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Natural killing of cytomegalovirus-infected fibroblasts by human mononuclear leucocytes

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SUMMARY

The ability of human peripheral blood mononuclear (MN) cells to lyse uninfected and cytomegalovirus (CMV) infected human fibroblasts was determined in a ^{51}Cr-release assay. Maximal release was obtained with 6-day infected fibroblasts incubated with MN cells for 24 hr. A linear relationship existed between E/T ratios of 12·5:1 to 100:1 and lysis of CMV-infected targets. Donor immune status had no effect on the magnitude of killing of infected or uninfected targets. Killing was mediated by non-B, predominantly non-T, Fc receptor-bearing cells. Preincubation of effector cells with interferon enhanced killing of both CMV-infected and uninfected fibroblasts, but infected targets were more effectively killed. These results indicated a possible role for natural killer cells in recovery from CMV infection.

INTRODUCTION

Symptomatic cytomegalovirus infections occur primarily in newborns and in immunocompromised hosts, particularly recipients of bone marrow, cardiac and renal transplants (Starr, 1979). Such infections may be associated with considerable morbidity and occasional mortality.

It has been postulated that impairments in cell-mediated immunity may contribute to the severity or duration of CMV infections. Several *in vitro* assays, including assays for lymphocyte proliferation and interferon production, have been used to assess cellular immunity to CMV.

A preliminary report of leucocyte-mediated killing of CMV-infected targets has also appeared. However, the effector cell involved was not well defined (Diamond *et al.*, 1977). It is known that incubation of natural killer (NK) cells with interferon results in augmented killing of certain target cells (Santoli, Trinchieri & Koprowski, 1978). The purpose of the present study was two-fold: (1) to define further the effector cell involved in killing of CMV-infected targets and (2) to determine whether interferon augments killing of CMV-infected targets. This latter question is of particular importance in view of the possible use of interferon for prophylaxis or therapy of severe CMV infection (Cheeseman *et al.*, 1979; Meyers *et al.*, 1980).

MATERIALS AND METHODS

Cell cultures and virus pools. Human embryonic foreskin fibroblasts, FS-4 strain, were obtained at

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passage level 12 from Dr Kurt Paucker, Medical College of Pennsylvania, and maintained in Eagle's minimum essential medium (MEM) supplemented with 7·5% fetal calf serum (FCS, Flow Laboratories, Rockville, Maryland). At passage level 14, cells were frozen in the presence of 10% dimethylsulphoxide (DMSO) and kept at −190°C until needed. Thawed cells were used up to passage level 22. Cell-free pools of the AD-169 strain of cytomegalovirus were prepared from monolayers of FS-4 cells infected at a multiplicity of infection of approximately 0·1. When approximately 90% of the cells exhibited cytopathic effect, the cells were removed with 2·5% trypsin, 0·1% EDTA, resuspended in MEM with 7·5% FCS and sonicated. Ten per cent DMSO was added, and aliquots were stored at −190°C until use.

Selection and serological status of donors. Donors consisted of healthy, young adult laboratory workers. Sera were screened for antibodies to CMV by means of a complement fixation (CF) assay with glycine-extracted CMV (Starr & Friedman, 1980). Sera that were negative in the CF assay were further tested for CMV antibodies by an anticomplementary immunofluorescence assay (Starr & Friedman, 1980).

Effector cell preparations. Mononuclear (MN) cells were obtained by buoyant density centrifugation on Ficoll–Hypaque (Böyum, 1968). Cells obtained were washed twice in Hanks' balanced salt solution (HBSS) and resuspended in RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 20% FCS, penicillin 125 units/ml and gentamicin 65·2 μg/ml. Monocyte- and B lymphocyte-depleted populations were prepared as previously described (Greaves & Brown, 1974). Briefly, nylon-wool columns were prepared by packing 300 mg of washed and dried nylon wool (Fenwall Leukopak, Travenol Laboratories, Deerfield, Illinois) into 10-ml plastic syringes. Thirty to 50 × 10^6 MN cells were incubated on the columns for 30 min at 37°C and then eluted in 30 ml of RPMI 1640 with 10% FCS at a rate of approximately 1 ml/min.

T lymphocyte-enriched and -depleted populations were prepared by separation on Ficoll–Hypaque gradients of cells rosetting with neuraminidase-treated sheep red blood cells (SRBC) from non-rosetting cells. Briefly, SRBC were incubated with *Vibrio cholerae* neuraminidase (GIBCO, Grand Island, New York) for 30 min. The treated SRBC were washed three times in HBSS. SRBC were added to 1 × 10^6 mononuclear cells to give a 50:1 ratio of SRBC to MN cells in a total volume of 3 ml. Mixtures were incubated at 37°C for 15 min, centrifuged at 200 ***g*** for 5 min and then incubated for an additional 45 min at 22°C. Cell pellets were carefully resuspended and subjected to Ficoll–Hypaque centrifugation at 200 ***g*** for 30 min at 4°C. Cells obtained from the interface were resuspended in HBSS. Pelleted cells were resuspended in HBSS, and SRBC were lysed by hypotonic shock. The cells were then subjected to a second cycle of SRBC rosette formation and Ficoll–Hypaque centrifugation.

MN cells were depleted of Fc receptor-bearing cells by adsorption on antibody-sensitized SRBC monolayers as previously described (Kedar, Landazuri & Bonavida, 1974). Briefly, SRBC were added to 25-cm^2 plastic flasks (Falcon, Oxnard, California) that had been pretreated with poly-L-lysine. After 30 min of incubation at room temperature the SRBC were lysed with distilled water, and rabbit IgG to SRBC (Cordis Laboratories, Miami, Florida) was added at a 1:50 dilution. After 30 min of incubation at 37°C, the erythrocyte–antibody (EA) monolayers were washed three times with PBS. Twenty million MN were added to each flask. After 20 min of incubation at room temperature, flasks were centrifuged for 5 min at 400 ***g*** and incubated for an additional 20 min. The non-adherent cells were removed and added to new flasks, and the adsorption procedure was repeated. Non-adherent cells were collected and counted. Control cell fractions were prepared by incubating MN on SRBC (E) monolayers that were not sensitized with antibody.

Cell identification. B lymphocytes were identified as cells exhibiting membrane fluorescence with fluorescein-conjugated F $(ab')_2$ fragments of goat anti-human immunoglobulin (Capell Laboratories, Cochranville, Pennsylvania) (Ross & Winchester, 1980). T lymphocytes were identified by rosette formation with neuraminidase-treated SRBC (Weiner, Bianco & Nussenweig, 1973). Monocyte–macrophages were detected by phagocytosis of latex particles (Dow Chemical Co., Indianapolis, Indiana) as previously described (Kohl *et al.*, 1977). Fc receptor-bearing lymphocytes were identified by rosetting with antibody-sensitized OxRBC (EA_{7S} rosettes) and by indirect immunofluorescence with heat-aggregated IgG and fluorescein-conjugated anti-human immunoglobulin (Shore, Melewicz & Gordon, 1977).

Preparation of target cells. Approximately 1×10^6 FS-4 cells were seeded in 25-cm^2 tissue culture flasks and incubated for 24 hr at 37°C. The monolayers were then infected with AD-169 virus at a multiplicity of infection of approximately 0·1. After 2-6 days of incubation, infected (FS-4-CMV) and uninfected FS-4 target cells were removed by exposure to trypsin–EDTA, washed once in HBSS containing 5% FCS and resuspended in 0·9 ml. One hundred microcuries of $Na^{51}CrO_4$ (New England Nuclear, Boston, Massachusetts) were added, and the cells were incubated at 37°C for 1 hr. Target cells were then washed four times in cold HBSS and resuspended in RPMI 1640 with 20% FCS.

Preparation of interferon. Mononuclear cells were obtained by Ficoll–Hypaque centrifugation of peripheral blood. Newcastle disease virus was added at a multiplicity of 10 to aliquots of 10×10^6 cells in 1-ml volumes of RPMI 1640 with 10% FCS. These mixtures were incubated for 18 hr at 37°C. Supernates were then harvested, centrifuged at 30,000 ***g*** for 30 min at 4°C, and brought to pH 2 with 1 N HCl. After 5 days incubation a 4°C, 1 N NaOH was added to bring the pH to 7, and the material was stored at −70°C until needed. The titre of this preparation was 40,000 units/ml in a previously described assay involving reduction of vesicular stomatitis virus cytopathic effect in FS-4 monolayers (Starr *et al.*, 1980).

Cytotoxicity assay. Target cells were distributed into 96-well flat-bottomed microtitre plates (Linbro, Hamden, Connecticut), 5×10^3 cells per well in a volume of 0·1 ml. Plates were then incubated for 3–5 hr at 37°C in a 5% CO_2 atmosphere. Various concentrations of effector cells in 0·1-ml volumes were added, and the plates were reincubated in 5% CO_2 for 20–24 hr. In certain experiments the effector cells and various concentrations of interferon were incubated for 1 hr and then added to the target cells. At the end of the incubation period, 100 μl of media were aspirated from each well without disturbing the cell pellet. Samples were counted using a gamma scintillation counter (LKB Wallac, Turku, Finland). Total releasable activity was determined by the addition of 1% Triton X-100. Spontaneous release for target cells was measured in the absence of effector cells. All determinations were done in triplicate. Per cent chromium release was calculated using the formula:

$$\frac{\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{spontaneous release}}$$

Specific release was determined by subtracting the per cent release with control target cells from the per cent release with infected targets. A lytic unit was defined as the number of effector cells required to cause 50% ^{51}Cr-release from target cells. The number of lytic units in an effector cell population was determined using experimentally derived graphs of E/T ratio versus per cent ^{51}Cr-release (Santoli & Koprowski, 1979).

RESULTS

Duration of target cell infection

FS-4 human fibroblasts were infected with AD-169 at a multiplicity of infection 0·1 for 2, 4 or 6 days prior to assay. A representative experiment is shown in Fig. 1. The magnitude of specific cytotoxicity at various effector-to-target cell (E/T) ratios was generally increased with longer duration of infection, with maximal activity detected against 6-day-infected target cells. Similar results were obtained in two additional experiments with different donors. Monolayers infected with CMV for more than 6 days yielded insufficient numbers of viable cells for use in cytotoxicity assays. In all further studies, 6-day-infected targets were used.

Kinetics of cytotoxicity (Fig. 2)

Mononuclear cells from four donors were incubated with infected and control targets at an E/T ratio of 50:1 for varying time periods. Increased ^{51}Cr-release from infected targets was detected as early as 15 hr and was generally maximal at 24 hr, while ^{51}Cr-release from uninfected targets was relatively stable during this time period. Subsequent experiments were harvested at 24 hr. With this

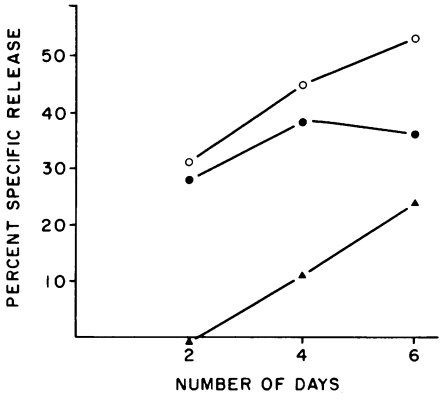

Fig. 1. Effect of duration of target cell infection on cytotoxicity. Three different effector-to-target cell ratios were used: 100:1 (o——o), 50:1 (●——●) and 25:1 (▲——▲).

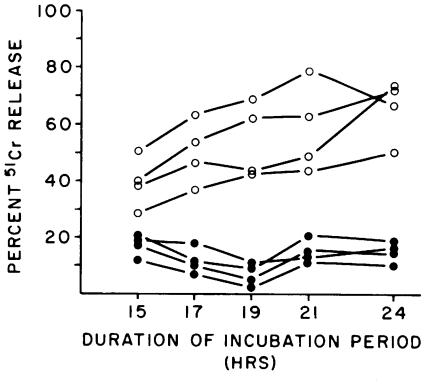

Fig. 2. Kinetics of cytotoxicity. MN cells of four individuals were incubated with uninfected (●——●) and infected (o——o) targets for the time periods indicated.

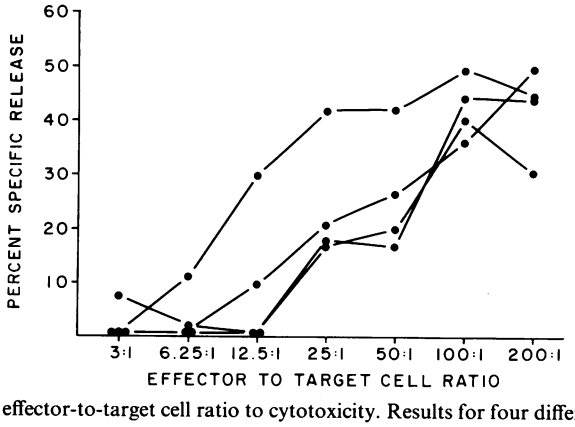

Fig. 3. Relationship of effector-to-target cell ratio to cytotoxicity. Results for four different donors are shown.

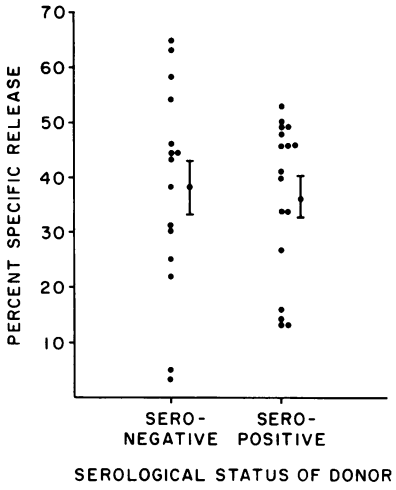

Fig. 4. Effect of donor serological status on cytotoxicity. Bars represent ±1 standard error of the mean.

incubation period, spontaneous ^{51}Cr-release was 39±5% (mean±s.d.) for control targets and 32±7% for CMV-infected targets.

Effector-to-target cell ratio

Mononuclear cells of four donors produced a linear rise in specific ^{51}Cr-release with increasing E/T ratios from 12·5:1 to 100:1 (Fig. 3). E/T ratios of 50:1 were used in subsequent experiments, except as otherwise stated.

Effect of donor immune status on cytotoxicity

To determine whether donor immune status influenced the magnitude of cytotoxicity, seropositive and seronegative normal adults were tested in the cytotoxicity assay. As shown in Fig. 4, there was

Table 1. Identification of effector cells mediating killing of CMV-infected FS-4

Donor serological status	Cell fraction	Per cent SRBC rosette-positive	Per cent sIg-positive	Per cent latex-positive	E/T*	Per cent ^{51}Cr-release	
						FS-4	FS-4-CMV
+	Ficoll–Hypaque	74	8·0	5·0	50:1	14	73 (800)†
	Nylon-wool NA‡	85	<0·5	1·0	50:1	12	55 (568)
	SRBC rosetting	89	—	—	50:1	11	35 (144)
	SRBC non-rosetting	2·5	—	—	6:1	13	49 (3,300)
−	Ficoll–Hypaque	77	5·0	17	50:1	0	69 (1,280)
	Nylon-wool NA	70	<0·5	3·0	50:1	11	77 (>1,600)
	SRBC rosetting	91	—	—	50:1	0	22 (64)
	SRBC non-rosetting	2·0	—	—	30:1	0	78 (>2,640)

*Effector-to-target cell ratio.

† Number in parentheses indicates lytic units per 10^8 lymphocytes against CMV-infected FS-4 targets.

‡ Non-adherent.

considerable variation in responses among normal adults. However, mean cytotoxic responses for seropositive and seronegative donors were not significantly different ($P > 0{\cdot}05$, Student's t test). Among seropositive donors, levels of complement-fixing and immunofluorescent antibodies to CMV did not correlate with the magnitude of specific cytotoxicity (data not shown).

Effector cell identification

Experiments were performed to identify the population of cells responsible for killing of CMV-infected fibroblasts. Mononuclear cells were incubated on nylon-wool columns as described in the Materials and Methods section. Non-adherent cells were largely depleted of B lymphocytes (Table 1) yet retained cytotoxic activity. The nylon-wool non-adherent cells were separated into rosetting and non-rosetting fractions. The number of non-rosetting cells recovered permitted testing at only low effector-to-target cell ratios. The data were therefore expressed as lytic units per

Table 2. Role of Fc receptor-bearing cells in killing of CMV-infected FS-4

Donor serological status	Cell fraction	Per cent EA rosette	Per cent agg. IgG*	Per cent ^{51}Cr-release†	
				FS-4	FS-4-CMV
+	Ficoll–Hypaque	16·2	n.d.	8	25
	E non-adherent‡	13·8	17·4	6	44
	EA non-adherent§	0·9	0·4	2	3·0
−	Ficoll–Hypaque	n.d.	11·0	17	50
	E non-adherent	n.d.	n.d.	16	52
	EA non-adherent	< 1%	1·5	12	1·0

* Percentage of cells positive for aggregated IgG.
† The E/T ratio was 50:1 for all cell fractions.
‡ Cells non-adherent to E monolayers.
§ Cells non-adherent to EA monolayers.

Table 3. Killing of uninfected or CMV-infected targets by effector cells preincubated for 1 hr with various concentrations of interferon

Expt	Target cell	Interferon concentration (units/ml)					
		0	50	100	250	500	1,000
1	Uninfected	32*				79	73
	Infected	56			92	85	89
2	Uninfected	14			36	28	21
	Infected	51			55	64	58
3	Uninfected	6	9	24	30	45	
	Infected	51	55	64	62	72	
4	Uninfected	4	12	15	28	34	
	Infected	36	49	59	62	48	

All experiments were done with E/T of 50:1, incubation for 24 hr.
* Per cent ^{51}Cr-release (mean of triplicate).

10^8 lymphocytes for comparative purposes. Non-rosetting cells, which were largely devoid of T lymphocytes, were greatly enriched for cytotoxic activity. Activity was reduced in the rosetting cell fraction, which contained 89–91% rosette-forming cells.

Additional experiments were done to determine whether the cytotoxic cells had receptors for the Fc portion of IgG. Fc receptor-depleted cell populations were prepared as described in the Materials and Methods section. As shown in Table 2, only a small percentage of cells non-adherent to EA monolayers had detectable Fc receptors. These cells were deficient in spontaneous cytotoxicity against K562 cells and in antibody-dependent cytotoxicity (ADCC) (data not shown). They were also markedly deficient in cytotoxicity against CMV-infected targets (Table 2), while cells non-adherent to E monolayers retained their cytotoxic activity.

Effect of preincubation of effector cells with interferon

Preincubation of effector cells with interferon for 1 hr enhanced killing of both uninfected and CMV-infected target cells (Table 3). Maximal killing of both target cells was achieved with interferon doses of 250 or 500 units/ml, but some enhancement was noted with doses as low as 50–100 units/ml. The per cent increase in natural killing due to interferon stimulation was greater for uninfected than for infected targets; however, infected cells were still killed to a greater extent. Kinetic studies indicated that enhanced killing of infected and uninfected targets by interferon-stimulated effector cells could be detected as early as after 4 hr of incubation (data not shown).

DISCUSSION

The cytotoxic activity detected in our assay system closely resembles that described by Diamond *et al.* (1977), although the two assays differ in details of method. Background leakage of ^{51}Cr from target cells was relatively high in our assay, but this did not adversely affect the detection of cytotoxicity. The high leakage might have been due to the use of small numbers of target cells in each well (Diamond *et al.*, 1977). The small number of effector cells required for our assay is advantageous for clinical studies, particularly those involving newborns or infants, since only 3–5 ml of blood is required.

The effector cell identified in our assay corresponds to the natural killer (NK) cell previously described by others (Herberman *et al.*, 1979). Depletion of nylon-wool-adherent cells demonstrated that neither B cells nor mature monocytes are the predominant cytotoxic cells. Although NK activity was found in both SRBC rosetting and non-rosetting fractions, the considerable enrichment of activity in the non-rosetting fraction suggested that the effector cells were predominantly non-T lymphocytes. It is not clear whether the activity found in the rosette-forming fraction was due to SRBC-binding cells or to contaminant non-rosetting cells. Activity in both rosetting and non-rosetting fractions has also been reported by Santoli & Koprowski (1979), for cytotoxicity against influenza-infected targets, and by Ching & Lopez (1979), for killing of herpes simplex-infected targets. The effector cell in our assay was shown to bear receptors for Fc portion of IgG, a previously described characteristic of NK cells (Herberman *et al.*, 1979).

The lack of dependence of cytotoxic activity on donor immune status further confirms that our assay detects NK activity. Similar enhanced killing of infected cells has been described for a number of other viruses including measles (Ault & Weiner, 1979), herpes simplex (Ching & Lopez, 1979), mumps (Anderson, Stejskal & Harfast, 1975) and influenza (Santoli *et al.*, 1978). We were unable to confirm the results of Thong *et al.* (1976) describing lysis of CMV-infected fibroblasts by leucocytes of seropositive, but not seronegative donors.

We have been unable to demonstrate enhanced cytotoxicity with addition of serum-containing antibodies to CMV (Starr *et al.*, unpublished observations). This was somewhat surprising since antibody-dependent cellular cytotoxicity has been described for a number of viruses including the closely related herpes virus, herpes simplex (Kohl *et al.*, 1977; Shore *et al.*, 1977).

Recently, two groups reported specific killing of CMV-infected fibroblasts by T lymphocytes from seropositive individuals (Sethi, Stroehmann & Brandis, 1980; Quinnan *et al.*, 1981). In both of these studies, T-cell cytotoxicity was detected only when target cells shared HLA determinants with

the effector cell donor. In the study of Sethi *et al.* (1980), long-term culture of lymphocytes with T-cell growth factor was necessary to detect this activity. It thus appears that T lymphocytes as well as NK cells can mediate killing of CMV-infected targets under appropriate *in vitro* conditions. We hypothesize that NK cells might serve an important role in primary CMV infection as a first-line defence acting before immune T lymphocytes and specific antibody appear.

The enhanced natural killing of virus-infected targets has been attributed to stimulation of natural killer cells by interferon produced in cytotoxicity assays by the mononuclear cells present (Santoli *et al.*, 1978). These authors also showed that preincubation of effector cells with interferon for 18 hr resulted in increased killing of both virus-infected and uninfected target cells. Both types of targets were killed with equal efficiency. Using a different experimental design with preincubation of effector cells for 1 hr, we demonstrated enhanced killing of both CMV-infected and uninfected targets. CMV-infected targets were killed to a greater extent than uninfected targets. Interferon-induced inhibition of susceptibility of uninfected targets to lysis by natural killer cells (Trinchieri & Santoli, 1978) may have contributed to our results since exogenous interferon was present throughout the assay.

Interferon therapy in humans has recently been shown to be associated with stimulation of natural killer cells (Huddlestone, Merigan & Oldstone, 1979). The concentrations of interferon required to stimulate enhanced killing in our assay are achieved in humans treated with interferon (Meyers *et al.*, 1980). Our results thus suggest an alternative mechanism by which interferon may act in prevention or treatment of CMV infections.

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