Inhibition of Herpes Simplex Virus Type 1 Replication in Fibroblast Cultures by Human Blood Mononuclear Cells

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Received 28 January 1985/Accepted 4 November 1985

An assay was developed to test the effect of human blood mononuclear cells (MNCs) on herpes simplex virus (HSV) replication. In this assay, human fibroblast monolayers were inoculated with HSV and then cultured with or without blood MNCs. Fewer HSV-infected cells were recovered from human fibroblasts cultured in the presence than in the absence of blood MNCs. This inhibition of viral replication by MNCs was independent of HLA matching between the MNCs and fibroblasts and persisted even when T cells were depleted by antibody and complement. However, depletion of Leu11⁺ MNCs either by panning or with antibody and complement reduced the ability of the cells to suppress HSV infection, whereas enrichment of Leu11⁺ cells by fluorescence-activated cell sorting increased the viral suppression. Depletion of OKM1⁺ MNCs also reduced the viral suppression. After coculturing of MNCs and HSV-infected fibroblasts for 3 days, alpha interferon (IFN) and gamma IFN were detected in the supernatants. Predepletion of Leu11⁺ MNCs reduced the amount of gamma IFN produced in these cultures. Incubation of the MNCs and HSV-infected fibroblasts with antibody specific for either alpha or gamma IFN resulted in reduced viral suppression. Preincubation of MNCs for 18 h with either interleukin 2 or alpha IFN or for 7 days with antigen increased the suppression of HSV infection. These results suggest that natural killer cells with the Leu11⁺ phenotype participate in the recognition of HSV-infected cells at a point sufficiently early to interfere with the spread of infection in vitro and may inhibit viral replication by natural killer cell cytotoxicity, by generation of interferon, and by lymphokine-activated killing.

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are each capable of lysing herpes simplex virus (HSV)-infected cells in vitro. CTLs may recognize viral antigens in association with major histocompatibility complex class I (18) or class II (22) antigens, depending on the nature of the virus-infected targets. NK cells appear unrestricted by major histocompatibility complex antigens and have specificity for virus-induced glycoproteins (2). T lymphocytes and NK cells can also generate lymphokines that can directly inhibit viral replication and can augment cellular antiviral immunity (6, 9, 13, 20, 21). For useful in vivo antiviral immunity to develop it seems likely that infected cells would have to be lysed early in the course of their infection, before a point had been reached at which target cell death would release large numbers of infectious virus. Conventional cytotoxicity assays give no indication of the extent to which virus may replicate or spread from cell to cell before the labeled target cells are lysed. We therefore developed a technique to quantitate the spread of virus infection in monolayers of human fibroblasts cultured with or without mononuclear cells (MNCs) of different HLA types. HSV was selected for these studies because its spread in tissue culture is predominantly through cell-to-cell contact. Furthermore, this virus causes significant morbidity in neonates and immunosuppressed individuals.

MATERIALS AND METHODS

Cell sources and separation. Blood was obtained from healthy adults after they had given informed consent. The serum was tested for the presence of antibody to HSV. The

MNCs were separated by Ficoll-Hypaque gradient centrifugation and then used in the various effector assays. Blood MNCs from the fibroblast donors were also HLA typed by using Terasaki trays.

Media. The complete medium used for all experiments other than the plaque inhibition assay was Dulbecco modified Eagle medium with glutamine (no. 10-331-26; Flow Laboratories, Inc.) with 10% heat-inactivated fetal calf serum, gentamycin sulfate (0.04 mg/ml), and sodium bicarbonate (3.7 mg/ml). The medium used for the plaque inhibition assay was Dulbecco modified Eagle medium containing glutamine, 1% heat-inactivated fetal calf serum, gentamycin sulfate, sodium bicarbonate, and L-arginine (25 mg/liter).

Cell fractionation. Four methods were used to selectively deplete specific lymphocyte subpopulations from unfractionated MNCs: (i) antibody and complement cytotoxicity, (ii) panning, (iii) cell sorting of specifically labeled MNCs by using the fluorescence-activated cells sorter, and (iv) Sephadex G10 monocyte depletion.

For antibody and complement cytotoxicity, MNCs were incubated on ice for 30 min with antibody (OKT3 for T-cell depletion or Leu11b for NK cell depletion), and then incubated for 60 min at 37°C with diluted rabbit serum (Low-Tox-H rabbit complement, no. CL331; Cedarlane Laboratories). Controls included incubation with complement alone.

Specific NK cell subsets were depleted from effector population by panning (16). Antisera specific for Leu11b (Becton Dickinson & Co.) (17), OKM1 (Ortho Reagents) (24), or HNK-1 (1) were incubated with MNCs for 30 min on ice. The cells were then washed three times, suspended in complete medium, and separated on petri dishes (no. 1007; Becton Dickinson Labware) precoated with goat anti-mouse

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FIG. 1. (a). Relationship between the MOI and the number of HSV-infected cells recovered from monolayers of human fibroblasts. Human embryo lung fibroblasts were inoculated with HSV at various MOIs. Three days later the fibroblast monolayers were trypsinized and seeded onto rabbit skin cell monolayers to determine the number of infectious centers. (b). Relationship between length of coinoculation of HSV and fibroblasts with and without MNCs and the number of HSV-infected cells recovered. HSV-infected human embryo lung fibroblasts (MOI, 0.01) were incubated with or without MNCs. At either 36 or 64 h after viral inoculation the number of HSV-infected cells was determined by plaque assay.

antibody (no. 4140 anti-immunoglobulin G [IgG] [Tago] for OKMI or no. 4152 anti-IgM [Tago] for Leu11 and HNK-1). Adherence was for 2 h on ice, and the nonadherent cells were recovered by rinsing the petri dishes with medium. These panning conditions depleted about 90% of the selected subset.

Separation of Leu11⁺ and Leu11⁻ MNCs was done by using the fluorescence-activated cell sorter (Coulter Epics C Clinical Flow Cytometer; Coulter Electronics, Inc., Hialeah, Fla.). MNCs were stained with fluoresceinated anti-Leu11a (no. 7523; Becton Dickinson) on ice for 30 min, washed, and then separated on the cell sorter. Separation was based on bit-mapping on lymphocytes (by using forward-angle light scatter and 90° light scatter parameters) and then gating on Leu11⁺ or Leu11⁻ MNCs. The selected populations were more than 85% viable after the cell sorting. The selected Leu11⁺ MNCs were greater than 85% Leu11⁺ upon reanalysis, and the selected Leu11⁻ MNCs were greater than 98% Leu11⁻ upon reanalysis.

Depletion of monocytes was done by passing MNCs over a Sephadex G10 column.

HSV plaque inhibition assay. The plaque inhibition assay comprised two steps. In the first step, a small amount of HSV was added to a monolayer of human fibroblasts and infection was allowed to proceed for 3 days in the presence or absence of MNCs. In the second step, the fibroblasts were recovered by trypsinization and seeded onto rabbit skin cell monolayers to determine the number of infectious centers (plaques).

Monolayers of human fibroblasts (either human embryo lung fibroblasts or fibroblasts grown from bone marrow aspirations of HLA-typed donors) (3) were grown to confluence in 1.6-cm-diameter wells (no. 25820; Corning Glass Works) or 3.5-cm-diameter wells (no. 76-058-05; Flow Laboratories) at a concentration of 50,000 fibroblasts per cm². After 24 h the fibroblast monolayers were infected with cell-free HSV (100 or 300 μ l in proportion to well size) at a

multiplicity of infection (MOI) of 0.01. The virus used for infection was the KOS strain of HSV that had been grown in rabbit skin cells, sonicated, and centrifuged. Infection of the fibroblasts was allowed to proceed for 90 min at 37°C with intermittent agitation, after which additional medium with or without MNCs was added. When MNCs were added, their concentration was 250,000 per cm² (effector/target ratio, 5:1) unless otherwise designated. After 3 days at 37°C in a 5% CO₂ humidified incubator, the monolayers were detached with 0.05% trypsin–0.02% EDTA in phosphate-buffered saline. Detached fibroblasts with or without MNCs were

TABLE 1. Effect of MNC-fibroblast HLA matching on suppression of HSV replication^a

Expt no.	MNCs added	PFU recovered (10 ³)	% plaque inhibition
1	None	278 $\pm$ 41	0
	HLA matched	1 $\pm$ 0.3	99
	HLA mismatched	20 $\pm$ 4.3	93
2	None	172 $\pm$ 19	0
	HLA matched	60 $\pm$ 14	65
	HLA mismatched	40 $\pm$ 17	76
3	None	240 $\pm$ 34	0
	HLA matched	152 $\pm$ 26	37
	HLA mismatched	162 $\pm$ 22	33
4	None	320 $\pm$ 40	0
	HLA matched	200 $\pm$ 22	38
	HLA mismatched	200 $\pm$ 24	38

^a Four experiments were done with different HLA-matched and -mismatched MNC-fibroblast combinations. Each experiment involved the use of a different HLA-typed fibroblast as target. The HSV-infected fibroblasts were cultured for 3 days with or without the MNCs, and then the number of HSV-infected cells was determined by plaque assay. Results of PFUs recovered are the mean $\pm$ 1 standard error (SE) for duplicate samples that were plaqued in triplicate.

TABLE 2. Lack of requirement of T cells for suppression of HSV infection in fibroblast monolayers^a

MNCs added	PFU recovered (10 ³)	% Plaque inhibition
None	1,085 ± 67	0
MNCs only	189 ± 28	83
MNCs + complement	103 ± 40	90
MNCs + anti-OKT3 + complement	138 ± 61	87

^a HSV-infected human embryo lung fibroblasts were cultured for 3 days with or without MNCs. The MNCs had been previously treated with monoclonal anti-OKT3 and complement, complement alone, or medium alone. Then the number of HSV-infected cells was determined by plaque assay. Results of the PFU recovered are the mean ± 1 SE for duplicate samples that were plaqued in triplicate.

centrifuged at 200 × *g* and suspended in 1 ml of medium. The fibroblasts were seeded in log dilutions onto confluent monolayers of rabbit skin cells in 16-mm-diameter wells. These cultures were overlaid with methylcellulose and incubated for 72 h before being fixed and stained with 1% crystal violet. PFU were counted with a dissecting microscope, and the results were expressed as the number of plaques derived from the original fibroblast cultures. Percent viral plaque inhibition by effector cells was calculated as follows: [(number of PFU with medium alone – number of PFU with effector cells)/number of PFU with medium alone] × 100.

Antigen or lymphokine stimulation of MNCs. To prestimulate MNCs with viral antigens, MNCs were incubated for 7 days with HSV or varicella-zoster virus antigen (23) in RPMI 1640 and 10% autologous serum. Also, 5-ml cultures of MNCs (4 × 10⁶/ml) were incubated with human recombinant *Escherichia coli*-derived interleukin 2 (250 U/ml) (no. 01010; AMGen Biologicals), human recombinant *E. coli*-derived alpha interferon (IFN-α) (5,000 U/ml) (kindly provided by Hoffman-La Roche, Inc./Genentech, San Francisco, Calif.), or medium alone for 18 h in conical tubes (no. 2095; Becton Dickinson). These prestimulated cells were then washed and used as effectors in the HSV plaque inhibition assay.

IFN assays. Supernatants generated during the 3-day incubation of MNCs and HSV-infected fibroblasts were tested for IFN. Live HSV in the supernatants was inactivated by UV light. Then IFN-α or IFN-γ activity was tested by using an IFN neutralization assay in which the supernatants were incubated with or without either anti-IFN-α (no. G026-502-568; National Institutes of Health) or anti-IFN-γ (no. G-34-501-565; National Institutes of Health) for 1 h at 37°C and then tested for antiviral activity by coculturing with HSV-infected fibroblasts. Three days later the fibroblasts were trypsinized and seeded onto rabbit skin cell monolayers to determine the number of PFU. Percent viral plaque inhibition by the supernatants was calculated as follows: [(number of PFU with medium alone – number of PFU with supernatant)/number of PFU with medium alone].

IFN-γ in the supernatants was measured directly by a radioimmunoassay (Centocor, Malvern, Pa.).

RESULTS

Reduced virus recovery from monolayers incubated with MNCs. In preliminary experiments, we varied MOI, the number of MNCs, and the duration of incubation to determine the optimal culture conditions. The results in Fig. 1a show a linear correlation between the logarithms of MOI and PFU virus recovery over the range tested. The highest MOI (0.01) was therefore selected to determine whether virus replication could be inhibited. Figure 1b shows an increasing

disparity in virus recovery from cultures with or without a fivefold excess of MNCs over fibroblasts (i.e., effector/target ratio, 5:1) through 3 days of culture. Cytopathic effects in the fibroblast monolayer became visible after 4 or more days in culture in the presence of MNCs. Effector/target ratios of 10:1 were associated with partial detachment of the monolayers after 3 days in culture. Since cytopathic effects and monolayer detachment made it difficult to isolate single cells, we used a 3-day culture and effector/target ratios of 5:1 in all subsequent experiments.

Using a fixed MOI (0.01) and effector/target ratio (5:1), we tested MNCs from 15 HSV-immune (seropositive) adults in the plaque inhibition assay. The plaque inhibition for MNCs from different individuals ranged from 18 to 99%. The plaque inhibition for MNCs from a given individual tested in different experiments on different days varied up to 25%. However, the relative differences between individuals remained the same in each experiment. MNCs from two HSV-nonimmune (seronegative) individuals tested in four separate experiments resulted in HSV plaque inhibition ranging from 27 to 67%.

In a separate experiment, the MNCs were washed off the fibroblast monolayer and plated onto rabbit skin cells to determine whether they transferred significant amounts of virus. The mean percentage of infected MNCs was 0.86%, with a range of 0.004 to 1.6%. These percentages are consistent with another report (19) and suggest that virus transfer in association with MNCs makes little contribution to the total number of plaques.

Lack of requirement for HLA matching between MNCs and fibroblasts. Using a fixed MOI (0.01) and effector/target ratio (5:1), we compared fibroblast-MNC combinations which were either completely mismatched or were matched for two or three class I major histocompatibility complex antigens. The results (Table 1) show that in MNC-fibroblast combinations sharing no HLA A or B antigens, the MNCs suppress HSV infection. In fact, HLA-matched or unmatched MNCs, when tested against the same HLA-typed fibroblasts, showed comparable viral suppression. This result suggested that class I restricted T cells were not required for the interference with HSV replication measured by our assay. A requirement for T cells was also tested by comparing unfractionated MNCs with MNCs depleted of T cells by antibody and complement lysis. The results (Table 2) indicate that T-cell depletion did not interfere with the MNC effector function. To determine which cells were required we undertook experiments in which individual subsets were depleted by panning.

Effect of MNC subset depletion by panning. The panning procedure itself caused a small decrease in the ability of MNCs to inhibit HSV replication (Table 3). In all experiments in which the MNCs were depleted of Leu11⁺ cells there was less suppression of HSV infection (*P* = 0.05 by Wilcoxon paired *t*-test). The same trend was observed when the MNCs were depleted of OKM1 cells, but predepletion of HNK-1⁺ cells did not reduce the viral suppression. Monocyte depletion of MNCs by Sephadex G10 resulted in a reduction of viral suppression ranging from 3 to 40%. To fully evaluate the role of the Leu11⁺ cells in HSV plaque inhibition, we undertook experiments in which MNCs were either depleted of Leu11⁺ cells by monoclonal antibody and complement or enriched for Leu11⁺ cells by the fluorescence-activated cell sorter.

Effect of Leu11⁺ MNCs on viral replication. The activity of Leu11⁺ cells was tested first by comparing unfractionated MNCs with MNCs depleted of Leu11⁺ cells by monoclonal

TABLE 3. Effects of MNCs depleted with different anti-NK cell antibodies and panning on HSV infection in fibroblast monolayers^a

Expt no.	% HSV plaque inhibition for:					
	Control	MNCs	Control panned MNCs	Leu11-depleted MNCs	OKM1-depleted MNCs	HNK-1 depleted MNCs
1	0 ± 8	88 ± 2	83 ± 1	54 ± 6		
2	0 ± 17	77 ± 1	47 ± 15	16 ± 6		
3	0 ± 13	75 ± 3	68 ± 3	55 ± 3		
4	0 ± 16	75 ± 4	68 ± 7	43 ± 7		
5	0 ± 8	83 ± 2	83 ± 2	-17 ± 8		80 ± 3
6	0 ± 8	46 ± 6	39 ± 4			43 ± 1
7	0 ± 8	70 ± 3	71 ± 1			60 ± 7
8	0 ± 4	73 ± 8	63 ± 10		5 ± 14	
9	0 ± 10	49 ± 3	17 ± 6		-2 ± 6	
10	0 ± 6	57 ± 11	62 ± 2		42 ± 23	
11	0 ± 14	84 ± 2	79 ± 2		48 ± 6	
Mean	0 ± 10	71 ± 4	62 ± 6	30 ± 14	22 ± 13	61 ± 6

^a MNCs were stained with the designated specific monoclonal antibodies and then incubated for 2 h at 4°C in petri dishes that had been precoated with goat anti-mouse antibody. The nonadherent cells were then cultured with HSV-infected human embryo lung fibroblasts for 3 days, and then the number of HSV-infected cells was determined by plaque assay. Results of the percent plaque inhibition are the mean ± 1 SE for duplicate samples that were plated in triplicate.

antibody and complement. The results (Table 4) indicate that depletion of Leu11⁺ cells markedly reduced the antiviral effect of the MNCs. Also, separation of Leu11⁺ and Leu11⁻ cells by the cell sorter showed that Leu11⁺ cells were more potent inhibitors of viral replication than were Leu11⁻ cells (Table 5). In fact, when the Leu11⁺-enriched population was used, only 20% of the normal number of MNCs were needed to provide comparable levels of plaque inhibition (i.e., 50,000 MNCs per cm² instead of 250,000 MNCs per cm²).

Amplification of viral suppression by pretreatment with interleukin 2, IFN-α, or antigen. If NK cells are responsible for the observed interference with the HSV replication, then their effect might be augmented by prior exposure to lymphokines. To test this, we cultured MNCs for 18 h with interleukin 2, IFN-α, or medium alone. The results (Table 6) indicated that either interleukin 2 or IFN-α can increase the anti-HSV activity of MNCs. When MNCs were stimulated with HSV antigen or an unrelated antigen (varicella-zoster virus) for 7 days, HSV suppression was again enhanced (Table 6), presumably as a result of endogenous lymphokine production. MNCs cultured for 7 days without antigen were not suppressive.

Generation of IFN-α and IFN-γ by culturing MNCs with HSV-infected fibroblasts. Supernatants from 3-day cultures of MNCs and HSV-infected fibroblasts were collected, UV inactivated, and then tested for antiviral activity by adding

them to HSV-infected fibroblasts and then determining the number of infected cells in a plaque assay 3 days later. These supernatants produced up to 95% plaque inhibition. Supernatants generated from cultures lacking either HSV or MNCs had no antiviral activity (data not shown). Since IFN can augment NK cell activity and can also directly inhibit viral replication, we tested culture supernatants for the presence of IFN-α and IFN-γ. The presence of IFN-α and IFN-γ in the supernatants was initially detected by using an IFN neutralization assay (Table 7). The amount of IFN-γ was then quantitatively measured by a radioimmunoassay (Table 8). Predepletion of Leu11⁺ MNCs by using antibody and complement resulted in decreased IFN-γ production, whereas Sephadex G10 depletion of monocytes resulted in increased IFN-γ production. MNCs from HSV nonimmune (seronegative) individuals did produce IFN-γ, but not as much as produced by MNCs from HSV-immune (seropositive) individuals.

Effect of antibody specific for either IFN-α or IFN-γ on viral suppression by MNCs. Since IFN-α and IFN-γ were present in culture supernatants, we tested the effect of neutralizing these lymphokines on subsequent viral suppression. The results (Table 9) indicate that antibody specific for either IFN-α or IFN-γ did reduce the level of viral suppression. However, if MNCs were exposed to IFN-α before culture, then incubation of the prestimulated MNCs and HSV-infected fibroblasts with anti-IFN-α resulted in only minimal reduction in percent plaque inhibition (Table 10).

TABLE 4. Effect of anti-Leu11 and complement treatment of MNCs on HSV infection in fibroblast monolayers^a

MNCs added	PFU recovered (10 ³)	% Plaque inhibition
None	58 ± 7	0
MNCs only	4 ± 2	93
MNCs + complement	8 ± 3	86
MNCs + anti-Leu11 + complement	36 ± 5	38

^a Two separate experiments were done with MNCs from different individuals. In each experiment, MNCs were first treated with monoclonal anti-Leu11b and complement, complement alone, or medium alone and then washed. These effector cells were then cultured for 3 days with HSV-infected human embryo lung fibroblasts. The number of HSV-infected cells was then determined by plaque assay. Results of the PFUs recovered are the mean ± 1 SE for quadruplicate samples that were plated in triplicate.

TABLE 5. Effect of Leu11⁺ and Leu11⁻ MNCs on HSV infection in fibroblast monolayers^a

MNCs added	PFU recovered (10 ³)	% Plaque inhibition
None	102 ± 8	0
Leu11 ⁺ MNCs	48 ± 3	53
Leu11 ⁻ MNCs	92 ± 5	10

^a Two experiments were done in which MNCs were stained with fluoresceinated anti-Leu11a and then processed on the cell sorter. Parameters were set to separate Leu11⁺ cells and Leu11⁻ cells. These separated MNCs were then cultured for 3 days with HSV-infected human embryo lung fibroblasts (50,000 MNCs per cm²). The number of HSV-infected cells was then determined by plaque assay. Results of the PFU recovered are the mean ± 1 SE for quadruplicate sampling that were plated in triplicate.

TABLE 6. Amplification of HSV infection suppression by lymphokine and antigen stimulation^a

Cells added to culture	PFU recovered (10 ³)	% Plaque inhibition
None	66 ± 3	0 ± 4
Untreated MNCs	46 ± 5	30 ± 7
IL-2-Pretreated MNCs	20 ± 3	70 ± 4
IFN-α-Pretreated MNCs	28 ± 5	58 ± 7
None	40 ± 2	0 ± 7
Fresh MNCs	19 ± 1	78 ± 7
HSV-prestimulated MNCs	1 ± 0.2	96 ± 5
None	19 ± 1	0 ± 6
Fresh MNCs	4 ± 1	77 ± 5
VZV-prestimulated MNCs	2 ± 1	91 ± 3

^a MNCs were pretreated by exposure to IL-2, IFN-α, medium alone, HSV antigen, or VZV antigen. These MNCs were then washed and incubated for 3 days with HSV-infected human embryo lung fibroblasts. The number of HSV-infected cells was then determined by plaque assay. Results of the PFU recovered are the mean ± 1 SE of triplicate samples plaqued in triplicate.

DISCUSSION

Lysis of HSV-infected cells by NK cells is an established phenomenon (5), and under certain culture conditions HLA-restricted lysis of infected targets by T cells can also be detected (18, 22). Neither of these experimental techniques gives any indication of whether virus production in the target cells is reduced. The approach we have used differs from those previously adopted in that a relatively low effector/target ratio is required, the target fibroblasts are infected at a very low MOI, and the endpoint is the number of virus-infected fibroblasts recovered from the culture after 3 days of incubation. The differences in the amount of virus between cultures with and without MNCs first became apparent after 24 h and was still increasing at 72 h of culture. This is a much longer time than the 4- or 8-h incubation periods conventionally used for CTL and NK assays and may reflect both the slower interaction of effector cells with a limiting number of infected targets and the generation of lymphokines with subsequent lymphokine-activated killing.

TABLE 7. Generation of IFN-α and IFN-γ by coculturing MNCs and HSV-Infected fibroblasts^a

Soluble factor tested	PFU recovered (10 ³)	% Plaque inhibition
Medium	96 ± 5	0
Supernatant	5 ± 3	95
Supernatant after neutralization with anti-IFN-α	46 ± 2	52
Supernatant after neutralization with anti-IFN-γ	19 ± 1	80
Supernatant after neutralization with anti-IFN-α and anti-IFN-γ	90 ± 5	6

^a HSV-infected human embryo lung fibroblasts were incubated with MNCs for 3 days. The supernatants from these cultures were collected, UV inactivated, and then tested for antiviral activity. Preincubation of the supernatant with antisera specific for IFN-α, IFN-γ, or both IFN-α and IFN-γ reduced the antiviral activity. Results of the PFU recovered are the mean ± SE of duplicate samples plaqued in triplicate.

TABLE 8. IFN-γ content of supernatants generated by coculturing MNCs and HSV-infected fibroblasts^a

MNCs Added	HSV added	IFN-γ content (IU/ml)
No MNCs	+	1
Unfractionated immune MNCs	—	10
Unfractionated immune MNCs	+	95
Immune MNCs treated with Anti-Leu11 + complement	+	50
Immune MNCs treated with complement alone	+	80
Immune MNCs Sephadex G10 depleted	+	130
Nonimmune MNCs	+	28

^a HSV-infected human embryo lung fibroblasts were incubated for 3 days with untreated or fractionated MNCs from either seropositive (immune) adults or seronegative (nonimmune) adults. The supernatants from these cultures were collected, UV inactivated, and then tested for IFN-γ content in a radioimmunoassay.

The 5:1 ratio of MNCs to fibroblasts we used is substantially lower than the 50:1 or 100:1 ratio conventionally used in CTL assays. Fitzgerald et al. (8) also recently reported an *in vitro* technique that demonstrates inhibition of HSV replication by NK cells, but this assay requires an effector/target ratio of 800:1 during the 14-h incubation period. Their requirement for a high effector/target ratio may reflect the independence of their inhibitory effect on lymphokine production (i.e., without lymphokine-activated killing, a higher effector/target ratio is needed to produce an inhibitory effect). The need for a low effector/target ratio in our assay is of potential practical advantage in the investigation of anti-HSV immunity of infants, when the volume of blood available for testing may be limiting.

The inhibition of HSV replication by MNCs in our assay did not require CTLs, as evidenced by the following: (i) the persistence of antiviral effects in cultures depleted of T cells by antibody and complement; (ii) the generation of antiviral activity by MNCs from nonimmune individuals; and (iii) the persistence of antiviral effects in cultures in which effectors and target were not HLA matched.

Evidence for a role of Leu11⁺ cells in providing viral suppression included the following: (i) depletion of Leu11⁺ cells by panning or antibody-complement lysis resulted in decreased viral suppression; and (ii) enrichment of Leu11⁺ cells by the cell sorter resulted in enhanced viral suppression. Leu11⁺ MNCs were also found to play a role in the production of IFN-γ in these cultures. Djeu et al. (6) had previously demonstrated that large granular lymphocytes can generate IFN-γ. The antiviral effect of MNCs bearing the NK-associated Leu11 antigen is consistent with the view

TABLE 9. Effect of coinubating MNCs and anti-IFN sera on HSV infection in fibroblast monolayers^a

MNCs added	Antisera added	PFU recovered (10 ³)	% Plaque inhibition
—	None	58 ± 7	0
+	None	4 ± 2	93
+	Anti-IFN-α	52 ± 4	10
+	Anti-IFN-γ	46 ± 7	21

^a Two experiments were done in which HSV-infected human embryo lung fibroblasts were cultured for 3 days with or without MNCs and with or without antisera specific for either IFN-α (no. G-26-502-56; NIH) or IFN-γ (no. G-34-501-565; NIH). The number of HSV-infected cells was then determined by plaque assay. Results of the PFU recovered are the mean ± 1 SE of quadruplicate samples plaqued in triplicate.

TABLE 10. Effect of preincubation of MNCs with IFN- α on HSV infection in fibroblast monolayers^a

MNCs added	Antisera added	PFU recovered (10 ³)	% Plaque inhibition
None	None	82 $\pm$ 4	0
Untreated	None	10 $\pm$ 4	88
Untreated	Anti-IFN- α	52 $\pm$ 8	36
Preincubated with IFN- α	None	2 $\pm$ 2	98
Preincubated with IFN- α	Anti-IFN- α	12 $\pm$ 4	85

^a MNCs were incubated with IFN- α or medium for 18 h. The cells were then washed and incubated for 3 days with HSV-infected human embryo lung fibroblasts and with or without antisera specific for IFN- α . The number of HSV-infected cells was then determined by plaque assay. Results of the PFU recovered are the mean $\pm$ ISE of duplicate samples plaqued in triplicate.

that this lymphocyte subset can mediate first-line defense against rapidly spreading HSV infections. Since NK cells are heterogeneous in phenotype and function (2, 7), other NK-associated antigens were evaluated. OKM1 depletion resulted in decreased viral suppression, and this might reflect the specificity of this monoclonal antibody for both NK cells and monocytes. In contrast, HNK-1 depletion did not reduce the viral suppression. This is consistent with previous reports that the HNK-1⁺ cells may not be the most functionally active subpopulation of NK cells (12, 14, 15).

NK cell cytotoxicity is known to be amplified by lymphokines (10, 11, 20), and we were able to reproduce this effect by preexposure of our cultures to either interleukin 2 or IFN- α . The increased suppression of HSV infection by MNCs that had been precultured with viral antigen may also have resulted from lymphokine release, as the effect did not appear to be virus specific. We have reported comparable amplification of HLA-unrestricted cytotoxicity to varicella-zoster virus-infected targets by prestimulation of MNCs with an irrelevant antigen (R. A. Bowden, M. J. Levin, R. H. Giller, T. G. Tubergen, and A. R. Hayward, Clin. Exp. Immunol., in press), and there are many other examples of lymphokine-activated killing (4, 10, 11). We did demonstrate that IFN- α and IFN- γ are produced during the 3-day cultures and that neutralization of their activity with specific antisera decreased the capacity for viral suppression. The direct inhibitory effect of IFN on viral replication is well described. The observation that the enhanced viral suppression resulting from preexposure to lymphokine could only be minimally blocked by subsequent exposure to anti-IFN suggested that IFN could also function by augmenting NK cell activity.

Therefore, these results suggest that Leu11⁺ NK cells can play a fundamental role in the inhibition of HSV viral replication. These MNCs can recognize virally infected cells and then directly mediate NK cell cytotoxicity. The lymphokines that are generated by MNCs responding to virally infected cells can also enhance the NK cell cytotoxicity. In fact, the Leu11⁺ MNCs can participate in the generation of IFN which can subsequently enhance NK cell cytotoxicity and have direct antiviral effects. The plaque reduction approach we describe provides a means for investigating these various responses of MNCs to HSV-infected cells. Whether this approach will more accurately reflect *in vivo* immunity to HSV than conventional cytotoxicity assays is yet to be determined. We are currently investigating this possibility in the context of subjects with low HSV-specific T-cell responses such as immunosuppressed individuals and neonates.

ACKNOWLEDGMENTS

We are grateful to L. Pizer and M. Levin for supplies of virus and helpful discussions, to M. Herberger for excellent technical assistance, and to Judy Franconi and Julia Gunnerson for preparing the manuscript.

These studies were supported by Public Health Service grants AI18941 and HD13733 from the National Institutes of Health and by a Clinical Research Grant from the National Foundation, March of Dimes.

LITERATURE CITED

1. Abo, T., M. D. Cooper, and C. M. Balch. 1982. Postnatal expansion of the killer and natural killer cell population identified by the monoclonal HNK 1 antibody. *J. Exp. Med.* 155:321-326.
2. Bishop, G. A., S. D. Martin, S. A. Schwartz, and J. C. Glorioso. 1984. Human natural killer cell recognition of herpes simplex type 1 glycoproteins: specificity analysis with the use of monoclonal antibodies and antigenic variants. *J. Immunol.* 133:2206-2214.
3. Bowden, R. A., L. McGarren, A. R. Hayward, and M. J. Levin. 1984. Use of bone marrow fibroblasts to prepare targets for an HLA restricted cytotoxicity assay system. *J. Clin. Microbiol.* 20:696-700.
4. Brooks, C. G. 1983. Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. *Nature (London)* 305:155-158.
5. Ching, C., and C. Lopez. 1979. Natural killing of herpes simplex virus type 1-infected target cells: normal human responses and influence of antiviral antibody. *Infect. Immun.* 26:49-56.
6. Djeu, J. Y., N. Storieg, K. Zoon, G. J. Stanton, T. Timonen, and R. B. Herberman. 1982. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *J. Exp. Med.* 156:1222-1234.
7. Fitzgerald, P. A., R. Evans, D. Kirkpatrick and C. Lopez. 1983. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1 infected fibroblasts and K562 erythroleukemia targets. *J. Immunol.* 130:1663-1667.
8. Fitzgerald, P. A., M. Mendelsohn, and C. Lopez. 1985. Human natural killer cells limit replication of herpes simplex virus type 1 *in vitro*. *J. Immunol.* 134:2666-2672.
9. Green, J. A., T. Yeh, and J. C. Overall, Jr. 1981. Sequential production of IFN- α and immune-specific IFN- γ by human mononuclear leukocytes exposed to herpes simplex virus. *J. Immunol.* 127:1192-1196.
10. Grimm, E. A., A. Mazunder, H. Z. Zang, and S. A. Rosenberg. 1982. Lymphokine activated killer cell phenomenon. Lysis of natural killer resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* 155:1823-1841.
11. Herberman, R. B., J. R. Ortaldo, and G. Bamard. 1979. Augmentation by interferon of human natural and antibody dependent cell mediated cytotoxicity. *Nature (London)* 277:221-223.
12. Itoh, K., A. B. Tilden, K. Kumagai, and C. M. Balch. 1985. Leu11⁺ lymphocytes with natural killer activity are precursors of recombinant interleukin 2-induced activated killer cells. *J. Immunol.* 124:802-807.
13. Kasahara, T., J. Y. Djeu, S. F. Dougherty, and J. J. Oppenheim. 1983. Capacity of human large granular lymphocytes to produce multiple lymphokines: interleukin 2, interferon, and colony stimulating factor. *J. Immunol.* 131:2379-2385.
14. Lanier, L. L., C. J. Benike, J. H. Phillips, and E. G. Engleman. 1985. Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens. *J. Immunol.* 134:794-801.
15. Lanier, L. G., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu11 antigens. *J. Immunol.* 131:1789-1796.

16. Mage, M. G., L. L. McHugh, and T. L. Rothstein. 1977. Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods* 15:47-56.
17. Phillips, J. H., and G. F. Babcock. 1983. NKP-15: a monoclonal antibody reactive against purified human natural killer cells and granulocytes. *Immunol. Lett.* 6:143-149.
18. Sethi, K. K., I. Stroehmann, and H. Brandis. 1980. Human T cell cultures from virus-sensitized donors can mediate virus-specific and HLA-restricted cell lysis. *Nature (London)* 286:718-720.
19. Teute, H., R. Braun, H. Kirchner, H. Becker, and K. Munk. 1983. Replication of herpes simplex virus in human T lymphocytes. *Intervirology* 20:32-41.
20. Trinchieri, G., M. Matsumoto-Kobayashi, S. C. Clark, J. Seehra, L. London, and B. Perussia. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 160:1147-1169.
21. Welsh, R. M. 1984. Natural killer cells and interferon. *Crit. Rev. Immunol.* 5:55-93.
22. Yasukawa, M., and J. M. Zarling. 1984. Human cytotoxic T cell clones directed against herpes simplex virus infected cells. I. Lysis restricted by HLA class II MB and DR antigens. *J. Immunol.* 133:422-427.
23. Zaia, J. A., P. L. Leary, and M. J. Levin. 1978. Specificity of the blastogenic response of human mononuclear cells to herpesvirus antigens. *Infect. Immun.* 20:646-651.
24. Zarling, J. M., and P. C. Kung. 1980. Monoclonal antibodies which distinguish between human NK cells and cytotoxic T lymphocytes. *Nature (London)* 288:394-396.