THE PATHOLOGY AND PATHOGENESIS OF GRAFT-VERSUS-HOST DISEASE

PATHOGENESIS AS STUDIED BY THE INTERACTIONS BETWEEN MACROPHAGES AND LYMPHOID CELLS IN CELL CULTURE

by

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SUMMARY

THE PATHOLOGY AND PATHOGENESIS OF GRAFT-VERSUS-HOST DISEASE

G.V.H.D. was produced by the intravenous inoculation of viable lymphoid cells from adult parental strain mice into the corresponding adult F_1 hybrids. The animals appeared normal for 2-3 weeks and thereafter became progressively wasted and appeared ill.

In the (CBA x C57BL)F_1 hybrid strain, the mice died of the disease in 20 days. Serial histopathological studies were made on normal and G.V.H.D. mice. The mice were sacrificed on the 5th (early stage), 10th, 15th (intermediate stage) and 20th day (late stage) of the disease.

During the early stage, the lymph nodes and spleen were markedly enlarged. The para-cortical areas of the lymph nodes showed marked expansion and increase in cellularity. Many large pyroninophilic blast cells and macrophages were seen in these areas. The malpighian bodies of the spleen also showed similar changes. Many cells in the para-cortical areas of lymph nodes and the malpighian bodies of spleens were seen in mitosis. Lymphophagocytosis was also noted in the lymph nodes. Multinucleate giant cells were seen in the lymph nodes and the red pulp of the spleen. In the intermediate stage of the disease, the lymph nodes were smaller in size in comparison to the control nodes. Histologically, the normal architecture of the lymph nodes was completely lost and the follicles were not seen. Large areas of lymph nodes and malpighian bodies of the spleen were replaced by macrophages, which were/

were seen to form sheets of tissue. In the late stage of the disease, the lymph nodes were extremely small and were almost entirely replaced by macrophages. The macrophages which replaced the malpighian bodies of the spleen were seen to undergo degeneration and were finally replaced by a structureless, hyaline, eosinophilic material. Special stains showed that this material was amyloid mixed with a certain amount of fibrin.

Pathological changes were also noted in the non-lymphoid organs. The bone-marrow was hypercellular in the early stages whereas in the intermediate and late stages it became progressively hypoplastic and many areas had sheets of macrophages. The alimentary tract and the organs embryologically-derived from it also showed remarkable changes. The small intestine had many mononuclear cells and macrophages in the lamina propria and in the submucosa. The epithelial cells of the villi had undergone degeneration and necrosis and many villi appeared incomplete with exposure of the lamina propria to the lumen of the intestine. In the $B6AF_1/J$ mice with G.V.H.D. in which the disease lasted for 35-40 days, the intestine showed marked loss, atrophy, fusion and irregularity of the villi. The muscle coat also was thin compared to that of control mice and the appearances were similar to human idiopathic steatorrhoea. The liver showed an increase in size and number of Kupffer cells and large accumulations of periportal infiltrates of mononuclear cells were seen. The pancreas and salivary glands also showed a loss of their specialized cells and the presence of large numbers of macrophages. The thymus became progressively/

progressively depleted of thymocytes and macrophages were found in large numbers. The kidney and the heart had prominent accumulations of mononuclear cells but no parenchymal damage was seen. The zona glomerulosa and the adjacent zona fasciculata of the adrenals showed cytolytic degenerative changes while the rest of the fasciculata showed lipoid depletion. The changes in the adrenals are probably due to non-specific stress. In G.V.H.D. mice inoculated with colloidal carbon, the cells in the lymph nodes, cells lining the sinuses of the liver, the cells replacing the pancreas and salivary glands, were found to be loaded with carbon. Hence these cells are macrophages. Pathological studies thus show the massive proliferation of macrophages. The damage to the intestine also suggests that this lesion may allow normal or abnormal amounts of endotoxins to enter the bloodstream of immunologically-deficient G.V.H.D. mice.

CELL CULTURE STUDIES

The review of the literature shows that lymphoid cells of animals may be sensitized by tissue antigens, soluble antigens (bovine serum albumin) or stimulated by non-specific agents such as phytohaemagglutinin. The specifically sensitized lymphoid cell or macrophage will interact with the corresponding target cell and cause death of the latter cells. The non-specifically stimulated lymphoid cells also may interact with histo-incompatible target cells to cause their lysis. The lymphoid cells sensitized to soluble antigens when triggered by the specific antigen release biologically active molecules/

molecules which may destroy target cell fibroblast monolayers. These reports show the importance of cell-mediated immune reactions in biological processes and so far there are no reports on similar studies in G.V.H.D. Because my histopathological studies revealed the importance of the macrophage in the tissue pathology of this disease, I investigated further their properties in a series of cell culture experiments.

The Interaction Between Macrophages and Lymphoid Cells in G.V.H.D.

Peritoneal macrophages were cultured from mice with G.V.H.D., from normal mice syngeneic with the strain developing the disease and from parental strain mice. To these macrophages lymphoid cells were added from the three strains mentioned above. Since serum factors are important in immune reactions, these cells were interacted in normal mouse serum (normal F_1 hybrid or parental serum) or in serum from G.V.H.D. mice. Thus G.V.H.D. peritoneal macrophages were interacted separately with either normal F_1 hybrid, parental or G.V.H.D. lymphoid cells in normal or in G.V.H.D. serum. Normal F_1 hybrid and parental macrophages were also interacted with lymphoid cells in a similar manner. The results of these experiments were as follows:-

1. G.V.H.D. macrophages were relatively big and hyperactive cells in comparison to syngeneic normal macrophages. G.V.H.D. serum has the capacity to alter the morphology and function of normal F_1 hybrid macrophages so that they appear and function as G.V.H.D. macrophages.
2. Normal F_1 hybrid and parental macrophages do not interact with/

with normal F_1 hybrid and parental lymphoid cells when cultured in normal serum.

3. Normal F_1 hybrid and parental macrophages interact with normal F_1 hybrid but not with parental lymphoid cells when cultured in G.V.H.D. serum. The interaction was seen as massive adherence of lymphoid cells to macrophages.

4. Cell-mediated death of peritoneal macrophages was observed when the following pairs of cells were interacted in normal serum.

   (a) Normal F_1 hybrid macrophages + G.V.H.D. lymph node cells.

   (b) Parental macrophages + G.V.H.D. lymph node cells.

   (c) G.V.H.D. macrophages + normal F_1 hybrid lymph node cells.

   (d) G.V.H.D. macrophages + G.V.H.D. lymph node cells.

In cell interactions (a) and (b) when G.V.H.D. lymph node cells were replaced by normal F_1 hybrid or parental lymphoid cells, death of the macrophages was not observed. Hence G.V.H.D. lymph node cells can be regarded as 'sensitized' cells.

Similarly, in cell interactions (c) and (d) when normal F_1 hybrid lymphoid or G.V.H.D. lymph node cells were replaced by parental lymphoid cells, no cell death occurred. Hence G.V.H.D. peritoneal macrophages may also be regarded as 'sensitized' cells.

My results are consistent with the view that the 'sensitized cells' are of parental origin although direct proof is lacking.

5. Prevention of Cytotoxic Death

Cell death was noted in the four pairs of cell interactions described/

described above. However, if the interactions took place in G.V.H.D. serum instead of in normal serum, there was complete preservation of the morphology of the macrophages. Hence G.V.H.D. serum had protected macrophages from cell death.

These studies show that macrophages play an important part in cell-mediated reactions in G.V.H.D. and also that macrophages from G.V.H.D. animals react against syngeneic tissue.

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CHAPTER I

Within the last decade G.V.H. reaction or disease has created almost as much interest and attention as the conventional host-versus graft or homograft reaction.

Certain requirements need to be satisfied for the production of Graft versus Host Reaction or Graft versus Host Disease. They are:-

(1) The graft must contain immunologically competent cells.

(2) The host must possess important transplantation isoantigens that are lacking in the graft donor, so that the host appears foreign to the graft and is therefore capable of stimulating it antigenically.

(3) The host itself must be incapable of mounting an effective immunological reaction against the graft, at least for sufficient time for the latter to manifest its immunological capabilities.

Thus Graft-versus-Host Disease (G.V.H.D.) may develop in any situation where sufficient viable immunologically competent cells are transplanted to animals which are unable to reject them for some reason or other and yet contain major tissue (histocompatibility) antigens which are absent from the donor. The transplantation of viable immunologically competent cells into hosts which cannot effectively eliminate them and yet contain major tissue antigens which stimulate the donor cells, produces a clinical syndrome in which the major signs and symptoms are wasting and/or retardation of growth, hunched back, ruffled fur, splenomegaly, atrophy of the lymph nodes and diarrhoea. To describe this condition Billingham and Brent (1957) /

(1957) coined a descriptive term "Runt Disease". Since then many workers have made use of the same experimental model but using hosts whose immunological capacities are suppressed by some means or other. The resulting changes in the hosts has been called by various names such as Transplantation disease, Homologous wasting disease, Allogeneic disease, Secondary disease and F_1 hybrid disease. In all these instances, the hosts always received viable cells in which a proportion of the cells are immunologically competent.

In transplantation immunology, unfortunately, two or more terms are used to mean the same thing. The table in the next page shows the internationally accepted terms as they stand today.

TABLE I

TERMINOLOGY FOR INTRASPECIES AND INTERSPECIES RELATIONSHIPS

(After Gorer, Loutit and Micklem, 1961)

Noun (e.g. grafts)	Adjective	Relating to:
autograft	autologous autochthonous	same individual
isograft isogenic graft syngeneic graft	isologous isogenic syngeneic	individuals of the same inbred strain (genetically identical)
homograft allogeneic graft	homologous allogeneic	individuals of the same species (genetically different)
heterograft xenograft	heterologous heterospecific xenogeneic	different species of animals

Inbred strain: A strain which has resulted from the mating of brother and sister for 20 or more generations.

Iso-antigens: Tissue antigens which characterize individuals, or an inbred strain, from genetically different members of the same species.

Iso-antibodies: These are antibodies produced by immunization with iso-antigens.

(A) THE VARIOUS EXPERIMENTAL MODELS OF GRAFT-VERSUS-HOST DISEASE

The models may be divided into six categories:-

1. Adult - Neonatal Experimental Model

In this model the host is either foetal or newborn; the capacity of the immune response is too feeble for a large number of foreign strain donor cells to be rejected. G.V.H. reaction or disease may develop in this situation, with or without the accompanying induction of specific immunological tolerance.

2. Parent - F_1 Hybrid Experimental Model

When the host has inherited tolerance of the donor's antigens, the injection of a first generation hybrid (F_1) between two inbred strains with donor cells obtained from either of the parental strains produces this condition. The two homozygous parents of the F_1 hybrid must differ at the H-2 histocompatibility locus for the production of this disease in most instances.

3. Graft-Versus-Host Reactions in Adult Tolerant Animals

In this situation the neonatal recipients, (not more than 24 hours old) are injected intravenously with donor strain viable cells which contain the foreign tissue antigens. The recipients develop tolerance to the tissues of the donor animals as shown by the prolonged survival of donor skin graft transplants in adult life. In an immunogenetic sense these animals are comparable to F_1 hybrids. They are fully susceptible to G.V.H.D. if inoculated with normal immunologically competent cells from the original donor (Martinez et al, 1961; Billingham and Silvers, 1961b).

4./

4. Secondary Disease in Radiation Chimeras

This occurs when the host's immunological reactivity has been severely depressed by agents such as X-irradiation or immunosuppressive drugs. The most studied example in this category is "secondary disease", a syndrome which develops following transplantation of foreign bone marrow to a lethally irradiated recipient.

5. Parabiosis

Parabiosis is the grafting of a whole animal onto another, hence either partner plays two rôles at the same time, i.e. each partner acts as a host as well as a graft. In its typical form it leads to a considerable difference in size of the two partners, one becoming larger and the other smaller.

6. G.V.H.D. in Adult Allogeneic Animals

Each of these six biological systems will be discussed in greater detail in the next section.

1. Adult - Neonatal Experimental Model

In this example adult lymphoid cells from one strain of animal are injected into neonatal animals (within 24 hours of birth) of another strain. The dose of lymphoid cells required varies between 1-10 million cells; the intravenous route is more efficient in the production of the disease and the orbital branch of the anterior facial vein or the sigmoid sinus are the blood vessels employed for the dissemination of the cells. The intra-peritoneal route is also commonly used although less efficient. Cells injected by the intramuscular/

muscular or subcutaneous route do not produce the disease.

Certain strain combinations result in a more frequent production of the disease. With the mouse strain combination DBA/1-C57BL/6, as few as 500,000 splenic cells are regularly effective and even 50,000 cells can sometimes cause the disease (Russell, 1962). With the Lewis-BN rat strain combination, a million lymph node cells will kill a third of the hosts.

The injected animals continue to grow normally for about a week or two and then some of them may become emaciated and die soon afterwards while the rest linger on as emaciated chronic runts for months. This condition was first produced by Billingham and Brent (1959) while they were attempting to produce tolerance in new-born mice by the injection of adult lymphoid cells.

2. Parent - F_1 Hybrid Experimental Model

In 1954 Medawar drew attention to the fact that grafts transplanted to F_1 hybrid mice from members of either of their parental inbred strains were genetically qualified to react against their hosts. Thus, a lymphoid graft from a homozygous (AA) donor will react against a host, which is the first generation hybrid (F_1) between the donor strain and a different homozygote (BB), i.e. to a hybrid of the genotype (AB); the usually adopted symbolism being $A \rightarrow A \times B$. On the assumption which all evidence so far has supported, that transplantation antigens are determined by dominant genes, it follows that $A \rightarrow A \times B$ is a situation in which the hybrid host/

host cannot reject the graft by immunological means, even if it is old enough to possess immunological competence in general. This is so because the graft does not possess any antigens which are foreign to the host. Conversely, of course, the graft can react against the host, since the latter contains antigens which are derived from the BB parent of the hybrid.

Making use of the above experimental model, Kaplan and Rossion (1959), Oliner et al (1961) and Fiscus et al (1962) have shown that immunologically competent parental cells injected into F_1 hybrid mice or rats will produce a syndrome similar to or identical with that seen in the adult-neonatal system. This condition is also called by some as F_1 hybrid disease. However, with some strain combinations, the adult F_1 hybrids are found to be much more resistant than 3-week old hosts of similar genetic combination. These resistant hybrids can frequently be made susceptible to attack by parental strain cells by prior sub-lethal X-irradiation. It is believed that this manoeuvre destroys some of the host's lymphoid tissue, creating more "vacant space" in the lymphoreticular system for the inoculated parental cells and their progeny. It may well be that presensitized parental cells are capable of doing this for themselves.

Cole and Garver (1960) have reported the interesting observation that (C57BL x A)F_1 hybrids 6-15 months old are more susceptible to G.V.H. reactions following inoculation of adult A strain spleen cells than are similar F_1 hybrids 3-4 months old. These authors attribute the/

the increased susceptibility of the older mice to poor regenerative capacity of their lymphoid tissue.

The acquired refractoriness of genetically tolerant F_1 hybrids to systemic G.V.H. reactions (assuming as cause the actual failure of parental cells to colonize and survive) possess superficial similarities to the syngeneic preference or allogeneic inhibition phenomenon described by Hellström (1961, 1963, 1964). They have described the failure of certain variant mouse lymphomas phenotypically identical with the parental strain to grow in semi-isologous F_1 hybrids. X-irradiation of the adult F_1 hybrid (450-500 rads.) did not render them more susceptible. Hellström et al (1964) as a result of in vitro experiments are of the opinion that contact of these lymphoma cells with the foreign H.2 antigens of the F_1 hybrid inhibited their growth. It is not known whether or not these tumours would grow without any inhibition in F_1 hybrids a few days old. (McBride, 1966).

In the case of Host-versus-Graft reactions, extremely minute antigenic differences are sufficient to reject the graft. In contrast in G.V.H. reaction, the host and the graft must differ by strong histocompatibility antigens. In inbred mice, it had been noted that strong histocompatibility antigens are determined at the H-2 locus. It is not possible, under normal circumstances to produce G.V.H.D. by antigens which are determined by genes other than those at the H-2 loci. However, G.V.H. reaction is possible if the donor, which does not differ at the H-2 locus, is pre-immunized against the host. Thus, if the homozygous male and female parents have/

have the same alleles at the H-2 loci, i.e. similar as regards H-2 antigens but different at other loci such as at H-1 or H-3, then cells from one of the parental donor immunized against the antigens of the other parental strain are capable of producing G.V.H.D. in the corresponding F_1 hybrid.

In the classical parent-F_1 hybrid (with the H-2 difference) combination, the host cannot react against the graft because of its genetic tolerance of the donor antigens. Two exceptions to this rule can, however, be made (Howard, 1961):-

(i) If the donor is male, it will possess a weak antigen determined by the Y chromosome which is absent from an F_1 hybrid female recipient.

(ii) If the donor strain is replaced by another related strain of the same H-2 group, (e.g. an exchange between strains CBA and C3H) the donor will possess relatively weak antigens which are not present in the recipient.

In both situations (those studied have been C57BL$\delta \longrightarrow$(C57BL x CBA)$F_1 \varphi$ and CBA$\longrightarrow$(C57BL x C3H)F_1 respectively), G.V.H. reaction will develop in spite of the possession of weak foreign antigens by the donor cells (Howard, 1961 and 1964).

In the case of the male-specific antigen, an anti-donor reaction does not seem to take place, as the recipient will subsequently accept male skin grafts indefinitely. This is a reflection of the relative ease with which immunological tolerance of the male-specific antigen can be induced in adult mice (Mariani, Martinez,/

Martinez, Smith and Good, 1959).

Nevertheless, tolerance is not essential for G.V.H. reaction to occur in experiments of this type. In the combination CBA → (C57BL x C3H)F_1 where the antigenic stimulus to the recipient is stronger than in the previous case, it was found that CBA skin grafts showed accelerated rejection when applied after 3-4 weeks. The presence of anti-donor immunity was thereby clearly established, in spite of the donor cells producing the usual G.V.H. reaction.

It is, in the above instance, possible to potentiate the anti-donor immunity by pre-immunization of the host against the weak antigens of the prospective donor cells by means of skin grafting. Little or no G.V.H. reaction was found by means of phagocytic index determinations, following the injection of donor strain spleen cells into recipients which had previously rejected a skin graft carrying the foreign antigens of the donor. The effect of pre-immunization was such that reinforcement of potential weak anti-donor immunity occurred to such a degree that it was possible to inhibit the donor cells. The smaller the antigenic difference between the donor and the recipient, the greater is the effect of pre-immunization (Simonsen, 1962).

Acquired Refractoriness to G.V.H. Reactions in the Adult Mouse

In certain parent/adult F_1 hybrid combinations, survivors from G.V.H. reactions have been found to be refractory to the effect of a second injection of parental strain spleen cells (Fox and Howard, 1963). This phenomenon has been studied in some detail by Howard (1963)/

(1963) in (C57BL x CBA)F_1 hybrids by means of changes in the phagocytic index together with evidence from discriminant spleen analysis and the use of cells carrying a chromosome marker. The main finding was as follows:- 3-7 weeks after the injection of C57BL spleen cells into (C57BL x CBA)F_1 mice the phagocytic index had returned to values within or slightly above the normal range. No second phagocytic response was observed when a further injection of C57BL spleen cells was given.

Could this effect be due to acquired immunity to weak antigens specific to the parent strains? No evidence for such an interpretation could be obtained. First attempts to transfer this effect either passively with serum or with adoptively transferred spleen cells were totally unsuccessful. Secondly, by using a CBA strain containing the translocation chromosome marker (T6) it was found that C57BL cells progressively replaced recipient cells in the spleen so that within two weeks or so all the dividing cells were of this genotype, (Fox, 1962). If this observation applies to lymphoid tissues generally, the explanation for this could be that the second injection of C57BL cells will "home" to sites devoid of F_1 cells for them to react against.

3. Graft-versus-Host Reactions in Adult Tolerant Animals

Animals can be made immunologically tolerant of the transplantation antigens of a foreign donor strain by the neonatal injection of viable donor cells which carry these antigens (i.e. by the injection of marrow or spleen cells of the donor). These injected cells for one/

one reason or another are incapable of producing G.V.H. reaction in the neonatal recipients and these animals are in an immunogenetic sense comparable to the F_1 hybrids. These neonates in adult life are fully susceptible to G.V.H.D. if injected with normal lymphoid cells from the original donor strain, (Billingham and Silvers, 1961b; Martinez et al, 1961).

4. Secondary Disease in Radiation Chimeras

Mice and certain other animals exposed to usually lethal doses of X-irradiation may recover if they are given a transfusion of viable bone marrow cells from either isologous or homologous donors (Micklem and Loutit, 1966). Studies on marrow cell "grafts" (with various techniques to identify the injected donor cells) have revealed that these cells become established mainly in the bone marrow and to some extent in the spleen, replacing the host's radiation depleted myeloid and lymphoid tissues, thus producing a state of chimerism. The successful rehabilitation of heavily irradiated animals with marrow of homologous or even heterologous origin, e.g. rat marrow cells to irradiated mouse, demonstrates the inability of the irradiated hosts to respond.

When the irradiated host derives its haematopoietic graft from isologous donors, it has been found that the majority of the restored animals will survive for a long time. However, if bone marrow infusions of homologous or heterologous origin are employed, survival is usually not prolonged. Mice which have usually recovered from the/

the primary effects of the irradiation frequently develop a "secondary" wasting disease that culminates in death within a few months. Since the host's lymphoid tissues are repopulated by the cells of the donor, the concept of graft-versus-host reaction for this disease was put forward, (Trentin, 1956). Further work by Trentin and others produced strong evidence in favour of the Graft-versus-Host reaction concept. The various findings sustaining this are:-

(i) The innocuous nature of marrow from isologous donors.

(ii) The innocuous nature of haematopoietic tissue from homologous infant or foetal donors.

(iii) The increased incidence of secondary disease in situations where the antigenic disparity between donor and recipient is slight, if the donor has been pre-sensitized.

(iv) In homologous rabbit irradiation chimeras suffering from secondary disease there is a positive Coombs' test and selective lysis of host red cells, (Porter, 1960).

The pathology of secondary disease resembles that of G.V.H.D. produced in the Adult-Neonatal or in the Parent-F_1 hybrid system. But secondary irradiation disease should be considered in a class of its own since the immunological attack takes place in a host previously subjected to radiation damage which complicates the picture.

5./

5. Parabiosis

The technique of parabiosis, the grafting of one complete individual on to the body of another was first introduced more than a century ago by Paul Bert (1864). It was rediscovered, named and popularized by Sauerbruch and Heyde (1908). It has long been known that when genetically dissimilar animals are parabiotically united, vascular anastomosis rapidly becomes established at the lines of union. These anastomoses may be of short duration or may remain patent for variable periods, if not indefinitely. This will depend on the degree of histo-incompatibility between the parabionts. A lethal wasting syndrome, known as parabiosis intoxication frequently develops in which the intoxicated partner loses weight and becomes severely anaemic and its unaffected partner gains weight and becomes polycythemic. Pathological changes observed in "intoxicated" subjects closely resembled those encountered in (G.V.H.D.)F_1 hybrid disease. The clarification of the aetiology of this condition required the use of genetically defined partners. A useful combination for investigation are those in which the F_1 hybrids are united with a partner of one of their parental strains. This would result in antigenic stimulation in one direction and the parental cells will interact with the F_1 hybrid, causing damage to it. In this parent - F_1 hybrid combination it is the F_1 hybrid which is susceptible to parabiotic intoxication. There is little doubt that a Graft-versus-Host reaction occurs in the F_1 hybrid, where the latter/

latter is attacked from within by the immunologically competent cells derived from its partner's blood.

Soon after the parent and its F_1 hybrid is parabiosed, it seems that an early initial shunting of blood occurs from F_1 hybrid to parent as the result of the establishment of a vascular pressure gradient and that the immunological reaction on the part of the parental strain partner helps to sustain this gradient, (Hilgard et al, 1963).

In mice, in situations where both parabionts are alike at the H-2 locus, overt parabiotic intoxication is not encountered and tolerance of its partner's foreign transplantation antigens may ultimately be induced in the potentially reactive partner.

6. G.V.H.D. in Adult Allogeneic Animals

When adult lymphoid cells are injected from adult donors of one strain into adult mice of another strain, the donor cells are destroyed by a host against graft reaction (Homograft reaction) before the donor cells get a chance to react against the host.

However, very high doses of cells from specifically presensitized animals will often succeed in causing the disease where similar numbers of cells from normal animals prove harmless. This occurs in strains which differ at the H-2 locus. Thus 3 to 6 lymphoid equivalents or about 700-1500 million cells - from presensitized donors, can cause fatal graft-versus-host disease which is accompanied by an impaired ability to reject skin grafts from the donor strain (Najarian and Feldman, 1962).

Whether/

Whether this impairment of the host's response is due to destruction of its own lymphoid tissue, to the induction of tolerance or to neutralization of its response by the overwhelming numbers of donor cells present, is not known.

The pathogenesis in all these six conditions is not fully known. G.V.H.D. in the simplest and easily analysable form is found only in the adult-neonatal and the parent-F_1 hybrid experimental models. In these two models the host animals are unable to destroy the transplanted donor tissue due to natural reasons and where no artificial procedures had been adopted to suppress their capacity to destroy the transplanted donor tissue.

In my thesis I shall limit the term Graft-versus-Host Disease (G.V.H.D.) to the study of the above two experimental models.

Since G.V.H.D. is produced by the transplantation of donor lymphoid cells from homologous animals, the next section will deal with the donor cellular inoculum.

(B) THE CELLULAR DYNAMICS OF G.V.H.D.

1. The Cellular Inoculum; Influence of Cell Dosage and Cell Type

The dosage of the lymphoid cell inoculum is an important factor in determining whether or not it will cause G.V.H. disease, low doses being less successful. It is more common for recovery to occur with low than with high doses. Furthermore, the minimal number of lymphoid cells required to cause G.V.H.D. in the majority of hosts varies according to the strain combination employed. With the mouse strain/

strain combination DBA/1 → C57BL/6, as few as 500,000 splenic cells are regularly effective and even 50,000 cells can sometimes cause the disease, (Russell, 1962). With the Lewis-BN rat strain combination, a million node cells will kill about a third of the hosts.

Careful comparisons on the quantitative basis of the runt-inducing potency of cell suspensions prepared from different tissues show that it is related directly with its content of lymphocytic cells, (Billingham and Silvers, 1961a). Bone marrow is almost innocuous and can frequently be used to induce tolerance of skin homografts with little risk of inciting overt runt disease, whereas spleen, leukocyte concentrates prepared from peripheral blood (predominantly lymphocytes), lymph node cells, and thoracic duct cells represent inocula of increasing potency. Thymocytes are anomalous: in mice these cells cause runt disease only fitfully, and in rats they are virtually harmless, even at very high doses.

The lymphoid cells from foetal or neonatal mammalian and avian donors (obtained from the blood) are completely harmless probably because of the immaturity of the cells. Likewise, in the Lewis-BN rat strain combinations, splenic cells from neonatal donors afford a perfectly innocuous tolerance-conferring stimulus.

2. The Dynamics of the Donor Cell Inoculum

In the Adult-Neonatal system and in the Parent-F_1 hybrid system, the inoculated donor cells (by the intravenous and the intra-peritoneal routes) circulate in the blood stream and settle down predominantly/

predominantly in the lymphoid organs of the host (Nakic et al, 1967; Gowans, 1962). In my histopathological study mononuclear cell infiltrates were also found in the various non-lymphoid organs such as the liver, intestines, mesentery, omentum, pancreas, salivary glands, bone marrow and the dermis of the skin. Very few, if any, are found in the brain and spinal cord.

Most of the donor cells "home" in the lymph nodes and in situations where lymphoid aggregates are normally found such as in the Peyer's patches. It is significant, that although splenomegaly is a very constant finding, the donor cells are not found to the same degree as in the lymph nodes after the first three days (Davies and Doak, 1960; Nakic et al, 1965; Nowell and Defendi, 1964). The former two groups of workers utilized the T6 marker chromosomes and the latter the sex chromosome to determine the proportions of the donor and host cells in the lymphoid organs.

Why donor lymphoid cells "home" in the lymphoid tissues of the host is not known. Perhaps there must be some selective mechanism operating for these cells to settle down in organs which are physiologically natural to them. As described under "Organ Specific Growth Stimulation", the microsomal and supernatant fractions of the adult spleen localize predominantly in the spleen and this may throw some light on the homing instinct of lymphoid cells.

The inoculated lymphoid cells leave the blood stream through the post-capillary veins of the lymph nodes as shown by Gowans, although its passage out of the post-capillary venule has not been directly demonstrated./

demonstrated.

3. The Cells Capable of Causing G.V.H.D.

Normally G.V.H.D. is produced by the injection of lymphoid cells prepared as monocellular suspensions from the lymph node, spleen, thoracic duct lymph or blood. The identification of the donor cells, in these suspensions, that are responsible for the induction of G.V.H. reactions is of some importance. They are cell-types which can interact with antigens and initiate a primary immune response against them. Cells of this kind, whose potential immunological reactivity has not yet been realised, have been termed "immunologically competent cells".

The ability of cells from the thoracic duct lymph and peripheral blood to cause G.V.H. reactions in rodents shows that circulating lymphocytes are immunologically competent cells (Billingham et al, 1960; Gowans et al, 1961; Gowans, 1962; Gowans and McGregor, 1965). Terasaki (1959) fractionated the leucocytes in the blood of adult chickens and showed that the ability to cause splenomegaly in embryo chicks was related to the number of lymphocytes and not to the number of monocytes in the inoculum. Lymphocytes are usually sub-divided on grounds of morphology and size into "small", "medium" and "large", although from a functional point of view each group is a heterogeneous population of cells. Many workers have attempted to determine the type of lymphocyte responsible for the reaction, i.e., whether it is the large or small lymphocyte.

The cell-type in the blood and lymph causing G.V.H. reactions

in/

in rodents was first assumed to be the large lymphocyte. Cole and Garver (1961) exposed F_1 hybrid mice to 500 rads of total body irradiation and then injected them intra-peritoneally with blood from parental donors which had been pretreated with Cortisone. G.V.H. activity was attributed to "large mononuclear cells" which made up an abnormally high proportion of the leucocytes in the blood of these pretreated donors. On the other hand the ability of thoracic duct cells to mediate G.V.H. reactions in rats does not depend upon the presence of large lymphocytes in the inoculum. When the rat thoracic duct was cannulated, the number of large lymphocytes increased on successive days after cannulation. It is possible to collect lymph with a high proportion of large lymphocytes by this technique and it was shown by Gowans that the ability to kill newborn and F_1 hybrid rats bore no relation to the number of large lymphocytes in the inocula. Further, inocula of thoracic duct cells in which the proportion of large lymphocytes had been greatly reduced (0.05%) were as effective as the normal mixture of lymphocytes from the thoracic duct (about 5% large lymphocytes) in killing F_1 hybrid rats. The experiments show that either the large lymphocytes have no ability to produce G.V.H. reaction or that the numbers injected were not sufficient to demonstrate it. Since very few large lymphocytes migrate into the nodes and spleen after intravenous injection into F_1 hybrid rats, it is very unlikely that these cells could play a significant part in the proliferative and destructive changes seen.

There is now very strong evidence that the immunologically competent/

competent cells present in the thoracic duct lymph and blood of rodents are small lymphocytes. Cowans (1962) prepared inocula rich in small lymphocytes by incubating rat thoracic duct cells in vitro for 24 hours at 37°C. This procedure results in the large lymphocytes dying more quickly than small lymphocytes. When these cells which contained predominantly small lymphocytes were inoculated (200 million cells) every member of the young adult F_1 hybrid rats died of G.V.H.D.

Hildemann et al (1962) separated small lymphocytes from the peripheral blood of mice by filtering heparinized whole blood through a tightly packed plug of glass wool. The filtrate contained 96-100 per cent small lymphocytes. An intracardial injection of less than one million small lymphocytes caused runt disease and death in a proportion of allogeneic newborn hosts.

Thus, there is sound evidence that small lymphocytes from rats and mice can initiate G.V.H. reactions in genetically appropriate recipients.

4. Transformation of Small Lymphocytes into Other Cell Types

Cowans (1962) injected parental strain thoracic duct lymphocytes into F_1 hybrid rats and studied the early histological changes which occurred in the spleen. They produced a fatal form of G.V.H.D. in F_1 hybrid rats by inoculating the parental thoracic duct lymphocytes. The most prominent early histological change occurred between 12 and 24 hours in the white pulp of the spleen in the periarteriolar zone of small lymphocytes. In this region cells were seen which possessed/

possessed an intensely pyroninophilic cytoplasm, a prominent pyroninophilic nucleolus and a pale vesicular nucleus with a fine chromatin structure. These cells were termed non-committally "large pyroninophilic cells". They increased in number between 24 hours and four days and mitotic figures were frequent. An autoradiographic study of the tissues of F_1 rats 24 hours after the inoculation of radioactively-labelled thoracic duct cells led to the conclusion that some of the injected small lymphocytes had transformed within 24 hours into large pyroninophilic cells.

The number of labelled large pyroninophilic cells at 24 hours made up only a small proportion of the totally labelled small lymphocytes (less than 5%) in the lymph nodes and spleen of the animal. By 72 hours no label remained in any of the pyroninophilic cells and it was assumed that the radio-activity had been diluted by cell division to a level that could not be detected by autoradiographs. However so many labelled small lymphocytes remained that it was clear that they were not a continuous source of new large pyroninophilic cells. The small lymphocytes transformed rapidly or not at all from which it may be inferred that small lymphocytes consist functionally of a heterogeneous population; some respond, most do not.

According to Gowans and McGregor (1965) the spleen appeared to be the preferential site for the transformation of the small lymphocyte into large pyroninophilic cells. The transformation was detected to a small extent in the lymph nodes but its extent was trivial in comparison with the spleen. The preference which the transforming/

transforming lymphocytes appeared to show for the spleen may be due to the fact that the spleen is the first organ where most of the donor lymphoid cells initially seem to settle down. Porter and Cooper (1962) made similar studies to Gowans and McGregor (1965) but used the adult-neonatal experimental model instead of the parent-F_1 hybrid system. They investigated the fate of radio-labelled adult rat thoracic duct lymphocytes in new-born rats. They also concluded that the donor small lymphocytes transformed into large pyroninophilic cells, but claimed that at 24 hours after the inoculation, instead of less than 5%, as was noted by Gowans and McGregor (1965), 30% of all the labelled cells had transformed into large pyroninophilic cells. No preferential transformation was seen in the spleen.

5. Transformation into Macrophage

By making use of an abnormal chromosome marker (the translocation chromosome T6) it is possible to identify the dividing donor cells from host cells. This technique had been made use of by Howard et al (1965), who demonstrated (1966a) the predominance of cells of donor karyotype in mice undergoing G.V.H. reaction. They noted that macrophage-type cells derived from donor thoracic duct lymphocytes appeared in the liver. Further studies by these groups of workers (Boak et al, 1968) making use of a combination of techniques such as the T6 chromosome marker, radioactive labelling of donor cells and the use of such colloidal suspensions as carbon, thorotrast and iron, showed that about 2% of the Kupffer cells in the liver were in mitosis and that of these, about 95% of them were of donor karyotype.

The/

The donor cells also take up colloidal particles and thus behave like macrophages. Further, by making use of thoracic duct small lymphocytes which were 98.5% pure, these workers were able to demonstrate that in mice undergoing G.V.H. reaction, 95% of the dividing cells were of donor origin and exhibited phagocytosis. Thus, it is very likely that the small lymphocyte is the cell-type in the donor inoculum which has the capacity to transform into macrophage-like cells. Even so, the possibility that this precursor is contained within the small percentage of large lymphocytes contaminating the small lymphocyte suspension used, had not been formerly excluded.

Using the same technique to identify the cell type and phagocytic capacity of the donor cells, Howard et al (1969) have shown that macrophages of donor karyotype are present in the lungs and peritoneum of mice undergoing G.V.H. reaction. They initiated G.V.H. reaction in normal adult (C57BL x CBA - T6T6)F_1 mice by the injection of C57BL spleen or glass wool filtered lymph node cells or thoracic duct lymphocytes. Pulmonary alveolar and peritoneal macrophages were isolated for karyotype analysis 11 days later and found to contain substantial proportions of dividing cells of donor origin.

6. The Significance of the Large Pyroninophilic Cell

The transformation of small lymphocytes into large pyroninophilic cells during G.V.H.D. appears to be a specific response to the foreign histocompatibility antigens of the host. No transformation has been observed in the spleen of syngeneic recipients nor in F_1 hosts/

hosts when the parental lymphocyte donors had been made immunologically tolerant of the recipients' tissues by a neonatal injection of F_1 bone marrow. The large pyroninophilic cells divide rapidly to produce lymphocytes of progressively decreasing size.

Pyroninophilia is a general indication of accelerated RNA synthesis. Binet and Mathé (1962) examined by the electron microscope the lymph nodes of mice undergoing G.V.H. reaction. They found that the pyroninophilic cells of these lesions differ from plasma cells by lack of endoplasmic reticulum although they are rich in ribosome content. By light microscopy the pyroninophilic cells at the fourth day are an immature cell type which in the following few days develop into histiocytes. It is very likely that pyroninophilic cells in G.V.H. reactions can develop from both the graft and the host. The former has been virtually proved in the experiments by Gowans (1961), and the latter is strongly implied by the fact that at the peak of splenomegaly in the mouse, almost all the multiplying cells may be of host origin and at the same time, many of these are pyroninophilic, (David and Doak, 1960).

7. The Cellular Responses in the Later Stages of G.V.H.D.

The changes occurring in the lymphoid tissues of the host during the first 72 hours are well documented but what happens to the donor cells in the later stages of the disease is not completely known. The spleen is the most frequently investigated organ as regards the donor and host cell components in mice undergoing G.V.H. reaction. Initially it was thought that splenomegaly in mice undergoing G.V.H. reaction/

reaction was due to donor cells since donor cells were identified and seen to proliferate in the early stage of the disease. However, quantitative cytological studies, making use of chromosomal markers on the spleen cell populations of host previously inoculated with homologous lymphoid cells, have now shown that splenomegaly in mice, rat and chicken, must be attributed in large measure to proliferation of host cells.

Following the neonatal inoculation of A strain mice with splenic cells from CBA/T6 donors, Zeiss and Fox (1963) found no evidence of donor cell proliferation in the spleens of one or two-day old hosts, but the dividing donor cells were consistently demonstrable during the following 3-10 days period. After this period there was a rapid proliferation of host cells although a minority of donor cell mitosis was still demonstrable at 60 days.

In Lewis rats injected at birth with BN lymph node cells or leukocytes, Nowell and Defendi (1964) found that proliferating donor cells were abundant in the host's nodes over the first 21 days. However, they were much less common in the spleen, which was nevertheless, markedly enlarged. These workers concluded that while the donor cell proliferation makes a substantial contribution to the enlargement of the nodes, splenomegaly is principally due to proliferation of host cells. They also noted a correlation between the frequency of dividing donor cells and the severity of runt disease.

In a cytological analysis of the splenomegaly occurring in adult/

adult (CBA x C57BL)F_1 mice inoculated with CBA spleen cells - a situation that should preclude any possible antigenic stimulation of host cells - Fox (1962) observed that an early but short-lived burst of donor cell mitosis occurred but subsequent cell proliferation was almost entirely of host origin, although there was a persistent low level of donor cell mitotic activity. In a study of 13-day chick embryos injected with adult donor blood, Owen, Moore and Harrison (1965) found that three days after inoculation, when the spleen weight had increased by a factor of 2-3, an overwhelming proportion of the dividing cells were of host type, though during the following three days the donor cells formed an increasingly high proportion of the dividing cells (up to 90%) so that they may have made a substantial contribution to the ultimate enlargement of the spleen. The evidence that the proportion of dividing donor and host cells at any time was approximately constant was of considerable potential significance, irrespective of the spleen weight, suggesting that both donor and host cell proliferation are interdependent events.

What provokes the massive host response is still a matter of conjecture. It cannot be an antigenic stimulus involving transplantation antigens of the inoculated cells. Most authorities regard it as some kind of protective mechanism set in motion by the effects of the initial G.V.H. reaction - its incitants may be products of cell damage or destruction, though no convincing evidence of any large scale destruction of cells has been consistently observed in the/

the lymphoid organs of animals suffering from homologous diseases (Billingham, 1966-67).

8. The Fate of Immunologically Competent Cells After Grafting to a Foreign Host

The previous account shows that the donor cells do not behave in a uniform manner in different species and in different host/donor combinations. The fate of the donor cells may be considered under three headings.

(i) The Grafted Cells Maintain Indefinitely Their Reactivity Against Their Hosts.

If this is the case, then the donor cells would proliferate and their cellular descendants would also react against the host. Such a hypothesis has been tested by transferring lymphoid cells from animals undergoing G.V.H. reaction to normal syngeneic recipient animals. Cells from these animals have in turn been transferred to normal syngeneic recipients.

This important type of experiment has seldom been carried out with a positive result. Siskind et al (1960) inoculated CBA lymphoid cells into A/Jax mice, and spleen cells were taken from surviving first recipients, at 8 weeks after injection, when they were obvious runts. On injection of these cells into a new batch of newborn A/Jax, 74% of the animals died. This seemed to represent an accelerated course of runt disease because the death rate of the first recipients was only 56%. Since the numbers of grafted cells were similar in the two transfer generations, the suggestion is one of active immunization. No more than two transfers have been done in/

in either of the two strain combinations that they used in these experiments. Dineen (1961) did the same type of experiment in two other combinations of mouse strains but found only little runting by the first passage and none by the second. He interprets his results as an indication of acquisition of tolerance of the graft towards the host but has no proof that the donor cells of the chimaeric spleens retained immunological competence towards other antigens. In summary, there is as yet no sufficiently analysed case to substantiate the theory that the grafted cells can pursue their attack on the host indefinitely.

(ii) The Grafted Cells Die in their Host

This has been shown by Boyse (1959) and Gorer and Boyse (1959) in their strain combination adult C57BL $\rightarrow$ adult (C57BL x A)F_1. C57BL cells from donors which had received a single injection of human blood cells before grafting were challenged with the same antigen after grafting to the hybrid host, in order to determine if the secondary response of agglutinin formation would occur. A primary response would indicate that the host lymphoid cells were responsible for its formation. When challenged 5 to 7 days after injection of donor cells, the secondary response occurred in two cases. When the challenge was made at days 8-27 after grafting, there was no or a questionable secondary response. Thus, these experiments show that secondary response is seen by one week but not after a longer interval of time. The suggestion is therefore that the donor cells had not persisted after one week; at least, that the/

the cells had not been functionally active.

(iii) Intermediate Possibilities of Behaviour

It is quite possible that there are numerous possible fates for grafted immunologically competent cells, different from the two extremes already mentioned. One of the intermediate possibilities is that the cells lose their reactivity against the host. In instances such as the inoculation of A cells pre-immunized towards C57BL cells, to (C57BL x A)F_1 hybrids, the host spleen cells are replaced entirely by graft spleen cells, as measured by the cytotoxic antibody technique. In spite of this fact, the spleen cells could not transfer the disease to new F_1 hybrids.

(C) LOCAL GRAFT-VERSUS-HOST REACTIONS

The G.V.H. reactions described so far have been of a systemic nature. However, it is possible to have G.V.H. reaction taking place locally, at the site of inoculation of homologous immunologically competent cells.

1. Intracutaneous G.V.H. Reactions

When normal homologous lymphocytes are injected into the skin of outbred or F_1 hybrid guinea-pigs, the development of a local cutaneous inflammatory reaction of delayed onset (normal lymphocyte transfer reactions) has been noted by Brent et al (1962). These reactions have been shown to be due to the stimulation of the injected lymphocytes by the iso-antigens of the host and their intensity provides a measure/

measure of the iso-antigenic disparity between the recipient of the cells and the cell donor. When cells are transferred from pre-sensitized donors, the "transfer reactions" are more intense and their development is accelerated. It was also noted that blood lymphocytes were 2 to 5 times more active than lymph node cells in causing normal lymphocyte transfer reactions, (Brent and Medawar, 1963).

2. Intrarenal Graft-versus-Host Reactions

It has been shown that if lymphoid cells from parental strain rats are injected beneath the renal capsule of F_1 hybrid hosts a local invasive and destructive reaction develops which may destroy up to 25% by weight of the affected renal parenchyma (Elkins, 1964, 1966). There is a close histopathological similarity between this lesion and that which develops in primary renal homograft. Studies by chromosomal marker techniques reveal that the inoculated lymphocytes give rise to a rapidly proliferating population of haemoblast-like cells during the active phase of the reaction. These cells appeared to play an active rôle in the pathogenesis of the lesion and they probably act as the precursors of the immunological effector cells (Gowans and McGregor, 1965). On day 7 after inoculation of parental strain cells into an F_1 hybrid kidney, practically all the dividing cells were of donor origin. These reactions were self-limiting, involving neither the contra-lateral kidney nor any other organs and tissues. During the early developmental phase, i.e., up to/

to 7 days, these reactions could be transferred to the kidneys of secondary F_1 hosts isologous with the primary hosts but not into hosts of the original (parental) donor strain, which implies that the donor cell not only persists, but also that it possesses the ability to continue an effective G.V.H. reaction for at least 7 days.

The cellular basis of these G.V.H. reactions is much more complex, in that prior irradiation of potentially susceptible F_1 hosts was found to inhibit the development of G.V.H. reactions. Elkins (1966) concluded that development of the lesions in the host kidney depends upon the continuous interaction of the specifically reactive donor-type cells with an immunologically non-specific population of host mononuclear cells. My hypothesis, developed further in the cell culture section, is that the injected lymphocytes confer on the host macrophages an ability to react against their own tissues.

(D) AETIOLOGY OF G.V.H.D.

1. Organ Specific Growth Stimulation

Historically, several of the described changes, notably the spleen enlargement in chicken embryos after grafting of adult spleen fragments on to the chorio-allantoic membrane, were known long before their nature as G.V.H. reactions was realised. It was thought, because of the superior age of the donor tissue, that this reaction represented the stimulation of the embryo by the adult donor cells, and was believed to be "organ specific growth stimulation".

At that time genetically distinct strains of mice had not been developed. Although some splenic enlargement occurs when adult isologous spleen cells are grafted to the chicken embryo, the degree of enlargement is not so great as when allogeneic splenic tissue is grafted. This difference in the splenic enlargement is easily detected when Simonsen's discriminant spleen assay analysis is used to detect the magnitude of splenomegaly. However, the fact that "organ specific growth stimulation" may occur, at least to a very small degree, cannot be entirely discounted, especially with regard to the splenomegaly. Some workers, although accepting the occurrence of G.V.H. reactions, appear to think that organ specific growth stimulation may still be involved in the aetiology of the splenomegaly, (Ebert, 1959; Van Alten et al, 1959; Ebert and De Lanney, 1960). The above phenomenon cannot be entirely discounted as Davis and Doak (1960) have clearly demonstrated host cell proliferation in the spleen of mice undergoing G.V.H. reaction.

The concept of organ specific growth stimulation is connected with the fact of specific localization of "transplanted" subcellular constituents. Ebert (1954) preinjected donors with S^{35}-labelled methionine and grafted the spleen or kidney fragments of these donors on to the chorio-allantoic membrane of the chick embryo. He found that the radioactivity from spleen grafts localized predominantly in the embryonic spleen, whereas labelled kidney material was mainly found in the embryonic kidney. The embryonic spleen was also found to be greatly enlarged. He thought he could exclude a significant migration/

migration of whole cells and believed that adult proteins or protein-fractions have been incorporated into the specific embryo organ. Spleen enlargement was considered to reflect increased proliferation of embryonic spleen cells, consequent upon this extra supply of prefabricated units.

In later experiments it has been demonstrated that microsomal and supernatant fractions of these organs can also be taken up predominantly in spleens and kidneys respectively. It was also shown that nuclear or mitochondrial fractions do not show selective incorporation.

Although splenic enlargement was noted in these experiments, the degree of enlargement does not reach such high proportions as seen in G.V.H. reactions. It seems quite possible that a relatively organ-or-tissue-specific incorporation of intravenously injected microsomal and supernatant fractions is a genuine phenomenon. It is at least an interesting hypothesis and this phenomenon may explain the so-called homing tendency of intravenously disseminated cells.

2. The Infection Theory

Here the assumption is made that G.V.H.D. is caused by a pathogen carried within or among the donor spleen cells - a pathogen to which the donor is adapted but which runs riot in the neonatal host tissue. Direct attempts to transmit the hypothetical pathogen have failed. Thus, three A/J mice which had been injected with CBA/J cells and which were dying of typical G.V.H. disease, were killed, and cell suspensions prepared from their spleens and livers were/

were injected into the ten members of two newborn A/J litters. In this situation, all mice remained in perfect health and were found to be normal in every respect when autopsied 3 months later. Also, in the strain combination C57BL→ A, in which all injected subjects succumb soon after injection, uninjected subjects remained perfectly healthy although they were constantly exposed to the loose faeces of their supposedly infected littermates. Heat treatment (48.5°C for 20 minutes) is known to kill mammalian tissue cells but is unlikely to inactivate most bacteria and viruses. It was found that inoculation of heated adult CBA/J-spleen cells into newborn A/J mice did not produce any mortality or lymphoid hypoplasia in the recipient animals. In contrast, injection of viable lymphoid cells in similar dosage in similar strain combinations produced 51% mortality and complete tolerance in the survivors. It was also found that heparinised donor plasma and also subcutaneous injection of spleen cells do not produce the disease.

Further, the demonstration that mice kept under germ-free conditions are as likely to develop G.V.H.D. as animals kept under normal laboratory conditions argues strongly against an infection hypothesis. However, one cannot entirely rule out a viral aetiology, where the virus may be introduced with the cellular inoculum or the virus gaining entry at a stage when the animal is immunologically debilitated.

3. The Theory of an Immunological Reaction by the Injected Cells Against their Hosts.

There is sufficient evidence to maintain the theory that G.V.H. disease/

disease is the consequence of the interaction of the injected viable donor lymphoid cells with the tissue antigens of the host which are foreign to it.

The evidence is as follows:-

(i) The injected homologous cells exist in the host for varying periods of time, the duration of existence varies in different species combination.

(ii) The injection of parental strain lymphoid cells into the corresponding F_1 hybrid produces the disease whereas the injection of F_1 hybrid lymphoid cells into the parental strain is quite innocuous. In this latter situation the parental tissues do not provide any foreign tissue for the F_1 lymphoid cells to interact with.

(iii) In the Adult-Neonatal system, mice can be protected from G.V.H.D. if they receive, together with the homologous cells, adult isologous spleen cells. The adult isologous cells have the capacity to interact with the inoculated donor cells and prevent it from damaging the host.

(iv) The severity and frequency of G.V.H.D. is enhanced if sensitized homologous spleen cells are injected, i.e., cells from donors already sensitized to host tissue antigens.

(v) The injection of cells from tissues other than the lympho-reticular system does not produce G.V.H.D.

(vi)/

(vi) The severity and incidence of G.V.H.D. is largely determined by antigenic differences between donor and host strains.

(vii) Embryonic lymphoid cells, though adept in producing tolerance, fail altogether to produce G.V.H.D. (Billingham and Brent, 1959).

Discussion on the Aetiology of G.V.H.D.

1. The Necessity of an Antigenic Stimulus

It was shown by Simonsen that the splenomegaly at 10 days after intra-peritoneal injection of newborn inbred mice with adult spleen cells from foreign strains was not found if host and donor belonged to the same inbred strain. This means that maturity of the donor alone was not sufficient to cause splenomegaly in immature recipient mice. A genetical and hence antigenic, difference was also necessary. Essentially the same requirements were demonstrated in newborn inbred chicks (Cock and Simonsen, 1958) and in embryonic chicks, (Isacson, 1959).

2. The Direction of the Antigenic Stimulus

This is best illustrated by inoculating parental strain lymphoid cells (A) into their corresponding F_1 hybrids (AB), i.e. A→(AxB). Here the 'B' antigens of the host stimulate the donor (A) cells. Hence the direction of antigenic stimulation is towards the graft. In the reverse situation where (AxB) cells are inoculated in A, i.e. (AxB)→A, the direction of antigenic stimulation/

stimulation is towards the host since the 'B' antigens of the donor stimulate the lymphoid cells of the host (A).

This effect was clearly shown by Billingham and Brent (1959) making use of strains AU and A where adult spleen cells from A → AU newborn gave a 100% incidence of G.V.H.D. whereas adult spleen cells from (AUxA) → A newborn resulted in no G.V.H.D.

3. Evidence from Pre-immunized Donors

Lymphoid cells sensitized to the foreign antigens of the host tissue produces G.V.H.D. in a much more severe form and in a shorter duration of time than the corresponding normal donor lymphoid cells. This is simply the general principle of secondary response where the sensitized lymphoid cells of the donor are exposed to the same antigens of the host for the second time. This fact strongly supports the immunological basis for G.V.H.R. at least in its early stages.

The fact that inoculation of lymphoid cells from AB hybrids into A hosts, results in an immune response in the A hosts further confirms the immunological basis for these reactions.

(E) WAYS OF ASSAYING G.V.H. REACTIONS

G.V.H. reaction or disease should be considered as a clinico-pathological syndrome and thus there are structural and functional alterations taking place in animals undergoing G.V.H. reaction. This would mean that there is no single satisfactory method of assaying these reactions and thus various assays are devised to measure/

measure the particular parameter that one is interested in. The following account gives the various assay procedures adopted to measure G.V.H. reactions.

1. Mortality Assay

This may be the most simple way of assaying the reaction but not the most sensitive since G.V.H.D. may run a milder course and end in spontaneous recovery.

Mortality rates between 5th-30th day after grafting has been used as the end point of analysis by Siskind et al (1959, 1960). They found the death rate to vary with strain combination and dosage of grafted cells. In the rat adult-neonatal experimental model Billingham et al (1960) noted a relationship between cell dosage and death rate whereas the time of death appeared very little dependent on cell dosage.

This method is useful mainly to demonstrate the enormous variability in "antigenic strength" between the various combinations of mice since death versus survival should be convincing evidence of this.

2. Weight Gain Assay

Russell (1960) has made use of the well known weight retardation in runt disease as an assay system. The method consists in daily weighing of the injected animals from the first day of birth. Normal syngeneic mice serve as controls but litter-mates of the test animals would best serve as controls.

The weight of control animals increased approximately linearly with/

with time, up to 30 days of age. The test animals, however, gained weight normally for 5-8 days, at which time an abrupt cessation of weight gain occurred. A weight plateau then supervened until the death of the animal.

Since G.V.H.D. is not always lethal, this is more sensitive than mortality assay. It is particularly useful for the experiment Russell performed, i.e., attempt to attenuate the course of runt disease.

3. Spleen Assays

Spleen assays of G.V.H. reaction exist in various modifications, all based on the splenomegaly which occurs after grafting of donor cells (the peak of splenomegaly occurs at some 6-16 days after grafting but depending on the strain combinations and cell dosage it may occur even later).

This assay has been used in chickens and mice as the main end point in the analysis of G.V.H.R. Simonsen (1957) compared absolute spleen weights from test animals with corresponding spleen weights from non-injected controls of the same age.

It was later found in inbred chickens that spleen weights of non-injected controls were positively correlated with body weights. The ratio of the spleen weight to the total body weight was then used as a measure of spleen enlargement and this was called relative spleen weight.

As a further refinement, Simonsen (1960) calculated the index of spleen enlargement for each experimental animal by dividing its relative/

relative spleen weight, as defined above, by the mean of the relative spleen weights for litter-mate controls. An index of 1.00 is thus by definition indicative of no change in spleen weight, whereas an index of 2.00 represents a doubling of spleen weight.

The calculation of spleen indices has the virtue of making results obtained from different litters more comparable because variation in spleen weight between litters is bigger than the intra-litter variation. The minimum spleen index which is considered significant in Simonsen's laboratory is 1.30.

It is necessary that the spleen index be calculated at the time of maximal splenomegaly which is about 8-10 days after the inoculation of cells. After this time, the growth retardation will occur and may interfere with the reliability of the spleen assay (Simonsen, 1962).

For most assays the use of F_1 hybrid litters is advantageous since, unlike homozygous hosts, the animals do not rapidly become refractory to the injected donor cells. They may be used as recipients up to 10 days of age.

4. The Discriminant Spleen Assay

This is an adaptation of the spleen assay described above, so as to make it possible in a mixture of immunologically competent cells of different origin, to measure the activity of one kind only. It follows that this is of particular interest for analysis of chimaeric tissues. How this is possible is that infantile F_1 hybrids older than newborns are sufficiently mature to inactivate grafted/

grafted foreign cells by a conventional host reaction. But, if the grafted cells are derived from one of the two parental strains, they will produce the usual G.V.H. reaction. Hence, a mixture of two such kinds of cells will be differentially treated after grafting to the hybrids; only the parental cells will be allowed to react against the host (Simonsen, 1962).

5. The Phagocytosis Assay

This assay was devised by Howard (1961a) and is based upon his observation that hyperphagocytic activity of the reticulo-endothelial system is associated with the proliferative phase of graft-versus-host reactions in mice. The assay quantitatively measures reticulo-endothelial stimulation. Colloidal carbon is injected intravenously and its rate of clearance from the blood stream is determined by taking periodic samples of blood from the retro-orbital venous plexus. It was found that colloidal carbon was cleared from the blood stream by the reticulo-endothelial system at an increased rate. The blood clearance of colloidal carbon follows an exponential function so that phagocytic index 'K' is determined by

$$\frac{\text{Log. concentration a} - \text{Log. concentration b}}{Tb - Ta}$$

where Ta and Tb = time in minutes at a and b. The index increased during the second week after grafting, reached a peak at the 14th to 15th day and declined sharply thereafter. A normal level was usually reached again by 20-40 days.

The phagocytic index is expressive of G.V.H. reaction for the following/

following reasons:

(i) The increased index is accompanied by other signs of G.V.H. reactions.

(ii) Immunisation of the donor by previous skin grafting from the other parental strain of the hybrid resulted in an increased index some 3-4 days earlier than found with normal donors.

(iii) Phagocytosis can also be stimulated by the reverse type of grafting, from the hybrid donor to parental strain host - here the host is stimulated by the antigens of the graft. In this instance the rise in phagocytic index is much less pronounced, began much earlier and had almost subsided by day 7 when the phagocytic index during G.V.H. reaction first began to rise.

(iv) The injection of isologous cells (providing no antigenic stimulus either way) gave no phagocytic index at any time from days 5-20.

These facts show that the increased phagocytic index in G.V.H. reaction is not due to any non-specific kind of stimulation.

6. Chorio-Allantoic Membrane Assay

This assay was developed by Burnet and Boyer in 1961. It depends upon the fact that if a suspension of immunologically competent cells is placed in contact with the chorio-allantoic membrane of a 12-day chick embryo, a number of white focal lesions developed/

developed within 4-5 days. These lesions are formed by the lymphocyte reacting against the foreign antigens of the host.

By varying dilutions of cell suspensions and using adequate numbers of embryos per dose, a linear dose-response relation is found with an average of one focus per 2×10^4 white blood cells applied to the membrane. The data presented by Burnet and Boyer (1961) show a very marked scattering of counts obtained from different membranes in response to the same cell inoculum. The authors point out, themselves, that this method has obvious limitations for quantitative work.

(F) PREVENTION OF GRAFT-VERSUS-HOST DISEASE

1. By Immunological Mechanisms

The immunological prevention of G.V.H. disease in newborn infants is brought about by one of three means:

(i) By the intravenous or intra-peritoneal inoculation of adult lymphoid cells syngeneic with the neonatal host.

(ii) By the intravenous or intra-peritoneal inoculation of serum antibodies formed in adult members of the host strain, and directed against the antigens of the donor strain, (Siskind and Thomas, 1959; Siskind et al, 1960; Russell, 1960).

(iii) By immunological enhancement.

(i)/

(i) Protection with Isologous Adult Spleen Cells

Here the isologous adult spleen cells probably assist the largely incompetent newborn host to reject the adult homologous lymphoid cells which are inoculated into it.

Siskind and co-workers found a marked protective effect of isologous adult spleen cells injected intra-peritoneally half an hour after the i.p. injection of homologous spleen cells. The two inocula consisted of approximately equal numbers of lymphoid cells (8-12 million cells). The experiment was done with three different strain combinations. The non-protected group had 100% mortality and the protected group had 38% mortality. Isologous adult liver cells did not have any effect. If the "protective" injection of cells is given 24 hours after the homologous cells, no protection was observed (95% mortality). Using a different strain combination (DBA/1→ C57BL/6) Russell found a similar protective effect, as determined by the weight gain assay. He also found that protection occurred even after 48 hours, although it was not as effective as if given earlier. If protection was offered as late as day 5 or 6, no beneficial result occurred.

In two runting animals, protection was attempted by inoculating isologous immune cells given on the 4th day after inoculation of homologous lymphoid cells. This protected the animals quite well till the 20th day. On the 23rd day, the weight of both animals declined but it began to rise again on the 25th day when another inoculation of isologous immune lymphoid cells was given. This result/

result may indicate not only the interaction that is going on between the two cellular types but also the persistence of the inoculated donor cells.

Similarly Silvers and Billingham (1969) studied the protective effect of normal and sensitized lymph node cells, using rats of the Lewis and BN isogenic strains (Ag-B locus incompatible) on the neonatal BN rats developing runt disease, brought on by the injection of adult Lewis lymphoid cells. Following a standard intravenous or intra-peritoneal inoculation of 20 million Lewis node cells into neonatal BN hosts, the animals develop cutaneous lesions by 11-15 days and death inevitably takes place within 20 days. When a similar number of adult BN lymph node cells are administered via a different vein or when BN cells are admixed with an otherwise harmful Lewis lymph node cell, the animals do not develop G.V.H. disease. It was found that the timing of the "protective" injection was very vital; precedence or delay by as little as 4 hours resulted in great impairment of protection. When the harmful and the protective cell injections are given at 24 hour intervals, no protection was noted. The authors suggest the necessary condition for the protection of the host by unsensitized isologous lymphoid cells is that they establish a prompt and intimate confrontation with the homologous target cells. When isologous adult lymphoid cells, sensitized to the donor tissue were injected in the same dosage as normal cells, protection of 92% of the animals was noted even when injected on the third day after the injection of homologous cells. These findings suggest that adult/

adult isologous lymphoid cells provide the immunologically vulnerable neonatal host with a ready-made functionally developed, normal immunological apparatus, to combat the harmful homologous cells.

(ii) Protection with Iso-immune Sera

Using the C57BL/6-DBA/2 combination Siskind et al (1960) found that passive transfer of serum from adult DBA/2 mice, which had been immunized against the donor tissue antigens, could minimize the attack by the grafted cells. The anti-serum was given as a daily intra-peritoneal injection in increasing dosages from days 2-9 after grafting. Litter-mate controls received identical treatment with normal DBA/2 serum. Mortality from G.V.H. disease was 5/18 in the group treated with antiserum as compared with 14/15 in the control group. Russell (1962) has also found a similar beneficial effect of specific anti-donor antiserum in abrogating the harmful effects of the donor lymphoid cells on neonatal mice and rats. Further there is suggestive evidence of a natural passive transfer of protection in the case of rats and mice, born of specifically sensitized mothers (Siskind and Thomas, 1959).

The results of Kren et al (1960, 1962) and of Silvers and Billingham (1969) indicate that isoantisera may be more effective in inhibiting G.V.H. reactivity than sensitized lymphoid cells. Silvers and Billingham inoculated BN neonatal rats by the I.V. route 20 million Lewis lymph node cells. When the animals were 10 days old, each of the 6 subjects received for 5 consecutive days i.p. injections of 0.2 ml. of BN anti-lewis antiserum. All the six animals/

animals grew normally, although two of them received further injections on day 15 and 17. Serum from normal, unsensitized BN donors has no protective effect.

It was also noted by these workers that when the animals appear clinically ill, the specific anti-donor antiserum has the capacity to prevent a fatal outcome in a percentage of these animals. Thus, 10, 13 day old 'runts' were injected daily with BN anti-Lewis serum until they either died or attained the age of 22 days. After this no antiserum was administered. Although all subjects manifested some tautness or lesions of the skin when this treatment was initiated and 6 died when they were 15-19 days old, 4 lived to adulthood, after considerable retardation of their growth. In comparison to this, all 6 BN rats injected daily with normal BN serum, from 6 days of age, died on the 16th day.

This shows that antiserum at the dosage levels administered, is a much more effective therapeutic agent than isologous normal lymphoid cells from normal or even specifically sensitized isologous adult donors.

(iii) Protection by Immunological Enhancement

Immunological enhancement has been defined as the "successful establishment or prolonged survival of an allogeneic graft", (Kaliss, 1962). Initially, enhancement was believed to be a feature associated with certain types of tumours but now there is ample evidence that this phenomenon is also applicable to normal tissues, (Hutchin, 1968; French and Batchelor, 1969). Enhancement can be induced in the host animal/

animal which is to receive a graft by one of two ways:

(a) By active immunization of the host by tissue homogenates from an animal which is intended to be the donor of the graft.

(b) By passive immunization of the host by iso-immune serum prepared against the tissue of the donor animal.

Voisin and Kinsky (1962) applied the principle of enhancement to the amelioration of G.V.H.D. in neonatal mice. They induced G.V.H.D. in neonatal A strain mice by the inoculation of adult CBA spleen cells. To one group of these animals, immune serum prepared by immunizing adult CBA mice against lyophilised newborn strain A tissue, was administered. To the second group of mice, which received the same number of adult CBA lymphoid cells, the same amount of normal CBA serum was administered. To the third group of control normal A strain mice, no cells or serum was administered. It was found that in the first group of animals (which received specific immune serum) 41% of the animals died of G.V.H.D. while in the second group 63% of the animals died of G.V.H.D. In the control group, no animals died. Thus, in his experiments, in the animals which were given specific anti-host immune serum, there were less deaths. This was attributed as being due to the phenomenon of immunological enhancement.

These workers (Voisin et al, 1967, 1968) also attempted to protect against G.V.H.D. in the parent-F_1 hybrid experimental model by /

by inducing enhancement actively and passively. Although the results of active enhancement are disputable, passive enhancement seems to protect a percentage of mice undergoing G.V.H.D.

However, the administration of donor lymphoid cells and specific anti-host antiserum do not always result in enhancement (noted by protection from development of G.V.H.D.). Batchelor and Howard (1965) found that administration of anti-host antiserum to animals inoculated with donor lymphoid cells, may result in the serum acting either synergistically with the donor lymphoid cells (thus aggravating the disease) or antagonistically (thus protecting them) depending on the strain combination employed. Thus with the strain combination C57BL spleen cells $\rightarrow$ $(C57BL \times CBA)F_1$ the anti-host serum acted synergistically with the donor lymphoid cells while in C57BL spleen cells $\rightarrow$ $(C57BL \times A)F_1$, there was evidence of protection against the disease.

The results of these experiments show that enhancement could play a part in protection against G.V.H.D.

2. Prevention by Non-Immunological Mechanisms

The mortality from G.V.H.D. can be reduced surgically by the removal of the spleen. Haller (1964) reported that if neonatal C57BL mice were splenectomised within a few hours of intravenous inoculation of adult CBA spleen cells, the mortality from G.V.H.D. was reduced by half, though no protective effect was seen if the spleen was removed before the injection of donor cells. This would mean that the donor cells primarily settle down in the spleen and initiate/

initiate the immune response. Biozzi et al (1964) studied the influence of splenectomy of the F_1 hybrid inoculated with the parental strain cells. In this case, some reduction in the severity of the G.V.H. reaction occurred if splenectomy was done before the injection of cells. If the splenectomy was performed 2 days after the injection of donor cells, there was a striking reduction of mortality rate and by 8 days there was only some reduction in mortality compared to the control group. These authors consider that splenectomy before the injection of donor cells tends to reduce the effective number of donor cells that can settle in the host, whereas splenectomy after inoculation removes a large focus of the reaction. Amethopterin, which had been shown to reduce the incidence of secondary disease in mouse irradiation chimeras has also proved effective in protection of mice from developing G.V.H. disease (Russell, 1962). The surviving mice were shown to be cell chimeras and were specifically tolerant of skin grafts from the donor strain. The reports on the capacity of 6-mercaptopurine in reducing the severity of G.V.H.R. are not very consistent. Barnes et al (1966) found that the incidence of G.V.H.D. was reduced from 82% to 55% when the drug in a total dose of 60 mg/kg body weight was given over a 6 day period, whereas Schwartz et al (1965) found that this drug causes an acceleration of the disease. Schwartz et al (1965) found that methotrexate treatment suppressed the disease when given at a time when active disease is ordinarily expected. These authors found that total body X-irradiation (400 rads) led to extreme susceptibility/

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susceptibility to G.V.H.D. and that prednisolone ameliorated G.V.H.D. only when given at the same time as parental cells.

However, the reports on the capacity of non-immunologic agents to suppress G.V.H.D. are not sufficient, although this aspect of the study may throw some light on the prevention and treatment of G.V.H.D. in human beings who are given bone marrow or lymphoid cells for certain immunological and haematopoietic disorders.

(G) OCCURRENCE OF RUNT (G.V.H.D.) DISEASE IN HUMAN INFANTS

When it was first known that tolerance can be induced in neonatal animals by the inoculation of viable adult homologous lymphoid cells, it occurred in the minds of many investigators that tolerance could also be produced in prenatal or newborn infants. If this were possible, the tolerant child could in later years have a transplant of some tissue or organ from the donor whose cells were used to produce tolerance. With this end in view, a few newborn babies were injected intramuscularly with nearly 300 million of their parent's leukocytes. None of them became tolerant (Woodruff, 1957). However, Fowler et al (1960) reported marked prolongation of graft survival in some infants transfused at birth with fresh blood for neonatal jaundice and challenged with skin from the blood donor a few days later. None of the subjects have suffered any harm but no deliberate attempts have been made to produce tolerance in infants for fear of inducing G.V.H.D.

There are two situations in which transplantation of cells from blood/

blood or bone marrow is carried out for therapeutic reasons, at the present time. In these two situations, if the transplanted cells are presented with foreign antigens in the host, the possibility of G.V.H.D. cannot be excluded. This hazard can be eliminated as soon as simple and more accurate methods of histocompatibility matching become available.

The two situations are:

(a) Treatment of immunological deficiency diseases such as Swiss type of agammaglobulinaemia or in certain haematological diseases such as aplastic anaemia and acute leukaemia. Here the affected individuals are transplanted with cells from the blood, bone marrow, foetal thymus and foetal liver (Hathaway et al, 1966; Hong et al, 1968; Graw et al, 1970). Here, although immunological restitution is possible, some of the survivors develop graft-versus-host disease which is fatal. An example of a patient dying of G.V.H.D. is that of an 8 month old boy with lymphopaenia (Swiss type), immunological deficiency syndrome and intractable oral moniliasis. The patient had a lymphopaenia and an allogeneic skin graft was not rejected. The lymphocytes did not show any response to in vitro stimulation by typhoid antigen or P.H.A. After transfusion with maternal blood, allogeneic foetal thymus and foetal liver cells (from a male and female foetus) the W.B.C. and lymphocyte counts rose and the previously stable skin graft was rejected completely. The moniliasis disappeared and the in vitro challenge with typhoid antigen resulted in cellular proliferation of lymphocytes. Unfortunately G.V.H. reaction supervened and the patient died from massive/

massive bilateral haemorrhagic pneumonia.

Graw et al (1970) treated seven patients with acute lymphatic leukaemia with a four-day cyclophosphamide regimen or total body irradiation (950-1000 rads). Following this, bone marrow transfusion from HL-A matched donors was given. Three of the seven patients developed G.V.H. reaction ranging from mild to severe degree.

(b) It is an accepted practice now to transfuse with packed red blood cells to foetuses who are victims of Rh sensitization. The intricate technique involves the transfusion of blood through the uterus of the mother and into the peritoneal cavity of the foetus. In this situation also, the lymphocytes of the blood donor can interact with the foreign antigens of the foetus and thus could lead to a G.V.H. reaction in the foetus (Liley, 1963). It is possible to administer repeated intra-peritoneal transfusions of packed blood cells (up to 375 ml) from week 26 of gestation onward (Bowman and Friesen, 1964) and followed by post-partum transfusions. Thus, these repeated transfusions which have immunocompetent cells have the capacity to induce tolerance in the foetus, at least to some degree. However, since large numbers of immunocompetent cells are transfused, it is not surprising that one case of human G.V.H.D. has been reported as a result of this procedure (Naiman et al, 1966). In this case, chromosome studies on peripheral blood lymphocyte cultures have established the existence of chimerism with respect to the cells donated by the father. If the leukocytes are removed before transfusion, which is possible, G.V.H. reaction can be prevented.

1./

1. Possible Natural Occurrence of G.V.H.D.

There is sufficient evidence that Rh sensitization in infants is due to the passage of foetal erythrocytes into the maternal circulation through the placenta. Less is known of the passage of maternal cells into the foetal circulation although there are reports that malignant melanomas sometimes cross the placenta and successfully establish themselves in the foetus. It is thus conceivable that motile maternal cells of the leukocyte series may also pass through the maternal-foetal barrier though it is very difficult to detect this (Billingham, 1964).

If the maternal lymphoid cells enter the foetal circulation, depending on the timing and the magnitude of the postulated infiltration, the foetus may (1) be sensitized by the maternal transplantation antigens which it does not possess, enabling it to destroy the maternal cells. (2) If it is exposed to much larger doses of maternal cells, it may respond by becoming tolerant but it is a rare phenomenon.

Since the peripheral blood contained immunologically competent cells, this indicated that the natural occurrence of a G.V.H.D. syndrome was at least a theoretical possibility.

In 1965 Kadowaki et al reported on a male infant, apparently normal at birth, who began to have various infections after a month with cessation of weight gain, after 3 months. There were abnormalities of the skin, including peculiar lympho-histiocytic infiltrations, transiently enlarged lymph nodes and a significant XX/XY chimerism apparently/

apparently restricted to lymphoid cells of the blood. The baby died after 16 months. G.V.H. reactivity by cells of maternal origin was held to have been partially responsible for this disease and the investigators considered the possibility that primary thymic arrest may have contributed to the host's non-reactivity to the alien cells of maternal origin.

(H) THE CAUSES OF 'RUNTING SYNDROME' CAUSED BY AGENTS OTHER THAN BY VIABLE IMMUNOLOGICALLY COMPETENT CELLS

Since G.V.H.D. was first described in 1957, several at least outwardly similar wasting syndromes have been described in mice and other species subjected to totally unrelated treatments that could not possibly cause G.V.H.D., (Billingham, 1966-67). These causes will now be discussed to consider as far as possible, the extent to which they may represent similar end results attained through different means.

Many workers in the past have seriously considered the possibility that bacterial or viral pathogens, either introduced together with the homologous lymphoid cell inoculum or subsequently gaining entrance into the immunologically deficient host, may cause wasting disease and/or death, (Isacson, 1959). Although the bulk of the evidence weighed highly against the possibility that G.V.H.D. was causally related to infection, it was not quite decisive. However, the demonstration that mice maintained under germ-free conditions are just as susceptible to G.V.H.D. as are normally maintained/

maintained mice, is virtually decisive (McIntire, Bell and Miller, 1964), although it does not entirely rule out the possibility of a viral aetiology.

1. Bacterial Infection and Runting

Brooke (1964) has shown that inoculation of infant mice with Salmonella typhimurium leads to failure to gain weight, splenomegaly and hepatomegaly, associated with areas of necrosis and ultimately death.

It was also noted that this pathogen can be transmitted together with the lymphoid cell suspensions. The factors that are significant in Salmonella induced runting being (1) the number of organisms inoculated, (2) the age of the hosts; susceptibility declining with host age.

There are two criteria by which Salmonella runting can be differentiated from G.V.H.D.

(i) Salmonella runting is associated with hypertrophy of lymphoid tissues whereas G.V.H.D., in its later stages, is associated with lymphoid tissue depletion.

(ii) G.V.H.D. in the adult-neonatal system is preventable by injection of adult isologous lymphoid cells into neonatal hosts together with, or soon after, the inoculation of the potentially harmful allogeneic lymphoid cells, whereas this procedure exerts no protective effect against Salmonella induced runting.

2. Vaccine-induced Wasting Disease

Ekstedt/

Ekstedt and Nishimura (1964) described a wasting syndrome in neonatal mice. They injected by the intra-peritoneal route sterile bacterial vaccines from Staphylococcus aureus and Group A (Type 30) Streptococci. The initial injection was before the animals were 24 hours old and the subsequent course of 18 injections were given every other day for up to 5 weeks. As soon as the injections were stopped, animals which had apparently developed as runts, gained weight and became indistinguishable from normal animals. Severely affected animals displayed marked lymphoid atrophy.

It is noteworthy that injections given 48 hours after birth were innocuous. It was also of significance that germ-free mice were much more resistant to runting than mice maintained conventionally. These authors put forward the view that exposure of these immature animals to such multiplicity of antigens results, in some unknown manner, in depletion of their lymphoid tissues. The enhanced resistance of germ-free mice to this vaccine-induced runting raises the question whether the disease is caused by environmental pathogens in immunologically debilitated animals. It is interesting to know whether isologous adult lymphoid cells would protect those animals which became severely affected by this vaccine treatment.

3. Virus-Induced Wasting Disease

Vandeputte and De Somer (1965) have shown that inoculation of high doses of polyoma virus into neonatal mice of some strains causes a wasting syndrome that, even in its finest details, is very difficult to differentiate from G.V.H.D. (immunological runting).

Inoculation/

Inoculation of very young mice with lymphocytic choriomeningitis virus in mice may also produce a transient form of runting (Hotchin and Weigand, 1961).

4. Subcellular Fractions and Runting

Stanley and Keast (1967) report on preliminary investigations which suggest that subcellular fractions may be able to induce runting indistinguishable from G.V.H.D. (homologous disease). A light mitochondrial fraction prepared from reovirus type 3 induced lymphoma (2731/L) has produced both lymphomas and a runting syndrome. The fraction is apparently cell-free and the runting potential seems to be closely linked with the ribonuclease (RNase) sensitivity of the fraction.

5. Chemical Induction of Runting Syndromes

Miller and Davies (1964) cite the use of 19-nortestosterone to retard the development of the bursa in the chick embryo; this results in a wasting syndrome similar to homologous disease (G.V.H.D.). Batchelor and Chapman (1965) report on thymic involution and lymphopaenia developing as a result of oestradial implantation in mice. The results of the hormone treatment can be modified to give an acute runting syndrome if immunization against an antigen is commenced shortly after the hormone injection. The authors record that the acute disease resembles "runt" disease. It is suggested that the artificial immunization increases lymphocyte turnover and that this accelerates the appearance of the acute disease.

Administration of a single subcutaneous injection of 0.25 mg. of/

of cortisol acetate to newborn mice results in a fatal wasting disease that appears like G.V.H.D. The weights of spleens and thymuses of affected animals are markedly subnormal.

Reed and Jutila (1965) find that the mortality in cortosol acetate-treated neonatal germ-free mice is significantly lower than that in conventionally reared animals. It was also found that the mortality in the germ-free treated animals was raised following monocontamination with Escherichia coli, a component of the normal flora of conventionally maintained mice. This would mean that the normal flora of healthy animals cannot be completely dismissed as possible aetiologic agents in at least some of the wasting syndromes considered. Harmless commensals may become pathogenic in immunologically debilitated or otherwise altered hosts.

6. Post-Natal Thymectomy Disease

When the thymuses are removed at birth an interesting disease develops in mice, and to a lesser and more variable extent in rats and hamsters (Parrott and East, 1964; Miller, 1965). In the mouse, the earlier the thymectomy, the higher the incidence of the wasting syndrome which overtakes its victims when they are a few weeks to several months old. The disease is fatal and closely resembles immunological runting (G.V.H.D.) in its finer details. The affected subjects display hyperplasia of the reticulo-endothelial system, with increased phagocytic activity, diminished immunological competence, terminal lymphoid atrophy and trophic disturbances. The possible causes which could account for this syndrome are infection, auto-immunity/

auto-immunity and loss of some thymus-specific growth-promoting factor. It was found that germ-free neonatally thymectomised mice exhibit none of the signs of the disease for up to 8 months, (Wilson et al, 1964; McIntire et al, 1964). However, if they are contaminated and "conventionalised" at any stage, they develop the disease within 4-8 weeks. Failure to detect pathogenic bacteria in the diseased animals cannot exclude the possibility that a viral or rickettsial agent may be responsible.

It has been mentioned that immunological runt disease (G.V.H.D.) might be due to an early immunologically procured destruction of the thymus, (Parrott and East, 1964). An argument against this possibility is that while immunological runt disease (G.V.H.D.) occurs in germ-free mice (McIntire et al, 1964) that of post-thymectomy wasting syndrome does not. It had also been noted that the post-thymectomy wasting syndrome can be abolished by subsequent inoculation of isologous or homologous thymus grafts (Stutman et al, 1967).

7. Wasting Disease Induced by Heterologous Antilymphocyte Serum

Monaco et al (1966) have shown that a wasting syndrome, similar to homologous disease (G.V.H.D.) and to many of the wasting syndromes described above, develops in adult mice chronically treated with rabbit-antimouse lymphocyte serum. After several weeks the animals become severaly wasted and display progressive weight loss, hunched backs, severe alopecia and variable degrees of diarrhoea associated with profound atrophy of the lymphoid organs. Although the treatment results in elevated levels of circulating corticosterone which/

which contribute to the lympholytic effect, the authors are of the opinion that the latter effect is due to destruction by antiserum.

In none of the syndromes described above, are there at present valid grounds for considering that lymphoid cell per se is sufficient to cause the wasting conditions associated with it. Among the various hypotheses advanced to account for the pathogenesis of these syndromes are: misdirection of immune process in deficient lymphoid tissues, "asynchronous molecular synthesis and deprivation of a postulated trophocytic function of lymphocytes", (Billingham,1966-67).

OBJECTIVES OF THIS THESIS

1. Studies on the Histopathology of G.V.H.D.

There are only a few reports dealing with the histopathology of graft versus host disease. The changes in the lymphoid tissues of rats with G.V.H.D. are fairly clear in the early stages (first 72 hours). Gowans (1962) had shown, using auto-radiographic labelling techniques that the inoculated donor lymphoid cells transform into large pyroninophilic blast cells which subsequently divide into small lymphocytes.

The importance of host cell proliferation in G.V.H. reactions was first demonstrated in chickens by Biggs and Payne (1959) who identified approximately 50 per cent of dividing cells in enlarged female spleens (4 days after inoculation of cockerel blood)as being of host karyotype. In mice, Davies and Doak (1960) using the T6 translocation chromosome marker, estimated the proportion of donor cells among all dividing cells in the spleen. They found that the vast majority of proliferating cells were of host origin. Howard et al (1961) induced G.V.H.D. in the parent - F_1 hybrid model and during the phase of spleen enlargement estimated the proportion of donor cells among all immunologically competent cells by the discriminant spleen assay. By employing 2-strain combinations they noted that 86-100 per cent of the immunologically competent cells were of host origin 1-58 days after the inoculation of parental cells. Howard (1961a) had also shown that in mice undergoing G.V.H.D. the phagocytic index was increased 2-10 fold two weeks after the inoculation of/

of the donor cells. These results show that not only the donor cells show evidence of proliferation but also that of the host. Billingham and Streilein (1969) said that "it is difficult to account at the moment for the massive host cell proliferation in the lymphoid organs and the question remains unresolved whether the incitants are products of antecedent local G.V.H. reactions". It appears that the host cell proliferation occurs in the later stages after the inoculation of donor cells, at a time when the animals manifest evidence of clinical disease.

My preliminary histopathological studies showed that the lymphoid tissues of the G.V.H.D. animals were replaced almost entirely by macrophages and the possibility existed that the massive host cell proliferation was the result of multiplication of macrophages. Hence I decided to make a serial study of the histopathological changes in G.V.H.D. not only to study the tissue lesions in all the organs but also to investigate the purpose of this massive host cell proliferation in the pathogenesis of this disease.

2. Cell Culture Studies in G.V.H.D.

It is known that macrophages play an important rôle in many biological reactions such as chronic infections, homograft rejection, tumour immunity, hypersensitivity and auto-immunity. The rôle of macrophages in G.V.H.D. is not clear; they may play a primary rôle in the pathogenesis of the disease. As regards the role of donor cells in G.V.H.D. there is as yet no sufficiently analysed case to substantiate the theory that the grafted cells can pursue their attack/

attack on the host indefinitely (Simonsen, 1962, p.414). The number of donor cells present in mice with G.V.H.D. is variable; this implies that continued proliferation of donor cells is not necessary in order for the host to experience the most severe of G.V.H. reactions, (Gowans and McGregor, 1965). It is possible that the donor lymphoid cells can cause an altered state of reactivity in the macrophages of animals with G.V.H.D.

My histopathological examination of the tissues of G.V.H.D. mice shows a massive proliferation of macrophages in the lympho-haematopoietic organs with loss of lymphoid cells in lymph nodes. Phagocytosis of lymphocytes or the presence of cell necrosis was not seen in these organs. It may be that the macrophages are responsible for the depletion of lymphocytes in lymph nodes. Certainly there is sufficient evidence in the literature to demonstrate the destructive ability of macrophages. Thus, Weiser et al (1965) had shown that specifically sensitized peritoneal macrophages from mice inoculated intraperitoneally into appropriate mice, produced a fulminating disease in which the animals died in 3-4 days. Tsoi and Weiser (1968) had shown the capacity of immune macrophages to specifically suppress the growth of Sarcoma 1 cells in irradiated intact animals. Granger and Weiser (1964, 1966) had shown that sensitized macrophages had the capacity to destroy specific target fibroblasts in vitro, by a non-phagocytic contact mechanism. Investigating further on the mechanism of action of these cells Weiser et al (1969) had shown that sensitized macrophages possess surface cytophilic antibodies/

antibodies which can be destroyed by trypsin or eluted by heating at 56°C. Immune macrophages "disarmed" of antibody by trypsin can be "rearmed" with antibody by exposure to "immune serum" or heat-elute of immune macrophages. A better understanding of the behaviour of macrophages in G.V.H.D. is made possible by *in vitro* techniques where macrophages from normal or G.V.H.D. animals could be cultured in test tubes in either normal or diseased animals' serum. Koltay *et al* (1965) had shown that the serum of mice undergoing G.V.H.D. had low levels of serum immunoglobulins and also showed the presence of antibodies directed against the host tissue (in the parent - F_1 hybrid experimental model). Thus it is possible that macrophages in G.V.H.D. could interact directly with lymphoid cells and replace them. The central hypothesis underlying my experimental work is that the macrophage of G.V.H.D. has cell-destructive properties.

CHAPTER II

THE PATHOLOGY OF GRAFT-VERSUS-HOST-DISEASE (G.V.H.D.)

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(A) MATERIALS AND METHODS

1. Animals

These were inbred strains of mice. Two strain combinations were used, the first being C57BL females and the first generation hybrid (C57BL x CBA)F_1 derived from mating C57BL males and CBA females, (the parental strains C57BL and CBA were obtained from 'Animal Carriers', London, and the F_1 offsprings were the result of breeding them in the Department of Pathology, University of Edinburgh.) The second strain combination was C57BL/6J males and B6AF_1/J hybrid males (produced by mating C57BL/6J males and A/J females) and was obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A.

The animals were 6-8 weeks old at the time of the experiment and weighed between 20 - 25 g. They were kept in plastic cages (six animals in each cage) and were fed with Spillers autoclaved diet and water. The temperature of the room was thermostatically controlled and maintained between 20° - 25°C.

2. The Production of G.V.H. Disease

The C57BL♀ and C57BL/6J♂ were the donors of lymphoid cells and (C57BL x CBA)F_1 and B6AF_1/J♂ were host animals which were injected with the donor cells (parental lymphoid cells) i.e.

C57BL♀ ⟶ (C57BL x CBA)F_1 males or females.

C57BL/6J♂ ⟶ B6AF_1/J♂

3. Bleeding of the Parental Strains

The mouse is held in the left hand and the thumb and forefinger holds the mouse by the scruff of the neck using sufficient pressure to/

to keep it immobile but not to choke it. The body of the animal is held in the palm of the hand. By making use of a Pasteur pipette with a fairly fine point, the end is inserted into the retro-orbital venous plexus of the eye. It is possible to collect between 0.4 - 0.6 ml. of blood from each animal by this procedure.

4. Preparation of Lymphoid Cell Suspensions for Injection into the Prospective F_1 Hybrids

After killing the animal by cervical dislocation, the fur is well swabbed with 70 per cent ethylalcohol. A horizontal cut is made on the ventral body skin with a pair of scissors and the skin is pulled away towards the head and tail end of the animal. The inner aspect of the integument is thus exposed in which the inguinal, and submandibular lymph nodes are visible. The axillary and brachial lymph nodes are found in the axilla and in the outer surface of the upper limb. A cut is made on the abdominal muscle and the mesenteric, para-aortic lymph nodes and the spleen are removed. The lymph nodes and spleen are collected under sterile conditions in a Petri dish containing tissue culture medium 199 and 10 units of Heparin per ml. The lymph nodes and spleen are cut into small pieces and teased at the same time, using a fine pair of forceps and scalpel. The suspension is then transferred to a loose fitting ground-glass tissue homogenizer and the cells released from the connective tissue framework by using gentle pressure. The suspension containing the cells was filtered through a fine-mesh stainless steel sieve to remove the coarse tissue debris. The tissue culture medium containing/

containing the lymphoid cells is then allowed to remain in a beaker for about 10 minutes thus permitting the relatively fine tissue fragments to settle on the bottom. This is very important since the fine tissue fragments can embolise in the capillaries of the lungs of the host animals and cause death.

The supernatant was pipetted into conical centrifuge tubes and spun in a refrigerated (4°C) M.S.E. centrifuge at 50 g. for 10 minutes. At the bottom, the lymphoid cells form a pellet and the supernatant was pipetted off. It is very necessary to pipette off this supernatant since it contains cytoplasmic constituents which are toxic to the host animal when given by the intravenous route.

The cell pellet was brought into suspension again by adding a small volume of heparinized tissue culture medium 199. This is done by mixing the suspension with a Pasteur pipette. The suspension was then drawn into a plastic syringe through a fine bore needle (23 G) and was gently ejected out of it into a small beaker. This procedure was repeated two or three times till a suspension of single lymphoid cells was obtained.

Now, using a standard haemocytometer and improved neubauer cell counting chamber, the total cell count was made and the suspension adjusted to the volume which contained the required number of cells (see Enumeration of Lymphoid Cells).

To determine the viability of the lymphoid cell suspension, one drop of 0.4 per cent Trypan blue and one drop of the suspension was mixed on a glass slide. After a few minutes the dead cells appeared as/

as large spherical light-bluish stained cells. The viable cells are smaller and are unstained. A total of 100 cells was counted using the counting chamber and the number of dead cells was determined. At the end of two hours after the removal of lymph nodes from the donor mice, 90-95 per cent of the cells were viable.

5. Enumeration of Lymphoid Cells

Apparatus: An improved Neubauer cell counting chamber and a standard red cell pipette of known capacity. The lymphoid cell suspension was drawn into the pipette to the 0.5 mark, and the white cell diluting fluid was drawn up to the 101 mark and the contents thoroughly mixed. The diluting fluid was made up as follows.

Glacial acetic acid ..... 1.5 ml

1 per cent solution of gentian violet in water.. 1.0 ml

Distilled water ..... 98.0 ml

Method: The counting chamber and the special thick coverslip supplied with it were cleaned and the coverslip placed centrally over the bar of the counting chamber. The fluid in the pipette was thoroughly mixed and about one-third of the contents of the bulb was expelled and discarded. The fluid in the pipette was allowed to run under the coverslip by capillary attraction. Bubbles must be avoided and the fluid just fill the space under the coverslip between the two grooves.

Counting: All the leucocytes contained in an area of 4 sq.mm. were counted and the average for 1 sq.mm. obtained. With the Neubauer ruling the four squares, each of 1 sq.mm., at the corners of the chamber/

chamber were used. Since the depth of the chamber is 1/10 mm., the number of cells found on 1 sq.mm. is that contained in 1/10 c.mm. of diluted blood.

Calculation: The average number of cells in the whole of the ruled area of the counting chamber was the number present in 1/10 c.mm. of diluted lymphoid suspension. Since the dilution of the lymphoid suspension is 1/200, the number of cells counted must be multiplied by 2,000.

6. Technique of Injection into Animals

Each (CBA x C57BL)F_1 hybrid received lymphoid cells obtained from two donor mice (140-160 million cells) and each B6AF_1/J mouse received lymphoid cells from three donor mice (250-300 million cells). The former group of animals received the cells intravenously in a single injection in a volume of 0.3 ml. while the latter group were injected intravenously on two consecutive days using a dose of 125 x 10^6 cells in a volume of 0.3 ml. Preliminary experiments indicated that in the first strain combination, the animals became emaciated and diedby 20 days whereas in the second strain combination the animals diedby 30-35 days when the above dose schedule was used.

The host animal was enclosed in a small cylindrical wire mesh cage which was fixed on to a retort stand. The tail was warmed with cotton wool soaked in water. The injection was given very slowly through one of the lateral tail veins. This procedure was repeated with the rest of the experimental animals. Twenty-four animals were inoculated in this manner and a similar number of animals were kept as/

as controls.

The control and test animals were weighed daily for two weeks before the injection of cells, and daily thereafter. At the time of weighing the animals were examined for their gait, the condition of the fur and signs of diarrhoea. The control animals were also examined at the same time.

The first batch of six animals was killed on the 5th day after the injection of cells. The second batch was killed on the 10th day, the third batch on the 15th day and the last batch on the 19th or 20th day. Serial pathological study was made on the (CBA x C57BL)F_1 hybrids which were inoculated with C57BL cells.

7. Tissues for Histology

The organs and tissues were examined naked eye and notes were made describing them. Small pieces of tissue were then taken from the organs and were fixed in 10 per cent buffered neutral formalin for 12 hours and then fixed in 5 per cent corrosive sublimate for 48 hours.

The following organs and tissues were removed and fixed: lymph nodes (inguinal, axillary, submandibular, mesenteric and lumbar), liver, spleen, kidney, pancreas, the alimentary tract, mesentery, ovaries or testes, thymus, lung, heart, sternum, intercostal muscle, skin, brain, thyroid, salivary gland, adrenal and pituitary.

Similar organs and tissues were removed from five normal litter mates and were fixed in a similar manner.

8. Injection of Colloidal Carbon into Normal and GVHD Mice

Materials: /

Materials: 1. A glass and metal tuberculin syringe. This is preferable to the all-glass kind. Slight pressure on the metal plunger enables the graduation marks to be easily seen despite the presence of carbon.

2. A beaker of warm water and cotton wool for warming the tail of the mouse.

3. A suspension of carbon in a stoppered tube at 37°C. The carbon is obtained from Gunther Wagner, Pelikan Werke, Hanover, Germany, and is stored in a stock solution of 64 mg/ml with a small amount of gelatin. The stock solution is melted in boiling water, thoroughly shaken and diluted to a concentration of either 8 mg/ml or 16 mg/ml.

Three normal (CBA x C57BL)F_1 mice and three (CBA x C57BL)F_1 mice 12 and 17 days after the inoculation of parental cells were used to inoculate carbon.

Method of Injection: To decide the dose of the carbon, the weight of the mouse was divided by 100, e.g. the dose of carbon for a 21 g. mouse is 0.21 ml. The carbon was diluted to a concentration of 16 mg/ml and on day 1 all the nine mice were injected subcutaneously into the four flanks with 0.8 ml. of carbon. This would result in the carbon passing up the lymphatics into the axillary and inguinal lymph nodes. On days 2, 3 and 4 each animal received 0.2-0.25 ml of carbon consecutively each day. The injection was given into the lateral tail vein and the first injection was given at the root of the tail so that the veins would be visible in the distal part of the tail/

tail to give the second and third injections.

9. Processing of Tissues

Lymph nodes and endocrine glands were processed by hand; the procedure was as follows:

1.	Methylated spirits	-	1½ hours at 9 o'clock
2.	Alcohol	-	1 hour
3.	Alcohol	-	1 hour
4.	Chloroform	-	1 hour
5.	Chloroform	-	1 hour
6.	Wax	-	1 hour
7.	Wax	-	1½ hours

Embedded at 5 o'clock.

The rest of the tissues were processed in the continuous running Histokinette (Hendrey Relays and Electrical Equipment Ltd., Bath Road, Slough, Bucks.)

The procedure was as follows:

1.	70% spirit	-	6 hours
2.	Methylated spirit	-	1 hour
3.	74 O.P. (99.18%)	-	1 hour
4.	74 O.P.	-	1 hour
5.	Absolute Alcohol	-	1 hour
6.	Absolute Alcohol	-	1 hour
7.	Equal parts of Alcohol and Chloroform	-	1 hour
8.	Chloroform	-	1 hour

9./

9. Chloroform - 1 hour

10. Benzene - 1 hour

11. Wax - 1 hour

12. Wax - 1 hour

Embedding was done the following morning.

Cutting of Tissues

The wax blocks were then cut on a Cambridge rotary rocker microtome and the sections cut at 5μ.

Staining of Tissues

The sections were routinely stained by haematoxylin and eosin. The special stains only will be described.

M.S.B. (Martius Scarlet Blue) Method for Fibrin (after Lendrum et al, 1962; see Drury and Wallington, 1967).

Fixation

Primary or secondary formol-sublimate, up to 8 weeks.

Sections

Thin paraffin sections.

Technique

1. Sections are taken to water.
2. Stain nuclei with celestine blue - haemalum.
3. Rinse in tap water.
4. Differentiate nuclei in 0.25 per cent hydrochloric acid in 70 per cent alcohol.
5. Wash well in tap water.
6. Rinse in 95 per cent alcohol and stain with 0.5 per cent martius yellow in 95 per cent alcohol containing 2 per cent phosphotungstic acid for 2 minutes.

7./

7. Rinse in distilled water and stain in 1 per cent brilliant crystal scarlet G in 2.5 per cent acetic acid for 10 minutes (see note 4).

8. Rinse in distilled water and treat with 1 per cent phosphotungstic acid to fix and differentiate the red stain for up to 5 minutes.

9. Rinse with distilled water and stain in 0.5 per cent soluble blue in 1 per cent acetic acid for up to 10 minutes.

10. Rinse in 1 per cent acetic acid, blot, dehydrate in absolute alcohol, clear in xylene and mount in a synthetic resin medium.

Results

Nuclei - black; erythrocytes - yellow; fibrin - red; connective tissue - blue.

Notes

1. In the absence of the recommended fixation, Lendrum et al advise that sections be treated with trichlorethylene (de-greasing) for 24-48 hours immediately after de-waxing of sections in xylene. This may with advantage be followed by mordanting for a few hours in alcoholic picric acid containing 3 per cent mercuric chloride.

2. Thin, flat sections are essential, it being difficult to remove the red stain from dense collagen in thick sections.

3./

3. The method is highly selective for fibrin though not specific; Paneth cell granules and other cytoplasmic inclusions may stain red. Bone may also retain the red stain.

4. Brilliant crystal scarlet 6R has several synonyms. They are acid red 44, crystal ponceau 6R, Naphthalene scarlet 6R, ponceau 6R.

Methyl Violet - Crystal Violet Method (after Jurgens, 1875; see Drury and Wallington, 1967)

Fixation - Not critical

Carnoy, formal-sublimate and 10 per cent formal-saline are suitable but long immersion in the latter may cause failure of stain.

Sections

Paraffin or frozen sections are suitable, the latter giving superior results. Cryostat sections of fresh tissue are particularly recommended.

Technique

1. Take sections to water.
2. Stain on 1 per cent aqueous methyl or crystal violet for 2-5 minutes.
3. Wash in water and examine with the microscope (see note 1).
4. Differentiate in 0.5 - 1 per cent acetic acid until Amyloid is purplish-red or red in good contrast with the blue - violet staining of nuclei and normal tissue.

5./

5. Wash in running tap water for at least 5 minutes.

6. Drain the slide and while the section is still moist, mount with modified Apathy's gum syrup (note 5). Ring coverslip with nail varnish.

Results

Amyloid - purplish-red to red.

Nuclei, cytoplasm and connective tissue - shades of blue - violet.

Notes

1. It is necessary to examine sections microscopically at stage 3 because in some cases, little or no differentiation in acid is required, amyloid showing bright and sharp metachromatic staining at this point.

2. It is not possible to dehydrate sections and mount in resinous media because the stain is readily soluble in alcohol. Mallory (1938) however, quotes Mayer's method whereby paraffin sections are floated on the surface of the stain, differentiator and water, before being affixed to slides, de-waxed in xylene and mounted in a resinous medium.

3. Numerous modifications of the above basic method have been devised. All aim at avoiding fading by preventing 'bleeding' of the stain in the aqueous mounting media. With the mounting medium recommended above and protection of the slide from strong light, there should be little fading for some years.

4./

4. Blue glass filters should be removed from microscope or light source when examining metachromatic staining.

5. Apathy's (1892) Mountant (R.I. 1.52)

Preparation

Pure gum arabic (crystals, not powder) 50 g.

Pure cane sugar ..... 50 g.

Distilled water ..... 50 ml.

Thymol ..... 0.05 g.

Dissolve with the aid of gentle heat and store in tightly stoppered jars or screw-cap collapsible tubes.

Alkaline Congo Red Method for Amyloid (Puchtler, Sweat and Levine, 1962; see Drury and Wallington, 1967).

Fixation

Carnoy's fluid or ethanol are recommended but results are adequate after formalin and mercuric chloride fixatives.

Sections

Paraffin or frozen sections

Preparation

Alkaline solution

Stock solution: 80 per cent alcohol saturated with sodium chloride.

Working solution: Add 0.5 ml. 1% aqueous sodium hydroxide to 50 ml. of the stock solution. Filter and use within 15 minutes.

Technique

1. Take sections to water.

2./

2. Stain nuclei in alum haematoxylin.

3. Wash, differentiate, and wash in water.

4. Treat with alkaline solution for 20 minutes.

5. Stain in Congo red for 20 minutes.

6. Dehydrate with three brief rinses with absolute alcohol.

7. Clear in xylene and mount in a synthetic resin medium.

Results

Nuclei - blue; amyloid - deep pink to red; elastic fibres and some cytoplasmic granules may be stained pink to red.

Notes

1. This method is highly selective and requires no differentiation.

2. Both stock solutions will keep for several months.

3. Freshly prepared Congo red solution should be allowed to stand for 24 hours before use. It must be saturated with the dye.

4. The working solutions are not stable.

Thioflavine T Method for Amyloid (see Drury and Wallington, 1967).

Fixation

10 per cent formal-saline is suitable. Fixatives containing salts of heavy metals are less satisfactory.

Sections

Paraffin or frozen sections may be used.

Technique

1. Sections are taken to water.

2./

2. Stain with alum haematoxylin for 2 minutes to quench fluorescence of nuclei. Differentiation is not necessary.
3. Wash briefly in water.
4. Stain in freshly filtered 1 per cent aqueous thioflavine T for 3 minutes.
5. Rinse in water.
6. Treat with 1 per cent acetic acid for 20 minutes (this is to reduce background fluorescence).
7. Wash well in running water.
8. Mount in Apathy's gum syrup.

Results

Amyloid and mast cells fluoresce bright yellow against an olive-green background.

Notes

1. Solutions of thioflavine T do not keep indefinitely and a control section should always be used.
2. Sections should be thin; with thick sections, bone may fluoresce strongly and extension of treatment with acetic acid and water may be required.
3. Hobbs and Morgan (1963) have favourably compared results using this technique with those of the other methods for amyloid but McKinney and Grubb (1965) stated that false positive results may be given by hyaline substances due to the persistence after staining/

staining of their strong autofluorescence.

The Methyl Green - Pyronin Stain for DNA and RNA (Pappenheim, 1899, 1901; Unna, 1902, 1913; see Drury and Wallington, 1967).

The following modification (Trevan and Sharrock, 1951) is suitable for material fixed in neutral formalin.

Fixation

Neutral formalin and other fixatives, including alcohol, Carnoy and alcoholic formalin.

Sections

Paraffin sections

Preparation

Solution A

Make a 2 per cent aqueous solution of methyl green and extract all the methyl violet with chloroform. Wash the solution with several changes of chloroform in a separating funnel until the chloroform remains colourless. This washed solution is stable and can be used as a stock solution from which the following is prepared fresh:

2 per cent aqueous methyl green (chloroform washed) .... 10 ml.

5 per cent aqueous pyronin Y 17.5

Distilled water ..... 250 ml.

Solution B

M/5 acetate buffer solution, pH 4.8. Mix 119 ml. of 0.1 M sodium acetate with 81 ml., 0.1 M acetic acid. The original method also used orange G1 (31 ml. of 1 per cent aqueous orange G1 in 200 ml of the buffer solution) but this is not essential.

The/

The working solution is prepared by mixing equal volumes of solutions A and B in a Coplin jar.

Technique

1. Take paraffin sections to water.
2. Rinse in distilled water and blot dry.
3. Stain for 20-30 minutes in the working solution of methyl green and pyronin.
4. Rinse in distilled water for a few seconds and blot lightly until almost dry. Some pyronin is removed during this washing and the correct time should be determined by trial and error.
5. Dehydrate in acetone for not more than one minute.
6. Rinse in equal parts of acetone and xylene and clear in pure xylene.
7. Mount in a synthetic resin medium.

Results

DNA (Chromatin) - green or blue green.

RNA (Nucleoli) - rose red; (granules) - dark rose red; (plasma cell cytoplasm) - purple.

(B) RESULTS

1. Changes in Weight of Control and Test Mice Inoculated with Parental Lymphoid Cells

A graph was drawn with days after the inoculation of cells as abscissae and the weights of the mice as ordinate. The (C57BL x CBA)F_1 mice inoculated with parental lymphoid cells (test mice) showed (Fig. 1) that the average of six test mice had a significantly different weight curve, compared to that of the average of five control mice. Initially there was a fall in weight of 2 g. over the first three days and thereafter the animals gained weight till day 6 and then the weights fell off again till day 10. From then on, the trend was one of progressive loss of weight even though there were two small peaks of increase in weight. From day 14 onwards a significant loss of weight was noted in comparison to the control animals. On day 20 the differencesin weight between the test and control animals were most marked and they differed by as much as 6 g.

The B6AF_1/J hybrid mice inoculated with C57BL/6J (parental) lymphoid cells showed a similar pattern of weight changes as the first strain of animals (Fig.2). The weights of the control and the test mice were not significantly different till day 8 after the inoculation of cells. From then on until day 25 there was a significant difference in weight between the control and test mice.

The difference in the graphs of the two strains of mice was that, in the latter combination, the disease process lasted longer/

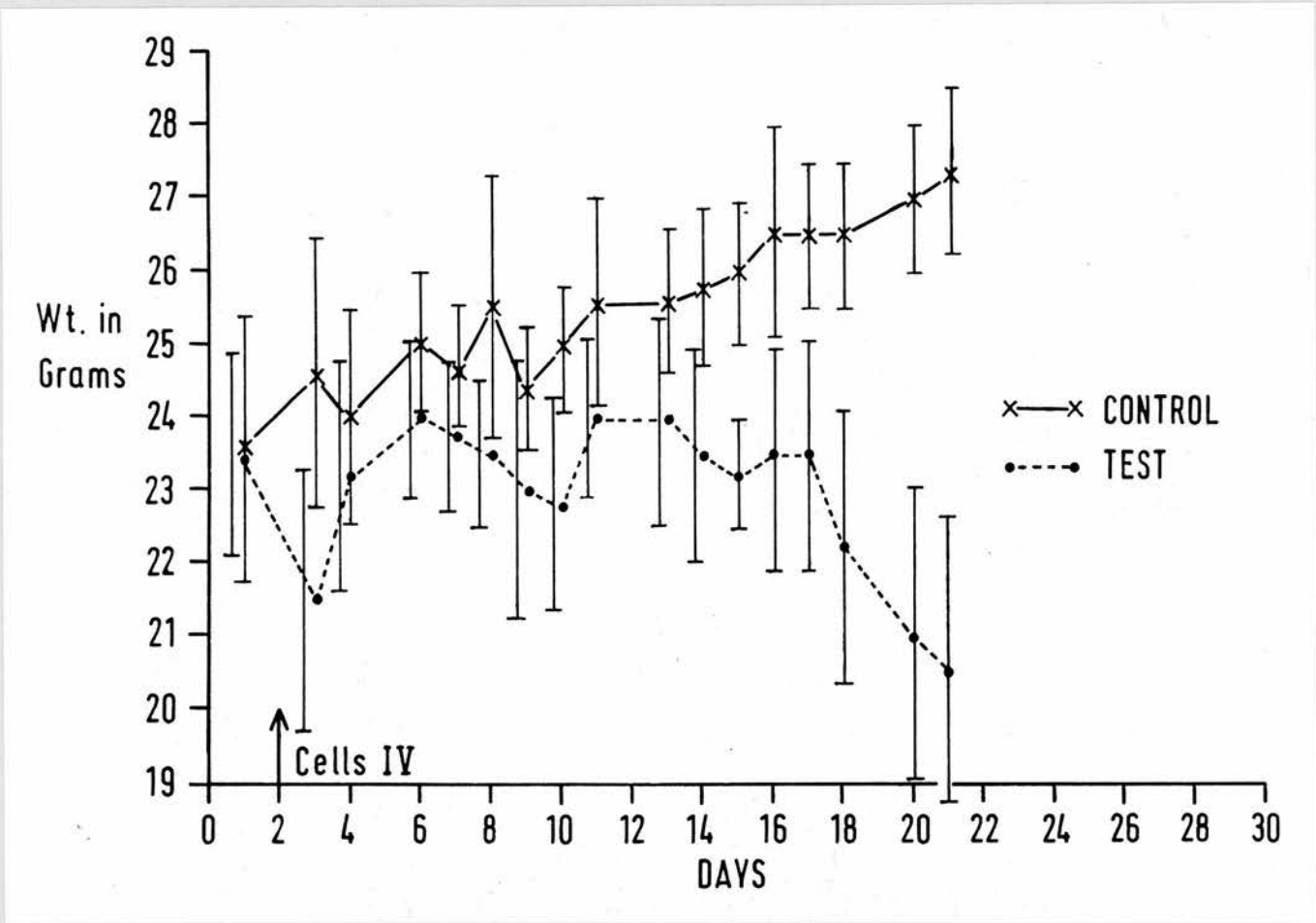

Fig. 1. Changes in weight with time of an average of 5 normal (CBA and C57BL)F_1 mice and that of 6 syngeneic mice with GVHD.

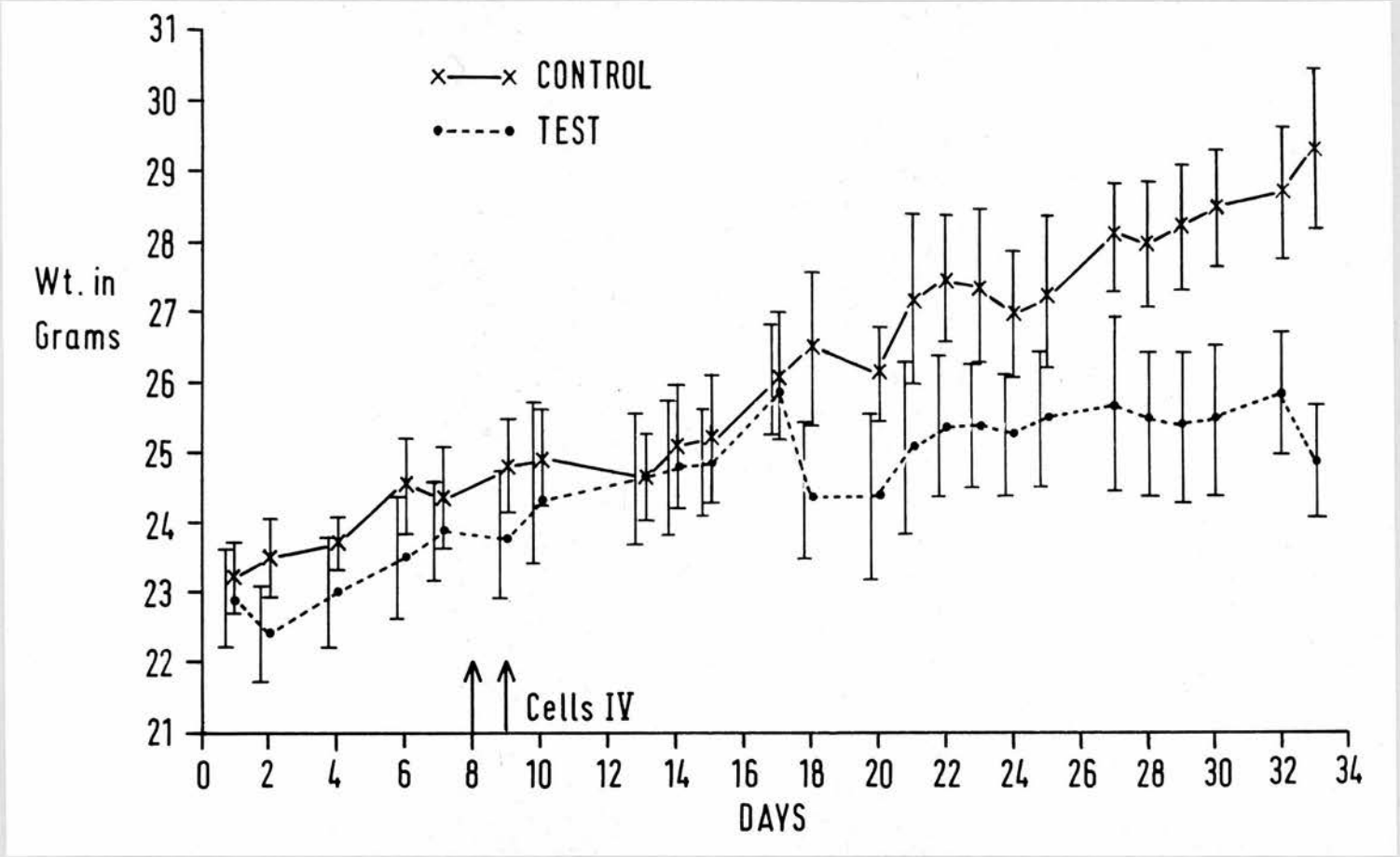

Fig. 2. Changes in weight with time of an average of 12 BSAF$_1$/J normal mice and that of 13 syngeneic mice with GVHD. The mice were sacrificed on day 25 after the inoculation of parental lymphoid cells. Other experiments showed that from day 25 onwards a progressive loss of weight occurred in the test mice.

THE INDIVIDUAL WEIGHTS (IN GRAMS) OF (C57BL x CBA)F_1 NORMAL MICE, THEIR MEAN WEIGHTS AND THE STANDARD DEVIATION

Day	Wt.of 1st mouse	Wt.of 2nd mouse	Wt.of 3rd mouse	Wt.of 4th mouse	Wt.of 5th mouse	Mean wt. of 5 mice	SD+
1	22	27	22	24	23	23.6	1.89
2							
3	24	28	23	25	23	24.6	1.89
4	23	27	22	24	24	24.0	1.54
5							
6	27	25	25	24	24	25.0	1.09
7	24	24	26	24	25	24.6	0.89
8	25	25	29	24	25	25.6	1.78
9	24	24	24	24	26	24.4	0.89
10	25	24	26	26	24	25.0	0.89
11	25	24	26	25	28	25.6	1.41
13	24	27	26	26	25	25.6	1.09
14	25	27	27	24	26	25.8	1.14
15	28	26	25	25	26	26.0	1.09
16	29	26	25	26	27	26.6	1.41
17	28	27	27	25	26	26.6	1.09
18	27	28	27	25	26	26.6	1.09
20	28	26	28	26	27	27.0	1.00
21	28	25	28	28	28	27.4	1.26

THE INDIVIDUAL WEIGHTS (IN GRAMS) OF (C57BL x CBA)F1 GVHD MICE, THEIR MEAN WEIGHTS AND THE STANDARD DEVIATION. LYMPHOID CELLS WERE INOCULATED ON DAY 2

Day	Wt. of 1st mouse	Wt. of 2nd mouse	Wt. of 3rd mouse	Wt. of 4th mouse	Wt. of 5th mouse	Wt. of 6th mouse	Mean Weight of six mice	SD+
1	26	23	23	22	22	23	23.5	1.41
2			CELLS INOCULATED					
3	25	19	21	21	22	21	21.5	1.87
4	26	21	24	22	23	23	23.2	1.58
6	24	23	26	23	23	25	24.0	1.14
7	25	22	24	24	23	25	23.8	1.09
8	25	22	24	23	23	24	23.5	1.09
9	26	20	23	23	22	24	23.0	1.81
10	25	20	23	23	23	23	22.8	1.51
11	26	22	24	24	24	24	24.0	1.14
13	26	22	23	25	23	25	24.0	1.41
14	26	22	24	23	22	24	23.5	1.48
15	24	22	23	24	23	23	23.2	0.70
16	26	21	23	24	23	24	23.5	1.58
17	26	22	22	25	23	23	23.5	1.58
18	26	20	21	22	22	22	22.2	1.87
20	25	18	20	21	22	20	21.0	2.12
21	25	18	20	19	21	20	20.5	2.28

THE INDIVIDUAL WEIGHTS (IN GRAMS) OF B6AF$_1$/J NORMAL HYBRID MICE
THEIR MEAN WEIGHTS AND THE STANDARD DEVIATION

Day	Wt. 1st mouse	Wt. 2nd mouse	Wt. 3rd mouse	Wt. 4th mouse	Wt. 5th mouse	Wt. 6th mouse	Wt. 7th mouse	Wt. 8th mouse	Wt. 9th mouse	Wt. 10th mouse	Wt. 11th mouse	Wt. 12th mouse	Mean wt. of 12 normal mice	SD±
1	24	24	22	22	24	24	24	21	22	24	24	24	23.2	1.12
2	24	23	24	22	22	24	24	24	23	23	24	24	23.5	0.86
4	25	23	24	22	22	24	21	26	24	25	24	25	23.7	1.44
6	26	22	26	27	23	24	24	25	24	24	26	24	24.5	1.44
7	27	23	26	22	25	24	24	23	26	23	25	24	24.3	1.47
9	23	23	27	24	26	27	26	24	24	25	23	26	24.8	1.47
10	26	27	25	23	27	24	25	26	26	23	24	23	24.9	1.44
13	26	26	23	24	26	24	26	25	25	23	24	24	24.6	1.15
14	28	22	24	27	24	25	22	26	26	27	24	26	25.1	1.85
15	27	23	23	24	26	26	27	22	26	24	26	28	25.2	1.82
17	28	25	27	23	27	24	27	28	26	28	26	24	26.1	1.85
18	28	27	23	29	26	23	28	27	26	29	28	24	26.5	2.12
20	27	28	28	24	25	25	27	26	24	26	27	26	26.1	1.32
21	24	28	26	23	30	27	30	26	28	27	29	30	27.2	2.23
22	27	30	26	28	25	24	30	29	25	29	30	28	27.5	1.98
23	25	29	25	27	24	30	28	28	30	26	30	28	27.4	2.16
24	28	30	24	25	28	24	27	28	26	27	30	28	27.0	1.94
25	27	30	25	26	24	28	27	30	29	27	30	28	27.2	2.02
27	29	28	28	26	25	29	29	30	29	30	30	27	28.2	1.55
28	27	25	26	25	30	28	28	29	30	31	28	29	28.0	1.87
29	28	30	25	26	26	30	30	29	30	30	28	28	28.3	1.77
30	27	26	31	28	27	26	30	30	29	28	30	30	28.5	1.73
32	31	28	24	28	29	28	32	28	31	30	28	29	28.8	1.89
33	28	32	25	31	28	29	30	32	32	28	29	30	29.5	2.08

THE INDIVIDUAL WEIGHTS (IN GRAMS) OF B6AF$_1$/J HYBRID GVHD MICE, THEIR MEAN WEIGHTS AND THE STANDARD DEVIATION. ON DAY 8 and 9 LYMPHOID CELLS WERE INOCULATED I.V.

Day	Wt. 1st mouse	Wt. 2nd mouse	Wt. 3rd mouse	Wt. 4th mouse	Wt. 5th mouse	Wt. 6th mouse	Wt. 7th mouse	Wt. 8th mouse	Wt. 9th mouse	Wt. 10th mouse	Wt. 11th mouse	Wt. 12th mouse	Wt. 13th mouse	Mean wt. of 13 mice	SD±
1	24	24	24	20	22	22	23	23	22	24	22	24	23	22.9	1.48
2	21	21	22	20	23	21	24	24	20	24	22	24	23	22.4	1.49
4	21	23	24	20	24	22	25	25	23	25	21	24	22	23.0	1.62
6	22	23	23	19	23	23	26	26	23	25	23	25	24	23.5	1.84
7	25	24	25	20	24	22	24	24	23	24	23	25	24	23.9	1.48
8						C E L L S	G I V E N	I.V.							
9	24	23	24	19	23	23	25	26	25	25	23	26	24	23.8	1.75
10	24	23	24	20	24	24	25	27	26	26	24	26	24	24.3	1.73
13	25	24	26	21	24	22	26	28	26	26	24	25	24	24.6	1.80
14	25	24	24	20	25	23	26	27	26	27	24	27	26	24.8	1.90
15	26	24	26	21	25	23	26	26	26	26	24	26	26	24.8	1.52
17	26	24	26	22	24	23	26	26	27	28	26	26	26	26.0	1.71
18	26	24	25	22	23	22	25	25	27	29	24	25	26	24.4	2.05
20	26	24	26	23	20	20	23	26	26	29	25	26	25	24.4	2.50
21	27	25	28	23	20	20	25	26	28	28	25	26	27	25.1	2.59
22	26	25	27	23	22	22	25	28	27	29	26	27	26	25.4	2.18
23	27	25	27	23	25	23	26	26	27	29	26	27	26	25.4	1.84
24	26	24	26	22	25	23	26	26	26	29	25	26	25	25.3	1.75
25	27	26	27	22	24	23	25	28	26	29	25	26	24	25.5	1.98
27	29	27	28	21	24	22	26	29	27	29	24	26	26	25.8	2.51
28	28	26	27	22	23	22	25	28	25	28	24	27	26	25.5	2.06
29	29	24	27	22	23	22	26	27	26	27	26	26	27	25.4	2.13
30	29	24	27	21	24	23	26	26	26	28	26	27	26	25.5	2.15
32	28	25	27	22	24	24	27	28	26	27	26	26	27	25.9	1.86
33	27	26	27	21	24	23	25	25	26	26	24	26	24	24.9	1.73

longer (30-35 days for 80 per cent of the mice to die of the disease).

2. Clinical Examination of Animals with G.V.H.D.

The animals appear physically normal till the 6th day in the (C57BL x CBA)F_1 test mice and up to day 18 in the B6AF_1/J strain. Thereafter the animals developed a ruffled fur which was most obvious during the terminal stages of the illness. The fine gloss of the integument seen in the control mice is not noted in mice with disease. As they begin to waste, they appear to be less active and seem not to be interested in the surroundings. They tolerate handling and interference much more than control mice. The wasting when marked was confined mainly to the thorax, neck and face. The neck seems to be 'drawn in' so that it appears short. The back became hunched and the mice keep their four feet close to each other and this results in the development of the hunched posture. The animals also walk with a characteristic mincing gait. They appeared emaciated and clinically 'sick' even though the weights had gone down by only 2 or 3 g. Diarrhoea is an uncommon feature and the packed cell volume was not markedly decreased in these strain combinations.

Examination of the leucocytes during the terminal stages of the disease showed that they had a normal or slightly increased leucocyte count but the total lymphocyte count in six animals was reduced by 50 per cent on the 15th day after the injection of cells.

3. Macroscopic and Microscopic Appearances of Normal Mouse Tissues and Tissues from G.V.H.D. Mice

The Functional Regions of a Normal Lymph Node

Six/

Six regions in the lymph nodes can be distinguished (Fig. 3).

Cortex: This is divided into an outer cortex and a mid and deep cortex.

(a) Outer cortex - This consists of a small rim of closely packed small lymphocytes. The follicles are present in this region.

(b) Mid and deep cortex - This part is found between the outer cortex and the medulla. It occupies a relatively large area of the node. It has a uniform appearance and the lymphocytes are spread out in a loose wide sheet rather than as nodular aggregations of lymphocytes. This area has been called the "para-cortical" area by Cort and Turk (1965).

(c) Follicles - These are seen as condensations of small lymphocytes in the outer cortex. The cells are closely packed together and appear as nodules which are distinct from the surrounding cortex.

(d) Germinal centres - In some follicles early germinal centre formation is seen. These are lightly staining, cellular areas inside the follicle. The cells are large, have vesicular nuclei and are regarded as lymphoblasts.

(e) Medulla - This area is found between the hilum and the deep cortex. It consists of cords of lymphocytes which are packed between the many sinuses found in that region.

(f) Sinuses - The cortical sinuses are seen very easily and in some nodes a few sinuses are seen in the para-cortical areas. Macrophages line these sinuses and a few of them are also seen scattered throughout the para-cortical areas.

Macroscopic/

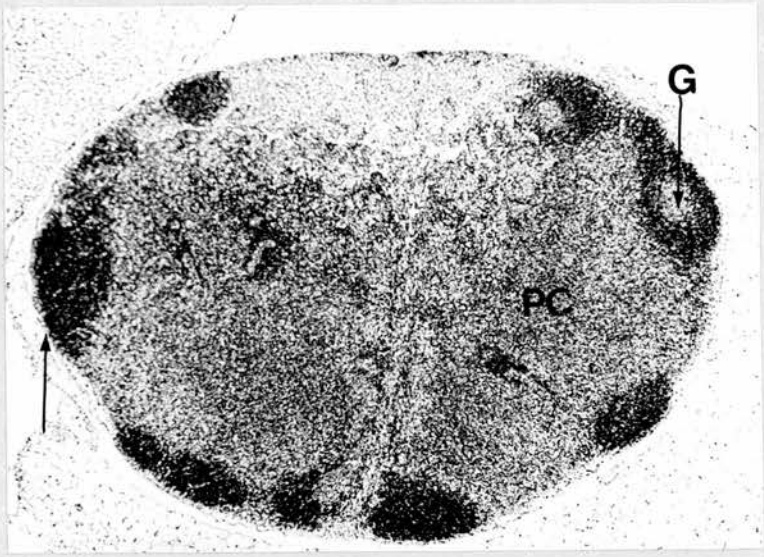

Fig. 3. Normal (C57BL x CBA)F_1 mouse lymph node. This shows the lymphoid follicles in the outer cortex (arrow) and germinal centres (G) are seen in two of them. The mid- and deep cortex ('paracortical area', P.C.) contains loose sheets of lymphoid cells.

H. and E. x 50

Fig. 4. Part of a lymph node from a 5 day old GVHD mouse. This shows the marked expansion of the 'paracortical' area (P.C.).

H. and E. x 110

Macroscopic Appearance of Lymph Nodes in G.V.H.D.

The lymph nodes removed on the 5th day after the inoculation of parental lymphoid cells were twice as large as those from normal control animals. They were normal in shape and colour but were firmer than usual. The lymph nodes examined from animals sacrificed on the 10th and 15th day after the inoculation of cells, were three-quarters the size of the corresponding lymph nodes. Their shape was normal; they were usually adherent to surrounding tissue which sometimes made their removal difficult. They were amber-coloured and firm. Lymph nodes removed from animals during the terminal stages of G.V.H.D. were extremely small and were visible only as small yellowish-brown spots. They were firm and adherent to the surrounding fibro-fatty tissue.

Microscopic Appearance of Lymph Nodes from G.V.H.D. Mice

Histological examination of the lymph nodes removed on the 5th day from mice undergoing G.V.H.D. showed a marked expansion and an increase in cellularity of the para-cortical areas (Figs. 4, 5). The lymphoid cells were seen as diffuse sheets or as closely packed groups of dark, round cells. In some areas clusters of many large cells 2-3 times the size of small lymphocytes were seen. These cells had big vesicular nuclei and had the appearance of lymphoblasts (Figs. 6, 7). Methyl green-pyronin staining showed these cells had an intensely pyroninophilic cytoplasm, a prominent pyroninophilic nucleolus, and a large pale vesicular nucleus with a fine chromatin structure. I have called these cells "large pyroninophilic cells" (Figs. 8, 9).

Examination/

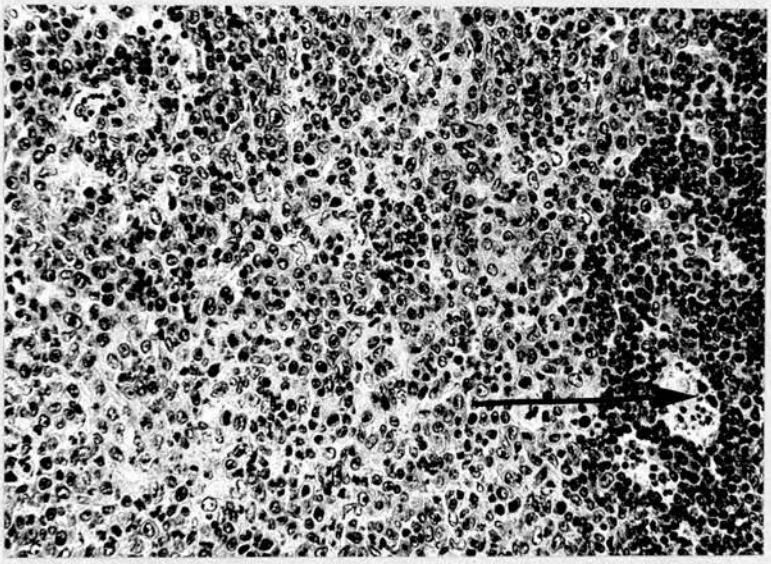

Fig. 5. High power of Fig. 4 showing the 'paracortical area'. The cells are large, have vesicular nuclei and abundant cytoplasm. The adjacent lymphoid nodule shows tingible bodies (arrow).

H. and E. x 250

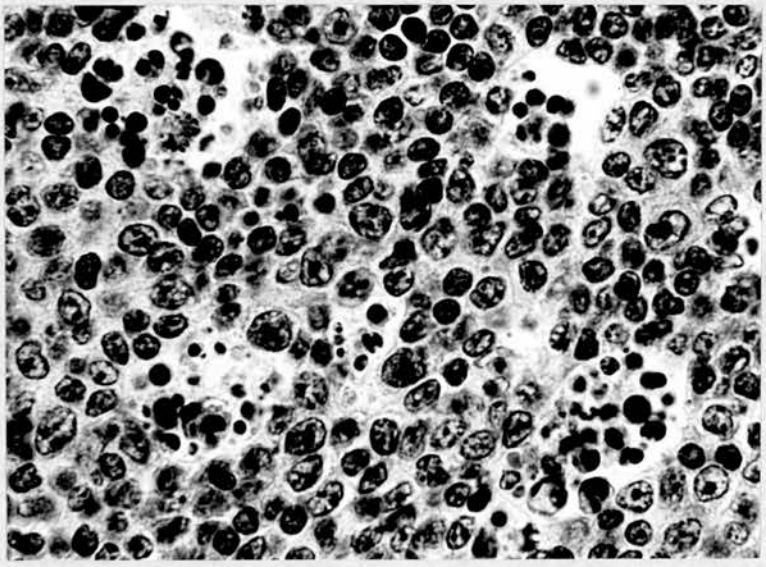

Fig. 6. The early stage of GVHD. The 'paracortical area' of a lymph node. The cells have vesicular nuclei and some have prominent nucleoli.

H. and E. x 750

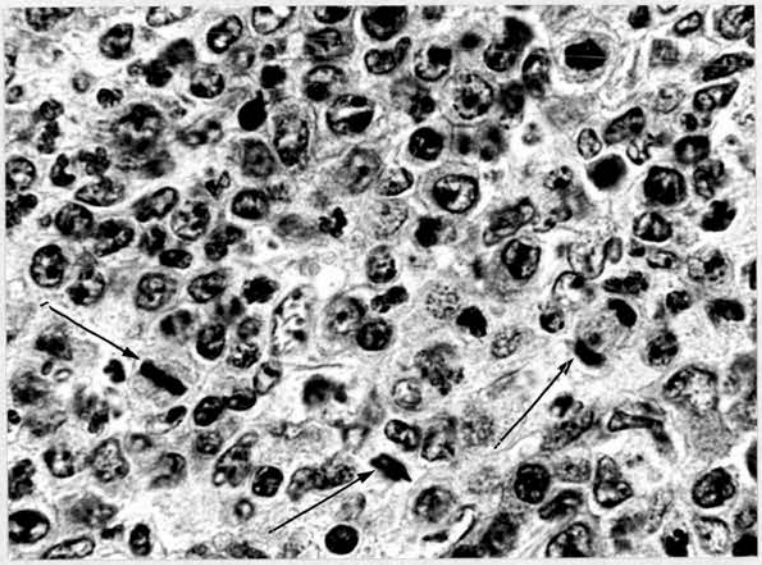

Fig. 7. Higher power view of part of Fig. 6. The cells are large with oval nuclei. They have abundant granular cytoplasm. These cells appear like lymphoblasts. Three of these cells are clearly seen in mitosis(arrows).

H. and E. x 800

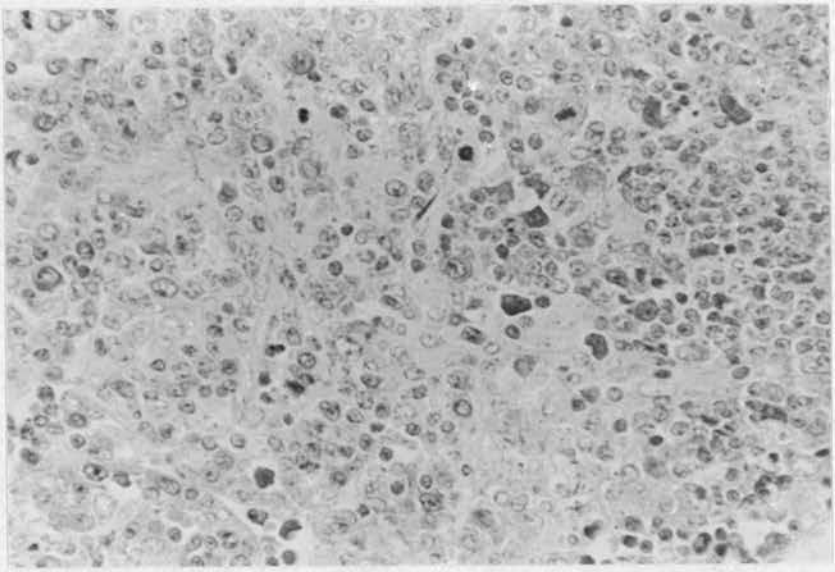

Fig. 8. Paracortical area of lymph node of mouse with GVHD (5 days). Most of the cells do not show pyroninophilia.

M.G.P. x 350

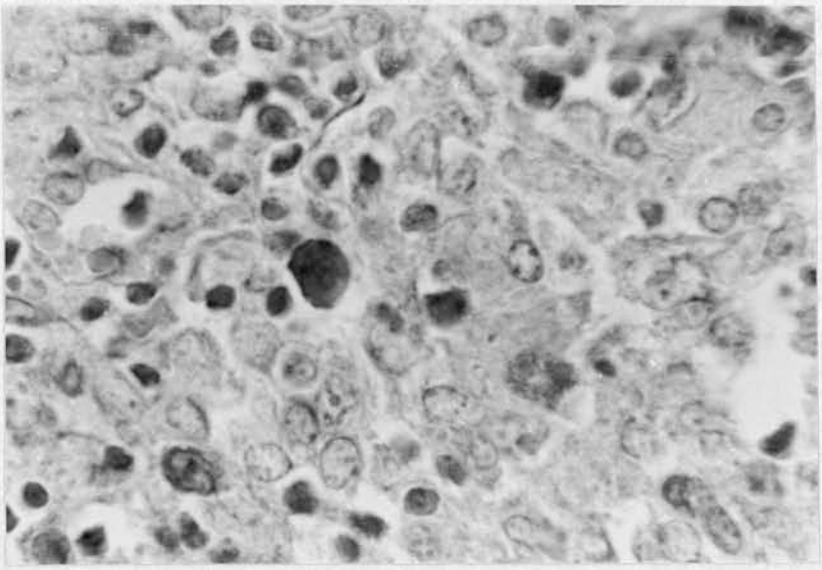

Fig. 9. High power view of part of an area from Fig. 8. This shows pale staining macrophages and cells probably lymphoblasts which show marked pyroninophilia.

M.G.P. x 800

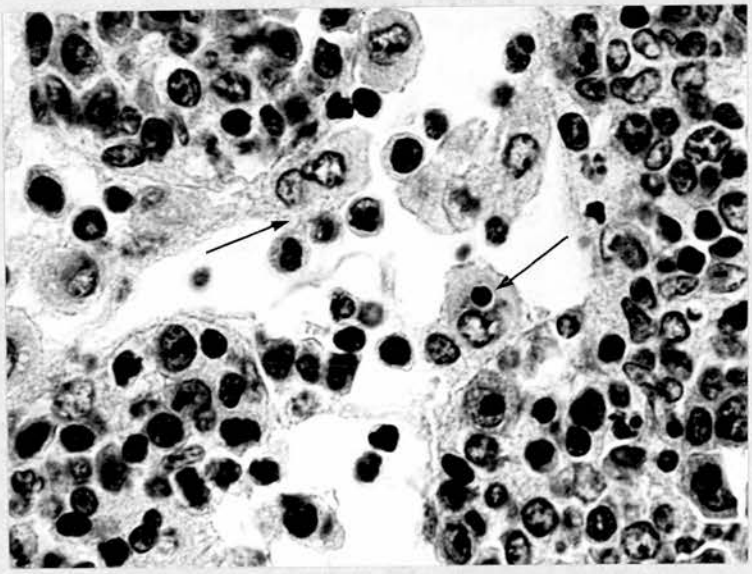

Fig. 10. Shows a sinus in the 'paracortical' area. Two lymphoid cells are seen in intimate contact with a macrophage (arrow). Another macrophage shows lymphophagocytosis (arrow). Lymph node from a mouse with GVHD (early stage).

H. and E. x 800

Examination of the sinuses in the para-cortical areas revealed many macrophages. The sinus lining cells were prominent and the lumen of the sinuses contained clusters of typical macrophages, usually found in small groups of 5-15 cells. At this stage of the disease, the para-cortical areas showed three cell types.

(a) Small dark round lymphocytes.

(b) Clumps of pyroninophilic cells with small lymphocytes surrounding them. The sections showed 6-8 areas of these clumps of pyroninophilic cells in the para-cortical area of the node.

(c) Clusters of macrophages intermingling with the pyroninophilic cells or with small lymphocytes. Many small lymphocytes are seen in intimate contact with the macrophages. Some macrophages show evidence of lymphophagocytosis (Fig.10). The lymphoid follicles were well preserved at this stage.

In the intermediate stage of the disease, 10-15 days after the inoculation of parental lymphoid cells, the lymph nodes had lost their normal architecture (Fig.11). The cortical, para-cortical and medullary areas no longer appear as separate entities. In most lymph nodes, the follicles were seen to be completely or partially replaced by macrophages. The bizarre morphology of the macrophages are seen clearly in the lining sinuses (Fig.12). The para-cortical areas were also almost replaced by macrophages (Fig.13). The medulla contained many macrophages and in addition had large numbers of/

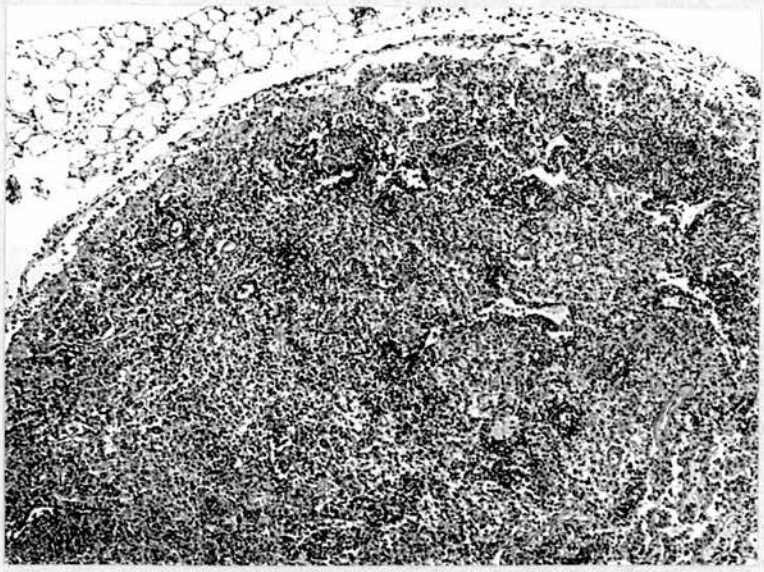

Fig. 11. Lymph node taken from mouse during the intermediate stage of GVHD. The lymphoid follicles have disappeared.

H. and E. x 60

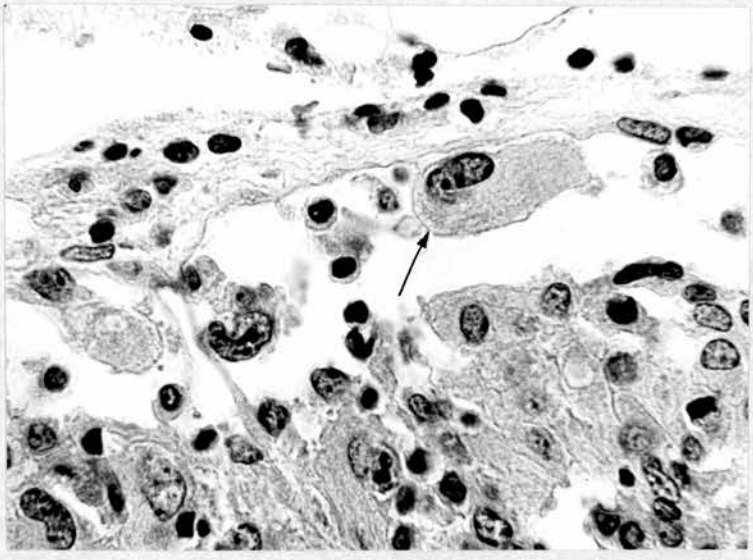

Fig. 12. Cortical area of lymph node of mouse with GVHD (intermediate stage). This shows the bizarre morphology of the macrophages (arrow) which is a feature of the intermediate and late stage of the disease.

H. and E. x 700

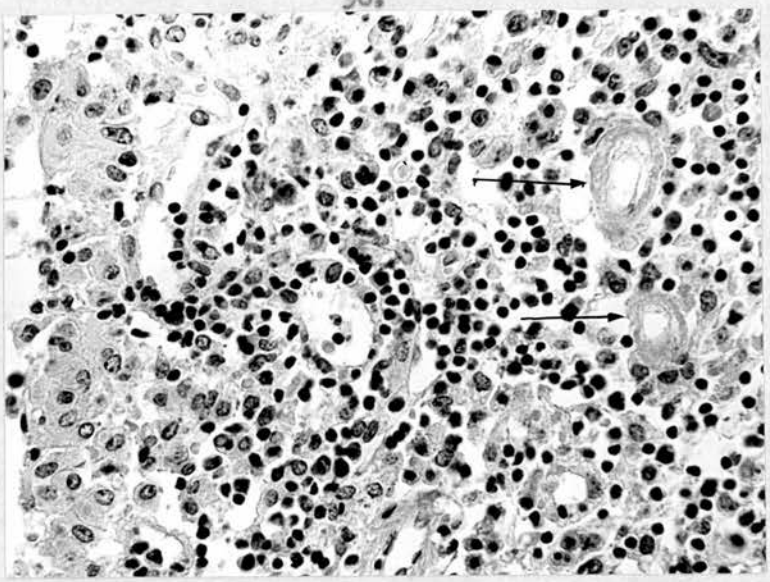

Fig. 13. Paracortical area of lymph node of mouse with GVHD (intermediate stage). This shows the gradual depletion of lymphoid cells with replacement by macrophages. The circular rings seen are blood vessels whose walls appear eosinophilic and "hyalinised" (arrows).

H. and E. x 60

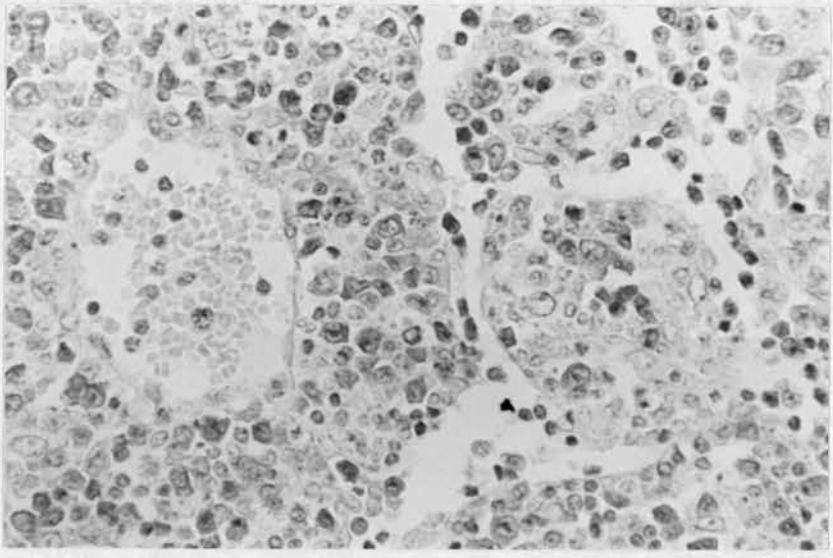

Fig. 14. Medullary area of lymph node of mouse with GVHD (late stage). This shows large numbers of pyroninophilic cells and macrophages.

M.G.P. x 500

of pyroninophilic cells (Fig.14). The medullary area also displayed prominent dilated lymphatic channels (Fig.15). The picture at this stage was one of complete or partial loss of architecture of the lymph nodes accompanied by diffuse proliferation of pyroninophilic cells and macrophages which replace 50-75 per cent of the lymphoid tissue.

Methyl green-pyronin staining of the lymph nodes at this stage showed islands of macrophages and abundant large pyroninophilic cells. Multinucleate giant cells were also seen throughout the lymph node but they were few in number. They had 5-10 nuclei per cell and were arranged mostly around the periphery of the cell (Fig.16). These cells had abundant cytoplasm which was eosinophilic and finely granular.

In the terminal stages of the disease process, the lymph nodes were very small and the lymphoid tissue was atrophic (Fig.17). The lymph node was almost entirely replaced by macrophages which were seen as one continuous sheet of cells with few small round lymphocytes between them (Fig.18). In some areas the fibroblasts had increased in number and an appearance similar to granulation tissue was seen. Some lymph nodes also showed areas of acellular pink hyaline material similar to that seen in the malpighian body of the spleen of G.V.H.D. mice (Fig.19)

Macroscopic Appearance of Normal and G.V.H.D. Spleen

The spleens of normal control animals were an inch long and 0.2 inch wide. They were elongated, dark red and the vascular hilum/

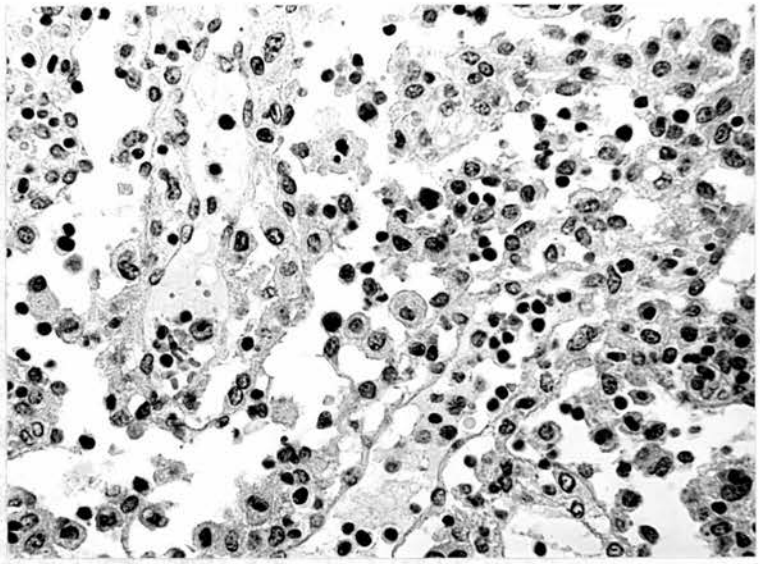

Fig. 15. Medullary area of lymph node of mouse with GVHD (intermediate stage). This shows the prominent lymphatic channels, strands of macrophages and many mononuclear cells.

H. and E. x 425

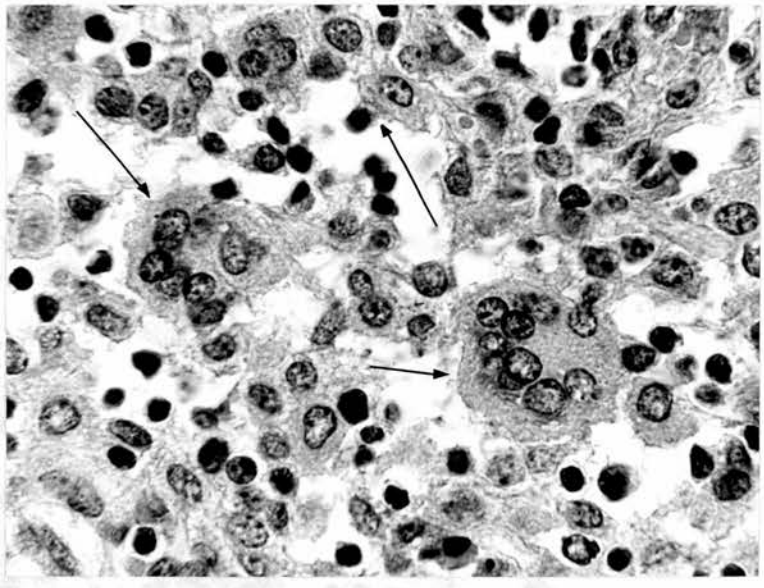

Fig. 16. Paracortical area of lymph node of mouse with GVHD (intermediate stage). This shows two multinucleate giant cells (arrows). The appearances of the cytoplasm are similar to those seen in the macrophages. The small dark round cells seem to be adherent to these macrophages (arrow).

H. and E. x 700

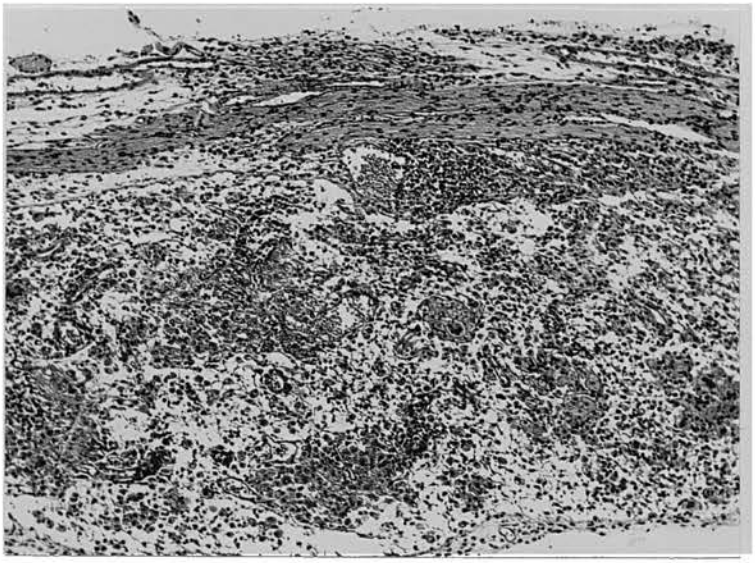

Fig.17. Mesenteric lymph node of a mouse with GVHD (late stage). The node is markedly hypocellular. Islands of macrophages are seen scattered throughout the node.

H. and E. x 70

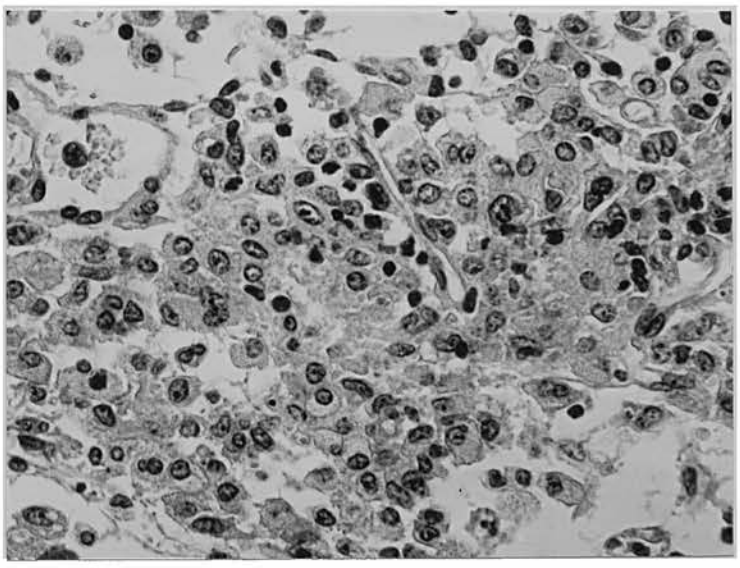

Fig.18. Higher magnification of the islands of macrophages shown in Fig.17. These cells are seen to form a sheet of tissue.

H. and E. x 450

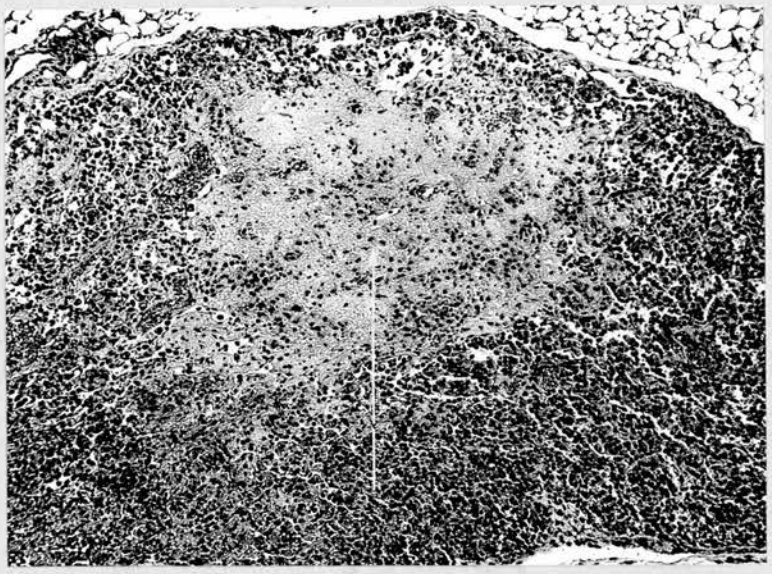

Fig. 19. Lymph node from a mouse with GVHD (late stage). An irregular almost structureless area is seen (arrow). This area has a similar appearance to the areas seen in the malpighian bodies of spleens of late GVHD mice.

H. and E. x 100

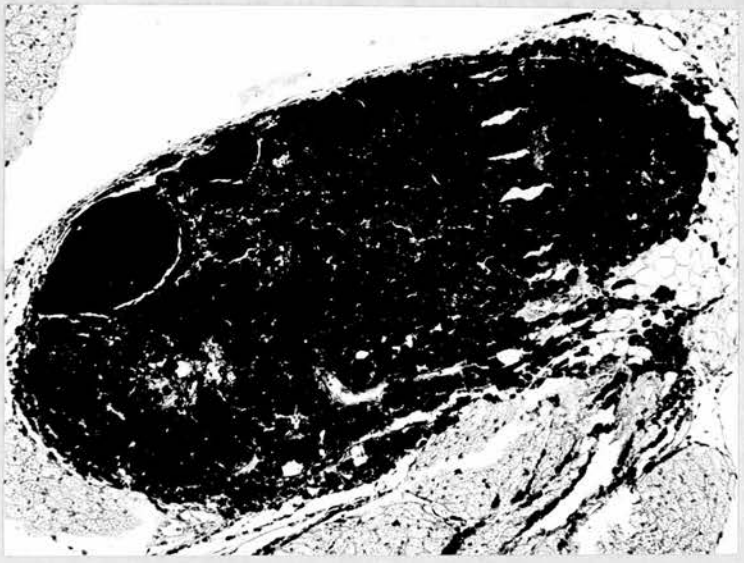

Fig. 20. Lymph node of mouse with GVHD (late stage). This shows the phagocytosis of subcutaneously inoculated colloidal carbon by almost all the cells of the lymph node. Hence these cells are macrophages.

H. and E. x 100

hilum was at the centre of the visceral surface. The spleens from control animals were uniformly small.

The G.V.H.D. spleens examined on the 5th day after the inoculation of cells were normal in shape and were one and a half times the size of the control spleens. They were dark red and firmer than the control spleens. The spleens removed on the 10th and 15th day after the inoculation of the parental cells were twice the normal size. They were much firmer than the control spleens and the cut surfaces presented pale whitish areas surrounded by red pulp. The spleens of terminal stage animals were normal in size or slightly larger. They were red in colour with irregular elevated whitish-yellow areas on the surface.

Microscopic Appearance of Normal Spleen

Two morphological areas are distinguished: the 'white pulp' forming the malpighian bodies with prominent arterioles present either centrally or eccentrically. The malpighian bodies represent nodular enlargement of the perivascular sheath of lymphoid tissue. The cuff of lymphoid cells close to the arteriole is known as the 'thymus-dependent' region of the malpighian body since neonatal thymectomy results in depletion of small lymphocytes in this area; this area is believed to be concerned with cell-mediated immune reactions. The periphery of the malpighian body is probably concerned with humoral antibody production (Parrott and Mousa, 1966; Parrott et al, 1966).

The red pulp is composed of sinusoids and the cords of Billroth; the/

the latter has a heterogeneous population of cells which are concerned with haematopoiesis and phagocytosis. Macrophages are seen in abundance lining the sinuses and in the areas surrounding the malpighian body.

Microscopic Appearance of G.V.H.D. Spleens

The spleens examined on the 5th day after the inoculation of cells showed marked proliferative and reactive changes. The malpighian bodies were slightly larger and more cellular than in the control spleens (Fig.22). The periphery of the malpighian body contained numerous large cells with basophilic cytoplasm and large oval vesicular nuclei. Many of these cells were in various stages of mitosis (2-10 cells in each malpighian body) and appeared morphologically as lymphoblasts. Methyl green-pyronin staining demonstrated the presence of large round pyroninophilic cells in the substance of the malpighian bodies. The pyroninophilic cells were less than 1.0 per cent of the total malpighian body.

The 'red pulp' was much more cellular than normal. The most prominent cells were small dark round lymphocytes and intermingling with them were large prominent 'blast' cells which were also found in large numbers (Fig.23). The macrophages were seen in the periphery of the malpighian bodies but they were far outnumbered by the rest of the lymphoid cell population at this stage. The venous sinuses appeared to contain numerous red cells and many macrophages were seen lining them. Many multinucleate giant cells were seen and in several places they formed large clusters (Fig.24). These cells had/

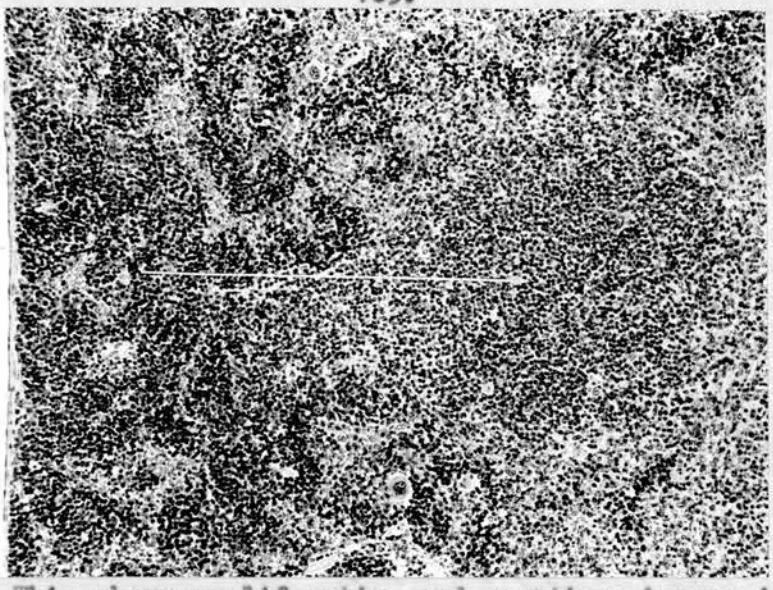

Fig. 22. This shows proliferative and reactive changes in the spleen. A lymphoid follicle (arrow) is present at the right side of the photograph. Prominent dark staining cords of lymphocytes are seen in the sub-capsular region. Spleen of mouse with GVHD (early stage).

H. and E. x 120

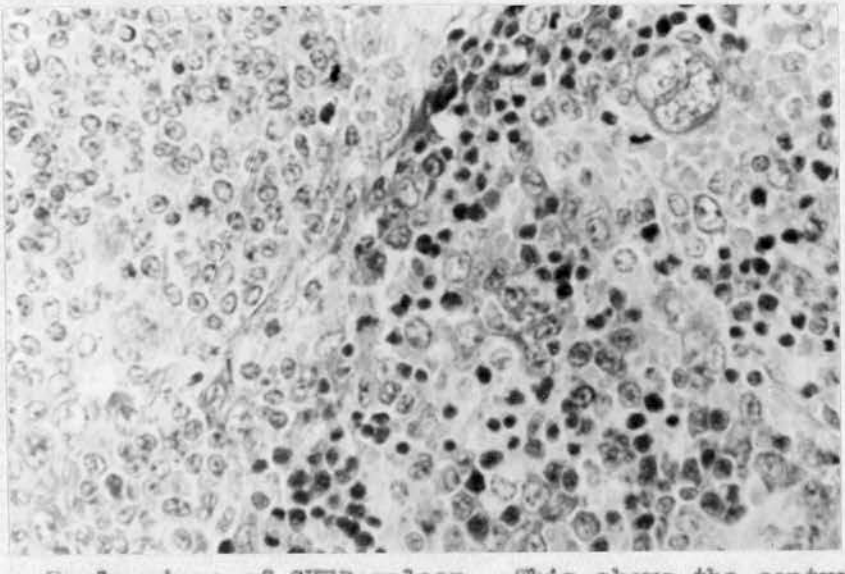

Fig. 23. Early stage of GVHD:spleen. This shows the contrasting appearances of the malpighian body and the 'red pulp'. The former area is pale staining while the latter area has many pyroninophilic and dark round cells.

M.G.P. x 500

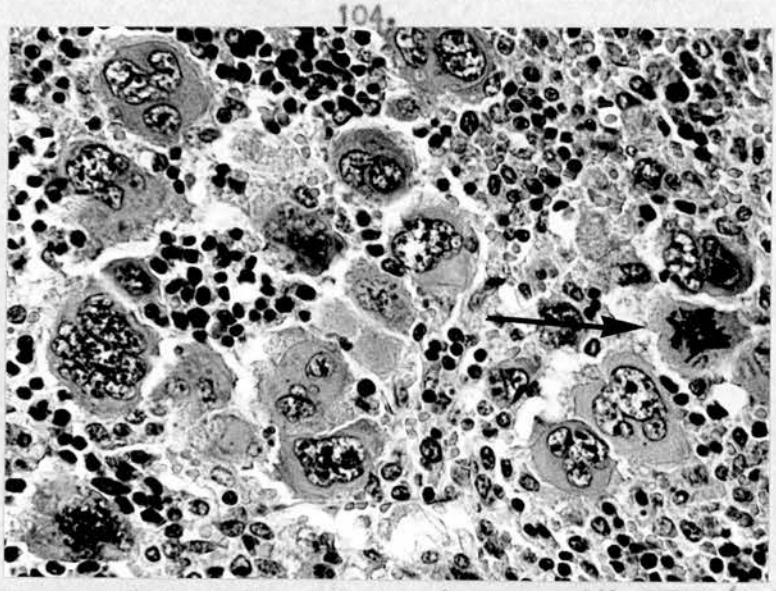

Fig.24. 'Red pulp' of the spleen of mouse with GVHD (intermediate stage). This shows an island of multinucleate giant cells. One cell is seen in division (arrow).

H. and E. x 450

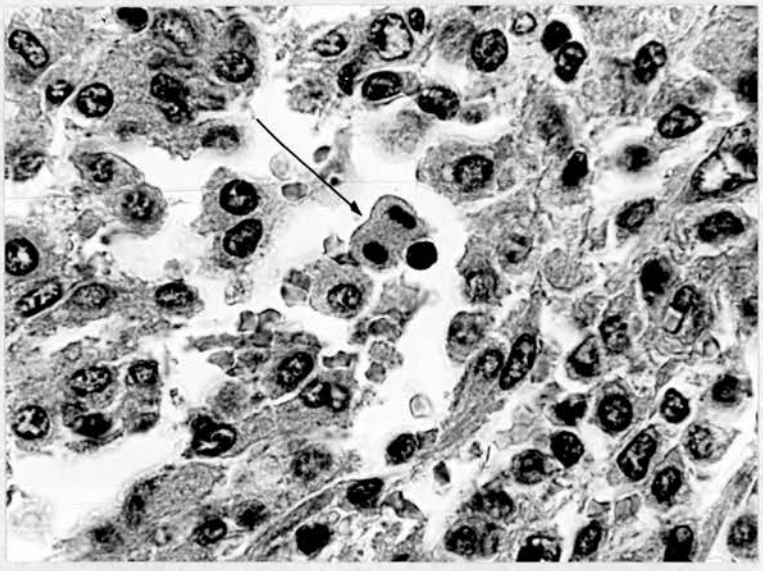

Fig.25. An area of the malpighian body of the spleen of mouse with GVHD (intermediate stage). One cell near the centre is in division (arrow). This cell is probably a macrophage since almost all the cells in this area are macrophages.

H. and E. x 800

had 3-20 nuclei situated for the most part centrally. Many cells containing 'doughnut' nuclei were seen and these were probably precursors of the erythropoietic and myelopoietic series.

Methyl green-pyronin staining showed that about 60-70 per cent of the red pulp was composed of pyroninophilic cells which in many areas were in intimate relationship with small round lymphoid cells. The multinucleate giant cells also appeared pyroninophilic. The pyroninophilia of the 'red pulp' was a very striking feature in contrast to the 'white pulp', which was almost colourless by comparison (Fig.23).

The spleens of the animals sacrificed on the 10th and 15th day after the inoculation of parental lymphoid cells showed that the architecture of the malpighian bodies were markedly altered. The 'red pulp' was relatively unaffected. In a few malpighian bodies macrophages were seen to extend into it and form a 'band', thus breaking up the malpighian bodies. Some of these cells were in mitosis (Fig.25). In the rest of the areas, the macrophages had entered the malpighian bodies and were seen to partly or completely replace them (Fig.26, 27). These macrophages had joined each other by cytoplasmic processes forming an interweaving network of cells which were arranged around the central arteriole. In a later stage these cells showed the early stages of degeneration and necrosis (Figs. 28, 29).

Methyl green-pyronin staining at this stage showed that the malpighian bodies were replaced by macrophages, which did not take the/

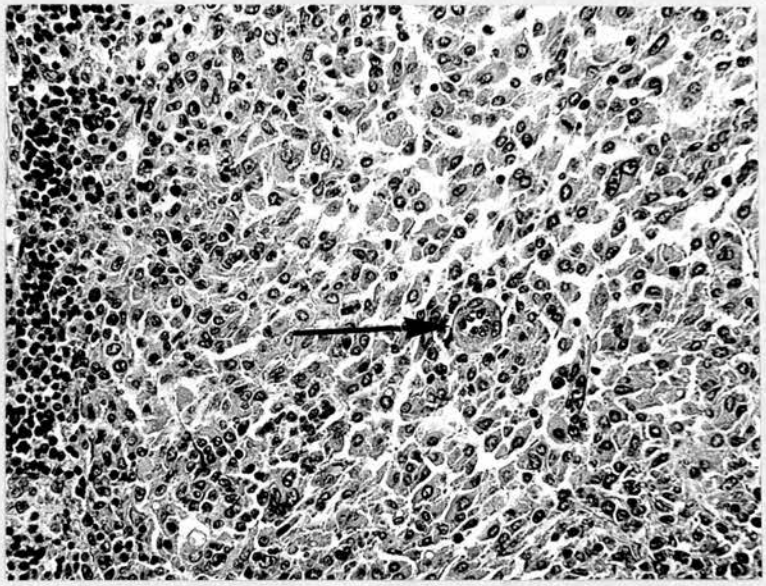

Fig. 26. Spleen from GVHD mouse (intermediate stage). This shows the complete replacement of the perivascular sheath of lymphoid tissue with macrophages. The central arteriole (arrow) marks the usual site of perivascular lymphoid sheath. A collection of dark staining lymphocytes is present at the left of the photograph.

H. and E. x 300

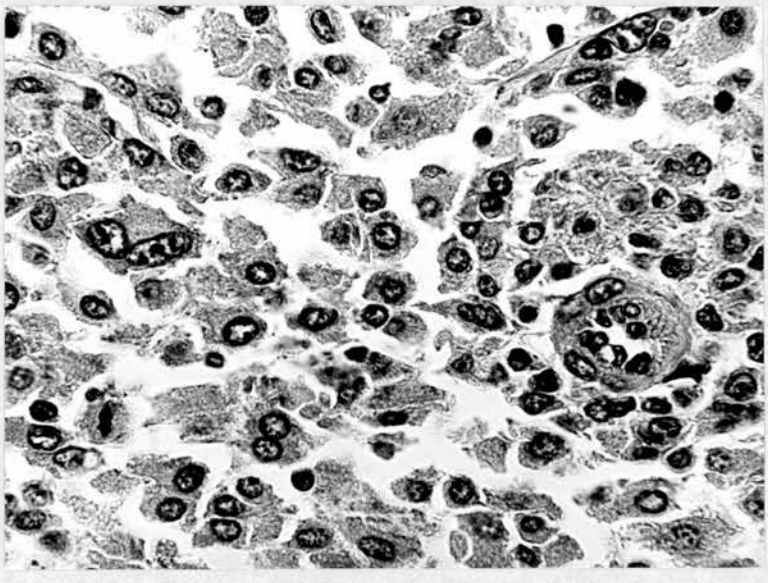

Fig. 27. This is a high power view of Fig. 26 which shows the central arteriole and macrophages quite clearly.

H. and E. x 600

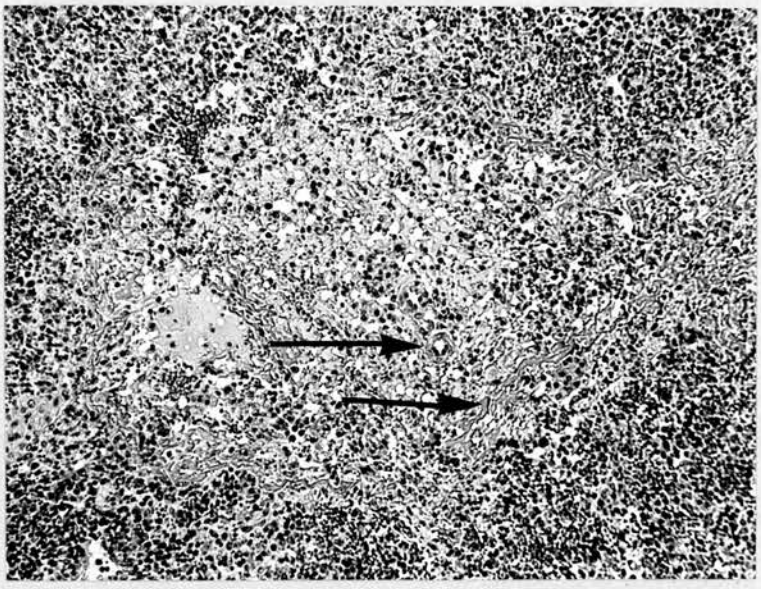

Fig.28. Intermediate stage GVHD spleen showing the perivascular lymphoid sheath of the spleen (arteriole arrowed). The area is hypocellular and contains macrophages and lymphoid cells. Acellular structureless hyaline material is present (arrow). These foci of early "breakdown" of architecture are widely distributed throughout the follicles at this time.

H. and E. x 130

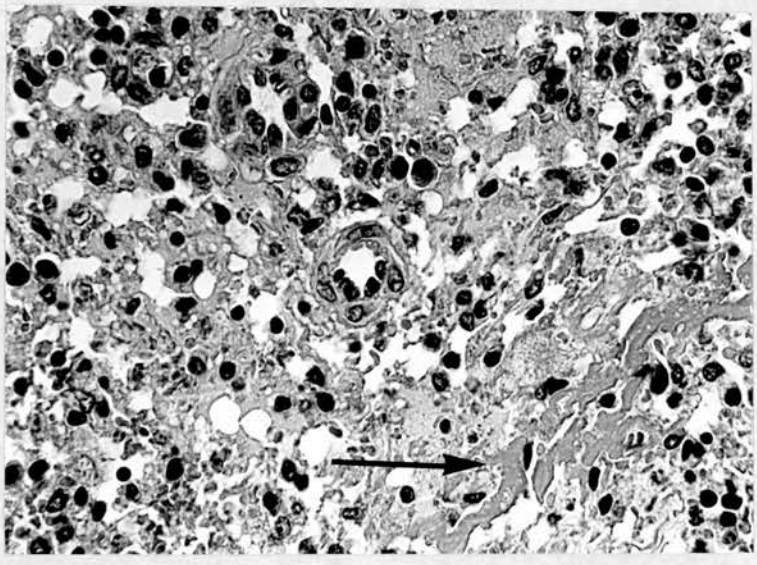

Fig.29. High power view of Fig.28 showing thick strands of hyaline eosinophilic material (arrow). The question whether this represents insudative fibrin or the formation of amyloid from the degenerating macrophages is discussed in the text. Intermediate stage GVHD spleen of mouse.

H. and E. x 450

the pyronin stain. In marked contrast to this the red pulp was almost packed with pyroninophilic cells with some small round lymphoid cells in close contact with them. No macrophages were seen in the red pulp (Fig.30). Many clusters of pyronin-staining multinucleate giant cells were also seen.

The spleens of animals sacrificed during the late stage of the disease showed that the macrophages which were replacing the malpighian bodies were undergoing a gradual process of degeneration and death. In many areas, the pale-pink cytoplasmic remnants of the macrophages were seen to form a whorled mass of material and in between them some degenerating nuclei and a few mononuclear cells were seen (Figs.31, 32). Methyl green-pyronin did not stain these areas. The 'red pulp' had the same cell population as was seen in the earlier stages. Methyl green-pyronin staining showed that 70 per cent - 80 per cent of the cells were pyroninophilic. Special stains were done to determine the nature of the acellular fine-granular hyaline material which occupied the areas of the malpighian bodies. M.S.B. staining of the spleens (Martius Scarlet Blue method for fibrin; see Carlton's Histological Technique by Drury and Wallington, 1967) showed that these areas stain partly fibrin. Methyl violet-Crystal violet method (after Jurgens, 1875), and alkaline congo red method for amyloid (Puchtler, Sweat and Levine, 1962; see Carlton's Histological Technique by Drury and Wallington, 1967) do not show a positive reaction for amyloid. However, thioflavine T staining and examination by the fluorescent microscope (see/

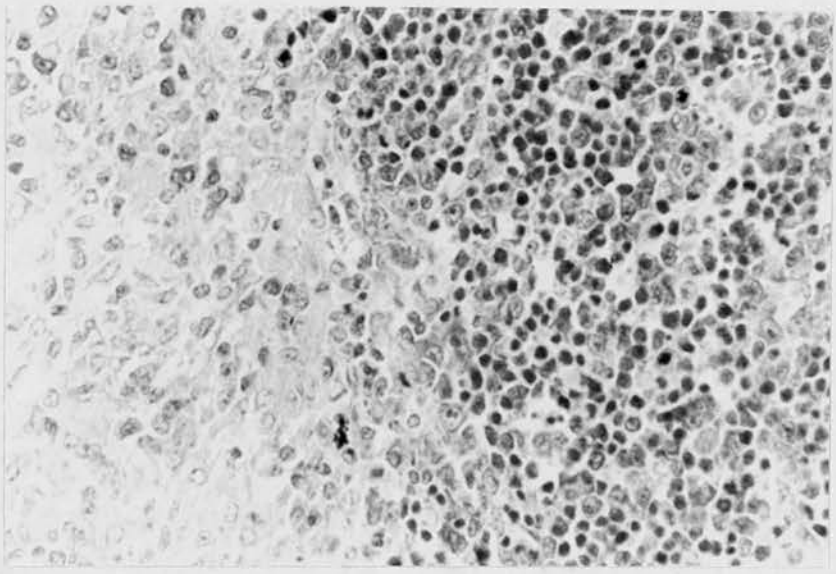

Fig. 30. Spleen from a GVHD mouse (intermediate stage). The 'red pulp' has abundant pyroninophilic cells and the malpighian body is composed of pale staining macrophages.

M.G.P. x 350

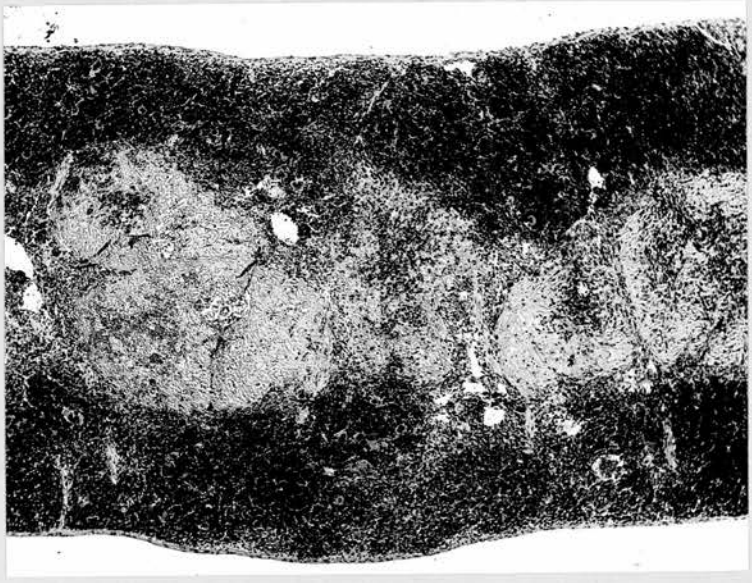

Fig. 31. Spleen of a mouse with GVHD (late stage). This shows extensive replacement of malpighian bodies by acellular, pale staining, confluent masses of hyaline material.

H. and E. x 50

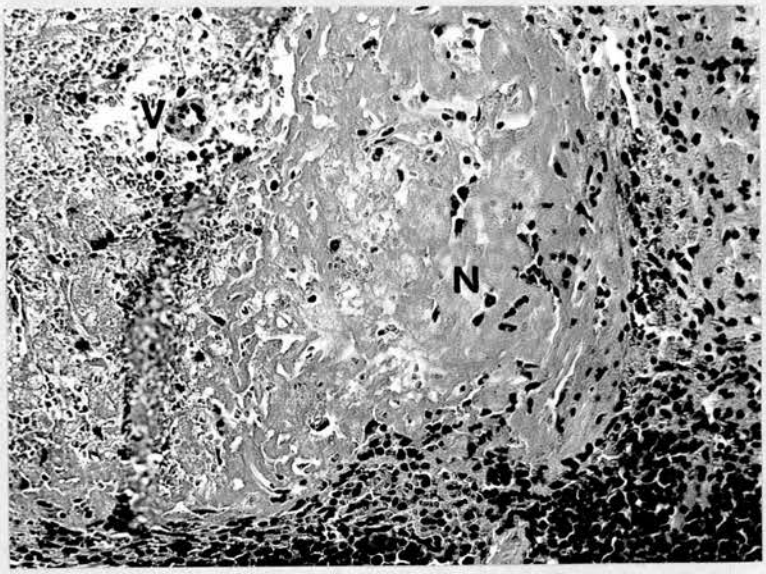

Fig.32. Spleen of a mouse with GVHD (late stage). This shows nodule (N) of hyaline material closely related to vessel (V).

H. and E. x 225

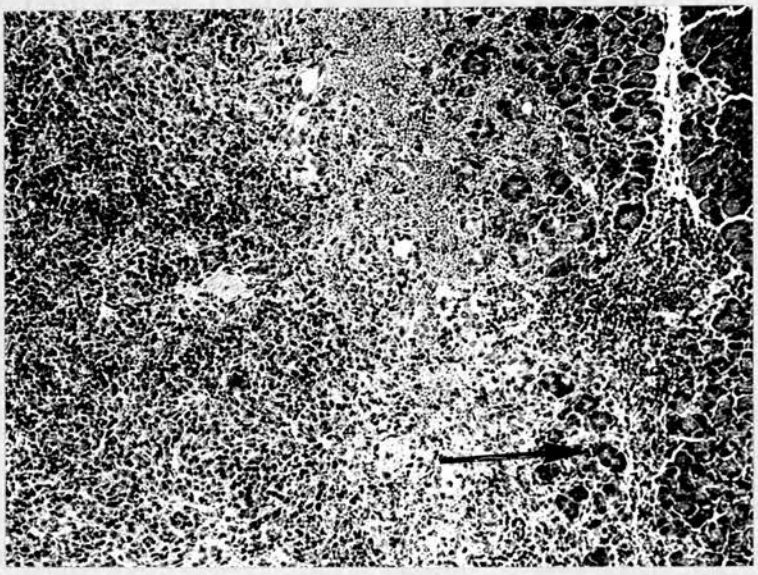

Fig.33. Spleen and pancreas of a GVHD mouse (late stage). This shows the splenic tissue on the left and mononuclear cells are seen infiltrating from the spleen into the pancreatic acini which seem to be undergoing degeneration (arrow). The capsule of the spleen is not seen.

H. and E. x 120

(see Carlton's Histological Technique by Drury and Wallington, 1967) shows evidence of bright fluorescence in areas occupied by the pinkish eosinophilic material; the control slides with amyloid also had the same intensity of fluorescence and slides of normal spleen did not show any fluorescence. It was thus very likely that this material was primary amyloid (West and Murphy, 1965) mixed with a certain amount of fibrin.

Examination of spleens of animals of the $B6AF_1/J$ strain taken during the terminal stages of the disease, showed that two out of six spleens had macrophages and other mononuclear cells infiltrating through the splenic capsule (Fig.33). These cells were seen to extend into the substance of the pancreas as sheets of cells and were replacing the pancreatic acini. This infiltration of cells from the spleen to the pancreas had a resemblance to tumours directly infiltrating from one organ to another.

Macroscopic Appearance of the Liver in G.V.H.D.

The livers of the animals sacrificed on the 5th and 10th day after the inoculation of parental cells, were normal in shape, size, colour and texture. The livers of the animals sacrificed on the 15th and 20th day were paler than usual but were otherwise normal.

Microscopic Examination of the Liver in G.V.H.D.

The general architecture of the liver was well preserved. The lobules were easily made out. The hepatocytes were normal in size and shape.

The sinuses were not dilated and some contained small dark mononuclear/

mononuclear cells. The Kupffer cells lining the sinuses presented a marked difference compared to the control livers on low power examination. They were much larger than normal and appeared as plump spindle-shaped cells with a prominent nucleus and abundant cytoplasm.

The periportal regions were infiltrated by mononuclear cells. These cells were around the bile ducts, portal vein and hepatic artery. In many regions, they formed large collections of cells and the adjacent hepatocytes appeared to undergo atrophy. In some periportal areas, the peripheral hepatocytes were markedly thinned out and the periportal macrophages were virtually enclosing them (Figs. 34, 35).

Examination of the liver after the intravenous injection of colloidal carbon shows that the Kupffer cells are markedly enlarged and numerous compared to the normal controls. The increased activity is seen by the massive uptake of colloidal carbon by these cells compared to the cells from control mice. The periportal macrophages had not taken up carbon.

Methyl green-pyronin staining did not stain the cells in the sinusoids and in the periportal areas. Thus most of the cells in the latter region had the morphological appearance of macrophages and had also not taken up the pyronin stain.

These changes were seen in mice at all stages of the disease without any apparent change in the amount of the infiltrate.

No subcapsular necrosis of liver cells and no fatty change was seen/

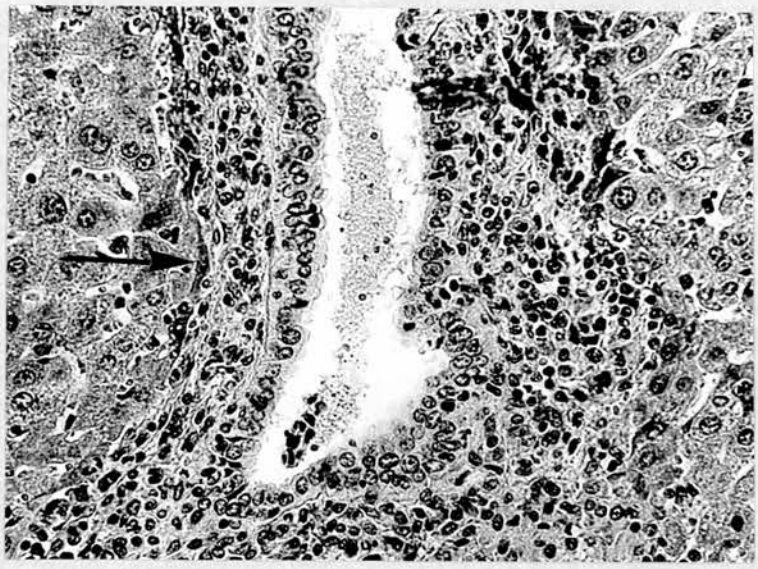

Fig.34. Liver in GVHD. This shows the large collection of cells around the bile duct and the adjacent hepatocytes appear atrophic (arrow).

H. and E. x 275

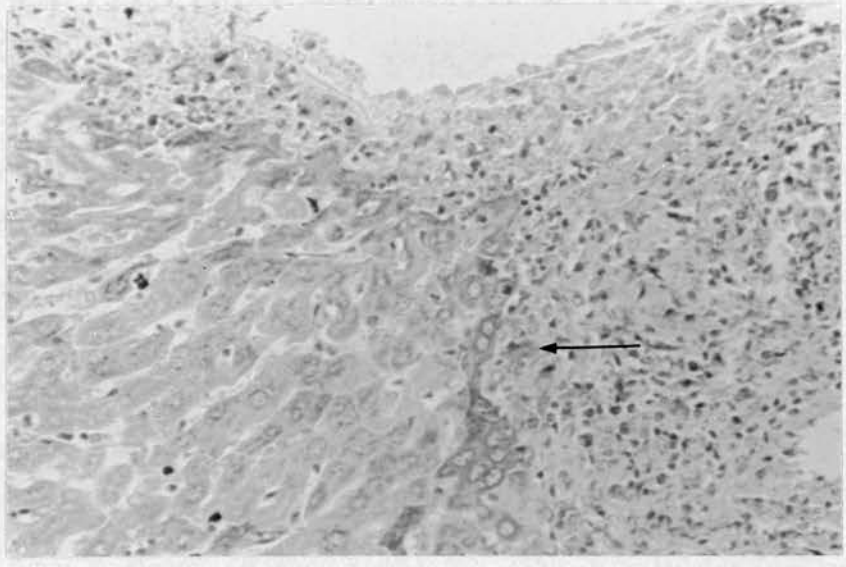

Fig.35. This shows the periportal accumulation of cells in the liver of GVHD animals. These cells are not pyroninophilic and appear like histiocytes. The adjoining liver tissue is atrophic and in one area one hepatocyte is clearly seen among the periportal infiltrate (arrow).

M.G.P. x 180

seen. The central vein, hepatic artery, hepatic vein and the bile duct appeared normal.

Macroscopic Appearance of the Thymus in G.V.H.D.

The thymuses examined from animals sacrificed on the 5th day had a normal appearance and size. The thymuses from mice sacrificed on the 10th and 15th day after the inoculation of cells were about half the size of the control mice. Thymuses from mice taken during the terminal stages of the illness were very small.

Microscopic Appearance of the Thymus in G.V.H.D.

The test animals sacrificed on the 5th day after the inoculation of cells had a distinct cortex and medulla but the cortex showed some depletion of thymocytes compared to normal. The test animals sacrificed on the 10th and 15th day showed marked loss of thymocytes in the cortex and the medulla appeared relatively more cellular. Thus many thymuses presented an 'inverted' type of appearance (Fig.36, 37). The thymuses of the 20th day test mice had a marked distortion of architecture and cortex and medulla could not be distinguished. These had many macrophages, reticulum cells, few round mononuclear cells and many fibroblasts. Methyl green-pyronin stain did not show the presence of pyroninophilic cells in these thymuses.

Macroscopic Appearance of the Pancreas in G.V.H.D.

The pancreas of the animals sacrificed on the 5th and 10th day after the inoculation of cells appeared normal compared to the pancreas from control animals. The pancreas of the animals sacrificed/

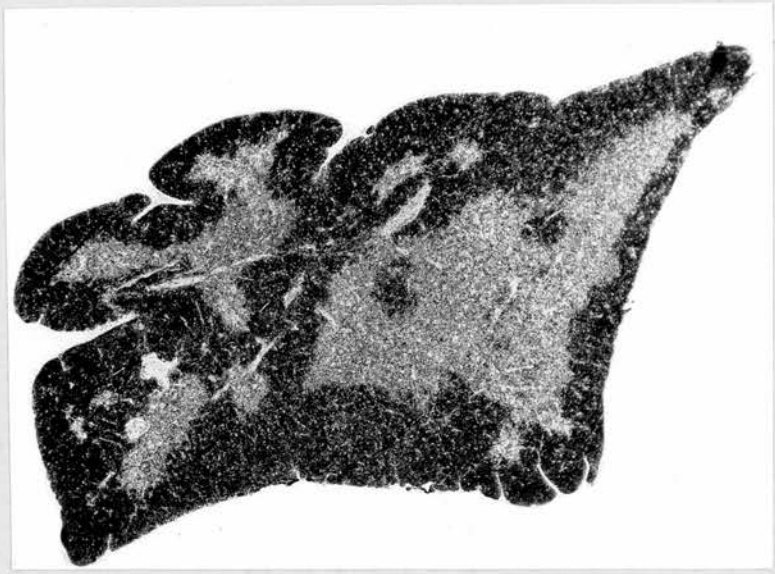

Fig. 36. Lobe of a thymus of a normal mouse. This shows the pale medulla and the dark cortex.

H. and E. x 25

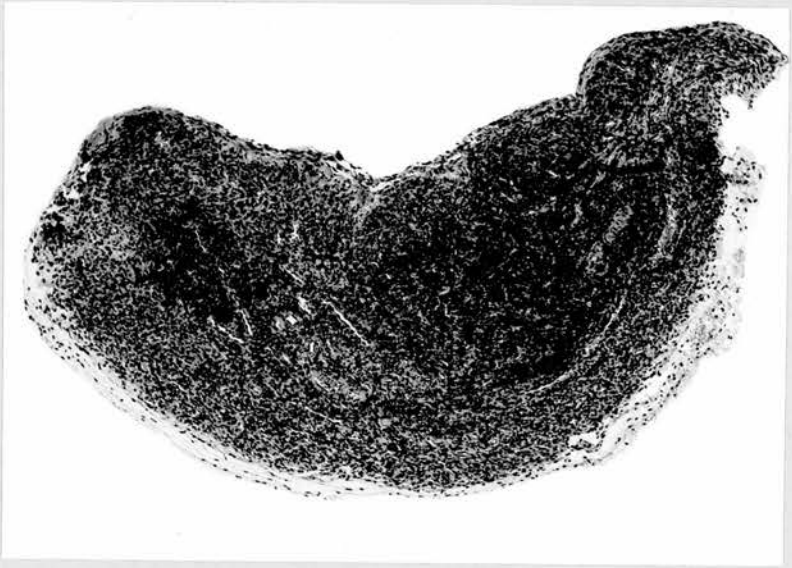

Fig. 37. This shows the lobe of a thymus from a mouse in the late stage of GVHD. The cortex is greatly depleted of thymocytes and the medulla is darker than the cortex. Thus the thymus presents an 'inverted' type of appearance.

H. and E. x 65

sacrificed on the 15th and 20th day after the inoculation of cells were whitish-yellow in colour and gelatinous.

Microscopic Appearance of the Pancreas in G.V.H.D.

The pancreas of animals sacrificed on the 5th day after the inoculation of cells showed massive accumulations of dark mononuclear cells which were found mainly along the connective tissue septae, blood vessels and around the islets of Langerhans. Examination of the pancreas of animals sacrificed on the 10th, 15th and 20th day after the inoculation of cells showed a cellular infiltrate around blood vessels, smaller ducts, acini and around islets. In many areas these infiltrates had accumulated as large masses of cells and were compressing the acini and islets. These latter structures in these areas appeared small and seemed to undergo varying degrees of atrophy and destruction (Fig.38). This infiltrate of cells was also seen to extend into the pancreas from the neighbouring lymph nodes. The infiltrate was composed of macrophages and pyronino-philic cells. On an average, 5-10 per cent of the pancreatic tissue appeared damaged and hence serious functional disturbances of the glands would not be expected. Examination of the pancreas of G.V.H.D. animals inoculated with colloidal carbon in the intermediate and terminal stages showed that the infiltrating cells had phago-cytosed massive amounts of carbon (Figs.39, 40).

Therefore there can be no doubt that these cells are mononuclear phagocytes.

Macroscopic Appearance of the Salivery Glands in G.V.H.D.

The/

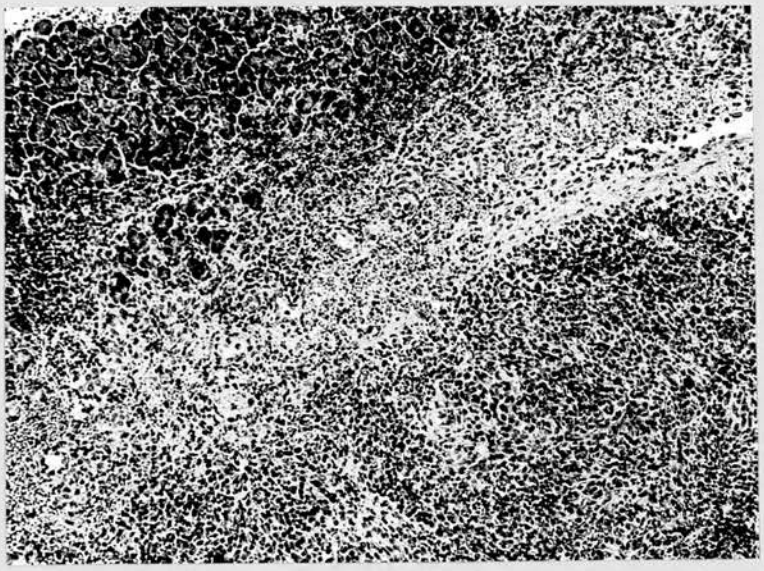

Fig.38. This shows the extensive infiltration of the pancreas by mononuclear cells. These cells have replaced the pancreatic tissue in the bottom left of the photograph.

H. and E. x 120

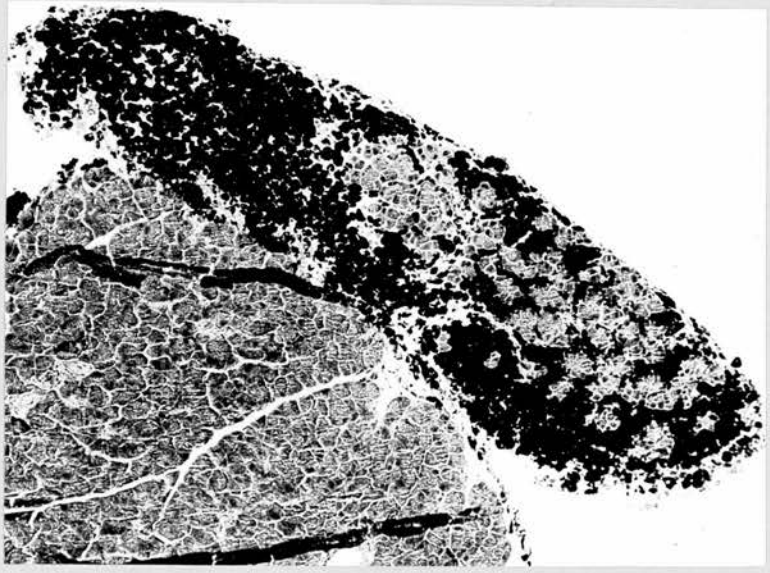

Fig.39. This shows the pancreas of a carbon-inoculated GVHD mouse. The cells infiltrating and replacing the pancreas have ingested large amounts of colloidal carbon. Hence these cells are macrophages.

H. and E. x 110

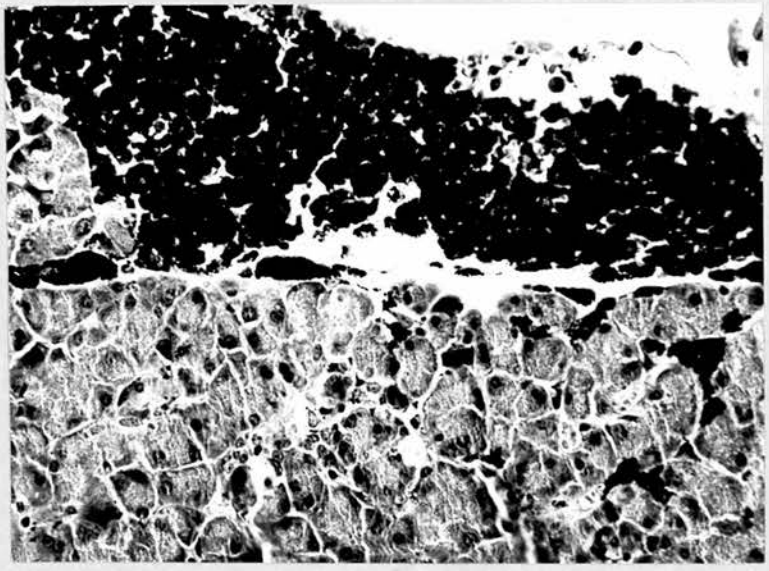

Fig.40. High power view of Fig.39 showing the carbon-laden macrophages.

H. and E. x 300

Fig.41. Salivary gland of a mouse with GVHD. There is extensive destruction of the acini but the ducts are intact. Small dark round cells are seen mostly around blood vessels (arrow). Macrophages are also seen diffusely scattered in the damaged areas (arrow).

H. and E. x 140

The salivary glands were normal in size and shape compared to the control animals. However, the glands appeared yellowish-brown in colour whereas the control glands were light yellow.

Microscopic Appearance of Salivary Glands in G.V.H.D.

The animals sacrificed on the 5th and 10th day after the inoculation of parental lymphoid cells showed focal infiltrations of dark mononuclear cells. These cells were seen between the acinar tissue and around the ductules and venules. The salivary glands of the 15th and 20th day animals were involved quite markedly. There was marked infiltration of lymphoid cells and macrophages.

Methyl green-pyronin staining showed in addition the presence of pyroninophilic cells. The acini were damaged to such an extent that the tissue contained merely the ducts, cell infiltrates and fibrous tissue (Fig.41). The salivary glands which were predominantly mucous in type, did not show any change.

Macroscopic Appearance of the Alimentary System in G.V.H.D.

The tongue, fauces, pharynx, oesophagus do not show any changes compared to control animals. The stomachs of animals examined during the terminal stages of the disease were somewhat small and contained undigested food material. The jejunum, ileum and caecum had localized areas of dilatation of about an inch in length; creamy fluid material could be seen through them. Exposure of the lumen confirmed the presence of this creamy material and the wall of the intestine in these areas appeared thinner than normal. The Peyer's patches were markedly atrophic. The colon and rectum did not/

not show any change. The localized areas of dilatation in the small bowel were seen in all stages of the disease in the (C57BL x CBA)F_1 strain.

Microscopic Appearance of the Alimentary System in G.V.H.D.

The small intestine was involved in all stages of the disease. Mild to moderately severe lesions were seen in the early and intermediate stages. In the late stages there was in addition evidence of some reparative reactions. Although the lesions were distributed in a patchy manner throughout the length of the intestines, the ileum was more affected than the jejunum. The lesions noted in the jejunum and ileum were similar. Examination of normal ileum showed normal villi, normal submucosa and normal muscular and serous coats (Fig.42). The Peyer's patches had large collections of lymphoid cells and many germinal centres were seen in them (Fig.43).

Studies on (C57BL x CBA)F_1 and B6AF_1/J mice with G.V.H.D. showed that the tissue lesions were similar except in the latter strain where more reparative reactions were noted.

The lesions seen in the two strains will be described separately.

Microscopic Changes in the Alimentary System of (C57BL x CBA)F_1 G.V.H.D. Mice

The outstanding change noted was damage to the villi. The epithelial cells lining the villi showed degeneration and necrosis. Often masses of epithelial cells, consisting of 5-10 cells of the superficial part of the villi were separated from the rest of the villi and were embedded in mucus and intestinal contents. The tips

of/

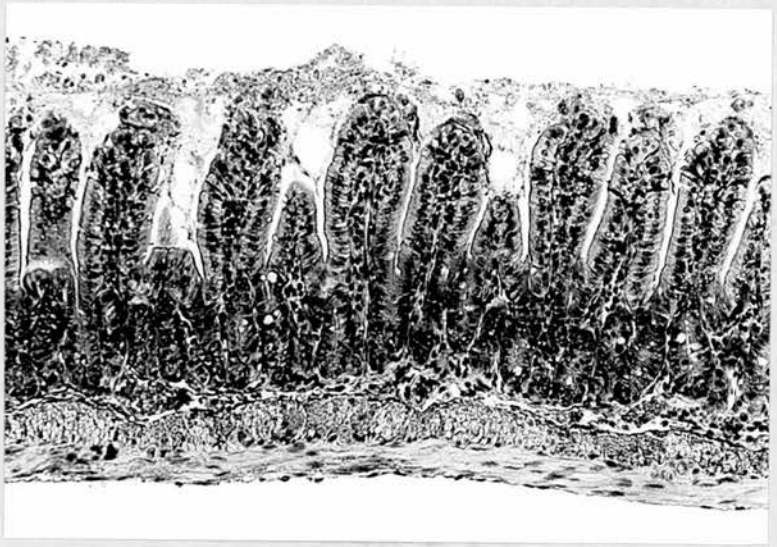

Fig. 42. A low power view of the normal ileum (longitudinal section). The villi, the submucosa, the muscular and serous coats are clearly seen.

H. and E. x 140

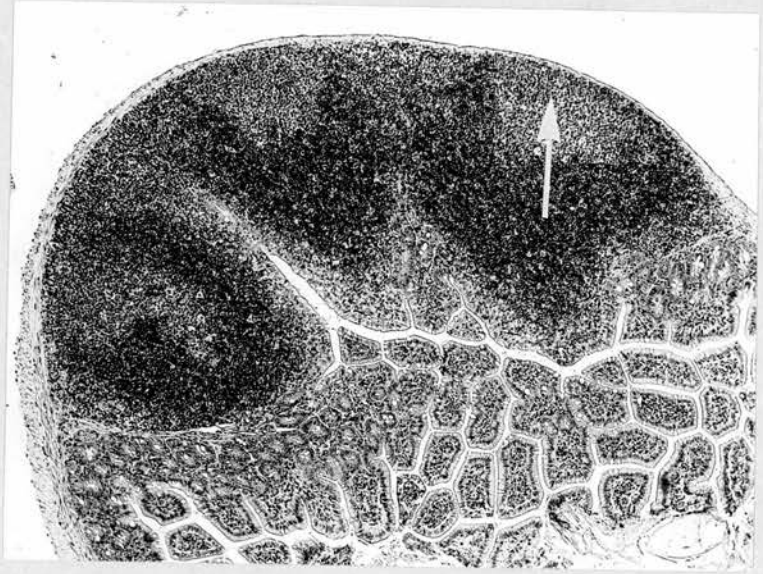

Fig. 43. Peyer's patch in the ileum of a normal mouse. Germinal centres (arrow) are seen within the lymphoid tissue.

H. and E. x 55

of the villi in the damaged areas did not have the normal covering of epithelial cells and hence the lacteals appear to be exposed directly to the lumen. The number of goblet cells in the undamaged part of the villi also appear to be decreased in number. Damage was also noted to the submucosal glands in the duodenum and to the crypts of Lieberkühn.

In many other areas the villi appeared clubbed. In the mucosa and submucosa many dark round mononuclear cells were seen. Similar cells were also seen in the lamina propria. Groups of lymphoid cells were also seen in the muscle coat and in the subserosal region.

Animals sacrificed on the 15th and 20th day after the inoculation of parental lymphoid cells in addition to the above changes showed the presence of macrophages in the mucosa and submucosa (Figs.44, 45, 46, 47). A few pyroninophilic cells were also seen. The Peyer's patches in the early stages were hypercellular and in the intermediate and late stages, macrophages had almost entirely replaced these patches (Figs.48, 49).

The colon and rectum showed an increase in the amount of mononuclear cells in comparison to the control organs. However no parenchymal damage was noted.

Changes in the Alimentary System of B6AF$_1$/J Hybrid G.V.H.D. Mice

Dissecting Microscope Appearance

The jejunum and ileum showed quite remarkable changes, seen specially during the latter half of the life of the animals. Initial examination of the whole of the alimentary tract was made in ten animals/

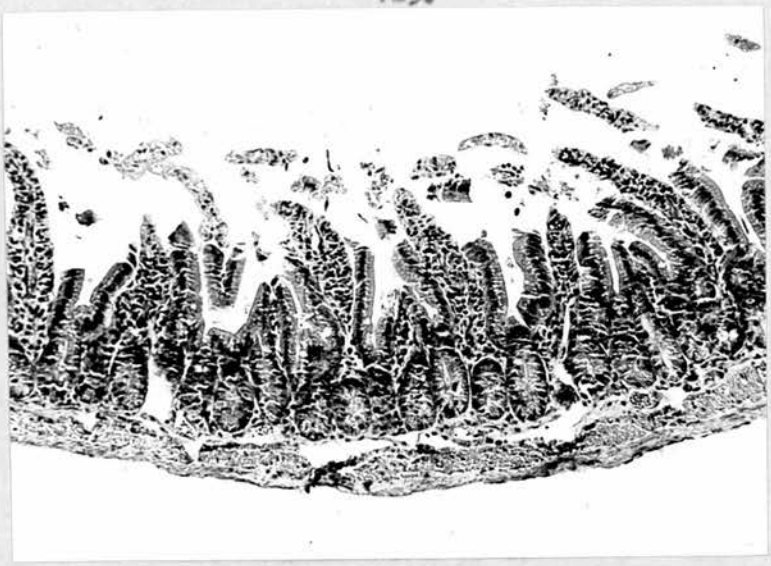

Fig. 44. The ileum of a (C57BL x CBA)F_1 GVHD mouse. The epithelial cells of the upper half of the villi are absent and the lamina propria are exposed to the lumen.

H. and E. x 130

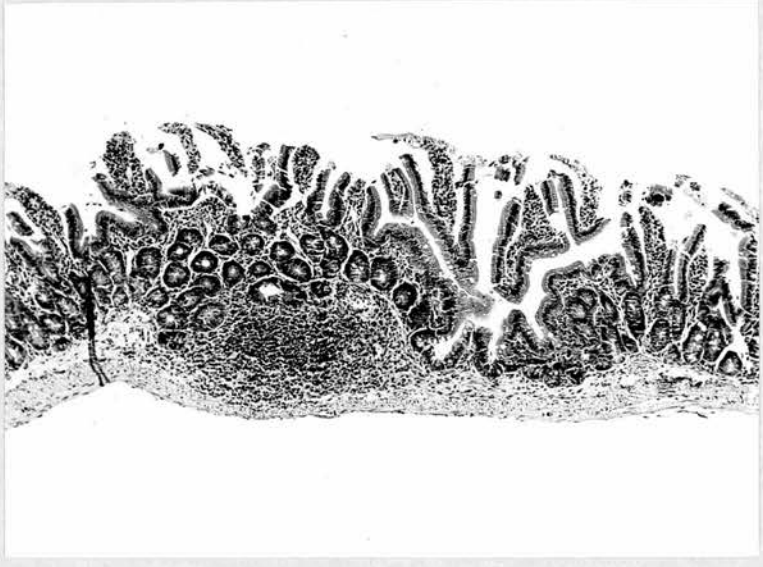

Fig. 45. Ileum of a (C57BL x CBA)F_1 GVHD mouse. The villi are irregular, incomplete and shortened. The Peyer's patch is markedly diminished in size.

H. and E. x 70

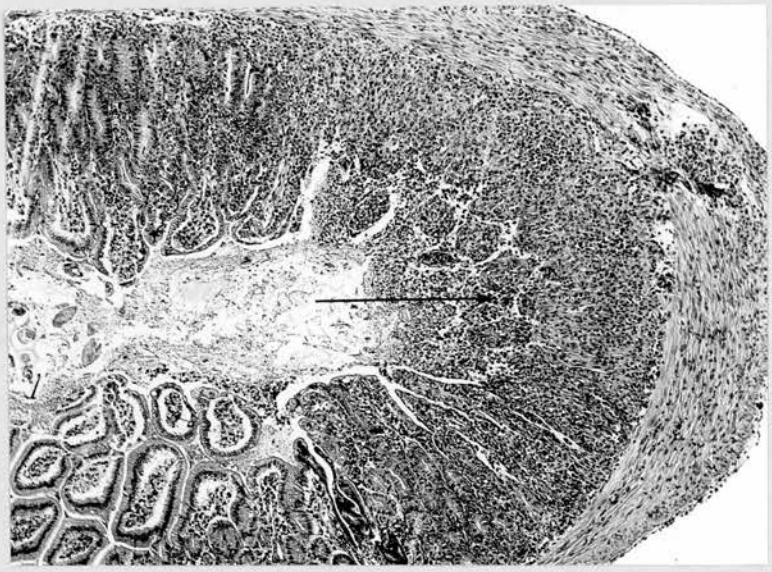

Fig.46. Transverse section of the ileum of a (C57BL x CBA)F_1 mouse. This shows an area (arrow) where the mucosa and submucosa is severely damaged.

H. and E. x 60

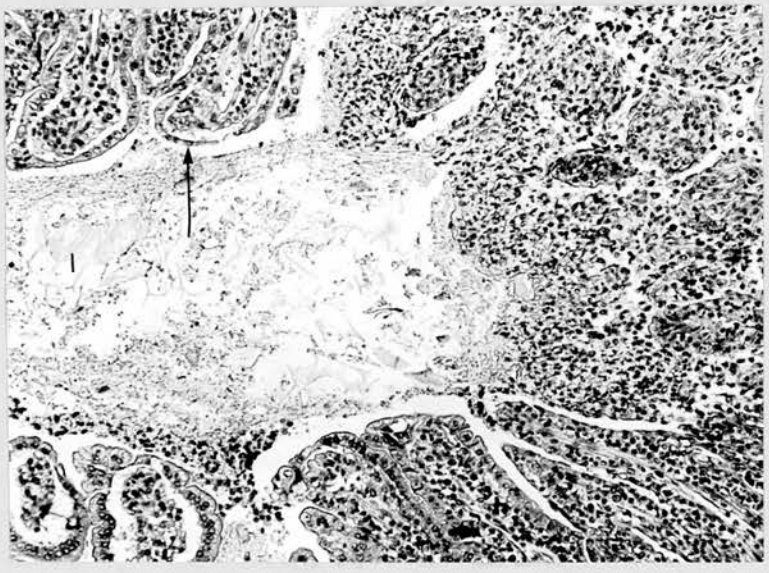

Fig.47. High power view of Fig.46. This shows clearly the acute necrotic changes in the mucosa and submucosa. The cuboidal epithelial cells of the villi are seen adjoining the necrotic area (arrow). Numerous neutrophilic polymorphonuclear cells have invaded the necrotic area.

H. and E. x 130

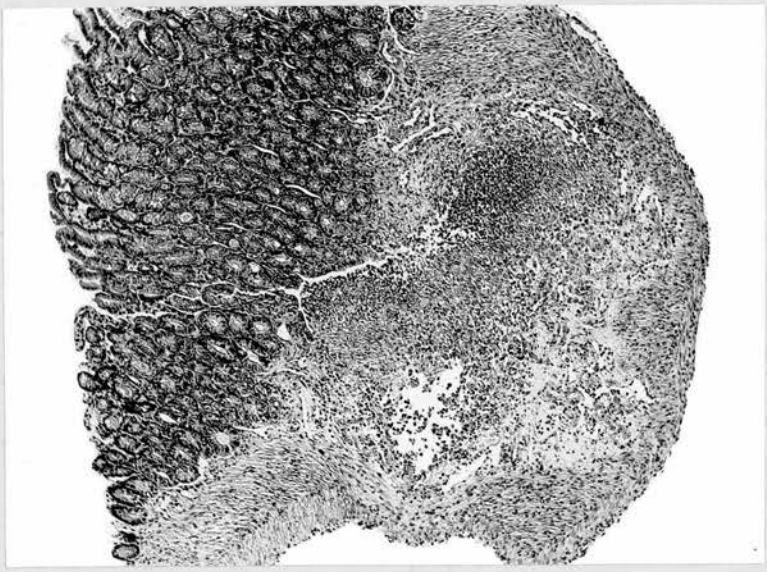

Fig. 48. Ileum of a GVHD mouse. This shows extreme atrophy of the Peyer's patch and formation of granulation tissue.

H. and E. x 55

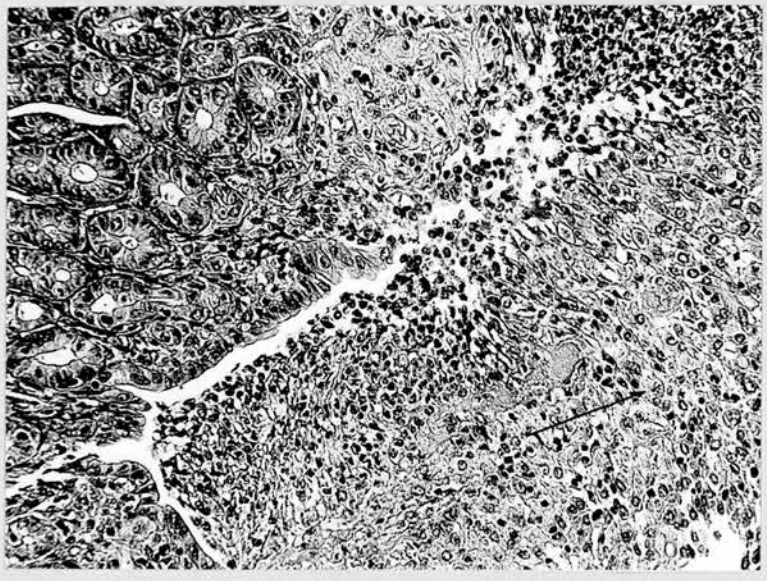

Fig. 49. High power view of Fig. 48. This shows the replacement of the lymphoid tissue by macrophages (arrow).

H. and E. x 200

animals using the dissecting microscope at a magnification of x 24. The alimentary tract was cut into 5 inch lengths and was placed on a filter paper and examined using reflected light. Examination of the mucosa of normal intestines showed finger-like projections of the villi and the whole mucosa was uniformly symmetrical (Fig.50). The jejunum and ileum of the diseased animals showed many irregular 'punched out' areas, and in some places the intestines had a honeycomb-like appearance. The walls of the intestines appeared extremely thin in these areas. In other areas, prominent irregular elevated ridges of mucosa were seen (Fig.51). The appearances seen are similar to the flat and featureless jejunal mucosa of patients with idiopathic steatorrhoea, (Booth et al, 1962).

Light Microscopic Appearance:

The jejunum and ileum were markedly damaged compared to the control intestines. There was obvious attenuation of the villi (Fig.52). In many areas, there was complete destruction of villi including the crypts of Lieberkühn and damage to the submucosal glands in the duodenum was also noted. Fusion of villi was a common feature. The picture was one of destruction as well as of repair. The epithelium lining the fused villi was cuboidal. In the lamina propria and in the submucosa chronic granulation tissue formation was seen. In these areas many mononuclear cells, macrophages and fibroblasts were seen. The circular and longitudinal muscle was much thinner over areas of mucosal damage. The elevated ridges which were seen under the dissecting microscope appeared as elevated/

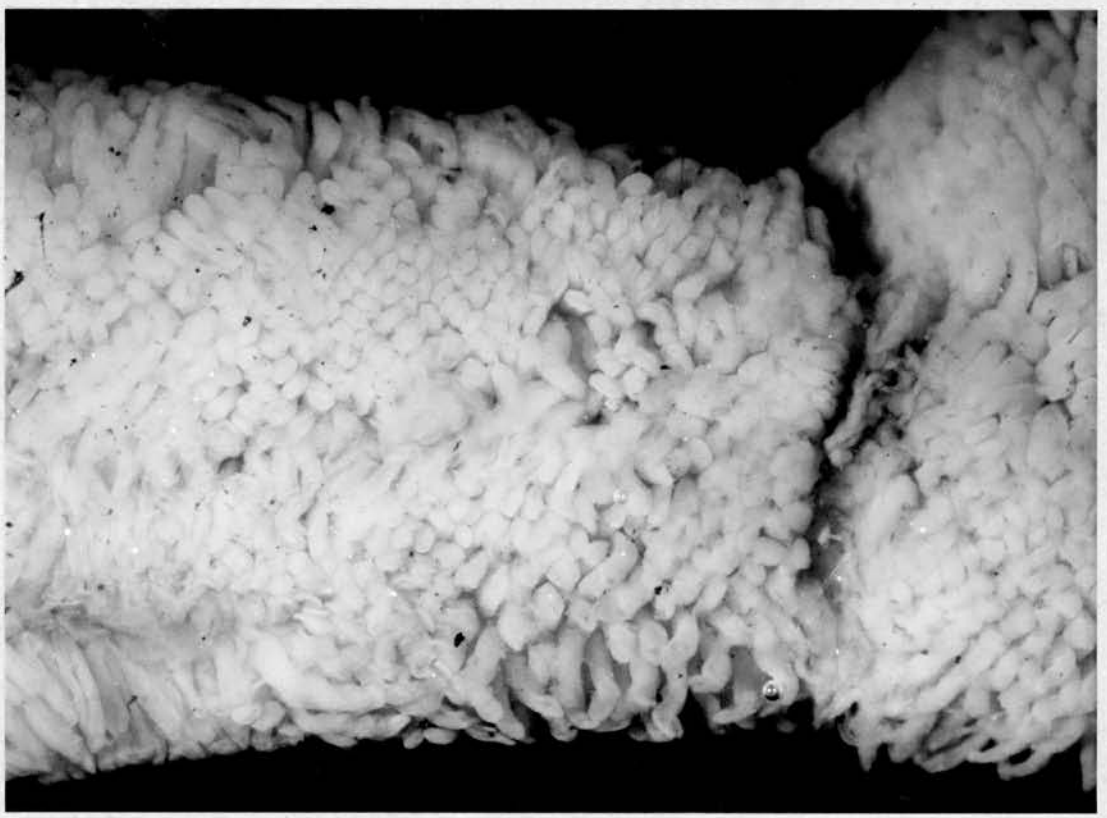

Fig. 50. This shows the ileum of a normal 8 week old $B6AF_1/J$ male mouse. The villi are seen as finger-like projections.

x 24

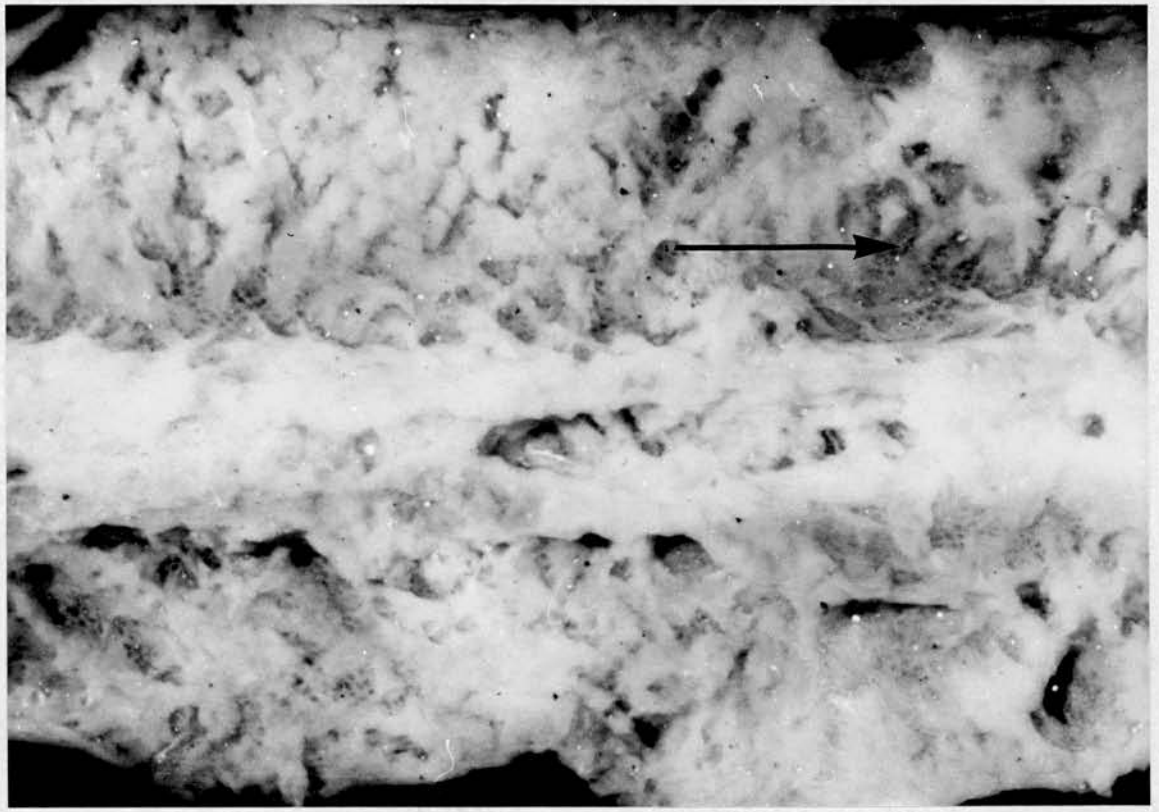

Fig. 51. This shows the ileum of an 8 week old $B6AF_1/J$ male GVHD mouse (late stage). There is evidence of subtotal and total villus atrophy. The muscular coat is extremely thin and transparent in places (arrow). The horizontal bar represents a ridge of fused villi.

x 24

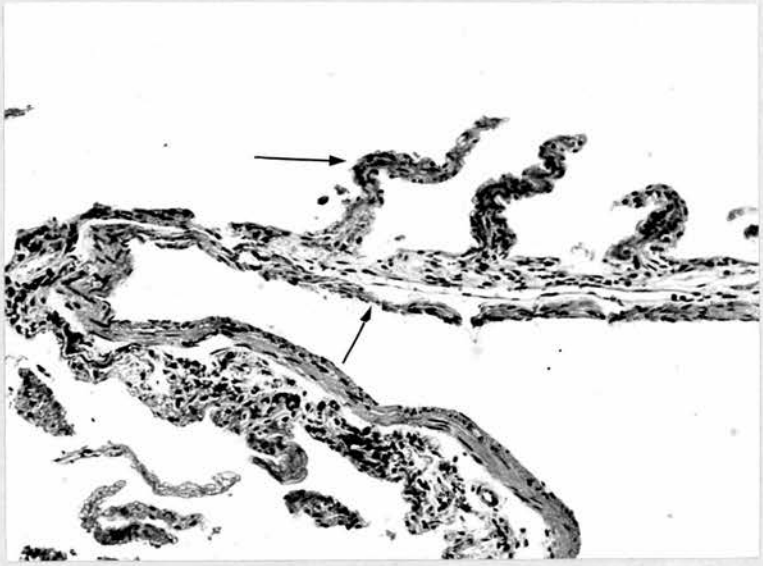

Fig. 52. A low power view of a section taken from tissue shown in Fig. 51. Many villi appear absent and the three seen are in subtotal villus atrophy (arrow). The submucosa and the muscular coat are thinned (arrow).

H. and E. x 140

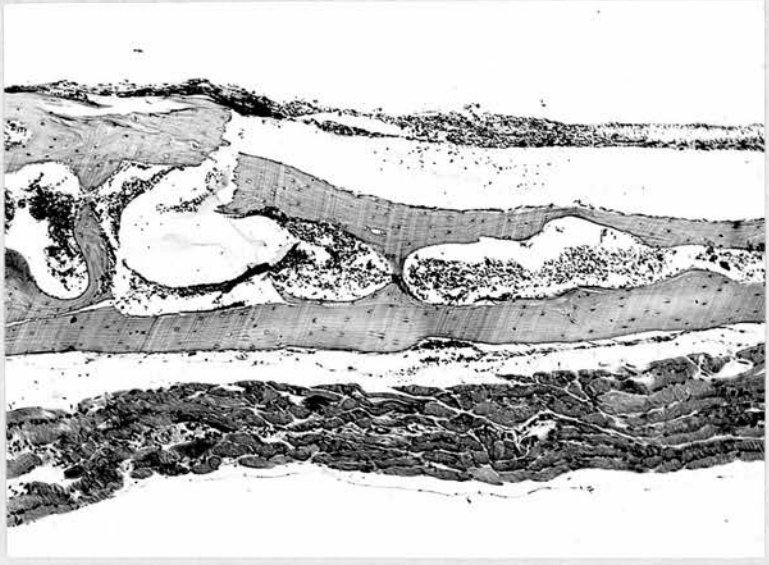

Fig. 53. Sternum of a mouse with GVHD. This shows the extreme loss of cellularity of the marrow.

H. and E. x 70

elevated areas lined by irregular fused villi.

The Peyer's patches were replaced by macrophages. The appearances were those of a slow and steady damage to the mucosa with an irregular and ineffective attempt to restore the mucosa to normal.

Similar changes were seen in two human infants dying of G.V.H.D. (Hathaway et al, 1965). The small intestine was devoid of lymphoid tissue and the ileum showed severe atrophic villi. The lamina propria contained focal infiltration of histiocytic cells.

Examination of the alimentary tract of animals inoculated with parental lymphoid cells but which did not develop significant weight loss, showed virtually no intestinal pathology when examined by the dissecting and light microscope.

Macroscopic Appearance of Bone Marrow in G.V.H.D.

A piece of skull bone from the occipital region, the sternum and the lower thoracic vertebrae were examined. The bones appeared normal but the marrow was less red than the marrows of control animals.

Microscopic Appearance of Bone Marrow in G.V.H.D.

The bone marrows of the animals sacrificed on the 5th day after the inoculation of parental cells were hypercellular. In addition to the normal erythropoietic and myelopoietic cellular precursors, equal numbers of small dark round mononuclear cells were seen. These latter cells were not seen in the control bone marrows. The marrows of animals sacrificed on the 10th, 15th and 20th day after the/

the inoculation of cells showed a progressive hypocellularity where the normal haematopoietic and myelopoietic elements were depleted and in their place were many macrophages. These macrophages were similar to those seen in the lymph nodes and spleen. Clusters of megakaryocytic cells were also seen in the early stages (Figs. 53, 54, 55, 56, 57).

Macroscopic Appearance of the Kidney in G.V.H.D.

The kidneys of the animals sacrificed on the 15th and 20th day were more pale than usual. The shape, size and texture were similar to the kidneys from control animals.

Microscopic Appearance of the Kidney in G.V.H.D.

No significant changes were observed in the animals sacrificed on the 5th day after the inoculation of cells. The animals sacrificed on the 10th, 15th and 20th day of the disease showed evidence of prominent accumulations of small dark mononuclear cells around the intralobular and interlobular arteries. Small collections of cells were also seen in the periglomerular and peritubular areas (Figs.58, 59). The cortex and medulla of the kidneys were normal and the cortico-medullary junction was clear. The pelvis appeared normal. No damage to the parenchymal cells of the kidneys were seen.

Macroscopic Appearance of the Heart in G.V.H.D.

Examination did not show any changes in the size, shape, colour or texture of the organ. The tricuspid, mitral, aortic and pulmonary valves were normal. The thickness of the right and left ventricles/

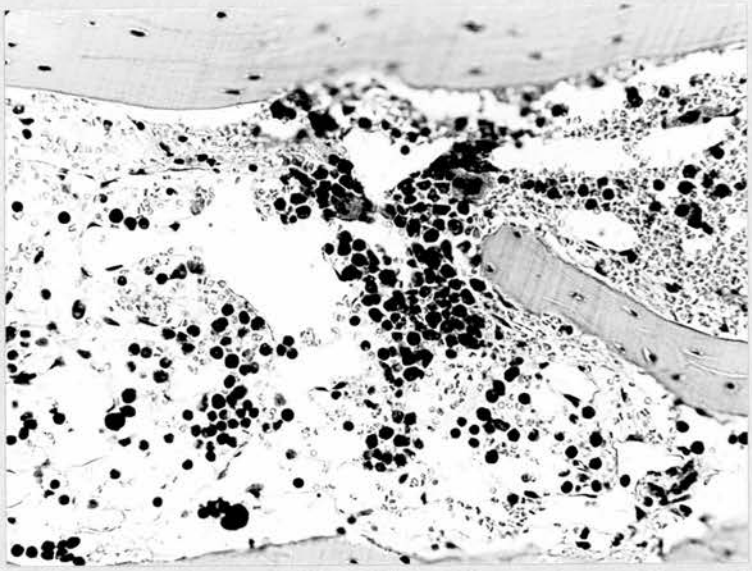

Fig. 54. High power view of Fig. 53. This shows the extremely hypocellular marrow and the presence of a few clumps of dark mononuclear cells.

H. and E. x 300

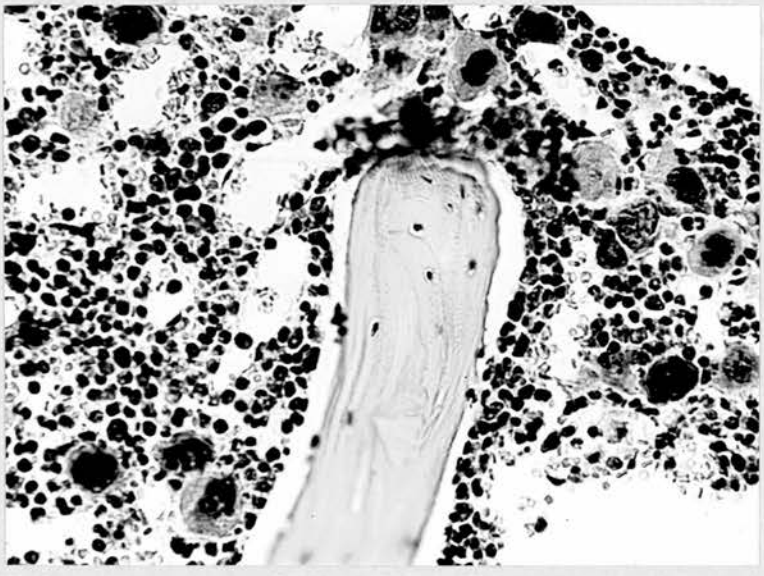

Fig. 55. High power view of Fig. 54. This shows the morphology of the dark mononuclear cells; dispersed among them are large megakaryocytic cells.

H. and E. x 350

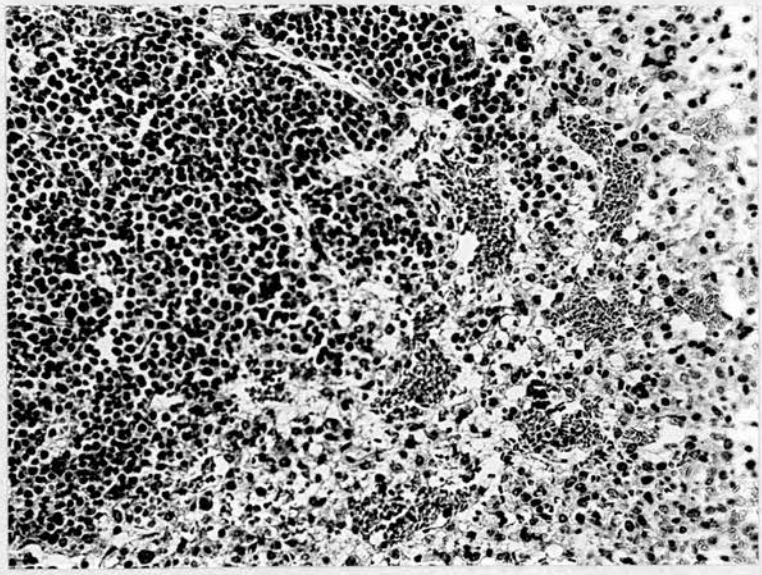

Fig. 56. Sternal marrow of GVHD mouse. This shows the dark mononuclear cells on the left and large light-pink masses of macrophages on the right. Many sinuses in this region are seen to contain red cells.

H. and E. x 225

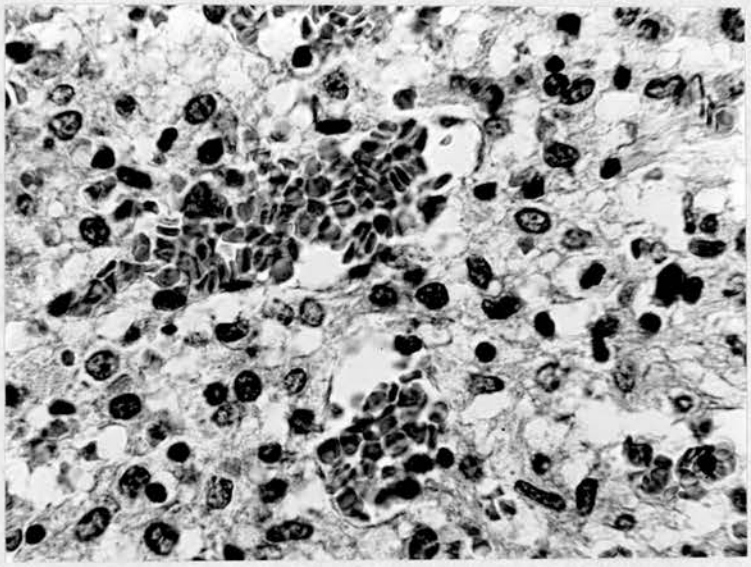

Fig. 57. High power view of the macrophages seen in Fig. 56. These cells form a syncytial mass of tissue. They have prominent nuclei and abundant pale pink cytoplasm.

H. and E. x 600

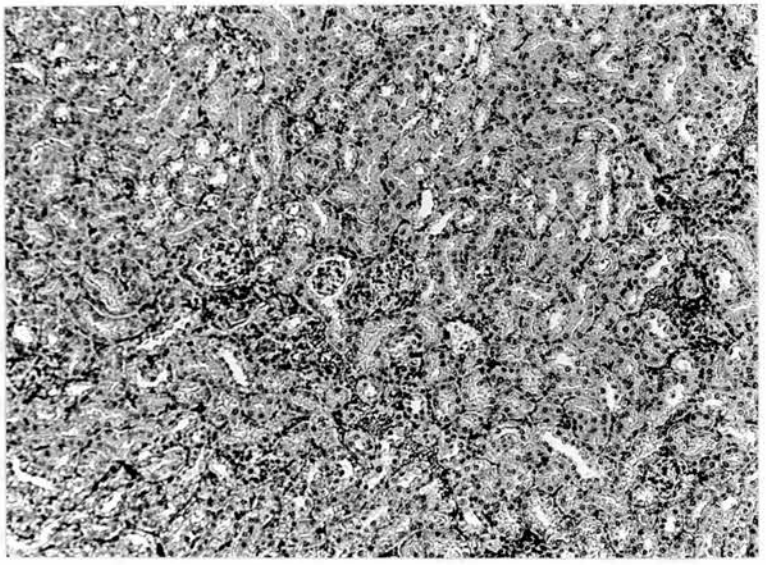

Fig.58. Cortex of the kidney of a GVHD mouse. This shows the infiltration of dark mononuclear cells into the interstitium of the tubules. The cells are distributed diffusely between the tubules and around the glomeruli.

H. and E. x 120

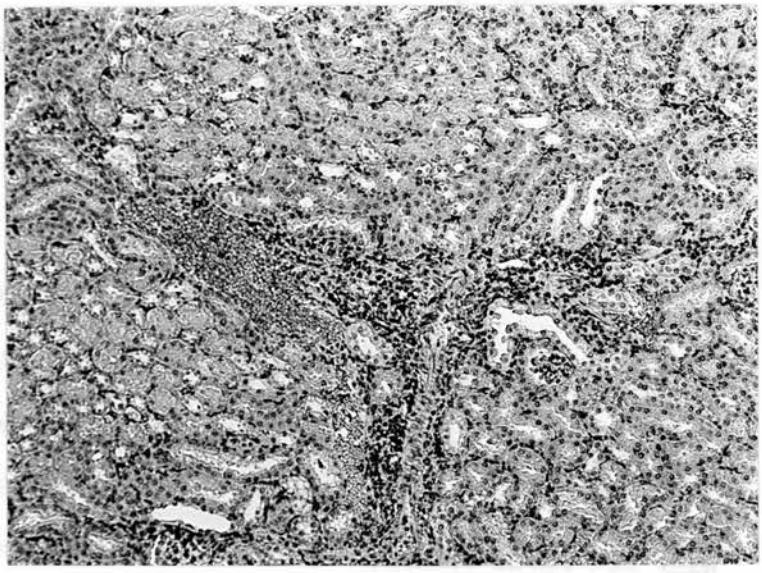

Fig.59. Medulla of the kidney of a GVHD mouse. This shows the presence of large numbers of dark mononuclear cells around an interlobar artery.

H. and E. x 120

ventricles were similar to the ventricles of control animals. The lumen of the ventricle contained clots of blood but no thrombi were seen.

Microscopic Appearance of the Heart in G.V.H.D.

There were small foci of mononuclear cells around the venules between the muscle fibres in the region of the insertions of the mitral and tricuspid valves. The muscle fibres around these cells contained deeply eosinophilic areas. In some foci, one or two muscle fibres appeared more lightly stained and vacuolated and had probably undergone cloudy degeneration. The changes noted in the heart are not impressive.

Macroscopic Appearance of the Adrenal Glands in G.V.H.D.

The animals sacrificed on the 5th day after the inoculation of cells had adrenal glands which were enlarged by about 25 per cent compared to the glands of the control animals. They were of a normal shape, colour and consistency. The glands of the animals examined on the 10th, 15th and 20th day after the inoculation of cells did not show any change in comparison to the corresponding controls.

Microscopic Appearance of the Adrenal Glands in G.V.H.D.

In the normal adult mouse the adrenal gland is composed of the cortex and medulla. The cortex is made up of the zona glomerulosa, which is rich in lipid and lies under the capsule. Next to this is the zona fasciculata, the cells of which contain abundant lipid and this zone constitutes more than half of the cortex. Most of them have clear or/

or vacuolated cytoplasm (called clear cells) in Haematoxylin and Eosin preparations. Some cells are eosinophilic with very little lipid (called compact cells). Both these cell types are diffusely scattered throughout the zona fasciculata. The zona reticularis is next to the fasciculata. These cells are much more eosinophilic and poor in lipid, which is scattered in fine droplets throughout the cytoplasm of the cell. In H. and E. preparations the distinction between zona glomerulosa and fasciculata is not clear. In many areas the zona fasciculata is adjacent to the capsule and hence the zona glomerulosa does not form a continuous outer layer.

The adrenals of G.V.H.D. mice sacrificed on the 5th day of the disease had enlarged zona glomerulosa and fasciculata but the latter areas were more prominent than the former. Most of the cells were clear cells containing lipid and the rest were compact cells. The medulla of the gland did not show any change from the controls. The adrenals of G.V.H.D. mice sacrificed on the 10th day did not show any significant change.

The adrenals of G.V.H.D. mice examined on the 15th and 20th day of the disease showed distinct changes. The zona glomerulosa and the adjacent fasciculata were completely depleted of lipids and cytoplasm and were small. Many other cells had pyknotic nuclei, diminished amount of lipids and pale cytoplasm, thus showing cytolytic degenerative changes. The solid cords of cells of the rest of the zona fasciculata showed lipid depletion and the cytoplasm was more eosinophilic (like compact cells). In between the cords of cells/

cells, many elongated oval nuclei are seen and these are probably reticulo-endothelial cells lining the sinuses of the vascular system. In the outer cortex they were found intermingling with the lipid depleted degenerating cells of the zona glomerulosa and fasciculata. Many macrophages and mononuclear cells were also seen in the junction between the zona reticularis and the medulla. These cells appear to extend into the latter two areas (Fig.60).

The brain, skeletal muscle, bone, cartilage, thyroid, pituitary, ovaries, testis, lungs and skin were examined macroscopically and microscopically in all the stages. No significant changes were seen in comparison to the tissues from control mice.

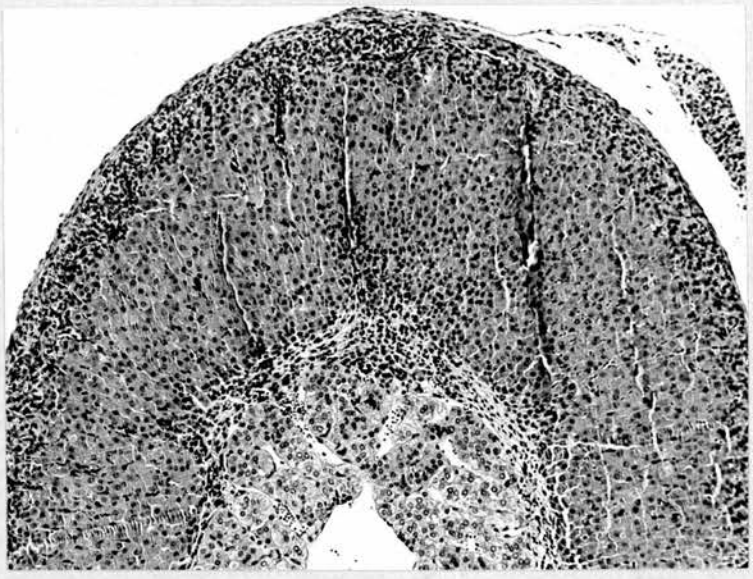

Fig.60. Adrenal gland of a GVHD mouse. The architecture of the zona glomerulosa and the fasciculata appears distorted and shows cytolytic degenerative changes. The rest of the zona fasciculata shows marked lipoid depletion. Many mononuclear cells are seen in between the zona reticularis and the adrenal medulla.

H. and E. x 120

TABLE 2

The number of mice with lesions and the degree of changes in the various organs of animals undergoing G.V.H.D. on the 5th day (Stage 1) after the inoculation of parental lymphoid cells.

Tissues	No. of Animals	No. of Animals with lesions	Infiltration by cells
Lymph node	6	6	+
Spleen	6	6	+
Liver	6	6	+
Thymus	6	0	-
Bone marrow	6	3	+
Intestine	6	6	+
Salivary gland	6	5	+
Pancreas	6	4	+
Kidney	6	1	+
Heart	6	1	+
Adrenal	6	0	-

- = No infiltration by lymphoid cells and/or macrophages.

+ = Infiltration by lymphoid cells and/or macrophages.

TABLE 3

The number of mice with lesions and the degree of changes in the various organs of animals undergoing G.V.H.D. on the 10th day (Stage II) after the inoculation of parental lymphoid cells.

Tissues	No. of Animals	No. of Animals with lesions	Infiltration by cells and degree of damage
Lymph node	6	6	++
Spleen	6	6	++
Liver	6	6	+
Thymus	6	6	+
Bone marrow	6	2	+
Intestine	6	4	++
Salivary gland	6	2	+
Pancreas	6	6	++
Kidney	6	6	+
Heart	6	2	+
Adrenal	6	0	-

- = No infiltration by lymphoid cells or macrophages.

+ = Infiltration by lymphoid cells and/or macrophages.

++ = Infiltration by cells with minimum to moderate damage to tissue.

TABLE 4

The number of mice with lesions and the degree of changes in the various organs of animals undergoing G.V.H.D. on the 15th day (Stage III) after the inoculation of parental lymphoid cells.

Tissues	No. of Animals	No. of Animals with lesions	Infiltration by cells and degree of damage
Lymph node	6	6	+++
Spleen	6	6	+++
Liver	6	6	+
Thymus	6	6	++
Bone marrow	6	4	++
Intestine	6	4	++
Salivary gland	6	4	++
Pancreas	6	6	++
Kidney	6	3	+
Heart	6	2	+
Adrenal	6	2	++

- = No infiltration by lymphoid cells and/or macrophages.

+ = Infiltration by lymphoid cells and/or macrophages.

++ = Infiltration by cells with minimum to moderate damage to tissue.

+++ = Infiltration by cells with moderate to severe damage to tissue.

TABLE 5

The number of mice with lesions and the degree of changes seen in the various organs of animals undergoing G.V.H.D. on the 20th day (Stage IV) after the inoculation of parental lymphoid cells.

Tissues	No. of Animals	No. of Animals with lesions	Infiltration by cells and degree of damage
Lymph node	6	6	+++
Spleen	6	6	+++
Liver	6	6	+
Thymus	6	4	+++
Bone marrow	6	4	++
Intestine	6	6	++
Salivary gland	6	3	++
Pancreas	6	5	++
Kidney	6	5	+
Heart	6	2	+
Adrenal	6	3	++

- = No infiltration by lymphoid cells and/or macrophages.

+ = Infiltration by lymphoid cells and/or macrophages.

++ = Infiltration by cells with minimum to moderate damage to tissue.

+++ = Infiltration by cells with moderate to severe damage to tissue.

TABLE 6

The sequence of changes seen in the various tissues of mice with G.V.H.D. when examined at various stages of the disease.

Tissues	Stage I	Stage II	Stage III	Stage IV
Lymph node	+	++	+++	+++
Spleen	+	++	+++	+++
Liver	+	+	+	+
Thymus	-	+	++	+++
Bone marrow	+	+	++	++
Intestine	++	++	++	++
Salivary gland	+	+	++	++
Pancreas	+	++	++	++
Kidney	+	+	+	+
Heart	+	+	+	+
Adrenal	-	-	++	++

- = No infiltration by lymphoid cells and/or macrophages.

+ = Infiltration by lymphoid cells and/or macrophages.

++ = Infiltration by cells with minimum to moderate damage to tissue.

+++ = Infiltration by cells with moderate to severe damage to tissue.

Stage I = 5th day
Stage II = 10th day
Stage III = 15th day
Stage IV = 20th day

(C) DISCUSSION

Lymph Nodes

The normal lymph node can be divided into two functional areas depending on the response to antigenic or chemical stimuli. White (1960), in his studies on the primary response to a soluble antigen noted the development of plasma cells of varying grades of maturity in the medulla without in many cases any changes in the outer cortex, follicles or 'para-cortical' areas. He was of the opinion that the cells concerned in antibody formation were mainly confined to the medulla. Subsequently, other workers showed that the germinal centres and plasma cell proliferation in the medulla were associated with the humoral antibody response, (Turk and Oort, 1967; Parrott and Sousa, 1966; Cottier et al, 1967).

The term 'para-cortical zone' (mid and deep cortex) was introduced by Oort and Turk (1965) as a result of studies on the auricular lymph nodes of guinea-pigs after initial sensitization with the chemical sensitizing agent Oxazolone. They found that the area in the lymph node between the outer cortex and medulla had enlarged and contained an increased proportion of large pyroninophilic cells. No changes were noted in the outer cortex, follicles and in the medulla. Similar changes were noted in the 'para-cortical areas' of the draining regional lymph nodes of homografted rabbits (Scothorne and McGregor, 1955). Oort and Turk were of the opinion that the para-cortical areas of lymph nodes were associated with specific cell-mediated immunity or delayed hypersensitivity whereas the germinal centres/

centres and plasma cell proliferation in the medulla were associated with humoral antibody response. The para-cortical areas of lymph nodes had also been called 'thymus-dependent areas' by Parrott et al (1966). These workers found that mice thymectomized at birth, showed a depletion of lymphocytes in the lymph nodes, primarily restricted to the para-cortical area, leaving the outer cortex, follicles and medulla relatively unaffected. Autoradiographic studies made by them also demonstrated that thymus cells localize almost exclusively in the thymus-dependent areas (para-cortical) of the lymph nodes. These studies show that lymph nodes have two functionally different regions.

Fig.21 demonstrates the behaviour of lymph nodes during various types of antigenic stimulation; primary response.

The lymph nodes of mice undergoing G.V.H.D. on the 5th day after the inoculation of parental lymphoid cells, showed an expansion and hypercellularity of the para-cortical areas. Pyroninophilic blast cells were seen in large numbers in the medulla and in the para-cortical area. The germinal centres were normal in appearance and new germinal centre formation was not seen.

Pyroninophilic cells were seen in the para-cortical areas of lymph nodes in guinea-pigs after initial sensitization with the chemical sensitizing agent Oxazolone (Oort and Turk, 1965). The increase in large pyroninophilic cells was mainly confined to the para-cortical area of the node and this was associated with delayed hypersensitivity reactions. Hence the para-cortical area of the lymph/

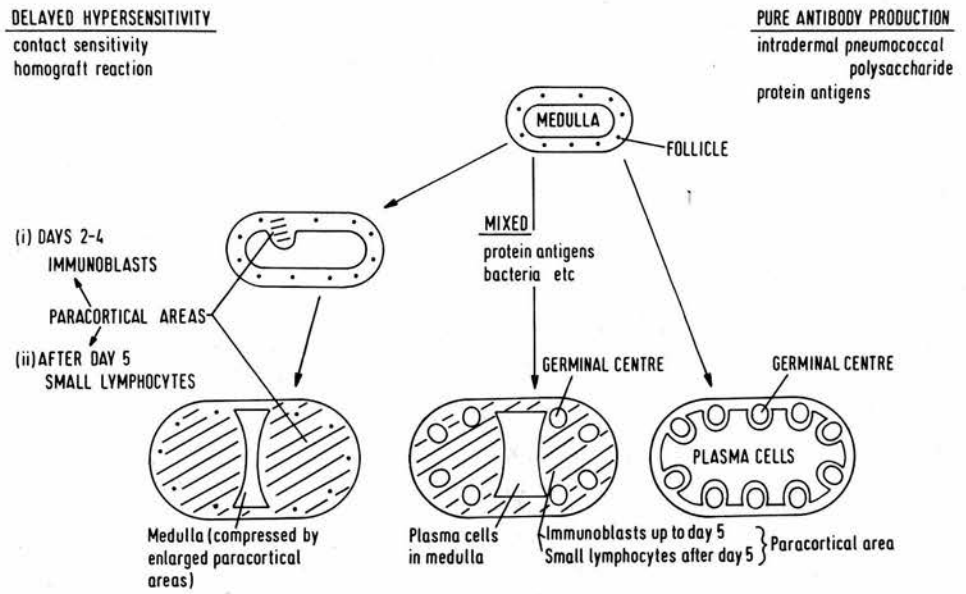

Diagram of the behaviour of lymph nodes during various types of antigenic stimulation: primary response.

Fig.21. Diagram taken from the book 'Delayed Hypersensitivity' by J.L. Turk, p.152.

lymph node was associated with specific cell-mediated immunity.

The presence of pyroninophilic cells in the para-cortical area of the lymph nodes of mice with G.V.H.D. thus may reflect reactions of cell-mediated nature. Gowans (1962) has shown the presence of large numbers of radio-labelled thoracic duct lymphocytes in the cortex of lymph nodes of F_1 hybrid rats inoculated with radio-labelled parental cells. He has also demonstrated by means of the above technique the transformation of parental small lymphocytes into large pyroninophilic cells. Hence the parental lymphoid cells could be expected to transform into large pyroninophilic cells in the para-cortical area of the lymph node of G.V.H.D. mice. Since pyroninophilic cells are found in the para-cortical areas in cell-mediated immune reactions, it is possible that the pyroninophilic cells in the para-cortical areas of the lymph nodes in G.V.H.D. are also involved in cell-mediated reactions.

Pyroninophilic cells are also seen in the medulla of G.V.H.D. lymph nodes. The medulla is normally involved in humoral antibody production and this is reflected by the presence of plasma cells of varying degrees of maturity (White, 1960). It may be that the many pyroninophilic cells seen in the medulla of G.V.H.D. lymph nodes are precursors of plasma cells. The presence of macrophages in the lymph nodes of G.V.H.D. mice was one of the significant features noted. These cells had a characteristic morphology and they phagocytosed colloidal carbon in significant amounts. It is not possible to distinguish the parental and F_1 hybrid cells in my histopathological studies. The macrophages were initially found in the para-

cortical/

para-cortical areas and steadily replaced the cells of the G.V.H.D. lymph nodes so that in the terminal stages of the disease they almost completely replaced the node. These cells were also seen to be in mitosis in many areas. They were non-pyroninophilic and many cells showed nuclear pleomorphism. Lympho-phagocytosis was another feature that was noted in these nodes. Some of these macrophages were in the form of giant cells with many nuclei and abundant cytoplasm.

In the early phases of G.V.H.D. the nodes had moderate numbers of pyroninophilic cells and macrophages while in the late stages most of the cells were macrophages. Howard et al (1969) and Boak et al (1968) have shown by use of the combination of techniques such as the use of chromosome marker, auto-radiography and colloidal dyes, that a proportion of donor lymphoid cells in mitosis have macrophage-like properties, as seen by the uptake of colloidal particles. This was noted in the liver, peritoneum and lungs of G.V.H.D. mice. Hence it is possible to speculate that a proportion of pyroninophilic cells seen in the para-cortical area of G.V.H.D. lymph nodes may have transformed into macrophages and perhaps the excess foreign antigens of the F_1 hybrid host could be a stimulus for these cells to transform.

Spleen

The increase in spleen weight was an important finding in G.V.H.D. In the strain studied by me the spleen was maximally enlarged/

enlarged ten days after the inoculation of parental cells. In the early stages of the disease the 'white pulp' was hypercellular and many mitotic figures were seen. The malpighian bodies contained pyroninophilic cells (less than 1 per cent) and most of these were confined to the periphery while the rest were around the central arteriole. In the intermediate stage of the disease, there was steady diminution of the number of lymphocytes and pyroninophilic cells while at the same time the macrophages were progressively increasing in numbers until finally they completely replaced the whole follicle. At this stage the appearances of the 'red and white pulp' were most remarkable. The 'red pulp' was packed with prominent red-staining pyroninophilic cells while the 'white pulp' was of a pale-pink colour containing only macrophages. These colour differences made the junction between these two regions quite distinct. Thus the appearances of the malpighian bodies of the spleen were similar to that observed in the lymph nodes.

The 'red pulp' which initially showed small round cells and pyroninophilic cells in almost equal numbers had in the final stages pyroninophilic cells to an extent of 70 per cent. Thus it is possible that the donor and the host were responsible for the increase in these cells. The lymphoid cell suspension was prepared from the spleen and lymph nodes. It may be that the 'red pulp' of the parental spleen had 'homed' in the 'red pulp' of the F_1 hybrid. It was also significant that macrophages which were abundant in the red pulp of the normal spleen were absent in the red pulp of G.V.H.D. spleens./

spleens. It was hence possible that the host macrophages had migrated to the malpighian bodies and had caused the destruction of those cells. The presence of clumps of large multinucleate giant cells in the 'red pulp' was also a regular feature. These cells were pyroninophilic and they could be megakaryocytes derived from the parent or F_1 hybrid or both. Since the presence of 'foreign body' giant cells is a regular feature at sites of inoculation of non-antigenic material, it may very well be that these cells represent, in part at least, a form of host response to the harmful non-antigenic parental cells.

In the terminal stages, the macrophages which were present in the malpighian bodies appeared to have undergone degeneration and death, leaving the cytoplasmic materials as a pale pink homogeneous finely granular mass. It is very likely that this material is primary amyloid which normally may not stain with the conventional stains for amyloid except with thioflavine T. The fibrin or "fibrinoid" which was also found in these areas may have come from the blood vessels in these regions, by a process of insudation.

The changes seen in the spleen are thus a part of a generalised change that is seen in the lymphatic tissues of G.V.H.D. animals.

Liver

Like the lymph nodes and spleen, the liver also showed definite changes in all the animals examined. The increase in size and number of Kupffer cells in G.V.H.D. animals correlates well with the increased/

increased function of rapid clearance of intravenously injected colloidal carbon. It was noted that cells in the periportal tract which were non-pyroninophilic and which had the appearances of macrophages, had not phagocytosed colloidal carbon. It is possible that the inoculated colloidal carbon had been taken up by the Kupffer cells and thus the periportal macrophages were not presented with any colloidal particles for phagocytosis. An alternative possibility is that these may be reticular cells.

The Role of the Thymus in the Pathogenesis of G.V.H.D.

There are no reports in the literature to mention that the thymus undergoes initial enlargement before it begins to atrophy. Hildemann et al (1964) noted that as early as the first day after the inoculation of donor cells the thymus underwent diminution in weight at a time when the lymph nodes and spleen showed a significant increase in weight. These workers were of the opinion that the thymic changes preceded that of the peripheral lymphoid organs and it was argued that the depletion of lymphocytes in lymphoid organs, seen in the intermediate and late stages, were secondary to the thymic atrophy. Thus the possibility was put forward that G.V.H.D. was due to 'immunological thymectomy' where the thymus was primarily destroyed by the inoculated donor cells and the changes seen in the rest of the tissues of G.V.H.D. animals were as a result of the destruction of the thymus. (Neonatal thymectomy in mice produces a wasting syndrome which bears a similar resemblance to the wasting of G.V.H.D. animals). However,/

However, thymic involution based on immunological assault in the G.V.H. reaction appeared unlikely because Gowans and Knight (1964) and Harris and Ford (1964) had observed that peripheral lymphoid cells did not reach the thymus in their pathway of recirculation within the lymphoid tissue.

In contrast to the above view Heim et al (1967) was of the opinion that the changes in G.V.H.D. were independant of thymic involution and that the changes seen in the latter organ were probably the result of adrenal hyperactivity. Thus these workers studied the effect of adrenalectomy on the thymus gland in mice undergoing G.V.H.D. They adrenalectomized a group of F_1 hybrid mice and inoculated parental lymphoid cells and also inoculated parental lymphoid cells into a group of non-adrenalectomized syngeneic mice. Both groups exhibited the typical clinico-pathological findings of G.V.H.D. but the adrenalectomized mice did not develop any destructive changes in the thymus. Thus it appears that the destruction of the thymus in G.V.H.D. is due to the presence of the adrenal gland. These workers conclude that the thymic involution observed in G.V.H. reaction does represent immunological thymectomy but results from the stress of an immunologically induced disease.

My examination of the thymuses in G.V.H.D. showed the presence of significant numbers of macrophages, a marked reduction of thymocytes and an absence of pyroninophilic cells. These appearances were closely similar to those seen in the lymph nodes. It/

It is my hypothesis that the macrophages of the thymus have developed an altered reactivity towards 'self' and have caused the depletion of thymocytes. The absence of pyroninophilic cells was a very notable feature.

Pancreas

Walters (1966) noticed severe granulomatous pancreatitis in neonatal mice with G.V.H.D. He found that sheets of lymphocytes and macrophages arising from the lympho-reticular aggregates of the covering serosa spread as an inflammatory pannus throughout the pancreatic interstitial tissue and ultimately only the degenerating remnants of acini, islets and ductules were found to remain. Gorer and Boyse (1959) using pre-immunized donor cells to produce G.V.H.D. in adult F_1 hybrids, noticed extensive infiltration of the omentum, pancreas and patches of fat necrosis. It must be noted that these workers inoculated the donor cells by the intra-peritoneal route. These workers had not described the infiltration of macrophages into the pancreas as was described by me.

The Alimentary Tract in G.V.H.D.

It was noted that when the G.V.H.D. animals did not develop wasting, no intestinal change occurred. It was also observed that all the animals which appeared ill and wasted had intestinal pathology. In the strain (C57BL x CBA)F_1 hybrid which developed a more severe form of the disease, intestinal lesions were more of a destructive nature with less evidence of reparative tissue changes.

On/

On the contrary, in the B6AF$_1$/J hybrid animals, the disease process ran a longer clinical course before death. In these animals the repair of the intestinal damage did not restore the architecture to normal. Similar changes are seen in human Idiopathic Steatorrhoea, (Booth et al, 1962). These results show that damage to the intestinal mucosa was one of the factors in the causation of wasting. This damage could cause loss of proteins from the intestinal vessels. Thus Cornelius (1970) studied the loss of plasma proteins into the bowel of mice with G.V.H. reactions and compared the results with the histological changes in the alimentary tract. By tagging $^{51}Cr\ Cl_3$ to plasma proteins, he found that the loss of $^{51}Cr\ Cl_3$ into the faeces was considerably greater than in control mice and histological damage was detectable in every runting animal. Hypoalbuminaemia was also noted in these animals. This author was of the opinion that protein loss into the intestinal tract was one of the factors responsible for the generalised wasting in G.V.H.D.

Wasting may also result from inadequate intake of a balanced diet. The food and water intake was investigated from day 6 after the inoculation of cells to day 23. From day 6 to 13, the test mice ingested less amounts of food and water compared to control mice (Table 7). From day 13 to 23 the test and control mice ingested almost the same amount of food and water even though the test animals were losing weight gradually (Table 8).

Wasting in G.V.H.D. may also result from other causes. The damage to the intestinal mucosa could result in easy access of normal and/

TABLE 7

FOOD AND WATER INTAKE OF B6AF$_1$/J NORMAL AND GVHD (TEST) MICE FROM DAY 6 to 13 AFTER INOCULATION OF PARENTAL CELLS

Mice	Number of mice	Food Intake	Water Intake	Amount of food per mouse for 7 days	Amount of water per mouse for 7 days
GVHD (Test)	13	190 g.	175 ml	14.6 g.	13.5 ml
Normal	12	265 g.	290 ml	22.0 g.	24.0 ml

TABLE 8

FOOD AND WATER INTAKE OF B6AF$_1$/J NORMAL AND GVHD (TEST) MICE FROM DAY 14 to 23 AFTER INOCULATION OF PARENTAL CELLS

Mice	Number of mice	Food Intake	Water Intake	Amount of food per mouse for 10 days	Amount of water per mouse for 10 days
GVHD (Test)	13	425 g.	400 ml	33.5 g.	30.0 ml
Normal	12	420 g.	330 ml	35.0 g.	27.5 ml

and pathogenic bacteria to the wall of the intestine and cause infection of the gut. This infection could then spread systemically in the immunologically deficient animal. However histological examination of the tissues did not show evidence of systemic infection. Investigation of the plasma of G.V.H.D. mice by Billingham and Brent (1959) showed that infection cannot be transferred by plasma. They inoculated newborn mice with lightly spun heparinized plasma from G.V.H.D. mice and found it to be entirely innocuous.

The rôle of endotoxin in causing wasting disease had been investigated by many workers. Howard (1961b) had shown that in mice undergoing G.V.H.R. the resistance to shigella dysenteriae type I endotoxin was reduced at a time when these mice showed maximum phagocytic activity. Similar studies were made by Keast (1968). He produced the wasting of G.V.H.D. in neonatal mice by the inoculation of allogeneic lymphoid cells. When a similar batch of mice were treated regularly with Neomycin sulphate gavages so as to reduce the bacterial flora of the gut, the wasting was found to be markedly reduced. It was found that less than 20 per cent of the animals survived in the G.V.H.R. groups, whereas 75 per cent of the animals survived to 70 days in the G.V.H.R. group receiving Neomycin sulphate gavaging. Histopathological changes in G.V.H.R. animals receiving Neomycin sulphate were also less acute. The animals examined from day 21 to 30 showed minimal lesions in the liver, pancreas and small intestine (Keast and Walters, 1968). Neomycin gavage may exert its beneficial action by preventing growth of the bacterial flora of the gut/

gut and this would mean reducing the amount of endotoxin that was produced in the gut. It was possible that the damaged gut in G.V.H.D. allowed abnormal amounts of endotoxin into the circulation and this would lead to wasting and generalized toxaemia. It had been shown by Keast (1968) that inoculation of bacterial endotoxin into normal mice produced runting and that this had many features similar to G.V.H.D.

Another possibility was that lymphocytes had a part to play in maintaining the vitality of normal gut epithelium. Gowans (1962) had shown by radio-labelling techniques that the large lymphocytes derived from the thoracic duct of parental strain rats 'home' in the stroma of the villi of the intestines.

It is also reported that the lymphocytes exert a trophic effect on the growth of rapidly proliferating cells (Loutit, 1962). Hence the lymphocyte aggregates found throughout the ileum and large intestine may be a source of growth factors. Some evidence suggests that this is true. Isologous lymphocytes appear to be necessary to maintain vitality of the intestinal epithelium after irradiation (Delorme, 1961). However, decrease in regeneration of bowel epithelium occurs in irradiated mice after the injection of homologous spleen cells, (Nowel and Cole, 1961). It may be that colonization of the gut of G.V.H.D. mice with parental lymphoid cells alters the proper inter-relationship that normally exists between isologous lymphoid cells and gut epithelium. This may in turn cause damage to the gut epithelium.

The/

The Consideration that the Alimentary Tract and the other Associated Glandular Structures are Primary 'Target Organs' in G.V.H.D.

The results show that the main pathological changes were confined to two main groups of systems.

1. Lympho-haemopoietic system.
2. Alimentary system and glands that are embryologically derived from it. The crypts of Lieberkühn are glands derived from the intestinal epithelium and found in the mucosa. The submucosal glands present in the duodenum also develop from the same epithelium. The salivary glands, liver and pancreas (exocrine) also develop as out-pouching from the alimentary system and their ducts maintain the connection with the alimentary system, (Ham and Leeson, 1961).

It was noteworthy that the non-lympho-haemopoietic organs that were involved in G.V.H.D. were the intestines including the glands contained within their walls as well as the salivary glands, liver and pancreas which were embryologically derived from it. It was noted in these organs that there was damage to parenchyma and also that macrophages, a few pyroninophilic and mononuclear cells were present. Hence these structures are probably important target organs in mice with G.V.H.D. In rats with G.V.H.D. severe skin lesions were a conspicuous finding and it was postulated that the skin in this species could be a primary target organ for allogeneic lymphoid cells, (Billingham, 1966-67).

Experimental studies on allografts of intestine show that the mucosa/

mucosa of the gut is an early target tissue in the rejection phenomenon. The mucosa showed maximal early damage and the central lacteals were also found to be dilated and associated with the damage, (Preston et al, 1965; State, 1969). All this evidence tends to suggest that the gut and structures derived from the gut may be primarily involved in the interaction between it and parental lymphoid cells. However the damage to the pancreas, liver and salivary glands was not serious enough to reduce the functions of these organs. On the contrary, the intestinal damage could lead to, as discussed above, loss of plasma proteins and absorption of excessive amounts of endotoxins which could lead to wasting and toxaemia in these lymphocyte-depleted mice.

Bone Marrow

Githens et al (1968) studied the bone marrow changes in mice undergoing G.V.H.D., from birth to 4 weeks of age. It was noted that a significant proportion of animals develop marrow abnormalities. In the early stages myeloid hyperplasia was noted followed by marked hypoplasia or aplasia during the 2nd and 3rd week. A characteristic infiltrate of the marrow with lymphocytoid, plasmacytoid and histiocytic cells was frequently seen by these workers. The marrows examined by me also showed hypoplasia and in addition there were sheets of macrophages replacing the normal marrow tissue.

Kidney and Heart

Cornelius et al (1968) rendered 'A' strain mice tolerant of (A x C57BL/1)F_1 hybrid tissue by a heavy dose of total body irradiation/

irradiation and injection of hybrid spleen cells. Thus these animals had an F_1 hybrid 'lymphoid system'. To these animals 'A' strain spleen cells were injected and these animals developed haematological and pathological features similar to animals with G.V.H.D. In these animals renal and cardiac changes were seen. The kidney had poorly outlined pale areas. Homogeneous glomerular deposits were seen which were positive for periodic acid-Schiff and Congo red. Tubular atrophy was widespread and severe. Myocardial necrosis was seen in almost half the tolerant mice injected with pre-immunized donor cells. These changes are considered to be non-specific as the inoculated 'A' spleen cells cannot interact with the 'A' strain kidney and heart cells of these tolerant mice.

The mononuclear cell infiltration and the minimal parenchymal damage to the kidney and heart noted by me in mice with G.V.H.D. are probably of no significance.

Adrenal Gland

The zona glomerulosa and fasciculata were found hypertrophic during the early stages of the disease. This would mean that these zones of the gland were hyperactive probably as a non-specific response to prolonged stress, (Russfield, 1967). It is possible that this hyperactivity has in turn led to exhaustion. The changes noted in the intermediate and terminal stages of the disease support this view. The reaction of the adrenal cortex to stress and trauma had been extensively studied in human beings, (Symington, 1969).

The/

The most outstanding change that occurred in the adrenal cortex to stress of a known intensity and lasting for a definite period of time was the variable distribution and size of the lipid globules. In addition Symington et al (1955) took into consideration the significance of such factors as alteration in cell-type, presence of degenerative cell changes, the distribution of RNA and non-specific cytoplasmic enzymes in assessing the changes that occur in stress.

In the zona glomerulosa and the adjoining fasciculata, the most severe changes were seen while the rest of the fasciculata showed lipid depletion. Such changes are seen in adrenals in conditions such as post-operative states complicated by haemorrhage or infections, in acute generalized peritonitis and in severe fatal burns. Hence the changes seen in the adrenals in these states must necessarily involve severe adrenal stress. The changes seen in the adrenals in the intermediate and late stage of O.V.H.D. are due to the state of stress probably caused by slow progressive cell damage.

CHAPTER III

THE PATHOGENESIS OF G.V.H.D; STUDIES ON THE INTERACTION BETWEEN MACROPHAGES AND LYMPHOID CELLS BY CELL CULTURE TECHNIQUES

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(A) HISTORICAL ACCOUNT AND REVIEW OF LITERATURE ON THE INTERACTIONS OF CELLS AND TISSUES IN CULTURE

The technique of tissue and cell culture was developed in the last century, as early as 1885, when Wilhelm Roux maintained the medullary plate of the chick embryo in warm saline for a few days. Since then, great interest has been shown by workers all over the world and this resulted in many refinements of the original techniques but until the 1940's the studies were mainly focussed on the mechanism of growth and differentiation of tissues, as studied by the embryologists at that time. Most of the early investigators made use of pieces of animal tissue as culture materials, (explants).

Just as much as tissues could be grown in vitro, tissues could also be grown in vivo by implanting fragments of tissue from one animal to another (transplantation). This development of transplantation is naturally to be expected as the techniques of tissue culture and transplantation have many factors in common. Murphy (1916) transplanted adult chicken spleen fragments on to the chorio-allantoic membrane of the chick embryo and noticed the enlargement of the spleen of the embryo. Since then, transplantation had aroused a good deal of interest but it was only in the 1950's that a serious attempt was made to study the mechanism and control of the homograft reaction. At that time, workers in transplantation biology made use of the technique of tissue and cell culture to study the mechanism of rejection of tissue grafts.

For purposes of clarity and convenience, information on cell culture can be classified into three broad groups. These are:-

1./

1. Experiments based on the principle that lymphoid cells or macrophages sensitized by donor histoincompatibility antigens have the capacity to destroy donor target cells in culture. These fall into the following sub-groups -

(a) Experiments which study the mechanism of rejection of normal tissues transplanted within the same species and in between species.

(b) Experiments which study the interaction between tumour cells and sensitized lymphocytes or macrophages.

(c) Experiments which study auto-immunity in human beings.

(d) Experiments which study experimental auto-immune conditions induced in animals.

2. Experiments which study the interaction between target cells (in cell culture) and lymphoid cells which are non-specifically stimulated by various agents such as phytohaemagglutinin (P.H.A.), xenogeneic antibody, poke-weed mitogen, streptolysin-O and staphylococcal filtrate.

3. Experiments which study the capacity of lymphocytes sensitized to soluble antigens (such as bovine serum albumin, ovalbumin and P.P.D.) to cause cell death in cell culture when incubated with the specific antigens. This model has been used for the <u>in vitro</u> study of the mechanism of hypersensitivity.

1. The historical account dealing with the significant landmarks in the evolution of the knowledge in the first group will now be discussed.

When/

When a tissue such as skin is transplanted from one animal to another animal of a different strain or species, the transplanted skin after an initial "take" dies within 10-14 days. Thus the host animal was able to cause death of the cells of the grafted tissue. At the site of this reaction, many cell types were seen such as lymphocytes, macrophages and plasma cells. It was hence naturally assumed that the small round cell (lymphoid cell) played at least some part in the destructive process. Thus an attempt was made by early workers to grow fragments of tissue such as skin and the specifically sensitized regional lymphoid fragments in culture in order to study the cellular interaction.

The early work by four workers was found to be not very encouraging. Harris (1943) first made a brief reference to growing explants from two species together in the presence or absence of cytotoxic antiserum. No cytotoxic effect was noted and his paper deals mainly with the cytotoxic effect of heterologous antisera on explants in tissue culture.

The experiments of Medawar (1948) also did not throw much light on the <u>in vivo</u> rejection phenomenon. Using rabbits, he cultured fragments of lymph node or spleen from homografted animals, in intimate contact with the skin from the original donor. He noticed no adverse effect either on mitotic or migratory activity of the cultured epidermis. Contact between the donor skin and recipient lymph node was particularly intimate; in some cases the lymph node was completely invested by the migrating epidermal cells. Medawar used/

used spleen fragments and only in a few experiments were lymph node fragments from the draining lymph nodes used. At that time, it was not known that the regional lymph nodes play a major part compared to spleen, in the genesis of the immune response to orthotopic skin homografts.

The experiments of Weaver et al (1955) involved the use of diffusion chambers. The target cells and the specifically sensitized spleen cells in diffusion chambers were placed into the peritoneal cavity of isologous hosts. They noted death of the target cells but their attempt to demonstrate this cell death in vitro resulted in failure. The cause of the failure may be that the lymphocytes were unhealthy. It is also mentioned that the large volume of fluid necessary to maintain a given number of cells in vitro may have resulted in too great a dilution of substances released by cells and necessary in a certain critical concentration for a positive result. Another possible cause of the failure is that the lysis of homograft cells involves the release from "immune" lymphocytes of antibodies which are active only in the presence of complement. It could be that the absence of lysis of target cells is due to the presence of insufficient complement.

Further refinements to the in vitro study of heteroplastic associations such as rat lymph node and mouse ureter or mouse lymph node and rat ureter was made by Trowell (1959) who showed that such associations survive normally in culture for six days and that rat tissues could be cultured satisfactorily for four days in contact with/

with lymph nodes from mice previously immunized by injections of rat tissue. He also did not observe any cytotoxic death in his in vitro studies.

Using more refined techniques of culture, Scothorne et al (1960) cultured fragments of lymphoid tissue in close apposition to each other. The regional lymph node fragments from mice which were immunized with donor skin was cultured in intimate side by side contact with similar node fragments from the original donor of the immunizing grafts. They did not notice any significant difference between the control and test cultures. Cultures of nodes from autografted or unoperated mice were set up as controls.

The first successful in vitro demonstration of cytopathic effect on target cells by immune lymphoid cells was by Govaerts (1960). He was chiefly concerned with the search for circulating (humoral or cellular) antibodies in dogs that had received a kidney homograft. One donor dog kidney was transplanted to the recipient dog. The second kidney of the donor animal was removed 3 days after transplantation and small fragments of the kidney cortex were incubated with 0.25% trypsin solution, and the supernatant which contained the kidney cells was centrifuged, washed and suspended in culture medium containing calf and horse serum. The cells were cultured in stationary tubes at a concentration of 800,000 viable cells per ml. (1 ml. per tube). To these cultures thoracic duct lymphocytes from recipient animals were added in the presence of serum obtained from the recipient dog at the time of or 4 days after graft rejection.

It/

It was noticed that fibroblast-like cells were not affected but most of the epithelial-like cells were retracted, round and somewhat agglutinated; their cytoplasm was granular with some vacuoles and many of the cells were surrounded with lymphocytes. These alterations were increased by complement, in the presence of which some cells were completely destroyed. In the absence of recipient serum or with recipient serum obtained before transplantation, some retracted epithelial-like cells with granular cytoplasm were observed.

The success by Govaerts in demonstrating the cytotoxic activity of lymphocytes from 'sensitized' dogs to the donor cells in vitro could be due to the use of monocellular suspensions of dissociated cells where close association between target and aggressor cell is possible. In explant cultures such close contact is not possible.

Since then, many research workers interested in various fields of biology and medicine made use of similar experimental techniques as used by Govaerts and demonstrated this cytotoxic effect (on target cells) by lymphoid cells specifically immunised towards the target tissue. Thus Rosenau and Moon (1961) studied the effects of 'sensitized' lymphocytes of BALB/C mice on homologous cells (fibroblasts) of the 'L' strain, originally derived from a C3H mouse. The cells were grown in milk dilution bottles in 90 per cent medium 199 and 10 per cent OK serum. Rosenau was the first to describe by in vitro technique the initial clustering of lymphocytes around target 'L' cells. This was followed by marked and progressive cytopathogenic changes in the 'L' cells which finally resulted in destruction/

destruction of most of these cells. These changes were observed in the absence of complement. The serum of the sensitized mice did not contain humoral antibodies demonstrable by precipitation, immunodiffusion or fluorescent antibody techniques. There was also, no demonstrable antibody bound to the 'L' cells, as determined by the fluorescent-antibody technique. The main findings in Rosenau and Moon's work was the necessity for close contact between aggressor lymphoid and target cells initially with the subsequent lysis of the target cells in the absence of complement.

Stuart (1962) demonstrated the capacity of normal mouse lymphocytes to adhere to monolayers of HeLa cells and cause cytopathic changes, after incubation for 48 hours and the optimum dose of lymphoid cells required was 100 million. Immunized spleen cells produced a similar but more rapid and severe effect. This cytotoxic effect was not noticed when heat killed lymphocytes were added. This work shows in addition to demonstration of cell death in the heterologous system, that viable lymphocytes are necessary for the interaction.

Rosenau and Moon (1962) demonstrated that hydrocortisone when incubated with the target and sensitized lymphocytes, although not preventing the adherence of the sensitized lymphocytes, nevertheless had a partial effect in reducing the cytopathogenic effects of the sensitized lymphocytes on the 'L' cells. Thus hydrocortisone appears to partially block the interaction between the two cell types, although the recognition of the homologous cells by the sensitized/

sensitized lymphocytes was not prevented.

An experimental model for the study of autosensitization reactions *in vitro* was devised by Koprowski and Fernandes (1962). These authors found *in vivo* techniques to have its limitations in the detailed study of Experimental Allergic Encephalomyelitis. Tissue culture technique was used as a tool to determine the immunological reaction of the host to brain tissue antigens. They prepared monolayers of glial cells on coverslips placed in Petri dishes, from newborn puppies and the cells were grown in 10% calf serum. As a source of 'sensitized' lymphocytes, rats were immunized with guinea-pig spinal cord suspension in complete Freund's adjuvant. The 'sensitized' lymph node cells were incubated with the cells from the brain in 5% CO_2 at 37°C and the sensitized cells were noted to adhere to glial elements like oligodendroglia, astrocytes, and not to the fibroblasts. About 10-15 'sensitized' cells agglutinated to each glial cell and the reaction was most intense on the third hour of contact. No agglutination was noted with either normal lymphoid cells or lymphoid cells prepared from rats sensitized with Freund's adjuvant alone. The authors name this reaction 'Contactual Agglutination'. On the sixth day of incubation, the brain cultures originally exposed to sensitized cells consisted entirely of fibroblastic type cells. The glial elements had disappeared, presumably destroyed by contact with the sensitized cells.

Thus the sensitized lymphoid cells acted specifically towards the/

the glial elements of the brain culture and this was further confirmed by an experiment in which the same sensitized cells did not interact with the cells of the kidney (mainly fibroblasts) obtained from the same puppy. The specificity in this experimental model, unlike those described so far, seems to be directed towards an organ rather than to the species, since rat lymph node cells sensitized to guinea-pig spinal cord reacted with glial cells of puppy brain but not with fibroblasts. Thus the cell interactions here are 'organotypic' and not species specific.

Taylor and Culling (1963) studied the cytopathic effect in vitro of sensitized homologous and heterologous spleen cells on fibroblasts. They demonstrated that the cytopathic changes can be induced in fibroblasts by contact for a short time with specifically sensitized living spleen cells. It was also shown that normal control lymphocytes, killed sensitized lymphocytes and spleen cells "non-specifically" sensitized to bovine serum albumin (B.S.A.), had a growth stimulating effect on the fibroblasts. The study of smear preparations of sensitized spleens indicated that the cell populations seen are similar to those seen in secondary immune response and 50% of the cells harvested were pyroninophilic suggesting that they were potential antibody producers. These authors speculate that the death of the fibroblasts could be due to the secretion of humoral antibody by these washed sensitized cells, during the period of incubation with the target cells.

Wilson (1963) made among other investigations, a quantitative study/

study of the destruction of homologous target tissue by immunologically activated lymphoid cells. He cultured cells from the kidney cortex obtained from 3-6 day old rat or mouse donors, in culture medium containing 10% calf serum. Sensitized lymphoid cells from rats and mice were added to the appropriate kidney cultures and incubated for 48 hours. The number of viable residual kidney cells were enumerated by preparing nuclear isolates of the target cells. The media were discarded (or saved in some cultures for enumeration of lymphocytes), the tubes gently rinsed three times with Hanks' salt solution and 1 ml. of 2% (W/V) citric acid was introduced into each tube. After 45-60 minutes incubation at 37°C the tubes were thoroughly agitated for a few seconds to suspend the kidney cell nuclei. One-half ml. of 0.1% (W/V) crystal violet dye (in 2% citric acid) was added to each tube and the nuclei were counted in a standard haemocytometer. The large round or oval kidney cells were easily distinguished from the smaller hyperchromatic nuclei of the lymphocytes. The number of lymphocytes present in the media was also determined in some experiments.

When the sensitized lymphoid cells and target cells were incubated for 48 hours, significant numbers of the latter were destroyed and this had been made the basis of a test system to establish the following points.

(1) The effect of sensitized lymphocytes is not one of mitotic inhibition. Homologous, non-dividing target cells are destroyed in the presence of sensitized lymphocytes;/

lymphocytes; whereas target cells cultured with normal lymphoid cells are unharmed.

(2) Neither complement nor immune serum is required for this reaction.

(3) Lymphocytes sensitized to a different set of transplantation antigens do not exert any deleterious effect on isologous target cells, i.e. the effect is immunologically specific.

(4) Lymphocytes obtained from the thoracic duct of homografted rats are fully as effective as immune lymph node cells in destroying donor strain kidney cells.

(5) No evidence of "allergic" cell death on the part of immunologically activated lymphocytes in contact with homologous versus isologous target kidney cells was obtained.

(6) Those target kidney cells which do not succumb to the effects of homologous sensitized lymphoid cells, do not represent a resistant population. It was suggested that the crude culture conditions impose a limit on the optimum performance of lymphoid cells in culture.

Granger and Weiser (1964) reported the capacity of 'immune' peritoneal macrophages from C57BL/6K mice to cause specific adherence and destruction of donor target cells derived from A/Jax mice./

mice. Both the interacting cells died and cell destruction apparently resulted in a non-phagocytic mechanism involving intimate cell contact rather than the effect of humoral antibody. Target cells were either normal fibroblasts or macrophages. Cell destruction was determined by the plaque technique on monolayer cell cultures. This is the first report of the capacity of macrophages from mice immunized with donor tissue to cause cytotoxic death of donor target cells.

Granger and Weiser (1966) further demonstrated that normal peritoneal macrophages treated with specific humoral antibody, derived from immune donors do not interact with donor target cells to produce cell destruction. The specificity of the 'immune' macrophage-target cell interaction was demonstrated by the fact that macrophages immunized towards various histoincompatible antigens other than target 'L' cells did not cause specific adherence and cytolysis of target 'L' cells. A specific haemagglutinin responsible for specific adherence was eluted from well-washed immune macrophages by heat treatment. In another experiment it was noted that metabolic inhibitors such as Actinomycin-D and Chloramphenicol, while not preventing adherence, inhibited cytolysis of target cells by specific 'immune' macrophages. Thus adherence between the target cell and 'immune' macrophage may be a passive phenomenon determined by an immunological interaction between the membrane of the two cell types. Target cell destruction requires perhaps further interactions between the cells, where the biosynthetic activity of the 'immune'/

'immune' macrophages may be necessary.

Stuart (1967) studied the interaction between macrophages and normal lymphocytes. It was noted that CBA peritoneal macrophages did not interact with C57BL mouse lymphocytes when incubated in normal CBA mouse serum whereas in immune CBA serum, marked adherence and phagocytosis was noted. This shows the importance of specific immune serum in altering the behaviour of macrophages towards nucleated cells.

Brondz (1964) demonstrated that immune lymphocytes obtained by immunizing different strains of mice with homologous sarcoma cells, when washed and transferred to monolayer cultures of these sarcoma cells or donor macrophages, adhere to the cells in culture and destroy them in 2-4 days. When cultured with normal lymphocytes, no cell death was seen. This cytotoxicity was dependent on the dose of lymphocytes and was effected in the absence of complement and immune serum. This interaction resulting in target cell death occurred only if there was an H-2 antigenic difference.

Ginsburg and Sachs (1965) devised a new experimental model which involved addition of normal rat lymphoid cells to monolayer cultures of mouse embryo cells. It was found that the rat lymphocytes transformed into large pyroninophilic cells (LPC's) after 5-7 days in culture. The cells transformed into blast cells in 3-4 days and underwent a series of mitotic divisions which finally culminated in the formation of large pyroninophilic cells (LPC's). The cells were also found to lyse the mouse embryo monolayers.

Hence/

Hence in this system the lymphocytes are "sensitized" *in vitro* to the transplantation antigens present on the surface of the mouse embryo cells. This system is in contrast to the techniques of previous workers where the lymphoid cells are sensitized in the intact animal by injecting into them transplantation antigens. These workers in later years made further refinements in culture methods and in the quantitative evaluation of the degree of lysis (Ginsburg, 1968; Berke *et al*, 1969b; Berke *et al*, 1969c). The rate of lysis was determined by three ways: measuring ^{51}Cr release from lysed fibroblasts, by direct count of surviving fibroblasts and by fixation and staining for examination of area of lysis. The kinetic analysis of the reaction indicated that one LPC was required to lyse one fibroblast and that each fibroblast died independently as a result of a single hit by a large pyroninophilic cell (LPC). The lysis of a target cell starts immediately when cell contact between LPC and fibroblast is accomplished. The number of LPC's are not decreased during the lytic reaction. Hence LPC's are not subjected to an "allergic death" after exerting a lytic effect on target cells. A quantitative study of the specificity of the lytic reaction with regard to the antigens of the target cells indicated that LPC's recognise strain differences of the mouse fibroblasts.

Perlmann and Broberger (1963) cultured freshly isolated foetal human colon cells and exposed them to white blood cells (mainly lymphocytes) from children with ulcerative colitis or from healthy controls. The colon cells were labelled with ^{32}P-orthophosphate or ^{14}C-aminoacids./

^{14}C-aminoacids. Exposure of the colon cells to patients' white cells led to a rapid isotope release, significantly higher than that obtained with normal white cells. For obtaining a significant cytotoxic effect of the patients' white cells, the presence of 10 to 20 per cent of fresh guinea-pig or human serum in the incubation medium was required (complement). The above experimental model had been made use of by many workers to study the pathogenesis of human and animal auto-immune-like states. In the case of humans, the target cells are grown in culture and the lymphoid cells of the patient suffering from the auto-immune disease are added to the culture and incubated.

In animal models, explants of the target animal tissue are grown in culture and lymphoid cells from animals suffering from the corresponding experimental auto-immune disease are added and incubated.

Thus trigeminal ganglia of foetal rats (Winkler, 1965) or glial cells prepared from neonatal rats (Berg and Källén, 1963) were cultured with lymphoid cells from animals suffering from experimental allergic neuritis or encephalomyelitis. This resulted in demyelination of nerve fibres in the former culture and destruction of the glial cells in the latter culture. The culture of explants of limb-girdle muscle of foetal Sprague-Dawley rats with lymphoid cells from rats sensitized to rabbit pelvic skeletal muscle also resulted in marked cytotoxic death of the explants (Kakulas, 1966a, 1966b).

Similarly/

Similarly, thoracic duct lymphocytes from rats with experimental auto-immune thyroiditis exerted a destructive effect on explants of thyroid tissue from normal rats of the same strain (Björklund, 1964). Ling et al (1965) did not observe any cytotoxicity to thyroid cell monolayers when incubated with lymphocytes from animals sensitized with thyroid antigens. Rose et al (1963) observed the inhibition of growth of rabbit thyroid cell cultures when incubated with rabbit lymphoid cells sensitized to guinea-pig thyroid antigens. Control lymphoid cells from rabbits injected with unrelated antigen (bovine serum albumin) produced similar effects but a larger number of these control cells were required to cause damage of a similar degree.

Experiments of a similar nature were performed using appropriate human target cells and lymphoid cells from patients diagnosed clinically as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and ulcerative colitis. In rheumatoid arthritis and in systemic lupus erythematosus, exudate mononuclear cells from synovial joints caused cytotoxic death of cultured human fibroblasts or amnion cells (Braunsteiner et al, 1964; Hedberg and Källén, 1964a; Hedberg and Källén, 1964b). However, Hedberg (1967), as a result of studies on 113 patients, found that the cytotoxic effects inflicted by mononuclear cells were obtained predominantly in a small series of S.L.E. and S.L.E.-like syndromes and that only one-third of rheumatoid arthritis patients, predominantly those with nodules, showed cytotoxic effects. It is necessary to note that the/

the exudate mononuclear cells need not necessarily be sensitized lymphocytes. Berg and Källén (1964) added mononuclear cells from patients with multiple sclerosis to monolayers of rat glial cells and cytotoxicity of the glial cells was caused by cells from 11 out of 33 multiple sclerosis patients. Cells from normal individuals did not cause this effect.

2. Target Cell Destruction Caused by Non-Specifically Stimulated Lymphocytes

Holm et al (1964) demonstrated for the first time that lymphoid cells need not be specifically stimulated to kill target cells. He demonstrated that normal lymphoid cells would kill target cells in tissue culture if non-specifically stimulated by PHA (a crude extract from the bean Phaseolus vulgaris), provided the cells are homologous with regard to their H-2 antigens. In this experiment primary cultures of foetal human kidneys (obtained from legal abortions) or kidneys from new-born Sprague-Dawley rats were set up in Eagle's medium supplemented with 15% heat inactivated calf serum. The cells were labelled with carbon-14 thymidine. Lymphoid cells were isolated from heparinized human peripheral blood or from the blood of normal Sprague-Dawley rats. Varying numbers of these lymphoid cells ($1\text{-}10 \times 10^6$) were mixed with labelled target cells (10^5) in roller tubes. 0.1 ml. of PHA extract was added to some of the tubes. The tubes were fitted loosely with screw-caps, placed at 45° angle and incubated at 37°C for 18 hours in an atmosphere of air containing 5% carbon dioxide. (The PHA extract was prepared by extracting 40 grams of/

of crushed Phaseolus vulgaris beans with 200 ml. of 0.15M phosphate buffer (pH7.2) at 4°C for 24 hours. The suspension was centrifuged at 4500 r.p.m. for 30 minutes and the supernatant dialysed in the cold for 24 hours against several changes of phosphate buffer).

The results of these experiments show that the isotope released (due to cell death) was always higher in the presence of PHA than in its absence. The magnitude of this effect depended on the ratio of lymphoid to target cells. As a rule only a slight cytotoxicity was obtained with a ratio of 10 : 1, whereas a ratio of 100 : 1 gave a pronounced cytotoxicity. Although the results of these experiments are suggestive of the capacity of PHA to induce cytotoxicity on lymphoid cells, the target cells used with the different lymphoid cell ratios were different and in one of two experiments where the ratio of lymphoid cells to target cells was 100 : 1, there was no noticeable difference between the results of the test and control. In another experiment the lymphoid cells were placed in cell-impermeable millipore chambers and placed in Petri dishes which contained PHA and target cells. The experiments were done under the same standard conditions as before, and no cytotoxicity was noted. This would mean that PHA-stimulated normal lymphocytes do not release a diffusible cytotoxic factor. Perhaps if the cell ratio of 100 : 1 had been used the results may have been different as with a ratio of 10 : 1 a mild degree of cytotoxicity was noted when the target and lymphoid cells were mixed in the presence of PHA. The cause of the cytotoxicity may be due to:

(1)/

(1) A passive rôle played by the lymphoid cells where these cells block the surfaces of target cells, thus depriving the latter of nutrients or by inducing various metabolic disturbances.

(2) The lymphoid cell may play an active part in producing cell damage. The damage may be due to contact between the target and lymphoid cell, the contact being mediated in some manner by PHA. The mere contact between target cell and lymphoid cell of the same species is not enough to cause cell death. This is shown by the fact that non-specific aggregation brought about by polylysine did not result in obvious cell death (Rosenau, 1963). It is possible that the lymphoid cells are 'stimulated' by PHA and this may be related to its mitogenic activity.

Möller (1965) also demonstrated the cytotoxicity of lymphoid cells towards histoincompatible target cells in the presence of PHA. However, in the control culture tubes a notable degree of cytotoxicity was noted even though PHA was not added to the culture medium. When the calf serum was replaced by the foetal calf serum the cytotoxic effect was markedly reduced. When the calf and foetal calf serum were separately agglutinated with red cells from strain A mice, a titre of $^{1}/_{16}$ was obtained with the serum of the older calves and no agglutination was noted with foetal calf serum. Thus/

Thus it is possible th t heterologous antibodies to mouse tissue present in older calf serum caused mixed agglutination of non-immune lymphoid cell and target cell resulting in close cellular contact and subsequent cell death. Further experiments were performed to test this possibility by adding normal allogeneic lymphoid cells to target cells previously incubated with immune rabbit serum strongly reactive against mouse cells. A marked cytotoxic effect was noted unlike control cultures without immune rabbit serum and unlike controls where syngeneic lymphoid cells and immune rabbit serum were added to target cells. Thus heterologous antiserum to mouse tissue has the capacity, like PHA, to bring about agglutination between lymphoid and target cells. Möller also added semi-isologous F_1 hybrid lymphoid cells to the parental target cells in the presence of heterologous antibody. In this system the normally accepted conventional immune response cannot occur since the parental target cells do not have any antigens which are foreign to the F_1 hybrid lymphoid cells. Hence in this experimental system a primary immune response cannot be induced by the heterologous antibodies stimulating the lymphocytes. As controls, isologous lymph node cells (i.e. parental) were added. It was noted that the semi-isologous F_1 hybrid lymph node cells caused pronounced cytotoxicity to parental target cells. Similar experiments were done in the presence of PHA, instead of heterologous antiserum and similar results were noted. The cytotoxic effect caused by normal cells aggregated to the targets was as strong as that observed with immune lymphoid/

lymphoid cells and between 25% and 90% of the target cells were killed.

Further work on contact-induced cytotoxicity between parental and semi-isologous F_1 hybrid cells was performed by Möller and Möller (1966). In one experiment they cultured sarcoma cells derived from parental strain mice and also embryo cells of the parental strain. To these monolayers, semi-isologous F_1 hybrid lymphoid cells were added in the presence of PHA. Marked cytotoxicity to the monolayers was noted which was not seen in the controls (i.e. semi-isologous F_1 hybrid target and semi-isologous F_1 hybrid lymphoid cells incubated with PHA). In a second experiment parental lymphoid cells were added in the presence of PHA to monolayers of F_1 hybrid embryo cells. A marked degree of cytotoxicity was noted. The explanation offered to the first experiment was that cell death of parental target cells occurred because of isoantigenic differences. Parental target cells interacted with the foreign antigens present in the membrane of the semi-isologous F_1 hybrid lymphoid cells, and this resulted in the death of the former cells. Hence in this experimental model isoantigenic differences were responsible for cell death. In the second experiment, if isoantigenic differences were to be the cause of death, then one should expect the F_1 target cells to survive and the parental lymphoid cells to die, because the foreign antigens present on the surface of the target cell should interact with the membrane of the parental lymphoid cell. However, the result of the experiment is that the target/

target cells die. The explanation that is offered by these authors is that even though the parental lymphoid cells do not have antigens which are foreign to the semi-isologous F_1 hybrid target cells, yet there is a structural difference between the two membranes of the cells that results in the death of the F_1 hybrid target cells. Hence in this experiment it is the structural difference that causes cell death. In the situations where there are no antigenic or structural differences between the two reacting cell types, as in parental target cells interacting with parental lymphoid cells or F_1 hybrid target cells interacting with F_1 hybrid lymphoid cells, no cell death occurred in the presence of PHA. These authors are thus of the opinion that cell death occurs *in vitro* between interacting lymphoid and target cells due to antigenic and/or structural differences between the membranes of the interacting cells. It is also their view that PHA or heterologous antiserum helps to effect close contact between the two cell types.

A logical consequence to the above hypothesis of contact-induced cytotoxicity is that not only lymphoid cells would be competent to mediate cytotoxicity but also other cell types containing foreign histocompatibility antigens, provided that the cells are brought into close contact with the target cells. Further support for this view might be provided by the work of Hellström *et al* (1965). They found that homozygous tumour cells grow in a higher frequency and after a shorter latency period when small numbers of tumour cells are transplanted to syngeneic hosts than to 'semi-syngeneic'/

'semi-syngeneic' F_1 hybrids. This phenomenon has been designated by the term 'syngeneic preference' in relation to the homozygous host and 'allogeneic inhibition' with reference to the F_1 hybrid host (Hellström, 1963, 1964).

This difference was not abolished by pre-irradiation of the F_1 hybrids (to abolish any possible immune response) and also by pre-treatment of the F_1 hybrids prior to grafting with normal or tumour cells of the homozygous strain. Thus allogeneic inhibition could not be due to reaction of the F_1 host towards any hypothetical antigens which might have been present in the homozygous cells and hosts but absent from the F_1 hybrid. Further, tissue culture experiments by Hellström showed that cell extracts or homogenates containing foreign histocompatibility antigens inhibited the growth of explanted target cells, suggesting that allogeneic inhibition *in vivo* might be due to the contact between the grafted tumour cells and the foreign isoantigens of the host. This hypothesis was subjected to further test by neutralization experiments. Here lymphoma cells were exposed *in vitro* to spleen or tumour cells containing foreign H-2 antigens and their outgrowth in syngeneic hosts was measured after subsequent implantation. It was found that allogeneic and F_1 hybrid spleen cells inhibited the outgrowth of lymphoma lines as compared to syngeneic spleen cells.

Möller and Möller (1966a, 1966b) performed another series of *in vivo* experiments to test the validity of the hypothesis that antigenic and/or structural differences between cells will result in cell/

cell death, as seen in tissue culture. They mixed neoplastic cells with syngeneic or allogeneic X-irradiated (3000 rads) lymphoid cells respectively in the presence of PHA, and the mixtures were inoculated into sublethally irradiated hosts (the hosts being syngeneic with the neoplastic cells). It was found that allogeneic lymphoid cells caused a pronounced inhibition of tumour growth, as compared to the growth of neoplastic cells treated with syngeneic lymphoid cells. This was also true if allogeneic lymphoid cells were of F_1 hybrid origin, unable to carry out immunological reactions against the parental target tumour cells.

Lundgren et al (1968) demonstrated that non-immune human lymphocytes caused destruction of human fibroblast monolayers cultured from skin in the presence of PHA. In this system, lymphocytes were able to kill allogeneic as well as autochthonous fibroblasts in the presence of PHA. It is suggested by these workers that the actual target cell destruction is immunologically non-specific. These workers also found that human polymorphonuclear leucocytes caused detachment of allogeneic and autochthonous human fibroblast monolayers. The cells were detached from the surface of the culture vessel into the medium and the detached target fibroblasts could be cultivated from the supernatant. Thus, although plaques were seen in monolayers, the target cells were not filled but merely detached from the surface. Further work on the cytotoxic effect of non-immunized human lymphocytes was studied by Lundgren and Möller (1969) where the lymphocytes were added to human fibroblast/

fibroblast monolayers of different genotypes. Non-immunized lymphocytes were not cytotoxic to the various target cell genotypes provided the lymphocyte suspensions were free from contaminating granulocytes. When PHA was added to the culture of lymphocytes and fibroblasts, the lymphocytes became strongly cytotoxic. The cytotoxicity was equally expressed on allogeneic and autochthonous fibroblasts. PHA stimulation of lymphocytes results in augmentation of cytotoxicity, increased synthesis of RNA, DNA and protein and also blast transformation. By studies using various metabolic inhibitors like Mitomycin-C (inhibits DNA synthesis), Actinomycin-C (inhibits DNA dependent RNA synthesis), X-irradiation, Methyl Prednisolone (inhibition of RNA and DNA synthesis), it was shown that cytotoxicity and other manifestations of PHA stimulation are probably independent processes.

Streptolysin O or anti-lymphocyte serum also acted like PHA; caused cytotoxicity of normal human lymphocytes and induced it to destroy fibroblasts of allogeneic and autochthonous genotype. These results suggest that expression of cytotoxicity is an immunologically non-specific process caused by stimulated lymphocytes. When the lymphocytes have acquired a cytotoxic potential they do not discriminate between the target cell genotype. The specificity of the cellular immune reactions is probably confined to the immunological recognition step, initiating the cytotoxic potential. This recognition step is by-passed if the lymphocytes are stimulated by PHA or other non-specific stimulators.

Holm/

Holm (1967) demonstrated that only cells of the lymphoid cell series could exert cytotoxic effect on allogeneic Chang cells (human liver cells grown in tissue culture) in the presence of PHA. Chicken red cells, rat kidney cells, fibroblasts from mouse, Chang cells, Burkitt's lymphoma cells,did not cause cytotoxicity of Chang liver cells. Hence cells of the lymphoid series are the immunologically competent cells. The lymphocytes survived the contact with allogeneic target cells as determined by release of isotope from radio-labelled lymphocytes. It was also noted that lymphocytes were not damaged (in mixed lymphocyte cultures, where either of the two sets of lymphocytes were radio-labelled in different sets of experiments) when exposed to a 100-fold excess of allogeneic lymphocytes for up to 72 hours. Holm and Perlmann (1967a) found that the human lymphocytes were not cytotoxic to syngeneic fibroblast cultures as noted by Lundgren and Möller (1969). They also found that the cytotoxicity of the lymphocytes on allogeneic fibroblasts was dependent on the concentration of the PHA in the culture medium and cell damage was maximal at concentrations of 4-8 µl PHA/ml. It was also noted that pre-treatment of lymphocytes with crude filtrate of Staphylococcus aureus damaged Chang target cells.

Holm (1967); Holm and Perlmann (1967a) investigated the relationship of the cytotoxic activity of the lymphoid cells to its various synthetic and metabolic functions. If the lymphocytes play an active rôle in the cytotoxic reaction, it could be expected that contact with foreign cells induces in them synthesis and/or release of/

of some effector substance leading to target cell damage. If this is true, it should be possible to inhibit the cytotoxicity of the lymphocytes by treating them with antimetabolites. Hydrocortisone inhibited the cytotoxic reaction when 10-100 µg/ml. was present in the incubation mixture. This range of concentration also reduced the incorporation of 14C-thymidine into lymphocytes cultivated with PHA (reduced lymphocyte stimulation). Inhibitors of protein synthesis such as puromycin and parafluorophenylalanine seemed not to reduce the cytotoxicity of the lymphocytes towards target Chang cells. RNA synthesis was impaired in lymphocytes treated with 1-10 µg/ml. of Actinomycin-D while their cytotoxicity measured at 3-6 hours of incubation with the target cells was not inhibited. Thus, under these conditions the cytotoxic reaction was not related to RNA synthesis. Dinitrophenol reduced the cytotoxicity of the lymphocytes towards target cells. Dinitrophenol impairs regeneration of high energy phosphate intermediates and uncouples oxidative phosphorylation. This would lead to less efficient utilisation of energy-yielding processes of the cells, and suggest that energy-yielding processes of the cells are involved in cytotoxic reactions. The cytotoxicity of the lymphocytes was partially or completely inhibited by iodoacetate in concentrations which inhibit anaerobic glycolysis. Antimycin-A irreversibly blocks the electron transfer step between cytochrome B and C and thus blocks oxygen consumption and glycolysis of lymphocytes. The inhibition of cytotoxicity of target cells by Antimycin-A was correlated with the inhibition of respiration/

respiration of the lymphocytes. This suggests that energy acquired from aerobic processes is involved in the effector mechanisms of the cytotoxic reaction. These results are compatible with the assumption that the PHA-induced cytotoxicity of human lymphocytes depends on energy requiring processes but is independent of RNA and probably protein synthesis.

In contrast to the above experiments Berke et al (1969a) had shown that PHA blocked the sensitization of rat lymphocytes to mouse cell antigens. These workers demonstrated that rat lymphocytes when grown on mouse embryo fibroblast monolayers transformed and differentiated into large pyroninophilic cells. These cells when added to similar monolayers caused lysis of the mouse embryo fibroblasts. When PHA was added to cultures of rat lymphocytes and mouse embryo fibroblasts the former cells were predominantly medium-sized lymphoid cells and not large pyroninophilic cells. These medium-sized lymphoid cells when added to similar monolayers of mouse embryo fibroblasts, were not able to bring about significant lysis. Thus PHA, in this instance, seemed to prevent the sensitization of rat lymphoid cells to foreign tissue antigens. The experimental model used by these workers simulates the in vivo condition more closely in that rat lymphocytes are cultured together with mouse cells and there results a natural differentiation of the lymphoid cells. This reaction is highly specific because the 'sensitized' rat lymphocytes can interact only with the specific mouse cells. PHA added under these conditions seems to block this specific sensitization. PHA stimulates/

stimulates lymphoid cells to transform and differentiate but it may well be that the resulting cell populations are different from the cell lines produced as a result of specific stimulation by tissue antigens.

The next major advance in the studies on the mechanism of cell death in cell interactions was by Granger and co-workers, (Granger and Williams, 1968; Williams and Granger, 1968; Kolb and Granger, 1968; Granger and Kolb, 1968). They cultured lymphocytes from several mammalian species (from mouse, hamster, cat and from humans). The lymphoid cells of these species when cultured in vitro with PHA or xenogeneic antibody (rabbit anti-mouse serum) transformed into 'blast' cells and at the same time developed the capacity to cause cytotoxic death of target cells derived from the mouse, cow and humans. It was also noted that when lymphoid cells were cultured for 48 hours at 37°C, the supernatants had the capacity to cause cytotoxic death of cell lines derived from many species of animals. They called this soluble toxic factor 'lymphotoxin'. Thus lymphotoxin-like substances are found in the supernatants of cultures of PHA-treated lymphocytes from humans, mouse, cat and hamster. The action of this lymphotoxin is not specific since it causes cytotoxic death of cells grown in vitro derived from many species of animals other than from which the lymphotoxin is prepared.

3. Experiments Devised to Study the Capacity of Lymphocytes Sensitized to Soluble Antigens, like BSA, Ovalbumin and PPD to Cause Cell Death in Tissue Culture when Incubated with the Specific Antigen

In/

In this category, lymphoid cells sensitized with heat-killed tubercle bacilli or hen-egg albumin, both in complete Freund's adjuvant, were incubated with isologous and allogeneic target embryo fibroblasts and the corresponding antigen. A significant degree of cell death was observed in the isologous and allogeneic systems (Ruddle and Waksman, 1967). Granger et al (1969) similarly incubated lymphocytes sensitized to bovine serum albumin and P.P.D. with the corresponding antigen in culture tubes for 72 hours. The cell-free supernatant was then added to indicator cell cultures for 48 hours and cell destruction was observed in these cultures. These workers thus postulate that the initiating step(s) in this reaction is highly specific, but the effect on cells surrounding the stimulated lymphocytes is non-specific. The incubation of the sensitized lymphocytes with the corresponding antigen probably results in the release of a non-specific cytolytic lymphotoxin.

Graft-versus-Host Reactivity In Vitro

One cardinal manifestation of Graft-versus-Host Reactivity in vivo can also take place ab initio in vitro. Auerbach and Globerson (1966) had shown that following the addition of a suspension of splenic cells from adult C3H mice to very small splenic explants from perinatal (C x C3H)F_1 mice, a marked enlargement of the explant occurred within 3 to 4 days. The addition of splenic cells from isogenic donors was ineffective. Histological changes within the enlarged spleen grafts closely simulate those which occur in the spleens/

spleens of animals suffering from G.V.H.R.

This review of the literature confirms the value of cell culture experiments in the investigation of cell-mediated immunity. It also reveals that so far no one has applied these techniques to the study of G.V.H.D. My account of the histopathology of this condition has emphasized the role of the macrophage and accordingly a series of cell culture experiments has been designed to study the function of this cell in G.V.H.D.

(B) MATERIALS AND METHODS

The control mice used in these experiments belonged to C57BL and $B6AF_1/J$ hybrid strains. G.V.H.D. was produced in $B6AF_1/J$ hybrid mice by the intravenous inoculation of parental cells - as described in Chapter II. On the twenty-first day after the inoculation of parental lymphoid cells, the mice showed significant weight loss compared to the control animals. The control and G.V.H.D. mice were first bled. The preparation and sterilization of the sera will be described later.

1. Collection of Peritoneal Cells from G.V.H.D., Parental and Normal F_1 Hybrid Mice

The technique for collection and culture of peritoneal macrophages was that described by Stuart (1967a). The mice with G.V.H.D. are sacrificed by cervical dislocation, the fur thoroughly swabbed with 70 per cent alcohol and a small horizontal cut is made on the ventral body skin of each mouse. By placing the thumb and forefinger of each hand on either side of the cut, the skin is retracted towards the head and tail end of the body. The mice are placed on a paper towel, under a plastic hood. A 10 or 20 ml. sterile plastic syringe with a sterile 21G (medium bore) needle is filled with Wellcome tissue culture medium 199 containing 10 international units of heparin per ml. The exposed ventral body wall of the mouse is sterile and the needle is inserted through the midline of the body wall into the peritoneal cavity. The needle is inserted obliquely at an acute angle of about 30° to the horizontal, to a depth just enough/

enough to enter the peritoneal cavity and extreme care is taken not to puncture the gut. If the gut is punctured, the animal, syringe and needle, containing the culture medium is discarded and a new needle, syringe and culture medium is used. About 4-5 ml. of culture medium is injected into the peritoneal cavity of each mouse (usually injected into 5 or 6 mice at a time). Now the paper towel on which the mice are laid is gently "rocked" from side to side a few times so as to detach the peritoneal cells from the serosal surface and get them into the culture medium. The needle is then inserted into the upper left flank of the abdomen, posterior to the spleen and the needle is gently swung outward, while still inside the peritoneal cavity. This results in the formation of a pocket-like space in which the culture medium containing the cells collects itself. The medium is then aspirated and transferred into a sterile measuring cylinder.

After pooling all the peritoneal cells from the mice, a cell count is done, using the WBC counting pipette, WBC dilution fluid and the counting chamber (Improved Neubauer Counting Chamber). By adding the required amount of culture medium 199, the cell concentration is adjusted to a million cells per millilitre. To this suspension, normal F_1 hybrid, parental or G.V.H.D. serum is added so that the final suspension in culture medium 199 (C.M.199) contains 5 per cent serum.

In a similar manner, peritoneal cells were harvested from normal F_1 hybrid and parental mice. These macrophages were also cultured/

cultured in G.V.H.D., normal F_1 hybrid and parental serum.

2. Technique of Culture of Peritoneal Macrophages

1 ml. of the suspension of G.V.H.D. peritoneal cells is transferred into each pre-sterilized rimless pyrex tube (16 x 125 mm.) containing a "flying"coverslip (Chance No.1, 19 x 6 mm.). The tubes are stoppered with white rubber bungs (Esco Rubber Ltd.) and incubated at 37°C (at an angle of 5°C from the horizontal) in a plastic rack which is constructed to hold 36 tubes at an angle of 5° from the horizontal.

Similarly normal F_1 hybrid (syngeneic with the strain developing the disease) and parental peritoneal cells are incubated for 18 hours (overnight). The tubes are then examined under the low power of a light microscope for growth of the cells. In a standard culture, the macrophages settle down on the "flying" glass cover slip and adhere to it in between 1-2 hours. In 4-6 hours the macrophages which were initially ovoid or spherical in appearance had spread out and appeared spindle-shaped. When examined after 18-24 hours, the cells were well spread out.

Sterilization of House Sera

The blood is collected in a centrifuge tube and allowed to clot. After 2 hours the blood is separated from the sides of the tube to allow the blood clot to retract. After a further period of 4 hours the blood is spun in a refrigerated M.S.E. centrifuge at 1000 r.p.m. for 10 minutes. The supernatant serum is pipetted off and transferred into a bijoux bottle. Since the serum collected from mice was/

was of limited amount (about 2 ml.) the loss during preparation and sterilization was minimized by diluting the serum with C.M.199 in equal volume.

The diluted serum is collected in a 10 ml. sterile plastic disposable syringe and the nozzle of the syringe is fixed into the top of the upper compartment of the sterile metal millipore filter chamber. A sterile bijoux bottle collects the sterile serum that comes down from the lower compartment of the filter (the "filter" which sterilizes the serum is placed in between these two compartments supported on a fine mesh steel sieve). The sterile serum is collected in 1 ml. volumes and stored in a deep freeze at -20°C after labelling and dating. On the day of the experiment the bottle containing the serum is thawed and checked for sterility.

3. Preparation of Lymphoid Cell Suspensions for Addition to Monolayer Cultures of Macrophages

Normal F_1 hybrid mice are sacrificed by cervical dislocation. The fur is well soaked in 70% methyl alcohol and a cut is made on the ventral body skin and the pelt is retracted to expose the body wall and limbs. The inguinal, axillary and submandibular lymph nodes are removed with fine sterile forceps and transferred into a sterile Petri dish containing a small amount of C.M.199 with 10 i.u.'s of heparin per ml. A cut is made in the ventral abdominal muscle and the mesenteric, lumbar and iliac lymph nodes are removed. Using a fine scalpel, the nodes are cut into very small pieces and then tranferred to a loose-fitting ground glass tissue homogenizer. Using/

Using gentle pressure, the cells are separated from the connective tissue stroma of the node and the supernatant is decanted using a fine mesh steel sieve. The coarse particles are allowed to sediment to the bottom and the supernatant is collected and a cell-count done. The cell concentration is adjusted to 5 million cells per ml.

Addition of Lymphoid Cells to G.V.H.D. Macrophages in Culture

G.V.H.D. macrophages were grown in 5 per cent normal F_1 hybrid serum (10 tubes). To the first set of two tubes nothing was added and these were used as controls to study the morphology of macrophages. The second set of tubes received 0.1 ml. of a 5 per cent suspension of haemolysin-coated human erythrocytes. To the third set of tubes 0.1 ml. of 5 million normal F_1 hybrid lymphoid cells were added. To the fourth set of tubes 0.1 ml. of 5 million parental lymphoid cells were added, and to the final two tubes 0.1 ml. of 5 million G.V.H.D. lymphoid cells were added.

G.V.H.D. macrophages were also grown in 5 per cent parental (10 tubes) and G.V.H.D. serum (10 tubes). These tubes were also treated in an identical manner to those macrophages grown in normal F_1 hybrid serum i.e. the first set of two tubes received nothing, the second set of tubes received haemolysin-coated human erythrocytes, the third set of tubes received normal F_1 hybrid lymphoid cells, the fourth set of tubes received parental lymphoid cells and the final two tubes received G.V.H.D. lymphoid cells.

Addition of Lymphoid Cells to Normal F_1 Hybrid and Parental Macrophages

Similar/

Similar to the experiments with G.V.H.D. macrophages, normal F_1 hybrid macrophages were grown in 5 per cent normal F_1 hybrid serum, parental serum and G.V.H.D. serum (10 tubes in each sera). The second, third, fourth and fifth sets of tubes received haemolysin-coated human erythrocytes, normal F_1 hybrid, parental and G.V.H.D. lymphoid cells as in the previous experiment.

The macrophages from parental animals were also grown in the three different sera (10 tubes in each sera) and were treated with particles in a similar manner to those described above, i.e. in each sera the first set of tubes received nothing, the second set received haemolysin-coated human erythrocytes, the third set received normal F_1 hybrid lymphoid cells, the fourth set parental lymphoid cells and the last set G.V.H.D. lymphoid cells.

Incubation of the Cultures

The duplicate sets of tubes containing macrophages and haemolysin-coated human erythrocytes were incubated for two hours while the tubes containing macrophages and lymphoid cells were incubated for four hours.

Fixation and Staining of the Monolayers

After the incubation of the lymphoid cells with the macrophages, the culture tube racks are removed from the incubator and the rubber stoppers of the tubes are removed. The culture medium is aspirated out after gentle shaking of the tubes. About 1 ml. of normal saline is added to the tubes and the saline is aspirated out. To each tube 1 ml. of 100 per cent methanol is added and left for 10 minutes to fix/

fix the cells. The methanol is aspirated out and about 1 ml. (1/200 dilution) of Giemsa (Improved Giemsa "R.66" from George T. Gurr, Ltd.) is added to each tube and the stain is left for 12 hours (overnight). The stain is now aspirated out and 1 ml. of distilled water is added. The 'flying' coverslips are then removed from the tubes, using a very fine pair of forceps, taking care that the surface of the coverslip containing the monolayer of cells is facing upwards. The coverslips are passed through water, 70 per cent, 90 per cent and 100 per cent ethyl alcohol, and then through xylol to remove the alcohol. The coverslips are now placed on a labelled filter paper and subsequently mounted on labelled glass slides using DPX. Finally the slides are allowed to dry and then examined under the microscope.

4. General Plan for Experiments

SERUM FROM	CELLS ADDED				
	Control	Haemolysin-coated Erythro-cytes	Normal F_1 Hybrid Lymphoid cells	Parental lymphoid cells	GVHD Lymph-oid cells
Normal F_1 Hybrid Mice	□□	□□	□□	□□	□□
Parental Strain Mice	□□	□□	□□	□□	□□
GVHD Mice	□□	□□	□□	□□	□□

Normal/

Normal F_1 hybrid, parental and G.V.H.D. macrophages were cultured in normal F_1 hybrid, parental and in G.V.H.D. serum. To these erythrocytes, normal F_1 hybrid, parental and G.V.H.D. lymphoid cells were added. The two small rectangles in each square represent duplicate cultures.

This experiment was done twice using different batches of the same strain of animals.

5. Technique of Preparation of Haemolysin-Coated Human Erythrocytes for Studies on Maximum Phagocytic Activity of Macrophages.

2 ml. of blood is collected from a volunteer and immediately transferred into a centrifuge tube containing normal saline. The suspension is centrifuged to sediment the red cells. The supernatant is removed with a pasteur pipette using a rubber teat, further normal saline is added and the cells are again resuspended. This procedure is repeated twice to remove all the plasma proteins. Finally, to 1 ml. of packed red cells 19 ml. of normal saline is added to make a 5 per cent suspension.

(i) Preparation of Haemolysin

Haemolysin was prepared and stored in small volumes in the deep freeze to be used on the day of the experiments. Three rabbits were injected with human erythrocytes. The immunization schedule was as follows:- human erythrocytes were washed thrice in saline and each rabbit was injected intramuscularly with 0.5 ml. of 5 per cent suspension of erythrocytes weekly for 6 weeks. 3 weeks after the beginning of the immunization schedule 1 ml. of blood was removed from/

from the marginal ear vein of each rabbit and the haemolysin titre was estimated separately. At the end of 6 weeks, good antibody titre was found in the three rabbits. The animals were then exsanguinated by cardiac puncture under Nembutal anaesthesia. The blood was then pooled, allowed to clot and the serum was separated by centrifugation and sterilized by millipore filtration.

(ii) Technique of Determination of Haemolysin-Titre

Using small test tubes arranged and labelled in a test tube rack, doubling dilutions of the anti-serum was prepared (each tube having a volume of 0.1 ml.). To this an equal volume of 5 per cent suspension of thrice washed human erythrocytes and 0.1 ml. of fresh guinea-pig serum was added and the results were read after one hour. Haemolysis was noted up to a dilution of 1/64.

(iii) Preparation of Haemolysin-Coated Erythrocytes

To 2 ml. of 5 per cent suspension of thrice washed human erythrocytes an equal volume of 1/10th dilution of haemolysin was added (original titre 64) and incubated for half an hour at room temperature. Examination under the microscope confirmed that the erythrocytes had agglutinated into small clumps. This suspension was added in 0.1 ml. volumes to test tubes containing macrophages to determine their maximum phagocytic activity.

6. Preliminary Experiments were Performed to Determine the Following:-

1. The concentration of mouse serum required for growth of a healthy monolayer.

2./

2. The concentration of macrophages necessary to form a uniform monolayer.
3. The optimum number of lymphoid cells required for interaction with macrophages.
4. The optimum incubation time for the interaction between macrophages and lymphoid cells.

(i) Experiment to Determine the Concentration of Mouse Serum Required for Normal Growth of Macrophages in Culture

Normal F_1 hybrid macrophages at a concentration of a million cells per tube were grown in 10 per cent, 7.5 per cent, 5 per cent, 2.5 per cent and 1 per cent normal F_1 hybrid mouse serum at 37°C for 18 hours, (4 culture tubes in each concentration). To two of these tubes in each concentration of mouse serum nothing was added and these were used to study the morphology and number of macrophages per coverslip. To the remaining two tubes in each concentration of mouse serum 0.1 ml. of haemolysin-coated human erythrocytes (5 per cent suspension) was added. The cultures were incubated for a further period of 2 hours and then washed with saline, fixed in methanol for 10 minutes and stained with Giemsa.

(ii) Experiment to Determine the Concentration of Peritoneal Macrophages Required for Sufficient Growth in Coverslips

Normal F_1 hybrid macrophages were grown in varying concentrations in culture medium 199 with 5 per cent normal F_1 hybrid serum. The first two tubes received 0.5 million macrophages, the second, third, fourth and fifth sets of duplicate tubes received 0.75, 1.0, 1.25 and 1.50 million cells respectively. The tubes were incubated at/

at 37°C for 18 hours. The coverslips were then washed in normal saline, fixed in methanol and stained with Giemsa.

(iii) Studies on the Time and Dose Relationship of the Interaction Between Macrophages and Thymocytes

These experiments were done to decide on the number of thymocytes that should be added to monolayers of macrophages and also the time for which these cell-types should be interacted.

The plan of the experiment was to grow macrophages in human serum, normal mouse serum and iso-immune anti-thymocyte serum. To these cultures, thymocytes were added in varying concentrations and incubated for 4 hours. In a second experiment, a constant number of thymocytes were added and incubated for varying intervals of time. These two experiments should indicate (1) the optimum number of thymocytes required to interact with a million macrophages, (2) the time for which the cell-types should be interacted for optimum results.

Preparation of Anti-Thymocyte Serum

Thymuses were removed from C57BL mice and thymocytes were prepared. These cells were inoculated intraperitoneally into six Tuck's T.O. strain mice (a closed colony), each mouse receiving a weekly injection of 150 x 10^6 cells for 4 weeks. A week after the last injection, the mice were bled retro-orbitally and the blood was pooled. The serum was then separated and an equal volume of packed thrice washed mouse erythrocytes was added and incubated for 1 hour at 37°C. This procedure was repeated twice to remove the haemolysin present/

present in the serum. The serum was then sterilized using a millipore filter.

Culture of C57BL Macrophages in Normal and Iso-immune Serum

One million C57BL macrophages were cultured in each of 36 tubes; 12 tubes contained 5 per cent pooled "O" human serum, 12 tubes contained 5 per cent anti-thymocyte serum (the original titre of anti-thymocyte serum was 128) and 12 tubes contained 5 per cent normal C57BL mouse serum.

The macrophages were incubated overnight and to the first two tubes in each sera nothing was added. To the second, third, fourth, fifth and sixth sets of tubes in each sera (2 tubes in each set), 1×10^6, 2.5×10^6, 5×10^6, 7.5×10^6, 10×10^6 thymocytes were added. The tubes were incubated at $37^\circ C$ for 4 hours and then washed gently with normal saline. The coverslips were taken out, fixed in absolute methanol and stained with Giemsa.

To determine the optimal time of incubation, experiments were done as described above where macrophages were grown in anti-thymocyte serum for 18-24 hours and 5×10^6 thymocytes were added and incubated at $37^\circ C$. Two tubes were incubated for one hour, two for 2 hours, two for 3 hours, two for 4 hours, two for 5 hours and two for 6 hours. The coverslips were then removed, washed, fixed and stained with Giemsa.

(c) RESULTS

1. Normal F_1 Hybrid Macrophages; Morphological Appearances in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

The morphology of normal F_1 hybrid macrophages grown in syngeneic, normal or parental serum were similar. The macrophages were either stellate-shaped or spindle-shaped, and some of them were ovoid. An occasional multinucleate giant cell was seen. The cytoplasm was blue in colour and the nucleus was round or ovoid and had a vesicular appearance (Fig.61).

In the presence of G.V.H.D. serum, the macrophages were larger and appeared as plump spindle-shaped, stellate-shaped or as ovoid cells. The cytoplasm was more darkly stained (violet-blue) and histochemical examination employing the standard coupling Azo dye technique for Acid Phosphatase (Pearse, 1968) showed the presence of increased amounts of acid phosphatase compared to the macrophages in normal F_1 hybrid serum. The nucleus also was more darkly stained and many more macrophages had more than one nucleus (2 to 4). The macrophages had the same appearance as G.V.H.D. macrophages (Figs.62, 63).

The Capacity of Normal F_1 Hybrid Macrophages Grown in Normal F_1 Hybrid, Parental or G.V.H.D. Serum to Phagocytose Haemolysin-Coated Human Erythrocytes

Normal F_1 hybrid macrophages grown in normal syngeneic or parental serum had phagocytosed haemolysin-coated human erythrocytes; about 70-75 per cent of the macrophages had phagocytosed the erythrocytes (Fig.64). The macrophages grown in G.V.H.D. serum are much/

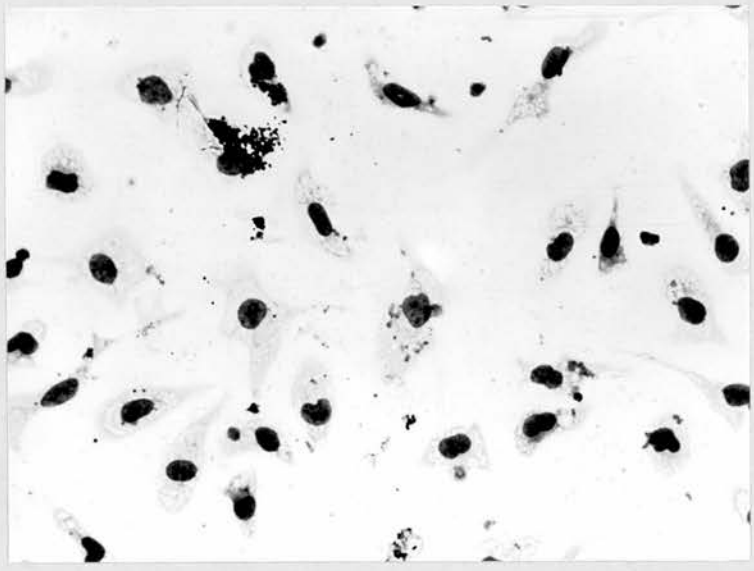

Fig.61. The morphology of normal F_1 hybrid macrophage monolayer after 18 hours in culture. The cells were grown in 5% normal serum. The macrophages are adherent to glass and have spread out. They have ovoid nuclei and abundant cytoplasm. Multinucleate cells are conspicuous by their absence.

Giemsa x 450

Fig.62. The morphology of GVHD macrophages cultured in 5% GVHD serum. They have relatively large nuclei and their cytoplasm appears more darkly stained. Autologous lymphoid cells are seen adherent to some of these macrophages (arrows). Similar appearances were noted when GVHD macrophages were grown in normal serum and when normal F_1 hybrid macrophages were grown in GVHD serum.

Giemsa x 450

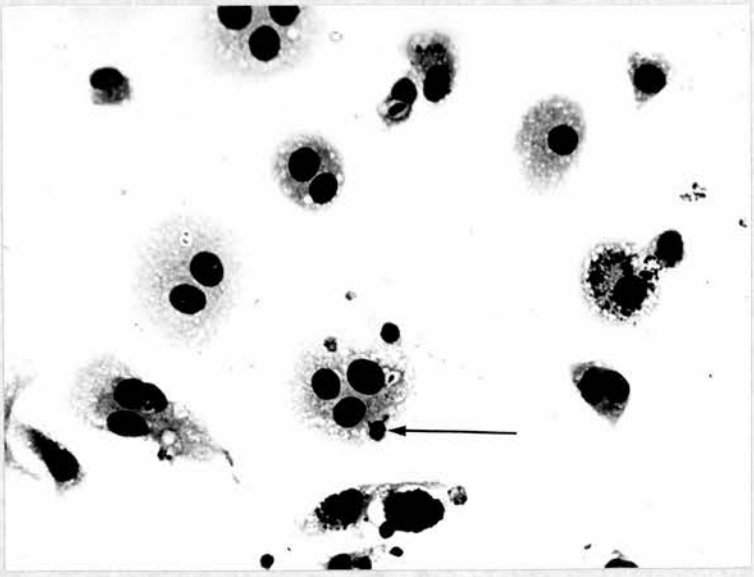

Fig.63. GVHD macrophages cultured in 5% GVHD serum. This field shows binucleate and trinucleate macrophages. Adherence and phagocytosis of autologous lymphocytes are also seen (arrow).

Giemsa x 450

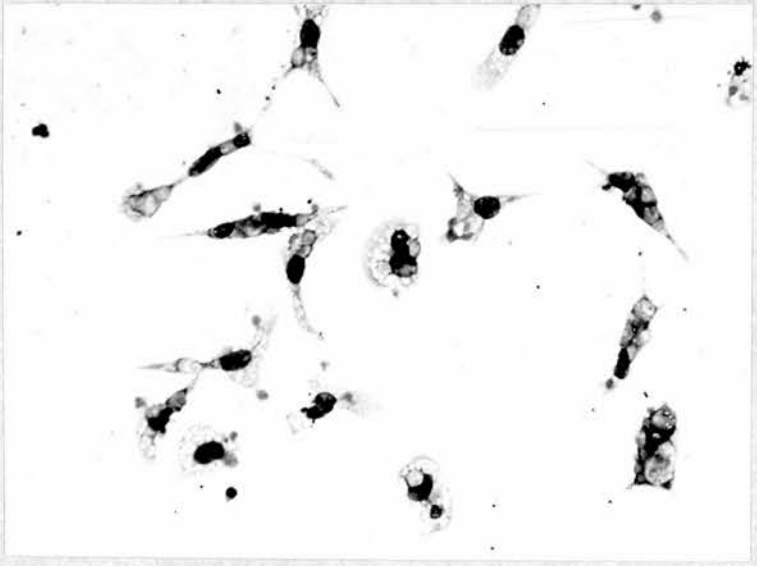

Fig.64. Normal F_1 hybrid macrophages cultured in 5% normal serum. This shows the maximum phagocytic capacity of these macrophages to ingest haemolysin-coated human erythrocytes.

Giemsa x 450

much more hyperactive and 90-95 per cent of the macrophages had ingested the erythrocytes. In addition, the latter macrophages had ingested many more erythrocytes than the macrophages cultured in normal F_1 hybrid and parental serum (Fig.65).

The Interaction of Normal F_1 Hybrid Macrophages with Normal F_1 Hybrid Lymphoid Cells in Syngeneic Normal, Parental and G.V.H.D. Serum

Normal F_1 hybrid macrophages did not interact with syngeneic normal lymphocytes in syngeneic normal or parental serum. In each coverslip about 5 per cent of the macrophages show evidence of one or two lymphoid cells adhering to its processes (Fig.66). In contrast to this, in G.V.H.D. serum about 80-90 per cent of the macrophages show massive adherence with syngeneic normal lymphoid cells. The surface of the macrophages are more or less completely invested by these lymphoid cells and about 1-20 lymphoid cells had adhered to the macrophages (Fig.67).

The Interaction of Normal F_1 Hybrid Macrophages with Parental Lymphoid Cells in Syngeneic Normal, Parental or G.V.H.D. Serum

Normal F_1 hybrid macrophages did not interact with parental lymphoid cells in syngeneic normal and in parental serum. The amount of adherence noted was less than 5 per cent. The interaction of normal F_1 hybrid macrophages with parental lymphoid cells in G.V.H.D. serum was also one of very little adherence (5-10 per cent) (Fig.68).

The Interaction of Normal F_1 Hybrid Macrophages with G.V.H.D. Lymphoid Cells in Syngeneic Normal, Parental or G.V.H.D. Serum

The interaction between normal F_1 hybrid macrophages and G.V.H.D. lymphoid/

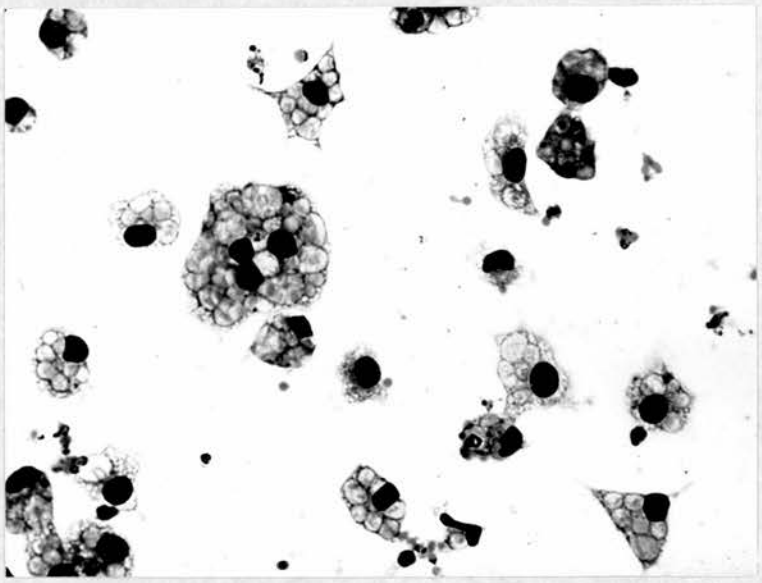

Fig.65. GVHD macrophages cultured in 5% GVHD serum. This shows the maximum phagocytic capacity of these macrophages to ingest haemolysin-coated human erythrocytes. The macrophages are hyperactive. Normal F_1 hybrid macrophages incubated in 5% GVHD serum show similar phagocytic capacity.

Giemsa x 450

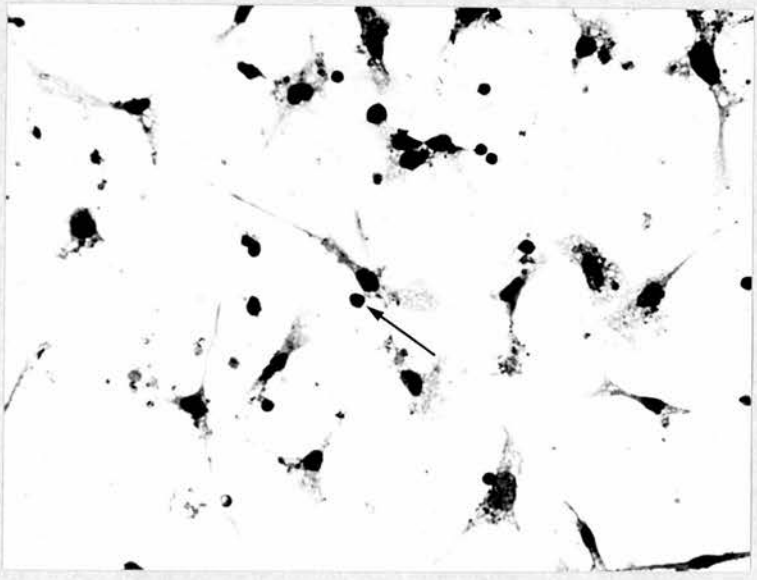

Fig.66. Normal F_1 hybrid macrophages cultured in 5% normal serum and incubated with normal lymphoid cells. An occasional lymphoid cell had adhered to the macrophages (arrow).

Giemsa x 450

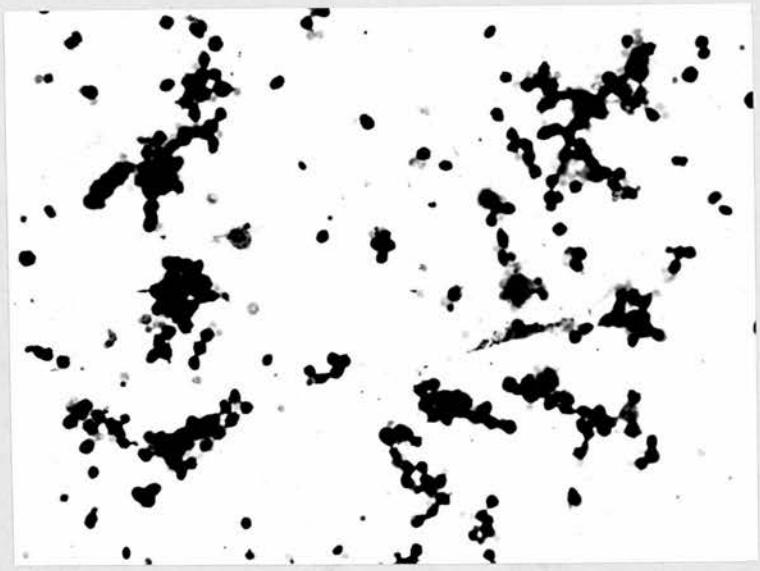

Fig.67. Normal F_1 hybrid macrophages cultured in 5% GVHD serum and incubated with normal F_1 hybrid lymphoid cells. The lymphoid cells have adhered to syngeneic macrophages in large numbers.

Giemsa x 450

Fig.68. Normal F_1 hybrid macrophages cultured in 5% GVHD serum and incubated with parental lymphoid cells. Very little adherence is noted.

Giemsa x 450

lymphoid cells in syngeneic normal and parental serum results in death of the macrophages. The coverslip which had a healthy monolayer was depleted of nearly 50 per cent of the cells and the remaining macrophages appeared as irregular, purplish blotches, containing disintegrated cytoplasm and nuclei. Some of these degenerated and dead macrophages had the G.V.H.D. lymphoid cells adherent to its surface.

In contrast to this, when these two cell types were interacted in G.V.H.D. serum, the macrophages appeared morphologically normal and the G.V.H.D. lymphoid cells had adhered to the macrophages. The adherence was not massive and more lymphoid cells were stuck to the coverslip than to the macrophages.

2. Parental Macrophages; Morphological Appearances in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

The morphological appearances of parental macrophages cultured in normal F_1 hybrid and parental serum were similar to the morphological appearances of normal F_1 hybrid macrophages cultured in normal F_1 hybrid and in parental serum. The cells were spindle-shaped, stellate shaped and some were ovoid.

The morphological appearances of parental macrophages grown in G.V.H.D. serum were similar to normal F_1 hybrid macrophages grown in G.V.H.D. serum. The cells were larger than normal, had a deeply staining cytoplasm and large vesicular nuclei. Few multinucleate cells were also seen (2-4 nuclei).

The Capacity of Parental Macrophages Grown in Normal F_1 Hybrid, Parental and G.V.H.D. Serum to Phagocytose Haemolysin-Coated Human Erythrocytes

Parental/

Parental macrophages grown in normal F_1 hybrid and parental serum phagocytose haemolysin-coated human erythrocytes to the same degree as normal F_1 hybrid macrophages cultured in the same two sera mentioned above. In G.V.H.D. serum, the parental macrophages show evidence of increased phagocytosis of haemolysin-coated erythrocytes, similar to normal F_1 hybrid macrophages cultured in G.V.H.D. serum.

The Interaction of Parental Macrophages with Normal F_1 Hybrid Lymphoid Cells in Normal F_1 Hybrid, Syngeneic Normal and in G.V.H.D. Serum

Parental macrophages do not interact with normal F_1 hybrid lymphoid cells in normal F_1 hybrid or syngeneic normal serum; about 5 per cent of the macrophages had adhered to one or two lymphoid cells.

In G.V.H.D. serum massive adherence was noted between the two cell types. The cell surfaces of the macrophages were almost covered by normal F_1 hybrid lymphoid cells. On an average about 2-30 lymphoid cells had adhered to the macrophages.

The Interaction of Parental Macrophages with Parental Lymphoid Cells in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

No interaction was noted between parental macrophages and parental lymphoid cells in normal F_1 hybrid or parental serum. The adherence was less than 5 per cent and one or two parental lymphoid cells had adhered to the macrophages.

In G.V.H.D. serum, the reaction was one of very little adherence; 5-10 per cent of the lymphoid cells had adhered to the macrophages.

The Interaction between Parental Macrophages and G.V.H.D. Lymphoid Cells in Normal F_1 Hybrid, Parental or in G.V.H.D. Serum.

In/

In normal F_1 hybrid and in parental serum, the interaction between G.V.H.D. lymphoid cells and parental macrophages resulted in death of 50 per cent of the macrophages. The dead macrophages appear as dark purplish masses and the cytoplasmic and nuclear details cannot be made out. The surviving macrophages appear morphologically normal and cytoplasmic and nuclear details can be made out (Fig.69).

In G.V.H.D. serum, no cell death had occurred and many lymphoid cells had adhered to the macrophages. The type of adherence was not massive and lymphoid cells were notably stuck to the coverslip rather than to the macrophages. The cytoplasmic and nuclear details of the macrophages were easily made out.

3. G.V.H.D. Macrophages; Morphological Appearance in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

In all these three sera, G.V.H.D. macrophages had the same morphological appearance. In comparison to normal syngeneic or parental macrophages, these cells were larger, had abundant dark blue cytoplasm and prominent oval nuclei. Relatively many cells (1-2 per cent) were found to contain 2-4 nuclei per cell. Most of the cells (90 per cent) appeared as stellate or plump spindle-shaped cells. Some appeared as round or ovoid cells.

The Capacity of G.V.H.D. Macrophages in Normal F_1 Hybrid, Parental or G.V.H.D. Serum to Phagocytose Haemolysin-Coated Human Erythrocytes

In all these three sera G.V.H.D. macrophages showed evidence of increased phagocytosis, far in excess of that seen with normal F_1 hybrid/

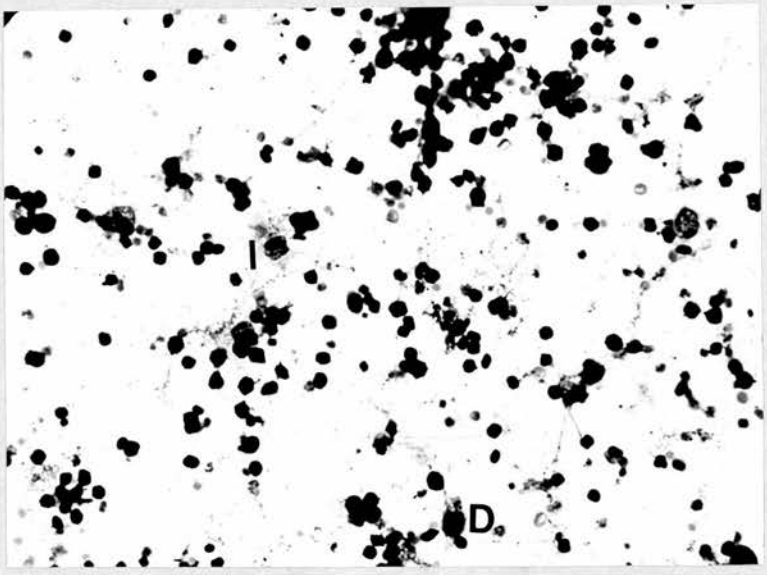

Fig.69. Parental macrophages cultured in 5% normal serum and incubated with cells from GVHD lymph nodes. Some macrophages are morphologically intact (I) while others had undergone degeneration and cytotoxic death (D).

Giemsa x 450

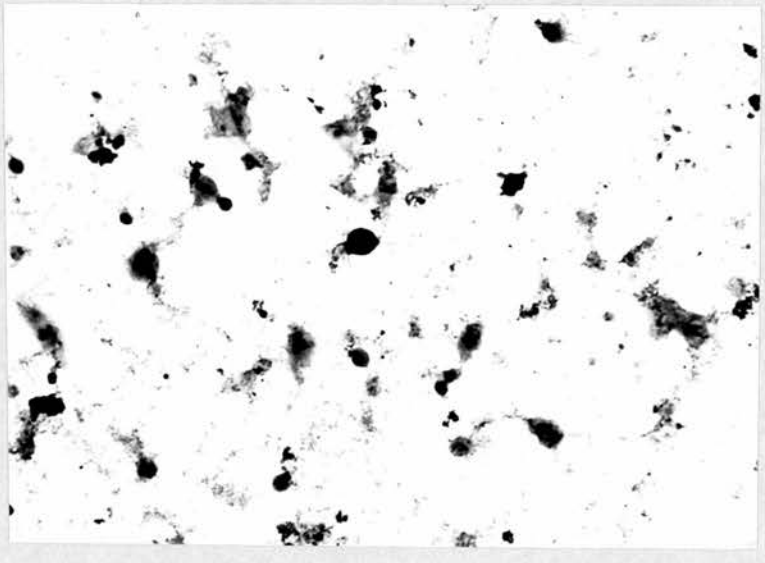

Fig. 70. GVHD macrophages cultured in 5% normal serum and incubated with normal F_1 hybrid lymphoid cells. The interaction between these two cell-types has resulted in cytotoxic death of the macrophages. Similar appearances were noted when normal F_1 hybrid or GVHD macrophages were interacted with cells from GVHD lymph nodes in normal serum.

Giemsa x 450

hybrid or parental macrophages cultured in normal F_1 hybrid or parental serum. On an average 90-95 per cent of the macrophages had taken up 5-30 erythrocytes per cell.

The Interaction of G.V.H.D. Macrophages with Normal F_1 Hybrid Lymphoid Cells in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum.

The result of the interaction of the above two cell types is the same in normal F_1 hybrid and in parental serum; the macrophages had undergone cytotoxic death. The coverslips showed death of 95-100 per cent of the macrophages. Many cells had granular cytoplasm and densely hyperchromatic nuclei (30-40 per cent). The rest of the cells appeared as completely disorganized cellular debris. The lymphoid cells had also adhered to a few macrophages (Fig.70).

In contrast to the above, when the cell types were interacted in G.V.H.D. serum, there was complete preservation of the morphology of the macrophages and 2-3 lymphoid cells had adhered to the macrophages.

The Interaction of G.V.H.D. Macrophages with Parental Lymphoid Cells in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

When the above two cell types were interacted in normal F_1 hybrid and parental serum 80 per cent of the macrophages appeared morphologically as normal uninjured cells and no adherence was noted between the cell types (Fig.71). In G.V.H.D. serum, the macrophages were morphologically normal and no adherence was seen.

The Interaction of G.V.H.D. Macrophages with G.V.H.D. Lymphoid Cells in Normal F_1 Hybrid, Parental and in G.V.H.D. Serum

When/

Fig.71. GVHD macrophages cultured in 5% normal serum and incubated with parental lymphoid cells. Neither cell death nor adherence was noted. Hence GVHD macrophages selectively interacted with normal F_1 hybrid lymphoid cells and not with parental lymphoid cells.

Giemsa x 450

When the above two cell types were interacted in normal F_1 hybrid and parental serum, all the macrophages in the coverslips had undergone cytotoxic death (100 per cent death). Most of the cells (80-90 per cent) appeared as irregular purplish masses and the coverslips contained irregular cytoplasmic fragments. Some of the macrophages had granular cytoplasm with small, deeply staining nuclei.

The results were just the opposite when these two cell types were interacted in G.V.H.D. serum. There was complete preservation of the morphology of the macrophages and no cytotoxic death was seen. The lymphoid cells had adhered to the macrophages (2-5 lymphoid cells per macrophage). Many more lymphoid cells were stuck to the coverslip rather than to the macrophages.

TABLE 9

Normal F_1 Hybrid Macrophages

SERUM FROM	CELLS ADDED				
	Morphology	Maximum Phagocytic Capacity	Normal F_1 Hybrid Lymphoid Cells	Parental Lymphoid Cells	G.V.H.D. Lymphoid Cells
Normal F_1 Hybrid Mice	Normal Size	Normal	No Adherence	No Adherence	100% Cell Death
Parental Mice	Normal Size	Normal	No Adherence	No Adherence	100% Cell Death
G.V.H.D. Mice	Larger than Normal	Increased	Massive Adherence	No Adherence	No Cell Death

The morphology and maximum phagocytic capacity of normal F_1 hybrid macrophages and its interaction with normal F_1 hybrid, parental and G.V.H.D. lymphoid cells in syngeneic normal, parental and G.V.H.D. serum.

TABLE 10

Parental Macrophages

SERUM FROM	CELLS ADDED				
	Morphology	Maximum Phagocytic Capacity	Normal F_1 Hybrid Lymphoid Cells	Parental Lymphoid Cells	G.V.H.D. Lymphoid Cells
Normal F_1 Hybrid Mice	Normal Size	Normal	No Adherence	No Adherence	50% of cells have died
Parental Mice	Normal Size	Normal	No Adherence	No Adherence	50% of cells have died
G.V.H.D. Mice	Larger than Normal	Increased	Massive Adherence	No Adherence	No Cell Death

The morphology and maximum phagocytic capacity of parental macrophages and its interaction with normal F_1 hybrid, parental and G.V.H.D. lymphoid cells in normal F_1 hybrid, syngeneic normal and G.V.H.D. serum.

TABLE 11

G.V.H.D. Macrophages

SERUM FROM	CELLS ADDED				
	Morphology	Maximum Phagocytic Capacity	Normal F_1 Hybrid Lymphoid Cells	Parental Lymphoid Cells	G.V.H.D. Lymphoid Cells
Normal Hybrid Mice	Larger than Normal F_1 Hybrid	Increased	100% Cell Death	No Cell Death	100% Cell Death
Parental Mice	Larger than Normal F_1 Hybrid	Increased	100% Cell Death	No Cell Death	100% Cell Death
G.V.H.D. Mice	Larger than Normal F_1 Hybrid	Increased	No Cell Death	No Cell Death	No Cell Death

The morphology and maximum phagocytic capacity of G.V.H.D. macrophages and its interaction with normal F_1 hybrid, parental and G.V.H.D. lymphoid cells in normal F_1 hybrid, parental and in G.V.H.D. serum.

TABLE 12

The Growth and Functional Activity of Macrophages Cultured in Mouse Serum

Macrophages	1% mouse serum	2.5% mouse serum	5% mouse serum	7.5% mouse serum	10% mouse serum
Morphology	Moderate growth	Normal growth	Normal growth	Moderate growth	Poor growth
Maximum Phagocytic capacity (P max C)	50% of macro-phages are active	60% of macro-phages are active	70% of macro-phages are active	40% of macro-phages are active	20% of macro-phages are active

The Concentration of Mouse Serum Required for the Normal Growth of Macrophages in Culture

The macrophages grown in 2.5% and 5% mouse serum grew uniformly as a monolayer and exhibited good function: 60-70 per cent had phagocytosed one or more erythrocytes. In 1%, 7.5% and 10% mouse serum the growth and function of the macrophages were found to be unsatisfactory (Table 12). Hence 5% mouse serum was the optimal concentration for growth of the macrophages.

The Concentration of Peritoneal Macrophages Required for Sufficient Growth in Coverslips

The tubes which had a million cells formed a uniform evenly spaced layer of macrophages. Hence, a million macrophages per tube was the optimal concentration for cell culture experiments.

Studies on the Time and Dose Relationship of the Interaction between Macrophages and Thymocytes.

The macrophages cultured in pooled 'O' human and normal mouse serum did not show significant adherence to the thymocytes (less than 5 per cent). The macrophages cultured in anti-thymocyte serum had adhered markedly to the thymocytes. This was best made out in the tubes containing 5 million thymocytes (Table 13). In the tubes containing 7.5 and 10 million thymocytes, the whole coverslip was completely covered by thymocytes and macrophages so that interpretation of their reactivity was difficult. One and 2.5 million thymocytes were not sufficient to demonstrate adherence to the same extent as in the tubes containing 5 million thymocytes. Hence, 5 million thymocytes was the optimum number to study cell interactions/

interactions under these experimental conditions.

The optimal time for incubation of macrophages and thymocytes was found to be between 3 and 4 hours. Hence, in the main cell culture experiments the cells were interacted for 4 hours (Table 14).

These results show that 5 per cent mouse serum, 1 million macrophages, 5 million lymphoid cells and an incubation time of 4 hours are the optimum conditions to perform the main experiments on cell interactions.

TABLE 13

The Interaction of C57BL Macrophages with Thymocytes in Human, Normal Mouse and in Anti-Thymocyte Serum

Serum	Control	1×10^6 Thymocytes	2.5×10^6 Thymocytes	5×10^6 Thymocytes	7.5×10^6 Thymocytes	10×10^6 Thymocytes
Pooled 'O' Human Serum	Good Growth	Adherence + -	Adherence + -	Adherence + -	Death of 50% of Macrophages	Death of 50% of Macrophages
Normal Mouse Serum	Good Growth	Adherence ±	Adherence ±	Adherence ±	Death of 60% of Macrophages	Death of 60% of Macro-
Anti-Thymocyte Serum	Good Growth	Adherence ±	Adherence ++	Adherence +++	Adherence cannot be determined	Adherence cannot be determined

TABLE 14

The Time of Incubation of Normal C57BL Macrophages (1×10^6) and Thymocytes (5×10^6) Cultured in Normal and in Anti-Thymocyte Serum

Serum	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	6 Hours
Normal	Adherence ±	Adherence ±	Adherence ±	Adherence ±	Adherence ±	Adherence ±
Anti-Thymocyte	Adherence ±	Adherence +	Adherence +++	Adherence +++	Adherence ++	Adherence ++

(D) DISCUSSION

Terminology

1. "Normal Serum". This refers to either serum from normal F_1 hybrid or parental mice.

2. "Lymphoid Cells". The designation lymphoid cells rather than lymphocytes has been used because the cells were obtained from lymph nodes and spleen and hence the effector cell population contain not only lymphocytes heterogeneous by themselves but monocytes and macrophages as well (Perlmann and Holm, 1969). Also G.V.H.D. lymph nodes were taken on the 21st day after the inoculation of parental cells. Histopathological examination showed that many of these cells were macrophages. These cells may be of parental or F_1 hybrid origin or both.

The Morphology of Macrophages

In normal serum and in pooled 'O' human serum normal F_1 hybrid and parental macrophages presented an almost identical appearance. It was possible to reproduce the same morphological appearances when the experiments were repeated months later. The morphology of the cells in normal serum was taken as the standard base-line in the cell culture experiments. The cells were spindle or stellate-shaped and some of them were ovoid. The cytoplasm was light blue and the oval nuclei appeared violet when stained by Giemsa's method.

The G.V.H.D. macrophages grown in either normal or G.V.H.D. serum were quite different. These cells were much bigger, appear-

appearing as plump spindle-shaped, stellate or oval cells. They had dark blue cytoplasm and cytoplasmic organelles appeared to take up more of the stain. The nuclei were relatively big and hyperchromatic. About 1-2 per cent of the cells were very large with abundant dark blue cytoplasm and contained between 2-4 nuclei per cell.

When normal F_1 hybrid macrophages were cultured in G.V.H.D. serum, their morphology changed and they resembled G.V.H.D. macrophages. This action of G.V.H.D. serum appears to be specific since the serum had no effect on peritoneal macrophages from another strain of mouse. Peritoneal macrophages derived from the "TO" strain of mice were grown in syngeneic normal and G.V.H.D. serum. The macrophages had the same morphology in both the sera and G.V.H.D. serum exerted no demonstrable effect. It is known that macrophages sensitized to bacteria are larger and have many more intracytoplasmic organelles than do unstimulated cells, (Hard, 1970). One possible explanation for the change in morphology of normal F_1 hybrid macrophages is that G.V.H.D. serum may contain anti-F_1 hybrid antibodies, (Koltay et al, 1965). How exactly anti-F_1 hybrid antibodies (G.V.H.D. serum) cause an increase in size of normal F_1 hybrid macrophages is not known. Histochemical studies on acid phosphatase levels of macrophages in cell culture (Azo-dye method, Pearse, 1968) demonstrated that normal F_1 hybrid macrophages grown in G.V.H.D. serum have an increased content of this enzyme compared to macrophages grown in syngeneic normal serum.

Thus/

Thus the more deeply stained cytoplasmic areas in G.V.H.D. macrophages and in G.V.H.D. serum-treated normal F_1 hybrid macrophages may be due to the increased size or numbers of lysosomes present in them. Hence G.V.H.D. serum may in some manner cause these macrophages to synthesize enzymes and perhaps other cytoplasmic constituents.

The Functional Activity of Peritoneal Macrophages

Normal F_1 hybrid and parental macrophages cultured in normal serum phagocytose haemolysin-coated human erythrocytes to their maximum capacity. However, when the above macrophages were grown in G.V.H.D. serum in the same concentration, they phagocytosed erythrocytes in greater numbers and metabolized these cells more rapidly. It is known that G.V.H.D. macrophages are hyperactive and have increased content of lysosomal enzymes, (Good, 1969; Waksman, 1969). G.V.H.D. macrophages irrespective of the type of serum in which they were grown showed the same degree of enhanced phagocytic capacity for haemolysin-coated human erythrocytes as normal F_1 hybrid and parental macrophages grown in G.V.H.D. serum. Hence G.V.H.D. serum increases the functional capacity of normal macrophages.

The Interaction Between Normal Macrophages and Normal Lymphoid Cells in Normal Serum

Normal F_1 hybrid peritoneal macrophages cultured in normal serum do not interact with syngeneic normal lymphoid cells. This result is to be expected as these two cell types have the same tissue antigens. Similar results were obtained by Stuart (1967) when normal/

normal macrophages were interacted with syngeneic normal lymphoid cells in normal serum. Similarly normal F_1 hybrid macrophages did not interact with parental lymphoid cells in normal serum. Here again the latter lymphoid cells do not have any foreign antigens for the macrophages to interact with. The non-interaction was manifested by non-adherence of the lymphoid cells to the macrophages. Similar results were obtained between the interaction of parental macrophages and syngeneic normal lymphoid cells grown in normal serum. In this interaction, again, the lymphoid cells have the same tissue antigens as the macrophages. Parental macrophages also did not interact with normal F_1 hybrid lymphoid cells in normal serum although the latter cells had antigens which were foreign to it. It is possible that serum factors are essential for the occurrence of this interaction.

The Interaction of Normal Macrophages with Normal Lymphoid Cells in G.V.H.D. Serum

Normal F_1 hybrid macrophages interacted with syngeneic normal lymphoid cells in G.V.H.D. serum. This interaction was seen in the form of massive adherence of the lymphoid cells to the macrophages. This is in direct contrast to the non-adherence of the two cell types when they were interacted in normal serum. Hence the G.V.H.D. serum has exerted its effect in such a manner that normal F_1 hybrid macrophages interacted with syngeneic cells. This could be considered as an instance of auto-reactivity.

A similar result was noted in the interaction between parental macrophages/

macrophages and normal F_1 hybrid lymphoid cells in G.V.H.D. serum. The explanation for these interactions might be that the inoculated parental lymphoid cells produce antibodies against F_1 hybrid tissue which are cytophilic for macrophages. These macrophage cytophilic-antibodies may be attached to the membranes of these macrophages and cause adherence of normal F_1 hybrid lymphoid cells. In contrast to this no adherence was noted between the macrophages and parental lymphoid cells in G.V.H.D. serum. Hence this reaction was specific for normal F_1 hybrid lymphoid cells implying the "cytophilic-antibodies" were produced against F_1 hybrid antigens only.

In experiments on the morphology of G.V.H.D. peritoneal macrophages cultured in G.V.H.D. serum, it was noted that these cells had adhered to and phagocytosed autologous peritoneal lymphoid cells, (Figs.62, 63).

If these results could be applied to the <u>in vivo</u> situation, it may be interpreted that the F_1 hybrid macrophages interact with autologous lymphoid cells in mice with G.V.H.D.

Cell-Mediated Death of Macrophages

Cell-mediated death noted as partial or complete destruction of the macrophage monolayers occurred when the following pairs of cells interacted in normal serum.

(a) Normal F_1 hybrid Peritoneal Macrophages + cells from G.V.H.D. Lymph Nodes.

(b) Parental Peritoneal Macrophages + cells from G.V.H.D. Lymph Nodes.

(c) G.V.H.D. Peritoneal Macrophages + cells from G.V.H.D. Lymph Nodes.

(d) G.V.H.D. Peritoneal Macrophages + cells from Normal F_1 hybrid Lymph Nodes.

In each of these interacting pairs of cells, at least one cell type in each pair is derived from G.V.H.D. animals. If in the interacting pairs (a) and (b) cells from G.V.H.D. lymph nodes were replaced by either normal F_1 hybrid or parental lymphoid cells no interaction was noted. Similarly no interaction was noted when G.V.H.D. macrophages were interacted with parental lymphoid cells. This means that cells from G.V.H.D. lymph nodes and G.V.H.D. peritoneal macrophages may be sensitized cells which are capable of interactions which lead to death of the macrophage monolayer.

The mechanism of cell death which occurs when the sensitized and target cells interact is not known. All investigators are in agreement that viable lymphoid cells are required for the expression of cytotoxicity. It has been shown clearly that cells or cell extracts obtained by ultra-sound disintegration, freezing-thawing or heating to 56°C do not cause cytotoxicity of target cells (Stuart, 1962; Lundgren and Müller, 1969).

There/

There are three main views as to the mechanism of cell death.

(1) Cell Death Caused by "Membrane Interaction"

Möller (1965) and Möller and Möller (1965) have shown that cell death will occur when target cells are confronted with antigenically incompatible PHA-treated lymphoid cells. These workers are of the opinion that interaction between membranes is sufficient to cause cytotoxicity. Further evidence for this view was found by the demonstration that iso-antibodies produced in the target strain and directed against the allogeneic lymphoid cells specifically suppressed cytotoxicity. The iso-antibodies specifically blocked the antigenic determinants of the lymphoid cells and thereby interfered with the recognition of incompatibility by the target cells, (Möller, 1967). Lundgren and Möller (1969) using the metabolic inhibitor Actinomycin-C showed that increased rates of synthesis of RNA, DNA and protein were not directly associated or required for the expression of cytotoxicity. It was noted that the cytotoxic effect in the mono-layer could be detected at 4-8 hours at a time when RNA and protein synthesis had not been detectably initiated. These results tend to show that membrane activity alone was sufficient to cause cell death.

(2) Cell Death Caused by Antibodies

The sensitized or immune lymphocyte may be closely associated with the target cell and the former cells may secrete small amounts of antibodies (Karush and Eisen, 1962; Taylor and Culling, 1963). Since the cells are close to each other the concentration of antibody near the target cell will be high and these will combine with the specific/

specific antigens on the membrane of the target cells and cause cytotoxic death, in the presence of complement. However this reaction cannot explain the cytotoxic death of target cells caused by autologous non-specifically stimulated human lymphocytes.

(3) Cell Death Caused by Lymphotoxin

Granger and Kolb (1968) have shown that the membrane of the sensitised lymphocyte and the corresponding target cell first come into contact with each other and later membrane interaction occurs between the two cells. These events alone were found not sufficient to cause death of target cells. Williams and Granger (1969b) found that for cytotoxicity of the target cell, the PHA-activated lymphocyte required energy metabolism and protein synthesis. These studies were made using metabolic inhibitors which suppress RNA, DNA and protein synthesis. It was also shown by Granger and Kolb (1968) that the non-specifically and specifically sensitized lymphocytes release a soluble cell-free cytotoxic factor into the culture medium which they termed "lymphotoxin". This toxin killed cultured target 'L' cells. They also showed that lymphotoxin once released acts non-specifically, killing target cells of different species. The mechanism of action of lymphotoxin appears to be the result of lymphotoxin interaction on membrane sites which are common to many different cell-types (Williams and Granger, 1969a). The presence of similar effector molecules termed "lymphokines" have also been described by Dumonde et al (1969). These workers found that the various actions of sensitized lymphocytes are expressed by immunologically non-specific effector molecules the/

the generation of which is governed by cellular reactions of immunological specificity.

Studies by Kramer and Granger (1970) on the interaction between purified specifically sensitized peritoneal macrophages and the specific target cells have revealed that a cell-toxin was released into the culture medium during the mutual destruction of target and aggressor cells. In contrast, under similar conditions, normal peritoneal and starch induced macrophages obtained from the same strain of unimmunized mice were not capable of either inducing target cell destruction or cell-toxin release. This factor(s) appeared to act in a non-specific manner, for it was cytolytic for syngeneic, allogeneic and xenogeneic cells. Serum from goats immunized with preparations containing mitogen-induced lymphotoxin (LT) neutralised the cytolytic activity of the cell-toxin obtained from macrophages. These results suggest that macrophages can be specifically induced to release a non-specific cell toxin(s) which may be responsible for the non-specific destruction of both target cells and macrophages. The factor(s) was immunologically similar to lymphotoxin released by activated mouse lymphocytes.

In my experiments normal F_1 hybrid macrophages interacted with cells from G.V.H.D. lymph nodes. Cells from the latter may be derived from the original parental inoculum in addition to native cells of hybrid origin. However, cells from G.V.H.D. nodes functioned as sensitized cells and in this experimental model the capacity/

capacity for active sensitization was possessed by the parental and not by the F_1 hybrid cells. This indirect evidence suggests that cells from G.V.H.D. lymph nodes were at least partly of parental origin.

It was also noted that G.V.H.D. peritoneal macrophages interacted with normal F_1 hybrid and not with parental lymphoid cells. Here again G.V.H.D. peritoneal macrophages could function as actively sensitized cells only if they were of parental origin. Hence it is inferred from the above two pairs of cell interactions that G.V.H.D. peritoneal macrophages and cells from G.V.H.D. lymph nodes are sensitized cells and that at least a percentage of them are of parental origin.

However, explanation needs to be provided for the other two pairs of cell interactions.

Firstly it was noted that the interactions between parental macrophages and cells from G.V.H. D. lymph nodes resulted in death of 50 per cent of macrophages. This effect cannot have an immunological origin because the genetics of this experiment are such that the recipient cells are unable to react against donor antigens. My explanation is that although the G.V.H.D. lymph nodes are partly of parental origin, they already have been stimulated by contact <u>in vivo</u> with F_1 hybrid antigens. Consequently they have been triggered to release lymphotoxin which subsequently kills the parental macrophages.

Secondly, the interaction between G.V.H.D. peritoneal macrophages and cells from G.V.H.D. lymph nodes resulted in 100 per cent death/

death of macrophages. The greater number of sensitized cells would allow for a correspondingly greater release of lymphotoxins and explain the total death of the monolayer.

There are *in vitro* and *in vivo* experiments in the literature to support cell death caused by non-specific mechanisms.

Lundgren and Möller (1969) have shown that human lymphocytes stimulated by PHA develop cytotoxicity towards allogeneic and autochthonous fibroblasts. These workers are of the opinion that the specificity of the cellular immune reactions are probably confined to the immunological recognition step initiating the cytotoxic potential and that the expression of cytotoxicity is an immunologically non-specific process caused by stimulated cells.

Similar non-specific cytotoxic damage was also noted *in vivo* experiments. Elkins and Guttman (1968) orthotopically transplanted Lewis parental kidneys into (Lewis XBN)F_1 hybrid rats and these were accepted by the latter animals. They subsequently inoculated suspensions of Lewis strain lymphoid cells beneath the capsules of the transplanted Lewis kidneys of the F_1 hybrids. A local G.V.H. type reaction developed which destroyed significant amounts of the renal tissue. Clearly the reacting cells were Lewis lymph node cells confronted and stimulated by F_1 hybrid mononuclear cells circulating in the blood of the transplanted kidneys. The damage inflicted upon the latter therefore must necessarily be non-specific.

A possible explanation for the mechanism of cell death in the last two cell interactions is the suggestion put forward by Granger (1969,/

(1969, P397; and supported by Chase and Good). Granger has suggested that the destructive effect of the lymphocyte is intimately associated with what he refers to as a micro-environment. This would be defined as that area adjacent to an activated cell. This micro-environment would contain lymphotoxin which would lead to a nidus of total cellular destruction and as materials begin to diffuse away and become diluted there may be differential cytolysis based on the metabolic state of the cells in the adjacent area.

The above suggestion is also applicable to the first two sets of cell interactions which may take place with immunological specificity. The cells come into contact due to immunological specificity and the lymphotoxin released by the sensitized cells would be in very high concentration in the micro-environment of the macrophages leading to their death. Hence immunological specificity helps the sensitized macrophages or lymphoid cells to release lymphotoxin in high concentration on to the target cell with great precision.

Prevention of cytotoxic death by G.V.H.D. serum

It was noted, as described in the previous section, that cytotoxic death of macrophages occurred in two situations - (a) when normal F_1 hybrid, parental and G.V.H.D. macrophages interacted with cells of G.V.H.D. lymph nodes, and (b) when G.V.H.D. macrophages interacted with normal F_1 hybrid lymphoid cells. Cytotoxic death occurred when the above pairs of cells were interacted in normal serum. However when the above pairs of cells were interacted in G.V.H.D./

G.V.H.D. serum 100 per cent survival of the macrophages was seen. Clearly G.V.H.D. serum under the experimental conditions employed had completely protected the macrophages from death.

Similar results demonstrating the protective effect of specific immune serum on the cytotoxicity of target cells had been shown by many workers. Brunner et al (1967) found that high titre specific iso-antibody protected DBA/2 mastocytoma cells against the in vitro cytotoxicity of immune lymphoid cells. They suggest that the protection from cell death was due to competition of iso-antibody and lymphoid cells for the same antigenic sites on the target cell membranes.

Möller (1965a) cultured target tumour cells in normal mouse serum from mice and added specifically sensitized regional lymph node cells derived from an animal pretreated with a single immunizing injection 7 to 10 days earlier. After 48 hours incubation significant numbers of target tumour cells were killed. In another experiment (with suitable controls) specific antisera against the target cells were added and incubated with the specifically sensitized lymphoid cells. It was noted that the specific immune serum partially or completely inhibited the cytotoxic effects of immune lymphoid cells. Thus the humoral antibodies had antagonised the cytotoxic effect of immune lymphoid cells in the above experiments.

The protective effect of specific immune serum in preventing the cytotoxicity of target cells by specifically sensitized lymphoid cells is equivalent to the 'enhancement' phenomenon described in animal/

animal experiments. The term immunological enhancement applies to the indefinitely prolonged survival or the delayed regression of allografts as a consequence of the presence of specific antigraft antibody in the host (Kaliss, 1957a).

Three mechanisms by which antibodies might exert their enhancing effects have been postulated, (Billingham, Brent, Medawar, 1956).

(a) Afferent - by combining with antigen and thus preventing it from reaching the immunologically competent cells in an effective form.

(b) Central - by acting directly on the immunologically competent cells and thus inhibiting their sensitization and/or antibody synthesis.

(c) Efferent - by combining with the antigenic determinants on the target cells and thereby protecting them against the damaging effect of specifically sensitized cells.

Thus in my experiments, G.V.H.D. serum acted at the 'efferent' level preventing the cells from G.V.H.D. lymph nodes from coming into close contact with the membrane of the macrophages or by neutralizing any lymphotoxin that may be liberated from sensitized cells.

The Role of G.V.H.D. Serum and G.V.H.D. Macrophages in the *in vivo* situation

G.V.H.D. macrophages caused severe cytotoxic reactions in normal serum. If these reactions had taken place in G.V.H.D. animals,/

animals, evidence of severe cell destruction should be seen. However, histopathological studies failed to reveal signs of acute cell damage. In the *in vivo* situation, it is possible to foresee various homeostatic and inhibitory mechanisms operating preventing this acute cell damage. This rôle is possibly played by factors present in G.V.H.D. serum.

(E) GENERAL DISCUSSION

Graft-versus-Host Disease was produced in adult (CBA x C57BL)F_1 and B6AF_1/J hybrid mice by the intravenous injection of parental lymphoid cells. Detailed histopathological studies indicate that the following systems are primarily involved.

(a) The Lympho-Haemopoietic System.

(b) The Alimentary System and associated structures embryologically derived from it (liver, exocrine, pancreas and salivary gland).

Histopathological studies on the lymph nodes during the early stages indicate a marked enlargement of the 'paracortical' areas of the nodes. This is probably due to the 'homing' of the parental lymphoid cells although no distinction could be made between the parental and F_1 hybrid cells in my studies. The 'paracortical' areas also had numerous pyroninophilic blast cells and macrophages, many of which were seen in mitosis.

It may be that these pyroninophilic blast cells had in the later stages transformed into macrophages. The malpighian bodies of the spleen also showed changes similar to that in the lymph node during the early stages of the disease. These bodies were initially more cellular and few pyroninophilic-blast cells and many macrophages were seen in them. In some, clusters of macrophages were seen extending into the malpighian body from the periphery. In the early stage, the intestine, liver, pancreas, salivary gland and bone-marrow also/

also showed infiltration by dark mononuclear cells; similar infiltration by cells was not seen in the tissues of control mice. It is hence probable that parental lymphoid cells had also settled in non-lymphoid organs.

In the intermediate stage of the disease, the main feature was the marked alteration in the architecture of the nodes. The lymphoid follicles were not seen and the whole node was almost replaced by macrophages. Many small lymphoid cells were adherent to these cells and very few pyroninophilic cells were seen in the cortex and paracortical areas. However the medulla contained large numbers of pyroninophilic cells and macrophages. It may be that these pyroninophilic cells were precursors of plasma cells. The spleen in the intermediate stage showed complete replacement of the malpighian bodies with macrophages; no pyroninophilic cells were seen. The bone-marrow was hypoplastic with loss of its erythroid and myeloid elements and was replaced with uniformly dark round mononuclear cells, multinucleate giant cells and macrophages. The intestine, pancreas and salivary gland showed evidence of infiltration of dark mononuclear cells and macrophages. These organs were also damaged to varying degrees but the damage to the mucosa of the intestine may be significant in the pathogenesis of the disease. The liver showed increase in number and size of Kupffer cells and the prominent periportal accumulation of mononuclear cells had the morphological appearance of macrophages.

In the late stage of the disease the lymph nodes were grossly atrophic/

atrophic and histologically the nodes were replaced by sheets of macrophages. The malpighian bodies of the spleen also showed the complete replacement of the lymphoid elements by macrophages. However the macrophages had joined each other and were seen to form whorls around the central arteriole. In a later stage these cells had undergone gradual cell death and eosinophilic hyaline masses were seen in their place. Special stains showed that these masses were amyloid mixed with fibrin. In the late stage the intestine, pancreas, salivary gland and bone-marrow showed changes similar to that seen during the intermediate stage. Changes were also seen in the thymus and adrenal gland. The thymus was progressively depleted of thymocytes with concurrent replacement by macrophages and fibroblasts. The adrenal gland showed cytolytic degenerative changes in the outer zona glomerulosa and adjacent fasciculata. The rest of the fasciculata was markedly depleted of lipid. The changes seen in the thymus may be due to the hyperactivity of the adrenal gland and the latter gland itself had shown signs of exhaustion during the later stages of the disease. An alternative possibility is that the macrophage proliferation of the thymus is part of the overall increase of the macrophage population observed in the lymphoid tissues of G.V.H.D. animals. Since no strong proof is available that the circulating pool of lymphocytes pass through the thymus, it may be that G.V.H.D. serum had altered the behaviour of macrophages to interact with autologous thymocytes. Hence the histopathological studies show the following results.

(a)/

(a) Extreme depletion of lymphoid cells.

(b) Damage to intestine and to the glands derived from it.

(c) Marked proliferation of macrophages.

Histopathological studies had indicated that loss of lymphoid tissue and proliferation of macrophages are important events in the pathogenesis of this disease. Hence cell culture experiments were planned to study the interaction between macrophages and lymphoid cells. These studies indicated that serum from G.V.H.D. animals had the capacity to alter the behaviour of normal syngeneic macrophages. In this serum the macrophages were bigger than normal and were more hyperactive. Macrophages from normal animals (syngeneic with the strain which develop G.V.H.D.) cultured in G.V.H.D. serum had interacted with syngeneic normal lymphoid cells. This was seen as massive adherence of the lymphoid cells to the macrophages. Similar adherence was not seen when these cell types were interacted in normal serum. This altered reactivity is probably due to the presence of anti-F_1 hybrid cytophilic antibodies in G.V.H.D. serum. It is possible that a similar interaction may be one of the mechanisms which may operate in mice with G.V.H.D.

Cells from G.V.H.D. lymph nodes in the intermediate and late stages of the disease were noted to be predominantly macrophages. These cells are 'sensitized' cells since they interact with peritoneal macrophages and cause their death. The capacity to cause death of normal peritoneal macrophages was not noted with normal lymphoid cells. Cells from G.V.H.D. lymph nodes caused death of normal F_1 hybrid/

hybrid and the interaction between normal F_1 hybrid lymphoid cells and G.V.H.D. peritoneal macrophages also resulted in death of the latter cells. These are specific immunological reactions and show that cells from G.V.H.D. lymph nodes and G.V.H.D. peritoneal macrophages are probably of parental origin since the capacity for active sensitization is possible by these cells alone. However, cells from G.V.H.D. lymph nodes also caused death of 50 per cent of normal parental macrophages and this reaction cannot have an immunological basis if the former cells were of parental origin. The lymphotoxin theory (Granger and Kolb, 1968) has been invoked to explain some of these reactions.

The four pairs of cytotoxic interactions described earlier occurred in normal serum (normal F_1 hybrid or parental serum). However, complete preservation of the morphology of the macrophages was noted when the above interactions took place in G.V.H.D. serum. Hence G.V.H.D. serum prevented the cell death caused by 'sensitized' cells. This protective effect is equivalent to the 'enhancement' phenomenon seen in animals transplanted with an allograft and at the same time inoculated with specific anti-sera prepared against the allograft.

Furthermore, G.V.H.D. serum may alter normal macrophages to react with autologous lymphoid and other parenchymal cells. The 'sensitized' macrophages present in these mice may in addition cause cell destructive reactions.

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