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THE EFFECTS OF CIRCULATING ISO-ANTIBODY ON
NEOPLASTIC CELL POPULATIONS.

June D. Wakefield



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INTRODUCTION

One of the major challenges in biology has been to maintain tissue in a state of continual growth after it has been grafted to another organism. Successful transplantation has been difficult to achieve in higher animals and at one time, it appeared unlikely that these attempts would ever be successful. This was not due simply to transplantation, for tissue would grow readily when replaced in the original animal (autotransplantation). Later it was realised that tissue would grow in other animals, providing that they were genetically identical to the donor. If the tissue was placed in another member of the same species (homotransplantation) or in a different species (heterotransplantation), the graft was almost invariably rejected. It was only after inbred mouse strains had been developed that the genetical basis of transplantation was established (63), (106).

The homograft reaction leading to graft rejection is not fully understood (75) though certain features are common to all reactions. The host develops a refractory state which persists in the animal for considerable periods of time. Further grafts from the same donor are rejected more rapidly than the first and grafts from unrelated donors elicit primary forms of response (29), (87).



The reaction is highly specific and bears all the characteristics of an immune response. Humoral antibody appears in the serum at the time of graft rejection (52) and immune host cells are actively produced in response to the tumour (95). In the past, both of these responses have been considered as possible mediators of graft rejection (125), but at present, the prevalent view is that immune cells are solely responsible (88).

There is considerable evidence that immune lymphoid tissue contains all the immunological properties necessary for graft rejection (21), (92). Algire and his coworkers have shown recently that foreign graft cells could survive indefinitely in an animal and that graft rejection would proceed only when host cells had access to the graft (121). It will be shown later that this evidence is not entirely convincing, and that the immunological efficiency of the host cells may be due to their producing humoral antibody rather than that the cells bear unique qualities. Lymphoid cells are known to produce antibody (38), (97).

Humoral antibody can be isolated and studied relatively easily. It can be shown that the antibody produced is directed specifically against the antigens of the graft tissue and in many instances, the antibody is directly cytotoxic to the graft cells in vitro. However, antibody

alone cannot produce cytotoxic changes (60), and the binding of the antibody on to the cells is only the initial step towards the damage of the cells (84). A complex sequence of chemical reactions follow the binding of antibody and these involve accessory humoral and possibly cellular factors.

The complexity of this reaction may account for the apparent variability in the activity of antibody, in vivo; frequently only antibody was added to the system (123). In some instances, antibody appears to have no effect on graft cells (23), (92). Other studies have shown that antibody inhibits the growth of certain tissues in vivo (56). The effect has been a limited one, and there was always the possibility that host cells had participated actively in the rejection.

If antibody is to be considered seriously as a factor in graft rejection, it must be shown that antibody is cytotoxic towards the graft cells in vivo, in the absence of graft cells. This formed the basis of the present study.

The present study followed closely the incidence and activity of the antibody produced in the mouse during the active rejection of certain lymphoma cells. It was hoped to establish that isoantibody was produced before there

were visible signs of graft rejection and to confirm that the antibody was cytotoxic to the tumour cells, in vitro.

Later, the effects of the antibody directly on the tumour cells in vivo were studied. The tumour was grown in isolation, in Algire diffusion chambers, in the peritoneal cavity. This prevented contact between graft and host cell but was no barrier to the diffusion of antibody.

Once the incidence and distribution of antibody during the rejection of the graft was known and the direct effects of antibody on isolated graft cells were determined, a detailed study was made on the changes occurring to the graft cells during their active rejection in the peritoneum.

These studies should indicate whether humoral antibody could participate significantly in the destruction of certain neoplastic cells in the mouse.

A SURVEY OF THE LITERATURE

Genetics of Transplantation

Attempts to transfer tumours were made sporadically throughout the nineteenth century but the tumours did not grow in the new host. In 1889, Morau showed that neoplastic tissue could be transferred serially from one animal to another, for he maintained a mouse carcinoma through seventeen generations (94). It was not until Beebe and Ewing (1906) showed that they were propagating a lineage of living cells and not an infectious agent, that the concept that tumours were serially transplantable gained wide acceptance (19), (62).

The successful transfer of tumour transplants continued to be highly unpredictable. In some animals a tumour would grow continuously, killing the host; in others, the tumour would grow transiently and then disappear, while in still others, the tumour would not grow at all. Jensen, a Danish biologist, tried to gain some insight into the causes for this variability, by defining the activity of a series of individual tumours (68). He transferred one tumour from a white mouse into nearly nine hundred white or grey mice, and by using such large numbers could show that the grey mice were noticeably

more refractory. He suggested that the fate of the tumour graft was in some way connected to the 'race' of the host. Tyzzer (116) confirmed these findings and Loeb and Fleischer concluded that "variations in susceptibility to inoculated tumours among different strains of the same species are due to inheritable causes" (81).

To test this hypothesis, animals and tumours would have to be of known and uniform genetic constitution. The stocks of mice then held were genetically heterogeneous. It was only after Little (78), Strong (113) and Cloudman (37) deliberately initiated inbreeding in their mouse stocks, that reproducible data on transplantation was gained.

It was found that tumours arising in an inbred line would usually grow in animals of the same line, but would not grow, or showed only temporary growth in mice of unrelated lines (114). A large number of combinations was tried and deliberate crosses were made between inbred lines. These results were reviewed by Bittner (25) and Little (79). There was no doubt that the success of a graft depended upon the presence of certain genes, mutually in graft and host.

Snell formulated certain 'laws' of transplantation:

" a. A tumour grows progressively in one hundred per cent of the animals on the inbred strain in which it originated.

b. It fails to grow, or grows temporarily and then regresses in unrelated strains.

c. It grows in one hundred per cent of the first generation (F1) animals where one parent is from the susceptible strain.

d. It grows in a fraction of second generation (F2) or of F1 mice crossed to the resistant parent, the fraction varying with the stocks and tumours used" (105).

Snell has made many further contributions to the immunogenetics of transplantation in mice. By applying these laws he has been able to define certain genetic relationships between strains precisely and quite consistently. In 1948, he coined the term "histocompatibility" factors for the genes determining tumour antigenicity (105). These histocompatibility genes appear to be inherited as dominants and that at least 6 or 7 loci and probably 14 or more loci are involved.

Nevertheless, this work rested upon the assumption that the tumour in its transplantation behaviour reflected the genetic and antigenic constitution of its strain of origin.

The first direct evidence for this assumption was provided by Gorer, when he showed that the serum of an animal, that had rejected a tumour, was capable of agglutinating red blood cells from the donor strain (50). This implied that tumour tissue and certain normal tissues shared a common antigen.

Further experiments showed that if the antigen was found to be on the red cells of one strain and not on those of a second, it was possible to decide the antigenic type of the back cross and second generation (F2) animals. If these animals were challenged with tumour from the first strain, the tumour generally grew only in those mice which had the antigen. The genes determining the presence of antigen appeared to be identical to those determining the fate of the graft. Gorer chose to call the gene he could define histocompatibility II (H-2), since it appeared to coincide with antigen II previously demonstrated in serum from rabbits immunised against mouse blood (51). Subsequently, it was shown that the H₂ locus was linked to the genes for kinky and fused on the ninth linkage group (3). Other H factors were soon found, H₁ on the first linkage group (107) and H₃ on the fifth (108).

The H-2 antigens are "strong" in the sense that tumours will rarely grow if there are H-2 differences between graft and host. This does not hold true necessarily when differences exist between some of the other known antigens such as H-1 or H-3 (106).

This serological technique provided a necessary independent check on the transplantation method for detecting H genes. It had certain advantages, not only was it a more direct and less cumbersome technique but it was potentially a more delicate indicator of genetic variation. Repeated injections of tumour into the same mice could produce hyperimmune sera containing antibody reacting against even quite minor antigens. Gorer and Mikulska greatly increased the sensitivity of the test (57). It became apparent that the H-2 'locus' contained a whole complex of factors, and the number of histocompatibility antigens present in each mouse strain was high (7). These antigens are considered as expressions of co-dominant genes in the sense that both factors are expressed in the heterozygote.

Isoantigenic differences play a major role in the transplantation process. Tumours which possess the same antigens as the recipient are accepted and conversely

where there is an antigenic difference between the donor and the host, the host rejects the tumour. This is an expression of immunity. Woglom (1929) (125) believed that tumour immunity was different from other immune states but this has been disproved (50). Tumours share some antigens with other tissues and are rejected in the same way as other tissues or foreign antigenic material (11).

The Immune State

The sequence of events following the injection of foreign neoplastic tissue in a non-compatible animal is quite characteristic. There is a well defined latent period in which the graft appears healthy and the cells grow quite rapidly. After about five days, if there is a major H₂ antagonism between graft and host, inflammatory changes ~~may~~ occur around the graft area and the graft cells begin to degenerate. These changes increase in intensity until all the graft cells are killed; often the rejection is so rapid that the death of the graft cells is completed in forty eight hours. This is followed in some cases with a fibrous encasement of the graft cells. In all cases the graft is eventually resorbed (54).

Early workers were well aware that the refractory state was highly specific and that it persisted in the

X
animal for a prolonged period of time (125). In 1903, Jensen had noticed that an abortive tumour might make an animal immune, for after rejection many animals would not support even a temporary growth of the same tumour (68). Two years later, Gaylord and Clowes showed that the immune state could be increased by persistently giving the same antigenic stimulus to the animal. A form of vaccination was produced (47). In the same decade, Bashford used normal tissues, red cells and epithelial cells, from the same animal to produce immunity against a tumour of the same strain (16). Most of the basic methods for evoking a state of active immunity were known early in the century even though Gorer's explanations were thirty years away (51).

The mechanisms effecting immunity were much more difficult to assess. During active immunity, one of the most obvious changes occurs locally in the area of the graft. There is a marked accumulation of different cell types at different stages of the immune response (67) (122) and often the cellular response varies according to the nature of the graft (54) (41). Other characteristic changes occur in organs distant from the graft site, but having a vascular or lymphatic connection with the graft bed. Frequently there is considerable hypertrophy of the drain-

ing lymphnode, of the spleen and of the liver and this is associated with the development of immunity. There is increased activity of the reticuloendothelial system in general (20).

Early workers tried to establish certain patterns of cell response that might be related to different phases of rejection. Da Fano detailed some of the changes that took place after the sub-cutaneous injection of red cells (39). There was a rise in the number of white cells circulating in the blood; at first these were lymphocytes, but by the fourth day, plasma cells and macrophages had entered the graft area.

Murphy (95) and Woglom (125) studied the cell responses to a variety of tissues and agreed essentially with Da Fano's claim that the lymphocyte was the main cell associated with the development and distribution of rejection. Darcy claimed that the plasma cell was equally important (40). Loeb was convinced that the tissue breakdown was due to a strictly cellular response (80). Though he commented that the mere presence of host cells in a breaking down graft was no evidence that they were causally connected with the rejection.

Loeb emphasised the limitation of the histological

approach. There is no doubt that the cellular response is vital and that it is essential to identify the cell types involved, but the significance of the presence of a cell at any one time cannot be interpreted until the function of each cell is known.

Haaland felt that the refractory state or "immune principle" set in before the first tumour was destroyed (61). Antibodies often appeared immediately after graft rejection, and it was tempting to assume that these substances were causally related to the state of increased resistance.

In bacteriology, it had been known for some time that immune serum contains an active factor against the infecting organism (98). By 1897 some of its special features were known and Durham was writing on the "Special action of serum of highly immunised animals". The antibody was highly specific, agglutinating *S. typhi* but not *E. coli* (43).

Bordet (1898) showed that normal tissue could induce a similar response, for red blood cells were agglutinated and lysed by the blood of another species previously injected with that blood (26). Von Dungern produced a tissue specific antibody, an epitheliolysin, by injecting

guinea pigs with epithelium from cattle (42).

Tissue specific antibodies could be produced at will, but the functional significance of these sera had not been proved. Pearce warned that 'Although such substances - agglutinins and precipitins - may be present they do not indicate cytotoxic activity' (99).

A more direct approach towards understanding the specific functioning of these antisera was made by Metchnikov (89). He produced a spermatoxin by injecting guinea pigs with rabbit testicle. The immune guinea pig serum was injected back into rabbits. He could show by direct histological examination that the testes had ceased to function.

This approach was a great advance, but histological examination could demonstrate only the extent of the damage, by itself, it could not indicate the way in which the changes were produced.

Lambert and Hanes used new tissue culture techniques to show that heterologous immune serum inhibited the growth of cells in vitro (72). Niven confirmed and extended these observations (96). She was more interested in the detailed cytological changes occurring to the graft cells, and the changes in cell permeability accom-

panying the damage of the cells. She showed that the presence of a third factor, in addition to the cell and antibody was essential, if damage and ^{not} mere agglutination of the cell was to take place. Earlier, Buchner (1889) had suggested that a heat labile serum factor was necessary for bacteriolysis (32). Now this third factor is termed complement.

Once the cytotoxic activity of these antibodies had been established in vitro, the potential therapeutic value of antibody had to be considered seriously. Passively transferring antibody to an animal bearing a tumour rarely had any effect on the outcome of the tumour growth (103) (see 125). Indeed, most investigators failed to find any effect with antibody and rejected its importance in the homograft reaction (125).

The climax came through the persistent efforts of Lumsden (83). Under certain conditions he was able to use antibody to induce the rejection of a tumour growing in vivo. The conditions were weighted in favour of the antibody. The tumour was recently transplanted into the foot region; the antibody was injected locally. Attempts were made to maintain the antibody in the area, either by constriction of the limb or by local infiltration with

adrenalin (82). The importance of this outstanding contribution to the transplantation field was to some extent marred by Lumsden's own enthusiasm. He was convinced that he could produce an antiserum specifically against malignant tissue, irrespective of the genotype of the donor tissue and host. This claim was not substantiated and many people failed to accept the significance of his earlier findings (100).

In the mid 1930's, Gorer established the antigenic relationship between red cells and tumour tissue (51). His work centred upon the action of antibody. At first he used it merely as a tool to establish the genetic identity of his cells. Later he found that the same serum if mixed with tumour would prevent the growth of the tumour in vivo (52). The serum seemed to act both as a haemagglutinin and as a protective antibody. These may have been manifestations of the activity of a single antibody (5), or the serum may contain two antibodies with the same antigenic specificity (52). It is still not certain.

Gorer's work, then and now, is the pivot around which centres all modern thinking on the activity of humoral antibody in graft rejection (8), (111), (123).

He was aware that workers were getting conflicting results with antibody, and the controversy over Lumsden was at its height. Much of the trouble lay in the biological variability of the experimental material. Often the genetic relationship between graft and donor was ill defined. Large solid tissue grafts were used and the antibody was of no known activity. It was little wonder that the antibody had no effect (103). Gorer used dissociated tumour cells, and assessed the strength of his antibody by haemagglutinin titre. Using these tools, he devised a series of techniques that would reflect the true activity of the antibody. These formed the basis for subsequent advances.

Gorer and O'Gorman (59) replaced the old tissue culture methods with a simpler method of assaying cytotoxic activity in vitro. The cytotoxicity test took advantage of tumour cells growing in the dissociated ascites form. The death of the individual cell could be followed, for the permeability of the cell wall would increase, allowing trypan blue to enter the cell readily. Mouse would not complement its own system in vitro but Guinea Pig serum was a satisfactory source of complement.

On the other hand, in the neutralization test the

antibody was added to the cell in vitro and the actual viability of the population was checked by transferring the cells to a susceptible animal (53). Viable cells would grow and the time of the appearance of the tumour gave some rough indication of the number of viable cells in the population.

It was possible to kill practically all the cells of a population with quite small volumes of antibody. Indeed, Gorer showed that there was a definite dosage relationship between the volume of antibody used and the number of graft cells affected (56). Burmester confirmed this activity in another species, using fowl leukotic cells (33), and Kidd confirmed that other tissues were sensitive when he showed that the rabbit Brown Pearce carcinoma was killed with antibody (70). Certain cells however, were relatively less sensitive to antibody. Normal rabbit dissociated epithelial cells needed prolonged exposure to antibody before death occurred (24).

Leukotic cells were particularly vulnerable to attack by antibody, but these tests measured the maximum potential cytotoxic activity of an antibody. Even in the neutralisation test most of the binding of antibody to the cell probably occurred in vitro. Gorer and Amos

wanted to find out whether any cytotoxic effects would be shown when the tumour and antibody first met in vivo (56). As a prelude to the next series of experiments, Amos (5) established that passively transferred isoantibodies were stable in vivo and that they could persist and bestow protective action for several days after the injection. The antibody was introduced into the general circulation rather than locally in the graft site.

Gorer and Amos (56) found that they could inject the antibody from seven days before to forty eight hours after the injection of the tumour cells and obtain complete suppression of growth of the cells. These experiments were performed in incompatible animals for the antibody would not be absorbed on to the host tissue; thus the antibody C3H anti E.L. 4 was injected into C3H mice. There were definite limitations to the effectiveness of these antisera, but if 0.1 ml. was given twenty four hours prior to the injection of the tumour cells, the growth of up to two million leukotic cells could be prevented. In the untreated controls, the tumour grew progressively to a palpable size for the first eight days.

When the cells were grown in the susceptible strain, say E.L. 4 in C57Bl the antibody was relatively less effective, 0.1 ml. would prevent the growth of 500-750 thousand cells; with a larger number of cells, the time of appearance of the tumour was only delayed, one million tumour cells would not appear for 10-15 days, but then would grow progressively and kill the host (56).

The growth of other leukotic tumours was inhibited by passively transferred antibody (10). If other tissues were used, particularly those which appear to be relatively insensitive to antibody in vitro from the neutralisation experiments, the results were equivocal. Billingham and Brent have used massive doses of antibody of unknown titre with normal skin homografts and did not succeed in sloughing the grafts (23). Chutna on the other hand, claimed to have been successful (35), (36). He used both iso and hetero antibody and injected it by the intraperitoneal route. Stetson approached the matter cautiously; rather than inject the antibody systemically he has given the antibody locally (110), (111), or he has used vasodilators locally in the hope of increasing the concentration of antibody in the graft area. With both systems he has successfully increased the rate at

which grafts were sloughed.

The experiments with passively transferred antibody indicate that antibody is involved in the rejection of both skin and of tumour, but do not prove that the effects are mediated entirely by antibody. In the experiment of Gorer and Amos and especially in those of Chutna and of Stetson and his colleagues, antibody accelerated a reaction that would have occurred naturally within a few days, for they used a homologous system which is inherently capable of rejecting the graft. Much more convincing evidence came from experiments in which antibody was injected back into the strain of mice compatible with the tumour. Here the tumour never normally regressed and natural immune mechanisms were entirely inadequate, yet the tumour could be suppressed with isoantibody.

There are, however, doubts as to the importance of antibody in the homograft reaction. These come from three considerations. Frequently there has been failure to detect antibody after the rejection of a graft (115), (117); sometimes, it seems that the timing of the first appearance of antibody is too late to account for the destruction of the graft (93). In certain circumstances serum was unable to transfer the immune state to non-immune animals,

whereas minced immune lymphoid tissue from appropriate hosts could confer a high degree of immunity (92).

Specific antibodies can be detected in the sera of animals following the rejection of a graft, providing that sufficiently sensitive techniques are used (12). After the primary graft rejection, the antibodies are often transient and of low titre (54). Evidence for the slightness of such a response may be quoted from Gorer, of the experiments of Swisher and Young (115). They noted that after a single and a second transfusion of red blood cells into a dog that these cells were being destroyed at an increasingly rapid rate, but no antibody could be detected. It was only after the third transfusion that a positive Coombs antiglobulin test was obtained. In this situation the bench tests were relatively poor indicators of a biologically active destruction mechanism.

Mitchison and Dube (93) could only detect haemagglutinating antibody at a time appreciably later than when the rejection phase was at its height, and they concluded that the antibody production was purely a side effect. On the other hand, Gorer, O'Gorman and Mikulska (58) repeated these experiments and showed that Mitchison and Dube's methods of detecting antibody were relatively

insensitive. The titres obtained by Gorer were consistently much higher; for example, a negative result compared to a titre of 1:1000. Using special methods, Gorer could detect antibody of a low titre on the third day and with ease on the fourth day.

It would seem likely that antibody is produced by the animal prior to rejection of the graft, but only the haemagglutinating activity of the antibody is established (58).

More directly related and of weighty importance in transplantation immunity, was the finding of Mitchison that he could confer a state of apparent immunity on a normal mouse, with injections of lymphoid tissue from an actively immune animal (92). The injected mouse behaved as if it were immune and of the same specificity as the actively immune mouse. This was termed a state of adoptive immunity.

They were able to elicit the breakdown of a graft of Sarcoma 1 with the injection of specific immune lymphoid tissue and yet failed to confer any heightened resistance against the graft by means of quite massive amounts of hyperimmune serum.

Experiments of this nature have been repeated in a

number of ways. Winn used dissociated lymph node cells, either mixed with the tumour or introduced systemically (123). There was no doubt that immune cells were relatively superior to serum in conferring immunity. In some cases there appeared to be an absolute distinction.

This depends upon the nature of the graft. Lymphoid cells can be destroyed with passively transferred serum; cells similar to Sarcoma I appear to be resistant to antibody (123). Mitchison was aware of these differences when he proposed that graft rejection is mediated solely by cells (92), in view of his own work on antibody and its appearance in graft rejection. He felt that antibody was probably irrelevant. The cells, he thought, may well have transferred a bound form of protective antibody resembling that in tuberculin sensitivity. Mitchison emphasised the similarity between the homograft response and various allergic hypersensitive states. Earlier, Burnet and Fenner made a similar suggestion that perhaps the skin homotransplant reaction was essentially a delayed hypersensitivity response (34).

Amos (8) has recently pointed out that Mitchison's experiments do not define the actual roles of antibody and cells, for in using immune lymphoid cells, Mitchison

transferred almost as complex a situation as existed in the immune host.

It is technically extremely difficult to define the cell type actually transferring the adoptive immunity. On the other hand, it is possible to culture single cells in vitro and to show that plasma cells produce humoral antibody (97) and there are other techniques to suggest that lymphocytes may produce antibody (38). These cells are certainly carried over in the lymph node in adoptive transfer. Lymphoid cells may be washed quite free of antibody at the time of transfer, but it is possible that they could resume the production of antibody in the fresh host. It is difficult to say whether the adoptive immunity is produced by humoral or sessile forms of antibody.

Similarly it is very difficult to interpret the cellular reactions occurring around any graft. It is just these cells, the lymphocytes, plasma cells and histiocytes that actively proliferate around the graft. It is not known whether some are liberating high concentrations of antibody locally in the graft bed (53), or whether some only produce a sessile form of antibody which requires actual cell contact before death takes place (21).

Weaver and his colleagues emphasise the fact that immune cells are needed for the homograft reaction to take place, and that cell contact is perhaps important (121). They show dramatic photographs showing immune cells and host cells in contact with each other, with both cells dying or dead. Particular importance has been placed on these results for they formed part of a larger inquiry in which an imaginative new technique was introduced, which, it seemed, would be able to separate the action of host cells from that of humoral antibody in graft rejection (101).

Algire, Prehn and Weaver devised a porous chamber in which graft cells could be grown in isolation from the host cells, but nourished by the extra-cellular fluids in the peritoneum (2). By using membranes of different pore size, they could manipulate the experimental design so that host cells could enter or be excluded from the chamber.

They compared the effects on graft tissue of placing the chambers in immunised and non-immunised animals. Chambers containing mouse tissue were placed in rats previously immunised against mouse. The tissue was destroyed irrespective of the pore size of the chamber. This sug-

gests that humoral hetero-specific antibody alone is quite capable of killing the graft tissue. However, when they used a mouse homologous situation, the tissues were not killed unless the pore size was sufficiently large to allow the host cells to enter (121).

They thus concluded that cellular factors were of prime importance in graft rejection.

Unfortunately in certain of the homograft situations used, both graft and host belonged to the same antigenic class, (H-2d), and thus the immune stimulus would be relatively slight. Moreover, no attempt was made by these workers to determine the antibody level in the system (101). Thus their interpretation cannot be entirely accepted.

In summary, although host cells proliferate and invade the graft area during rejection and can readily transfer immunity, the effective cells are just those which produce or transport antibody (44), (45). The graft situation in which host cells are present is complex and the fact that antibody is known to be cytotoxic to certain graft cells in vitro, makes it very difficult to identify the factors responsible for the rejection of the graft.

Now that graft cells can be grown in a simpler environment without the contact with host cells, the effect of one possible factor - antibody on the cells - can be studied.

From these studies it should be possible to determine whether antibody has any direct effect on graft cells if there are no host cells present. If such an effect is established, the extent and nature of the response will be determined together with an understanding of the conditions necessary for the changes to take place.

MATERIALS AND METHODS

Experimental design

Mouse strains

Tumours

Production of antibody

 Detection of haemagglutinins

 Detection of cytotoxic antibodies

Assay of mouse complement

Growth of tumour cells in the peritoneal cavity

 Sampling procedure

Growth of tumour cells in the diffusion chamber

 Construction of the chamber

 Experimental chamber procedure

 Sampling of the chamber

EXPERIMENTAL DESIGN

Mouse ascites tumour cells were used in all the experiments to be presented. This type of cell is unique in that it will grow as a suspension of single cells in the peritoneal cavity (102) or in the diffusion chamber (13). Single cells are more uniformly exposed to antibody and accessory factors than are the cells in solid transplants. There is no solid stroma and no intimate connection with blood vessels. The tumour cells can be recovered easily without damage from manipulation, and the surrounding fluid is available in quantity for examination. Ascites lymphomas have been studied almost exclusively, since they appear to be very sensitive to antibody and therefore are well suited to the study of any early changes which result in the death of the grafted cells.

Two techniques have been used. In the first, a state of active immunity was induced in the mouse. A counted suspension of cells was injected directly into the peritoneal cavity. The animals were killed after a predetermined interval; the animals were bled and the ascites fluid removed. The peritoneal cavity was washed out repeatedly with large volumes of saline to recover the remaining cells.

This part of the study would give information in three

areas. It would be possible to compare the time of appearance of antibody in the serum with that in the actual vicinity of the graft. The total number of cells in the peritoneum could be determined and by making smears of the exudate, the percentage of tumour cells to host cells could be reasonably established. This part of the study will also show the extent of the damage occurring to the tumour cells, both the percentage of cells fully coated with antibody and the cells already dead.

In the second part of the study, the tumour cells were protected from host cells by placing the cells in diffusion chambers, and the experiments were designed to find out if passively transferred antibody would produce changes similar to those seen in the first group. The sampling and analysis of the cells was essentially the same as with the tumour grown in the peritoneum.

The effect of antibody on the tumour population could be shown in a number of ways. Where the antibody reaction had proceeded to completion, the affected cells were completely lysed. The extent of this reaction could be gained, by comparing the number of intact cells in a treated population with those in a control. Those cells which were killed, but not lysed, could be detected by adding trypan blue. Finally, those cells which had reacted with antibody

and had become sensitized, but to which no overt damage had occurred, could be detected by adding an additional source of complement.

In any study on the effect of antibody on a cell population, it is essential to measure the antibody level attained locally. The reaction will proceed only in the presence of complement; thus estimates of the level of free antibody and of complement in the peritoneal fluid surrounding the chamber and in the chamber itself have been carried out.

Materials and methods.

Mouse strains (109).

The mouse strains used in these studies possess a high degree of genetic uniformity and have been maintained by deliberate inbreeding. The DBA/2, C3H and the A/Ha strains were bred in the mouse colony of Dr. T. S. Hauschka and the DBA/1 mice in the Roswell Park colony.

DBA/1 dilute brown, coat colour aabbCCdd, histocompatibility genotype H-2q was originally isolated by Little in 1909 and was carried by Strong as the D strain. It has been known at various times as the Dbr, dbr, dba and DBA.

DBA/2 dilute brown, coat colour aabbCCdd, H-2d. This originated from the Dbr stock.

C3H/St, coat colour AABCCDD, H-2k was originally isolated by Strong in 1920 from the cross Bagg albino x DbA.

A/Ha white, coat colour aabbccdd, H-2a originally isolated by Strong from the cross Cold Spring Harbor albino x Bagg albino.

Tumours.

The QL1 lymphoma arose in the DBA/1 strain. The Dalton DBA/2 thymoma and the L1210 are both lymphosarcomas arising in the DBA/2 strain. The L#2 lymphoma, originated by Shelton, is grown in A/Ha mice. The tumours were maintained by the weekly transfer of cells, by sterile technique, to stock mice of the strain in which the tumour originated.

Antibody.

The production of iso antibody. Iso antibodies were produced by immunizing one strain with tissue from a second strain. Antibodies of high titre and strong avidity were produced by hyperimmunization. A course of four injections of antigen (in this case tumour tissue) were given to the hosts over a period of two months. The animals were bled by cardiac puncture ten days after the last injection of the antigen. Small volumes of the sera were lyophilized and stored at -70°C. The activity of the sera was assessed in a number of ways.

The detection of haemagglutinating antibodies.

The dextran - human serum technique (57)

Method

Blood was collected from the retro-orbital sinus and

was immediately mixed with 3.8% sodium citrate. The cell suspension was gently centrifuged and washed, once in sodium citrate and once in a mixture of 50% sodium citrate and 50% saline. The final red cell suspension was made to approximately 2.5% in a mixture of equal volumes of saline and human serum. This human serum had previously been absorbed free of any naturally occurring anti-mouse antibodies.

The antibody was serially diluted in 2% Dextran 'Intra-dex' in saline. 0.025 ml. of the standard cell suspension was added to an equal volume of each dilution of antibody. These tubes, together with control tubes containing the red cell suspension with the 2% Dextran alone, were incubated for one hour at 37°C. The cell suspension was streaked on a slide. The strongly agglutinated cells could be read by eye, but since the arbitrary scoring sets an 'end point' at that agglutination just visible to the naked eye, microscopic confirmation of the lower values was necessary.

This technique was a very sensitive indicator of the strength of each antibody. Strong antibodies with a known titre of 1-4000 (i.e. antibodies diluted to one part in 4000 of Dextran giving an 'end point' agglutination) were needed for the passive transfer studies. The technique employed had the ability to detect very low concentrations of antibody in certain tissues fluids during the homograft reaction.

The detection of cytotoxic antibodies.

Amos has shown that certain antigens on the red cells are shared by white cells, and that these white cells will agglutinate in the presence of specific antibody. Gorer has used this observation as the basis of quite a different technique. If complement was added to such an agglutinated suspension, the cells were irreversibly damaged.

Method.

Usually Guinea Pig serum was used as the source of complement. These sera were screened, and only the sera with very low cytotoxic activity against mouse cells were used. The sera were diluted to 66% in Mammalian Ringer.* Ascites tumour cells were collected into excess Mammalian Ringer mixed well and gently centrifuged. A standard suspension of 500,000 cells in 0.1 ml. Mammalian Ringer was made.

The antibody was serially diluted in Mammalian Ringer and 0.025 ml. of both the cell suspension and the Guinea Pig serum were added to each volume of antibody. Individual controls were necessary since the Guinea Pig serum, the antibody or even the Mammalian Ringer alone may have caused a number of the cells to die during the time of incubation.

*Composition of Mammalian Ringer

NaCl	8.2 gram	made up with 1000 ml. of twice distilled water.
KCl	0.2 gram	
CaCl ₂ ·2H ₂ O	0.2 gram	

The test was incubated for twenty minutes at 37°C.

Occasional affected cells in a population were completely lysed and in some cases total counts of all the cells in the test were made. The great majority of affected cells, however, were not lysed; changes in the permeability of the cell wall took place and this formed an adequate measure of the activity of the antibody. Usually there was marked cytoplasmic swelling and this was accompanied by a loss of rigidity of the cell wall. The cell appeared to collapse on the glass slide, producing an increased definition of the nuclear membrane.

This change in permeability could be seen more easily if a basic dye such as Trypan Blue was added to the suspension. Rapid changes in the cell took place; the dye was quickly absorbed by the damaged cell, particularly by the nucleus. This became swollen, often causing the outer cytoplasmic-membrane to rupture.

Unaffected cells did not take up the stain. A cell suspension often contained a low percentage of dead cells present in any normal ascites population. These cells were usually small and shrunken and though they stain intensely with the dye, this was not accompanied by swelling of the cell.

Assay of mouse complement.

Assays of complement usually use red cell lysis as a

criterion of complement activity. It is known that the lysis of red cells requires both antibody and complement and that if the concentration of cells and the dilution of antibody remain constant, then the degree of lysis of the cells is proportional to the concentration of complement present. The classical complement assay uses a rabbit anti-sheep haemolysin system and the activities of complement from a variety of species have been determined.

It was extremely difficult to detect any complement activity in mouse serum by this method, although there must be complement produced in the mouse for lysis of cells does occur in vivo. It was felt that mouse complement might complement a mouse lytic system rather more adequately. A mouse anti-human haemolysin was produced by injecting human group O cells into DBA/1 or Ha ICR/Swiss mice. Complete lysis of human red cells could be achieved with this antibody providing the concentrations of the mouse complement were high.

Method

Human group O red cells were washed twice in saline and the concentration of cells adjusted so that if they were completely lysed in a volume of 1.2 ml. of distilled water, the amount of haemoglobin released could be read

on a Coleman spectrophotometer to give an optical density of 0.58 at a wave length of 415. The serum containing the mouse anti human antibody was heated to 56°C. to remove the complement present. The antibody was diluted to one in five with saline and added to an equal volume of the red cell suspension. These red cells had become fully coated with antibody after they had been incubated for twenty minutes. The suspension was centrifuged at 4°C., the supernatant removed and the cells resuspended in saline to their original volume. 0.05 ml. of these cells were mixed with 0.05 ml. of the complement dilution and incubated at 37°C. for a further twenty minutes. The contents of the tubes were shaken every few minutes during the incubation period.

The tubes were centrifuged at 400 r.p.m. for one minute. 0.05 ml. of the supernatant was removed, care being taken not to disturb the pellet of unlysed red cells. Distilled water was added to the supernatant to a volume of 1.2 ml. Since it was known that under these conditions 100% lysis gave an optical density of 0.58, variations in the degree of lysis and therefore of the levels of complement could be measured in terms of relative optical density.

Growth of the tumour cells in the peritoneal cavity.

Sampling procedure.

Initially, 20 million L1210 or 60 million QL1 tumour cells in 0.2 ml. of Mammalian Ringer were injected into the peritoneal cavity, care being taken that there was no leakage from the injection site. Each day, the stage of growth of the tumour was assessed. A sample was taken both of the ascites fluid and serum for the presence of antibody. An aliquot of the cells in the peritoneal cavity was taken to estimate the percentage of tumour cells in the population.

The following procedure was adopted:

The animal was killed with chloroform and pinned to a small wooden block. A blood sample was taken by cardiac puncture. The body wall was cut midventrally. The peritoneum was palpated to mix the cell contents. A small volume of cells was withdrawn from the peritoneal cavity, the sample was centrifuged and the ascites fluid removed. Part of the sample was mixed with 5% bovine serum albumin and smears of the ascites cells were made. These were stained with Wright's stain for one minute and in a mixture of one part stain to two parts distilled water for fifteen minutes.

Another cell sample was taken to study the viability and the degree of sensitisation of the cells. The remaining cells were placed in a beaker. The peritoneal cavity was flushed free of all cells with large volumes of saline; 60-100 ml. were used according to the growth of the tumour. The number of cells taken for the two samples was usually very small so that a count of the cells washed from the peritoneum gave a reasonable estimate of the total number of cells growing in the peritoneum.

L1210 and QL₁ cells migrate from the peritoneal cavity and invade other tissues. In this study, the growth of the tumour population is used merely as an indication of the development of immunity in the animal, rather than to indicate the total number of tumour cells in the animal.

Growth of tumour cells in diffusion chambers.

Construction of the chamber.

Nitrocellulose filters of known pore size were bonded to the upper and lower faces of a stout supporting lucite ring 16 mm. in diameter and 5 mm. deep. This completed a chamber with an internal volume of 0.3 ml. that could be made impermeable to the passage of cells. This depended upon two factors: efficient bonding of the membranes to the lucite by means of acriloid cement and the pore

size of the membrane. The H.A. filter made by the Millipore Corporation has an average pore size of 0.45μ and this has been used throughout the study. The pores were large enough to allow nutrients to pass into the chamber and yet small enough to prevent the passage of cells. Any major leaks in the assembly of the chamber were detected by checking the completed chambers with compressed air under water.

A small entry hole was drilled in the lucite ring. This was closed by means of a small tapered lucite peg and sealed with a fast drying cement made by dissolving Millipore filter in acetone. The chambers were placed vertically in racks in petri dishes. These and the pegs were sterilised overnight in a 2.5 litre dessicator containing 1.9 ml. ethylene oxide. Afterwards the petri dishes were allowed to stand in a warm atmosphere for at least twenty four hours to enable the gas to escape.

Experimental chamber procedure.

The ascites cell suspension was freshly prepared in Mammalian Ringer and kept on ice. The initial concentration of cells was determined and was kept within the range of half to one million cells in 0.1 ml.; 0.2 ml. aliquots were placed in each chamber. Great care was taken to

preserve absolute sterility throughout the assembly of the chambers. Contaminating bacteria grow unchecked in the chambers and kill the tumour cells. The cell suspension was mixed continually so that each chamber received the same number of cells. This was checked by counting the remaining cell suspension after completion of the chambers and comparing the count to the original. The completed chambers were placed in animals immediately.

Host mice of known genotype were anaesthetised with an intramuscular injection of sodium pentobarbital (Nembutal, 0.8 mg. per 1 gm. animal weight). The ventral abdominal wall was shaved, swabbed with iodine and a mid-ventral incision was made through both the body and peritoneal walls. A chamber was placed gently into the peritoneum. Sterile Mammalian Ringer was usually injected into the peritoneum to bathe the chamber, and the peritoneal wall was sewn with a running suture of Ethicon nylon. The body wall was usually closed with 11 mm. Michel clips although it was found that where large volumes of fluid were to be injected two or three days after this operation, a tight suture with nylon was needed to ensure no leakage of the injected fluids through the wound.

Sampling of the chamber.

The chamber was removed from the peritoneum and the

outside carefully swabbed free of cells. The chamber was held so that one filter could be slit upwards to form a lid and the fluid was removed with a syringe. Usually a fibrous clot remained in the chamber; this was placed separately in a tube so that the interior of the chamber could be rinsed free of cells. Cells tend to grow in the clot and so it was important to titrate the clot in excess Mammalian Ringer. All the washings were pooled and a total cell count made. Histological sections showed that relatively few cells remained in the clot. The total cell counts gained in this way are probably plus or minus 5% of the actual population in the chamber. This inaccuracy does not interfere with the interpretations of the results and is certainly no less accurate than the technique described earlier - that of washing cells out of the peritoneal cavity.

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RESULTS

PART I. EVIDENCE FOR THE APPEARANCE OF ANTIBODY DURING THE HOMOGRAFT REACTION

Three tumour host combinations were used: QL₁ cells were grown in C3H mice; L1210 cells were grown in DBA/1 and C3H mice. Nine separate experiments were undertaken in which a standard dose of tumour cells were injected initially into the peritoneum. The growth of the tumour cells was checked every 24 hours throughout the homograft reaction. The L1210 and QL₁ tumours have very similar growth curves. Thus, Graph 1 represents the composite findings of the nine experiments, so that the general features of the reactions are shown.

1. Growth of the tumour cells in the peritoneum.

The tumour grew rapidly in the peritoneum and fluid accumulated until Day 6. At this time the animals were greatly distended and the volumes of ascites fluid often exceeded 2 ml. By Day 7, there was a decline in the tumour population. This decline continued rapidly through Day 8, and by Day 9 there were practically no tumour cells remaining in the peritoneal cavity.

If the results of two individual experiments are studied - Graph 2 and 2a - it will be seen that although the cell counts were reasonably similar in the active growth

phase of the tumour, Day 5 and Day 6, there were fluctuations in the cell count on the seventh day. This would appear to be a reflection of the slightly different timing of rejection in the individual animals. Rejection of L1210 seemed at a height on Day 7 in C3H and DBA/1 hosts. By the eighth day, there were few tumour cells in any of the animals.

2. The appearance of haemagglutinins.

Both the serum and ascites fluid were tested on the day of sampling against red cells of the appropriate genotype. It will be seen from Graph 1 that there was a trace of antibody in the serum on Day 4 and on Day 5. The antibody had increased in titre by Day 6. From Graph 1 it appeared that there was a drop in the strength of the circulating antibody on the seventh day. Afterwards, the titre of the antibody rose continually until Day 9, when it reached a plateau.

There was some variation in the level of antibody found in the serum of animals sampled on the same day (Graph 2 and 2a). This was particularly marked on Day 7 when the rejection of the tumour was at its height. It appeared that the animals with a higher titre of free antibody were in a more advanced stage of rejection.

There was no detectable antibody found in the peritoneum until the rejection of the graft was almost com-

pleted and there were few tumour cells remaining in the peritoneum (Table 1). Later the antibody titre rose sharply (Graph 1). At this stage the fluid in the peritoneum was rapidly disappearing, and it was technically difficult to check whether the titre of the antibody rose continuously and approached the level in the serum.

3. The appearance of cytotoxic antibody.

Although the presence of antibody could be shown by agglutination, this form of activity has little direct significance, and it was necessary to show that there was a correlation between rejection and the appearance of cytotoxic antibody.

Direct cytotoxicity tests occasionally detected antibody by the eighth day after injection and regularly by the ninth and tenth days (Table 2). If low titred antibody occurred earlier, it could not be detected since the anti-complementary action of concentrated mouse serum prohibits the testing of undiluted antibody. Various modifications of the technique were devised.

Modification A.

Tumour cells from a compatible host were incubated with excess undiluted or slightly diluted control or test serum for twenty minutes at 37°C. The tubes were lightly

centrifuged and the supernatant discarded. This removed most of the mouse serum and the anticomplementary effect of the residual serum was negligible. Guinea Pig serum was then added as the source of complement and the tubes were incubated for a further 20 minutes.

Using this modification, cytotoxic activity could be detected in the serum of two out of three experimental animals on the seventh day. Serum from a stock C3H mouse was not cytotoxic.

Modification B.

Another technique modified the target cells. Cells partially coated with antibody were used instead of fresh ascites cells. These cells were still viable (Table 4, stage 1). It was thought that experimental serum with a relatively low titred antibody might be able to complete the death of these cells, whereas it would be unable to kill unsensitised cells.

These pre-sensitised cells were washed free of excess antibody and were used instead of stock cells in the cytotoxicity test (Table 4, stage 2). This test showed that 22.5% of the sensitised cells died if complement was added on its own. When the experimental serum at a dilution of 1/4 was added, 65% of the cells died. There was no activity in the ascites fluid.

This confirmed that cytotoxins could be found in the serum on Day 7.

Modification C.

Later a more efficient source of complement was found than Guinea Pig serum. Human sera from multiple myeloma patients helped to detect cytotoxic activity as early as Day 5; that is, well before there were visible signs of graft rejection.

Stock tumour cells were pre-sensitised, either with one volume of the test serum from Day 5, or with normal mouse serum (Table 5). The supernatant was removed and the pre-sensitised cells tested with four different sources of complement, three multiple myeloma sera and Guinea Pig serum.

The Guinea Pig serum was ineffective. All three multiple myeloma sera detected the sensitisation caused by the test serum. These sera did not affect stock cells incubated with normal serum.

4. The extent of the antibody pool at the time of graft rejection.

Cytotoxic titres of 1/40 and 1/80 can be found in the serum on the seventh day, if multiple myeloma serum is used as complement. On day seven, the rejection

of the tumour is at its height. Amos has shown that the total ^{extracellular} fluid volume of the mouse is approximately 2.0 ml. (9), and thus the total concentration of antibody at the time of sampling can be determined.

From the cytotoxicity tests (see methods section) it can be shown that where:

0.025 ml. antibody diluted to 1/40 kills 125,000 cells in 0.025 ml. Mammalian Ringer

then

2.0 ml. of the same antibody undiluted would kill 400 million cells.

This suggests that at the time of graft rejection, providing that there was adequate complement in the animal, that there was sufficient antibody to kill all the graft cells present. The rate of antibody production was not known.

5. The state of the tumour cell population during the rejection phase.

The cells in the peritoneum represent a mixed population of tumour and a variety of host cells and the proportion of these depends upon the stage of the homograft

reaction at the time of sampling (41). At the height of the tumour growth, the cell population was predominantly neoplastic. During the early stage of rejection the proportion of tumour cells was very high even though the total number of cells was dropping rapidly. Since in this thesis, interest was in the state of the tumour populations, only populations which were predominantly tumourous were considered. The state of the total population, therefore, was more likely to reflect the state of the neoplastic cell than the host cell.

Cells removed from the animal on Day 5 were as sensitive as the stock tumour in their response to the immune serum being produced against them. They showed an interesting difference from the stock tumour in their response to myeloma serum (Table 5). The serum from multiple myelomatous patients was not cytotoxic towards stock tumour cells, but when added to cells removed from the experimental animals, it killed a proportion of these cells. This finding suggested that a proportion of the cells in the peritoneum were coated with antibody on Day 5. It had been shown already that cytotoxic antibody was detectable in the serum by the fifth day after inoculation.

An indication of the extent of this sensitisation

throughout the homograft reaction can be gained by considering the state of the individual populations, on successive days, in the nine experiments. A population was considered to show evidence of sensitisation, if added complement caused an increase of at least 10% dead in the population.

The results in Table 6 indicate that many of the populations were sensitised during the critical period of graft rejection. A proportion of the cells had sufficient antibody to be killed if complement was added. Other cells might have been inadequately coated with antibody but such cells could not have been detected by this technique. Some populations of QL₁ had very high rates of sensitisation where over 50% of the cells were sensitised.

The tumour populations also showed signs of irreversible damage, for there was an increasing number of dead cells in the experimental populations, particularly in the later stages of rejection. Healthy tumour populations growing in a susceptible strain rarely contain more than 5% dead cells.

On Day 5, there were no populations in which more than 10% of the cells were dead. By Day 8, 25% of the populations contained more than 10% dead cells. This

percentage was never spectacular; there were rarely more than 15% dead cells in a single population.

SUMMARY - PART I.

These direct observations on graft rejection in the peritoneum show that cytotoxic antibody was produced in the system, before the graft was rejected, and that sufficient antibody was produced to kill all the graft cells present in the animal at the time of graft rejection. Antibody could not be detected in the immediate vicinity of the graft; that is, in the ascites fluid until practically all the tumour cells had disappeared. This may have been due partly to the binding of antibody on to the surface of the tumour cells. There was evidence that a proportion of the tumour cells became fully sensitised and that there was an increase in the number of dead cells in the population in the rejection phase.

PART II - THE USE OF DIFFUSION CHAMBERS TO DEFINE THE
ROLE OF ANTIBODY IN GRAFT REJECTION.

1. The growth of tumour cells in diffusion chambers.

A series of tumours have been grown successfully in diffusion chambers. The tumours often have characteristic rates of growth, but the growth curves all have essentially the same features (Graph 3), (13).

After an initial inoculum of approximately one million cells, a rapid drop in the population always occurred. After twelve to twenty-four hours, the population recovered and began to divide, often at a rate comparable to that of the ascites growing freely in the peritoneum. After this logarithmic phase of growth, the rate of growth declined, often to a mere replacement of dying cells in an overcrowded population.

The growth of the tumour in the chamber had the advantage of being independent of the genotype of the host mouse. A tumour grew equally well in chambers placed in the peritoneum of resistant and susceptible mice (Graph 4). This suggested that the possible natural immune factors were of little importance in affecting the rate of growth of the tumour in chambers. This was important for the tumour in the experimental series had to be grown in resistant hosts, hosts of the same genotype in which the

antibody was prepared.

It was important to establish consistency in the reproducibility of population counts in different chambers in the same experimental situation. It can be seen from the graphs plotted for seven different experiments, that there was reasonable agreement in the normal growth of DBA₂ thymoma in the same experimental series and between series (Graph 5).

2. The effects of single injections of antibody.

The DBA₂ thymoma was particularly suitable for these experiments. It has a rapid and continuous growth phase extending over a period of days and its cells are known to be antibody sensitive. From numerous experiments on the cytotoxic effect of antibody against this tumour in vitro, the numerical relationship between the amount of antibody and cell damage was known. As little as 0.2 ml. of antibody would produce permeability changes in five million lymphoma cells. This was an expression of the activity of the antibody in vitro, using Guinea Pig serum as a source of complement. These results were of some value when considering the possible doses of antibody to be injected for the in vivo experiments (Table 9).

0.1 ml. DBA₁ anti DBA₂ antibody was injected intraperitoneally into animals bearing chambers containing

DBA₂ thymoma. Other animals were injected with 0.1 ml. normal mouse serum. Normal mouse serum had no effect on the growth of the populations in the chambers; the tumour cells divided rapidly throughout the experimental period (Graph 6).

The populations receiving antibody were damaged severely. Within six hours of the injection, the total population had dropped from approximately 2.5 million to 1.2 million cells; half the cells were lysed and 50% of the cells remaining in the chambers were dead. Chambers sampled at twenty-four hours showed that the treated populations were beginning to recover. Only 20% of the cells were dead, and in the last eighteen hours, the size of the population had doubled. Chambers sampled at forty-eight and seventy-two hours showed that the rate of growth in the experimental populations was equal to that in the control populations.

Antibody was injected when the tumour cells were in a more advanced stage of growth; a lytic effect was seen providing that sufficient antibody was given. This dosage effect was more apparent when different volumes of the antibody were given at the same point on the growth curve (Graph 7). Here, the lytic effect was directly related to the volume of antibody given, but in no instance did the

antibody completely lyse the population. The damage caused by antibody was always maximal within 24 hours. Recovery and continued growth of the population took place to restore the rate of growth to that of the control population.

3. The effects of multiple injections of antibody.

If a relatively large volume of antibody (0.3 ml.) was given to 1,000,000 DBA₂ cells at the beginning of their active growth phase, great destruction took place initially, and even at 24 hours there was little sign of the residual population recovering. A further injection of 0.3 ml. of antibody was given to two chambers in the hope that complete destruction of the population might occur. Recovery and growth of these tumours populations was delayed but took place 1-2 days after the last injection of the antibody (Graph 8).

In the next experiment (Graph 9) the antibody injections were given daily for four days beginning on the second day of the growth phase. Twenty four hours after the first dose of antibody, the treated population totalled one million. The number of tumour cells remained constant during the course of the antibody injections. No further lysis of the population was caused by injecting additional antibody. Indeed, three days after the antibody injections

stopped, the recovery of the tumour became apparent and the following day the rate of growth was comparable to that in the control populations. Only a proportion of the tumour population was therefore susceptible to the action of antibody. The apparent resistance to the effects of antibody by the residual cells and their subsequent growth, might have been due to two factors:

1. Insufficient antibody in the chambers.
2. Selection of an antibody resistant population.

Experiments were devised to study these possible factors.

4. The detection of antibody in the chamber.

a. Haemagglutinins.

Haemagglutinating antibody could be detected easily in the chamber two to four hours after its injection into the peritoneum (13). During the course of successive antibody injections, the titre in the chambers rose steadily and antibody was present, even four days after the last injection of antibody (Graph 9). At this time, the antibody level in the chamber was almost as high as in the serum, suggesting that relatively little antibody was needed to sensitise the tumour cells in the chamber. The sensitisation does not affect the rate of growth. Many sensitised populations, in spite of being bathed in antibody, were dividing at as rapid a rate as

control untreated populations.

b. The presence of cytotoxic antibody in the chamber.

It was known that immune serum has both haemagglutinating and cytotoxic properties. It was possible that these were properties of two distinct antibodies with the same genetic specificity and although there was excess haemagglutinin in the chamber fluid, cytotoxic antibodies might have been lacking. Chamber fluids from certain stages in the multiple injection series were studied for cytotoxic activity.

The chamber fluids were tested, in vitro, against fresh DBA₂ thymoma cells, using Guinea Pig serum as a source of complement (Table 8). Cytotoxic antibody was detectable in the chamber fluids, 24 hours after the first injection of antibody, until at least three days after the last injection of antibody. At no time during this period was cytotoxic antibody lacking from the chambers. The activity present in each individual chamber was according to in vitro studies, more than sufficient to kill the tumour cells growing in the chamber (Table 9). There was considerable cytotoxic activity in the serum.

5. Studies to determine whether cells resistant to

antibody have been selected from the population.

a. The sensitivity of the residual cells remaining in the population to antibody in vitro.

The residual cells were taken from the chamber that had received four injections of antibody and was sampled three days after the last injection. The activity of these cells was compared to that of two control populations that had had no previous contact with antibody. Antibody and complement were added in vitro (Table 10).

The cells from the chamber in the antibody treated host were badly damaged already for they showed a high degree of staining without further treatment. If complement was added to this population, the great majority of the rest of the population died. This suggested that antibody was bound to these cells in vivo for the complement alone was not cytotoxic. The addition of fresh antibody to the system did not increase the number of damaged cells in the population even though this antibody, in the presence of complement, was highly active against the control cells. There appeared to be a few refractory cells in all tumour populations.

b. The growth of previously sensitised cells in

lightly immunised animals.

Several sensitised populations were subjected to a further selection pressure provided by growing the cells in DBA₁ animals lightly immunised against the DBA₂ thymoma.

This might have indicated whether the failure of the antibody to kill all of the cells in the chamber was due to the emergence of an antibody resistant population. Such a resistant cell type would be favoured when transplanted into lightly immunised hosts.

c. A comparison of the susceptibility of the "passaged" DBA₂ thymoma with the stock tumour to cytotoxic antibody.

The results of the in vitro cytotoxicity test (Table 11) suggested that the "passaged" line might be more antibody resistant than the stock DBA₂ thymoma. The experimental tumour, at this time, had been passaged through five generations of immunised animals.

Both tumours were grown in chambers. Both grew at approximately the same rate, and when the populations reached one million, 0.3 ml. antibody was injected into animals from each group. The chambers were sampled twenty four hours later. The populations in each group were markedly susceptible to the antibody. The next generation of "passaged" cells were tested again, in vivo, and

showed a similar degree of susceptibility (Graph 10).

SUMMARY - PART II

Antibody has considerable immediate lytic and damaging effects on a tumour population, growing in a diffusion chamber. Some of the cells did not lyse and in fact grew in the presence of antibody. These cells were sensitised with antibody and became irreversibly damaged if complement was added in vitro.

It did not appear that the population of cells remaining the chamber was inherently resistant to the action of antibody.

PART III - THE ROLE OF COMPLEMENT IN AUGMENTING THE
EFFECTS OF ANTIBODY IN VIVO.

Lysis is known to be a two stage process needing specific antibody binding at the antigenic sites, followed by the fixation of complement (84). It may be that there was insufficient complement in the chambers to achieve the full cytotoxic effects of the antibody.

A population of cells that had been subjected to a series of daily injections of antibody was studied. Here the maximum lytic effects of the antibody would have been reached; the cells would be fully sensitised with antibody and there would be free antibody in the chamber. Complement would be added at this stage. Guinea Pig serum was used for it was known to be a reliable source of complement.

1. The effects of a single injection of complement.

Animals received three daily injections to receive a total of 0.9 ml. antibody. Varying doses of Guinea Pig serum were given at the same time as the last injection of antibody (Graph 11).

Guinea Pig serum alone had little effect on an untreated population. There was also very little difference between populations treated with antibody alone and those which had received additional small volumes of Guinea Pig

serum - 0.2 ml. When larger volumes of Guinea Pig serum were injected there was a considerable increase in lysis compared with the controls, the intensity of effect appearing to be directly related to the volume of Guinea Pig serum given. Practical difficulties were encountered when larger volumes of serum were given. Attempts were made to inject 2.0 ml. but the serum leaked through the suture wound making it impossible to know the volume of Guinea Pig serum remaining in the peritoneum.

The residual cells in these experimental chambers were fully sensitised with antibody, for they became irreversibly damaged when additional Guinea Pig serum was added in vitro.

Mouse serum though ineffective as a source of complement, in vitro, probably has some activity in vivo, since in the early stages of antibody injection, lysis does take place in the chambers. Additional injected mouse serum may, therefore, be able to enhance the lytic system in vivo.

Freshly drawn mouse serum from DBA₂ mice was injected into antibody treated animals bearing chambers. The result was interesting; not only did the mouse serum cause pronounced lysis, but the degree of lysis was comparable

to that produced by the same volume of Guinea Pig serum (Graph 12). It was known that in vitro, mouse serum partially inactivated Guinea Pig complement and that much of the activity of the Guinea Pig serum may be lost in vivo. These results suggested that small amounts of complement augmented the effect of antibody and that mouse serum supplied this complement as efficiently as did the Guinea Pig serum (Graph 13). Normal mouse serum added to the unsensitised cell control population had no detectable effect.

2. A detailed study of the effects of antibody and complement.

The earlier experiments have established that antibody could cause lysis of the tumour population, and that complement could augment this effect. In all these experiments, the chambers were sampled at twenty four hour intervals, so that the general features of the reaction were apparent. In the following experiment the effects of antibody and complement were watched in detail.

The lymphoma L#2 was used. The antibody was heated at 56°C. for twenty minutes to remove the heat labile components of complement present in the immune serum. Preliminary testing in vitro showed that the heated antibody

was moderately cytotoxic, in the presence of fresh complement. 0.4 ml. antibody was therefore injected (Graph 14).

It could be shown that nearly 50% of the population had lysed within two to four hours of the injection of the antibody. The remaining cells in each population were fully sensitised with antibody. Antibody alone appeared to have no further effect and the population remained stable for the next four hours. Eight hours after the injection of the antibody, 1 ml. of fresh mouse serum was injected into each of these animals. A chamber taken one hour later showed that half the remaining population had lysed and by two hours practically the whole population was lysed. Meanwhile, in the control injected with antibody alone, the population showed mitotic activity, and the cells began to increase in number.

3. The effects of multiple injections of complement.

The previous experiment suggested that practically the whole tumour population could be destroyed if the complement level in the system was raised. Multiple injections of complement were given to sensitised populations, where there was known to be excess antibody in the chambers.

0.2 ml. of antibody was given to a population of L#2 on Day 3 and Day 4 (Graph 15). Twenty-four hours after the

last injection the antibody group remained at a total of two million cells, whereas the cell control population had reached a total of eight million cells. By the following day the experimental population had broken free from the restraint of the antibody, in spite of there being antibody with a titre of 256 in the chambers. This confirmed earlier experiments in which after initial lytic effects, a population would grow in the presence of antibody.

Some animals were given a series of daily injections of complement, in addition to the antibody (see injection schedule, Graph 15); the tumour cells did not recover. Four days after the last injection of antibody, these populations remained at 2 million, whereas the populations treated with antibody alone or with mouse serum alone had the same high cell number as the untreated populations.

Even under these experimental conditions, it had not been possible to destroy the tumour population. It was known that excess antibody had been injected, for it could be detected in the chambers. A total of 4 ml. normal mouse serum had been injected into each mouse; it was assumed that this would boost the level of available complement in the peritoneum, but unfortunately at this point, there was no satisfactory method of assaying mouse

complement in vitro. There was no means of knowing how much complement remained active and had actually entered the chamber.

4. The in vitro assay of mouse complement.

The level of complement in mouse serum cannot be determined by the classical rabbit anti sheep haemolysin system. Even diluted mouse serum will not haemolyse sheep cells previously sensitised with rabbit anti sheep haemolysin. This is in contrast with the serum of many other species where the complement levels are so high, that the serum has to be diluted several hundred fold, in order to determine the dilution at which the serum no longer complements the reaction (22).

Eventually, it was found that mouse serum would complement a mouse antihuman red cell lytic system. Even when the mouse serum was tested at one part in two often the red cells were not completely haemolysed. The complement levels in the peritoneal fluid were considerably lower and there was practically no activity at all in the chamber fluid (Graph 16).

This was the most physiological system that could be devised to test mouse complement. It could not detect complement in the chamber though undoubtedly some comple-

ment was present.

The complement levels were determined after the animals had received 1 ml. volumes of freshly collected mouse serum. It was hoped that these injections would boost the complement in the peritoneum and therefore the complement in the chambers to levels measurable by this technique. Graph 16 shows that there was no detectable increase in the levels of complement in the peritoneum or the chamber fluid.

It is known that this test will detect only complete complement. There are four components of complement, and lack of any one component prevents lysis. Mouse serum may, therefore, only be lacking in certain complement components (31) or it may be that certain inhibitors are activated in vitro (85).

Attempts to concentrate mouse complement by salt precipitation or by ethanol fractionation were unsuccessful. This was probably due to the extreme lability of complement and the initially low level of complement (or certain components) in the mouse, relative to other species (77).

SUMMARY - PART III

Passively transferred complement can increase the damaging effects of antibody on a tumour population in a

chamber. Neither antibody alone, nor antibody with additional complement can destroy the whole population. The initial level of complement in the chamber appears to be very low, and even the addition of complement to the system does not result in a detectable increase in the level of complement in the chamber. Some complement obviously did enter the chamber, for there is initial lysis of the population when antibody alone is added. The addition of guinea pig serum and normal mouse serum produced an increased effect.

Lack of complement appears to limit the full potential effect of antibody.

DISCUSSION

There is no doubt that the isoantibody circulating in the immune host has activity against the tumour. Gorer and O'Gorman have shown cytotoxic properties in vitro (59), and this has been confirmed by Amos and Wakefield (15), Winn (123) and others. Gorer and Amos had previously shown that such antibody would suppress the growth of tumour cells in vivo (56). From neither set of experiments was it possible to say that antibody did in fact kill tumour cells in vivo. In one case, heterologous complement was added to the system in vitro; the other, although in vivo, did not preclude any activity by the host cells.

The evidence presented here shows that antibody is able to damage tumour cells directly within the animal and that this damage can take place in the absence of host cells. A large proportion of the cells are completely lysed; other cells die, but either do not lyse or have not reached that stage at the time of sampling.

The fact that antibody is active but quite dependent upon an effective source of complement has been emphasised by the techniques used (see 60). Antibody kills a large number of cells in the chamber, but a residual

population remains that will grow rapidly even in the presence of antibody and in spite of the cells having antibody bound to their surfaces. Direct measurements showed that there was insufficient complement in the system.

The chamber itself was not anticomplementary. The membranes are to some extent a barrier to the diffusion of complement, but the failure to demonstrate complement either in the peritoneal fluid, or in the chamber, after the injection of large amounts of normal serum into the peritoneal cavity, suggests that either the complement is leaving the peritoneum very rapidly or else it is being inactivated. An inhibitor of mouse complement has recently been described by Mayer and his colleagues (85). Attempts to boost complement were successful in that the potential of the antibody was raised by the addition of normal serum, but it cannot be said that the maximal effect of antibody has been demonstrated until it can be shown that complete complement is present in excess.

The lytic action of antibody was very marked in most experiments, but it proved impossible to destroy the whole population. Although this was partly due to the lack of complement it may also have been due to the

refractory state of some of the cells. It is known from cytotoxicity tests in vitro that the cells in any ascites population vary in their susceptibility to antibody and complement. Often from 2-6% of the ascites cells seem quite insensitive and Waksman has suggested a similar range of susceptibility in a red cell population (119). It is likely that these are the immature cells of the population, for it is known that the cellular antigens take time to fully develop (119).

Earlier, Hauschka has shown that immunologically resistant variants can be selected from a tumour (64). Hauschka and Amos showed that long transplanted tumours frequently shift from a predominantly diploid to a polyploid population and that often these populations are progressively less vulnerable to immunological attack (6), (65). Perhaps it is this nucleus of relatively inert cells in each population that can survive multiple injections of antibody.

It is known that these cells will continue to grow when transplanted to the peritoneal cavity of incompatible mice even if the new host is immunised mildly. Since these cells have antibody bound to their surfaces, this refractory state may be due to the cells having

lost the ability to bind mouse complement. On the other hand, this effect may be due merely to the low level of complement in the abdominal cavity of the new host. This would prevent a significant degree of lysis.

To test the hypothesis that these cells were refractory, five serial transfers of the population were made through weakly immunised hosts. In vitro, there was no detectable shift in the sensitivity to antibody; the tumours grown in chambers showed the same relative susceptibility to lysis as did the stock tumour. This suggests neither the ability of the cells to bind antibody nor the ability to fix mouse complement was permanently impaired.

These experiments show that even with lymphoma cells known to be antibody sensitive, there are variations in the response of the individual cells to the antibody. It cannot be said that this is due to the inherent mosaic character of the population, for it is certainly due in part to the technical difficulties in producing adequate complement levels at the reaction site.

There is marked variation between tissues in their susceptibility to antibody; lymphomas are vulnerable (112);

carcinomas, sarcomas and skin show less sensitivity (24), (123). These tissues are very likely to vary in their capacity to bind antibody and complement.

Hauschka and Amos have shown that within a mixed population of diploid and polyploid cells, that the polyploid cells often have relatively fewer antigenic sites than their diploid counterparts (65). Winn has shown that similar differences occur between tissues. He believes that Sarcoma I has only 4% of the antigenic sites of 6C3HED (123), and this would limit severely the amount of antibody that could be bound by Sarcoma I. On the other hand, some tumours can absorb antibody, so that clearly antigenic sites are present and accessible, and yet the cells are refractory (123). This may be due to variations in the ability of antibodies to bind complement, for Goodman has shown that differences do exist (49). Complement fixation involves the sequential binding of all the four components of complement, and damage does not occur until this sequence of fixation is complete. The components are often unstable and if the reaction does not proceed to completion within a few minutes, individual factors may decay or dissociate from the complex. The fixation of C^1 in adequate amounts and

the availability of all the other components is essential for lysis (84). Mayer has stressed that providing the whole sequence of binding is completed, a cell might lyse even if only one site is affected (84). The cell is more vulnerable if it has a greater number of sites, and lysis will be more rapid if more sites are affected.

Variations in the response of different types of tumour to the cytotoxic activity of antibody have been shown by the in vitro test, but these variations may be of significance only in so far as they reflect the actual behaviour of the cells in vivo. It is believed that cytotoxic activity is more of a functional measurement of antibody than other in vitro tests, such as those involving agglutination, but it is recognized that cytotoxic activity is only one rather restricted measure of humoral immunity. Other humoral factors present in the animal may not be effective in the in vitro test. The in vitro effect is certainly less effective than that in vivo, for whereas complete lysis of many of the cells occurred in the chamber experiments, most of the cells in the in vitro tests had increased permeability and were presumably dead, but did not lyse. As a corollary to this, Kidd showed that heteroantibody and complement

did not affect Brown Pearce carcinoma cells even after eighteen hours in vitro (70). Yet the same antibody transferred in vivo completely inhibited the growth of such cells.

Because of these drawbacks, many workers have preferred to study transferred immunity in, in vivo situations, not only to get a more accurate idea of the susceptibility of the individual cells to antibody, but also to mimic the conditions during the active rejection of a whole tissue graft. These are two quite different goals and sometimes, in the interpretation of results, this has led to a misunderstanding of the action of antibody.

The physiological conditions in vivo are an obvious improvement over the test tube, but the animals are not actively immune; they merely supply the environment in which the antibody and cells meet.

One of the major differences between the actively and the passively immunised animal is that in the former, there are acute vascular changes resulting in increased permeability. These might allow antibody to flow more freely into the graft area. In the passively immunised animal, this reaction does not occur and the concentration

of antibody in the graft site or the degree of penetration into the graft is not known. Gorer and Amos have shown that, although passively transferred antibody prevented the growth of leukotic cells, the growth of relatively few leukotic cells was inhibited (56). There was great excess of antibody circulating in tumour bearing animals, but relatively little antibody was absorbed by the tumour (5). It was Amos' opinion that much of the antibody did not reach the graft.

Another possible difference is that the complement may not be produced at the same rate as in the actively immune animal. Indeed, Winn has shown recently that the effect of antibody in the neutralisation test is greatly improved if complement is injected into the experimental animals (123). If neither antibody nor complement reach the individual graft cells in adequate concentrations, it is little wonder that antibody is not thought to affect the growth of certain grafts.

Technical difficulties such as these have plagued investigations into the relationship between graft and host. The failure of passively transferred antibody to cause the regression of a skin graft (23) has been extrapolated to the conclusion that humoral antibody

plays no part in skin graft rejection (86). This conclusion is unwarranted because the conditions in the passively transferred animal do not approach those in the actively immunised host.

Dissociated epidermal cells show some sensitivity to antibody in vitro (24) and now that Stetson has used topical applications of vasodilator substances, it is possible to provoke an accelerated rejection of skin grafts in passively immunised but not in normal mice (110). Thus one of the possible technical objections have been overcome. It is not known what effect artificially raising the level of complement in the graft area would have on the apparent activity of antibody.

It would seem that most cells appear to be susceptible to antibody, but this conclusion has been reached by a series of techniques designed to define the potential activity of antibody rather than to define the in vivo situation in the course of graft rejection. It is, therefore, by no means proved that antibody elicits graft rejection.

The present studies do show, however, that cytotoxic antibody was produced by the actively immune animal. It was present in the animal before there were visible

signs of graft rejection and at this time, there was sufficient total antibody in the serum to kill the graft.

The graft appeared to absorb the antibody actively, for while there were tumour cells in the peritoneum, there was no antibody detectable in the vicinity of the graft, even though the level of antibody in the serum was quite high. Immediately after the tumour disappeared from the peritoneum, antibody was detected locally, and it increased rapidly in concentration. The sudden appearance of antibody in the peritoneum could be due either to a transient increase in permeability of the peritoneal wall or to the fact that antibody was being formed in the peritoneum throughout the rejection phase.

Gorer noticed a sudden drop in the serum titre on Day 6, during the rejection of E.L. 4 in the subcutaneous site, such as would be associated with a sudden change in permeability in the area of the graft (58). It is interesting that in these current experiments with L1210 in the peritoneal site that a similar drop was noticed on Day 7 at the height of the rejection phase.

These results contrast markedly with the results of Amos in the passively immune state. Here, if the tumour

was placed subcutaneously, little antibody was absorbed on to the tumour cells (5).

There was some indirect evidence that antibody becomes bound to the tumour cells during their rejection. An increasing number of the cells become sensitised and in certain instances, e.g. QL₁ in C3H animals practically the whole tumour population was affected, at the height of rejection. The fate of these affected cells was not known. It was difficult to gauge whether any direct lysis occurred as in the diffusion chamber, but many of the tumour cells were found to be dead at the time of sampling. Some of these cells were morphologically normal, but a few showed gross degenerative changes.

It is not known what part immune host cells play in these reactions. It is known that many histiocytes invaded the graft area during rejection. These cells may be cytolytic; they were certainly phagocytic. Where the histiocyte response was sluggish and the cells were rather inactive, there was often an accumulation of pycnotic cells (131). This underlines the fact that the histiocyte acts as a scavenger, but there may be less obvious methods of host cell participation. It is by no means certain that histiocytes ingest only dead cells,

sensitised cells or quite healthy tumour cells may be vulnerable to attack.

The evidence for antibodies as a factor in graft rejection may be restated: cytotoxic antibodies are formed before the visible onset of graft rejection; the antibody titre rapidly increases as the tumour mass decreases; the tumour cells are found to be sensitised before they are destroyed. By suitable methods, many types of cells and not just lymphomas (71), can be shown to be sensitive to antibody.

Graft rejection is a complex procedure and since there is no evidence to show how closely the situation at the graft site resembles any experimental in vivo system, it is justifiable to consider the animal's response against different types of antigen. Much of the evidence for the activity of the host cells comes from investigations into the response against tuberculin or other simpler antigens. There are two sharply defined reactions. Some antigens such as picryl chloride elicit an immediate inflammatory response when injected into an immune host (73); others do not provoke a response for 24 to 48 hours (see 27). This difference in timing is quite characteristic and seems to reflect fundamental

differences in the nature of the two immune responses. Since the immediate response is mediated by serum and the delayed type only by cells there is some similarity between an animal response to graft tissue and to simpler antigens (74).

It is widely held that tissue grafts, notably those refractory to antibody, elicit a delayed hypersensitivity reaction, and that antibody plays no role in graft rejection (88). Hildemann and Medawar have cited the points in favour of this idea (66). The most cogent evidence stems from the fact that the rejection of many tissues is mediated only by immune cells and this was supported by the early work in diffusion chambers (121). This claim must be softened.

Algire's early work in diffusion chambers now should be considered evidence that the 'immune cell' is relatively superior to antibody in mediating graft rejection (1). His work on a heteroimmune system (2) and this present work in an isoimmune situation show that when antibody is passively transferred, a diffusion chamber does not prevent damage occurring to the graft.

It is not even certain whether delayed type hypersensitivity is a unique immune state. There is some

recent evidence to suggest that the delayed response is only a reflection of incomplete immunity; that is, a stage in the development of complete immunity (104). Leskovitz and Waksman have shown that often, injection of the antigen by the intradermal route produces both an immediate and a delayed response, whereas the same antigen will elicit only an immediate response involving complete antibody when injected intravenously (76).

Grafts particularly of skin are placed most frequently in the intradermal site. There is evidence that these skin grafts produce delayed type response (30) although Milgrom has shown that skin grafts also elicit certain immediate type responses (91). Further evidence from patients lacking gamma globulin and therefore having negligible or no antibody producing capacity, show that they can tolerate skin homografts indefinitely. Yet these patients are able to acquire delayed hypersensitivity against simpler antigens (48).

It is premature to consider the skin homograft reaction purely as a delayed type of response, and the evidence from leukotic grafts suggests quite the reverse. It would seem more likely that in the rejection of tissue grafts both immune cells and humoral antibody play a

role, and that the relative importance of each response depends on the nature of the grafted tissue. This would give the animal the most efficient and versatile defence system.

Studies on the cellular mechanism of graft rejection are, however, usually carried out with complex mixtures of cells. The lymph node cells used in the adoptive transfer studies of Mitchison and by others are a mixture of cell types ranging from multipotential reticulum cells to mature lymphocytes. The apparent superiority of this lymphoid tissue over humoral antibody may be due to the concentrated levels of humoral antibody known to be produced by lymphoid cells. A seldom considered possibility is that some of the cells produce complement in the area of the graft (18). The effectiveness of the lytic action of antibody is dependent on complement, and since the site of formation of complement remains unknown, this possible function of the immune cells needs to be stressed.

At this stage neither the concept of delayed hypersensitivity nor the realisation that the immune cell is involved in graft rejection increase the understanding of how the final immune state is achieved.

Immune cells have been studied in simpler model systems and there is evidence to suggest that the reaction is most intense where host cells come into direct contact either with antigen (see 120) or antibody (9). This provides strong presumptive evidence for the participation of host cells. Favour first showed the property of 'lympholysis', when he found that tuberculin would cause the lysis of granulocytes and lymphocytes of sensitised animals, providing complement was present (46). Many cells of the reticulo-endothelial system are known to possess this property (see 120).

On the other hand, there is evidence that where both the antigen and the antibody are extracellular, lysis of certain cells can occur; Middlebrook and Dubos have shown that red cells will adsorb antigen on their surfaces and that the cells will lyse in the presence of the appropriate antibody (90). This has been confirmed on many occasions (28). Recently, Amos has shown a similar effect involving host histiocytes. Host histiocytes can adsorb or ingest tumour antigen and these cells will lyse if host anti tumour antibody is added in vitro (9).

Thus many host cells have the potential and are known to lyse easily. It is possible that this effect

though extracellular is a major determinant of graft rejection (8). The reaction shown by Amos only occurred at a critical stage in graft rejection in the local area of the graft.

Wissler and Flax have suggested that the antibody may protect the host cell (124) by damping down an allergic response. They have suggested that the antibody might actively neutralise the free antigen in the area of the graft, so that the immune cell is able to act directly on the graft cell.

Antibody does act synergically with immune cells in vivo. Batchelor, Boyse and Gorer have shown this effect experimentally, and have indicated that the effect is of major importance in graft rejection (17). Although in certain situations both antibody and cells can transfer immunity independently, they have shown that the effect can be far greater if the antibody and cells are injected simultaneously. Gorer does not attempt to explain synergism, although he emphasises that the effect is destroyed if too much antibody is transferred (55). It would seem that if the equilibrium between the immune cell and the antibody is disturbed the regression of the tumour may be delayed or even aborted. It is possible that this

is the reason why passively transferred antibody can enhance the growth of a tumour in certain experimental situations (69).

All these experimental systems have defined individual properties of the immune system. There is evidence that antibody can have a direct effect on the graft cell, that antibody and host cells can act synergically on the graft cell and that it is possible for the immune cell to cause the death of the graft cell by direct contact. All this evidence is fragmentary and the relative importance of these effects in any specific homograft situation is not known. These effects may reflect quite different mechanisms of homograft rejection.

Studies on the actively immune animal suggest that different mechanisms may be involved. Hildemann and Walford have shown recently that where the genetic differences between graft and host were very slight, it took several months for the homograft to be rejected. This chronic rejection elicited quite a characteristic cellular response (67). For a long time, Gorer has emphasised that the host cellular response is determined mainly by the nature of the graft (54).

Lymphomas elicit quite a characteristic cellular

response. Gorer notes that in this Type 3 graft 'that few host cells entered the reaction in the early stages, on the sixth day, there is a very marked exudation reaction together with considerable cytolysis of the malignant cells. This continues, but from about the ninth day onwards is accompanied by histiocytic invasion. The histiocytes function largely as scavengers' Gorer considered that the lymphomas were particularly vulnerable to antibody attack (54).

The present study has gathered considerable support for this view. The rejection of the lymphomas followed Gorer's description closely. It was shown that antibody was produced in the animal early, that the antibody could lyse the cells directly and that theoretically, there was sufficient antibody in the system to cause the death of all the graft cells present. This study did not determine the extent of host cell participation in graft rejection and the precise role of antibody in the active rejection of these grafts has still to be determined. The participation of antibody in the Type 1 and Type 2 grafts of Gorer is even less certain.

The importance of this study is that it established that antibody is able to damage certain graft cells

directly, and that this effect is dependent upon the presence of complement. Many other cell types are known to bind antibody, but until the level of humoral complement in the test system is known and the cells that produce complement are recognised, the full effect of humoral antibody in the homograft reaction will not be known.

SUMMARY

1. Antibody production appeared to be a characteristic feature of the homograft reaction in the mouse. Iso-antibody was produced before there were visible signs that the graft cells were being destroyed.

2. At the time of rejection, there was sufficient antibody in the animal to kill all the graft cells present. Many of the graft cells were fully sensitised with antibody during the rejection phase.

3. The iso-antibody produced was directly cytotoxic to the leukotic graft cells. This could be shown most clearly, in vivo, by using diffusion chambers. Here, the antibody caused damage and considerable lysis of the graft cells.

4. Lysis took place in two well defined stages. Antibody became bound to the graft cells, but in spite of this, the cells remained viable and multiplied in the presence of the antibody. If complement was added, the cells were irreversibly damaged and most of the cells lysed immediately.

5. Not all the graft cells in the chamber were destroyed. This appeared to be due to the low levels of complement in the chamber, rather than to variability in the susceptibility of the individual cells to humoral antibody.

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TABLE 1. The incidence of antibody in the serum and peritoneum during the active rejection of tumour cells from the peritoneal site.

Fluid tested	Over 1×10^6 tumour cells in peritoneum			Less than 1×10^6 tumour cells in peritoneum		
	Antibody present	Antibody absent	Not tested	Antibody present	Antibody absent	Not tested
Serum	6*	0	0	8	0	0
Peritoneal Fluid	0	6	0	4	1	3

* Number of Animals tested.

TABLE 2. The detection of cytotoxic antibody by the standard cytotoxicity test.

Control tubes cells mixed with			Experimental tubes cells mixed with antibody and complement			
Mammalian Ringer	Comple- ment	Anti- body	dilutions of antibody			
			1/8	1/16	1/32	1/64
9*	7	8	48	43	17	6

Antibody Serum from a C3H animal injected with L1210 tumour eight days earlier.

Cells L1210 cells, 500,000 in 0.1 ml. of Mammalian Ringer.

Complement Serum from selected Guinea Pigs diluted 66% with Mammalian Ringer.

* The % dead cells in each population.

TABLE 3

Detection of cytotoxic antibody
Modification A

	Control tubes cells mixed with			Experimental tubes cells presensitised with 3 vols, serum.
	Mammalian Ringer	Complement	Antibody 3 volumes	Complement added.
Serum #1	8*	6	9	73
" #2			10	89
" #3			7	14
Normal C3H serum			6	6

Antibody Serum #1, #2 and #3 from C3H mice injected with L1210 tumour cells seven days earlier.

Cells Stock L1210 tumour cells, 500,000 cells in 0.1 ml.

Complement Guinea Pig serum diluted to 66% with Mammalian Ringer.

* The percentage of dead cells in a count of 200 cells.

TABLE 4 The detection of cytotoxic antibody.
Modification B.

Stage 1

Control tubes cells mixed with			Experimental tubes cells mixed with antibody and complement				
Mammalian Ringer	Complement	Anti-body	dilutions of antibody				
			1/5	1/10	1/20	1/40	1/80
< 1*	1	5	95	73	20	4	2

Antibody Hyperimmune DBA1 anti DBA2 antibody.
Cells Stock L1210 cells.
Complement Guinea Pig serum diluted 66% with Mammalian Ringer.

Stage 2

	Control tubes cells mixed with			Experimental tubes cells mixed with antibody and complement		
	Mammalian Ringer	Complement	Anti-body 1/4	dilutions of antibody		
				1/4	1/8	1/16
Serum	5	22.5	0.5	65	52	36
Ascites			9.0	32	28	27
Normal DBA1 serum			6.0	24	22	25

Antibody Serum and ascites from a pool of DBA1 mice injected with L1210 tumour 7 days earlier.
Cells L1210 cells presensitised with DBA1 anti DBA2 antibody at 1/20.
Complement Guinea Pig serum diluted to 66% with Mammalian Ringer.
* The % dead cells in a count of 200 cells.

TABLE 5

A comparison of 1. The relative susceptibility to cytotoxic antibody of stock tumour cells and tumour cells that have grown in an incompatible host for five days.

2. The relative efficiency of two sources of complement in the cytotoxicity test.

Cells	Control tubes Cells presensitised with 1 vol. normal mouse serum.					Experimental tubes Cells presensitised with 1 vol. experimental serum.				
	M.R.	Complement added				M.R.	Complement added			
	G.P.S.	M.M.1.	M.M.2.	M.M.3.	G.P.S.	M.M.1.	M.M.2.	M.M.3.		
A	1.0*	2.0	1.5	2.5	3.5	5.0	8.0	95.5	84.5	100.0
B	2.0	3.0	23.0	10.5	7.5	4.0	10.0	87.5	67.5	96.0

Experimental serum

Serum from C3H animals injected with QL1 tumour five days earlier.

Cells

A Stock QL1 tumour cells.
B QL1 cells from the same C3H animals from which the serum was taken.

Complement

G.P.S. Normal Guinea Pig serum diluted to 66%.
M.M.1.2 and 3. Three samples of Human serum from Multiple Myeloma patients, diluted to 66%.

M.R.

Mammalian Ringer.

*

The percentage of dead cells in a count of 200 cells.

TABLE 6 The state of sensitisation of the L1210 tumour populations during the rejection phase.

	Days after injection of tumour into the peritoneum				
	4	5	6	7	8
% populations showing evidence of sensitisation	0	0	50	33	62
Number of animals in each sample	6	8	16	18	8

A population is considered to show evidence of sensitisation if there is a 10% increase in death, when complement is added to the cells.

Both Guinea Pig and Multiple Myeloma serum were used as sources of complement. These were selected to be free from toxicity against stock tumour cells.

TABLE 7 The incidence of dead cells in L1210 tumour populations during the rejection phase.

	Days after injection of tumour into peritoneum				
	4	5	6	7	8
% populations with more than 5% dead	36	37	33	39	50
10% dead	0	0	11	17	25
Number of animals in each sample	6	8	18	18	8

TABLE 8

The detection of cytotoxic antibody in the chamber fluids.

Experimental procedure			Antibody detected could damage *	Number of cells in the chamber at the time of sampling
Number of injections of antibody	volume of antibody injected	time of sampling		
2	0.2ml	24hrs.	$>6.4 \times 10^6$	2.3×10^6
3	0.2ml	24 "	$>6.4 \times "$	$1.7 \times "$
3	0.2ml	72 "	$>7.2 \times "$	$4.2 \times "$
0	none	-	1.6×10^5	$4.9 \times "$

Strength of the antibody injected*.

0.2ml would damage 19×10^6 tumour cells.

0.6 " " " 57x " " "

*The activity of the chamber fluids was determined by the in vitro cytotoxicity test, where heterologous complement was present in excess (59). The number of cells in each tube is approximately 125,000 and since 0.025 ml. antibody of known dilution is added, a 'destructive index' can be determined.

TABLE 9 A comparison of the theoretical cytotoxic effects of the antibody injected, based on the cytotoxicity test, with the actual damage caused to the target populations.

Experiment	Theoretical damage of		to Actual damage the chamber cells			Approximate number of tumour cells in the chamber at the time of injection of antibody.
	Total serum	Chamber fluid	Lysis	Damage	Total	
A	4.6*	0.46	1.3	0.3	1.6	0.7 - 1.9
B	>19.0	>1.9	1.0	0.25	1.25	0.4 - 1.9
C	>32.0	>3.2	2.0	0.1	2.1	0.7 - 2.5

* Denotes the size of the population affected by the antibody in millions of cells.

The theoretical activity of the serum and the chamber fluid was determined by means of the in vitro cytotoxicity test.

TABLE 10 A comparison of the relative susceptibility to antibody of a series of tumour cell populations.

	Control tubes cells mixed with			Experimental tubes cells mixed with antibody and complement		
	Mammalian Ringer	Comple- ment	Antibody 1/5	dilutions of antibody 1/5 1/10 1/40		
Cells A	9*	5	16	100	87	11
" B	12	5	13	95	85	18
" C	49	81	43	88	79	77

Antibody DBA₁ anti DBA₂ thymoma.

Cells A Stock DBA₂ ascites cells.
 B DBA₂ thymoma cells grown in a chamber.
 C DBA₂ cells grown in a chamber to which
 0.8ml. antibody was added.

Complement Guinea Pig serum diluted to 66% with
 Mammalian Ringer.

* The percentage dead cells in a count of
 200 cells.

TABLE 11 A comparison of the relative susceptibility of stock and 'passaged' DBA₂ thymoma cells to cytotoxic antibody.

	Control tubes cells mixed with			Experimental tubes cells mixed with antibody and complement			
	Mammalian Ringer	Comple- ment	Antibody 1/5	dilutions of antibody			
				1/5	1/10	1/20	1/40
Cells A	8*	10	7	95	98	98	95
" B	1	1	3	32	70	64	46

Antibody DBA₁ anti DBA₂ thymoma.

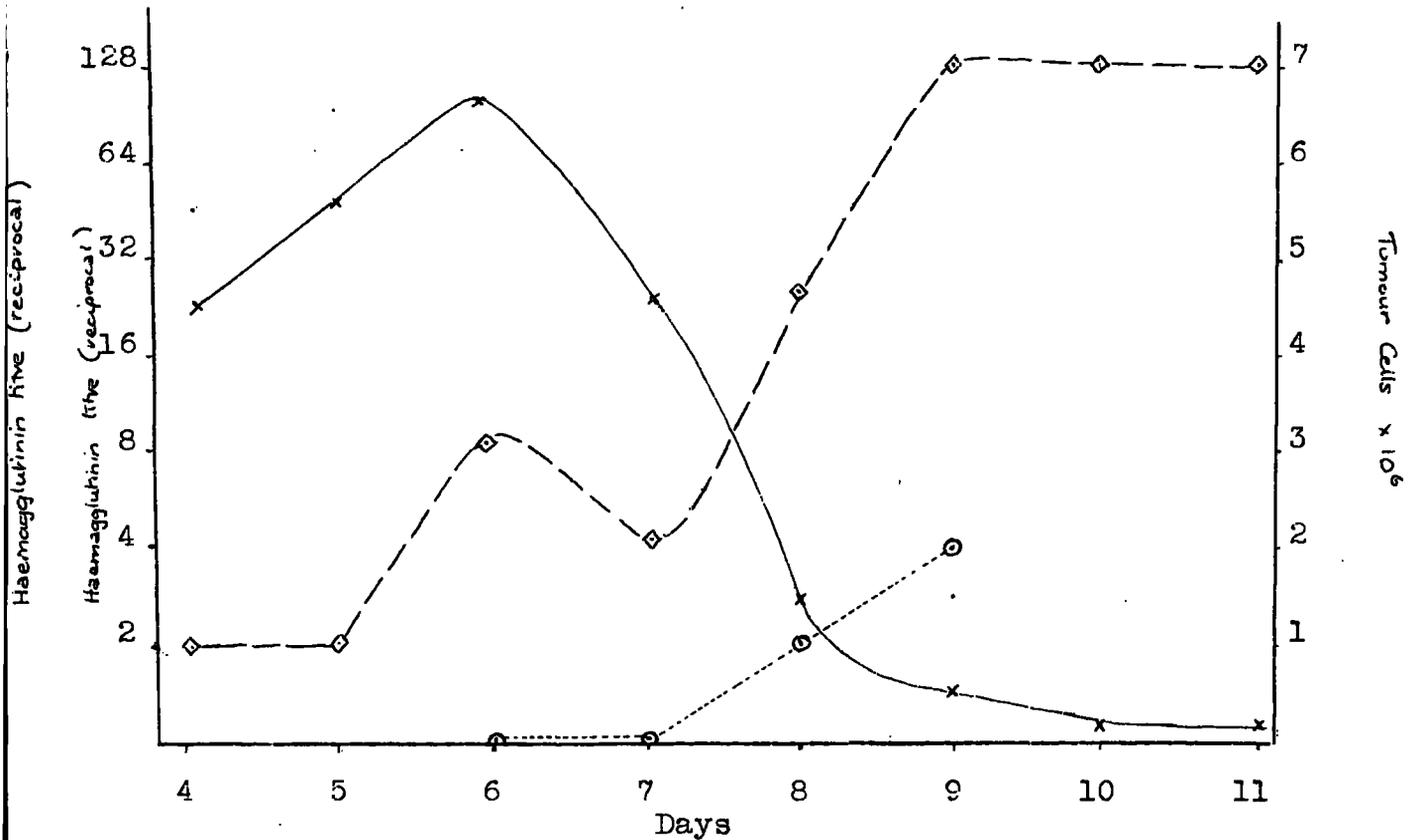
Cells A Stock DBA₂ ascites cells.
 B 'Passaged' cells. This line was derived from the residual cells of a few chambers after treatment with antibody. The line had been maintained by passaging the cells through weakly immunised DBA₁ animals for five generations.

Complement Guinea Pig serum diluted to 66% with Mammalian Ringer.

* The percentage dead cells in a count of 200 cells.

GRAPH 1

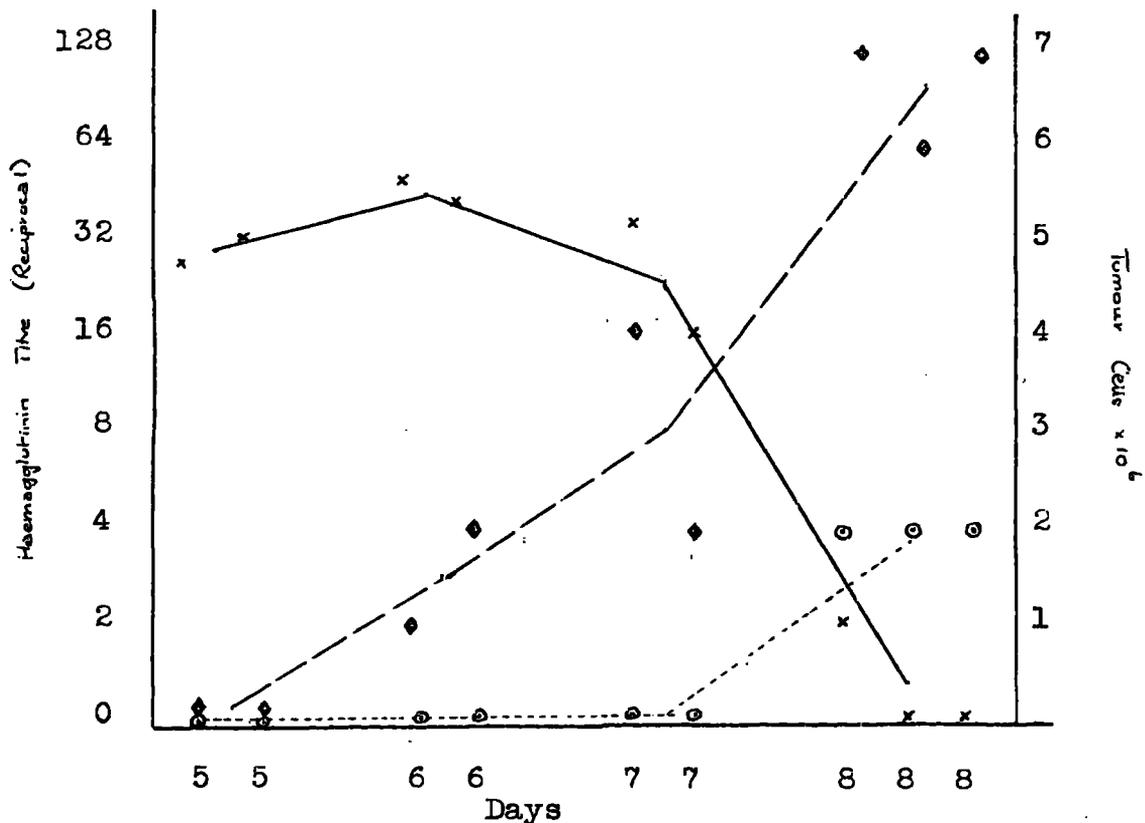
The timing of the appearance of haemagglutinins in relation to tumour growth.



Key x—x Tumour population
 ◇—◇ Antibody titre in the serum
 ○-----○ Antibody titre in the peritoneum
 Each point represents the average for that day.

<u>Size of samples</u>	<u>Days</u>							
	4	5	6	7	8	9	10	11
Tumour population and serum.	5	21	29	32	20	12	3	5
Ascites fluid	-	-	8	24	15	7	-	-

GRAPH 2 To show the level of antibody in the serum and peritoneal fluid of individual DBA₁ animals, during the rejection of 20 million L1210 cells.

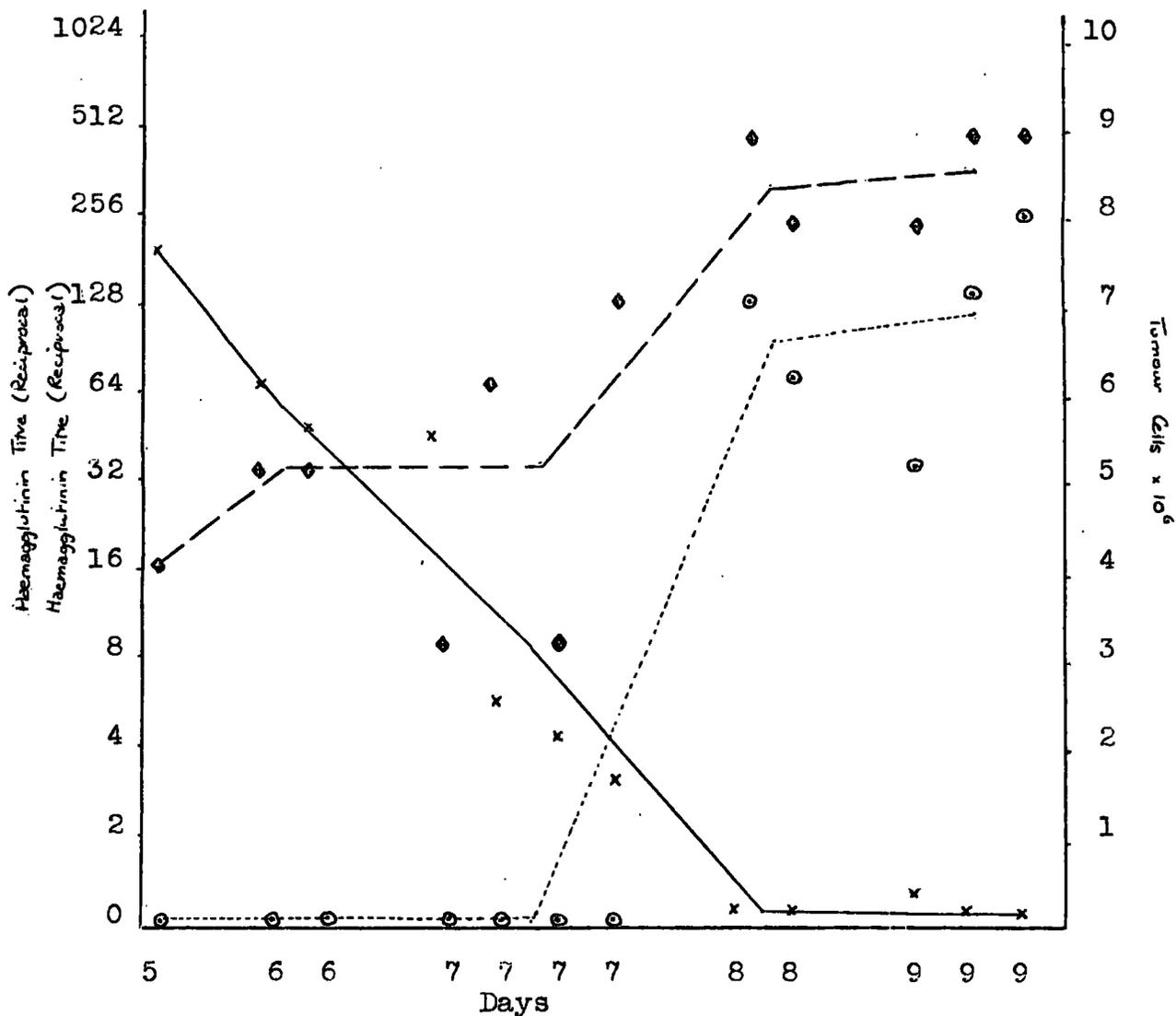


Key

- x—x Tumour population
- ◆—◆ Antibody titre in the serum
-○ Antibody titre in the peritoneum

The lines are drawn from the average value for each day.

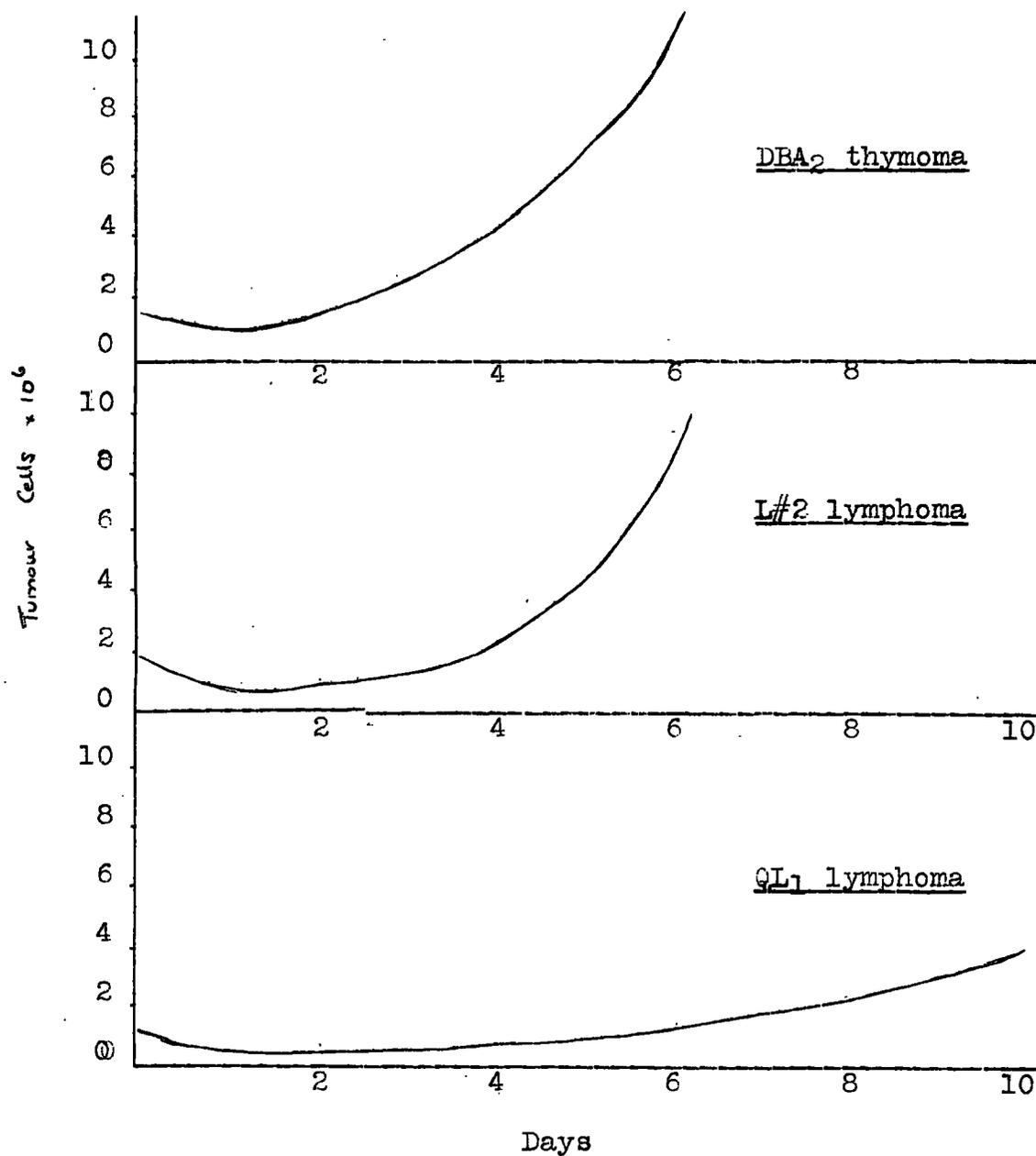
GRAPH 2a To show the level of antibody in the serum and peritoneal fluid of individual C3H animals, during the rejection of 20 million L1210 cells.



Key x—x Tumour population
 ♦—♦ Antibody titre in the serum
 ○—○ Antibody titre in the peritoneum.

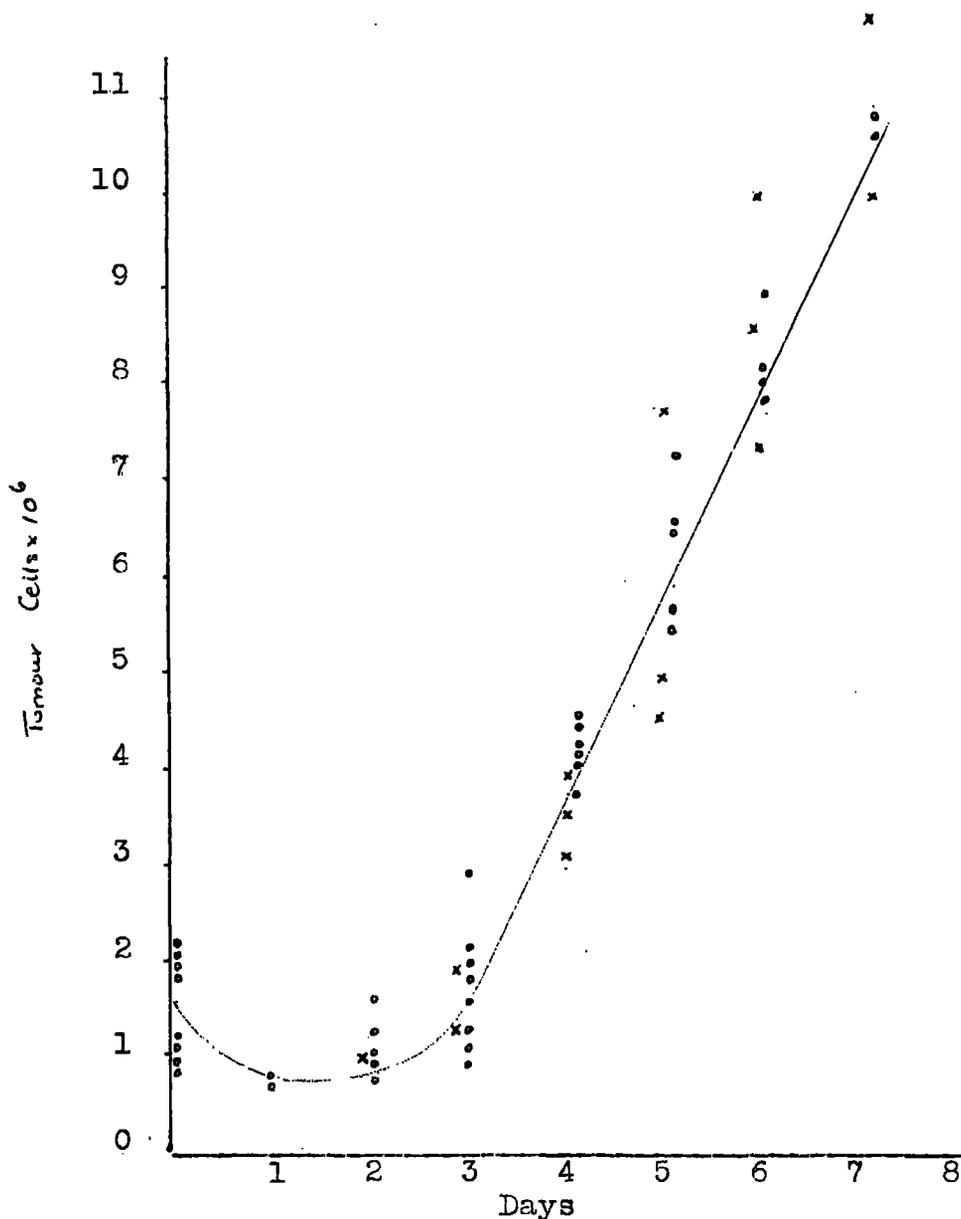
The lines are drawn from the average value for each day.

GRAPH 3 The growth curves of three ascites tumours
growing in diffusion chambers



GRAPH 4 and 5

Details of the growth curve of
DBA₂ thymoma



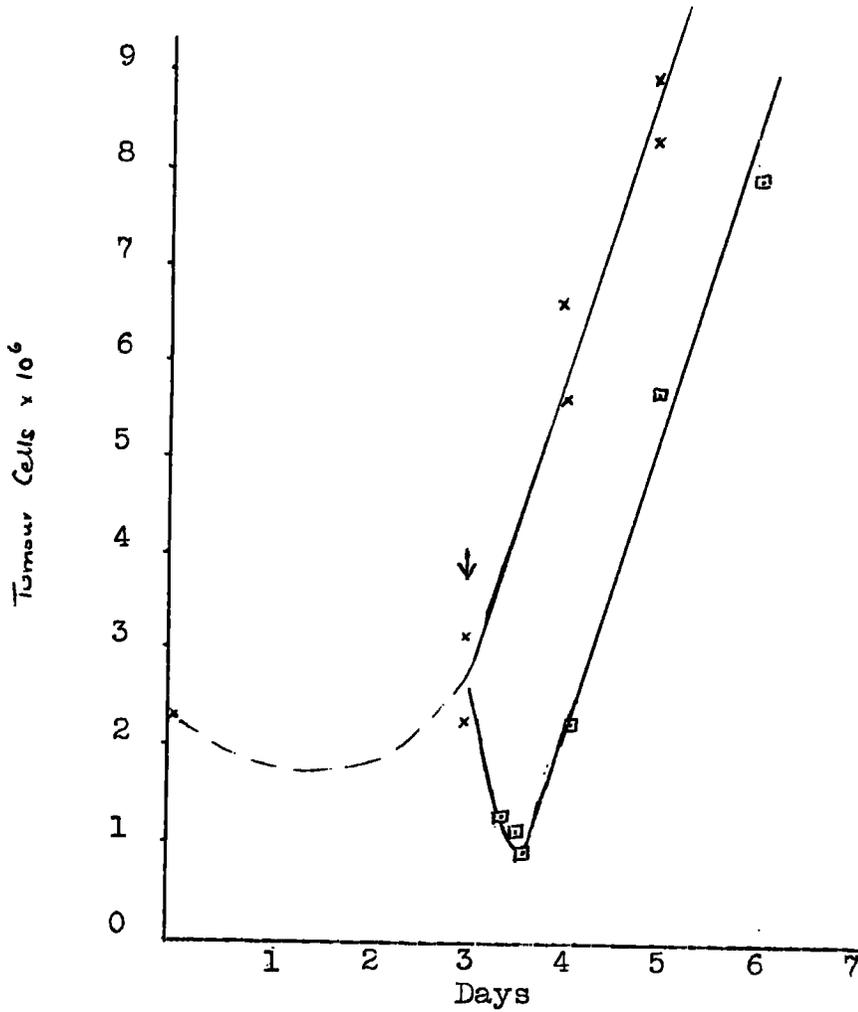
Key

- Chambers in DBA₁ animals
- x Chambers in DBA₂ animals

Each point represents the cell count in a single chamber. Five sets of chambers were placed in DBA₁ animals and two in DBA₂ animals.

GRAPH 6

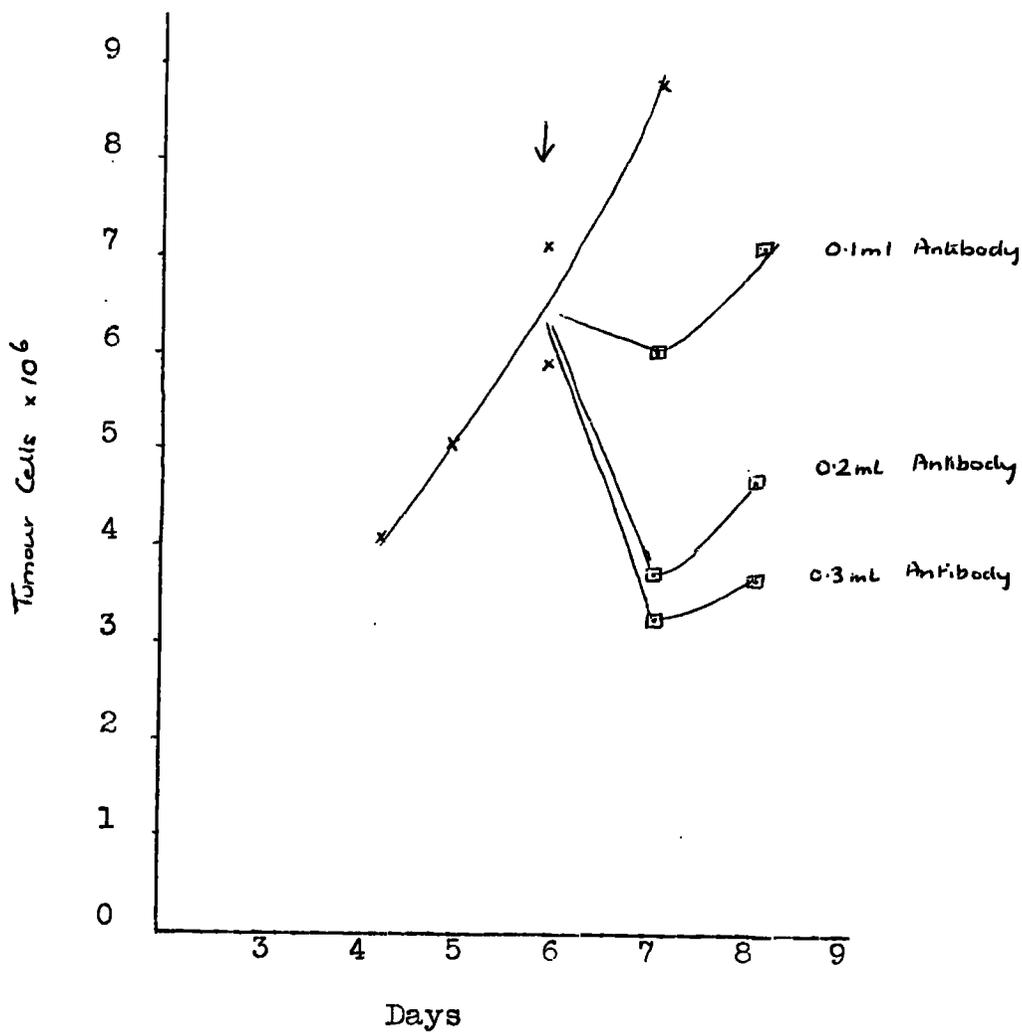
The effect of a single injection of antibody on a DBA₂ thymoma population



□—□ Animals injected with 0.1 ml. DBA₁ anti DBA₂ antibody
x—x Animals injected with 0.1 ml. normal mouse serum.
↓ Time of injection.

GRAPH 7

A comparison of the effect of different volumes of antibody on DBA₂ thymoma populations.

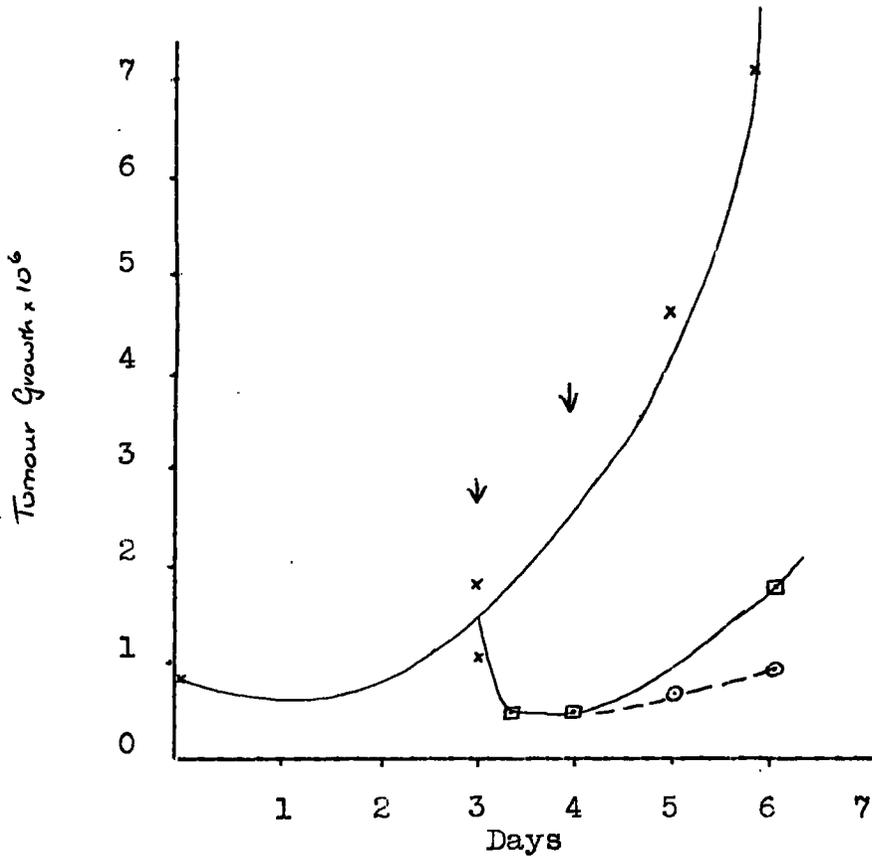


—x— No injections of antibody
—□— Antibody treated populations

↓ Time of injection of antibody

GRAPH 8

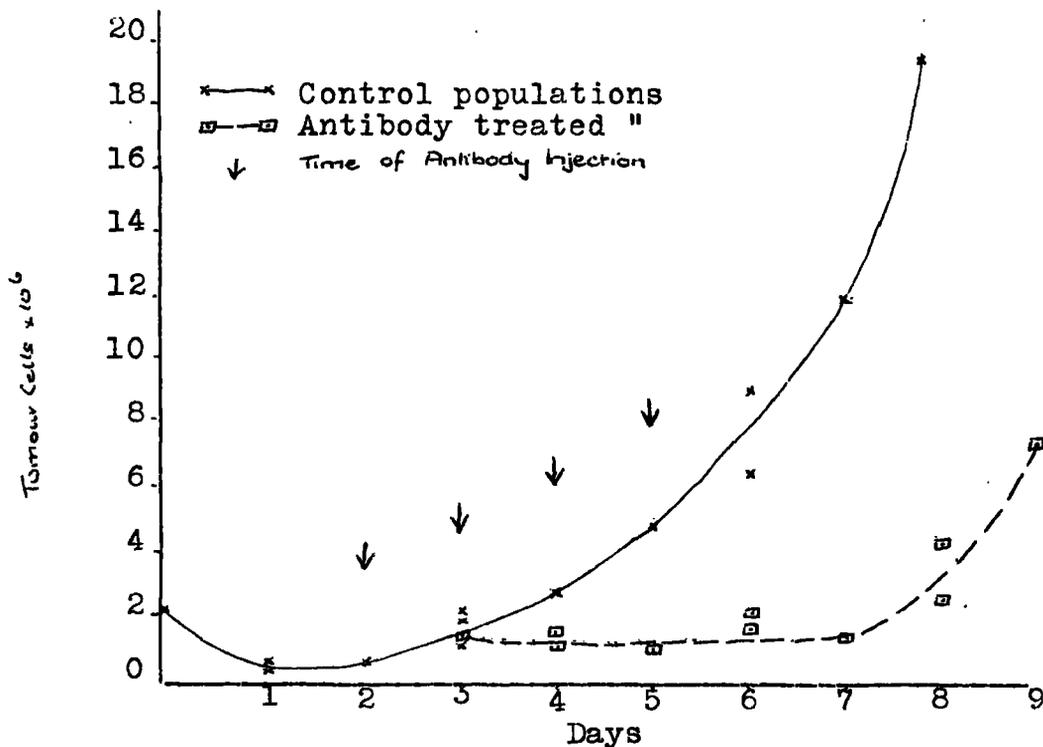
Antibody injected on two successive days to a growing DBA₂ thymoma population.



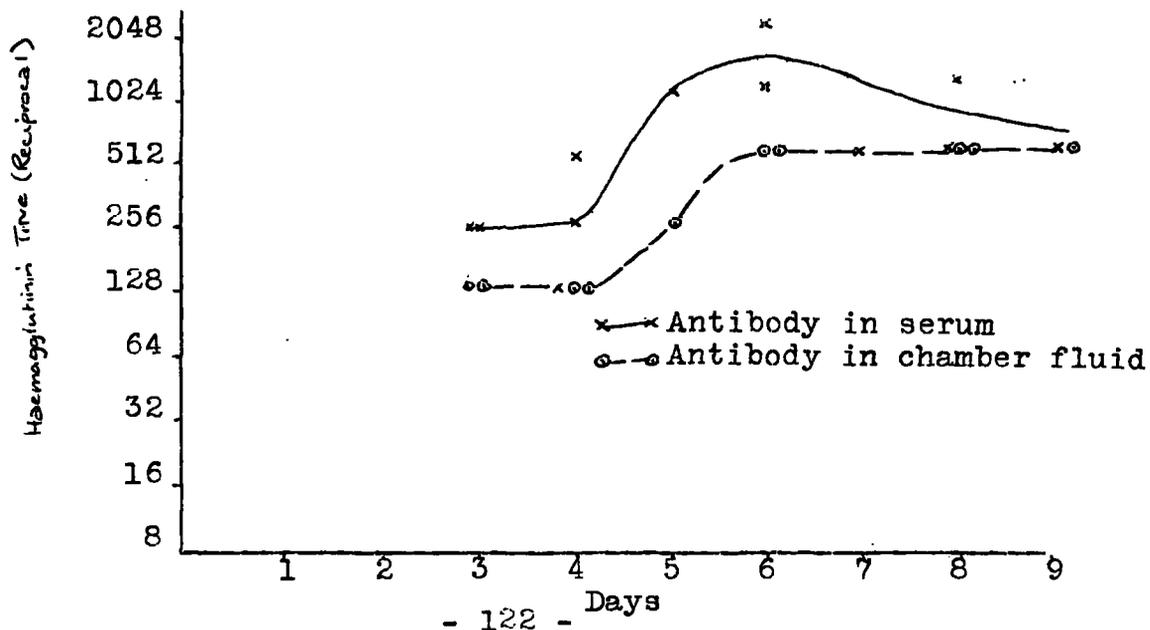
- x — x Control populations
- — □ Treated with 0.3ml. DBA₁ anti DBA₂ antibody on Day 3.
- — ○ Treated with 0.3ml. DBA₁ anti DBA₂ antibody on Day 3 and Day 4.
- ↓ Time of injection of antibody.

GRAPH 9

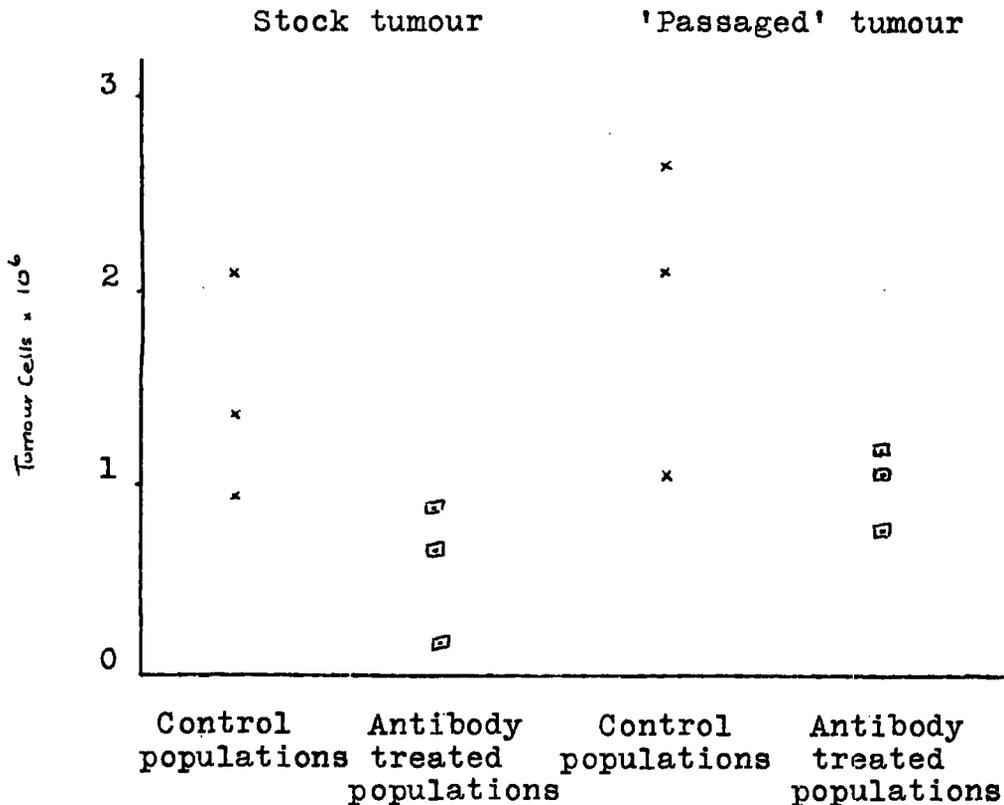
The effect of injecting antibody on four successive days.



The levels of antibody in the serum and chamber fluid of the animals in the above experiment.



GRAPH 10 A comparison of the antibody sensitivity of two DBA₂ thymoma lines, the stock line and a line possibly containing antibody resistant cells.

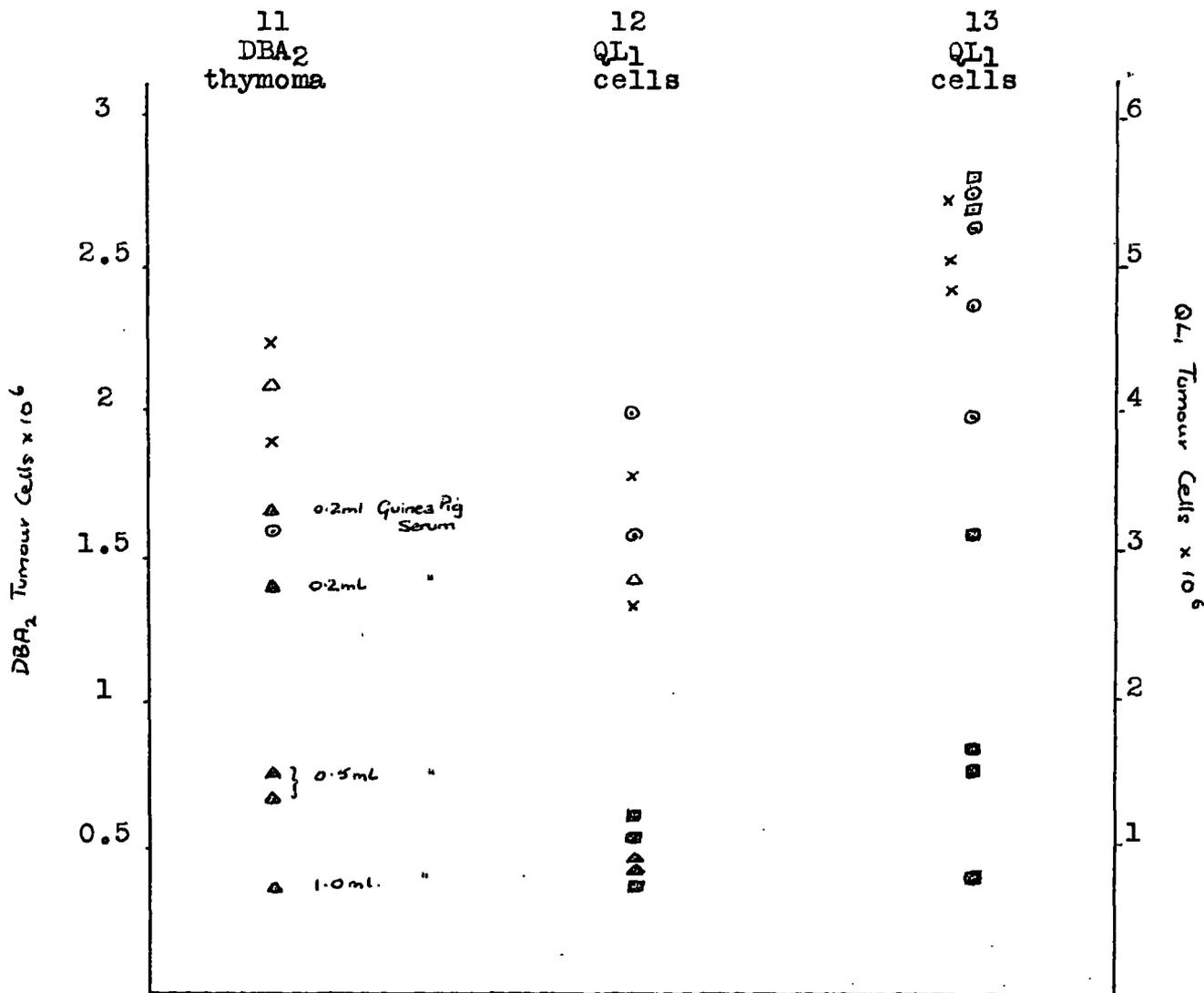


'Passaged' tumour. Several sensitised populations were subjected to a selection pressure by growing the cells in DBA₁ animals lightly immunised against the DBA₂ thymoma. The population tested had been transferred for six generations.

Control chambers received 0.3ml. Mammalian Ringer by intraperitoneal injection.

Experimental chambers received 0.3 ml. AHa anti DBA₂ thymoma antibody by intraperitoneal injection.

GRAPHS 11,12,13. The effect of adding either Guinea Pig Serum or Normal Mouse Serum to populations previously treated with antibody.



Pretreated with 0.9 ml. antibody Guinea Pig Serum injected 24 hours before sampling

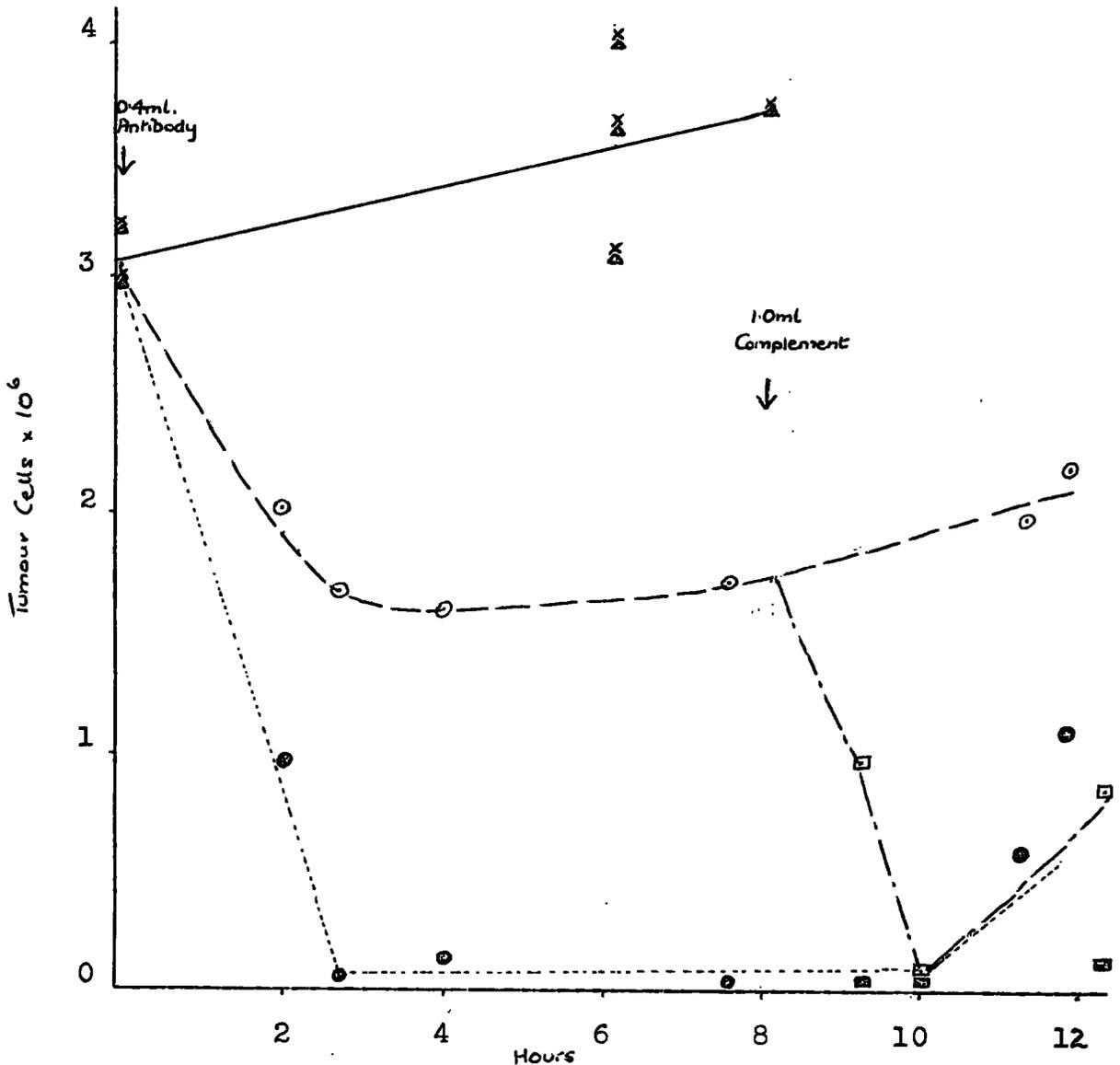
Pretreated with 0.5ml. antibody 1 ml. Guinea Pig serum or 1 ml. Normal Mouse serum injected

Pretreated with 0.4 ml. antibody 1 ml. Normal Mouse serum injected

- Key**
- x Cell control
 - o Antibody injected alone
 - Δ Guinea Pig Serum " " " "
 - ◻ Normal Mouse " " " "
 - Δ Antibody and Guinea Pig serum injected
 - ◻ Antibody and Normal Mouse serum injected

Graph 14

A detailed study of the effects of antibody and complement on a neoplastic population.



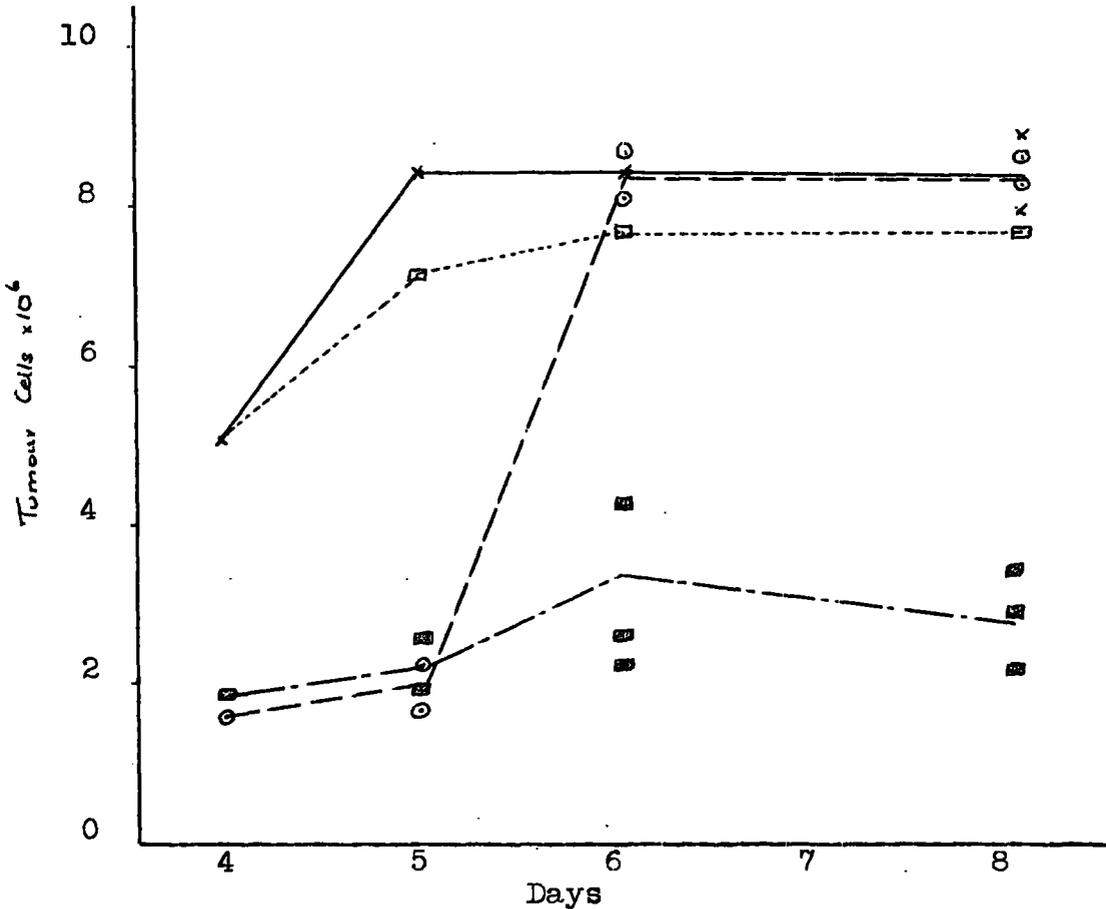
Total undamaged cells

Total unsensitised cells

- x— Cell controls
- o— Treated with antibody
- Treated with antibody and complement

- Cells controls
- Treated with antibody
- Treated with antibody and complement

GRAPH 15 The effect of multiple injections of mouse complement on sensitised neoplastic populations



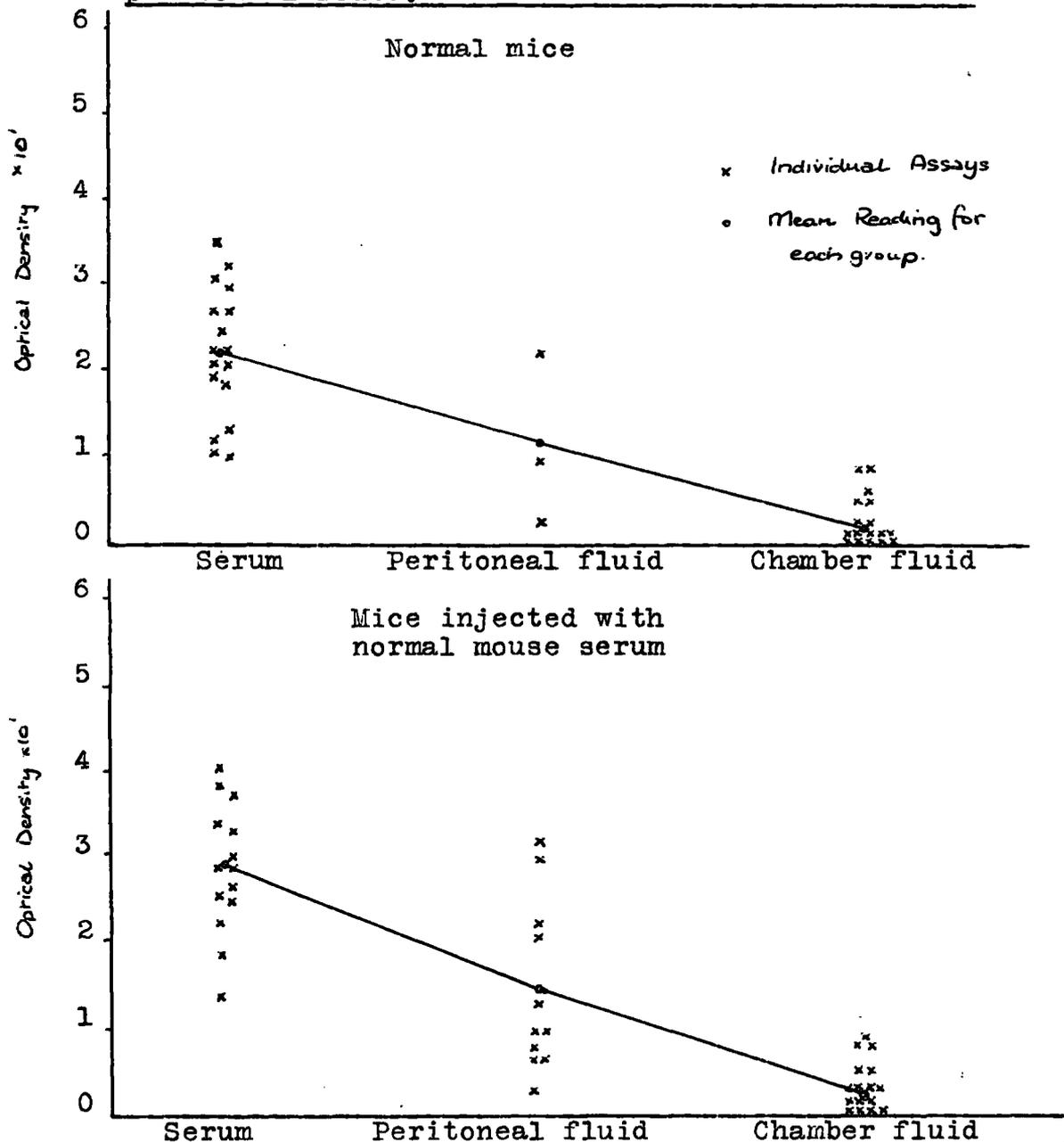
Key

x—x	Cell control	□—□	Treated with mouse serum
o—o	Treated with antibody	■—■	Treated with antibody and mouse serum

Injection and sampling procedure

	Day							
	1	2	3	4	5	6	7	8
Injection of Antibody			0.2	0.2ml.				
" Mouse serum			0.8	0.8	0.8	0.8	0.8ml.	
Sampling				S	S	S		S

GRAPH 16 The levels of complement in the serum, peritoneal fluid and chamber fluid of normal mice and mice injected with normal mouse serum by the intra-peritoneal route.



All the fluids were diluted 1/2.
The red cell suspension was standardised so that 100% haemolysis would give an Optical Density reading of 56 on the Coleman spectrophotometer.

