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Macrophage Dependent Protection of Tumour Cells

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Summary. Rat lymphoma cells sensitized with antibody and incubated with normal macrophages were shown to become resistant to damage from cytotoxic antibody and complement or cytotoxic lymphocytes. Both lymphoma specific antibody and normal macrophages were required to produce protection. The cytotoxic effect of lymphocytes or antibody with complement and the protective effect of antibody with macrophages was assessed by ^{51}Cr release and by loss of capacity of lymphoma cells to transfer the disease to syngeneic rats. No cytotoxic effect could be attributed to macrophages from sensitized allogeneic rats. Inhibition of phagocytosis by Cytochalasin B did not destroy the capacity of macrophages to protect lymphoma cells. Sodium fluoride, however, did block the protective effect.

It is envisaged that this protective phenomenon may be of importance in understanding some aspects of growth of tumours *in vivo* despite the presence of immune cytotoxic mechanisms.

INTRODUCTION

The role of macrophages in relation to tumour growth has recently excited much interest. A number of reports have indicated an effector role for macrophages against tumour cells after both specific (Evans and Alexander, 1970; Tsoi and Weiser, 1968) and non-specific (Keller and Jones, 1971) sensitization of the host. The cytotoxic mechanism in most cases appears to depend on direct contact with the target cells as originally described against fibroblast and L cell monolayers (Granger and Weiser, 1964). In one system a co-operative effect with immune lymphocytes has been described (Evans and Alexander, 1972) which seems analogous to that described against certain bacteria (Mackaness, 1969)

We have studied the role of macrophages in relation to the rejection of an allogeneic lymphoma in rats. Both cytotoxic lymphocytes and antibody, lytic in the presence of complement, can be demonstrated *in vitro* from an allogeneic strain of rats which have received tumour. These have been shown to be highly effective cytolytic agents against the lymphoma *in vitro*. Contrary to the reports described above no effector role for the macrophage can be demonstrated in this system. Instead, when tumour cells are sensitized with antibody, macrophages have been found to inhibit both the described effector mechanisms. The mechanism and implications of this unusual aspect of macrophage function in relation to tumour growth are discussed.

MATERIALS AND METHODS

The model used in these studies is a lymphoma with an overt leukaemic phase which

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grows in the syngeneic black hooded PVGc strain of rats. It was kindly provided by Dr W. L. Ford and Dr B. Roser of the William Dunn School of Pathology, Oxford and has been maintained by serial passage in the syngeneic strain. At intervals of 3 months animals are injected with the original tumour stored in liquid nitrogen to minimize antigenic changes. The time of development of the leukaemic phase is proportional to the size of the initial inoculum.

The allogeneic albino 'Agus' strain of rats is able to reject an inoculum of tumour in excess of 10^9 cells and in immunized rats cytotoxic lymphocytes and complement lytic antibodies can be demonstrated.

1. *^{51}Cr labelling of tumour cells*

Tumour cells were obtained from syngeneic animals during the blood borne phase of the disease from either the peritoneal cavity by peritoneal washout or the blood by isopycnic centrifugation (Perper, Zee and Mickelson, 1968).

Labelling was carried out at a concentration of 10–20 million cells in 1 ml in HEPES buffered basal medium (HBBM) supplemented with 10 per cent foetal bovine serum (10 per cent FBS) (Biocult Laboratories, Glasgow) by incubation with 100 μCi Na_2 $^{51}CrO_4$/ml (Radiochemical Centre, Amersham, Bucks) for 1 hr at 37°. The cells were washed twice in 25 ml minimal essential medium (MEM) and resuspended in HBBM with 10 per cent FBS. All *in vitro* studies were carried out in duplicate or triplicate in $3 \times \frac{1}{2}''$ plastic tubes (M & H Plastics, Romford, Essex).

2. *Direct killing by lymphocytes*

Cytotoxic lymphocytes were obtained from the peritoneal cavity of immunized Agus rats 3 days after the 3rd i.p. injection of 10^8 tumour cells given at weekly intervals. The peritoneal cells were washed in MEM and resuspended in HBBM+10 per cent FBS, macrophages were then removed from other cells by incubation for 2 hr at 37° on 30-ml columns of 3-mm diameter glass beads. After incubation the cells were eluted, washed in MEM and resuspended in HBBM containing 10 per cent FBS preparatory to use. Optimal killing of tumour cells occurred when 1×10^5 tumour cells in 1 ml were added to 2×10^6 immune peritoneal cells in 1 ml and incubation carried out for 8 hr at 37°. Controls consisted of non-immune Agus lymphocytes treated in the same way as the immune lymphocytes.

At the end of the incubation period the tubes were centrifuged at 400 ***g*** for 5 minutes and half (1 ml) of the supernatant removed. This and the tube containing the remaining supernatant and cells were counted separately in an automatic gamma counter. Total activity released is equivalent to twice the activity in supernatant tube only and this is expressed as a percentage of the activity in both tubes.

$$\text{Per cent } ^{51}\text{Cr released} = \frac{2 \times (\text{Activity in supernatant tube} - \text{background counts})}{\text{Sum of activity in both tubes} - \text{background counts}} \times 100.$$

Percentage specific cytotoxicity is calculated as described by other workers (Brunner, Mauel, Cerottini and Chapuis, 1968). The difference between the ^{51}Cr release from cultures with immune lymphoid cells and non-immune lymphoid cells is expressed as a percentage of the maximum percentage ^{51}Cr release from the tumour cell minus the percentage ^{51}Cr release from cultures with non-immune lymphoid cells.

$$\text{Per cent specific cytotoxicity} = \frac{\text{Per cent } {}^{51}\text{Cr release with immune lymphoid cells} - \text{per cent } {}^{51}\text{Cr released with non-immune lymphoid cells}}{\text{Maximum per cent } {}^{51}\text{Cr releasable from cells} - \text{per cent } {}^{51}\text{Cr released with non-immune lymphoid cells}} \times 100.$$

3. *Antibody-induced complement lysis*

Antisera were prepared in Agus rats by i.p. injection of 10^7 tumour cells followed by injection of 10^8 cells 10 days and 17 days later. Antisera were harvested 3 days after the last injection. The complement lytic titre of such sera is usually 1 : 128–256 taking the end point as the dilution of antiserum at which the ^{51}Cr release is 10 per cent above that occurring in the presence of complement alone. (The specificity of these antisera in terms of complement lytic activity is directed mainly against the histocompatibility antigens on the PVG tumour cell not shared by the Agus strain of rats. Extensive absorption of the sera against normal PVGc tissues removes most of the complement lytic activity with titres of approximately 1 : 8 remaining.)

Optimal complement lysis of the tumour cell measured in terms of maximal ^{51}Cr released from the cells occurs with antisera at a concentration of 1 : 10 and fresh rat serum at a final concentration of 1 : 2 as a source of complement. These conditions were used in experiments where protection from complemented mediated lysis was assessed. Sera used for sensitization in macrophage protection experiments were heat inactivated at 56° for 30 minutes.

4. *Peritoneal exudate cells*

Peritoneal cells used in the protection studies were obtained from the peritoneal cavity of Agus rats 3 days after i.p. injection of 10 ml of 3 per cent bacterial peptone. They were washed in MEM and resuspended in HBBM with 10 per cent FBS preparatory to use. Differential counts of these cell preparations show that macrophages account for at least 70 per cent of the cells present, the remainder being lymphocytes and granulocytes. For clarity this population of peritoneal cells used as a source of protective cells will be referred to in the text as peritoneal exudate macrophages (PEM) although it is recognized that other cells are present.

5. *Protection of tumour cells by macrophages*

The experiments illustrated in Fig. 1 were carried out in two stages. Firstly, sensitization and incubation of the tumour cells was carried out at 37° with the number of Agus PEM shown; secondly addition of the effector system.

With cytotoxic cells as the effector system, tumour cells (10^5 in 0·5 ml) were presensitized with Agus antiserum raised against the PVGc tumour at a concentration of 1 : 80 and then incubated for 45 minutes with the PEM (0·5 ml) prior to the addition of the cytotoxic Agus peritoneal lymphocytes (2×10^6 in 1 ml). The degree of tumour cell damage was then assessed as already described. (In previous studies inhibition of the effect of cytotoxic lymphocytes had been demonstrated by antiserum to the tumour cell at dilutions less than 1 : 40 but no impairment of their cytotoxic action was seen at the concentration used for presensitization in these experiments.)

When complement lysis was used as the effector system, tumour cells (10^5 in 0·1 ml) were sensitized with Agus antiserum as above at a concentration of 1 : 10 in 0·1 ml and

incubated for 1½ hours with the PEM (0·1 ml). This time of incubation has previously been shown to be the period at which maximum protection occurs (Hersey, 1972). At this time 0·2 ml of the lytic solution, consisting of unheated Agus antiserum diluted 1:4 in fresh normal Agus serum, was added and incubation continued for 1 hour. One millilitre of MEM was added, the tubes centrifuged at 400 *g* for 5 minutes and 1 ml of supernatant removed for counting. Total radioactivity released in this case is equivalent to the activity in the 1 ml of supernatant, multiplied by 1·5. Percentage ^{51}Cr release and percentage specific cytotoxicity was calculated as before.

6. In vivo *transfer studies*

The development of the lymphoma in syngeneic animals receiving the tumour cells described in the text was assessed by counting the total number of white blood cells (wbc)/ml in a 10-μl sample of blood taken from the tail vein of the rats with a 10-μl Eppendorf pipette. Counts were assessed by use of a Coulter counter model 'D' and the animals were recorded as developing the lymphoma when a wbc count above 35000/cmm was seen. The route of transfer used in these studies was by intracardiac injection in a volume of 1 ml.

RESULTS

1. PROTECTION OF TUMOUR CELLS

Fig. 1 illustrates the protective effect of PEM upon the two cytotoxic mechanisms operating against antibody-sensitized tumour cells *in vitro*. The results are the means with standard errors for three experiments. In both systems the degree of protection is seen to depend on the number of PEM added to cultures, however protection against complement lysis is seen with as few as 3×10^4 PEM and is almost maximal with $1{\cdot}25 \times 10^5$ PEM or at a ratio of approximately 1:1 with the tumour cells. The protection against cytotoxic lymphocytes is less efficient than that against complement lysis and approximately sixteen

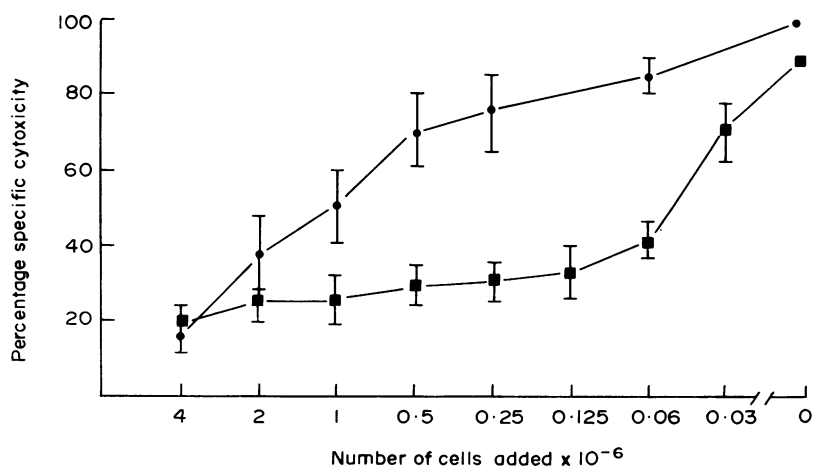

FIG. 1. Inhibition by macrophages of cytotoxity against antibody-sensitized rat lymphoma cells (10^5). (●) Cytotoxicity induced by lymphocytes (2×10^6). (■) Antibody induced complement lysis. The number of peritoneal exudate cells added to the cultures is shown along the abscissa. The results are means with standard errors for three separate experiments.

times more PEM are needed to achieve the same degree of protection. Whether this difference indicates the presence of two protective mechanisms has not been answered in these studies.

The methods used for induction of the macrophages does not appear to be important for this protective function. PEM from the peritoneal cavity of Agus rats immunized i.p. with the PVGc lymphoma appear as capable of this protective function as PEM induced by bacterial peptone.

2. DEPENDENCE ON SENSITIZING ANTIBODY

The protective effect of macrophages against complement lysis can be shown to be dependent on sensitization of the tumour with Agus anti-PVGc tumour sera by incubating the tumour cells with increasing dilutions of the antisera in the first incubation step with

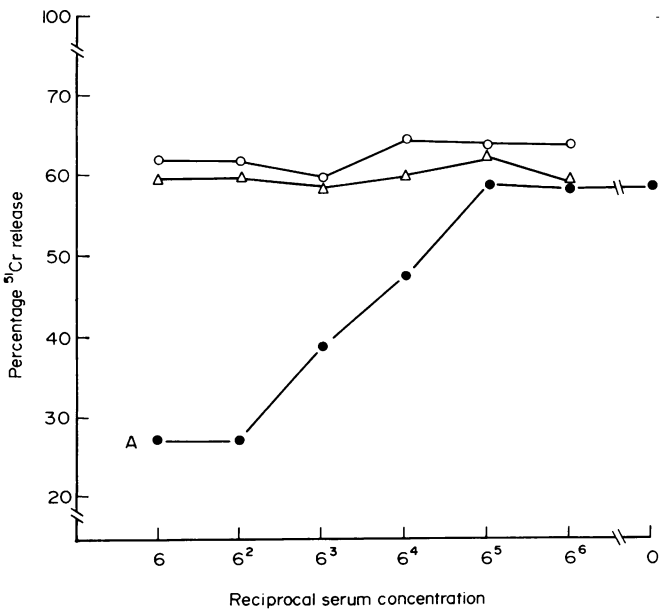

FIG. 2. The requirement of tumour-specific antibody for protection of lymphoma cells by macrophages against complement lysis. The experiment was carried out in two stages and the results illustrate the ^{51}Cr release occurring in the second stage after the addition of the complement lytic system described in the text. The first stage consisted of incubation of 10^5 lymphoma cells with 10^6 macrophage with (●) antiserum, (▲) normal serum or (○) incubation with antiserum in the absence of macrophages.

PEM. Fig. 2 shows that when the lytic system (antibody and complement) is added to tumour cells after they have been incubated with macrophages at the concentrations of antiserum shown, the protection against complement lysis is lost at low concentrations of antibody. Controls where normal heat inactivated Agus sera is used at the same concentrations, or where tumour cells are incubated in antisera at the same concentrations but in the absence of macrophages, do not show any inhibition of complement lysis. (Some qualification of this latter statement is needed in that pre-incubation with some antisera in the absence of macrophages has been noted to cause a marginal (5–10 per cent) reduction in ^{51}Cr release in the subsequent lysis step.)

3. *In vivo* TRANSFER STUDIES

One of the questions arising from the observations in Figs 1 and 2 is whether the protection is real in terms of tumour viability or whether the presence of macrophages may merely interfere with the release of ^{51}Cr from effete cells. This question was answered by transferring the contents of the 'protected' cell cultures at the end of the 2nd incubation period with cytotoxic lymphocytes or the complement lytic system, to groups of animals syngeneic with the tumour cells. If the tumour cells in cultures with antibody and macrophages were protected and viable, then animals given these cultures should develop leukaemia earlier than control groups. The extent of the differences in time of onset is taken as a measure of the number of viable cells transferred.

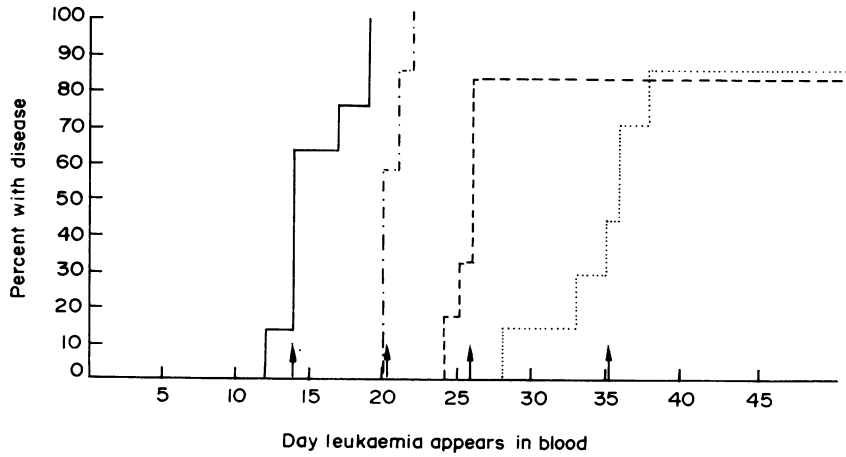

FIG. 3. Protection of lymphoma cells by macrophage as assessed by *in vivo* viability of protected cells in syngeneic rats. The time of onset of leukaemia is recorded in groups of eight rats (two experiments) given tumour cells which had been treated in the following way before transfer into rats. (——) Tumour (2×10^6) incubated alone, no complement lytic system added. (·—·—·—·) Tumour sensitized with antibody, incubated (protected) with macrophages (4×10^6) then exposed to complement lysis. (– – –) Tumour sensitized with antibody then exposed to complement lysis. (· · · · ·) Unsensitized tumour cells incubated with macrophages (4×10^6) before addition of complement lytic system. The arrows indicate the median time of onset of leukaemia in each group.

Fig. 3 illustrates the pooled data from two such experiments where complement mediated lysis was used as the effector system. Each group represents eight animals and each rat in the group received cultures initially containing 2×10^6 tumour cells. The results appear clear. The median time of development of the tumour in animals receiving cultures in which tumour cells had been sensitized and incubated with macrophages prior to addition of the lytic system, was 20 days. This is significantly different ($P > 0{\cdot}05$) from the median of 26 days and 35 days for the control groups in which tumour cells were incubated with antibody alone, or peritoneal cells alone respectively before addition of the lytic system. The group of animals receiving tumour cells not treated with the lytic system have a median development time of 14 days. In terms of the number of tumour cells growing out from the inoculum we infer from previous experiments relating time of development of leukaemia to number of cells in initial inoculum, that approximately 10^3–10^4 viable tumour cells were transferred in the test group compared with 10^2 in the unprotected control groups. Attention is also drawn to the greater time taken for leukaemia to develop in animals given allogeneic

peritoneal cells. When this protective effect of the allogeneic cells against the tumour is taken into account the difference between the time of onset in the groups given tumour only and the group given tumour cells protected with antibody and macrophages, is made even smaller.

In vivo transfer experiments have also been carried out where cytotoxic lymphocytes are the effector system. In one experiment with four rats per group, the group receiving cultures in which 10^5 tumour cells were incubated with antibody and normal macrophages (as well as the cytotoxic lymphocytes) for 8 hours, developed leukaemia on transfer between 26–33 days. In the control groups; the rats receiving cultures in which peritoneal cells only were added to the tumour cells and cytotoxic lymphocytes, developed the tumour between 34–38 days. In the group given tumour cells incubated with antibody alone, only 1 of the group developed the tumour at all.

4. THE MECHANISM OF PROTECTION OF TUMOUR CELLS BY MACROPHAGES

Initial evidence on this aspect was obtained from three sources. Firstly, histological studies with macrophage cover slip cultures showed numerous tumour cells within macrophages and when macrophages were removed by absorption on glass bead columns the protective effect of peritoneal cells was lost. Moreover, when sodium fluoride (NaF), a known metabolic inhibitor of phagocytosis (Cohn, 1966) was added at a concentration of 10 mM to the cultures inhibition of the protective effect was seen. These studies suggested phagocytosis as the mechanism operating to protect tumour cells. However, two further studies have made this conclusion unlikely.

The first of these consisted of correlating in quantitative terms the histological studies with the ^{51}Cr release data. It is possible to quantitate precisely the number of tumour cells being protected by macrophages by estimating the difference in radioactivity released from tumour cells incubated with and without opsonizing antibody when a lytic system is added (as for the study illustrated in Fig. 2). If this difference in released radioactivity is divided by the radioactivity known to be released per cell on lysis of the cell, it is possible to estimate the number of cells protected. When studies of this sort were carried out and cytocentrifuge preparations made of the cultures in parallel, it was found that only 20 per cent of the cells known to be protected could be seen within macrophages. Most of the tumour cells appeared to be merely adherent by light microscopy.

The second study consisted of experiments using two inhibitors of phagocytosis which have different modes of action. NaF is a known metabolic inhibitor whereas the second inhibitor—Cytochalasin B—(Cyto B) is thought to act on the contractile microfilaments of the macrophage (Allison, Davies and de Petris, 1971). Both these agents have been shown to inhibit the phagocytosis of sheep red blood cells (SRBC) under similar experimental conditions as used for the protection studies with tumour cells. In these studies (Hersey, 1972) it has been shown that inhibition of phagocytosis of SRBCs can be demonstrated with NaF at concentrations of 10 mM or above and with Cytochalasin B at concentrations 2·5 μg/ml or above.

Fig. 4 demonstrates the results of parallel experiments in which NaF and Cytochalasin B at the concentrations shown were incubated with tumour cells, PEM and antibody in the first stage of the test. The results illustrated are the per cent ^{51}Cr release occurring in the second stage of the test when antibody and complement are added. In Fig. 4a it is seen that as increasing concentrations of NaF are added the protection afforded the tumour

cells by incubation with macrophages and antibody in the first step against subsequent complement lysis is lost. The maximum inhibitory concentration being 10 mM. However, in Fig. 4b it is seen that at concentrations of Cyto B known to inhibit phagocytosis only slight (10 per cent) inhibition of the protection against complement lysis is seen. (The controls in the study shown by the discontinuous (upper) lines illustrate the ^{51}Cr released from tumour cells incubated in the first step in the absence of antibody. With NaF some inhibition of complement lysis is seen at high concentrations. The controls in both 4a and b illustrated by the dashed (lower) lines illustrate the effect of the inhibitors on ^{51}Cr release in the absence of the addition of complement.

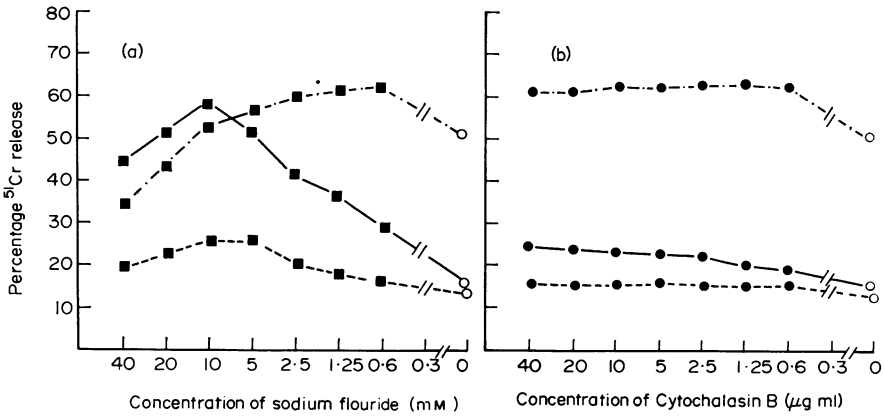

FIG. 4.(a) Inhibition of tumour cell protection with NaF. 1st step: Incubation of 10^5 lymphoma cells with 10^6 macrophages and (—■—■—) antiserum to the tumour cell or (■-·-·-·-■) with normal serum, both at a final concentration of 1 : 20, together with NaF at the concentrations shown. 2nd step: Addition of complement lytic system to the cultures except (■- - -■) which represents cultures with no complement added. Maximum inhibition of ^{51}Cr release is seen at a concentration of 10 mM.

FIG. 4(b)Absence of inhibition of protection with Cytochalasin B. Experimental procedure and symbols as with NaF.

DISCUSSION

The studies reported here represent an extension of initial observations made during investigation for the presence of cytotoxic lymphocytes in immune allogeneic rats. The cytotoxic effect of lymphocytes from these animals could only be poorly demonstrated until peritoneal exudate cells were absorbed on glass bead columns during purification procedures. After absorption it became evident that cytotoxic lymphocytes were present which were highly effective in causing the *in vitro* destruction of tumour cells.

The inhibitory role of macrophages against the cytotoxic mechanisms revealed in these studies is rather unusual in view of the usual role of the macrophages as part of the host defences. The reason for this effect can only be speculative at present. It seems possible that some tumour cells are not susceptible to the cytotoxic functions of the macrophage and in the absence of a cytotoxic effect these same functions (e.g. pinocytosis) may interfere with the operation of other cytotoxic mechanisms available to the host.

Initial studies on the actual mechanism of protection have suggested that the process is largely independent of the phagocytic function of the macrophages but do indicate that the process depends upon an active metabolic step in the macrophage or the tumour cell. The protection against complement lysis has similarities to the phenomenon of 'antigenic

modulation' described for the TL antigen on mouse leukaemic cells after exposure to antibody (Old, Stockert, Boyse and Kim, 1968) in terms of the kinetics of the process and the *in vitro* steps required. It seems possible in the current studies that surface antigens and antibody are concentrated at the site of adherence to the macrophage so that they are not exposed to the action of added antibody and complement in the second stage of the test system. The work of Taylor, Duffus, Raff and De Petris (1971) has clearly shown that such movement of antigen and antibody around the cell surface of lymphocytes can occur in the production of the capping phenomenon. In the present studies the hypothesis is that the lattice formed by attachment of the antibody on the tumour cells to the surface of the macrophage, causes the movement of this area to one pole of the cell. In this respect the surface of the macrophage may be acting in much the same way as visualized for anti-immunoglobulin molecules.

The question arises as to the implications of these studies in regard to the growth of tumours. Sites in the body where macrophages are numerous, such as the spleen, lymph nodes, peritoneal cavity, liver and bone marrow may provide a protected environment against immune destruction to tumours which are not susceptible to killing by macrophages. Certainly the growth of lymphomas as well as metastases from other tumours are favoured at many of these sites. The recovery of viable tumour cells from the peritoneal cavity of immune allogeneic rats in our own studies and from the peritoneal cavities of immune allogeneic mice for periods up to 39 days after transfer (Britton, 1971) lends some support to this hypothesis. This particular role described for macrophages may add some understanding of the reasons for continued growth of tumour cells in the face of immune mechanisms of rejection and provide an explanation for the growth of tumours in particular sites in the body.

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