TRANSPLANTATION AND IMMUNOPATHOLOGY OF THE LANDSCHÜTZ ASCITES TUMOUR

by

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GENERAL INTRODUCTION

The Significance of Studies with Transplantable Tumours

One of the outstanding defects in the literature on transplantation immunity is the contradiction and lack of uniformity in terminology. It is therefore necessary to start with the definitions of certain terms which have recently been almost universally accepted (Snell 1958) and which will be used throughout the present work.

An inbred strain is a strain which results from the mating of brother and sister for 20 generations or more.

Autotransplant is a graft of the individuals own tissue and transplanted in the same individual.

Isotransplant is a graft between members of the same inbred strain.

A Homotransplant is a graft between two individuals of the same species but who are genetically different.

Heterograft is a graft between members of different species.

Iso-antigens are tissue antigens which characterise an individual or an inbred strain from a genetically different individual or another strain. This is clearly a misnomer and such antigens should correctly be called homologous antigens. However the term iso-antigen has

been used so long that it is better to preserve it. (Snell 1958).

Alternative nomenclature is given by Gorer et al (1961) and is shown in the accompanying table.

Transplantable tumours are either specific or non-specific to a particular strain of animal. This applies particularly to the mouse where inbred strains are available. The specificity to the strain is in obeyance to the laws of transplantation immunity as formulated by Snell (1958). The non-specific tumours on the other hand are less exacting and will grow in alien homologous hosts. The Landschütz ascites tumour is an example of the non-specific or homotransplantable tumours. A lot of the earlier works on tumour transplantation was carried out with homotransplantable tumours or with tumours of very low strain specificity. Though a great deal of information was obtained the major advances in the problem of tissue specificity had to await the development of inbred strains and specific tumours.

The interplay of various factors, some genetic and others environmental, determine the fate of a grafted tissue. It is therefore necessary to survey the genetic and environmental aspects of tissue transplantation. This will be followed by a consideration of the homograft reaction and the antigens involved in it, with particular

Proposed revisions of terminology after Gorer, Loutit and Micklem

Nouns			Adjectives		
Old terms	Proposed New terms	Alternative New terms	Old terms	Proposed New terms	Alternative New terms.
autograft	autograft	-	autologous	autoplastic autochthonous	-
isograft	Syngeneic homograft or graft	isogenic homograft or graft	isologous	syngeneic	isogenic
Homograft general sense	Homograft	-	Homologous	Allogeneic	-
Homograft connoting genetic diversity	Allogeneic homograft or graft	-	-	-	-
Heterograft	xenograft	Heterospecific graft Interspecific graft Heterograft	Heterologous	Xenogeneic	Heterospecific Heterologous

reference to transplantable tumours. Finally some pitfalls and drawbacks in the use of such tumours will be pointed out.

Genetic Factors Involved In Tumour Immunity

Much of the early work on tumour immunity was concerned with the morphological reactions around grafted tumours and the induction of resistance to such tumours (Woglom 1929). The important role of genetic factors in determining susceptibility or resistance to homotransplants was, however, considered in the early part of this century. Thus Tyzzer (1909) investigated the inheritance of susceptibility to tumour grafts in mice. He demonstrated that a tumour arising in the Japanese Waltzing mice grew progressively in 100% of these mice but in noneof the common tame mice. The first filial generation obtained by crossing the Japanese and the outbred mice were susceptible. The importance of heredity in susceptibility of the rat to a transplantable tumour was stressed by Levin and Sittenfield (1910).

Tyzzer (1916) extended his studies with the waltzing and the common mice further. He found different degrees of susceptibility of the first filial, second filial generations and complete resistance in the backcross

between the F1 generation and the common mice. He concluded that susceptibility or resistance depended on the presence of complex independantly inherited unit factors.

Fleisher and Loeb (1913) stated that the differences in susceptibility to the growth of inoculated tumours in various strains of the same species of animals were due to inheritable constitutional causes.

Little and Tyzzer (1916) using a transplantable tumour in parental - F1 and F2 hybrid system, thought that the number of inheritable factors determining susceptibility to their tumour was between 12 and 14 factors.

Little and Johnson (1922) found that the inheritance of susceptibility to implants of splenic tissue was similar to that of the Japanese Waltzing tumour. They concluded ".....in all probability susceptibility to transplants of splenic tissue depends on the same general principles of heredity found to apply in the case of tumour tissue, namely multiple mendelizing factors".

The most definite evidence bearing on the problem of genetics of transplantation of tissue has been obtained with the use of inbred strain of mice. Prominent recent investigators in this field are Gorer and Snell. Gorer (1937 - 1938 - 1942) found that the genes which determine

the fate of a transplant also determine iso-antigenic differences. An antigen II, was found to be shared between erythrocytes of strain A mouse and its fixed tissues and this antigen was found to be important in immunity to tumour transplants. The locus of the histocompatibility gene determining this antigen was called H_2 locus. This was found to be the most important locus in determining the fate of a graft though other loci were identified. Snell (1948) has estimated the number of H loci in all strains of mice to be six or seven and probably in the neighbourhood of 14.

As a result of his own contribution and the work of several investigators, including the pioneer work of Tyzzer and Little, Snell (1958) formulated the genetic laws of tissue transplantation. He made use of tumour transplantation in inbred strains of mice, their F_1 and F_2 generations and backcross to either parent. These laws are:

(1) Transplants within a single inbred strain (isotransplants) grow progressively in all strains.

(2) Transplants between strains (Homoiotransplants) do not grow or grow temporarily and then regress.

(3) Transplants into hybrid animals where one parent is inbred and susceptible grow progressively and kill all hosts.

(4) Transplants into F_2 mice and into backcross mice from mating F_1 and resistant parent grow in a fraction of the hosts only. The susceptible percentage may vary within wide limits according to the strain of mice and the tumour employed.

(5) A tumour originating in F_1 hybrid will grow in all F_1 hybrids but not in members of either parent strain. It will grow in a fraction of F_2 and backcross mice.

(6) A tumour originating in F_2 or later hybrid generations will grow in all F_1 mice.

A tumour will grow progressively only if the recipient carries certain dominant genes which are represented in the original donor of the tumour. The number of dominant genes required for the growth of tumours in several strains has been worked out (MacDowell and Richter 1932, Furth et al 1944, Gorer 1937, 1942). The histocompatibility genes, with rare exceptions, determine not only the fate of a tumour homograft but also a homograft of normal tissue (Little and Johnson 1922, Kaliss and Robertson 1943).

So far 4 histocompatibility loci have been described in the mouse; H_1 (Snell and Kelton 1953) H_2, H_3 (Snell et al 1955) and a locus associated with the Y chromosome (Eichwald and Silmser 1955).

The most important locus is the H_2 locus which is a

"strong" locus. This means that a graft differing from the host at the H_2 locus will not grow in that host. A tumour differing at other histocompatibility loci may grow progressively in the host.

The H_2 locus was further analysed and was found to be quite complex and contains at least 11 alleles (Allen 1955, Amos et al 1955).

It is worth stressing that there are some exceptions to the laws of tumour transplantation. Thus there are recorded examples where an isologous tumour of recent origin grew progressively in some of the isologous mice (Edwards et al(1942) Stewart et al(1947). Snell (1958) attributed the failure of tumour to grow in the strain of origin to the occurrence of some degree of heterozygosity in the inbred strain as a result of mutations. He could not exclude the possibility of tumour - specific antigens as an alternative explanation in some cases. Exception to the second law of tumour transplantation is the homotransplantation to the so called privileged sites like the eye and the brain. It is known that transplants in those sites will grow even in the presence of major differences in histocompatibility. An important exception to the transplantation laws are the homotransplantable or non-specific tumours which grow in several alien strains.

In summary then the fate of a tumour homograft is

determined by genes which control the iso-antigenic structure of the tumour. The most important of these genes is the H_2 locus. Incompatibility reactions occur when any one or more of the histocompatibility alleles present in the donor is absent in the host.

Environmental Factors Affecting Tumour Transplantation.

Although the genetic differences between a tumour and the host are the most important factors in determining the success or failure of a tumour graft there are other non-genetic factors which are of considerable importance. Thus the site of tumour inoculation as mentioned above is important. The volume of tumour inoculated is also important. Thus Klein et al (1950) working with the Ehrlich ascites tumour found that 400,000 cells were the minimum number required to produce growth in 100% of mice inoculated. Even with isotransplants the dosage of the inoculum is critical. Gross (1943) used a chemically induced sarcoma in the same inbred strain and showed that the incidence of regression was inversely proportional to the inoculated dose. However, Furth and Kahn (1937) were able to transmit a leukaemia with a single cell.

The age of the tumour used as a source of the inoculum may also be important. Thus Zahl and Drasher (1947) found

that with sarcoma 180 the optimal time for obtaining the most viable cells was from 7 day old tumours. These cells yeilded better growths than the cells from a 2 or 12 day old tumour.

In any work involving tissue transplantation the degree of maturation of the host's immune mechanism is important. It is well established that tolerance to a foreign tissue may be acquired by mammals in utero or in early neonatal life. Thus the age of the host may affect the fate of a graft. Gross (1950) showed that a leukaemia of A K origin did not grow in 2-6 months old C_3H mice while growing in 97% of C_3H mice 1-7 days old.

The transplantation of endocrine tissue can be more exacting in its requirements. There is some evidence for example that the transplants of the thyroid and the adrenals are facilitated by deficiency of thyroid and adrenal tissue respectively. (Ingle and Cragg 1939, Ingle and Higgins 1938). This perhaps depends on the increase in the pituitary stimulating hormones in thyroidectomised or adrenalectomised animals. A similar hormonal dependence may explain, at least in part, differences in growth of a given isologous tumour in animals of different sexes. Thus Foulds (1947) found that mammary tumours originating in female mice grow better in females than in males of that strain. The growth of the tumour in the males was

improved by the administration of oestrogens.

The Nature of Tumour Homograft Immunity

Since the beginning of this century it was established that under certain circumstances animals could be rendered resistant to transplantable tumours. It was demonstrated by Paul Ehrlich that, with a few exceptions, animals bearing a transplantable tumour were immune to further re-inoculation. Clowes (1905) showed that mice in which a homologous tumour had regressed were immune against re-inoculation with a homologous tumour. Russell (1912) inoculated mice with a transplantable tumour, allowed the tumour to grow, then excised it. After an interval he re-inoculated the mice with this tumour. He found that in the case of two tumours, this procedure did not immunize the animals but with a third neoplasm there was a regression of the re-inoculated tumour in the majority of animals. Gay (1909) found that rats bearing an early growth of Flexner Jobling sarcoma were immune to re-inoculation of that tumour. Haaland (1910) was able to immunize mice against a transplantable tumour by pre-treatment with living embryo tissue. Many of these early workers tried to immunize animals against transplantable tumours, through pre-treatment with killed tissues. Several were

confronted with the curious finding that animals thus treated were rendered hypersusceptible to tumour re-inoculation (Flexner and Jobling 1907, Haaland 1910, Leitch 1910). Thus arose the idea that dead tissue could not immunize and that living tissue was necessary. This early period in the history of tumour transplantation marks the birth of some fundamental concepts which led to the recognition of the homograft reaction and the second set response. The basis for the important role of the lymphocyte in homograft destruction is to be found in the pioneer observations of Wade (1908) and Da Fano (1912). The science of immunogenetics stems from the fundamental observations of Tyzzer (1909). The phenomenon of tumour enhancement, the mechanism of which is still controversial, was first described by Flexner and Jobling more than half a century ago.

Although authors at that time wrote about natural immunity and "concomitant immunity" to tumour graft very little was known about its nature. However several hypothesis were put forward. Thus Paul Ehrlich (1906) introduced the theory of athrepsia to explain the mechanism of homotransplant rejection. This theory supposed that the unsuccessful graft was unable to obtain from the host essential nutrients. This theory was attacked by Ehrlich's contemporaries (Haaland 1911, and Bashford and Russell (1910).

Leo Loeb (1917) explained the homograft rejection on the theory of individuality differentials. According to him each individual had peculiar characteristics of a chemical nature in his tissues and body fluids. When tissues from an individual were transplanted to a foreign host the individuality characteristic of the graft evoked a host response which was mainly lymphocytic. The host individuality differential was toxic to the graft.

Yet other workers like Russell (1908) were impressed by the fact that a transplantable tumour in resistant host was not furnished with stroma and blood vessels necessary for its nutrition and explained homograft resistance along this line.

Several workers thought that immunity to a grafted tumour was similar to that of bacterial infection. This is shown by early attempts to discover antibodies in grafted animals. Michaelis and Fleischmann (1905) demonstrated a haemolytic "antibody" produced by inoculating mouse liver cells in heterologous hosts; this antibody was bound by liver cells and mouse carcinoma and they concluded that these had common receptors.

Lambert (1914) found that a serum prepared in the guinea pig against rat sarcoma was toxic to tumour cells in culture. A serum prepared in the guinea pig against

rat embryo skin was also toxic to the tumour. There is no doubt however that these early workers were preparing antiserum to species specific antigens. Lambert and Hanes (1911) found that the growth of a rat sarcoma in vitro was not inhibited by serum from tumour bearing rats.

There were several attempts to discover the nature of the immunizing antigen and its distribution in the tissues of the homologous host. Bashford (1913) concluded that all tissues could immunize but embryo skin produced the highest degree of immunity. Itami (1926) concluded that there were some variations in the immunizing power of tissues derived from the different germ layers. He also showed that the immunizing power of the blood was due to the white cells. According to several reports living tissue was necessary for immunization (Haaland 1910). There were several conflicting reports as to the effectiveness of killed or disintegrated tissue as opposed to living tissue in producing immunity. The work of later authors like Barrett (1958) and Kaliss (1957) are significant. Barrett found that homologous blood could induce resistance against isologous tumour and that both the white cells and red cells were effective in inducing immunity. The presence of intact or minimally injured red cell ghosts was necessary for inducing the resistance. He concluded "some degree of architectural integrity was required for the effective action of this antigen". Disintegration of

tumour cells prior to inoculation robbed them of the immunizing effect. Algire et al (1957) showed that the implantation of homologous tissue in cell impervious filters prevented the development of immunity probably because of the fact that a cell fragment of the required size did not reach the reactive centres of the host. However Kaliss (1957) found that the immunizing power of lyophilized tissue depended on the dose. Large doses produced inhibition while small doses abrogated the tumour immunity.

The work with transplantable tumours continued and workers realized that the immunity to homotransplantable tumours was non-specific (Adervont 1932). The later workers utilized inbred strains and emphasized the specificity of the reactions with such strains. Thus Lewis (1940) tested immunity to tumours induced in these different strains of mice and found the immunity was strain specific. However a tumour arising in one strain of mouse could immunize against another tumour arising in that strain. The specificity of the homograft reaction to normal tissues was demonstrated by Medawar (1944).

Cellular and Humoral Factors in the Homograft Reaction.

As a result of advances in immunogenetics and

improvement in serological methods great advances were made in the study of the homograft reaction. It is now known that the reaction depends on iso-antigenic differences between the host and the graft. These in turn are determined by histocompatibility genes and the laws of transplantation immunity hold true for normal as well as tumour tissue. Gibson and Medawar's observation (1943) that a second graft was destroyed more quickly than a first graft was confirmatory evidence that the homograft reaction was immunological. This phenomenon was known as the second set response. Lumsden (1938) and Gorer (1937-1938) had already demonstrated the appearance of haemagglutinins in the sera of animals in which an incompatible graft was regressing.

The association of lymphocytes with homograft destruction was known for a long time (Wade 1908, Da Fano 1912, and Murphy 1926). The work of Murphy is particularly important. He first observed that heterotransplantation of mammalian tumour was successful in the avian embryo in the first few days of its life only. He transplanted tumour with adult chicken tissue to embryos and found that adult spleen and marrow were effective in destroying the tumour. Other adult tissues had no effect on the tumour. Further experiments showed that when the rat lymphatic tissue was depleted by x-rays chicken and mouse tumour grew progressively in the x-ray treated rats.

It was the investigation of Mitchison (1953, 1955) and Mitchison and Dube (1955) that clearly showed the importance of the cellular response of the host in homograft immunity. These workers showed that immunity to a tumour homograft could be transferred by lymph node cells draining a regressing graft. Serum or whole blood failed to transfer such immunity. The sera however contained haemagglutinins directed against the red cells of the original donor of the tumour.

Potter et al (1938) found that mice could be immunized against a transplantable leukaemia. The spleens and livers from such animals could transfer this immunity to unimmunized members of the same strain.

Further studies by Mitchison (1955) have shown that the transfer of the immunity by lymph node cells was specific. By transferring the lymph nodes at intervals after tumour grafting the time of appearance of the immunity was studied. It was found that there was no activity in lymph nodes taken at 3 days, that the activity was maximal at 5 and 10 days but then disappeared at 15 and 20 days.

The work of Weaver et al (1955) with tumour grafts in diffusion chambers also stressed the important role of lymphoid cells in homograft immunity.

What is then the relative significance of humoral antibody and cellular factors in homograft rejection?

Brent, Brown and Medawar (1958) stated "the evidence in favour of the cellular hypothesis is partly direct and partly circumstantial; much of it is evidence against the participation of serum antibodies rather than evidence which demands the acceptance of a cellular hypothesis in its own right". Leukaemic homografts were inhibited by passive transfer of an antiserum prepared in alien host (Gorer 1942). The problem of homograft immunity was however further complicated when it was discovered by Kaliss and Molomut (1952) that under certain circumstances the passive transfer of antiserum produced enhancement of tumour growth.

It is well to remember that the lymphocyte does not play the dominant role in the destruction of all tumour grafts. With some tumours, particularly the ascites, sarcomas and lymphomas, histiocytes appear to be most important (Amos 1961, Gorer 1961, Baker et al 1962). The homograft reaction is quite complex and perhaps there is an interplay of several factors. As Amos (1962) has pointed out "Many different antibodies and several types of immune cells working against a background of variable accessory factors form the variety of responses that comprise "the homograft reaction".

The Antigenic Structure of Tumours: A comparison of specific and heterotransplantable tumours.

A question of fundamental importance in the biology of the neoplastic process is that of the antigenic structure of the neoplastic cell. A tumour cell contains several types of antigens; species specific, strain specific and organ specific antigens. The association of viruses and other infecting agents is an additional complication especially in the case of transplantable tumours. Whether a tumour cell contains antigens which are lacking in the tissues of the host or a "cancer" specific antigen is a debatable point. The literature on this issue is quite extensive and full of contradictions. A great fallacy stems from the failure to recognize transplantable tumours as experimental models quite different in many respects from spontaneous tumours. Woglom (1929) reviewed the literature on tumour immunity and remarked "The tumour problem in brief is a tissue problem, resistance being directed against the tumour graft as a strange tissue, merely, and not connected with any neoplastic qualities which the graft happened to possess".

The most significant evidence for the existence of a tumour specific antigen would be the demonstration of immunity to a spontaneous or induced tumour in the individual of origin of that tumour, or failing that, in the isologous inbred strain. There are several reports

that this has been achieved. Thus MacDowell et al (1934) used small inocula of a transplantable leukaemia and succeeded in immunizing the isologus strain of mice against subsequent challenge with large inocula of the same tumour. Their tumour however had been transplanted over 400 times. A similar but weaker immunity was reported by Marshak and Erf (1941) for a transplantable tumour of strain Strong A origin. The tumour had been transplanted for 40 generations. Gross (1945) immunized C3H mice against a sarcoma (Sa I) that had been chemically induced in that strain. The immunity was apparently specific since mice immunized agains Sa I were quite susceptible to other induced and spontaneous tumours of the same strain. He concluded that the immunity acquired against tumours was directed specifically against the immunizing tumour and was not caused by genetic differences between the cells of the host and those of the animal in which the tumour originated. The view that transplanted tumours are homografts and the the rules governing their fate are dependent on iso-antigenic differences between them and the alien host have already been summarized above. Dulaney and Arnesen (1949) found that an antiserum prepared in rabbits against a mouse leukaemia was much more effective against it than sera prepared against normal mouse tissues. Kingester and Fisher (1954) confirmed the findings of Dulaney and

Arnesen.

Several workers have reported the existence of a specific antigen in malignant tissue by using in vitro serological methods. Thus Kidd (1940) reported the existence of a distinctive substance associated with the Brown Pearce rabbit carcinoma. This substance was capable of fixing complement when extracts of the tumour were mixed with the sera of certain rabbits bearing the tumour. The antigen was absent from extracts of normal rabbit tissues. This was confirmed by Ellerbrook et al (1952). Jacobs and Houghton (1941) found doubtful results with complement fixation tests carried out with sera of animals bearing the Brown Pearce tumour and extracts of that tumour. Hoyle (1940) showed that an alcoholic extract of three mouse neoplasms fixed complement when mixed with the sera of mice bearing those tumours. The antigen from the tumours was lipoidal in nature and though the antigen from different tumours was closely allied, it was absent from normal mouse tissues. Hoyle however was not able to find a distinctive antigen in three spontaneous mouse neoplasms.

Other methods were devised to detect tumour specific antigens. Thus the method of anaphylaxis following desensitization was used. The principle underlying this method is to render guinea pigs sensitive to a nucleo-protein fraction obtained from a tumour; the animals are then completely desensitized by the intravenous

administration of normal tissues. The desensitized animals are then challenged with a nucleoprotein from the tumour tissue. An anaphylactic reaction indicates the presence in the preparation of the tumour tissue of an antigen which is lacking in the normal tissues. The results of the application of the method to different tumours are summarized by Zilber (1958). Several workers demonstrated specific tumour antigens using this method.

Suffice it to mention at this point that Fink et al (1953, 1955) sensitized an inbred strain of mouse to a transplantable tumour that arose in that strain. When the sensitized mice were challenged with tumour tissue they responded by a state of shock.

A major criticism of most of the fore-mentioned work is the fact that the tumours used had been transplanted for several generations. This may lead to antigenic changes in the tumour cells as was discussed above. The second criticism is the fact that inbred strains may develop some degree of heterozygosity (Snell 1958). Thus as a result of a change in the tumour, the inbred strain of both a previously genetically compatible tumour may become different from its host. This may wrongly be thought a cancer antigen.

Gorer and Amos (1956) produced passive immunity against the isologus C57 BL leukosis EL4 by the use of

iso-immune sera prepared in foreign strains. The possibility that the antibody was directed against iso-antigens was considered. It was found that absorption of the antisera with C57 BL liver did not remove the antitumour effect. Absorption with EL4 tissue however removed most of the activity. The antigen present in EL4 and absent in normal C57 BL tissue was termed x antigen. Amos and Day (1957) demonstrated x antigen in three different mouse leukoses. They could find no cross reactions between the different x antigens. The tumours used were however old transplantable tumours and the possibility that the x antigen was a product of mutation in the tumour could not be excluded. Gorer et al (1962) made further studies of x antigens in chemically induced and spontaneous leukaemias of recent origin. The x antigen was again demonstrated. They concluded that the x antigen was either a distorted product of a normal cell found specifically in leukaemic tissue or it might have represented a quantitative increase in normal cell antigenic component. Davies (1963) showed that purified mouse histocompatibility antigens prepared from leukaemic cells, carried the specificity of the x antigen. The active immunity produced in the mouse against the isologous leukaemia was very weak and "was insufficient to establish active immunity by x specificity". Klein et al (1960) produced evidence for the existence of antigenic differences between chemically

induced tumours and their primary hosts. The induced tumour was operatively removed from the primary host and maintained in isologous mice. The primary host was treated with its own irradiated tumour cells, followed by a challenge with viable cells of the same tumour which had been maintained in isologous hosts. The resistance was however relative rather than absolute and broke down when the dose of viable cells was progressively increased. It is reasonable to conclude that with the chemically induced tumours and certain spontaneous leukaemias there is an antigen which is absent from normal tissues. With other spontaneous tumours the existence of tumour specific antigens is doubtful.

The antigenic structure of homotransplantable tumours

It is clear that transplantable tumours are essentially homografts. Compatibility between the graft and the host occurs when both have the same gene determined iso-antigens. This statement, however, needs qualification in the case of the non-specific or homotransplantable tumours which required the ability to grow across some degree of genetic non-identity i.e. in alien homologous hosts.

Several hypotheses have been suggested to explain the

lack of strain specificity of homotransplantable tumours. One possible factor was that these tumours had lost specific iso-antigens. It was thought that the simplified antigenic composition of the tumour cells was produced by a chromosomal imbalance. (Hauschka and Levan 1953). The strains specific tumours were found to be composed of a near diploid chromosomal complement; the homotransplantable tumours had modal numbers near the tetraploid region. Although strain specific neoplasma were composed of a near diploid number of chromosomes, it was shown that most contained about 5% tetraploid cells. (Hauschka et al 1956). Occasionally a spontaneous change occurred in the tumour and the tetraploid cells formed the major part of the tumour. Such tumours lost their strain specificity. The change could be induced by growing a tumour in an alien host. Under these circumstances the heteroploid cells were at an advantage because of the loss of specific iso-antigens. They were therefore immunologically selected. Hauschka et al (1956) studied the $6C_3$ HED ascites lymphosarcoma (of C_3H origin) and three sublines of that tumour. They showed that the parent $6C_3$ HED tumour was strain specific, was highly virulent and its chromosome number was diploid. A subline was adapted in DBA/2 mice and this was shown to be less virulent and its chromosomes were near the tetraploid number.

It is well known that the H_2 locus in the mouse determines the iso-antigens of the tissues of the mouse as well as the antigens of the red cells. (Gorer 1938, 1942). Thus when tissues of a mouse A are injected in a genetically different mouse B haemagglutinating antibodies are produced which are absorbed specifically by A tissues (including tumours of A) and which will agglutinate A red blood cells. Amos (1956) showed that diploid tumours of a given strain of mouse were more effective in absorbing haemagglutinating sera against that strain than tetraploid tumours. This is in support of Hauschka's idea of loss of antigens in tetraploid cells. The nature of the change in tetraploid cells is unknown. It is to be remembered that homograft immunity is largely influenced by H_2 antigens present on the cell surface. Hauschka et al (1956) accordingly put forward the possibility that in polyploid cells there was a crowding out of the strong H_2 antigens on the cell surface by other weaker antigens. This "crowding out" was due to the chromosomal imbalance in the polyploid cells. Other possible explanations were mentioned but could not be excluded.

Feldman and Sachs (1957, 1958) have challenged the immunoselection theory advanced by Hauschka to explain the homotransplantability of tumours. They used three tumours, $6C_3$ HED, MC_1M and MC_1A which are tumours of strain C_3H mice but which showed different degrees of

homotransplantability. Foreign strains challenged with these tumours produced antibodies that agglutinated C_3H erythrocytes. They proved that the intensity of haemagglutinin production by a foreign host challenged with a tumour was directly proportional to the intensity of the immunity as measured by the degree of host resistance to tumour homograft. Using these criteria they found that the homotransplantable tumours produced a stronger degree of immune response than the strain specific tumours. They thought that this was evidence against the theory that homotransplantable tumours were of low antigenicity. On the contrary they produced data which proved that homotransplantable tumours elicited a stronger homograft response than strain specific tumours; but they were able to resist the reaction they elicited. They attributed this to the fact that non-specific tumours emitted a greater quantity of extra-cellular antigen which was able to neutralise "antibody" response before it reached the tumours cells. Feldman and Sachs (1958) compared the immunogenetics, transplantation immunity and chromosomal structure of the strain specific $6C_3$ MED tumour and two sublines which were homotransplantable and adapted in other strains. They showed, by the differential absorption of red cell agglutinins that the two sublines had lost some of their agglutinogens. Though some antigenic components of the H_2 antigens were not represented in the adapted

tumour cells, the total haemagglutinin response was not reduced. With some further experiments they showed that the homograft response elicited by the sublines was stronger than that elicited by the original tumour.

Barrett and Deringer (1950, 1952, 1953), described a form of decreased specificity in a tumour which was different from that described by Hauschka. A mammary carcinoma of C_3H origin was transplanted into F_1 hybrid, (C_3H x resistant strain). When the tumour was transferred from the F_1 hybrid to the backcross between the F_1 hybrid and the resistant parent there was an increase in takes. The change could always be produced when the tumour was passaged in F_1 hybrids in a total of more than 20 attempts. The increased transplantability of the tumour did not extend to foreign genotypes and there was no change in the invasiveness of the tumour. A change occurring in the tumour as a result of passage through the F_1 hybrid animal is known as the Barrett-Deringer phenomenon.

Klein and Klein (1956) investigated the nature of the Barrett-Deringer adaptation. They attributed the phenomenon to cellular adaptation induced in the tumour in the environment of the F_1 hybrid rather than to a selection of pre-existing cell variants with decreased antigenicity. They based their argument on the fact that the change occurred in the completely susceptible F_1 host, was regularly produced and after only a short residence

there.

There are several other reported and apparently different methods of decreasing the specificity of a tumour. Thus Koprowski (1955) induced an adaptation of a strain C_3H lymphoma to adult Swiss mice by inoculating the tumour first in Swiss embryos. An important phenomenon which should be mentioned here is that of immunological enhancement of tumour whereby a strain specific tumour is made capable of growth in a genetically different host (Kaliss 1958). This phenomenon will be discussed in a later chapter.

It is clear that the loss of strain specificity is a complex problem which can be produced in a variety of ways. It is possible that different factors operate in different tumour host combinations.

Pitfalls of transplantable Tumours.

Infection.

Transplantable tumours form a rich soil for the growth of infecting agents. These may be bacterial, viral or fungal in nature. Bacterial infection in mouse neoplasms was studied by Eisen (1936) and Schrek (1936). The latter author found that infected transplantable tumours tended to have a longer incubation period, and the incidence of regressions was higher than in uninfected

tumours. Koprowska and Koprowski (1953) had demonstrated that transplantable tumours were good media for the growth of viruses. They studied the effect of 12 viruses on the Ehrlich ascites carcinoma. Some viruses were found to produce a marked inhibitory effect on the growth of the tumour. Other viruses failed to multiply in the tumour and had no effect on its growth. A third group of viruses, however, multiplied in the tumour cells but did not change its growth characteristics. Pearce and Rivers (1927) investigated the effect of a filtrable virus in the Brown Pearce tumour, on the host's response to that tumour. It was found that a more severe disease developed in the rabbits which were inoculated with a virus-bearing tumour, than in those that were inoculated with a virus-free tumour. They suggested that the increased malignancy in the former group was due to altered host response to the tumour as a result of the viral infection. De Bruyn (1949) described a virus contaminating a transplantable lymphosarcoma. This virus did not alter the growth of the tumour. Taylor and MacDowell (1949) also described a self reproducing agent, which they thought was a virus in a much transplanted mouse leukaemia. As Klein (1959) has emphasized, viral contamination may "complicate the results and interpretation of many studies on the growth, biochemistry and ultra structure of neoplastic cells. They may also lead to abnormal sensitivities to drugs or

other forms of treatment".

Changes in the character of the tumour.

There are many well documented cases where a physiological or even a morphological change in a transplantable tumour had occurred. It would therefore be unsafe to assume a uniformity in the biological behaviour of a much transplanted tumour. The changes in the antigenic structure have already been mentioned. It is well to emphasize again the fact that transplantable tumours are made up of a heterogenetic population of cells and under certain circumstances certain types of cells may be selected. For example Mottram (1936) found that different parts of a tar induced mouse papilloma produced strains of different rates of growth. Peacock (1964) produced three homotransplantable lines of tumours from different parts of the same spontaneous mouse tumour. The three lines showed completely different morphologies.

It is in the light of the facts mentioned in this introduction that I intend to interpret the experimental results obtained from studies with the Landschütz ascites tumour. Since this tumour arose in outbred mice and since it was transplanted as sublines in different laboratories, its antigenic composition is unknown. It falls in the category of homotransplantable tumours and is therefore subject to the pitfalls mentioned earlier. Its,

main advantage is that it is convenient to work with and the dosage is easily controlled. The study of tumour enhancement which will be mentioned in a later chapter, was quantitatively determined because tumour growth could be accurately assessed by cell counts and haematocrit readings. This is possible only with ascites tumours.

I regard the Landschütz tumour as a homograft differing from homografts of normal tissue in its higher degree of growth potential. It has been used in the present work as a convenient experimental model to study the physiology of graft - host relationship and the biology of cell death using immunological methods.

CHAPTER I

Changes in the Lymphoreticular tissues of mice bearing the Landschütz tumour

The Landschütz ascites tumour is a transplantable mouse neoplasm that grows in several strains. It arose spontaneously in a strain of Rockefeller white mice and was subsequently propagated by Landschütz in outbred mice and described as a reticulum cell sarcoma (see Tjio and Levan 1954). The latter authors made a careful and detailed study of the chromosomes of this tumour. They noted its resemblance to the Ehrlich carcinoma and thought that the two were related. The Landschütz tumour, however, is not as haemorrhagic as the Ehrlich carcinoma and is widely used for experimental chemotherapy. When the tumour is inoculated in the peritoneal cavity it produces a progressive accumulation of ascitic fluid and in about 7-12 days, depending on the dose inoculated, there is a visible abdominal distension. The ascitic fluid is rich in tumour cells. Its protein content is high and will clot spontaneously outside the body. The mice bearing tumour in their peritoneal cavity invariably die with ascites. The survival time depends partly on the strain of mouse. When the tumour is inoculated in the subcutaneous tissues it grows in the solid form as sheets of highly anaplastic cells. The morphology of the tumour will be described in the chapter dealing with the pathology of the Landschütz tumour.

Despite its progressive growth in several mouse strains, the Landschütz tumour is antigenically different and elicits a host reaction of varying strength depending on the strain in which it is transplanted. It will be seen in subsequent chapters of this work that DBA_2 strain of mice are more resistant to the tumour than A/Jax strain. The latter are more resistant than outbred mice. In this chapter evidence is presented for a host reaction during the progressive growth of the tumour in the inbred strain A mouse. The evidence is based on a study of the morphology and function of the lymphoreticular tissue of mice bearing the tumour. Changes in the weights and histology of the lymphoid organs of the hosts were followed as the tumour developed. These were correlated with the phagocytic function of the reticulo-endothelial system as measured by the rate of clearance of intravenously injected colloidal carbon.

Material and Methods

Mice; A 100 A/Jax (Porton) and 200 A/Cum (Cumberland Farms) sublines of the inbred strain A mouse weighing 16-18 g. were used.

Propagation of tumour; the Landschütz ascites tumour was maintained in an outbred but closed colony of white

mice for four years. The tumour was first introduced into A/Jax mice in December 1963 from the outbred mice. Since then it has been maintained within that strain by weekly transfer of fresh ascites fluid in a volume of 0.1-0.5 ml. The A/Cum mice were always inoculated from tumour maintained in A/Jax mice.

Tumour for inoculation into experimental mice was collected from ascitic stock mice under sterile conditions and added to 5 ml. of heparinized tissue culture medium 199 (Glaxo Laboratories). It was then filtered through 3 layers of sterile gauze and the number of cells in the filtrate counted with a haemocytometer. Appropriate dilutions were made with medium 199 and the required dose was administered intraperitoneally in a volume of 0.1 or 0.2 ml. or in some experiments subcutaneously.

Changes in the organs of mice bearing tumour; 60 A/Jax mice were inoculated intraperitoneally with a dose of 10^5 ascites cells. The animals were weighed every 2 days and inspected daily for ascites. Ascites was indicated by an increase in weight of 3 g. over a period of 2 days or by visible abdominal distension.

Groups of mice were sacrificed at 5, 10, 20 and 25 days after tumour inoculation. The animals were first bled from the neck vessels and the blood was used for haematological investigation as described below. A

detailed autopsy was then performed. The spleens, livers, thymuses and adrenals were weighed and fixed in neutral formol saline for histological examination. Splenic dabs were made, fixed in formol alcohol and stained by the Unna Pappenheim method. Some animals bearing the tumour were allowed to die naturally and the organs were inspected and weighed at death. The carcass weight was compared with the initial weight.

The effect of the site of the tumour growth on the host response; 7 A/Jax mice were inoculated subcutaneously in the right flank with 10^7 ascites cells in 0.1 ml. volume. The fluid was cultured aerobically and anaerobically on blood agar plates. Six days later the animals were sacrificed; the regional lymph nodes, spleens and local tumour were removed and fixed in neutral formol saline for histological examination.

Estimation of the phagocytic function of the reticulo-endothelial system in mice bearing the Landschütz tumour; following the inoculation of 10^5 ascites cells I-P. in 25 A/Cum mice the phagocytic function of the reticulo-endothelial system was determined by the carbon clearance method (Biozzi et al 1954). Colloidal carbon (Gunther, Wagner, Hanover) was injected intravenously in a dose of 8 mg/100 g. of body weight. Samples of 0.025 ml. of blood were removed from the retro-orbital venous plexus at 3

minute intervals over a period of 15 minutes. The blood was added to 2 ml. of distilled water. The samples were read in an E.E.L. colorimeter using a red filter; the optical density is directly proportional to the concentration of the carbon. The log of the optical density (log C) was plotted against time on ordinary graph paper. The slope of the straight line thus produced is the phagocytic co-efficient K and is given by the formula

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

C = concentration of carbon in the blood and T is the time in minutes.

The intravenously injected carbon is removed mainly by the macrophages of the liver and the spleen. To avoid variation due to the size of the animal the corrected phagocytic co-efficient α was calculated from the formula

$$\alpha = \sqrt[3]{K \times \frac{W}{WLS}}$$ where

W = body weight and WLS is the combined weight of the liver and the spleen.

Determination of the correct value of K in ascitic mice; Ascitic mice were injected with an overdose of carbon calculated for the combined body weight and ascites. When the mouse was sacrificed the peritoneal cavity was emptied of fluid and the carcass weight was determined; the

corrected value of K was calculated from the formula

$$K = \frac{K_1 \times (W + A)}{W}$$ where

K_1 = phagocytic co-efficient as calculated for the ascitic mouse. W = carcass weight.

Phagocytic function in A/Cum mice bearing tumour but treated with immune isologous spleen cells; the phagocytic function was determined in the following groups of mice.

(1) 15 mice bearing 10^5 ascites cells but treated with 500 x 10^6 isologous immune spleen cells 48 hours later. The spleen cells were derived from mice that had been inoculated with 10^5 tumour cells I-P. 10 days before. The method of preparation of the spleen cells will be described in the next chapter.

(2) 13 mice inoculated with 500 x 10^6 immune cells only. This group will be called treated controls.

(3) The phagocytic function in the above two groups was compared with that of normal A/Cum mice. This group will be referred to as normal controls.

The spleens, liver, thymuses and adrenals from animals used in the carbon clearance experiments were weighed and examined microscopically.

Haematological changes in mice bearing tumour; A/Jax

mice were inoculated with 10^5 ascites cells I-P., bled and sacrificed at 5, 10, 15, 20 and 25 days as described above. The blood was added to 0.025 ml. of heparin and the haemoglobin, total red cell count, packed cell volume and total white blood count and differential were determined. Similar estimations were done on 3-5 normal control animals included with each group of test mice.

The haemoglobin was determined by the oxyhaemoglobin method using an E.E.L. colorimeter with a green filter. A standard haemoglobin curve was constructed by plotting the optical densities of different dilutions of pooled normal blood from 10 normal mice. All haemoglobin estimations were read on this curve and the values of test and control animals for each day were compared.

The P.C.V. was determined in micro-haematocrit tubes centrifuged at 4500 R.P.M. for 45 minutes.

Coombs' antiglobulin test; Antiglobulin serum. Rabbit antimouse serum (Wellcome Labs.) was absorbed with an equal volume of packed mouse red cells which had been washed three times in physiological saline. Absorption was carried out at 37°C for 60 minutes. Normal A/Jax mouse red cells or cells from animals bearing tumour were washed three times in normal saline. The cells were packed by centrifuging at 3000 R.P.M. for 10 minutes and made to 10% suspension in normal saline and were then ready

for use in the antiglobulin test.

The antiglobulin test was carried out in agglutination tubes by adding 0.2 ml. of antiglobulin serum to an equal volume of 10% washed red cells from mice bearing tumour.

Control tubes contained antimouse serum and normal A/Jax red cells. The tubes were incubated at 37°C and agglutination was read by pattern and microscopically.

Marrow smears were made from the right femur and stained with Leishman stain.

Bacteriological examination; 4 A/Jax mice were inoculated with 10^5 ascites cells I-P. Ten days later the animals were sacrificed and under sterile conditions the peritoneal cavity was exposed. The ascitic fluid from each mouse was plated directly on two nutrient agar and two blood agar plates. One agar and one blood agar plate was incubated aerobically at 37°C; the other two were incubated anaerobically at 37°C.

Aerobic and anaerobic cultures were similarly prepared from the cut surface of the spleens of the above mice. The spleens of 4 normal control mice were also cultured.

Results

Changes in organ weights of mice bearing intraperitoneal tumour; The spleen showed an increase in weight in the early stages of tumour growth. Fig. 1 shows that in A/Jax mice the spleen reached a maximum size 10 days after tumour inoculation. It then became smaller until at 25 days it was nearly normal. A similar change in spleen weight was observed with A/Cum mice (Table I). Spleens from mice bearing a 10 day growth of tumour contained 260×10^6 cells per spleen. Control spleens yielded 160×10^6 cells per spleen.

In a combined series of 46 A/Jax and A/Cum mice the liver weights were not altered significantly (Table II).

The thymus decreased in weight as the tumour grew and in the late stages showed complete atrophy (Fig. 2 and Table I). This was found in both A/Cum and A/Jax mice but especially in the latter. The greatest loss in thymic weight occurred between 15 and 20 days after inoculation of tumour. The variation of A/Cum thymus weights was probably related to age differences.

There was no significant change in adrenal weight of mice bearing the tumour (Table III).

Morphological changes in the lymphoreticular tissues of mice bearing the tumour; the spleen showed no obvious

changes five days after the intraperitoneal inoculation of tumour. At 10 days the Malpighian bodies were larger than normal. Fig. 3 shows that the normal Malpighian body contained small cells with a rounded darkly staining nucleus. The cytoplasm was scanty. Fig. 4 shows that the Malpighian body of a spleen from mice bearing a 10 day growth of the tumour was made up of larger cells. The nucleus was larger, irregular, vesicular and contained a single prominent nucleolus. The chromatin was dispersed in irregular clumps or was condensed in a thin rim on the nuclear membrane. Mitotic activity was increased. The cytoplasm was moderate in amount and agranular. The red pulp at 10 days contained foci of proliferating cells with an eccentric or central vesicular nucleus and plentiful pyronophilic cytoplasm.

Small spherioles of pyronophilic material 1-3 μ in diameter were seen in the sinusoids of the spleen. These spherioles were homogeneous and opaque. They were entirely separate from the erythrocytes and nucleated cells. Mature plasma cells were not seen. 15 days after tumour inoculation the Malpighian bodies were still active. The red pulp then contained an increased number of granular leucocytes.

In the late stages of tumour growth (20 and 25 days) the splenic activity was markedly decreased. The

Malpighian bodies were small and the red pulp showed a marked degree of venous congestion. Many of the nucleated cells in the sinusoids were granular leucocytes.

Five days after tumour inoculation the livers of most animals were normal although in some cases the Küpffer cells were stimulated. At 10 days the Küpffer cells were larger and the cytoplasm more abundant and branched than usual (Fig. 5). The normal pattern is shown in Fig. 6. The Kupffer cell changes were best seen after the intravenous injection of colloidal carbon. At this time immature monomuclear cells appeared. They were seen as small clusters of 8-10 cells or sometimes as individual cells in the sinusoids. Larger foci were situated in the portal tracts in intimate relation to a bile duct (Fig. 7) or vein. When situated in the portal tract these foci were well developed and measured up to 100 U in diameter. Very rarely these foci developed near a central vein. These monomuclear cells showed a variable morphology and measured between 7-10 μ. The nuclei were round or slightly indented. Sometimes they were vesicular with a single prominent nucleolus; in other cells the chromatin was dispersed in minute masses. The cytoplasm was scanty. When the foci were situated in portal tracts, fibroblasts, macrophages and occasional lymphocytes were also seen. In the case of monomuclear cell foci in the vicinity of a vein, the adjacent vascular endothelium was sometimes

swollen (Fig. 8).

Mature plasma cells in the liver were seen in only one mouse which had multiple tumour deposits in that organ. Scattered in the sinusoids an occasional megakaryocytic type of cell was seen. These cells measure about 20 μ in diameter, have a lobulated vesicular nucleus and abundant agranular cytoplasm. In some animals there was an increase in mitotic activity (Fig. 9), in the hepatic parenchyma cells at 10 days. At 15 days the Küpffer cells were still active although the foci of mononuclear cells were less prominant. At 20 and 25 days after tumour inoculation the Küpffer cells appeared normal. Foci of mononuclear cells were no longer seen. The sinusoids and blood vessels now contained polymorphonuclear leucocytes.

Changes in the lymphoid tissue in response to subcutaneous tumour growth; the tumour grew as a solid mass showing extensive ischaemic necrosis and a peripheral chronic inflammatory reaction (Fig. 10). Moderate numbers of plasma cells were seen as well as lymphocytes, macrophages and mast cells (Fig. 11). The regional lymph nodes were enlarged and reactive. The marginal sinus and sinusoids in the depth of the gland were lined by cords of histiocytic cells one to three layers in thickness (Fig. 12). The follicles were large, showed prominent germinal

centres and increased mitotic activity (Fig. 13). At this time plasma cells were not seen in the lymph nodes. The spleens and liversfrom mice bearing subcutaneous tumour were normal.

Phagocytic function in mice bearing tumour.

Fig. 14 shows that when the tumour was inoculated in the peritoneal cavity of A/Cum mice there was a rise in K in the early stages of tumour development and that in the late stages of tumour growth K returned to normal levels. The corrected phagocytic index α was elevated 10 days after tumour inoculation. (Table I).

When the tumour growth was suppressed by passive immunisation there was no variation in phagocytic function (Fig. 15). Treated control mice showed no change in K or α .

Haematological changes in animals bearing tumour.

Table IV shows that there was a slight terminal fall in haemoglobin concentration. There was a polymorphonuclear leukocytosis which was maximal 15 days after tumour inoculation but was still evident at 25 days. The other

data were normal apart from an elevation in the number of monocytes at 15 and 20 days. The bone marrow showed an increase in the white cell series.

Bacteriological examination; no organisms were grown from the aerobic or anaerobic cultures of the ascitic fluid. Cultures from the spleens were also negative.

Discussion

It is well established that homotransplantable tumours excite a host response (Klein 1959, Snell 1958). This response is probably a homograft type of reaction directed against the transplantation antigens of the original donor of the tumour. These may have been modified by either loss or acquisition of antigens. There is also the likelihood of viral or bacterial contamination of the tumour although it has not been possible to isolate aerobic or anaerobic bacteria from the Landschütz tumour. The possibility of an associated virus cannot be excluded. The mice bearing the tumour developed a leukocytosis around the tenth day and the possibility of an infection still remains despite repeated negative bacterial cultures. Polymorphonuclear leukocytosis has also been noted with other transplantable tumours (Lewis 1937, Blumenthal 1941). It will be seen in the next chapter that spleen cells removed from mice bearing the Landschütz tumour for ten

days are immunologically active against the tumour which could be inhibited by the passive transfer of such cells into isologous hosts. It is therefore reasonable to assume that the host response is wholly or in part specifically directed against the tumour and probably depends on the existence of iso-antigenic differences between the two. Whatever the host response may be the above results show that the Landschütz tumour excites a partial resistance in strain A mice. This reaction is soon overcome by this rapidly proliferating tumour.

When the tumour was implanted in the dermis the host reaction was confined to the subcutaneous tissues, and regional lymph nodes. This reaction was manifest in the early stages of tumour growth and advanced tumours elicit little or no reaction.

A tumour in the peritoneal cavity caused a more generalised host response. This was probably due to a wider dissemination of the antigen. The histological changes in the spleen of mice bearing the tumour in the peritoneal cavity for 10 days are consistent with antigenic stimulation and antibody synthesis. Marshall and White (1950) showed the appearance of germinal centres in the spleens of rabbits injected intravenously with antigen. Miotic activity was obvious in such centres. Wissler et al (1960) reported on the appearance of germinal centres in the spleens of rats injected with Salmonella typhi. The

appearance of the pyronophilic cells in the red pulp of the spleens of mice bearing tumour was evidence that these cells were actively engaged in protein synthesis.

Pyronophilic cells were noted by Scothorne and McGregor (1955) in the lymph nodes draining skin homografts in rabbits. Scothorne (1957) believed that the pyronophilic cells originated from reticulum cells.

Fagraeus (1948) described the development of pyronophilic cells in the spleens of rabbits injected with foreign antigen. These cells eventually matured to plasma cells. It is worthy of note that mature plasma cells were not seen in the spleen although they appeared early around a subcutaneous tumour.

The nature and significance of the small pyronophilic spherioles in the splenic simusoids is uncertain. Their morphology and staining properties are similar to that of the cytoplasm of the surrounding pyronophilic cells. It is therefore probable that they are in fact masses of cytoplasm derived from such cells although the possibility that they are an artifact produced during fixation and processing cannot be entirely excluded. If we accept that they are derived from antibody forming cells then the spherioles may represent a form of antibody transport.

The change in the liver in the early stages of tumour growth indicates antigenic stimulation. This is seen in

the marked degree of hypertrophy of the Kupffer cells and the presence of immature mononuclear cells. The exact origin of these cells cannot be determined. They probably arise from activated endothelial cells of the hepatic veins and sinusoids. These cells did not phagocytose colloidal carbon given intravenously and their morphology is quite distinct from tumour cells.

In the later stages of tumour growth there were no signs of stimulation in the liver or the spleen. The collapse of the lymphoreticular response in mice bearing advanced tumour might have been caused by the terminal poor nutritional state of the mice. A contributory factor could be antigenic overload from the increasing volume of tumour with consequent immunological paralysis.

Morphological changes in the reticulo-endothelial system of animals bearing transplantable tumours has attracted the attention of several workers. Wade (1908) was probably the first to attribute tumour destruction to lymphocytes. He noted that a regressing transplantable sarcoma of the dog was surrounded by lymphocytes. The important role of the lymphocyte in the destruction of tumour homografts in mice and rats was emphasized by Da Fano (1912). Loeb (1919) showed that normal tissue homografts were destroyed by lymphocytes. As a result of extensive study of the problem of homograft destruction Murphy (1926) implicated the lymphocyte as playing the major role.

Borghi (1928) and Calo (1932) noted proliferation of the Kupffer cells of the liver and changes in the spleens of animals bearing tumours. An increase in spleen weight of rabbits bearing the Brown-Pearce carcinoma was reported by Brown and Pearce (1932). Twort and Lasnitski (1932) reported a similar finding in rats bearing the Jensens rat sarcoma. More recent work confirmed and extended these earlier observations. Parsons et al (1947) described changes in the lymphoid organs of rodents bearing tumours. Albert, Johnson and Pinkus (1954) investigated the effect of both transplanted and spontaneous mammary gland carcinomas on lymph nodes and the spleen. They found that homologous tumour transplants increased the weight of these organs and their utilisation of radio-active phosphorus. Black and Speer (1955) noted changes in lymph nodes of mice bearing spontaneous tumours. Baruah (1960) showed that a transplantable carcinoma and a carcinogen induced sarcoma of the rat were accompanied by splenomegaly and increase in the size of the regional lymph nodes. Old et al (1960) demonstrated splenomegaly in mice bearing sarcoma 180 and Ehrlich ascites tumour. They also noted hepatomegaly and splenomegaly in spontaneous mammary carcinoma.

The thymic atrophy in mice bearing tumour is difficult to explain. The atrophied thymus showed no pathological change apart from loss of thymocytes. Thymic atrophy was noted by Larinow (1932) in rodents bearing tumours.

Hilf et al (1960) showed that the thymic atrophy occurring in Swiss mice bearing sarcoma 180 was associated with progressive tumour growth and hypertrophy of the adrenal. Mice bearing the Landschütz tumour showed no increase in adrenal weight. Savard and Homburger (1949) showed that thymic atrophy in mice bearing sarcoma 180 was not prevented by hypophysectomy and suggested that thymic atrophy was not mediated through the pituitary adrenal axis. The thymic involution observed with the Landschütz tumour does not seem to be related to a poor nutritional state of the tumour bearing animals. The thymus showed marked atrophy at a time when the tumour was small, the animals showed no weight loss and appeared well. In outbred mice where the degree of resistance was lower, the same degree of thymic atrophy did not occur. Accordingly this thymic depletion may have an immunological basis caused by a progressive demand on the lymphoreticular tissue by increasing amounts of antigens liberated from the neoplasm. (It is noteworthy here that Begg (1951) showed that while force feeding rats bearing tumour prevented weight loss, it did not prevent the systemic effects of the tumour). The morphological changes in the Küpffer cell system showed an initial stimulation and subsequent return to normal appearances. This was confirmed by measurement of the R.E.S. phagocytic function. It was shown that stimulation of phagocytic function occurred and reached a maximum ten

days after tumour inoculation. In the late stages of tumour growth the phagocytic function was normal. This is in agreement with the findings of Old et al (1960) for the Ehrlich ascites tumour in mice. Halpern et al (1960) found no change in phagocytic function with Ehrlich tumour growing in the peritoneal sac. Stern and Duwelius (1958) showed increased phagocytic stimulation in rats bearing subcutaneous Lewis lymphoma. It seems likely that the extent of phagocytic response is largely influenced by the intensity of host versus graft reaction especially since Howard (1963) has demonstrated activation of the phagocytic system during a graft versus host reaction.

The degree of host response to this tumour depends on the duration of growth and histological examination in the late stages adequately fails to reveal a previous state of host reaction. Bacterial or viral contamination of experimental murine tumours remains important in the interpretation of a lymphoreticular reaction. Because the activated lymphoreticular cells used in these experiments were able to inhibit the ascites tumour, it seems reasonable to conclude that the histological and functional response described here is directly related to the antigenicity of the tumour.

Phagocytic indices K and α with organ weights from mice given 10^5 ascites cells (A/Cum. mice).

No. of mice	Time after tumour (in days)	Average K	Average α	Average spleen wt. (in gms.)	Average liver wt. (in gms.)	Average thymus wt. (in gms.)	Wt. of thymus of control of same age. g.
6	5	035	4.7	0.13	1.17	0.05	0.05
10	10	049	5.0	0.18	1.20	0.028	0.029
6	15	034	4.3	0.12	1.18	0.012	0.045
4	21	021	3.3	0.10	1.13	0.003	0.028
15 Normal Controls		032 ± .011	4.5 ± .28	0.10 ± 0.01	1.07 ± 0.08	.39 ± .008	

± = S.D.

TABLE II

Changes in liver weight in Strain A mice inoculated with 10^5 ascites cell I-P.

Strain A/Cum.			Strain A/Jax		
No. of Mice	Time after 10^5 (days)	Weight of liver g.	No. of mice	Tissue after 10^5 (days)	Weight of liver g.
6	5	1.17	5	5	0.85
10	10	1.20	6	10	1.18
6	15	1.18	4	15	1.14
4	21	1.13	5	20	0.99
Average liver weight for 15 normal controls = 1.07 ± 0.08 g.			Average liver weight for 14 normal controls = 0.90 ± 0.36		

± = S.D.

TABLE III

Adrenal weight in A/Jax Mice Bearing Tumour

No. of Mice	Time after 10^5 ascites cells (days)	Av. Adrenal weight mg.	Average adrenal weight 10 normal controls.
13	10	5.5±1.3	3.9 ± 0.9
5	20	4.4	

± = S.D.

Table IV

Changes in the peripheral blood of mice bearing tumour I-P.

No. of mice	Days after Tumour	Hb %	P.C.V. %	R.B.C. millions per cub. mm.	Total white cells	Neutrophils	Lymphocytes	Monocytes
5	5	—	41	5.7	3,700	761	2585	206
4	10	89	43	7.2	6,600	2567	2604	257
4	15	80	43	6.2	12,600	9965	2190	545
5	20	80	45	8.9	10,600	7057	2630	767
4	25	72	41	8.0	11,050	7600	3270	173
Average value for 10 normal mice		92	45	7.6	4,350	740	2709	260

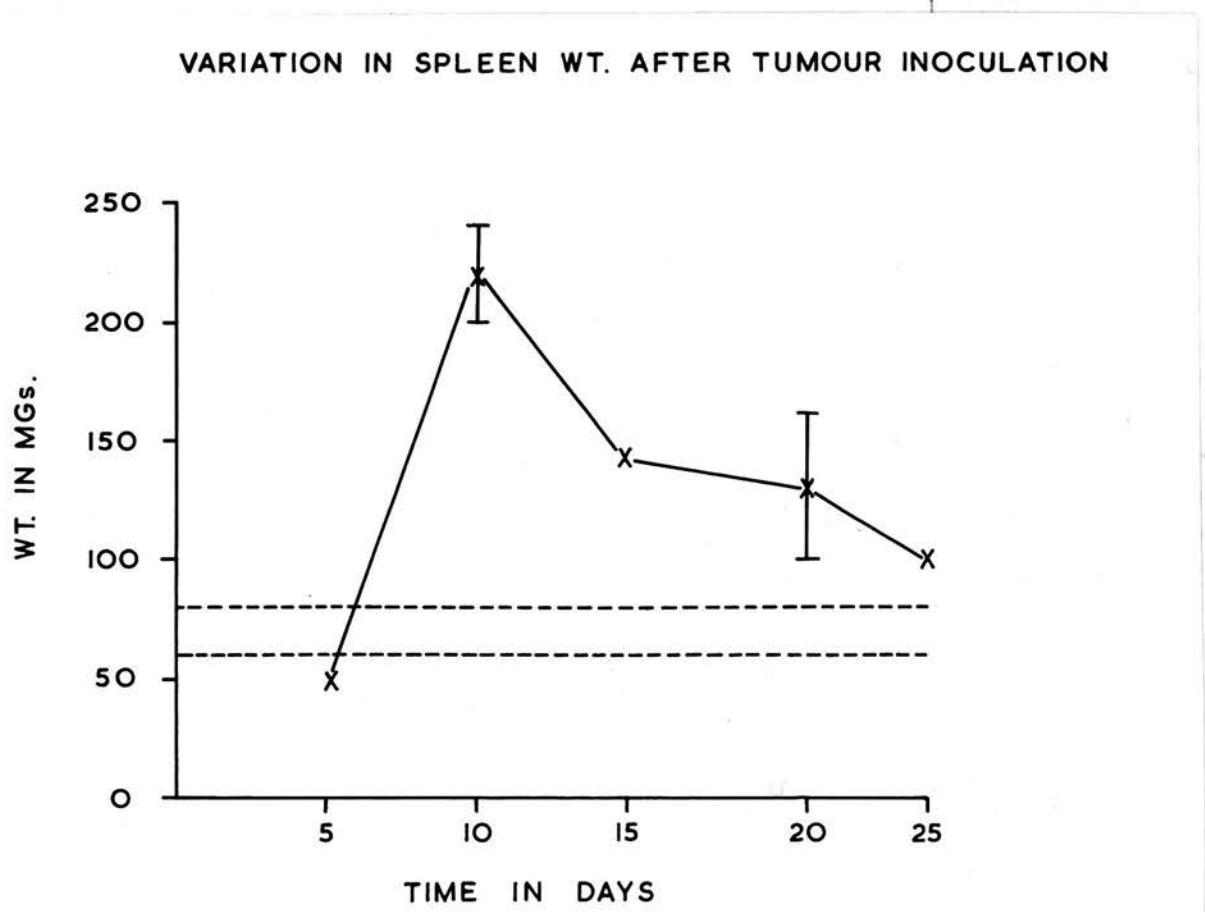

Fig. 1: Average weight of the spleen in groups of 8 mice after intraperitoneal injection of 10^5 cells. The dotted lines show the limits of standard deviation of 19 controls.

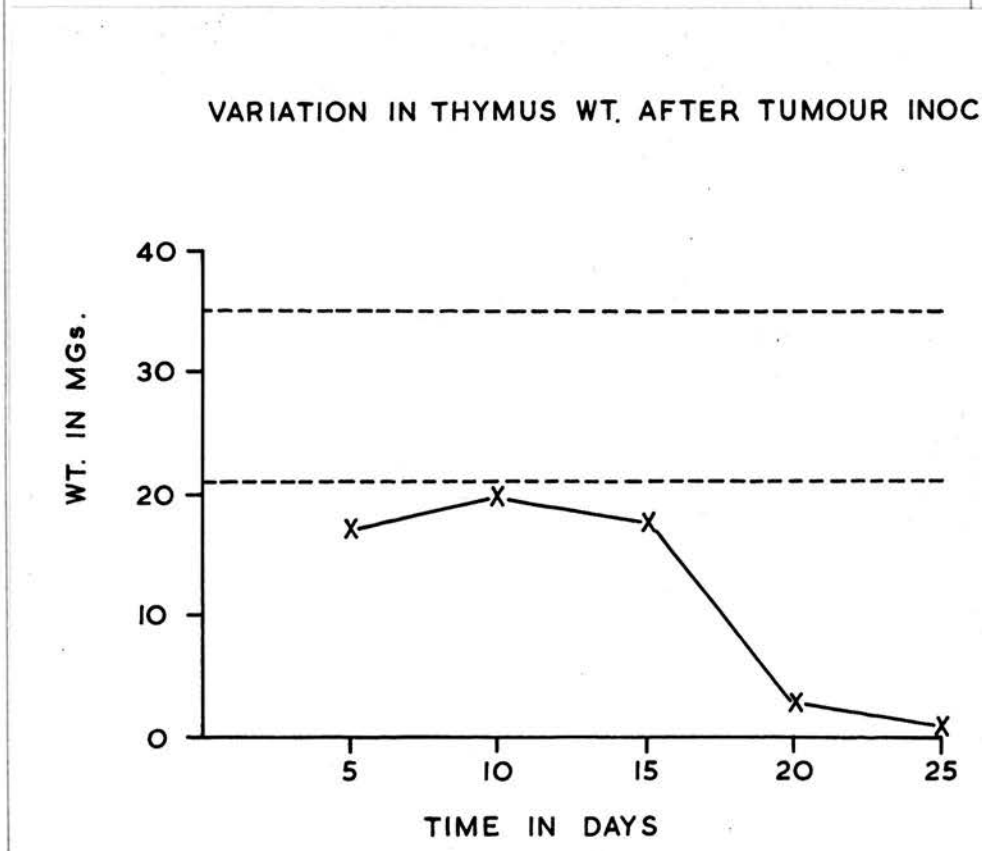

Fig. 2: Average weight of the thymus in groups of 5 A/Jax mice after intraperitoneal injection of 10^5 ascites cells. The dotted lines show the limits of standard deviation of 10 controls.

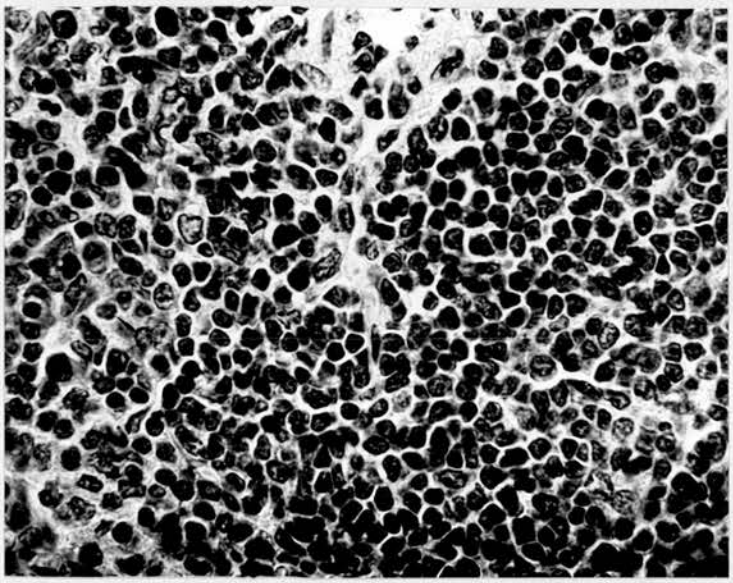

Fig. 3: Normal malpighian body of A/Jax spleen. H & E x 650.

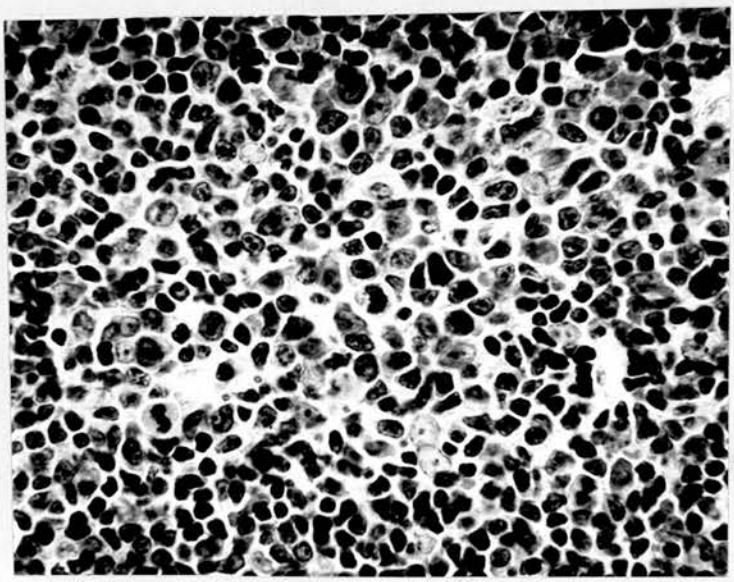

Fig. 4: Malpighian body of A/Jax spleen 10 days after intraperitoneal injection of 10^5 ascites cells. H & E x 650.

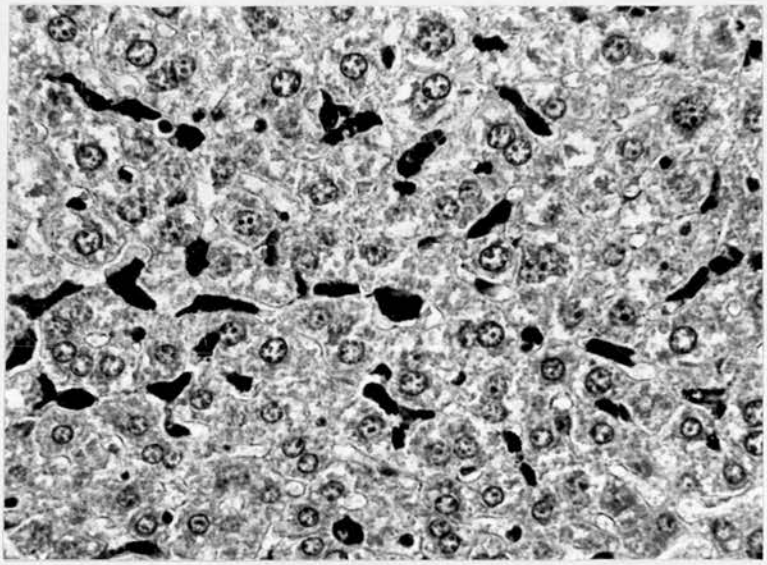

Fig. 5: Küpffer cells of animals bearing 10^5 ascites cells for 10 days and injected with carbon intravenously. H & E x 425.

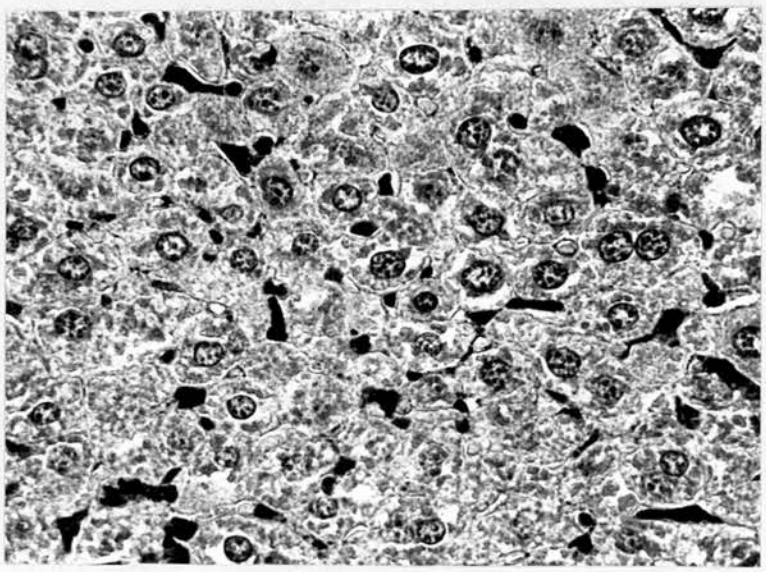

Fig. 6: Kupffer cells of normal animals after intravenous carbon. H & E x 425.

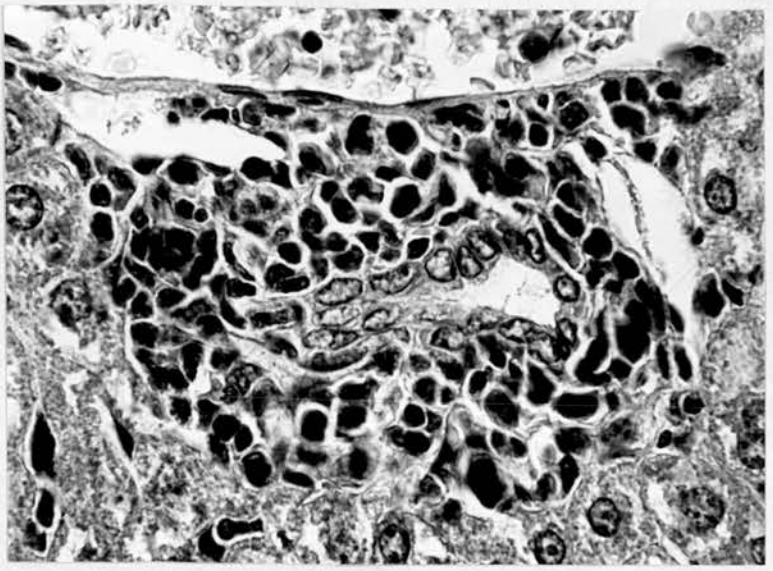

Fig. 7: Focus of immature mononuclear cells surrounding a bile duct.
H & E x 760

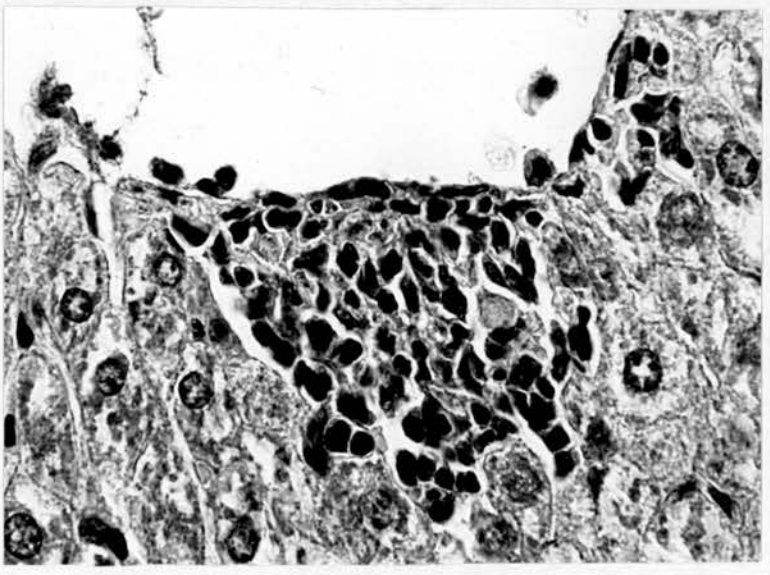

Fig. 8: Focus of immature mononuclear cells near a portal vein. H & E x 476.

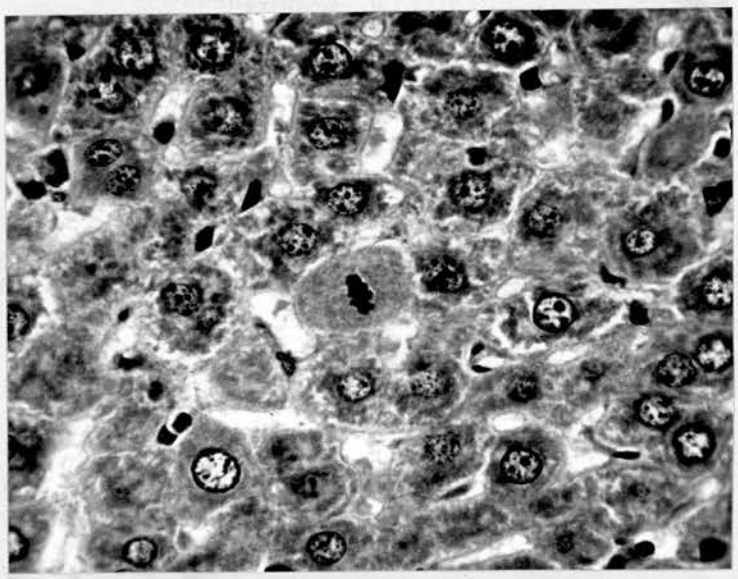

Fig. 9: Mitosis in liver cell of a mouse bearing a 10 day growth of 10^5 ascites cells by the intraperitoneal route. H & E × 1000

Fig. 10: Mononuclear cell reaction in the dermis to a subcutaneous tumour. H & E x 150.

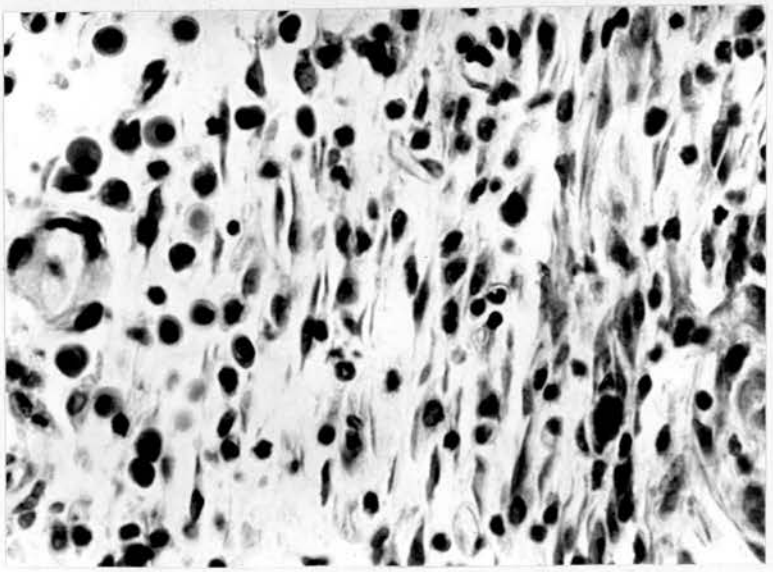

Fig. 11: Cellular reaction around a subcutaneous tumour. The reaction is composed mainly of lymphocytes and plasma cells. H & E x 575.

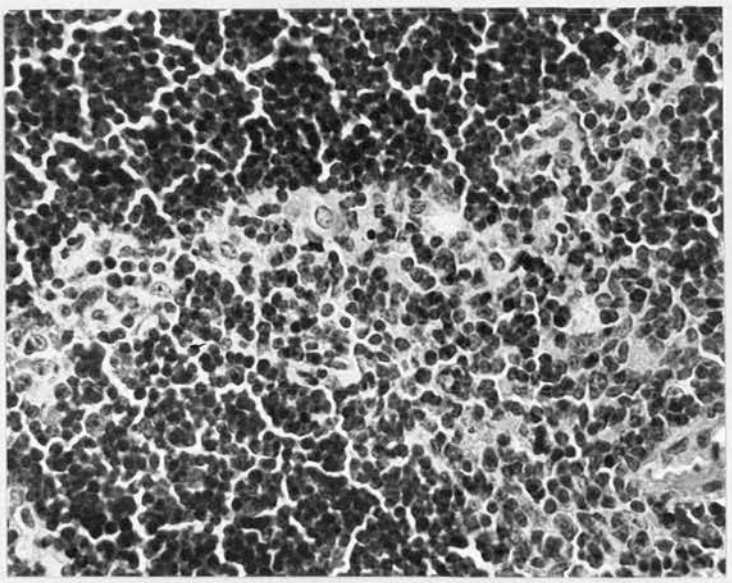

Fig. 12: Sinus cell hyperplasia in a lymph node draining a subcutaneous tumour.
H & E x 450

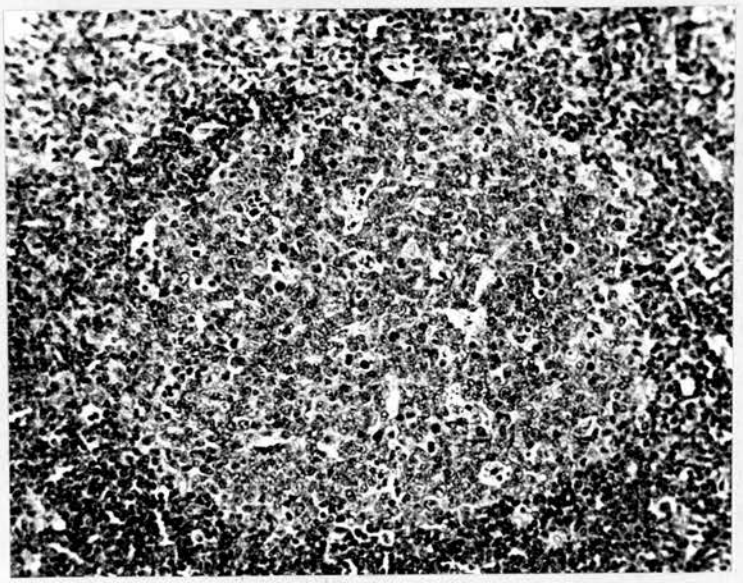

Fig. 13: Prominent germinal centre in a follicle of a lymph node draining a subcutaneous tumour. H & E x 200.

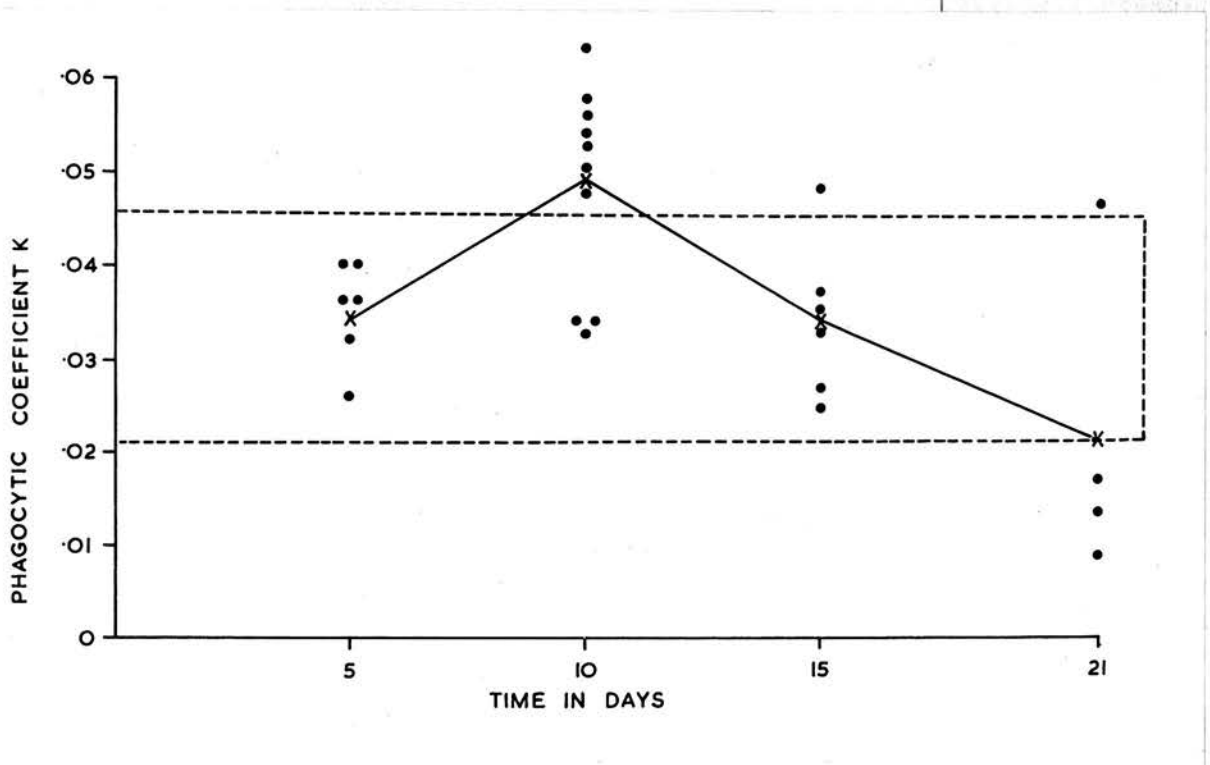

Fig. 14: Phagocytic co-officient K in A/Cum mice after I.P. inoculation of 10^5 ascites cells. The dotted lines show the limits of standard deviation of 15 normal controls. Each solid circle represents one mouse.

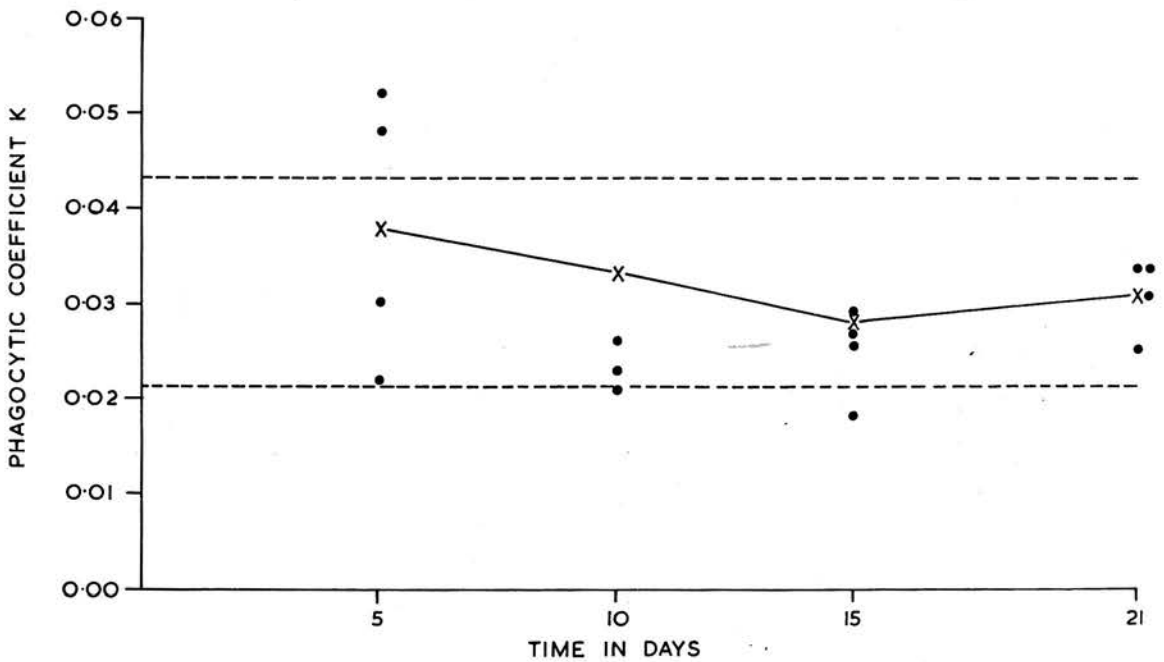

Fig. 15: Phagocytic co-efficient K in A/Gum mice inoculated with tumour and treated with spleen cells. The limits of standard deviation of K for 15 normal controls are shown between dotted lines. Each solid circle represents a single mouse.

CHAPTER II

Inhibition and enhancement of the Landschütz tumour by isologous and homologous cellular systems.

Introduction

The application of lymphoid cells transfer technique to the study of immunological phenomena has been known for a long time, particularly in the field of bacteriology. Thus in 1899 Deutsch transferred splenic tissue from guinea pigs which had been inoculated with typhoid organisms to normal guinea pigs and demonstrated the appearance of haemagglutinins in the sera of the recipients. Luckhardt and Becht (1911) demonstratedcirculating antibodies in the sera of dogs receiving splenic tissue of other dogs that had been inoculated with foreign red cells or typhoid organisms. More recently a major contribution was made by Landsteiner and Chase (1942) who showed that immunity to simple chemicals could be transferred from immune to normal guinea pigs by peritoneal cells obtained from the former. Chase (1945) was able to transfer tuberculin hypersensitivity by lymphoid cells, but not by sera, of tuberculin-sensitive guinea pigs.

Of more relevance to the present work is the transfer of immunity to tumour homografts by cellular systems. Potter, Taylor and MacDowell (1938) were the first to transfer immunity to a transplantable tumour by the use of isologous cells. The tumour used was a leukaemia which

arose in a C 58 mouse and was transplanted in the same strain (C 58) for over 400 generations. Starting with sublethal inoculations of isologous mice with the tumour, they increased the dose of tumour until the animals were rendered immune to a normally lethal inoculum. The spleen and liver cells from such animals could immunise isologous normal mice against a lethal dose of the tumour. Brncic et al (1952), using a similar method, transferred immunity to an AK leukaemia to normal members of the same strain.

Mitchison (1954) utilised a new technique to study the problem of tumour homograft immunity, particularly its effector mechanism. The technique which is generally known as the regrafting technique assesses the degree of immunity developing in a host inoculated with tumour by excising the graft and replacing it in an animal which is known to be susceptible to the tumour. The latter is used as an indicator of viability of the regrafted tissue. Mitchison implanted a tumour of C_3H origin subcutaneously in the mouse strains Ai and C57 BL. The tumour was excised at time intervals and re-implanted in a susceptible strain. The mean survival time of the tumour in the normal alien strain was 9.9 days; in immunised mice it was 2.1 days. Mitchison transferred the lymph nodes draining a regressing tumour to normal members of the same strain. These were challenged with tumour and 8 days after inoculation the tumour was excised and regrafted to a

susceptible strain to measure its viability. It was shown that the regional lymph nodes (nodes draining the graft) conferred immunity on normal isologous mice but serum and peritoneal exudate had no effect, on tumour growth. After the transfer of lymphoid cells, the immunity was firm at 4 days, weaker at 10 days, and disappeared in 24 days time.

In 1954 Billingham et al made a study of the nature of homograft immunity in relation to normal tissue. Their method of approach was similar to that of Mitchison although they used skin grafts and assessed the degree of immunity by visible inspection of the graft. A skin homograft from strain A to CBA mice had a median survival time of 10.8 days and inspection of the graft showed signs of regression at 8 days. When an animal that rejected a first graft was regrafted, the second graft was destroyed at an accelerated rate (second set reponse) and complete rejection occurred in 6 days. The vigour of this second set response waned gradually but was still detectable as long as 120 days after the first graft. Normal CBA mice (secondary hosts), which had been inoculated with regional lymph nodes and, to a lesser extent, spleen cells from actively immunized CBA (primary hosts) mice, behaved as if they themselves had been actively immunized. The lymphoid cells were usually transferred a day before the graft, though the optimum time was 3 days (before the skin graft). The cells did not confer any protection when transferred 2 days after the skin graft.

The route of administration was important; intraperitoneal, but not subcutaneous inoculations, could transfer immunity. Normal lymph nodes and lymph nodes from the contralateral side had no effect. Billingham and his colleagues inferred from their experimental data that lymphoid cells passively transferred from the primary host survived and were incorporated in the secondary host. Consequently the latter were immunised. This form of immunity was called adoptively acquired immunity. The principle of adoptive immunity has been used in the experiments to be described in this chapter.

Mitchison (1955) further extended his studies of adoptive immunity in relation to tumour grafts using sarcoma I in C57 BR/a hosts. Using the regrafting method he showed that lymph nodes from mice receiving a single immunising tumour dose were effective at 5 and 10 days after grafting. Nodes taken at 3, 15, or 20 days were ineffective. The immunising power of lymph nodes draining a second set graft, contrary to expectation, was much less.

It is noteworthy that in the above mentioned work the lymphoid cells were transferred to isologous hosts. Some workers tried to influence the growth of a tumour by the transfer of cells from an immunised homologous host to a susceptible recipient. Ellis and Kidd (1952) incubated Brown-Pearce carcinoma with lymphoid cells from rabbits

that had overcome and resisted re-implantation with this tumour. They found that after this treatment the cells failed to grow when implanted in the muscles of the susceptible hosts and since serum had no comparable effect they concluded that lymphoid cells of immune homologous hosts may act directly against tumour cells in vitro.

Wigzell (1961) reported immunological depression of tumour growth in F1 hybrid - parental strain systems. He found that the intraperitoneal injection of lymphoid cells of one parental strain into F_1 hybrid mice inhibited the growth of lymphoma cells of the opposite parental strain inoculated subcutaneously. The lymphoid cells were either immunised against the parental strain and inoculated simultaneously with the tumour cells or inoculated untreated 5 days before tumour cell inoculation. The inhibition was specific because when the lymphoma and the lymphoid cells were derived from the same strain the tumour inhibition was very slight, due to the wasted condition of the host. When the tumour tested was a sarcoma or a carcinoma the inhibition was very slight. The early death of some animals in this series following treatment with lymphoid cells was probably due to graft versus host disease. Wigzell suggested that the beneficial effect observed with lymphoma tumour might be due to the susceptibility of the latter to circulating antibody. In this connection the studies of Amos and Day (1957) are of direct relevance since they showed that

antibodies prepared by immunising mice of a resistant strain against a leukaemia or lymphoma would passively immunise mice of both resistant or susceptible strains.

Woodruff and Symes (1962) claimed that the growth of a transplanted mammary tumour in strain A mice might be retarded and sometimes destroyed by exposing the recipient to irradiation and then injecting homologous lymphoid cells from either a normal CBA or CBA mouse immunised against the A strain tumour. However, the death rates of both treated and control groups were similar.

It is clear that the immunity to homologous, normal and tumour tissue are basically the same and largely dependent on iso-antigenic differences between the transplanted tissue and the host. Hauschka (1952) in his review of tumour immunity has pointed out that "the mechanisms operating in transplantation immunity are not specifically neoplastic but are influenced to a large extent by iso-antigenic relationships".

All the evidence from the in vivo experiments points to the importance of lymphoid cells in the transfer of homograft immunity (lymphomas are an exception). The in vitro demonstration of a homograft response, however, has largely met with failure. Medawar (1948) cultured rabbit spleen and lymph node fragments, immunised against the skin of another rabbit, with epidermal cells from the latter. He

found no deleterious effect on the epidermal cells as judged by both the mitotic activity and the rate of migration of the epidermal cells. Weaver et al (1955) were also unable to demonstrate a reaction in vitro between epidermal cells and immune homologous spleen fragments. Scothorne and Nagy (1960) showed that fragments of regional lymph node from Swiss white mice carrying skin homografts from CBA donors did not exert any destructive effect when cultured in vitro in intimate contact with lymph node fragments from the original donor for four days. They pointed out "the repeated failure to demonstrate the homograft reaction in vitro in no way invalidates evidence from other sources bearing on the importance of lymphoid tissues in transplantation immunity; the failure means that we have not yet provided adequate in vitro conditions". This contrasts with the effect of heterologous spleen cells in vitro where Stuart (1962*b*) demonstrated cytopathic effects of immunised cells on their target. Rosenau and Moon (1962) showed that splenic lymphocytes sensitized to homologous cells destroy these cells in vitro, but non-sensitized lymphocytes were not cytolytic.

It was noted in chapter I that A/Jax mice inoculated with Landschütz tumour showed a marked degree of stimulation of the reticulo-endothelial system in the early stages of tumour growth. It seemed likely that spleen cells removed at the height of stimulation would confer adoptive immunity

on isologous animals bearing a smaller inoculum of tumour. The experiments described in the present chapter were designed to verify this hypothesis. For comparison homologous spleen cells, obtained from DBA_2 mice immunised in the same manner as isologous animals, were used.

Material and Methods

Mice - A total of 500 A/Jax (Porton) mice, 80 DBA_2 (Porton) and 29 outbred M.R.C. mice weighing 18-20 g. were used.

Propagation of tumour and inoculation of experimental mice; The Landschütz tumour was propagated in stock mice by the method described in chapter I. All A/Jax and DBA_2 mice used in the following experiments were inoculated with tumour maintained in the A/Jax strain. The experimental outbred mice received tumour that had been maintained in that strain.

Immunisation Procedures

Schedule I - 70 A/Jax mice were inoculated by the intraperitoneal route with two injections at weekly intervals of 10^5 tumour cells disintegrated by short exposure to ultra sound followed by a subcutaneous injection of a similar dose

of cells that had been frozen and thawed twice at -20°C. Seven days after the subcutaneous inoculation it was found that 52 mice developed ascites and the remainder did not. The mice were accordingly divided into two groups.

a) Group 1 included 52 mice that were ascitic. These are subsequently to be referred to as ascitic mice.

b) Group 2 included 18 mice that were not ascitic and these are named non-ascitic mice.

Schedule II - A/Jax mice were inoculated with 10^5 ascites cells I.P. Ten or twenty days later spleen cells and sera from these animals were used to treat other A/Jax mice inoculated with the tumour. The spleen cells and sera from such animals will be called 10 or 20 day immune spleen cells. In other experiments 10 day immune DBA_2 spleen cells and sera were prepared by injecting DBA_2 mice with 10^5 ascites cells I.P.

Preparation of spleen cells

The spleens of mice were removed with aseptic precautions. They were homogenised in tissue culture medium 199 using a loose fitting hand operated, all glass homogeniser. The cell suspension was then filtered through a fine mesh steel wire sieve. Cell counts were made and the required dose of nucleated spleen cells was made in a volume of 1-1½ ml.

The interval between preparation of the cells and their subsequent injection into mice did not exceed 35 minutes.

Sera

The mice were bled from the neck vessels. The serum was used fresh or was kept frozen at -20°C until required. The serum was always injected by the intraperitoneal route.

Inhibition of tumour growth by passive transfer of cells or serum

a) Effect of spleen cells and sera from ascitic and non-ascitic mice (immunisation schedule I). 500×10^6 spleen cells or 0.5 ml. of serum from either ascitic or non-ascitic A/Jax mice were inoculated by the I.P. route into other A/Jax mice injected 48 hours previously with 10^5 ascites cells I.P.

b) Effect of 10 day and 20 day immune A/Jax spleen cells and sera. A/Jax mice bearing a 48 hour growth of 10^5 ascites cells I.P. were inoculated with 500×10^6 10 day or 20 day immune spleen cells. Other mice bearing a similar dose of tumour were inoculated with 0.5 ml. of 10 day immune serum. The control groups included both untreated tumour bearing animals and animals given 500×10^6 normal A/Jax spleen cells. In some experiments the 10 day immune spleen

cells were homogenised in excess medium 199 and centrifuged at 1200 r.p.m. for 10 minutes. They were reconstituted to a standard dose of 500 x 10^6 per ml. These were then used to treat the mice bearing the tumour.

The minimum dose of 10 day immune spleen cells necessary for inhibition of the Landschütz ascites tumour; dilutions of homogenised 10 day immune spleen cells containing 500, 100 and 20 million nucleated spleen cells in medium 199 were prepared. These were inoculated into mice bearing 10^5 or 10^7 tumour cells for 48 hours. In some experiments the graded doses of spleen cells were inoculated into mice which were then challenged with 10^7 ascites cells I.P. 24 hours later.

The effect of 10 day immune DBA_2 spleen cells on the ascites tumour; A/Jax mice were inoculated with a dose of 10^5 ascites cells I.P. 48 hours later they were injected I.P. with 500, 100 or 20 million 10 day immune DBA_2 spleen cells.

The effect of spleen cells from outbred mice on the growth of the tumour; 29 outbred mice were inoculated with 10^5 ascites cells by the intraperitoneal route. 48 hours later 9 of these were inoculated with 500 x 10^6 spleen cells obtained from other outbred mice bearing a 10 day growth of 10^5 ascites cells I.P. Other animals were treated with 0.5 ml. of serum obtained from the spleen donors. The

remaining 10 mice were untreated controls.

Enhancement of tumour growth with immune serum

Effect of dosage and time of giving serum on tumour growth; 10 day A/Jax immune serum was given in doses of 0.5 or 0.1 ml. The serum was injected 48 hours after the intraperitoneal inoculation of 10^7 ascites cells. Control mice were inoculated with 10^7 tumour cells only or with 10^7 tumour cells followed by 0.5 ml. of normal A/Jax serum 48 hours later.

Analysis of ascitic fluid from mice showing enhancement; 17 A/Jax mice were inoculated I.P. with a dose of 10^7 ascites cells. 48 hours later 9 of these were given 0.5 ml. 10 day immune A/Jax serum I.P. 5 days after tumour inoculation all the mice were killed and divided into control and serum treated groups. The peritoneal cavities were washed with 1 ml. of heparinised medium 199 and the contents of each group was pooled. The total volume was measured. The nucleated cell count was estimated and differential counts made on smears stained by Leishman's method. Haematocrit tubes filled with ascitic fluid were centrifuged at 4000r.p.m. for 1 hour and the packed cell volume measured as a percentage. Solid tumour in the peritoneal cavity was weighed to the nearest mg. as were the spleens.

Effect of blockade of reticulo-endothelial phagocytic function on the growth of the ascites tumour

Blockade with ethyl palmitate; this was prepared as a 30% suspension by adding 2.5 ml. of ethyl alcohol to 15 ml. of ethyl palmitate at 45°C. The mixture was slowly added by pipette to 32.5 ml. of 1 per cent Tween 20 in 5 per cent dextrose water and rapidly homogenised in a blender for 5 minutes. The suspension was dialysed overnight at 37°C against excess 5 per cent dextrose water buffered with phosphate to pH 7.2. 0.15 ml. of the suspension per 10 g. of mouse body weight was injected intravenously into 14 mice. No mortality occurred as a result of injecting the ethyl palmitate and six hours later the mice were given 10^7 ascites cells I.P.

Blockade with cholesterol oleate; a 10 per cent suspension of cholesterol oleate was made in Tween dextrose water and 30 mg. of ester injected intravenously into 9 mice. 6 hours later they were inoculated with 10^7 ascites cells I.P. followed 48 hours later by a further I.V. injection of 30 mg. cholesterol oleate. No mortality occurred as a result of injecting cholesterol oleate.

Measurement of phagocytic function in mice showing tumour enhancement

The phagocytic function of the reticulo-endothelial

system was measured by the carbon clearance method as described in chapter I.

Phagocytic function was measured in the following groups of A/Jax mice.

1. 14 mice inoculated with 10^7 ascites cells only.
2. 16 mice inoculated with 10^7 ascites cells followed by 0.5 ml. 10 day immune A/Jax serum 48 hours later.
3. 12 mice inoculated with 0.5 ml. of 10 day immune serum alone.
4. 12 mice inoculated with 0.5 ml. of normal A/Jax serum.
5. 13 normal A/Jax mice.

A detailed necropsy was made on all animals dying from tumour. Animals surviving for 60 days after tumour inoculation were killed with chloroform and a postmortem examination made.

Results.

The effect of spleen cells and sera from ascitic or non-ascitic mice on the ascites tumour; Table I shows that spleen cells derived from ascitic mice were much more effective in inhibiting the growth of the tumour than were those from animals which did not develop ascites.

The serum from ascitic mice prolonged life and converted the tumour to the solid form. It was more active than the serum from non-ascitic animals. All untreated animals developed ascites around the 12th day and died on an average in 28 days.

Table II shows that the spleens of mice immunised with either viable (ascitic mice) or non-viable (non-ascitic mice) tumour cells were significantly larger than normal spleens.

The effect of 10 day immune spleen cells on the tumour; Table III shows that 500 x 10^6 10 day immune spleen cells produced almost complete inhibition of the tumour inoculum. All 18 animals survived 60 days and at autopsy 16 mice were free from tumour. Normal spleen cells did not inhibit the tumour and promoted the growth of solid masses. Fig. 1 shows the weight curves for ascitic mice compared with normal controls. The increase in weight of animals bearing tumour parallels the progressive accumulation of ascites. Mice bearing tumour and treated with immune spleen cells show a normal weight curve except for a temporary initial loss in weight (Fig. 2).

The minimum tumour inhibiting dose of A/Jax spleen cells. Table III shows that 100 million cells produced partial inhibition of a 48 hour growth of the tumour. Although all the treated mice eventually showed solid

tumour, none became ascitic and their average survival time was considerably prolonged. The most significant effect of 20 million immune spleen cells is the partial or complete conversion of the tumour to the solid form.

The effect of normal spleen cells on the tumour; Table III shows that mice treated with normal spleen cells died about the same time as the untreated controls. Five of six treated animals developed both ascites and solid tumour. Postmortem examination showed more abundant tumour than in the untreated controls.

Comparison of the inhibitory action of 10 day and 20 day immune spleen cells; Table IV shows that the 10 day immune spleen cells are more effective in inhibiting the growth of tumour than 20 day immune spleen cells. The average weight of 10 day and 20 day spleens was 0.22 g. and 0.13 g. respectively. The average weight of normal spleens was 0.07 g.

The effect of 10 day immune spleen cells on 10^7 ascites cells; Table V shows that the effect of immune spleen cells on 10^7 ascites cells depends on the dose of the lymphoid cells and the time of their administration. When the cells were given 24 hours before the tumour there was some inhibition with both the 500 and 100 million spleen cells. When given after tumour inoculation, only the highest dose of spleen cells had any effect. It is important to note

that in this experiment tumour enhancement was not observed.

Inhibition of the tumour with 10 day immune DBA_2 spleen cells; Table VI shows that 10 day DBA_2 spleen cells markedly inhibited a 48 hour growth of the Landschütz tumour. This inhibition occurred even with a small dose of 20 million spleen cells.

The effect of 10 day immune serum on 10^5 ascites cells; Table VII shows that sera from 10 day immune mice had little effect - part of the tumour was converted to the solid form.

The effect of homologous spleen cells from outbred mice on the growth of the tumour; Table VIII shows that the spleen cells and sera from outbred mice bearing 10^5 ascites cells I-P. for 10 days, had no effect on the tumour when passively transferred to mice bearing 10^5 ascites cells. Normal spleen cells from outbred mice had no inhibitory effect on the growth of the tumour (see chapter III).

Enhancement of tumour growth

Enhancement with passively transferred serum; Table IX shows that mice bearing a 48 hour growth of 10^7 ascites cells and treated with 0.5 ml. of immune serum died

significantly earlier than tumour bearing controls. The smaller dose of 0.1 ml. had no enhancing effect. Normal serum has no enhancing effect.

Analysis of ascitic fluid from mice showing enhancement; Table X shows that the fluid obtained from tumour bearing mice treated with 10 day immune serum had higher packed cell volume and was more cellular than fluid obtained from mice given tumour alone. Differential counts showed that the increase in cells was due to tumour cells.

The effect of reticulo-endothelial blockade on the growth of the tumour; Table XI shows that cholesterol oleate and ethyl palmitate did not enhance tumour growth. Animals treated with ethyl palmitate showed widespread splenic necrosis at postmortem, and delay of splenic regeneration (fig. 3).

Reticulo-endothelial phagocytic function in mice showing enhancement; Fig. 4 shows that mice inoculated with 10^7 ascites cells show an elevation of phagocytic index K which is still present at 8 days. Mice treated with serum and showing enhancement also had elevated values for K (fig. 5). The corrected phagocytic indices also showed a corresponding increase above normal. Five days after serum inoculation the values of ascitic controls and enhanced mice were 5.2 and 4.4 respectively whereas normal controls averaged 3.3. Immune serum alone had mild

blocking effect on R.E.S. function (fig. 6), and normal serum gave results in the lower limits of normal (fig. 7) probably because of altered haemodynamics. The spleens of animals showing enhancement are smaller than those of mice bearing unenhanced tumour (fig. 8).

Discussion

The results show that the passive transfer of suitably immunised isologous spleen cells inhibited the growth of the tumour. The manner of immunisation was important since the biological activity of lymphoid cells obtained from animals immunised with disintegrated tumour cells was very much inferior to those obtained from animals immunised with living cells. It seems that the disintegration of the tumour cells with ultra-sound destroys antigens essential for proper immunisation of homologous hosts. Since the spleens from non-ascitic mice were significantly larger than normal spleens, it is almost certain that they were responding to antigens other than those operating in the induction of transplantation immunity. On the other hand ascitic mice were exposed to a higher concentration of antigen than mice immunised with disintegrated tumour cells.

It has been demonstrated (chapter I) that A/Jax mice inoculated with the Landschütz ascites tumour showed evidence of stimulation of the lymphoreticular tissues. This activity was at its maximum 10 days after tumour inoculation but diminished in the late stages of tumour

growth (at 20 days). A stimulated spleen showed an excess of immature pyronophilic mononuclear cells. The present results have shown that ten-day immune spleen cells had a higher inhibitory action on the tumour than spleens from animals bearing the tumour for 20 days. About 90% of mice bearing 10^5 ascites cells and treated with 500 million ten day immune spleen cells were free of tumour at autopsy sixty days later.

The inhibitory effect of the spleen cells on the tumour is almost certainly immunological. Other non-specific modes of action of the lymphoid cells can be excluded. That the effect was not due to competition between the spleen cells and tumour cells for some important nutrient was shown by the lack of activity of normal spleen cells. It could, however, be argued that the immunised spleen cells were metabolically more active than normal spleen cells. On the other hand, spleen cells from mice immunised with disintegrated tumour cells were of about the same size as those from ascitic mice, yet they did not inhibit the tumour. This proves that the activity of the former was due to the development of a specific type of immunity.

It is accepted that the immunity against transplantable tumours is essentially a homograft type of immunity, largely governed by iso-antigenic differences between the graft and the host. The Landschütz tumour may be

conveniently regarded as a homograft with a high degree of growth potential. Because of the rapid proliferation of the cells, the homograft immunity is overcome. This immunity, however, can be passively transferred to other mice bearing a small inoculum of tumour which is fatal in 100% of untreated mice. The degree of inhibition depends not only on the quality of the spleen cells but on the ratio of spleen cells to tumour cells. Thus, in the experiments in which the dose of tumour cells was kept constant and the number of spleen cells varied, the degree of protection was directly proportional to the number of spleen cells given. It is notworthy that enhancement with pre-immunised lymphoid cells did not occur. It will be seen in chapter III that under similar circumstances with heterologous spleen cells, enhancement was obtained. Conversely, heterologous serum did not enhance tumour growth whereas 10 day immune isologous serum produced enhancement. These experiments emphasise the importance of a titration of the tumour and the inhibiting agent.

The spleen cells have an agglutinating effect on the tumour and probably a cytotoxic action as well. So far it has not been possible to demonstrate toxicity in vitro with cells, serum or a mixture of the two. The inhibitory activity of these isologous lymphoid cells is remarkably specific since they are accepted by the recipient as self. The only foreign tissue encountered by the donated cells is

the tumour, and this situation permits an immunological reaction between the two. The DBA_2 spleen cells were more effective in suppressing the tumour growth than the A/Jax spleens, despite the longer persistence of the latter in the host. This means that the DBA_2 lymphoid cells were better immunised and supports the finding that DBA_2 mice survive slightly longer than A/Jax host when inoculated with the ascites cells. The difference in response between the two different strains is consistent with the view that the antigenic differences of the Landschütz tumour are largely physiological and determined by genetic relationships between host and tumour. Perhaps the main advantage of isologous spleen cells is their specificity and freedom from toxicity. In the case of primary tumours, either spontaneous or induced, one would expect the administration of suitably prepared isologous cells to modify the natural history of the growth, provided that an antigenic difference was present.

Normal spleen cells failed to inhibit the tumour and at necropsy enhancement had occurred, judging by solid masses of exuberant growth as well as ascites. This may be due to a tumour-stimulating property of lympho-reticular tissue (Szent Gyorgi et al 1962) or to low grade immunological reaction by the transfused cells against the tumour.

The spleen cells from outbred mice did not inhibit tumour growth. This indicates that the level of immunity against the tumour is lower in outbred mice than in A/Jax

and DBA_2 strains.

The activity of serum depended on the manner of immunisation, the dose and the time of administration in relation to tumour inoculation. The inhibiting action of serum from mice given the first schedule of immunisation is unusual. It must be pointed out, however, that the immunity was partial and the tumour was converted to the solid form. Consequently the mice survived longer. This is in favour of the theory that in homograft immunity there is in fact no qualitative difference between the activity of lymphoid cells and serum.

The effector mechanism in the homograft reaction is still controversial. There are four possibilities; the homograft immunity may be mediated by classical antibodies, by cell bound factors analogous to delayed hypersensitivity reactions, by both serum antibodies and cell bound activity or by an immunological mechanism peculiar to this reaction. The last view has been expressed by Thomas (1959).

The role of circulating antibody in homograft rejection caused a great deal of controversy. Gorer (1958) has shown that in the mouse iso-antibodies of the H_2 system were regularly found before the onset of homograft destruction. In vitro such antibodies, in the presence of complement, were cytotoxic to normal cells from the spleen,

lymph nodes and bone marrow. Cells from 5 different cases of mouse leukaemia were found to be susceptible to the action of such iso-antibodies although the cells of a sarcoma appeared to be completely resistant. Amos (1962) pointed out that special techniques might be needed to demonstrate cytotoxic activity of iso-immune serum to carcinoma and sarcoma cells because of the use of foreign complement. Mouse serum had an anticomplementary effect on guinea pig serum. This fact entailed exposing the tumour cells to undiluted mouse serum washing and then adding complement. Using this technique Amos and Wakefield (1961) demonstrated cytotoxic action of iso-antibody on carcinoma and sarcoma cells.

Besides cytotoxic antibodies demonstrated in vitro iso-antibodies with different properties can also be detected. These may be different antibodies or one single antibody with different activities depending on the conditions of the tests. Thus in reponse to homografts erythrocyte agglutinating (Gorer and Mikulska 1954); leukocyte agglutinating (Amos 1953) and complement fixing antibodies (O'Gorman 1960) were found. It is important to remember that the action of iso-antibody in vitro is stronger with dispersed cells than with cell clumps or solid tissue. For example Gabourel and Fox (1959) showed that iso-antibodies were not toxic to L cells in clumps; later Gabourel (1961) reported strong toxic changes on

isolated L cells growing in diffusion chambers.

Some workers were unable to demonstrate the cytotoxic action of iso-antibody in vitro. Miller (1956), for example, could not find toxic changes in epithelial cells exposed to the action of iso-antibody in vitro. O'Gorman (1960) attributed this to an inadequate complement supply.

Most of the in vitro work was performed on the iso-antibodies of the H_2 system of the mouse. Amos 1962 pointed out that reactions with mouse iso-antibodies in vitro are often difficult to perform, "Because certain activities had not been demonstrated, it was often thought that they did not occur..... The emphasis in the past has therefore been largely upon activities of the immune cell itself and upon the delayed type of response which, in the tuberculin system, is not mediated by conventional humoral antibodies". One of the limiting factors in the mouse system is the fact that mouse serum in vitro, is deficient in complement. Brown (1943) believed that it lacked the $C^1 2$ complement component. That mouse serum complement is active in vivo is shown by experiments with diffusion chambers (Algire et al 1957).

Boyse, Old and Stochart (1962) thought that iso-antibody cytoxicity to transplantable tumours could always be demonstrated in vitro provided the correct proportions of cells and sera were used. They demonstrated toxic

changes in two ascites sarcomata which previous experience showed to be resistant to the action of H_2 iso-antibody. This was due to the use of cells suspensions more dilute than those commonly used in cytotoxicity tests.

Möller and Möller (1962) have shown that the cytotoxic effect of an iso-antibody on cells in vitro is proportional to the amount of iso-antigenic receptors on the surface of the cell. They showed that tumour cells which were partially or completely resistant to the action of a single iso-antibody could be made sensitive to humoral iso-antibodies provided that they were treated with a mixture of antisera produced in different donor genotypes against the same target genotype; each individual genotype reacts against a different antigenic site on the cell.

With the in vivo activity of iso-antisera there is no doubt that lymphomas are highly susceptible to the action of humoral iso-antibody (Gorer and Amos 1956). Carcinomata and sarcomata are regarded by many workers to be, on the whole, insensitive to the action of humoral antibody. In some cases this failure could be due to inability to raise adequate concentration of antibody in the graft. With a high antibody concentration ensured, Amos and Wakefield (1959) could lyse both a carcinoma and a sarcoma, when growing in diffusion chambers in the peritoneal cavity of the mouse.

The importance of ensuring a high concentration of iso-antibody around a graft will be realised from the work of Gorer and Kaliss (1959) who showed that with certain iso-antibodies a low concentration may enhance tumour growth.

Stetson and Demopoulos (1958) were impressed by the fact that large numbers of plasma cells were demonstrated, by Darcy (1949, 1952), in the bed of skin homografts and in the lymph nodes draining a homograft, by Scothorne (1957). They argued that if these cells were producing antibody against the graft, then the concentration of antibodies in the graft would be higher at the site of production than in the serum. Then for purely quantitative reasons, these antibodies in the serum may be found ineffective in transferring homograft immunity. Stetson and Demopoulos (1958) showed that when rabbits or mice were hyperimmunised against a skin homograft, a subsequent graft was rejected without the establishment of vascular connections (white graft reaction). This type of homograft immunity could be passively transferred with hyperimmune serum injected at the same time as the graft. Stetson and Jensen (1960), however, showed that an antiserum with a high degree of cytotoxicity in vitro had no effect when injected into mice bearing a skin graft for 2-4 days. They believed that this was due to the fact that iso-antibody cannot reach an established graft in

sufficient quantity. Amos (1955) had already demonstrated that in mice bearing tumours, the rate of removal of iso-antibody from the circulation was the same as in normal animals. Stetson and Jenson (1960) increased the circulation to established skin grafts by topical application of xylene. When this was done in animals that had already received intravenous injections of cytotoxic antiserum, graft breakdown occurred in the painted areas.

As was mentioned in the general introduction to this work the participation of lymphoid cells in homograft immunity has been known for a long time (Wade 1908, Da Fano 1912 and Murphy 1926). Immunity to tumour homografts (Mitchison 1955) and to normal skin grafts (BILLINGHAM et al. 1954) could readily be transferred with lymphoid cells. The puzzling questions are the relationship of lymphoid cells to classical antibodies in homograft reactions, the type of cell involved and the mode of its action. Most authors agree that there is a synergism between "immune" cells and circulating antibody (Snell 1957); but few believe that the two antagonise each other (Batchelor 1963). Most favour the lymphocyte as the cell involved in the mediation of homograft immunity, because of its well known immunological competence (Lancet 1962). Thoracic duct lymphocytes are mediators in graft versus host reactions and destruction of skin homografts. (Gowans et al 1963). Weaver et al (1955) stressed the importance of the lymphocyte

in tumour rejection.

Woodruff et al (1963) believed that thoracic duct lymphocytes from immune rats could inhibit the Landschütz tumour. This tumour is highly susceptible to hetero-antibody (see chapter III). The protocols of the experiments of Woodruff et al indicate that some of the effect may be due to circulating antibody since the lymphocytes were not completely washed. A critical experiment, which should have been included, is to demonstrate the effect of the supernatant from the injected lymphocytes on the growth of the tumour. Delrome and Alexander (1964) reported the inhibition of a chemically induced fibrosarcoma of the rat by thoracic duct lymphocytes.

With some tumours the histiocyte appears important in graft destruction (Amos(1960) Baker et al (1962).

The actual mechanism of the action of the cells is poorly understood. They may owe their activity to antibodies of the type found in the serum. Many investigators believe that lymphocytes elaborate a special form of antibody (cell bound antibody), the nature of which has not yet been demonstrated.

The homograft reaction is a complex one and Gorer (1956) has demonstrated three histological variants in the mouse. It is probable that these are different types of

homograft immunity depending upon the graft-host combination. It seems reasonable to assume that in homograft rejection both antibodies and immune cells play a part. There is no support for the assumption that immune cells carry a special type of cell-bound antibody. The function of cells appears to be their ability to create a local concentration of antibody around the graft, higher than that in the serum. In the present work, when mice were properly immunised, a serum has been obtained whose activity approached that of immune cells.

It is paradoxical that serum removed from mice at the time of their maximum resistance should enhance the growth of the Landschütz tumour. The enhancement described in these experiments was characterised by transmission of isologous serum given after inoculation of tumour. Furthermore, it could be accurately measured by haematocrit estimations. The most striking feature of the enhanced animals was the small size of the spleen. The usual splenomegaly which develops in response to tumour was almost completely suppressed by serum.

It will be seen from chapter III that enhancement of the Landschütz tumour followed the passive transfer of heterologous lymphoid cells. This form of enhancement was attributed to the profound depression of the immunological status of the host, as judged by diminished phagocytic function and partial suppression of antibody

response to foreign red cells. Because of iso-antigenic differences between the ascites tumour and the host, this depression was sufficient to remove the immunological reaction of the host to the tumour, and enhancement was possible.

The enhancement observed with the passive transfer of iso-antiserum in A/Jax mice was quite different from that obtained with heterologous cells. The degree of depression of phagocytic function by immune serum was limited to the first 24 hours after serum transfer. The treatment of mice with lipids known to produce depression of phagocytic function (Stuart et al 1960, Shivas and Frazer 1959) was not followed by tumour enhancement. It follows therefore that depression of phagocytic function by itself is not sufficient to explain enhancement. Splenic repair in lipid-treated mice was markedly retarded and accorded well with the finding of lymphoreticular and thymic atrophy in the later stages of tumour growth. Despite extensive splenic necrosis, lipid-treated animals had the same survival time as the controls, and one must assume that the abdominal lymph nodes play an important role in the reaction to this ascites tumour.

The enhancement of the Landschütz tumour by passively transferred antiserum is perhaps related to immunological enhancement described by Kaliss and Molomut (1952). These authors showed that enhancement of tumour growth could be

produced by the passive transfer of homologous immune serum. They used a tumour of strain A origin which normally regressed in strain C57 BL/6Ks. When C57 black mice were treated with an antiserum prepared against strain A normal or tumour tissue, prior to tumour inoculation, there was a marked enhancement of tumour growth, noted by the progressive growth of the tumour in the alien strain. This form of enhancement was defined as immunological enhancement. (see chapter IV).

The mechanism of action of the antiserum in the production of enhancement is still debateable. Kaliss (1958) put forward the theory that antiserum acted directly on the tumour and altered it in such a manner that it could survive despite the hostile environment of the host. Feldman and Globerson (1960) postulated that antiserum stimulated antigen production by the tumour. The excess antigen then neutralises the "cytotoxic immune response" provided by the host and thus protects the tumour. On the other hand, Snell (1956) and Snell et al (1960) believed that enhancement was due to "walling off" of the graft's antigens by antiserum so that the immune centres of the host were not stimulated. Brent and Medawar (1962) thought that enhancement was due to a central inhibition of the immune response of an unknown nature. Chantler and Batchelor (1964) believed that enhancement of tumour growth was due to suppression of cellular reactivity by excess circulating

antibody.

The present work indicates that immunological enhancement of the Landschütz tumour is due to suppression of the central immune mechanism of the host. The most likely explanation of this is the combination of antigen with an excess of antibody. Because it has not been possible to detect these "antibodies" by complement fixation, gel diffusion or in vitro cytotoxicity tests, they were present only in minute quantities. If this is so, it is difficult to see how peripheral sequestration of antigen can explain the diminished splenic reaction and a more fundamental mechanism involving a central failure of antibody synthesis (Uhr and Baumann 1961)may operate.

TABLE I

The Effect of spleen cells or sera from ascitic or non-ascitic immunised mice on ascites tumour

Dose of Tumour	No. of mice	Treatment	No. with Ascites	Average onset of Ascites (days)	Apparent cures at Necropsy	Survival of Remainder (days)
10^5	23	Nil	23	11.7 ± 0.7	Nil	28 ± 6.7
	10	Spleen cells from non-ascitic mice	10	10.8	0	43
	11	Spleen cells from ascitic mice	2	34	6	-
	5	0.5 ml. serum from non-ascitic mice	4	12	1	31.5
	5	0.5 ml. serum from ascitic mice	0	0	1*	all sacrificed at 60 days

* 4 mice had solid tumour in peritoneal cavity.

TABLE II

Changes in the spleen weights of A/Jax mice immunized with tumour cells by the I-P. route

No. of mice	Immunization	Spleen wt. (g) and its s/m
19	nil	0.07 ± 0.01
20	non viable tumour cells	0.13 ± 0.04
18	viable tumour cells	0.17 ± 0.03

TABLE III

The effect of normal or immune strain A spleen cells on a 48 hour growth of 10^5 ascites cells given I.P.

No. of mice	Dose of spleen cells	No. with ascites	Onset of ascites (days)	No. with solid tumour dead before 60 days	Mice surviving for 60 days		Average sur of remainde (days)
					With solid tumour	Without solid tumour	
23	nil	23	11.5 ± 1	nil	nil	nil	28 ± 6.5
6	500 $x10^6$ normal spleen cells	6	10.5	5	nil	nil	24
18	500 x 10^6 10 day immune spleen cells	nil	-	0	2	16	All sacrifi at 60 days
5	100 x 10^6 10 day immune spleen cells	nil	-	2	3	nil	46.0
5	20 x 10^6 immune 10 day spleen cells	3	14	5	nil	nil	36.5 range 32-35

± = S.D.

TABLE IV

The in vivo effect of spleen cells, harvested at different times during tumour development, on the ascites tumour.

Dose of tumour	No. of mice	Treatment	No. with ascites	Average onset of ascites (days)	No. with solid tumour	No. free of tumour at 60 days	Survival of remainder (days)
10^5	23	nil	23	11.5 ± 1	nil	nil	28 ± 6.5
	8	500×10^6 10 day Immune spleen cells	0	0	1	7	All sacrificed at 60 days
	5	500×10^6 20 day Immune spleen cells	0	0	3	2	All sacrificed at 60 days.

TABLE V

The effect of graded doses of 10 day A/Jax immune spleen on A/Jax mice inoculated with 10^7 ascites cells.

Time spleen cells administered	Dose of spleen cells	No. of mice	No. with ascites	Average onset of ascites (days)	No. with solid tumour	Apparent cures	Survival of remainder (days)
24 hours before tumour	500×10^6	3	1	11	2	1	42 x 53
	100×10^6	4	3	9	2	1	32
48 hours after tumour	500×10^6	7	5	12 (range 8-27)	6	1	23
	100×10^6	9	8	8	9	nil	25
	20×10^6	5	5	11	4	nil	26
untreated controls		17	17	8.5 ± 1	nil	nil	22 ± 3.5

± = S.D.

TABLE VI

The in vivo effect of 10 day immune DBA_2 spleens on a 48 hour growth of 10^5 ascites cells in A/Jax mice.

Dose of DBA_2 spleen cells	No. of A/Jax mice given 10^5 ascites cells I-P.	No. with ascites	Average onset of ascites (days)	No. with solid tumour	Apparent cures	Average survival of remainder (days)
500×10^6	9	nil	-	1	8	All sacrificed at 60 days
100×10^6	11	nil	-	3	8	All sacrificed at 60 days
20×10^6	10	2	18	3	5	30
nil	23	23	11.5 ± 1	nil	nil	28 ± 6.5

± = SD.

TABLE VII

The in vivo effect of 10 day immune serum on ascites tumour

Dose of Tumour	No. of mice	Treatment	No. with ascites	Time of onset of ascites (days)	No. with solid tumour	Apparent cures	Survival of remainder (days)
10^5	23	nil	23	11.5 ± 1	nil	nil	28 ± 6.5
	4	0.5 ml. of 10 day JAx serum	4	11	4	nil	23 (range 22 - 27)
	10	0.5 ml. of 10 day JA_2 serum	9	13.5 ± 2	8	nil	27 ± 4.5

± = S.D.

TABLE VIII

The effect of spleen cells or sera from outbred mice on a 48 hours growth of 10^5 ascites cells.
Donors of cells and serum had been bearing 10^5 ascites cells I-P. for 10 days.

Dose of Tumour	No. of mice	Treatment	No. with ascites	Onset of ascites (days)	Survival (days)
10^5	9	500×10^6 spleen cells	9	8 ± 0	20 ± 6
	10	nil	10	10 ± 0	21.5 ± 3
	10	0.5 ml. serum	10	11 ± 0	21 ± 3

TABLE IX

The in vivo effect of A/Jax serum given 48 hr. after 10^7 ascites cells

Treatment	No. of mice	Dose of serum (mls.)	No. of mice with ascites	Average onset of ascites (days)	Average Survival (days)
10^7 ascites cells and immune serum	4	0.5	4	6	12
	4	0.1	4	7	19
10^7 ascites cells and normal serum	5	0.5	5	7	20
10^7 only	17	nil	17	8.5 ± 1	22 ± 3.5

TABLE X

Enhancement of tumour growth by passive transfer of 10 day immune A/Jax serum (0.5 ml.)
Fluid analysed 5 days after tumour inoculation.

	No. of mice	Dose of tumour	Treatment	Volume of ascites ml.	P.C.V. %	Tumour cells per mouse	Wt. of solid tumour (mg.)
Exp. 1.	5	10^7	serum	8.4	-	320 x 10^6	210
	5	10^7	nil	6.7	-	250 x 10^6	nil
Exp. 2.	4	10^7	serum	9.8	20	370 x 10^6	-
	3	10^7	nil	6.7	12	280 x 10^6	-

TABLE XI

The effect of cholesterol oleate and ethyl palmitate on the host response to the tumour.

Dose of tumour	No. of mice	Treatment	No. with ascites	Time of onset of ascites (days)	Survival (days)
10^7	9	cholesterol oleate	9	8 ± 0	20 ± 1
	14	ethyl palmitate	14	8 ± 0	26 ± 4
	17	nil	17	8.5 ± 1	22 ± 3.5

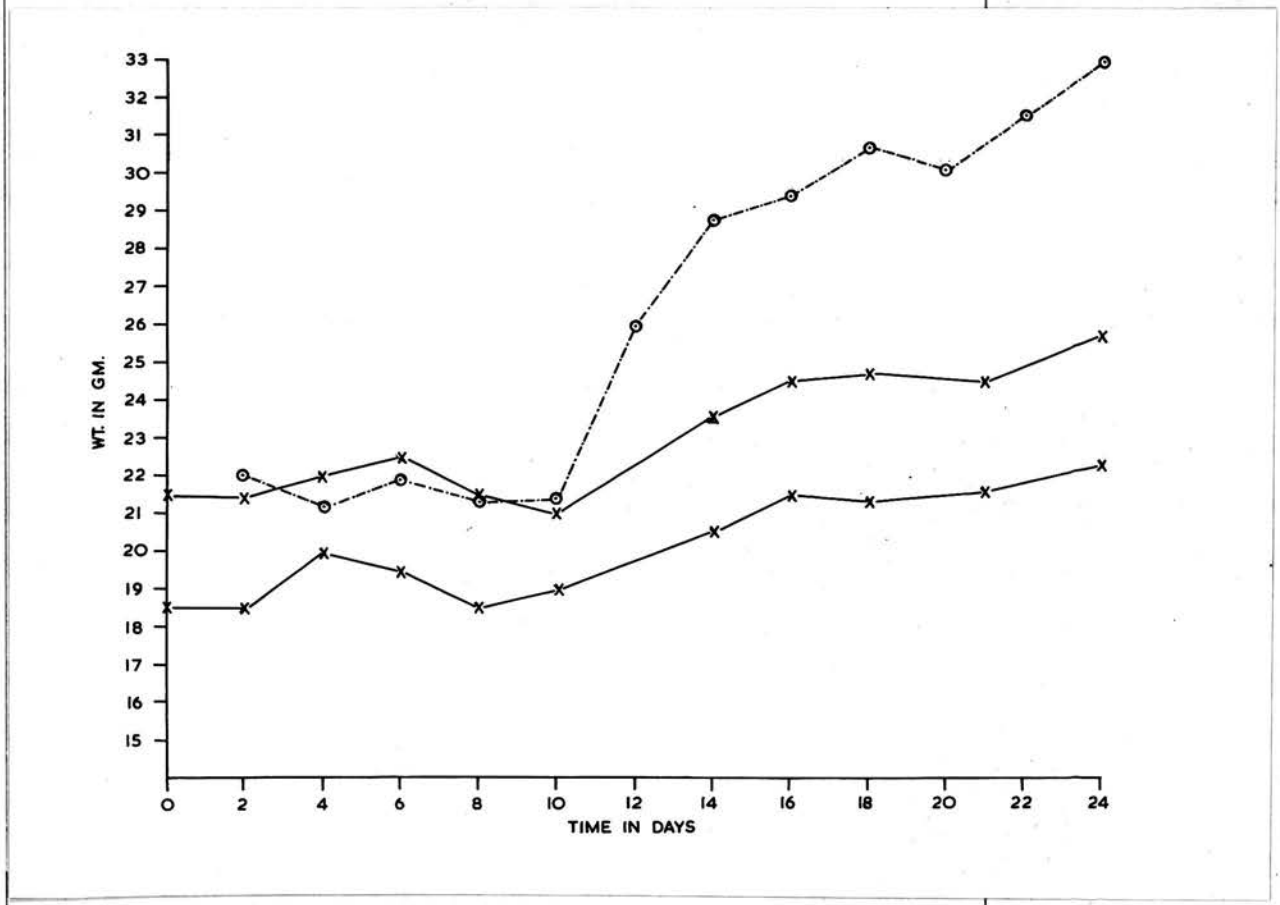

Fig. 1: Weight curves of normal and tumour bearing animals. The crosses indicated the limits of standard deviation of 20 normal controls and the circles the average weight of 10 mice of a similar age and sex inoculated with 10^5 ascites cells I.P.

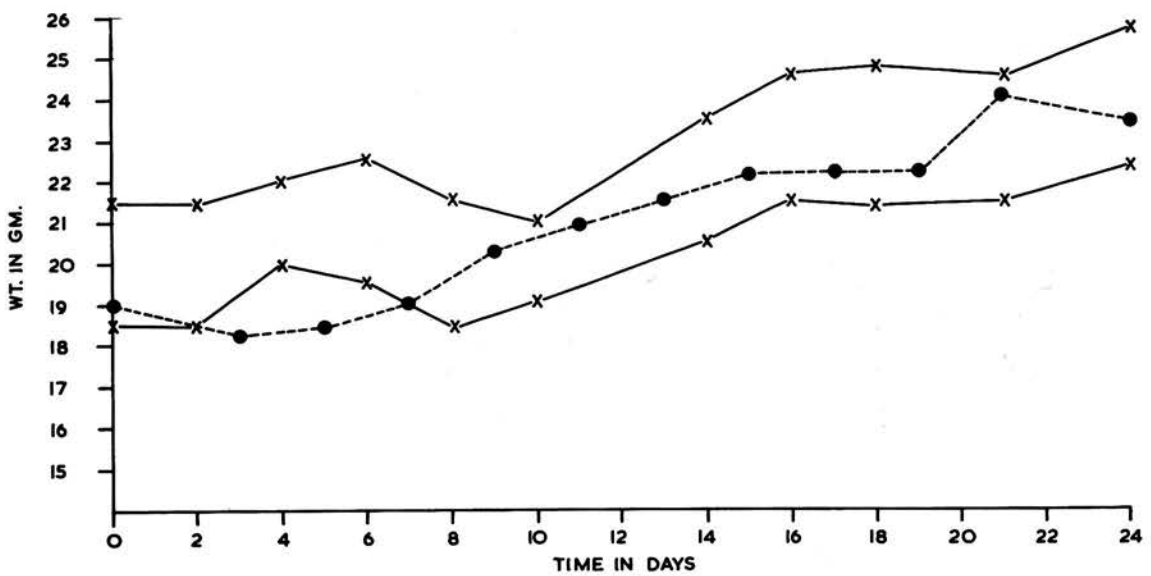

Fig. 2: Weight curve of 8 mice (solid circles) inoculated with 10^5 ascites cells I.P. and treated 48 hours later with 500 million 10 day immune isologous spleen cells. Weights of 20 normal controls taken from Fig. 1 shown by crosses.

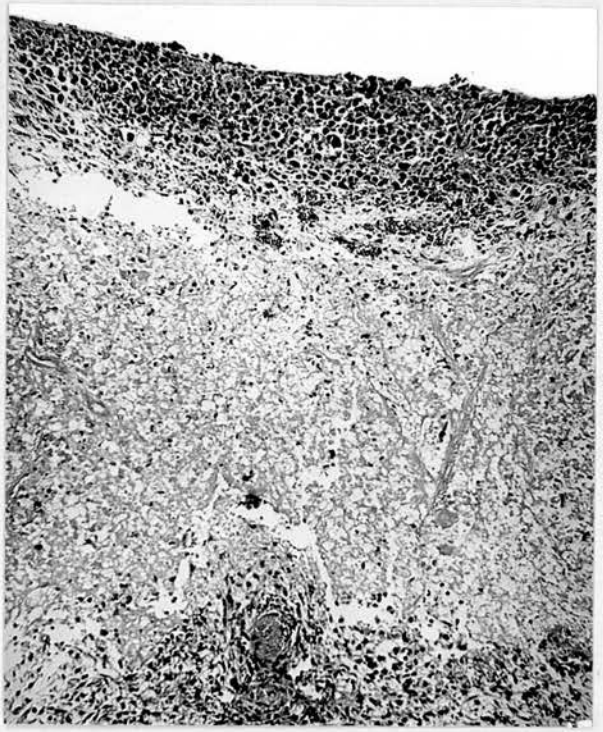

Fig. 3: Widespread necrosis in the spleen of a mouse treated with ethyl palmitate I.V. and tumour I.P., no signs of regeneration. H & E x 55.

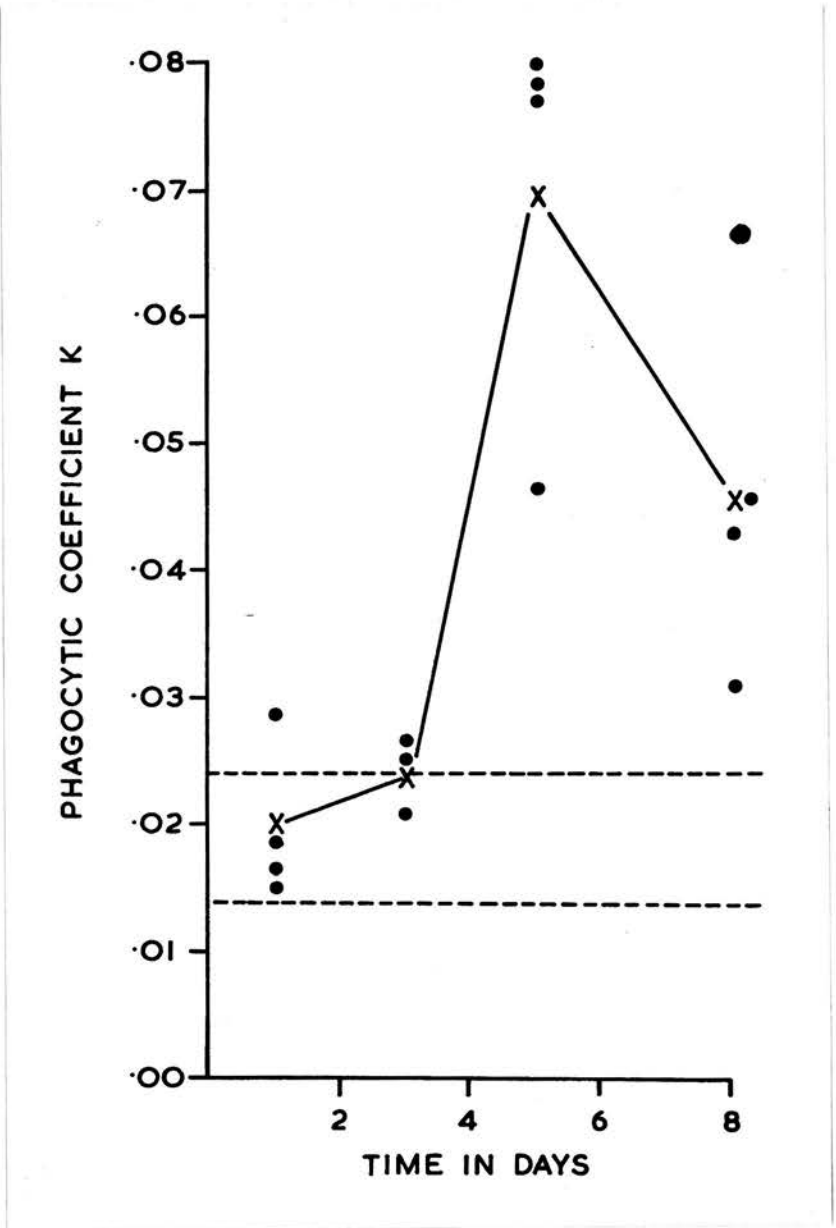

Fig. 4: Phagocytic co-efficient K in A/Jax mice inoculated with 10^7 ascites cells I.P. The dotted lines show limits of standard deviation of 13 normal controls. Each circle represents a mouse.

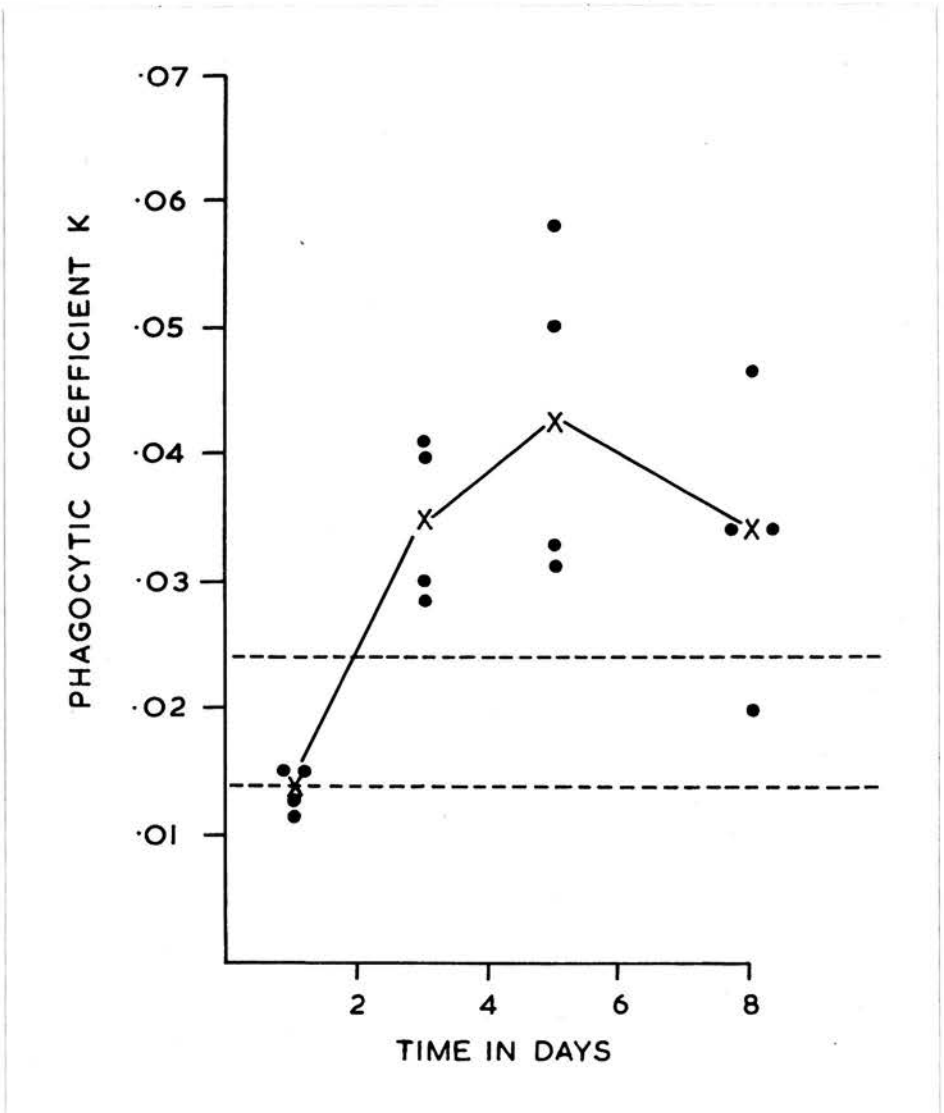

Fig. 5: Phagocytic co-efficient K in enhanced A/Jax mice inoculated with 10^7 ascites cells I.P. and immune serum. The dotted lines show limits of standard deviation of 13 normal controls. Each circle represents a mouse.

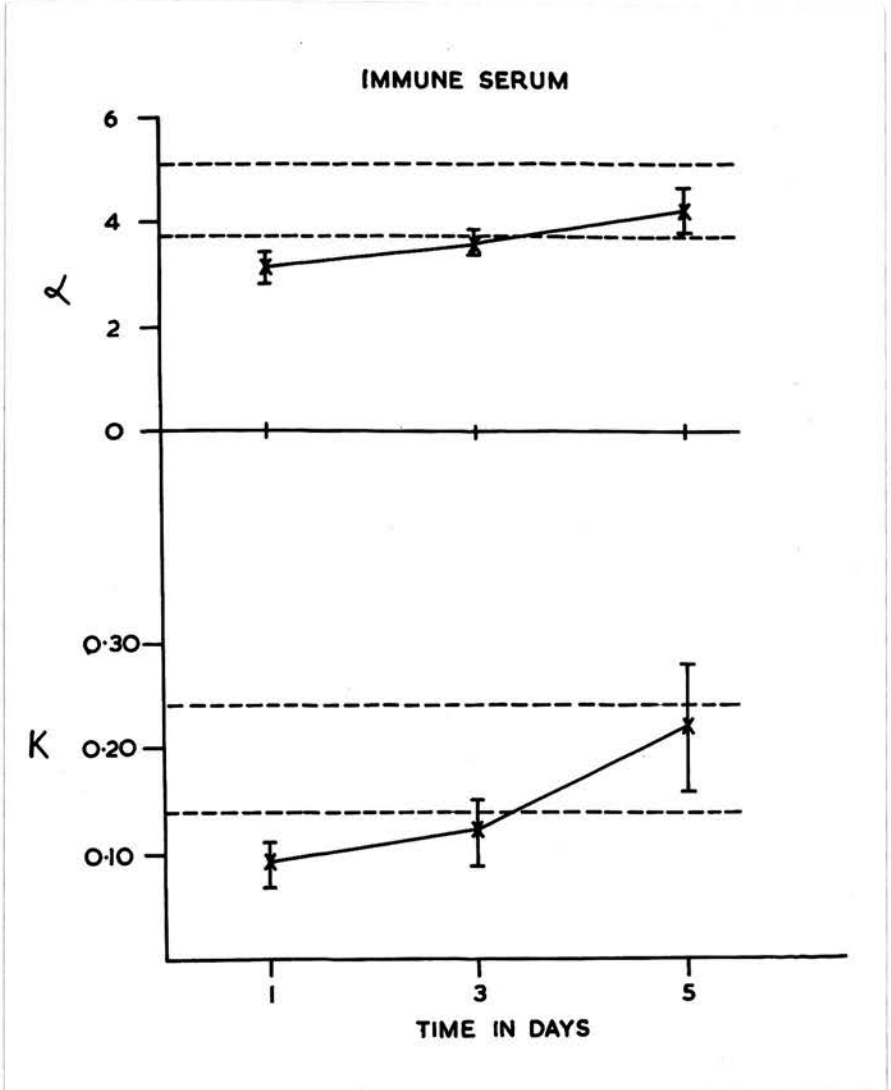

Fig. 6: Phagocytic co-efficient K and corrected phagocytic co-efficient α in mice treated with isologous immune serum. The dotted lines show the limits of standard deviation for 13 normal controls. Each cross represents the average of 4 animals.

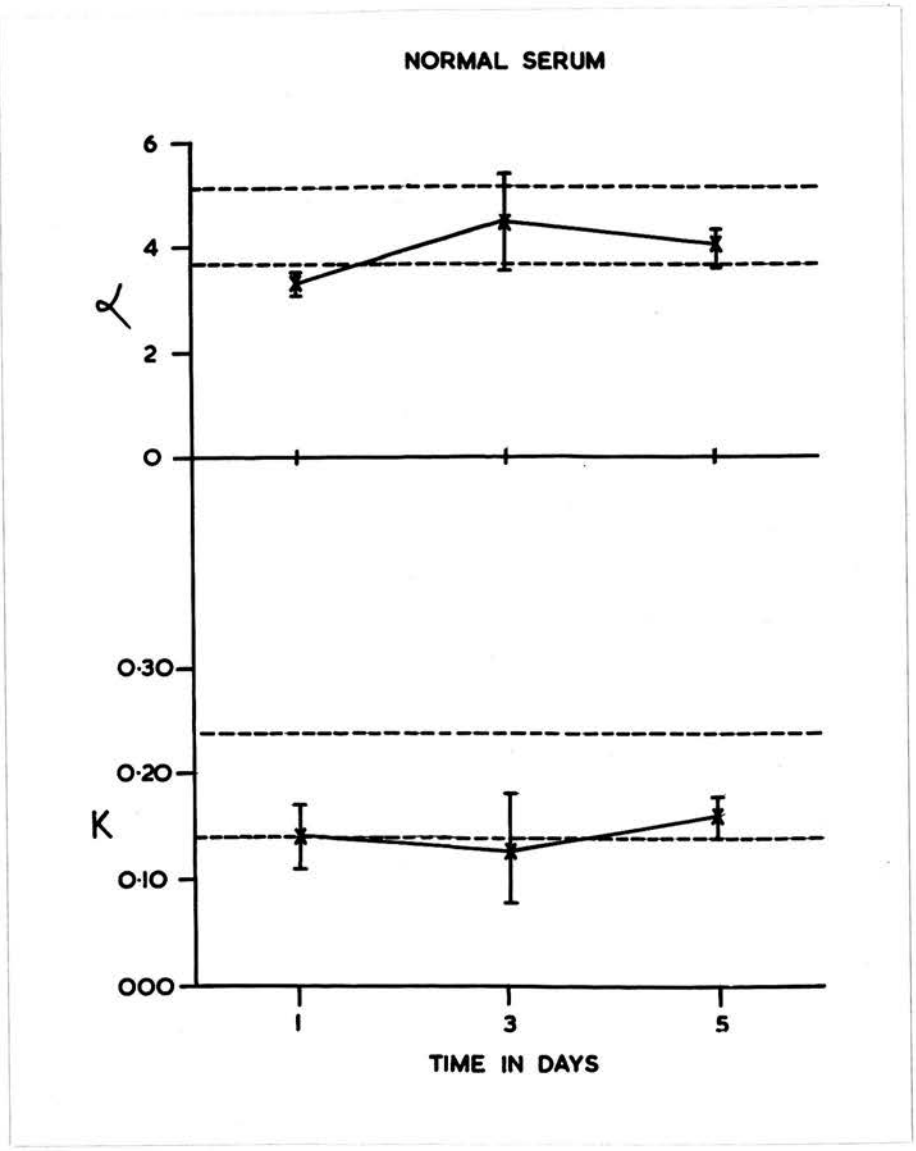

Fig. 7: Phagocytic co-efficient K and corrected phagocytic co-efficient α in mice treated with normal isologous serum. The dotted lines show the limits of standard deviation for 13 normal controls. Each cross represents the average of 4 animals.

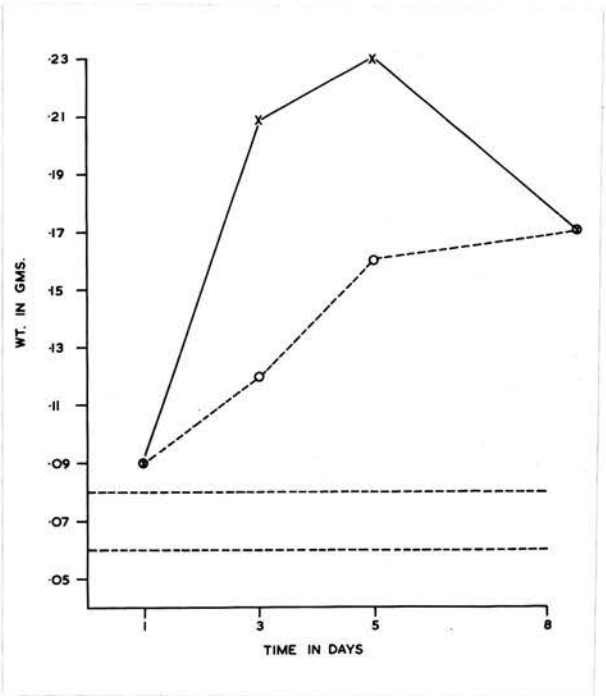

Fig. 8: Effect of passive transfer of 0.5 ml. 10 day immune A/Jax serum on spleen weight of isologous tumour bearing mice.

x - x tumour bearing controls (20 mice)

0——0 enhanced animals (20 mice)

The parallel dotted lines show the limits of standard deviation for 19 normal mice.

CHAPTER III

Inhibition and Enhancement of the Landschütz tumour by heterologous cellular system

Introduction

In the previous chapter inhibition and enhancement of the Landschütz tumour was compared in homologous and isologous systems. It was pointed out that the use of the isologous system offered a high degree of specificity. In this chapter immunization against the Landschütz tumour has been carried out in a completely foreign species. Cells and sera from immunized rabbits were passively transferred, under various experimental conditions, into mice inoculated with tumour. Inhibition or enhancement of tumour growth was demonstrated with the heterologous cells depending on the experimental manipulation. The conditions under which the enhancement occurred are discussed and contrasted with immunological enhancement obtained by the passive transfer of iso-antibody (chapter II).

It is well recognised that the transplantation of cells in a foreign host excite a strong immune reponse. The sera of animals inoculated with heterologous tissue contain antibodies which can easily be demonstrated by both in vitro and in vivo methods. Theyusually have a broad spectrum of specificity.

The cytotoxic activity of heterologous antibodies in vitro has been known for a long time. Lambert and Hanes (1911) demonstrated that a mouse sarcoma could grow

in normal rat serum but failed to grow in serum obtained from rats which had previously been inoculated with tumour. Lambert (1914) showed that such antisera were non-specific. When guinea pigs were immunized with rat embryonic skin, then their sera were toxic to rat skin and rat tumour tissue. Lumsden (1924 , 1925 , 1926) performed a series of in vitro experiments using several tumours, particularly Jensen's rat sarcoma and carcinoma M 63. He showed that heterologous antisera were cytotoxic to tumour cells and the effect was specifically anti-neoplastic rather than anti-donor tissue. His work was not confirmed by Pybus and Whitehead (1929). Niven (1929) prepared an extremely potent antiserum by repeated inoculations of rabbits with normal mouse embryo. She found that her antiserum was cytotoxic to a variety of normal mouse tissue and mouse tumours in the presence of complement. The antiserum in addition to its cytotoxic activity had slight degree of haemolytic, precipitating and complement fixing action.

Pomerat and Anigstein (1945 a and b) and Pomerat (1945) prepared sera in foreign species against lympho-reticular tissues and found that the antisera reacted with a wide variety of normal donor tissue as well as tumours. Stuart (1962a) showed that an antiserum prepared in the rabbit against guinea pig leucocytes contained haemolysin, Forssman and a variety of organ/antibodies. The antiserum

specific

produced inhibition of motility and phagocytosis in vitro. Thus the early observations on the cytotoxic action of heterologous antibodies were extended by later workers who also confirmed their wider spectrum of action. Also with advances in technology, it was possible to study the ultrastructure of cells damaged by heterologous antibody (Goldberg and Green 1959) and the biochemical lesions in such cells (Flax 1956).

With the in vivo action of heterologous immune sera the reports in the literature are less clear cut and are sometimes conflicting. One of the early in vivo experiments was neutralisation tests whereby the tumour was incubated with antiserum in vitro and later re-inoculated into a susceptible animal. Nather, (1923), treated rabbits three times, intraperitoneally, with a saline suspension of a mouse carcinoma. The sera of such rabbits did not lower the growth power of that tumour when mixed with it prior to inoculation. More recently Green (1946), using the neutralisation test showed that the serum of rabbits immunized by heterotransplants of mouse breast tumour was toxic to that tumour. By using properly constructed diffusion chambers in the peritoneal cavity of animals Algire et al (1957) demonstrated the importance of serum antibody in heterograft destruction. Nungester and Fisher (1954) reported the in vivo inhibition of a lymphosarcoma of the mouse treated with heterologous antiserum. Horn (1956) did not succeed in

inhibiting the growth of Ehrlich mouse carcinoma by the passive transfer of antiserum, prepared in the rabbit.

The relationship of spleen to tumour growth had interested several early workers probably in analogy to the significance of that organ in bacterial immunity and the fact that the spleen was a rare site for metastases. Thus Frankl (1913) observed that the mixing of spleen cells with tumour cells prior to inoculation, inhibited the growth of tumour. Mottram and Russ (1917) reported similar findings. More recently other lymphoid cells were used. Ellis and Kidd (1952) incubated Brown-Pearce carcinoma cells with lymphoid cells from rabbits that had overcome Brown-Pearce carcinoma and later resisted implantation with the tumour. They found that after this treatment the cells failed to grow when implanted in the muscles of susceptible hosts. Of more relevance to the experiments in this chapter is the demonstration of Stuart (1962b) that heterologous spleen cells were toxic to Hela cells grown in culture. He also demonstrated that the Landschütz tumour was partially inhibited by the passive transfer of rat spleen cells. Latterly (El Hassan and Stuart 1963) rabbit spleen cells were used because they seemed to exert a greater antitumour effect.

In this chapter the inhibition and enhancement of the Landschütz tumour by rabbit lymphoid cells will be

described. The disadvantage of this system is its inherent incapacity to elucidate basic problems related to host-tumour antigenic relationship. Its advantages are the precision and reliability of its antitumour effect which may give some insight into the mechanism of cell destruction by immunological mechanisms, and the elucidation of graft host relationship.

Material and Methods

Rabbits; rabbits of either sex, and weighing between 1 and 2 kilograms, were used.

Mice; a total of 2500 outbred male mice weighing between 17 and 22 gm. obtained from an M.R.C. closed colony were used in these experiments.

Propagation of tumour; Tumour was propagated in stock mice as was described in chapter I.

Inoculation of tumour in the required dose in experimental mice was performed in exactly the same manner as was described in chapter I. The mice were inspected daily, and weighed every 2 days, to the nearest gram. The onset of ascites was shown either by a gain in weight of 5 grams over a period of 2 days, or by obvious abdominal distension.

Controls; the total number of controls was 469 mice. In each experiment, the control group included at least 10 animals given tumour cells only. Because of the similarity of response in these groups the data havebeen pooled, with the exception of Table IV where the control group developed tumour rather later than usual.

IMMUNIZATION PROCEDURE; rabbits were injected with either ascites cells or a suspension of B.C.G. Three immunization schedules were used with the ascites cells. Fresh ascites fluid was collected in heparinised medium 199 and washed in 3 changes of sterile physiological saline with centrifugation at 4°C, at a speed of 1,200 R.P.M. The final centrifugation was made at this speed for 8 minutes, and the tumour deposit made to all10% v/v suspension, using medium 199 as the diluent.

The first immunisation schedule was used for the majority of experiments, viz. day 0, 0.5 ml. 10% ascites cells i/v followed by 2 intraperitoneal injections of 1 ml. on the 3rd and 7th days. The spleens and thymuses were used 7 days after the last injection. Subsequently, in this chapter, cells, or sera obtained from these rabbits are referred to as "immune".

The second immunization schedule was used to determine the optimal time for harvesting cells or serum. The rabbits were given a single intravenous injection of 0.5 ml.

of 10% ascites cells, and their serum and spleens were used 3, 7, 15 or 30 days after that injection.

The third immunization schedule was for the preparation of hyperimmune serum for serology. The first injection was 0.5 ml. 10% ascites cells i/v, followed by 0.25 ml. of a similar suspension in Freund's complete adjuvant intramuscularly. Booster doses, without adjuvant, were given at weekly intervals i/m, for 8 weeks. There were six rabbits in this group.

Immunization of rabbits with B.C.G; rabbits immunized with B.C.G. were given a single i/v injection of freeze-dried B.C.G., in a dose equivalent to 7.5 mg. wet weight of bacteria. The spleen cells from these animals were used as a control for non-specific effects consequent to immunization.

Preparation of lymphoid cells and sera; the spleens and thymuses from normal or immunized animals were removed with aseptic precautions. Cell suspensions from these organs were prepared by the method used for mouse spleen cells and described in chapter II.

Disintegration of spleen cells; freshly homogenised spleen cells were disintegrated by ultra-sound for 1-2 minutes. The cells were kept at $4^{\circ}C$, during and after disintegration.

Preparation of washed immune spleen cells and supernatant from immune spleen cells; rabbits were immunized with tumour according to schedule I. The spleens were homogenised in medium 199 and the suspension adjusted to 500 million cells in 2 ml. The suspension was centrifuged in large tubes at 1000 R.P.M. for 5 minutes. The supernatant was removed and respun at 3000 R.P.M. for 10 minutes. It was found that 1.25 ml. of supernatant were equivilant to supernatant from 500 million immune cells. The cells deposited after the first centrifugation were suspended in excess medium 199 spun at 1000 R.P.M. for 10 minutes and the supernatant discarded. The cells were finally resuspended in 20% normal rabbit serum in medium 199. The final concentration of the suspension was 500×10^6 cells per $1\frac{1}{2}$ ml.

Absorbing immune serum with normal rabbit spleen cells; A suspension of normal spleen cells was made in the usual manner. This was centrifuged for 5 minutes at 1000 R.P.M. and supernatant discarded. The cells were resuspended in 40 ml. of hyperimmune rabbit serum and the mixture was incubated at 37°C. After one hour incubation the suspension was centrifuged for 5 minutes at 1000 R.P.M., the supernatant discarded and the cells resuspended in 2% normal rabbit serum. This mixture was spun again at the same speed to remove unabsorbed immune serum. After removing the supernatant the cells were finally resuspended

in 20% normal rabbit serum.

Serum; rabbits immunized with ascites cells according to the first schedule were bled from the external ear vein, the evening before or on the morning of removing the spleens. Serum from a group of rabbits was pooled and either used on the same day or stored at -20°C until required. Hyperimmune serum was obtained from rabbits immunized according to schedule 2.

Demonstration of tumour inhibition

(1) Inhibition with cells; groups of mice were inoculated with 10^5, 10^6, 10^7 or 10^8 ascites cells by the I-P. route. They were injected 48 hours later with 500 x 10^6 spleen cells from either normal or immune rabbits. A comparison of intact and disintegrated immune spleen cells was made by inoculating tumour bearing mice with disintegrated immune spleen cells.

The effect of washed immune spleen cells and the supernatant from immune spleen cells was determined. Mice bearing a 48 hour growth of 10^5 ascites cells I-P. were inoculated with 500 x 10^6 washed immune spleen cells or the supernatant from that number of spleen cells.

To find the minimum inhibiting dose of immune spleen

cells mice bearing a 48 hour growth of 10^5 ascites cells I-P. were inoculated with 100 million or 25 million immune spleen cells.

Do normal rabbit spleen cells absorb humoral antibody and inhibit tumour growth? To answer this question mice bearing 10^5 ascites cells I-P. were inoculated with 500×10^6 spleen cells that had been incubated with hyperimmune serum as described above.

In some experiments mice bearing tumour were inoculated with thymocytes from normal or immune rabbits.

The effect of normal mouse spleen cells was compared with heterologous lymphoid cells. Mice bearing 10^5 ascites cells were treated 48 hours later with 500×10^6 normal intact or disintegrated mouse spleen cells.

(2) Inhibition with serum; Immune rabbit serum prepared according to the first immunisation schedule was injected I-P. in doses of 1, 0.5, or 0.1 ml. into mice inoculated 48 hours previously with 10^5 ascites cells. Control mice bearing tumour were inoculated with 1 ml. of normal serum. The effect of 1.0 ml. of immune serum was studied in mice given either 10^6 or 10^7 ascites cells I-P. 48 hours before.

Comparison of the effect of immune spleen cells and serum at time intervals following a single intravenous injection of ascites cells.

Spleen cells and serum from rabbits given a single intravenous injection of tumour (immunization schedule 2) were harvested at intervals of 3, 7, 15 and 30 days. The cells were injected in a dose of 500×10^6 into mice given 10^5 ascites cells 48 hours previously. A single injection of serum was given in doses of 1.0 ml., 0.5 ml., or 0.1 ml. to mice similarly inoculated with tumour.

Demonstration of tumour enhancement.

In these experiments both heterologous and homologous cells were used. Groups of mice were inoculated with 500×10^6 normal rabbit spleen cells 5, 2, and 1 day before an intraperitoneal injection of 10^7 ascites cells. Two other groups of mice were given the same dose of spleen cells one and two days after injection of tumour. The controls were injected with ascites cells only and all mice were given the same tumour from a single donor.

Immune heterologous spleen cells were given in a similar dose 24 hours before the intraperitoneal inoculation of 10^7 ascites cells. In a confirmatory experiment mice were given heterologous or homologous spleen cells 24 hours

before the inoculation of 10^7 ascites cells. 4 days later the peritoneal fluid from test and control groups was added to 10 ml. of heparinised medium 199. The packed cell volume was measured in haematocrit tubes and differential cell counts made on Giemsa stained smears.

Measurement of growth rate of tumour from "enhanced" animals when injected into normal mice; one group of mice was injected with 10^7 ascites cells from mice showing enhancement and another with the same number of cells from control tumour bearing mice for that experiment. On the sixth day the mice were sacrificed and the fluid analysed as described.

Serology

Measurement of antibody in sera of rabbits injected with ascites cells.

Ascites cell agglutination; 0.2 ml. volumes of ascites cells in a concentration of 30,000 to 40,000 cells per mm^3. were added to doubling dilutions of an equal volume of immune rabbit serum. Dilutions of normal rabbit serum in phsyiological saline served as controls. The tubes were incubated at 37^0C for 30 minutes and the highest serum dilution showing strong agglutination was the end point.

Erythrocyte agglutination; sera were inactivated at

56°C for 30 minutes. Normal mouse red cells were washed three times in physiological saline and made to a 1% v/v suspension. This suspension was added to normal and immune serum prepared in doubling dilutions in perspex agglutination trays. These were incubated at 37°C for 45 minutes and then left on the bench for a further 45 minutes. They were read under the low power of the microscope. Compact groups of 8-12 cells were taken to indicate agglutination.

Washed sheep red cells were similarly prepared, the controls in this instance were normal rabbit serum and immune rabbit serum inactivated and absorbed with an equal volume of packed sheep red cells for 1 hour at 37°C.

Preparation of antigens; for other serological tests the tissues used were ascites tumour cells, mouse liver, spleen and normal serum. The tissues were cut into thin slices, washed with ice cold saline, minced and washed again. A 20 per cent tissue suspension in 0.9 per cent saline was homogenised for 3-5 minutes at 4°C and then submitted to ultrasonic disintegration for 2-3 minutes. The suspension was spun at 2000 R.P.M. for 30 minutes and the supernatant used as antigen.

Complement fixation; A standard technique using 2½ M.H.D. 50 of complement was used. Complement was always titrated in the presence of antigen. The end

point was taken at 50 per cent haemolysis.

Gel diffusion. A double diffusion technique after Oakley and Fulthorpe (1953) was used. The bottom layer consisted of antiserum and agar, the middle of 0.8 cm. column of agar alone and the top antigen and agar.

The in vitro cytotoxicity of immune serum; 0.2 ml. volumes of ascites cells in a concentration of 20,000 cells per c.mm. were added to doubling dilutions of an equal volume of immune rabbit serum. 0.1 ml. of fresh guinea pig serum was added to each tube. The tubes were incubated at 37°C for 30 minutes. Dilutions of normal rabbit serum in physiological saline with complement and tumour cells in saline with and without complement served as controls. After incubation cytotoxicity was read under phase contrast microscopy. Tumour cells showing toxic changes were swollen and had "blisters" forming on the surface. The nuclear membrane was sharply defined. The end point was taken at the dilution where 50% of the cells showed toxic changes.

Tanned cell haemagglutination; A standard technique (Boyden, 1951) was used. In every titration the controls included tanned cells + test serum and antigen coated cells + saline. The optimal concentration of antigen for coating the cells was determined in every case. Sheep red cells were used in the test and sera were routinely absorbed.

Effect of heterologous cells on the capacity of the host to form antibodies against other antigens.

Groups of mice were given either 500 x 10^6 immunised or normal spleen cells I-P. followed 24 hours later by 0.1 ml. of a 10 per cent suspension of washed sheep red cells (Burroughs Wellcome formolised cells), intravenously. Blood samples were taken from the retro-orbital venous plexus and sheep red cell agglutinating antibody titres measured.

Doubling dilutions of serum starting from 1 in 8 were made in 0.1 ml. volumes and an equal amount of 1 per cent red cell suspension added in perspex dilution trays; the mixtures were incubated at 37°C for 45 minutes and at room temperature for a further 45 minutes. The agglutination was read by naked eye and microscopically.

Effect of heterologous spleen cells on the phagocytic function of the reticulo-endothelial system (R.E.S.).

Following the I-P. administration of 500 x 10^6 immune spleen cells the phagocytic index K and the corrected phagocytic index α were determined, at intervals of 1, 3 and 5 days. The carbon clearance method was used as described in chapter 1.

Thiotepa (Triethylenethiophosphoramide)

This was given I-P. to groups of ten mice in a single dose of either 2, 4, or 8 mg/Kilo of body weight. The animals had been injected I-P. 48 hours previously with either 10^5, 10^6 or 10^7 ascites cells.

Autopsy examination

All animals were subjected to autopsy examination and tissues were fixed in 10% neutral formol saline for histological examination. Treated animals surviving for 90 days were chloroformed and detailed postmortem examination was carried out.

Results

In the series of 202 untreated control mice (Table I) it is seen that the majority of mice developed ascites by the tenth day after tumour inoculation and died with remarkable consistency around the twentieth day. In the higher dose of tumour inoculum these figures were slightly shortened. On the other hand animals treated with immunised spleen cells developed ascites much later, if at all, and their survival time was considerably prolonged. This applied only to the tumour dose of 10^5 or 10^6 ascites

cells and no beneficial effect was noted with the higher doses of 10^7 and 10^8. In the latter instances the mice appeared worse than did the controls and this suggested acceleration of tumour growth although the statistics of the survival times do not support this impression. Spleen cells from non-immunised rabbits had little or no effect when given after the tumour.

The weights of mice inoculated with 10^5 ascites cells intraperitoneally showed a sudden increase about the tenth day due to accumulation of ascitic fluid (Fig. 1). The weight curve of mice given 10^5 ascites cells and treated with immunised spleen cells showed an initial drop in weight but afterwards remained within the limits of standard deviation for weights of normal mice. Weight curves of mice bearing 10^5 ascites cells and treated with normal rabbit spleen cells also showed an initial weight loss to a similar degree but developed ascites at the same time as untreated control animals inoculated with 10^5 ascites cells (Fig. 2).

Table II shows that when the immune spleen cells were disintegrated by ultrasound their capacity to delay the onset of ascites or extend survival times was markedly reduced. In the low tumour dose range of 10^5 ascites cells there was a slight increase in survival time, partly contributed to by one animal which survived for 55 days

with solid tumour.

The effect of washed immune rabbit spleen cells and supernatant from immune spleen cells on tumour growth; out of 10 mice bearing a 48 hour growth of 10^5 ascites cells and treated with 500 x 10^6 washed immune spleen cells, 6 of the mice survived for 90 days and the remainder survived for an average of 50 days. The supernatant from 500 x 10^6 immune rabbit spleen cells had no effect on the growth of the ascites tumour inoculated in a dose of 10^5 ascites cells I-P. 48 hours previously.

The minimum inhibitory dose of spleen cells; Table III shows that 100 x 10^6 and 25 x 10^6 immune rabbit spleen cells had no effect on the growth of the tumour. Consequently the effective dose of 500 x 10^6 was used in all experiments while the dose of tumour was varied as described above.

The capacity of normal rabbit spleen cells, incubated with immune serum, to inhibit tumour growth.

All 9 mice bearing 48 hour growth of 10^5 ascites cells and treated with rabbit spleen cells that had been incubated with immune serum, died with tumour at the same time as the untreated tumour bearing controls.

The effect of thymocytes on the tumour; thymocytes from normal or immune rabbits had no effect on the growth of the tumour (Table IV).

The effect of normal mouse spleen cells on the growth of the tumour; Table V shows that normal mouse spleen cells had no effect on the growth of the tumour.

The effect of B.C.G. immunised rabbit spleen cells on the growth of the tumour; All 11 mice bearing a 48 hour growth of 10^5 ascites cells I-P. and treated with 500 x 10^6 B.C.G. immunised rabbit spleen cells, developed ascites and died at the same time as untreated controls.

The effect of immune rabbit serum (schedule 1) on the ascites tumour; Immune rabbit serum was remarkably free from toxicity to the mice. Large amounts were more effective than small in inhibiting the tumour (Table VI). No enhancement of tumour growth was noticed with the smaller dose of serum. With 1 ml. of immune serum some mice were free of tumour at 90 days. It is noteworthy that some mice treated with high serum doses survived longer than the controls but eventually died of solid tumour. As the amount of antiserum was decreased the percentage of animals dying with tumour ascites increased. Within these dose limits there was a correlation between increase in dose of serum and conversion of the tumour to the solid form.

In another experiment 1 ml. of normal rabbit serum had no effect on 10^5 ascites cells inoculated 48 hours previously.

Table VII shows that 1 ml. of immune serum had no effect on a tumour dose of 10^7 ascites cells inoculated in mice 48 hours previously. With the smaller dose of 10^6 ascites cells 8 out of 9 mice developed solid tumour. These mice survived for more than twice as long as the controls possibly because of conversion of the tumour to the solid form.

Optimal time for collection of immune serum and spleen cells from rabbits immunized with a single intravenous injection of ascites cells; The day 3 serum was inert in all doses (Table VIII). Both the 7 and 15 day sera showed marked activity even in the 0.1 ml. dose. The 30 day serum showed a diminished potency although the largest dose of 1.0 ml. prevented ascites in 6 out of 7 mice. It is noteworthy that 4 of these non-ascitic mice developed solid tumour. Solid tumours were also obtained with 7 day immune serum, where their frequency increased as the dose of serum decreased.

Spleen cells harvested at intervals after a single intravenous injection showed different effects (Table IX) on the ascites tumour. Cells taken at 3 or 30 days had no cytocidal effect whereas those obtained on the seventh day showed the greatest degree of inhibition noted in these experiments. 9 out of 9 mice were free from tumour at necropsy 90 days after inoculation of ascites cells.

Enhancement of the Landschütz tumour

Table X shows that normal rabbit spleen cells injected into mice by the intraperitoneal route produced enchancement of tumour when the latter was inoculated 1 or 2 days after treatment with the spleen cells. The treated groups developed ascites more rapidly and died much sooner than the controls. When the animals bearing a high dose of tumour were subsequently treated with spleen cells the degree of enhancement was not so marked. Although the statistics do not reveal a significant difference in survival time between control and test groups, the latter appeared to develop ascites more rapidly than usual.

In another experiment a similar degree of tumour enhancement was obtained in mice treated with 500×10^6 immune spleen cells 24 hours before 10^7 ascites cells.

Analysis of the fluid obtained from mice treated with rabbit spleen cells 24 hours before an intraperitoneal injection of 10^7 ascites cells, showed a bigger volume of ascitic fluid and more tumour cells than the controls (Table XI). A similar increase in tumour cells was also observed in mice treated with mouse spleen cells but not with normal rabbit serum. Differential counts on the ascitic fluid from all groups showed that the increase in cell numbers was due entirely to the presence of tumour cells. It is noteworthy that animals given

spleen cells alone yielded only 14 x 10^6 cells. These were mainly peritoneal macrophages.

Table XII shows that tumour cells removed from enhanced animals and inoculated into normal mice grew at the same rate as did the controls. Both the volume of the ascites fluid and number of tumour cells in test and control groups were remarkably similar when removed and counted 6 days after injection of ascites cells.

Phagocytic activity of the reticulo-endothelial system in mice showing enhancement.

Fig. 3 shows that the phagocytic indices in animals treated with spleen cells were reduced for three days. Recovery commenced by the 5th day when some animals showed an increased index and others were still depressed.

Response of mice pretreated with rabbit spleen cells to sheep red cells.

Treatment with lymphoid cells delayed the synthesis of antibody to foreign red cells (Fig. 4). This was most evident after the injection of spleen cells from immune rabbits. Normal spleen cells also diminished the antibody titres but to a less degree.

Serology

The serology showed that the 14 day immune serum (schedule 1) was relatively inert in the in vitro tests. Low titres were recorded with complement fixation (Table XIII) and the tanned cells (Table XIV) gave negative results. The immune serum agglutinated mouse red cells to a titre of 1/16 and sheep cells to 1/2. After absorption with sheep red cells these titres were reduced to 1/8 and zero respectively. The cytotoxicity test titres were the same as the complement fixation titres.

Thiotepa; (Table XV) shows that thiotepa had no inhibitory action on the ascites tumour even when administered in the high dose of 8 mg/Kilo.

Discussion

It is clear that immune rabbit spleen cells could inhibit the growth of the Landschütz ascites tumour when the latter was inoculated in doses of 10^5 or 10^6 cells 48 hours before. The spleen cells must be intact to produce this effect. This inhibitory action of immunised cells may be interpreted in several ways. It is conceivable that the spleen cells, introduced in great excess to the peritoneal cavity, produced their effect by

competing with the ascites cells for nutrients. This does not seem to be the case since no effect was observed with either normal cells or those derived from spleens stimulated by the injection of B.C.G. Enzymes and products from necrotic spleen cells cannot be of significance in this system because disintegrated normal lymphoid cells had no effect on the tumour.

Since the supernatant from immune cells was ineffective in suppressing the tumour, and washed immune spleen cells behaved like unwashed cells it follows that carry over of circulating antibody does not account for inhibition of the tumour. This view is further supported by the experiments with normal cells soaked in immune serum which showed that these cells had no measurable inhibitory activity. While disintegration of the immune cells markedly reduced their inhibitory action it did not abolish it completely; the average survival of mice treated with disintegrated immune cells being slightly prolonged. This was probably due to antibody released during the disintegration of the spleen cells. The marked difference of activity between disintegrated and intact cells stresses the importance of the latter in tumour rejection under these experimental conditions. However, one cannot eliminate the possibility of enzymatic degradation of antibody caused by disintegration of the cells. The lack of activity of the rabbit thymocyte is

not surprising since most workers are agreed that little or no antibody is formed in the thymus.

One cannot tell which type of cell in the spleen is responsible for inhibition of the ascites tumour although the lymphocyte is most likely in view of its well known immunological competence. (Lancet 1962). As long ago as 1912 Da Fano noted the importance of the lymphocyte in the rejection of solid transplantable tumours. More recently Ellis and Kidd (1952) and Weaver (1957) have stressed the important role of the lymphocyte. However the lymphocyte does not play the dominant role in the destruction of all tumours. In the rejection of the ascites sarcomata and lymphomas histiocytes appear most important. (Amos, 1961; Journey and Amos 1962; Gorer 1956). The latter made a histological study of the mechanisms of rejection of tumour homografts and found that the host's cellular response varied with different types of tumours. In some the host reaction was predominantly of lymphocytes and plasma cells. In other tumours, and especially the ascites sarcoma inoculated subcutaneously, histiocytes were intimately associated with the tumour cells during their rejection. However with leukaemic cell homografts the host reaction was mainly exudative and the tumour cells were destroyed before host cells were seen at the site of injection.

The minimum number of immune heterologous spleen cells

required to inhibit 10^5 ascites cells was not accurately determined. 100×10^6 immune spleen cells were not effective. This was in marked contrast to the effect of isologous and homologous spleen cells where smaller doses of immunised cells were effective. (Chapter II). The effect of DBA_2 spleen cells was particularly striking; 20 million cells produced marked inhibition in homologous hosts. The differences in the killing capacity of heterologous and homologous lymphoid cells may be due to differences in survival in the recipients. A contributory factor could be the production of some degree of graft versus host reaction in mice receiving heterologous cells. In these experiments there were no obvious signs of toxicity in mice transfused with heterologous cells. Graft versus host disease would be a grave danger in homologous or heterologous systems if the cells are allowed to persist in the host. This complicated the experiments of Wigzell (1961) who transplanted parental cells into F_1 hybrid animals bearing tumour. Woodruff and Symes (1962) encountered graft versus host disease when they treated mouse mammary carcinoma with homologous lymphoid cells. The immune rabbit serum was also quite effective in inhibiting the Landschütz ascites tumour and there was a curious discrepancy between the high in vivo activity and the extremely low in vitro titres obtained by sensitive immunological methods. Heterologous antisera are devoid

of specificity and the sera of rabbits immunised against the Landschütz tumour reacted in vitro with several of the mouse normal organs.

There are several reports in the literature on the use of heterologous serum in studies with transplantable tumours. These studies were performed both in vitro and in vivo and reports on the latter were sometimes contradictory. Niven (1929) described cytotoxic changes in cultures of normal and neoplastic mouse tissues produced by hyperimmune serum prepared in the rabbits. Toxic changes in the cells were shown by arrest of mitotic activity, swelling of the nuclei, Brownian movement of cytoplasmic granules and "blebbing" of the cytoplasm. Lumsden (1925) showed that serum from rabbits and rats immunised with mouse tumour tissue was toxic for explants of the tumour but not for explants of other normal tissues. Pybus and Whitehead (1929) found that the sera of rabbits immunised with mouse tumour was also toxic to mouse heart and kidney cells. Harris (1943) immunised rats against a pure strain mouse sarcoma. These sera were toxic to the tumour cells in culture as well as other normal mouse tissues. Cytotoxicity with normal tissues was demonstrable whether these were of the same strain of mice that donated the tumour for immunisation or of a foreign strain. More recently Mountain (1955) described the effects of rabbit anti-HeLa cell serum on these cells in tissue culture in

the presence of complement. Goldstein (1957) immunised rabbits against 4 different human cell strains of normal and malignant origin. He showed that the antisera had species but not tissue specificity. Goldberg and Green (1959) showed that the exposure of Krebs ascites tumour cells to immune rabbit serum and complement produced biochemical changes in the cells. They also studied the ultrastructure of cells incubated with antiserum. Heated antiserum caused changes in the cell membrane which became foamy and folded. The multiple finger-like processes observed were believed to represent two sites of attachment of antibody. In the presence of complement the antibody produced severe damage to the interior of the cells. Chemical studies on cells injured by heterologous antibody and complement showed striking changes. The cells became more permeable and Potassium leaked out and Sodium passed in more rapidly (Green et al 1959). Ellem (1958) using the Ehrlich carcinoma cells showed that immunologically injured cells lost phosphate. Amino acids were also lost from the cells (Green et al 1959). Kalfayan and Kidd (1953) and Colter et al (1957) reported loss of RNA from cells damaged by immune serum and complement.

Metabolic changes in antibody-complement treated cells were also detected. Thus, Flax (1956) showed that the utilization of glucose in such cells was interrupted while

the metabolism of succinate was unimpaired. This could be due to loss of soluble glucolytic enzymes from the cytoplasm and retention of the particulate succinic dehydroganose system of the mitochondria (Green and Goldberg 1960). Colter et al (1957) found that Ehrlich ascites carcinoma and 6C 3HED ascites tumour, incubated with antibody and complement, could not utilise C^{14} labelled glycine.

Thus in vitro methods indicate that heterologous antibody and complement act primarily on the cell membrane producing increased permeability. This allows the passage of water and electrolytes into the cell with consequent swelling and escape of macromolecules from the cells.

Varying degrees of success in inhibiting tumours were reported with the use of heterologous sera in vivo. Nettleship (1945) reported regression produced in the Murphy lymphosarcoma treated in vivo with antiserum. Mungester and Fisher (1954) reported the inactivation of a mouse lymphosarcoma by the passive transfer of heterologous antibody prepared in the rabbit. Mice were inoculated with serum at the same time as the tumour followed by 3 doses of serum at 48, 96, and 144hours. When the serum administration was delayed for 96 hours after tumour inoculation no protective effect was observed. Flax (1956) showed that the injection of gamma globulin from rabbits immunised against Ehrlich ascites

tumour could prolong the life of mice to about twice that of the controls. Horn (1956) in a detailed analysis of the Ehrlich ascites cell, prepared an antiserum to the intact ascites cell or its fractions. Repeated injections of these sera in mice given tumour did not greatly alter the mean survival time. Levi (1963) made an antiserum against AKR/Jax mouse leukaemia in rabbits rendered tolerant to normal AKR/Jax tissues. This serum only slightly prolonged the life of mice bearing the lymphosarcoma.

The marked in vivo activity of immune rabbit serum against the Landschütz ascites tumour made it possible to investigate the relative role of immune cells and serum in tumour inhibition. After a single intravenous dose of fresh intact ascites cells into rabbits the activity of the cells and serum parallelled each other within the dose used. This was maximal 7 days after the immunising injection. Thereafter the activity of the cells gradually diminished while the serum was still effective to some extent 30 days later. It is reasonable to assume that the antibody generated by the spleen cells was released into the blood and that circulating and cell bound antibody are qualitatively the same. This view is consistent with the morphological findings in immunised spleens which show cells with pyronophilic cytoplasm and also small spherioles of pyronophilic material, presumably derived from the latter,

and lying free in the lumen of vessels. The failure of this experimental model to distinguish between circulating and cell bound antibody may be a characteristic of the heterologous system. On the other hand the method of immunisation and nature of the antigen may be critical. The importance of the manner of immunisation has been already discussed in the isologous system. (Chapter II).

The necropsy findings show that prolongation of survival time as an index of tumour inhibition must be regarded with caution. In some cases the treatment with cells or serum converted the tumour from the ascitic to the solid form and altered completely the natural history of the disease. Untreated mice died from the progressive accumulation of fluid and tumour cells within the peritoneal cavity; this led to respiratory difficulties, compression of the intestine and terminal malnutrition. With the solid type of growth death was usually caused by the local penetration of a hollow viscus or large bowel obstruction from a tumour growing in the root of the mesentery.

Tumour enhancement was first suspected when mice were inoculated with a large dose of tumour (10^7 cells) followed by 500×10^6 spleen cells 48 hours later. Though their survival was not significantly altered they appeared to develop ascites earlier than the untreated controls, and clinically appeared worse. When the spleen

cells were given before the tumour there was a drastic reduction in the survival times of the mice. This was confirmed in other experiments by finding a higher tumour volume in mice showing enhancement than in untreated tumour bearing controls. A similar degree of enhancement was noted in mice treated with normal mouse spleen cells. The behaviour of immunised rabbit spleen cells given before 10^7 ascites cells differed markedly from that of isologous immune spleen cells transferred under similar conditions. Whereas the former produced enhancement, isologous spleen cells conferred some degree of immunity (chapter II).

Enhancement with heterologous cells is different from immunological enhancement produced by the passive transfer of specific antiserum (Kaliss and Molomut 1952). Immunological enhancement of the Landschütz tumour has already been mentioned in chapter II and its possible mechanism was discussed. Because the enhancement observed with rabbit spleen cells was demonstrated equally well with either immune or non-immune cells it could not be due to carry over of immune serum. Since the transfusion of heterologous cells depressed profoundly the immunological status of the host as judged by the diminished phagocytic co-efficient K and partial suppression of antibody response to foreign red cells, one may conclude that this is sufficient to temporarily inhibit the host response to any iso-antigenic differences between it and the ascites tumour.

This removal of immunological constraint, even for a short time, was sufficient to enhance the growth of the tumour cells. That enhancement could not be maintained on subsequent homografting of the tumour is in favour of the theory that the treatment acted on the host rather than on the graft.

The enhancement reported in the literature after treatment of the hosts with vital dyes and certain colloids may be similar to enhancement with heterologous cells. These agents are known to block the reticulo-endothelial system. Ludford (1932) showed that the treatment of mice with trypan blue, vital red or an inorganic colloid prior to tumour transplantation led to abrogation of resistance of the mice to the tumour. Foulds (1932) noted that rabbits treated with trypan blue were more susceptible to the Brown-Pearce tumour. Andervont (1932, 1936) working with mouse sarcoma 180 noted that injections of trypan blue before tumour inoculation in mice immunised against this tumour, led to a lowering of their resistance. He also showed that tumours of strain A or C_3H origin which normally regressed in strain D mice, grew progressively if the latter were treated with trypan blue prior to tumour inoculation. Saphir and Appel (1943) showed that prolonged administration of trypan blue in rabbits immunised against the Brown-Pearce carcinoma destroyed their immunity. The trypan blue was started 3 days before tumour inoculation

and continued daily for 15 days.

Kaliss and Borges (1952) compared the effect of prior injections of lyophilized tumour tissue and trypan blue on the resistance of the host to tumour transplants. They utilised a strain specific mouse tumour that normally regressed in alien mouse strains. They found that trypan blue alone had no effect, while lyophized tumour tissue abrogated the resistance. They noted however a synergestic action between lyophilized tissue and trypan blue in preparing animals for enhancement.

The blockade of the phagocytic function by inert colloid is of short duration and is often followed by a phase of hypertrophy (Stiffel 1959). In all the work cited on enhancement no measurement of phagocytic function was made and R.E.S. blockade was only assumed.

Table I

The effect of 5 × 10^4 immunized or normal intact rabbit spleen cells on graded doses of ascites cells given to mice by the I-P. route.

	Dose of Tumour	No.of Mice	Treatment	No. with Ascites	Time of onset of ascites (days)	Apparent cures	Survival of the remainder (days)
Untreated control mice	10^5	97	Nil	97	11 ± 2	Nil	21.5 ± 4
	10^6	43	Nil	43	10 ± 3	Nil	20 ± 3.5
	10^7	43	Nil	43	7.5 ± 2.3	Nil	19 ± 4.2
	10^8	19	Nil	19	5.8 ± 2.2	Nil	15 ± 5.7
Mice treated with cells	10^5	36	Immune cells	6	36.5 range 13-65	23	52
		13	Non-immune cells	13	10.8 ± 2.8	Nil	26.5 ± 3.5
	10^6	18	Immune cells	12	9 ± 3	6	25 ± 8.5
		16	Non-immune cells	16	9.1 ± 2.6	Nil	20.5 ± 5.2
	10^7	24	Immune cells	24	6.0 ± 1.0	Nil	18 ± 4
		10	Non-immune cells	10	5.3 ± 1.0	Nil	16.7 ± 5.4
	10^8	9	Immune cells	9	4.3 ± 1.0	Nil	13.8 ± 5.4
		9	Non-immune cells	9	5.4 ± 0.7	Nil	17.6 ± 3.6

± = S.D.

Table II

TABLE

The effect of disintegrated immune or non-immune rabbit spleen cells on graded doses of ascites tumour given to mice by the I-P. route.

Dose of Tumour	No. of Mice	Treatment	No. with Ascites	Time of onset of ascites (days)	Apparett cures	Average survival of remainder (days)	Survival of controls (days)
10^5	14	Immune cells	13	13 ±	Nil	29.6 ±	21.5
	11	Non-immune cells	11	12.9 ± 4.1	Nil	21.4 ± 4.1	
10^6	9	Immune cells	9	10 ± 1.4	Nil	21 ± 3.6	20
	9	Non-immune cells	9	9 ± 1.9	Nil	19 ± 6.3	
10^7	10	Immune cells	10	7.3 ± 1.1	Nil	18.2 ± 5	19
	10	Non-immune cells	10	7.5 ± 1	il	18 ± 5	

TABLE III

The effect of 25 or 100 million immune rabbit spleen cells on ascites tumour cells inoculated into mice by I-P. route

Dose of tumour	No. of mice	Dose of spleen cells	No. with ascites	Average onset (days)	Survival (days)
10^5	17	100 10^6	17	1.5 ± 2	6 ± 5
	18	25 x 10^6	18	10 ±0.5	25 ± 3
	8	nil	9	9 ± 0.5	21 ± 6

Table IV

The effect of 50×10^6 thymocytes from non-immune or immune rabbits on 10^5 ascites cells given to mice by the I-P. route.

Dose of tumour	No. of Mice	Treatment	No. with Ascites	Average onset	Average survival
10^5	10	Nil	10	15.0 ± 2.3	27.1 ± 3.5
10^5	10	Non-immune Thymocytes	10	16.1 ± 4.1	26.4 ± 3.8
10^5	9	Nil	9	10.3 ± 1.3	21.0 ± 6.1
10^5	8	Immune Thymocytes	8	11.3 ± 0.8	21.0 ± 3.3

± = S.E.

Table V

Effect of Normal mouse spleen cells on ascites tumour.

Dose of Tumour	No. of Mice	Treatment	No. with Ascites	Average onset of Ascites	Average Survival	Average onset of ascites of controls	Average Survival of Controls
10^5	16	500×10^6 Intact Mouse spleen cells	16	12 ± 1.9	24.3 ± 4.4	11 ± 2	21.5 ± 4
10^5	13	500×10^6 Disintegrated mouse spleen cells	13	10.2 ± 2.5	20.3 ± 2.6		

Table VI

The in vivo effect of graded doses of immune rabbit serum on 10^5 ascites cells given to mice 48 hours previously by the I-P. route.

Treatment	No. of Mice	No. with Ascites	Average onset of Ascites (days)	No. with Solid Tumour	Apparent cures	Survival of Remainder (days)
Untreated control animals from Table I	97	97	11 ± 2	Nil	Nil	21.5
1.0 ml. serum	16	1	13	9	6	16.5
0.5 ml. serum	10	3	23.3	7	2	51
0.3 ml. serum	9	5	15	6	Nil	40
0.1 ml. serum	10	8	13.2	2	Nil	26.2

± = S.D.

TABLE VII

The effect of 1.0 ml. of immune serum on graded doses of ascites tumour cells given 48 hours previously to mice by the I-P. route

Dose of Tumour	No. of mice	No. with Ascites	Average onset of Ascites	No. with Solid Tumour	Apparent cures	Average Survival of remainder (days)	Survival in days of untreated controls from Table I
10^5	16	1	13	9	6	46.5	21.5 ± 4
10^6	9	1	15	8	1	52	20 ± 3.5
10^7	9	9	14	nil	nil	24	19 ± 4.2

t = S.D.

Table VIII

Relation of time of collection of immune serum to its in vivo effect on 10^5 ascites cells given to mice I-P. Serum collected at intervals of 3, 7, 15 and 30 days after a single I.V. injection into rabbits of mouse ascites cells.

Dayof collection of Serum	1 ml. serum						0.5 ml. serum						0.1 ml. serum						Survival in days of 97 untreated controls (from Table I)
	No. of Mice	No. with Ascites	Average onset of Ascites Days	No. with Solid Tumour	Apparent cures	Survival of remainder	No. of Mice	No. with Ascites	Average onset of Ascites Days	No. with Solid Tumour	Apparent cures	Survival of remainder	No. of Mice	No. with Ascites	Average onset of Ascites Days	No. with Solid Tumour	Apparent cures	Survival of remainder	
3	9	9	16.5	Nil	Nil	32.5	9	8	18	Nil	1	25.6	9	9	10.8	Nil	Nil	21	
7	8	Nil	Nil	1	7	Free of tumour sacrificed at 90 days	8	1		2	5	39	9	3	20	3	4	47	21.5
15	8	1	54	1	6	57	10	1 Terminal		2	7	60	8	5	23	Nil	3	41	
30	7	1	16	4	2	34	9	6	16	2	1	38	10	8	13.2	1	1	39.5	

Table IX

Effect of immunised rabbit spleen cells harvested at intervals of 3, 7, 15 and 30 days after a single injection of ascites cells.

Time in days	No. of Mice	No. with Ascites	Average onset of Ascites	Average Survival	Cures
3	9	9	12 ± 6 Range 9-25	18 Range 12-37	Nil
7	9	Nil	-	Sacrificed at 90 days	9
15	8	3	40 days Range 16-58	62	4
30	7	6	13 days Range 12-32	21.7	1

± = S.D.

Table X

Enhancement of ascites tumour by lymphoid cells. Mice were treated with 500×10^6 normal rabbit spleen cells at various time intervals before or after the intraperitoneal injection of 10^7 ascites cells.

Time before 10^7	No. of mice	Average onset of ascites (days)	Average survival (days)
5 days	10	4.4 ± 2.5	13.3 ± 5.1
2 days	9	4 ± 0	11.1 ± 2.4
1 day	18	5 ± 1	10.5 ± 3.6
Untreated control given 10^7 ascites cells only	43	7.5 ± 2.3	19 ± 4.2
Time after 10^7			
1 day	9	4.4 ± 0.8	19.5 ± 6.4
2 days	10	5.3 ± 1.0	16.7 ± 5.4

± = S.D.

Table XI

Analysis of the Ascites Fluid obtained from mice treated with heterologgus or homologous tissue 24 hours before intraperitoneal inoculation of 10^7 ascites cells. The fluid was taken 4 days after injection of tumour.

No. of mice	Treatment given 24 hours before 10^7 tumour cells	Volume of Ascites fluid in mls.	Packed cell volume	Average No. of Ascites cells per mouse.	Weightof solid tumour (mgs.)
10	Nil (control)	11	20%	101×10^6	Nil
7	500×10^6 normal rabbit spleen	20.5	19%	339×10^6	600
7	500×10^6 Mouse spleen cells	18.0	22%	283×10^6	Nil
10	1 ml. normal rabbit serum	13.0	25%	148×10^6	Nil

Table XII

Analysis of Ascites Fluid obtained from mice infected with tumour from mice showing tumour enhancement. Fluid was analysed 6 days after intraperitoneal injection of 10^7 Ascites cells.

No. of Mice	Treatment	Volume of Ascites Fluid	Packed cell volume	Cells per mouse
6	10^7 tumour cells from enhanced mice	22 mls.	16%	257×10^6
6	10^7 tumour cells from unenhanced mice	23 mls.	16%	283×10^6

Table XIII

Titres obtained with mouse tissues and rabbit antiserum by complement fixation.

Antigen	14 day serum	hyperimmune serum
disintegrated ascites cells	8	64 - 128
supernatant from disintegrated cells	2	128 - 256
kidney	4 - 8	256 - 512
liver	2	128
spleen	2 - 4	256 - 512
mouse serum	trace	trace

Table XIV

Titres obtained with mouse tissues and rabbit serum by tanned cell haemagglutination.

Antigen	14 day serum	hyperimmune serum
ascites cell supernatant	2	10,000 +
kidney	nil	nil
liver	nil	320
spleen	nil	640
mouse serum	nil	1280

Table XV

The effect of Thiotepa on the growth of the ascites tumour.
(Data for controls same as table I)

Dose of Thiotepa	Dose of Tumour	No. of Mice	No. with Ascites	Average onset of Ascites (days)	Average Survival (days)
2 mg/Kilo	10^5	10	10	12.4 ± 1.3	27.3 ± 1.2
	10^6	10	10	10.2 ± 1.1	23.7 ± 3.5
	10^7	10	10	10.2 ± 4.7	20.3 ± 4.9
4 mg/Kilo	10^5	9	9	12.4 ± 1.1	25 ± 3.9
	10^6	10	10	11.4 ± 1.3	23.8 ± 3.3
	10^7	10	10	9 ± 0	17.9 ± 2.8
8 mg/Kilo	10^5	9	9	12.6 ± 3.4	23.9 ± 5
	10^6	9	9	8 ± 2.1	17.2 ± 4.7
	10^7	10	10	7 ± 2.1	19 ± 4.9

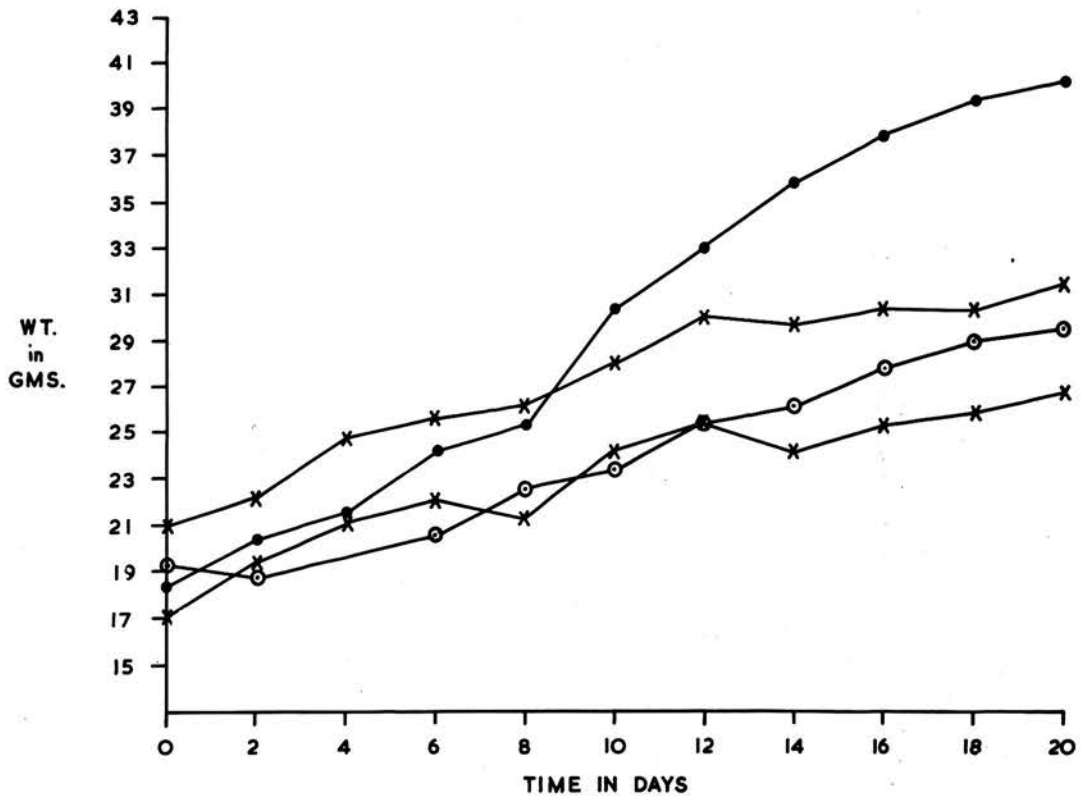

Fig. 1: Weight curves of mice treated with immune cells.

Limits of standard deviations of weights of 29 normal mice.

Mice given 10^5 ascites cells and treated 48 hours later with 500 x 10^6 immune intact rabbit spleen cells.

Mice given 10^5 ascites cells only (38 mice).

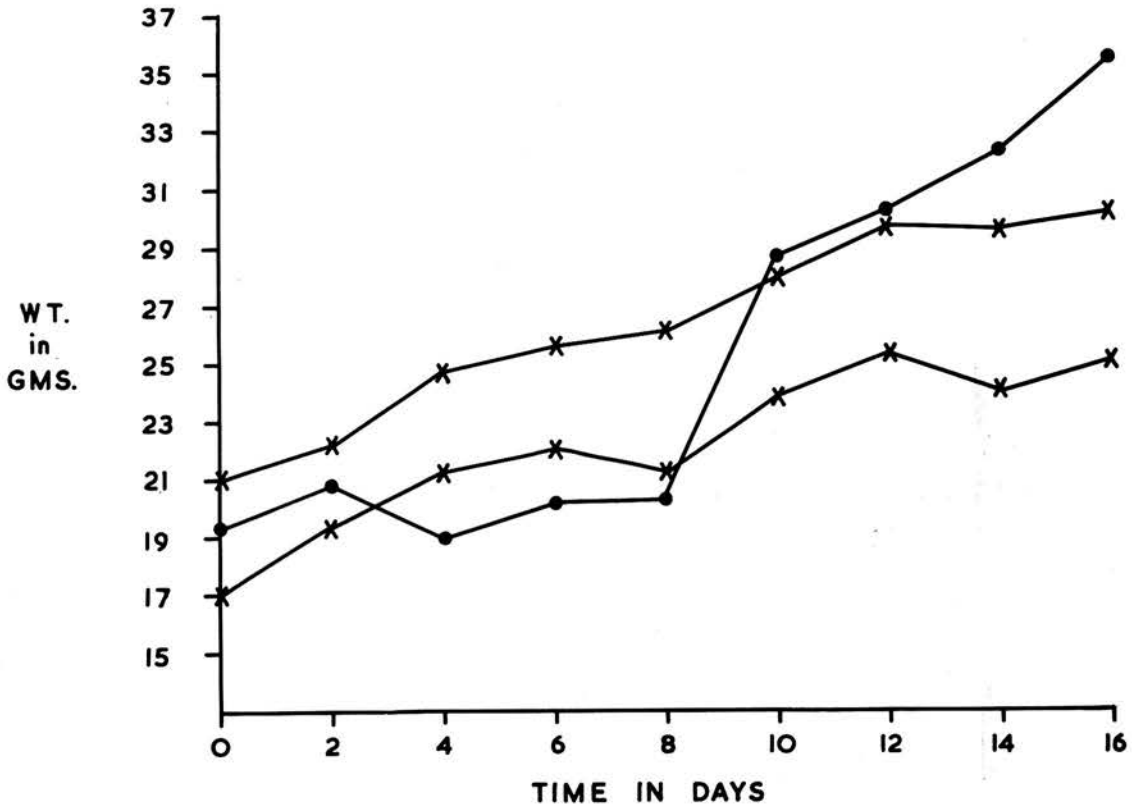

Fig. 2: Weight curves of mice treated with normal rabbit cells.

Limits of standard deviation of weights of 29 normal mice.

Weight curves of mice given 10^5 ascites cells intraperitoneally and treated 48 hours later with 500 x 10^6 normal intact spleen cells.

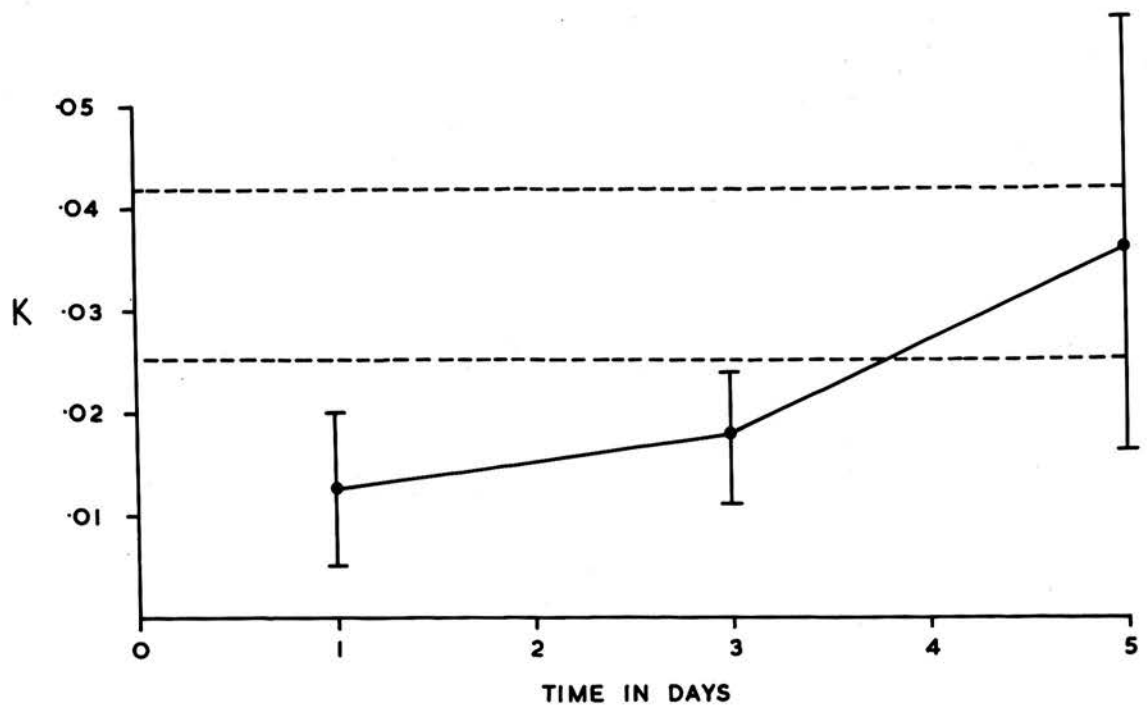

Fig. 3: Response of mice treated with normal rabbit spleen cells to intravenous injection of colloid carbon. Each point represents the mean of K for a group of seven mice. The dotted lines are the limits of standard deviation for 20 control mice.

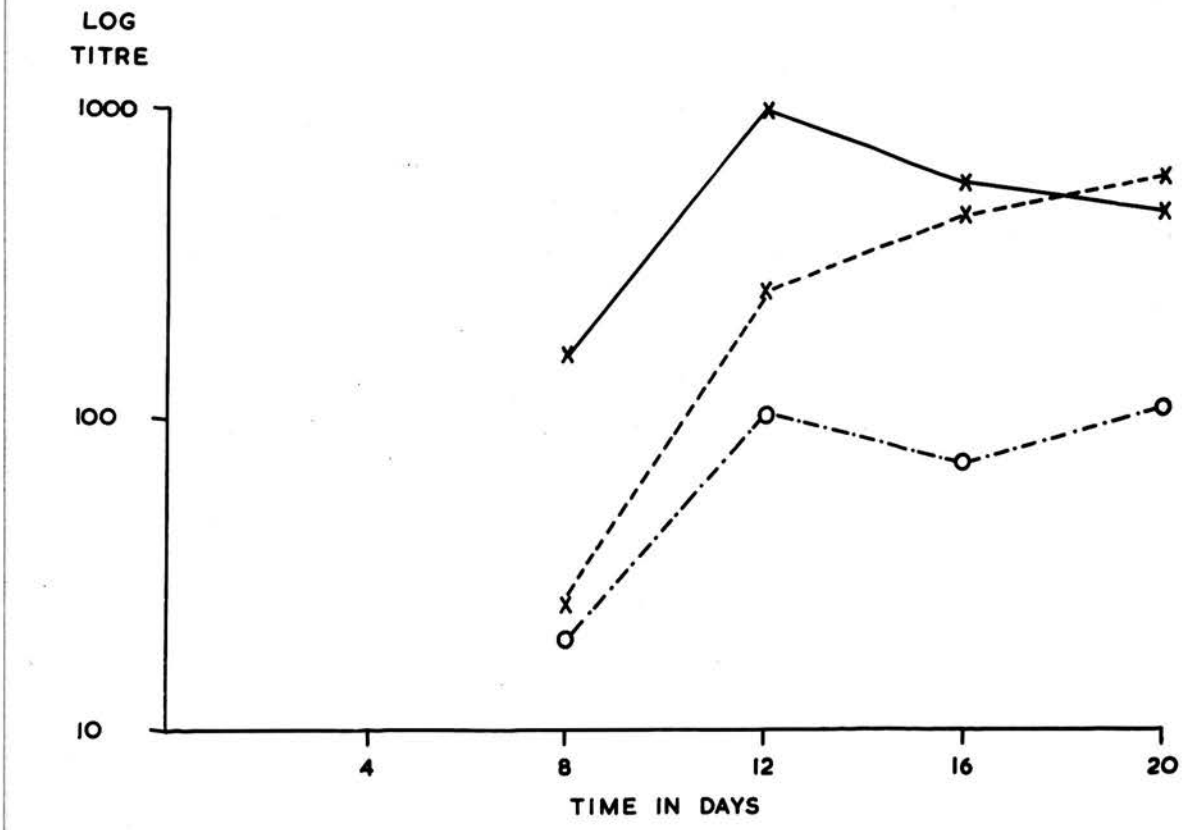

Fig. 4: The response of mice treated with rabbit spleen cells to a subsequent intravenous injection of sheep red cells.

Controls given sheep red cells only (15 mice).

Mice given normal rabbit spleen cells 24 hours before sheep red cells (7 mice).

Mice inoculated with immune rabbit spleen cells 24 hours before sheep red cells (8 mice).

CHAPTER IV

Enhancement of tumour growth. Historical background.

Over the past sixty years it has been increasingly apparent that under certain experimental conditions the growth of a tumour homograft is facilitated by appropriate conditioning of the host. This phenomenon has been described under various names in the literature viz., acceleration of tumour growth, enhancement and the XYZ effect. In the case of the strain specific tumours enhancement occurs when such a tumour breaks through the homograft barrier and grows progressively in an alien strain in which it normally regresses. With the less strain specific tumours, enhancement is detected when increased numbers of "takes" of tumour homotransplants result, or when the tumour grows more rapidly with or without increased mortality to the host. There are different means of producing enhancement, and though the conditions leading to it may be different the end result is the same. Broadly speaking there are specific and non-specific causes of enhancement. In the former category falls immunological enhancement (Kaliss 1958), which is due to contact between the grafted tumour and specific antiserum against the tissues of the strain of origin of that tumour. Immunological enhancement of the Landschütz tumour by the passive transfer of antiserum has already been described in chapter II.

There are several causes of non-specific enhancement of tumour. It was observed in chapter III that the passive transfer of heterologous spleen cells could, under certain experimental conditions, enhance tumour growth in mice. That was a form of non-specific enhancement dependent on the depression of the immune reponse of the host. Other forms of enhancement and related phenomena will be discussed later.

Early work on tumour enhancement.

In the early part of this century there were several attempts to immunise animals against transplantable tumours. The impetus for this work was the discovery that the regression of a tumour homograft could render the animal immune to a second graft of the same tumour. Several workers tried to immunise animals against tumour by using dead tumour cells or extract of tumour tissue. They found that under these circumstances the growth of some tumours was facilitated and a state of hypersusceptibility was induced in the host. Flexner and Jobling (1907) were the first to notice tumour enhancement while working on a transplantable sarcoma of the rat. They found that the injection of a heated sarcoma emulsion into rats 10 to 30 days prior to their inoculation with live sarcoma cells led to an increase in the number of tumours developing in

such treated animals as compared with the controls. The effect was most marked if the rats were repeatedly injected with the heated tumour emulsion.

Gaylord (1908), however, using the Buffalo spindle cell sarcoma of the rat in a manner similar to Flexner and Jobling was unable to demonstrate any enhancing effect on that tumour when hostswere pretreated with heated tumour material. This was perhaps the first indication, which was confirmed by later workers, that the phenomenon of enhancement could be demonstrated in certain tumour host combinations and not in others. Gaylord (1908), however, succeeded in producing enhancement of a transplantable mouse tumour by pretreating mice with tumour or normal mouse tissue.

Haaland (1910) showed that mice treated with disintegrated tumour tissue and challenged 15 to 20 days later with the same but viable tumour, developed larger tumours than the untreated controls. Enhancement of tumour growth could also be produced by prior treatment with disintegrated embryo tissues. When mice were treated with live embryonic tissue prior to tumour challenge, the animals were immunised against the tumour. The experiments were repeated using normal spleen, liver or whole blood and again it was evident that pretreatment with disintegrated tissue produced enhancement, but intact tissue immunised against homologous tumour.

Leitch (1910) produced enhancement of mouse carcinoma 63 by pretreating the host with a saline extract of the tumour. The mice received three inoculations of the extract at 10 day intervals. 24 hours after the last injection, they were challenged with living tumour. A week later 78% of the mice pretreated with the tumour extract developed tumours, but only 33% of the controls did so. Chambers and Scott (1924) accelerated the growth of the Jensens rat sarcoma by pretreating rats with the solid residue of tumour emulsion that was allowed to autolyse at 42°C. Bisceglie (1926) injected mice on each of 4 alternate days with 0.25 ml. of cell free Berkefeld-Kerze filtrate of a transplantable mouse tumour, and challenged them10 days afterwards with living tumour. He found that the extract markedly enhanced tumour growth.

In the thirties, Casey, who was the first to coin the term XYZ effect to the phenomenon of enhancement, reported in several papers his work on the problem of tumour enhancement. In 1932 Casey produced enhancement of the Brown-Pearce carcinoma of the rabbit by pretreating the animals with a saline emulsion of a tumour that had been placed in the ice box for 3 weeks. The experiments were repeated 7 times though the tumour emulsion was then prepared from tumour stored under paraffin in the ice box. Two months after a challenge with living tumour all animals pretreated with non-living tumour emulsion had

tumours. Only 68% of the unconditioned controls developed tumours. In the former group the tumours were larger, mortality was greater and metastases more common than in the controls.

Casey (1933, 1934a) showed that the enhancing or XYZ effect was specific. Thus he showed that an extract of the Brown-Pearce carcinoma did not enhance the growth of the Bashford tumour of the mouse.

The hypersensitivity induced in rabbits by homologous tumour enhancing material persisted as long as seven months after the cessation of the enhancing treatment (Casey 1934b).

The Brown-Pearce carcinoma, investigated by Casey, usually regressed when it was inoculated intracutaneously and was only propagated by testicular inoculation. When rabbits were treated with homologous tumour emulsion, intracutaneous tumour inoculations grew progressively and even metastasised (Casey 1934c).

It would be misleading to assume that pretreatment of host with non-living tumour tissue always produced enhancement of a subsequently inoculated living tumour. There are several recorded examples of immunity to a transplantable tumour induced by pretreatment with tissue extract. This in fact is not surprising, since several different factors in the design of an experiment determine the occurrence of either inhibition or enhancement. This

fact will be elaborated later in this discussion. Suffice it to mention at this point that the antigenic relationship of the tumour and the host, the dose of tissue extract used for conditioning of the host and the interval between such treatment and challenge with living tumour inoculum are all important. It is therefore not surprising to find that some authors reported increased immunity in animals pretreated with tumour extract. Bridre (1907) increased immunity to a tumour homograft by pretreating the hosts with tumour extract. The tumour he used was a mouse neoplasm that regressed in about 50% of mice inoculated. When mice were given three injections of tumour extract and challenged with living tumour 32 or 34 days after the first injection, the incidence of tumour regression was 100%. Kopinow (1920) reported that tumour immunity was induced in mice pretreated with boiled tissue of that tumour. Inhibition of the Ehrlich mouse carcinoma was produced by the pretreatment of mice with an extract from the tumour (Domagk and Hackman 1935). Sugiura and Benedict (1931) immunised rats against Sugiura rat carcinoma by a heated tumour emulsion inoculated before viable tumour cells.

Recent work on tumour enhancement: The development of the concept of immunological enhancement

It is clear from the above resumé that the early work

on tumour enhancement was performed on random-bred animals. Further developments in the field of enhancement were made possible only by the use of inbred animal strains, the development of the genetics of transplantation immunity (Little 1914, Gorer 1937, 1938, and Snell 1948) and the improvement of the in vitro serological techniques for the detection of iso-antibody (Gorer and Mikulska 1954).

Lewis and Lichtenstein (1936) were the first to produce enhancement of a strain specific tumour. The tumour, which was a carcinoma induced by 1 : 2 : 5 : 6 dibenzanthracene in strain A mice, was inoculated every 5 days into 24 Bagg albino mice in which it normally regressed. 19 of these mice developed one or more tumours, some of which were transplantable into normal Bagg albino mice. It is noteworthy that Lewis and Lichtenstein used living tumour to prepare the mice for enhancement.

Snell et al (1946) showed that stimulation or inhibition of transplanted tumours in animals pretreated with non-living tumour material, was dependent to a large extent on the tumour and stocks used. They demonstrated that when strain C57 BR, C57 BL or BALB/c mice were repeatedly injected with lyophilized strain A cardinoma 1509/a, they became more susceptible to a subsequent challenge of the same tumour. But a similar series of inoculations of C57 BL myeloid leukaemia C1498 failed to condition the inbred strains for enhancement.

Kaliss and Newton (1949) showed that the enhancement due to prior inoculation of lyophilized tumour tissue was dependent on the dose of the lyophilized tissue, at least in some tumour host combinations. They used a mouse mammary tumour of C57 black origin which grew progressively in about 50% of normal C_3H Jax mice. Treatment of C_3H Jax mice with lyophilized C57 black tumour prior to inoculation with live tumour produced different results, depending on the dosage of the lyophilized tissue. Small doses produced inhibition, whereas large doses enhanced the tumour growth.

Kaliss and Snell (1951) found that the prior injection of an alien mouse strain with lyophilized normal tissues, derived from the strain of origin of a tumour, could produce enhancement of that tumour when subsequently inoculated. They therefore concluded that the growth enhancing agent or agents were present in normal as well as in tumour tissues.

Kaliss and Avnet (1950) compared the enhancing effect of fresh tumour homogenate and the ultrafiltrate of the homogenate and concluded that the latter was devoid of ehhancing activity.

The observations on the tumour-enhancing effect of tissue extract raised the question as to whether these extracts contained some growth-stimulating factor or whether they produced their effect through an altered tumour-host

relationship. In an attempt to answer these questions, Kaliss postulated that the enhancement encountered in his experimental procedure was due to an immune mechanism. He based his argument on the fact that small doses of lyophilized tissue produced immunity while larger doses led to abrogation of immunity. This suggested that an immune phenomenon, rather than cancer-stimulating substances, was involved. Support for this conclusion was furnished by the demonstration of Kaliss and Molomut (1952) that enhancement of a tumour homograft could be mediated through the passive transfer of antiserum. The enhancing sera were prepared in rabbits or alien strain of mice against a tumour of strain A mice. Prior injection of these antisera in C57 Black/6Ks led to progressive growth of subsequent inoculation of two specific strain A tumours. These tumours regressed in untreated C57 Black/6Ks mice.

The enhancing effect of the antiserum was associated with the globulin fraction and was most concentrated in the γ globulin (Kaliss and Kandutsch 1956). That the activity of the antisera was not due to recovered homologous tissue extract used in the immunisation procedure was proved by the fact that the physical and chemical properties of the antiserum and tissue extracts were different. The activity of the antiserum was not impaired by treatment with sodium periodate, or heating to 56°C, but was destroyed by heating at 100°C. Tissue extract producing enhancement

were destroyed by these procedures.

An interesting observation made by Kaliss (1955a) was that the injections of cortisone along with tissue extract abolished the ability of the latter to induce enhancement. This was attributed to the suppression of antibody production which was thought to be necessary for enhancement.

Snell et al (1948) made a detailed study of the tumour "immunity" induced in mice with lyophilized tissues. They investigated the degree of immunity as influenced by 4 different variables viz. tumour type, host strain, source and dosage of the lyophilized tissue. They used 7 different inbred mouse strains and 9 different tumours to provide lyophilized tissue for injection or fresh tumour tissue for inoculation. Thus 45 different combinations were tested. Mice were given a series of injections of lyophilized tumour tissue or, in a few cases, of lyophilized normal tissue, and inoculated with living tumour 10 days after the last injection. In the unconditioned controls the tumours showed moderate growth followed by regression. In the mice pretreated with lyophilized tissue, the results depended on the particular host-tissue combination used. In some combinations growth of the tumours was almost completely inhibited; in others, it was stimulated to the point where 70 to 100 per cent of the mice died. One tumour gave strong inhibition or stimulation according to the strain in which it was tested. In a few cross tests

between different tumours there were indications of some specificity. As a result of this extensive work, the authors concluded that the host-tumour combination was the significant factor in determining whether inhibition or stimulation was produced. They, however, could not exclude the importance of other factors not revealed by their system.

It is clear that in most of the work on enhancement non-living tissue was used to prepare the host for enhancement. Kaliss, however, in 1955, (Kaliss 1955b) showed that enhancement of a tumour could be produced experimentally by repeated injections of living tumour in incompatible hosts. Living sarcoma I, which is indigenous to strain A mice, was inoculated in C57 BL/6Ks mice in which it regressed. Two months later the mice were challenged with the same tumour and in a significant number the tumour survived. When the interval between the two successive tumour inoculations was reduced to one or two weeks, the second graft regressed.

In Gorer's experience with 8 transplantable tumours, enhancement of a tumour by repeated inoculation of viable tumour cells was not possible (Gorer 1956).

Snell (1955) studied the specificity of the enhancing effect in inbred strain of mice. He used mice of known geno-type with respect to the H_2 locus. Using different

combinations of enhancing lyophilized material, hosts and tumours, he showed that enhancement could occur if the tumour and enhancing tissue shared a common histocompatibility factor which was lacking in the host. He concluded therefore, that the enhancing substance must be a product of the H_2 locus and that it was probably iso-antigen.

There is a direct correlation between the enhancing effect of an iso-antiserum and its ability to agglutinate the red cells of the tumour-donor. The enhancing effect could be completely abolished by absorption with homologous tumour and reduced by absorption with donor red cells, Kaliss (1958). This indicated a probable identity between the enhancing antibody and H_2 antibodies. Kaliss (1958), however, pointed out that this relationship was not proven beyond all doubt. In fact the BP8 tumour of C_3 H_1 mouse origin and of H_2K antigenic complex could be enhanced by iso-antiserum prepared in strain A mice. The latter strain has all the H_2 antigens present in C_3H mice. Kaliss thought, therefore, that the antigens responsible for the enhancement in this particular situation could be due to undetermined H_2 antigens or perhaps to incompatibility at loci other than the H_2 locus.

The factors leading to immunological enhancement are complex and as yet not completely understood. For example, different tumours of the same strain of mouse may respond differently to the enhancing treatment (Kaliss 1958). The

same tumour also may show different degrees of enhancement in different hosts. Also, in a single strain females sometimes show a higher degree of resistance to enhancing procedures than males.

The antigenic difference between two mouse strains is not, by itself, enough to ensure the possibility of enhancing a tumour from one strain into the other. Thus Kaliss and Bryant (1958) have shown that sarcoma I of strain A origin grew in normal C_3 H/Ks mice for a short time but eventually regressed. When C_3 H/Ks mice received 5 inoculations of sarcoma I at intervals, the mice maintained a heightened resistance. However, despite this increased resistance, the mice were producing an antibody which could, on passive transfer to normal C_3 H/Ks mice, enhance a subsequently inoculated sarcoma I.

In summary, the phenomenon of tumour enhancement was observed in the early part of this century. Recent investigators confirmed and extended early observations. All this work culminated in the recognition of immunological enhancement, largely due to the work of Kaliss and his collaborators. Kaliss (1958) defined immunological enhancement as "the successful establishment of a tumour homograft and its progressive growth (usually to death of the host) as a consequence of the tumour's contact with specific antiserum in the host". The antiserum may be actively produced by injecting the host with tissues from

the strain of origin of the tumour, or by passive immunisation with hetero- oriso-antisera.

Immunological enhancement is species-specific and to some extent strain-specific. It is produced most readily if the enhancing tissue and the tumour donor share antigens controlled by the H_2 histocompatibility locus, and which are absent in the recipient.

Thus, in immunological enhancement specific humoral antibodies play a fundamental role. Their mode of action, however, is a mystery. Several hypotheses have been put forward and these will now be discussed.

Mechanism of immunological enhancement

Immunoselection hypothesis

Hauschka et al (1956) put forward the theory of immunoselection in order to explain why some tumours may acquire the property of progressive growth in a genetically incompatible individual. They showed that most tumours contained cells with different chromosomal number. Heteroploid cells were thought to be least antigenic (see general introduction to this thesis) and in a foreign host environment were selected. Kaliss (1957, 1958) put forward three arguments against the immunoselection hypothesis.

(1) Tumours from enhanced mice do not usually show progressive growth in untreated mice.

Whereas this is mainly true there are intances where progressive growth of an enhanced tumour occurred when it was inoculated in unconditioned animals. Thus Casey et al (1951a) were able to propagate enhanced mouse carcinoma EO 771 into normal foreign mice.

(2) Kaliss showed that when a mouse was inoculated with two successive grafts of an incompatible tumour, the second was destroyed while the first became enhanced. To exclude the possibility that the second graft might have been more specific than the first, Kaliss performed experiments whereby the same tumour was the source for the first inoculum in one experiment and second graft in another; still the second graft was destroyed but the first grew progressively. Immuno-selection should be operative on both tumours.

(3) If antiserum induced a selection, it would be expected that higher doses of serum were more effective than smaller doses in producing enhancement. With some tumours, enhancement was more easily produced with smaller than with larger doses of antiserum.

Central inhibition hypothesis

According to this view, the antiserum reacts with the central immune mechanisms of the host and specifically inhibits their reactivity against the grafted tumour.

Kaliss (1957) thought that central inhibition was an unlikely cause of enhancement. His argument was based on observations made from experiments performed with sarcoma I in the incompatible strain C57 BL/6Ks mice. He showed that when a mouse was inoculated with the same tumour on two successive occasions and if the interval between the grafts was two weeks or less, the inhibitory immunological reaction produced as a result of the first graft could not be overcome with passive transfer of antiserum administered along with the second graft.

Mitchison and Dube (1955) have shown that the enhanced state could be abolished by the introduction of lymphoid cells suitably and specifically immunised against the grafted tumour and isogenic with the recipient. The results with passively transferred normal cells were equivocal. If passively transferred isologous normal cells could be shown to abolish the enhanced state then this would favour central inhibition hypothesis.

Snell et al (1960) showed that lymph nodes taken from actively immunised mice were twenty times more successful in opposing the growth of sarcoma I than lymph nodes taken

from mice which had undergone pretreatment with specific enhancing antisera followed by tumour inoculation. Although this proves that the antiserum interferes with the development of an immune response it does not conclusively show where the antiserum acts. One could argue that it acts centrally on the reaction centres but it is also conceivable that it neutralises the antigen in the graft and prevents it from reaching the lymph nodes.

Brent and Medawar (1962) working with skin grafts have shown that a specific antiserum injected into animals could prevent sensitization of such animals by tissue extracts; the serum was injected 2 or 3 days after the extracts. If the antigens were exposed to the antisera in vitro, the antigens could still sensitize in vivo. They concluded "antiserum probably affects the process of sensitization itself, acting by a central inhibition of unknown character rather than by obstructing the afferent or efferent pathways of the response".

The results with the Landschütz ascites tumour agree with those of Brent and Medawar. It has been shown (chapter II) that enhancement of the Landschütz tumour occurred when serum was inoculated 48 hours after tumour. The spleens of enhanced mice were much smaller than those of mice bearing tumour alone, indicating a central inhibition.

The efferent inhibition hypothesis

Kaliss advanced the hypothesis that immunological enhancement of a tumour was due to a physiological alteration in the tumour occurring as a result of contact with an antiserum specifically directed against it. This physiological change in the tumour allows it to survive despite the hostile environment of the host. He found that an enhanced tumour showed a greater rate of growth, not only in the alien strain, but also when it was retransplanted into the strain of origin. In support of his hypothesis, Kaliss (1958) cites the experiments of Gorer (1942) in which the growth of sarcoma and a lymphoma were accelerated when exposed to antiserum in vitro before inoculation into appropriate hosts. Kaliss (1958) also demonstrated that the growth of sarcoma I incubated with iso-immune serum in vitro overnight, and then inoculated into C57 BL/Ks hosts, grew progressively; fresh untreated tumour did not. However, when the tumour was incubated with normal serum or even with saline it also grew progressively in normal C57 BL/Ks mice.

Brent (1961) pointed out that the physiological change, as envisaged by Kaliss, does not allow the tumour to overcome the second set response of the homograft reactions as the tumour from enhanced animals did not grow in mice which had been presensitized with living tumour 7 to 14 days previously.

Feldman and Globerson (1960) showed that enhanced tumours elicited a homograft reaction but despite this they grew progressively in foreign hosts. They postulated that a passively transferred antiserum caused a tumour homograft to emit more antigens. The excess soluble antigens reacted with the homograft response evoked by the tumour and "neutralised" it. The tumour was therefore protected from the hostile environment of the host and enhancement was possible.

The afferent inhibition or blockade hypothesis

Some investigators postulated that enhancement of tumour growth was due to blockade of the afferent side of the immune response. They believed that passively transferred antiserum prevented the antigen from reaching the reactive centres of the host. In 1956 Billingham, Brent and Medawar put forward the theory that the tissues of the mouse had two types of antigens which were determined by the same histocompatibility genes. These two sets of antigens were respectively, transplantation antigens (in the nucleus), and haemagglutinogens (in cytoplasm). Humoral antibodies against these antigens reacted with both antigens. In the preparation of tissues to produce enhancement the transplantation antigens were inactivated. On injecting this tissue extract into mice

a haemagglutinating antibody was produced which cross reacted with transplantation antigens of a subsequent living tumour transplant and thus blocked them and prevented them from reaching the reactive centres of the host. However, these same authors failed to confirm the presence of two types of antigens in nucleated cells (Brent 1961).

Snell (1956) also postulated an afferent inhibition to explain immunological enhancement. According to his theory circulating antibodies were fixed to the graft and therefore produced "walling off" of the tumour antigens and prevented them from reaching the regional lymph nodes. In support of this theory Snell et al (1960) showed that normal lymphocytes of the strain of origin of the tumour injected into conditioned mice abolished the enhancement of a subsequently inoculated tumour. In their interpretation of this finding they believed that the highly mobile lymphocyte could not be fixed by the antibody and could therefore reach and stimulate the reactive centres of the host.

There are several factors contradicting this "walling off" hypothesis. First the amount of antiserum required to produce enhancement, in certain circumstances, is extremely small. Also there is no direct evidence that tumour cells do not reach the regional lymph nodes. At

least the direction of lymph flow favours their transport to the regional lymph nodes. As Kaliss (1962) has pointed out enhancement could occasionally be produced even if antiserum was given 7-10 days after tumour inoculation by which time the immune centres of the host should have been stimulated.

Experiments carried out by Kaliss (1958) have shown that tumour from enhanced mice was an effective immune stimulus and would generate a second set response. When mice were inoculated with enhanced tumour, they destroyed a second inoculum of an unenhanced tumour more efficiently.

Snell et al (1960), however, put forward evidence in support of the theory that enhancing antiserum blocked the development of cellular immunity which is mainly involved in homograft rejection. In these experiments the effect of iso-immune serum on the immune response of both humoral and cellular types was measured. Foreign strain lymphoid cells were injected into mice which responded by the formation of haemagglutinins. When antiserum against the injected lymphoid cells was mixed with these cells before inoculation in foreign hosts almost no additional antibody was formed. Next, cellular immunity against a tumour was assayed: antiserum given at the same time as the graft tumour depressed the cellular immunity, while antiserum given one or more days after the immunising tissue

also resulted in a lower level of cellular immunity. The authors therefore concluded that passively transferred humoral antibody depressed cellular immunity.

Lastly, Gorer (1961) pointed out that enhancement could, perhaps, be explained by the interference of humoral antibody with the function of sensitized cells. This view supposed that homograft immunity was similar to delayed hypersensitivity reactions, mediated by cells. It is known that delayed hypersensitivity diminishes with the development of humoral antibody. Gorer's concept is shared by Batchelor (1961, 1963).

The nature of "the enhancing agent" in normal and tumour tissues.

There were several attempts to identify the agent present in normal or tumour tissue which conditioned a host for enhancement.

The enhancing material was found in mechanically disintegrated tissue (Haaland 1910); autolysed tissue (Chambers and Scott 1924); frozen tissue (Casey 1932); lyophilized normal or tumour tissue (Kaliss and Snell 1951) and fresh tissue extract (Shear et al 1954).

Casey (1936) demonstrated that the enhancing material obtained from refrigerated homogenized Brown-Pearce

carcinoma could pass through a Berkefeld filter. Later however Casey et al (1951b) showed that Berkefeld or Seitz filtration of fresh tumour did not contain evidence of the specific XYZ or enhancing factor. They therefore concluded that the material was either bound in some combination in fresh tumour and activated when tumour was stored in the cold or that it was part of a large molecule which was not Seitz filtrable. Similar results were obtained by Kaliss and Avnet (1950) when using fresh tissue and fresh tissue filtrate to enhance a mouse carcinoma.

Shear et al (1954) showed that the enhancing material obtained from a centrifuged saline extract of freshly homogenized tumour was non-dialyzable and did not pass through a Seitz filter. By differential centrifugation the enhancing material was found to be associated with both the mitochondrial and microsomal fractions of the cell. Thus it was not specifically associated with a particular cell organelle. One possibility was that its presence in 2 cell fractions was due to contamination. The other possibility was that the size of the enhancing agent was intermediate in size between the mitochondria and the microsomes.

The enhancing material in the lyophilized tissue prepared by Snell (1952) withstood heating at 100°C, with only a slight loss of activity. When the material was

fractionated, however, its stability to heat was less. This was attributed to a protective action of the lyophilized tissue on the enhancing agent or to the possibility that the process of fractionation brought about separation of a thermolabile fraction.

Shear et al (1954) however found that the accelerant material extracted from fresh tissue retained its activity when exposed at 45°C for 30 minutes, but was destroyed by heating at 56°C for a similar period of time.

Miroff et al (1955) found that heat denatured tumour tissue contained an accelerating material. This material reduced the transplantation and killing times of the Z mammary carcinoma inoculated in the strain of origin. (It is noteworthy that the tumour had been transplanted for 40 generations in the strain of origin). The accelerating agent was extractable by ether and acetone and was therefore strongly suspected to be lipoid in nature. The material gave negative reactions with tests for proteins and carbohydrates.

Green and Wilson (1956) showed that the extractable enhancing agent was greater in frozen and thawed tumours than in fresh tissue. They also believed that two factors constituted the enhancing agent, a lipoprotein and a phospholipid. Shear et al (1954) showed that the enhancing material obtained from a mouse mammary carcinoma

was probably a lipoprotein.

Kandutsch (1957) investigating the chemical nature of the enhancing material found that it was labile to heat, 90% phenol, 80% alcohol and to digestion with trypsin. This suggested that it had a protein component. Other tests suggested the presence of a carbohydrate component. He found hexosamine in all active fractions and this suggested that the two fractions were associated in the form of a mucoprotein.

Snell (1958) pointed out that the H_2 antigens were concerned in homologous tissue enhancement. The H_2 antigens are also blood group antigens. Since the antigenicity of the ABO blood groups is due to mucopolysaccharides, it is reasonable to assume that the enhancing agents are of a similar nature.

The unresponsive states and their relationship to immunological enhancement

The immunological reactivities of an animal can be suppressed specifically or non-specifically. Specific suppression means that the animal fails to react to a particular antigen though reacting normally to other antigens. These specific unresponsive states have been classified by Medawar (1960) into five main categories. These were immunological tolerance; unresponsiveness in

irradiated animals exposed to antigen shortly after irradiation; the Sulzberger-Chase phenomen; Feltons immunological paralysis and unresponsiveness in adult animals due to exposure to high doses of foreign protein antigen.

It is noteworthy that the immunological enhancement of transplantable tumours was not classified with these unresponsive states. Although immunological enhancement is highly specific, it has its own peculiarities. For example in immunological enhancement produced by pre-treatment of the host with lyophilized tissue, the animal does respond to the antigen, at least by forming circulating antibody.

Immunological enhancement and immunological tolerance

For a long time after Paul Ehrlich formulated his theory of "Horror Autotoxicus" biologists were in the dark as to why an animal does not normally respond immunologically to its own tissues. In 1949 Burnet and Fenner postulated that there was a period in an animal's early life during which its immunological mechanism was immature. During this period it regards any potentially antigenic material as self and will not react against it. The non-recognition of antigenicity could also be extended to foreign antigens

coming in contact with the animal during this early period. Thus they were able to explain Owen's observation, in 1945, of erythrocyte mosaic pattern in adult dizygotic twin cattle as being due to continued presence of haemopoietic cells exchanged between the twins through vascular placental anastamosis in utero. Billingham et al (1953) confirmed the theory of Burnet and Fehner using the experimental model of skin homografts in inbred strains of mice. They coined the term "tolerance". This is now defined as the unresponsiveness specifically induced by contact of a potential antigen with an organism in utero or early postnatal life (Medawar 1960).

The principle of actively acquired immunological tolerance was utilized to adapt transplantable tumours to foreign strains. This was "enhancement" but on a different basis to immunological enhancement. Thus Koprowski (1955) rendered ICR mice susceptible to a foreign strain specific lymphoma and a rat hepatoma by making the ICR tolerant of tumour donor tissue.

Adaptation of tumour homografts to thymectomised animals

Miller (1962) has shown that there was a marked reduction in the immunological reactivities of mice thymectomised early in life. This was manifested, among

other things, by the acceptance of a foreign tumour graft even when the two differed at the H_2 locus. Good et al (1962) have also shown that transplantation of a strain specific mammary tumour of the mouse could be successful in a thymectomised alien strain.

Steroids and enhancement

The effect of steroids on tumour growth varies with different types of tumours (Sugiura et al 1950). Foley and Silverstein (1951) showed that the resistance of C F_1 mice to C_3H mice lymphosarcoma was markedly decreased if the C F_1 mice were treated with cortisone. They suggested that the decreased resistance was probably due to depression of the lymphoid tissue and immune responses in the steroid treated animals. Howes (1951) showed that cortisone treatment of the recipients led to the successful homotransplantation of a mouse adenocarcinoma in a strain in which it normally regressed.

To conclude, these apparently different forms of enhancement, mention should be made of the interesting work of Abd El Ghaffar (1963). He produced accelerated growth of sarcoma 180 in vitro and in vivo by using small doses of an alkylating agent or an antimetabolite. He postulated that small doses of the drugs inhibited the growth of a small proportion of the tumour cells. Death of these cells

led to the stimulation of the rest of the tumour "either through humoral factors released from the dead cells or through some form of biological overcompensation". The author suggested the term "chemical enhancement" for this biological phenomenon.

Contributions of the present work to the phenomenon of enhancement.

(1) An ascites tumour was used to study enhancement. This made possible an accurate quantitative study of enhanced tumour growth.

(2) A new type of enhancement due to the passive transfer of heterologous and homologous spleen cells was described (chapter III). The passive transfer of lymphoid cells was found to depress R.E.S. phagocytic function, and the capacity of the host to react to a particular foreign antigen. Enhancement was attributed to the depressed immune response.

(3) Immunological enhancement of the Landschütz ascites tumour was observed with the passive transfer of isologous immune serum 48 hours after tumour inoculation (chapter II). It was found that the spleens of enhanced animals were significantly smaller than those of animals bearing

unenhanced tumour. The significance of this finding in the interpretation of the mechanisms of enhancement was discussed.

(4) Enhancement of tumour growth can follow the passive transfer of normal isologous spleen cells. With this treatment the tumour was partly converted to the solid form.

CHAPTER V

The Pathology of the Landschütz Tumour

Introduction

The changes in the lymphoreticular tissues of mice bearing the Landschütz tumour have already been described (chapter I). It was noted that in the peritoneal cavity the tumour grows mainly in the ascitic form. The tumour changes readily from the ascitic to the solid form after treatment with serum or lymphoid cells (chapters II and III). Conversion from the ascitic to the solid form alters completely the natural history of the disease. In this chapter the necropsy findings in mice dying from either the solid or ascitic types of the neoplasm are described.

Material and Methods

The material is based on the necropsy examination of animals used in the experiments described in the preceding chapters. It comprised 800 white mice from a closed colony (M.R.C. endocrinology research unit strain). 200 A/Jax Porton strain and 80 DBA_2 porton strain. The white out-bred mice were used in experiments involving treatment with heterologous cells and sera (chapter III). The A/Jax mice were donors and recipients of isologous cells and sera. DBA_2 mice were donors of homologous cells used in the treatment of A/Jax Porton mice bearing tumour

(chapter II).

In all the experiments the mice dying naturally of tumour or those that were sacrificed were subjected to a detailed postmortem examination. The peritoneal cavity was inspected for the presence of ascitic fluid. Solid tumour when present was measured and its position noted. In animals treated with spleen cells the peritoneal cavity was inspected for the presence of "splenicules" (chapter VI). The spleen, liver and thymus were weighed. All the tissues were fixed in 10% formol saline followed by 12-24 hours in saturated corrosive sublimate. Paraffin sections were made and stained with Mayer's haemalum and eosin. Reticulin was demonstrated by an improved silver method (Slidders et al 1958) and fibrin by the acid Picro Mallory technique (Lendrum 1949).

Results

Influence of mouse strain on the growth of the tumour; There was a distinct variation in the rate of growth of the Landschütz tumour when inoculated in the dose of 10^5 ascites cells by the intraperitoneal route in different strains of mice. Outbred mice developed ascites by the 12th day and died with remarkable constancy around the 20th day (chapter III). A/Jax mice were more resistant and lived for approximately 30 days (chapter II).

The results of passive transfer of spleen cells showed that the DBA_2 spleen cells could inhibit the tumour to a greater extent than A/Jax spleen cells (chapter II).

Pathological changes in untreated mice inoculated with tumour; the mice inoculated with tumour in the peritoneal cavity appeared well for a time and then in 7-12 days, depending on the dose inoculated, developed visible abdominal distension due to ascites formation. Particularly in the outbred mice there was oedema of the subcutaneous tissue. As the fluid accumulated in the peritoneal cavity the animals looked more ill and their coats were rough. Terminally their movement was sluggish and their skin was cold. A terminal watery diarrhoea was sometimes observed. Fig. 1 shows a mouse in the late stages of tumour growth compared with a mouse treated with spleen cells. The peritoneal cavity of mice dying with tumour contained 10-15 ml. of ascitic fluid. The fluid was rich in tumour cells, protein and a lipid material, that could block the pores of a Seitz filter. Irregular gelatinous masses of necrotic tumour cells were sometimes found floating in the ascitic fluid. Occasionally the abdominal organs were covered by a thin layer of fibrin. Invasion of the organs was never noted although in some mice tumour cells were seen in the veins and sinusoids of the liver (Fig. 2). The changes in the lymphoid organs of mice bearing tumour have already been described (chapter II).

It was noted that there was stimulation of the lymphoid organs in the early stages of tumour growth but in the late stages the hosts resistance collapsed. The spleens of mice bearing tumour, for example, increased to about three times their normal weight 10 days after tumour inoculation but at death were either normal or smaller than normal. Fig. 3 and 4 compare the red pulp of the spleen at 10 days and at death respectively. The former is cellular whereas the latter shows congestion and hypocellularity.

Necropsy findings in mice treated with serum or lymphoid cells; Ascites was not common in this group and the animals died of solid infiltrative growth. This made it possible to study the morphology of the tumour cells and their mode of spread in the tissues. The tumour cells measured 12-14 μ in diameter and had large oval or round nuclei (Fig. 5). The nuclear membrane was thin and distinct. The nuclei contained numerous discrete small basophilic chromatin masses and sometimes round homogeneous eosinophilic bodies were present. They varied in number from 1 to 4 and measured about 2 μ in diameter although by coalescence they formed quite large assymmetrical masses. The cytoplasm was faintly eosinophilic and rather opaque; it did not contain granules. Mitoses were frequently seen. The morphology of the tumour cells in the ascitic fluid was similar to tumour cells growing in the solid form although the chromatin material in the nuclei of the former was

more uniformly distributed (Fig. 6). A conspicuous feature of large solid tumours was the presence of central necrosis which contained irregular haematoxyphil bodies that varied in size from 5 to 20 μ (Fig. 7). They did not stain by Von Kossa method and were Feulgen positive. Some large tumours with moderate degree of necrosis showed that the surviving tumour had a parvascular distribution (Fig. 8). The tumour did not form reticulin and fig. 9 shows preformed reticulin in a paravertebral metastases. Fig. 10 shows the normal reticulin around the muscle fibres.

The commonest site for solid tumour was the root of the mesentery (Fig. 11) where scirrhous tumour encircled the bowel and led to intestinal obstruction. In two animals, one treated with heterologous and the other with isologous spleen cells, tumour in the root of the mesentery had infiltrated the common bile duct. The gall bladder was distended with bile (Fig. 12) and the animals were jaundiced. A tumour in the root of the mesentery occasionally infiltrated radicles of the portal vein and produced varicosities and thrombosis (Fig. 13). Visible metastases in the liver were rare but were occasionally seen.

The second commonest site of solid tumour growth after the root of the mesentery was the pancreas (Fig. 14) which may be completely replaced by tumour. Infiltration of the stomach might lead to perforation and peritonitis (Fig. 15). This was an uncommon complication although a few tumour cells in the gastric mucosa were occasionally seen (Fig. 16).

The pelvis of some mice contained a mass of solid tumour which obstructed the ureters with consequent unilateral or bilateral hydronephrosis. Infiltration of the kidney by tumour was not common, occurring only in three animals. Tumour in the kidney infiltrated around the glomeruli (Fig. 17) and in one mouse there was evidence of tubular necrosis (Fig. 18).

In some treated mice, particularly A/Jax strain, there were solid tumour masses in the fat of the testicular omentum with multiple isolated or confluent masses of tumour on the posterior abdominal wall and in the paravertebral muscles (Fig. 19).

Two treated mice in the series developed paraplegia. The first was an outbred mouse inoculated with 10^7 ascites cells and 0.5 ml. of immune rabbit serum. About 40 days after treatment the mouse developed a left sided intra-abdominal firm and immobile swelling (Fig. 20). The swelling gradually increased in size and the mouse developed paraplegia. This animal was chloroformed and at autopsy there was a mass of solid tumour replacing the left kidney (Fig. 21). There was also a separate tumour mass in the root of the mesentery infiltrating the posterior abdominal wall and paravertebral muscles with secondary compression of the spinal cord (Fig. 22). Fig. 23 shows tumour infiltrating the paravertebral muscles; the muscle

fibres show swelling and proliferation of the sarcolemma. A cellular reaction composed of lymphocytes and spindle shaped cells was also seen. The vertebral periosteum in the vicinity of the tumour was thickened and hyperplastic (Fig. 24).

While blood born metastases were rare, spread by the lymphatic system was not uncommon (Fig. 25 and 26). Fig. 27 shows a metastases in a lymph node which had started in a marginal sinus. Lymph nodes containing tumour showed reactive changes characterised by increased proliferation of immature mononuclear cells (Fig. 28). This however was not always the case and tumour deposits might be found in lymph nodes without any reactive changes (Fig. 29).

Discussion

The conversion of the tumour to the solid form had completely changed the natural history of the tumour. It was observed (chapter I) that the Landschütz tumour was originally described as a sarcoma. Tjio and Levan (1954) on the bases of detailed chromosomal studies noted the resemblance of the tumour to Ehrlich carcinoma and thought the two were related. The histological observations described here are in agreement with Tjio and Levan's

suggestion regarding the histogenesis of the Landschütz tumour. It was observed that the tumour was well vascularised and tumour cells radiated from well formed blood vessels. The tumour spread by lymphatics and when present in lymph nodes it formed discrete masses; it grew in solid cords or sheets. The absence of pericellular reticulin did not help and cytologically one cannot confidently distinguish between reticulum cell sarcoma and carcinoma although perhaps the rather coarse chromatin pattern favours the latter diagnosis. It is noteworthy that the "nucleoli" seen in tissue sections had an unusual morphology and may in fact be viral inclusion bodies. No viral agent was demonstrated in a Seitz filtrate of the tumour.

The necropsy findings in animals incompletely treated with either lymphoid cells or serum show that they died from the local complications of solid tumour such as intestinal obstruction, peritonitis, pancreatic insufficiency jaundice, paralysis and widespread metastases. This agglutination of the ascites cells meant that the animals lived longer than the untreated controls and that the usual parameters of tumour growth such as the time of onset of ascites and survival time were both inaccurate and misleading. The persistent growth of the tumour in the solid form long after exposure to an immunological constraint is difficult to explain but teleologically

represents a defence reaction by the neoplasm against unfavourable host reaction.

The finding of different growth rates in different mouse strains indicates different degrees of host resistance. This is probably due to the fact that the antigen of the ascites cell is probably physiological and not a pathological mutant which would be expected to evoke a similar response in different hosts.

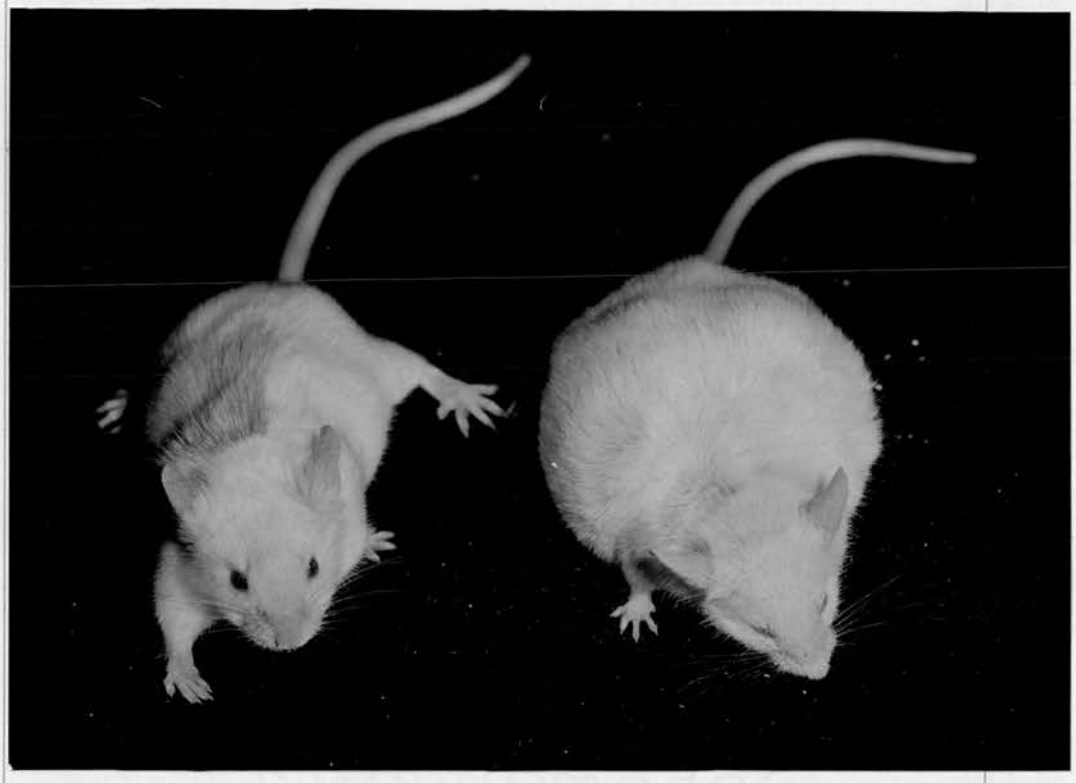

Fig. 1: The mouse on the right is bearing a 20 day growth of 10^5 ascites cells by the intraperitoneal route. Note the swollen abdomen and the roughened coat. The mouse on the left had been inoculated with the same dose of tumour but was treated with heterologous spleen cells; it looks normal.

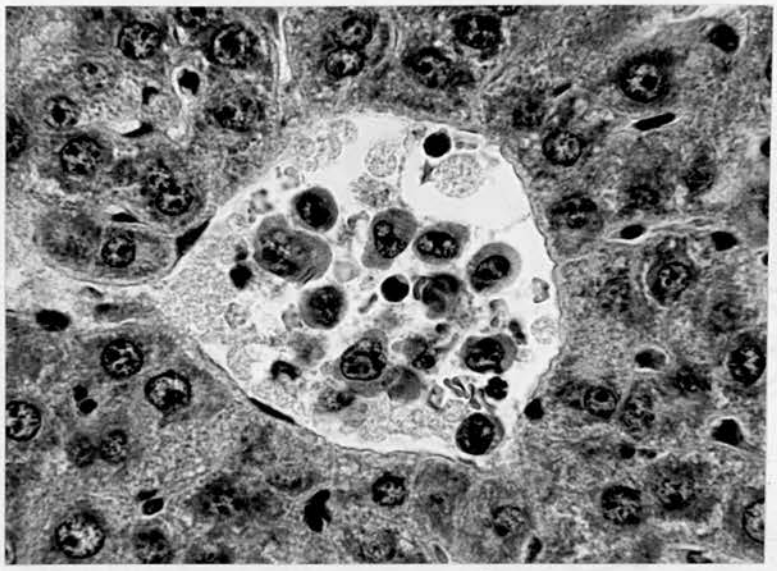

Fig. 2: Tumour cells in a central vein of the liver.
Untreated mouse dying of ascites.
H & E x 700.

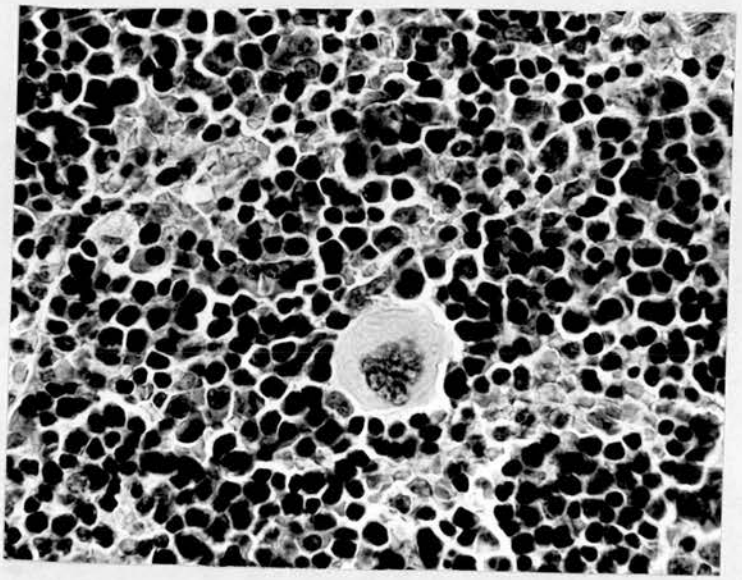

Fig. 3: Splenic red pulp of an A/Jax mouse bearing a 10 day growth of 10^5 ascites cells I.P. Note the marked degree of cellularity. H & E x 450.

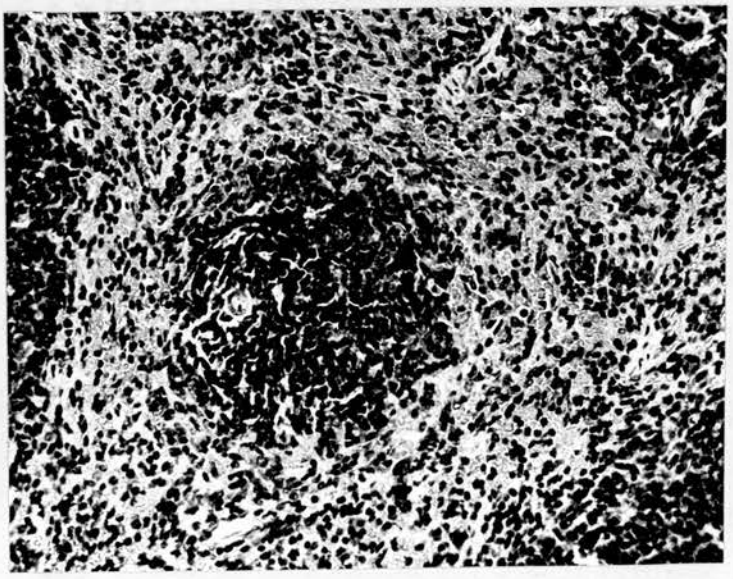

Fig. 4: Spleen of an A/Jax mouse dead with ascites. Note the small Malpighian body and the surrounding red pulp showing venous congestion. H & E x 250.

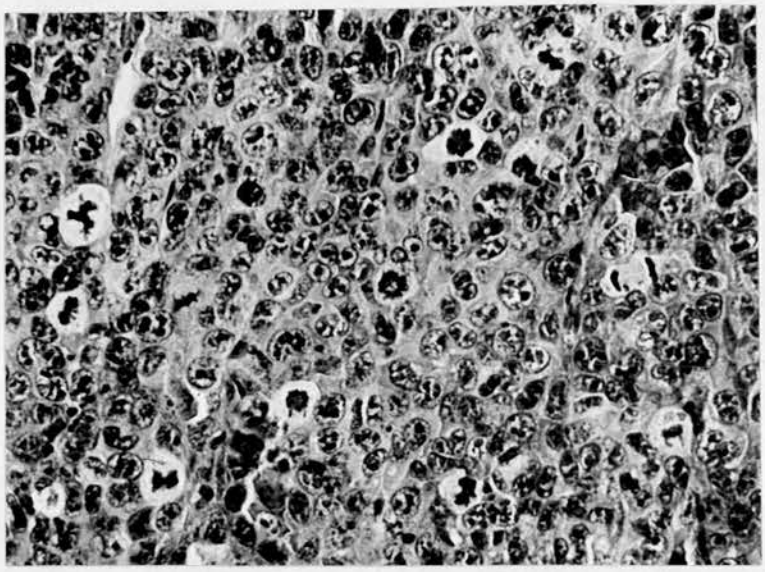

Fig. 5: Solid tumour growth in the paravertebral muscles. H & E x 450.

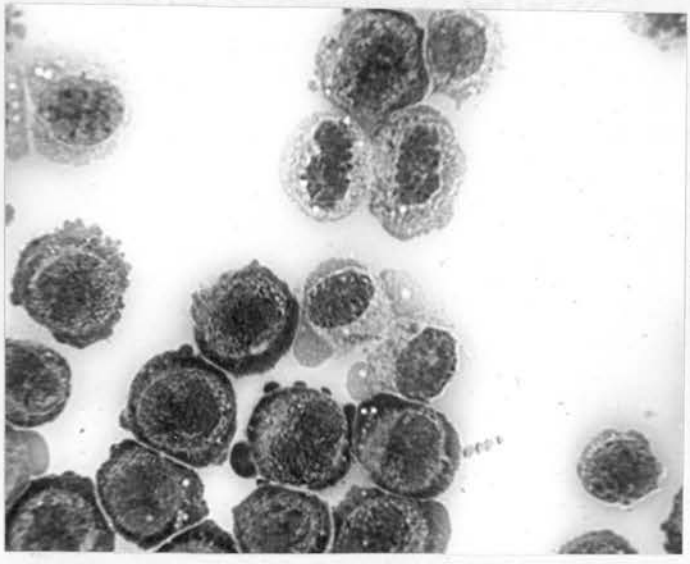

Fig. 6: A smear of the Landschütz tumour growing in the ascitic form.
Leishman stain x 750.

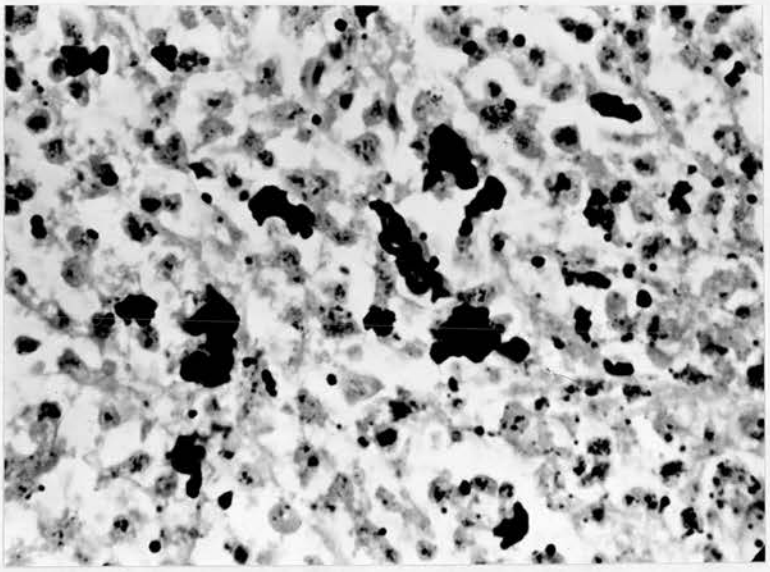

Fig. 7: Haematoxophil bodies in the centre of a necrotic tumour. H & E x 500.

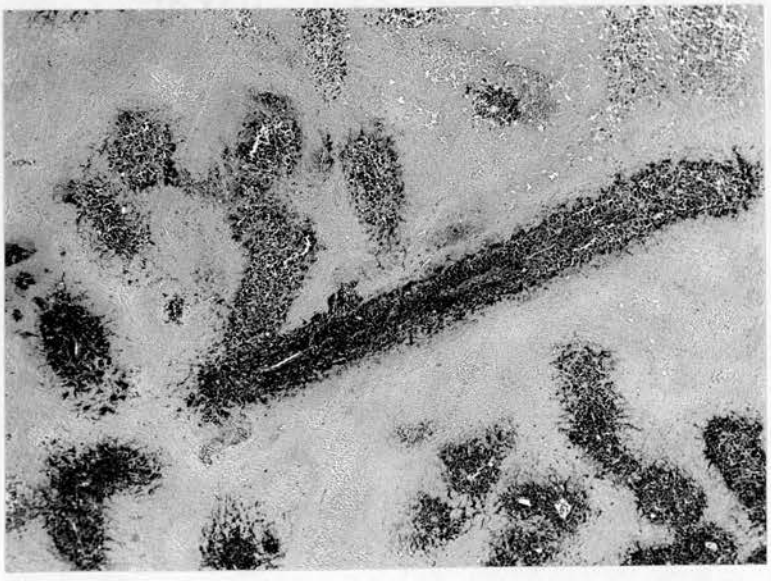

Fig. 8: Para-vascular distribution of viable tumour. H & E x 50.

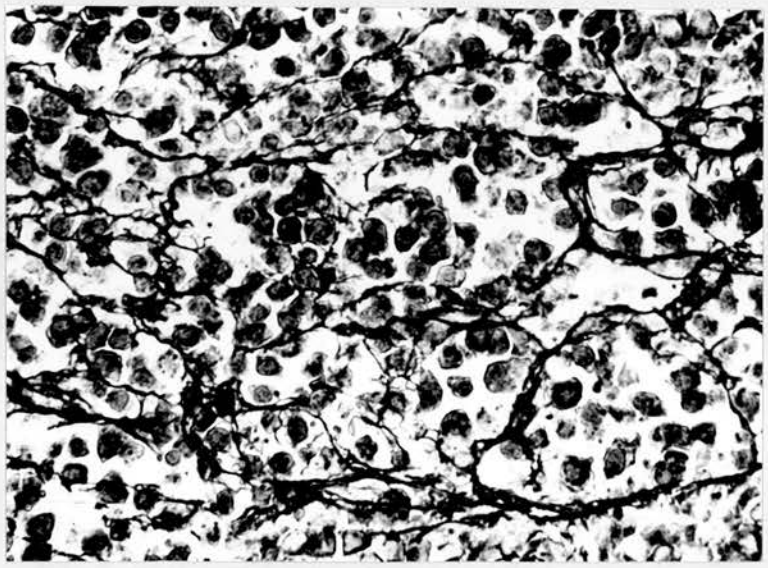

Fig. 9: Preformed reticulin around tumour cells infiltrating the para-vertebral muscles. Retic. stain x 450.

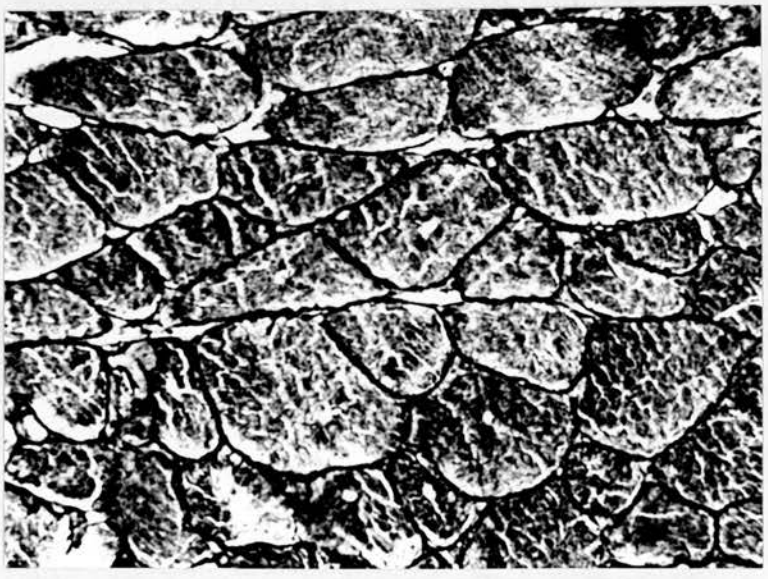

Fig. 10: Normal reticulin pattern of para-vertebral muscles. Retic. stain x 450.

Fig. 11: Solid tumour growth in the route of the mesentery.

Fig. 12: Solid tumour in route of mesentery (A) obstructing the common bile duct. Note the marked distension of the gall bladder (B).

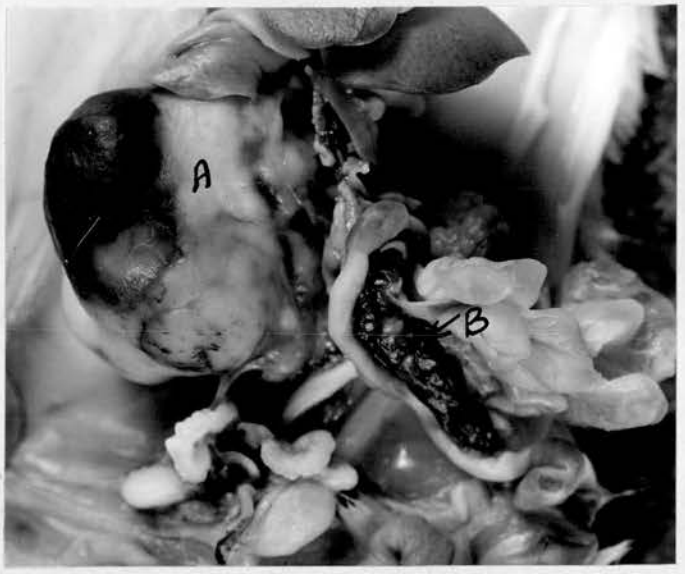

Fig. 13: Solid tumour in route of the mesentery infiltrating the right kidney (A) and radical of the portal vein (B). The latter are varicose and thrombosed.

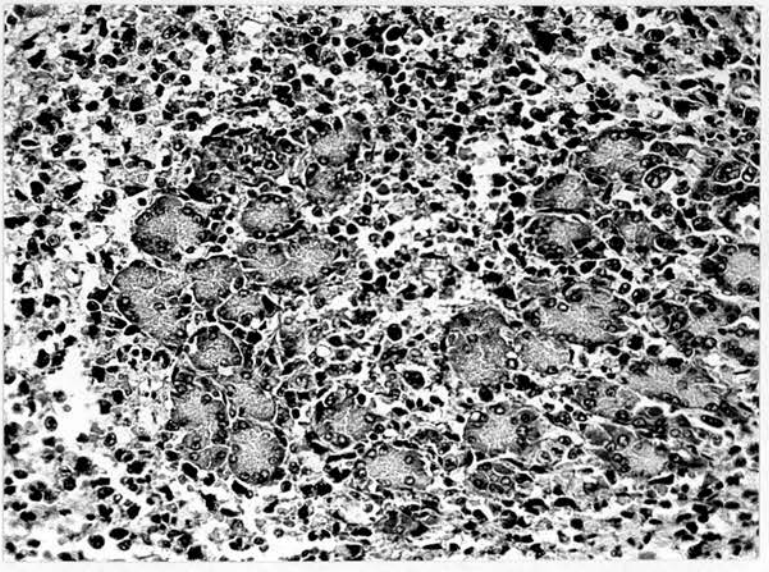

Fig. 14: Infiltration of the pancreas with solid tumour. Note the isolated groups of surviving glands. H & E x 225.

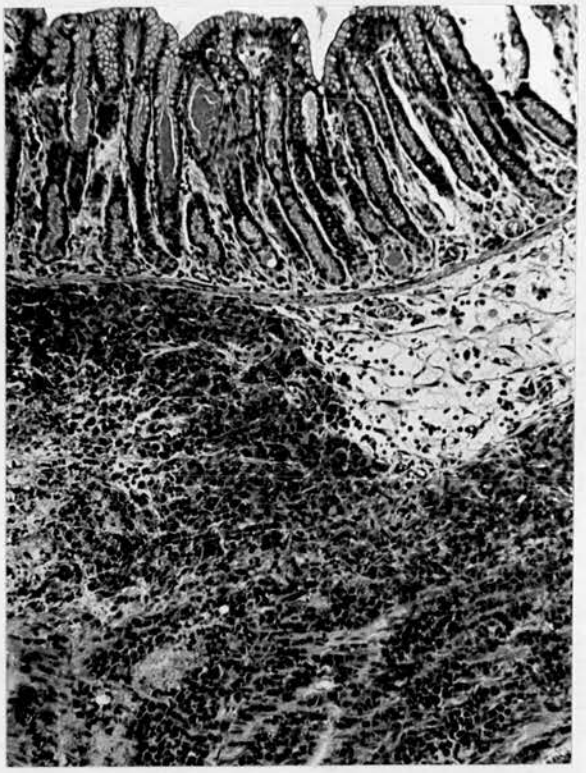

Fig. 15: Massive infiltration of the stomach with tumour. This mouse died of gastric perforation. H & E x 130.

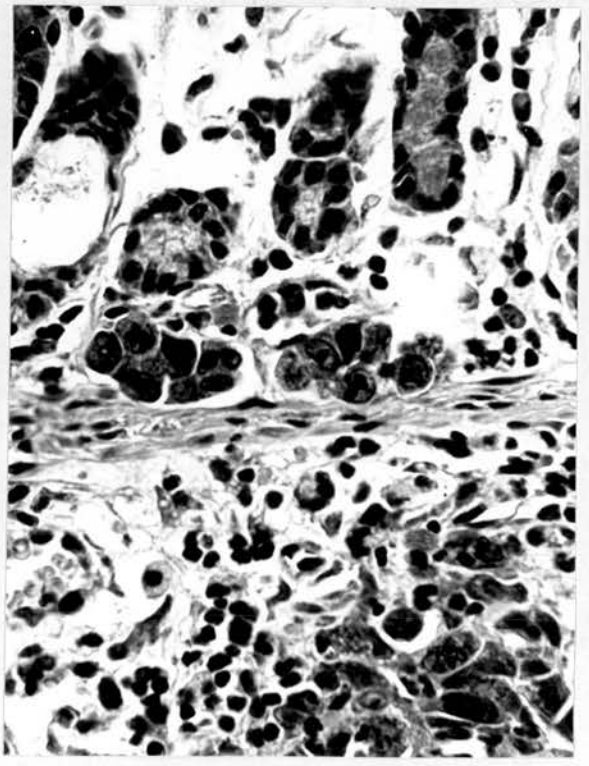

Fig. 16: Deposits of tumour cells in the mucosa of the stomach. H & E x 550.

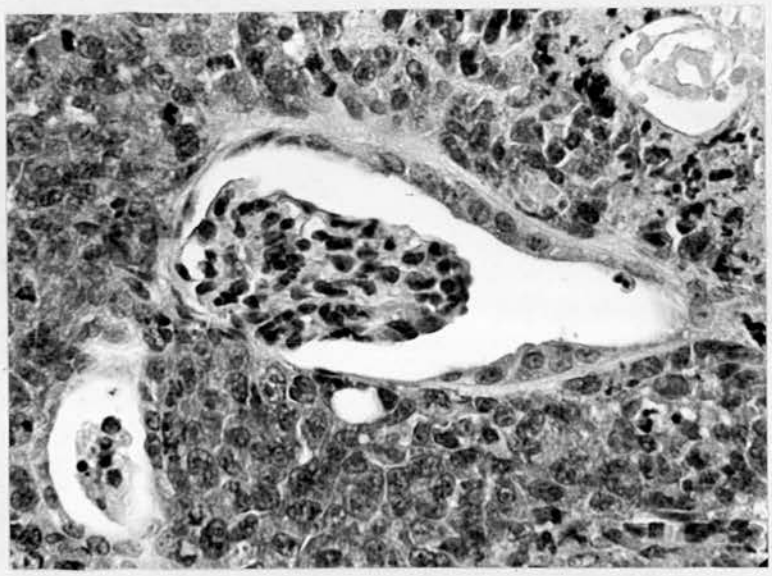

Fig. 17: Glomerulus surrounded by tumour.
H & E x 450.

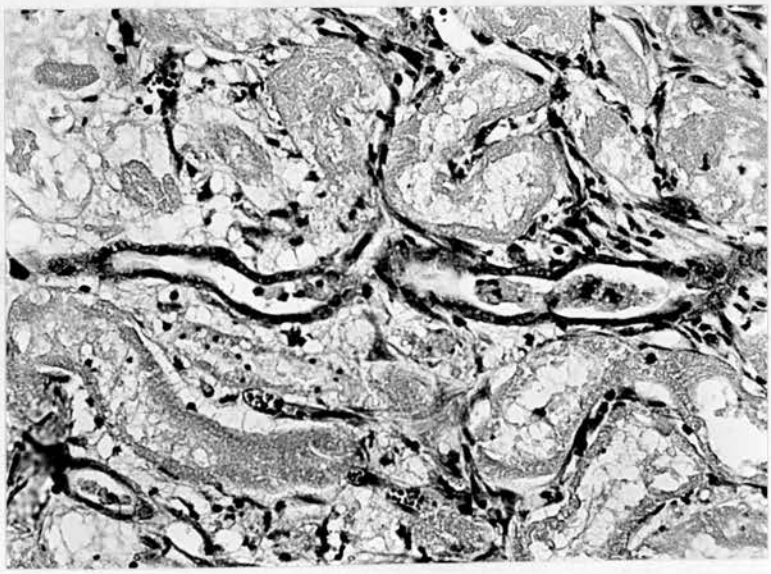

Fig. 18: Kidney showing acute tubular necrosis. Most of the tubules show complete necrosis of the lining cells. The two tubules in the middle of the picture are lined by flattened newly generated epithelium. H & E x 225.

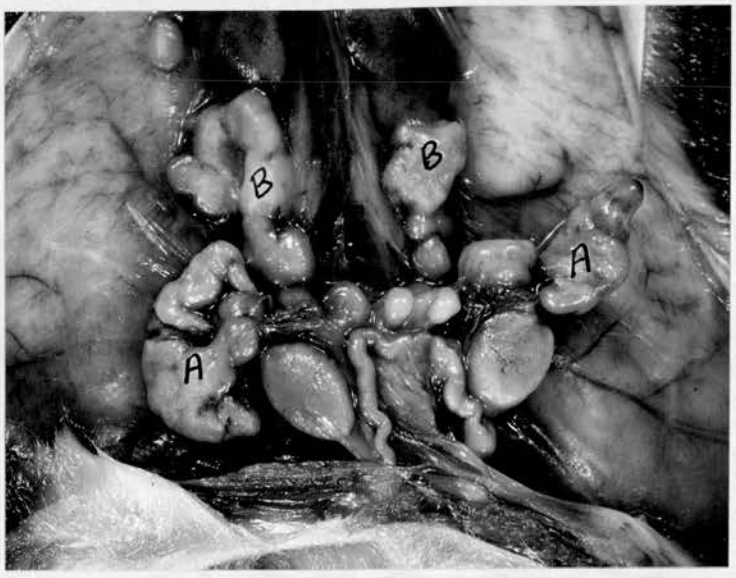

Fig. 19: Solid tumour masses in the fat of the testicular omentum (A), and the posterior abdominal wall (B). A/Jax mouse bearing tumour and treated with normal isologous spleen cells.

Fig. 20: An outbred mouse bearing tumour and treated with heterologous spleen cells. 40 days after treatment the mouse developed paraplegia. Note the extended hind legs and the bulging tumour mass in the left flank.

Fig. 21: This is the same mouse shown in fig. 20. The intra-abdominal mass is seen replacing the left kidney.

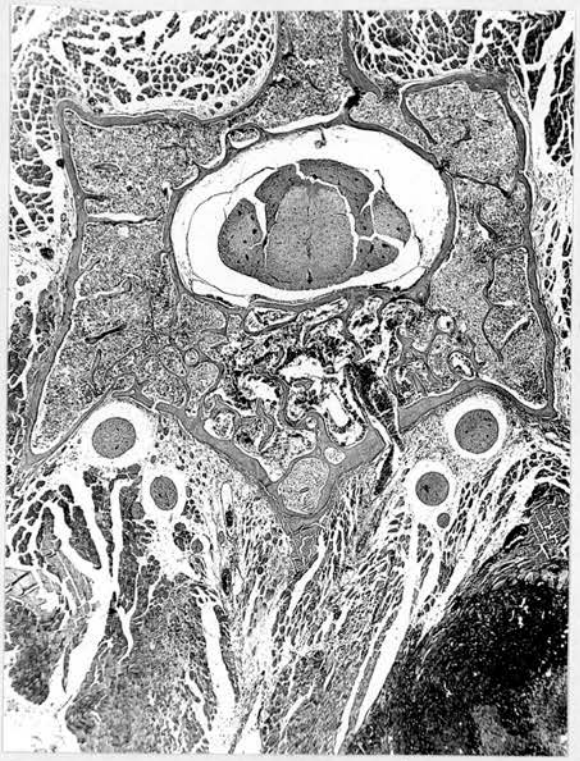

Fig. 22: Solid tumour growth infiltrating the para-vertebral muscles. The tumour is the dark mass in the bottom right hand corner. H & E x 25.

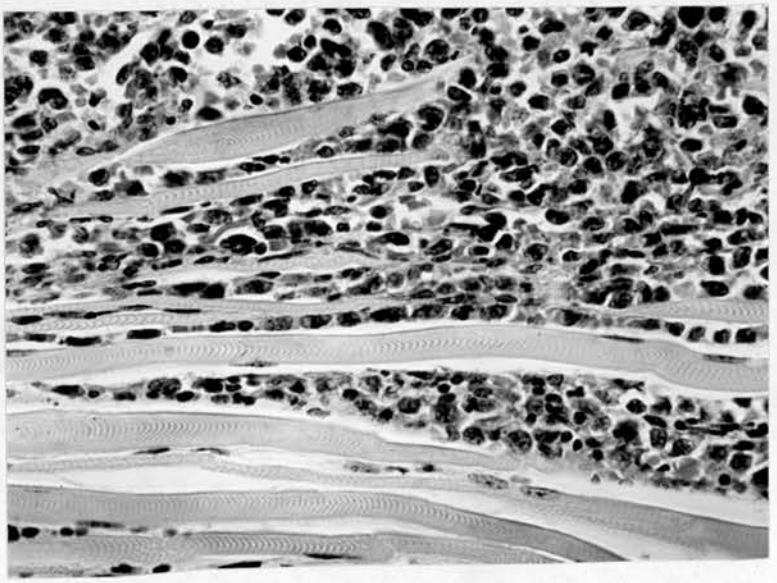

Fig. 23: Solid tumour infiltrating and destroying the para-vertebral muscles.
H & E x 400.

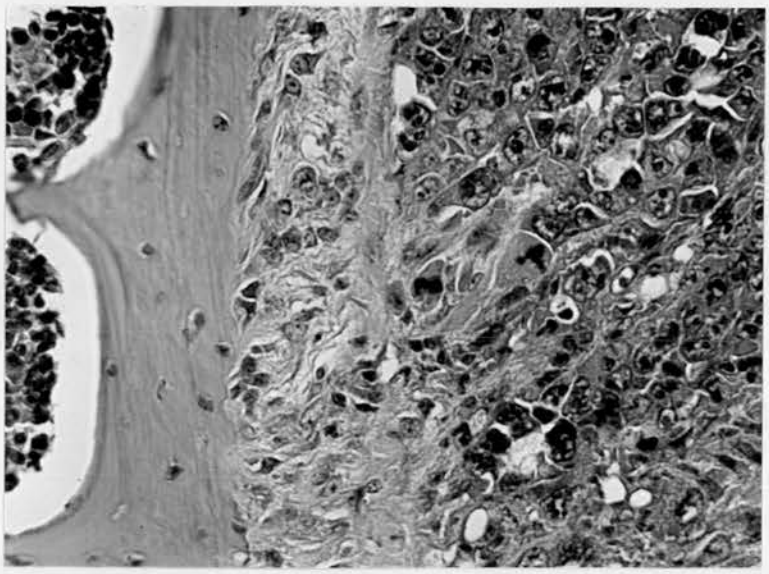

Fig. 24: Vertebral periosteum infiltrated with tumour. Note the thickened periosteum. H & E x 400.

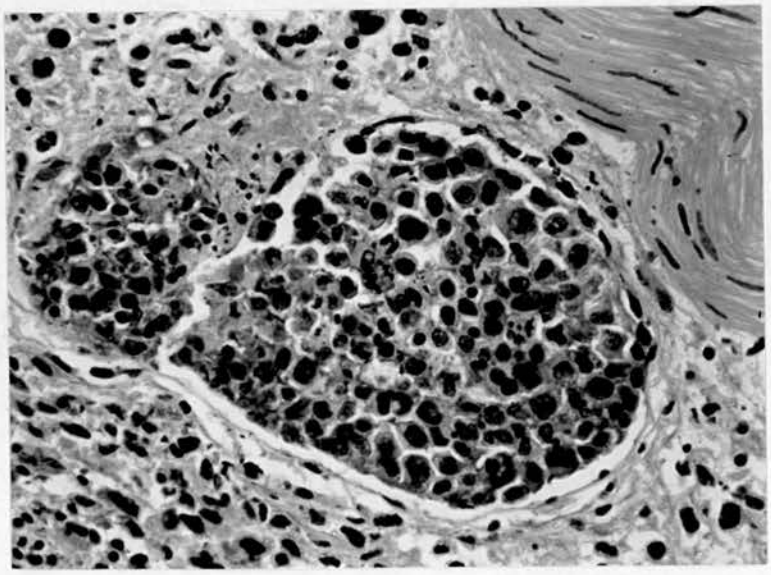

Fig. 25: Solid tumour mass in a large lymphatic. H & E x 400.

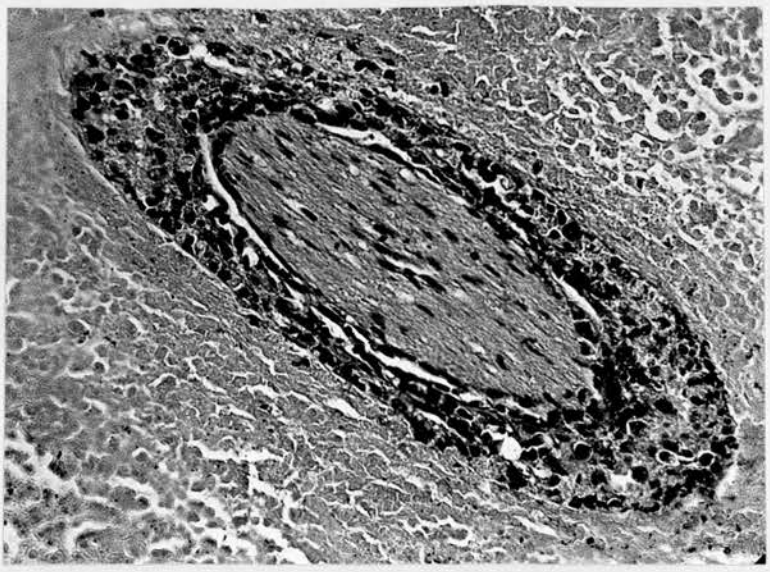

Fig. 26: Spread of the tumour by the perineural lymphatics. H & E x 225.

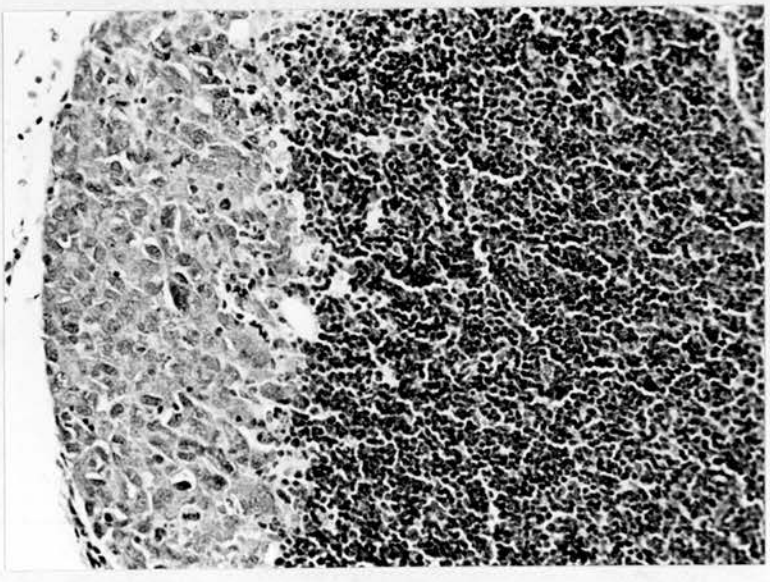

Fig. 27: Metastatic tumour deposit in the marginal sinus of a lymph node. H & E x 225.

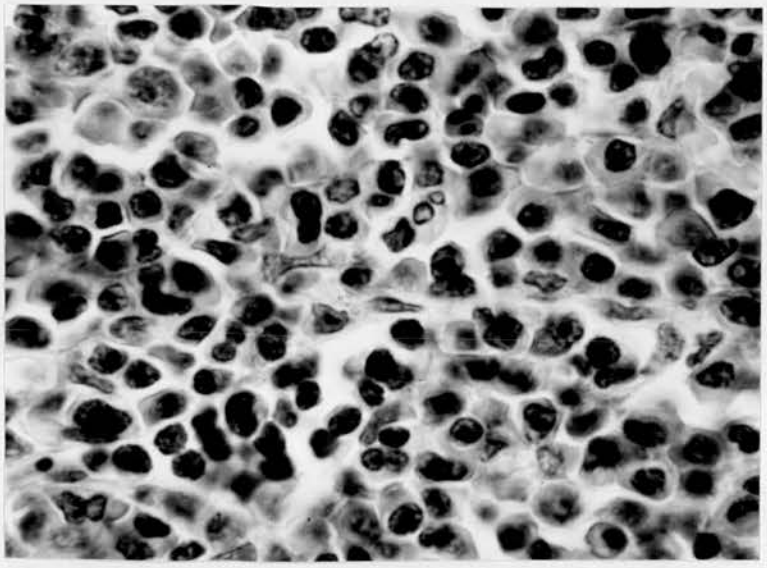

Fig. 28: Immature mononuclear cell reaction in a lymph node invaded by tumour. (Tumour cells are not shown in the picture). H & E x 1000.

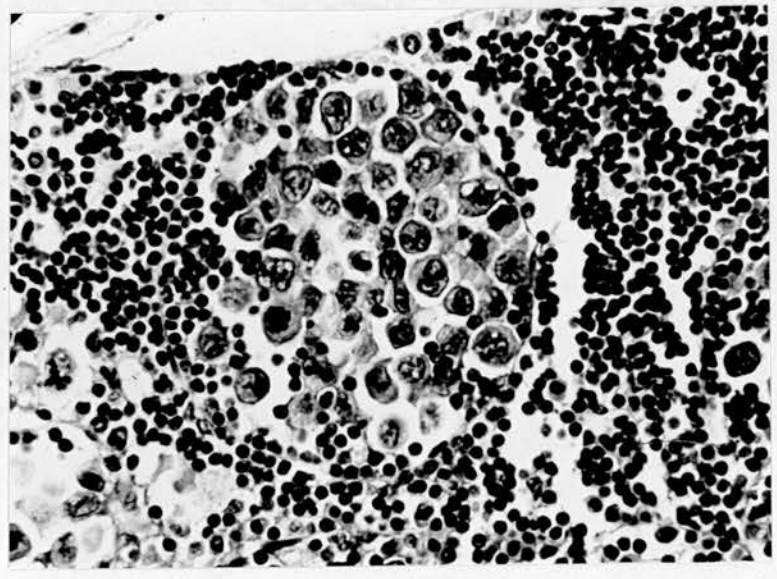

Fig. 29: A mass of solid tumour in a lymph node. There is no reaction to the tumour. H & E x 450.

CHAPTER VI

The fate of passively transferred spleen cells.

Introduction

In previous chapters spleen cells have been used to inhibit the growth of the Landschütz tumour. During this work it was observed that mice treated with isologous spleen cells developed "splenicules" in the peritoneal cavity. By contrast the peritoneal cavity of mice inoculated with heterologous or homologous spleen cells contained necrotic masses of tissue. This chapter describes the fate of isologous, homologous and heterologous spleen cells passively transferred to the peritoneal cavity of the mouse.

Material and Methods

The fate of passively transferred isologous spleen cells; the material was obtained from the autopsy examination of the animals used in the experiments carried out in chapter II. In all A/Jax mice treated with isologous spleen cells the peritoneal cavity was carefully examined for the presence of "splenicules". Their size, position and relation to solid tumour masses were noted. The tissue was fixed in 10% formol saline followed by 12-24 hours in saturated corrosive sublimate. Paraffin sections were made and stained with Mayer's haemalum and eosin. Reticulin was demonstrated by the method described

in chapter V.

Study of the evolution of the splenicules; 30 strain A mice were inoculated with 10^5 ascites cells I-P. and 48 hours later were treated with 500 x 10^6 immune isologous spleen cells which were obtained from mice bearing a 10 day growth of 10^5 ascites cells given intra-peritoneally. The method of preparing the immune spleen cell suspension was the same as the one described in chapter II. The treated (i.e. test) mice were sacrificed in groups of 5 animals at 3, 8, 13, and 18 days after passive transfer of the spleen cells. The evolution and formation of splenicules was followed macroscopically and microscopically. Control mice received 500 x 10^6 immune isologous spleen cells only and were sacrificed at the same time-intervals as the test animals. Some of the mice in the test and control groups were allowed to survive for 60 days then chloroformed and the peritoneal cavity examined for splenicules.

The fate of passively transferred homologous and heterologous spleen cells; homologous spleen cells were DBA_2 cells passively transferred in A/Jax mice bearing tumour (chapter II). The heterologous spleen cells were rabbit spleen cells used in the treatment of outbred mice bearing tumour (chapter III).

Results

The fate of passively transferred isologous spleen cells; the majority of animals bearing tumour, treated with isologous cells and autopsied 60 days later, had splenicules present. These were often situated in the fat of the testicular omentum, the omentum and occasionally on the surface of the small intestine (Fig. 1). The size varied from pin-points to 3 or 4 mm. in diameter. The colour was similar to that of the spleen. They differed from accessory splenicules in their lack of encapsulation. Accessory spleens in the mouse are usually single, rounded and situated in the region of the tail of the pancreas.

Table I shows that splenicules were found in mice that had been inoculated with tumour and a high dose of spleen cells. The presence of solid tumour at the time of postmortem examination, however, was not essential for the persistence of splenicules. Normal A/Jax mice receiving 500×10^6 immune isologous spleen cells showed no splenicules. The animals bearing tumour and treated with normal isologous cells died with massive tumour which was partly ascitic and partly solid in form. Only one mouse out of 4 in this group showed the presence of splenicules.

Histology of a fully developed splenicule; All the cellular elements seen in the adult normal mouse spleen were represented in the splenicule although the relationship of the various elements to each other was somewhat

disturbed. The lymphoid cells, consisting mainly of mature lymphocytes, were arranged in a follicular manner, mainly at the periphery (Fig. 2). The follicle formation was best seen in the reticulin preparations where coarse concentric fibres marked the periphery of the follicle (Fig. 3). Inside some of these "follicles" erythrocytes were seen among the lymphocytes. Proper lymphoid follicles with a follicular artery, and indistinguishable from Malpighian bodies of mouse spleen, were also seen. In the more central part of the splenicule, lymphocytes and other immature mononuclear cells were arranged in irregular foci in a vascular tissue reminiscent of the red pulp of the spleen. Other cells were also seen, including megakaryocytes (Fig. 4), occasional plasma cells and monomuclear cells with a horse-shoe shaped nucleus. Unlike accessory spleens, capsules and trabeculae were never seen. Fatty tissue was sometimes incorporated in the splenicules and helped in differentiating between them and accessory spleens.

The evolution of the splenicules; at 3 days after the passive transfer of the isologous spleen cell suspension, the test and control mice showed the same appearances. In several of the splenicules there was a central area of necrotic tissue surrounded by a zone of large immature mononuclear cells. These cells had rounded vesicular nuclei with prominent nucleoli and a moderate amount of agranular cytoplasm (Fig. 5). Thus these cells were morphologically reticulum cells. Occasionally the cells were spindle

shaped. Scattered among the large mononuclear cells were plasma cells and mature lymphocytes. The latter were more frequently seen in the surrounding fat at a distance from the mononuclear cells. A conspicuous feature of the splenicule at 3 days was the presence of several thin-walled large capillaries containing blood (Fig. 6). These large vascular channels were reminiscent of a cavernous haemangioma. An extraordinary thing to find, even at 3 days, was the presence of proper well formed lymphoid follicles in one splenicule (Fig. 6). In two splenicules from the test animals, small islands of tumour cells were seen. At 8 days the general morphology of the splenicule was similar to that seen at 3 days. With small splenicules, however, the necrotic material had been absorbed and replaced by the tissue described above which grew from the periphery. Haemosiderin, containing macrophages and cells with a foamy cytoplasm were also seen.

At 13 days the splenicules obtained from mice receiving spleen cells only showed less cellularity than mice receiving spleen cells and tumour (Fig. 7). The main cells were fibroblast-like cells and haemosiderin containing macrophages. A splenicule from a test mouse at 13 days showed more cellularity (Fig. 8); the main cell was the lymphocyte.

At 18 days splenicules from test mice were mature and had proper follicles at the periphery. In some of these a

follicular artery with hyalinised wall was seen. The centre of the splenicule was formed of clefts and spaces lined by endothelial cells and containing erythrocytes. A few scattered lymphocytes were seen. Megakariocytes were rarely seen.

The fate of passively transferred homologous and heterologous spleen cells; Mice treated with homologous or heterologous spleen cells never showed any splenicules. The transferred cells were destroyed, leaving only an encapsulated mass of necrotic tissue (Fig. 9).

Discussion

The finding of splenicules in the peritoneal cavity of mice was curious, since the spleens were homogenised, filtered and this supension of individual cells injected through a size 20 needle. It is clear from the results presented here that splenicules persisted only in animals receiving tumour and spleen cells. There was a close association between the spleen cells and solid tumour, at least in some animals. By contrast, heterologous and homologous spleen cells were completely destroyed and, in the late stages, only encapsulated necrotic masses of tissue were found in the peritoneal cavity.

The series of events culminating in the formation of splenicules was similar in many respects to the changes occurring in splenic autografts. Manley and Marine (1917) were the first to perform successful splenic autografts in rabbits. Small pieces of the spleen were implanted in the subcutaneous tissues; these regenerated and had all the morphological and functional characteristics of an active spleen. As a result of further experiments, Marine and Manley (1920) thought that in the splenic autografts each element regenerated independently. Danchakoff (1918), using chicken spleen grafts on the allantoic membrane of chicken embryo, believed that the reticulum cell gave rise to granular leukocytes. Perla (1936) made a study of the regeneration of splenic autografts in the rat. He noted that autotransplants underwent rapid degeneration in the first 24 hours; only a thin zone of reticulum cells survived. These cells penetrated the central necrotic zone. Lymphocytes were seen on the third or 4th day and complete regeneration occurred in 12 to 21 days. Perla believed that the reticular cells were the precursors of all the structural elements of the spleen. Calder (1939) gave a detailed description of the regeneration of splenic autografts in mice and rats and concluded that reticulum cells gave rise to the mature cells in the spleen. Cameron and Rhee (1959) confirmed and extended the work of Calder. They noted that as early as two days after splenic autograft in the rats, the reticulum cells were forming

thin-walled blood vessels which established vascular connection with the host's blood vessels by the third day. By the fifth day lymphocytes appeared in relation to blood vessels and at about the 14th day definite Malpighian follicles were seen. In about 4 weeks the autograft resembled a normal spleen.

The work cited above on the regeneration of autografts utilised slices of spleen in which degeneration occurred as a result of ischaemia. With the present system a suspension of isologous spleen cells was injected in the peritoneal cavity. Under such circumstances it is probable that the cells had a better chance to survive than cells in a solid graft. It seems that, following the passive transfer of isologous spleen cells, some of the lymphocytes persist; other cells die or were already dead when injected into the peritoneal cavity. Reticulum cells proliferate and differentiate into the various cell types that are normally present in the spleen. The cells of the splenicule are not derived from the host since splenicules occurred only in the isologous system and homologous spleen cells were completely destroyed, leaving only amorphous encapsulated debris.

There are two puzzling questions which are difficult to answer. Firstly, what is the nature ofthe stimulus for the formation of splenicules? Secondly, how do cells that have been completely dissociated from each other reconstitute

a highly complex organoid structure? In answer to the first question, it is noteworthy that the presence of tumour cells in the present system was necessary for the persistence of the splenicules. It was shown by several workers that splenectomy was a strong stimulus to the growth of splenic autografts (Marine and Manley 1920, Metcalf 1963). Splenectomy may produce its effects by creating a functional demand for splenic tissue. This demand may also be created by the presence of an antigenic stimulus, in the present system, tumour cells.

It is even more difficult to answer the second question. It is conceivable that spleen cells function as a unit and somehow the unit reconstitutes itself even when the cells have been dispersed. Cameron and Rhee (1959) suggested that the spleen was made up of growth centres or "fields". To quote their own words - "in our opinion the experiments in which we show that the complete spleen may be divided up into many small grafts which grow into miniature spleens, despite the initial destruction of much of their substance, suggests that the organ consists of a number of growing centres or fields. These possess no definite anatomical location, for the spleen may be sub-divided at random with the same end result". They could not define the minimum number of cells required to constitute a field. From the present study as few as 20 million cells led to the formation of splenicules.

It is relevant at this point to recall the development of splenicules in man. It is well recognised that splenicules may follow rupture of the spleen. Faltin (1911) reported a case where splenicules were found in the peritoneal cavity of a boy six years after splenectomy for splenic rupture. More recently Shaw and Shafi (1937) Stobie (1947) Jancho and Anderson (1939) have reported more cases. It is generally agreed that the splenic nodules that follow injury to the spleen are due to autotransplantation of splenic tissue.

TABLE I

Development of splenicules in normal or tumour bearing mice. (x = average survival)

No. of mice	Tumour dose	Treatment (isologous spleen cells)	No. with solid tumour	No. with splenicules	P.M. at
5	Nil	500 x 10^6 10 day immune cells	Nil	Nil	60 days
11	10^5	500 x 10^6 10 day immune cells	2	11	60 days
5	10^5	100 x 10^6 10 day immune cells	5	Nil	46x
4	10^5	20 x 10^6 10 day immune cells	4	Nil	36x
5	10^5	500 x 10^6 20 day immune cells	5	5	60 days
4	10	500 x 10^6	all had ascites and solid tumour	1	

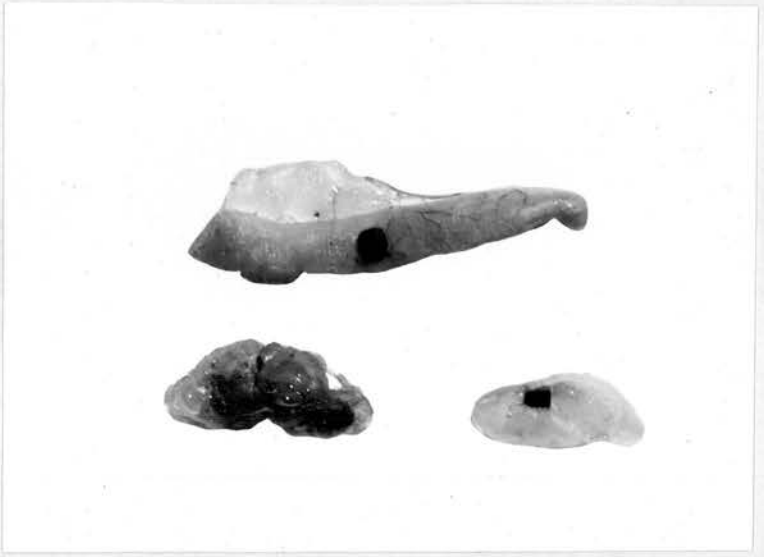

Fig. 1: Splenicules on the surface of the intestine (above) and in the fat of the testicular omentum (below).

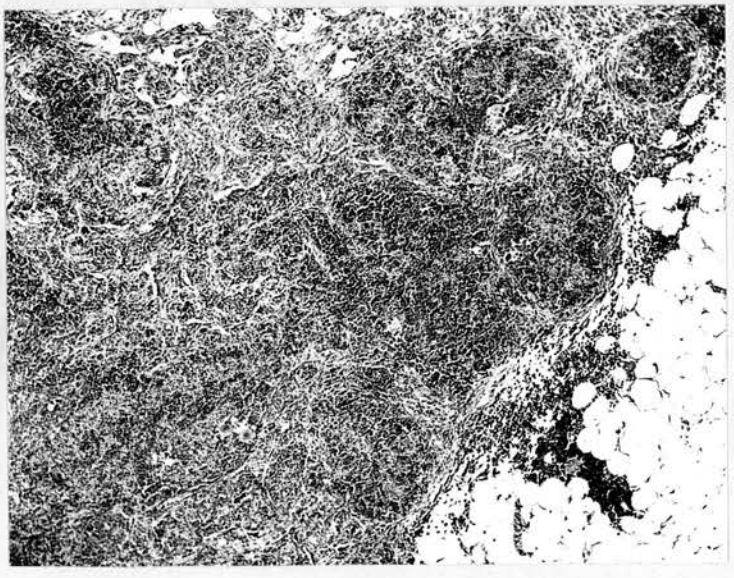

Fig. 2: Splenicule 60 days after passive transfer of isologous spleen cells. Note extension of lymphoid tissue into the fat and the follicular arrangement of the cells at the periphery. H & E x 80.

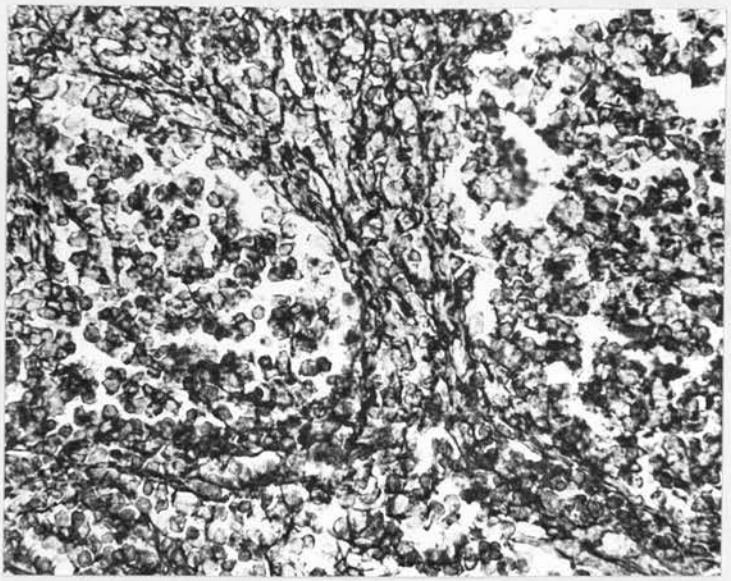

Fig. 3: Concentric coarse reticulin fibres at the periphery of two adjacent follicles. 60 day old splenicule.
Retic. stain x 400.

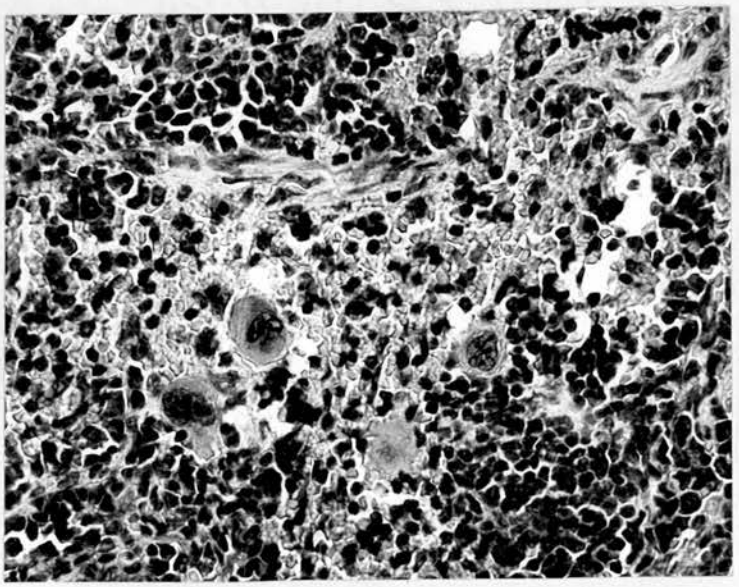

Fig. 4: Splenicule at 60 days showing three megakaryocytes. H & E x 450.

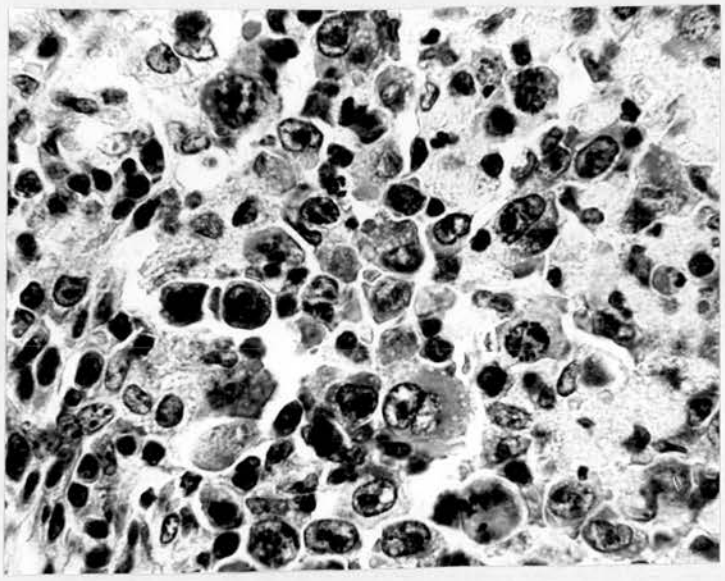

Fig. 5: Splenicule at 5 days showing a focus of large immature mononuclear cells.
H & E x 650.

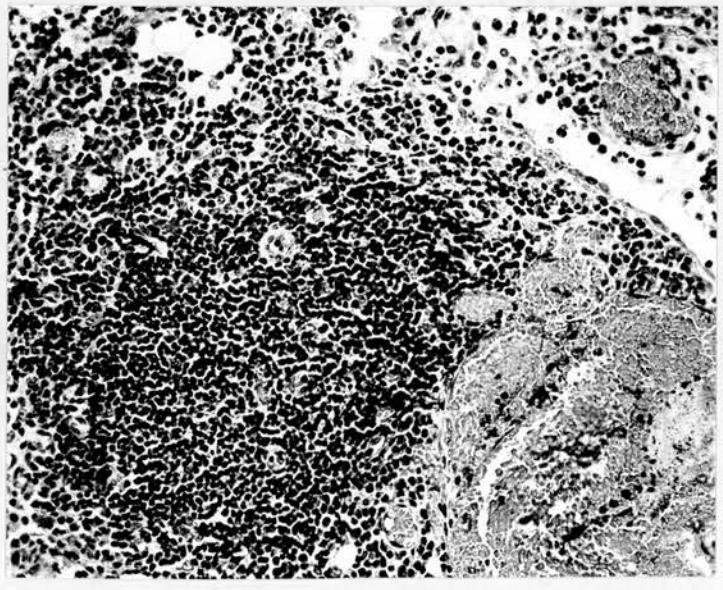

Fig. 6: Splenicule at 3 days. Large vascular channels are seen in the bottom right hand corner. Note the well-formed lymphoid follicle on the left.
H & E x 200.

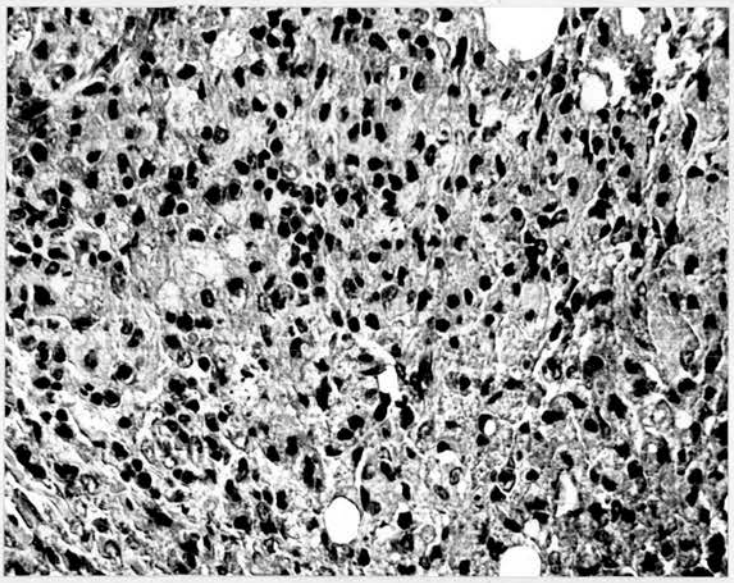

Fig. 7: 13 day splenicule from control mice receiving spleen cells only. This shows much less cellularity than fig. 8. H & E x 450.

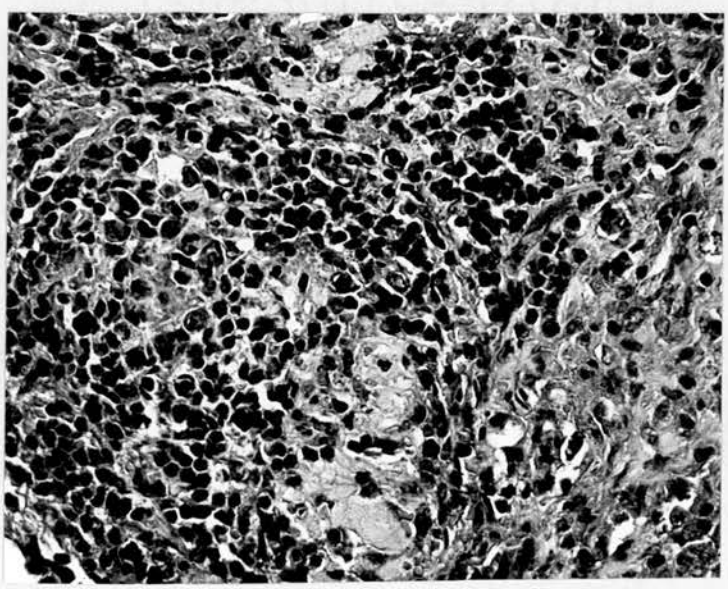

Fig. 8: 13 day splenicule from test mice receiving tumour cells and spleen cells. Note that the splenicules are markedly cellular. No tumour cells are seen. H & E x 450.

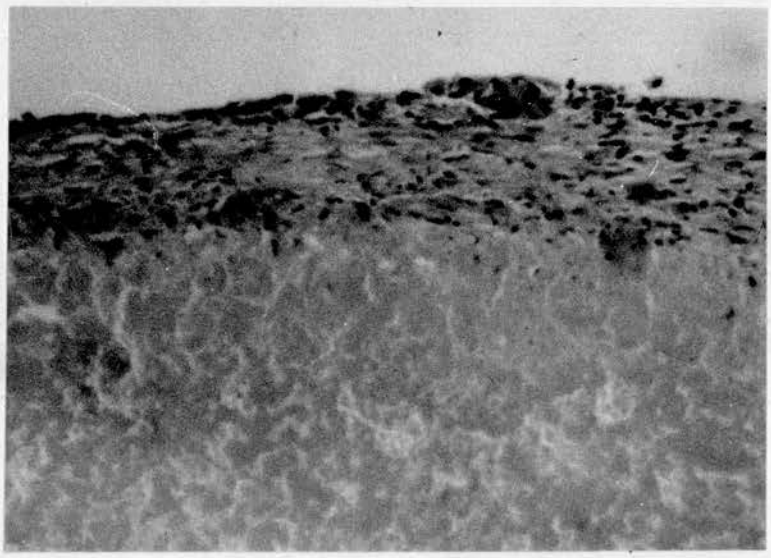

Fig. 9: Encapsulated necrotic tissue found in peritoneal cavity of mouse treated with homologous spleen cells by the I.P. route. H & E

SUMMARY

The Landschütz ascites tumour is a rapidly proliferating mouse neoplasm that grows progressively in several strains of mice. It was used in the present work as an experimental model to study the interaction between a rapidly proliferating cell and the lymphoreticular tissues of homologous and heterologous hosts. The tumour was regarded as a homograft of a high degree of growth potential and was found to be convenient for the study of the biology of cell death using immunological methods. Particular attention was paid to the following aspects.

(1) The natural history of the disease in untreated animals.

(2) The role of cells and serum in adoptive immunity and enhancement of tumour growth.

(3) The fate of passively transferred spleen cells.

(4) The specificity of isologous, homologous and heterologous systems.

Animals; the following animals were used in the present study.

300 Outbred mice from an M.R.C. closed colony.

700 of the inbred strain A mouse.

80 of the inbred strain DBA_2 mouse.

400 Rabbits of a mixed, commercial stock.

Transplantation of the tumour in a homologous system.

The natural history of the tumour in the different mouse strains was studied. It was shown that the rate of tumour growth and the survival of the hosts were dependent on the strain in which the tumour was inoculated.

Changes in the lymphoreticular tissues of mice bearing the Landschütz tumour; A detailed study of the interaction between the tumour and the host was undertaken in the strain A mouse. The tumour evoked reactive changes in the spleen and lymph nodes of this strain. The liver showed an increase in size of the Kupffer cells and the carbon clearance technique revealed an increase in phagocytic function. This phase of reticulo-endothelial system stimulation was maximal at the tenth day after tumour inoculation and subsequently returned to normal as the tumour grew progressively. Treatment with isologous cells inhibited the activation of the R.E.S. The thymus showed complete atrophy at postmortem examination. Bacteriological cultures of the ascitic fluid were sterile and the changes in the lymphoreticular histology were mainly due to iso-antigenic differences between the host and the tumour.

Inhibition and enhancement of the Landschütz tumour by an isologous and a homologous cellular system; Adoptive immunity and enhancement of the tumour were studied by transferring spleen cells or sera from suitably

immunised animals to appropriate recipients bearing tumour. The degree of immunity was markedly influenced by the quality of the lymphoid cells transferred. This depended on the manner of immunisation and the time of harvesting the cells. Isologous strain A cells persisted in the peritoneal cavity of the recipients as splenicules but did not exert as potent inhibitory effect as homologous DBA_2 spleen cells. Cells from outbred mice had no inhibitory activity. Isologous serum could inhibit the tumour if correctly prepared but as a general rule immune isologous serum greatly enhanced tumour growth. The serum caused enhancement when given two days after tumour inoculation. The spleens of mice showing tumour enhancement were smaller than those of animals bearing unenhanced tumour. The significance of this new finding in the interpretation of the mechanism of immunological enhancement was discussed. The R.E.S. phagocytic function of both enhanced and tumour bearing controls was above normal. R.E.S. blockade and lipid induced splenic necrosis did not enhance tumour growth. Enhancement of tumour growth was also produced by passive transfer of normal isologous spleen cells.

Inhibition and enhancement of the Landschütz tumour by heterologous cells or sera; Heterologous spleen cells obtained from rabbits immunised against the Landschütz tumour exerted a marked inhibitory effect on the growth

of the tumour in vivo. Intact spleen cells from immunised rabbits were effective in suppressing a 48 hour growth of 10^5 or 10^6 ascites cells in outbred mice. Thiotepa was without effect in similar circumstances. It was emphasized that with a tumour dose of 10^7 cells treatment with lymphoid cells produced enhancement particularly when administered before the tumour. This was noted with homologous and heterologous cells. Mice inoculated with heterologous lymphoid cells showed a reduction of R.E.S. phagocytic function as judged by the carbon clearance test and impaired antibody production to the injection of sheep red cells. This form of enhancement was new in its mode of production and was attributed to depression of the host R.E. system.

Immune heterologous serum also inhibited tumour growth and sometimes converted it to the solid form. Following a single inoculation of rabbits with tumour the antitumour activity of both lymphoid cells and serum was assessed over a period of time. The conclusion was reached at that in this system there was no qualitative difference between circulating antibody and "cell bound" activity.

The drawback of the heterologous system was its non-specificity. Using different in vitro serological methods, it was shown that the rabbit anti-tumour serum had a broad spectrum of specificity and cross reacted with several normal mouse tissues.

It was observed that inadequate treatment of the tumour with cells or sera led to conversion of the tumour to the solid form and completely altered the natural history of the disease. Treated animals died from local complications of solid tumour such as intestinal obstruction, pancreatic insufficiency, uraemia, jaundice, paralysis or widespread metastases. This agglutination of the ascites cells meant that animals survived longer than the untreated controls. The importance of recognising this in the assessment of the effect of a tumour inhibitory against was stressed.

The fate of passively transferred spleen cells; It was found that passively transferred isologous spleen cells persisted as splenicules in the peritoneal cavity of the recipients. The mode of formation of the splenicules and their histogenesis was discussed.

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APPENDIX I

APPENDIX I

SEROLOGICAL METHODS

Tanned cell Haemagglutination

Ref. 1) Boyden, S.V. 1951 J. Exp. Med., 93, 107.

2) Stavitsky, A.B. 1954 J. Immunol., 72, 360.

3) Stuart, A.E. Ph.D. Thesis Edinburgh University 1958.

PRINCIPLE

When sheep red cells are treated with suitable concentration of tannic acid, they are rendered capable of absorbing certain protein molecules from solution in saline. Red cells treated in this way and washed are agglutinated by the homologous anti-protein sera. Thus sera can be titrated for antibodies against antigens absorbed on the cells exposed to tannic acid.

MATERIAL AND METHODS

(1) 4% suspension of washed sheep erythrocytes (Wellcome Labs.) are washed 3 times in saline and resuspended in buffered saline PH 7.2.

(2) Preparation of erythrocytes with tannic acid. To a 4% suspension of washed cells add an equal volume of tannic acid strength 1/20,000 diluted in saline PH 7.2. Stand at room temperature for 30 minutes, centrifuge gently and wash three times in buffered saline, and resuspend to give a 2% suspension.

ABSORPTION OF ANTIGENS

An equal volume of suitably diluted antigen is added to 2% of solution of red cells and the mixture allowed to stand at room temperature for 30 minutes. The sensitized red cells are now washed three times with 1/200 normal rabbit serum. The cells are resuspended in 1/200 N.R.S. in saline PH 7.2 to give a 1% suspension.

(The method of preparing mouse antigens was described in chapter III. Three concentrations of antigen were used for absorption on tannic acid treated erythrocytes, viz. 1/50, 1/250 and 1/500).

Prior to use normal rabbit serum has been inactivated at 56°C for 10 minutes and then absorbed with its own volume of washed (x3) packed sheep cells for 30 minutes at 37°C.

EXPOSURE OF CELLS TO TEST SERUM

1. Set out 2 rows of 11 tubes for each test serum; place 0.1 ml. serum diluted 1/10 in the first tube of each row and thence forth run doubling dilutions to a titre of 1/10,000.
2. To the front row add 0.1 ml. of tanned antigen coated cells. To the back row add 0.1 ml. of tanned cells.
3. Saline Controls: In two tubes place in each 0.1 ml. tanned antigen coated cells and 0.1 ml. saline; in another two tubes place in each 0.1 ml. saline and

0.1 ml. of tanned cells.

4. Normal serum controls; tubes are set as for test serum but using normal rabbit serum.

Shake all tubes vigorously and leave at room temperature for 2-4 hours.

IMPORTANT PRACTICAL POINTS

Tannic acid:

A stock solution of 1 g. in 200 cc. of buffered saline PH 7.2 may be kept at 4°C for not more than 3 weeks. If commercial grade tannic acid is used make up fresh each time. Cells treated with tannic acid must be washed three times to remove excess acid which can act as ambo-ceptor and causes haemolysis.

Red cells:

Sheep cells are stored at 4°C for a week. If during preliminary washing the cells show excessive haemolysis they should not be used.

Normal rabbit serum:

This prevents lysis and aids in the resuspension of red cells after centrifugations.

The rabbit serum should first be absorbed with packed sheep cells before use.

Absorption of antigens:

Manipulate the cells gently after exposure to antigens. Free antigen will completely inhibit the haemagglutination reaction.

SERA

Test sera are stored at 4°C or if deep frozen (at -20°C) are thawed only once and used. Sera which are turbid may be infected. Infected sera must not be used as bacterial haemagglutinations will interfere with the reaction. Therefore examine suspect sera under phase contrast (high magnification) for organism.

ANTIGENS

Make sure that the concentration of antigen used does not by itself cause haemagglutination.

SALINE

Phosphate buffer to PH 7.2.

TUBES

3" x $\frac{3}{8}$".

Agar Gel Diffusion

Modified Oudin tube technique.

References;

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PRINCIPLE

If antigenic mixtures are allowed to diffuse from one side and the corresponding antiserum from the other side into a central column of agar, dises or lines of precipitation are produced if antigen and antibody concentration are suitable. The exact position of the dise will depend on the relative concentrations of antigen and antibody in their respective mixtures, and on their rates of diffusion in the agar gel. Since the diffusion rates are not likely to be the same for all the antigens, though they may be for all the antibodies, and since the relative concentrations of antigen and antibody are unlikely to be the samefor all antigen-antibody systems present, it is likely that a separate line will be produced by each such system present in sufficient concentration.

MATERIAL AND METHODS

(1) Melt agar solution (2% in 0.9% saline) in boiling water bath and then place in water-bath at 42-45°C.

(2) Mix equal volumes (0.2 ml.) of test serum and agar in 3 x ½ " tube.

(3) Pipette a column of test-serum - agar about 1.5 cm. into a 5 x 0.6 cm. tube at room temperature using a long pasteur pipette. Take care not to touch the side of the tube with the pipette.

(4) When the layer of test-serum -agar has set a 0.8 cm. column of a mixture of equal parts of 0.9% Nacl and agar solution at 42°C is pipetted on top of the serum-agar.

(5) When the saline-agar column has set add the antigen layer on top of it. The antigen layer consists of equal volumes of agar solution and antigen. The length of this layer is the same as the test-serum - agar column.

(6) Cork the tubes and leave at room temperature. The tubes are examined daily.

RESULTS

Antigen and antibody diffuse slowly through the agar/saline (i.e. the middle layer) and lines of precipitate appear when antigen and antibody meet in correct proportions.

IMPORTANT PRACTICAL POINTS

(1) Never touch the sides of the tubes with the pasteur pipette when transferring the different re-agent-agar mixtures.

(2) Allow each layer to set before adding the next.

(3) Warm the pasteur pipette before transferring the agar.

Complement fixation test

PRINCIPLE

Dilutions of serum are mixed with complement and various antigens. If an antigen - antibody reaction takes place complement is fixed. This is demonstrated by testing for residual complement. Sheep cells sensitized with anti-sheep cell haemolytic serum are added which are lysed in the presence of complement. Where there is no lysis complement must have been fixed in the first reaction.

MATERIALS

Perspex plates, agglutination trays (W.H.O.), obtainable from Messrs. Prestware Ltd., Southdown Works, Kingston Road, Raynes Park, London S.W.20.

Automatic Pipettes to deliver 0.1 ml. obtainable from Messrs. R.B. Turner & Co., 9 Eagle Street, London, W.C.1.

Buffered normal saline PH 7.2

Sheep red cells (Wellcome Labs.).

Haemolytic Serum (Wellcome Labs.).

Complement. Fresh guinea pig serum.

Titration of haemolytic serum and complement

Titrate these as a chessboard, in perspex plates.

Prepare a series of dilutions with a 20% difference in concentration of complement between each.

Add 0.2 ml. of saline to each cup (to represent 0.1 ml. of serum and 0.1 ml. of antigen in the test proper).

Deliver 0.1 ml. volumes of complement dilutions into the cups of perspex plates as in the example shown below.

Stand the plates at 37°C for 1½ hours; cover to prevent evaporation.

Prepare a series of doubling dilutions of haemolytic serum. Mix each with an equal volume of 4% washed sheep cells. Sensitize the mixture for 10 minuts in water-bath at 37°C or at room temperature for at least 30 minutes.

Deliver 0.1 ml. volumes of sensitized cells as in the example.

Incubate for 30 minutes at 37°C. Shake after about 15 mimutes and again on removal from the incubator.

Place the plates in the refrigerator overnight or for several hours until the cells have settled. Remove from the refrigerator. Readings may be made at one or within a few hours.

Dilutions of Haemolytic Serum	Dilutions of Complement									
	30	36	43	51	61	74	88	107	128	C
25	0	0	0	0	0	tr	1	3	3	4
50	0	0	0	0	0	0	tr	2	3	4
100	0	0	0	0	0	0	tr	2	3	4
200	0	0	0	0	tr	1	2	3	3	4
400	tr	tr	tr	tr	1	1	2	3	4	4
800	1	1	1	2	2	3	4	4	4	4
C	4	4	4	4	4	4	4	4	4	4

Readings

0 = no cells or complete lysis
tr = 10% cells.
1 = 25% cells.
2 = 50% cells.
3 = 75% cells.
4 = 100% cells, or no lysis.

The optimal sensitizing dose (O.S.D.) of haemolytic serum is in that dilution which gives complete lysis with the highest complement dilution. In the above example the optimal sensitizing dilution is about 1:75 and this batch of haemolytic serum should be used at that dilution.

The 50% dose of complement (HD_{50}), is in the highest dilution which gives 50% lysis (reading 2). In the test proper complement should be used at 2.5 times the HD_{50}. In the above example the HD_{50} is at the 1:107 level and this complement should be used at 1:43 (2.5 x 1/107).

The complement fixation test

(1) Inactivate the complement in the sera to be tested by heating in a water-bath at 56°C for ½ hour. This may also remove anticomplementary activity of the serum.

(2) Deliver reagents into the cups of perspex plates as follows:

0.1 ml. of serial doubling dilutions of serum

0.1 ml. of complement ($2\frac{1}{2}$ HD_{50})

0.1 ml. of antigen at its optimal dilution.

Set up the following controls:

Serum

0.1 ml. of serial doubling dilutions of serum.

0.1 ml. complement ($2\frac{1}{2}$ HD_{50}).

0.1 ml. antigen.

Antigen

0.1 ml. saline

0.1 ml. complement ($2\frac{1}{2}$ HD_{50}).

0.1 ml. antigen.

Incubate the plates at 37°C for 1½ hours to allow fixation of complement.

Sensitize the washed 4% sheep cells by mixing with an equal volume of haemolytic serum at itsoptimal sensitizing dilution either in water-bath at 37°C for 10 minutes or at room temperature for at least 30 minutes.

Add to each cup 0.1 ml. of 2% optimally sensitized

sheep cells.

Re-incubate for 30 minutes to allow lysis to occur. Shake after about 15 minutes incubation and again at the end of the 30 minutes.

Place the plates in the refrigerator overnight or for several hours until the cells have settled.

Remove from the refrigerator. Readings may be made at once or within a few hours.

APPENDIX II

APPENDIX II

Attempt to prevent thymic atrophy in A/Jax mice bearing tumour.

A/Jax mice inoculated with 10^5 ascites cells intraperitoneally show an initial phase of stimulation of the lymphoreticular tissue followed by a collapse in the later stages of tumour growth (Chapter I). The thymus shows progressive atrophy; the reduction in thymic weight is very marked between the 10th and 15th days after tumour inoculation and in animals dying of tumour the thymus has virtually disappeared.

The experiment described here was designed to answer two questions:

(1) Can thymic atrophy be prevented by replacement therapy?

(2) Can the involution of the lymphoid tissue in the late stages of tumour growth be prevented by treating animals with isologous thymocytes?

Material and Methods

Animals - strain A/Jax mice weighing 18-20 g.

Preparation of thymocytes; Thymuses were removed under aseptic precautions, homogenised and counted as described earlier for preparation of spleen cell suspension.

(Chapter II). Thymocytes for I.V. injection were washed once in excess tissue culture medium 199 and resuspended in this medium. Thymocytes inoculated I.P. were not washed.

60 mice were inoculated with 10^5 ascites cells I.P. 10 days later 24 mice received 50 x 10^6 thymocytes by the I.V. route and a similar dose I.P. 5 days later 6 of these animals and 6 untreated tumour bearing controls were sacrificed and the thymus, spleens, inguinal lymph nodes, axillary lymph nodes and livers weighed. 15 days after tumour inoculation 15 mice received a second dose of 50 x 10^6 thymocytes I.P. 8 animals were sacrificed at 20 days and 24 days after tumour inoculation respectively. Organs were weighed as described above and the weights were compared with untreated controls bearing tumour for 20 or 24 days.

Results

The accompanying table shows that atrophy of the thymus and the terminal fall in spleen weight could not be prevented by treating tumour bearing animals with isologous thymocytes. The axillary and inguinal lymph nodes at 20 days, however, were larger in thymocyte treated mice.

Comment

The important role of the thymus in the development of immunological competence is now well recognised. Thymectomy of the new-born mouse leads to depletion of the lymphocyte population and several defects in the immunological reactions of the mature animal (Miller 1962). Such mice show atrophy of the spleen and lymph nodes and marked deficiency of germinal centres and plasma cells and die from a wasting syndrome. On the other hand thymectomy in adult life is generally associated with negligible effects (Miller 1963). If, however, an adult animal is thymectomised and irradiated, grave defects in the immune response results. This suggests that the thymus of an adult animal is required to restore immunological potential. It is conceivable therefore that the progressive growth of the tumour in some way produces depletion of the lymphoid tissue (see chapter I). The thymus would theoretically be needed to restore such a severe depletion. The results shown here demonstrate clearly that thymocyte treatment was ineffective in preventing atrophy of the lymphoid tissue. This may be due to a low dosage, a late start of the treatment or a rapid utilisation of the lymphocytes. It is also possible that dissociated thymocytes are ineffective and an intact thymic graft is required. Miller 1964 has shown that the injection of thymocytes in a suspension did not restore the immunological

capabilities of neonatally thymectomised animals; these, however, were easily restored with a thymic graft.

The enlargement of the axillary and inguinal lymph nodes in thymocyte treated animals is significant. Further studies are needed to evaluate the importance of this finding.

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Changes in organ weights of mice bearing tumour treated or untreated with thymocytes

No. of Mice	Dose of Tumour	Treatment	Time of autopsy (days after tumour)	Av. Thymus weight g.	Av. spleen weight g.	Av. Inguinal L. nodes weight g.	Av. Axillary L. nodes weight g.	Av. Liver weight g.
20	Nil	Nil	-	0.032 ± 0.005	0.90 ± 0.02	0.0050 ± 0.0017	0.0080 ± 0.0026	1.14 ± 0.2
6	10^5	Nil	10	0.038	0.19	-	-	-
6	10^5	Nil	15	0.016	0.14	0.0140	0.013	1.38
8	10^5	Nil	20	0.015	0.11	0.0090	0.011	1.38
7	10^5	Nil	24	0.005	0.13	0.0060	0.007	1.43
6	10^5	Thymocytes	15	0.012	0.17	0.0150	0.012	1.50
6	10^5	Thymocytes	20	0.006	0.12	0.0170	0.021	1.32
6	10^5	Thymocytes	24	0.006	0.12	0.007	0.012	1.38