INFECTION AND IMMUNITY, June 1978, p. 608-612
0019-9567/78/0020-0608$02.00/0
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Vol. 20, No. 3
Printed in U.S.A.

Innate Cytotoxicity of CBA Mouse Spleen Cells to Sendai Virus-Infected L Cells

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Received for publication 11 October 1977

The presence in the spleens of unsensitized CBA mice of cells that are spontaneously cytotoxic for Sendai virus-infected L cells was confirmed. This innate cytotoxic activity to virus-infected cells was shown to exhibit some *H-2* restriction. Partial identity of only the *D* end of the *H-2* gene complex between the target and effector cells was required to produce cytolysis. Attempts to characterize the kind of cell active in this system indicated that neither the θ antigen nor the surface immunoglobulin markers were present. Furthermore, the cells appeared to have no adherent or phagocytic properties. The relationship between the effector cells responsible for innate cytotoxicity to virus-infected cells and the natural killer (NK) cells spontaneously cytotoxic for certain tumor cells is discussed.

Lymphoid cells that are spontaneously cytotoxic for certain tumor cell lines have been found to occur naturally in both humans and rodents. In human systems, spontaneous cytotoxic activity directed against Burkitt lymphoma and melanoma cell lines have been found to depend upon the action of unsensitized, non-T cells (4, 16). More recently, these effector cells have been characterized as immunoglobulin-bearing lymphocytes secreting lymphotoxin (18).

The existence of spleen cells naturally cytotoxic for tumor cells has been reported in a number of mouse systems that utilize a wide variety of tumor cell lines as target cells (8, 9, 14, 21). The effector cells active in these studies were initially characterized as small lymphocytes lacking the characteristics of any hitherto recognized lymphoid cell type (11, 15). More recently, a new cell surface antigen, selectively expressed on this natural killer (NK) cell, has been described (7).

In a previous publication (1), Anderson et al. reported a further example of naturally occurring cytotoxic activity in unsensitized donors. Splenic lymphocytes from CBA mice were shown to be cytotoxic for syngeneic L929 cells infected with Sendai virus, but to have no significant effect upon uninfected L929 cells. This paper describes the characteristics of the cells responsible for this innate cytotoxicity.

MATERIALS AND METHODS

Target cells. The fibroblast cell line L929 (L cells), derived from C3H mice, was used throughout this study as a source of target cells. These cells were grown and maintained as previously described (1). The Sendai strain of parainfluenza type 1 virus was prepared as described previously (1).

Sendai-infected cells were obtained by inoculating confluent monolayers of L cells with 10 50% tissue culture infective doses of virus per cell and incubating them overnight at 37°C.

Animals. Mice of the following inbred strains were obtained from colonies at The London Hospital Medical College: CBA, A/Jax, A.BY, A.SW, and A.CA. Strain B10.HTT and C3H.OH mice were the generous gifts, respectively, of M. Hetherington of the Clinical Research Centre, Harrow, England, and R. Shreffler of the University of Michigan Medical School, Ann Arbor.

Uninfected mice of each strain lacked detectable antibodies to Sendai virus, having hemagglutination inhibition titers of less than 4 throughout the study.

T-cell-deprived mice were prepared by surgical removal of the thymus at 4 weeks of age. Ten days later, they were exposed to 850 rads of X-irradiation and immediately reconstituted with an intravenous injection of 5×10^5 syngeneic bone marrow cells. Mice found to have thymic remnants postmortem were discarded from the study.

Preparation of effector cell suspensions. Spleen cell suspensions were prepared from the pooled spleens of three mice as previously described (1).

Spleen cells were depleted of T cells by incubation in medium containing 2.5% anti-θ serum (Searle Diagnostic, High Wycombe, England) for 1 h at room temperature; control suspensions were incubated with heat-inactivated fetal calf serum. After being washed in medium, the cells were suspended in a 1:10 dilution of preserved guinea pig complement (Wellcome Reagents Ltd., Beckenham, England) and held for a further 45 min at room temperature. After being washed, the cells were resuspended to a concentration of 10^7/ml of medium. Indirect immunofluorescent staining for θ antigen indicated that 90 to 95% of all T

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cells were removed by this method.

Immunoglobulin-bearing cells were selectively removed from spleen cell suspensions by incubation on nylon wool columns as described by Julius et al. (12). The efficiency of depletion was monitored by indirect immunofluorescent staining, which demonstrated that between 85 and 95% of the initial immunoglobulin-bearing cells were lost, while the proportion of θ-positive T cells increased from 38 to 84%.

Phagocytic macrophages were selectively depleted from spleen cell suspensions by incubation with carbonyl iron powder and passage over a magnet. Adherent cells were removed by incubation on columns of glass beads (10). At least 70% of macrophages were removed from cell suspensions treated with either technique, as judged by neutral red dye uptake. Indirect immunofluorescent staining for surface immunoglobulin and θ antigen indicated that the proportions of B and T cells were unaffected by incubation of the splenocytes with carbonyl iron. Incubation upon columns of glass beads, however, resulted in a slight increase in the percentage of T cells, together with a decrease in the numbers of B cells.

Cells bearing receptors for complement were removed from spleen suspensions in the following way. Erythrocyte-antibody-complement (EAC) complexes were prepared by the method of Arnaiz-Villena et al. (2). Equal volumes of EAC and spleen cells (10^7/ml) were mixed in siliconized glass tubes, centrifuged at 75 $\times$ g for 5 min, and held at room temperature for a further 15 min. When half of the supernatant had been removed, the cell pellet was gently resuspended, and the rosetted cells were removed from the suspension by fractionation in a solution of Ficoll (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline and Triosil (Nyegard and Co., Norway) at a 2.7:1 (vol/vol) ratio (10). Three volumes of the cell mixture were layered onto 1 volume of the Ficoll-Triosil mixture and centrifuged at 1,000 $\times$ g for 10 min. The cells collected from the interface were treated with ammonium chloride to remove the remaining erythrocytes and washed twice in medium before being resuspended to a concentration of 10^7/ml. Controls consisting of a mixture of untreated erythrocytes and lymphocytes, or a suspension of lymphocytes alone, were also centrifuged in Ficoll-Triosil mixture and treated in the same way.

Rosette formation was monitored by staining with toluidine blue and determining the ratio of free lymphocytes to rosetted lymphocytes. Control preparations of lymphocytes incubated with washed erythrocytes only failed to form rosettes, whereas 30 to 40% of the lymphocytes formed rosettes upon incubation with the EAC complex.

After fractionation of EAC rosettes on Ficoll-Triosil, cells harvested from the interface were shown to be free of complement receptor-bearing lymphocytes, since no further rosettes were formed upon reincubation with EAC complexes. In contrast, control cell suspensions incubated either with or without control erythrocytes formed comparable numbers of EAC rosettes before and after fractionation in Ficoll-Triosil.

Cytotoxic assay. The ^{15}C release assay of cytotoxicity against Sendai-infected L cells was carried out as described previously (1). ^{51}Cr release was estimated in 10 replicate wells, and the percent cytotoxicity was calculated by subtracting the percentage of the total ^{51}Cr released spontaneously from target cells in the absence of spleen cells from the percentage released from target cells in the presence of spleen cells. Student's t test was used to assess the significance of results.

RESULTS

Determination of optimum assay conditions. Results indicating that incubation of unsensitized spleen cells from normal CBA mice with Sendai-infected L cells consistently results in significant levels of cytotoxicity have been obtained previously by using a spleen cell:target cell ratio of 10:1 (1). To determine the optimum spleen cell:target cell ratio for the measurement of this naturally occurring "innate cytotoxicity," spleen cells from 10 6-week-old, male CBA mice were pooled and incubated for 20 h with either Sendai-infected or uninfected L cells at concentrations ranging from 100 spleen cells to 1 spleen cell per target cell. The amount of ^{51}Cr release from Sendai-infected cells was found to increase with the concentration of spleen cells (Fig. 1). Spleen cell:target cell ratios of 5:1 and above resulted in significantly greater cytotoxicity to Sendai-infected cells than to uninfected L cells ($P < 0.001$). However, with ratios in excess of 10:1, progressively increasing quantities of radiolabel were released from uninfected L cells.

The greatest difference in ^{51}Cr release from Sendai-infected and uninfected L cells was found to occur with a spleen cell:target cell ratio of 10:1, and this ratio was therefore used in all subsequent experiments.

In a series of 15 consecutive experiments, pools of spleen cells were obtained from three mice, and their innate cytotoxicity was shown to range between 11.4 and 24.0%, with a mean value of 15.8%. The variation was independent of the sex of the mice and of age within the range of 4

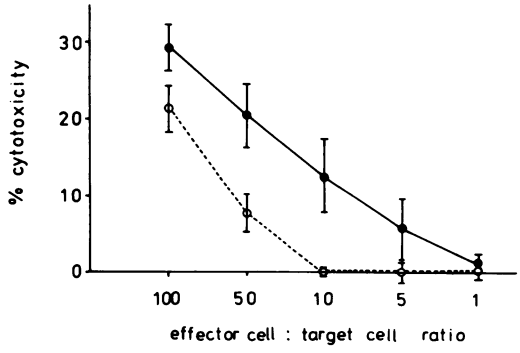

FIG. 1. *Innate cytotoxic activity (mean of 10 replicates ± 95% confidence limits) of unsensitized CBA mouse spleen cells against* (●) *Sendai virus-infected L cells and* (○) *uninfected L cells.*

to 15 weeks. When spleen cell suspensions from six female CBA mice (aged 5 weeks) were prepared and tested individually, appreciable variation was again found (range, 13.3 to 22.2%; mean, 18.8%).

Strain distribution of innate cytotoxicity. The target cell line L929 was originally derived from C3H mice (20). The cells used in this study have remained histocompatible with the mouse strain CBA (F. Garrido and M. J. Anderson, unpublished data). To determine whether histocompatibility between spleen cells and target cells is required for the expression of innate cytotoxicity, the cytotoxic effect of spleen cells from various strains of mice against Sendai-infected L cells was examined (Table 1).

Mice of strains A.SW, A.CA, and A.BY, sharing no portion of the *H-2* genome with L cells, exhibited no cytotoxicity for either Sendai-infected or uninfected L cells. Similarly, splenocytes of A/Jax mice were found to be unreactive, although they shared loci *K, I-A,* and *I-B* with L cells.

In contrast, the spleen cells of mice of both B10.HTT and C3H.OH strains, sharing partial identity with L cells in the right-hand *D* end of the *H-2* complex, were found to be significantly cytotoxic for Sendai-infected L cells, although uninfected L cells remained undamaged.

Characteristics of effector cells in innate cytotoxicity. The importance of T cells in innate cytotoxicity was next investigated. First, the spleen cell cytotoxic activity of thymus-deprived (TXBM) CBA mice was compared with that of whole CBA mice. In three separate experiments, no significant reduction of the cytotoxicity of spleen cells from TXBM mice was found, and in two cases there was a significant increase in the innate cytotoxicity of the TXBM mice (Table 2). Second, spleen cell suspensions were treated with anti-θ serum and complement, and the cytotoxicity was compared with the same suspension treated with fetal calf serum and complement. These studies showed that there was a tendency for treatment with anti-θ serum to enhance rather than reduce innate cytotoxicity, confirming that T cells were not active in this system (Table 2).

To investigate the possibility that innate cytotoxicity is mediated by antibody-dependent cell-mediated cytotoxicity, the effect of filtration of normal spleen cells through nylon wool columns was studied. Fractionation by this technique was found to deplete both immunoglobulin-bearing lymphocytes and adherent cells. However, in spite of the loss of these cell types, no significant difference in cytotoxicity was observed between depleted and whole spleen cell suspensions (Table 3).

The role of macrophages was further investigated by removing phagocytic cells by carbonyl iron treatment and by removing adherent cells on glass bead columns. Neither treatment re-

TABLE 1. *Cytotoxic effect of spleen cells from various strains of mice against Sendai-infected L cells*

Mouse strain	Allele	% Cytotoxicity against:	
		Sendai-infected L cells	Uninfected L cells
CBA	*k*	19.7[a]	0.2
C3H.OH	*d*	20.5[a]	3.5
B10.HTT	*t3*	7.4[a]	0.4
A/Jax	*a*	1.1	0
A-BY	*b*	0	0
A-CA	*f*	0	0
A-SW	*s*	0	0

[a] Significant cytotoxicity ($P < 0.001$) using Student's *t* test to compare ^{51}Cr release in the presence or absence of spleen cells.

TABLE 2. *Effect of T-cell depletion upon innate cytotoxicity*

% Cytotoxicity of spleen cells from:		% Cytotoxicity of spleen cells treated with:	
TXBM mice	Control mice	Anti-θ serum and C[a]	Normal serum and C[a]
12.4	9.2[b]	15.0	12.4
20.5	13.7[b]	27.0	24.0
20.0	21.0	17.1	11.1

[a] Guinea pig serum as a source of complement.
[b] Significant difference ($P < 0.001$) in cytotoxicity between TXBM and control mouse splenocytes.

TABLE 3. *Effect of various depletion procedures upon innate cytotoxicity*[a]

Depletion procedure	% Innate cytotoxicity of:	
	Depleted cell suspension	Control cell suspension
Nylon wool filtration	14.9	15.7
	16.3	14.4
	20.0	21.7
Carbonyl iron	16.8	12.3
	7.9	6.9
	14.7	14.4
Glass bead filtration	7.2	4.8
	8.5	13.9
	4.0	8.0
EAC rosette	15.0	17.7[b]
	11.9	12.0[b]
	26.9	31.4[b]

[a] In none of these experiments were the differences between cytotoxic activity of control and depleted cell suspensions statistically significant.
[b] Control cells were incubated with washed erythrocytes before Ficoll-Triosil fractionation.

sulted in a significant change in the innate cytotoxicity of spleen cells to Sendai-infected L cells (Table 3).

Cells bearing receptors for the third component of complement were removed from normal spleen cell suspensions by rosetting with EAC complexes and separating the rosettes from nonrosetted cells by centrifuging in Ficoll-Triosil. As a control, spleen cell suspensions were incubated with washed erythrocytes alone and then centrifuged in Ficoll-Triosil. The cytotoxicity of cell suspensions from which complement-receptor-bearing, EAC rosetted cells had been removed was not significantly different from that of the control cells (Table 3).

DISCUSSION

The results of this study confirm that spleen cells from normal, unsensitized mice may express innate cytotoxicity against virus-infected syngeneic cells. This activity was originally observed in CBA mice (1). The results reported here show that innate cytotoxicity was found in all CBA mice tested, but that there was appreciable variation in its intensity between individual animals. The number of virus-infected target cells killed by the normal CBA mouse spleen cells is dependent upon the ratio of spleen cells to these target cells; increasing numbers of spleen cells bring about the death of increased numbers of target cells. It is of interest that, at spleen cell:target cell ratios in excess of 10:1, significant and increasing quantities of ^{51}Cr were released from uninfected control L cells. This may be due to the action of products of lymphocyte metabolism being present in toxic amounts at high spleen cell concentrations.

The expression of specific T-cell-mediated immunity to virus infections has been shown to be dependent upon histocompatibility between target cells and effector cells (5, 6). The possibility of a similar *H-2* restriction operating in the expression of innate cytotoxicity to virus-infected cells was examined by studying mice with *H-2* types differing from the *H-2*^{k} type of L929 cells. The spleen cells of those mouse strains lacking any *H-2* identity with L cells were not cytotoxic to either Sendai-infected or uninfected L cells, suggesting that shared *H-2* type might be important. Splenocytes of A/Jax mice were similarly incapable of spontaneously killing Sendai-infected L cells, indicating that identity of the *K* end of the *H-2* gene complex (*K, I-A, I-B, I-J*) was insufficient for the expression of innate cytotoxicity. However, the spleen cells of B10.HTT mice showed significant cytotoxic activity against Sendai-infected L cells, suggesting that identity in only the *I-C* and *S* regions of the *D* end of the *H-2* complex may be sufficient. Interestingly, identity in only the *D* region may also lead to the expression of innate cytotoxicity as demonstrated in the splenocytes of C3H.OH mice.

Recent work described by Becker et al. (3) has indicated a lack of *H-2* homology in the lysis of various tumor cells by unsensitized mouse spleen cells. However, the results reported here indicate that the expression of innate cytotoxicity to virus-infected nontumor cells may depend upon partial *H-2* identity between effector and target cells. In this respect, this system is similar to cell-mediated immunity to acute virus infections (5, 6). However, the results of this study indicate that the nature of the effector cells in the two systems is very different. In assays of conventional T-cell-mediated immunity to virus infections, depletion of T cells by either thymectomy, irradiation, and bone marrow reconstitution, or treatment with anti-θ serum and complement results in the elimination of cytotoxic activity. In contrast, neither of these procedures produced any decrease in innate cytotoxicity of unsensitized spleen cells.

Antibody-dependent, cell-mediated cytotoxicity (ADCC) has been shown to occur in the presence of minute amounts of antibody (18). The possible existence in the spleen of small numbers of B cells secreting antibodies that are specific for Sendai virus-infected L cells was therefore examined. Depletion of nylon wool-adherent and immunoglobulin-bearing cells from the spleen failed to reduce cytotoxic activity in contrast to results obtained in antibody-dependent, cell-mediated cytotoxic systems (14), suggesting that antibody-dependent, cell-mediated cytotoxicity was not a component of this innate cytotoxicity. Moreover, the specific removal of complement receptor-bearing lymphocytes, another procedure known to reduce cytotoxicity in antibody-dependent, cell-mediated cytotoxic systems (21), had no effect.

The alveolar macrophages of unsensitized calves have been shown to be spontaneously cytotoxic for calf kidney cells infected with parainfluenza type 3 virus (20). However, mouse splenic macrophages do not appear to contribute detectable activity to the killing of Sendai-infected L cells, since selective depletion of both glass-adherent and phagocytic cells does not depress cytotoxicity in this system.

The effector cells operating in the innate cytotoxicity to Sendai-infected cells thus appear to be nonadherent and nonphagocytic. They carry neither surface immunoglobulin nor the θ antigen and, in addition, bear no receptors for the third component of complement. These properties are similar to those of the cells described by

Herberman et al. (11) and the NK cells described by Kiessling et al. (15). These authors have described the phenomenon of natural or innate cytotoxicity with activity against tumor cells. This is the first report of natural cytotoxicity directed toward virus-infected cells. Whenever the derivation of tumor target cells has been known, it has appeared that only virus-induced and not chemically induced tumors are susceptible to natural cytotoxicity. This observation, together with the work reported here, suggests that the presence of virus infection may be of prime importance in determining the susceptibility of cells to lysis by unsensitized NK lymphocytes. Indeed, preliminary results have been obtained indicating that infection of L cells with the Kunz strain of influenza A renders these cells similarly susceptible to innate cytotoxicity. The importance of NK cells in immune surveillance against both virus-induced tumors and virus infections generally is likely, therefore, to be considerable and worthy of further study.

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