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The Distribution of Antibody and Antibody-Producing Cells After Immunization with Xenogeneic Cells

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Summary. After intradermal injection of xenogeneic cells, antibody producing cells were demonstrated in the regional lymph nodes and peritoneal fluid as early as 5 days post-immunization. By 19 days antibody-producing cells were also found in the bone marrow, spleen and peripheral blood. They were not found in significant numbers in the thymus or non-regional lymph nodes. Antibody was demonstrable in the blood as early as 4 days post-immunization and reached maximum titre at 19 days. Both IgG and IgM were recovered from the thoracic duct lymph after intravenous injection. The significance of these findings is discussed in relation to the rejection of solid tumours and grafts.

INTRODUCTION

Immunologically specific damage to a line of xenogeneic target cells by lymphocytes from immunized rats requires the participation of two populations of lymphoid cells. One population produces IgG specific for target cell antigens but is not itself cytotoxic, whilst the other population is cytotoxic to target cells whose antigens are complexed with specific antibody (MacLennan and Harding, 1970). Antibody dependent, lymphocyte-mediated cytotoxicity has also been found in allogeneic systems, including human (MacLennan, Loewi and Howard, 1969), duck (Bubenik, Perlmann and Hâsek, 1970), and mouse (Möller, 1965).

Most of this work has been done *in vitro* and further evidence to show that this mechanism acts *in vivo* is required. The mechanism *in vivo* would depend upon the availability of the sensitizing antibody and of effector lymphocytes. In this paper we present data showing the distribution and the relative number of specific antibody-producing cells in lymphoid organs at various times after immunization. We also present data which show that this antibody is found in the peripheral circulation, and that it can be recovered from thoracic duct lymph after intravenous injection. The significance of these data is discussed in relation to the rejection of solid tumours and grafts.

MATERIALS AND METHODS

Target cells

The target cells used in this study were Chang liver cells obtained from Flow Laboratories (Irvine, Scotland). They were grown in 8-oz medical flat bottles with Eagle's

minimum essential medium for suspension cultures supplemented with 10 per cent foetal bovine serum, 1 per cent fresh isotonic glutamine, 1 per cent non-essential amino acids, penicillin 200 units/ml, and streptomycin 100 μg/ml. Single cell suspensions of Chang cells were obtained by shaking the bottles at least once every 48 hours, removing the medium and suspended cells, and replenishing with fresh medium.

In that part of this study which is concerned with the demonstration of IgM in the thoracic duct the target cells used were fresh sheep red blood cells (SRBC), which had been washed four times in normal saline to remove fragile cells.

Animals

Adult F_1 hybrid rats from inbred Agus and PVG strain parents were used throughout this study. The rats were between 2 and 6 months of age but were matched within each experiment.

Mode of immunization

Chang cells were washed four times in normal saline. The rats were immunized with 10^7 Chang cells emulsified in Freund's complete adjuvant given as multiple intradermal injections over the shoulders.

Anti-SRBC serum was prepared by injecting rats with 2×10^8 SRBC given intravenously into the tail vein. The serum was taken 14 days post-immunization.

Lymphoid populations and sera

The following cell populations were harvested at 5, 12, 19 and 27 days post-sensitization: axillary lymph nodes (regional lymph nodes), lymph nodes from the paratracheal region (non-regional lymph nodes), spleen cells, bone marrow cells, thymus cells and peritoneal exudate cells.

Solid lymphoid organs were processed to produce a single cell suspension by chopping with a scalpel blade, crushing the resulting pieces with artery forceps and suspending in minimum essential medium (MEM).

The heparinized peripheral blood was centrifuged and the serum kept for assay. The cells were resuspended to original volume with MEM and the erythrocytes and polymorphs sedimented with an equal volume of 3 per cent gelatine (Coulson and Chalmers, 1964).

The peritoneal fluid was obtained by peritoneal washing with MEM. The fluid obtained was natural, and not previously induced by bacteriological peptone or other agents.

The bone marrow cells were obtained by washing out the femurs with MEM using a syringe and needle.

All cell populations were washed four times in MEM to remove passively absorbed antibody from their surface. The cell populations were finally suspended in MEM with 10 per cent foetal bovine serum (MEM-FBS). The concentration of intact mononuclear cells was assessed in these final suspensions by phase contrast microscopy.

Blood was collected from the sensitized rats by cardiac puncture on days 3, 4, 5, 8, 12, 19 and 27. The serum was heat inactivated for 30 minutes at 56° and stored at −20°.

Preparation of antisera for diffusion study

No attempt was made to purify the anti-Chang IgG as we have previously shown in

our assay system that only IgG is effective at inducing cell mediated cytotoxicity (MacLennan, Loewi and Harding, 1970).

A pool of high titre anti-SRBC serum was used as the source of IgM. Ammonium sulphate was added dropwise to 5 ml of this serum to give a final concentration of 60 per cent. The precipitate was centrifuged at 2000 *g* for 30 minutes, and washed in 60 per cent ammonium sulphate before being separated again at 2000 *g*. The washed globulin precipitate was then suspended in a minimum volume of phosphate buffered saline and dialysed against two changes of phosphate buffered saline. This preparation was then fractionated on a Sepharose 6B (Pharmacia, Uppsala, Sweden) column of 600-ml bed volume with an upward flow rate of 10 ml per hour. Previously Dextran 2000 had been passed through the column to determine the void volume. The IgM elution point was determined by Mancini estimation of eluted human serum and the fraction coincident with this was concentrated and retested to show sensitizing activity.

Cytotoxicity test procedure

Target cell damage was assessed by release of ^{51}Cr sodium chromate from labelled target cells. Five to 10×10^6 target cells were incubated at 37° for 45 minutes in 1 ml of MEM-FBS containing 100 μC ^{51}Cr sodium chromate (Radiochemical Centre, Amersham). The target cells were then washed four times in MEM before being finally suspended in MEM-FBS.

Target cells (2×10^4) and non-immune spleen cells (3×10^6) were added to each tube. Either lymphoid cells (at concentrations of 10^6, 3×10^5 and 10^5) or sera (dilutions from 10^{-1} to 10^{-6}) were added to certain tubes. The total volume in each tube was 2 ml. Incubation was for 18 hours in polystyrene tubes at 37° in a moist atmosphere of 5 per cent CO_2 and 95 per cent air. At the end of the incubation period the tubes were spun at 300 *g* for 15 minutes at 4°. Half the supernatant was then taken off into another tube. The percentage ^{51}Cr sodium chromate release from the target cells was calculated by counting the activity in the original and the supernatant tubes.

Cytotoxicity

For present purposes this is defined as the percentage of ^{51}Cr sodium chromate release from target cells in the presence of non-immune spleen cells without immune lymphoid cells or immune serum subtracted from the percentage of ^{51}Cr sodium chromate release with immune lymphoid cells or immune serum.

Spontaneous release of ^{51}Cr sodium chromate from Chang cells at 18 hours is 20–28 per cent. Chang cells disrupted at the beginning of incubation by twice freezing and thawing release 75–85 per cent of the label by 18 hours.

RESULTS

SERUM LEVEL OF ANTIBODY IN RELATION TO TIME

Groups of two rats were sensitized against Chang cells as described in the methods. All sera were tested together in the same experiment. The antisera were tested at dilutions of 10^{-1} to 10^{-6}.

Sensitizing antiserum was first detectable at 4 days (Fig. 1). The characteristic dilution curve previously described (MacLennan, Loewi and Harding, 1970) which shows a pro-zone, a peak, and a falling off of activity was first seen on day 5. The maximum

sensitizing titre was at 19 days (Fig. 1), but the highest level of cytotoxicity was induced by serum taken at 5 days. Although the pro-zone effect can be partially mimicked by non-immune serum especially after heating at 56° for 30 minutes (MacLennan *et al.*, 1970), the increasing extent of the pro-zone with time after immunization in these experiments, suggests that enhancing antibody may also play a part in the prozones associated with the later sera.

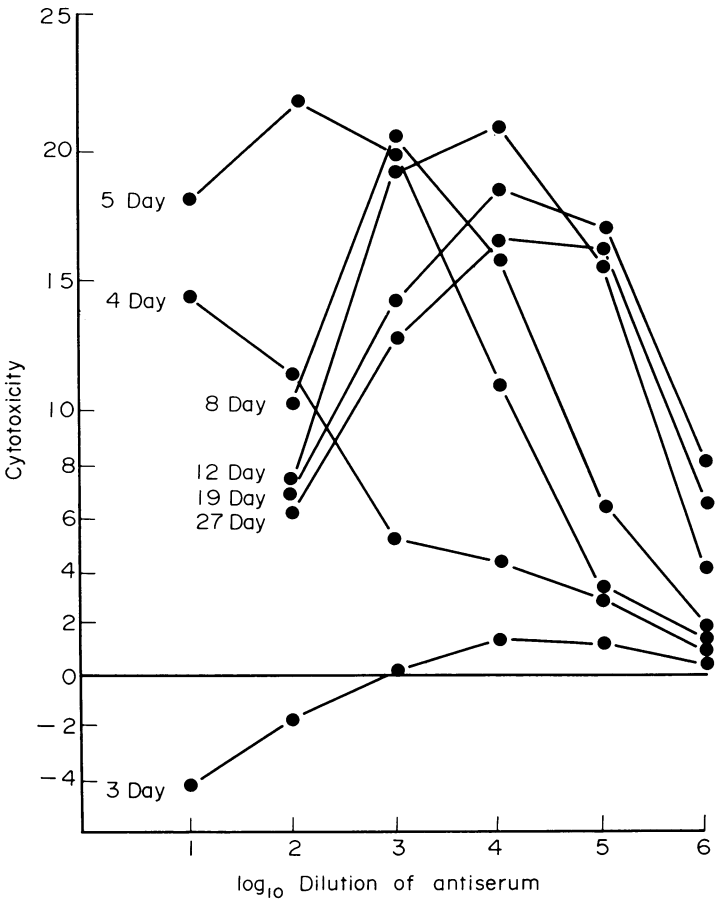

FIG. 1. The ability of antisera from rats at various days post-immunization to induce cell killing by normal spleen cells. Cultures contained 2×10^4 target cells and 3×10^6 splenic lymphocytes. Each point is the mean of results given by sera from two rats.

Distribution of antibody-producing cells

In preliminary experiments we tested thoracic duct lymphocytes and lymph from immune rats. We found that the lymph possessed sensitizing activity. When the thoracic duct lymphocytes were serially washed they showed no sensitizing activity after two washes. However, local lymph node cells retained their sensitizing activity even after five washes (the maximum number tested). From this it seems reasonable to assume that we were measuring real activity and not absorbed activity after washing the lymphoid

populations four times before testing as described in the methods. As we have never found thoracic duct lymphocytes to possess sensitizing activity after thorough washing we have not included them in this study.

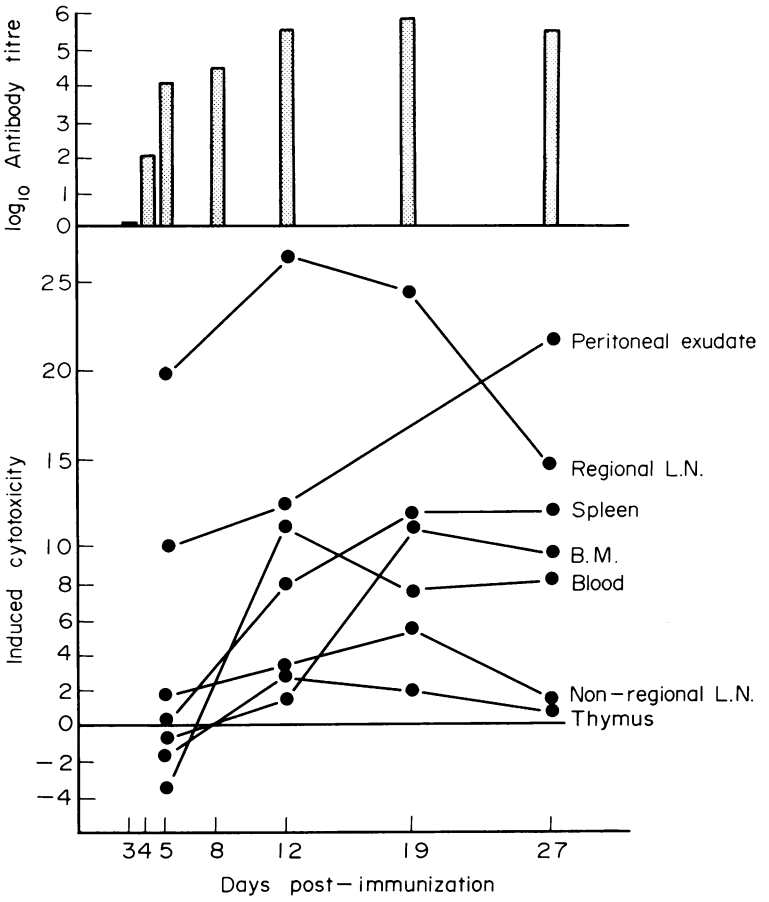

FIG. 2. The capacity of lymphoid populations from rats at various days post-immunization to induce target cell killing by normal spleen cells. This is compared with the sensitizing antibody titre. Culture conditions as Fig. 1 except that 10^6 lymphoid cells from immunized rats were added instead of antibody. Each point has been corrected for the contribution of effector cells by the immune lymphoid cells and is therefore a measure of the production of sensitizing antibody. Points are the pooled results of lymphoid populations from two rats.

The various lymphoid populations were first tested on day 5 and it can be seen from Fig. 2 that at this time only the regional lymph node cells and the peritoneal cells have appreciable antibody-producing activity. Thus as early as day 5 antibody-producing cells are found in a tissue space distant from the site of antigen injection. Although previous experiments have shown that sensitizing antibody, in the absence of antigen, is not cytophilic for lymphocytes (MacLennan *et al.*, 1969), the possibility of cytophilic antibody being carried over by the macrophages or neutrophils in the peritoneal exudate had to be excluded. Therefore peritoneal cells from normal rats were incubated with 1:4 sensitizing antibody (titre $> 1:10^5$) at 37° for 45 minutes. 1×10^6 of these peritoneal cells

failed to sensitize Chang cells to damage by splenic lymphocytes after two washes in MEM. As our immune lymphoid cells were washed four times it would seem unlikely that the activity of the peritoneal cells can be attributable to the presence of cytophilic antibody on peritoneal macrophages.

On subsequent days the other lymphoid populations are seen to develop antibody-producing activity as is shown in Fig. 2. It should be noted that thymus cells and non-regional lymph node cells never develop appreciable antibody-producing capacity. The level of activity demonstrated in thymus and non-regional lymph node cells could be produced by a contamination with 1–3 per cent peripheral blood.

Spleen cells, bone marrow cells and peripheral blood lymphocytes all develop antibody-producing activity by day 19. It is interesting that in other experiments, where the immunization was by the intra-peritoneal route, earlier and higher antibody production is seen in the spleen.

DIFFUSIBILITY OF SENSITIZING ANTIBODY

Experiments were designed to test the diffusibility of sensitizing IgG and IgM antibody through the tissues. As it is impractical to measure antibody in the tissues the model used was the diffusion of antibody from the blood into the thoracic duct lymph.

A thoracic duct cannula was inserted into a rat (Bollman, Cain and Grindley, 1948) and the lymph collected for 30 minutes to provide a zero time sample. A sample of blood was also taken at this time from an indwelling catheter in the jugular vein. A 2-ml sample of either the serum containing IgG sensitizing antibody or the purified anti-SRBC IgM was then injected through the jugular catheter. This route ensures that any leakage would not have been into the drainage area of the thoracic duct. The thoracic duct lymph was then collected over the next 2 hours. A blood sample was taken at 2 hours. The lymph was centrifuged to remove cells. Lymph and serum from zero time and two hours were tested as described under cytotoxicity test procedure.

It can be seen from Fig. 3 that both IgG and IgM appear in the thoracic duct lymph. Both fractions were found to have approximately 5 per cent of the activity found in the serum of the perfused rat. Thus it appears that IgG and IgM both diffuse from the blood into the thoracic duct at similar rates.

DISCUSSION

Bubenik *et al.* (1970) have provided some evidence to show that antibody can induce cell mediated rejection of allografts in ducks. Although this observation *in vivo* was highly suggestive of rejection by the cytotoxic action of lymphocytes towards antibody sensitized target cells the evidence was indirect. From other observations (MacLennan and Loewi, 1970; MacLennan and Harding, 1970) it is clear that effector cells can reach sites of inflammation. The present study was designed to investigate the accessibility of antibody to grafts. We have investigated the distribution of antibody-producing cells and the diffusibility of antibody.

As expected the lymph nodes draining the site of antigen injection were the first sites from which antibody-producing cells could be demonstrated. The significant antibody production by cells from the peritoneal fluid was more noteworthy. Möller and Wigzell (1965) have produced evidence which suggests that antigen is required for the production of plaque-forming cells to sheep red blood cells in the spleen. It is most unlikely that

antigen became selectively concentrated in the peritoneal cavity of our rats as there is no direct anatomical pathway described from the skin over the shoulders to the peritoneal cavity. Consequently it is probable that the antibody producing cells or their direct precursors migrated into the peritoneal cavity following contact with antigen elsewhere.

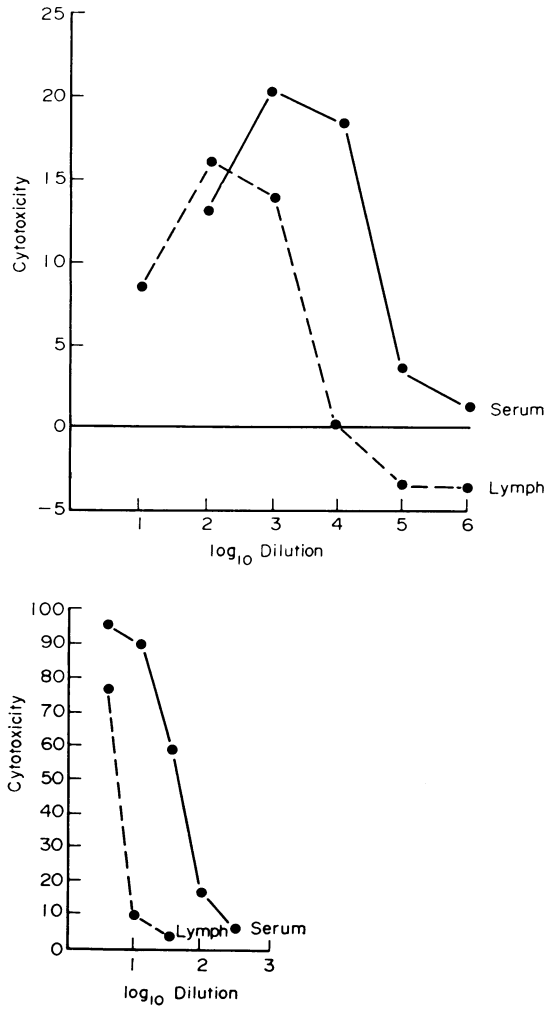

FIG. 3. The relative titres of cytotoxic antibody in the blood and thoracic duct lymph 2 hours after the passive intravenous transfer of antibody. Above: IgG with the capacity to induce lymphocyte mediated target cell (Chang cell) damage. Below: IgM with complement dependent target cell (SRBC) lytic activity.

We offer no explanation as to why there should be an apparent predilection of antibody-producing cells for the peritoneal cavity rather than for instance the spleen or non-regional lymph nodes. Investigation of this point may uncover factors concerned with the homing of antibody-producing cells.

The second set of data which we have presented deals with the diffusibility of sensitizing antibody from the circulation. We chose as our model the diffusion of antibody

from the blood into the thoracic duct lymph. Wasserman and Mayerson (1952) showed that protein in the thoracic duct lymph is wholly derived from plasma protein which initially mixes with the extravascular protein pool before being taken up by the lymph ducts. Protein injected into the blood equilibrates with the thoracic duct lymph in 7–13 hours. Thus although when we collected our lymph sample at 0–2 hours the serum to lymph ratio was 20:1, this is probably not the final equilibrated concentration. Vogel and Stoeckert (1963) in rats found that the equilibrated serum to lymph concentration of γ-globulin in the thoracic duct was 1·5:1. Mayerson, Wolfram, Shirley and Wasserman (1960) showed that dextran fractions of mean molecular weight greater than 100,000 appear in lymph in concentrations almost independent of molecular size. This they suggest may be due to the presence of large pores in the capillaries, but a process of cytopemphis may be involved. Our finding that IgG and IgM diffuse into the thoracic duct lymph at similar rates is in agreement with this earlier finding. Wasserman, Loeb and Mayerson (1955) stated that high molecular weight dextran is probably not evenly distributed throughout the extravascular space. An explanation for this is given by Mayerson *et al.* (1960) in which they produce evidence which suggests that certain capillary beds have a higher proportion of the large pores which permit the passage of macromolecules than others. Thus the concentration of IgG and IgM in the thoracic duct lymph may not necessarily be representative of the average concentration of these molecules in the extravascular space throughout the body, but only representative of the concentration in the drainage area of the thoracic duct.

From our data and from the other work cited it appears that various components required for antibody-induced lymphocyte-mediated cell damage should be able to reach extravascular target cells.

It is somewhat surprising that antibody is seldom considered in the process of graft rejection despite the fact that antibody appears capable of reaching the graft in sufficient amounts for rejection to occur. There may of course be other reasons why antibody does not participate in graft rejection. Antibody may be neutralized by soluble graft or tumour antigen. This could apply to low titre complement dependent lysis, but not to lymphocyte mediated lysis, where we have found that a serum dilution in excess of 10^5 induces target cell injury *in vitro* in the presence of effector lymphocytes (MacLennan *et al.*, 1970). A more plausible possibility is that inhibition is brought about by soluble antibody–antigen complexes. This mechanism has been shown to inhibit this sytem by the complexes competing for receptors for immunoglobulin on the cytotoxic lymphocytes (MacLennan, 1972). Another possibility is that cell antigens are covered by enhancing antibody.

Although it has often been said that antibody is not capable of inducing graft rejection it is possible that this has not been investigated under the right circumstances. Different types of graft may be rejected by different mechanisms. Thus Brunner *et al.* (1968) has reported that the damage to DBA/2 mastocytomas mediated by lymphocytes from C57 Bl mice is exclusively attributable to thymus-dependent lymphocytes and antibody is not involved. We confirmed his finding in mice, but in rats using the same target cell we found that antibody-induced lymphocyte-mediated damage is operative. So lymphocyte mediated damage of this target cell is by different mechanisms in allogeneic and xenogeneic situations. Hamilton and Gaugas (personal communication) have recently shown humoral rejection of hamster skin grafts in mice. We feel that a wider analysis of graft rejection may implicate antibody-induced lymphocyte-mediated cell damage as an effector mechanism in certain situations.

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