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Cytotoxic Effects *In Vitro* by Lymphoid Cells from Specifically Tolerant Animals

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Summary. Mice of strain A were made tolerant to cells from (A × CBA)F_1 mice by neonatal injection of spleen cells. Lymphoid cells from tolerant animals carrying (A × CBA)F_1 skin grafts for 1–6 months were competent to cause destruction of both (A × CBA)F_1 and CBA fibroblast target cells in tissue culture in the presence of PHA. Normal A lymphoid cells were cytotoxic to both targets, whereas (A × CBA)F_1 lymphocytes did not kill the syngeneic F_1 targets, but were effective against the CBA fibroblasts. Lymphocytes from H-2 incompatible strains were cytotoxic to both target cell genotypes. These experiments demonstrate that the A lymphocytes in animals tolerant to (A × CBA)F_1 were effective in causing destruction of the target cells, indicating that PHA-induced cytotoxicity by incompatible cells is independent of specific immunological recognition processes.

INTRODUCTION

Normal mouse lymphoid cells have the capacity to kill histo-incompatible normal and neoplastic target cells *in vitro* in the presence of certain substances, such as heterologous antiserum or phytohaemagglutinin (PHA) (Holm, Perlmann and Werner, 1964; Holm and Perlmann, 1965; Möller, 1965). No effect is obtained on syngeneic mouse target cells in parallel experiments (Holm and Perlmann, 1965; Möller, 1965). PHA is a necessary prerequisite for expression of cytoxicity. Cytotoxicity is most likely caused by a non-immunological reaction, since F_1 hybrid lymphoid cells are capable of killing parental type target cells, in spite of the fact that F_1 cells cannot react immunologically against the parental target cells (Möller, 1965). Further characteristics of the reaction have been summarized in several recent publications (Möller, 1967; Möller and Möller, 1966, 1967). Although the mechanism of cytotoxicity is unknown as yet, it seems established that it is initiated by target cell contact with foreign histocompatibility antigens carried by the lymphocytes.

A different mechanism of cytotoxicity appears to operate in human systems. PHA is known to cause morphological transformation of human lymphocytes *in vitro*, and also to stimulate their protein, RNA and DNA synthesis (Nowell, 1960; Sell, Rowe and Gell, 1965). Human lymphocytes are not cytotoxic by themselves, but after stimulation by, e.g. PHA they exert cytotoxicity on allogeneic (Holm and Perlmann, 1967) and autochthonous target cells as well (Möller and Lundgren, 1969). Various other non-specific stimulants, such as anti-lymphocytic serum and streptolysin are also competent to transform the lymphocytes into a cytotoxically active stage. Furthermore, contact between lymphocytes from immunized individuals and the specific antigen, and also contact between allogeneic lymphocytes triggers lymphocyte cytotoxicity (Lundgren, Collste and Möller, 1969).

Although triggering of cytotoxicity is usually accompanied by morphological transformation and increased DNA, RNA and protein synthesis in the lymphocytes, the latter events can be completely suppressed without affecting cytotoxicity (Lundgren *et al.*, 1969).

The mixed lymphocyte culture response, which triggers cytotoxicity by human lymphocytes, has been found to be absent in animal systems if the lymphocytes are taken from animals made specifically tolerant to the allogeneic partner in the culture (Wilson, Silvers and Nowell, 1967). Thus, the initiating event in this lymphocyte stimulation appears to be related to immunological recognition.

As an attempt to study further the possible relationship of cytotoxicity to immunological recognition by normal lymphoid cells, experiments were performed with lymphocytes derived from animals made specifically tolerant to the target cells.

MATERIALS AND METHODS

Mice

The following inbred strains were used in the experiments: A/Sn (H-2^a), CBA (H-2^k), A.CA (H-2^f), A.SW (H-2^s) and the (A × CBA) (H-2^a) × (H-2^k) F_1 hybrid strains.

Tissue culture technique

Mouse embryo fibroblast cultures were initiated from embryos at the 19th to 21st day of gestation. The head and viscera of the embryos were removed. The rest of the foetuses was cut into small pieces with a pair of scissors. A 0·25 per cent trypsin solution was added to the pieces and was incubated at 37° while being stirred for 45 minutes. The supernatant containing free cells was removed and the cells were washed three times with BSS. The cells were cultivated in milk dilution bottles in a medium consisting of 15 per cent foetal calf serum (inactivated at 56° for 1·5 hours) in Eagle's solution in Earle's medium. After the first or second passage the cells were transferred to plastic Petri dishes (Nunc A/S, Roskilde, Denmark) and cultivated in 5 per cent CO_2 in air at 37°. When a scant monolayer without distinct orientation of the fibroblasts had developed, two different types of lymphoid cell suspensions were added to local pre-indicated regions of each plate in amounts of 0·1 ml containing $1–2 \times 10^7$ Trypan blue resistant cells. Prior to addition of the lymphocytes the medium was supplemented with PHA in a total concentration of 1 : 100 as described earlier (Möller and Möller, 1965).

Lymphoid cells

These were obtained from lymph nodes and spleens, by pressing the organs through a metal sieve into BSS salt solution. The cell suspensions were washed twice and thereafter diluted to a concentration of $1–2 \times 10^8$ Trypan blue unstained cells/ml.

Areas of cytotoxicity were scored after 48 hours by both macroscopic and microscopic examination. Macroscopically visible complete destruction of the monolayer at the site of lymphoid cell application was scored as ++, a clear microscopic target cell destruction as +. Doubtful or negative reactions were designated ± and −, respectively. Each experiment was read independently by at least two persons. Plaques giving a + score or more were considered positive. The number of positive sites was calculated for each lymphoid cell type in each experiment.

Immunological tolerance was induced in 12–48 hours old A/Sn mice by the intravenous injection of 20×10^6 normal (A × CBA)F_1 hybrid spleen cells. At 30 days of age the recipients were grafted with skin from (A × CBA)F_1 hybrid animals. Lymphoid cells

harvested from animals carrying a healthy skin graft 1–6 months after grafting were used in the experiments.

RESULTS

Mice of the A/Sn strain which are made tolerant to the (A×CBA)F_1 hybrid genotype are also tolerant to cells of the CBA strain, since all histocompatibility antigens are co-dominantly expressed (Goodman, 1965). Furthermore, animals made tolerant by lymphoid cell inoculation are chimeras, and therefore contain lymphoid cells of both their own and the donor genotype. According to the concept of contact-induced cytotoxicity outlined above spleen cells from the tolerant animals, containing both A and (A×CBA)F_1 lymphoid cells, would cause destruction of CBA target cells, since both the A and the (A×CBA)F_1 hybrid cells contain isoantigens, which are absent from the CBA targets.

However, if cells from the tolerant animals are added to (A×CBA)F_1 hybrid monolayer cells, the F_1 hybrid cells present in the tolerant animals cannot cause destruction, since they are syngeneic with the target cells. The host lymphocytes in the tolerant animals are specifically unresponsive to CBA antigens and, therefore, cannot react immunologically against targets of this genotype or the F_1 hybrid. It has been demonstrated earlier, however, that normal parental lymphoid cells kill semi-syngeneic F_1 hybrid target cells in the presence of PHA. Therefore, it was tested whether strain A parental lymphoid cells, which were made immunologically tolerant to CBA isoantigens would be capable of causing destruction of CBA and (A×CBA)F_1 hybrid target cells.

Lymphoid cells were derived from strain A animals tolerant to (A×CBA)F_1 cells, as well as from normal A, CBA and (A×CBA)F_1 hybrid animals. The lymphoid cells were tested for their ability to cause destruction of CBA and (A×CBA)F_1 hybrid fibroblast embryo cells in tissue culture in the presence of PHA. As additional controls, lymphoid cells from the H-2 incompatible strains A.CA and A.SW were prepared and tested on the same target cells.

The results of ten different experiments are given in Tables 1 and 2. It was found that lymphoid cells from normal A, (A×CBA)F_1, A.CA and A.SW mice were all capable of killing CBA target fibroblasts in the presence of PHA, whereas syngeneic CBA cells did not cause any visible destruction. As expected, lymphoid cells from the tolerant A mice were as efficient as normal A and (A×CBA)F_1 cells to cause target cell destruction of the CBA fibroblasts.

Analogous experiments were carried out on (A×CBA)F_1 target fibroblasts. Syngeneic normal F_1 hybrid cells were not capable of causing significant destruction of the F_1 hybrid

TABLE 1

CYTOTOXICITY OF NORMAL LYMPHOID CELLS DERIVED FROM VARIOUS STRAINS ON CBA EMBRYO FIBROBLAST CELLS *in vitro* IN THE PRESENCE OF PHA

Lymphoid cells from strain:	No. of plaques in experiment				Total
	No. 1	No. 2	No. 3	No. 4	
A	1/4	3/3	3/4	3/4	10/14
CBA	0/4	0/3	0/3	0/3	0/13
(A×CBA)F_1	4/4	2/3	5/5	3/3	14/15
A tol. (A×CBA)F_1		3/4	3/4	3/4	9/12
A.SW	3/5			4/4	7/9
A.CA		2/3	4/4		6/7

The total number of 'plaques', indicating cytotoxicity is given for each experiment.

TABLE 2

CYTOTOXICITY OF NORMAL LYMPHOID CELLS DERIVED FROM VARIOUS STRAINS ON (A × CBA)F_1 HYBRID EMBRYO FIBROBLAST CELLS *in vitro* IN THE PRESENCE OF PHA

Lymphoid cells derived from strains:	No. of plaques in experiment						Total
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	
A	3/4	2/3	4/4	3/3	4/4	0/4	16/22
CBA	3/3	4/4	4/4	3/3	4/4	4/4	22/22
(A × CBA)F_1	2/5	0/5	2/4	1/3	0/3	0/4	5/24
A tol. (A × CBA)F_1	5/5	2/3	3/4	3/4	3/3	4/4	20/23
A.SW				3/3	3/3	4/4	10/10
A.CA	3/3	3/3	4/4				10/10

The total number of 'plaques', indicating cytotoxicity is given for each experiment.

target cells, whereas the allogeneic lymphocytes from strain A.SW and A.CA were strongly cytotoxic. Lymphocytes from normal A mice as well as from A mice tolerant to (A × CBA)F_1 cells were fully competent to inflict damage on the F_1 target cells *in vitro*, in spite of the fact that the tolerant animals, from which the lymphoid cells were derived, carried healthy (A × CBA)F_1 skin grafts for 1–6 months. Since normal (A × CBA)F_1 lymphoid cells did not cause destruction of the (A × CBA)F_1 targets, it follows that the cytotoxic effect on these targets obtained with lymphoid cells from the tolerant strain A animals must be caused by host lymphoid cells, which are tolerant to the target cells and incapable of rejecting skin grafts from these animals. These findings support the previous conclusions that contact-induced cytotoxicity as demonstrated with histo-incompatible cells *in vitro*, is caused by target cell contact with lymphoid cells carrying foreign histocompatibility antigens, and that it is independent of the ability of immunological reactivity by the lymphoid cells.

DISCUSSION

The 'allogeneic inhibition' concept is defined as the inhibition of growth of parental strain tumours in semi-syngeneic F_1 hybrids as compared to their growth in the syngeneic parental strain (Hellström, 1963; Hellström and Hellström, 1967). This phenomenon is considered to be of non-immunological nature as outlined elsewhere. Most likely it is caused by tumour cell confrontation with foreign isoantigens in the recipients. The finding that non-sensitized lymphocytes could cause cytotoxicity on allogeneic but not syngeneic target cells in the presence of PHA (Möller, 1965) was interpreted as an expression of the allogeneic inhibition phenomenon *in vitro*. This conclusion was based on the finding that 'genetically tolerant' F_1 hybrid cells were cytotoxic to parental targets. Furthermore, it was demonstrated that blocking of the foreign isoantigens on the lymphocytes by isoantibody suppressed cytotoxicity (Möller, 1967), thus supporting the concept that cytotoxicity was related to target cell confrontation with foreign antigens on the lymphoid cells.

It has been clearly established that the mechanism of cytotoxicity discussed above does not represent the only pathway of cytotoxicity following lymphocyte–target cell interaction *in vitro*. In other species (human, rats and chickens) lymphocytes which have been stimulated in various ways, are cytotoxic both to allogeneic and syngeneic (or autochthonous) target cells (Ruddle and Waksman, 1967; Perlmann, Perlmann and Holm, 1968; Möller and Lundgren, 1969). Lymphocyte stimulation could be achieved by certain

substances such as PHA, by contact between sensitized lymphocytes and the corresponding antigen or by lymphocyte confrontation with antigen–antibody complexes or with allogeneic lymphocytes (Möller and Lundgren, 1969). These observations indicate the existence of at least two reactions leading to target cell death. One is represented by human lymphocytes that have been stimulated by one of several substances, and as a consequence become non-specifically cytotoxic to allogeneic and autochthonous target cells as well. Another pathway of cytotoxicity (allogeneic inhibition) appears to be largely independent of lymphocyte stimulation, and is only mediated by incompatible lymphoid cells. This conclusion is supported by studies by Ax, Malchow, Zeiss and Fischer (1969). With time-lapse microcinematography they have been able to show that PHA-induced cytotoxicity by normal incompatible mouse lymphoid cells is achieved without any morphological or functional signs of lymphocyte stimulation.

We have also shown that F_1 hybrid mice made immunologically incompetent by adult thymectomy followed by whole body irradiation and repopulation with syngeneic bone marrow cells, are still competent to cause allogeneic inhibition of a transplanted parental strain tumour (Möller, Lapp and Lindholm, 1969). These data indicate that allogeneic inhibition, exhibited *in vivo* or *in vitro*, is a reaction which is independent of immunological recognition processes.

Wilson *et al.* (1967) have studied whether lymphoid cells from immunologically incompetent animals were susceptible to stimulation by allogeneic lymphocytes *in vitro*. They found that lymphoid cells from adult rats made tolerant by the inoculation of allogeneic cells at birth were unable to respond in a mixed culture *in vitro* against lymphoid cells from the strain used to induce tolerance. Recently, Schwarz (1968) has confirmed the findings of Wilson *et al.* (1967) in a critical study. It is obvious, therefore, that the recognition process involved in mixed lymphocyte cultures is different from that operating in the cytotoxic effects described above. Furthermore, cytotoxicity by 'tolerant' lymphocytes does not appear to be efficient against allogeneic skin grafts. So far, the allogeneic inhibition phenomenon has been demonstrated only with suspended neoplastic or lymphoid cells *in vivo* and not with skin or organ grafts, and the phenomenon may be of limited importance as an effector mechanism in graft rejection in these cases. However, as pointed out before, the phenomenon may have a normal function as a surveillance mechanism by eliminating arising neoplastic cells by direct cell contact.

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REFERENCES

Ax, W., Malchow, H., Zeiss, I. and Fischer, H. (1969). 'The behaviour of lymphocytes in the process of target cell destruction *in vitro*.' *Exp. Cell Res.* (In press).

Goodman, J. W. (1965). 'A search for parental antigens not expressed in F_1 hybrid mice.' *Transplantation*, **3**, 190.

Hellström, K. E. (1963). 'Differential behaviour of transplanted mouse lymphoma lines in genetically compatible homozygous and F_1 hybrid mice.' *Nature* (*Lond.*), **199**, 614.

Hellström, K. E. and Hellström, I. (1967). 'Allogeneic inhibition of transplanted tumor cells.' *Progr. exp. Tumor Res.*, **9**, 40–76.

HOLM, G. and PERLMANN, P. (1965). 'Phytohaemagglutinin-induced cytotoxic action of unsensitized immunologically competent cells on allogeneic and xenogeneic tissue culture cells.' *Nature (Lond.)*, **207**, 818.

HOLM, G. and PERLMANN, P. (1967). 'Cytotoxic potential of stimulated human lymphocytes.' *J. exp. Med.*, **125**, 721.

HOLM, G., PERLMANN, P. and WERNER, B. (1964). 'Phytohaemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture.' *Nature (Lond.)*, **203**, 841.

LUNDGREN, G., COLLSTE, L. and MÖLLER, G. (1969). 'Cytotoxicity by human lymphocytes: antagonism between inducing processes.' *Nature (Lond.)*, **220**, 289.

MÖLLER, E. (1965). 'Contact-induced cytotoxicity by lymphoid cells containing foreign isoantigens.' *Science*, **147**, 875.

MÖLLER, E. (1967). 'Cytotoxicity by non-immune allogeneic lymphoid cells: specific suppression by antibody treatment of the lymphoid cells.' *J. exp. Med.*, **126**, 395.

MÖLLER, E. and MÖLLER, G. (1967). 'Current status of contact-induced cytotoxicity by histoincompatible cells.' *Histocompatibility Testing* (Ed. by E. S. Curtoni, P. L. Mattiuz and R. M. Tosi). Munksgaard, Copenhagen.

MÖLLER, G. and MÖLLER, E. (1965). 'Plaque-formation by non-immune and x-irradiated lymphoid cells on monolayers of mouse embryo cells.' *Nature (Lond.)*, **208**, 260.

MÖLLER, G. and MÖLLER, E. (1966). 'Interaction between allogeneic cells in tissue transplantation.' *Ann. N.Y. Acad. Sci.*, **129**, 795.

MÖLLER, E., LAPP, W. and LINDHOLM, L. (1969). 'Interference by isoantibodies on cytotoxicity by non-immune lymphoid cells.' *Biological Recognition Processes* (Ed. by R. T. Smith). (In press).

MÖLLER, G. and LUNDGREN, G. (1969). 'Aggressive lymphocytes and sensitive target cells: two pathways for cytotoxicity.' *Biological Recognition Processes* (Ed. by R. T. Smith). (In press).

NOWELL, P. C. (1960). 'Phytohemagglutinin—an initiator of mitosis in cultures of normal human leukocytes.' *Cancer Res.*, **20**, 462.

PERLMANN, P., PERLMANN, H and HOLM, G. (1968). 'Cytotoxic action of stimulated lymphocytes on allogeneic and autologous erythrocytes.' *Science*, **160**, 306.

RUDDLE, N. H. and WAKSMAN, B. H. (1967). 'Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity.' *Science*, **157**, 1060.

SCHWARZ, M. R. (1968). 'The mixed lymphocyte reaction: an *in vitro* test for tolerance.' *J. exp. Med.*, **127**, 879.

SELL, S., ROWE, D. S. and GELL, P. G. H. (1965). 'Studies on rabbit lymphocytes *in vitro*. III. Protein, RNA and DNA synthesis by lymphocyte cultures after stimulation with phytohemagglutinin, with staphylococcal filtrate, with anti-allotype serum and with heterologous antiserum to rabbit whole serum.' *J. exp. Med.*, **122**, 823.

WILSON, D. B., SILVERS, W. S. and NOWELL, P. C. (1967). 'Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationship of the proliferative response to the immunologic status of the donors.' *J. exp. Med.*, **126**, 655.