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Natural killing of fibroblasts infected with low-passage clinical isolates of human cytomegalovirus

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SUMMARY

Fibroblasts infected with most low-passage clinical isolates of human cytomegalovirus (CMV) were as susceptible to lysis by human natural killer (NK) cells as high passage AD-169-infected fibroblasts. NK lysis occurred despite the absence of detectable CMV-specific late membrane antigen(s) on the majority of the target cells infected with most of the low passage strains. The magnitude of NK lysis of different CMV-infected target cells did not correlate with their ability to induce IFN-α. NK cell-mediated lysis of cells infected with low-passage clinical isolates of CMV required both NK cells and HLA-DR$^+$ accessory cells, as previously shown for AD-169-infected target cells.

Keywords NK cytomegalovirus accessory cells

INTRODUCTION

Human cytomegalovirus (CMV) infections are an important cause of morbidity and mortality in immunocompromised patients, particularly recipients of transplants (Starr, 1979). Although cellular immune responses are thought to play a major role in recovery from CMV infections, the precise mechanisms involved have not been defined. Natural killing of CMV-infected target cells has been proposed as one possible mechanism contributing to recovery from CMV infections (Rook *et al.*, 1984; Starr *et al.*, 1984). Target cells infected with high-passage AD-169 strain CMV are lysed *in vitro* by peripheral blood NK cells from seropositive and seronegative individuals (Borysiewicz *et al.*, 1985; Kirmani *et al.*, 1981; Starr & Garrabrant, 1981). We now report that fibroblasts infected with low-passage isolates of CMV are, in most cases, also susceptible to lysis by human peripheral blood NK cells.

MATERIALS AND METHODS

Viruses

High-passage AD-169 strain CMV was obtained from the American Type Culture Collection, Rockville, MD. A stock prepared from infected human fetal fibroblasts (Flow-5000, Flow Laboratories Inc., McLean, VA) as described by Starr & Garrabrant (1981) had a titre of 10^6 plaque forming units per ml. Eight strains of CMV isolated from urine specimens of renal transplant recipients were provided at passage level 2 or 3 by Dr Stanley Plotkin, Children's Hospital of Philadelphia, Pennsylvania.

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Preparation of target cells

Flow-5000 cells in 25 cm^2 flasks were inoculated with the low-passage clinical isolates of CMV. When monolayers showed 90% cytopathic effect (CPE), the cells in each flask were trypsinized and used to inoculate uninfected fibroblasts in three 25 cm^2 flasks. Virus was passaged in this fashion until inoculated monolayers developed 90% CPE in 4–6 days. The cells of each 25 cm^2 flask were then used to inoculate one 75 cm^2 flask. For preparation of AD-169-infected target cells, cell-free AD-169 was added to monolayers of Flow-5000 cells in 75 cm^2 flasks at a multiplicity of infection of 0·1. When 90% of cells in 75 cm^2 flasks exhibited CPE after inoculation with AD-169 or low-passage strains, the monolayers were trypsinized, washed, and suspended in MEM (Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS), 2% vitamins, 2 mM glutamine and 10% dimethyl sulphoxide. Fractions were kept at −70°C for 12–16 h, transferred to the vapour phase of a liquid nitrogen freezer, and maintained at −190°C until use. All target cells were tested for mycoplasma and found to be negative.

Immunofluorescent staining

Frozen target cells were thawed and then tested for CMV-specific antigen expression using CMV-immediate early antigen specific murine monoclonal antibody (MoAb) E13 kindly provided by Ronald Colimon (Mazeron *et al.*, 1983), CMV-late antigen specific murine MoAb 4E1 kindly provided by Giuliano

Furlini, Wistar Institute (Furlini *et al.*, 1987), and polyclonal human CMV-seronegative and seropositive serum pools. Individual human sera were selected for the pools on the basis of the absence or presence of CMV-specific antibodies as detected by anticomplementary immunofluorescence (Kettering *et al.*, 1977). Membrane antigens on target cells were detected using a technique described by Middeldorp, Jongsma & The (1986). Live target cells ($2{\cdot}0 \times 10^5$) were exposed to optimal dilutions of antibodies, as determined in preliminary studies, and incubated for 1 h on ice. Following two washes with precooled phosphate-buffered saline (PBS) fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-human Ig or goat anti-mouse IgG) was added. After 1 h incubation on ice the cells were washed three times with precooled PBS, mounted in 50% glycerol-PBS and examined in a fluorescent microscope (Carl Zeiss, Germany). For the detection of intranuclear immediate early antigens, target cells were fixed with acetone for 20 min at $-20°C$ and then incubated with a 1:100 dilution of E13 for 1 h. The target cells were then washed twice and incubated for 1 h with FITC-conjugated goat anti-mouse IgG. After three additional washes they were mounted and read. For both live and fixed preparations, 200 total cells were counted and the intensity of fluorescence of positive cells was scored on a scale of 1–4.

Preparation of effector cells

PBMC of healthy seropositive (No. 1 and 3) or seronegative (No. 2, 4, and 5) adult donors were obtained by Ficoll-Hypaque density gradient centrifugation. The serological status of each donor was determined by tests for anticomplementary immunofluorescence. Adherent cells were removed from PBMC by incubation on FBS-coated plastic Petri dishes as described by Kumagai *et al.* (1979), and the non-adherent PBMC obtained were used in all experiments.

Antibody plus complement (C′)-mediated lysis

Murine MoAb anti-Leu-11b (IgM) which recognizes the low-affinity Fc receptor (CD16 antigen) on neutrophils and on a subpopulation of granular lymphocytes known to contain most of the NK activity against K562 cells (Perussia *et al.*, 1984) was purchased from Becton Dickinson & Co., Mountain View, CA. MoAb B33.1 (IgG2a) which recognizes a non-polymorphic determinant of the HLA-DR molecule was provided by Giorgio Trinchieri, Wistar Institute (Perussia *et al.*, 1982). Antibodies were added to 5×10^6 PBMC in a total volume of 1 ml of RPMI 1640 containing 10% fetal bovine serum (FBS). Final dilutions based on preliminary experiments were: anti-Leu-11b, 0·1 μg/ml; and B33.1, 1:100. After 1 h incubation at room temperature, cells were centrifuged (100 *g* for 10 min), resuspended in 1 ml of baby rabbit C′ (Pel-Freeze Biologicals, Rogers, AZ), diluted 1:2 and incubated for 1 h at room temperature. The cells were centrifuged, the supernatant was removed, and treatment with C′ was repeated. The cells were then washed twice and resuspended in RPMI 1640 containing 10% FBS. Viability was determined by trypan blue exclusion. Treatment with C′ alone had no effect on cell viability and reduced spontaneous NK activity by $< 15\%$.

Cytolytic assay

Eighteen hour ^{51}Cr release assays for NK cell cytotoxicity against uninfected and CMV-infected targets were done as described by Bandyopadhyay *et al.* (1986). The effector-to-target cell ratios ranged from 25:1 to 100:1. All determinations were done in triplicate. Percent ^{51}Cr release was calculated using the formula: percent ^{51}Cr release = [ct/min experimental) − (ct/min spontaneous)]/[(ct/min total) − (ct/min spontaneous)] × 100 where spontaneous release was that obtained from target cells incubated with medium alone and total release was that obtained from target cells incubated with 1% Triton X-100. In 18 h assays spontaneous release from uninfected, AD-169-infected or low-passage CMV-strain-infected targets never exceeded 35% of total release.

Interferon assay

Supernatants from wells of NK assays were collected at the end of the 18 h incubation period, inactivated by exposure to ultraviolet light and quantified for antiviral activity using a cytopathic effect inhibition assay described by Arenzana-Seisdedos, Virelizier & Fiers (1985). Bovine cell line MDBK, which detects human IFN-α, but not human IFN-γ (Arbeit, Leary & Levin, 1982; Gresser *et al.*, 1974) was used as the indicator line. Titres were calculated in international units (IU) based on results obtained with the National Institutes of Health reference standard for IFN-α (G-023-901-527).

RESULTS

Expression of CMV antigens by infected target cells

Eighty to 90% of target cells infected with AD-169 or with low-passage clinical isolates demonstrated strong nuclear fluorescence when fixed and then stained with MoAb E13, which reacts with a CMV immediate-early antigen (Mazeron *et al.*, 1983) (Table 1). When unfixed targets were tested for membrane fluorescence, 80% and 75% of AD-169-infected cells were positive when stained with MoAb 4E1, which reacts with a late CMV antigen, or with polyclonal human serum, respectively. In contrast, with both antisera, the percentage of low-passage strain-infected target cells demonstrating membrane fluorescence was considerably lower in most cases (Table 1). None of the antibodies used stained uninfected cells, and control antibodies, murine ascites fluid and seronegative human serum, did not stain infected cells.

Susceptibility of target cells infected with low-passage isolates of CMV to PBMC mediated lysis

Preliminary experiments showed that similar killing curves were obtained for AD-169-infected and several low-passage strain-infected target cells with E:T ratios ranging from 25:1 to 100:1. In all subsequent experiments, E:T ratios of 50:1 were used. Uninfected target cells were variably lysed by PBMC from different healthy donors, but overall the magnitude of lysis of uninfected target cells was low. With AD-169-infected and most (five of eight) low-passage strain-infected target cells, mean lysis was significantly higher than that of uninfected target cells ($P < 0{\cdot}05$ for each comparison) (Table 2). Lysis of two other low-passage strain infected target cells was higher than that of uninfected target cells, but statistical significance was not achieved. The magnitude of killing of AD-169-infected and low-passage strain-infected target cells by PBMC of seropositive and seronegative donors was similar. Target cells infected with low-passage strain 2590 were not efficiently lysed by PBMC. With

Table 1. Immunofluorescent staining of targets inoculated with CMV strains

Target	Passage level of CMV for each target	Percent positive cells and intensity of immunofluorescence in the presence of various antisera					
		E13*		4E1†		Human‡	
		% Positive cells	Intensity§	% Positive cells	Intensity§	% Positive cells	Intensity§
Virus-infected							
AD-169	>250	90·0	4+	80·0	1–3+	75·0	1–3+
5809	4	85·0	4+	25·0	1–3+	15·0	1–2+
5723	7	90·0	4+	85·0	1–3+	70·0	1–3+
2590	7	87·0	4+	28·0	1–3+	20·0	1–2+
2786	6	80·0	4+	15·0	1–3+	12·0	1+
5852	7	90·0	4+	20·0	1–3+	10·0	1–2+
4237	10	90·0	4+	85·0	1–3+	80·0	1–3+
1356	7	90·0	4+	25·0	1–3+	10·0	1–2+
1354	7	85·0	4+	30·0	1–3+	10·0	1–2+
Uninfected	—	0·0	—	0·0	—	0·0	—

* MoAb E13, which reacts with an immediate-early CMV antigen, was added at a dilution of 1:100. Nuclear fluorescence was detected using fixed cells.

† MoAb 4E1, which reacts with a late CMV antigen, was added at a dilution of 1:100. Membrane fluorescence was detected using unfixed cells.

‡ Human polyclonal serum pool was added at a dilution of 1:30. Membrane fluorescence was detected using unfixed cells.

§ Fluorescence intensity was graded on a scale of 1–4.

Table 2. Cytotoxicity mediated by PBMC against CMV-infected targets*

Expt.	Donor†	Percent ^{51}Cr release with various target cells									
			Infected with various CMV strains								
		Uninfected	AD-169	5809	5723	2590	2786	5852	4237	1356	1354
1	1	4·2	34·2	37·5	17·8	6·7	18·7	23·4	20·1	22·1	25·7
2	2	9·8	44·4	42·0	24·6	11·8	17·0	25·6	27·6	37·1	38·0
	3	20·0	48·7	44·4	29·6	13·4	22·7	44·1	29·8	36·8	39·9
3	4	12·1	23·3	35·4	16·7	11·9	19·9	19·6	30·2	20·0	19·2
	5	20·0	39·9	38·7	47·6	17·8	41·9	35·3	24·8	50·3	46·1
1–3	1–5	13·2‡ ± 6·8	38·1§ ± 9·9	39·6§ ± 3·6	27·3 ± 12·5	12·3¶ ± 3·9	24·0 ± 10·2	29·6§ ± 9·9	26·5§¶ ± 4·2	33·3§ ± 12·4	33·8§ ± 11·0

* E:T ratios were 50:1.

† Donors 1 and 3 were seropositive for CMV, while 2, 4 and 5 were seronegative.

‡ Mean ± s.d.

§ $P < 0·05$ compared with results with uninfected targets, Student's *t*-test.

¶ $P < 0·05$ compared with results with AD-169-infected targets, Student's *t*-test.

this target cell ^{51}Cr release was comparable to that obtained with uninfected target cells and significantly lower than that obtained with AD-169-infected target cells ($P < 0·05$).

Identification of leucocytes participating in NK lysis of CMV-infected target cells

We have demonstrated previously that NK cell-mediated lysis of AD-169-infected fibroblasts requires the presence of two phenotypically distinct leucocyte populations: NK cells and accessory non-B, non-T, non-NK, non-monocytic peripheral blood leucocytes which bear HLA-DR molecules on their surface (Bandyopadhyay *et al.*, 1986). We determined whether accessory HLA-DR$^+$ cells were also needed for NK lysis of low-passage CMV-infected target cells. Treatment of PBMC with anti-Leu-11b, an MoAb which reacts with NK cells, plus C′ or with anti-HLA-DR plus C′, markedly reduced NK activity against target cells infected with AD-169 or each of five low-passage CMV-isolates tested (Table 3). When HLA-DR-de-

Table 3. Cytotoxicity mediated by PBMC, Leu-11b$^-$, and HLA-DR$^-$ cells against CMV-infected targets

Expt. no.	Targets	Percent ^{51}Cr release in the presence of various effector cells*			
		PBMC (C′ treated)†	Leu-11b$^-$†	HLA-DR$^-$†	Leu-11b$^-$/HLA-DR$^-$‡
1	Uninfected	2·6	0·0	9·9	8·9
	AD-169-infected	29·7	0·0	0·0	28·4
	1354-infected	28·3	0·0	4·8	25·5
	1356-infected	11·0	4·0	0·0	15·6
2	Uninfected	3·3	2·2	3·1	6·8
	AD-169-infected	25·0	3·9	2·3	23·6
	5723-infected	18·3	5·8	5·0	17·7
	5852-infected	19·5	0·0	0·6	12·1
	5809-infected	42·5	4·7	3·3	33·5

* Leu-11b$^-$ and HLA-DR$^-$ cells were prepared as described in Materials and Methods. Different donors were used in experiments 1 and 2.

† The E:T ratio was 50:1.

‡ Equal numbers of Leu-11b$^-$ and HLA-DR$^-$ cells were mixed and added to assays at a total E:T ratio of 100:1.

Table 4. IFN concentrations in supernatants of NK assays

Expt. no.	Donor†	IFN concentrations in supernatants of assays containing various targets*								
		Uninfected	Infected with various CMV strains							
			AD169	5809	5723	2590	2786	5852	4237	1356	1354
1	1	<1·0	16·6	66·6	266·2	133·1	8·3	66·6	66·6	133·1	133·1
2	2	<8·3	29·6	118·4	473·6	236·8	14·8	59·2	118·4	59·2	118·4
	3	<8·3	14·8	59·2	236·8	118·4	14·8	29·6	29·4	29·6	59·2
3	4	<4·2	4·2	ND	133·4	66·7	4·2	33·4	ND	16·7	33·4
	5	<4·2	33·4	ND	533·8	266·9	8·3	66·7	ND	66·7	266·9
1–3	1–5	—	19·7‡ ± 11·8	81·4§ ± 32·2	328·7§ ± 168·5	164·4§ ± 84·2	10·1 ± 4·6	51·1§ ± 18·2	71·5§ ± 44·6	61·1 ± 45·2	122·2§ ± 90·7

* IFN-α was detected using MDBK cells as described in Materials and Methods. Concentrations are expressed as IU/ml.

† The donors and their serological status are the same as in Table 2.

ND, not done.

‡ Mean ± s.d.

§ $P < 0·05$ compared to results with AD-169-infected targets, Student's *t*-test

pleted cells were added to Leu-11b-depleted cells, NK activity against target cells infected with AD-169 or low-passage CMV isolates was largely restored (Table 3). These results indicate that NK cells mediate lysis of low-passage CMV isolate-infected target cells, and that the presence of accessory HLA-DR$^+$ cells is also required for lysis to occur.

Interferon concentrations in supernatants of assays for NK against CMV-infected target cells

We have demonstrated previously that IFN-α produced by accessory HLA-DR$^+$ cells stimulated NK cells to mediate lysis of AD-169-infected target cells (Bandyopadhyay *et al.*, 1986). Experiments were done to determine whether IFN-α was also produced during NK cell mediated lysis of low-passage strain-infected target cells. Supernatants harvested at the end of NK assays were tested for IFN-α. Supernatants of infected or uninfected target cells incubated without effector cells did not contain detectable amounts of IFN-α (result not shown). PBMC incubated with uninfected target cells did not produce detectable IFN-α (Table 4). PBMC incubated with target cells infected with either AD-169 or one of the eight low-passage CMV isolates tested, produced substantial amounts of IFN-α. Interestingly, IFN-α levels obtained when PBMC were incubated with targets infected with six of the eight low-passage CMV isolates tested were significantly higher than the level obtained when AD-169-infected target cells were used (Table 4). With one low-passage strain, 2786, levels of IFN-α were similar to those obtained with AD-169-infected target cells. The magnitude of

NK lysis of different CMV-infected target cells did not correlate with the levels of interferon detected in assay supernatants ($r=0{\cdot}3033$, $P>0{\cdot}05$).

DISCUSSION

Natural killing of CMV-infected fibroblasts has been described by several groups (Borysiewicz *et al.*, 1985; Kirmani *et al.*, 1981; Rook *et al.*, 1984; Starr & Garrabrant, 1981). In most of these studies only target cells prepared from the highly passaged AD-169 strain of CMV were used. Waner & Nierenberg (1985) tested target cells infected with Davis, another highly passaged laboratory strain of CMV, and found them to be resistant to NK lysis.

The present study indicated that fibroblasts infected with low-passage clinical isolates of CMV were, with one exception, as susceptible to lysis as AD-169-infected target cells. The effector cells responsible for lysis of both low passage and AD-169-infected target cells were identified as NK cells on the basis of depletion of activity after treatment of effector cells with anti-Leu-11b, an MoAb which reacts with NK cells (Perussia *et al.*, 1984), plus C′.

The magnitude of lysis of CMV-infected target cells was similar when effector cells of seronegative or seropositive donors were used. Individual donors demonstrated similar patterns of lysis of the different CMV-infected target cells indicating a lack of strain specificity. Both of these results are consistent with previous observations that NK lysis of CMV-infected target cells does not appear to require presensitization of donors (Starr & Garrabrant, 1981).

Immunofluorescent staining was done to determine the percentage of target cells that expressed CMV antigens as a result of virus infection. Most fibroblasts inoculated with AD-169 and low-passage strains expressed intranuclear immediate-early CMV antigen indicating that infection had occurred. With most of the low-passage-strain-infected target cells late CMV antigens were expressed on fewer cells than with AD-169-infected target cells. These results are of interest in view of earlier observations that low-passage strain CMV-infected fibroblasts release much less infectious virus than high-passage ones (Plotkin *et al.*, 1975). The reduced membrane fluorescence observed with low-passage-strain infected fibroblasts may be correlated, at least in part, with less release of virus extracellularly from such fibroblasts compared to that from AD-169 infected fibroblasts.

Despite reduced expression of late CMV antigens on the membranes of low-passage strain infected fibroblasts, these cells were as susceptible to NK lysis as AD-169-infected fibroblasts which expressed greater amounts of membrane antigen. One possible explanation is that the low-passage strain infected fibroblasts may express CMV early antigens (EA). It was previously shown that AD-169-infected fibroblasts treated with phosphonoacetic acid to block late antigen expression were as susceptible to NK lysis as untreated infected targets (Borysiewicz *et al.*, 1985). Studies are in progress to determine whether low-passage strain-infected target cells express CMV EA on their membranes. Alternatively, the presence of CMV membrane antigens may not be required for NK lysis of infected target cells to occur. Borysiewicz *et al.* (1985) have published several observations which tend to support this possibility: (1) uninfected fibroblasts inhibit the lysis of AD-169-infected fibroblasts in cold target inhibition studies; (2) effector cells allowed to absorb to uninfected fibroblasts were depleted of NK activity against AD-169 infected targets; and (3) polyclonal human antisera against CMV failed to inhibit NK lysis of CMV-infected targets. These observations suggest that structures found on uninfected fibroblasts may be involved in NK lysis of infected targets. A recent study by this same group suggests that the transferrin receptor is not involved (Borysiewicz, Graham & Sissons, 1986).

We have previously shown that HLA-DR$^+$ accessory cells are required for NK lysis of AD-169-infected targets (Bandyopadhyay *et al.*, 1986). These cells were further characterized as non-B, non-T, non-NK, non-monocytic cells which produce IFN-α when incubated with CMV-infected targets. The IFN-α then stimulates NK cells to mediate lysis of infected targets. In the present study we have shown that HLA-DR$^+$ accessory cells are also required for NK lysis of low passage strain-infected targets. Presumably, as in the case for AD-169-infected targets, the role of HLA-DR$^+$ cells is to produce IFN-α. No correlation was found, however, between the amounts of IFN-α produced and the magnitude of NK lysis of different CMV-infected targets. These results suggest that the magnitude of lysis depends on variables in addition to stimulation of NK cells by IFN-α. An interesting observation was the greater induction of IFN-α by low-passage strain infected fibroblasts than by AD-169-infected fibroblasts. Considering the results of immunofluorescent staining, the expression of viral antigens on infected target cell membranes does not appear to correlate with the ability of targets to induce IFN-α production. It is of interest in this regard that highly purified CMV virions are poor inducers of IFN-α (Starr *et al.*, 1980). Taken together these results suggest that one or more virus-specified or induced, but non-structural, moieties induce HLA-DR$^+$ cells to produce IFN-α.

One of the low-passage-strain-infected target cell lines (2590) was poorly susceptible to NK lysis. This result was similar to that previously reported for Davis-infected targets (Waner & Nierenberg, 1985), results that we have confirmed (unpublished results). Davis strain infected targets induced levels of IFN-α similar to those induced by AD-169-infected target cells (Waner & Nierenberg, 1985). In our experiments strain 2590-infected target cells induced higher levels of IFN-α than did AD-169-infected target cells. Thus, decreased susceptibility of NK lysis could not be attributed to decreased IFN-α production. The mechanism of resistance to lysis of these two CMV-infected targets is currently unknown.

In summary, we have shown that target cells infected with most low passage strains of CMV induce IFN-α and are susceptible to NK lysis. Low passage CMV strain-infected target cells have also been shown to be susceptible to killing by cytotoxic T lymphocytes (Schrier & Oldstone, 1986). These observations increase the probability that both types of cytotoxic activity occur *in vivo*.

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