ALLOGRAFT REJECTION

and the

RETICULOENDOTHELIAL SYSTEM


A thesis submitted for the degree of Doctor of Medicine.

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RETICULOENDOTHELIAL SYSTEM.

A study of the changes in Reticuloendothelial system activity
associated with allograft rejection and anti-lymphocyte serum
therapy.
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This thesis contains no material previously submitted by me for a degree in any University, and to the best of my knowledge contains no material previously published by another person except where due reference is made in the text.

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ABSTRACT

While further evidence has been provided that skin allografts stimulate the reticuloendothelial system as indicated by an increased rate of clearance of $^{32}$P labelled *Salmonella typhimurium* (C5) from the circulation, it has been shown that the magnitude of this increase is directly related to the size of the graft employed.

Splenomegaly was observed in these allografted mice and this as with the increased rate of clearance of labelled bacterial particles occurred just prior to macroscopic rejection of the graft. Splenectomy did not, however, alter the subsequent survival of the graft or the changes in the activity of the reticuloendothelial system. The distribution of the radioactive label indicated that the phagocytic cells of the liver were primarily responsible for the increased rate of clearance observed. This increased rate of clearance which reflected one aspect of the activity of the reticuloendothelial system was not paralleled by an increased ability of such an animal to produce antibodies when challenged with another antigen.

The demonstration of changes in phagocytic activity following removal of the graft at varying intervals and the relationship of this response to macroscopic and histologic graft rejection suggests that the release of antigens from the graft stimulates the reticuloendothelial
system rather than graft rejection per se. This, however, does not appear to be a direct effect of the antigens on the cells of the reticulo-endothelial system but results from a factor released by the lymphoid cells after interaction with the antigen.

These studies have also shown that the measurement of phagocytic activity may be of value in indicating the presence of a host versus graft reaction.

The utilisation of heterospecific anti-lymphocyte serum and its associated immunosuppressive properties has made it possible to investigate the phagocytic activity of animals clearly incapable of rejecting an allograft. The initial step was to establish a satisfactory time, dose and route of administration for the antiserum. These studies revealed that the time of commencement of the antiserum in relation to the day of grafting as well as the dose were of importance to the subsequent survival of the graft, whereas the route of administration was unimportant.

Apart from achieving prolongation of allograft survival the antiserum used in the various experiments was also found to produce marked hyperplastic changes in the lymph nodes and spleens with the appearance of large numbers of pyronin-positive cells. The observation that these changes correlated well with allograft survival suggested a relationship between this histologic picture and
the disturbed immune function.

The clearance studies indicated that the antiserum treated animals had an adequately functioning reticuloendothelial system. However, commencing a given antiserum regime prior to antigenic challenge was more effective than the same regime commenced after this challenge, suggesting that the antiserum had a significant effect on the initiation of the immune response.

Two important practical points have emerged; firstly antiserum treated animals were not more susceptible to infection; secondly, while it was not possible to produce tolerance to a purified protein the simultaneous administration of anti-lymphocyte serum and antigen resulted in a significantly reduced secondary response to the same antigen.
INTRODUCTION AND REVIEW OF LITERATURE

It is in the realms of mythology and legend that we gain the first glimpses of man's interest in joining portions of separate individuals, although at this time such a task was clearly the special province of the deities. In these legends and myths we see the appearance of a host of strange composite animals, one of the most striking being the chimaera of Greek mythology. This was without doubt a most formidable female, with the head of a lion, the body of a goat and the tail formed by that of a dragon. One may perhaps say that these creatures were the creation of the dream world of ancient mankind and quite devoid of reality. However, the idea that portions of separate individuals could be joined had been provided. This, plus an awakening of a desire to replace lost or damaged parts of the human body, started man on his quest to turn mythology into reality.

The appearance of grafting procedures can be traced back for some seven or eight centuries B.C. (cited Davis, 1941), although it is evident that the majority of the grafts described were autografts. Nevertheless, throughout recorded medical history there have been isolated reports of successful allotransplants, such as the successful skin grafts performed by the Sicilian surgeon Branca (cited Calenzio, 1503) in the 15th century and the experiments with teeth performed by Ambrose Pare (cited Fauchard, 1786) in the sixteenth and John
Hunter (1771) in the 18th century. While these reports may now be looked upon with some scepticism in the light of greater experience and experimentation, they at least testify to man's persistent interest in the problem of grafting tissues between separate individuals.

The 19th Century saw a great expansion both in interest and activity in the field of tissue transplantation. During this century auto and allotransplants of almost every conceivable type of tissue were attempted, often with very varied and at times controversial results. The attainment of successful transplants using this wide range of tissues necessitated, in many instances, the appearance of new and more sophisticated surgical techniques. In 1824 Reisenger performed the first successful corneal transplant and towards the end of that century Jaboulay and Briau (1896) performed the first free arterial transplant, to list but two of the advances that were made.

Consequently, the literature of this century contains many references to new techniques and the improvement and development of older techniques for the grafting of tissues. This improvement in technical ability was unfortunately in no way paralleled by increased reports in the literature of successful allotransplants. Indeed, it was becoming apparent to the majority of workers in this field that there was a definite barrier where allotransplants were concerned and that this barrier could not be overcome by technical excellence alone.

Faced with these persistent and unexplained failures with allo-
transplants the 20th. century saw the beginning of attempts to analyse and investigate this problem in a systematic fashion.

Prior to this time, although realising that the allografts did not survive, there had been no real attempt to provide any explanation for this phenomenon. In 1906 Ehrlich put forward this theory of athrepsia to explain why these allotransplants failed to survive. His idea was that allotransplants perish because they are unable to obtain a constant supply of essential nutrition from the host. This was based on his failure (1906, 1908) to obtain further neoplasms by the reinoculation of the tumour cells into animals that were already bearing rapidly growing tumours. The concept also suggested that the failure of the secondarily inoculated cells to survive was due more to a property of the initial tumour rather than that of the host.

This theory of Ehrlich's was not met with general acceptance by any means and was criticised by Bashford and Russell (1910), Haaland (1911) and Levin and Sittenfield (1911), the latter group interpreted Ehrlich's work as indicating that "immunity to cancer growth consists in the lack of food athrepsia."

These criticisms of Ehrlich's work were answered by Apolant (1911) who was particularly scathing about the article by Levin and Sittenfield (1911). He pointed out that they had confused "the actively produced tumour immunity with that of athrepsia."

He went on further to say that Ehrlich had always sharply
separated the two immunities.

These reports were all based on experiences with tumours, and it was Shone (1912a, b) and Borst (1913) who first suggested that the failure of allotransplants of normal tissues could be explained along similar lines, although Borst did not believe that this was the entire answer.

In the same year that Apolant (1911) was extolling the value of athrepsia, there appeared in the literature reports by Lambert and Hanes (1911) and Carrel and Burrows (1911) of successful cultivation of tissues in vitro using media containing both homologous and heterologous plasma. This discovery led to the eventual waning of the theory of athrepsia which lost further ground with reports by Murphy (1926), Krebs, et al. (1930), Furth, et al. (1933) and Richter and McDowel (1935) that total body irradiation rendered mice susceptible to tumour transplants and leukaemias. Similar results using trypan blue as the modifying agent were obtained in rabbits by Saphir and Appel (1943).

Arising also from studies on tumours was the idea that the secondary transplants failed because the host did not provide an adequate blood supply and suitable supporting stroma. This was supported by the findings of Russell (1908a, b) and of Woglam (1912). This concept has received little further experimental verification and in opposition Medawar (1948) showed that in specifically immunised
animals, a graft placed in the anterior chamber of the eye, is destroyed only if invaded with blood vessels.

The second major attempt to explain the phenomenon of allograft rejection also appeared in the early nineteen hundreds. According to this hypothesis there was an innate resistance to allografts and this resistance was mediated by humoral factors. The protagonists of this theory suggested that there was a marked similarity between this response and the resistance exhibited to incompatible homologous erythrocytes. Indeed, it was further extended to the point where some believed the same mechanism to be involved in both instances and that allografts would survive permanently if host and donor had the same blood groups. Such a concept was supported by a number of reports of successful allotransplants, (Davis, 1917, 1927; Masson, 1918; Shawan, 1919; Baldwin, 1920 and Dyke, 1922), especially of skin, where the host and donor had the same blood groups. When reviewing this question in 1951, Longmire and Smith were most sceptical of these reports in the light of further clinical experience. Studies by Medawar (1946) showed that there was no antigenic relationship between red cells and skin in rabbits, and later Woodruff and Allan (1953) showed that skin allografts still break down even when the donor and host are indistinguishable with respect to all the demonstrable red cell antigens. In the mouse it has been shown that H-2
antigens are present on red cells but in spite of this Medawar (1959) failed to provoke immunity to skin grafts with erythrocytes. Presumably this is because the relevant antigens are not present on the red cells in sufficient quantity, or because they are not present in an immunogenic form. At present although it is acknowledged that erythrocyte compatibility is of significance in human renal transplants, this is on the basis that haemagglutination may occur within the graft, if the donor and recipient are of incompatible blood groups (Starzl, 1964).

It was the cellular aspect of this phenomenon that next came under notice. Leo Loeb was for many years the principal worker in this field and provided a vast amount of experimental data (1921, 1930, 1937 and 1945) to support his idea that the local infiltrating cells were of considerable importance in allograft rejection. However, as both Dempster (1951) and Woodruff (1960) have pointed out Loeb's general theory is difficult to follow. Woodruff summarises his work by saying that essentially there were two postulates, firstly, that all tissues have a common chemical characteristic which was termed by Loeb "the individuality differential", and secondly two factors were responsible for the destruction of the allotransplant. Primarily it is the "differential" of the transplant which evokes in the host a cellular response, the accumulated cells of which are directly injurious to the graft and secondarily the
"differential" of the host exerts a toxic effect on the transplants.

The observations of Loeb were further supported by the work of Murphy (1926) and Blumenthal (1941). It is interesting to note that these were the first attempts to carry out any careful histological studies on grafts undergoing rejection. However, it was this heavy reliance upon histological appearances that prompted criticism by Dempster (1951) on the grounds that the interpretation of histological patterns was indeed difficult and also, in his hands, the mesenchyme cell response to skin allografts was highly inconsistent. Prior to this, Loeb's theories had, to a degree, been challenged by the work of Morgan Harris (1943). Using a variety of tissues, which included heart, kidney and spleen from mice and rats, he was unable to demonstrate any evidence of toxic phenomena in his in vitro system. Surprisingly, interest then appeared to diminish where the in vitro investigation of cell cell interaction was concerned until the nineteen sixties.

At this point it is necessary to mention the studies by Tyzzer (1909), Little and Tyzzer (1916), Little and Strong (1924) and more recently Snell (1948) amongst others on inbred animals and F₁ hybrids. While not providing any definite theory as regards allograft rejection it was their work on the genetics of histocompatibility and the development of inbred lines of animals, that has provided the raw materials and the genetic background for the subsequent studies on
allograft rejection, especially at the cellular level.

The development of tissue culture techniques, by Govaerts (1960) and Rosenau and Moon (1961) for the demonstration of cell mediated immune reactions reawakened interest in cell cell interactions and provided a model for the further study of the role of cellular immunity in tissue transplantation. Once this technique had been established a number of workers, Brondz (1964), Govaerts (1960), Granger and Weiser (1964), E. Möller (1965b), Perlmann and Broberger (1963), Rosenau and Moon (1961), and Wilson (1963) rapidly demonstrated that presensitised lymph node cells were capable of damaging both normal and neoplastic target cells in tissue culture. Their studies showed that this reaction was much slower than in the case of target cell antibody systems. In the antibody system, the cells were destroyed in one hour, the cell systems required 24-48 hours to produce their effect.

In these cell cell interaction systems it was found that complement was not necessary and the effect could not be explained on the basis of diffusible substances, as the separation of lymphoid cells from the target cells by millipore filters (Rosenau, 1963) completely inhibited target cell damage. In all of the reports a constant factor appeared necessary for the demonstration of cytotoxicity. This was close contact between target cell and the lymphoid cells.
This work was further extended when it was shown that non-immune lymphoid cells could inhibit the growth of allogeneic target cells (Holm, et al., 1964; Holm and Perlmann, 1965; E. Moller, 1965a; and Moller and Moller, 1965). This reaction did not appear to occur as readily as in the case of the sensitised lymphoid cells, as it was first necessary to aggregate the non-immune cells to the target cells with phytohaemagglutinin or heterologous antibodies. Aggregation alone, however, could not explain this phenomenon (Holm and Perlmann, 1965; E. Moller, 1965a). Reports have appeared (Bach and Hirschhorn, 1965; Hirschhorn and Ripps, 1965 and Ginzburg and Sachs, 1965) where these aggregating factors have not been added, but the lack of syngeneic controls make it difficult to evaluate the importance and significance of their results.

Perhaps the most significant and outstanding discovery that has emanated from this tissue culture work has been the discovery by Moller and Moller (1965) and E. Moller (1965a, 1966) that $F_1$ lymphoid cells were fully competent to cause pronounced target cell damage yet according to the consensus of opinion (Woodruff, 1960), $F_1$ cells are genetically incompetent to carry out immunological reactions against parental cells. If this is so then it is as Moller and Moller (1966) point out "unlikely that immunological reactions of conventional types are involved in target cell destruction." This
has been further supported (Moller and Moller, 1966; Bergheden and Hellstrom, 1966) with the finding that not only lymphoid cells but also allogeneic neoplastic cells of either lymphoid or fibroblastic origin can destroy target cells in vitro. The implication of this work is that certain biochemical and synthetic processes may be stimulated in lymphoid cells after contact with foreign histocompatibility antigens, and that these processes are triggered by the non-immunological recognition of the foreign antigens. The bulk of the evidence at present for such an hypothesis rests upon relatively few in vitro studies. Such agents as complement and antibody, which apparently are unnecessary may still, of course, be present in very small amounts. Whether this is so, and of importance, remains unanswered as do many other questions relating to this phenomenon. Nevertheless, from the results already obtained, these studies appear to open an important avenue for the further investigation of allograft rejection and recognition at a cellular level. Furthermore, it is the first experimental evidence that has in any way seriously challenged the acceptance of active immunity as the most satisfactory explanation of the phenomenon of allograft rejection.

This theory of active or acquired immunity arose, or perhaps more accurately, 'came of age', with the work of Medawar (1944,
1945). In a series of experiments, that have now become classics in the field of transplantation, he clearly demonstrated the involvement of inflammatory cells and blood vessels in the rejection of primary allografts, and that a second graft from the initial donor was rejected more rapidly than the first.

The reason for suggesting that this theory 'came of age' with Medawar's work was that many years previously an hypothesis put forward by the opponents of Ehrlich's 'athrepsia' to account for the destruction of tumour transplants (Bashford, et al., 1908; Russel, 1912; and Bashford, 1913) and also allotransplants of embryonic and adult tissue (Rous, 1910; Underwood, 1914; and Holman, 1924) was indeed very similar to this concept. These earlier workers failed, however, to define or demonstrate the development of immunity in the same concise manner with respect to allotransplants as did Medawar.

It was the demonstration of this now so called 'second set phenomenon' that more than anything else has indicated the importance and significance of immunological mechanisms in allograft rejection.

Medawar's work has by no means provided the only evidence that acquired immunity is the most likely explanation for the resistance demonstrated by the host to an allotransplant. The subsequent findings relating to the specificity of this "transplantation
immunity", the kinetics of its development, and the effects of agents known to suppress the immune response are all in accord with this theory. It has, however, been the ability to transfer sensitivity by means of cells (adoptive immunity), the production of "immunological tolerance" and the increasing evidence for the presence of antibodies, that have provided the most important corroborative evidence in support of this concept.

The determination of the relative importance of cellular and humoral factors in the mechanism of allograft rejection has provided a source for constant investigation and argument ever since Medawar propounded this theory of active immunity. It was the initial attempts to investigate these factors that provided the first demonstration of adoptive immunity. Experimenting with tumours Potter, Taylor and MacDowell (1938) demonstrated that they could confer immunity with spleen and liver cells taken from hyperimmunised animals of the same strain. Similar findings were reported by Brncic, Hoecker and Gasic (1952). Mitchison (1954, 1955) also using tumour grafts as his experimental model showed that immunity could be transferred by means of cells from the draining lymph nodes but not by the contralateral nodes, the spleen, or the serum from these animals.

Mitchison (1955) in attempting to explain this phenomenon suggested several possible mechanisms. The two he most favoured were, firstly, that cells are immunologically activated before
transfer and confer immunity by continuing to function in the host, and secondly, that processed material either in the form of transformed antigen or as an enzyme system is handed to the lymphoid cells of the host and thus activates them immunologically.

With respect to the first idea, reports by Najarian and Feldman (1962a, 1963) and McKhann (unpublished – cited Møller and Møller, 1967) would suggest that this is unlikely. The former workers using immune lymphoid cells labelled with tritiated thymidine were unable to detect significant numbers of these cells in the graft bed even though the graft was heavily infiltrated with lymphoid cells. Their findings do not necessarily negate the original idea, as an important unknown in this system is the number of donor cells necessary to trigger the destructive changes that occur in the graft. It is also possible that the donor cells may be destroyed in the reaction with consequent loss of label and failure to demonstrate an accumulation of the labelled cells.

Experimental verification for the second theory has also been lacking, but recently experiments with other immune systems have provided evidence to support the presence of such transfer factors. This evidence as well as the role of the reticuloendothelial system in such a process is discussed later.

Regarding the transfer of immunity Billingham, Brent and Medawar (1954) showed that a similar situation held good for normal
tissues and they too stressed the point that immunity could not be transferred by serum factors. Further studies by Gowans, Gesner and McGregor (1961) and Billingham, Silvers and Wilson (1962) have shown that the transfer of immunity can also be achieved using thoracic duct lymphocytes and blood leukocytes. These observations naturally provided strong supporting evidence for the protagonists of cellular immunity. Such a concept was further supported by the apparent similarity between this reaction and the reaction of delayed hypersensitivity (Burnett and Fenner, 1953; Brent, Brown and Medawar, 1958; Hasek et al., 1961 and Good et al. 1962).

The other main line of investigation has, of course, arisen from attempts to establish the significance of humoral factors in allograft rejection.

The earliest workers to demonstrate the presence of any antibody were Lumsden (1931, 1938) and Gorer (1937, 1938) who were able to detect the presence of red cell agglutinins in animals that had received tumour transplants. Initially Gorer was inclined to believe that there was some correlation between the level of agglutinins and allograft rejection but further work by him (1947, 1948) revealed that there was no such relationship between the two.
The main factor that has caused many workers in this field to be sceptical concerning the potential value of humoral factors in this reaction has been the failure to transfer immunity with serum (Billingham, Brent and Medawar, 1954; Billingham and Brent, 1956; and Brent and Medawar, 1962). These results are, perhaps, not as conclusive as they first appear as Stetson and Demopoulos (1958) and Stetson and Jensen (1960) have been able to transfer immunity in mice and rabbits by means of serum from hyperimmunised donors. This has been further supported by Steinmuller (1962) using various strains of inbred rats. The clarification of this situation, therefore, appears to be of considerable importance for the theoretical understanding of this process.

While general agreement has not been achieved in the case of organised normal tissue grafts, there has been greater agreement where dissociated cell grafts are concerned. Amos and Day (1957), Siskind, Leonard and Thomas (1960) and Loutit and Micklem (1961) have shown that serum factors from immunised animals are capable of bringing about the destruction in vivo of both lymphoid and tumour cells.

In such a situation, where attempts were being made to determine the significance of humoral factors, it appeared that experiments in diffusion chambers would provide valuable and
possibly conclusive evidence. Unfortunately, once again no
definite conclusions can be drawn due to the failure of various
workers to obtain uniform results. Initial experiments by Algire
(1954), Algire et al., (1954, 1957) showed that there did appear to
be prolonged survival of allografts in these chambers. These tissues,
as Woodruff (1960) states, appeared to be "decidedly underprivileged"
and it was not surprising, therefore, that Amos and Wakefield (1958,
1959) and Wakefield and Amos (1958) were able to show that iso-
antibodies and complement could only penetrate these chambers with
some difficulty. If the levels of these factors were made equivalent
then allografts were destroyed (Gorer and Boyse, 1959; Gabourel,
1961; and Moller, 1963). It is, nevertheless, important to realise
that tumour cells were used by these workers and that Algire (1959)
and Gorer and Boyse (1959) have shown that not all cells were
equally susceptible; notably the non neoplastic cells.

Using this experimental model in reverse, that is to say,
placing sensitised cells in millipore chambers into grafted animals,
Najarian and Feldman (1962a, b) and Kretschmer and Perez-Tamayo
(1962) were able to show accelerated rejection of the test grafts.
In their discussion Najarian and Feldman were reluctant to equate
this diffusible agent in any way with humoral factors as they were
unable to transfer this effect by means of serum from the animals
that had provided the sensitised cells.

These results are contrary to those of Rosenau (1963) who was unable to demonstrate, in a tissue culture system, the presence of any toxic diffusible substance. The only way that one could reconcile these results would be to suggest that the diffusible substance of Najarian and Feldman was active in the in vivo situation because of the additional effect of either a serum factor or cells which were not present in the Rosenau model.

The other factor that for many years weighed heavily against the acceptance of a possible role for antibodies in this reaction had been the failure to demonstrate their presence in animals rejecting fixed tissue grafts. In the initial experiments this failure may have been related more to inadequate or low sensitivity techniques rather than to a lack of antibodies. In the mouse, frequently used in such experiments, the strong anti-complementary activity (Woodruff, 1960) and the presence of "incomplete" or blocking antibodies, (Stetson, 1963) unless specifically overcome, would seriously affect the common assay systems based on target cell destruction.

In 1963 Stetson reviewing the situation pointed out that while there were initial failures there had been numerous more recent reports of successful detection of antibodies in a wide variety
of experimental animals. Nelson (1961) and Fujii and Nelson (1963) have suggested, that the failure to passively transfer immunity by serum from animals bearing organ allotransplants was due to the antibodies cross reacting with the host tissue, which prevented them from appearing free in the circulation. This theory was, to a degree, supported by the findings of Hager et al., (1964) and also Altman (1963) who by injecting immune serum directly into the renal artery, and thus preventing cross reaction, was able to demonstrate accelerated rejection of renal grafts. More recently Najarian and Perper (1967) with immunofluorescent and radioiodinated gamma globulin studies have been able to demonstrate marked cross reactivity with host tissues. Their results also indicated that second set rejection may be mediated entirely by humoral antibody.

The evidence then, for the presence of humoral antibodies, now appears to be undeniable. The question that remains unanswered where allograft rejection is concerned is whether humoral immunity behaves in a synergistic, antagonistic or completely unrelated fashion with respect to cellular immunity.

The histological picture and the demonstration of adoptive immunity have indicated that cellular immunity plays a most important role, in allograft rejection, but by what mechanism these cells produce allograft destruction is far from clear. It has been suggested
that this is achieved by means of "cell bound" antibody and this would appear to be a most realistic compromise but, unfortunately, the evidence from tissue culture systems (Brondz, 1964; E. Moller, 1965; Rosenau and Moon, 1961; and Wilson, 1963) would suggest that this "cell bound" antibody is not the same as humoral antibody. This idea of "cell bound" antibody which has now gained wide acceptance originated by extrapolation from studies on the mechanism of delayed hypersensitivity reactions (Smith, 1960; Lawrence, 1956; Lawrence et al., 1960; Hasek et al., 1961; and Amos, 1962).

The establishment of this experimentally has been difficult, as all experiments aimed at detecting cell bound immunity by adoptive transfer or neutralisation tests are necessarily performed in the presence of antibody producing cells. Merril et al. (1960) attempted to detect cell bound antibody in vitro using peritoneal cells from rabbits and although increased cell death was demonstrated the effect may have been related to humoral antibodies synthesised by the immunised cells. The most convincing experimental evidence in support of "cell bound" antibody was firstly the work by Berriam and Brent (1958) who were able to demonstrate cell bound immunity in a mixed in vitro and in vivo system based on the capacity of the cells to inactivate antigenic extracts. This was further substantiated by Brent and Medawar (1962) who were unable to
demonstrate a similar phenomenon with humoral antibody.

The other evidence has been provided by the tissue culture experiments discussed previously where it had been shown that immune cells could destroy target cells in the absence of complement. The effect was, therefore, presumed to be due to the actions of "cell bound" antibodies as opposed to humoral antibodies as it has been clearly shown that complement is necessary for the cytotoxic action of humoral antibodies.

The difficulties in attempting to define a particular role for either cellular or humoral factors where allograft rejection is concerned are, perhaps, highlighted by the studies on immunological enhancement. The phenomenon of passive enhancement initially described by Kaliss (1958) was at first thought by him to be due to an effect on the transplant, but now appears more likely to be due to the antisera interfering with the immunological response of the recipient (Snell et al., 1960; Brent and Medawar, 1962; G. Moller, 1963a).

The work of Mitchison and Dube (1955) and Klein and Sjögren (1960a, b) indicated that immune lymphoid cells could abolish the enhancing activity of antiserum whereas at the other extreme, Batchelor et al., (1960) using a carefully balanced system, were able to demonstrate a synergistic effect between humoral
antibodies and immune lymphoid cells with respect to their growth inhibiting effects.

The Möllers (1965) when reviewing this phenomenon suggest that the contradictory findings, because of the complexity of the test systems and the involvement of different immunological forces could possibly be explained on the basis of quantitative factors. While such a comment is particularly pertinent to the phenomenon of enhancement, enhancement per se illustrates clearly a situation where there appears to be a complex relationship between the two types of immunity. Whether such a relationship occurs in vivo for fixed tissue grafts remains a subject for further investigation.

The other main piece of evidence that has pointed to the immunological nature of the allograft reaction has been the demonstration of immunological tolerance. It has been shown (Burnet and Fenner, 1949; Billingham, et al., 1953) that the introduction of an antigen under certain conditions will induce a state of specific unreactivity (tolerance) instead of a state of immunity. It was initially thought that tolerance could be produced only in immunologically immature animals. However, it would appear that the factor controlling whether tolerance is produced or not is the dose of antigen given. Brent and Gowland (1962, 1963), Gowland (1965) were able to show that even in
neonatal animals small doses of antigen would produce sensitisation as opposed to tolerance, whereas Shapiro et al. (1961) and Martinez et al. (1962) were able to show that large doses of cells given as a single or as repeated doses could produce a state of tolerance in mature adults. This relationship between dose and the establishment of tolerance has been more clearly demonstrated where purified protein antigens have been used. Dresser (1962) found that very small doses of bovine gamma globulin in the microgram range could induce tolerance in mice. Further studies by Mitchison (1964) indicated that unresponsiveness could be achieved using either small or large doses of antigen, and that doses in between immunised, and so rendered the animals hyperactive. Subsequently, in a series of experiments with protein antigens Dresser and Mitchison (cited Mitchison, 1967) have found the low dose lies in the 1-10 μg concentration range and that this concentration of antigen has to be maintained over a period of approximately ten weeks by repeated injections for a high degree of paralysis to be produced. Mitchison (1967) has suggested that this paralysis is achieved by more direct access to the lymphocyte and that in immunisation a different and far more complex pathway is taken with the likely involvement of the macrophage. It would appear, therefore, that transplantation tolerance as well as being
produced by large amounts of antigen may also be produced by small amounts. The experimental verification of this may rest, however, upon the preparation of transplantation antigens in a suitable purified form.

The Reticuloendothelial System in Tissue Transplantation

The role of the reticuloendothelial system in transplantation immunity has only received sporadic attention, although its activity in other immune mechanisms has been more closely observed. Largely from the latter observations it would appear that the reticuloendothelial system in combination with antibody could destroy grafted cells. In support of this Gorer (1958) reported phagocytosis of tumour cells in solid transplants and Amos (1960) demonstrated extreme phagocytic activity in the rejection of some ascites tumours. He was able to show that phagocytosis could account for the disposal of $10^8$ cells at any given time. Later in 1962 Amos was still prepared to acknowledge the potential role of this system in the removal of dissociated cells, but he was reluctant to support anything more than a scavaging role where fixed tissue grafts were concerned.

Old, et al. (1963) have also shown macrophages to be very prominent in the rejection of ascites tumours. It was further shown by Bennett, et al. (1963) that tumour cells could be phagocytosed by 'immune' peritoneal cells. This property could be abolished by washing these
peritoneal cells and the washings could then convert normal macrophages to immune cells, presumably indicating the presence of opsonic antibody.

The present knowledge regarding the function and role of the reticuloendothelial system in other immune systems is also far from precise, although recent experimental information has helped to establish more clearly some of the functions of this system. One of the problems associated with elucidating these functions has been the diffuse nature of the system and its diversity of cell type and potential activity. The demonstration of the cellular uptake of particles had been achieved in the late eighteen hundreds by a number of workers including Kupffer (1878) and Wyssokowitsch (1886), but it was Metchnicoff (1887) who provided the first suggestion that these cells were involved in host defence systems or in what we would now consider immune mechanisms.

Indeed, at that time there was a verbal battle between the supporters of humoral defence systems and cellular defence systems. The two opposing factions were, to a large extent, reconciled by the works of Denys and Leclef (1895) and Wright and Douglas (1903) who showed that the phagocytosis of bacteria was increased in the presence of humoral factors.

Following the elaboration of this widespread system of
phagocytic cells by Metchnicoff, attempts were made to study more closely the structure and function of the system by making use of the phagocytic activity of the cells. The initial attempts were made by Ribberts (1904) and Goldman (1909) using a series of dyes discovered by Ehrlich. The uptake of these dyes resulted in the vital staining of the cells. This approach was further expanded by Aschoff and Kiyono (1913) using lithium carmine as the particle and later further developed by Aschoff (1924). It was essentially these latter studies that provided a precise histological demonstration of this system and the development of the idea of a "reticuloendothelial system".

The studies using uptake of particles as the criterion for demonstrating the cells of the reticuloendothelial system were supported by the findings of Marshall (1956). Previously in 1932 Rio-Hortego had shown that cells of this system had a marked affinity for silver and other metals. Using this technique Marshall was able to demonstrate a marked similarity between the distribution of cells with this property and the cells that had been demonstrated by means of particle uptake.

The other important line of investigation that was pursued was based on attempts to quantitate the activity of this system. It had been shown by Bull (1915) and Manwaring and Coe (1916) that
bacteria were removed from the circulation of rabbits but that the rate varied depending on the organism used. Subsequently Foot (1919) and Wislocki (1924) carried out histological studies on rabbits injected with carbon in an attempt to quantitate the uptake in the various organs and they found marked uptake in liver, spleen and kidney. Further studies by Drinker and Shaw (1921) using manganese dioxide and Cappell (1929, 1930) using colloidal iron provided added evidence that established the significance of the liver in particular and, to a lesser extent, the spleen in quantitative studies on the removal of particles from the circulation.

In attempts to compare the in vivo phagocytosis of particles, one of the important discoveries was the appearance of a mathematical formula that could be used to calculate the rate of phagocytosis. In 1921 Fenn from his studies using carbon and quartz derived a formula for the rate of phagocytosis

\[ K = \frac{1}{t} \times \log \frac{A}{(A - X)} \]

where A equals the total number of particles, and X the number of particles ingested at time t. It was also apparent from this work and from that of Maxfield and Mortensen (1941) that this was a random process, the rate of phagocytosis depending on the chance collision between the particle and the cell.
It has, however, been Benacerraf, Halpern and their colleagues who, by virtue of their extensive in vivo studies of the reticuloendothelial system that have been able to provide the most precise quantitative methods for determining the activity of this system.

In the studies described by Halpern et al. (1953) carbon was used as the particle. The amount of carbon in suspension at a given time was measured by spectrophotometry after lysis of the red cells with dilute sodium carbonate. From the experimental results obtained they were able to derive an expression for the determination of the phagocytic index \( K \). The value for this index was determined by plotting the \( \log_{10} \) of the concentration of particles against time using an equation similar to that previously proposed by Fenn:

\[
K = \log \frac{C_1}{C_2} - \log \frac{T_2}{T_1}
\]

in which \( C_1 \) and \( C_2 \) were the carbon concentrations at time \( T_1 \) and \( T_2 \).

In order to quantitate the activity of the reticuloendothelial system with respect to viable particles, Benacerraf et al. (1959) developed a technique by which bacteria could be grown in a phosphorous free medium to which radioactive phosphorous (\( ^{32}P \)) in the form of orthophosphate had been added. It was found that
when the bacteria grew they incorporated the label into their structure, and consequently when injected intravenously into animals it was possible to measure the rate of their removal from the circulation by assaying blood samples for radioactivity. It was found that the radioactivity of the blood decreased according to an exponential function of the time down to about ten per cent of the amount injected. The derivation of the phagocytic index, therefore, utilised the same formula as that for colloidal particles.

Following the development of these more precise techniques it was possible to confirm that the functional activity with respect to a particle varied from species to species but that the organ distribution was similar. In order to compare more accurately the phagocytic index between animals it was found (Biozzi et al., 1953) that the organ weights must be taken into account. To this end a formula was derived for the corrected index

\[ \alpha = \frac{W}{WLS} 3^{\sqrt{K}} \]

where \( W \) is the weight of the animal and \( L \) and \( S \) the weights of the liver and spleen and \( K \) the phagocytic index.

Not only has this phagocytic ability provided a very valuable means for the investigation of this system, the inherent ability to remove particles, in particular bacteria from the
circulation has encouraged many to suggest that there is an important relationship between immunity and the reticuloendothelial system. In spite of the ability to demonstrate bactericidal effects of the reticuloendothelial system in the presence of antibody, (Jenkin, 1962) attempts to alter the phagocytic activity of this system and hence immunity have been disappointing (Howard, 1958).

Attempts have also been made to correlate changes in humoral antibody formation with changes in the functional activity of the reticuloendothelial system. It has been reported by Cutler (1960) using Zymosan and Halpern (1959) with BCG that the resultant stimulation of the reticuloendothelial system by these agents had achieved an increase in the humoral antibody response to a given antigen.

This implied important relationship has not been met with universal acceptance due to conflicting reports by Howard and Woodruff (1961) and Barrie and Cooper (1964). It is difficult, however, to assess the relative significance of these and other results in this field due to the very different methods by which the reticuloendothelial system has been stimulated and also the timing, the method and type of antigenic stimulation employed.

More recently there has been suggested a more indirect role for the macrophage in immunity and antibody formation, but never-
theless a role that might be critical as far as immune mechanisms are concerned. Garvey and Campbell (1957) suggested that the macrophage acted as a storehouse for antigenic fragments and that this was necessary for the production of antibody over a long period of time. Although many workers including Nossal and Makela (1961) have stressed the role of the "Memory Cell" in such a situation, when reviewing the literature in 1963 Garvey and Campbell still felt, that on the evidence available, the retention of small foreign molecular structures could play an important role in the stimulation and modification of protein biosynthesis. Along similar lines Lapresle (1955) and Sorkin and Boyden (1962) suggested that the macrophage may solublise and degrade particulate antigens and in doing so provide them in a form that is capable of initiating and maintaining antibody production.

The evidence in favour of the lymphoid series of cells being responsible for antibody production (Nossal, 1958; Attardi, et al., 1959; Amos, 1962) appears undeniable but this idea, that the macrophage initially receives the antigen and then in some way provides the stimulus for the lymphoid cells to synthesise antibody, has been gradually gaining ground.

Fishman (1959, 1961) using an in vitro system has been able to show that bacteriophage T2 alone cannot stimulate lymph node cells
to produce antibody. However, if the phage particle is incubated with macrophages and the cell free extract added to the lymph node cells then antibody is produced. Further studies by Fishman and Adler (1963) have shown that the activity of this extract resides in a fraction which possesses the properties of ribonucleic acid. More recently Adler et al. (1966) have shown that macroglobulin synthesised by lymphnode cells from one rabbit after the addition of a ribonucleic extract from antigenically stimulated peritoneal cells from a second rabbit, has the allotypic determinants characteristic of the immunoglobulins found in the second animal. The gamma globulins produced were found to have the same determinants as the globulins of the donor of the lymph node cells. This, therefore, provides strong evidence that the nucleic acids from these peritoneal cells can direct the synthesis of immunoglobulins in lymph node cells.

Fishman, et al. (1964) pointed out that this was an in vitro system which demonstrated a pathway of antibody synthesis, which may be one of several alternatives, but to extrapolate this to antibody formation in vivo is of necessity still speculative. Support for this hypothesis in an in vivo model is by no means clear cut and in many instances is essentially circumstantial. Dresser (1962) showed that if the particulate fraction of bovine gamma globulin
was removed by centrifugation then the remaining material is far less antigenic. This suggests that the larger particulate fraction being more readily trapped by the phagocyte induces antibody formation by first entering the macrophage. White (1963) and Nossal, Ada and Austin (1963) and subsequently in a series of papers from the Hall Institute have shown the localisation and retention of antigen for a long period of time in a specific type of cell in the lymph node follicle, the dendritic macrophage. It has been suggested that because of its intimate contact with the lymphoid cell, this cell may be involved in the antibody response. Nossal (1964) and his colleagues further supported such an idea when they failed to demonstrate any of the labelled antigen in the antibody forming cell.

Perhaps one of the most important in vivo studies with respect to this problem has been the recent work of Galily and Feldman (1967). From previous studies the suppression of immunological activity in animals receiving total body X-irradiation had been attributed to damage to lymphoid cells. On the basis of histological evidence (Bloom, 1948; Brecher, et al., 1948) and clearance studies (Barrow, et al., 1951; di Luzio, 1955; Benacerraf, et al., 1959) the reticuloendothelial system appeared to be radioresistant. On the other hand, reports by Gordon, et al. (1955) and Donaldson, et al. (1956) suggested that although the
phagocytic capacity was not altered the "digestive" ability of these cells may be reduced. In their system Galily and Feldman (1967) were able to demonstrate that animals receiving 500r total body irradiation failed to produce antibody to a given antigen. If, prior to injection, the antigen was incubated with macrophages and the cell antigen mixture injected into animals, then antibody could be detected. Macrophages from irradiated animals were incapable of producing this response.

There has, therefore, been this increasing amount of evidence to support the suggestion that the macrophage may play a most important role in the initiation of antibody production and, more generally, in the initiation of immunity. Whether the macrophage plays a similar role with respect to transplantation immunity of course remains highly speculative. It might be argued that in the case of adoptive immunity where lymph node fragments and cells are transferred, (Billingham, et al., 1954) one is also transferring these dendritic macrophages described by Nossal and his colleagues. These cells, therefore, once in their new host could continue to provide the stimulus for the development of immunity. Such an idea is to a degree supported by the failure of Najarian and Feldman (1962) to demonstrate the localisation of sensitised lymphocytes in animals bearing allografts undergoing adoptive
destruction. However, the finding that thoracic duct lymphocytes (Gowans, et al., 1961) could confer adoptive immunity, and after perfusion through a kidney confer sensitivity (Strober and Gowans, 1965) together with the cell cell interaction experiments previously discussed, must argue strongly against such a mechanism.

Nevertheless, one of the major difficulties associated with any of these cell mediated and cell transfer experiments has been to ensure that the cell population is of a single type. This problem was highlighted by Howard (1961) and Howard, et al. (1964) who provided evidence that thoracic duct lymphocytes under strong antigenic stimulation assume the appearance and function of phagocytic cells. Therefore, on this basis these objections may not be as strong as they first appear.

When considering the possible role of the macrophage and the reticuloendothelial system in transplantation immunity it is of interest to note that the dose of irradiation, 500r, used by Galily and Feldman (1967) to produce this apparent disruption of macrophage "digestion", is very similar to the dose of 450r used by Medawar (1963) to produce significant prolongation of skin graft survival. Other reports by Dempster, et al. (1950) and Micklem and Brown (1961) although not using doses that correspond as closely have also demonstrated graft prolongation. More recently in relation
to this possible "digestive" role by macrophages in vitro studies by Jones (1966) have provided some evidence that macrophages process transplantation antigens into a form optimal for mitotic stimulation in mixed leukocyte cultures.

These findings are pertinent to the afferent arc of graft rejection. In the efferent arc, the role of the macrophage in the rejection of ascites tumours as previously cited appears undeniable, but their role in the rejection of solid grafts appears more tenuous. Gorer (1960) drew attention to the infiltration of tumour grafts by macrophages and the findings of Wiener, Spiro and Russell (1964) indicate that some of the mononuclear cells infiltrating first set skin allografts possess features of both lymphocytes and macrophages. It has also been shown by Granger and Wieser (1964) that immune macrophages in vitro can destroy target cells. For this to occur phagocytosis is not necessary and this property appears to be related to the presence of a cell-bound antibody more firmly adherent than that described by Bennet, et al. (1963) as it cannot be removed by washing.

The studies associated with agents known to alter phagocytic activity have provided some evidence regarding the role of the reticuloendothelial system in allograft rejection. Unfortunately, it is not always apparent whether the afferent or efferent limbs of
this rejection process have been affected. In 1932 Foulds and Ludford (1932) showed that by blockading the reticuloendothelial system with colloidal suspensions they could demonstrate increased susceptibility to tumours. Further studies by Lehmann and Tammann (1925, 1926), Blumenthal (1941), Brent and Medawar (1962) and Medawar (1963) demonstrated that the survival of skin allografts could also be prolonged by these agents. On the other hand, negative findings were reported by Loeb (1945) and more recently by Fisher and Fisher (1964), who also found that the reticuloendothelial system was stimulated by the rejection of the graft and that this stimulatory effect was capable of overcoming the blockading effects of thorotrast.

Other investigators have attempted to accelerate graft rejection by stimulating the reticuloendothelial system. It has been reported (Bradner, et al., 1959; Old, et al., 1959; 1961) that zymosan and BCG inoculation prior to tumour transplants results in the accelerated rejection of these grafts. This was not as evident with normal tissues as Balner, et al. (1962) also using BCG as the stimulating agent were unable to demonstrate accelerated rejection of skin grafts. They suggested that this failure was possibly due to the difficulty of detecting accelerated rejection in an already rapid process. In support of this they found that using sex specific grafts they could show accelerated rejection. This effect was abolished
if the animals were splenectomised. From their observations they concluded that the results may be due to relatively non specific stimulation of reticuloendothelial elements which may be responsible for antigen recognition and immunological responses.

This, perhaps, adequately sums up the extent to which anyone at the present time could implicate the reticuloendothelial system in allograft rejection. However, considering the evidence in favour of allograft rejection being mediated by immune mechanisms, and the increasing support for the role of the reticuloendothelial system in these mechanisms it would appear both important and valuable to further investigate the role of the reticuloendothelial system in allograft rejection.

**Antilymphocytic Serum**

There have appeared in the literature over the last few years an increasing number of reports regarding the production and activity of so-called antilymphocytic serum, which has been raised in members of one species against lymphoid cells taken from a member of another species. Humphrey, in 1967, suggested that this agent perhaps has something in common with Ehrlich's "magic bullet". Therefore, for practical purposes such an agent must selectively suppress only specifically desired aspects of the immune response. This antiserum certainly has immunosuppressive properties and, indeed, Medawar
(cited - Humphrey, 1967) has stated that it is the only true immuno-
suppressive agent. If this is so, then not only is it a valuable agent
in its own right, but also this remarkable property makes it of great
potential value in the elucidation of the mechanisms by which allo-
grafts are rejected.

The first worker to show any interest in the production of an
antileukocytic serum was Metchnicoff in 1899. While there
appeared many reports subsequently, the only two of significance
were the reports by Chew and Lawrence (1937) using guinea pigs
and Cruickshank (1941) using rats, that a heterologous antisera
could severely depress the peripheral lymphocyte count.

The interest in the use of antisera then languished until
Woodruff (1960), investigating the role of the small lymphocyte in
graft rejection, suggested that anti-lymphocyte serum could be used
to suppress these cells, and as a result modify allograft rejection.
Such an approach he hoped would provide more definite information
concerning the role of the lymphocyte in this process. In this
attempt he was unsuccessful for neither could he maintain a
lymphopenia, a factor which he, at that time, thought was of the
utmost importance, nor could he demonstrate prolongation of
allograft survival.

It is interesting to note, especially in view of the resemblance
between delayed hypersensitivity and the allograft reaction, that Humphrey (1955) and Inderbitzen (1956) were able to suppress Arthus type and delayed type hypersensitivity reactions by using antisera. This work was further extended by Waksman, et al. (1961) whose results agreed with the previous workers, but their antisera, which so successfully suppressed these delayed type hypersensitivity reactions produced only marginal prolongation of graft survival. While showing definite evidence of damage to the lymphocytes, they were unable to find any evidence of primary destruction of lymph node cells, although Moore (1959) had demonstrated the localisation of radio-iodine labelled antibodies in the lymph nodes utilising a comparable serum.

It was the report by Woodruff and Anderson (1963) that provided the first indication of the real potential of this agent where the allograft reaction was concerned. They were able to show marked prolongation of survival of skin grafts in rats treated with heterologous antisera. This effect was further enhanced by prior thoracic duct drainage. Since then there has appeared a series of papers, Gray, et al. (1964), Jeejeebhoy (1965), Gray, et al. (1966), Monaco, et al. (1966a), Levey and Medawar (1966a, b) Starzl, et al. (1966, 1967), Woodruff, et al. (1966) that have all indicated that heterologous antisera raised against lymphoid cells is a very
powerful immunosuppressive agent as judged by its ability to prolong allograft survival and to depress humoral antibody formation.

While these results have represented a great deal of experimental work, there is still no general agreement on the nature of the properties which provide anti-lymphocyte serum with this remarkable ability to depress immune mechanisms. It has been clearly established by Monaco, et al. (1966a), James and Anderson (1967), James and Medawar (1967) and by Starzl, et al. (1967) that the activity lies in the globulin fraction of this serum and although James and Anderson (1967) have found some activity in the macro-globulins from horses the majority of the activity as reported by the other workers was in the gamma globulin fraction.

Since the activity resides in the gamma globulin fraction of the serum this strongly suggests that the active principle is an antibody. This being so the specificity of this serum has attracted the attention of a number of workers. The earlier studies of Moore (1959) suggested that it may be specific for lymph node cells. Gray, et al. (1966) demonstrated that the activity could be removed by absorbing with lymphoid cells but not with liver, kidney or red blood cells. More recently Levey and Medawar (1967) have confirmed this report although earlier (1966b) the latter workers had shown some absorption with lung and kidney cells. Further studies by Monaco,
et al. (1967) have shown that anti-lymphocyte sera gave precipitin lines against extracts of lymphoid and non lymphoid cells, but absorption by non lymphoid cells could not remove all of the precipitin lines.

However, it has been demonstrated by Levey and Medawar (1966b) that anti-lymphocyte serum is not directed against antigens peculiar to lymphoid cells. This has been confirmed by Jooste (unpublished - cited Levey and Medawar, 1967) who was able to raise a weak anti-lymphocyte serum using cultured mouse fibroblasts as the antigen. Nevertheless, it appears that lymphoid cells produce the best antiserum. This may, as Levey and Medawar (1967) have suggested, be due either to the presence of a greater number of appropriate antigens and/or because lymphoid cells are good immunogens. In the attempts to produce this antiserum most workers have stressed the point that living lymphoid cells, or viable cells as judged by their ability to exclude such dyes as trypan blue, should be used as the immunising agent. This may not be as important as first thought as Levey and Medawar (1967) using cell fractions, in particular the crude insoluble lipoprotein fraction, have been able to produce a highly effective antiserum.

Methods for quantitating the activity of the antiserum have been devised using both in vivo and in vitro systems. The in vivo
assays (Levey and Medawar, 1966a) have been based on the ability of a certain dose of antiserum to prolong skin graft survival or to prevent a graft versus host reaction in neonatal animals (Brent, et al., 1957). The in vitro assays (Gray, et al., 1966 and Woodruff, et al., 1966, 1967) have been based on the ability of the antiserum to produce leukoagglutination or cytotoxicity in the presence of complement. Unfortunately, there appears to be little correlation between the in vitro and in vivo assays. This lack of correlation naturally presents a considerable problem, especially to the groups who are attempting to use this agent at a clinical level. Summing up this situation Humphrey (1967b) suggested "that one should measure the activity against lymphocytes in vitro in the hope that this will correlate with the biological effect, but that it is over optimistic to suppose it will correlate well."

The mode of action of the anti-lymphocytic serum has aroused considerable speculation. The idea that the lymphocyte is the target cell has been widely accepted, due largely to the evidence reviewed by Gowans (1965) and Gowans and McGregor (1965), that allograft reactions are mediated by lymphocytes. It was initially thought by Woodruff (1960) that lymphocyte depletion was necessary if the antiserum was to be effective. However, further studies by Woodruff (1963) and Abaza, et al. (1966) showed that there appeared
to be little correlation between the peripheral lymphocyte count and the survival of an allograft. In separate studies Monaco, et al. (1966a, b and 1967) found a persistent lymphopenia in the blood and lymphoid tissues. This lymphoid depletion in their experiments has correlated closely with graft survival and they have, therefore, concluded that their antisera is effective by virtue of lymphocyte depletion. These findings have not been confirmed by Starzl (1967) in human patients, nor do Levey and Medawar (1967) find similar changes in their serum treated mice. Indeed, they find a degree of hyperplasia of all the lymphoid organs similar to that reported in guinea pigs by Chew and Lawrence (1937). This, appears to become a critical point where the understanding of the mode of action of anti-lymphocyte serum is concerned. Unfortunately, it is extremely difficult to compare and assess the relative significance of these results, due firstly to the variation in the method of preparation, and secondly to the undoubted heterogeneity of the antibodies that are involved.

Another theory, put forward by Russe and Crowle (1965), is that the antiserum acts on a thymus target and, thereby, prevents the production of some humoral factor which is vital for the maintainance of lymphocyte function. Parrott (1967) although finding the thymus reduced in size could not detect any changes in the architecture of
the gland. Earlier studies by Parrott, et al. (1966) and Parrott and De Sousa (1967) had suggested that there were certain areas in a lymph node that were "thymus dependent". In the antiserum treated mice it was these areas that Parrott (1967) found depleted of cells, and it was this finding that led her to suggest that the anti-lymphocytic serum had produced an "immunological thymectomy". Similar observations have been reported by Turk and Willoughby (1967) and Turk (1967) in the lymph nodes of guinea pigs treated with antiserum. Monaco, et al. (1967) on the other hand noted the depletion of these small lymphocytes, but reported the loss of cortical lymphocytes and the normal follicular pattern as well.

The other notable histological finding has been related to the presence of pyroninophilic blast cells. It had been shown with solid tissue allografts (Scothorne and McGregor, 1955; Burwell, 1962) and in contact sensitivity (Oort and Turk, 1965) that the first cytological reaction in the draining lymph node was the appearance of these pyroninophilic cells. Studies by Parrott (1967) and Parrott and De Sousa (1966) led them to suggest that as this reaction does not occur in neonatally thymectomised mice it was reasonable to relate the failure of these animals to reject grafts to the absence of small lymphocytes and this blast cell proliferation. In the antiserum treated animals, painted with oxazolone or skin
grafted, Parrott (1967), Turk (1967) and Monaco, et al. (1967) have all been able to demonstrate these cells; although reduced in number, they are not as dramatically reduced as the small lymphocytes. Whether this finding is of significance is uncertain due to the limited data available. The presence of these cells may represent a limited response by the serum treated animals, whereas in the animals thymectomised at birth no such response occurs. This could simply reflect the inability of the serum to produce a total "immunological thymectomy", and, indeed, Monaco, et al. (1965, 1966b) have shown that adult thymectomy appears to potentiate the action of anti-lymphocyte serum.

The other major attempt to provide some explanation for the mode of action of this serum has been provided by Levey and Medawar. The first hypothesis put forward by them (1966a) was that the antisera acted by "blind folding" the cell. The antisera achieved this by coating the lymphocyte with rabbit immunoglobulin and thus prevented it from recognising the antigen. Further studies by Levey and Medawar (1966b) have shown that the incompetence persists through at least one cell division indicating that this blind-fold theory is unlikely to be the complete explanation. Likewise, the results of Brent, et al. (1967) indicate that the unresponsiveness to skin homografts is in force at a time when relatively few cells
could be expected to show any visible signs of coating. This failure to detect coating may, of course, be related to the sensitivity of the methods for detecting the presence of small amounts of immunoglobulin. Levey and Medawar (1966b) also noting the appearance of large blast like cells in animals treated with antisera suggested that this may represent lymphocytes that had undergone "sterile activation". The idea being that the cells had been induced to undergo blast formation with cell division but without specific immunological commitment, similar perhaps to the in vitro activation of lymphocytes reported by Grasbeck, et al. (1963, 1964).

Medawar (1967) has also suggested that one may think of this antiserum as a competitive antigen. The basis for this being that rabbit gamma globulin is a powerful antigen but a poor immunogen and, therefore, tends to produce tolerance. While the active constituent of the serum can be thought of as antibody it could also become an antigen for which every lymphocyte has receptors. This may then result in the production of a large population of tolerant cells which presumably cannot act with any other antigen. One of the problems associated with any such hypothesis is that it must assume that a tolerant cell possesses certain properties, when on the basis of the experimental evidence available, such an assumption is in itself highly speculative.
In spite of the lack of understanding of the mode of action of this antisera in the experimental model the use of this agent at a clinical level appears most promising. The reports, in particular, those of Starzl (1967) using the gamma globulin fraction of the serum in conjunction with immunosuppressive agents have indicated that the addition of this agent improved the early course of human renal allotransplants. The potential dangers associated with the use of this agent therapeutically are, however, far from trivial.

In general to use any agent at a clinical level whose mode of action is as uncertain as anti-lymphocytic serum, presents a considerable problem, and when this agent is such a powerful immunosuppressive, the problem becomes even more manifest.

More specifically in renal allotransplants it has been shown by Seegal, et al. (1962) that animals may develop a Masugi-type nephritis. Although Iwasaki, et al. (1967) whilst demonstrating their globulin fraction to be in no way specific have found no evidence of acute nephritis in their experimental animals. Perhaps a greater risk is associated with the possible production of a serum sickness nephritis. The changes associated with this type of nephritis have been previously described by Dixon, et al. (1961). At present there is experimental evidence from Iwasaki, et al. (1967) that clearly indicates the capability of their serum, if given
for prolonged periods of time to produce similar renal lesions.

It would appear from this and from Starzl, et al. (1967) and Gray, et al. (1966) that prolonged administration of this antiserum is undesirable. Therefore, with respect to the future use of this antiserum the report by Monaco, et al. (1966) appears to be of particular significance. They were able to show in adult thymectomised mice, treated with a short course of antiserum, followed by donor antigens in the form of living lymphoid cells, marked prolongation of subsequent test skin allografts. The results indicated a state of tolerance and indeed the animals with the permanently surviving grafts were shown to have associated lymphoid chimerism. Whether the ultimate role for this antiserum will be in the development of tolerance to transplantation antigens remains speculative. However, it is becoming increasingly evident that the potential value of antiserum clinically and experimentally in both suppressing and unravelling the mechanisms of allograft rejection, necessitate the further investigation of all aspects of this agent.
CHAPTER II

MATERIALS AND METHODS

Experimental animals:

The animals used in all experiments were maintained in air-conditioned rooms (22°C) and allowed food and water ad libitum.

Mice. The average body weight of the animals used was from 20-22 gm. and as far as possible animals were matched for age and sex, especially where grafts were being exchanged between animals.

The strains used in this study were:

1. A strain of outbred Swiss white mice.
2. Inbred strains Balb/C, C57Bl, AKR and B10D2 - maintained and bred in the laboratories of the Department of Surgery, University of Adelaide.
3. F1 hybrid mice: these were obtained by crossing C57Bl males with Balb/C females.

Rabbits. Laboratory flop eared rabbits were used extensively for the production of the antisera.

Bacterial strains:

The following strains were used:

1. *S. typhimurium* (C5) a virulent strain for mice,
   
   LD50 = 100 organisms (Furness and Rowley, 1956).
(2) *S. typhimurium* (M206) an avirulent strain for mice,
LD50 = 10^6 organisms (Jensen, 1929).

(3) *E. coli* CV an avirulent strain for mice.

(4) *Listeria monocytogenes* a virulent strain for mice,
LD50 = 5 \times 10^5.

The bacterial strains were kindly supplied by Professor D. Rowley of the Department of Microbiology.

The pure cultures of these strains were provided on nutrient agar slopes, with the exception of *Listeria monocytogenes* which was supplied on an enriched medium (Brain heart broth with 2% agar). The agar slope cultures were all stored at 4°C. After a given slope had been opened ten times it was discarded and another slope used.

**Radioactive isotope labelling of bacteria.**

The *Salmonella typhimurium* (C5) was grown in a supplemented casimino acid medium which had been previously described by Benacerraf, *et al.* (1959). The medium after adjustment of its pH to 7 was autoclaved in 50 ml. amounts.

To label the bacteria 1 milli-curie of P^{32} as orthophosphate was added to 50 ml. of the above medium in a 500 ml. flask. This was inoculated with 0.1 ml. of a 6 hour shaker culture of the organism in nutrient broth and the inoculated medium agitated in a hot room at 37°C for 18 hours. The bacteria were recovered by
centrifugation for 10 minutes at 5,000 r.p.m. and washed three times in 50 ml. of physiological saline. Finally, they were resuspended in a small known volume of the casamino acid medium.

The concentration of bacteria required was $10^9$ organisms/ml. This was obtained by measuring the optical density (O.D.) using a Shimadzu photoelectric spectrophotometer, model Q.R.50 at a wavelength of 675 milli-microns. It had been shown (Reade, 1964) that an O.D. of 0.16 at this wavelength represented a concentration of $10^9$ organisms/ml. After adjustment of the concentration to $10^9$ organisms/ml, the suspensions were kept at 4°C and were not used for more than five days. Prior to each use the bacteria were rewashed.

**Opsonisation of bacteria.**

Before all clearance studies the $^{32}$P labelled *S. typhimurium* (C5) were opsonised with normal pig serum. This procedure as well as increasing the sensitivity of the system also eliminated the possibility that opsonins naturally occurring in the mouse serum could influence the rate of clearance.

To opsonise the bacteria equal volumes of the pig serum and the bacterial suspension were mixed and kept at 37°C for 30 minutes. Initially, the mixture was then centrifuged at 5,000 r.p.m. for ten minutes and deposited bacteria washed three times with 15 ml. of sterile physiological saline. Following the washing the bacteria were
resuspended in saline to their original volume. However, clearance studies in both normal and experimentally modified mice indicated that the rates of clearance were not significantly altered if unwashed bacteria were used and in the latter studies this step was frequently omitted.

**Opsonic activity of mouse serum.**

In order to ensure that changes in the rate of clearance were not due to opsonins in the mouse serum, sera from normal and experimentally modified animals were tested for possible opsonic activity.

Bacteria after opsonisation with pig serum were washed and resuspended as above. These opsonised bacteria were added to an equal volume of the mouse serum under investigation. The procedure following this was the same as that described for the opsonisation with pig serum. Bacteria thus treated were subsequently injected into normal animals and the rate of clearance determined.

**Bacterial clearance studies.**

The technique used for these studies was similar to that described by Benacerraf, *et al.* (1959). In all the studies a volume of 0.2 ml. of the opsonised isotopically labelled suspensions of *S. typhimurium* (C5) was injected into the tail vein of mice using a 1 ml. tuberculin syringe fitted with a 25 gauge needle. After the
injection, a standardised 0.02 ml. pipette was used to take blood samples from the retro-orbital venous plexus of each mouse at minute intervals. The blood samples were immediately pipetted onto planchettes cut from plastic backed chromatography paper and once dry the radioactivity of each sample was estimated using a Phillips proportional counter Model PW4200. To obtain $K$, the phagocytic index, the log of the counts per minute were plotted against the time.

In the Introduction, reference was made to the work by Benacerraf, et al. (1959). This indicated the blood clearance of viable particles to be exponential with time and while this was so the expression

$$K = \log \frac{C_1 - C_2}{T_2 - T_1}$$

applied. However, when the level fell below ten per cent of the injected dose the bacteria were cleared more slowly. Figure I shows the initial part of a typical clearance curve for normal Balb/C mice injected with the opsonised isotopically labelled bacteria. This was also typical for all the strains of mice used. Here it can be seen that for nearly the first ten minutes the curve is in fact linear and, therefore, the equation relating to $K$ will apply. In view of this, for the subsequent experimental studies, it was decided to bleed the
FIG I  CLEARANCE OF $^{32}$P LABELLED S. TYPHIMURIUM (C5) IN NORMAL BALB/C MICE.
animals at minute intervals for a period of five minutes, thus ensuring that the relationship held good.

**Organ distribution of isotopically labelled bacteria.**

The amount of radioactivity was determined in the livers, spleens and lymph nodes of normal, grafted and antisera treated mice. Animals injected with the P³² labelled bacteria were sacrificed by dislocation of their cervical vertebrae five minutes after injection. The livers and spleens were removed, cut into small pieces using fine scissors and the tissue pieces washed in physiological saline in an attempt to remove excess blood. Following this they were digested in 10 per cent NaOH in a water bath at 100°C for 30 minutes and the radioactivity measured by placing 0.1 ml. samples on planchettes, and counting, as previously described for the blood samples.

The amount of radioactivity injected was determined by submitting 0.2 ml. of the bacterial suspension to the same treatment. The amount recovered in each organ was expressed as the percent of the radioactivity injected.

**Skin grafting in mice.**

The technique described below for skin grafting mice was used in all experiments irrespective of the size or type of graft employed. The size of the grafts varied from 0.5 cm.² to 8 cm.².

The animals were anaesthetised by an intraperitoneal injection
of sodium pentabarbitone (May and Baker 60 mgm./ml.). The dose employed was 60 mgm./Kilo which meant that each 20 mgm. mouse received approximately 0.2 ml. of a 1 in 20 dilution of the standard solution in sterile normal saline. This dose adequately anaesthetised the mice for a period exceeding thirty minutes. Following the surgical procedures the mice were placed on a hot plate maintained at 32°-35°C in order to combat the hypothermia induced by the barbiturate.

Anaesthetised animals after being clipped along the dorsal surface, were pinned out on a cork board as described by Billingham and Silvers (1961) and shaved with a safety razor. The skin was cleaned with 70% alcohol and once dry the area of skin to be removed was outlined using a No. 11 scalpel blade, care being taken, while doing this not to penetrate the panniculus carnosus. A corner of the skin so outlined was lifted and the skin separated by blunt dissection from the underlying panniculus carnosus. Figure 2 shows a piece of skin being removed. During this step every effort was made to ensure that the vessels of the panniculus carnosus were preserved.

Donor skin.

In most cases it was found more convenient to sacrifice a mouse, remove its skin completely and use this to provide a series
Fig. 2. Skin removal from Balb/C mouse showing preservation of panniculus carnosus and associated vessels.

Fig. 3. Balb/C and hybrid (C57Bl x Balb/C) skin trimmed and cleaned of fascial tissue prior to grafting.
of grafts. The skin when removed was placed in a sterile petri dish which contained cotton wool plugs previously moistened in sterile saline and immediately prior to grafting the skin was cut to size. It was found that the best results were achieved if the graft was slightly smaller (10-15 per cent) than the deficit it was to cover as this allowed the graft to be placed under slight tension. It was also noted that highly pigmented areas of skin consistently failed to take and were, therefore, not used in any of the experimental studies. Figure 3 shows two pieces of skin trimmed and scraped clean of any adherent fascial tissue immediately prior to grafting.

The grafts were sutured in position using firstly a single 5/0 silk suture in each corner after which the edges were brought into apposition with a continuous 5/0 suture. Contrary to most reports (Woodruff, 1960; Billingham and Silvers, 1961) it was found that a dressing was unnecessary when this technique was employed.

Figure 4 shows an isografted mouse at four and nine days. No overt evidence of infection was found in any of the animals nor did the mice eat or damage the unprotected grafts. Apart from the advantage of being able to continuously observe the grafts the mice were in no way restricted by dressings or plaster jackets. All grafts were subjected to daily visual and tactile inspection. The criteria used to determine rejection being essentially the same as those
Fig. 4. Balb/C mouse with isograft at day 4 and day 9.
described by Billingham and Silvers (1961). To establish an end point a graft was considered to be rejected when there appeared to be less than 20% surviving epithelium. Figure 5 shows a C57Bl allograft at day 7, shortly before rejection and the same graft (Fig. 6) at day 9 now showing definite signs of rejection.

Table I lists the median survival times for the first set skin grafts in the various combinations used.

In the range of graft sizes employed it was found that although graft size per se did not materially alter the subsequent survival of an allograft there was a definite tendency for the largest allografts to survive the longest.

Graft removal.

To determine whether the transfer of antigenic information as opposed to graft rejection, was responsible for stimulating the reticuloendothelial system, studies were performed on animals that had had their grafts removed after various intervals. The grafts were readily removed from anaesthetised animals by firstly cutting the sutures and then separating the grafts from the host by blunt dissection. To protect the raw area thus exposed the edges were either drawn together with 5/0 silk sutures or an artificial covering provided by means of a nobecutane spray. Neither of these procedures per se were found to significantly alter the activity of the reticuloendothelial
Fig. 5. Balb/C mouse bearing C57Bl allograft at day 7

Fig. 6. Same animal as in Fig. 5, now at day 9 showing allograft rejection.
TABLE I

Median survival time of primary skin allografts

<table>
<thead>
<tr>
<th>Combination</th>
<th>Number of animals</th>
<th>Median Survival time</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 Bl to Balb/C</td>
<td>100</td>
<td>8.5</td>
<td>+1.1</td>
</tr>
<tr>
<td>F₁ to Balb/C</td>
<td>50</td>
<td>8.5</td>
<td>+1.2</td>
</tr>
<tr>
<td>AKR to B₁₀ D₂</td>
<td>75</td>
<td>10.0</td>
<td>+1.3</td>
</tr>
<tr>
<td>Balb/C to C57 Bl</td>
<td>35</td>
<td>9.6</td>
<td>+1.2</td>
</tr>
<tr>
<td>Balb/C to CBA</td>
<td>25</td>
<td>11.3</td>
<td>+1.2</td>
</tr>
</tbody>
</table>
Splenic tomv in mice.

Mice were splenectomised firstly to provide spleen cells and secondly to provide a source of animals for further studies on the activity of the reticuloendothelial system. Anaesthetised mice were clipped and shaved along their left flank after which the skin was carefully cleaned with 70% alcohol. Particular care was taken to ensure asepsis throughout the surgical procedures as splenectomised animals were found to be more susceptible to infection than normal animals.

A one centimetre left subcostal incision was made and the spleen delivered into the wound by means of an aneurysm needle. The splenic pedicle and an anastomotic vessel from the stomach were ligated with 5/0 silk, divided and the spleen removed. The wound was closed in two layers using interrupted 5/0 silk sutures.

Washing of glassware.

All glassware used in the preparation of cell suspensions and the collection of sera was cleaned by boiling for 30 minutes in Calgon (Albright and Wilson Aust. Pty. Ltd.) and sodium metasilicate. After boiling it was carefully washed and rinsed in glass distilled water, allowed to dry, and finally autoclaved prior to use.
Preparation of cell suspensions.

Lymphoid cells

Cell suspensions were prepared from lymph nodes, spleens and thymuses. The tissues were removed under sterile conditions from animals that had been sacrificed by cervical dislocation, or in the case of some spleens in the manner described above. In most instances it was found preferable to use weanling mice as the source of the thymocytes due to the increased size and cellularity of the thymus in these animals.

The tissues after removal were placed in sterile petri dishes containing cold (4°C) 0.15M saline. The collected tissues were then pressed through a fine nylon mesh (equivalent to 50 mesh stainless steel) with a large sterile centrifuge tube packed with ice. The expressed tissue pulp was subsequently suspended in 0.15M saline as described by Billingham and Silvers (1961). Following suspension the cells were washed three times in 0.15M saline and after the last wash resuspended in a suitable volume of the saline. All cell suspensions were used as soon as possible, and no suspensions over two hours old were used, irrespective of the viability count.

Peritoneal cells

The technique used to obtain mononuclear cells from the
peritoneal cavity of mice was similar to that described by Rowley (1958) and Freeman and Vasa (1958). Mice were killed by cervical dislocation and pinned out on a cork board. After cleaning the abdominal surface with 70% alcohol the skin was reflected to expose the abdominal wall. Using a 0.2 ml. syringe fitted with a 2 inch 19 gauge needle 2.0 ml. of Hank's solution (prepared according to the method described by Weller, et al. (1952) was injected into the peritoneal cavity. The abdomen was then gently massaged for about one minute and the injected fluid withdrawn. It was usually possible to recover 1.5 ml. of fluid containing approximately $6 \times 10^6$ cells/ml. of which 80-90% appeared to be macrophages.

**Viability Counts.**

In order to assess the viability of the cells in these suspensions, their ability to exclude the vital dyes, eosin (0.1%) and trypan blue (0.05%) was determined. The technique used was based on that described by Abaza and Woodruff (1966). Immediately prior to use a suitably diluted suspension of the cells was drawn up to the first mark of a white count pipette. The pipette was then filled with the vital dye, shaken for one minute and after standing for a further two minutes the cells were counted in a Neubaur haemocytometer. If the number of stained cells exceeded 15% of the total count the suspension was discarded.
Cell counts.

(i) **Peripheral white cell counts.**

Peripheral white cell counts were performed on both normal and serum treated mice. The blood was obtained from the retro orbital venous plexus by means of a glass pipette and immediately added to a glass tube in which either heparin (5,000iu/ml.) or 3% EDTA had been allowed to dry in the cold. After agitating the blood for 30 seconds it was diluted 1 in 20 in a white count pipette with a diluting fluid described by Whitby and Britton (1957). Following the dilution of the blood the pipette was gently agitated for several minutes and then allowed to stand for five minutes before the cells were counted in a haemocytometer.

(ii) **Differential counts.**

Differential white cell counts were performed on Leishman stained smears. A minimum of 200 cells being counted on each occasion.

(iii) **Bacterial counts.**

To determine the total number of bacteria in a given broth culture, counts were performed in a Petroff Hausser counting chamber. The method used for counting the bacteria in this chamber was essentially the same as that described by Cruickshank (1960). The number of viable bacteria present in a broth culture was
determined by plating out a known volume of the broth onto an appropriate medium. After incubation at 37°C overnight it was possible to estimate the number of viable bacteria/ml. by counting the number of colonies that appeared.

The estimation of the dilution necessary to give a certain number or organisms/ml. was facilitated by finding that a 10 ml. overnight broth culture of the organisms used, routinely gave between $10^8$ and $10^9$ organisms/ml.

Collection of sera.

(i) Mouse serum.

Mice were bled from their retro orbital venous plexus and the blood so obtained allowed to clot at room temperature in conical glass centrifuge tubes. The clotted blood was cooled (4°C for 30 minutes) to encourage clot retraction and so improve the yield of serum. The final step was to gently ring the clot with a fine glass rod to prevent adherence and centrifuge at 1,500 r.p.m. for 5 minutes. The serum was then pipetted off and used either immediately or stored in 3.0 ml. amounts at -20°C until required.

(ii) Pig serum.

The serum was prepared from the blood of healthy pigs freshly slaughtered at the Adelaide Metropolitan Abbatoirs. The serum following collection was handled and stored in a manner identical
with that described for the mouse serum.

**Antibody production.**

The ability of splenectomised, grafted and antiserum treated mice to produce antibody in response to the introduction of certain antigens was determined.

(i) **Antigens employed.**

The antigens used were sheep erythrocytes (obtained from the Institute of Medical and Veterinary Science) and bovine serum albumin fraction V (Commonwealth Serum Laboratories).

(ii) **Immunisation of mice with sheep erythrocytes.**

Groups of mice containing 8-10 per group were injected intravenously with 0.2 ml. of a 1% suspension of washed (3 times in normal saline) sheep erythrocytes. The animals were subsequently bled every second day and the haemagglutination titre of the serum determined. The total antibody as well as the amounts of macro-globulin (19S) and gamma globulin (7S) agglutinating antibody were determined. The separation of the two fractions was achieved by incubating the serum with an equal volume of 0.1M 2-mercapto-ethanol at 37°C for 60 minutes. The 2-mercapto-ethanol reducing the 19S antibody and rendering it biologically inactive, whereas the 7S antibody being resistant to reduction remained active.
(iii) **Estimation of the haemagglutination titre.**

The titrations were carried out in clean perspex haemagglutination trays. A volume of 0.2 ml. of a 1% suspension of washed sheep erythrocytes was added to doubling dilutions of the mouse serum under test in 0.2 ml. of normal saline. The trays after incubation at 37°C for 60 minutes were placed in the cold (4°C) for two hours. It was found that the cooling of the trays frequently produced a more definite end point. The dilution in the last well in which the cell pattern differed from the control pattern being taken as the end point.

(iv) **Immunisation of mice with bovine serum albumin (BSA).**

In these experiments doses of BSA ranging from 0.8 μgm to 12.5 mg. were employed. The BSA was dissolved in sterile saline and the volume of saline used adjusted, so that the desired dose was contained in a volume of 0.125 ml. In all instances the BSA was emulsified with an equal volume of Freund's incomplete adjuvant prior to its subcutaneous injection. Certain animals were challenged 28 days following the initial injection with an intraperitoneal injection of 20 μgm of BSA in 0.2 ml. of normal saline.

(v) **Estimation of anti BSA antibody levels (Precipitation).**

The level of anti BSA antibody in the serum of mice was determined by two methods. The first method used was similar to
that described by Reisner and Sobey (1962). Agar double diffusion plates were prepared by pouring 12 ml. of Iov agar (oxoid) (0.8% in normal saline) into clean 50 mm. glass petri dishes and after cooling a series of wells were made in the plates. The wells, shown in Fig. 7, were arranged so that the centre of each well was 11 mm. from that of the adjacent well.

The centre well was filled with a standard, (obtained from Dr. D. Hardy, Department of Microbiology, containing 5.4 μgm antibody/ml.) wells 1, 3 and 5 with 0.025% BSA and wells 2, 4 and 6 with the unknown serum samples. These plates were then incubated at 37°C for one hour and after being left overnight at room temperature, in a moist atmosphere, were examined for precipitin lines. The plates in Fig. 7 also show typical precipitin lines which could be compared with the control plates containing serial dilutions of the standard. The amount of antibody in any sample could, then, be expressed as being equivalent to that present in a certain dilution of the standard. The main disadvantage of this method was that adequate precipitin lines were not produced after the standard had been diluted 1 in 256. This consequently limited the sensitivity of the system where small amounts of antibody were involved.

In an attempt to quantitate more accurately the amount of antibody produced a second method was used. This was based on the
Fig. 7. Double diffusion plates showing arrangement of wells and the precipitin lines produced by BSA and anti-BSA antibody interaction.
ability of the serum to agglutinate sheep red cells onto which BSA had been diazotised.

(vi) **Diazotisation of BSA onto sheep erythrocytes (Haemagglutination).**

The method used for the diazotisation of the BSA onto the sheep red cells was based on those described by Rangel and Repka (1965) and Hyslop and Roeder (1966) for the diazotisation of protein onto red blood cells. Five ml. of a 4% suspension of sheep erythrocytes, washed three times in phosphate buffer (equal volumes of 0.15M NaCl and 0.15M phosphate buffer pH 7.4) were mixed in the cold for thirty minutes with an equal volume of an 0.2% solution of the BSA in the same buffer. The Bis-diazo-benzidine (BDB) was prepared by dissolving 100 mgm. of benzidine di HCl in 10 ml. of 0.025N hydrochloric acid. After cooling on ice 2 ml. of 0.5N NaNO₂ in water were added and the mixture allowed to stand in an ice bath for a further 30 minutes with occasional shaking.

To the cells and BSA, 0.9 ml. of a 1 in 15 dilution of the freshly made BDB in the phosphate buffer was added and the mixtures agitated continuously at room temperature for 30 minutes. Finally, after centrifugation in the cold and three washes with the buffer the cells were resuspended to 1% in the buffer which contained 0.1% gelatin for stabilisation. The cells were used immediately and the method for determining the haemagglutination titre was the same as
that described for normal sheep erythrocytes.

Histological studies.

The tissues subjected to histological examination were skin, liver, lymph node and spleen. These tissues were taken from normal, grafted and serum treated animals either under anaesthesia or immediately after death. Once removed from the animal the tissues were fixed by placing them immediately in a suitable volume of 10% formal saline.

After a period of at least 24 hours in the fixative the material to be sectioned was trimmed and embedded in wax (M.P. 56°C) and the blocks so obtained sectioned with a rotary microtome at a thickness of 3-4 microms. Finally the sections were floated onto glass slides and after drying were stained with either haematoxylin and eosin or methyl green pyronin. The stained sections were mounted and the mounting medium (Gurr's Xam) solidified by placing the slides in a 37°C incubator. The sections were examined and photographed with a Leitz 'photomicroscope' using a range of powers to 500 times magnification.

Carbon.

The carbon for the histologic studies was prepared according to the technique described by Biozzi et al. (1953). The concentration of carbon employed was 16 mgm./ml. and it was contained in 2 per
cent gelatine at pH 7.4. Each animal was injected intravenously with 0.2 ml. of this suspension and sacrificed five minutes later at which time the liver and spleen were removed for histologic examination. The stock solutions and the syringes were maintained at 37°C to prevent solidification, which tended to occur if the solutions were kept at room temperature.

Preparation of Rabbit anti mouse lymphocyte serum.

The cell suspensions for the immunisation of the rabbits were prepared from the thymuses and lymph nodes of both Balb/C and Swiss white mice as previously described. The method of raising the antiserum was similar to that described by Levey and Medawar (1966a). Each adult rabbit received an initial intravenous injection of $5 - 10 \times 10^8$ viable cells which was followed 14 days later by a second intravenous injection of a similar number of cells. Seven days following this injection the rabbits were anaesthetised with sodium pentobarbitane (60 mgm./Kilo) and bled out by cardiac puncture using a 2" 19 gauge needle and a 50 ml. syringe.

While this method produced a biologically active antiserum it was subsequently found that a better antiserum could be produced if the initial injection was given intraperitoneally and the second intravenously. The same cell numbers were employed in both cases.

The serum was prepared from the whole blood in a manner
essentially the same as that described previously for mouse serum. When collected the serum was decomplemented by heating at 56°C for 30 minutes, and Seitz filtered and stored in aliquots of 3 ml. at -20°C until required for use.

Absorption of anti mouse lymphocyte serum.

In most instances it was found unnecessary to absorb the antiserum. However, occasional batches of the serum were toxic, with death occurring in the treated mice within 36 hours of the first injection. This toxicity could be abolished by absorbing the serum overnight in the cold (4°C) with an equal volume of washed mouse erythrocytes.

In other studies relating to the activity of the antiserum, absorption was carried out using washed lymph node, spleen, liver and kidney cells. Again the serum was absorbed overnight at 4°C with an equal volume of the particular tissue under investigation. After absorption the serum was recovered by centrifugation at 1,500 r.p.m. for 10 minutes.

Assay of anti mouse lymphocytic serum.

Prior to using the antiserum for experimental studies it was necessary to assay for activity. The initial method of assay was based on the ability of the serum to produce lymphocyte agglutination. The first workers to make use of this property to provide an assay
system were Gray et al. (1966) and Abaza and Woodruff (1966).

**Leucocyte agglutination.**

The procedure followed was the same as that described previously for haemagglutination. 0.2 ml. of a washed suspension of viable lymphocytes, thymocytes or peritoneal cells containing $10^7$ cells/ml. was added to doubling dilutions of the serum in 0.2 ml. of Hanks solution. The trays after incubation at $37^\circ$ for 60 minutes were cooled and read by placing a small sample from each well onto a slide and examining as a wet preparation under high power. The scoring for agglutination was the same as that described by Abaza and Woodruff (1966). The titre recorded was the last dilution of the antiserum capable of producing greater agglutination than the normal rabbit serum controls. The titres obtained were usually 1 in 256 or 1 in 512.

While such a system has the advantage of giving a rapid indication of potential activity it in no way indicates possible toxicity. It can also be seen in Table II that the lymphocyte agglutination titre in some instances did not correlate at all well with the biologic activity of the serum as indicated by graft prolongation. However, all sera capable of prolonging allograft survival were found to produce significant lymphocyte agglutination.
TABLE II

Comparison of lymphocyte agglutination titre and biologic activity as indicated by allograft survival.

<table>
<thead>
<tr>
<th>Lymphocyte agglutination titre of six different sera</th>
<th>Median survival time (MST) of 2 cm.$^2$ C57 Bl to Balb/C skin grafts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:64</td>
<td>19.8 $^+$ 2.1</td>
</tr>
<tr>
<td>1:64</td>
<td>11.9 $^+$ 1.8</td>
</tr>
<tr>
<td>1:256</td>
<td>24.5 $^+$ 2.5</td>
</tr>
<tr>
<td>1:256</td>
<td>13.6 $^+$ 1.4</td>
</tr>
<tr>
<td>1:512</td>
<td>12.4 $^+$ 1.7</td>
</tr>
<tr>
<td>1:512</td>
<td>26.3 $^+$ 1.9</td>
</tr>
</tbody>
</table>

Each animal was given 0.5 ml. of antiserum subcutaneously on days -2, +2, +5 where day of grafting was day 0.

MST for normal rabbit serum treated controls 8.5 days $^+$ 1.1 days.
Skin graft assay.

This form of assay, favoured by Levey and Medawar (1966), depends upon a given dose of the antiserum to produce a certain prolongation of allograft survival. It was found (Chapter IV) that 0.5 ml. of antiserum given subcutaneously on day -2, +2 and +5, where the day of grafting is day 0, could prolong the survival of a skin graft in the combination used C57Bl to Balb/C from 8.5 days to a minimum of 17 days. In view of this it was decided that unless a serum given in this dosage could produce this degree of prolongation it would not be used for further studies. Although this is a very arbitrary criterion, when the effect of antiserum on other parameters in mice was measured there was no doubt that it possessed the important property of prolonging allograft survival.
CHAPTER III

The effect of skin allograft rejection on the activity of the Reticulo-endothelial system.

The presence of phagocytic cells and the general anatomy of the reticuloendothelial system has been known for many years, but the understanding of the function of these cells and their interaction with the other systems of the body is far from precise. To some workers these phagocytic cells represent little more than scavengers, always eager and available to remove unwanted debris. Others, however, would attribute to these cells more sophisticated roles such as the degradation of antigen and the induction of host immune responses.

It has, nevertheless, been this inherent phagocytic activity that has provided a means by which the activity of the system can be measured (Halpern, et al., 1953) and also modified. Attempts to blockade this system using a variety of agents such as trypan blue, indian ink, and thorotrast, have been made by a number of workers including Roberts (1929), Jungeblut (1930), Jaffe (1931) and McMaster (1953). These workers have reported that animals thus blockaded exhibit a reduced ability to form antibody to a number of antigens.

Similar blockading procedures in animals bearing allotransplants have produced prolonged graft survival (Brent and Medawar, 1961;
which is perhaps suggestive of a common pathway where these immune responses are concerned.

Unfortunately, in neither situation has general experimental agreement been achieved, but the discrepancies observed would appear to be related to the variety of techniques employed and the uncertain degree of blockade produced. Fisher and Fisher (1964) in an attempt to determine more definitely the relationship of reticuloendothelial blockade to transplant survival found that allograft rejection per se stimulated the phagocytic activity of the reticuloendothelial system. The resultant increased activity was capable of overcoming the blockading effect of thorotrast. Therefore, as Fisher and Fisher (1964) point out, although the grafts were rejected, in reality, adequate blockade did not exist.

The findings of the above workers relate to a host versus graft reaction. In the reverse situation the macrophage also appears prominent. The hepato-splenomegaly produced in graft versus host reaction has been attributed to the accumulation of these cells by Gorer and Boyse (1959). Howard (1961) showed that the phagocytic activity was markedly increased in these animals. The demonstration of this greatly increased phagocytic activity led Howard (1961) to suggest that the measurement of the phagocytic activity could provide
a sensitive in vivo test for graft versus host reactions.

As a result of the work of Fisher and Fisher (1964) it would seem that this stimulatory effect of an allograft could readily account for the failure to prolong allograft survival by blockading the reticuloendothelial system. It does not, however, account for the reports where prolongation has been achieved. It was, therefore, in an attempt to clarify this situation and to more accurately define the role of the reticuloendothelial system in allograft rejection that the following studies were performed. At the same time, extrapolating from Howard's (1961) results, it was also possible to assess whether the determination of the phagocytic activity could provide a successful in vivo test for host versus graft reactions. In the clinical situation such a test would be of the utmost value in the management of patients with renal allografts.

Phagocytic activity in allografted animals.

To investigate the phagocytic activity in allografted mice four strains of inbred animals were used. Each animal was given a 6-8 cm.$^2$ full thickness skin graft. Balb/C and $B_{10}D_2$ mice being the recipients and C57Bl and AKR the respective donors. The phagocytic activity was then determined in these animals over a period of 28 days at one to two day intervals by measuring the rate of clearance of labelled S. typhimurium C5 from the circulation. The experiment was
controlled by isografted and non-grafted members of the test strains.

Each of the points shown in Figures 8 and 9 represent the average of results obtained from a minimum of four animals. Similar numbers of animals were used in all the subsequent clearance studies. The phagocytic index K for the grafted animals has been expressed as a percentage of that for the normal animals, with K for these animals being taken as 100%. The reason for expressing K in this way was that the K for normal Balb/C mice was \( .12 \pm 0.1 \) whereas for \( B_{10}D_2 \) it was \( .11 \pm .01 \). Consequently the changes in phagocytic activity could be more readily compared between the strains if the results were expressed in this manner.

In the combination AKR to \( B_{10}D_2 \) (Fig. 8) there was little change in the phagocytic activity for the first 5-6 days. Following this, however, it can be seen that the clearance rate increased in the allografted animals and remained so for some 14 days by which time the grafts had been completely rejected. Similar results (Fig. 9) were obtained from the other combination (C57Bl to Balb/C) although the elevation occurred 24-48 hours earlier in these animals. The results obtained in both cases tend to suggest that this is a biphasic response with an initial peak of activity at approximately the time of graft rejection and a second peak occurring after the grafts had been completely rejected. In the control isograft animals there were
FIG 8. Rate of clearance of S. Typhimurium C5 in allografted mice.
FIG 9. Rate of clearance of S. Typhimurium C5 in allografted mice
fluctuations initially in activity but an analysis of variants has shown a significant difference between the response of this group as compared with those animals that received an allograft.

It was also found that prior opsonisation of the bacteria with serum taken from these allografted animals did not alter their rate of clearance in normal animals, thereby indicating that the changes observed in phagocytic activity were not caused by the presence of non-specific opsonic factors in the serum of the allografted mice.

The temporal relationship between allograft rejection and changes in phagocytic activity.

The rejection times, based on macroscopic appearances, in the combinations used were \( 10.0 \pm 1.2 \) days for AKR to \( B_{10}D_{2} \) and \( 8.8 \pm 1.1 \) days for C57Bl to Balb/C. Therefore, from the results shown in Figures 8 and 9 the increase in phagocytic activity occurs approximately 24-48 hours prior to macroscopic rejection.

In an attempt to correlate these changes in phagocytic activity with graft rejection at a cellular level the allografts and isografts from Balb/C mice were subjected to histologic examination. Within four days there was a definite cellular infiltration present in the isografts as well as the allografts and it was not until six days after grafting that the infiltration in the allografts was significantly greater and different from that found in the isografts. The infiltration at
this stage was predominantly a mononuclear one. Even so, in certain areas polymorphonuclear cells were prominent, but at this early stage these cells did not appear to be as prominent as suggested by Titus and Shorter (1962).

In spite of this infiltration the allografted epithelium still appeared to be relatively healthy. The most dramatic changes occurred in the epithelium after the seventh day. Figure 10 shows a section of a skin allograft at eight days with destruction of the epithelium and the cellular infiltration characteristic of rejection. A higher power (Fig. 11) of the same section shows an area in which polymorphonuclear as well as mononuclear cells were present. Figure 12 shows a sloughing graft at 12 days. It can be seen that a marked cellular infiltration was still present, and in many instances, at this stage and slightly later, large numbers of polymorphs were found. The presence of these cells was possibly related to infection and to a degree of infarction occurring in the sloughing grafts.

By combining the histologic and clearance studies it would appear that the first peak in Figures 8 and 9 has a close temporal relationship with graft rejection, whereas the second peak may be more directly related to the reparative process and possible infection following the death and sloughing of the graft. In general, these studies indicate that while the changes in phagocytic activity
Fig. 10. A C57Bl skin allograft at day 8 showing epithelial destruction and the cellular infiltration. (H & E x 125).

Fig. 11. Cellular infiltration present in an 8 day C57Bl to Balb/C allograft. Both mononuclear and polymorphonuclear cells are present. (H & E x 500).
Fig. 12. A sloughing C57Bl allograft at day 12.
(H & E x 125).
preceded both macropscopic rejection and histologic evidence of marked epithelial damage, they do not precede the cellular infiltration invariably associated with allograft rejection.

**Dose dependency of changes in phagocytic activity.**

In view of the relatively large grafts employed in the above experiment (approximately 20% of the surface area) further studies were carried out to ascertain whether graft size was important. The size of the grafts employed in this experiment were 1 cm.\(^2\), 3 cm.\(^2\) and 8 cm.\(^2\).

The results obtained when these animals were subsequently cleared are shown in Figure 13. It can be seen that the timing and direction of the response were the same irrespective of the graft size, but the magnitude and duration of the response appeared to be related to the size of the graft employed. These results would, therefore, indicate that this is a dose dependent phenomenon.

**Phagocytic activity in animals bearing F\(_1\) grafts.**

In view of the considerable increase in phagocytic activity produced during graft versus host reactions (Howard, 1961) it appeared to be of some importance to ensure that the changes observed in the present studies were due entirely to a host versus graft reaction. To this end the phagocytic activity was determined in Balb/C mice bearing 6-8 cm.\(^2\) F\(_1\) skin grafts.
B10.D2 mice bearing AKR primary allografts - Effect of graft size

FIG 13. Rate of clearance of S.Typhimurium C5 in allografted animals
The pattern of changes produced (Fig. 14) in the phagocytic activity were essentially the same as those found in Balb/C mice bearing a correspondingly sized graft from the other parent. These animals also showed a similar degree of splenomegaly but as before there was no hepatomegaly. Using this experimental model in reverse it was found that $F_1$ mice bearing parental grafts behaved in a manner similar to isografted controls with no splenomegaly or hepatic enlargement. These results would, therefore, confirm that this increase in activity occurs as a result of a host versus graft reaction.

**Organ changes during skin allograft rejection.**

In the introduction mention was made of the findings by Gorer and Boyse (1959) and Howard (1961) that marked hepato-splenomegaly was produced during graft versus host reactions. To ascertain whether similar changes occurred during host versus graft rejection the organs classically associated with the reticuloendothelial system were examined. The livers, spleens and lymph nodes were removed from $B_{10}D_2$ mice bearing 6-8 cm.$^2$ iso- or allografts at various times after grafting and their weights determined, immediately after blotting by weighing on a Shimadzu L5-1 Balance. It was found that there was no significant change in the weights of the livers obtained from either group. The results obtained from weighing the spleens are shown in Figure 15. It was found that there was a significant increase in the
BALB/C mice bearing 6-8 cm$^2$ F1 (BALB/C x C57 Bl.) grafts

FIG 14. Rate of clearance of S. Typhimurium C5 in allografted mice
spleen weights of these allografted animals. This increase occurred quite rapidly over a period of one or two days preceding macroscopic rejection. Superimposed on Figure 15 is the phagocytic index $K$ obtained prior to splenectomy showing that the changes in spleen weights in most instances closely paralleled the changes in phagocytic activity. It is also of note that these splenic changes occurred prior to graft breakdown at a time when isografts and allografts were almost identical macroscopically. This would tend to preclude bacterial or other irrelevant antigens as a cause for the initial splenomegaly. It is nevertheless not possible to preclude these agents as a cause for the prolonged response.

The increase in spleen weight also appeared to be related to the size of the allograft employed. It can, however, be seen in Figure 16 that this relationship was not as marked as in the clearance studies. The small grafts which produced only a marginal increase in phagocytic activity have produced a proportionately much greater increase in spleen weight.

The weights of the draining lymph nodes were not found to be as greatly increased as had been previously described in mice (Micklem and Loutit, 1966) and in rabbits (Scothorne and McGregor, 1955). Whereas the former workers had reported a fourfold increase in weight, the maximum increase found in this series was twofold.
FIG 15. Spleen weights expressed as % body weight
$B_{10}D_{2}$ mice bearing AKR primary allografts

FIG 16. Spleen weights expressed as % body weight
The increase in weight of the lymph nodes was not related to graft size. The only difference observed between large and small grafts was that with the former more nodes were affected due to the greater area of lymphatic drainage involved. Apart from this the timing and duration of these weight changes were similar to those described previously for the spleen, although, it was not uncommon to find within four days of grafting a fifty per cent increase in the weight of the local lymph nodes.

**Organ distribution of radioactivity.**

Benacerraf, *et al.* (1959) have shown that the phagocytes of the liver are almost exclusively responsible for the removal of well opsonised bacteria from the circulation. Furthermore, from this and a number of other studies it would appear that irrespective of the particle used the liver and spleen together are responsible for the uptake of 90% of the injected dose. Consequently, having demonstrated this increased phagocytic activity in allografted animals the following studies were performed to ascertain whether these organs were still primarily responsible for the increased rate of clearance of the bacteria. To achieve this the level of radioactivity was estimated in the livers and spleens taken from Balb/C mice that had been sacrificed 5 minutes after the injection of $2 \times 10^8$ labelled bacteria.

The amount of radioactivity recovered from each organ was
expressed as a percentage of the dose injected, and the results shown in Figure 17 for any one day represent the average of estimations performed on a minimum of eight organs. It was found that in the animals showing increased phagocytic activity (K.18, .19) the liver had taken up over 70% of the injected dose within five minutes, whereas the livers from animals with normal phagocytic activity (K.12) had removed less than 50% of the injected dose in the same time. The spleens on the other hand did not appear to be significantly involved as they accounted for only 4% of the injected dose in normal animals, increasing to 6-8% in the animals with increased phagocytic activity.

It would appear, therefore, that in allografted animals it is the phagocytic cells of the liver that are mainly responsible for the increased clearance rates, and that the spleen although increased in size plays a relatively minor role.

**Phagocytic activity in splenectomised mice.**

Halpern (1959) when reviewing the function of the reticuloendothelial system pointed to the remarkably constant finding of hepatic and splenic hypertrophy in animals that had a hyperactive reticuloendothelial system. In the present studies no hepatomegaly was observed but splenomegaly was clearly present in animals with increased phagocytic activity. However, it was evident from
FIG 17. % RECOVERY OF RADIOACTIVITY FROM LIVER AND SPLEEN AFTER 5 MINUTES.
the organ distribution studies that in spite of its increase in size
the spleen was not primarily responsible for the increased rate of
clearance. Nevertheless the possibility of a less direct relationship
could not be dismissed and it was in an attempt to investigate this
possibility that the following studies were performed.

It can be seen from the results shown in Figure 18 that
during the period of investigation the phagocytic activity of
splenectomised animals was not significantly different from that
in the sham operated controls, although it was noted that both
groups tended to clear slightly more rapidly than normal animals.
In the allografted animals it became rapidly evident that splenectomy
did not alter the subsequent survival time of the grafts. To establish
that splenectomy had indeed produced some alteration in immune
responsiveness antibody levels were measured in these animals
after the intravenous injection of 0.2 ml. of a 1% suspension
of sheep erythrocytes. The ability of these animals to produce
both macroglobulin and gammaglobulin antibodies was clearly
reduced. The highest titre recorded in the splenectomised animals
being 1 in 32, whereas the normal and sham operated animals
gave titres of 1 in 512 or more.

The clearance studies in mice that had received a 6–8 cm.$^2$
allograft on the day of splenectomy revealed that the overall response
FIG 18. Rate of clearance of S. Typhimurium C5 in splenectomised mice
(Fig. 19) was essentially the same as that found in non-splenectomised animals. It was noted, however, (Fig. 19) that the control isografted splenectomised animals showed a transient increase in phagocytic activity five to seven days after grafting, but the phagocytic activity of the allografted group was still significantly different ($p < 0.01$).

The other feature of the results shown in Figure 19 that attracted attention was that the increased rate of clearance in the allografted splenectomised animals was not maintained for as long as in the non-splenectomised controls.

Since these animals had been grafted on the day of splenectomy, it appeared possible that a time factor was involved. This was not subsequently confirmed as clearance studies performed on animals allografted 21 days and 35 days after splenectomy showed these animals to have increased phagocytic activity.

It is evident from these results that the changes in phagocytic activity and spleen size could well be produced by a common factor, although the spleen per se was not responsible for the heightened phagocytic activity.

**Transfer of antigenic information - its relationship to changes in phagocytic activity.**

It has been assumed in this and other studies that the changes in phagocytic activity were directly related to graft rejection. As
FIG 19. Clearance rate of S. Typhimurium C5 in splenectomised and grafted mice
these changes in activity preceded macroscopic graft rejection the following experiments were performed to investigate whether the transfer of antigenic information and the development of sensitivity could have some bearing on this response.

Balb/C mice bearing 6-8 cm.\(^2\) allografts were chosen as the experimental animals and the grafts were removed two or four days after grafting by the method described in Chapter II. The phagocytic activity of the animals whose allografts had been removed at two days was not significantly different from the control groups, which were composed of animals that had had either a correspondingly sized piece of skin or isograft removed. Initially as can be seen from Figure 20 there was no significant alteration in phagocytic activity but by the tenth or twelfth day all groups did show some heightened activity. This response was almost certainly related to such factors as infection and tissue repair.

On the other hand, animals whose allografts had been removed at four days were found to have an increased phagocytic activity (Fig. 21). This response was similar both in timing and magnitude to that initially found in animals whose grafts had been left in situ. The control groups as can be seen failed to show any such response. It was also noted that the spleens taken from animals whose allografts had been present for four days were two to three times heavier than
BALB/C MICE BEARING 6cm² C57 BL. ALLOGRAFTS

- ◦ Sham operated
- ◇ Allografted
- ○ Isografted

FIG 20. RATE OF CLEARANCE OF S. TYPHIMURIUM (C5)
GRAFT REMOVED DAY +2
BALB/C MICE BEARING 6 cm² C57 BL. ALLOGRAFT

- • sham operated
- □ iso-grafted
- ■ allo-grafted

FIG 21. RATE OF CLEARANCE OF S. TYPHIMURIUM (C5) GRAFT REMOVED DAY +4
those taken from any of the other groups. As before no significant changes were observed in the liver weights.

The results shown in Figures 20 and 21 were obtained from animals that had been nobecutane treated after graft removal. Similar results were also obtained using the suturing technique (see Chapter II).

The other observation which appeared to be of some significance was that animals, after four days of contact with the allograft rejected a second graft, given two weeks later, in an accelerated fashion (8.5 ± 1.1 days to 6.4 ± 1.0 days - 10 animals), whereas the group which had two day contact with the graft failed to do so and rejected their second graft in a first set fashion.

It would seem from the results shown above that the initial peak of activity can occur irrespective of the presence of the graft, although a certain minimum time of contact was necessary. In this experiment it was four days. The other interesting and possibly related finding was that four days of contact with the allograft were necessary for the development of allograft sensitivity.

**Stimulation of the reticuloendothelial system by adoptive transfer.**

The previous studies described in this chapter have indicated that the liver macrophages are primarily responsible for the changes observed in the phagocytic activity of the allografted animals.
Therefore, as these cells are remote from the site of graft rejection it appeared that it might be possible to transfer this effect to another animal.

If this were possible then the elements most likely to achieve this transference would appear to be either in the serum, or associated with lymphoid cells.

Serum, lymph node and spleen cells were taken from normal Balb/C mice, as well as from animals bearing 6-8 cm$^2$ isografts or C57Bl allografts. In all instances the cells and serum were obtained six days after grafting. This time was chosen as the previous studies had shown that in this combination the phagocytic activity increased markedly between day 5 and 6 and remained at this level for several days.

Groups of Balb/C mice (6-8 per group) were injected intravenously with either 0.5 ml. of serum, $5 \times 10^7$ lymph node cells or $5 \times 10^7$ spleen cells from the various donors and the phagocytic activity determined two and four days later. Apart from the fact that cells from all sources produced a slight increase in phagocytic activity (Fig. 22) no other significant changes were observed.

Further attempts to transfer this effect were made using larger doses of serum and cells. As such doses were not well tolerated intravenously the route of administration was changed to intraperitoneal.
FIG 22. Animals receiving intravenous injection
Groups of Balb/C mice were now given either 1.0 ml. of serum or the cells from eight draining lymph nodes or two spleens. In each case these doses were taken from two animals. Where normal and isografted animals were the donors, the same volume of serum was used, but because of the greater size of the lymph nodes and spleens from the allografted animals twice as many lymph nodes and spleens were used in order to make the dose equivalent on a weight basis. The injected animals were cleared as before on day two and four.

Once again it was found (Fig. 23) that the injection of serum irrespective of the source had little effect on the phagocytic activity. However, the animals that had received cells from the allografted donors showed a significant increase in phagocytic activity (p< 0.01) on day 4 when compared with the other groups. It was also noted that the animals that received spleen cells from the allografted donors had developed splenomegaly by day four but that those receiving the lymph node cells had not. No alteration was noted in the liver or spleen sizes in serum treated animals.

Finally, attempts were made to determine whether this dose of cells had been capable of producing adoptively acquired immunity. The same strain of animals and cell dosage were used as in the clearance studies and the animals were given 2 cm.² C57Bl skin grafts 3 days after the injection of cells. Day 3 was chosen as
FIG 23. Animals receiving intraperitoneal injection
Billingham, Brent and Medawar (1954) using a similar system had found their results to be more strongly positive if the transferred lymphoid tissue remained in the new host for 3 days prior to grafting. It was found that the animals receiving cells from the allografted donors did reject their allografts more rapidly but it was only a marginal increase, $7.8 \pm 1.0$ days as opposed to $8.5 \pm 1.1$ days - from 8 animals.

It would appear that both lymph node and spleen cells when taken from these animals with an increased phagocytic activity were capable of transferring this effect to normal animals. While this could only be achieved by intraperitoneal and not intravenous inoculation the failure using the intravenous route could well be related to quantitative factors.

**Summary.**

The studies described in this chapter provide further evidence that skin allografts stimulate the reticuloendothelial system. Furthermore it has been shown that the magnitude of the response bears a definite relationship to the size of the graft employed. The larger the graft, the greater the increase in phagocytic activity.

These changes in activity while preceding macroscopic rejection by 24-48 hours, did not precede the cellular response
associated with the allograft reaction. Even so, it would appear that the measurement of the phagocytic activity could still provide a useful **in vivo** indicator of a host versus graft reaction. However, it is quite clear that the particle used, *S. typhimurium* C5, is only suitable if the animal can be sacrificed.

The spleen, although prominent because of its morphological changes, did not significantly influence phagocytic activity or allograft survival. In contrast, no significant morphological changes were found in the liver, yet the distribution of radioactivity would indicate that it was primarily responsible for the changes in phagocytic activity.

The ability to demonstrate changes in phagocytic activity in the absence of the graft, and the timing of the response would indicate that it is most likely the release of antigens from the graft that stimulate the reticuloendothelial system as opposed to graft rejection **per se**. It is clear that the transfer of antigenic information has occurred at this early stage as animals rejected a second graft in an accelerated fashion after four days of contact with the primary graft.

The results from the transfer studies have revealed that this response is unlikely to have been produced by the direct effect of the antigens on the reticuloendothelial system. In the initial
experiments relating to adoptive immunity Mitchison (1954) and Billingham, et al. (1954) showed that the immunity produced was not due to the transfer of antigenic material. Since a similar experimental model has been used in the present studies it would appear equally improbable that significant amounts of antigen have been transferred. The results would, therefore, be consistent with a more indirect route, where the antigen interacts with lymph node or spleen cells with the resultant production of a factor which then stimulates the reticuloendothelial system.
CHAPTER IV

Studies on the effect of heterologous anti-lymphocyte serum on the reticuloendothelial system in mice.

Human renal allotransplantation has increasingly become a therapeutic possibility for patients dying with renal failure. This clinical achievement has resulted from the use of agents capable of suppressing the constant and unrelenting process by which the host destroys foreign tissue. Unfortunately, all of these agents have their limitations and no regime yet tried appears to provide the ultimate solution to immunosuppression. Whether anti-lymphocytic serum can fulfil this role is a question which has recently been undergoing extensive investigation.

The ability of anti-lymphocytic serum to prolong allograft survival (Woodruff and Anderson, 1963, Monaco, et al, 1966), influence delayed hypersensitivity reactions (Waksman, et al, 1961) and suppress antibody formation (Monaco, et al, 1966, James and Anderson, 1967), has undoubtedly established it as a powerful immunosuppressive agent. These observations would suggest that it has the capacity to suppress all aspects of immunity, but as Gowans (1967) has pointed out, its most striking effect appears to be in relation to those allotransplant reactions involving strong antigenic
differences which are so difficult to suppress by existing regimes. This is amply supported by the finding of Levey and Medawar (1967) that as little as 0.25 ml. of an effective antiserum can prolong skin graft survival as efficiently as 600r of whole body irradiation.

While considerable data has accumulated concerning the properties of this serum the means by which it achieves its \textit{in vivo} effect is far from clear. The assumption that the target cell is the lymphocyte has been based largely on the evidence that allograft reactions are mediated through these cells (reviewed Gowans, 1965; Gowans and McGregor, 1965). The findings of Monaco et al. (1966, 1967) that their anti-lymphocyte serum produced a profound lymphopenia and that the persistence of this lymphopenia correlated well with graft survival provided further experimental support for such an hypothesis. In other studies, Turk (1967) and Parrott (1967) found only the 'thymic dependent' areas of the lymph nodes depleted of lymphocytes, whereas Levey and Medawar (1967) have described hyperplastic changes in these tissues as did Chew and Lawrence (1937).

In spite of the marked histologic differences, the \textit{in vivo} effects of the sera were remarkably constant especially in the prolongation of allograft survival. To reconcile these widely divergent observations it would appear necessary to postulate that anti-
lymphocyte serum has several *in vivo* effects, any of which is capable of disrupting the normal immune response, or as Levey and Medawar (1967) have suggested, to the presence of irrelevant antibodies. This wide divergence of experimental evidence on what appears to be a critical point, clearly indicates the numerous fundamental questions that remain unanswered where this agent is concerned. Perhaps the most important of which is whether the lymphocyte is the cell primarily affected.

The present studies were carried out to determine the effect of heterospecific antiserum on the activity of the reticuloendothelial system with the aim of further investigating the mode of action of anti-lymphocyte serum as well as the possible role of the reticuloendothelial system in allograft rejection. The initial step was to establish a satisfactory dose, route and time of administration for the antiserum. **Allograft survival I - the effect of the timing and the dosage of antiserum.**

The antisera raised in rabbits against Balb/C lymph node cells or thymocytes respectively were injected subcutaneously into the left shoulder region of adult Balb/C mice. In all subsequent experiments this site was used for the subcutaneous injections. The dose employed ranged from 0.25 ml. to 2.0 ml. with the maximum dose given at any one time being 1.0 ml. The efficacy of a particular dosage regime was judged by its ability to prolong the survival of a 2 cm.² C57Bl skin
allograft.

The data obtained from this experiment is summarised in Table III and it can be seen that by increasing the dose the survival time of the graft increased and multiple doses were more effective than a single dose. The time of administration of the antiserum in relation to grafting was also important. Administering the antiserum two days prior to grafting was more effective than giving the same dose (1.5 ml.) on the day of grafting or two days later. Nevertheless, there was a limit to the improvement in survival achieved by commencing the antiserum prior to grafting. Animals receiving the first dose on day -4 rejected their grafts more rapidly than animals given the first dose on day -2.

While the results shown in Table III are for animals treated with anti-lymphocyte serum an essentially similar pattern was obtained using anti-thymocyte serum.

From these initial studies it was found that by giving a total dose of 1.5 ml. in 0.5 ml. doses on days -2, +2, and +5 where the day of grafting was day 0, it was possible to prolong the survival of a skin graft in the combination used, (C57Bl to Balb/C) from 8.5 days to a minimum of 17 days. It was decided that unless this degree of prolongation could be achieved by a particular batch of serum it would not be used in further studies. Although, this is an arbitrary
The effect of timing and dosage of anti-lymphocyte serum.

<table>
<thead>
<tr>
<th>Regimen of injection</th>
<th>Median Survival time (MST) of 2 cm.$^2$ C57Bl allografts</th>
<th>Regimen of injection</th>
<th>MST of 2 cm.$^2$ C57Bl allografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 ml. at -2</td>
<td>11.2</td>
<td>0.5 ml. at -2, +2, +5</td>
<td>22.9</td>
</tr>
<tr>
<td>0.25 ml. at 0</td>
<td>10.0</td>
<td>0.5 ml. at 0, +4, +7</td>
<td>18.6</td>
</tr>
<tr>
<td>0.25 ml. at +2</td>
<td>9.8</td>
<td>0.5 ml. at +2, +6, +9</td>
<td>15.5</td>
</tr>
<tr>
<td>0.5 ml. at -2</td>
<td>12.6</td>
<td>0.5 ml. at -2, +2, +5, +8</td>
<td>27.4</td>
</tr>
<tr>
<td>0.5 ml. at 0</td>
<td>11.5</td>
<td>1.0 ml. at day -2</td>
<td>15.5</td>
</tr>
<tr>
<td>0.5 ml. at +2</td>
<td>11.2</td>
<td>1.0 ml. at day 0</td>
<td>13.0</td>
</tr>
<tr>
<td>0.5 ml. at -4, 0, +3</td>
<td>20.6</td>
<td>1.0 ml. at day +2</td>
<td>12.5</td>
</tr>
</tbody>
</table>
criterion when the effect of the antiserum on the other parameters in mice was assessed, there was no doubt that the serum was effective in prolonging allograft survival.

Allograft survival II - the effect of the route of administration of the antiserum.

The following experiment was performed to investigate whether the route of administration of the serum was important in prolonging allograft survival. 0.5 ml. of the antiserum was injected either intravenously, intraperitoneally or subcutaneously into Balb/C mice on days -2, +2 and +5 with each animal receiving a 2 cm.² C57Bl skin allograft on day 0.

It was noted that the antiserum was appreciably more toxic when given intravenously or intraperitoneally than subcutaneously. This toxicity appeared to be related to a factor directed against mouse erythrocytes as prior absorption with these cells abolished this effect. Consequently, only absorbed serum was used in these studies.

Once this toxicity was avoided it was found that the survival times of the allografts were not significantly different in the three groups (Table IV). In spite of the fact that these results have indicated the various routes to be equally effective, it appeared preferable to administer the antiserum subcutaneously as this route minimised the toxic side effects and in most instances obviated the
TABLE IV

The effect of the route of administration of anti-lymphocyte serum (ALS)

<table>
<thead>
<tr>
<th>Route of administration of ALS</th>
<th>M.S.T. of 2 cm.$^2$ C57Bl skin allografts (8 animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>22.5</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>23.4</td>
</tr>
<tr>
<td>Subcutaneously</td>
<td>23.1</td>
</tr>
</tbody>
</table>
necessity for prior absorption with erythrocytes.

**Allograft survival III - the effect of allograft size.**

The experiments of Medawar (1944, 1945) and Lehrfeld and Taylor (1953) indicated that small skin allografts survived longer than large ones. These results have been generally accepted, although, Zotikov and Budik (1960) reported that in rats skin allografts which were greater than one third the surface area of the animal survived longer than those of smaller size.

In the subsequent experiments since large skin allografts (20-30% of the surface area) as well as small grafts were to be employed it appeared important to establish whether the size of the graft affected its subsequent survival in antiserum treated animals. Balb/C mice were grafted with 0.5, 1.0, 4.0 and 6-8 cm.\(^2\) pieces of C57Bl skin and each animal received a total of 1.5 ml. of antiserum in three divided doses. It is evident from the results shown in Table V that there was no significant difference between the survival times of the various grafts, which would suggest that in tissue grafting the dose of foreign antigens released is unlikely to be important, where the efficacy of this agent is concerned.

**Allograft survival IV - the specificity of antiserum.**

The production of this antiserum against lymphoid cells taken from inbred strains of mice has tended to foster the idea that antiserum
TABLE V

Survival time of C57Bl allografts on Balb/C mice treated with anti-lymphocytic serum.

<table>
<thead>
<tr>
<th>Graft size</th>
<th>Median Survival Time C57Bl to Balb/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 cm.²</td>
<td>24.5 ± 2.3</td>
</tr>
<tr>
<td>1.0 cm.²</td>
<td>22.8 ± 2.0</td>
</tr>
<tr>
<td>4.0 cm.²</td>
<td>22.0 ± 1.8</td>
</tr>
<tr>
<td>6-8 cm.²</td>
<td>23.7 ± 1.6</td>
</tr>
</tbody>
</table>

Balb/C mice were given 0.5 ml. of antiserum on day -2, +2, +5. Number of animals per group - 6.
is a relatively specific agent. From a practical viewpoint it appeared important to establish whether such an agent could be employed satisfactorily in an outbred situation and if the antiserum was not specific for lymphoid tissues, to determine which other tissues were affected by its administration.

To determine whether there was strain specificity, antiserum raised against Balb/C lymphocytes was used in an attempt to prolong the survival of Balb/C skin allografts on C57Bl and CBA mice. The data obtained from this experiment is summarised in Table VI and shows that the antiserum does not appear to possess marked strain specificity as comparable prolongation was achieved using an antiserum raised against cells from an unrelated strain. This has been further confirmed by the observation that antiserum raised against lymphocytes from outbred Swiss white mice can significantly prolong allograft survival in that strain, as well as being capable of trebling the survival time of grafts in the combination C57Bl to Balb/C when given in the same dose as used previously.

**Tissue specificity.**

The anti-lymphocytic serum was absorbed with spleen, lymph node, liver kidney and red cells by the method described in Chapter II. The capacity of a particular tissue to absorb out the biologic activity of the antiserum being indicated by the subsequent
Median Survival Times (MST) of 2 cm. \(^2\) allografts in mice treated with antiserum (ALS) and normal rabbit serum (NRS).

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Donor</th>
<th>Recipient</th>
<th>Treatment</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>C57Bl</td>
<td>Balb/C</td>
<td>NRS</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>C57Bl</td>
<td>Balb/C</td>
<td>ALS</td>
<td>22.5</td>
</tr>
<tr>
<td>10</td>
<td>Balb/C</td>
<td>C57Bl</td>
<td>NRS</td>
<td>9.6</td>
</tr>
<tr>
<td>8</td>
<td>Balb/C</td>
<td>C57Bl</td>
<td>ALS</td>
<td>21.5</td>
</tr>
<tr>
<td>10</td>
<td>Balb/C</td>
<td>CBA</td>
<td>NRS</td>
<td>10.6</td>
</tr>
<tr>
<td>9</td>
<td>Balb/C</td>
<td>CBA</td>
<td>ALS</td>
<td>26.8</td>
</tr>
</tbody>
</table>

Each animal was given 0.5 ml. of ALS or NRS on days -2, +2, +5.

Day of grafting = 0.
reduced ability of the serum to prolong the survival of a 2 cm.² allograft in the combination C57Bl to Balb/C. The results of the assays are shown in Table VII.

Although the lymphoid cells, volume for volume, were capable of producing significantly greater inactivation, the other tissues were capable of producing some reduction in the biologic activity of the serum. This would almost certainly indicate that this antiserum is directed against components of these cells as well as those of lymphoid cells. Attempts were also made to inactivate the antiserum by prior absorption with peritoneal cells, but because of the difficulty in obtaining sufficient cell volumes it was not possible to absorb adequate amounts of serum for grafting studies. The only observation of possible relevance to this question was that the leukoagglutination titre produced by a given serum sample was the same irrespective of whether lymphoid or peritoneal cells were used.

**Phagocytic activity in anti-lymphocyte serum treated mice.**

As a result of the ability of anti-lymphocytic serum to prolong skin allograft survival in mice it has been possible to investigate the activity of the reticuloendothelial system in animals clearly incapable of rejecting allografts.

Balb/C mice were injected subcutaneously with either 0.5 ml. or normal rabbit serum or antiserum on days 0, +4, and +7 and the
TABLE VII

Inactivation of anti-lymphocytic serum by absorption with tissues and cells.

<table>
<thead>
<tr>
<th>Absorbent (overnight at $4^\circ$C)</th>
<th>Median Survival Time (MST) for absorbed serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>23.5</td>
</tr>
<tr>
<td>erythrocytes</td>
<td>22.5</td>
</tr>
<tr>
<td>spleen cells</td>
<td>11.2</td>
</tr>
<tr>
<td>liver</td>
<td>16.5</td>
</tr>
<tr>
<td>kidney</td>
<td>17.6</td>
</tr>
<tr>
<td>lymph node cells</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* Animals were given 0.5ml. of the absorbed serum on days -2, +2, +5.

Day of grafting - 0

MST for normal rabbit serum treated animals 8.5 days.
phagocytic activity determined at two to three day intervals by measuring the rate of clearance of labelled *S. typhimurium* C5 from the circulation as described in Chapter II. The results obtained from this experiment are shown in Fig. 24. It was found that animals treated with anti-thymocyte serum exhibited increased phagocytic activity compared with untreated and normal rabbit serum treated controls. This increased activity was first observed 24 hours after the initial injection and remained so for some 10-12 days following treatment. Figure 25 shows that a similar response occurred when anti-lymphocyte serum was used. The other feature of potential significance was that the phagocytic activity had returned to normal, or near normal, prior to the expected time of graft rejection.

From studies to determine the amount of radioactivity in the livers and spleens taken from these animals it appeared that the liver was primarily responsible for the increased uptake of particles. The effect of anti-lymphocytic serum on the reticuloendothelial system - morphological studies.

In the course of the previous experiment it was noted that the spleens of the antiserum treated animals were markedly increased in size. As these changes were most pronounced further studies were performed to investigate this phenomenon, and to ascertain whether
FIG 24 RATE OF CLEARANCE OF S. TYPHIMURIUM(C5) IN SERUM TREATED MICE

- - Normal rabbit serum
- - Rabbit anti-mouse thymocyte serum

K (PHAGOCYTIC INDEX)

SUBCUTANEOUS INJECTION OF 0.5mls SERUM TIME IN DAYS
FIG 25 RATE OF CLEARANCE OF S.TYPHIMURIUM(C5) IN SERUM TREATED MICE

- - Normal rabbit serum.
- - Rabbit anti-mouse lymphocyte serum.

SUBCUTANEOUS INJECTION OF 0.5mls SERUM

TIME IN DAYS
other organs associated with the reticuloendothelial system were similarly affected.

Balb/C mice were given either anti-lymphocyte serum or normal rabbit serum, the dosage and schedule of administration being the same as that used in the clearance studies. These animals were then sacrificed at varying intervals and their livers, spleens and superficial lymph nodes removed and, after blotting to remove excess blood, weighed on a Shimadzu L5-2 balance.

Liver weights.

The liver weights were expressed as a percentage of the body weight. It can be seen from Fig. 26 that there was no change in the ratio of the liver weight to body weight in normal rabbit serum treated animals, but that there was an increase in this ratio in the antiserum treated animals.

Spleen weights.

The most marked changes were observed in the spleen weights. The spleens from both anti-lymphocyte and anti-thymocyte serum treated animals were 4-6 times heavier than those from the control animals (Fig. 27). This meant that the weight had increased from 130-140 mgm. in normal animals to 750-900 mgm. in the antiserum treated animals within a period of 8-9 days.
FIG 26 LIVER WEIGHTS OF SERUM TREATED MICE EXPRESSED AS % OF BODY WEIGHT.
FIG 27  SPLEEN WEIGHTS OF SERUM TREATED MICE EXPRESSED AS % OF BODY WEIGHT.
Lymph node weights.

The lymph node weights were also increased in the antiserum treated animals. The nodes weighed were 4 axillary and 2 femoral. These nodes were easily distinguished and removed in the strain of animals used. From Figure 2 it can be seen that there was an increase in the weight of the nodes from normal rabbit serum treated mice. This was due to a marked increase in the weight of the nodes draining the area into which the serum had been injected. However, in the antiserum treated mice all nodes were enlarged. This did not apply only to these nodes, as the mesenteric nodes which were also weighed showed a similar increase. These changes were abolished by absorption of the antiserum with lymphocytes, but were unaffected if the antiserum was absorbed with mouse erythrocytes. In all instances the timing of the weight changes were similar. The increase first became apparent on the fourth to fifth day after the commencement of treatment and clearly outlasted the course of antiserum. As with the changes in phagocytic activity the weights were returning to normal prior to the anticipated time of allograft rejection.

It appeared from these results that a correlation existed between increased phagocytic activity, increased organ weights and survival of allografts. Therefore, by giving larger doses of
FIG 28

WEIGHT OF AXILLARY AND FEMORAL LYMPH NODES (6) IN SERUM TREATED ANIMALS.
antiserum over a longer period of time, as well as increasing the survival time of the allografts these other effects should also be prolonged. To test this, groups of Balb/C mice (5-6 per group) were given either normal rabbit serum or antiserum, with the dose ranging from 0.5 ml. to 2.5 ml. per animal. These animals then received a 2 cm.$^2$ C57Bl skin graft or were left for 18 days when they were cleared with labelled *S. typhimurium* C5 and their organ weights determined. The results obtained from this experiment are shown in Table VIII. As expected from the data presented earlier in this chapter the groups of mice given one, two or three injections were not significantly different from the controls after 18 days. On the other hand, animals given four and five injections as well as exhibiting a greater survival time for their allografts were found to have an increased phagocytic activity and spleen weight at this time. Although not shown in Table VIII the lymph node weights in these latter groups were also found to be significantly increased. These observations are, therefore, in accord with the suggestion that there is a correlation between allograft survival and the changes found in phagocytic activity and organ size.

**The effect of antiserum on the reticuloendothelial system - morphological studies II.**

While it has been demonstrated that marked changes occur
TABLE VIII

Effect of prolonged administration of anti-lymphocyte serum (ALS) and normal rabbit serum (NRS) on MST of C57Bl skin grafts on BALB/C mice.

<table>
<thead>
<tr>
<th>Regimen of injection</th>
<th>Phagocytic index Day + 18</th>
<th>Spleen Weight mgm.</th>
<th>MST of C57Bl skin grafts on BALB/C mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALS</td>
<td>NRS</td>
<td>ALS</td>
</tr>
<tr>
<td>1 x 0.5 ml. at -2</td>
<td>.12</td>
<td>.13</td>
<td>120</td>
</tr>
<tr>
<td>2 x 0.5 ml. at -2, +2</td>
<td>.13</td>
<td>.12</td>
<td>150</td>
</tr>
<tr>
<td>3 x 0.5 ml. at -2, +2, +5</td>
<td>.13</td>
<td>.13</td>
<td>230</td>
</tr>
<tr>
<td>4 x 0.5 ml. at -2, +2, +5, +8</td>
<td>.15</td>
<td>.12</td>
<td>320</td>
</tr>
<tr>
<td>5 x 0.5 ml. at -2, +2, +5, +8, +11</td>
<td>.16</td>
<td>.13</td>
<td>550</td>
</tr>
</tbody>
</table>

* Day of grafting day 0
in the size and weight of certain organs taken from antiserum treated animals, these studies in no way afforded any information concerning the possible cause of these changes. In an attempt to bridge this hiatus the tissues involved were subjected to histologic examination. Balb/C mice that had received the same regimen of injections of antisera or normal rabbit serum as those in the clearance studies were sacrificed at varying intervals after the commencement of treatment. Their livers, spleens and superficial lymph nodes were removed and after fixation and sectioning were stained with either haematoxylin and eosin or methyl green pyronin. To more clearly delineate the phagocytic cells, especially in the livers, some animals were injected intravenously with 0.2 ml. of a gelatin suspension of carbon containing 16 mg. of carbon per ml. These animals were sacrificed five minutes after injection and their tissues processed in the same manner as the non injected animals.

No significant changes were observed in the livers taken from antiserum treated animals. The normal architecture was preserved and in the carbon injected animals the number and distribution of the phagocytic cells appeared unaltered. It should, however, be pointed out that the particle size of the carbon precluded the possibility of seeing individual particles by light microscopy and that the particles only become visible on agglomeration. This
meant that as far as this technique was concerned only cells capable of phagocytosing a quantity of carbon could be identified as constituent cells of the reticuloendothelial system.

In contrast to the findings in the livers, marked histologic changes were observed in the spleens. Although some spleens showed vascular congestion the increase in spleen weight and size appeared to be due almost entirely to an increase in cell numbers, with the most striking feature being the appearance of large numbers of cells closely resembling plasma cells. These cells became prominent initially three to four days after the commencement of the antiserum and in Fig. 29 large numbers of these cells can be seen between the lymphoid follicles of a spleen taken from an antiserum treated mouse at day six. From Fig. 30, it can be seen that while these cells were pleomorphic a constant finding was a large nucleus and in many instances abundant cytoplasm. Mitotic figures were also a constant finding in these areas. Contrary to the reports by Monaco et al. (1966a, b, 1967) there was no marked evidence of lymphoid depletion in any of the sections examined. In some sections, however, there did appear to be a reduction in the number of follicles. While the extent of this reduction was difficult to assess due to the marked proliferation of this other cell type, it never appeared to be greater than fifty
Fig. 29. Section of a spleen taken from an antilymphocyte serum treated animal at day 6 showing the cellular proliferation between the lymphoid follicles. (H & E x 125).

Fig. 30. Cellular proliferation in the spleen taken from an antiserum treated mouse at day 6. (H & E x 500).
per cent.

No similar changes were found in the normal rabbit serum treated animals where the only feature of note was an increase in the number and size of the follicles.

At the time when the spleens were returning to their normal size there was definite evidence of cell death and in these sections large multi-nucleate giant cells were prominent. With this particular dosage schedule the histology had returned to normal within 28 days.

In the lymph nodes the picture was again one of marked cellular hyperplasia. It was evident that the predominant cell type (Figs. 31, 32) was similar to that observed in the spleens. These cells, which also appeared at approximately the same time as their splenic counterparts, were very prominent in the medulla and paracortical areas with the resultant disruption of the normal architecture of the node. While the hyperplastic changes were by far the most striking feature, it was nevertheless evident that there had been a reduction in the number of lymphocytes in these areas. The cortical areas, on the other hand, appeared essentially normal with no evidence of follicle or lymphocyte depletion. A like response was found in all the nodes taken from these antiserum treated animals irrespective of their site.

No similar changes were found in the nodes taken from normal
An axillary lymph node taken from an antilymphocyte serum treated animal at day 6 showing the cellular proliferation. (H & E x 125).

Fig. 31.

An axillary lymph node taken from an antilymphocyte serum treated animal at day 6. Large cells similar to those in the spleen are present with an apparent loss of small lymphocytes in these areas. (H & E x 500).

Fig. 32.
rabbit serum treated animals with the exception of the nodes draining the site of injection. In these nodes, cell proliferation was evident especially in the medulla, with the appearance of cells similar to those found in the antiserum treated animals.

The hyperplasia found in the lymph nodes, as in the spleen did not persist. There was almost complete restoration of normal architecture prior to what would have been the expected time of allograft rejection. However, before the re-establishment of a normal histologic picture, there was again evidence of loss of cells and cell death with the concurrent appearance of multi-nucleate giant cells similar to those found in the spleen.

Many of the large cells in the haematoxylin and eosin stained sections appeared to be similar, if not identical, to the immunoblasts described by Oort and Turk (1965) and Turk (1967). This observation was further substantiated by the finding that many of these cells were pyroninophilic. Figures 33 and 34 show typical areas of these cells, with many of them demonstrating marked pyroninophilia, in the spleen and lymph node of an antiserum treated animal at day six. It would seem, therefore, that this antiserum provides an immunological stimulus capable of inducing blast cell formation without the subsequent appearance of small lymphocytes.
Fig. 33. Large numbers of pyronin positive cells in the spleen of an antiserum treated animal at day 6. (Methyl green-pyronin x 500).

Fig. 34. Similar numbers of pyronin positive cells in an axillary lymph node taken from the same animal. (Methyl green-pyronin x 500).
Peripheral white cell count in anti-lymphocyte serum treated animals.

In view of the hyperplasia previously described in this chapter and the lack of evidence of lymphocyte depletion it appeared important to determine the effect of this antiserum on the peripheral white cell count. The same regimen of injections was employed as in the previous studies and both differential and total white counts were performed on blood samples taken from the retro-orbital venous plexus of the mice as previously described in Chapter II.

The total white cell counts obtained are shown in Fig. 35. In accord with the findings of other workers a pronounced fall in the peripheral white count was found to occur within four hours. It was also noted that although the white count remained depressed during the period of administration of the antiserum it had returned to normal well before the animals were capable of rejecting their allografts.

The differential white cell count clearly indicated that the lymphocyte was preferentially affected; the polymorphonuclear leukocyte count being essentially unaltered.

Allograft rejection in antiserum treated animals.

The results obtained in Chapter III have indicated that there is a significant increase in the phagocytic activity of animals rejecting skin allografts. To further establish this relationship
FIG 35   PERIPHERAL WHITE CELL COUNT IN RAMLS TREATED BALB/C MICE

WCC (x10^3/mm³)

- - Normal rabbit serum
- - Rabbit anti-mouse lymphocyte serum

TIME IN DAYS

SUBCUTANEOUS INJECTION OF 0.5mls RAMLS.

TIME IN DAYS
the phagocytic activity was determined in antiserum treated animals that were subsequently allowed to reject their allografts.

The grafts employed in this experiment were 4 cm.² isografts and C57Bl allografts on adult Balb/C mice. These mice were also given either 0.5 ml. of normal rabbit serum or antiserum on days -2, +2 and +5 and the phagocytic activity was determined at two day intervals by measuring the clearance of *S. typhimurium* C5 from the circulation. It was noted that the isografted antiserum treated animals exhibited a similar pattern of phagocytic activity to non-grafted animals indicating that grafting *per se* did not influence the phagocytic activity in these animals.

From the results shown in Fig. 36 it can be seen that initially both groups of allografted animals had an increased rate of clearance. In the normal rabbit serum treated animals this was presumed to be due to the combined effects of graft rejection and the rabbit serum, both of which have been previously shown to stimulate the reticulo-endothelial system. Likewise, in the allografted antiserum treated animals the change was consistent with that observed with antiserum alone.

Subsequently, in both groups the phagocytic activity returned to normal. When the antiserum treated animals rejected their grafts then their phagocytic activity was once again found to be
Figure 36

Rate of clearance of *S. typhimurium* (c5) in serum treated mice.

Serum given -2, +2, +5
increased. This increase, as with animals that had received only allografts, preceded macroscopic rejection. The fact that the antiserum treated animals rejected their grafts over a greater time range than untreated animals has tended to make this relationship less pronounced (Fig. 36) since each point represents the average of values obtained from three or four animals.

The other important feature of this experiment was that in the antiserum treated animals the allografts were completely healed in and growing hair prior to rejection. This would further exclude such non-specific factors as trauma and infection as a possible cause for the increased phagocytic activity.

**Allograft rejection in antiserum treated animals - histologic studies.**

It has been previously shown in this chapter that the antiserum as well as prolonging allograft survival produced a marked hyperplasia of lymphoid tissues. The following histologic studies were performed to investigate the pattern of cellular changes occurring in the graft bed and to determine whether the cell type so prominent in the lymphoid tissues was significantly involved.

In this experiment four groups of Balb/C mice were used, two of which were given 4 square cm. skin isografts, and the remainder received similar sized C57Bl allografts. One isografted and one allografted group received 0.5 ml. of normal rabbit serum on days -2,
+2 and +5 and the remaining groups received the same dosage of the antiserum. The animals were sacrificed at two day intervals, their grafts removed and fixed prior to sectioning and staining with haematoxylin and eosin or methyl-green pyronin.

In this study the most dramatic differences became evident six to eight days after grafting. In the allografts taken from the normal rabbit serum treated animals there was a marked cellular infiltration by day six and definite evidence of graft death by day eight. On the other hand it can be seen (Fig. 37) that in the antiserum treated animals although there was a definite cellular infiltration the graft was still quite healthy at day eight. This infiltration initially appeared to be of significance but Fig. 38 shows that even isografted animals can exhibit a pronounced cellular infiltration at this stage, which would suggest that the response is related to trauma. It can also be seen from Figures 39 and 40 that in both the allografts and the isografts the infiltrating cells were predominantly mononuclear. In the haematoxylin and eosin section some of the cells appeared similar to the large cells found in the lymphoid tissues. However, at no stage was it possible to demonstrate the presence of pyronin positive cells.

By day twelve the allografted animals receiving normal rabbit serum had almost completely rejected their grafts (Fig. 41), whereas
Fig. 37. A C57Bl allograft taken from an antiserum treated Balb/C mouse at day 8. (H & E x 125).

Fig. 38. An isograft taken from a serum treated Balb/C mouse at day 8. (H & E x 125).
Fig. 39. The cellular infiltration present in a C57Bl allograft taken from an antiserum treated Balb/C mouse at day 8. (H & E x 500).

Fig. 40. The cellular infiltration present in an isograft taken from a serum treated Balb/C mouse at day 8. (H & E x 500).
Fig. 41. A C57Bl allograft at day 12, from a normal rabbit serum treated host - graft rejected. (H & E x 125).

Fig. 42. A C57Bl allograft at day 12, from an antilymphocyte serum treated host - graft surviving. (H & E x 125).
the allografts from the antiserum treated animals (Fig. 42) still appeared healthy in spite of the cellular infiltration. This infiltration persisted unchanged until the animals eventually began to reject their grafts. Similar infiltrations were still present in the isografted animals twelve to sixteen days after grafting but they were not as prominent as they had been at day eight or ten.

When the antiserum treated animals eventually rejected their grafts the intensity of the cellular infiltration and the cell type involved (Figs. 43, 44) was the same as that observed in normal animals.

From these studies it would appear that the antiserum treated animals are incapable of providing the cellular response necessary for the destruction of the graft. The significance of the cellular response that does occur is somewhat clouded, firstly because of its apparent failure to damage the graft and secondly because a similar infiltration occurs in isografted animals especially when the graft is healing in.

**Summary.**

In accord with other studies relating to anti-lymphocyte serum it has been possible to produce marked prolongation of skin allograft survival using this agent. While the route of administration, apart from the avoidance of toxicity does not appear important, the dose and the time of administration of the antiserum in relation to the day of
Fig. 43. A C57Bl allograft at day 25 from an antiserum treated host - graft rejected. (H & E x 125).

Fig. 44. The cellular infiltration present in the above 25 day C57Bl allograft. (H & E x 500).
grafting is important. Increasing the dose clearly increased the efficacy of the antiserum and it appeared preferable although not vital to commence the antiserum several days prior to grafting. In the present studies it was not possible to demonstrate that the antiserum was directed exclusively against lymphocytes. However, the superior ability of the lymphocytes to absorb out the biologic activity of the antiserum suggested that they must provide more receptors for the active component of this antiserum than the other cells used for absorption.

The further investigation of the biologic activity of this antiserum by means of clearance studies revealed that the global phagocytic activity of antiserum treated mice was significantly increased. This enhanced activity firstly became apparent 24-48 hours after the commencement of the antiserum and was found to persist in spite of the cessation of antiserum treatment. Therefore, in view of the undiminished phagocytic activity it appeared that the recognition of an effete particle by the cells of the reticuloendothelial system was unimpaired in these antiserum treated animals.

From the histologic studies it was evident that although there was a depletion in the number of small lymphocytes in the medulla and paracortical areas of lymph nodes taken from antiserum treated mice, this antiserum failed to produce in the lymph node the profound
lymphopenia described by Monaco et al. (1966a, b). Indeed, the administration of this antiserum was characterised by the onset of hyperplastic changes in all lymphoid tissues, with the appearance of cells resembling primitive plasma cells, many of which were subsequently shown to be pyroninophilic. On the basis of the histologic studies it is not possible to say that these changes were responsible for the reduced ability of these animals to reject allografts. However, the finding that these animals only rejected their grafts at the time, or shortly after these tissues returned to normal would suggest that these changes were of functional significance.
CHAPTER V

Antibody production and immunity in allografted and antiserum treated mice.

Since the initial observations of Metchnikoff (1887) there has been a growing appreciation of the role played by the reticuloendothelial system in the process by which animals resist infection. Recently further experiments have suggested an extension of this role, namely that the reticuloendothelial system plays a vital part in the initiation of immune responses.

It has been shown that the stimulation of the reticuloendothelial system with either Zymosan (Cutler, 1960) or BCG (Halpern, 1959) results in increased resistance to infection and also increased antibody production. However, it has not been generally agreed that all agents capable of increasing phagocytic activity have these associated effects. This seems to be so where simple lipids have been used (Barrie and Cooper, 1964) and where the stimulation is produced by a graft versus host reaction (Howard and Woodruff, 1961). Thus agents capable of stimulating the reticuloendothelial system can be divided into two broad groups in which one group has the additional ability to produce an associated alteration in immune responsiveness. The experiments of Fisher and Fisher (1964) suggested that the stimulation produced by a host versus graft reaction was similar to
that produced by Zymosan or BCG. Hence one would also expect the rate of antibody production to be significantly increased.

In Chapter IV it was shown that the phagocytic activity was increased in animals given anti-lymphocytic serum. The exact cause and significance of this response remain uncertain due to the complex action of the antiserum. To ascertain whether this increased phagocytic activity was of significance in immune responsiveness, the ability of animals treated with the antiserum to resist infection and produce antibody was evaluated.

The antibody studies were subsequently extended to determine whether anti-lymphocytic serum could be of value in the establishment of specific immunological tolerance in adult animals. Monaco et al. (1966b) showed that preliminary thymectomy followed by antiserum treatment and later infusion of lymphocytes could induce a state of allograft tolerance. To achieve this, however, it was necessary to use lymphoid cells from \( F_1 \) hybrid mice to avoid a graft versus host reaction. Consequently from a practical viewpoint it would be preferable to use transplantation antigens instead of viable cells.

Antibody production in allografted mice.

In Chapter III it was shown that presence of a skin allograft resulted in an increase in phagocytic activity. To investigate the significance of this increased rate of reticuloendothelial clearance in
relation to immune responsiveness the ability of these animals to produce antibody was determined.

Balb/C mice bearing 6-8 cm$^2$ isografts or C57Bl allografts and normal animals were immunised with sheep erythrocytes and the total as well as the amounts of 19S and 7S antibody produced, determined at two day intervals, by the method described in Chapter II. To cover the period in which the changes in activity were observed the mice were given the red cells either 2, 4, 6, 8 or 10 days after grafting.

The results in Figure 45 show that the total antibody response of the skin allografted animals immunised six days after grafting was not significantly different from that shown by non grafted and isografted control animals. The response shown by this group was representative of that found in all the grafted animals, clearly indicating that although these animals had an increased reticuloendothelial clearance they did not have an enhanced ability to produce antibody.

**Susceptibility to infection of mice treated with anti-lymphocyte serum.**

During the studies described in Chapter IV it was noted that the antiserum treated animals remained perfectly healthy on the regimes of treatment that were used and it appeared that they were as resistant to infection as their normal counterparts. To investigate this further the animals were artificially infected with three organisms,
Animals injected with 0.2 mls of 1% SRBCs six days after grafting

![Graph showing antibody response to sheep erythrocytes in normal and grafted Balb/c mice.](image)

**FIG 45.** Antibody response to sheep erythrocytes in normal and grafted Balb/c mice
Listeria monocytogenes, S. typhimurium M206 and E. coli C.V.

The infecting dose was administered intraperitoneally and was the LD50 for the control group which had been established by injecting various doses of these organisms into groups of normal Balb/C mice.

The results in Table IX show that this same dose was also the LD50 for the antiserum treated animals. Smaller doses, viz a half and one tenth of the LD50, of both S. typhimurium M206 and Listeria monocytogenes were also tested but there was no evidence of any altered susceptibility to infection in the antiserum treated animals.

In Chapter IV it was also found that the time of administration of the antiserum in relation to the time of grafting was of significance in relation to the subsequent survival of the graft. However, it can be seen from Table X that altering the time of administration of the antiserum still failed to demonstrate any increased susceptibility to infection.

The antibody response to sheep erythrocytes in antiserum treated mice.

It has been established in Chapter IV that this anti-lymphocyte serum can prolong the survival of skin allografts, suggesting that it must possess powerful immunosuppressive properties, but from the preceding studies it does not appear to alter the host's resistance to infection. In view of this somewhat paradoxical situation it
TABLE IX

Survival of anti-lymphocyte serum treated Balb/C mice after infection with E. coli CV, S. typhimurium M206 and Listeria monocytogenes

<table>
<thead>
<tr>
<th>Organism injected</th>
<th>Treatment*</th>
<th>Survivors/Animals injected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> CV $10^7$ org.</td>
<td>ALS</td>
<td>8/15</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>7/15</td>
</tr>
<tr>
<td><em>S. typhimurium</em> M206 $5 \times 10^6$</td>
<td>ALS</td>
<td>9/15</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>8/15</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> $5 \times 10^5$</td>
<td>ALS</td>
<td>7/15</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>7/15</td>
</tr>
</tbody>
</table>

* The Balb/C mice were given 0.5 ml. of anti-lymphocyte serum (ALS) or normal rabbit serum (NRS) on days -2, +2, and +5, where the day of inoculation was day 0.
TABLE X

Survival of anti-lymphocyte serum treated Balb/C mice after injection with $5 \times 10^6$ S. typhimurium M206.

<table>
<thead>
<tr>
<th>Regimen of antiserum injections</th>
<th>Survivors Animals injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml. at day -4, 0, +3</td>
<td>7/15</td>
</tr>
<tr>
<td>0.5 ml. at day -2, 0, +2</td>
<td>9/15</td>
</tr>
<tr>
<td>0.5 ml. at day -2, +2, +5</td>
<td>8/15</td>
</tr>
<tr>
<td>0.5 ml. at day 0, +2, +4</td>
<td>10/15</td>
</tr>
<tr>
<td>0.5 ml. at day 0, +4, +7</td>
<td>8/15</td>
</tr>
<tr>
<td>0.5 ml. at day +2, +6, +9</td>
<td>7/15</td>
</tr>
</tbody>
</table>

Animals inoculated with M206 on day 0.
appeared necessary to investigate the pattern of immune responsiveness in these antiserum treated animals by testing their ability to produce antibody.

Balb/C mice, 6-8 per group, were immunised with washed sheep erythrocytes and the total as well as levels of 19S and 7S antibody were determined at two day intervals as described in Chapter II. The animals received the erythrocytes on day 0, with the antiserum or normal rabbit serum being given subcutaneously in 0.5 ml. doses on days -2, +2 and +5. The estimation of antibody titres were carried out on both pooled and individual serum samples.

In the initial experiments it was found that normal rabbit serum as well as antiserum treated mice had low haemagglutination titres. The failure of these mice to respond in the normal manner appeared to be related to the presence of antibodies in the rabbit serum which were capable of agglutinating sheep erythrocytes in vitro. To avoid this effect it was necessary to absorb all rabbit serum with equal volumes of sheep erythrocytes, and sera were not used, if after absorption they still produced agglutination when diluted two-fold.

The results obtained from this experiment are shown in Figure 46. It can be seen that giving absorbed normal rabbit serum did not alter the animals' response to sheep erythrocytes. On the
0.5 mls. of NRS or ALS given subcut. on days -2 +2 +5

- Non-treated
- Normal rabbit serum (NRS)
- Antilymphocyte serum (ALS)

FIG 46. Antibody response to sheep erythrocytes in serum-treated Balb/c mice
other hand, the administration of absorbed antiserum clearly suppressed the ability of these animals to produce antibody. Although antiserum treated animals were found to have a reduced ability to produce both 19S and 7S antibody, those that produced these fractions did so at the same time as their normal untreated counterparts. The other interesting feature was that from the eight antiserum treated animals whose sera were examined individually, only one animal failed to produce any detectable antibody. This would suggest that this agent can quantitatively modify the immune response without necessarily achieving complete suppression.

Antibody response to Bovine Serum Albumin in antiserum treated mice.

In these experiments a purified protein antigen (bovine serum albumin) was used to assess whether antiserum could assist the induction of tolerance to a defined antigen and also to determine whether the antigen dose was important. Groups of Balb/C mice containing 12 animals per group were injected subcutaneously at the base of the tail with one of the following doses of Bovine Serum Albumin (BSA) in Freund's incomplete adjuvant 0.8 µgms, 4 µgms, 20 µgms, 100 µgms, 500 µgms and 12.5 mgm. The groups were then divided into two with one half receiving 0.5 ml. of normal rabbit serum and the other half the same dosage of antiserum on days -2, +2, and +5, where the day of injection was day 0. The animals
were bled 28 days later and the amount of antibody in each serum sample estimated by the method described in Chapter II. After bleeding each animal received an intraperitoneal challenge dose of 20 µgms of BSA in 0.2 ml. of normal saline and seven days later the antibody levels were again determined in these animals. This dose of 20 µgms of BSA per se failed to produce a detectable antibody response in normal mice when given intraperitoneally. It was also determined that neither the normal rabbit serum nor the antisera used in these experiments contained detectable amounts of anti-BSA antibody.

From Figure 47 it can be seen that four weeks after immunisation all the normal rabbit serum treated animals, with the exception of those given 0.8 µgms of BSA had a detectable antibody response. On the other hand only three groups of the antiserum treated animals, namely those receiving 100 µgms, 500 µgms and 12.5 mgm. had responded, and in two of these (viz. those given 100 µgms and 500 µgms of BSA) the response was reduced.

The results using the precipitation technique (Fig. 48) and using the haemagglutination technique (Fig. 49) summarise the antibody response in these same animals seven days after the challenge dose. It can be seen that while there had been a general increase in the levels of antibody found in normal rabbit serum
FIG 47. Antibody response to sol. BSA in serum treated Balb/c mice at day 28.
FIG 48. Antibody response in serum treated Balb/c mice 7 days after challenge with 20μgms sol. BSA
HAEMAGGLUTINATION (BSA diazotised onto sheep erythrocytes)

- Normal rabbit serum
- Antilymphocyte serum

FIG. 49. Antibody response in serum treated Balb/c mice 7 days after challenge with 20 μgms sol. BSA.
treated animals, the group given 0.8 μgms of BSA still failed to produce a detectable response. In the antiserum treated animals while it was found that all groups, with the exception of the 0.8 μgm group had responded, their response was less than that observed in the normal rabbit serum treated controls. Furthermore, it appeared that the extent to which these animals did respond was related to the initial immunising dose, although no such relationship was evident in the normal rabbit serum treated animals.

While it could be argued that the failure of the antiserum treated animals to respond in the expected manner was due to the persistent effect of the antiserum this appeared unlikely for two reasons. Firstly, it was found that mice when grafted 28 days after the same course of antiserum treatment rejected their grafts in a predictable manner. Secondly, from Figure 50 it can be seen that giving the antiserum after the immunising dose of BSA was not as effective in suppressing the secondary response as giving the same dose prior to immunisation. If, therefore, the reduced secondary response was due to the persistent effect of the antiserum this result should have been reversed.

Summary

It has not been possible to demonstrate that animals whose reticuloendothelial system has been stimulated by a skin allograft
REGIMEN OF TREATMENT (Initial dose of sol. BSA given day 0.)

FIG 50. Antibody response in serum treated Balb/c mice 7 days after challenge with 20 µgms of sol. BSA.
have an enhanced capacity to produce antibody. This would suggest that the stimulation of the reticuloendothelial system produced during a host versus graft reaction is akin to that produced during a graft versus host reaction (Howard and Woodruff, 1961) and by simple lipids (Barrie and Cooper, 1964).

From the further studies to investigate the pattern of immune responsiveness in antiserum treated animals it was found that as well as having a reduced ability to reject their allografts these animals had a reduced capacity to produce antibody. However, using the same dose of antiserum it was not possible to elicit any evidence of increased susceptibility to infection in these animals. While such results may represent selective suppression of immune responses by the anti-lymphocyte serum, this is possibly not the result of fine discrimination and in these studies may only reflect the inability of the antiserum to adequately suppress the secondary immune response.

While it has not been possible to produce immunological tolerance in the present experimental model it has been possible to demonstrate a reduced secondary response in animals that were immunised with BSA while receiving anti-lymphocyte serum. The two factors which emerged as being important for this reduced secondary response were, firstly, the dose of antigen used for
immunisation and secondly the time of commencement of the anti-serum in relation to the time of immunisation. It appeared, therefore, that if anti-lymphocyte serum was to be of value in the induction of immunological tolerance in adult animals such a property would be facilitated by pre-treatment of the animal with antiserum and the use of small doses of antigen in the microgram range.
CHAPTER VI

Discussion

The importance of phagocytic cells in host defence systems was first suggested by Metchnicoff (1887). Since then evidence has accumulated to indicate that the reticuloendothelial system is important not only in resisting infection but also in the initiation of immune responses generally.

Halpern (1959) when reviewing the role and function of the reticuloendothelial system in immunological processes suggested that it had a double mechanism of action. The first, he believed, depended upon the ability of the cells of this system to phagocytose and subsequently destroy bacteria and foreign debris and the second which he termed a humoral process, depended largely upon the production of specific antibody. Little doubt now remains as to the validity of the first mechanism, but only the recent work of Adler, et al. (1966), Galily and Feldman (1967) and Mitchison (1967) has provided evidence that the reticuloendothelial system is significantly involved in the production of specific antibody and in a wider sense the initiation of immune responses. Therefore, although immune mechanisms appear to provide a link between allograft rejection and the reticuloendothelial system, it remains uncertain whether the
reticuloendothelial system is an integral part of the immune mechanisms associated with allograft destruction.

In support of such a role it has been shown that agents known to blockade the reticuloendothelial system can significantly prolong skin allograft survival (Lehmann and Tammann, 1925; Brent and Medawar, 1961; Medawar, 1963). Other workers, Loeb (1945), Stark (1951), however, have failed to demonstrate such a relationship. Unfortunately, these reports have all lacked quantitative functional studies and so in every instance the degree of blockade remains uncertain.

Fisher and Fisher (1964) found that skin allografts were capable of stimulating the reticuloendothelial system in rats and that the resultant stimulation could overcome the blockading effects of a given dose of thorotrast. While in the present studies it has been possible to demonstrate a similar effect in mice bearing large allografts between 20-30% of the surface area smaller grafts 4-5% of the surface area failed to produce a significant increase in phagocytic activity. If then the persistence of the reticuloendothelial blockade is important for the survival of the allograft it would seem that such a blockade can be more readily maintained in animals bearing small grafts. This may account for the inability of Fisher and Fisher (1964) and Loeb (1945) using large grafts and the ability of Brent and
Medawar (1961) and Medawar (1963) using small grafts to prolong allograft survival in blockaded animals.

The present studies suggest that the release of some factor from the graft rather than graft rejection per se, is responsible for the observed increase in phagocytic activity. Supporting this theory is the fact that the increased phagocytic activity occurred prior to macroscopic rejection and histologic evidence of gross graft destruction rather than simultaneously, or later, as would have been anticipated if this response were related to graft rejection and the release of cellular debris. Furthermore, it was observed that if an allograft was left in contact with the host for two days and then removed the animals failed to develop an increased phagocytic activity. These animals also failed to reject a second graft in an accelerated fashion suggesting that an inadequate amount of antigenic information had been released to achieve sensitisation. On the other hand, animals whose allografts had been present for four days were subsequently found to develop an increased phagocytic activity at the time expected if the graft had been present. Confirmation that significant amounts of antigenic information had also been transferred was evidenced by the ability of these animals to reject their second graft from the original donor in an accelerated fashion. Similar observations concerning the time of contact necessary for host
sensitisation and for the definite quantitative transfer of antigenic material necessary for sensitisation have been reported by McKhann and Berriam (1959) and Goulian et al. (1962).

The distribution of radioactivity associated with this increased phagocytic activity indicated that the liver was primarily responsible for the increased rate of clearance of the bacteria from the circulation. It is perhaps not surprising that the liver should appear prominent in this respect as Florey (1962) indicated that the liver was the major source of phagocytic cells. However, as these cells are remote from the site of the rejecting graft it is necessary to postulate the presence of some circulating stimulatory factor. Although it has been previously suggested that transplantation antigens were responsible for the changes in phagocytic activity the results of the transfer studies implied a more indirect pathway, in which the antigens by interacting with either the draining lymph node cells or the spleen cells produced a factor capable of stimulating the cells of the reticuloendothelial system. The ability of the lymph nodes as well as the spleen to produce this factor would account for the failure of prior splenectomy to obliterate this stimulation of the allografted animals. Although it may have been expected that the serum taken from these allografted animals should also produce some stimulation of the recipients reticuloendothelial system, the failure
to do so may have been related to quantitative factors or to the lability of the transference factor.

Unfortunately, it still remains uncertain what an increased rate of reticuloendothelial clearance means in terms of immune responsiveness. There have been many attempts to equate an increased rate of reticuloendothelial clearance with increased antibody production and the acceleration of immune mechanisms in general. Animals whose reticuloendothelial system has been stimulated by BCG, Zymosan or endotoxin have an enhanced ability to produce antibody. Whether this is a direct result of the increased rate of clearance of the antigenic material or the result of some other associated effect of these agents has not been clearly established. The observations of Howard and Woodruff (1961) Barrie and Cooper (1964) and Moller (1964) suggest that the latter explanation is more likely as in their studies they have been able to increase the rate of reticuloendothelial clearance by other means without being able to demonstrate an associated increase in immune responsiveness. Similarly in the present studies although an increase in the phagocytic activity of the reticuloendothelial system occurs in response to a skin allograft it has not been possible to establish that this increased rate of clearance is of functional significance in immune responsiveness when other antigenic stimuli were utilised in the presence of this
heightened activity.

Apart from the increase in phagocytic activity it was also observed that animals bearing skin allografts developed splenomegaly. These observations conflict with those of Gillette et al. (1966) who found no changes in spleen weight in mice bearing skin grafts comparable in size to those used in the present studies. However, with tumour grafts Adreine et al. (1955), Woodruff and Symes (1962) and Gillette et al. (1966) have all noted that splenomegaly occurred in the animals bearing these tumours. Furthermore, the maximal spleen size coincided with the period of maximal response to the allografted tissue. A similar temporal relationship has been observed in the present studies with normal tissues. The other interesting feature about this change in spleen size and weight was that in the present studies and also in the studies of Adreine et al. (1955) it appeared to be biphasic. The first peak occurred at the time of rejection and a second peak occurred later which appeared to be related to infarction, and infection of the sloughing graft. This 'biphasic response' may possibly explain the failure of Gillette et al. (1966) to observe these changes as they examined the spleens on only two occasions which were four days apart. It has also been possible to transfer this splenomegaly to non graft bearing animals by spleen cells or splenic extracts. Crosby (1962) suggested that in the
tumour bearing animals the spleen may be 'auto stimulated' by the elaboration of a factor, produced by the spleen cells, in response to contact with tumour antigens. From the present studies such an explanation also appears applicable to animals bearing non-neoplastic grafts.

The application of the results of the clearance studies to a model more closely related to clinical transplantation has not been possible due to the unsuitability of the particle used in the present studies. However, the observation that these changes in phagocytic activity occur prior to gross graft destruction indicate that the measurement of phagocytic activity may be of value not only as an indicator of a host versus graft reaction but also as an indicator of imminent graft destruction. The utilisation of this relationship at a clinical level could, therefore, be of considerable value as an aid to the detection of organ-graft rejection.

It remains to find a suitable particle which could be used on numerous occasions at short intervals: this would presumably be a radioactive particle of extremely short half life which is free of dangerous side effects.

The studies involving anti-lymphocyte serum treated animals revealed that these animals although incapable of rejecting skin allografts still had an adequately functioning reticuloendothelial
system as was indicated by their ability to remove particles from the circulation more rapidly than untreated controls. Therefore, in spite of the observation that anti-lymphocyte serum was capable of agglutinating macrophages in vitro this failed to disrupt the mechanism of recognition and removal of an effete particle. However, the inability to demonstrate a depression in phagocytic activity does not necessarily exclude this system of cells from the allograft reaction. The work of Galily and Feldman (1967) showed that a dose of radioactivity which was incapable of disrupting the phagocytic activity of the reticuloendothelial system, nevertheless, was capable of disrupting immune responses through damage to these phagocytic cells. Using the same dose of whole body irradiation (500r) as Galily and Feldman (1967) it has been possible to confirm (unpublished observations) that this dose of irradiation does not depress phagocytic activity but rather the animals so treated were found to have an increased rate of clearance. This same dose of irradiation was also capable of doubling the survival time of test grafts in the same combination of animals as used in the present studies. It would appear then that many of the phenomena associated with anti-lymphocyte serum such as the depression of antibody production, the facilitation of the development of tolerance and the prolongation of allograft survival could be explained on the basis of disruption of macrophage 'digestion'. In accord with this
theory the work of James (1967) suggested that the anti-lymphocyte antibody probably affected the sensitising or triggering phase of the immune response. The results of the studies with bovine serum albumin also support this view as commencing the antiserum prior to the administration of the antigen produced greater suppression of the immune response than a comparable dose given after the antigen. However, Levey and Medawar (1966a, b) showed that commencing the anti-lymphocyte serum after grafting could also prolong skin allograft survival. This result has been confirmed by the present studies, but it was also found that commencing a standard dosage regime of antiserum prior to grafting produced greater prolongation of allograft survival than when the same regime was commenced after grafting. Monaco, et al. (1966a) have found a comparable temporal relationship between the time of administration of their antiserum in relation to the day of grafting and the prolongation of graft survival. The ability of these various antisera to prolong graft survival may be due to their effect on sensitised cells, or it could reflect that a certain time interval must elapse between the application of the allograft and the release of adequate amounts of antigen to achieve sensitisation.

The most obvious morphological changes were observed in the lymphoid tissues of the anti-lymphocyte serum treated animals.
Both the anti-lymphocyte and the anti-thymocyte serum caused marked hyperplastic changes in all lymph nodes and spleens with the appearance of large numbers of pyronin-positive cells in these tissues. The livers of these animals increased in size and while this may have been due to a direct effect of the antiserum, it is more likely to have been a secondary response of the liver parenchyma, resulting from the intense synthetic activity taking place in the lymphoid tissues. That these changes could be of functional significance in immune responsiveness was supported by the finding that their presence correlated well with allograft survival. The conflict between these observations and those of Gray, et al. (1966) and Monaco, et al. (1966, 1967) make it difficult to evaluate the importance of these changes. This divergence of experimental results is not an isolated example and it would seem that the anti-lymphocyte sera currently being used may produce either hyperplastic changes, or lymphopenia and hypoplasia of lymphoid tissue, but despite this they all have the ability to prolong allograft survival. Consequently these histologic changes are of little help in assessing possible immune responsiveness. The numerous theories, viz. 'cytotoxic', (Gray, et al. 1966; Monaco, et al. 1966) 'blindfolding' (Levey and Medawar, 1966a) 'competitive antigen' (Levey and Medawar, 1967) 'sterile activation' (Levey and Medawar, 1966b) and
the 'anti-thymic' (Russe and Crowle, 1965, Parrott, 1967) highlight the difficulties in attempting to explain the mode of action of this agent on an histologic basis. The conclusion of Levey and Medawar (1966a) that no hypothesis satisfactorily explains the mode of action of this agent and that no theories are mutually exclusive still appears to be correct. Similarly the suggestion of these workers that the failure of the various groups investigating this agent to obtain uniform results could be related to the presence of irrelevant antibodies appears to be equally sound, especially in view of the heterogeneity of the antibodies that must constitute any one anti-lymphocyte serum. Consequently, the morphological changes that have, in general, provided the basis for the above theories could be artefacts.

Faced with this inability to provide an antiserum that is free from these 'contaminants' it would seem to be more fruitful to concentrate on the similarities between the various sera rather than to accentuate their differences when attempting to explain its mode of action. From all the reported experimental data the ability of antiserum to depress the peripheral white cell count would undoubtedly be its most constant effect. In almost every instance a profound fall in the peripheral lymphocyte count occurs within the first four hours. What happens after this is variable depending on the regime of
treatment and the type of serum employed. With some sera (Monaco, et al. 1967) the count remains depressed, whereas with others (Levey and Medawar, 1967; and in the present studies) the count rises. This observation naturally has suggested that gross lymphocyte depletion is not a prerequisite for this agent to be effective, however, it is not known whether there has been a qualitative as well as quantitative recovery of the peripheral lymphocytes. The observation of Taub (1967) and from the present studies that in antiserum treated animals the allografts become infiltrated with mononuclear cells without any evidence of tissue damage would suggest that qualitative changes have taken place in the lymphocyte population.

The suggestion of Russe and Crowle (1965) that the antiserum acts by neutralising a humoral factor produced by the thymus has received little further support. However, the observation of Parrott (1967) that there was a depletion of small lymphocytes in the 'thymic dependent' areas of the lymph nodes appears to be a relatively constant phenomenon and coupled with the results of the earlier studies by Parrott (1967) and Parrott and de Sousa (1966) provides a possible mechanism by which anti-lymphocyte serum could produce its biologic effect. Few other workers have stressed this seemingly important point possibly because of the difficulty in
assessing the degree of depletion of these cells and its significance when associated with the other more obvious histologic changes.

The other area where the various antisera have a similar effect has been previously discussed in relation to the involvement of the macrophage and the sensitising phase of the immune response.

In these studies several important practical factors have emerged in regard to the future utilisation of anti-lymphocyte serum especially in human allotransplants. Both thymocytes and lymphocytes have been found to be equally effective as the antigen when raising the antiserum, and the resultant sera appeared to be identical in their biologic effect. While the sera initially had been raised against cells from members of inbred populations and used on these same populations, subsequent studies revealed that highly effective sera could be produced using members of outbred and unrelated strains as both donor and recipient respectively. Therefore it should be possible to raise antiserum against human lymphocytes which would be effective in all recipients.

The most serious toxic side effects noted were related to the presence of antibodies which were characterised by their ability to agglutinate erythrocytes in vitro. Once these factors had been removed by absorption with erythrocytes the serum was found to be equally safe and effective whether administered intravenously,
intraperitoneally or subcutaneously.

Initially it appeared that because anti-lymphocyte serum was a powerful immunosuppressive agent the grafted animals would be more susceptible to infection. This, however, did not eventuate and attempts to demonstrate that antiserum treated animals were more susceptible to infection after the artificial introduction of bacteria were also unsuccessful. Whether this represents a degree of specificity in the effect of anti-lymphocyte serum or is related to the inability (Monaco, et al., 1966b, James and Anderson, 1967) to adequately suppress secondary immune responses is by no means certain. A more precise interpretation of these results does not at present appear possible due to the difficulty in defining the relative significance of such factors as polymorphonuclear leukocytes, antibodies, bactericidal power of the serum and the reticuloendothelial system in the mechanisms by which animals normally resist infection.

With the present regime of treatment it was not possible to produce tolerance in the antiserum treated animals. However, it was evident that the animals receiving the antigen at the same time as the anti-lymphocyte serum had a reduced ability to respond when subsequently challenged with the same antigen. This apparent suppression of the secondary immune response did not occur in every instance and it was clear that the initial immunising dose of bovine
serum albumin was of importance. The reduced secondary response being associated with immunising doses of less than 100 μgm. This, therefore, appears to be an important avenue for further investigation for as with the studies of Mitchison (1964) it may be possible to achieve complete tolerance by the judicious manipulation of the dose of antigen and the regime of antiserum treatment. The unique immunosuppressive properties of anti-lymphocyte serum may also obviate the necessity to provide the antigen in a purified form and so afford a method by which transplantation tolerance could be achieved with materials that are currently available.
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BIBLIOGRAPHY


DENYS, J. and LE CLEF, J. (1895). Sur le mechanisme de l'immunite chez le lapin vaccine contre le streptocoque pyogene. La Cellule, II, 175.


FOULDS, L. (1932). The effect of vital staining on the

FREEMAN, B. A. and VASA, L. R. (1958). Host parasite relationships
in Brucellosis. I. Infection of normal guinea-pig macro-
phages in tissue culture. J. infect. Dis., 102, 258.

FUJII, G. and NELSON, R. A. Jnr. (1963). The cross-reactivity and
transfer of antibody in transplantation immunity. J. exp. Med.,
118, 1037.

with the species S. typhimurium. J. gen. Microbiol, 15, 140.

Experimental studies on lymphomatosis of mice. Amer.
J. Cancer, 19, 521.

GABOUREL, J. D. (1961). Cell culture in vivo. II. Behaviour of L-
fibroblasts in diffusion chambers in resistant hosts. Cancer
Res., 21, 506.

GALILY, R. and FELDMAN, M. (1967). The role of macrophages in
the induction of Antibody in X-irradiated animals. Immunology,
12, 197.


Sarcoidosis. Progr. Allergy, 6, 187.


IWASAKI, Y. PORTER, K. A., AMOND, J. R., MARCHIORI, T. L.,
ZUHLKE, V. and STARZL, T. E. (1967). The preparation and
testing of horse anti-dog and anti-human antilymphoid plasma
124, 1.

JABOULAY and BRIAU (1896). Recherches experimentales sur la greffe
arterielle. Lyon. med., 81, 97.

JAFFE, R. H. (1931). The reticulo-endothelial system in immunity.
Physiol. Rev., 11, 277.

JAMES, K. (1967). Some factors influencing the ability of anti-
lymphocyte antibody to suppress humoral antibody formation.

JAMES, K. and ANDERSON, N. F. (1967). Effect of anti-rat
lymphocyte antibody on humoral antibody formation. Nature,
213, 1195.

JAMES, K. and MEDAWAR, P. B. (1967). Characterisation of anti-

JEEJEEBHOY, H. F. (1965). Immunological studies on the rat
thymectomised in adult life. Immunology, 9, 417.

JENKIN, C. R. (1962). An antigenic basis for virulence in strains of


MONAÇO, A. P., WOOD, M. L., van der WERF, B. A. and
RUSSELL, P. S. (1967). Effect of antilymphocyte serum in
mice, dogs and man. In Ciba Symposium Study Group on
Antilymphocyte serum. Ed. Wolstenholme, G. E. W. and

MOORE, R. (1959). Localisation of I\(^{131}\) labelled antirat lymph node

MURPHY, J. B. (1926). The lymphocyte in relation to tissue grafting,
malignant disease and tuberculous infection. Monogr.

transplantation immunity. I. Tritiated cells. II. Lymphoid

NAJARIAN, J. S. and FELDMAN, D. (1962b). Homograft rejection by
sensitised lymphoid cells in millipore chambers. Surg. Forum.,
13, 71.

NAJARIAN, J. S. and FELDMAN, J. D. (1963). Passive transfer of
transplantation immunity. III. Inbred guinea-pigs. J. exp.
Med., 117, 449.

NAJARIAN, J. S. and PERPER, R. J. (1967). Participation of humoral
antibody in allogeneic organ transplantation rejection. Surgery,
62, 213.


