RELATIONSHIP BETWEEN EXPRESSION OF
HERPES SIMPLEX VIRUS GLYCOPROTEINS
AND SUSCEPTIBILITY OF TARGET CELLS
TO HUMAN NATURAL KILLER ACTIVITY*

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One of the early defense mechanisms by which a host limits the spread of a viral
infection may involve the activity of natural killer (NK)1 cells. This lymphocyte
subpopulation possesses cytotoxic activity against a wide variety of targets, including
transformed, rapidly dividing, and virus-infected cells (1-3). NK cells have been
found in virtually all mammalian species tested, including nude mice, which lack
mature T lymphocytes (4-9). In man, as well as mouse, evidence is accumulating that
NK cells may play an important role in immunosurveillance against malignancies
and viral infections (10-13), and may also be involved in the regulation of growth
and differentiation of normal cells (2, 14, 15).

NK cells appear to share the general characteristics of nonadherent, large granular
lymphocytes, lacking complement (C) receptors and surface Ig, and possessing Fc
receptors (1). However, NK cells appear to comprise a heterogeneous population with
regard to expression of specific cell surface antigens (16-19), and may also be
heterogeneous in their ability to recognize antigenic structures on susceptible target
cells. Although a number of investigators have recently sought to define NK target
cell antigens (20-24), the recognition mechanism is still largely unknown. In the case
of virus-infected targets, recognition may be mediated through virus glycoproteins
expressed on the plasma membrane of infected cells. Several experimenters have
shown that not only whole virions, but specific viral proteins, can induce NK activity
(25-27). To investigate the relationship between expression of viral glycoproteins on
a target cell and its susceptibility to NK activity, we studied human NK cell activity

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† Abbreviations used in this paper: C, complement; CM, complete medium; CTC, cold target competitors;
2dG, 2-deoxy-D-glucose; EAC, erythrocytes coated with antibody and complement; EM, emetine HCl; gB,
gC and gD, glycoproteins B, C, and D; HEL, human embryonic lung fibroblasts; HSV-1, herpes simplex
virus type 1; IFN, interferon; KOSdG, cells infected with herpes simplex virus type 1, strain KOS, in the
presence of 2dG; NK, natural killer; PBML, peripheral blood mononuclear leukocytes; PBS, phosphate-
buffered saline, PFU, plaque-forming unit; syn LD70dG, cells infected with the HSV-1 mutant, syn LD70,
in the presence of 2dG.
against targets expressing various combinations of herpes simplex virus type 1 (HSV-1) glycoproteins.

Materials and Methods

Target Cells. Cells used as targets of natural cytotoxicity in these experiments included human embryonic lung fibroblasts (HEL), and the human transformed epithelial cell line, WISH. Target cells were grown and maintained as monolayers in Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY), with nonessential amino acids and 10% heat-inactivated fetal calf serum. The human leukemia cell line, K562, a highly sensitive target of human NK cells, was included in some experiments.

Virus Source and Infection Procedure. Wild type HSV-1, strain KOS, and mutant syn LD70 viruses were grown and titered as plaque-forming units (PFU) in African green monkey kidney cells (Vero), as previously described (28). Syn LD70 was isolated as a syncytial mutant from the KOS strain, and does not synthesize glycoprotein C (gC) (29). Monolayers of target cells were infected with virus as follows. Virus in 0.4 ml of culture medium was added to a monolayer containing 2 × 10⁶ cells at a multiplicity of 3 PFU/cell. Virus was allowed to adsorb to the cells for 1 h at 37°C, after which cells were overlaid with 4 ml of culture medium and labeled by the addition of 200 µCi of Na²⁶CrO₄ (200-500 Ci/g; New England Nuclear, Boston, MA) to the medium. Duration of the infection before use of the cells as targets was 18 h unless otherwise specified. Mock-infected targets were included in all experiments.

Inhibition of Protein Synthesis with Emetine HCl. After 1 h of virus adsorption, target cell monolayers (infected and mock infected) were overlaid with medium containing 0.1 mM emetine HCl (EM; Sigma Chemical Co., St. Louis, MO). The inhibitor was present for the remainder of the infection.

Inhibition of Glycosylation with 2-Deoxy-D-Glucose. Following virus adsorption, infected and mock-infected cell monolayers were overlaid with medium containing 10 mM 2-deoxy-D-glucose (2dG, Sigma Chemical Co.). The inhibitor was present for the remainder of the infection.

Effector Cells

Peripheral blood mononuclear leukocytes (PBML) were separated from heparinized venous blood of healthy adult donors by Ficoll-Hypaque density gradient centrifugation (30). Cells were washed three times with 0.85% NaCl and resuspended in 1-2 ml of RPMI 1640 culture medium (Gibco Laboratories) containing 5% heat-inactivated fetal calf serum, fresh glutamine (300 µg/ml), and antibiotics (hereafter referred to as complete medium, CM). Lymphocytes were depleted of adherent cells by incubation on a column of G10 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) according to the method of Ly and Mishell (31). Cells eluted from this column contained <1% esterase-positive or latex-ingesting monocytes. Following elution, cells were washed and resuspended to the desired concentration in CM. For some experiments, nonadherent cells were further treated by one of the following methods.

DEPLETION OF T CELLS. Nonadherent PBML were depleted of mature T cells by two methods. In the first, T and non-T cell populations were separated by the ability of the former to form rosettes with neuraminidase-treated sheep erythrocytes (32). In the second, cells were incubated for 30 min at room temperature at a concentration of 5 × 10⁶/ml in CM containing a 1:100 dilution of the monoclonal pan-T antibody OKT3 (Ortho Pharmaceutical, Raritan, NJ). Cells were then washed in serum-free medium, resuspended in medium containing rabbit C, and incubated for 1 h at 37°C. Following this treatment, the number of viable cells remaining was determined by trypan blue exclusion. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient, and viable cells were recovered at the interface.

DEPLETION OF B CELLS. B cells were removed from nonadherent PBML by two methods. The first employed the presence of complement receptors on B cells in forming rosettes of erythrocytes coated with antibody and C(EAC) (33). Cells were then centrifuged on a cushion of Ficoll-Hypaque, and nonrosetting cells were recovered at the interface. The second method depleted B cells by antibody plus C treatment as described above; the monoclonal anti-DR antibody OK-1a (Ortho Pharmaceuticals) was used at a 1:20 dilution. Both methods were tested for their effectiveness by examining control and B-depleted cell populations for the presence of Ig-bearing B cells, using direct immunofluorescence. Both methods were found to remove virtually 100% of B cells.

DEPLETION OF OKM-1+ CELLS. The monoclonal antibody OKM-1 (Ortho Pharmaceutical)
detects an antigen present on all peripheral blood monocytes and ~50% of the null cell fraction functionally identified as NK cells (34). It was thus of interest to determine whether treatment of monocyte-depleted effector cells with OKM-1 plus C would decrease spontaneous cytotoxicity against HSV-1 infected targets. Antibody plus C treatment was carried out as described above; OKM-1 was used at a 1:10 dilution.

**Percoll Enrichment of NK Effectors.** Nonadherent PBML were centrifuged on discontinuous density gradients of Percoll (Pharmacia Fine Chemicals), and effectors were recovered from the 37.5% layer (fraction 0, previously found by us to yield cells highly enriched for NK activity), the 42.5% layer (fraction 2), and the 47.5% layer (fraction 4), as previously described (35).

**Cytotoxicity Assay.** NK activity was measured by a direct 51Cr-release assay. Briefly, graded numbers of effector cells were added to 1 × 10⁴ labeled target cells in CM in triplicate cultures of 0.2 ml each. Assays were conducted in flat-bottom microtitration plates (Costar, Cambridge, MA). Plates were incubated at 37°C in a humid 5% CO₂, 95% air atmosphere for 18 h unless otherwise specified. At the completion of the incubation, 0.1 ml of culture supernatant was harvested from each well and counted. Percent cytotoxicity was calculated as follows: percent cytotoxicity = [(experimental release - spontaneous release)/(total counts/2 - spontaneous release)] × 100; where spontaneous release represents counts released from wells containing targets alone, and total counts represents counts contained in a 0.1 ml aliquot of labeled target cells (10⁴ cells). Total counts are divided in half in this calculation to account for the dilution factor present in the experimental and spontaneous release wells. Spontaneous release did not exceed 20% in these experiments. We further calculated a term called “virus-specific cytotoxicity.” This represents percent cytotoxicity (as calculated above) against virus-infected targets minus percent cytotoxicity against mock-infected target cells of the same type. Cytotoxicity against mock-infected targets was low in all our experiments; thus, we show NK activity as virus-specific cytotoxicity unless otherwise specified.

**Preparation of Cold Target Competitors (CTC).** CTC were grown and infected in the same manner as labeled targets; the cells were then detached from the monolayer with trypsin and washed. Targets were resuspended in 1.0 ml of 0.1% glutaraldehyde (Sigma Chemical Co.) and incubated at room temperature for 10 min in 17 × 100-mm polypropylene test tubes. On completion of fixation, cells were washed five times in PBS to thoroughly remove the glutaraldehyde before CTC were used in experiments.

**Interferon (IFN) Assay.** Culture supernatants were assayed for the presence of IFN by a modification of the colorimetric assay of Borden and Leonhardt (36). Briefly, 96-well flat-bottom microtitration plates (Costar) were seeded with WISH cells at a concentration of 3 × 10⁴/well in 0.1 ml. Serial IFN dilutions were added 8 h later in 0.1 ml. Included in all assays was a standard IFN-α preparation of known concentration, provided by Parke-Davis and Co., Detroit, MI. This preparation was previously standardized against National Institutes of Health reference standard #G023-901-527. After 18 h at 37°C, cells were infected with vesicular stomatitis virus at 200 PFU/well. After development of cytopathic effect (24 h), culture fluid was aspirated, cultures were washed with 0.85% NaCl, and overlaid with 0.2 ml of 0.015% neutral red in saline. Monolayers were incubated with neutral red at 37°C for 2 h, after which they were washed and cellular dye was eluted into 0.2 ml of a 1:1 solution of absolute ethanol and 0.1 M NaH₂PO₄. Eluted dye was transferred to clean microtiteration plates and measured at 550 nm in a Titertek Multiscan (Flow Laboratories, Inc., McLean, VA). For the series of dilutions of the standard IFN preparation, a line of best fit, plotting percent dye uptake versus U/ml of IFN, was determined by a programmed calculator (Texas Instruments Model 59). Titer of experimental samples were then determined by linear regression. This assay reproducibly detected as little as 4 U/ml of IFN.

**Pretreatment of Effector Cells with IFN.** NK effector cells were incubated at a concentration of 8 × 10⁶/ml in CM containing 5,000 U/ml of IFN-α, at 37°C for 1 h. We have previously found that this procedure gives maximal enhancement by IFN of NK activity (35). Following incubation, cells were washed before use in cytotoxicity assays.

**Results**

**Induction of NK Activity by HSV-1 Infection of Target Cells.** Uninfected HEL and WISH cells were poor targets for human NK cells (Fig. 1). Over an 18-h assay period,
Fig. 1. Increased susceptibility of NK targets following infection with HSV-1. WISH or HEL target cells were infected with HSV-1, strain KOS, for either 3 or 18 h before addition of effector cells. Mock-infected cultures were treated in an identical manner as infected cells, without addition of virus. K562 cells were uninfected. Results shown are representative of six similar experiments.

using a relatively high effector/target ratio (50:1), little cytotoxic activity was seen against either target. Following infection with HSV-1 (strain KOS), however, both targets became highly sensitive to spontaneous cytotoxicity. Cytotoxic activity against the infected targets increased steadily for the duration of the assay, and was initially higher when targets were infected for a longer duration (18 h vs. 3 h) before the assay. Similar results were obtained using human skin fibroblasts and KB cells, a transformed human epithelial cell line (data not presented).

Characterization of the Cytotoxic Cell. Blood donors for these experiments were healthy adults, most of whom are seropositive for HSV-1. Table I shows that we found no noticeable effect of donor serological status on amount of virus-specific cytotoxicity. We wished to determine, however, whether the cytotoxicity seen against infected targets in our assays required the presence of B or T lymphocytes. Effector cells are routinely depleted of monocytes before use, as described in Materials and Methods.
We removed B cells by EAC rosetting, or antibody plus C cytolysis using a monoclonal antibody directed against human Ia (DR) antigens. Results, presented in Table II-I, show that removal of B cells did not decrease the cytotoxic activity seen in our assays. Both seropositive and seronegative donors were used for these studies. Similarly, removal of T cells by E-rosetting or treatment with a monoclonal anti-T cell antibody plus C did not decrease, but enriched, cytotoxicity against infected targets (Table II-II). Although control, T-depleted, and purified T cell populations showed the same low levels of cytotoxic activity against mock-infected targets, T-depleted populations showed enhanced virus-specific cytotoxicity. Purified T cells showed little or no virus-specific cytotoxicity.

Since removal of B and T cells did not lower the cytotoxic activity seen in our assays, we used two methods to determine whether the cytotoxic effector cell possesses characteristics of the lymphocyte subpopulation functionally defined as NK cells. OKM-1 is a monoclonal antibody recognizing an antigen found on monocytes, macrophages, and ~50% of cells exhibiting NK activity against tumor targets (34). Since our effector cell population was already depleted of monocytes, cytolysis using OKM-1 plus C would remove only NK cells. Results in Table II-III show that following this treatment, the remaining cells possessed markedly reduced virus-specific cytotoxicity. We had previously used fractionation on discontinuous density gradients of Percoll to obtain a cell population enriched for NK activity against tumor targets (35). When we used cells banding in the fraction where highest antitumor cell NK activity was found, these cells also showed enrichment of virus-specific cytotoxicity (Table II-IV). The results thus suggest that the cytotoxic effector cell in our assays possesses the characteristics of an NK cell.

Requirement for HSV-1 Protein Synthesis in Induction of Virus-specific Cytotoxicity. Experiments were performed to determine whether viral protein synthesis is required
### Table II

**Characterization of the Effector Cell Cytotoxic to HSV-1-Infected Targets**

| Treatment of nonadherent effectors* | Control§ | B Depleted | T Depleted | Purified T Cells |
|-------------------------------------|----------|------------|------------|------------------|
| I. Removal of B cells               |          |            |            |                  |
| A. EAC§                             | 22       | 26         |            |                  |
| B. OK1A + C¶                        | 16       | 18         | 24         | 23               |
| II. Removal of T cells              |          |            |            |                  |
| A. OKT3 + C**                       | 25       |            | 24         | 20               |
| B. E-Rosetting††                    | 20       |            | 20         |                  |
| III. Removal of OKM1§§ cells       |          |            |            |                  |
|                                      | Control  | T Depleted | Purified T Cells |
|                                       | 10       | 17         | —           |
|                                       | 28       | 32         | —           |
|                                       | 15       | 25         | —           |
|                                       | 28       | 34         | —           |
| B. OKM1 + C                         | 35       | 55         | 0           |
|                                       | 7        | 24         | 4           |
|                                       | 8        | 20         | 0           |
| IV. Percoll fractionation||| | | |
|                                   | Control  | Fraction 0 | Fraction 2 | Fraction 4 |
|                                   | 20       | 44         | 10         | 0          |
|                                   | 19       | 33         | 18         | 4          |

* PBML were collected and separated as described in Materials and Methods. Monocytes (adherent cells) were removed by passage of PBML through a column of G-10 Sephadex.

† Virus-specific cytotoxicity = percent cytotoxicity (as calculated in Materials and Methods) against virus infected targets - percent cytotoxicity against mock-infected controls. Effector/target ratio was 20:1.

§ Control populations are nonadherent PBML.

¶ B cells were removed by rosetting with erythrocytes coated with antibody and C (EAC).

¶¶ OK1a is a monoclonal antibody recognizing human DR antigens and was used to sensitize cells before lysis with C.

§§ OKM1 is a monoclonal antibody recognizing an antigen present on human monocytes and a proportion of NK cells and was used to sensitize cells before lysis with C.

|| Density gradients of Percoll were used to obtain a population enriched for NK cells (fraction 0), as described in Materials and Methods.

in an infected target for development of sensitivity to NK cells. Following a 1-h adsorption of virus to target cells, cells were overlaid with either medium alone, or medium containing 0.1 mM EM, an irreversible inhibitor of protein synthesis (37). Although the low NK activity seen against mock-infected targets was not altered by treatment of the cells with EM, results shown in Fig. 2 demonstrate that when infection of targets occurred in the presence of EM, these infected targets were not susceptible to virus-specific cytotoxicity. HSV-1 protein synthesis is thus required in infected targets if these targets are to become susceptible to NK activity; adsorption
Expression of HSV-1 Glycoproteins on the Surface of Infected Target Cells

| Infecting virus | 2-Deoxy-D-glucose‡ |
|----------------|-------------------|
| KOS§           | gB, gC, gD        |
| LD70∥          | gB, gD            |

* Results were obtained from immunoprecipitation and polyacrylamide gel electrophoresis as well as antibody plus C-mediated immune cytolysis experiments, which are presented elsewhere (52).
† Infection carried out in the presence or absence of 10 mM 2dG, as described in Materials and Methods; gB, gC and gD refer to herpes-specific glycoproteins B, C, and D, respectively.
§ Wild-type strain of HSV-1.
∥ Syncytial mutant of KOS strain of HSV-1 that does not synthesize gC.

and penetration of the virus into the target cell is not sufficient.

Expression of Different HSV-1 Glycoproteins at the Surface of Infected Target Cells. HSV-1 glycoproteins are expressed on the surface of infected cells within a few hours after infection (38), and may thus have a role in the induction of NK activity. We examined this possibility by generating various combinations of major HSV-1 glycoproteins, glycoprotein B (gB), glycoprotein C (gC), and glycoprotein D (gD), on the surface of infected target cells in the following manner (shown in Table III). Cells infected with
wild-type HSV-1, strain KOS, express gB, gC, and gD at the cell surface. If infection is carried out in the presence of 10 mM 2dG, however, gB is not expressed on the cell membrane. If cells are infected with syn LD70, a C-minus mutant isolated from strain KOS (29), gC is not expressed on the cell surface. It thus follows that the only major viral protein on the surface of cells infected with syn LD70 in the presence of 2dG known to be involved in immunocytolysis reactions is gD. In this manner, we obtained target cells expressing gB, gC, and gD; gC and gD; gB and gD; or gD only.

Relationship between Expression of HSV-1 Glycoproteins on Infected Cell Surfaces and Sensitivity to NK-Mediated Cytolysis. The four types of infected targets described above were tested for their relative sensitivity to lysis by NK cells. Results are shown in Fig. 3. When either HEL or WISH cells were used as targets, similar results were found. Highest virus-specific cytotoxicity, at all effector/target ratios, was directed against targets infected with wild-type virus (strain KOS). An ~30% reduction in activity was consistently observed against targets infected with the mutant virus syn LD70, which fails to synthesize gC. Activity was also decreased ~30% against targets infected with KOS in the presence of 2dG (KOSdG), which do not express cell surface gB. A reduction in activity of ~60% was seen against targets infected with syn LD70 in the presence of 2dG (syn LD70dG); such targets express only an underglycosylated form of gD at their surface. We therefore conclude that expression of HSV-1 glycoproteins at the surface of infected cells strongly influences their relative susceptibility to NK-mediated cytolysis.

Ability of Targets to Serve as CTC. Compared to KOS-infected cells, KOSdG-, syn LD70-, or syn LD70 dG-infected targets may exhibit decreased sensitivity to NK cells due to decreased ability to bind NK effector cells. To test this hypothesis, we examined the ability of cells expressing various combinations of HSV-1 glycoproteins to act as CTC. CTC were obtained as described in Materials and Methods and tested for their ability to compete for killing of KOS-infected labeled targets. Results presented in Fig. 4 show that mock-infected CTC were unable to compete for killing, and the various types of infected CTC showed competitive ability paralleling their relative sensitivities as NK targets (Fig. 3). Results presented in Fig. 5 demonstrate that while KOS- and syn LD70-infected CTC competed equally well for killing of syn LD70-infected labeled targets, KOS-infected CTC blocked significantly more killing to KOS-infected labeled targets than did syn LD70-infected CTC. Table IV presents data obtained when KOSdG- and syn LD70-infected cells were used as labeled targets and CTC. KOS- and syn LD70-infected CTC showed similar ability to compete for killing of syn LD70-infected labeled targets, while KOSdG-infected CTC showed slightly decreased competition for these targets. Conversely, while KOS- and KOSdG-infected CTC showed equal ability to compete for killing of KOSdG-infected targets, syn LD70-infected CTC could not compete as well for these targets. The above differences were not statistically significant but were consistently observed in all experiments.

Quantitation of IFN in Culture Supernates. Supernates from experimental cultures were tested for the amount of IFN produced in the cultures during the 18-h assay period. Results are shown in Table V. Cultures containing either effector or target cells alone, whether uninfected or infected, did not produce detectable amounts of IFN. Similary, co-cultures of effectors and mock-infected targets did not produce IFN. Co-cultures of effectors and virus-infected targets produced significant amounts of
Fig. 3. Susceptibility of targets expressing various combinations of HSV-1 glycoproteins to NK activity. HEL (left) or WISH (right) cells were infected with wild-type HSV-1 (strain KOS) or the HSV-1 mutant γ32 LD70 virus in the presence or absence of 10 mM 2dG. Duration of the assay was 18 h. Results shown are representative of four similar experiments.
IFN. However, the amounts of IFN produced did not strictly correlate with the cytotoxic activity seen against a particular type of infected target. While KOSdG- and syn LD70-infected targets elicited similar NK activity, co-cultures of effectors and syn LD70-infected targets produced over twice as much IFN as did co-cultures of effectors and KOSdG-infected targets. Also, although NK activity against KOS-infected targets was 21% higher than activity against syn LD70-infected targets, the amount of IFN produced in co-cultures containing KOS-infected targets was no greater than the amount produced in co-cultures containing syn LD70-infected targets.

Effect of IFN Pretreatment of NK Effector Cells. Results presented in Table V demonstrate that co-cultures of effectors and HSV-1-infected targets produced substantial amounts of IFN. We thus wished to determine whether stimulation of effectors with an equivalent amount of exogenous IFN would cause NK activity against mock-infected targets to be as high as that against KOS-infected targets. Data presented in
Table VI show that this is not the case. Although IFN pretreatment of effectors increased cytotoxic activity against both mock- and KOS-infected targets, activity against mock-infected targets consistently remained much lower than that against KOS-infected targets, and virus-specific cytotoxicity did not decrease.

Discussion

The present study has demonstrated that HSV-1-infected targets are strong inducers of NK activity, and that relative NK susceptibility of such targets correlates with
**TABLE IV**
Relative Abilities of Different Infected Targets to Act as Cold Target Competitors (CTC) *

| CTC Added | Virus-specific cytotoxicity | Labeled targets infected with: |
|-----------|-----------------------------|-----------------------------|
|           | KOS | syn LD70 | KOSdG |
| None      | 59  | 29      | 27   |
| KOS|| (10)§ | 20 | 14 | 14 |
|         | (20) | 0      | 0    | 0    |
| syn LD70| (10)¶ | 26 | 16 | 18 |
|         | (20) | 5      | 0    | 0    |
| KOSdG    | (10) | 25     | 25   | 12   |
|         | (20) | 0      | 3    | 0    |
| syn LD70dG | (10) | 37     | 28   | 24   |
|         | (20) | 22     | 10   | 10   |

* Infection of labeled and "cold" (unlabeled) targets with KOS or syn LD70 viruses in the presence or absence of 2dG, as indicated, was carried out as described in Materials and Methods. Virus-specific cytotoxicity calculated as described in Materials and Methods. Results presented are representative of five similar experiments. § Numbers in parentheses represent ratio of unlabeled/labeled targets in co-cultures. Effector/labeled target ratio was 80:1. ¶ Wild-type HSV-1. ¶ Syncitial mutant of KOS strain of HSV-1 which does not synthesize gC.

their expression of HSV-1 glycoproteins on the cell surface. Previous studies have shown that infection of many types of cells, both in vivo and in vitro, with a wide variety of viruses can induce NK activity (3, 6, 8, 9, 13, 39-48). We observed a dramatic increase in NK activity against several previously resistant cell lines when these cells were infected with HSV-1. As most of our donors of effector cells have had prior exposure to HSV-1, it was important for us to establish that the cytotoxic activity seen in our assays was not due to the presence of monocytes, B lymphocytes, cytotoxic T lymphocytes, or herpes-specific antibodies. Our effector cell populations are routinely depleted of monocytes before each experiment; thus, these cells were removed from consideration as cytotoxic effectors. We found that donor serological status did not correlate with amount of virus-specific cytotoxicity (Table I), and removal of B cells by two different methods did not decrease activity against infected targets in our experiments (Table II). Although a number of investigators have found that antiviral antibody may contribute to spontaneous cytotoxicity (13, 41, 44), we conclude that B cells are not required for the cytotoxicity seen in our assays. Other investigators, using several different viruses, have also reported that antiviral antibody or B cells are not required for cytotoxicity against virus-infected cells (45-48). Similarly, when effectors were depleted of T lymphocytes by two different methods, T-depleted populations showed enriched virus-specific cytotoxicity, while purified T cells possessed little or no activity against infected targets. This parallels findings using
NATURAL CYTOTOXICITY AGAINST HERPES-INFECTED CELLS

TABLE V
Quantitation of Interferon in Culture Supernates of NK Effector Cells and HSV-1 Infected Targets

| Supernate from cultures containing: | Interferon | Cytotoxicity |
|------------------------------------|------------|-------------|
| (A) Effector cells alone           | <4        | --         |
| Target cells alone                | <4        | --         |
| mock-infected                     | <4        | --         |
| mock dG-infected                  | <4        | --         |
| KOS-infected                      | <4        | --         |
| KOSdG-infected                    | <4        | --         |
| syn LD70-infected                 | <4        | --         |
| (B) Effectors + targets           |           |            |
| mock-infected                     | <4        | 18         |
| mock dG-infected                  | <4        | 22         |
| KOS-infected                      | 2600      | 81         |
| KOSdG-infected                    | 1280      | 68         |
| syn LD70-infected                 | 2800      | 64         |

* Supernates were obtained from standard cytotoxicity assay using WISH target cells, as described in Materials and Methods. Effector/target ratio was 40:1. Data are representative of four similar experiments.

~: Interferon quantitation was performed as described in Materials and Methods.

TABLE VI
Pretreatment of Effector Cells with Interferon *

| Experiment | Target Cells§ | Percent Cytotoxicity‡ |
|------------|---------------|-----------------------|
|            |               | -IFN | +IFN |
| 1          | Mock-infected | 9    | 17   |
|            | KOS-infected  | 31   | 62   |
| 2          | Mock-infected | 12   | 13   |
|            | KOS-infected  | 39   | 49   |
| 3          | Mock-infected | 19   | 25   |
|            | KOS-infected  | 55   | 68   |
| 4          | Mock-infected | 18   | 25   |
|            | KOS-infected  | 67   | 76   |

* Pretreatment of effector cells carried out as described in Materials and Methods.
‡ Calculated as described in Materials and Methods. Effector/target ratio was 80:1.
§ HEL cells were used as targets.
¶ Wild-type HSV-1.

Although the studies mentioned above established that cytotoxicity against a virus infected cell can be mediated by a nonadherent, non-T, non-B lymphocyte, we wished to further determine whether the effector cell in our assays possessed characteristics of an NK cell. When putative NK cells were removed by OKM-1 monoclonal antibody plus C treatment, virus-specific cytotoxicity was markedly reduced. Also, when a population previously shown to be enriched for NK cells was obtained by density
gradient centrifugation (35), these cells showed enriched activity against infected targets. We thus conclude that the virus-specific cytotoxicity seen in our assays is effected by a cell possessing NK characteristics.

The most intriguing question about NK activity against virus-infected cells concerns the mechanism by which effectors recognize susceptible targets. If virus-specified proteins are required to mediate increased susceptibility of infected targets, blocking protein synthesis before the relevant proteins are made should also inhibit the development of susceptibility. We found that this was indeed the case; when protein synthesis was blocked during infection (Fig. 2), infected targets showed no greater susceptibility to NK cells than uninfected controls. These results did not, however, indicate whether viral proteins expressed in the plasma membrane of infected cells are important in determining their sensitivity as targets. To address this question, we used as targets cells infected in such a manner as to express different combinations of the major HSV-1 glycoproteins (Table III). Results shown in Fig. 3 indicate that viral glycoprotein expression on infected cell membranes appears to play an important role in mediating sensitivity of these targets to NK activity. An ~30% reduction in activity was seen against targets lacking either gB or gC, and an ~60% reduction in activity was observed against targets lacking both cell surface gB and gC.

Decreased NK activity against infected targets lacking one or more of the major HSV-1 glycoproteins in the plasma membrane could occur for a number of reasons. We thus performed cold target competition experiments to determine whether decreased cytotoxicity against a particular target is paralleled by decreased ability of such a target to bind NK effector cells. That this is the case is shown by results presented in Figs. 5 and 6 and Table IV. HSV-1 glycoproteins expressed on the plasma membranes of infected cells appear to act as recognition structures for NK effectors. Results shown in Fig. 5 and Table IV also suggest that it is not simply the total amount of viral glycoprotein expressed on target cell membranes which determines their relative NK susceptibility. Thus, KOS-infected CTC, although expressing the most viral glycoprotein, did not show greater competitive ability for killing of KOSdG- or syn LD70-infected labeled targets than did KOSdG- or syn LD70-infected CTC, respectively.

It is now well established that IFN can enhance NK activity against a variety of target cells (1, 35), including virus-infected targets (3, 25, 39). Some investigators have suggested that differences in susceptibility of target cells to NK activity can be explained solely by differing abilities of the targets to induce or produce IFN (3, 40). Several recent studies, however, have challenged this conclusion. Casali et al. (27) report stimulation of NK activity using purified measles virus glycoproteins without concomitant release of IFN. Similarly, Lee and Keller (50) found that the amount of exogenous IFN required to augment NK activity against murine cytomegalovirus-infected targets was greater than the amount produced in co-cultures of effectors and infected targets. Also, when production of IFN was blocked by addition of actinomycin D, no decrease in NK activity was seen. Kirchner et al. (43) reported that cultures of mouse peritoneal exudate cells produced IFN only in response to infectious HSV-1 virions, although both infectious and noninfectious virions induced increased NK activity.

Recently, Fitzgerald et al. (51) found that during NK assays against HSV-1-infected fibroblasts, levels of cytotoxicity and IFN generated did not correlate, and
when IFN-pretreated effector cells were used, there was still a preferential lysis of infected over uninfected target cells. In agreement with these authors, we found (Table V) that the amount of IFN generated in co-cultures of NK effectors and infected target cells did not correlate directly with the amount of NK activity seen in these co-cultures. We also found (Table VI) that pretreatment of NK effector cells with IFN did not decrease the virus-specific cytotoxicity seen in our assays. Data from our cold target competition experiments mentioned above also supports the concept that the NK reaction may consist of both IFN-dependent and IFN-independent mechanisms. We are currently investigating this further, using anti-IFN antiserum in our cytotoxicity assays.

The present findings strongly suggest that HSV-1 glycoproteins expressed in the plasma membrane of infected target cells may act as recognition sites for the binding and/or activation of human NK cells. We are presently using techniques of clonal analysis to examine more closely the possible recognition specificities of NK cells for HSV-1 glycoproteins.

Summary

Cells normally insensitive to human natural killer (NK) activity were rendered susceptible by infection with HSV-1. The cytotoxic effector cell was a nonadherent, non-T, non-B lymphocyte. Antibody plus complement treatment, using a monoclonal antibody that recognizes an antigen present on NK cells, removed much of the cytotoxic activity, and a density gradient fraction enriched for NK cells yielded cells of increased virus-specific cytotoxicity. It was concluded that the effector cell active against infected targets possessed characteristics of an NK cell.

Blockage of viral protein synthesis during infection inhibited development of increased susceptibility of infected targets to NK activity. When targets were infected with a mutant virus unable to produce viral glycoprotein C (gC), NK activity against these targets was reduced ~30% compared with activity against targets infected with wild-type virus. Similarly, activity against targets infected in the presence of 2-deoxyglucose (2dG), which prevents cell surface expression of viral glycoprotein B (gB), was also reduced ~30%. An ~60% reduction in activity was seen against targets infected with mutant virus in the presence of 2dG; these targets express gD, but neither gB nor gC.

When cells expressing various combinations of HSV-1 glycoproteins were used as both labeled targets and cold target competitors, it was found that the susceptibility of a particular target to NK activity was paralleled by its ability to act as a cold target competitor. This indicates that targets with decreased sensitivity to NK cells were less able to bind NK effectors. Further, the amount of interferon produced in co-cultures of NK effectors and infected target cells did not directly correlate with the amount of NK activity generated, and interferon pretreatment of effectors did not decrease virus-specific cytotoxicity. The present results suggest that HSV-1 glycoproteins expressed at the surface of infected targets may act as recognition structures for NK cells.

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