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Human Adenovirus-Specific CD8$^+$ T-Cell Responses Are Not Inhibited by E3-19K in the Presence of Gamma Interferon

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Adenovirus has considerable potential as a gene therapy vector, but recent animal data suggest that transduced cells are destroyed by adenovirus-specific cytotoxic T-lymphocyte (CTL) responses. Therefore, it will be important to develop strategies to evade adenovirus-specific CTL responses in humans. As a first step, an assay was developed to detect and characterize human CTLs directed against adenovirus. Adenovirus-specific CTL responses were demonstrated to be present in four of five healthy adults by in vitro stimulation of peripheral blood mononuclear cells with autologous fibroblasts infected with the adenovirus type 2 (Ad2) E3 deletion mutant Ad2$^+$ND1. Killing by adenovirus-specific CTLs was major histocompatibility complex class I restricted and was documented to be mediated by CD8$^+$ T cells. Wild-type-Ad2-infected cells were poor CTL targets compared with cells infected with the E3 deletion mutant because of the expression of E3-19K, an early viral glycoprotein which prevents transport of major histocompatibility complex class I antigens out of the endoplasmic reticulum to the cell surface. However, preincubation of targets with gamma interferon resulted in enhanced killing of wild-type-Ad2-infected cells, to levels comparable to those obtained with Ad2$^+$ND1-infected cells. Radioimmunoprecipitation analysis revealed that gamma interferon not only increased the synthesis of class I antigens but also allowed excess molecules to escape from the endoplasmic reticulum. It is concluded that E3-19K expression in adenovirus-infected cells inhibits human CTL recognition in vitro but that gamma interferon may help overcome the E3-19K effect during acute infection in vivo.

Adenovirus offers unique advantages as a gene therapy vector, including the ability to transduce and express recombinant proteins in a wide range of cell types. On a practical level, adenovirus vectors can also be readily prepared in large quantities in tissue culture. In particular, adenovirus-mediated gene therapy is being evaluated for the treatment of cystic fibrosis. Adenovirus vectors have been successfully used to express the cystic fibrosis transmembrane conductance regulator (CFTR) in mouse, cotton rat, and simian models, as well as in human bronchial xenografts in nude mice (5, 11, 45). In one short-term study with humans, CFTR was successfully expressed in adenovirus-transduced nasal mucosa without significant side effects (48). Several phase I clinical trials of adenovirus-mediated gene therapy are under way.

Recent studies with animal models, however, have suggested that the efficacy of adenovirus-mediated gene therapy may be limited by antiviral immune responses. Using a replication-deficient adenovirus vector with a deletion of the early region 1 (E1) (E1A and E1B) in a mouse model, Yang and colleagues have documented that adenovirus-specific CD8$^+$ T cells destroy transduced cells within 2 to 3 weeks (42). Presumably, in vivo, despite the absence of the E1A transactivating region in the vector, low-level expression of one or more viral proteins occurs in transduced cells which can be recognized by virus-specific cytotoxic T lymphocytes (CTLs). In support of the concept of T-cell-mediated destruction of adenovirus-transduced cells, recombinant-protein expression from an adenovirus vector persisted for up to 6 months in nude mice (44). Adenovirus-specific immune responses are likely to present an even greater problem in humans because more viral proteins may be expressed from deletion vectors in human cells than in mouse cells, in which adenovirus replication is restricted. In addition, most individuals have had prior exposure to this common virus and immune responses will already be primed.

Thus, there is a need to develop new strategies for vector design in order to evade adenovirus-specific CTL responses. CTL responses are primarily mediated by CD8$^+$ T cells which recognize endogenously synthesized viral proteins in association with major histocompatibility complex (MHC) class I antigens (46). Viral proteins are processed into peptides within proteasomes in the cytoplasm, and peptides are translocated to the endoplasmic reticulum (ER), where they are bound to newly synthesized class I molecules and transported to the cell surface. In contrast, CD4$^+$ T cells recognize exogenous viral proteins which are degraded into peptides in endosomal vesicles and bound to class II molecules (20). Therefore, one strategy for vector design is to insert a protein(s) which interferes with class I-restricted antigen presentation. Several viruses have evolved such proteins. For instance, adenovirus expresses an early region 3 (E3) protein, E3-19K, which specifically binds to class I molecules in the ER and prevents transport of class I antigens to the cell surface (6). As another example, herpes simplex virus interferes with antigen presentation by the action of an immediate-early protein, ICP47, which inhibits the transport of peptides to the ER (47). An alternative approach is to design vectors with additional deletions which eliminate epitopes crucial to CTL recognition. As a first step, the addition of a temperature-sensitive E2 muta-

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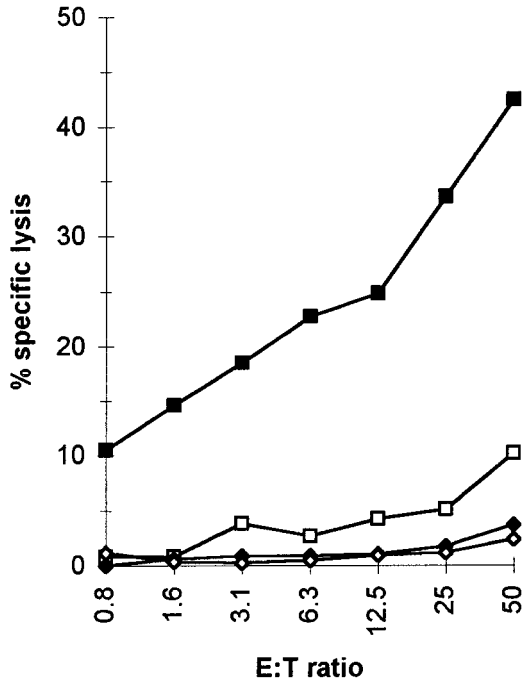

FIG. 1. Detection of adenovirus-specific cytotoxic responses in PBMC after in vitro stimulation. Donor 1 PBMC were enriched for $CD8^+$ T cells prior to stimulation with $Ad2^+ND1$ (ND1)-infected autologous fibroblasts for 3 weeks. Cytotoxicity against infected and uninfected autologous (auto) fibroblast targets was measured by a chromium release assay (percent specific lysis). HLA class I-mismatched infected and uninfected fibroblast targets from donor 2 were also tested as an allogeneic (allo) control. E:T ratio, effector/target ratio. ■, auto fibroblasts infected with ND1; □, allo fibroblasts infected with ND1; ◆, uninfected auto fibroblasts; ◇, uninfected allo fibroblasts.

tion to an E1-deleted adenovirus vector increased the survival of adenovirus-transduced cells in the mouse model (12). It is therefore important to identify immunodominant epitopes recognized by human adenovirus-specific CTLs.

Human adenovirus-specific cellular responses have not previously been studied. Adenovirus is a ubiquitous DNA virus which is a common pathogen in early childhood, and most individuals have serologic evidence of prior infection by age 10 (23). Adenovirus may establish persistent infections. As examples, adenovirus may be shed in feces for months to years postinfection in healthy individuals (18) and reactivation of adenovirus infection in immunocompromised hosts may lead to fatal disease (14). In light of the high seroprevalence of adenovirus and its ability to persist in vivo, we postulated that it would be possible to detect adenovirus-specific cellular immune responses in healthy adults. Recently, we confirmed that peripheral blood mononuclear cells (PBMC) from the majority of healthy adults exhibit adenovirus-specific $CD4^+$ T-cell proliferative responses (16).

We now report the identification of adenovirus-specific human $CD8^+$ T-cell cytotoxic responses in PBMC after in vitro stimulation with live infected, autologous fibroblasts. The ability of the adenovirus early glycoprotein E3-19K to interfere with presentation of antigen to adenovirus-specific CTLs was evaluated by using a panel of E3 deletion mutants. Although E3-19K expression blocked human CTL recognition of adenovirus-infected cells in vitro, this effect was overcome by pretreatment of targets with gamma interferon (IFN-γ). These findings suggest that the E3-19K-mediated inhibition of presentation of antigen to antiadenovirus CTLs may be counteracted in vivo.

MATERIALS AND METHODS

Study participants and HLA types. Skin biopsy and blood samples were obtained from five healthy adult volunteers in a protocol approved by the Medical College of Wisconsin Institutional Review Board. HLA typing was performed at the Blood Center of Southeastern Wisconsin with the following results: donor 1, A1, 3; B35, 37; donor 2, A2, 29; B44, 61; donor 3, A2; B44, 50; donor 4, A3, 28; B14, 38; and donor 5, A1, 34; B7, 35.

Cell lines. Human fibroblast cell lines derived from skin biopsy samples were grown in Dulbecco's modified Eagle medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2.5 μg of amphotericin B (Fungizone) per ml (cDMEM) and 10% fetal bovine serum (FBS). Fibroblasts were used from passages 2 through 12.

Other cell lines used were human lymphoblastoid B-cell lines (B-LCL), K-562 (CCL 2243; American Type Culture Collection [ATCC], Rockville, Md.), and A549 (CCL 185; ATCC). B-LCL were prepared from Ficoll-Hypaque-separated PBMC by incubation with supernatant from the Epstein-Barr virus-infected marmoset cell line B95-8 (CRL 1612; ATCC) in the presence of 1 μg of cyclosporin A per ml. B-LCL were used as feeder cells in the generation of adenovirus-specific CTLs. K-562, an erthyroleukemia cell line, was used as a natural killer (NK) target. Both B-LCL and K-562 were maintained in RPMI 1640 supplemented with 2 mM glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% FBS. Adenovirus stocks were prepared from virus-infected human lung carcinoma A549 cells as described below.

Viruses. The adenovirus type 2 (Ad2) prototype represents wild-type Ad2, and the $Ad2^+ND1$ mutant contains a deletion of the entire E3 region, which is substituted by a simian virus 40 (SV40) DNA fragment (26). The following E3 deletion mutants were derived from *rec*700, an Ad5/2/5 construct in which the Ad5 E3 region has been replaced by the Ad2 E3 region (41): *dl*704, containing a deletion of the 19-kDa open reading frame (ORF) (3); *dl*739, containing a deletion of the 6.7-kDa ORF (40); and *dl*763, containing deletions of both the 14.5- and 14.7-kDa ORFs (4). A549 monolayers were infected with each virus in cDMEM with 2% FBS and harvested after 3 to 4 days when a cytopathic effect of 4+ was present. Cells were subjected to three freeze-thaw cycles to release virus into the media, debris was removed by centrifugation, and supernatants were stored at −70°C. Each virus stock was titrated on fibroblast monolayers. The lowest dilution at which >60% of the cells stained positive by an adenovirus-specific immunofluorescent assay at 24 h postinfection was used to infect stimulator and target cells.

Generation of adenovirus-specific CTLs. Adenovirus-specific CTLs were prepared by using modifications of the technique of Riddell et al. for the generation of CTLs to cytomegalovirus (CMV) (34). Fibroblasts were trypsinized and adsorbed with $Ad2^+ND1$ virus stock for 1 h in suspension. Cells were washed with phosphate-buffered saline (PBS), placed in a 24-well plate at 10^5 cells per well in cDMEM with 10% human AB serum, and incubated at 37°C in a 5% CO_2 humidified atmosphere. At 24 h postinfection, Ficoll-Hypaque-purified autologous PBMC were added to the infected fibroblasts at a ratio of 30:1 in RPMI 1640 supplemented with 10% human AB serum, 10 mM HEPES, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, 2.5 μg of amphotericin B per ml, and 2.5×10^{-5} M 2-mercaptoethanol (CTL medium). After a 7-day incubation, cells were washed, and 3×10^6 cells were added to new wells containing 24-h-infected fibroblasts and 3×10^6 autologous irradiated PBMC (3,000 rad) or B-LCL (10,000 rad) as feeder cells. Recombinant human interleukin-2 (Cetus Corporation, Emeryville, Calif.) was added to cultures at a concentration of 5 U/ml on days 2 and 4 after restimulation. Cell cultures were tested for cytotoxic responses on day 14, or in some cases, effectors were restimulated a third time with infected cells, feeder cells, and interleukin-2 and then assayed on day 21.

Adenovirus-specific CTL assay. For use as infected targets, autologous and allogeneic HLA-mismatched fibroblast monolayers were adsorbed with the appropriate dilution of virus stock for 1 h, washed with PBS, and incubated in cDMEM and 10% FBS for 24 h. In some experiments, fibroblasts were pretreated 24 h prior to infection with 200 U of human IFN-γ per ml (Biogen, Cambridge, Mass.) and the cytokine was maintained in the media postinfection. Fluorescence-activated cell sorter (FACS) analysis confirmed that fibroblast targets expressed class I but not class II antigens; IFN-γ treatment increased the number of class I molecules and induced low-level class II expression. As controls, uninfected autologous and allogeneic fibroblasts, as well as K-562 cells, were also used as targets in each experiment. Target cells were labeled in suspension with ^{51}Cr (150 μCi) as sodium chromate for 1 h. After three washes with cDMEM plus 10% FBS, 5×10^3 labeled targets were added to triplicate microwells containing various numbers of effector cells in a final volume of 200 μl in CTL medium. Effector/target ratios ranged from 100:1 to 1.3:1. After a 4-h incubation, plates were centrifuged at $300 \times g$ for 5 min and 150 μl of supernatant was collected and counted in a γ-counter. Percent specific ^{51}Cr release was calculated as follows: [(cpm of sample − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release)] × 100, where cpm is counts per minute. Spontaneous release was measured from target cells incubated in medium alone, and maximum release was measured from target cells incubated with 2% 7× detergent (Flow Laboratories, McLean, Va.). Spontaneous release was less than 20% for all targets.

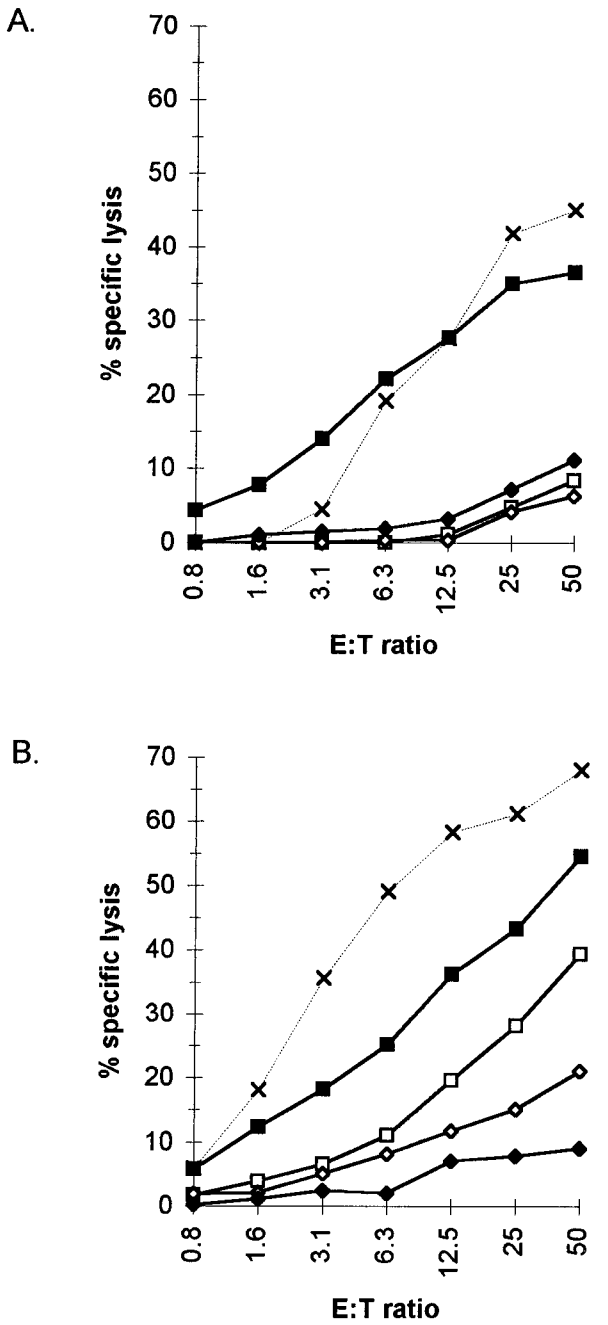

FIG. 2. Comparison of adenovirus-specific cytotoxic responses in bulk CTL cultures from two different donors. PBMC were depleted of $CD56^+$ cells prior to stimulation with $Ad2^+ND1$ (ND1)-infected autologous (auto) fibroblasts for 2 weeks. Cytotoxic activity against infected and uninfected fibroblasts and K-562 was measured by a chromium release assay (percent specific lysis). (A) Donor 3 effectors exhibit a high-level HLA class I-restricted antiadenovirus cytotoxic response; class I-mismatched fibroblasts (donor 4) were used as the allogeneic (allo) control. (B) Donor 4 effectors exhibit a low-level class I-restricted adenovirus-specific cytotoxic response and high-level background cytotoxicity against both K-562 and infected class I-mismatched fibroblasts (donor 3). E:T ratio, effector/target ratio. ×, K-562 cells; ■, auto fibroblasts infected with ND1; □, allo fibroblasts infected with ND1; ◇, uninfected allo fibroblasts; ◆, uninfected auto fibroblasts.

Enrichment of $CD8^+$ T cells. PBMC (10^8 cells) were incubated with 200 μl of CD8 microbeads (Miltenyi Biotech, Auburn, Calif.) at 4°C for 30 min, washed, and positively selected by using the MACS magnetic cell separator (Miltenyi Biotech). Purity of the $CD8^+$ T-cell-enriched preparation was >98% as measured by flow cytometry.

Depletion with MAb and complement. PBMC cultures were depleted of $CD56^+$ cells, $CD4^+$ T cells, or $CD8^+$ T cells with the monoclonal antibodies (MAbs) NKH1A (Coulter, Hialeah, Fla.), OKT4 (Ortho Diagnostics, Raritan, N.J.), and OKT8 (Ortho), respectively. Cells were suspended at 10^7/ml in RPMI with 5% human AB serum and the appropriate concentration of each MAb and incubated for 30 min at 4°C. Rabbit complement (Cedarlane, Hornby, Canada) was added at 35% of the final volume in the presence of 15 U of DNase (Sigma, St. Louis, Mo.) per ml and incubated at 37°C with mixing for 90 min. Cells were washed, suspended in Hanks balanced salt solution, and layered over a Ficoll-Hypaque gradient to remove nonviable cells. The efficiency of each depletion was confirmed by flow cytometry.

Immunophenotyping. Analysis of cell surface antigens was done by two-color immunofluorescent flow cytometry by standard direct-staining methods as previously described (16). MAbs were purchased as fluorescein or phycoerythrin conjugates from Becton Dickinson (San Jose, Calif.) and included antibody cocktails directed against CD45/CD14, CD3/CD4, CD3/CD8, and CD8/CD56. Nonspecific binding was monitored by using isotypic controls.

Adenovirus-specific immunofluorescent assay. Adenovirus-infected fibroblasts were trypsinized, washed, and applied to slides by using a cytocentrifuge (Shandon, Pittsburgh, Pa.). Slides were fixed in cold acetone and stained for late adenovirus proteins by using the Bartels antiadenovirus MAb and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (Baxter, West Sacramento, Calif.). Test slides were also stained with nonimmune mouse immunoglobulin as a negative control. Ad2-infected and uninfected A549 cells were used as controls for each assay.

Metabolic labeling and immunoprecipitation. Ad2 stock was adsorbed onto T25 fibroblast monolayers for 1 h, and cells were rinsed in PBS and incubated in cDMEM with 10% FBS overnight. Mock-infected monolayers were prepared as controls. After a 30-min incubation in prewarmed DMEM without methionine and cysteine (ICN, Costa Mesa, Calif.), cells were labeled from 23 to 24 h postinfection with 200 μCi of [^{35}S] Protein Labeling Mix (NEN, Wilmington, Del.) in amino-acid-depleted DMEM with 10% dialyzed FBS (Gibco, Gaithersburg, Md.). Monolayers were rinsed with PBS and incubated for an additional 3 h in cDMEM with 10% FBS containing a fourfold excess of cold methionine and cysteine. Cells were harvested and lysed in 300 μl of cold lysis buffer (1% Nonidet P-40 [NP40], 150 mM NaCl, 50 mM Tris [pH 8.0], 0.02% NaN_3), and insoluble debris was removed by centrifugation.

Prior to radioimmunoprecipitation assay (RIPA), lysates were adsorbed with normal rabbit sera and protein A-Sepharose (Pharmacia, Norwalk, Conn.). Specific rabbit antisera or mouse MAb was incubated with 100 μl of a 10% solution of protein A-Sepharose in lysis buffer for 1 h at 4°C. Equal volumes of each lysate were incubated with antibody-loaded protein A-Sepharose for 1 h at 4°C in a final volume of 400 μl in lysis buffer containing 2% bovine serum albumin. Immune complexes were washed three times in lysis buffer and eluted in sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.06 mM Tris [pH 6.8], 0.005% bromophenol blue, 0.1 M dithiothreitol) by heating to 95°C for 5 min. For analysis of carbohydrate moieties, immune complexes were eluted instead in 0.5% SDS–50 mM citrate buffer (pH 5.5) and then one-half of each specimen was incubated with 1,000 U of endoglycosidase H_f (Endo H) (New England Biolabs, Beverly, Mass.) at 37°C for 16 h and specimens were mixed with an equal volume of 2× sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and then subjected to fluorography with En^{3}Hance (NEN).

Antibodies. The following antibodies were used in the RIPA. The MAb W6/32 recognizes an HLA class I common determinant (1), a rabbit serum against the Ad2 E3-19K has been previously described (17), and a rabbit serum against purified Ad2 virions was purchased from the ATCC (VR-1079).

RESULTS

Adenovirus-specific CTL responses can be detected in PBMC after in vitro stimulation. PBMC from a healthy donor were stimulated with $Ad2^+ND1$-infected autologous fibroblasts for 2 weeks, as described in Materials and Methods, in order to amplify adenovirus-specific CTLs. $Ad2^+ND1$, which contains a deletion of the E3 region, was used to infect stimulators in order to avoid potential downregulation of MHC class I antigens by E3-19K early after infection. Autologous, infected fibroblasts, which constitutively express HLA class I but not HLA class II antigens, were used as both stimulators and targets in order to assay class I-restricted CTL responses. In initial experiments, CTL cultures exhibited higher levels of cytotoxicity against $Ad2^+ND1$-infected autologous fibroblasts than against uninfected autologous or class I HLA-mismatched fibroblasts. However, the level of cytotoxicity against infected HLA-mismatched fibroblasts was higher than it was against uninfected controls, suggesting the presence of non-

TABLE 1. MHC-restricted adenovirus-specific cytotoxicity is mediated by $CD8^+$ T cells

Effector treatment[a]	% Positive cells[b]			% Specific lysis[c]			
	$CD4^+$	$CD8^+$	$CD56^+$	Auto + ND1	Allo + ND1	Auto	K-562
None	30	58.4	25.3	49.1	39.7	45.4	75.3
OKT4, NKH1A, and complement	0.2	98.8	9.8	36.9	1.5	0	20.2
OKT8, NKH1A, and complement	88.9	2.4	ND[d]	0	5.7	5.4	ND

[a] Bulk CTL cultures were prepared by in vitro stimulation of donor 1 PBMC with $Ad2^+$ND1-infected autologous fibroblasts for 2 weeks. Effectors were depleted with MAb plus complement prior to assay.
[b] Cells were analyzed by flow cytometry, and results are listed as the percentage of viable cells that express each surface marker.
[c] Cytotoxicity against $Ad2^+$ND1 (ND1)-infected and uninfected autologous (Auto) fibroblasts was measured by a chromium release assay using an effector/target ratio of 25:1. Infected HLA class I-mismatched fibroblasts (donor 3) and K-562 were also tested as allogeneic (Allo) and NK controls, respectively.
[d] ND, not done because of inadequate cell numbers.

MHC-restricted activity. Therefore, PBMC were enriched for $CD8^+$ T cells by using the MACS magnetic cell separator prior to stimulation with infected, autologous fibroblasts. High levels of adenovirus-specific CTL killing were observed with $CD8^+$ T-cell-enriched cultures, with low-level background cytotoxicity against all control targets (Fig. 1). This experiment confirmed the presence of adenovirus-specific, HLA class I-restricted cytotoxicity in PBMC and suggested that this activity was mediated by $CD8^+$ T cells.

Adenovirus-specific CTL activity is detectable in four of five healthy adults. PBMC from five different donors were stimulated in vitro with $Ad2^+$ND1-infected autologous fibroblasts for 2 weeks and assayed for virus-specific killing. As an alternative to $CD8^+$ T-cell enrichment, which resulted in very low cell yields, PBMC were depleted of NK cells with anti-CD56 MAb and complement on day 1 in an attempt to reduce nonspecific killing. However, residual NK cells proliferated in the CTL cultures, as confirmed by FACS analysis, and all donors exhibited cytotoxicity against the NK target K-562. PBMC from donors 1 and 3 demonstrated high levels of adenovirus-specific cytotoxicity and low-level background cytotoxicity against HLA class I-mismatched targets; a representative assay is shown in Fig. 2A. In comparison, donors 2 and 4 exhibited low levels of MHC-restricted cytotoxicity against adenovirus-infected targets. As illustrated in Fig. 2B, effectors generated from donor 4's PBMC exhibited relatively high-level cytotoxicity against infected allogeneic control targets, which correlated with a higher level of cytotoxicity against K-562 than that shown in Fig. 2A. In all subsequent experiments, therefore, PBMC were depleted of NK cells on the day of the CTL assay. Cells from donor 5 had high levels of cytotoxicity against all targets, including uninfected controls (data not shown).

Adenovirus-specific CTL activity is mediated by $CD8^+$ T cells. The initial experiment utilizing $CD8^+$ T-cell-enriched PBMC suggested that $CD8^+$ T cells mediated MHC class I-restricted adenovirus-specific cytotoxicity. In order to confirm the identity of the effector cell population responsible for the adenovirus-specific killing, CTL cultures were tested after depletion of either $CD4^+$ or $CD8^+$ T cells. PBMC were stimulated with $Ad2^+$ND1-infected autologous fibroblasts for 14 days, and effectors were depleted of NK cells plus either $CD4^+$ T cells or $CD8^+$ T cells with the appropriate MAbs and complement prior to CTL assay. Nondepleted effectors were tested as a control. In this experiment, untreated effectors exhibited a high level of nonspecific cytotoxicity, which correlated with a high proportion of $CD56^+$ cells and high-level cytotoxicity to K-562 (Table 1). $CD56^+$ and $CD4^+$ T-cell-depleted effectors exhibited MHC-restricted adenovirus-specific cytotoxicity, whereas depletion of $CD56^+$ and $CD8^+$ T cells abolished cytotoxicity. Therefore, $CD8^+$ T cells are responsible for the MHC-restricted adenovirus-specific cytotoxicity detected in this assay.

Adenovirus-specific CTLs do not recognize input virion proteins. In order to determine whether adenovirus-specific CTLs recognized input virion proteins or required de novo synthesis of viral proteins, the ability of effectors to kill autologous fibroblasts after infection with $Ad2^+$ND1 for 1 h was compared with their ability to kill fibroblasts infected for 24 h. After a 1-h adsorption with virus, cells were incubated for an additional 1 or 24 h prior to being plated with effector cells for a standard 4-h Cr release assay. The 1-h time point was chosen to allow for uncoating of virions without the expression of new viral proteins. Previous studies have demonstrated that adenovirus

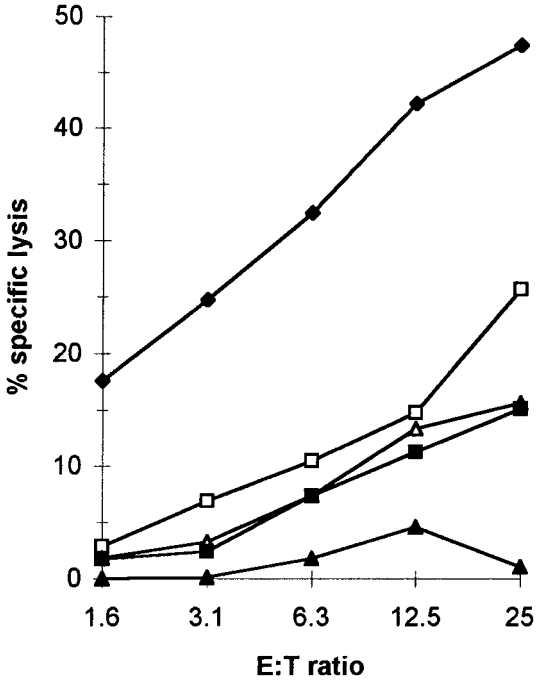

FIG. 3. Adenovirus-specific cytotoxic T cells do not recognize input virions. Antiadenovirus effectors were generated by stimulating donor 1 PBMC with $Ad2^+$ND1 (ND1)-infected autologous (auto) fibroblasts. The cytotoxic response against auto fibroblasts infected with ND1 for 1 h (input) was compared with that against standard 24-h-infected targets. Cytotoxic activity (percent specific lysis) was measured by a chromium release assay. HLA class I-mismatched fibroblasts from donor 3 were used as an allogeneic (allo) control. E:T ratio, effector/target ratio. ◆, auto fibroblasts infected with ND1; □, allo fibroblasts infected with ND1; △, uninfected allo fibroblasts; ■, auto fibroblasts infected with ND1 for 1 h (input); ▲, uninfected auto fibroblasts.

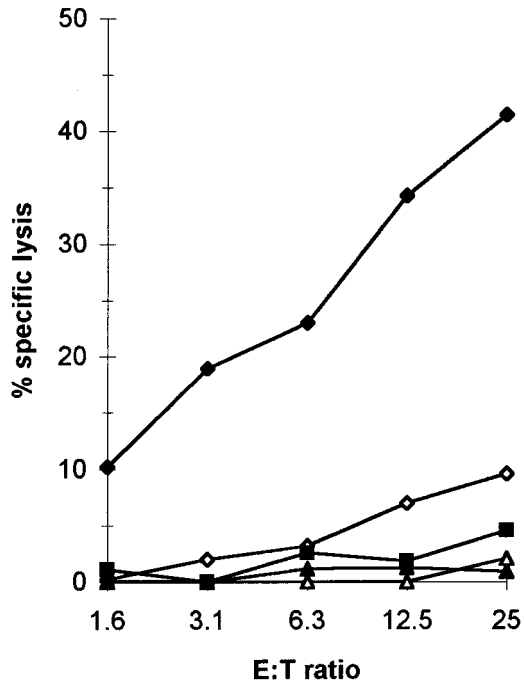

FIG. 4. Wild-type-Ad2-infected fibroblasts are not killed by adenovirus-specific cytotoxic T cells. Adenovirus-specific effectors were generated by stimulating donor 1 PBMC with Ad2$^+$ND1 (ND1)-infected autologous (auto) fibroblasts and tested for the ability to kill fibroblasts infected with Ad2. Cytotoxic activity (percent specific lysis) was measured by a chromium release assay. HLA class I-mismatched fibroblasts from donor 3 were used as an allogeneic (allo) control. E:T ratio, effector/target ratio. ◆, auto fibroblasts plus with ND1; ◇, allo fibroblasts infected with ND1; ■, auto fibroblasts infected with Ad2; △, uninfected allo fibroblasts; ▲, uninfected auto fibroblasts.

penetrates cells by receptor-mediated endocytosis and is rapidly released into the cytoplasm (7, 13). An immunofluorescence assay confirmed that the majority of 1-h-infected targets contained intracellular adenovirus proteins. As shown in Fig. 3, effectors exhibited specific cytotoxicity against standard 24-h-infected targets but did not kill 1-h-infected targets which contained only input viral proteins.

E3-19K expression inhibits adenovirus-specific CTL recognition. In all of the above-described experiments, adenovirus-specific CTLs were generated by using the E3 deletion mutant Ad2$^+$ND1 to infect both stimulators and targets. In order to test the effect of the E3 region on CTL recognition, the adenovirus-specific CTL cultures were tested against targets infected with wild-type Ad2 which express E3 proteins. Although effectors readily killed Ad2$^+$ND1-infected autologous fibroblasts, there was no detectable specific cytotoxicity against Ad2-infected autologous fibroblasts (Fig. 4). It was postulated that the lack of killing of wild-type-adenovirus-infected targets in comparison with killing of Ad2$^+$ND1-infected targets was related to the expression of the early viral glycoprotein E3-19K, which inhibits the transport of MHC class I molecules to the cell surface. The fact that Ad2$^+$ND1 contains an SV40 insert also made it possible, but unlikely, that the effectors were specific for SV40 proteins expressed by Ad2$^+$ND1 rather than adenovirus. Therefore, the CTL activities against fibroblasts infected with a panel of different E3 deletion mutants based upon the construct *rec*700 were compared. The parent *rec*700 and the mutants *dl*739 and *dl*763, which contain deletions of individual E3 region ORFs, all express E3-19K, whereas *dl*704 contains a deletion of the 19-kDa ORF alone and does not express E3-19K. As shown in Fig. 5, only the targets which did not express E3-19K were killed, i.e., the cells infected with either *dl*704 or Ad2$^+$ND1. These data confirm that the effectors are adenovirus specific and that the expression of E3-19K protects infected cells from being destroyed by human adenovirus-specific CTLs in vitro.

IFN-γ enhances killing in the presence of E3-19K. On the basis of data showing that IFN-γ increases class I transcription and assembly (27), it was postulated that IFN-γ might enhance killing of wild-type-Ad2-infected cells. This was tested by incubating fibroblast targets with IFN-γ (200 U/ml) prior to the CTL assay for 48 h, the time period by which cell surface class I expression peaks. At 24 h, fibroblasts were infected with wild-type Ad2 or mock infected as a control. Uninfected and Ad2-infected fibroblasts without IFN-γ pretreatment were used as controls. In contrast to untreated Ad2-infected cells, Ad2-infected cells which were pretreated with IFN-γ were killed at levels similar to those for Ad2$^+$ND1-infected targets (Fig. 6). RIPA determined that the levels of E3-19K were similar in Ad2-infected cells incubated with and without IFN-γ, which ruled out a direct effect of IFN-γ on E3-19K expression (data not shown). However, IFN-γ pretreatment reduced the proportion of adenovirus-positive cells detected by immunofluorescence assay (77 against 48%), which correlated with a partial inhibition of expression of late viral proteins as measured by RIPA.

IFN-γ allows class I molecules to escape E3-19K effect. The effect of IFN-γ on class I expression and processing in Ad2-infected fibroblasts was analyzed by RIPA and Endo H digestion. Fibroblasts with and without IFN-γ pretreatment were infected with Ad2 for 24 h, as described in Materials and Methods. Cells were then pulse-labeled with a mixture of

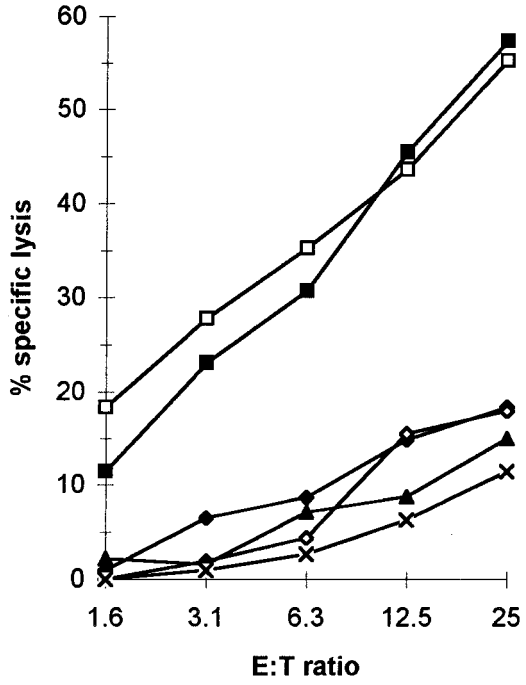

FIG. 5. Adenovirus-specific cytotoxicity is inhibited by the expression of E3-19K in infected targets. Antiadenovirus effectors (donor 1) were assayed for the ability to kill a series of autologous (auto) fibroblasts infected with different E3 deletion mutants. Cytotoxic activity (percent specific lysis) was measured by a chromium release assay. HLA class I-mismatched fibroblasts (donor 3) were used as an allogeneic (allo) control. E:T ratio, effector/target ratio. ■, *dl*704; □, ND1; ◆, *dl*739; ◇, *dl*763; ▲, *rec*700; ✕, allo fibroblasts infected with ND1.

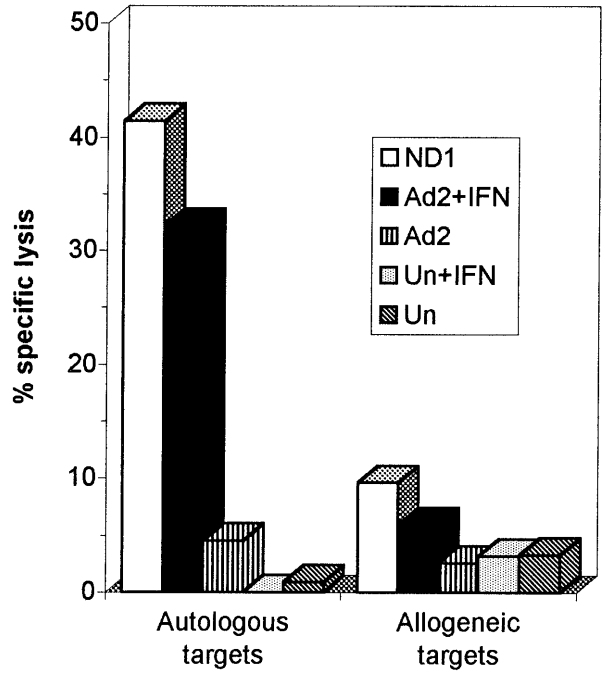

FIG. 6. Pretreatment of wild-type-Ad2-infected targets with IFN-γ allows recognition by adenovirus-specific cytotoxic T cells. Adenovirus-specific effectors were generated by stimulating donor 1 PBMC with Ad2+ND1 (ND1)-infected autologous fibroblasts. The abilities of effectors to kill Ad2-infected fibroblasts with and without pretreatment with IFN (200 U/ml) for 48 h were compared. Cytotoxic activity (percent specific lysis) was measured by a chromium release assay using an effector/target ratio of 25:1. Un, uninfected fibroblasts.

[35S]methionine and [35S]cysteine for 1 h, after which a 3-h chase with excess cold amino acids was done. As controls, uninfected cells with and without IFN-γ were also labeled. Class I molecules were immunoprecipitated with the MAb W6/32, and one-half of each sample was digested with Endo H. As shown in Fig. 7, uninfected fibroblasts synthesized a low level of normally processed class I heavy chains which became terminally glycosylated and Endo H resistant. In contrast, the class I heavy chains in Ad2-infected cells were retained in the ER and remained Endo H sensitive. The 25-kDa E3-19K glycoprotein, which contains two high-mannose carbohydrate moieties, was coimmunoprecipitated with class I and reduced to 19 kDa with Endo H. IFN-γ increased class I heavy-chain synthesis in both uninfected and infected cells approximately sevenfold (as quantitated by phosphorimager analysis). In addition, in Ad2-infected cells treated with IFN-γ nearly one-half of the class I molecules escaped from the ER and became Endo H resistant. These data, taken together with the ability of IFN-γ to render Ad2-infected cells sensitive to lysis by CTLs, indicate that IFN-γ not only increases class I expression in Ad2-infected cells but also allows a subset of class I molecules to evade the E3-19K effect and present viral antigens to CD8+ T cells.

DISCUSSION

This is the first study to document the presence of adenovirus-specific CTLs in humans. Human adenovirus-specific effectors were generated by stimulating PBMC from healthy adults with live, infected, autologous fibroblasts in order to allow presentation of newly synthesized viral peptides in association with class I antigens. Presumably, the adenovirus-specific cytotoxicity detected was the result of amplification of memory T cells generated from prior infection, which is consistent with our ability to detect strong adenovirus-specific proliferative CD4+ T-cell responses in most healthy adults in a previous study (17).

The adenovirus-specific cytotoxicity amplified from PBMC was documented to be MHC class I restricted and mediated by CD8+ T cells. Effectors killed adenovirus-infected autologous fibroblasts in preference to uninfected autologous fibroblasts and infected HLA class I-mismatched fibroblast controls. Enrichment of PBMC for CD8+ T cells increased adenovirus-specific cytotoxicity and reduced the background cytotoxicity against controls. Furthermore, depletion of CD8+ T cells with MAb and complement immediately prior to the CTL assay abolished cytotoxicity, whereas CD4+ T-cell-depleted effectors remained lytic. The adenovirus-specific CD8+ T-cell responses were presumed to be MHC class I restricted because effectors did not kill infected class I-mismatched fibroblasts, and the fibroblasts did not have detectable class II molecules by FACS analysis. Hence, this assay cannot detect class II-restricted cytotoxicity, and these results do not rule out the possibility that CD4+ antiadenovirus CTLs are present in PBMC.

PBMC from four of five healthy adults exhibited adenovirus-specific CTL responses, although some variation in the patterns of cytotoxicity was observed. The differences between the two high-level and the two low-level responders were likely due to differences in the frequencies of adenovirus-specific memory CD8+ T cells, as suggested by limiting-dilution analysis (31a). CTL cultures from two donors exhibited relatively high levels of cytotoxicity against infected allogeneic control targets. On the basis of the presence of cytotoxicity against the NK target K-562 and the ability to reduce background killing by depletion of CD56+ cells, this background non-MHC-restricted cytotoxicity was likely mediated by NK or lymphokine-activated killer (LAK) cells. LAK activity may be generated during short-term culture of CTLs in the presence of interleukin-2 (21), and LAK cells, but not untreated NK cells, have been demonstrated to kill adenovirus-infected fibroblasts (35). The CTL assay on cells from one donor was uninterpretable because of high levels of cytotoxicity to both uninfected and infected targets.

As a first step towards identification of viral epitopes recognized by human antiadenovirus CTLs, effectors were shown to be unable to kill autologous fibroblasts containing input virion

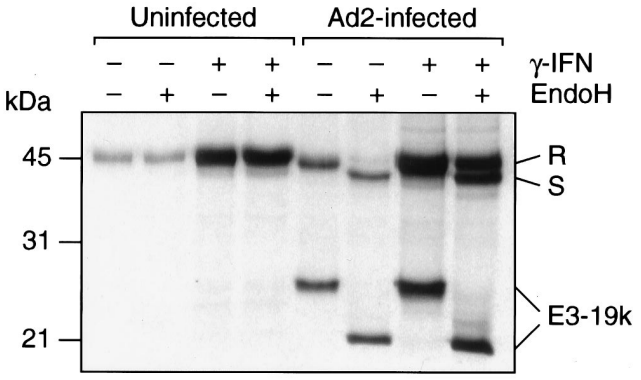

FIG. 7. IFN-γ counteracts the ability of E3-19K to inhibit processing of nascent HLA class I molecules. Ad2-infected and uninfected fibroblasts with and without pretreatment with IFN were labeled with [35S]methionine and [35S]cysteine for 1 h and then subjected to a cold chase. Equal volumes of cell lysates were immunoprecipitated with antibody against class I with and without Endo H digestion and analyzed on a 12% polyacrylamide gel. R and S, Endo H-resistant and Endo H-sensitive class I heavy chains, respectively.

proteins only. Although class I-restricted responses classically require endogenous synthesis of proteins, there are examples of enveloped viruses such as CMV and herpes simplex virus whose input virion proteins are endogenously processed via the cytoplasmic pathway and represent the primary CTL epitopes (34, 38). There is, however, little information about CTL responses to nonenveloped viruses such as adenovirus. In the present study, adenovirus-specific effectors did not lyse targets adsorbed with virus for 1 h and then incubated for an additional 1 h prior to the CTL assay, whereas 24-h-infected targets were killed. Adenovirus is internalized via receptor-mediated endocytosis and rapidly released into the cytoplasm. Therefore, adenovirus input proteins may not be accessible for processing with class I antigens via the cytoplasmic pathway, or it may be that such processing requires a longer incubation period. Alternatively, antiadenovirus effectors may primarily recognize early regulatory viral proteins rather than structural proteins. Further studies will be needed to distinguish between these possibilities.

The early viral glycoprotein E3-19K was documented to inhibit human adenovirus-specific CTL responses in vitro. E3-19K, which is localized to the ER, blocks MHC class I-mediated antigen presentation by its ability to bind to nascent class I MHC molecules and prevent transport of class I molecules to the cell surface. Therefore, adenovirus-specific CTLs were amplified from PBMC by using the E3 deletion mutant $Ad2^+ND1$ to infect both stimulators and targets in order to bypass the E3-19K effect. We subsequently documented that effectors killed wild-type-Ad2-infected cells more poorly than $Ad2^+ND1$-infected cells and confirmed this to be the result of the expression of E3-19K by testing a panel of mutants with deletions of individual E3 ORFs. These data are consistent with results obtained in other in vitro studies in which E3-19K was used to block presentation of SV40 or influenza virus antigens with H-2 to CTLs in mice (8, 37). In addition, using a nonreplicative murine model of human adenovirus infection, Gooding and colleagues showed that E3-19K inhibited adenovirus-specific $CD8^+$ CTL responses in vitro (33). Those authors found that E3-19K expression inhibited CTL recognition of infected cells from inbred mouse strains whose H-2 antigens bind to E3-19K, but not from strains whose H-2 antigens do not associate with E3-19K. In contrast, E3-19K blocks the transport of all HLA class I antigens which have been tested, although differences in the affinities of E3-19K for binding to different HLA class I antigens have been documented (2, 15). The fact that E3-19K successfully blocked killing of the human antiadenovirus CTLs described in this study also confirms that the cytotoxicity was HLA class I restricted.

Preincubation of targets with IFN-γ, however, counteracted the E3-19K effect and allowed killing of wild-type-Ad2-infected cells. Similarly, Sparer et al. found that antiadenovirus mouse CTLs recognized wild-type-Ad2-infected mouse cells if pretreated with IFN-γ (36). In our study, it was documented that IFN-γ did not affect E3-19K expression, although partial inhibition of late viral protein synthesis was observed. RIPA analysis confirmed that IFN-γ increased class I expression in Ad2-infected fibroblasts. Moreover, Endo H studies revealed that, in contrast to the situation with untreated Ad2-infected cells, in which all class I molecules remained in the ER, nearly one-half of the class I molecules in IFN-γ-pretreated Ad2-infected cells escaped the ER and became terminally glycosylated. Korner et al. have shown that IFN-γ partially restores cell surface expression of class I molecules in E3-19K-transfected 293 cells, a human embryonic kidney cell line transformed by the E1 region (28). These data suggest that IFN-γ blocks E3-19K-mediated inhibition of antigen presentation to $CD8^+$ CTLs by increasing cell surface expression of class I molecules despite the presence of E3-19K. IFN-γ stimulates transcription of both class I and class II genes, as well as those encoding proteins involved in peptide processing and transport to the ER (19). The ability of IFN-γ to counteract the E3-19K effect is most likely the result of increasing the ratio of class I to E3-19K molecules within infected cells. The viral glycoprotein does not appear to interfere with peptide binding to class I molecules (8), so that an effect of IFN-γ on the availability of peptides is unlikely to play a major role. However, this study cannot rule out the possibility of a contribution from the induction of low-level class II expression in fibroblast targets which could allow killing by $CD4^+$ CTLs if present.

These data provide a possible explanation for the paradox of the ability to generate an effective adenovirus-specific CTL response after natural infection in humans and the ability of E3-19K to inhibit CTL recognition in vitro. In murine models, E3-19K also does not appear to inhibit antiviral CTL responses in vivo. For example, mice infected with recombinant vaccinia virus expressing E3-19K generated vaccinia virus-specific $CD8^+$ CTLs, despite the fact that E3-19K blocked CTL recognition of vaccinia virus-infected cells in vitro (9). In addition, E3-19K did not interfere with the ability of animals to clear the vaccinia virus infection. Although there is evidence that primary CTL responses may be generated by processing of exogenous viral antigens with class I molecules within dendritic cells in vivo (29), thus bypassing the E3-19K effect, this hypothesis alone would not explain the ability of such CTLs to recognize adenovirus-infected cells which express E3-19K. In vivo, IFN-γ and perhaps other cytokines produced in response to acute viral infection may enable infected cells to counteract the E3-19K effect on antigen presentation. There is evidence that IFN-γ is produced in response to adenovirus infection in the mouse model (43), and supernatants from human adenovirus-specific $CD4^+$ T-cell cultures contain interferon activity (31a).

There is a precedent for the importance of IFN-γ in the antiviral CTL response in vivo as illustrated in the murine CMV (MCMV) model. MCMV-specific CTLs recognize a pp89 immunodominant epitope in vivo, but CTL responses cannot be detected in vitro because of the expression of an immediate-early MCMV gene that specifically blocks transport of peptide-loaded class I molecules (10). Similar to what was observed in our experiments with antiadenovirus CTLs, recognition of infected cells by MCMV-specific CTLs was conferred by pretreatment of targets with IFN-γ (22). Moreover, in the same study, the importance of IFN-γ in vivo was confirmed by adoptive transfer experiments which showed that CTL activity was blocked by treating animals with antibody against IFN-γ.

An alternative explanation for the apparent lack of effect of E3-19K in acute infection, unrelated to the hypothesis of an IFN-γ effect, is that the level of inhibition of class I expression by E3-19K in most infected cells in vivo may be insufficient to block antigen presentation. It is possible that the level of E3-19K expression in infected cells in vivo is lower than the level in in vitro studies in which experimental infection of targets with adenovirus at a high multiplicity of infection allows high levels of E3-19K production in excess of the level of class I molecules and that in vivo, free class I molecules remain available to present viral antigens. Therefore, antiadenovirus CTLs may be able to kill most infected cells in vivo because the cells express either relatively low levels of E3-19K molecules or high levels of class I molecules.

Adenovirus-specific CTL responses are likely important for recovery from acute adenovirus disease in humans. Most cases of disseminated disease have occurred in patients with im-

paired cell-mediated immunity, such as bone marrow transplant recipients, solid-organ transplant patients, and patients with cancer and AIDS (14, 30, 31). No antiviral treatment is available, and invasive disease may be fatal. The identification of adenovirus-specific human CTL responses opens up the possibility of using cloned antiviral CTLs as immunotherapy, as has been done for CMV (39). A recent case report of a procedure in which donor leukocytes were successfully used to treat an invasive adenovirus infection in a bone marrow transplant recipient suggests that cellular immunotherapy may be of benefit (24). We have been able to clone adenovirus-specific $CD8^+$ T cells from two donors (31a), and further analysis of these T-cell clones is under way.

CTL responses to adenovirus, however, may interfere with the efficacy of gene therapy with adenovirus vectors in humans. It is anticipated that adenovirus-mediated gene therapy will stimulate memory adenovirus-specific T cells, as well as induce the development of new responses to transduced cells. One strategy proposed to evade the CTL response to transduced cells is to express E3-19K in the adenovirus vector in order to block class I-restricted antigen presentation. The E1-deleted vectors presently being used in gene therapy experiments do not express significant amounts of E3-19K. However, on the basis of the present study, expression of E3-19K at wild-type levels may not be sufficient to inhibit presentation of viral antigen to CTLs in vivo. Hence, it may be necessary to delete immunodominant epitopes recognized by human adenovirus-specific CTLs from gene therapy vectors. The early region E1A has been found to be an important CTL target in both adenovirus-transformed (25) and nonproductive adenovirus-infected mouse cells in some inbred strains (32). However, gene therapy experiments showed that mice generated CTL responses to E1-deleted vectors. We have recently found that human anti-adenovirus CTLs recognize targets infected with the E1A deletion mutant *dl*312 (data not shown). This observation confirms that there are other viral proteins expressed in cells infected with E1A-deleted vectors which can be recognized by CTLs. On the basis of our study, it should now be feasible to identify immunodominant epitopes recognized by human adenovirus-specific CTLs.

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