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Natural Killer Cell Lysis of Cytomegalovirus (CMV)-Infected Cells Correlates with Virally Induced Changes in Cell Surface Lymphocyte Function-Associated Antigen-3 (LFA-3) Expression and Not with the CMV-Induced Down-Regulation of Cell Surface Class I HLA

Jean M. Fletcher,* H. Grant Prentice,† and Jane E. Grundy2*

CMV and other viruses down-regulate the cell surface expression of class I HLA, and while this allows them to evade CTL, it may make infected cells more susceptible to lysis by NK cells, due to the failure to engage class I inhibitory receptors on the NK cell. We studied CMV infection and found that fibroblasts infected with virus strains Towne, Toledo, Davis, and C1FE were refractory to NK lysis, while those infected with strains AD169, C1F, or R7 were susceptible. All viral strains down-regulated class I HLA to a similar extent, and we concluded that there was no evidence for any correlation between the latter and susceptibility to NK lysis. In contrast, there was a strong correlation between NK killing of CMV-infected cells and cell surface levels of lymphocyte function-associated antigen-3 (LFA-3). Fibroblasts infected with the Towne, Toledo, Davis, and C1FE strains of CMV down-regulated LFA-3 expression and were refractory to lysis, while strains AD169, C1F, and R7 up-regulated LFA-3 and were susceptible to NK killing. U373 MG (malignant glioma) cells expressed constitutively high levels of LFA-3 and were sensitive to NK lysis when infected with any of the above-listed CMV strains. We estimated that a minimum of between 29,000 and 71,000 LFA-3 molecules per target cell were needed for NK susceptibility. The effects on LFA-3 expression were due to immediate early/early viral gene products. We also demonstrated that fibroblasts infected with the strains Towne, Toledo, Davis, and C1FE expressed a ganciclovir-sensitive late CMV gene product, which delivered an inhibitory signal to NK cells.

NK cells play a protective role in the host immune response to viruses, providing a first line of defense before the generation of a more specific T cell response (1). In addition to their ability to lyse virus-infected cells, NK cells can secrete IFN-γ, which has direct antiviral activity and which can also influence the subsequent T cell response (2). The most convincing in vivo experimental evidence for the protective role of NK cells in viral infection has come from the murine model of CMV infection (3, 4), in which both cytotoxic and cytokine-mediated effects have been shown to be important (5). In the human, there is laboratory evidence in support of a role for NK cells in defense against CMV infection/disease (6–9), and there is in vivo evidence to support this role in rare NK cell-deficient individuals who exhibit abnormal sensitivity to CMV as well as to other herpes viruses (10).

Despite evidence to support a protective role for NK cells in viral infection, the mechanism by which NK cells can recognize and kill virus-infected cells is unclear. Recent work has shown that the control of NK cell lysis is complex, depending on a balance of positive and negative signals (11). An important negative signal is the engagement of class I HLA on the target cell by HLA-specific inhibitory receptors on NK cells (12, 13). Thus, target cells that had lost or have low expression of class I molecules might fail to engage the relevant inhibitory receptor and would therefore be expected to be susceptible to lysis by NK cells. It has been suggested that it is the loss of class I HLA molecules from the surface of tumor cells (14) and possibly virus-infected cells (15, 16) that renders them susceptible to NK cell lysis, although for viral infections, the in vitro evidence to support this theory is lacking.

In vitro, CMV-infected fibroblasts have been shown to be targets for NK cells (24). However, unlike the conventionally used NK cell-sensitive targets, such as K562 cells, which are killed in a 4-h 51Cr release assay, NK cell-mediated lysis of CMV-infected fibroblasts required an extended 20-h assay before specific lysis was observed (24). This was thought to reflect a requirement for prior activation of the NK effector cells before target cell lysis could occur, possibly mediated via IFN-α released by DR+ accessory cells (25). The lysis of CMV-infected fibroblasts was mediated predominantly by CD16+CD3+ lymphocytes (24, 26), and the effector cells were shown to be a subset of the cells that lysed K562 target cells (26, 27).

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Departments of Clinical Immunology and Haematology, Royal Free Hospital School of Medicine, London, United Kingdom

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Address correspondence and reprint requests to Dr. Jane E. Grundy, Department of Clinical Immunology, Royal Free Hospital School of Medicine, London, NW3 2PF, United Kingdom.

Previous work from this laboratory showed that CMV-infected fibroblasts were refractory to lysis by in vivo-activated NK cells in a 4-h 51Cr release assay (28). These effector cells, from the peripheral blood of bone marrow transplant recipients, were able to lyse target cells infected with EBV (28). The failure to kill the CMV-infected target cells was found to be due to the lack of a reciprocal lymphocyte function-associated antigen-1 (LFA-1)-ICAM-1 interaction between effector cells and CMV-infected fibroblasts, an interaction that was present between effector cells and the EBV-infected lymphoblastoid target cells (28). Thus, the expression of adhesion molecules, as well as class I HLA, can affect lysis by NK cells. Interestingly, CMV infection itself alters the expression of adhesion molecules. We have previously found that CMV infection of fibroblasts increases the expression of ICAM-1 and LFA-3 (29, 30), with consequent increased binding of CD2+ lymphocytes (31), a subset that includes NK cells. Thus, CMV infection modulates the expression of a number of cell surface molecules that might alter the susceptibility of the infected cell to NK cell–mediated lysis. In this report, we have investigated the relative importance of CMV-induced changes in the cell surface expression of class I HLA, ICAM-1, and LFA-3 on the susceptibility of cells to lysis by NK cells. Our studies utilized a panel of virus strains and cell types with different susceptibilities to NK cell–mediated lysis to help analyze these parameters.

Materials and Methods

**Cell culture**

Hs68 foreskin fibroblasts were obtained from the European Collection of Animal Cell Cultures and were used between passages 20 and 35. Autologous skin fibroblasts were obtained by explantation from a normal skin biopsy and were used between passages 5 and 10. Human embryonic lung fibroblasts were isolated from fetal lung tissue by trypsin digestion (500 μg/ml) and were used between passages 170 and 180. Endothelial cells were isolated from umbilical cords by explantation. Good culture conditions for both were maintained in MEM supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FCS (all from Life Technologies, Paisley, U.K.). U373 MG (malignant glioma) astrocytoma cells were obtained from the European Collection of Animal Cell Cultures, maintained in MEM supplemented with 1% nonessential amino acids, 1 mM sodium pyruvate, and 10% FCS, and used between passages 12 and 18. All fibroblasts were grown in MEM supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FCS (all from Life Technologies, Paisley, U.K.). U373 MG (malignant glioma) astrocytoma cells were obtained from the European Collection of Animal Cell Cultures, maintained in MEM supplemented with 1% nonessential amino acids, 1 mM sodium pyruvate, and 10% FCS, and used between passages 170 and 180. Endothelial cells were isolated from umbilical cords as described previously (32), maintained in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 20% FCS, 50 μg/ml endothelial cell growth factor (Sigma, Poole, U.K.), and 20 IU/ml heparin, and used between passages 2 and 4. All cell lines were grown under standard cell culture conditions at 37°C in 5% CO2 in air and were confirmed to be mycoplasma free using the Mycoplasma T.C. Rapid Detection System kit (Gen-Probe, San Diego, CA).

**Virus**

The following strains of CMV were used: laboratory strains AD169 (passage 94), Davis (passage 76), Towne (passage 132), and the low passage clinical isolates Toledo (passage 12), C1F (passage 8), C1FE (8 passages in fibroblasts plus 7 passages in endothelial cells), and R7 (passage 4–7). The AD169, Davis, and Towne strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and the Toledo strain was a gift from Dr. Stuart Starr (Children’s Hospital of Philadelphia, Philadelphia, PA); R7 and C1F are clinical isolates that have been passed through fibroblasts, while C1FE was derived from C1F by subsequent passage through endothelial cells and has enhanced pathogenicity for the latter cell type (32). Virus stocks were harvested from the supernatants of CMV-infected human embryonic lung fibroblasts or endothelial cells at 5 to 7 days postinfection and stored at −80°C. Virus stocks were titrated using the plaque assay (33) and then were used to infect cells at a multiplicity of infection of 2 to 4. Virus stocks were confirmed to be mycoplasma free using the detection system described above.

**Infection of cells with CMV**

Cells were seeded into six-well plates at a density of 3 × 105 cells/well. On the following day, cells were either treated with medium alone or infected with CMV at a multiplicity of infection of 2 to 4. Cells were incubated with virus for 1 h and thereafter maintained in the appropriate medium containing 4% FCS. For endothelial cells, the infection was enhanced by centrifugal inoculation of the virus as described previously (32). In all experiments, at least 95% infection was obtained, as determined by flow cytometric analysis of CMV immediate early Ag (30) with the exception of endothelial cells, where such levels are difficult to achieve. In our experiments, viral DNA synthesis was inhibited by the addition of 50 μg/ml of the antiviral agent ganciclovir (Cymevene; Syntax, Maidenhead, U.K.) to the cell culture medium of fibroblasts following the usual 1-h adsorption period.

**Monoclonal Abs**

Flow cytometric analysis was performed with mAbs specific for: class I HLA, clone PA2-6 (hybridoma cells were obtained from the ATCC); ICAM-1, clone BB10/I1 (R&D Systems, Abingdon, U.K.); LFA-3, either clone AICD58.9 (Boehringer Mannheim, Germany) or clone BRIC-5 (Serotec, Kidlington, U.K.). Isotype matched, purified mouse IgG (Sigma) was used at the appropriate concentration as a negative control. The following Abs were added to cytotoxicity assays, where indicated, at a concentration of 10 μg/ml: CD94, clone HP-3D9 (PharMingen, San Diego, CA) and CD2, clone RPA-2-10 (PharMingen). The following Abs produced in this institute were used for the relative quantification by flow cytometry: RTF4 (IgG1 specific for CD4), RTF8 (IgGl specific for CD8) and 2D1 (IgG specific for CD45).

**Flow cytometry for the detection of cell surface molecules**

Human embryo lung or Hs68 skin fibroblasts were seeded into six-well plates at 3 × 105 cells/well, infected the following day, and thereafter maintained in MEM/4% FCS. At various times postinfection, the fibroblasts were detached by trypsinization and washed in PBS containing 0.1% sodium azide and 0.1% BSA. To aliquots of 1 × 107 cells, primary mAbs (or appropriate isotype controls) were added at their saturating concentrations for 30 min, followed by a FITC-conjugated Fab(ab), fraction of a sheep anti-mouse IgG Ab (Sigma). After fixation in 2% paraformaldehyde, samples were analyzed by flow cytometry. Median fluorescence intensity (MFI) values for the isotype controls (MFI < 10) were subtracted from those of the test Abs to correct for differences in fluorescence between infected and uninfected cells, and the results were then expressed as MFI values. Alternatively, for experiments performed at various times postinfection, the values at each time point were normalized by expressing the data as a percentage of that of the accompanying uninfected cell control, as described previously (30). The data shown represent the mean ± SD of triplicate samples.

**Relative quantification of cell surface molecules**

The relative number of cell surface molecules was quantified by flow cytometry (34, 35). Normal PBMCs were stained with CD4, CD8, and CD54 by indirect immunofluorescence and analyzed by flow cytometry. A standard curve of MFI vs molecules per cell was calculated according to the known number of CD4, CD8, and CD45 molecules on the relevant leukocyte population. In parallel, CMV-infected or uninfected fibroblasts or U373 MG cells were stained for LFA-3 or class I HLA, followed by the secondary Ab. The relative number of class I HLA or LFA-3 molecules was then determined from the standard linear regression curve.

**Cytotoxicity assays**

**Effector cells.** Heparinized venous blood from normal volunteers of known HLA class I types (Table I) was separated over Lymphoprep (Nycomed, Oslo, Norway) to obtain the peripheral blood mononuclear (PBMC) cell fraction that was used in most experiments at an E:T ratio of 50:1. The CD56+ fraction was positively selected using CD56 microbeads over a MACS column (Miltenyi Biotec, Cambridge, U.K.), the fraction that passed through the column was the CD56− fraction. The binding of CD56 mAb has been shown not to affect NK cell cytotoxicity (36). The separated subsets were then analyzed by flow cytometry using a CD56−phycocerythrin-conjugated Ab (Becton Dickinson, Mountain View, CA). The CD56− fraction was found to be 90% pure, while the depleted fraction contained <1% CD56+ cells. In some experiments, effector cells were activated by overnight incubation with 1000 U/ml of IFN-α (ProproTech, London, U.K.) or 1000 U/ml of IL-2 (R&D Systems), washed, and then added to radiolabeled target cells in a 4-h 51Cr release assay.

1 Abbreviations used in this paper: LFA-1, lymphocyte function-associated antigen; PBMC, peripheral blood mononuclear; MFI, median fluorescence intensity; MG, malignant glioma.
either 20 h or 4 h (for preactivated effector cells) at 37°C in 5% CO₂ in air.

\[ \frac{\% \text{ lysis}}{100} = \left( \frac{\text{sample release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \right) \times 100 \]

\[ \text{Percent lysis} = \frac{\text{sample release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100 \]

\[ \text{Assay.} \] PBM effector cells were added in triplicate to radiolabeled target cells at ratios of 50:1 to 1:25:1 in a volume of 100 μl and incubated for either 20 h or 4 h (for preactivated effector cells) at 37°C in 5% CO₂ in air. CD56⁺ effector cells were added in triplicate at a ratio of 5:1. The total 51Cr release was determined by the addition of 100 μl of 2% Triton X-100 and the spontaneous release by the addition of medium alone. The percent lysis was determined by counting a 50-μl aliquot of supernatant and applying the formula: % lysis = (sample release - spontaneous release) / (total release - spontaneous release) × 100.

\[ \% \text{ lysis} = \left( \frac{\text{sample release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \right) \times 100 \]

\[ \% \text{ lysis} = \left( \frac{\text{sample release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \right) \times 100 \]

**Target cells.** Allogeneic Hs68 or autologous skin fibroblasts were seeded at 3 × 10⁵ cells per well in six-well plates and then, on the following day, either infected with CMV or left uninfected. Before use as target cells, fibroblasts were detached, and 5 × 10⁴ cells were radiolabeled in 50 μl of PCS containing 20 μCi of ⁵¹Cr (Amersham, U.K.) for 1 h at 37°C. The radiolabeled cells were then washed twice and seeded at 10⁴ cells per well of a 96-well plate. In most experiments, the infected target cells were used at day 4 postinfection, as this was the time at which lysis was optimal, while infected cells were not showing advanced signs of cytopathic effect. Assay. PBM effector cells were added in triplicate to radiolabeled target cells at ratios of 50:1 to 1:25:1 in a volume of 100 μl and incubated for either 20 h or 4 h (for preactivated effector cells) at 37°C in 5% CO₂ in air. CD56⁺ effector cells were added in triplicate at a ratio of 5:1. The total ⁵¹Cr release was determined by the addition of 100 μl of 2% Triton X-100 and the spontaneous release by the addition of medium alone. The percent lysis was determined by counting a 50-μl aliquot of supernatant and applying the formula: % lysis = (sample release - spontaneous release) / (total release - spontaneous release) × 100.

**Statistical analysis** The two-tailed paired t test was used for all statistical analyses.

**Results**

**Fibroblasts infected with the Davis, Toledo, and Towne strains of CMV are resistant to lysis by NK cells**

Initially, we verified a previously reported finding that cells infected with the Davis strain of CMV were refractory to lysis by NK cells (26). Using Hs68 fibroblasts in a 20-h ⁵¹Cr release assay with PBM effector cells, we confirmed that, while AD169-infected target cells were lysed under these conditions, uninfected cells and fibroblasts infected with the Davis strain were not (Fig. 1A). The study was extended to another laboratory strain of CMV, Towne, and to the clinical isolates Toledo, C1F, and R7. Target cells infected with the Towne and Toledo virus strains (Fig. 1B) were not susceptible to NK-mediated lysis; however, fibroblasts infected with the clinical isolates C1F and R7 were killed (Fig. 1C). The differences in lysis were not due to disparate levels of infection, as a similar level of infection (≥95%) was obtained with all strains (data not shown). The data shown are for day 4 postinfection; however, similar data were obtained for days 3 through 5. This pattern of susceptibility or resistance to NK lysis was not restricted to a single donor-target cell combination, as we tested 11 donors of various HLA types against Hs68 target cells infected with the various strains and obtained similar results for all donors (Table I). Furthermore, susceptibility or resistance to NK cell-mediated lysis did not appear to be affected by HLA mismatching, as we found similar results in an autologous setting (Table I). The donor serologic status for CMV did not have any effect on the NK cell-mediated lysis of CMV-infected fibroblasts (Table I). The percent of the six CMV strains tested, cells infected with three strains were resistant to lysis by NK cells from a wide panel of donors.

**The lack of susceptibility of fibroblasts infected with the Towne, Toledo, and Davis strains to NK cell-mediated lysis lies at the level of target cell recognition/lysis rather than effector cell activation**

We considered whether the lack of susceptibility to lysis of fibroblasts infected with the Davis, Toledo, or Towne CMV strains was due to the inability of cells infected with these strains to activate NK cells or to differences in target cell recognition and lysis. To investigate these phases separately, the previously used 20-h ⁵¹Cr release assay was reduced to an overnight (16-h) activation phase followed by a conventional 4-h ⁵¹Cr release assay. PBM effector cells were activated either by overnight treatment with IL-2 or IFN-α or by coculture in a transwell system with uninfected fibroblasts or fibroblasts infected with either strain AD169 or Towne as prototypes of “NK-sensitive” and “NK-resistant” strains, respectively. These effector cells were then tested in a 4-h ⁵¹Cr release assay against ⁵¹Cr-labeled fibroblasts that were uninfected (Fig. 2A) or infected with strain AD169 (Fig. 2B) or Towne (Fig. 2C). Effector cells, which were treated with medium alone (control) or cocultured with uninfected cells, were not activated and failed to lyse any of the target cell types (Fig. 2, A–C). However, effector cells became activated by coculture with fibroblasts infected with either the Towne or AD169 viral strains and were able to lyse various target cells in a 4-h assay. IL-2 or IFN-α treatment also activated the effector cells, and when IFN-α-activated PBM effector cells were sorted into CD56⁺ and CD56⁻ populations, the CD56⁺ population was shown to be responsible for the lysis. Uninfected fibroblasts, which were not lysed in a 20-h assay due to a lack of NK cell activation, were rendered susceptible to lysis by activated NK cells in the 4-h assay (Fig. 2A). As in the 20-h assay, AD169-infected fibroblasts were also susceptible to activated NK cell-mediated lysis although, interestingly, to a lesser degree than that of uninfected target cells (Fig. 2B). However, fibroblast target cells infected with the

<table>
<thead>
<tr>
<th>Donor</th>
<th>CMV Status</th>
<th>Class I HLA Phenotype of Donor</th>
<th>Donor NK Group</th>
<th>Percent Lysis of Fibroblast Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>Cw</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>2.68</td>
<td>44.15</td>
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</tr>
<tr>
<td>2</td>
<td>+</td>
<td>2.24</td>
<td>7.35</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>2.32</td>
<td>18.27</td>
<td>2.12</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>2.36</td>
<td>35.58</td>
<td>3.16</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>2.3</td>
<td>14.58</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>1.7</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>7.12</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>7.7</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>7.7</td>
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<td>–</td>
<td>ND</td>
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<td>11</td>
<td>–</td>
<td>2.3</td>
<td>7.13</td>
<td>6.7</td>
</tr>
<tr>
<td>11'</td>
<td>–</td>
<td>2.3</td>
<td>7.13</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table I. The effect of class I HLA matching between effector and target cells on the NK cell-mediated lysis of CMV-infected and uninfected fibroblasts

- Donor 11 effector cells were used with autologous skin fibroblasts as target cells.
- NK group (1 or 2) of donor effector cells according to whether the HLA C alleles are recognized by the p58.1 or p58.2 inhibitory receptor, respectively.
- Percent lysis of radio-labeled Hs68 fibroblasts was measured using PBM effector cells at an E/T ratio of 50:1 in a 20-h ⁵¹Cr release assay.
- Cw160/02 split not determined; therefore, NK group cannot be predicted.
Towne strain of CMV were shown to be completely refractory to lysis by NK effector cells that had been activated by any of the above means (Fig. 2C). Similar data to that shown for the Towne strain were obtained for the Toledo and Davis strains of CMV (data not shown). Thus, our results indicated that the resistance to NK cell lysis of fibroblasts infected with the Towne, Toledo, and Davis strains of CMV was at the level of target cell recognition/lysis and not NK cell activation.

**Lack of correlation between CMV-induced cell surface class I HLA down-regulation on fibroblast target cells and their susceptibility to NK cell-mediated lysis**

As it has been suggested that the down-regulation of cell surface expression of class I HLA by CMV may render infected cells more susceptible to NK cell attack (15, 16), it was possible that the resistance to lysis of cells infected with particular strains of CMV described above might relate to an inability of these strains to down-regulate class I HLA. We thus compared the effect of infection of fibroblasts with the different CMV strains on the expression of cell surface class I HLA. Fibroblasts infected with AD169, C1F, Davis, Toledo, or Towne were analyzed by flow cytometry for class I HLA expression on day 3 postinfection. The results showed that CMV infection resulted in similar down-regulation of class I for all of the virus strains (Fig. 3A). It was possible that the kinetics of the down-regulation of class I might differ between the various CMV strains, and we therefore studied the cell surface expression of class I following infection of fibroblasts with the strains.
AD169, Davis, Toledo, and Towne from days 1 to 5 postinfection. The class I down-regulation followed a similar pattern for all of these virus strains, with maximal reduction in expression by day 3 postinfection (Fig. 3, B and C). Thus, we found no correlation between the NK susceptibility of cells infected with the various CMV strains and the extent of the down-regulation of class I HLA.

The relationship between the susceptibility to NK cell-mediated lysis and class I HLA expression following CMV infection of different cell types

Having demonstrated the resistance to NK cell-mediated lysis of fibroblasts infected with the Davis, Towne, and Toledo strains, we extended the study to other permissive cell types. In contrast to the findings for fibroblasts, U373 MG astrocytoma cells infected with either the AD169 or Towne strains were killed by IFN-α activated effector cells (Fig. 4A). However, as in the case of infected fibroblasts, the cell surface class I HLA expression was down-regulated by both viral strains (Fig. 4B). Similar data to that shown for the Towne strain were obtained for the Davis and Toledo strains (data not shown). Thus U373 MG cells infected with the Towne, Toledo, and Davis strains were susceptible to NK lysis, while fibroblasts were not, despite the down-regulation of class I HLA by these viral strains in both cell types.

We next studied the susceptibility to NK lysis of CMV-infected endothelial cells. The latter cell type is not permissive for the
AD169, Towne, or Davis strains of CMV, and therefore we studied infection with the endothelial cell tropic strain C1FE. While uninfected endothelial cells were susceptible to lysis by activated NK cells, infection with C1FE prevented lysis (Fig. 4A). To determine whether this effect was a function of the cell type or the virus strain, we also tested the susceptibility to NK lysis of fibroblasts infected with C1FE in the 4-h $^{51}$Cr release assay. In addition, we studied the strain C1F (from which C1FE had been adapted by passage in endothelial cells) in parallel, since we had previously found fibroblasts infected with this strain to be susceptible to NK lysis (Fig. 1C). While C1F-infected fibroblasts were lysed as before, those infected with C1FE were not (Fig. 4A), indicating that the endothelial cell adapted virus strain caused inhibition of lysis in both endothelial cells and fibroblasts. Class I HLA on the surface of fibroblasts infected with either C1F or C1FE was downregulated to a similar extent (Fig. 4B).

The results obtained from studying either different cell types infected with the same virus strain (fibroblasts or U373 MG infected with the Towne strain) or a single cell type infected with different strains of CMV (for example, fibroblasts infected with the C1F vs C1FE strains) all showed that there was no correlation between NK cell-mediated lysis of CMV-infected cells and the virally induced down-regulation of class I HLA.

**Fibroblasts infected with the CMV strains Davis, Toledo, and Towne down-regulate LFA-3**

We next investigated whether differences in the expression of the adhesion molecules ICAM-1 and LFA-3 may have been responsible for the differences in susceptibility to lysis of target cells infected with the various strains of CMV. Fibroblasts infected with AD169, Davis, Toledo, or Towne were analyzed by flow cytometry for the cell-surface expression of ICAM-1 and LFA-3 on days 1 to 5 postinfection (Fig. 5). As previously reported (29, 30), AD169-infected cells up-regulated LFA-3 with maximum induction by day 3 postinfection (Fig. 5B). Similar data were obtained for the other two NK-sensitive strains, C1F (Fig. 4C) and R7 (data not shown). In contrast, infection of fibroblasts with the Davis, Toledo, or Towne strains failed to up-regulate LFA-3 on the cell surface at any time postinfection, and indeed, LFA-3 levels were significantly ($p \leq 0.01$ for days 3–5 postinfection for all three strains) decreased compared with those of uninfected cells at each time point (Fig. 5, B and D). The data shown used the BRIC-5 mAb specific for LFA-3; however, similar results were obtained using the AICD58.9 clone. ICAM-1 expression was up-regulated by CMV infection, with a similar pattern shown for all of the virus strains tested (Fig. 5, A and C). The down-regulation of cell surface LFA-3 observed upon infection of fibroblasts with the Davis, Towne, and Toledo strains of CMV correlated with their lack of susceptibility to NK cell-mediated lysis (as shown above).

The expression of cell surface LFA-3 on different cell types infected with CMV

We investigated whether this correlation between reduced LFA-3 expression and resistance to lysis could be observed with the other cell types examined earlier. In the case of the U373 MG astrocytoma cells, the basal level of LFA-3 expression on uninfected cells was found to be very high in comparison to that found on fibroblasts (Fig. 4C). The level of LFA-3 on U373 MG cells was relatively unaffected by infection with the AD169 strain (Fig. 4C). As seen with fibroblasts, infection of U373 MG cells with the Towne strain of CMV, significantly ($p < 0.05$) reduced LFA-3 levels (Fig. 4C), although LFA-3 levels on these infected cells still remained well above the levels seen on uninfected fibroblasts (Fig. 4C) or on fibroblasts infected with the AD169 strain (data not shown).
Table II. Relative quantification of cell surface class I HLA and LFA-3 levels on fibroblasts and U373 MG cells infected with CMV

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Virus Straina</th>
<th>Susceptibility to NK Lysisb</th>
<th>Class I HLAc</th>
<th>LFA-3d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>Uninfected</td>
<td>+</td>
<td>&gt;217</td>
<td>71 ± 9</td>
</tr>
<tr>
<td></td>
<td>AD169</td>
<td>+</td>
<td>54 ± 2</td>
<td>142 ± 8</td>
</tr>
<tr>
<td></td>
<td>Towne</td>
<td>−</td>
<td>102 ± 6</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>U373 MG</td>
<td>Uninfected</td>
<td>+</td>
<td>&gt;217</td>
<td>&gt;217</td>
</tr>
<tr>
<td></td>
<td>AD169</td>
<td>+</td>
<td>&gt;217</td>
<td>&gt;217</td>
</tr>
<tr>
<td></td>
<td>Towne</td>
<td>+</td>
<td>&gt;217</td>
<td>153 ± 18</td>
</tr>
</tbody>
</table>

a Uninfected, AD169- or Towne-infected cells were analysed at 4 days postinfection.
b Target cell susceptibility to activated NK cell-mediated lysis in a 4-h 51Cr release assay.
c Cell surface levels of class I HLA or LFA-3 were analysed by flow cytometry. The results are given as the relative number of molecules per cell \( \times 10^{-3} \) (mean ± SEM) for the mean of three separate experiments.

down-regulation of LFA-3. The results are expressed as MFI values.

In the case of CMV infection of endothelial cells, the inability to be recognized by NK cells appeared to relate to the virus strain, since the endothelial-adapted C1FE strain also failed to render fibroblasts susceptible to lysis, while the related C1F strain did so (Fig. 4A). The relationship between NK lysis and LFA-3 levels could not be investigated in endothelial cells, since it was not possible to obtain high levels of infection in this cell type, so that “infected” cultures contained both infected and uninfected cells. Interestingly, however, we found that the C1FE strain significantly \( (p < 0.05) \) down-regulated LFA-3 expression in fibroblasts, while C1F significantly \( (p < 0.005) \) up-regulated LFA-3 as compared with uninfected fibroblasts (Fig. 4C). Thus, again, susceptibility to NK lysis correlated with the up-regulation of LFA-3, and inhibition of lysis with the down-regulation of LFA-3.

Relative quantification of cell surface LFA-3 and class I HLA levels

To determine the relative number of LFA-3 molecules per target cell required for NK cell-mediated lysis, we performed relative quantification of LFA-3 expression on uninfected fibroblasts or U373 MG cells or these two cell types infected with either the AD169 or Towne strains. Table II shows that the relative number of cell surface LFA-3 molecules on uninfected fibroblasts that were lysed by activated effector cells was \( \approx 71,000 \) molecules per cell. Infection of fibroblasts with the Towne strain resulted in a significant \( (p < 0.05) \) decrease in the number of LFA-3 molecules to \( \approx 29,000 \) molecules per cell, and these cells were not lysed by NK cells. All other combinations of cell type and virus strain had greater numbers of LFA-3 molecules than the uninfected fibroblast level, (Table II) and, like the latter, all were susceptible to NK cell-mediated lysis. This suggests that the critical number of LFA-3 molecules on target cells required for NK cell lysis is between 29,000 and 71,000 molecules per cell.

Table II also shows that there was no correlation between a low number of molecules of class I HLA and susceptibility to lysis by NK cells. Uninfected U373 MG cells that are sensitive to lysis had

>217,000 class I molecules per cell, while fibroblasts infected with the Towne strain of CMV, which had \( \approx 54,000 \) molecules per cell, were refractory to lysis.

mAb to CD2 reduced NK cell-mediated lysis

We examined the effect of blocking the CD2-LFA-3 interaction between effector and target cells on target cell lysis. Preincubation of IFN-α-activated effector cells with a mAb to CD2 significantly \( (p \leq 0.001) \) reduced the lysis of AD169-infected target cells \( (25.62\% \pm 1.1) \) in a 4-h 51Cr release assay, compared with similar treatment with an isotype-matched control Ab \( (39.6\% \pm 0.7) \). We concluded from these data that the NK cell-mediated lysis of CMV-infected fibroblasts is at least partially dependent on the interaction between CD2 and LFA-3 and, from the data described above, that such an interaction is dependent on a critical level of LFA-3 expression on the target cell.

Kinetics of CMV induced down-regulation of LFA-3

We studied this correlation between the lack of susceptibility to NK lysis and the down-regulation of LFA-3 as a function of time postinfection. Hs68 fibroblasts that were either uninfected or infected with the Towne strain for different time periods were used as target cells in a 4-h 51Cr release assay with IFN-α activated effector cells; in B, the cells were analyzed by flow cytometry for the cell surface expression of LFA-3. The results are expressed as MFI values.
Davis, Toledo, and C1FE strains in Figure 7. These data support the existence of a late CMV gene(s) expressed upon infection of fibroblasts with the Toledo, Towne, and Davis strains of CMV that inhibits lysis by NK cells.

In contrast, ganciclovir treatment of the cells infected with these virus strains did not affect the virally induced reduction in LFA-3 expression (Figs. 6B and 7B), indicating that the latter effect is due to viral immediate early or early genes, the expression of which is not inhibited by ganciclovir. In agreement with our previously reported finding, the up-regulation of LFA-3 by infection with CMV strain AD169 is not blocked and, indeed, is enhanced by ganciclovir treatment (30).

Discussion

In this study, we identified a number of CMV strains (Towne, Toledo, Davis, and C1FE) that could render infected fibroblasts resistant to lysis by NK cells, while infection with other strains (AD169, C1F, and R7) rendered the cells sensitive to NK killing. By separating out the activation and lysis stages involved in the killing of CMV infected cells, we demonstrated that the resistance to NK lysis of cells infected with the Towne, Toledo, Davis, and C1FE strains lay at the level of target cell recognition/lysis. We showed that contact between CMV-infected target cells and PBM effector cells was not required for NK cell activation and that activation was mediated via an as yet unidentified soluble factor.

The susceptibility of cells infected with the various strains of CMV to NK cell-mediated lysis was shown not to relate to differences in their ability to down-regulate the expression of class I HLA Ags, since all strains could do this to a similar extent. Furthermore, U373 MG astrocytoma cells infected with the Towne strain of CMV were killed by NK cells, despite the fact that they had much higher levels of class I HLA than Towne-infected fibroblasts, which were refractory to NK lysis. Our study could not, therefore, provide support for the "missing self hypothesis" (14, 37), which proposes that the down-regulation of class I renders cells more susceptible to NK cell lysis, due to the failure to engage class I-specific inhibitory receptors (12, 13) on the NK cell. Thus, despite a widely held belief that it is the down-regulation of class I HLA that makes virally infected cells more susceptible to lysis by NK cells, this was not the case in the cell types studied for CMV. These data raise the question of whether the well known phenomenon of IFN-mediated protection of uninfected cells from NK-mediated lysis is mediated via the up-regulation of class I HLA, as proposed elsewhere (38). We have shown here that the basal class I level on uninfected cells was insufficient to provide protection from lysis by activated NK cells; however, IFN treatment can increase class I levels by up to 10-fold, which could result in a sufficiently high level of class I to protect the uninfected cell from lysis. It is also possible that IFN treatment could result in the up-regulation of certain HLA alleles, some of which may be particularly effective in delivering inhibitory signals to NK cells.

In contrast to the lack of correlation between NK cell susceptibility and low levels of class I HLA, we found a strong correlation between sensitivity to NK lysis and cell surface levels of LFA-3. Fibroblasts which were infected with strains of CMV that down-regulated LFA-3 (Towne, Davis, Toledo, and C1FE) were refractory to NK cell lysis, while those that up-regulated LFA-3 (AD169 and C1F) were susceptible to lysis. U373 MG cells were susceptible to lysis regardless of which CMV strain was used for the infection; however, these cells expressed a relatively high basal level of LFA-3, and even after the down-regulation of LFA-3 following infection with the Towne, Toledo, C1FE, or Davis strains, they still had higher levels of LFA-3 than uninfected fibroblasts.
which were lysed by activated effector cells. We estimated that the minimum number of LFA-3 molecules per cell required for NK lysis was between 29,000 and 71,000. We were able to partially block NK cell lysis by the addition of a CD2-specific Ab, indicating that the CD2-LFA-3 interaction was a component of the target effector cell interaction, further supporting a partial role for LFA-3 in the NK-mediated lysis of CMV-infected cells.

It is possible that the LFA-3-CD2 interaction plays an important role in NK lysis of target cells other than those infected with CMV, since it was recently shown that transfection of the murine cell line P815 with LFA-3 conferred susceptibility to lysis by NK cell clones (11). At present, it is unclear whether the CD2-LFA-3 interaction merely provides increased adherence of NK cells to target cells or whether it also results in costimulation of the NK cell. The CD2-LFA-3 interaction is one of low affinity and the complexes are highly dynamic, continuously forming and dissociating (39). CD2 is believed to play a role in T cell activation (39), and it has been shown that it is possible to activate NK cells via CD2 (40–42). In the case of T cells, it has been suggested that the CD2-LFA-3 bridge, which has a relatively small span, might act to bring the T cell and APC together at the correct distance for the TCR-MHC interaction to occur (39). The CD2-LFA-3 interaction could play a similar role in bringing the NK cell in the right proximity to its target cell, but it is also possible that it could provide a positive signal to the NK cell. NK cell lysis is thought to depend on a balance between inhibitory and stimulatory signals (11), and although our knowledge of the inhibitory signals involved has increased vastly in recent years (12, 13), less is known about the positive signals. CD2 is one of a number of molecules that have been suggested to provide positive signals to NK cells upon ligation (42), others being CD69, CD16, NKRP1 (43–45), and more recently, the NK cell-specific p46 molecule (46).

All of the virus strains that we tested had an effect on LFA-3, either up-regulating or down-regulating its expression. These effects correlated with susceptibility or resistance to NK lysis, respectively. We showed that both the up-regulation and down-regulation of LFA-3 was mediated by CMV immediate early or early CMV genes, since neither of these effects was inhibited by ganciclovir treatment, which blocks viral DNA synthesis and late viral gene expression. Two of the strains (Toledo and C1FE) that down-regulated LFA-3 and renders infected fibroblasts (and in the case of C1FE, also endothelial cells) resistant to NK cell lysis were low passage clinical isolates, raising the question of whether in vivo some strains of CMV might evade NK lysis of infected cells. It will be of great importance to study more clinical isolates of CMV and to identify the viral genes that affect LFA-3 expression. Our studies also highlighted the fact that target cell type can profoundly affect NK cell lysis, so that the effects of viral infection on adhesion molecule expression and NK cell susceptibility in a particular cell type might be more important than in another. In addition, the way in which the NK cell is activated also appears to affect the susceptibility to lysis. In this study, we demonstrated that NK cells activated in vitro by exposure to factors from CMV-infected cells or treatment with IL-2 or IFN-α could lyse fibroblasts infected with CMV strain AD169 in a 4-h assay, whereas we had previously found that NK cells activated in vivo in bone marrow transplant recipients could not do so (28). As mentioned in the introduction, these in vivo-activated NK cells could lyse EBV-infected target cells, and a reciprocal LFA-1-ICAM-1 interaction was shown to be necessary for lysis (28). Thus, our data suggest that the way in which an NK cell is activated might also affect the requirements of effector-target cell interaction.

In addition to the effect on NK cell-mediated lysis by CMV-induced alterations in LFA-3 expression, we also demonstrated the existence of a virally induced inhibitory signal in fibroblasts infected with CMV strains Towne, Toledo, Davis, and C1FE. This inhibitory signal was mediated by a ganciclovir-sensitive late viral gene product. We cannot rule out the possibility that this represents the CMV-encoded UL-18 gene product (21, 22), which has been shown to confer NK resistance when transfected into a class I HLA-deficient cell line (23). Expression of the gene product has not yet been demonstrated in CMV-infected cells, and thus we do not know whether its expression would be expected to be blocked by ganciclovir treatment. The UL-18-induced inhibition of NK lysis was found to be mediated via the CD94 complex, as it was blocked by the addition of an Ab to CD94 (23). In our study, the same Ab failed to affect the resistance to NK cell lysis of fibroblasts infected with the Towne strain of CMV (data not shown); however, an alternative ligand for UL-18 has since been suggested (47). The identification of the CMV late gene responsible for the NK inhibitory signal associated with infection of fibroblasts with the Towne, Toledo, Davis, or C1FE strains must therefore await further investigation.

It is of interest that the inhibitory late gene signal discussed above did not seem to be effective in U373 MG cells, since infection of these cells with the Towne, Toledo, Davis, or C1FE strains rendered them susceptible to NK lysis. It is possible that the positive NK signal given by the high levels of LFA-3 expressed on U373 MG cells infected with these virus strains might have been sufficient to overcome any negative signal. This would be in keeping with current views of NK cell function, where susceptibility or resistance to lysis results from the outcome of a balance between positive and negative signals (11). Alternatively, the viral late gene product responsible for the NK inhibitory signal might not be functional in this cell type.

It is not clear whether infection with the AD169 or C1F strains also induces an inhibitory signal. The AD169 strain of CMV contains the UL-18 gene (21), and indeed it was the AD169 UL-18 gene that conferred the NK resistance in transfected cells discussed above. In support of the existence of an inhibitory signal, we usually observe a lower level of killing by activated effector cells of fibroblasts infected with the AD169 or C1F virus strains compared with uninfected cells (see Figs. 2 and 4A, respectively). This finding is in contrast to the situation for herpes simplex virus, where infected fibroblasts are preferentially lysed, even by activated NK cells (48, 49). Interestingly, no NK cell inhibitory signal has been described for this virus to date. It is possible that the high level of cell surface LFA-3 expression induced by infection with the AD169 and C1F CMV strains may provide a sufficiently positive signal to partially overcome an inhibitory signal. In contrast, the low level of LFA-3 expressed on fibroblasts infected with strains Davis, Toledo, and Towne is clearly insufficient to overcome the inhibitory signal.

In conclusion, in the cell types studied (fibroblasts, endothelial cells, and U373 MG cells), for seven different strains of CMV, the CMV-induced down-regulation of class I HLA did not appear to influence target cell susceptibility to lysis by NK cells. In contrast, there was a strong correlation between the level of cell surface LFA-3 expression and the susceptibility of the target cell to lysis. We propose that LFA-3 provides an important adhesion/costimulatory function for NK cell-target cell recognition/lysis.

The up-regulation of LFA-3 by the AD169 and C1F strains of CMV was shown to be mediated by immediate early/early viral gene products, as we have reported previously for the AD169 strain. Interestingly, others have shown that the susceptibility to NK lysis of AD169-infected fibroblasts required expression of only immediate early/early genes and that late gene expression was not necessary (27). This supports our hypothesis that the up-regulation of LFA-3 plays a role in determining the susceptibility to NK lysis of cells infected with the AD169 or C1F strains of CMV.
NK LYSIS OF CMV-INFECTED CELLS CORRELATES WITH LFA-3 EXPRESSION

In contrast, we have shown that both immediate early/early and late viral genes play a role in determining the resistance to NK lysis of fibroblasts infected with the Towne, Davis, Toledo, and CIFE strains. Initially, immediate early/early gene functions in these virus strains induce the down-regulation of LFA-3, which correlates with a decreased susceptibility to NK lysis compared with uninfected cells at early times postinfection. At later times postinfection, fibroblasts infected with these virus strains express a late gene product that provides an inhibitory signal to NK cells, thereby further increasing their resistance to lysis. We conclude that NK lysis of CMV-infected cells results from a balance between positive signals, including the LFA-3-CD2 interaction, and an as yet undefined inhibitory signal that results from the expression of a late CMV gene.

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References