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Specificity of Human Natural Killer Cells in Limiting Dilution Culture for Determinants of Herpes Simplex Virus Type 1 Glycoproteins

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The frequency and specificity of human cells with natural killer (NK) cytotoxic activity for herpes simplex virus type 1 (HSV-1)-infected targets was measured by limiting dilution culture. The frequency of NK cell precursors (NK-p) reactive with HSV-1-infected cells was 2- to 11-fold higher than that of NK-p reactive with mock-infected cells. The frequency of NK-p reactive with infected target cells lacking viral glycoprotein C or presenting an antigenically altered glycoprotein B was approximately twofold lower than that with wild-type virus-infected cells. Specificity analysis demonstrated that NK cells with a high statistical probability of being monoclonal were reactive with either glycoprotein B or C. These results provide the first evidence that cells with human NK activity possess clonal specificity for HSV-1-infected target cells.

Human natural killer (NK) cells lyse a wide variety of target cells without a demonstrable requirement for either prior antigen exposure or matching between effector and target cells at the major histocompatibility locus (17). This distinguishes NK activity from cytotoxicity mediated by antigen-primed T lymphocytes and led to the assumption that NK activity is nonspecific. However, recent studies suggest that cells with NK activity recognize specific structures on target cells and may possess clonal specificity for their targets (3, 17–19, 26, 29, 30).

To investigate the specificity of human NK cells, we used target cells infected with herpes simplex virus type 1 (HSV-1). The major HSV-1-encoded glycoproteins (gB, gC, gD, and gE) are expressed on infected cell surface membranes, and the physical and antigenic structure of many of these proteins has been at least partially characterized (reviewed in references 24 and 11a). This information provides an important advantage over the use of tumor target cells, most of which have not been well characterized for surface antigens. In addition, a number of laboratories have reported methods of genetically manipulating these glycoproteins in ways that change their ability to function in virus replication or to interact with the immune system (12, 24). In previous studies, a combination of a glycosylation inhibitor and a virus glycoprotein mutant was used to demonstrate that the preferential lysis of HSV-1-infected target cells by human NK cells correlates with the expression of gB and gC on target cell membranes (3) and is independent of interferon production (4). Bishop et al. (5) have recently reported that cells infected with antigenic variants of HSV-1 altered in individual epitopes of gB or gC show decreased susceptibility to NK activity and that monoclonal antibodies specific for gB or gC block NK activity against HSV-1-infected target cells. Here, we use short-term clones of cells with NK activity in limiting dilution (LD) culture to examine whether they possess clonal specificity for HSV-1 glycoproteins. Our results indicate that cell cultures having a high statistical probability of containing NK cells derived from a single precursor recognize specific viral glycoprotein determinants on HSV-1-infected target cells.

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MATERIALS AND METHODS

Preparation of short-term NK cell clones by LD. Peripheral blood mononuclear cells (PBML) were collected from healthy adult donors and separated by density gradient centrifugation on Ficoll-Hypaque (7). Donors both seronegative and seropositive for HSV-1 were used, since both Bishop et al. (3) and others (9, 20) have demonstrated that NK activity against HSV-1-infected cells does not correlate with donor serological status. After three washes with 0.85% NaCl, cells were suspended in 1 ml of RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 300 μg of fresh glutamine per ml, and antibiotics (complete medium [CM]). Adherent cells were removed by passage of PBML over a column of Sephadex G-10 (16). Cells eluted from this column contained <1% latex-ingesting or esterase-positive monocytes. Cells were washed after elution and layered onto a discontinuous density gradient of Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.), as previously described (6). Cells at the 40% interface, previously shown to be highly enriched for NK activity (6), were recovered and washed. Cells were counted and resuspended at a concentration of 5×10^6/ml in CM and depleted of T cells by antibody plus complement-mediated cytolysis, with the monoclonal antibody OKT3, as previously described (3). Viable cells were recovered after density gradient centrifugation on Ficoll-Hypaque. These cells (approximately 5% of the starting population) were seeded in 96-well round-bottom microtitration plates at concentrations varying from 40,000 to 1,250 cells per well in a volume of 0.1 ml per well, with 24 wells per cell concentration. Cells were cultured at 37°C in CM which contained 10% fetal calf serum, 10^{-5} M 2-mercaptoethanol, and a predetermined optimal concentration of interleukin-2-containing supernatant, pre-

TABLE 1. Frequency of human NK-p reactive with mock- or HSV-1 (KOS)-infected WISH target cells[a]

Expt no. and type of infection	Serological status of donor[b]	NK-p frequency, 1 in (range)[c]:	Ratio of frequencies[d]
1			
Mock	+	40,518 (24,994–106,937)	2.2
HSV-1 (KOS)		18,484 (12,821–33,115)	
2			
Mock	−	136,781 (76,776–626,211)	11.4
HSV-1 (KOS)		12,011 (9,262–17,079)	
3			
Mock		TL[e]	
HSV-1 (KOS)	+	3,276 (2,426–4,046)	
4			
Mock		7,671 (5,084–11,306)	3.5
HSV-1 (KOS)	+	2,182 (1,636–3,276)	

[a] LD cultures were carried out as described in Materials and Methods.
[b] Status was determined by virus neutralization assay.
[c] For each experiment, frequencies were statistically different by the global test for homogeneity of independent slopes ϕ at $P \leq 0.05$.
[d] Ratio of frequency measured on mock-infected target cells over frequency measured on target cells infected with HSV-1 (KOS).
[e] TL, Too low to estimate.

pared as described below. Cultures were fed with 0.1 ml of fresh medium on day 3 of culture and were tested on day 5.

Preparation of interleukin-2-containing supernatant. PBML were collected from two or more donors as described above, mixed, and given 1,000 R of gamma irradiation with a ^{60}Co source (Phoenix Memorial Laboratory, University of Michigan, Ann Arbor, Mich.). The cell concentration was adjusted to 10^6/ml in CM, and 1 μg of purified phytohemagglutinin-P (Burroughs Wellcome Co., Research Triangle Park, N.C.) per ml was added. Cells were cultured in roller bottles at 37°C for 48 h. The cells were then removed by centrifugation, mitogen was removed by passage over a concanavalin A-Sepharose column, and the supernatant was filtered and stored at −20°C until use. The optimal concentration of supernatant for supporting the expansion of LD cultures was determined for each preparation by the method of Gillis (10), with the interleukin-2-dependent cell line HT-2 (kindly provided by David Thomas). No feeder cells were included in these cultures, as it has been previously demonstrated that NK cells will clonally expand in the absence of feeder cells (25, 28). In contrast, the expansion of cytolytic T-cell clones has been shown to require feeder cell layers and continued stimulation with the priming antigen (25, 28).

Target cells. Cells from the human epithelial cell line, WISH, were used as target cells in these experiments. WISH cells were grown and maintained as monolayers in Eagle minimum essential medium with nonessential amino acids and 10% heat-inactivated fetal calf serum. The human erythroleukemia cell line, K562, was used as target cells in some experiments. K562 cells were grown in suspension culture in CM.

Viruses and infection procedure. Wild-type HSV-1, strain KOS, and the HSV-1 mutants, *syn* LD70 and the monoclonal antibody-resistant antigenic variant of wild-type HSV-1 KOS, *mar* B3.1, were grown in African green monkey kidney cells (Vero) and titers were determined as the number of PFU by methods described earlier (12). *syn* LD70 does not insert gC into host cell surface membranes (14). *mar* B3.1 was isolated on the basis of resistance to neutralization by a gB-specific monoclonal antibody and has an alteration in a distinct antigenic site of gB (S. D. Marlin, T. C. Holland, S. Highlander, M. Levine, and J. C. Glorioso, submitted for publication). Cells infected with *mar* B3.1 contain normal amounts of gB in their surface membranes but show a significantly reduced sensitivity to NK-mediated cytolysis. This reduction in susceptibility is equivalent to that seen in infected cells which completely lack surface membrane gB (5).

Monolayers of WISH cells were infected with virus by the following procedure. Virus in 1 ml of culture medium was added to monolayers in T-75 culture flasks at a multiplicity of 10 PFU per cell. Adsorption of virus was allowed to proceed for 1 h at 37°C. The monolayers were then overlaid with 9 ml of culture medium containing 500 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.). The infection was allowed to proceed for 18 h before use of the cells as targets. All experiments included mock-infected target cells.

Cytotoxicity assay. Effector cells of LD cultures were tested for cytotoxic activity on uninfected or HSV-1-infected WISH cells or K562 tumor cells. Each LD culture plate was split into four replica plates. Two fractions were tested on HSV-1 (KOS)-infected WISH cells to assess reproducibility of the split culture technique. Target cell specificity was determined by testing one fraction against uninfected WISH cells and one fraction against K562 cells or cells infected with an HSV-1 glycoprotein mutant. The exceptions to this scheme were experiments in which we compared NK activity against HSV-1 (KOS)-, *syn* LD70-, and *mar* B3.1-infected cells simultaneously (see Fig. 3 and 4). In this case, only one fraction was tested against HSV-1 (KOS)-infected cells, since all other experiments showed the split to be equal (see Fig. 2). At high concentrations of NK cell precursors (NK-p) plated in LD culture, all types of targets were lysed, demonstrating that all were approximately equal in ability to be lysed, and that, as shown previously (3), HSV-1-infected target cells were not more able to be lysed than uninfected cells.

The split cultures were washed once and suspended with mixing in 0.22 ml of CM with a multichannel pipette. Portions of 0.05 ml each were transferred into four V-bottom microtitration plates for assay. Labeled target cells at a concentration of 5×10^3 cells in 0.150 ml of CM were added to each well. For each type of target cell tested, 24 wells were included, containing labeled target cells alone plus 0.05 ml of CM. These wells were used to measure spontaneous ^{51}Cr release, which ranged from 5 to 20% of the maximum release from uninfected and infected target cells. At the end of the assay period (18 h at 37°C), plates were centrifuged at $500 \times g$ for 5 min, and 0.075 ml of supernatant from each assay mixture was harvested and counted by gamma spectrometry. Results were analyzed as described below.

Statistical analysis of results. A computer program was used to process all data, as previously described (15). The criterion for positive cytotoxicity for an experimental well was having counts per minute greater than the mean plus three standard deviations of the counts per minute of spontaneous release control wells for a given target. The precursor frequency of cells mediating NK activity was estimated by the maximum likelihood method, and the 95% confidence limits of these frequencies were calculated. Whether two frequencies differed at the significance level of $\alpha = 0.05$ was assessed by the global test for homogeneity of independent slopes (22). Closeness of fit to the Poisson distribution was determined by chi-square analysis, and probability was

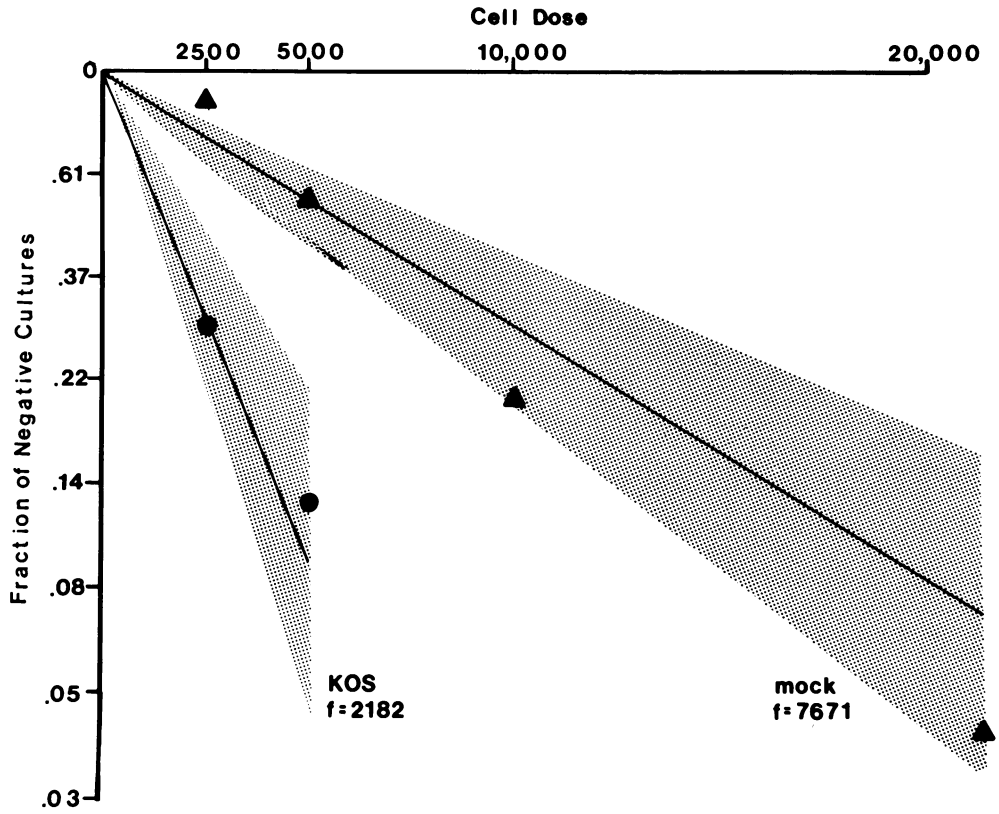

FIG. 1. Frequencies (f) of human NK-p tested on HSV-1 (KOS)-infected (●) and mock-infected (▲) WISH target cells (data are from experiment 4, Table 1). A T-cell-depleted, NK-cell-enriched population of PBML was cultured in LD with interleukin-2-containing supernatant. On day 5 of LD culture, an 18-h NK-cell-mediated cytolysis assay was performed on virus-infected and uninfected cells (see Materials and Methods).

assigned to each chi-square value. LD data are consistent with single-hit kinetics of the Poisson model, in which $P > 0.05$; the data presented in this study fulfill this criterion. The probability of monoclonality (P_M) for each LD cell concentration was also determined as described earlier (15).

RESULTS

NK-p recognize determinants of HSV-1-infected targets. We first examined whether the marked preferential lysis of HSV-1-infected target cells by NK cells in freshly isolated PBML (3–5, 9) is also seen with cells with NK activity in LD culture. Data presented in Table 1 and Fig. 1 show that this is indeed the case. Frequencies of NK-p reactive with uninfected WISH cells ranged from 1 in 7,671 to 1 in 136,781. In contrast, frequencies of NK-p reactive with HSV-1 (KOS)-infected cells ranged from 1 in 2,182 to 1 in 18,485 and were 2.2- to 11.4-fold higher than those reactive with mock-infected cells. The range of frequencies presented in Table 1 reflects the variation in NK activity seen in individual human lymphocyte donors as previously demonstrated (3).

Comparison of NK-p frequencies for K562 and HSV-1-infected targets. To examine whether T-cell-depleted effector cells grown in LD culture showed cytotoxicity characteristic of NK cells, LD cultures were also tested against K562 target cells, the cell line most often used as a target in experiments to demonstrate human NK effectors. Bulk cultures of human NK cells show a greater degree of lysis of K562 target cells than HSV-1-infected targets (5), and in the present experiments, this was reflected in higher frequencies of NK-p for K562 than for HSV-1-infected cells. In the experiments presented in Fig. 2, the NK-p frequency tested on K562 target cells (1 in 5,389) was significantly higher than the NK-p frequency tested on HSV-1-infected WISH cells (1 in 13,242). The specificity analysis of individual wells with NK activity presented in Fig. 2 demonstrated that the majority of cultures contain a mixture of clones which are cytolytic for either virus-infected cells, tumor cells, or other specificities. However, the most frequently occurring clones recognized the tumor cell target, since 67% of clones at an effector cell seeding dose of 5,000 ($P_M > 0.75$) lysed K562 cells but did not also lyse HSV-1 (KOS)-infected WISH cells (Fig. 2D). This finding indicated that the dominant effector cell specificity was for K562 cell surface determinants and validated the use of K562 cells as an indicator target cell type for the detection of human NK activity. Data shown in Fig. 2A also demonstrate that splitting of LD cultures into four aliquots does not lead to unequal distribution of NK-p, since two replica plates with HSV-1 (KOS)-infected targets yielded similar results.

Specificity of NK-p for HSV-1 glycoproteins. Previously, Bishop et al. (3) reported that target cells lacking either gB or gC show 30 to 50% less susceptibility to NK-mediated cytotoxicity than targets infected wth wild-type HSV-1 (KOS). Bulk cultures of NK effector cells show a reduction in cytotoxic activity against monoclonal antibody-selected variants of HSV-1 which produce antigenically altered forms of surface membrane gB. For example, NK lysis of *mar* B3.1-infected target cells was reduced 30 to 50% compared

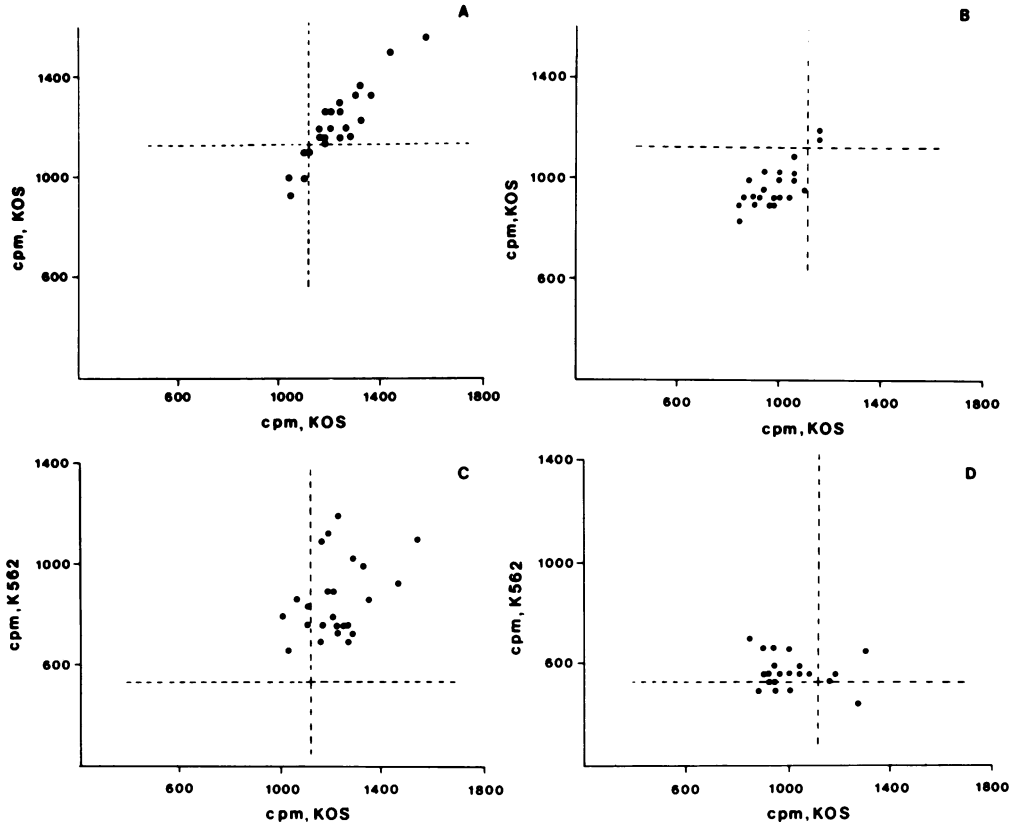

FIG. 2. Cytotoxic activity of NK clones (seeding dose of 10,000 cells per well, $P_M > 0.50$ [A and C] and 5,000 cells per well, $P_M > 0.75$ [B and D]). Clones were independently tested twice on HSV-1 (KOS)-infected WISH cells (A and B), once on mock-infected cells (data not shown), and once on K562 tumor cells (C and D). The same method as described in the legend to Fig. 1 was used. Dashed lines represent the criterion for positive cytotoxicity of an individual well against a given target, which is equal to cytotoxicity greater than mean spontaneous release plus three standard deviations above the mean (11).

with lysis of target cells infected with wild-type HSV-1. Although the mutant virus *mar* B3.1 was only altered in one epitope of gB, this particular alteration appears to affect NK recognition, since NK cells lyse *mar* B3.1-infected targets to no greater extent than targets which completely lack surface membrane gB (5). The results shown in Fig. 3 confirm and extend our earlier findings. NK-p tested on target cells infected with *syn* LD70, which lack surface membrane gC, show a frequency twofold lower (1 in 26,505) than the frequency of NK-p tested on HSV-1 (KOS)-infected targets (1 in 12,011). Similarly, the NK-p frequency on *mar* B3.1-infected target cells (1 in 26,407) was reduced approximately twofold. The NK-p frequency on mock-infected cells was more than 11-fold lower than that on virus-infected cells, confirming that virus-infected targets contained determinants highly visible to the NK-mediated effectors. Thus, the pattern of NK reactivity in LD culture closely parallels that previously seen with bulk cultures and indicates that cells in LD culture mediating NK activity possess specificity for gB and gC.

The specificity analysis of the NK cell wells from the experiment presented in Fig. 3 is shown in Fig. 4. In wells shown in Fig. 4A, B, and C, $P_M > 0.75$; in Fig. 4D, E, and F, $P_M > 0.95$. The majority of cultures cytolytic for target cells infected with wild-type HSV-1 (KOS) also lysed cells infected with the mutant viruses *syn* LD70 or *mar* B3.1. This finding indicated that the majority of NK clones recognized determinants shared by both wild-type and mutant virus-infected cells. However, some cultures contained HSV-1 (KOS)-specific clones of effector cells which appeared to recognize distinct HSV-1 glycoprotein determinants. In this experiment, five individual HSV-1 (KOS)-specific clones ($P_M > 0.75$) were cytolytic for *syn* LD70-infected cells but not *mar* B3.1-infected cells and were thus gB specific (Fig. 4D and F). Similarly, three virus-specific clones ($P_M > 0.75$) were cytolytic for *mar* B3.1- but not *syn* LD70-infected cells and were thus gC specific (Fig. 4E and F).

DISCUSSION

It has been previously demonstrated that unprimed human effector cells with NK activity can lyse target cells infected with a variety of viruses (reviewed in reference 29). The majority of these studies, including our own, were carried out with uncloned PBML as a source of NK effector cells. Long-term tissue culture clones of human cells with NK activity have now been obtained by a number of investigators (1, 2, 8, 13, 23, 27). All clones described to date, however, have been isolated and characterized primarily on the basis of their reactivity against tumor cell targets. Since very little is known about the membrane antigens on and possibly shared by these cells, it is difficult to draw conclusions about the specificities of these clones with NK activity. The use of virus-infected target cells, for which the virally encoded cell surface antigens are often well characterized and can be manipulated through genetic and biochemical techniques, provides a useful alternative model system for studies of NK recognition. With this system, we have shown

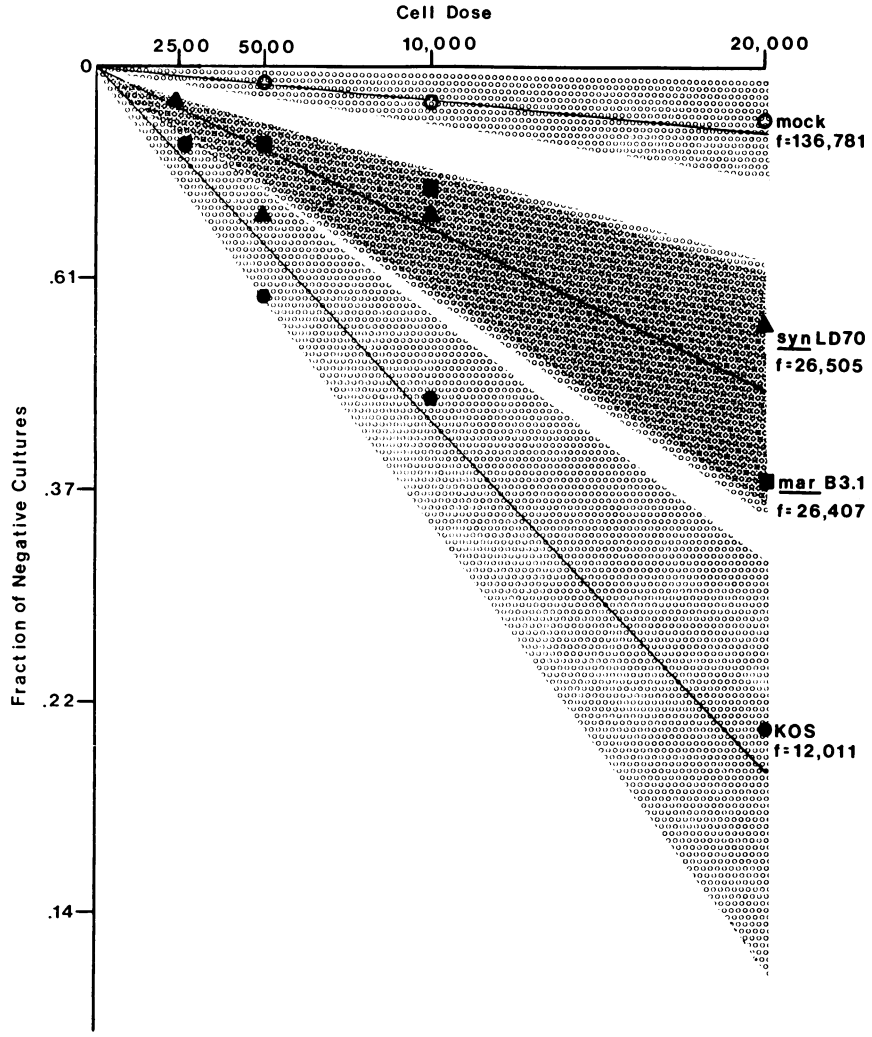

FIG. 3. Frequencies (f) of human NK-p tested on wild-type HSV-1 (KOS)-infected (●), *mar* B3.1-infected (■), *syn* LD70-infected (▲), and mock-infected (○) WISH cells. The same method as described in the legend to Fig. 1 was used with the exception that each of the split cultures was tested on different individual target cells.

that human NK cells specifically recognize HSV-1 glycoproteins on infected cell targets and that antigenic alterations in these glycoproteins can result in altered NK recognition (3, 5).

We have considered the possibility that virus infection in some nonspecific way perturbs the surface membranes of the normally NK-resistant target cells, causing them to become NK sensitive without NK cell recognition of specific viral determinants. This appears unlikely for the following reasons. (i) Monoclonal antibodies specific for gB or gC block NK activity against HSV-1-infected targets but only if the infected target expresses the appropriate cell surface glycoprotein (5). (ii) A monoclonal antibody recognizing a human cell membrane glycolipid blocks NK activity against the NK-sensitive K562 target but has no effect upon NK activity against HSV-1-infected target cells (5), arguing against the existence of a recognition determinant common to all NK-sensitive cells. (iii) The minor antigenic alteration in gB induced in the virus *mar* B3.1 strongly affects the ability of NK cells to recognize infected cells. However, it is unlikely to cause major alterations in infected cell membranes since the *mar* B3.1 mutation results in a single amino acid change in the external amino-terminal domain of gB (D. J. Dorney, S. Highlander, and J. Glorioso, unpublished data).

Because our earlier studies used uncloned effector cells, we determined whether cells mediating NK activity against HSV-1-infected cells possess recognition specificity for viral determinants at the clonal level. Only by studying the NK reactivity at the clonal level could we distinguish whether a given effector cell has recognition specificity for a single viral glycoprotein or whether HSV-reactive NK effector cells do not distinguish between the viral glycoproteins and recognize all HSV-1 antigens equally. Short-term clones of effector cells in LD assay were used to minimize the selective mechanisms operating in long-term cloning methods. These experiments can thus provide information on the fraction of clones possessing a particular reactivity in an individual population.

The major disadvantage of the short-term cloning method used in the present experiments is the inability to obtain larger numbers of cells from each clone for detailed analysis of its phenotype. However, all available evidence argues against the possibility that the clones studied here were cytotoxic T memory cells. First, the cells cloned exhibited a

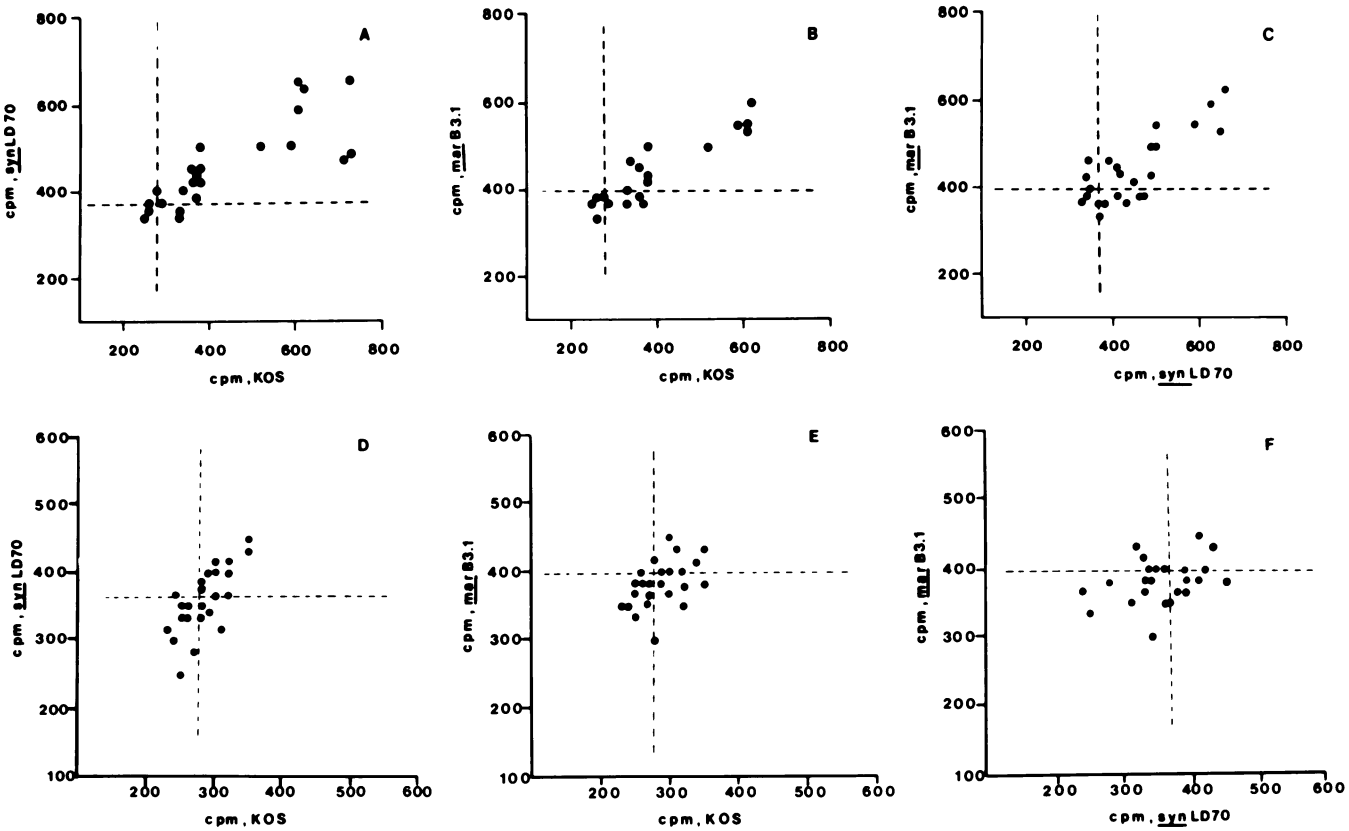

FIG. 4. Cytotoxic activity of HSV-1 (KOS)-specific NK clones (seeding dose, 5,000 cells per well [A, B, and C] and 2,500 cells per well [D, E, and F], $P_M > 0.95$). Clones were tested on KOS-infected, *mar* B3.1-infected, and *syn* LD70-infected WISH cells. Cytotoxicity on wild-type KOS-infected targets is shown on the abscissa, and cytotoxicity on mutant-infected targets is shown on the ordinate (A, B, D, and E). Panels C and F compare the coincidence of cytotoxicity on *syn* LD70-infected (abscissa) and *mar* B3.1-infected (ordinate) target cells. The data are from the experiment in Fig. 3. Dashed lines represent the same criterion for positive cytotoxicity as in Fig. 2.

high degree of reactivity with K562, the target cell used in most NK cell studies (Fig. 2). Although T-cell clones have been found under various conditions to lyse NK-sensitive tumor cell targets (1, 8, 21), such clones also often lyse NK-resistant targets, a phenomenon not observed here. NK-like activity of T-cell clones is seen after stimulation of the effector cells with allogeneic cells or mitogens (1, 8), neither of which was present during our cloning or assay procedures. In addition, our clones were grown in the absence of any feeder cells or specific (viral) antigen, which have been reported to be essential for the expansion of cytolytic T-cell clones (25, 28). The isolation procedures used here have been shown previously to enrich for cells mediating spontaneous cytotoxicity and to deplete T cells (3, 6). A final argument is that the effector cells used in the experiments presented in Table 1 (experiment number 2) and Fig. 3 and 4 were derived from HSV-1-seronegative individuals and were not matched with the target cells at the histocompatibility locus, providing further evidence that the cytotoxic effector cells were not memory T cells.

We have demonstrated here that cells mediating NK activity show clonally restricted recognition of HSV-1-infected target cells and that NK-p specific for determinants of single HSV-1 glycoproteins exist in normal human PBML of both seronegative and seropositive donors. These findings have interesting implications for the function of cells with NK activity in limiting the spread of viral infection. Since these cells in an unimmunized individual can specifically recognize viral determinants expressed early in infection, they can act to eliminate infected cells quickly and specifically. In addition, since Fig. 3 and 4 demonstrate the existence of clones of cells recognizing single viral glycoproteins, mutation in one or more virally encoded cell surface antigens would not prevent recognition and cytolysis of infected cells by NK cells. The isolation and long-term propagation of clones of cells with NK activity specific for HSV-1 glycoproteins should allow more detailed analysis of the phenotype and mechanism of action of these cells in eliminating virus-infected target cells.

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