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COMPETITION FOR RECEPTORS FOR IMMUNOGLOBULIN ON CYTOTOXIC LYMPHOCYTES

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SUMMARY

Target cell killing by lymphocytes can be induced by appropriate antibody complexed to target cell antigens. In this paper it is shown that this form of lymphocyte mediated cytotoxicity is susceptible to inhibition by third party immune complexes which compete with target cell bound antibody for receptors for immunoglobulin on the cytotoxic lymphocytes. The physical state of the complexes is investigated in relation to their inhibitory efficiency. Evidence is presented to show that soluble complexes which exist in antigen–antibody equilibrium or slight antigen excess are the most effective inhibitors.

No evidence could be obtained to support the hypothesis that soluble immune complexes can induce indiscriminate cytotoxic activity in lymphocytes. The biological significance of this effect is discussed in relation to chronic inflammatory diseases.

INTRODUCTION

The binding of antibody to cell surface antigens may render a cell susceptible to attack by a variety of cytotoxic systems. Effectors in such systems include complement, macrophages and certain types of lymphocyte. All these systems are triggered into action by determinants on immunoglobulin which are normally only effectively exposed when antibody complexes with antigen. This paper is concerned with the interaction of antigen complexed antibody with certain cytotoxic lymphocytes. Target cell killing mediated by lymphocytes and initiated by antibody bound to target cell antigens has been demonstrated frequently *in vitro* (Möller, 1965; MacLennan & Loewi, 1968; Holm & Perlmann, 1969; MacLennan & Harding, 1970a). A recent study has also provided strong evidence that this mechanism can bring about the rejection of skin grafts (Bubeník, Perlmann & Hašek, 1970). In this study the effect of third party free immune complexes on this type of cytotoxic reaction is investigated. It is shown that such complexes successfully compete with target cell bound antibody for receptors on cytotoxic lymphocytes and consequently protect the target cells from

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attack. No evidence could be found to support the hypothesis that such complexes might induce indiscriminate cytotoxic activity in lymphocytes.

METHODS

The experimental system which has been used involves the killing of antibody sensitized target cells by effector lymphocytes. It is emphasized that the cytotoxic lymphocytes in this system have affinity for determinants on immunoglobulin which is complexed to target cell antigens and not for the target cell antigens themselves.

Target cells. The target cells used in these experiments were a continuous human cell line, Chang cells.

Animals. F_1 hybrid rats of inbred parent strains Agus/PVG were bred in this department. The parent lines were obtained from the M.R.C. Animal Breeding Unit at Carshalton.

Effector lymphocytes. These are contained in suspensions of splenic lymphoid cells. Spleens were removed from non-sensitized rats. They were then finely chopped with a scalpel blade and further dissociated by crushing with artery forceps. These cells were then suspended in Eagle's minimum essential medium (MEM) and drawn up into a syringe through a 25-gauge needle. The spleen cells were then washed once in MEM before being finally suspended in MEM with 10% foetal bovine serum (MEM10). An aliquot of this suspension was then diluted 10 times in white cell counting fluid and the concentration of splenic lymphoid cells was then taken to equal the concentration of intact mononuclear cells as assessed by phase contrast microscopy.

Cytotoxicity test procedure. The system used is a modification of that first described by Holm & Perlmann (1967). Target cell damage was assessed by the release of ^{51}Cr from target cells. They were labelled by incubation with 100 μCi ^{51}Cr sodium chromate (specific activity 3–10 μg Cr/mCi, Radiochemical Centre, Amersham). Incubation was at 37°C for 45 min. The total volume was made up to 0·5 ml with MEM. Between one and six million Chang cells were labelled at one time. After incubation the target cells were washed four times in MEM before final suspension in MEM10. Cultures were made up with 2×10^4 intact target cells and 3×10^6 splenic lymphoid cells. Sensitization of the target cells was effected by the addition of 1 : 1000 rat anti-Chang antibody. This concentration of antibody rendered the target cells susceptible to maximum damage by effector lymphocytes without making them vulnerable to damage by complement.

Cultures were of 2 ml volume. Half the volume was made up with MEM10. The other millilitre was of phosphate buffered saline (PBS), pH 7·2. Immune complexes were made up in the PBS. Incubation of cultures was for 18 hr at 37°C in a moist atmosphere of 5% CO_2 in air. At the end of incubation the reaction was stopped by cooling to 4°C in a refrigerated centrifuge while sedimenting the cells at 300 *g* for 15 min. 1 ml was then pipetted from each tube, i.e. half the released counts. The residual millilitre contained the other half of the released counts plus the bound counts. By counting the radioactivity in both tubes it was possible to calculate the per cent ^{51}Cr released. Per cent inhibition ^{51}Cr release is taken as:

$$\frac{\text{The difference between \% } ^{51}\text{Cr released from sensitized target cells with and without inhibitor}}{\text{The difference between \% } ^{51}\text{Cr released from sensitized and non-sensitized target cells}} \times 100.$$

Antigens. Human serum albumin (HSA) puris was obtained from Koch Light (batch no. 0145t). Porcine gamma-globulin was also obtained from Koch Light (batch no. 9119t). The incorporation of dinitrophenol groups into porcine gamma-globulin was performed using 2·4 dinitrobenzenesulphonate following the method described in Williams & Chase (1967). The dinitrophenylated porcine gamma-globulin (DNP PGG) possessed approximately 40 DNP residues per molecule.

Antibodies. These were obtained from rats 21 days following the intraperitoneal injection of 10^{10} heat killed pertussis organisms with 3 mg of either HSA or DNP PGG; none of the antisera used in these experiments were heat inactivated. The method of sensitization of target cells used does not render them susceptible to complement mediated lysis. Sensitizing anti-Chang antibody was obtained from F_1 rats 14 days after intraperitoneal injection of 10^8 Chang cells.

Ammonium sulphate precipitation of HSA:anti-HSA complexes

HSA was iodinated by the iodine monochloride technique. Different quantities of ^{125}I HSA were added to equal quantities of antibody. All solutions were made up in a borate buffer, pH 8·5. Incubation of antigen with antibody was overnight at 4°C. At the end of incubation an equal volume of a saturated solution of ammonium sulphate in water at 4°C was added. The mixture was left for 30 min and then centrifuged at 1500 *g* for 30 min. 1 ml of supernatant was then removed and after counting the activity in both supernatant and residue tubes total counts and bound counts were calculated.

RESULTS

The effect of HSA:anti-HSA complexes on the cytotoxic activity of splenic lymphoid cells

1. *The effect when sensitized target cells are used.* Experiments were set up where various concentrations of antigen (HSA) and antibody (anti-HSA) were added to cultures of sensitized target cells and splenic lymphoid cells. The result of adding immune complexes to these cultures was to depress the cytotoxic effect of the splenic lymphoid cells towards the target cells. Antigen alone had no effect on cytotoxicity in the concentrations used in these experiments. Antibody alone if used fresh had negligible inhibitory activity; however, some inhibitory activity appeared where stored antisera were used. Antisera heated to 56°C for 30 min were highly inhibitory. Such inhibitory activity is attributable to the IgG component of serum (MacLennan, unpublished data). The inhibitory effect of HSA:anti-HSA immune complexes occurred over a wide range of antigen and antibody concentrations. The results of one experiment are shown in Fig. 1. It will be seen that increase in antibody concentration increased inhibition. Isoantigen lines rise continuously to the highest antibody concentrations. Following the isoantibody lines shows that there is an optimal inhibitory concentration of antigen for each antibody concentration. The peak of maximum inhibition was sharpest at high antibody concentrations. When lower antibody concentrations were used the antigen concentration required to produce maximum inhibition was less critical.

2. *The effect when non-sensitized target cells are used.* In several experiments using wide ranges of antigen and antibody concentrations no evidence was found suggesting that immune complexes could induce indiscriminate cytotoxic activity in lymphocytes. DNPPGG:anti-DNPPGG complexes and heat altered rat IgG, also excellent inhibitors of

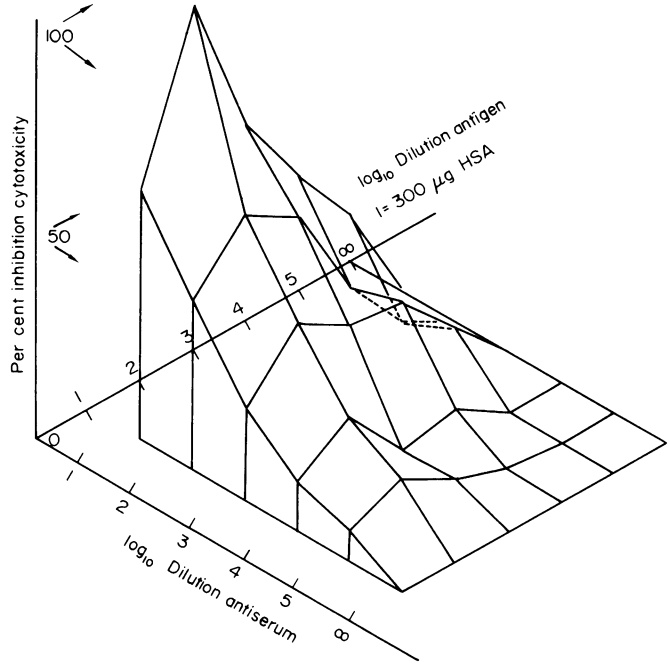

FIG. 1. Inhibition of antibody induced lymphocyte mediated cell damage by HSA:anti-HSA immune complexes. Each culture contained 2×10^4 target cells and 3×10^6 splenic lymphoid cells. All cultures were sensitized with 1:1000 anti-target cell antibody and in the absence of immune complexes target cell killing at 18 hr was reflected by 68% chromium release. Spontaneous chromium release from unsensitized target cells was 27%. Reduction of ^{51}Cr release to 27% in cultures with sensitized target cells was taken as 100% inhibition; 50% inhibition, therefore, was seen in cultures with sensitized target cells where the per cent ^{51} Cr release was 47·5.

lymphocyte killing of sensitized target cells, failed to induce indiscriminate lymphocyte mediated killing of non-sensitized target cells.

The effect of DNPPGG: anti-DNPPGG complexes on the cytotoxicity of splenic lymphoid cells towards sensitized target cells

Immune complexes of DNPPGG and anti-DNPPGG were found to have a similar inhibitory effect on the cytotoxic activity of lymphocytes towards sensitized target cells, as seen with HSA:anti-HSA. The inhibition was immunologically specific. No inhibition, above that seen with antibody only, was apparent when HSA was added to anti-DNPPGG or normal rat serum. The result of one such experiment is shown in Fig. 2.

Evidence relating to the physical state of antigen and antibody at optimal inhibitory ratios

1. *The degree of antigen binding.* The degree of inhibition of cytotoxicity was compared with the amount of antigen bound by antibody. HSA was labelled with ^{125}I and the amount of antigen bound to antibody was assessed by precipitation with ammonium sulphate. Bound but not free antigen is precipitated by half saturated ammonium sulphate at 4°C (Farr, 1958). Fig. 3 depicts an experiment where percentage inhibition of cytotoxicity is compared with percentage binding of antigen. In this experiment antigen concentration is

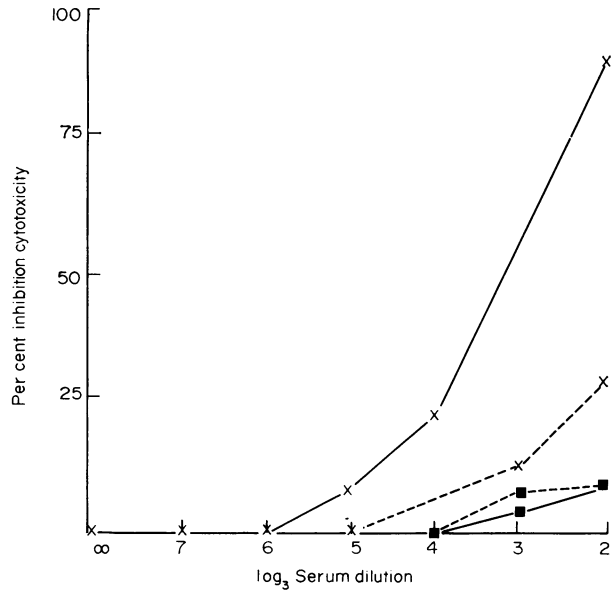

FIG. 2. The necessity for specific antibody to react with antigen in order to obtain inhibition of cytotoxicity. ×——×, anti-DNPPGG with DNPPGG; × – – ×, anti-DNPPGG only; ■——■, anti-HSA with DNPPGG; ■ – – ■, anti-HSA only. Other culture conditions as in Fig. 1. Starting concentration of DNPPGG was 30 μg/ml and of antibody 1:9; three-fold dilutions of each were made.

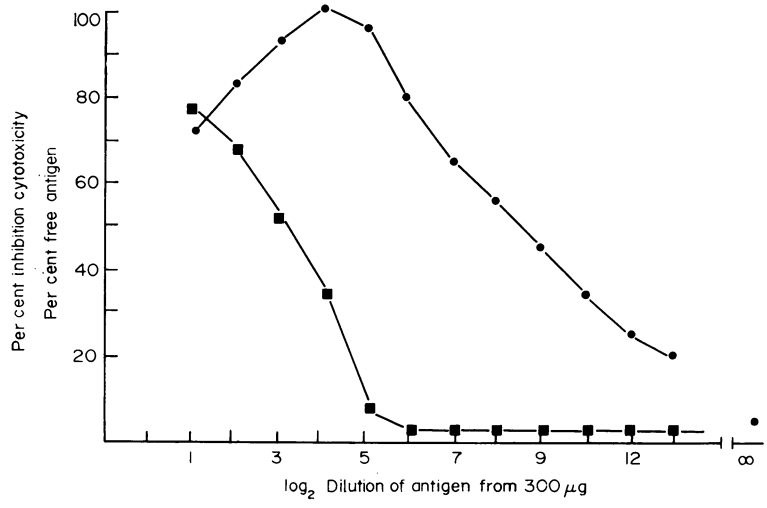

FIG. 3. The relationship of inhibition of cytotoxicity to the degree of antigen binding by antibody. All tubes have 1:10 anti-HSA. ■, Per cent of ^{125}I antigen not precipitated by half saturated ammonium sulphate; ●, per cent inhibition of cytotoxicity in relation to antigen concentration. Other culture conditions as in Fig. 1.

varied while antibody concentration is constant. It will be seen that maximum inhibition occurred at slight antigen excess. Moderate increase in antigen, however, reduced the efficiency of inhibition. These experiments were performed with fresh whole serum and no attempt was made to distinguish between antigen binding by antibody which reacts with cytotoxic lymphocytes and binding by other antibody types. Antibody capable of sensitizing target cells to lymphocyte mediated damage has been shown to be IgG (MacLennan, Loewi & Howard 1969; MacLennan, Loewi & Harding, 1970). Clearly the heterogeneity of the antibody necessitates caution in interpretating the results in these experiments.

2. *The solubility of inhibitory complexes.* Using 1:33 anti-DNPPGG and an optimum inhibitory concentration of DNPPGG the solubility of the inhibitory activity was assessed by centrifugation. The antigen and antibody were mixed in phosphate buffered saline, pH 7·2, and allowed to equilibrate for 2 hr at 20°C. Aliquots were then centrifuged at 3000 g_{max},

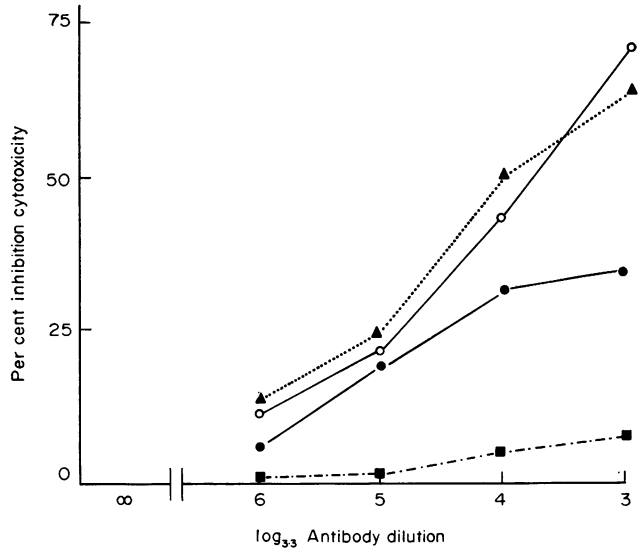

FIG. 4. The solubility of inhibitory immune complexes. The highest concentration of complex was made up from a mixture of 1:33 anti-DNPPGG and 30 μg DNPPGG, both of these are final culture concentrations. Dilutions were made of this mixture of antigen and antibody after centrifugation. ○, Uncentrifuged antigen–antibody mixture; ▲, after 20,000 g; ●, after 140,000 g_{max}; ■, antibody only. Other culture conditions as in Fig. 1.

20,000 g_{max} and 140,000 g_{max} for 90 min and the supernatants were assessed for inhibitory activity. Only the 140,000 g aliquot showed appreciable reduction in inhibition compared with the unspun tube (Fig. 4). Even after 140,000 g a considerable proportion of the activity remained in the supernatant.

DISCUSSION

The experiments described in the results show that the cytotoxic action of rat spleen cells

towards sensitized target cells is highly vulnerable to inhibition by third party soluble immune complexes. In the experiments of this study only crude spleen cell suspensions have been used as a source of effector cells. It has been implied that the cytotoxic agents are predominantly lymphocytic and it is therefore necessary to refer to the data which substantiates this suggestion. After extensive absorption of rat spleen cell suspensions by serial passage through glass bead columns the eluted suspension of cells showed only marginal decrease in cytotoxic capacity. In three experiments conducted in our laboratory each utilizing two spleens the cytotoxic activity of the eluted cells was a mean of 85% of that of the untreated population, standard deviation 14·3 (Harding, unpublished results). The absence of glass adherent cells from the eluates was checked by further culture for 12 hr on a tissue culture washed surface. Less than 1% of the original glass adherent population remained. We have previously presented positive evidence showing that highly purified lymphocytes are potent effectors in this system (MacLennan, 1970; MacLennan & Harding, 1970b). These results do not exclude the possibility that macrophages are minor effector agents in the spleen cells used in the current experiments. If they are then they can at most only represent 15% of the total cytotoxic capacity. It must be concluded that the suppression of cytotoxic activity by immune complexes seen in the experiments described in this paper is mainly the result of action on lymphocyte effectors. In a similar system, however, which uses fowl erythrocytes as target cells, both macrophages and neutrophils play a significant role in haemolysis (Perlmann & Perlmann, 1970). It is possible that cytotoxic lymphocytes have affinity for sensitizing immunoglobulin through an additional system which has affinity for both immunoglobulin and lymphocytes. Such a complicated mechanism is known to act as a link between IgM and macrophages. Macrophages appear to have little or no affinity for IgM *per se* but are able to recognize C'3 fixed as the result of interaction of complement components with the target cell bound IgM (Huber *et al.*, 1968). Such a mechanism does not appear to be of importance in the lymphocyte mediated cell damage described in this system for the following reasons. The immunoglobulin class responsible for inducing lymphocyte mediated cell damage of Chang cells is IgG (MacLennan *et al.*, 1969, 1970). Up to 1 mg/ml of IgG prepared from Chang cell sensitized rats fails to render the Chang cells susceptible to complement mediated lysis. Such IgG preparations, however, induce lymphocyte mediated cell damage to titres of between $1:10^5$ and $1:10^7$. It cannot be excluded from these results that C'1423 are being fixed without C'5–9. The following data are against this hypothesis. Lymphocyte mediated cell damage of Chang cells can be obtained equally in cultures containing fresh and heat inactivated serum, providing significant levels of heat altered IgG are avoided, v.s. If required, heat labile components of complement would have to be produced within the culture in experiments where only heated serum was added. As cytotoxicity towards sensitized target cells is not prevented by doses of puromycin which effectively suppress the production of sensitizing antibody it is hard to see how sufficient heat labile components of complement could be produced in the presence of this inhibitor of protein synthesis (MacLennan & Harding, 1970a). In addition the cytotoxic reaction is not accelerated by the addition of fresh serum to such puromycin treated cultures. Finally Perlmann *et al.* (1969) have shown that sheep red cells sensitized with anti-Forssman antibody are not damaged by purified lymphocytes and C'1423. The anti-Forssman antibody *per se* makes target cells vulnerable to complement but not lymphocyte mediated lysis.

Attempts during this study to induce indiscriminate cytotoxic activity with immune complexes failed. That the immune complexes were reacting with the cytotoxic lymphocytes, is

clear from the marked inhibition of antibody induced cytotoxic activity, which was achieved with such complexes.

Two recent publications have shown that immune complexes can initiate mitotic activity in non-sensitized lymphocytes (Bloch-Stacher, Hirschhorn & Uhr, 1968; Möller, 1969). Möller speculated that non-cell-bound immune complexes might also be capable of initiating indiscriminate cytotoxic activity in lymphocytes. As stated above, the current results do not support this hypothesis. In the present experiments extended sensitization periods were not used and it has not been excluded that increased cytotoxic activity might be seen after some days' pre-incubation with immune complexes. However, such experiments have been conducted by Stejskal & Perlmann (1971). They were only able to show specific sensitization of lymphocytes by primary incubation for 5 days with antibody and target cells. Antigenically distinct target cells were not killed as a result of this sensitization. Further studies are required to exclude sensitization simply as the result of carry over of antibody and target cell antigen complexes, a phenomenon described by this group after short-term cultures. Certainly lymphocytes show non-specific cytotoxic activity towards target cells after protracted incubation with antigen to which they had been previously sensitized (Holm & Perlmann, 1967). A similar effect is seen after lymphocyte stimulation with allogeneic lymphoid cells (Holm & Perlmann, 1967; Hardy *et al.* 1970). On the other hand, the association of blast transformation with cytotoxic activity when phytohaemagglutinin is the stimulant has been shown to be only superficial. Holm (1967) showed that DNA synthesis and protein synthesis could be blocked by chemical agents without seriously affecting phytohaemagglutinin induced cytotoxicity. Recent population studies in this laboratory have shown that the population of lymphocytes which is responsible for killing antibody sensitized target cells is distinct from that which responds mitotically to phytohaemagglutinin (MacLennan & Harding, 1970b; Harding *et al.*, 1971). In addition Briton, Perlmann & Perlmann (personal communication) have shown that phytohaemagglutinin induced cytotoxicity and blast transformation in the mouse are the properties of different populations of lymphocytes.

Inhibition of antibody induced lymphocyte mediated cytotoxicity by immune complexes is potentially of clinical interest. The presence of circulating immune complexes in a number of chronic inflammatory diseases is well recognized (Baumal and Broder, 1968). This may be associated with reduced complement levels (Hedberg, Lundh & Laurell, 1970). In this laboratory we have examined the serum of patients with inflammatory disease of the bowel and that of patients with rheumatoid arthritis for activity which suppressed antibody induced lymphocyte mediated cell damage. Activity which markedly suppresses the cytotoxic effect of human lymphocytes towards allogeneic sensitized target cells was found in some but by no means all patients (Jewell, Barnett & MacLennan, unpublished results). Preliminary chemical analysis is consistent with this activity being associated with immune complexes. In the presence of high concentrations of appropriate immune complexes it is hard to see how antibody dependent cytotoxic activity mediated by lymphocytes is not grossly impaired. Such paralysis of effector capacity, which might be paralleled with inhibition of phagocytosis, could be a significant factor in the chronicity of inflammation in these diseases.

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