REQUIREMENT FOR HLA-DR$^+$ ACCESSORY CELLS IN NATURAL KILLING OF CYTOMEGALOVIRUS-INFECTED FIBROBLASTS

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NK cells mediate spontaneous killing of tumor-derived cells, virus-infected cells, and certain normal cells (1, 2). This type of cytotoxicity does not require presensitization of the donor. For example, PBMC of individuals who are seronegative or seropositive for a given virus are equally able to lyse targets infected with that virus (3). Production of IFN by lymphocytes exposed to virus-infected target cells and subsequent stimulation of NK cells by IFN was originally proposed as the mechanism by which NK cells preferentially lyse virus-infected cells compared with uninfected ones (4). A primary role for IFN was challenged, however, by several authors who described a lack of correlation between magnitude of lysis and amounts of IFN detected in supernatant fluids (5, 6), an almost normal capacity of effector cells from patients with reduced ability to produce IFN to lyse virus-infected target cells (7), and the inability of anti-IFN antibodies when present during the NK assay to prevent lysis of virus-infected cells (5, 6).

The cells responsible for NK activity against normal and tumor-derived target cell lines were identified as a leukocyte subset, distinct from B and T cells and from myelomonocytic cells (2). This subset expresses the low-affinity Fc receptor (FcR)[1] for aggregated IgG (CD16 antigen), recognized by a series of mAbs (8). NK cells responsible for lysis of virus-infected target cells have not been fully identified. Fitzgerald et al. (9) reported that the NK cells able to lyse HSV-infected targets differed from those that lysed K562 cells, as treatment of PBMC with an mAb to HLA-DR plus C reduced their ability to kill HSV-infected fibroblasts, but not K562 cells. These authors concluded that these NK cell subsets could be distinguished on the basis of surface expression of HLA-DR antigen. However, few if any resting NK (CD16+) cells in healthy donors are HLA-DR$^+$ (10, 11), raising the possibility that in the experiments of Fitzgerald et al. (9), a HLA-DR$^+$, non-NK cell population was depleted.

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[1] *Abbreviations used in this paper:* CMV cytomegalovirus-infected; FcR, Fc receptor; FS4, foreskin fibroblast strain; NK(CMV-FS4), NK activity against CMV-infected FS4 targets; NK(FS4), NK activity against uninfected FS4 targets; NK(K562), NK activity against K562 targets.

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In this study we investigated the role of HLA-DR$^+$ cells in NK activity against virus-infected cells, using as targets (cytomegalovirus-infected) CMV-infected fibroblasts, which were previously shown to be susceptible to NK cell–mediated lysis (3, 12, 13). Our results indicate that treatment of PBMC with anti-HLA-DR antibody plus C reduces cytotoxic activity against CMV-infected targets, but not against K562, by depleting an HLA-DR$^+$,CD16$^-$ population that contains accessory cells that stimulate CD16$^+$ NK cells to exert cytotoxic activity against CMV-infected targets. A factor that stimulates NK cytotoxic activity is produced when such HLA-DR$^+$ cells are cocultivated with CMV-infected targets. This factor is identified as IFN-α.

Materials and Methods

Media and Reagents. RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) was supplemented with 2 mM glutamine, 125 IU/ml penicillin, 6.25 μg/ml gentamicin, and 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT). Eagle's modified MEM (Gibco Laboratories) was supplemented with 7.5% FCS, 2% vitamins, and 2 mM glutamine. rIFN-α with antiviral activity of $5.2 \pm 3.4 \times 10^6$ IU/ml was kindly provided by Patrick Trown, Hoffman-LaRoche Inc., Nutley, NJ.

Monoclonal and Polyclonal Antibodies. mAb anti-Leu-11b (clone G022, IgM; reference 8), which recognizes the low-affinity Fc receptor (CD16 antigen) on a subpopulation of granular lymphocytes known to contain most of the NK activity against K562 cells and on neutrophils, was purchased from Becton Dickinson & Co., Mountain View, CA. The preparation of antibody B33.1 (IgG2a, reference 14), which recognizes a nonpolymorphic determinant of the HLA-DR molecule, anti-monocyte (CD14 antigen) antibodies B52.1 (IgM, reference 15), B13.4 (IgM, reference 16), anti-pan T cell (CD5 antigen) antibody B36.1 (IgG2b, reference 14) was previously described (14, 15). The antigen defined by OKM1 mAb is present on monocytes and granulocytes (17). Anti–B cell BA1 (18) was purchased from Hybritech, Inc. (San Diego, CA). Antibody B133.3 (IgG1), which neutralizes human IFN-γ but not IFN-α or -β, was previously described (19). The tissue culture supernatant fluid used in the present study had a neutralizing titer of 32,400 U/ml. A sheep antiserum against human IFN-α (10^5 neutralizing U/ml) was obtained from Interferon Sciences Inc. (New Brunswick, NJ). A calf antiserum to human IFN-β (2×10^5 neutralizing U/ml) was obtained from ICN Radiochemicals, Irvine, CA.

Preparation of Target Cells. CMV-infected (CMV-FS4) and uninfected (FS4) fibroblasts were prepared as previously described (3). Briefly, human embryonic foreskin fibroblasts (FS4 strain, National Institutes of Allergy and Infectious Diseases, Bethesda, MD) were maintained in MEM and used at passage level 12–22. Monolayers of FS4 in 75-cm^2 flasks were infected with human cytomegalovirus strain AD-169 (American Type Culture Collection, Rockville, MD) at a multiplicity of infection of ~0.1. When ~90% of cells exhibited cytopathic effect (after 5–7 d), the monolayers were trypsinized, washed, and suspended in MEM containing 10% DMSO. Aliquots were kept at −70°C for 12–16 h, transferred to the vapor phase of a liquid nitrogen freezer, and kept there until use. K562 cells, kindly provided by Drs. Gertrude and Werner Henle (Children's Hospital of Philadelphia), were passaged in RPMI-1640 supplemented with 10% FCS, and cryopreserved as described above. Cell lines and target cells were routinely tested for mycoplasma and were found to be negative.

Preparation of Effector Cells. PBMC of healthy randomly selected seropositive or seronegative adult donors were separated by Ficoll-Hypaque density gradient centrifugation. Adherent cells were removed by incubation on FCS-coated plastic petri dishes (Corning Glass Works, Corning, NY) for 1 h at 37°C as previously described by others (20). Nonadherent PBMC were used in all experiments except those indicated, in which adherent cells, obtained by scraping the plastic dishes after extensive washing with PBS, were used. Cell viability of adherent and nonadherent populations as determined by trypan blue exclusion was >98%.

Antibody Plus C-mediated Lysis. Antibodies were added to 5×10^6 PBMC in a total volume of 1 ml of RPMI 1640 containing 10% FCS. Final dilutions based on preliminary experiments were: anti-Leu-11b, 1 μg/ml; B33.1/ascitic fluid, 1:100; B52.1/ascitic fluid, 1:200; B13.4/ascitic fluid, 1:200; OKM1/culture supernatant, 1:2; B36.1/culture supernatant, 1:4; BA1, 1 μg/ml. In selected experiments more than one antibody was added to PBMC. After a 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% FCS. Viability was determined by trypan blue exclusion. Treatment with C alone had no effect on cell viability and reduced spontaneous NK (CMV-FS4) by <15%.

Detection of Surface Markers by Direct Immunofluorescence. The proportion of NK, B, T, monocytic, and HLA-DR+ cells in the preparations treated with antibody and C was determined by immunofluorescence (flow cytofluorometry), as described (14). FITC conjugates of the following antibodies were used: CLB (anti-CD16), kindly provided by Dr. Tetteroo (Central Laboratory of the Netherlands, Amsterdam) for the detection of NK cells (21); OKT3 (anti-CD3) for detection of T cells (22); B1 for detection of B cells (23); B44.1 (anti-CD14) for detection of monocytes (15); and B33.1 for detection of HLA-DR+ cells. PBMC were resuspended in PBS (pH 7.2) containing 2% FCS and incubated with previously determined optimal dilutions of the FITC-conjugated antibodies for 30 min at 4°C. The cells were then washed three times and analyzed using a Cytofluorograf System 50 HH connected to a 2150 data handling system (Ortho Diagnostic Systems Inc., Westwood, MA). Cells were considered positive when their fluorescence intensity exceeded the threshold at which 99% of unstained cells had lower fluorescence intensity (14).

Nonspecific Esterase Staining. Nonspecific esterase stains were done as previously described (24).

Irradiation of Effector Cells. Aliquots of PBMC, Leu-11b−, or HLA-DR− cells (5×10^6/ml) were exposed at room temperature to 50 Gy from a cesium-137 source (Gammator M; Isomedix Inc., Parsippany, NJ). Control aliquots kept at room temperature were not irradiated. All cells were assayed within 1 h after irradiation.

Cocultivation of PBMC, Leu-11b−, and HLA-DR− Cells with Uninfected and CMV-infected Fibroblasts. PBMC, Leu-11b−, or HLA-DR− cells were cocultured with FS4 or CMV-FS4 for 18 h at 37°C at an E/T ratio of 50:1 (2.5×10^6 effector cells and 5×10^4 target cells per ml) in a total volume of 3 ml in 5-ml plastic tubes. Cell-free supernatant fluid was collected after centrifugation (100 *g* for 10 min) and kept at −70°C for subsequent determination of antiviral activity and ability to stimulate HLA-DR− cells to mediate NK (CMV-FS4).

IFN Titration. A previously described cytopathic effect inhibition assay was used to quantitate antiviral activity (25). Briefly, twofold serial dilutions of samples were incubated with WISH cells in microtiter plates for 24 h at 37°C. The monolayers were then challenged with vesicular stomatitis virus (2,500 PFC/well). After an additional 24-h incubation at 37°C, the monolayers were stained with 0.5% crystal violet/70% methyl alcohol. Each assay included cell and virus controls. Titers were calculated in IU based on results obtained with the National Institutes of Health reference standards for IFN-α (G-023-901-527) and IFN-γ (Gg23-901-530).

NK Cell Assays. NK cell assays against K562 cells and uninfected and CMV-infected fibroblasts were performed as described (3). Cryopreserved targets were thawed rapidly, washed twice, and resuspended in 0.2 ml of HBSS. 50 μCi of $Na_2{}^{51}CrO_4$ (407 mCi/mg spe act; ICN Radiochemicals, Irvine, CA) were added per $1-2 \times 10^6$ cells. After 1 h incubation at 37° C in 5% CO_2 with gentle shaking every 15 min, the cells were washed four times and then resuspended in RPMI 1640 with 10% FCS at a concentration of 5×10^4 cells/ml. 5×10^3 target cells in 0.1 ml of RPMI 1640 containing 10% FCS and various concentrations of effector cells in 0.1-ml aliquots were added to round-bottomed microtiter wells (Linbro Chemical Co., Hamden, CT) to yield the desired E/T ratios. All

TABLE I

Cytotoxicity Mediated by PBMC, Leu-11b$^-$, and HLA-DR$^-$ Cells Against Various Target Cells

Donor	Target cell FS4			Target cell CMV-FS4				Target cell K562		
	PBMC	Leu-11b$^-$*	HLA-DR$^-$*	PBMC	Leu-11b$^-$	HLA-DR$^-$	Leu-11b$^-$ + HLA-DR$^-$‡	PBMC	Leu-11b$^-$	HLA-DR$^-$
1	0.0	0.0	0.0	20.2	6.1	4.1	ND	38.3	5.7	28.0
2	11.8	0.6	10.6	31.1	11.8	3.8	ND	70.0	6.6	66.3
3	0.0	0.0	0.0	31.2	4.3	4.0	ND	66.2	2.3	64.5
4	10.8	4.0	13.5	35.0	10.5	2.6	ND	48.6	3.6	49.2
5	ND	ND	ND	30.1	3.4	0.0	22.2	ND	ND	ND
6	7.0	0.0	7.1	27.2	4.0	2.8	28.6	50.5	0.0	54.3
7	ND	ND	ND	45.3	2.3	0.0	42.3	ND	ND	ND
8	ND	ND	ND	28.5	7.4	2.8	23.0	ND	ND	ND
1–8§	5.9 ± 5.7	0.9 ± 1.7‖	6.2 ± 6.1	31.1 ± 7.1	6.2 ± 3.4¶	2.5 ± 1.6¶	29.0 ± 9.3	54.7 ± 13.1	3.6 ± 2.6¶	52.5 ± 15.4

Cytotoxicity (percent ^{51}Cr release) was assayed as described in Materials and Methods. E/T ratio, 50:1.

* Leu-11b$^-$ and HLA-DR$^-$ cells were prepared as described in Materials and Methods.

‡ Equal numbers of Leu-11b$^-$ and HLA-DR$^-$ cells were mixed and added to assays.

§ Mean ± SD.

‖ $p < 0.05$ compared with results with PBMC, paired t test (only data for donors 2, 4, and 6 were included in the analysis).

¶ $p < 0.001$ compared with results with PBMC, paired t test.

determinations were done in triplicate. Plates were centrifuged at 100 *g* for 6 min and then incubated for 18 h at 37°C in 5% CO_2. After incubation, 0.1 ml of supernatant was collected from each well without disturbing the cell pellet and counted in a gamma counter (4000; Beckman Instruments, Inc., Fullerton, CA). In selected experiments, additional 50 μl aliquots of supernatant were harvested from triplicate wells, pooled, and kept at −70°C for subsequent determinations of antiviral activity. The percent of ^{51}Cr release was calculated using the formula: percent ^{51}Cr release = 100 × [(cpm experimental) − (cpm spontaneous)]/[(cpm total) − (cpm spontaneous)] 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 detergent. In 18 h assays, spontaneous release from uninfected and CMV-infected targets never exceeded 35% of total release while spontaneous release from K562 cells never exceeded 30%.

Statistical Analysis. The two-tailed paired t test was used for all analyses.

Results

Cells Participating in NK Cytotoxicity against Different Targets. Uninfected FS4 cells were lysed at a low level of magnitude by PBMC from some, but not all, healthy donors, whereas CMV-infected FS4 cells were efficiently lysed by PBMC of all donors tested (Table I). To identify the cells participating in NK cell–mediated cytotoxicity, the surface markers of the effector cell populations active against uninfected fibroblasts, CMV-infected fibroblasts, and a tumor-derived target cell line (K562) were compared using the anti-CD16 (low-affinity FcR) antibody anti-Leu-11b and the anti-HLA-DR antibody B33.1. Treatment of PBMC with anti-Leu-11b plus C significantly reduced NK(CMV-FS4), NK(FS4), and NK(K562) (Table I and Fig. 1). In contrast, treatment of PBMC with antibody B33.1 plus C significantly reduced NK(CMV-FS4), but not NK(K562) or NK(FS4). Since the effect was consistent at all E/T ratios tested (Fig. 1), subsequent experiments were done using E/T ratios of 50:1.

To define further the HLA-DR$^+$ cells required for NK(CMV-FS4), PBMC were treated with mAbs specific for cells of different lineages plus C, as described in Materials and Methods. Depletion of T cells to <1.8%, as determined by

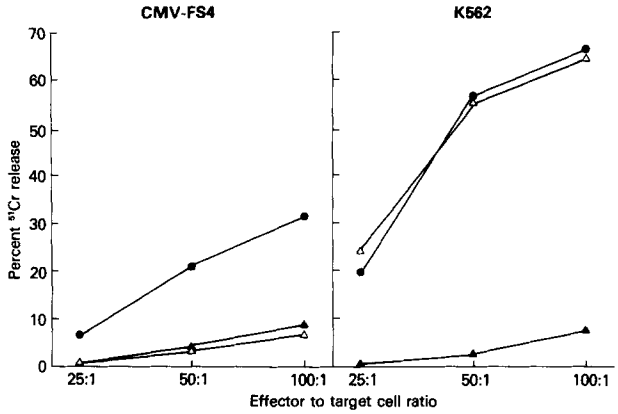

FIGURE 1. Effect of depletion of CD16(Leu-11b)$^+$ and HLA-DR$^+$ cells on NK (CMV-FS4) (*left*) and NK(K562) (*right*). PBMC were treated wtih C (●), anti-Leu-11b + C (▲), or B33.1 + C (△) as described in Materials and Methods, washed twice, and tested at different E/T ratios for NK activity. Results shown are representative of four similar experiments.

immunofluorescence (flow cytometry), had no effect on NK(CMV-FS4) (Table II, Exp. 1). Similarly, depletion of either B cells or monocytes to <0.1%, as determined by flow cytometry and nonspecific esterase staining respectively, or of both B cells and monocytes had no effect on NK(CMV-FS4) (Table II, Exp. 2). In another approach, monocytes and B cells were depleted from Leu-11b$^-$ cells with specific mAbs and C. When such populations depleted of monocytes (<0.5% esterase-positive), B cells, and NK cells were mixed with HLA-DR$^-$ cells at a ratio of 1:1 and tested for NK(CMV-FS4), activity was largely restored (Table II, Exp. 3). Finally, graded numbers of plastic dish adherent cells (>95% nonspecific esterase-positive) were added to HLA-DR$^-$ cells and the mixtures tested for NK(CMV-FS4) activity. While addition of as few as 2.5×10^4 Leu-11b$^-$ cells to HLA-DR$^-$ cells largely reconstituted NK(CMV-FS4), addition of as many as 2.5×10^5 adherent cells to HLA-DR$^-$ cells failed to reconstitute NK(CMV-FS4) (Table II, Exp. 4). Thus, these data indicate that HLA-DR$^+$ cells required for NK(CMV-FS4) were neither B or T lymphocytes, nor were they monocytes.

Since NK(CMV-FS4) activity was largely restored (Tables I and II) when Leu-11b$^-$ and HLA-DR$^-$ cell populations of individual donors were mixed, treatment with anti-Leu-11b or anti-HLA-DR plus C appeared to suppress cytotoxicity by acting on two different cell populations, one Leu-11b$^-$, HLA-DR$^+$ and the other Leu-11b$^+$,HLA-DR$^-$.

Leu-11b$^-$ and HLA-DR$^-$ cells were next combined at additional E/T ratios. NK(CMV-FS4) and NK(K562) rose as the percentage of HLA-DR$^-$ cells was increased from 0 to 80% and 0 to 50%, respectively (Fig. 2). There was no further increase in NK activity against the two targets after the percentage of HLA-DR$^-$ cells reached 97%. Maximal NK(K562) was still observed with 100% HLA-DR$^-$ cells. NK(CMV-FS4) was not mediated by HLA-DR$^-$ cells in the absence of Leu-11b$^-$ cells, but 3% Leu-11b$^-$ cells were sufficient to reconstitute maximal NK(CMV-FS4). Because <20% of the cells in Leu11b$^-$ preparations were HLA-DR$^+$ (not shown), <0.6% HLA-DR$^+$ cells were required to fully

TABLE II
Characterization of Cells Contributing to NK Against CMV-FS4

Exp.	Effector cells in NK assays	Percent ^{51}Cr release from CMV-FS4	
		Donor 1	Donor 2
1	PBMC	14.2	26.7
	B36.1$^-$	11.0	24.0
2*	PBMC	23.4	23.7
	BA1$^-$	22.7	25.8
	B52.1$^-$	23.2	22.6
	B13.4$^-$	20.4	39.5
	BA1$^-$,B52.1$^-$,B13.4$^-$	20.9	29.6
3‡	PBMC	19.3	ND
	Leu-11b$^-$	7.9	ND
	Leu-11b$^-$,BA1$^-$,B52.1$^-$,B13.4$^-$,OKM1$^-$	6.7	ND
	HLA-DR$^-$	1.4	ND
	Leu-11b$^-$ + HLA-DR$^-$	16.5	ND
	Leu-11b$^-$,BA1$^-$,B52.1$^-$,B13.4$^-$,OKM1$^-$ + HLA-DR$^-$	18.2	ND
4§	PBMC	31.5	25.7
	HLA-DR$^-$	4.5	1.0
	Leu-11b$^-$	7.8	6.1
	HLA-DR$^-$ + Leu-11b$^-$ (2.5×10^4)	36.3	17.7
	HLA-DR$^-$ + Leu-11b$^-$ (5×10^4)	36.6	23.9
	HLA-DR$^-$ + Leu-11b$^-$ (2.5×10^5)	34.0	21.4
	Adherent cells	<1.0	1.7
	HLA-DR$^-$ + adherent cells (2.5×10^4)	2.9	3.9
	HLA-DR$^-$ + adherent cells (5×10^4)	6.4	4.9
	HLA-DR$^-$ + adherent cells (2.5×10^5)	5.7	ND

* PBMC or PBMC depleted of subsets of cells as described in Materials and Methods were added to CMV-FS4 at an E/T ratio of 50:1.
‡ Final E/T ratios were 50:1 for all effector cell populations including mixtures. Mixtures consisted of equal numbers of depleted cell populations.
§ Graded numbers (as indicated in parentheses) of Leu-11b$^-$ cells or plastic dish adherent cells and 2.5×10^5 HLA-DR$^-$ cells were added to CMV-FS4.

reconstitute the ability of HLA-DR$^-$ cells to mediate NK(CMV-FS4). These results suggested that CD16(Leu11b)$^+$ cells were the effector cytotoxic cells for both NK(K562) and NK(CMV-FS4), while small numbers of HLA-DR$^+$ cells provided an accessory function for NK(CMV-FS4).

Effect of Irradiation on the Ability of PBMC, Leu-11b$^-$, and HLA-DR$^-$ Cells to Mediate NK(CMV-FS4). Our previous experiments suggested that Leu-11b$^+$ and HLA-DR$^+$ cells have different functions in mediating NK(CMV-FS4). We therefore determined whether these functions were equally sensitive to irradiation, which has been reported to reduce NK(K562) (26, 27). PBMC-mediated NK(CMV-FS4) was markedly reduced after irradiation with 50 Gy (Table III). Mixtures of irradiated HLA-DR$^-$ cells plus nonirradiated Leu-11b$^-$ cells had little or no NK(CMV-FS4) activity, whereas mixtures of irradiated Leu-11b$^-$ cells plus nonirradiated HLA-DR$^-$ cells mediated levels of NK(CMV-FS4) similar to those of mixtures of nonirradiated cells (Table III).

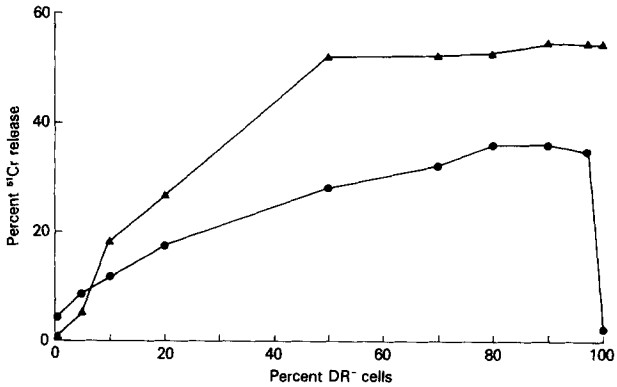

FIGURE 2. Effect of combining CD16(Leu-11b)$^-$ and HLA-DR$^-$ cells on NK activity against CMV-FS4 (●) and K562 (▲). CD16(Leu-11b)$^-$ and HLA-DR$^-$ cells, prepared as in Fig. 1 were mixed at varying ratios and added to targets at an E/T ratio of 50:1.

TABLE III

Effect of Irradiation of PBMC, Leu-11b$^-$, and HLA-DR$^-$ Cells on NK Cytotoxicity Against CMV-FS4 Target Cells

Effector cells*	Percent ^{51}Cr release
PBMC	45.3
PBMC (irradiated)‡	10.4
Leu-11b$^-$	2.3
HLA-DR$^-$	0.0
Leu-11b$^-$ + HLA-DR$^-$§	42.3
Leu-11b$^-$ (irradiated) + HLA-DR$^-$ (irradiated)§	3.3
Leu-11b$^-$ (irradiated) + HLA-DR$^-$§	33.1
Leu-11b$^-$ + HLA-DR$^-$ (irradiated)§	7.2

* E/T, 50:1.
‡ PBMC, Leu-11b$^-$, and HLA-DR$^-$ cells were irradiated with 50 Gy.
§ Equal numbers of Leu-11b$^-$ and HLA-DR$^-$ cells were added to the NK assay.

Supernatant Fluids from Cocultures of PBMC or Leu-11b$^-$ Cells with CMV-FS4 Contain IFN-α Which Stimulates HLA-DR$^-$ Cells to Mediate NK(CMV-FS4) and NK(FS4). Since HLA-DR$^-$ cells mediated NK(CMV-FS4) in the presence of small numbers of Leu-11b$^-$ cells (3%), we tested the possibility that Leu-11b$^-$ cells might secrete soluble factor(s) that stimulate NK cells present in HLA-DR$^-$ populations to mediate NK(CMV-FS4). PBMC, Leu-11b$^-$, or HLA-DR$^-$ cells were cocultured with FS4 or CMV-FS4 at a ratio of 50:1 for 18 h at 37° C. Cell-free supernatants were then collected and added at a final dilution of 1:2 in cytotoxicity assays using HLA-DR$^-$ or Leu-11b$^-$ cells as effectors. The magnitude of NK(CMV-FS4) mediated by Leu-11b$^-$ effector cells remained low despite addition of supernatants generated by coculturing PBMC, Leu-11b$^-$, or HLA-DR$^-$ cells with CMV-FS4 (results not shown). In contrast, when supernatants generated by coculturing PBMC or Leu-11b$^-$ cells with CMV-FS4 were added to HLA-DR$^-$ cells, NK(CMV-FS4) activity was completely restored (Table IV). NK(CMV-FS4) was not restored when supernatants generated by coculturing

TABLE IV

Effect of Supernatants Generated by Coculturing PBMC, Leu-11b$^-$, or HLA-DR$^-$ Cells with Uninfected or CMV-infected Targets on Cytotoxicity Mediated by Fresh HLA-DR$^-$ Cells Against CMV-FS4

Effector cells*	Supernatant added to NK assay‡	Antiviral activity (IU/ml) of the supernatant§	Percent ^{51}Cr release	
			Exp. 1	Exp. 2
PBMC	None	—	44.6	48.6
HLA-DR$^-$	None	—	8.4	18.6
HLA-DR$^-$	PBMC + FS4	<20	5.6	11.5
HLA-DR$^-$	PBMC + CMV-FS4	2,406	51.3	51.6
HLA-DR$^-$	Leu-11b$^-$ + CMV-FS4	2,406	58.2	46.8
HLA-DR$^-$	HLA-DR$^-$ + CMV-FS4	<20	5.8	ND

* PBMC and HLA-DR$^-$ cells were prepared as described in Materials and Methods. E/T, 50:1.
‡ The leukocyte-target cell combination used to generate each supernatant is shown. Leukocytes were cocultivated with target cells at a ratio of 50:1 for 18 h at 37° C as described in Materials and Methods. Aliquots of 100 μl of supernatants were added to NK assays to give final concentrations of 1:2.
§ Antiviral activity of supernatants was measured as described in Materials and Methods.

TABLE V

Effect of PBMC-CMV-FS4 Supernatant and rIFN-α on NK activity against FS4 and CMV-FS4

Exp.	Donors (*n*)	Effector cells*	Additive to medium	Percent ^{51}Cr release from:	
				FS4	CMV-FS4
1‡	5	PBMC	none	3.6 ± 4.1§	30.4 ± 15.2
		HLA-DR$^-$	none	4.5 ± 3.3	7.7 ± 6.6
		HLA-DR$^-$	supernatant	21.5 ± 8.4	39.7 ± 11.6
2‖	2	PBMC	none	4.0 ± 5.6	19.3 ± 9.9
		PBMC	supernatant	37.5 ± 2.2	52.8 ± 10.4
		PBMC	rIFN-α	40.4 ± 3.9	56.6 ± 13.4
		HLA-DR$^-$	none	0.0 ± 0.0	5.5 ± 7.7
		HLA-DR$^-$	supernatant	27.3 ± 0.5	43.5 ± 4.9
		HLA-DR$^-$	rIFN-α	20.8 ± 1.1	37.2 ± 9.3

* E/T, 50:1.
‡ 100 μl of the supernatant generated as described in Materials and Methods were added to NK assays to give final concentrations of 1:2.
§ Mean ± SD.
‖ Effector cells were incubated with supernatant (1:4 final dilution) or recombinant IFN-α (500 IU/ml) for 18 h, washed twice, and added to the NK assays.

HLA-DR$^-$ cells with CMV-FS4 or PBMC with FS4 were added to HLA-DR$^-$ cells (Table IV). In the presence of PBMC-CMV-FS4 supenatant, HLA-DR$^-$ cells also mediated NK(FS4), although the magnitude of lysis was considerably lower than that of NK(CMV-FS4) (Table V, Exp. 1). When HLA-DR$^-$ cells were pretreated with PBMC-CMV-FS4 supernatant or rIFN-α for 18 h, washed, and then tested for NK activity, lysis of both FS4 and CMV-FS4 targets was increased, although the magnitude of NK(FS4) was lower than that of NK(CMV-FS4) (Table V, Exp. 2).

When aliquots of serial, twofold dilutions of the supernatant generated by

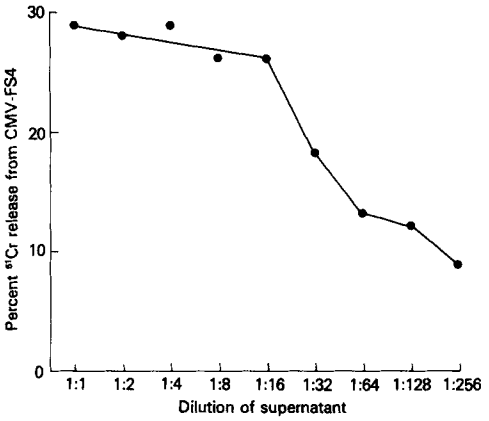

FIGURE 3. Effect of dilution of the supernatant generated by coculturing PBMC with CMV-FS4 on NK activity mediated by HLA-DR$^-$ cells against CMV-FS4. The generation of the supernatant and depletion of HLA-DR$^+$ cells were done as described in Materials and Methods. 100-μl aliquots of serial, twofold dilutions of the supernatant were added to NK assays containing HLA-DR$^-$ cells as effectors and CMV-FS4 as targets. The E/T ratio was 50:1. ^{51}Cr release was 4% with HLA-DR$^-$ cells alone and 24% with PBMC.

coculturing PBMC with CMV-FS4 were added to assays, a dose-response relationship was obtained with respect to HLA-DR$^-$-mediated NK(CMV-FS4) (Fig. 3).

Supernatants that restored NK(CMV-FS4) contained >2,000 IU/ml of antiviral activity, whereas supernatants unable to restore NK activity contained <20 IU/ml of IFN (Table IV). We investigated whether IFN in the supernatant was responsible for stimulating HLA-DR$^-$ cells to mediate NK(CMV-FS4). When the IFN-containing supernatant was diluted 1:4 and incubated with antiserum to human IFH-α sufficient to neutralize its antiviral activity, the ability of the supernatant to stimulate HLA-DR$^-$ cells to mediate NK(CMV-FS4) (Table VI) and NK(FS4) (not shown) was abolished. In contrast, incubation of the supernatant with antibodies to human IFN-β or γ sufficient to neutralize 2,000 IU of IFN-β or γ, respectively, had no effect on either the ability of the supernatant to restore NK(CMV-FS4) by HLA-DR$^-$ cells or its antiviral activity (Table VI).

Effect of Anti-IFN-α on PBMC-mediated NK(CMV-FS4). Since supernatants containing IFN-α stimulated HLA-DR$^-$ cells to mediate NK(CMV-FS4), experiments were done to determine whether IFN-α also contributed to NK(CMV-FS4) mediated by PBMC. Antibody to IFN-α was added to PBMC in microtiter wells before the addition of ^{51}Cr-labeled CMV-FS4. Addition of amounts of antibody sufficient to completely neutralize antiviral activity in supernatants did not affect the magnitude of NK(CMV-FS4) (Table VII).

Discussion

Resting peripheral blood NK cells from healthy donors do not express HLA-DR antigens; however, HLA-DR antigen expression is observed on NK cells induced to proliferate in vitro (10, 11) and on activated NK cells in peripheral blood of patients. NK cells are spontaneously cytotoxic, but their activity can be enhanced severalfold by various cytokines, e.g., IFN and IL-2 (28, 29). These

TABLE VI

Effect of Anti-IFN Antibodies on the Ability of PBMC-CMV-FS4 Supernatant to Stimulate NK Activity of HLA-DR⁻ Cells

Effector cells*	Supernatant added to NK assay	Treatment of supernatant‡	Antiviral activity (IU/ml) in supernatant after treatment§	Percent ^{51}Cr release from CMV-FS4
PBMC	–	–	–	16.8
HLA-DR⁻	–	–	–	5.6
HLA-DR⁻	+	medium alone	1203.2	24.6
HLA-DR⁻	+	anti-IFN-α	<20.0	5.9
HLA-DR⁻	+	anti-IFN-β	1504.2	24.7
HLA-DR⁻	+	anti-IFN-γ	1593.6	24.1

PBMC-CMV-FS4 supernatant was generated as described in Materials and Methods.

* PBMC and HLA-DR⁻ cells were obtained as described in Materials and Methods. E/T, 50:1.

‡ ~2,000 neutralizing units of anti-IFN-α, anti-IFN-β, anti-IFN-γ, or medium alone were incubated for 1 h at room temperature with equal volumes of supernatant diluted 1:4. Aliquots of 100 μl of each mixture were added to NK assays to give final dilutions of 1:2 (1:16 of original supernatant).

§ Antiviral activity was determined as described in Materials and Methods.

TABLE VII

Effect of anti-IFN-α Antibody on Cytotoxic Activity Mediated by PBMC Against CMV-FS4 and on IFN Production in the Cytotoxic Assay

Exp.	Percent ^{51}Cr release from CMV-FS4 in the presence of:*		Antiviral activity (IU/ml) of supernatants generated in the presence of:‡	
	Medium	Anti-IFN-α	Medium	Anti-IFN-α
1	37.0	33.6	600	<20
2	31.6	27.3	1,200	<20
3	41.2	30.2	246	<15
4	29.6	28.5	1,900	<15
5	26.1	23.9	300	<20
1–5§	33.1 ± 6.0	28.7 ± 3.6	849 ± 699	—

E/T, 50:1.

* 100 μl of anti-IFN-α (~2,500 IFN-α neutralizing units) or medium as added to microtiter plate wells before addition of effectors and target cells.

‡ Antiviral activity was determined as described in Materials and Methods.

§ Mean ± SD.

activated NK cells lyse NK-sensitive target cells with increased efficiency and can also lyse target cells that are poorly sensitive to the cytotoxic effect of nonactivated NK cells.

In the present study we showed that NK cell–mediated cytotoxicity against CMV-infected cells, unlike cytotoxicity against tumor-derived or normal target cells, depends on cooperation between two phenotypically distinct cell populations. NK(K562) and NK(FS4), as expected (8), were abrogated by treatment of PBMC with anti-CD16 (Leu-11b) antibody plus C, but not by anti-HLA-DR antibody plus C. Treatment of PBMC with either anti-HLA-DR or anti-CD16

(Leu-11b) antibody plus C reduced NK(CMV-FS4) to very low levels. Experiments in which mixtures of Leu-11b$^-$ and HLA-DR$^-$ cells mediated as much NK(CMV-FS4) activity as PBMC treated with C alone indicated that PBMC-mediated lysis of CMV-FS4 requires both CD16$^+$,HLA-DR$^-$ cells and HLA-DR$^+$,CD16$^-$ cells. When cell populations containing different ratios of the two cell types were tested for NK(CMV-FS4) activity, cytotoxicity was proportional to the number of HLA-DR$^-$ cells added, whereas <3% CD16$^-$ cells (corresponding to <0.6% HLA-DR$^+$ cells) were required for maximal NK(CMV-FS4) activity. These results indicate that the limiting cells in lysis of CMV-FS4 are HLA-DR$^-$ and suggest that HLA-DR$^+$ cells play an accessory role and stimulate CD16$^+$ effector cells to mediate NK(CMV-FS4) activity. Distinct roles for the HLA-DR$^+$ and CD16$^+$ populations were also suggested by the results of the irradiation experiments. The cytotoxic function of HLA-DR$^-$ cells was abolished by exposure to 50 Gy, as expected based on previously published data (26, 27), whereas the accessory function of Leu-11b$^-$ cells was radioresistant. Borysiewicz et al. (13) have shown that Leu-11$^+$ cells purified by cell sorting can lyse CMV-FS4. These results appear to contrast with those of our depletion experiments. However, this discrepancy might be explained by efficient enrichment of spontaneously cytotoxic cells in the positively selected Leu-11$^+$ population and, possibly, contamination of the Leu-11$^+$ population with small numbers of HLA-DR$^+$ cells, which we have shown to be sufficient, at levels as low as 0.6%, for optimal NK(CMV-FS4) activity. Furthermore, since we have observed (unpublished results) that treatment of PBMC with the anti-CD16 B73.1 antibody enhances cytotoxic activity of NK cells against various relatively NK-insensitive target cells, the results of positive selection experiments must be interpreted with caution.

The possibility that HLA-DR$^+$ cells might produce a soluble mediator that in turn stimulated CD16(Leu-11b)$^+$ cells to become cytotoxic was investigated. Cell-free supernatants of PBMC or Leu-11b$^-$ cells incubated with CMV-FS4 targets stimulated HLA-DR$^-$ cells to mediate NK(FS4) and NK(CMV-FS4) activity. In contrast, supernatants of HLA-DR$^-$ cells incubated with CMV-FS4 targets could not do so. A dose-response relationship was observed between dilutions of supernatants able to stimulate HLA-DR$^-$ cells and the magnitude of NK(CMV-FS4) activity, and maximal stimulation of cytotoxicity was observed when the supernatant was added at the final concentration of ≥3%.

Supernatants that stimulated HLA-DR$^-$ cells contained antiviral activity, and when anti–IFN-α antibody was added in sufficient quantities to neutralize this activity, the ability of the supernatants to stimulate cytotoxic activity of HLA-DR$^-$ cells was abolished. In contrast, addition of anti–IFN-β or anti–IFN-γ antibodies affected neither the antiviral activity of supernatants nor their ability to stimulate NK(CMV-FS4) activity. These data indicate that IFN-α present in the supernatants was responsible for stimulating CD16$^+$HLA-DR$^-$ cells to mediate NK(FS4) and NK(CMV-FS4). Negative selection studies indicated that the HLA-DR$^+$ cells that contributed to NK(CMV-FS4) activity did not have surface markers of B cells, T cells, or monocytes. It was of particular importance to determine whether monocytes might contribute to NK(CMV-FS4) since monocytes are both HLA-DR$^+$ and have been reported to produce IFN-α when

stimulated with certain viruses. However, PBMC depleted of monocytes with three mAbs plus C and containing <0.5% nonspecific esterase-positive cells could fully reconstitute NK(CMV-FS4), suggesting that monocytes were not necessary for reconstitution. Furthermore, purified adherent cells that contained >95% nonspecific esterase-positive cells could not reconstitute NK(CMV-FS4) when added to HLA-DR$^-$ cells, and supernatants of these assays did not contain detectable IFN-α. These findings strongly suggest that the HLA-DR$^+$ cells, which provide accessory function to Leu-11b$^+$ NK cells, are not monocytes. The HLA-DR$^+$ cells that provide the accessory function for NK(CMV-FS4) activity may be identical to the HLA-DR$^+$ peripheral blood leukocyte subset described by Perussia et al. (30, 31) as the major producer of IFN-α after exposure of PBMC to viruses or to virus-infected targets. This cell type has not yet been completely characterized; it represents a small proportion of nonadherent PBMC (<1%) (31), and has phenotypic and density characteristics similar to those of reported antigen-presenting cells, cells providing accessory functions for B cell stimulation (32), and cells stimulating autologous mixed lymphocyte reactions (33).

Our results show that IFN-α, which is able to stimulate CD16$^+$ cells to lyse CMV-FS4, is produced during interaction of PBMC with CMV-FS4, and that a CD16$^-$,HLA-DR$^+$ leukocyte subset is required for both production of IFN-α and lysis of CMV-FS4. Moreover our findings indicate that the HLA-DR$^+$ IFN-α-producing cells are present in excess in PBMC preparations from healthy donors, and that IFN-α is produced by HLA-DR$^+$ cells in contact with CMV-FS4 in a much higher concentration than needed for optimal NK(CMV-FS4) activity. These results suggest that the reported lack of correlation between concentrations of IFN-α in cultures and NK lysis of virus-infected target cells (5, 6) cannot be considered as evidence against a role for IFN in cytotoxicity activity. For the same reason, a correlation between the ability to PBMC from various types of patients to produce IFN-α and their ability to lyse virus-infected cells (7) should not be expected; rather, lysis of virus-infected cells should correlate with the number and activity of CD16$^+$ cytotoxic cells, which we have shown to be the limiting cells in the cytotoxic reaction. However, similar to results previously reported by Fitzgerald et al. (9) and by Bishop et al. (5) with respect to NK cytotoxicity against HSV-infected target cells, the presence in cytotoxic assays of quantities of anti–IFN-α antibody sufficient to neutralize extracellular IFN-α had no significant effect on NK(CMV-FS4) activity. There are several possible explanations for the inability of anti–IFN-α antibodies to inhibit NK(CMV-FS4) when added directly to the cytotoxicity assay. HLA-DR$^+$ cells may release a factor in addition to IFN-α that can activate CD16$^+$ NK cells; such a factor might not be demonstrable in supernatant fluids either because it is labile or because it is present at effective concentrations only in the intercellular spaces between contiguous cells. Alternately, HLA-DR$^+$ cells might activate CD16$^+$ cells by a mechanism that requires cellular contact but that also depends on IFN; both the antiviral and the cytotoxicity-enhancing effect of IFN can be transferred by direct cellular contact, with no requirement for extracellular IFN (34). However, the inability of anti–IFN antibodies to inhibit NK(CMV-FS4) does not exclude the possibility that the increased sensitivity of the infected target cells is due exclusively to the NK-enhancing effect of extracellular IFN-α. As discussed, IFN-

α is present in the supernatant fluid of cytotoxic assays at concentrations much higher than required for optimal NK(CMV-FS4) activity; it is possible that high concentrations of IFN-α in the intercellular spaces of cell pellets cannot be readily inhibited by the amounts of anti–IFN-α antibody used and thus activation of NK cells may occur before IFN-α is completely inactivated.

In conclusion, our data indicate that a subset of $CD16^-$,$HLA\text{-}DR^+$ leukocytes interacts with CMV-infected fibroblasts and activates cytotoxic $CD16^+$, $HLA\text{-}DR^-$ NK cells by releasing IFN-α, and possibly also by other mechanisms involving either labile soluble factors or cellular contact. These observations might explain why increased sensitivity of virus-infected fibroblasts to NK cells cannot be shown before 4–6 h of culture and is maximal at 18–24 h. CMV infection may also affect the sensitivity of target cells to the cytotoxic signal of NK cells and enhance the ability of NK cells to bind to target cells (unpublished data). However, the extent of lysis of CMV-infected target cells seems to be primarily determined by the accessory $HLA\text{-}DR^+$ cells, which after interaction with the virus-infected cells, nonspecifically activate cytotoxic NK cells. These activated NK cells lysed both uninfected and CMV-infected target cells, although in the present study the latter were lysed somewhat more efficiently. In other studies, cells infected with murine CMV or with HSV-1 have been shown to be less or equally sensitive to lysis, respectively, as compared with uninfected target cells (35, 36). Trinchieri et al. (28) showed that IFN, shown here to be produced by the $HLA\text{-}DR^+$ cells, might contribute to the selective killing of virus-infected cells by rendering uninfected cells, but not virus-infected cells, resistant to the lysis mediated by NK cells. Bukowski and Welsh (37) recently reported that the inability of IFN to protect virus-infected cells against lysis by NK cells correlates with NK cell–mediated antiviral effect in vivo. Such data strongly support the hypothesis that, in vivo, IFN renders NK cells selective for virus-infected target cells by acting both at the effector and at the target cell levels. Our present data indicate that an accessory $HLA\text{-}DR^+$ cell able to produce IFN-α and distinct from effector NK cells plays an important role in regulating the ability of the NK cells to lyse CMV-infected targets, and thus may contribute to resistance to infection with this virus.

Summary

The role of $HLA\text{-}DR^+$ cells in NK activity against CMV-infected FS4 foreskin fibroblasts and K562 erythroleukemia cells was examined. When nonadherent PBMC were depleted of either $HLA\text{-}DR^+$ or $Leu\text{-}11b^+$ cells by treatment with mAbs plus C, NK activity against CMV-FS4 target cells was markedly reduced. In contrast, depletion of $HLA\text{-}DR^+$ cells had no effect on NK activity against K562 target cells. When HLA-DR-depleted cells were added to Leu-11b-depleted cells, NK activity against CMV-FS4 was restored. Negative selection experiments indicated that the $HLA\text{-}DR^+$ cells contributing to NK activity against CMV-FS4 are not B or T cells, while negative and positive selection experiments excluded a role for monocytes. Experiments in which $HLA\text{-}DR^-$ and $Leu\text{-}11b^-$ cells were mixed in varying proportions indicated that NK(CMV-FS4) is mediated by $Leu\text{-}11b^+$ cells, while $HLA\text{-}DR^+$ cells provide an accessory function. Irradiation (50 GY) abolished the NK effector function of $Leu\text{-}11b^+$ cells, but not the accessory

function of HLA-DR$^+$ cells. The NK activity against CMV-FS4 of HLA-DR$^-$ cells was restored by the addition of rIFN-α or of cell-free supernatants generated by coculturing PBMC or Leu-11b$^-$ cells with CMV-FS4. The ability of these supernatants to restore NK activity of HLA-DR$^-$ cells was completely abrogated by the addition of neutralizing amounts of antibody to IFN-α. In related experiments, neutralization of IFN-α in NK assays had little or no effect on NK activity against CMV-FS4, suggesting that the accessory function of HLA-DR$^+$ cells might be mediated by alternative mechanisms in addition to the secretion of extracellular IFN-α.

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