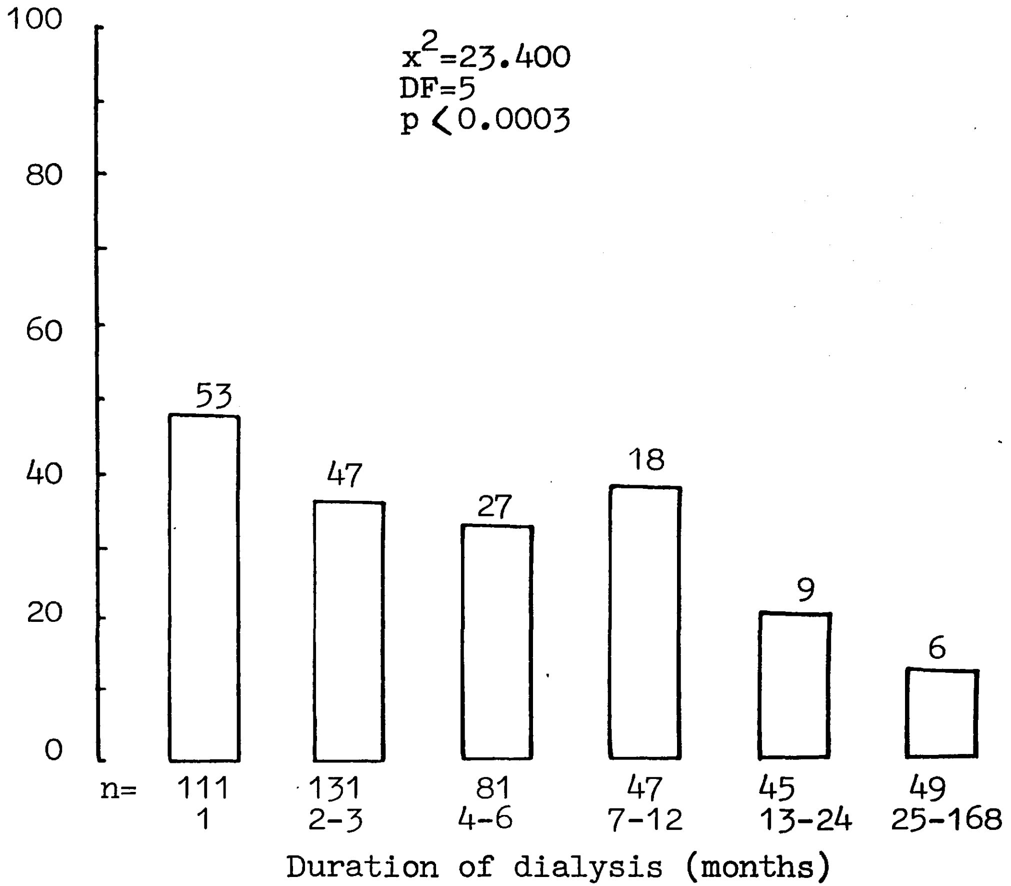


Figure 9.12. Percent of strong DNCB responders in 464 patients in relation to duration of dialysis prior to the test.

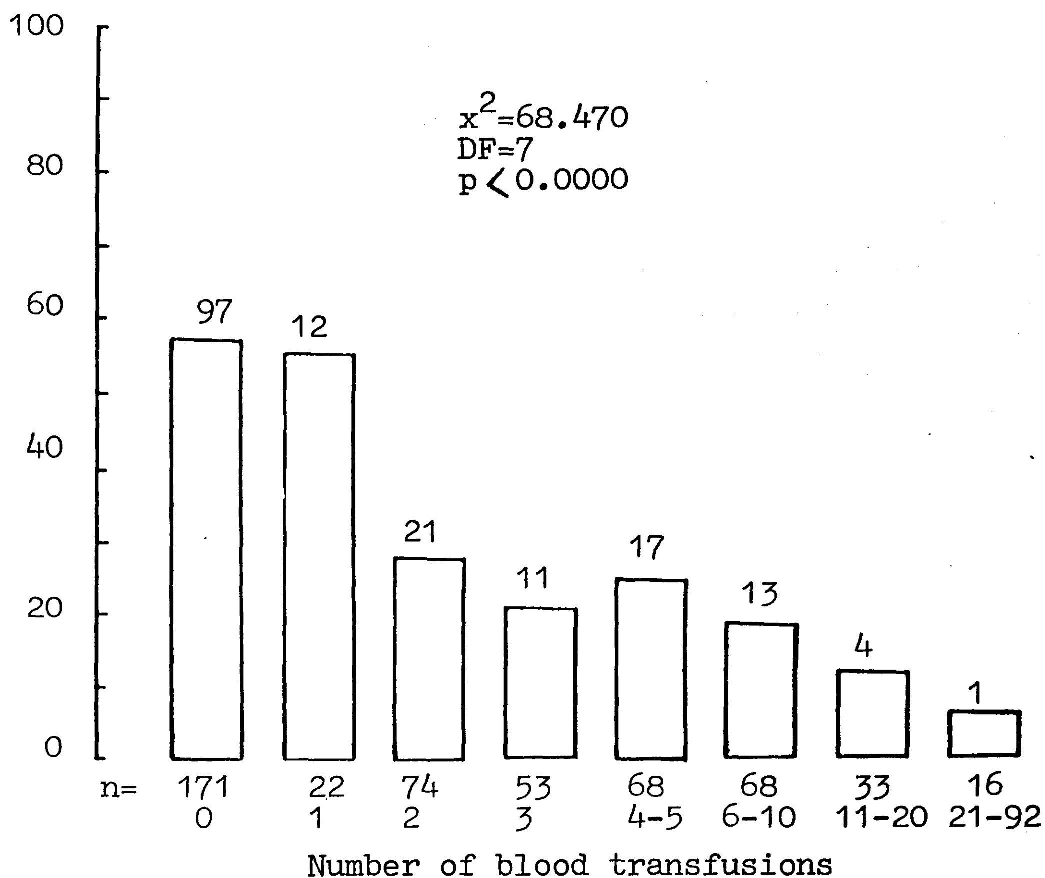


Figure 9.13. Percent of strong DNCB responders in 505 patients in relation to blood transfusions they received prior to the test.

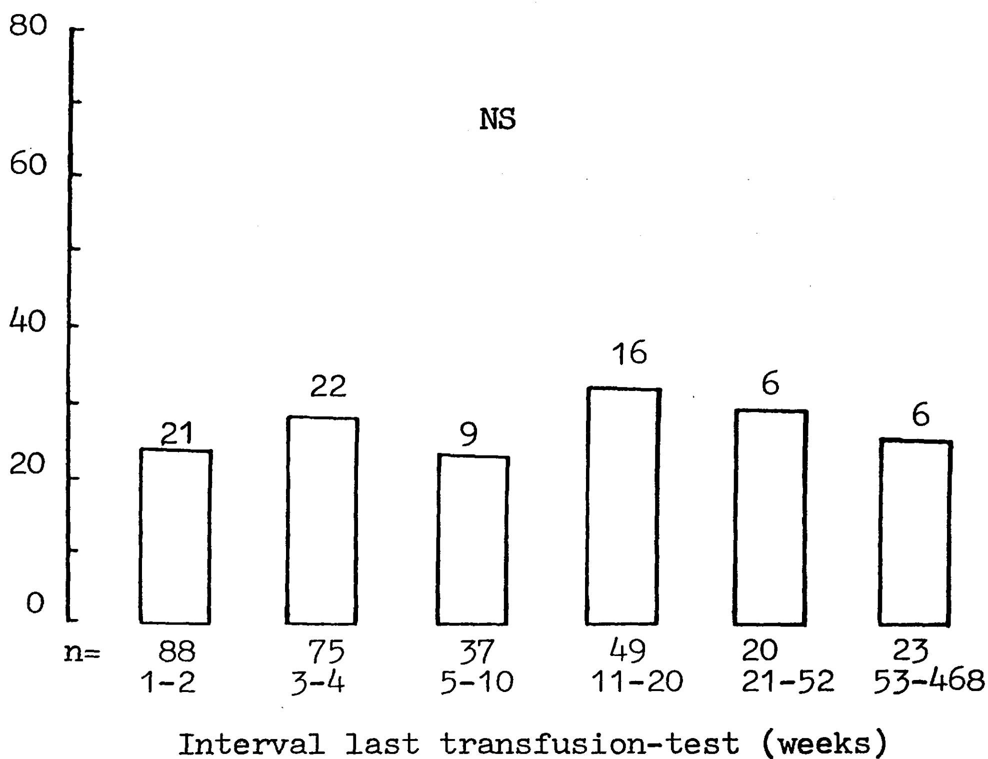


Figure 9.14. Percent of strong DNCB responders in 292 patients in relation to interval between last blood transfusion and sensitisation to DNCB.

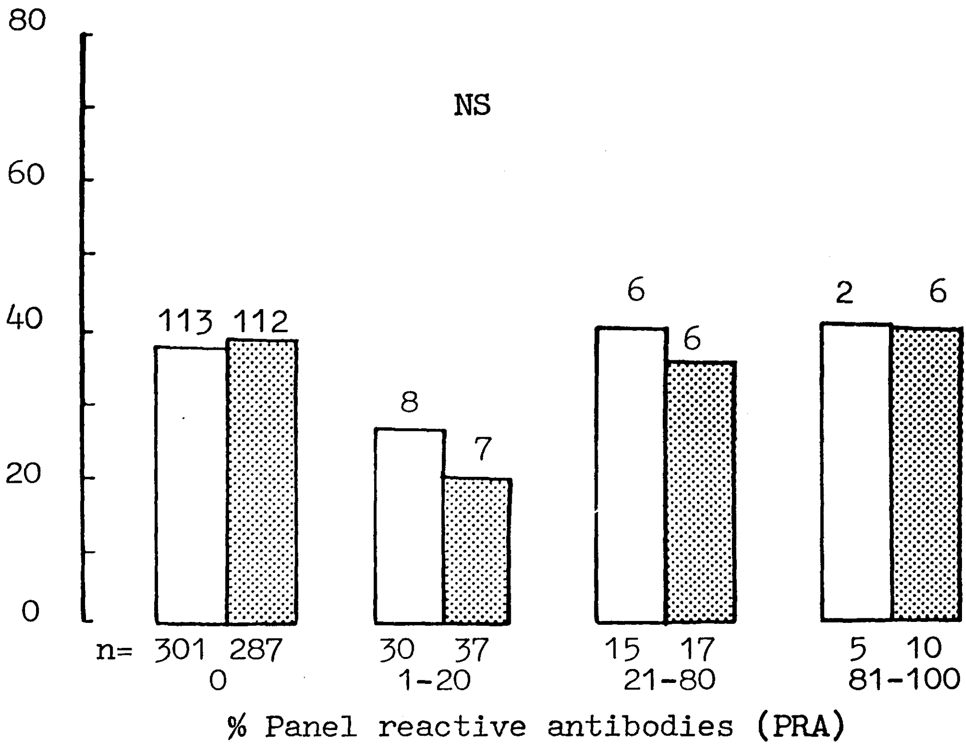


Figure 9.15. Percent of strong DNCB responders in 351 patients in relation to their PRA at the time of the test; open bars = current PRA, shaded bars = historical PRA.

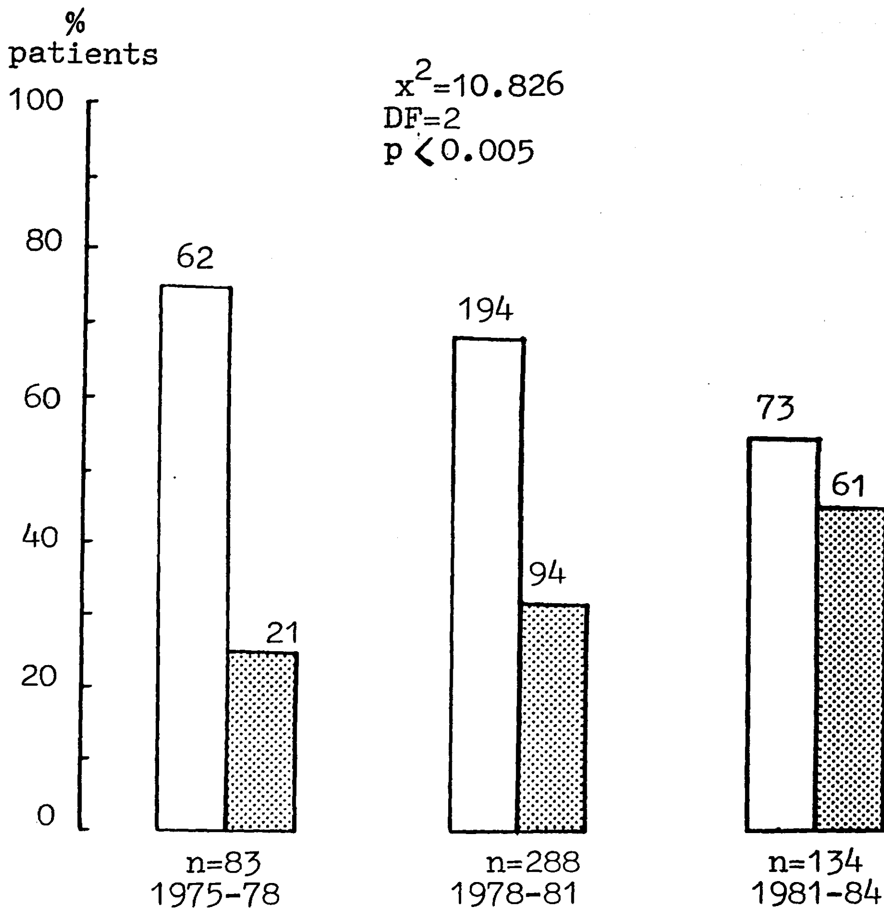


Figure 9.16. Percent of weak (open bars) and strong DNCB responders (shaded bars) in 505 patients in relation to the period they were tested.

TABLE 9.3. FREQUENCY OF HLA-DR ANTIGENS IN 362 DIALYSIS PATIENTS

	DR1	DR2	DR3	DR4	DR5	DR6	DR7	Blank
HLA DR +ve	51 14%	113 31%	111 31%	144 40%	30 9%	44 12%	99 27%	131 36%
HLA DR -ve	311 86%	249 69%	251 69%	218 60%	332 91%	318 88%	263 73%	231 64%

TABLE 9.4. FREQUENCY OF WEAK AND STRONG DNCB RESPONDERS IN RELATION TO THEIR HLA-DR ANTIGENS IN 362 DIALYSIS PATIENTS

	DR1	DR2	DR3	DR4	DR5	DR6	DR7	Blank
DNCB 0-3:								
HLA-DR +ve	33/51 65%	65/113 58%	67/111 60%	94/144 65%	20/30 67%	21/44 48%	60/99 61%	84/131 64%
HLA-DR -ve	189/311 61%	157/249 63%	155/251 62%	128/218 59%	202/332 61%	201/318 63%	162/263 62%	138/231 60%
DNCB >3:								
HLA-DR +ve	18/51 35%	48/113 42%	44/111 40%	50/144 35%	10/30 33%	23/44* 52%	39/99 39%	47/131 36%
HLA-DR	122/311 39%	92/249 37%	96/251 38%	90/218 41%	130/332 39%	117/318* 37%	101/263 38%	93/321 40%

*$x^2 = 3.905$, $p<0.05$, $p_{cor}<0.4$

TABLE 9.5. CORRELATION COEFICIENTS AND TWO-TAILED SIGNIFICANCE BETWEEN THE DNCB TEST, BLOOD TRANSFUSION, SEX, NEPHRECTOMY, DURATION OF DIALYSIS AND PERIOD WHEN THE TEST WAS PERFORMED

Pearson correlation coefficients (n=460)

Variable	Time period	Duration of dialysis	Nephrectomy	Sex	DNCB score
Duration of dialysis	-0.182 p=0.000				
Nephrectomy	-0.261 p=0.000	0.190 p=0.000			
Sex	0.000 NS	0.024 NS	0.066 NS		
DNCB score	0.160 p=0.001	-0.190 p=0.000	-0.096 p=0.04	-0.132 p=0.004	
Blood transfusion	-0.268 p=0.000	0.237 p=0.000	0.119 p=0.01	0.143 p=0.002	-0.316 p=0.000

TABLE 9.6 CORRELATION COEFFICIENTS BETWEEN THE DNCB TEST AND SEX, NEPHRECTOMY, DURATION OF DIALYSIS AND TIME PERIOD CONTROLLING FOR THE VARIABLE BLOOD TRANSFUSION

Pearson correlation coefficients (n=460)

Variable	Time period	Duration of dialysis	Nephrectomy	Sex
DNCB scores	0.083 NS	-0.125 p=0.007	-0.062 NS	-0.093 p=0.05

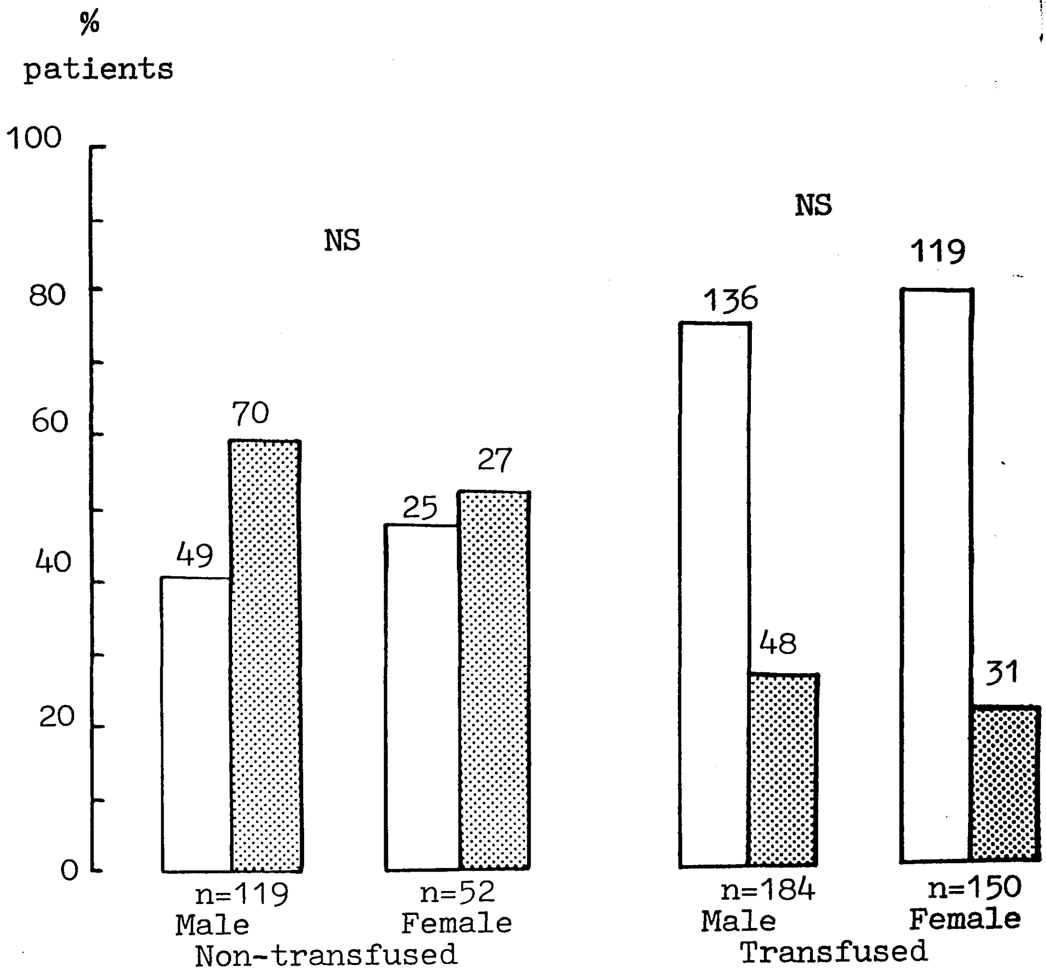


Figure 9.17. Percent of weak (open bars) and strong DNCB responders (shaded bars) in 171 patients sensitised to DNCB prior to any blood transfusion and 334 patients sensitised after one or more units of blood.

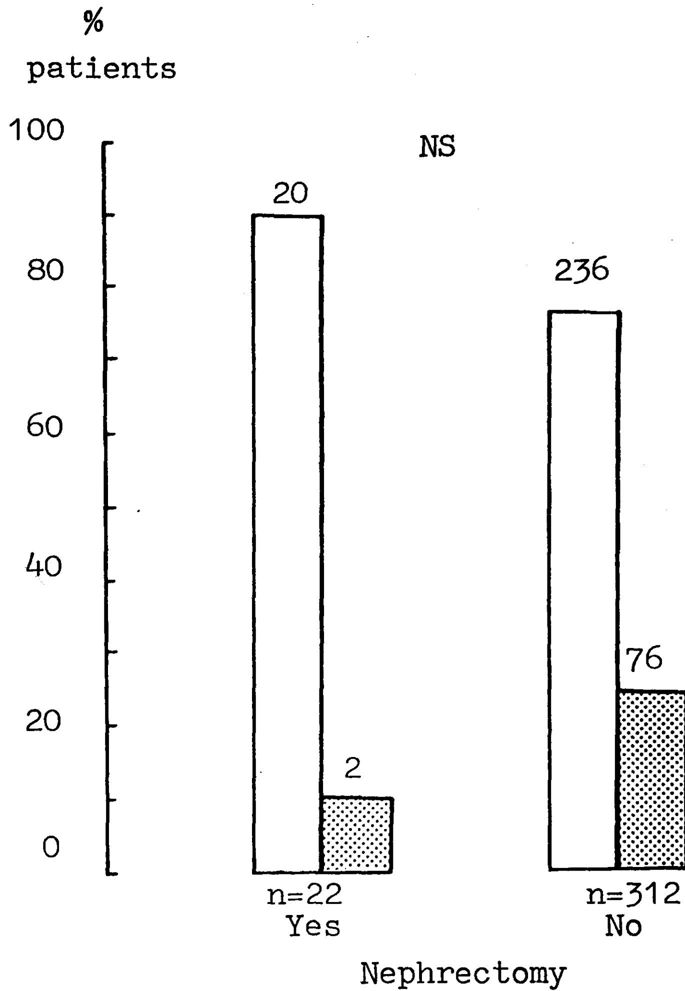


Figure 9.18. Percent of weak (open bars) and strong DNCB responders (shaded bars) in 334 with and without nephrectomy who were transfused prior to the test.

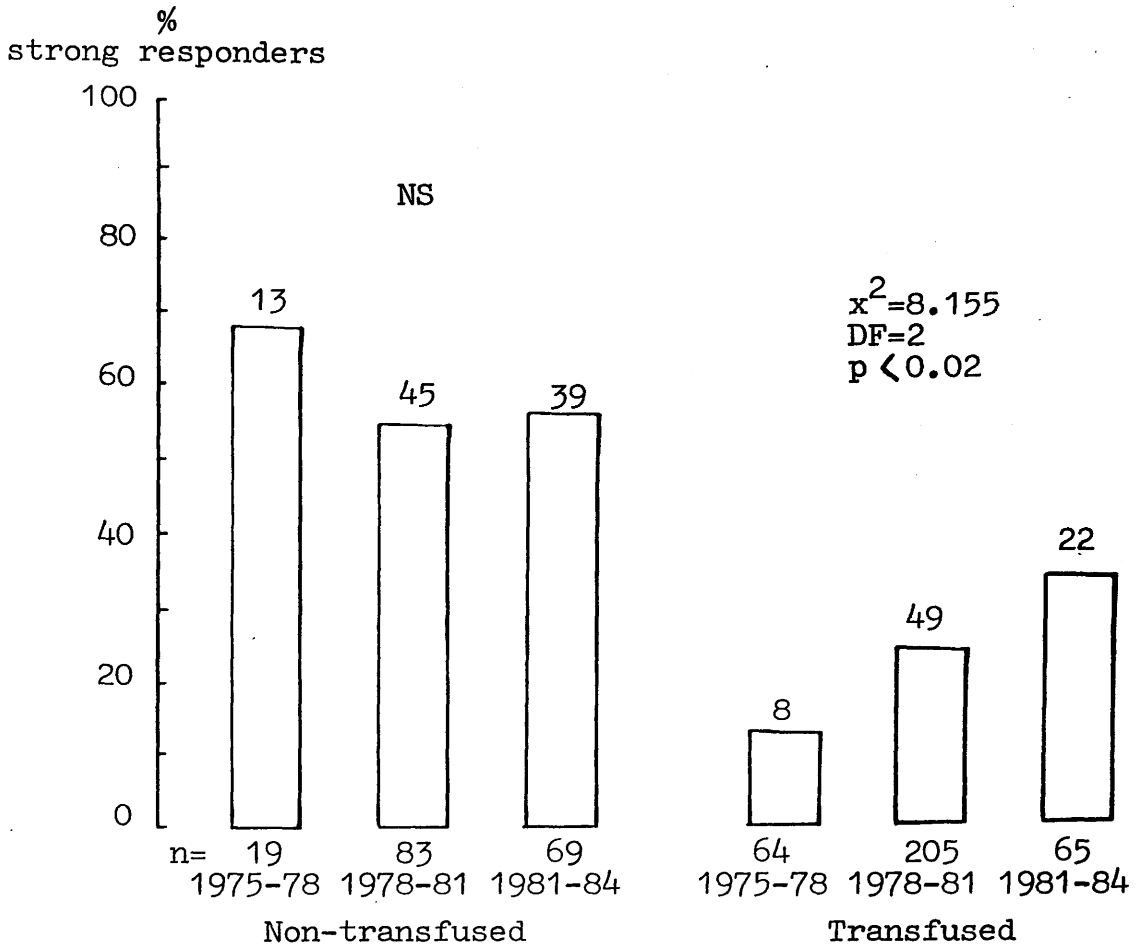


Figure 9.19. Percent of strong DNCB responders in the 171 and 334 patients who were sensitised to DNCB prior to any and after one or more transfusions respectively in relation to the period they were tested.

TABLE 9.7. DURATION OF DIALYSIS AND BLOOD TRANSFUSIONS PRIOR TO THE DNCB SKIN TEST

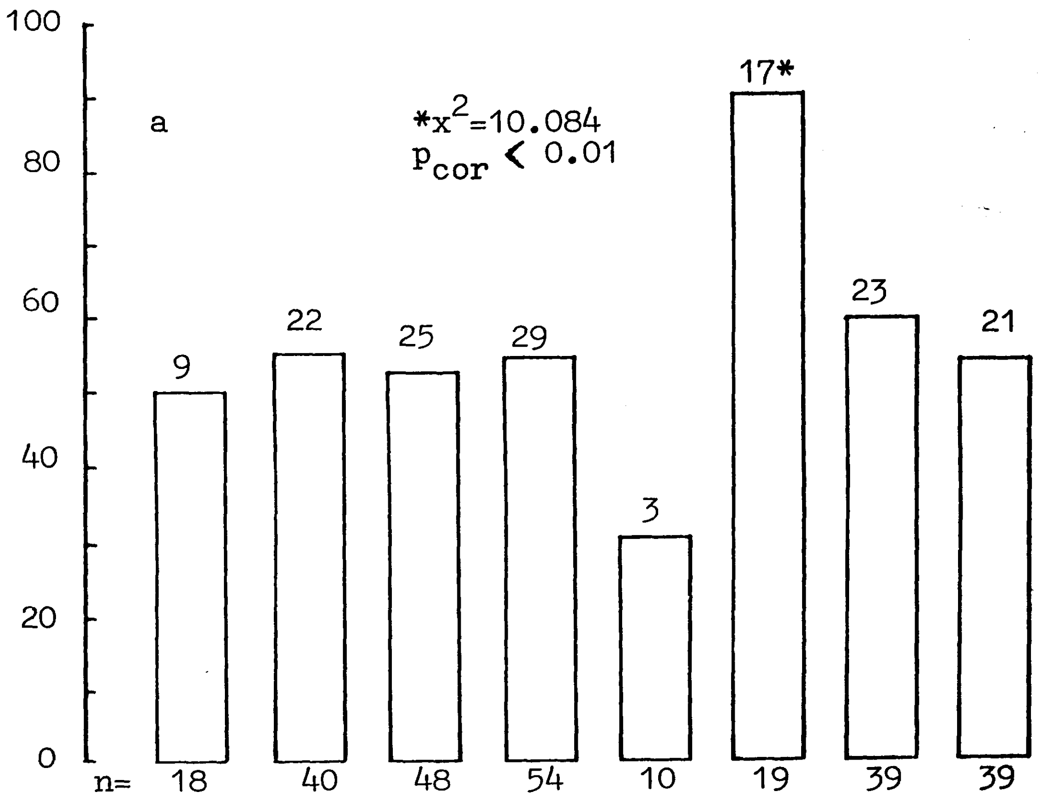
	Mean ± SD	
	Months of dialysis	Number of transfusions
For entire population (n=464):	10.5±17.5	4.3±7.3
Sensitised prior to BT (n=145)	3.9±7.5*	0.0
DNCB 0-3 (n=60)	4.1±7.2^{+}	0.0
DNCB >3 (n=85)	3.7±7.8^{+}	0.0
Sensitised after BT (n=319)	12.8±19.8*	6.4±8.1
DNCB 0-3 (n=243)	14.2±21.0(	7.1±9.0^{++}
DNCB >3 (n=76)	8.3±14.4(	4.3±3.8^{++}

*$p<0.0000$; $^{+}$NS ; ($p<0.02$; $^{++}p<0.002$

TABLE 9.8. ASSOCIATION BETWEEN HLA-DR ANTIGENS AND STRONG DNCB RESPONDERS IN 362 DIALYSIS PATIENTS

	DR1	DR2	DR3	DR4	DR5	DR6	DR7	Blank
Sensitised prior to BT (n=134):								
HLA-DR +ve	9/18 50%	22/40 55%	25/48 52%	29/54 54%	3/10 30%	17/19* 90%	23/39 59%	21/39 54%
HLA-DR -ve	66/116 57%	53/94 56%	50/86 58%	46/80 58%	72/124 58%	58/115* 50%	52/95 55%	54/95 57%
Sensitised after BT (n=228):								
HLA-DR +ve	9/33 27%	26/73 36%	19/63 30%	21/90 23%	7/20 35%	6/25 24%	16/60 27%	26/92 28%
HLA-DR -ve	56/195 29%	39/155 25%	46/165 28%	44/138 32%	58/208 28%	59/203 29%	49/168 29%	39/136 29%

*x^2=10.084, $p<0.0015$, $p_{cor}<0.01$



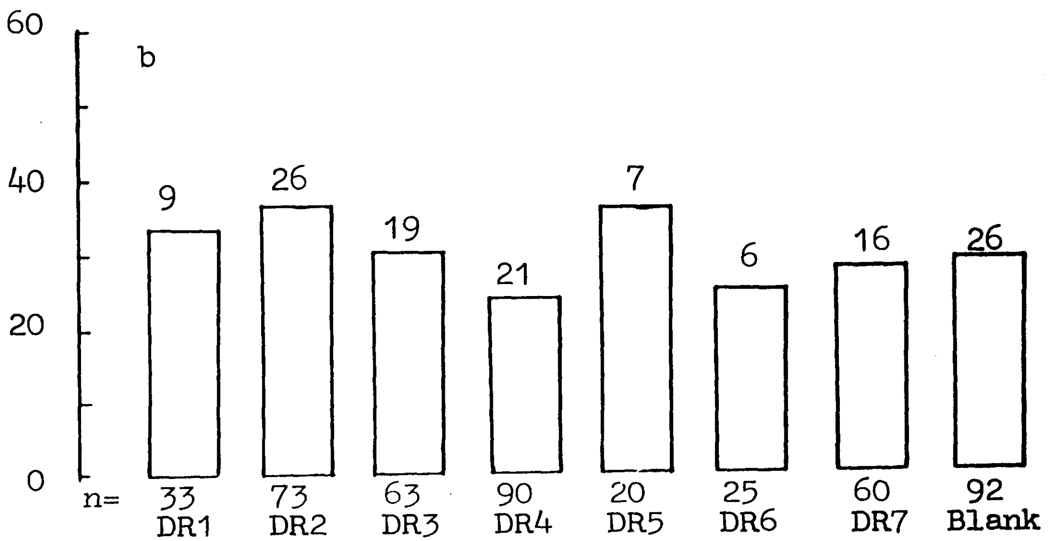


Figure 9.20. Percent of strong DNCB responders in relation to their HLA-DR antigens in 134 patients sensitised prior to any blood transfusion (a), and in 228 patients sensitised after one or more units of blood (b).

TABLE 9.9. DNCB SCORES AT FOLLOW-UP SKIN TESTING IN THE 505 PATIENTS

	DNCB scores (mean ± SD)			
	1st test	2nd test	3rd test	4th test
	n=505	n=380	n=259	n=35
For entire population:	3.2±3.6	3.9±4.4*	4.1±4.9*	5.5±6.1{
	n=171	n=145	n=97	n=17
Sensitised prior to BT	4.8±3.8	6.1±4.3*	6.7±4.9*	7.1±6.4{
	n=74	n=63	n=44	n=10
DNCB 0-3	1.4±1.2	2.6±2.8*	2.7±3.2$	3.4±5.3
	n=97	n=82	n=53	n=7
DNCB >3	7.4±2.9	8.7±3.3*	10.0±3.3*	12.3±3.6#
	n=334	n=235	n=162	n=18
Sensitised after BT	2.4±3.2	2.5±3.9	2.6±4.1	4.1±5.5
	n=255	n=172	n=123	n=12
DNCB 0-3	0.9±1.1	0.6±1.3*	0.6±1.9*	2.1±4.3
	n=79	n=63	n=39	n=6
DNCB >3	7.2±2.9	7.8±3.5+	8.7±3.2*	8.2±5.7

p values against respective 1st test: *p<0.0001; +p<0.006;

$p<0.008; {p<0.02; #p<0.03

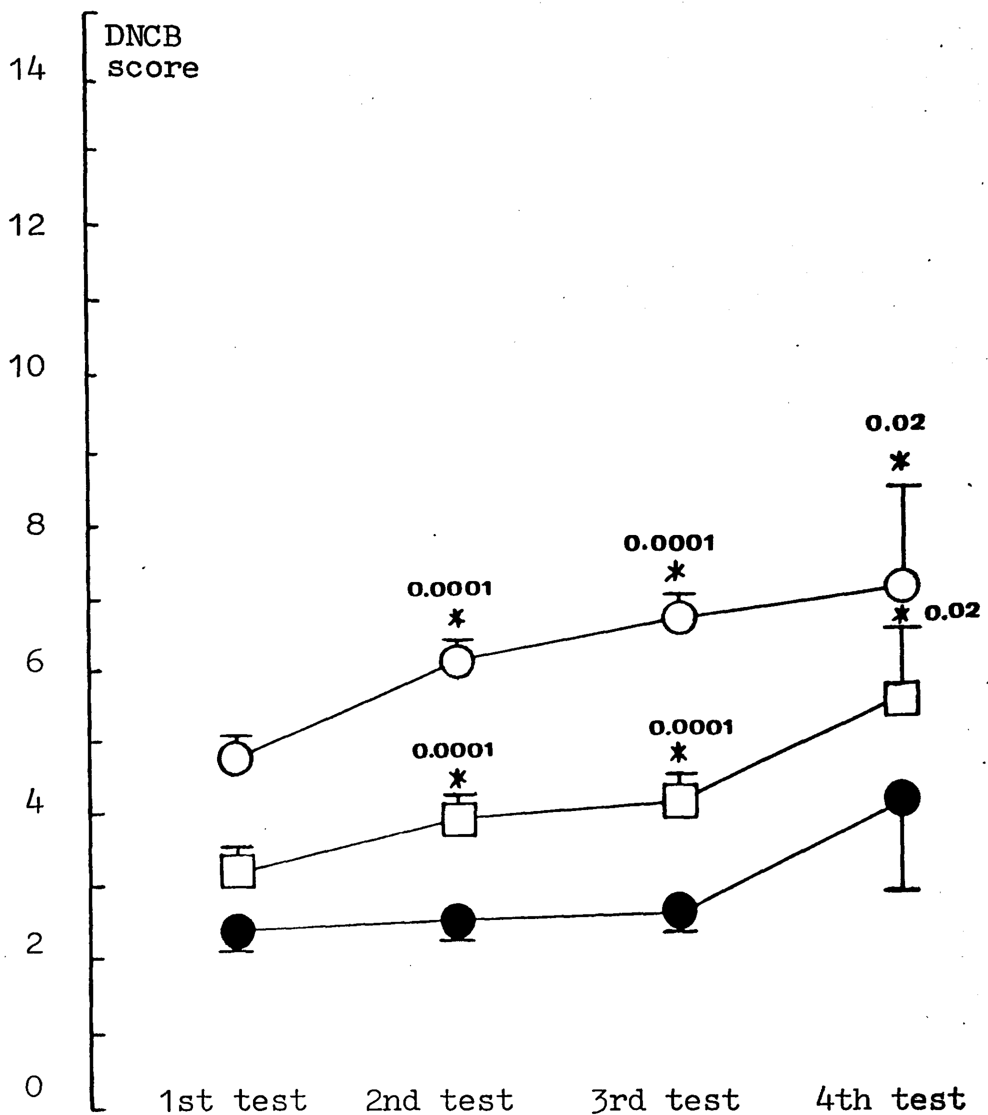


Figure 9.21. Mean ± SEM DNCB scores in the 505 patients (□), and in the breakdown groups of patients sensitised to DNCB before any transfusion (O) and after one or more units of blood (●); p values against respective 1st test; number of patients tested on each occasion are shown in Table 9.9.

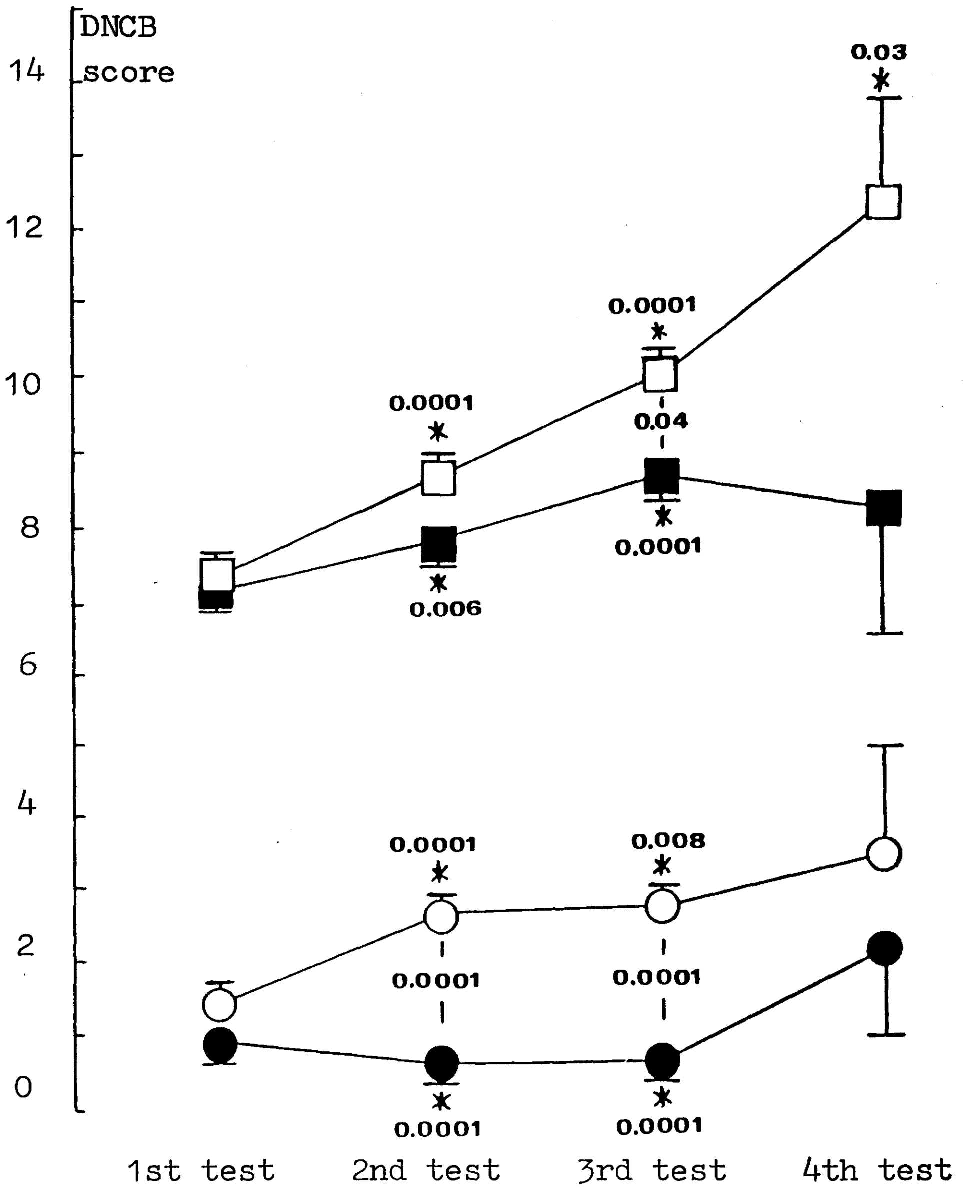
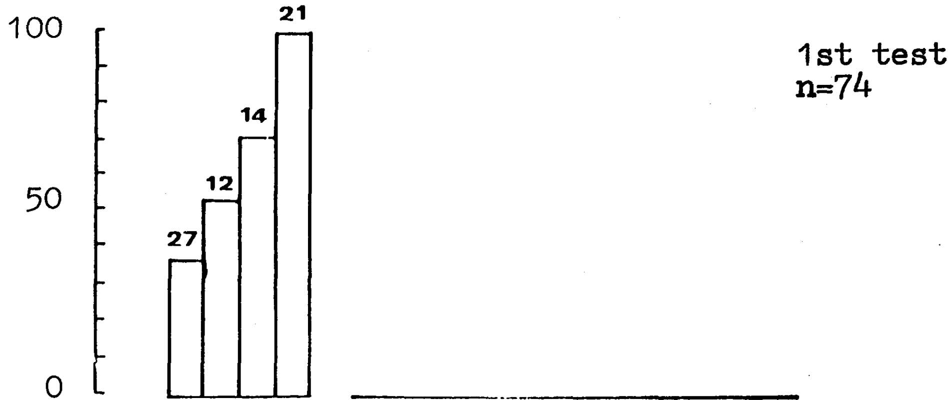
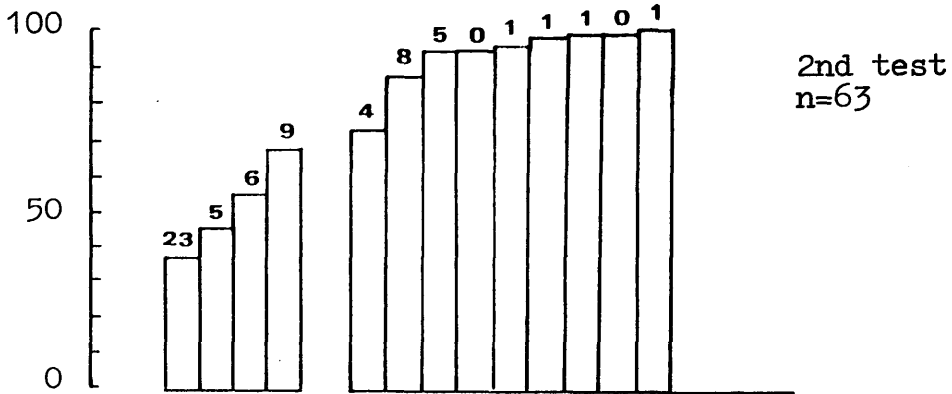


Figure 9.22. Mean ± SEM DNCB scores in the weak (circles) and strong responders (squares); open symbols = patients sensitised to DNCB prior to any transfusion, closed symbols = patients sensitised after one or more units of blood; p values against respective 1st test (*), and between groups; number of patients tested on each occasion are shown in Table 9.9.





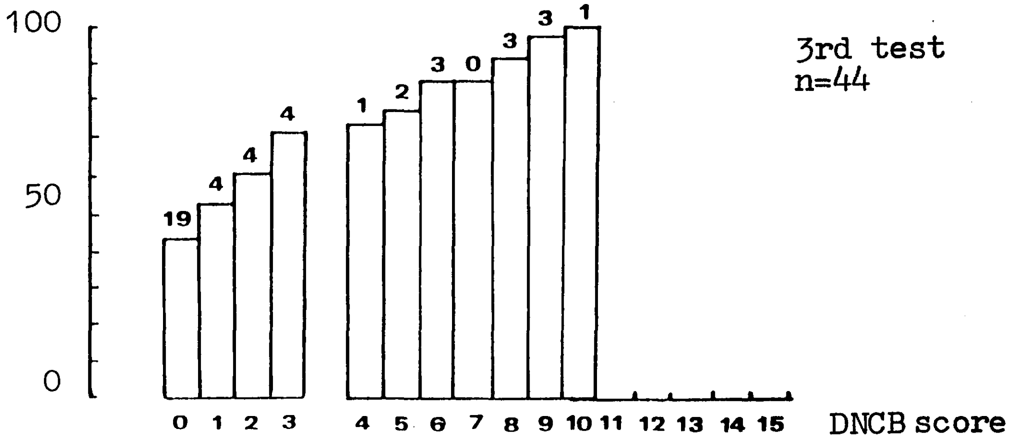
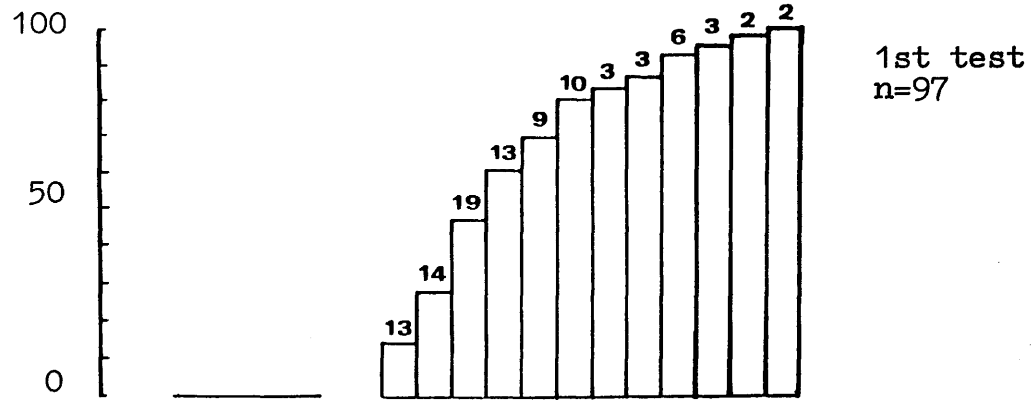
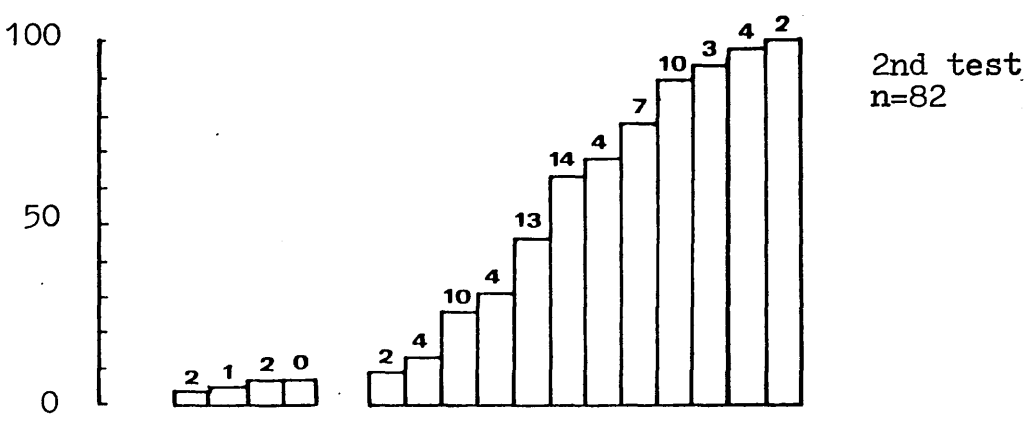


Figure 9.23. Cumulative percent of DNCB scores in 74 weak responders who were sensitised to DNCB prior to any transfusion indicating those who changed to strong responders on serial testing; numbers on top of the bars represent number of patients with the respective DNCB score.

cum %
patients





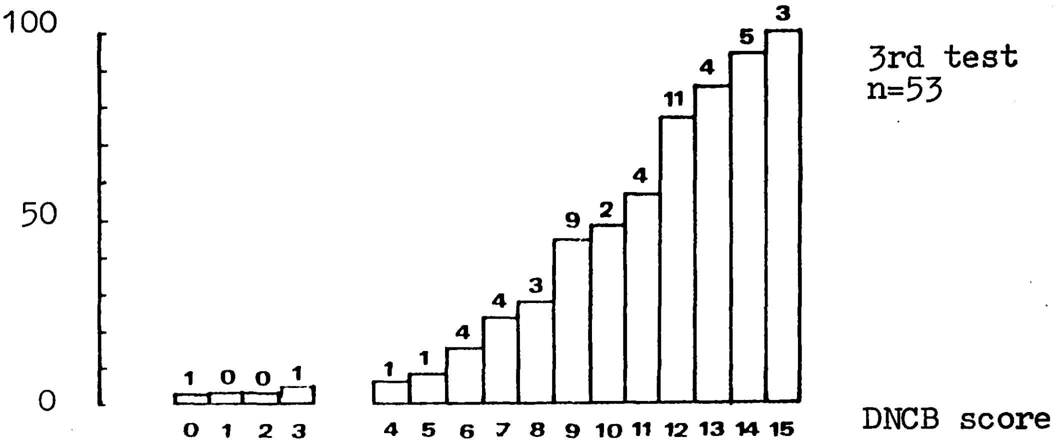
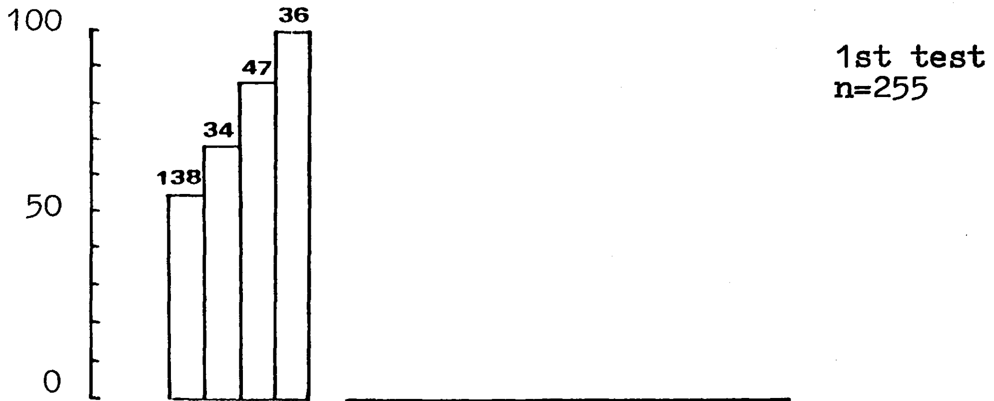
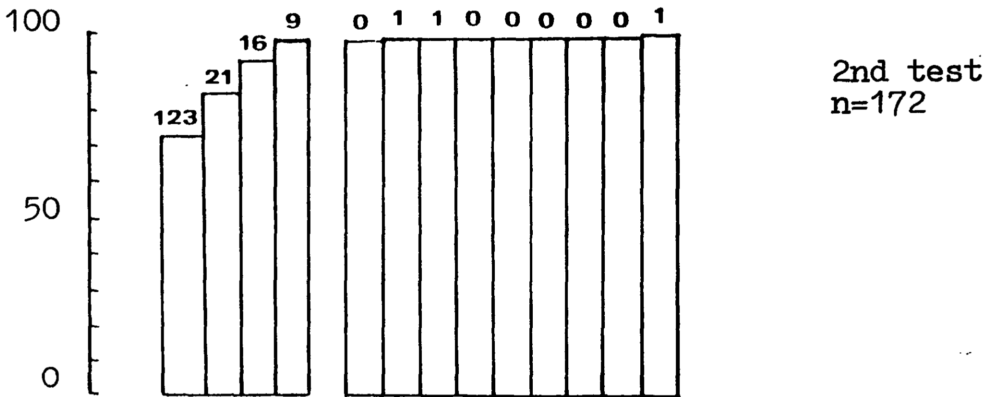


Figure 9.24. Cumulative percent of DNCB scores in 97 strong responders who were sensitised to DNCB prior to any transfusion indicating those who changed to weak responders on serial testing; numbers on top of the bars represent number of patients with the respective DNCB score.

cum %
patients





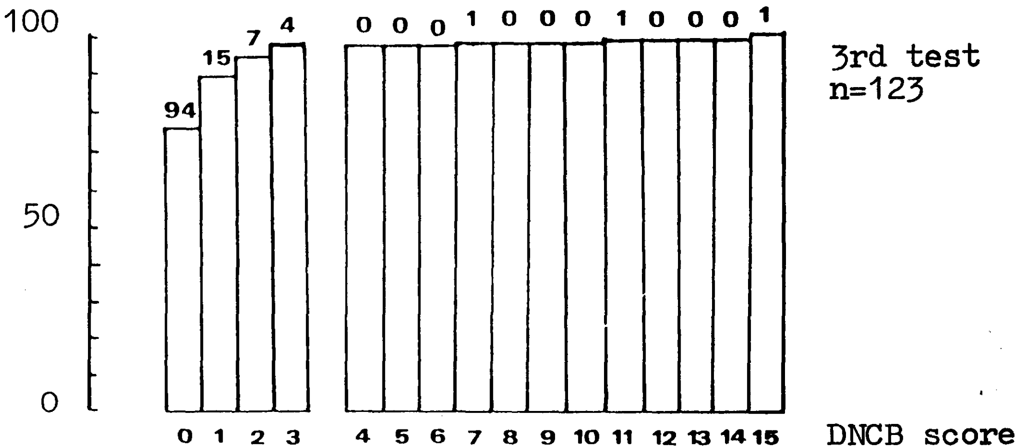


Figure 9.25. Cumulative percent of DNCB scores in 255 weak responders who were sensitised to DNCB after one or more units of blood indicating those who changed to strong responders on serial testing; numbers on top of bars represent number of patients with the respective DNCB score.

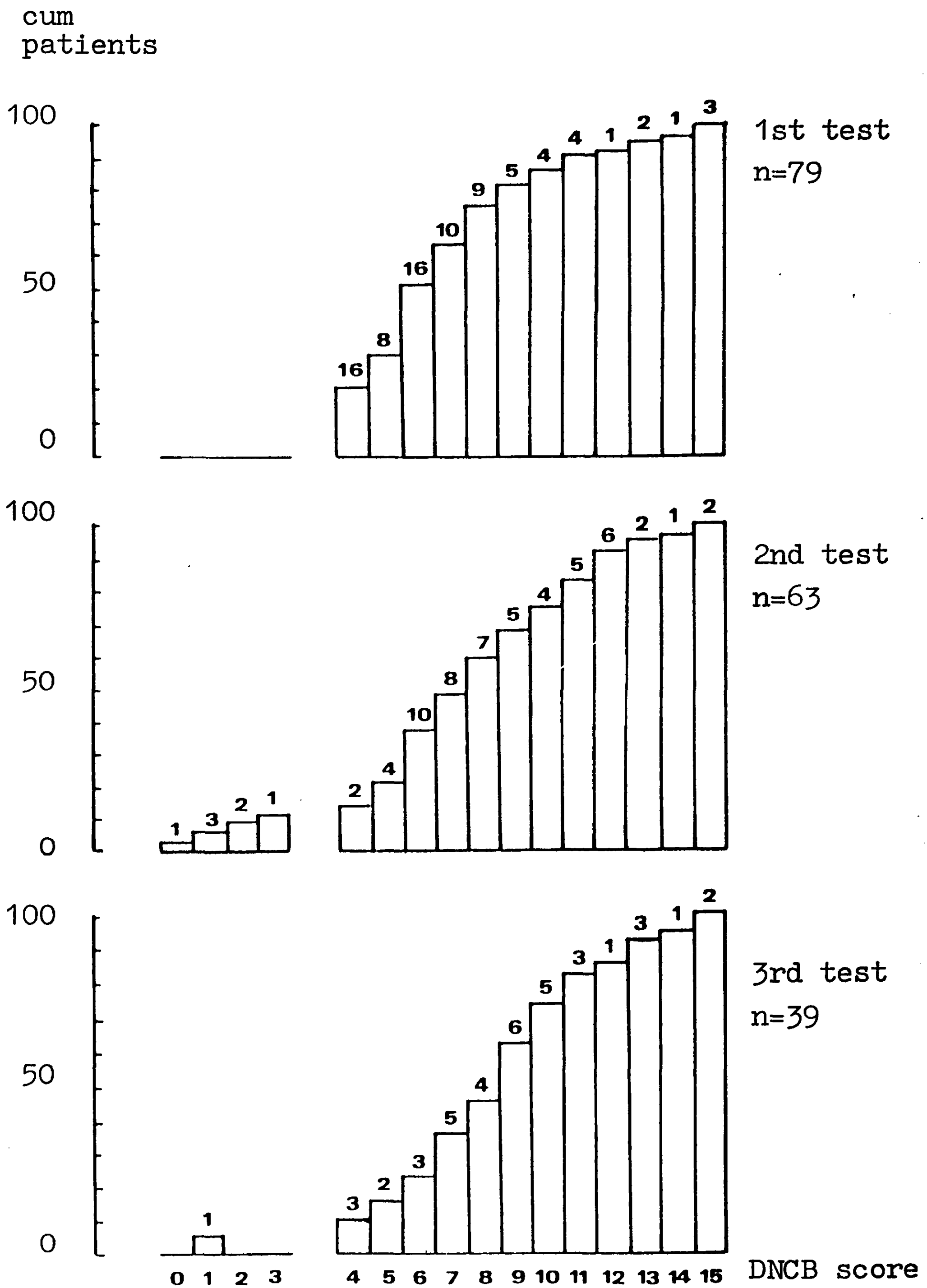


Figure 9.26. Cumulative percent of DNCB scores in 79 strong responders who were sensitised to DNCB after one or more units of blood indicating those who changed to weak responders on serial testing; numbers on top of the bars represent number of patients with the respective DNCB score.

TABLE 9.10 TIME INTERVALS BETWEEN FOLLOW-UP DNCB SKIN TESTS

	Months between tests (mean ± SD)		
	1st-2nd	2nd-3rd	3rd-4th
For entire population:	n=380 12.7±12.4	n=259 14.5±14.3	n=35 25.1±21.3
Sensitised prior to BT	n=145 11.2±12.9	n=97 11.1±12.3*	n=18 22.1±25.3
DNCB 0-3	n=63 12.1±14.9	n=44 12.8±14.8^{+}	n=11 17.8±20.7
DNCB >3	n=82 10.4±11.1	n=53 9.7±9.8}	n=7 28.7±31.8
Sensitised after BT	n=235 13.6±12.0	n=162 16.5±15.0*	n=17 28.4±16.1
DNCB 0-3	n=172 13.9±11.9	n=123 16.9±15.7^{+}	n=12 30.3±16.1
DNCB >3	n=63 12.9±12.6	n=39 15.1±12.9$^{\{}$	n=5 23.6±16.8

*$p<0.003$; $^{+}p<0.05$; $^{\{}p<0.004$

TABLE 9.11. BLOOD TRANSFUSIONS GIVEN DURING TIME INTERVALS BETWEEN FOLLOW-UP DNCB SKIN TESTS

	No of transfusions (mean ± SD)		
	1st-2nd	2nd-3rd	3rd-4th
For entire population:	n=380 5.5±9.7	n=259 5.5±9.6	n=35 9.1±12.8
Sensitised prior to BT	n=145 3.7±4.9*	n=97 4.8±7.4	n=18 7.0±12.2
DNCB 0-3	n=63 3.9±4.7^{+}	n=44 5.5±7.0	n=11 8.7±15.5
DNCB >3	n=82 3.6±5.1$^{(}$	n=53 4.2±7.8	n=7 4.3±3.5
Sensitised after BT	n=235 6.7±11.6*	n=162 5.9±10.7	n=17 11.3±13.4
DNCB 0-3	n=172 6.8±12.6^{+}	n=123 5.9±10.7	n=12 8.7±9.2
DNCB >3	n=63 6.1±8.1$^{(}$	n=39 5.6±11.0	n=5 16.5±19.3

*$p<0.004$; $^{+}$NS ; $^{(}p<0.02$

TABLE 9.12 CHARACTERISTICS OF WEAK AND STRONG DNCB RESPONDERS UNDERGOING TRANSPLANTATION UNDER AZATHIOPRINE AND PREDNISOLONE

	Weak responders (n=189)	Strong responders (n=95)	p value
Sex:			
Male	106 (56%)	69 (73%)	
Female	83 (44%)	26 (27%)	0.007
Age (mean ± SD)	38.9±13.6	39.7±12.3	NS
Duration of dialysis (months)	28.4±28.2	15.9±12.6	0.0001
No of BT (mean + SD)	11.7±13.1	7.1±8.7	0.0001
Type of Tx:			
Cadaveric	175 (93%)	87 (92%)	
Live	14 (7%)	8 (8%)	NS
No of Tx:			
1st	170 (90%)	90 (95%)	
2nd	14 (7%)	5 (5%)	
3rd	5 (3%)	0 -	NS
HLA-A mismatches:			
0	59 (31%)	36 (38%)	
1	91 (48%)	39 (40%)	
2	39 (21%)	20 (21%)	NS
HLA-B mismatches:			
0	65 (34%)	34 (36%)	
1	94 (50%)	49 (51%)	
2	30 (16%)	12 (13%)	NS
HLA-DR mismatches:			
0	62 (50%)	38 (48%)	
1	53 (43%)	31 (39%)	
2	8 (17%)	10 (13%)	NS

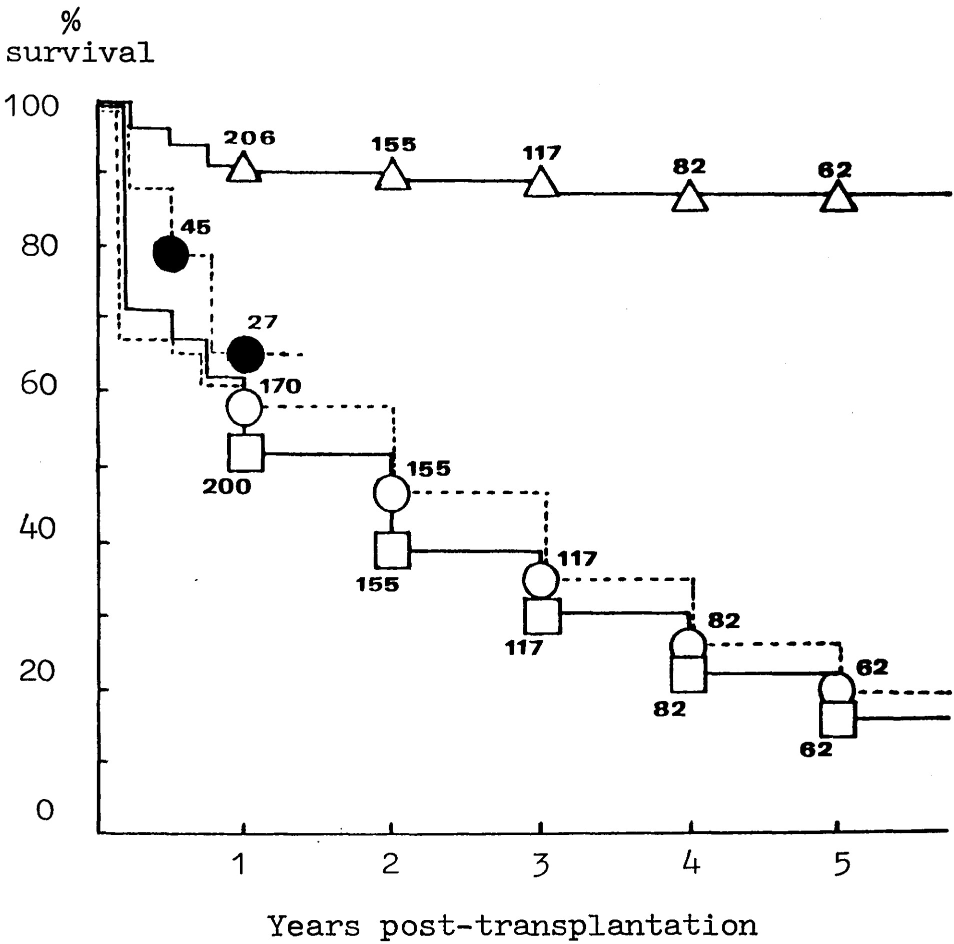


Figure 9.27. Actuarial patient and graft survival (GS) in the 344 transplants; △ = patient survival, □ = GS, all patients (n=344), O = GS, patients treated with azathioprine (n=284), ● = GS, patients treated with cyclosporin (n=57); all graft losses included, 3 patients with combined immunosuppressive regimen were excluded from GS analysis.

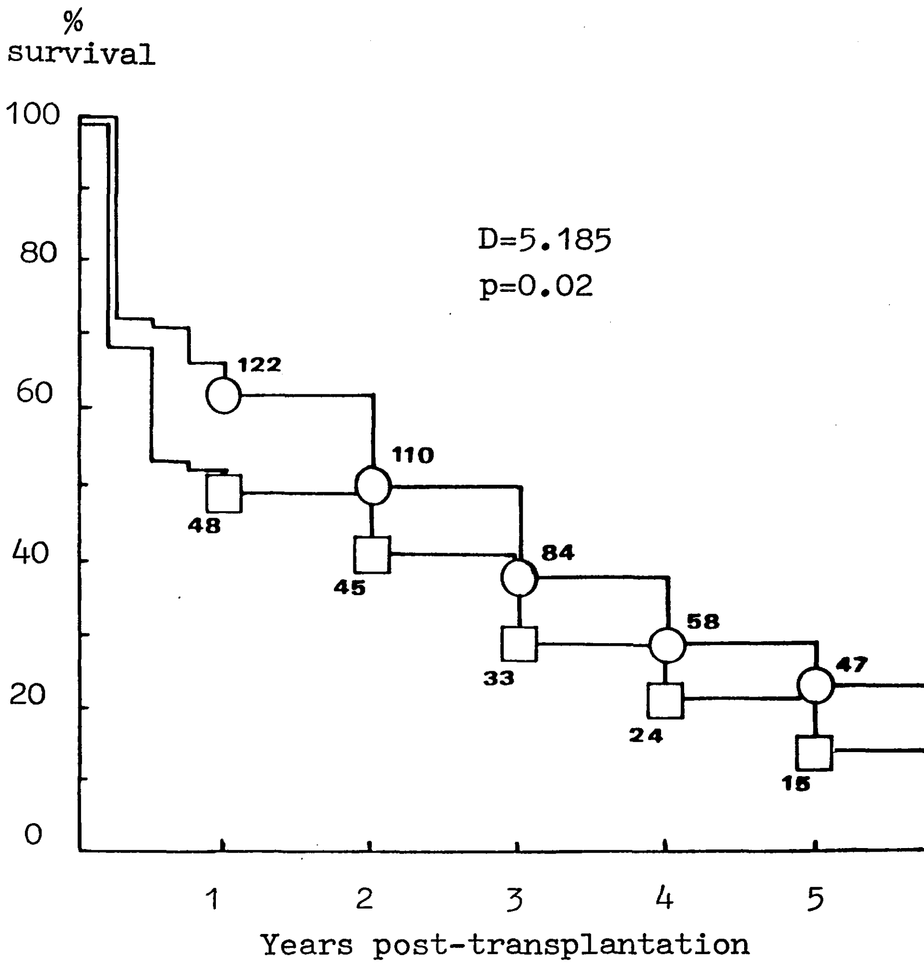


Figure 9.28. Actuarial graft survival in the 284 transplants treated with azathioprine; comparison between 189 weak (O) and 95 strong DNCB responders (□), as classed by the first DNCB test.

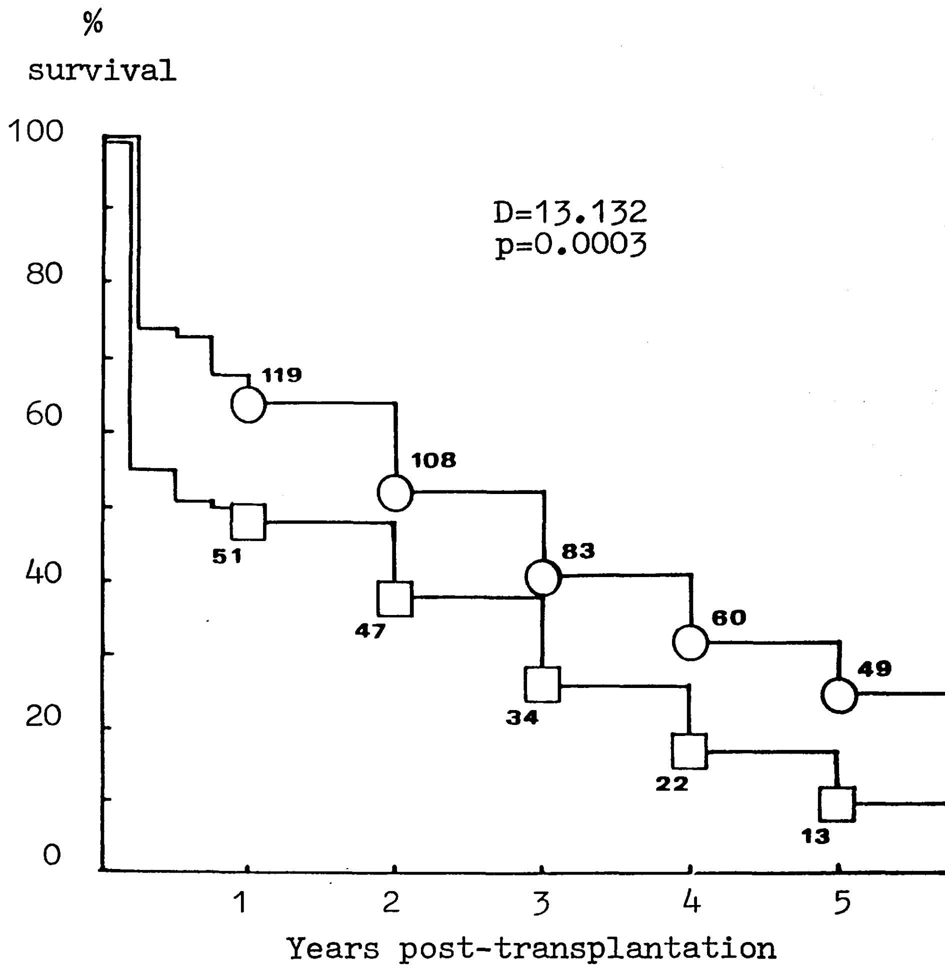


Figure 9.29. Actuarial graft survival in the 284 transplants treated with azathioprine; comparison between 179 weak (O) and 105 strong DNCB responders (□), as classed by the last DNCB test.

TABLE 9.13 CUMULATIVE GRAFT SURVIVAL IN WEAK AND STRONG DNCB RESPONDERS IN THE 57 TRANSPLANTS TREATED WITH CYCLOSPORIN

	Cumulative % graft survival (±SE)		
First DNCB test:	3 months	6 months	9 months
Weak responders (n=37)	89±5	84±6	65±8
Strong responders (n=20)	85±8	70±10	65±11
Last DNCB test:			
Weak responders (n=33)	88±6	82±7	67±8
Strong responders (n=24)	87±7	75±9	63±10

TABLE 9.14 CUMULATIVE GRAFT SURVIVAL IN WEAK AND STRONG DNCB RESPONDERS IN THE 284 TRANSPLANTS TREATED WITH AZATHIOPRINE IN RELATION TO THE NUMBER OF TRANSFUSIONS PRIOR TO TRANSPLANTATION

	Cumulative % graft survival (±SE)				
First DNCB test Weak responders:	1 year	2 years	3 years	4 years	5 years
0 BT (n=8)	50±18	38±17	38±17	25±15	-
1-5 BT (n=52)*	63±7	58±7	46±7	38±7	27±6
6-10 BT (n=63)	67±6	49±6	37±6	25±6	21±5
>10 BT (n=65)	58±6	46±6	34±6	26±6	25±5
Strong responders:					
0 BT (n=9)	33±16	33±16	33±16	33±16	33±16
1-5 BT (n=45)*	53±7	42±7	24±6	20±6	23±5
6-10 BT (n=28)	50±9	43±9	32±9	18±7	7±5
>10 BT (n=13)	46±14	38±14	38±14	23±12	15±10
Last DNCB test Weak responders:					
0 BT (n=8)	50±18	38±17	38±17	25±15	-
1-5 BT (n=45)+	73±7	71±7	58±7	49±8	36±7
6-10 BT (n=61)	67±6	49±6	38±6	26±6	21±5
>10 BT (n=65)	56±6	44±6	33±6	27±6	25±5
Strong responders:					
0 BT (n=9)	33±16	33±16	33±16	33±16	33±16
1-5 BT (n=52)+	46±7	33±7	17±5	13±5	8±4
6-10 BT (n=30)	50±9	43±9	30±8	17±7	7±5
>10 BT (n=14)	57±13	50±13	43±13	21±11	14±9

*D=3.358, p<0.06; +D=17.343, p<0.00001

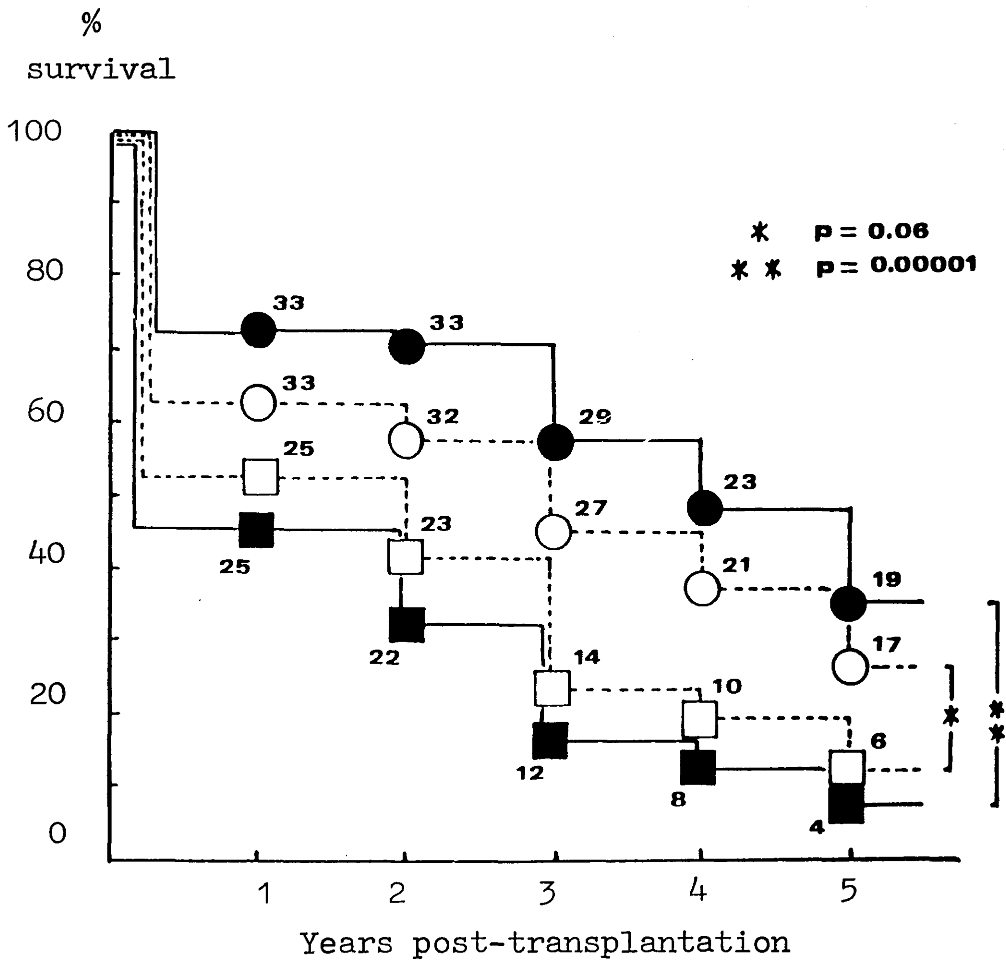


Figure 9.30. Actuarial graft survival in 97 transplants treated with azathioprine who had received 1-5 units of blood prior to transplantation; comparison between weak (O) and strong DNCB responders (□), as classed by the first (open symbols) and the last DNCB test (closed symbols).

TABLE 9.15 CUMULATIVE GRAFT SURVIVAL IN WEAK AND STRONG DNCB RESPONDERS IN THE 57 TRANSPLANTS TREATED WITH CYCLOSPORIN IN RELATION TO NUMBER OF TRANSFUSIONS PRIOR TO TRANSPLANTATION

	Cumulative % graft survival (± SE)		
First DNCB test			
Weak responders:	3 months	6 months	9 months
1-5 BT (n=13)	92±7	92±7	69±13
6-10 BT (n=14)	93±7	79±11	64±13
>10 BT (n=10)	80±13	80±13	60±16
Strong responders:			
1-5 BT (n=7)	86±13	71±17	57±19
6-10 BT (n=7)	86±13	57±19	57±19
>10 BT (n=6)	83±15	83±15	83±15
Last DNCB test			
Weak responders:			
1-5 BT (n=8)	88±12	88±12	75±15
1-6 BT (n=14)	93±7	79±11	64±13
>10 BT (n=11)	82±12	82±12	64±15
Strong responders:			
1-5 BT (n=12)	92±8	83±11	58±14
6-10 BT (n=7)	86±13	57±19	57±19
>10 BT (n=5)	80±18	80±18	80±18

TABLE 9.16 CUMULATIVE GRAFT SURVIVAL IN THE 284 TRANSPLANTS TREATED WITH AZATHIOPRINE AND IN THE 57 TRANSPLANTS TREATED WITH CYCLOSPORIN IN RELATION TO NUMBER OF BLOOD TRANSFUSIONS PRIOR TO TRANSPLANTATION

Azathioprine Group No. of BT:	Cumultative % graft survival (±SE)				
	1 year	2 years	3 years	4 years	5 years
0 (n=17)	41±12	35±12	35±12	29±11	18±9
1-5 (n=97)	60±5	51±5	36±10	30±5	21±4
6-10 (n=91)	62±5	47±5	35±5	23±4	16±4
>10 (n=79	56±6	45±6	35±5	26±5	23±5

Cyclosporin Group No. of BT:	3 months	6 months	9 months
1-5 (n=20)	90±7	85±8	65±11
6-10 (n=21)	90±6	71±10	62±11
>10 (n=16)	81±10	81±10	69±12

TABLE 9.17 CORRELATION COEFFICIENTS OF THE VARIABLES WHICH SHOWED A SIGNIFICANT ASSOCIATION WITH THE TRANSPLANT OUTCOME

Variable	Spearman correlation coefficients Transplant outcome All transplants	Azathioprine group	Cyclosporine group
Last DNCB test	0.121 n=344 p=0.025	0.141 n=284 p=0.02	NS
HLA DR mismatches	0.165 n=261 p=0.008	0.125 n=204 p=0.01	0.370 n=57 p=0.005
Cadaver/Live transplant	-0.118 n=334 p=0.03	-0.168 n=284 p=0.004	NS
No. of rejection episodes	0.343 n=217 p=0.0001	0.322 n=195 p=0.0001	NS
%PRA post transplant (peak)	0.510 n=305 p=0.0001	0.518 n=251 p=0.0001	0.437 n=51 p=0.001
Azathioprine/ Cyclosporine	-0.204 n=344 p=0.0001		
Hospital	NS	-0.126 n=284 p=0.03	NS
Time of 1st rejection episode	NS	-0.203 n=156 p=0.01	NS

DISCUSSION

In order to clarify the effect of blood transfusion in uraemic patients we set up the prospective controlled study which has been discussed in the first eight chapters of this thesis. Although a controlled study is the classical research design, restricting the observations to two groups has an inherent limitation which is to overlook the complexity and variability of the phenomena under investigation. The questions we have posed and aimed to provide an answer to were: a) whether individuals with a strong or weak reaction to DNCB differ in their response to blood transfusion and try to define the optimum number of transfusions for each group to achieve a beneficial effect; b) to see whether blood transfusion-induced changes in CMI responses could be monitored with simple skin tests, and c) to define possible factors determining the response to DNCB and assess the predictive value of this skin test in graft survival.

The research design necessary to provide experimental answers to the above questions would require comparison of more than two groups with more subjects in each group. Luckily more patients were available and were used in Chapter 9 to retrospectively analyse results of DNCB skin testing from a viewpoint suggested by the prospective study. This methodology proved useful and shed light on discrepancies reported in previous studies from our centre.

In the following four sections I shall discuss these points and try to put forward an answer to the questions we have raised, based on analysis of the results obtained from both the prospective and retrospective data.

The effect of blood transfusion on skin testing in uraemia

The first point which deserves attention is the finding of a higher proportion of strong DNCB responders in the group of dialysis patients who had not received prior transfusion. Among the 60 patients were 58% strong and 42% weak reactors (Table 1.1). Thirteen percent had no reaction on first testing and they are included among the weak reactors. These figures are strikingly different from our previous studies where strong DNCB responders were found to account for between 21% and 31% of patients (399-402,571). Retrospective analysis of DNCB testing in 505 patients, which has been described in

Chapter 9, showed that there were 35% strong responders, and the remaining 65% were weak responders, 33% being anergic (Table 9.2). This difference in the proportion of strong responders between patients described in Chapters 1 and 9 is attributed to the effect of blood transfusion, as there was an impressive decline in strong DNCB responders in Chapter 9 with the increasing number of patients transfused prior to the test (Figure 9.13). Among the 171 patients who were sensitised to DNCB prior to any transfusion, the proportion of strong responders (57%) was identical to that described in Chapter 1 (58%). With two previous transfusions there were fewer strong responders at 28% and the number fell to only 6% in the patients sensitised after more than 20 units of blood (Figure 9.13). From these findings it is evident that uraemia and blood transfusion had a synergistic effect in suppressing CMI response in these patients.

In our historic group of normal controls all 15 individuals had a strong DNCB response (score more than 3) as measured by our semiquantitative method, none of them being anergic, ie score 0 (Figure 9.1). Catalona and Chretien studied 143 healthy controls and found that 3.5% had impaired reactivity to DNCB, 2.8% of them being anergic (293). Rolley et al found 65 of 66 normal individuals with a positive response to DNCB (398). Only 24 of his 154 dialysis patients (16%) responded to DNCB and there were no significant differences between patient groups classified by their DNCB response with regards to various parameters including the number of known blood transfusions (398).

These results regarding DNCB response in normal individuals are in accord with our results but there is a discrepancy in the proportion of strong responders in dialysis patients and the results of the transfusion effect between Roley and ourselves. This discrepancy is an example of the numerous conflicting reports which have been produced in the study of immune responses in uraemia. Although there is general agreement that suppression of DTH is a manifestation of the uraemic syndrome, the proportion of non-responder patients varied widely from 28-87% using recall antigens (356, 406) and from 49-84% using the DNCB skin test (398, 406). These variations are due to differences in the protocols of sensitisation or other factors that might interfere with expression

of CMI by skin testing, such as malnutrition (286, 306, 307), infections (296, 297), surgery (379), immunosuppressive agents (286, 308, 309) and blood transfusion (393, 394, 410).

With regards to blood transfusion, Valderrabano et al noticed an increase in non-responders to recall antigens from 46% to 63% within three years of introducing a policy of deliberate blood transfusion in their unit (410). Russ et al using a protocol based on our earlier studies, confirmed our results by showing that the reactivity to DNCB of previously transfused patients was lower than those who had not been previously transfused (408). In order to circumvent the influence of the uraemia-induced immunodepression, Gerbase-DeLima et al studied the DNCB response in multi-transfused non-uraemic patients with a sickle cell disease and thalassaemia and found 50% and 60% responders respectively compared to over 90% of normal individuals (591).

These results provide evidence suggesting that the primary response to skin testing using DNCB and recall antigens in uraemic patients is affected by blood transfusion. Most of the reports which have been reviewed in the introduction regarding immune responses in uraemia have not taken into account this point. So, it seems probable that the effect of uraemia in inducing immunosuppression might have been overemphasised.

This issue becomes more evident when one reviews the results we obtained from the sequential skin testing. Patients who were immunised with DNCB and recall antigens prior to any transfusion gave stronger anamnestic reactions on repeating the test regardless of the amount of blood they had received or whether they were transfused at all (Figure 1.4, 1.6, 2.1, 2.2 and Table 9.9, Figure 9.22, 9.23). Only anergic patients tended to remain anergic and there is some evidence from Chapter 9 that weak responders who were sensitised to DNCB after one or more units of blood might have further depression of their reaction on repeating the test (Figure 9.22).

Indeed, both the individual DNCB reactivity and the proportion of strong responders was increased on repeating the test in patients who were sensitised to DNCB prior to blood transfusion (Tables 1.1, 9.9, and Figures 9.23, 9.26). These findings suggest that primary

immunisation to DNCB is a crucial event and blood transfusion may interfere with the response to it by exerting a suppressive effect on the afferent arm of the immune response.

An early study in 1962 on the immune response in uraemic rabbits is reminiscent of the importance of the timing of the primary sensitisation (343). Gowland et al found that severely uraemic rabbits failed to clear bovine albumin following primary sensitisation, but clearance was very effective when rabbits were sensitised before uraemia was established. Furthermore, an abnormal antibody response to bovine serum albumin occurred only when rabbits were immunised with the antigen while uraemic. Those animals which were immunised prior to the establishment of uraemia showed normal antibody responses when subsequently challenged with the antigen while uraemic.

Similar results to ours were obtained by Russ et al who skin-tested with DNCB 25 dialysis patients before and after a three month period in which three transfusions were given and found an increase in DNCB score (408). Increased reactivity to DNCB with transfusion has been also reported in rhesus monkeys (592).

Support for these stronger anamnestic reactions following rechallenge with DNCB has also been provided from in vitro studies of contact sensitisation in man with DNCB (253, 270, 601, 602). Miller and Levis reported that the in vivo primary response to DNCB correlated with the in vitro blastogenesis using DNCB to stimulate leukocyte cultures from DNCB-sensitised healthy subjects (253). DNCB rechallenge boosted the in vitro response and DNCB-induced blastogenesis which tended to remain elevated for longer than has been observed following primary sensitisation with DNCB (601). This increased blastogenic activity on rechallenge with DNCB was associated with significant lymphokine activity suggesting an amplifying mechanism of CMI involving recruitment of previously uncommitted lymphocytes (602). This was confirmed in uraemic patients by Hamilton et al in our centre who showed that leukocyte migration inhibition by a DNCB/RBC conjugate started to decline three months after the primary sensitisation to DNCB, but it was fully restored when patients were repatched (270).

Giacchino et al, in contrast, reported that three strong DNCB responders changed to weak when transfused but the number of patients was very small and it is not stated whether these patients had been transfused prior to the primary sensitisation to DNCB (593).

It appears from these observations that sequential skin testing does not reflect changes induced by blood transfusion, as we found stronger anamnestic reactions also in uraemic controls who were not transfused at all (Figures 1.4, 2.1). This is an important point because sequential skin testing has been applied in several clinical conditions as a means of testing restoration of CMI. It has been alleged that a few months of treatment by CAPD restores the response to DNCB to normal as opposed to the effect of haemodialysis (387). Our results suggest that CMI is comparable as measured by DNCB testing in these two groups and does not improve in CAPD patients when the blood transfusion effect has been taken into account (594). Follow-up skin testing gave anamnestic reactions which were comparable in the two groups when patients were stratified according to whether they had been transfused or not at the time of sensitisation to DNCB. It would be interesting to apply a similar approach in pulmonary and miliary tuberculosis (595, 596), in surgical patients (300) and following drug therapy with levamisole (300,597), cimetidine (598,599), NSAIDs (86,92,97,599) and zinc (600). Restoration of CMI as measured by skin tests using DNCB and recall antigens has been described in all these conditions. Interestingly, Diamanopoulos in his PhD thesis described restoration of the response to DNCB and PPD in two of seven dialysis patients using levamisole in our centre (597). Both these patients were sensitised before they commenced haemodialysis and prior to any blood transfusion.

Therefore, interpretation of DTH tests should be careful when the design of the experiment does not exclude factors which might influence the development of a specific immunological memory and subsequent secondary responses.

Many investigators have used more than one test to define CMI defects in uraemia and these have been reviewed in the introduction. Kunori et al used different antigens to study the in vitro lymphocyte reactivity in uraemic patients and observed different low responders

with each mitogen (373). d'Apice et al used different functional assays to investigate suppressor activity in uraemic patients and found discordant results, supporting previous suggestions that different assays measure different suppressor cell populations (392). In our patients, there was no correlation between the response to the DNCB test and the response to skin testing with four recall antigens, which suggests that the two tests define different subpopulations of responders and non-responders (Figure 2.3, 2.4). Diamanopoulos, however, in his thesis found a correlation between the DNCB and PPD test in 11 dialysis patients (597). These discrepancies most likely are due to differences in patient selection.

Factors determining the response to DTH in uraemia

DTH has been traditionally thought to reflect in vivo CMI responses which involve primarily two cell types, T helper cells and antigen presenting cells (APC). Numerous studies have described impaired DTH to skin test preparations in both non-dialysed (348, 356,366,397), and dialysed uraemic patients (334,349,348,356,366, 387,398-411) and in uraemic animals (412). However, this well established view has been challenged by considerable experimental evidence which suggests that uraemic lymphocytes are not intrinsically defective, but a suppressive host milieu or other exogenous factors such as blood transfusion, surgery, malnutrition, infections and drugs may all contribute to alterations in their reactivity both in man (286,296,297,306-309,379,393,394,399,402,410, 571) and animals (382-393,412,415).

In this thesis it has been shown that more than half of the uraemic patients who had never received a blood transfusion or renal allograft and who had recently been established on dialysis were capable of eliciting a normal DTH response to DNCB (Chapter 1). This finding partly supports the view that the endogenous immunosuppression due to uraemia may have been over emphasised.

In an earlier study we looked into factors determining the response to DNCB and found that sex, polycystic kidney disease, duration of dialysis and transfusion might influence the response to

the test (402). This, however, was not confirmed in the prospective study where age, sex, original disease, type of dialysis and parity of the patients did not relate to the response to DNCB (Table 3.1).

The number of patients in the prospective study is small, but a detailed analysis of factors determining the response to DNCB in 505 patients has clarified the controversy (Chapter 9.3.2). In a univariate fashion analysis the factors which showed a significant association with the response to DNCB were sex, nephrectomy prior to transplantation, time period when patients were tested, duration of dialysis and number of blood transfusions prior to the test. In a multivariate analysis, however, which excluded the interdependence and the overlapping effect of blood transfusion between these variables, the only significant variable proved to be blood transfusion (Table 9.5, 9.6, 9.7). The importance of the other variables was lost when patients were stratified in terms of whether they had been transfused or not at the time of sensitisation to DNCB (Figures 9.17-9.19).

Roley et al, also did not find any correlation between DNCB response and various parameters including age, sex, race, original disease, duration of dialysis, ABO and rhesus blood types, previous nephrectomy or splenectomy and cytotoxic antibodies (398). This is in agreement with our results, but they did not find that blood transfusion is a significant variable either. Maxwell et al also did not show any association between DNCB response and transfusion in diabetic uraemic patients (403). Rolley et al has classified his patients into responders and non-responders according to negative, positive, and early or irritant response (398). Maxwell et al designated his patients as being either positive or negative according to the induration present in an area where a challenge dose of 50 and 100ug was applied 14 days after the 2000ug sensitising dose (403). These protocols are different from our semiquantitative method where we use smaller challenging doses (399-402,571,574,575). This may account for the fact that these authors did not describe the transfusion effect on DNCB testing. Russ et al who adopted our own method of sensitisation reported results similar to ours (408). Valderrabano et al using seven recall antigens (Multitest) described an increase in the number with anergy from 46% in 1982 to 63% in 1985

after the introduction of a policy of deliberate transfusion in 209 patients awaiting transplantation and anergy was more frequent in patients who had received more transfusions (410). In contrast, Stoffner et al, utilising the Multitest in 253 patients, did not detect any association between DTH response and pre-transplant blood transfusions (414).

It can be recalled from the introduction that a number of metabolites were thought to be responsible for the inhibitory effect of uraemic sera on lymphocyte reactivity, namely methylguanidine, quanidinosuccinic acid, cAMP, corticosteroids, antileukocyte and lymphocytotoxic antibodies, and decreased levels of thymosin and vitamin B_6 coenzyme (317). Apart from certain physicochemical parameters, such as increased osmolality due to sucrose and urea, pH and increased sodium, potassium and calcium concentrations in human lymphocyte cultures inhibited PHA stimulation (603). The results from this thesis do not support any association between a number of haematological and biochemical parameters and the in vivo response to DNCB and recall antigen both prior to and after the transfusion of 5 or 10 units of blood (Tables 3.3-3.5). A couple of weak correlations which were observed were restricted, as the statistical significance was established at the 0.05 level and the number of computations is large enough to justify one in twenty parameters to come up as significant by chance. Another reason which limits the importance of these correlations is the restriction of the range of the studied variables within that seen in end-stage uraemia. Roley et al also did not find any association between DNCB response and peripheral WBC, serum urea and albumin levels (398), and the same conclusion was reached by Diamanopoulos (597).

Malnutrition has been implicated as a cause of depression of DTH (286, 306, 307), but it is unlikely that this was a major issue in our dialysis population. In a large population of 505 patients it is expected that nutritional deficiency would be equally distributed between weak and strong DNCB responders. CMI in geriatric patients as tested by a battery of tests including skin tests with DNCB and four recall antigens was found to be impaired (304). We did not find an association between age and the response to DNCB, but the number of patients exceeding the age of 65 was small (Figure 9.5).

Surgery has also been claimed to be associated with impaired DTH response in non-uraemic man (298-300, 302) and rats (379), but not in mice (412). Our results suggest that patients who had undergone nephrectomy and were not tested immediately after surgery proved to have depressed DNCB response because they had received blood transfusions prior to the test (Figures 9.7, 9.18). Among transplant patients with functioning grafts, who had the DNCB test while under immunosuppression two-thirds were anergic (score 0) and only one in ten had a strong response (Figure 9.1, 9.2). This confirms that immunosuppressive agents interfere with DTH responses (286, 308, 309).

An interesting finding was the association between HLA-DRW6 and the response to DNCB skin test. DRW6 positive patients were significantly more likely to be strong responders when tested prior to any blood transfusion as compared to DRW6 negative patients. This finding was confirmed both in the prospective study (Figure 3.6) and in the retrospective analysis with a larger number of patients (Table 9.8, Figure 9.20). In patients sensitised to DNCB after one or more transfusions, however the DRW6 effect was not found (Table 9.8, Figure 9.20). This evidence supports the concept that HLA-DRW6 is a marker of strong CMI, although this strong response can be modified by blood transfusion.

In 1941 Chase, on the basis of different degrees of contact sensitivity within groups of guinea pigs, showed by breeding experiments that the immune response of guinea pigs to DNCB skin sensitisation was influenced by hereditary factors (180). Since then it has been established that genetically controlled variations in immune response can be mediated by the immune response (Ir) genes (111, 114-117, 121-129). The evidence that DTH reactions are under the control of the Ir genes was provided by experiments using inbred strains of non-responder and responder animals which differed only at the I region of the MHC. These animals were tested for their immune responses to synthetic polypeptides either by quantitating an anti-hapten response using the antigens as carriers or by direct measurement of DTH to the antigens. As DTH reactions are T cell-dependent phenomena, it was suggested that the Ir gene control is linked to MHC and is expressed at T cell level (115, 128, 604).

The association between HLA-DRW6 and a strong response to DNCB in our patients is in accordance with this principle, as DR antigens are the human counterpart of murine Ia molecules, the Ir region in mice (604, 605).

A number of reports from Leiden have described HLA-DRW6 pre-transplant patients as high responders to certain renal allograft antigens (606, 607). Baldwin et al showed that DRW6 was a high-Ir gene for the production of antibodies against a non-HLA antigen present on renal endothelium and peripheral blood monocytes (606) and Hendriks et al reported that DRW6 positive individuals produce more anti-DR antibodies than DRW6 negative ones (607). These antibodies were mainly directed against antibodies present on B cells or monocytes and were associated with irreversible renal graft rejection; HLA-DR matching significantly improved graft survival only in DRW6-positive recipients (607). The DRW6 effect on graft survival was confirmed by several reports from other centres (408-412), but was not supported by Kerman et al (613). Opelz, analysing the CTS data, was also unable to demonstrate a significant DRW6 effect (614). However, because HLA-DRW6 is a heterogeneous and difficult to define antigen, he analysed separately the 9th Workshop-typed transplants and a strong DRW6 effect was apparent (614).

The Leiden group later examined the DRW6 donor effect on graft survival and reported that DRW6 in the donor has a beneficial effect on graft survival regardless of the match and the presence or absence of DRW6 in the recipient (615-617). They suggested that the presence of DRW6 or another class II determinant in linkage disequilibrium with DRW6 activated a suppressor cell circuit which inhibits allograft rejection. However, they were unable to propose a good mechanistic explanation for either the recipient or donor DRW6 phenomenon, as supporting proof provided by experimental and in vitro systems has been rather poor (reviewed in 604, 617). Lehner showed that HLA-DRW6 positive T cells in vitro have a higher affinity for some streptococcal antigens than DRW6 negative T cells (618). Sercarz et al described epitopes on artificial molecules which induced suppressor T cells in mice with particular H-2 haplotypes

(619), and suppressor T cell-dependent low responsiveness to microbial antigen has been associated with the DQW1 determinant (604, 620).

These studies clearly indicate the existence of human Ir genes, the HLA-D and HLA-DR molecules, which are similar to Ia antigens in mice, and can function as restriction elements determining reactivity to simple and complex antigens. HLA-DRW6 could be such an Ir gene regulating the interplay between the cellular components involved in the DTH reaction. From Table 9.8, however, it is evidence that the DRW6 positive strong responders represent only 23% (17 of 75) of the total number of strong responders among our group of patients who were sensitised to DNCB prior to any transfusion. Previous reports of family studies in humans suggested that the control of T cell proliferative responses to synthetic polypeptides appeared to be a polymorphic one, unlikely to be mediated by a single dominant Ir gene (604). The control of such a complex event as DTH may be mediated by an Ir trait coded for by gene(s) in high linkage disequilibrium with DRW6.

This influence of DRW6 on the DNCB response could not be demonstrated in the patients sensitised to DNCB after blood transfusion (Table 9.8). This, together with the overall lowered incidence of strong responders in the transfused group, implies that transfusion suppresses the induction of responsiveness to DNCB by interfering with the function of HLA-DR antigens as restriction elements in cellular interactions in the DTH reaction. We have recently reported that IgG-PFC responses induced by polyclonal activators were significantly suppressed in the PWM-driven system after 5 units of third-party blood in pretransplant patients (621), and the degree of suppression in both DRW6 positive and DRW6 negative patients was equal (575). This is in agreement with the results from Donnelly et al who found no difference in the degree of transfusion-induced plasma suppressive activity between DRW6 positive and DRW6 negative patients (546, 612). Thus, it appears that HLA-DRW6 is a marker of strong CMI response, although this strong response can be modified by blood transfusion.

The predictive value of the DNCB test in graft survival

In 1977 Rolley et al tested the CMI of 154 dialysis patients using the DNCB skin test and showed a correlation between the reaction to DNCB and the outcome of transplantation (398). He found a striking 78% graft survival at two years in non-responders to DNCB compared to 29% in responders. This result was supported by a series of studies from our unit (399-402, 571). Watson et al showed that graft survival at six months was 71% and 15% in the weak and strong DNCB reactors respectively (399), and this difference was reproducible in the follow-up reports (400-402). In these early studies it was noted that weak DNCB reactors required more blood transfusions to maintain their haemoglobin level and it seemed likely that the weak DNCB reactors had greater bone marrow depression than strong reactors which could not only reduce the number and function of lymphocytes but also reduce red cell production (399). This theory could have offered an explanation for the depressed CMI respond and the greater need for blood transfusion in the weak DNCB responders, but conclusive evidence to support this view was lacking. On the contrary, there was evidence to suggest that lymphopenia did not appear to correlate with suppressed DTH responses (366). Therefore the predictive value of the DNCB test in renal transplantation was partly linked to the blood transfusions effect (399), but later this effect was shown to be independent of the influence of the DNCB response, as the six month graft survival was better in all groups of weak reactors compared to strong reactors receiving no blood, frozen cells or whole blood/packed cells (402).

These first reports were based on data analysis from a period when patients were transfused only for reasons of clinical need, but from June 1979 a policy of deliberate transfusion prior to transplant was introduced. All patients received 5 units of concentrated red cells before being considered for transplantation. Another important change after 1981 was a deliberate policy to sensitise to DNCB more patients prior to any blood transfusion and see what happened with the follow-up skin testing, whereas in the early period the timing of the DNCB test was carried out irrespective of their transfusion status. In 1983 we compared the results of graft survival from transplants between 1975-79 and June 1979-81 and we found that the 6

month survival of weak DNCB reactors was comparable at 73% and 71% , whereas the survival in strong reactors improved from 36% to 61% (571). Thus DNCB testing appeared to have lost its predictive value for transplant outcome, because, with the adoption of deliberate pre-transplant transfusion the improvement in graft outcome was observed to occur almost entirely in the strong DNCB responder who appeared to require more transfusion than did weak reactors to improve graft survival (571).

Other workers had suggested that increasing amounts of blood improved graft survival (425, 426, 440, 443, 456, 457), but this was not so in some centres (446, 448, 459, 460). Briggs from our centre reported in 1978 that the beneficial effect of transfusion was no greater in patients transfused more than 5 units compared to those given less than 5 units (447). In 1983, we observed that graft survival in strong DNCB responders improved from 20% in those without any blood prior to transplantation to 38% in those receiving 1-5 units, and to 72% in those receiving more than 5 units (571). In the prospective study described in Chapter 4 the number of transplants was too small to justify firm conclusions, although it appeared that strong DNCB responders had more rejection episodes and the number of transfusion (5 or 10 units) did not seem to associate significantly with the transplant outcome (Tables 4.1, 4.2).

It has already been discussed that an important observation from follow-up DNCB skin testing was that patients eliciting a strong primary response retained and gave even stronger responses in subsequent tests regardless of blood transfusion status at the time of sensitisation to DNCB. Also, weak responders who were sensitised prior to any transfusion were more likely to show increased secondary responses, and as a result of this approximately one third of the latter patients changed to strong responders in follow-up tests. This is the most likely explanation for loss of the predictive value of the DNCB test we described in 1983 (571). When we analysed graft survival in patients treated with azathioprine and prednisolone, using both first and last DNCB test prior to transplantation, the predictive value of the test was best expressed with the last test (Figure 9.28, 9.29). A similar conclusion was reached by Russ et al (408). They showed that patients whose DNCB reactivity increased

when tested before and after a three month period in which three transfusions were given, had worse graft survival compared to those whose score did not change enough to cross the division between weak and strong reactors. It is interesting that many of our patients, particularly those tested prior to transfusion, experienced greater reactivity at 72-96 hours compared with the 48 hour reading which was used to classify them as weak or strong responders. This may reflect the immunosuppressive effect of uraemia and suggest that some of the patients who were not anergic and classed as weak responders at the first test, may be better labelled by a later than 48 hour reading or a second test. It appears that the discrepancy with regard to predictability of the DNCB test was not related to the amount of blood given to strong responders, but to the fact that one third of patients changed reactivity as a result of the policy to sensitise them to DNCB prior to blood transfusion (Figures 9.23, 9.26). This view is also supported by the finding that the weak DNCB responders had better graft survival compared to strong responders over a five year follow-up, and this effect was independent of the amount of transfusion and was most clearly shown in patients receiving 1-5 units of blood (Table 9.14, Figure 9.30).

Apart from Roley et al (398) and our own centre, several reports have confirmed the predictive value of DTH tests in graft survival in patients treated with azathioprine and steroids (403, 408-410). Others, however, did not show this ability to predict outcome. Light et al (406) and Harris et al (411) did not find any correlation between response to DNCB and transplant outcome. Both authors used different sensitisation protocols from ours, which included a smaller primary sensitisation dose and larger challenge dose on repatching. As a result of that, Light et al in 37 unselected dialysis patients had a higher proportion of strong responders (51%) compared to ours (35%) and the extensive use of antilymphocyte globulin in the strong DNCB responders may explain the discrepancy (406). The proportion of responders in the Portsmouth study was no different from ours, but in addition to the different sensitisation protocol they scored their patients in a different way which was based on a control group consisting of 6 normal volunteers

and 4 surgical patients (411). It is likely, therefore, that we may have defined a different subpopulation of responders and non-responders.

In transplant recipients treated with CyA we found that the DNCB test did not have any predictive value (Table (9.13). This is not surprising as CyA proved to be a powerful immunosuppressive agent with specific action on many T cell dependent functions both in experimental and clinical studies. CyA has been shown to inhibit the generation of cytotoxic T cells in humans, the GVHD, chronic inflammatory reactions, the humoral response to T cell dependent antigens, and DTH reactions (622-625). The latter two activities are mediated by T helper cells through inhibition of lymphokine production such as IL-2 and T cell differentiation factor (622, 623). With regard to DTH reactions, which primarily involve T helper cells and APC, CyA has also been shown to inhibit production of IL-1, colony stimulating factor, INF-γ and possibly other inflammatory lymphokines regulating the interaction between T helper cells and APC (622-625). Rabbit dendritic cells have been shown to be sensitive to CyA, although it is not clear whether the effect is a direct one on the dendritic cells or via a secondary effect of presentation of CyA to T cells by the dendritic cells (626). In clinical practice, Kerman et al showed that CyA improved allograft survival in strong responders designated as such by a battery of non-specific assays, including DTH skin testing (627). It can also be recalled that Light et al failed to describe any predictive value of the DNCB test in patients receiving another powerful immunosuppressive compound, ALG (406). It appears, therefore, that in the CyA era the DNCB test has no clinical application. However, in recent randomised studies, Wood et al tried to convert patients from CyA to azathioprine and 19% of the patients experience rejection leading in some to the need to restart CyA (554, 555, 628). It would be interesting to know whether these patients were strong responders.

Blood transfusion-induced immunosuppression in uraemia

Previous studies, which have been reviewed in the introduction, have shown that blood transfusion induces both non-specific immunosuppression mediated by suppressor cell activity or blocking

antibodies/complexes and specific unresponsiveness mediated by anti-idiotypic antibodies (391,393,394,458,495,516,531-546). The most favoured hypothesis is that transfusion alters the lymphocyte reactivity of uraemic patients due to an increase in non-specific suppressor activity via T suppressor cells or monocytes (391,393,394,531,534). As T suppressor cells regulate immunoglobulin (Ig) production (248-250,577,579), one means of assessing their role at the cellular level is to examine the production of Ig by PBMC in response to polyclonal B cell activators which differ in their dependence on suppressor cells. PWM-induced Ig synthesis is influenced by suppressor cells, whereas the response to SAC is not regulated by Con A-induced suppressor T cells (249,577).

In Chapter 6 we described the effect of third-party blood transfusion on IgG secreting cells both spontaneously and after stimulation with PWM and SAC using a PFC assay, and the results are summarised in section 6.3.5.

The first important point is that uraemic patients prior to transfusion had significantly lower spontaneous and SAG-IgG-PFC counts compared to normal controls, but the difference in PWM-IgG-PFC counts was not significant (Figure 6.5). This finding suggests that B cell function in non-transfused uraemic patients is comparable to normal and the defect in IgG secretion which was detected in the spontaneous and SAC-driven systems may be due to altered reactivity of other mediators involved in B cell activation. This observation was confirmed when we extended the in vitro analysis of B cell function to a larger number of patients (629a).

We showed that EBV-induced stimulation of B cells, which occurs in the absence of T cells (250,579), had normal PFC responses, indicating that uraemic B cells retain a normal potential to secrete Ig. This is in agreement with some previous studies which reported that primary and secondary antibody responses in uraemia are normal (reviewed in 317). The low proliferative responses to PHA in our patients confirmed previous reports of depression of T-cell dependent activity in uraemia (335,373,380,384,385,388,397). This finding indicates a defect in the T cell proliferation process. PFC responses to the T cell dependent activator, PWM, were normal in the

non-transfused uraemic patients, suggesting that T helper activity was intact. Unlike PWM, SAC interacts with B cells directly without stimulation of T cells and the resulting proliferation is T cell independent, but full differentiation of B cells depend on T cell help (248,577). This supports the concept that responses to SAC may require pre-activated T helper cells and the abnormal SAC-IgG-PFC responses in uraemia might be due to impaired pre-activation of T helper cells. The role of T suppressor cells in the uraemia-induced defect in T cell proliferation is rather unlikely, as SAC is little influenced by increased suppressor T cell activity, whereas responses to PWM should be profoundly affected (249). This view is in agreement with that of d'Apice et al who failed to support the possibility that suppressor cells are involved in the immunodeficiency of uraemia (392). On the other hand, Langhoff et al were able to show that addition of exogenous IL-2 normalised the impaired responses to PHA and Con A of uraemic lymphocytes (629). This finding suggests that IL-2 plays a central role in the impaired responses in uraemia. A very recent study from the same centre showed that accessory cell function and IL-1 activity was normal in uraemia whereas the defect in T cell proliferation was primarily linked to decreased IL-2 production (630). In another recent study the presence of an abnormally high proportion of pre-activated T cells, as measured by elevated concentrations of soluble IL-2 receptors, was held responsible for the impaired T-cell dependent response to hepatitis B vaccination of haemodialysis patients (631).

To conclude the discussion with regards to uraemia induced suppression, it seems from our results in uraemic patients prior to any transfusion, that the defect must lie within the T cell proliferation process. Other studies are in agreement with this view, and IL-2 activity seems to be primarily involved in this defect.

After blood transfusion in our patients, IgG production in the PWM-driven system was progressively suppressed (Figure 6.7). This suppression was significant after the second unit of blood and peaked after the fifth unit. In contrast, SAC-IgG-PFC counts remained basically unchanged during the transfusion course. The interpretation of these results is based on the differing effects of

PWM and SAC. As full differentiation of B cells to Ig production in response to SAC is dependent on helper T cells (248,577), and SAC-IgG-PFC counts were not affected by transfusion , it is assumed that blood transfusion does not affect helper T or B cell function. In contrast as PWM-induced B cell differentiation is influenced by suppressor T cells (249), it would be likely that the decreased PWM-IgG production after transfusion is due to activation of suppressor T cells.

This finding confirms results from other studies where T suppressor cells were involved in the transfusion effect. Smith et al described an increase in Con A induced suppressor cell activity in previously non-transfused uraemic patients after a transfusion with two units of packed red cells (393,394). This suppressor activity was not related to the number of suppressor T cells nor to the development of cytotoxic antibodies, and returned to normal by 20 weeks from transfusion. Lenhard et al, utilising MLC and Con A suppressor assays in previously non-transfused patients, showed that blood transfusion induced at least two different cell mediated immunoregulatory mechanisms (534). In the early post-transfusion period, non-specific immunosuppression was probably mediated by the action of monocytes, whereas in a later phase, increasing suppressor T-cell activity was demonstrable. Both effects seemed to be dependent on the number of transfusions and the time interval between them (632). In a more recent study in rats, Lenhard et al showed that multiple DST depressed subsequent antibody responses to the blood donor and this was transferable by suppressor T cells carrying the MRC OX8 marker (633). Klatzmann et al also studied the suppressive effect of blood transfusion in selected previously non-transfused uraemic patients. They found a sustained decrease in MLR after the second or third transfusion (634), and this is in agreement with our results. The induction of suppression appeared to be primarily non-specific, because the response to cells from the specific blood donor, as well as to third party stimulating cells, was generally inhibited (634). However, the existence of two different types of suppression was suggested, one of which was alloantigen non-specific and radiosensitive, whereas the second was

radioresistant and specific, through soluble mediators, for cells sharing at least one HLA-DR antigen with the blood donor who had been used for transfusion (634,635).

From these studies it is evident that suppressor T cells may be primarily involved in the blood transfusion effect, and our findings provide evidence that inhibition of Ig production resulting from transfusion is possibly mediated by suppressor T cell activity.

With regard to spontaneous IgG-PFC counts, there was an overall progressive increase after each transfusion (Figure 6.7), which was due to a significant increase in IgG production in 46% of the transfused patients (Figure 6.12). The reason for this is not clear, and it is likely that this increase in resting B cell activity reflects a specific or non-specific response to antigens present on the transfused cells. Regression analysis showed that there was no correlation between spontaneous IgG-PFC counts and cytotoxic antibodies developing after 5 or 10 units of blood (Tables 6.11, 6.12).

Within the main group of transfused patients there were subgroups with a high or low rate of IgG production both spontaneously and after stimulation with SAC or PWM (Table 6.4, Figure 6.11), but the DNCB skin test failed to identify them (Figure 6.11). Again this confirms a point which has been raised earlier, that different tests define different subpopulations of responders and non-responders (373). The same conclusion was reached in multitransfused non-uraemic patients, where no correlation was found between the response to DNCB and the response to PHA, cytotoxic antibodies and the MLC inhibitory effect (591). With regard to the degree of the transfusion-induced suppression of IgG production in the PWM-driven system, there was no difference between weak and strong DNCB responders. This finding provides an answer to one of the questions we posed when this experiment was planned. Thus, it appears that strong DNCB responders are equally immunosuppressed after transfusion compared to weak responders. Furthermore, whatever effect blood transfusion had on the individual weak or strong DNCB responders, ie enhancement, suppression or unresponsiveness, it was demonstrable by the fifth transfusion. Also further 5 units of blood

did not change significantly their overall reactivity, suggesting that strong DNCB responders do not seem to require more transfusions, as we had thought in an earlier study (571).

The prostaglandins (PGs) have been enjoying massive attention from the research community, particularly with regard to their role as regulators of immune responses. Although numerous studies have established the immunosuppressive effect of PGs under in vitro conditions (reviewed in 81,83, 5,86,92,94,101), most of the studies have been carried out in experimental models or healthy humans. PGE-mediated immunoregulation is abnormal in certain diseases (85, 92, 94, 101, 102) and very little is known about the complexity of such mechanisms in the uraemic state.

In 1976 Webb and Osheroff reported that following intravenous injection of sheep erythrocytes in mice there was a 20 to 80 fold increase in PGF2a in the spleen (636). This elevation of splenic GF2a level was blocked by indomethacin, and this inhibition enhanced DNA synthesis induced in MLR only in whole spleen cell cultures and not in cultures purified from adherent cells.

This was the first indication that PG-mediated alterations in immune response could be induced by infusion of blood products. At the same time there were few conflicting reports which linked graft survival with PGE1 in the rat, mouse and dog model (99,637,638). When the transfusion effect on graft survival was well established in the early 1980's, we thought it would be interesting to include measurement of PGE activity in our prospective blood transfusion protocol.

In a preliminary report which was published in 1985, we observed that transfusion induced an increase in PGE release, which was mainly due to the increase observed in weak DNCB responders (586). Strong DNCB responders produced concentrations of PGE comparable to those of weak DNCB responders only after transfusion of 8-10 units of blood. This finding fitted the hypothesis we had expressed then, that strong DNCB responders might require more blood to elicit a beneficial effect on graft survival (571).

The final results from this experiment have been described in Chapter 7, and only partly confirm the results from the preliminary study. The first point of interest was that the in vitro PGE

synthesis in Con A stimulated PBMC culture supernatants in previously non-transfused uraemic patients was comparable to that of the healthy controls (Table 7.2 and Figure 7.3). Lenhard et al also did not find any difference in pre-transfusion PGE2 and PGF2a release in 25 dialysis patients compared to normal controls (639). This suggests that the uraemia induced immunosuppression is mediated by mechanism(s) which might not involve PGE activity. Evidence to support this view comes from experimentally induced uraemia in rats. Alevy and Slavin found that adherent spleen cells from uraemic animals display a more potent suppressor activity than do control adherent cells (382). The suppression mediated by uraemic adherent spleen cells was not inhibited by indomethacin, as opposed to that of controls, and furthermore the uraemic adherent suppressor cells did not have Ia antigens, whereas control adherent cells had (382). Similar results indicating the presence of more than one suppressor mechanism in rats have been reported by Mattingly et al (640) and Folch and Waksman (641). In human PBMC, Rice et al described three suppressor cell systems, only one of which is dependent on PG synthesis (642). In patients with sarcoidosis there is evidence suggesting that there may be at least two adherent cell subsets inducing suppression only one of which is mediated by PG (643,644).

In uraemic patients, Langhoff and Ladefoged demonstrated that their lymphocytes had a 3 to 4-fold increase in sensitivity to methylprednisolone compared to normal controls which caused 50% suppression of T cell proliferation in response to PHA and Con A (645). This inhibitory effect is thought to be mediated through inhibition of IL-2 release (629), and the difference in the inhibitory effect of steroids on IL-2 production between uraemic patients and controls suggests differing interactions at a cellular level. Smith et al, however, showed that the addition of flurbiprofen to the Con A enhancement cultures of dialysed and normal subjects caused a significant decrease in suppressor cell activity indicating that the suppression seen both in dialysed and normal controls is mediated by PGE (393).

From these studies it is evident that there may be more than one mechanism regulating responses of immunocompetent cells, and which mechanism prevails might depend on specific in vitro conditions or a disease state.

Blood transfusion in our uraemic patients induced a significant increase of PGE synthesis which was more pronounced after the 3rd unit of blood (Figures 7.3, 7.5). The presence, however, of the increased PGE release was not associated with the PWM-IgG-PFC suppression which was observed following transfusion of one to ten units of blood (Table 7.6). In vitro studies using human cells have not clearly defined a role for PGE in the regulation of B cell responsiveness. Goodwin et al reported that PGE2 had minimal inhibitory effect on PWM-induced DNA synthesis in cultures of unseparated human lymphocytes, whereas significant inhibition of PHA and Con A induced ^{3}H-thymidine incorporation was noted (646). Stobo et al showed heterogeneity in the response of human PBMC to PGE, as high density cells were very sensitive to PGE-mediated suppression whereas the proliferative response of low-density cells was enhanced by PGE (100). Thompson et al provided further evidence of a function dichotomy in PGE sensitivity of B cell subsets in response to PWM or staphylococcus aureus (SA)-stimulated cultures (647). PGE suppressed SA-induced B cell proliferation but not PWM-stimulated B cell DNA synthesis, because PGE did not inhibit the production of BCGF activity from PWM-stimulated T cells. This suggests that the secretion of some, but not all, regulatory lymphokines produced by activated T cells may be inhibited by PGE, as for example happens with the secretion of IL-2 (581-585).

These varied effects of PGE were described in normal human PBMC and indicated the complexity of regulatory interactions between macrophages, T cells and B cells. With regard to uraemic patients Lenhard et al, like us, found that transfusion of three units of blood induced a dose dependent increase of PGE concentrations in LPS-stimulated PBMC which inhibited MLR responses (639). They attributed the transfusion induced suppression to activation of a short lived T suppressor cell by PGE.

Fisher et al reported that PGE at high concentrations in normal humans may activate a short lived radiosensitive T suppressor cell which displayed the same characteristics as Con A-activated T suppressor cells (648). On the other hand, the existence of a PG-producing suppressor cell in healthy subjects has been identified in a population of glass adherent mononuclear cells which suppressed T-cell mitogenic activity (646).

It can be recalled that Klatzmann et al, when studying the beneficial effect of transfusion in uraemic patients, suggested the existence of two types of suppression, one which was non-specific and radiosensitive and the other specific and radioresistant (634,635). van Rood and Lenhard et al also tried to implicate a two-step process (an early non-specific and a later specific) in order to reconcile differing findings with regard to the transfusion effect (458,534, 632).

Our results support this possibility as we did not observe any correlation between PGE concentrations, which are known to exert some suppressive activity, and transfusion induced suppression of IgG production in the PWM-driven system. Roy et al in a Con A enhancement assay also were unable to associate suppressive activity induced by 1-5 units of blood with PGE2 release in culture supernatants (649). These findings are in agreement with failure of flurbiprofen to produce significant decrease in suppressor activity to Con A enhancement cultures of lymphocytes from transfused patients in the study of Smith et al (393).

The difference in PGE concentrations between weak and strong DNCB responders prior to and after 1-10 transfusions was not significant in our patients (Table 7.3, Figures 7.6, 7.7). Furthermore, transfusion of a further five units of blood did not have any significant effect in PGE concentrations compared to the initial five units in the whole group of patients (Table 7.4, Figure 7.8) nor in the weak and strong DNCB groups individually (Table 7.5). These findings are not in accord with the results in our preliminary report (586), apparently because of the difference in the number of patients and the large intersubject variation in PGE concentrations before and after transfusion (Figure 7.4). These results imply that PGE concentrations did not interfere with the expression of DTH

responses to DNCB in our patients. Furthermore, to the extent that the transfusion effect is mediated by PGE concentrations, strong DNCB responders do not appear to acquire stronger suppressive activity compared to weak DNCB responders following transfusion regardless of the amount of blood. In other words, using PGE concentrations as a measure of transfusion-induced suppression, it appears that strong DNCB responders do not require more blood to achieve suppression comparable to that of weak DNCB responders. This again does not confirm the hypothesis we proposed in 1983 (571).

As mentioned before, in the mid 1970's there were conflicting reports in experimental animal models which had associated graft survival with PGE (99, 637, 638). More recent reports suggest that PGE enhances graft survival. Strom and Carpenter reported that treatment of rats with a PGE1 derivative with a prolonged half-life resulted in a near complete protection of renal grafts from immunologic damage, despite withholding immunosuppressive therapy until day 4 following transplantation (650). Campbell et al implanted a pump in a canine model and continuously delivered PGE1 into the renal transplant artery (651). Although allograft failure was not prevented, there were striking differences in the histology between treated and untreated transplants. PGE1 perfusion resulted in the appearances of large numbers of polymorphonuclear leukocytes, but few lymphocytes, whereas in the control allografts, the picture was typical of lymphocyte-mediated acute rejection.

In our study, patients who were subsequently transplanted and had a successful outcome showed a significant increase in PGE levels after the last elective transfusion, whereas in a small number of patients who rejected their graft, such an increase was not observed (Figure 7.10). Roy et al, who also was unable to directly associate the suppressive activity after transfusion with PGE2, found 84% graft survival at one year in those whose suppressive Con A ratio was more than 5 after transfusion, compared to 56% among those with a Con A ratio less than 5 (649). These results, although inconclusive, suggest that PGE might be an important mediator in transfusion-induced immunoregulatory events.

The effect of blood transfusion on serum complement levels after transfusion was described in Chapter 8. There was no measurable effect in the entire group of patients, nor in the weak and strong DNCB groups individually. Complement has an immunoregulatory role in antibody induction and generation of memory B cells (reviewed in 107), but we did not find any correlation between complement levels and IgG production either spontaneously or after SAC or PWM stimulation before or after transfusion.

The last point to be discussed in this thesis is the development of cytotoxic antibodies following transfusion, which has been described in Chapter 5. We were aiming to define the optimum number of transfusions in weak and strong DNCB responders required to achieve a beneficial effect on graft survival, while keeping the risk of sensitisation to a minimum. The risk of broad sensitisation was negligible after 3 or 4 units of blood but was increased with more transfusions particularly in women. This is in agreement with previous studies (491-496, 509-513). The sensitisation rate was not significantly different between weak and strong DNCB responder patients (Table 5.1), but the latter patients developed cytotoxic antibodies earlier after transfusion. After 5 units of blood both the rate and level of PRA were comparable in the two groups (Table 5.2, Figures 5.2-5.5). Gerbase-DeLima et al also did not find any association between DNCB and cytotoxic antibodies in multitransfused non-uraemic anaemic patients (591). Early development of PRA before the 5th transfusion has been associated with worse graft survival compared to those sensitised beyond the fifth transfusion and it was thought that the former patients represent a group of high responders (493). In our study the DNCB test detected this group of high responders.

The beneficial effect of transfusion in our study seemed to have occurred after the first three transfusions, since PWM-IgG suppression and PGE release were significantly increased by then. As there were no differences in suppressor activity between weak and strong DNCB responders after the 3rd unit of blood, and the risk of sensitisation increased with further transfusion in both groups, we established a policy of giving three units of blood to our pre-transplant patients after obtaining these results.

This study was based on the hypothesis that strong DNCB responders might require more blood in order to achieve a beneficial graft survival. The results did not confirm this hypothesis, but shed light on discrepancies, provided useful observations on the application and interpretation of DTH skin tests, and further established the presence of uraemia and transfusion induced suppression in these dialysis patients. Fisher (652) has claimed that '.....the null hypothesis is never proved or established, but is possibly disproved in the course of experimentation. Every experiment may be said to exist only in order to give the facts a chance of disproving the null hypothesis'.

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