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The Role of Immunoglobulins in Lymphocyte-Mediated Cell Damage, *in vitro*

II. THE MECHANISM OF TARGET CELL DAMAGE BY LYMPHOID CELLS FROM IMMUNIZED RATS

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Summary. Lymph node cells from Black Hooded (BH) rats immunized with Freund's complete adjuvant (CFA) are only poorly cytotoxic to target cells treated with target cell specific antibody. Normal spleen cells are highly cytotoxic to antibody coated target cells. When 10^6 lymph node cells, from rats immunized with Chang cells in CFA, are mixed with 10^7 spleen cells from unimmunized rats, the mixture is 8·6 times more cytotoxic than the sum of the cytotoxicities of the two lymphoid cell populations alone. Immune lymph node cells were no more cytotoxic in the presence of 5 per cent fresh guinea-pig serum than in the presence of heat inactivated guinea-pig serum. Immune spleen cells are prevented from damaging target cells by antagonists of protein synthesis such as Puromycin. Puromycin, however, does not alter the cytotoxic effect of either immune or non-immune spleen cells on antibody coated target cells. From these experiments it is tentatively suggested that lymphoid cells from immunized animals mediate target cell damage by a reaction involving firstly immunologically specific responder cells which synthesise antibody and secondly non-specific effector cells which damage the antibody coated target cells. Evidence is presented which suggests that stable soluble factors may not be responsible for mediating target cell damage in this system.

INTRODUCTION

Target cell damage *in vitro* by lymphoid cells from the spleen, peripheral blood and lymph nodes of specifically immunized animals has repeatedly been shown to be significantly greater than that produced by lymphoid cells from unimmunized controls (Rosenau and Moon, 1964; Brunner, Mauel, Cerottini and Chapuis, 1968; MacLennan and Loewi, 1968). It has also been shown that lymphoid cells, especially those from the spleen and peripheral blood, are capable of severely damaging target cells treated with target cell specific antibody (Möller, 1965). This phenomenon has been described in detail in the preceding paper. MacLennan, Loewi and Howard (1969) have shown that in humans this type of antibody separates with IgG and that it is unable, *per se*, to cause target cell damage even in the presence of complement.

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The present paper describes experiments which provide evidence to support the hypothesis that target cell damage by lymphoid cells from immunized animals requires firstly the participation of specifically acting antibody producing cells, and secondly the action of non-immune effector cells which damage the target cell once it is complexed with antibody. Evidence is also provided which suggests that target cell damage in this system may not be mediated by soluble factors.

METHODS

Target cells

Both L strain fibroblasts and Chang cells were obtained from Flow Laboratories. The method of culture and preparation of single cell suspensions has been described in the preceding paper.

Animals

Adult Black Hooded (BH) rats inbred for more than thirty-five generations were obtained from Animals Supplies Ltd. Animals used were between 3 and 6 months of age.

Immunization

(a) *For lymph node cells.* BH rats were immunized with 10^7 Chang cells in Freund's complete adjuvant (CFA) given as multiple intradermal injections in the back. These animals were killed at 6–7 days and the lymph nodes from the inguinal and scapular regions were removed.

(b) *For spleen cells.* BH rats were injected intraperitoneally with 10^8 Chang cells. Spleens were harvested at 2–4 weeks.

Preparation of spleen cells and lymph node cells

These were removed aseptically from bled out rats. They were cut finely with scalpel blades and the resulting pieces were crushed with artery forceps. The cells were suspended in Parker 199 (Glaxo) with 10 per cent foetal bovine serum (Flow batch 40077)(PFB 10), filtered through loosely packed gauze and washed twice in PFB 10. They were enumerated in white cell counting fluid under phase contrast microscopy in a haemocytometer.

Cytotoxicity test procedure

This was carried out as described in the preceding paper.

Cytotoxicity

This is defined as the percentage ^{51}Cr release without lymphocytes subtracted from the percentage ^{51}Cr release with lymphocytes.

Puromycin was obtained from Nutritional Biochemicals Corporation.

RESULTS

THE CYTOTOXIC EFFECT OF IMMUNE AND NON-IMMUNE SPLEEN AND LYMPH NODE CELLS ON CHANG CELLS

Spleen cells from BH rats, taken 4 weeks after immunization by intraperitoneal injection of 10^8 Chang cells, showed a mean cytotoxicity of 29 (twenty-two experiments),

SD 9·25. Control spleen cells showed a mean cytotoxicity of 9·4 (twenty experiments), SD 4·5. BH rat lymph node cells, harvested 6 days after multiple intradermal injection of 10^7 Chang cells in CFA, showed a mean cytotoxicity of 14·5 (eight experiments), SD 6·1. Control lymph node cells from animals immunized with CFA only showed a mean cytotoxicity of 2·3 (eight experiments), SD 1·6.

COMPARISON OF THE CYTOTOXIC EFFECTOR CAPACITY OF NORMAL SPLEEN CELLS AND LYMPH NODE CELLS FROM CFA IMMUNIZED RATS

Anti-Chang antibody from BH rats immunized 3 weeks previously by intraperitoneal injection of 10^8 Chang cells was added to a dilution of 1 : 1000 to cultures of 10^7 lymphoid cells and 10^5 Chang cells. Cultures containing spleen cells showed an increase in cytotoxicity, on addition of antibody, to 22·4 from 8·6 (mean six rats). Cultures containing lymph node cells from rats immunized 6 days previously with CFA showed a basic mean cytotoxicity of 2·4 which was raised to 7·4 (six experiments), on addition of antibody.

From this observation it would appear that lymph node cells from our animals immunized with CFA contain relatively few cells which are capable of effecting damage to Chang cells. The spleen, however, appears to be relatively rich in such cells.

INDUCTION OF CHANG CELL DAMAGE BY NON-IMMUNE SPLEEN CELLS WITH IMMUNE LYMPH NODE CELLS WHICH ARE NOT *per se* CYTOTOXIC

It was considered possible that immune lymph node cells were only poorly cytotoxic to Chang cells because they lacked non-specific effector cells. To test this, 10^6 immune lymph node cells were mixed with 10^7 normal spleen cells, a population rich in effector cells. The immune lymph node cells by themselves were essentially non-cytotoxic to Chang cells. Normal spleen cells were only slightly cytotoxic to Chang cells. In combination these lymphoid cells were 8·6 times as cytotoxic (mean of three experiments, lymphoid cells pooled from three animals in each experiment) as the sum of the cytotoxicity of the two populations individually ($P<0·001$) (see Fig. 1). This result would appear to confirm the hypothesis that immune lymphoid cells are not cytotoxic *per se* but make target cells susceptible to damage by non-immune effector lymphocytes.

FAILURE OF COMPLEMENT TO REPLACE NORMAL SPLEEN CELLS AS AN EFFECTOR AGENT IN CHANG CELL DAMAGE INDUCED BY IMMUNE LYMPH NODE CELLS

One possible explanation of the above experimental findings is that the immune lymphoid cells produced antibody capable of lysing Chang cells in the presence of complement and that the normal spleen cells acted by synthesizing complement components. To test this, 10^6 immune lymph node cells were incubated with Chang cells in the presence of 5 per cent fresh guinea-pig serum. Chang cells were not damaged in these cultures (see Fig. 2). In control cultures with 5 per cent BH rat anti-Chang antibody and 5 per cent fresh guinea-pig serum the Chang cells were completely destroyed.

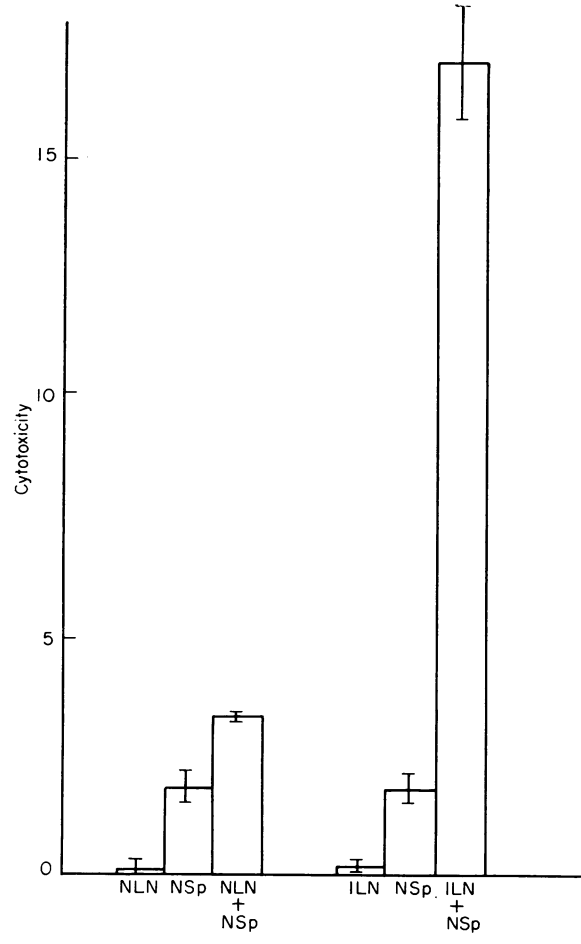

FIG. 1. The effect of 10^6 normal and 10^6 immune lymph node cells on the cytotoxicity of 10^7 normal spleen cells towards 10^5 Chang cells. NLN = 10^6 lymph node cells from BH rats immunized with CFA only; NSp = 10^7 spleen cells from unimmunized BH rats; ILN = 10^6 lymph node cells from BH rats immunized with CFA and Chang cells.

INHIBITION OF IMMUNE SPLEEN CELL DAMAGE TO CHANG CELLS BY AN ANTAGONIST OF PROTEIN SYNTHESIS. FAILURE OF THIS AGENT TO INFLUENCE LYMPHOCYTE-MEDIATED CELL DAMAGE INDUCED BY TARGET CELL SPECIFIC ANTIBODY

Puromycin has been shown to block protein synthesis by preventing the formation of polypeptides at the ribosome (Simpson, 1962). As such, it has been shown to block the production of antibody in that it inhibits the formation of Jerne plaques (Strander, 1966). Holm (1967) has shown that puromycin fails to inhibit phytohaemagglutinin-induced cytotoxicity by human peripheral blood lymphocytes towards Chang cells. Brunner *et al.* (1968) showed that cyclohexamide, which also interferes with protein synthesis, inhibited the cytotoxic action of immune BALB/c mouse spleen cells towards DBA_2 monocytoma cells. These findings were confirmed in the following experiment. Immune BH rat spleen cells were inhibited from damaging Chang cells by the presence of 10 μg/ml of puromycin

(see Fig. 3). Cytotoxicity at 5 hours was reduced by 62 per cent (mean of three experiments), $P<0{\cdot}001$. If the immune spleen cells were incubated with 1 : 1000 BH rat anti-Chang antiserum, the cytotoxic effect on Chang cells was greater than in cultures with immune spleen cells only. The addition of10 μg/ml of Puromycin to cultures of immune spleen cells plus antibody did not significantly alter the cytotoxic effect on Chang cells. Puromycin at 10 μg/ml did not affect the spontaneous release of ^{51}Cr from Chang cells.

From this experiment it would appear that although the effector stage of Chang cell damage by lymphoid cells is not dependent upon protein synthesis, the specific action of immune spleen cells is. These findings, therefore, are consistent with, but are not conclusive in proving, the hypothesis that immune spleen cells act primarily by producing antibody which sensitizes Chang cells to damage by non-specific effector cells.

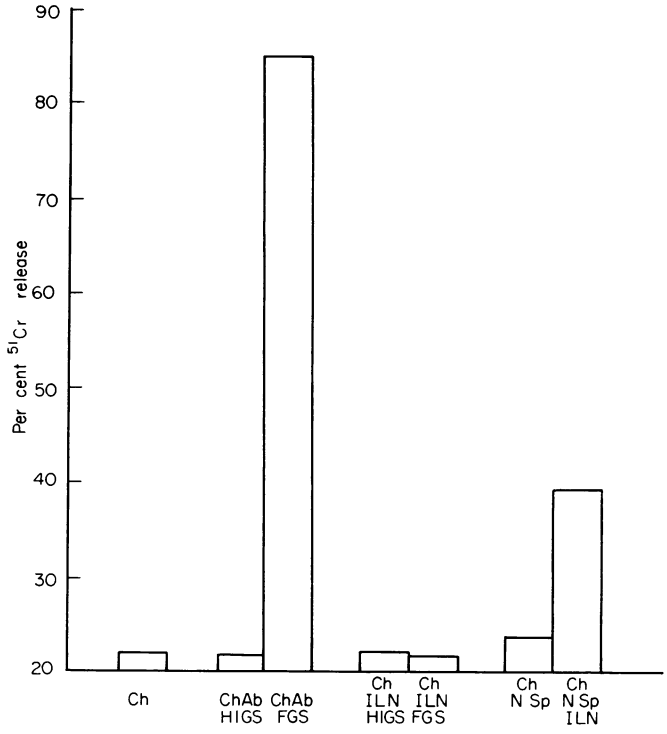

FIG. 2. The inability of complement to replace normal spleen cells as an effector agent in Chang cell damage induced by immune lymph node cells. Ch = 10^5 ^{51}Cr-labelled Chang cells; ChAb = 10^5 ^{51}Cr-labelled Chang cells in 5 per cent BH rat anti-Chang serum; ILN = 10^6 lymph node cells from BH rats immunized with CFA and Chang cells; NSp = 10^7 spleen cells from unimmunized BH rats; FGS = 5 per cent fresh guinea-pig serum; HIGS = 5 per cent guinea-pig serum heated for 45 minutes at 56°.

THE MECHANISM OF CHANG CELL DAMAGE PRODUCED BY NON-IMMUNE EFFECTOR CELLS

Granger and Kolb (1968) have published reports describing the presence of a cytotoxic factor in supernatants of cultures of lymphoid cells and target cells treated with target cell specific antibody. Their assay of cytotoxicity depended upon measuring failure of incorporation of labelled amino acids into target cells. They compared this with the uptake of

label in control cultures. In order to investigate the possibility that such a soluble cytotoxic factor might be important in producing target cell damage in our system, the following experiment was performed.

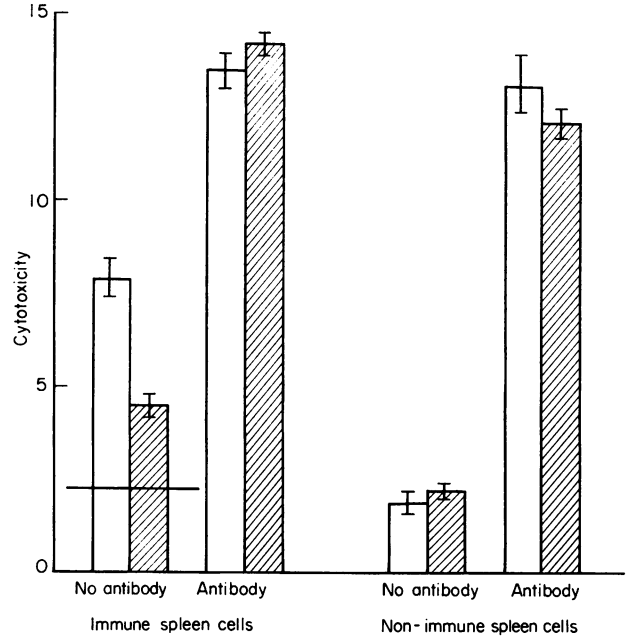

FIG. 3. The effect of Puromycin on lymphocyte mediated cell damage. The horizontal line in immune spleen cell cytotoxicity without antibody is drawn at the level of cytotoxicity of pooled normal spleen cells without antibody. Hatched columns represent cultures with 10 μg/ml Puromycin. Cultures were incubated for 5 hours only. There was no pre-incubation of cells with Puromycin or antibody; these agents were only present during the 5 hours of culture.

Cultures were set up with 10^7 non-immune spleen cells and 10^5 unlabelled Chang cells in the presence of 1 : 1000 BH rat anti-Chang antiserum and 10^5 ^{51}Cr labelled L strain C_3H mouse fibroblasts. The ^{51}Cr release from the L cells at 24 hours was the same in cultures with and without antibody, four out of four experiments. When the Chang cells were labelled with ^{51}Cr the cytotoxicity in cultures with anti-Chang antibody was three to four times greater than in those without antibody. If the excess Chang cell damage demonstrated in the presence of anti-Chang antibody had been produced by a soluble factor such as that described by Granger, one would have expected to detect increased release of ^{51}Cr from the L cells. L cells cultured with BH rat spleen cells are susceptible to increased damage when BH rat anti-L cell serum is added.

DISCUSSION

Combining the findings of experiments in this paper and those in the previous paper, it is possible tentatively to suggest a pathway by which *in vitro* damage by lymphoid cells from specifically immunized rats towards Chang cells occurs (see Fig. 4).

Firstly, antigenic sites on Chang cells evoke antibody production by sensitized lymphocytes. This antibody will then combine with the antigenic sites on the target cell, rendering the target cells susceptible to damage by non-specific effector cells.

Theoretically specific antibody, which does not react with non-specific effector cells, could protect the target cells from damage by both masking their antigens and by saturating the sites to which antibody, effective at evoking non-specific lymphocyte damage, could become attached. We have failed to find convincing evidence for such specific enhancing antibody in our system, but Brunner and his co-workers have described such a factor in serum from BALB/c mice which had been immunized with DBA_2 mastocytoma target cells. The evidence which has been presented is derived entirely from *in vitro* assay of cytotoxicity and this must be borne in mind before discussing the possible *in vivo* implications of these findings. If these mechanisms do apply *in vivo*, then it would appear that a substantial proportion of lymphoid cells are not specific immunological units responding to antigens but are non-specific effector cells. This would appear to fit in well with

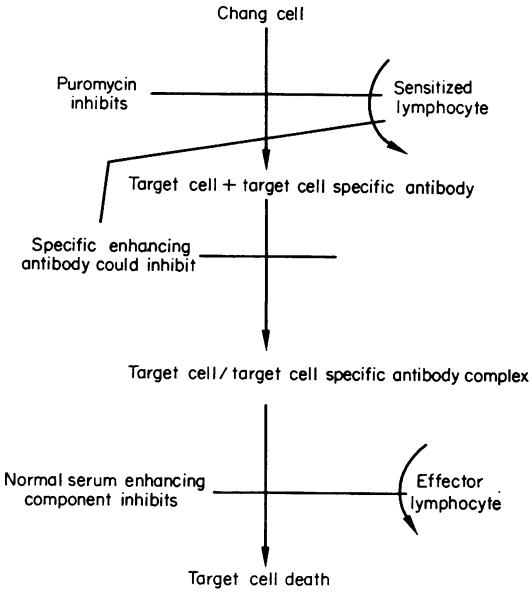

FIG. 4. Proposed pathway by which Chang cells may be damaged by lymphoid cells from immunized animals.

the observations of Najarian and Feldman (1963) and Turk and Oort (1963) who transferred delayed hypersensitivity with labelled lymphoid cells. They showed that the proportion of transferred cells playing an active part in delayed hypersensitivity in recipients was less than 1 per cent of the mononuclear population in the lesion. One possible explanation of these findings is that immunological information had been conveyed to host cells. On the other hand, a very few antibody-producing cells could by the mechanisms described in this paper initiate a delayed hypersensitivity lesion, for the process is extremely economical in its antibody requirements. Ward, Remold and David (1969) have shown that lymphoid cells from immunized animals produce soluble factors in the presence of antigen, which are chemotactic to macrophages. A number of workers including Dumonde *et al.* (1969) have shown that in similar circumstances a factor is produced which initiates lymphocyte division. Clearly these factors would tend to dilute out further the few cells which initiated the lesion by specific reaction with antigen.

It is clear from the work of Granger and his co-workers that non-immune lymphocytes produce, after several hours, a soluble cytostatic agent, when they are incubated with antibody coated target cells. Ruddle and Waksman (1968) have described the appearance of similar activity in the supernatants of cultures of rat lymph node lymphocytes incubated in the presence of a soluble antigen to which they had been sensitized. This cytostatic activity was only evident after 17 hours. The evidence presented in the last experiments described in this paper suggests that the factor or factors responsible for these effects may not be the agent responsible for lymphocyte mediated cell damage in our experiments.

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