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Adenovirus-Pulsed Dendritic Cells Stimulate Human Virus-Specific T-Cell Responses In Vitro

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Adenovirus infections cause significant morbidity and mortality in immunocompromised patients, yet little is known about the immune response to adenovirus infections. We established a system for the generation of a cytotoxic immune response to adenovirus in vitro. Cytotoxic T cells (CTLs) were derived from normal donors by using peripheral blood dendritic cells as antigen-presenting cells. The CTLs were found to contain a mixture of effector cells that recognized virus peptides in the context of both class I and class II antigens. Endogenous viral gene expression was not required to sensitize cells to lysis by adenovirus-specific CTLs. CTLs raised against subgroup C adenovirus type 5 can lyse cells infected with subgroup B adenovirus type 11, indicating that viruses of different subgroups have epitopes in common. This system holds promise for defining the human immune response to adenovirus, including characterization of the viral protein(s) against which the response is generated, and the identity of the effector cells. Such studies are in progress.

There are approximately 50 serotypes of human adenovirus, which are divided into six families based on immunological, molecular, and functional criteria (37). Physically, adenovirus is a medium-sized icosahedral virus containing a double-stranded, linear DNA genome which, for adenovirus type 5 (Ad5), is 35,935 bp (6). To initiate infection, adenovirus binds to a receptor whose identity is unknown (7, 35) and then is internalized following binding to a second protein, which has recently been identified as the vitronectin receptor (15, 17, 39). The virus is internalized in endosomes, and the viral core is released and migrates to the nucleus, where viral RNA transcription is initiated.

The majority of people (>85%) have circulating antibodies against several of the common adenovirus serotypes by the time they reach adulthood (32). Infected individuals develop immunity to adenovirus and retain lifelong immunity to the virus (8, 32), as the virus remains in the body in a latent form (8, 19). Adenovirus infections are rarely severe in healthy children or adults but can be life threatening in immunocompromised individuals. For example, a recent review of 201 patients who received T-cell-depleted allogeneic bone marrow from HLA-mismatched or HLA-matched, unrelated donors found that 42 (21%) shed adenovirus in urine or stools. The percentage was higher in the pediatric population (31.3 versus 13.6%). Thirteen patients had clinical symptoms of adenovirus infection, and seven died of disseminated disease (9). Adenoviruses from five of the six subgroups were found. The association between severe adenovirus infection and immunodeficiency suggests that adenovirus is normally controlled by the cellular arm of the immune response. There are no antiviral compounds that are effective against adenovirus infections, and it may be possible to adoptively transfer the cellular immune response to control adenovirus infections in immunocompromised individuals, as has been done for Epstein-Barr virus (27).

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Little is known about the cellular immune response to adenovirus in humans. A recent study (10) indicated that healthy individuals who were seropositive for adenovirus also had cell-mediated memory responses, as demonstrated by the proliferation of helper T cells in response to purified adenovirus virions in vitro. A second recent study (34) demonstrated that purified viral capsid proteins could stimulate proliferation of peripheral blood mononuclear cells in vitro, but the identity of the responding cells was not determined. In a series of investigations, Cook and colleagues have studied the role of NK cells in the killing of adenovirus-infected human cells and have shown that adenovirus sensitizes rodent, but not human, cells to NK cell killing (28–31). These differences were ascribed to downregulation of major histocompatibility complex class I antigens on the cell surface of rodent, but not human, cells following virus infection (29). These studies cast doubt on the validity of using human adenovirus infection of mice as a model system. The major cytotoxic T-lymphocyte (CTL) response against adenovirus-infected rodent cells is directed against early viral proteins, either E1A (21, 28, 31) or E2A (18, 22). This contrasts with preliminary findings on humans that show that viral capsid antigens are the targets of proliferative responses (10, 34). To characterize the key components in the human immune response to adenovirus infection, it is necessary to establish an in vitro system derived from humans to perform the necessary studies. In this report, we describe the establishment of an in vitro system that permits induction and analysis of the human CTL response to adenoviruses.

MATERIALS AND METHODS

Viruses. Ad5 strains *dl*312 (E1A$^-$) (Tom Shenk) and *dl*7001 (E3$^-$) (W. S. M. Wold) were grown on either 293.1 (E1A strain) or KB spinner cells (wild-type and E3 strains) as previously described (16). Virus was purified by CsCl density gradient centrifugation. All virus stock titers were determined on 293.1 cells.

Generation and culture of dendritic cells. Peripheral blood and skin biopsy samples were obtained from four healthy adult donors. HLA types were as follows: donor A, A2,11; B7,8; Cw$^-$; DR15,3; donor B, A2,3; B13,35; C4,−; DR1; donor C, A1,1; B8,57; Cw6,w7; DR7,17; donor D, A1,11; B7,22; Cw3; DR1,1.

Dendritic cells were generated by the procedure of Romani et al. (25). Briefly, Ficoll-Hypaque-purified peripheral blood mononuclear cells (MNCs) were seeded on 24-well plates (1.5-cm-diameter wells) at 10^6 per well and allowed to

adhere for 2 h. Nonadherent cells were removed and frozen for later use. Adherent cells were then cultured for 4 to 5 days in 800 U of granulocyte-macrophage colony-stimulating factor (Immunex, Seattle, Wash.) per ml and 500 U of interleukin-4 (Peprotech, Rocky Hill, N.J.) per ml. Ten to fifty percent of the resulting cells exhibited the large, irregular morphology typically associated with dendritic cells. Cytofluorographic analysis revealed that these cells were $CD3^-$, $CD14^-$, $CD19^-$, and HLA DR^+.

Generation of adenovirus-specific CTLs. The dendritic cells were pulsed with gradient-purified adenovirus in 2 ml of serum-free medium at a multiplicity of 100 for 1 h. The dendritic cells were then irradiated (3,000 cGy) and seeded into 24-well plates at 5×10^5 per well. The nonadherent MNCs were then added at 10^6 per well to achieve an MNC-dendritic-cell ratio of 2:1. After 10 days, the cocultures were harvested on Ficoll gradients, resuspended at 10^6 per well, and restimulated with virion-pulsed, irradiated dendritic cells again at a ratio of 2:1. Four days later, the nonadherent cells were assayed either immediately or after 2 to 3 days of culture with 5 to 10 U of interleukin-2 (Cetus, Emeryville, Calif.) per ml.

Chromium release assay. Dermal fibroblasts were derived from skin biopsies and used as targets in cytotoxicity assays. Fibroblasts were cultured in 100 U of gamma interferon (Genentech, South San Francisco, Calif.) per ml for 2 days and then infected with adenovirus at a multiplicity of infection of 100 to 200 PFU per cell. After 24 h of incubation, the cells were trypsinized, labeled with ^{51}Cr, and seeded in triplicate in 96-well flat-bottom plates at 5×10^3 per well. The CTLs were then added, at the effector-to-target ratios indicated in the figures, 16 to 18 h before harvesting of supernatants.

In several experiments, actinomycin D was included to inhibit RNA transcription. Fibroblasts were incubated with 20 μg of the drug per ml for 30 min prior to infection. CTL assays were performed exactly as described in the preceding paragraph, except for the presence of actinomycin D in the medium. Brefeldin A (BFA) was added in some experiments at a concentration of 1 μg/ml 30 min prior to virus infection and was then present throughout the remainder of the assay at the same concentration.

Immunophenotyping and sorting. A FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.) was used to determine the phenotypes of the CTL preparations. Cells were stained with pairs of monoclonal antibodies directly conjugated with fluorescein isothiocyanate or phycoerythrin, which recognized the cell surface molecules CD4, CD8, CD56 (Dako, Carpinteria, Calif.), T-cell receptor γδ (Becton-Dickinson), and T-cell receptor αβ (T Cell Diagnostics, Cambridge, Mass.), and then combined in pairs for dual fluorescence analysis. In several experiments, the CTL population was depleted of $CD56^+$ cells by cell surface staining and sorting on a FACStar (Becton-Dickinson).

[^{3}H]thymidine uptake experiments. Nonadherent peripheral blood MNCs were seeded in 96-well round-bottom plates at 10^4 per well. Dendritic cells that were either uninfected or infected with adenovirus and irradiated were then added at the ratios indicated in the figure legends. After 10 days, [^{3}H]thymidine was added at 10 μCi/ml and incubated for 6 h. Cells were harvested (Harvester 96 Mach II; TomTec, Orange, Conn.), and the incorporated counts were measured.

Protein and RNA gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Anderson et al. (1). The gels contained 12.5% acrylamide and 0.1% bisacrylamide. RNA was extracted by the method of Chomczynski and Sacchi (5) and probed for E2A RNA with the *Nar*I DNA fragment of Ad5, which includes nucleotides 22612 to 23913 of the viral genome.

RESULTS

Adenovirus-pulsed dendritic cells cause T-cell proliferation. Blood dendritic cells are known to be superb antigen-presenting cells (APCs) (25), but their permissiveness for adenovirus infection was unknown. Preliminary experiments with various multiplicities of infection and staining for either early or late adenovirus proteins demonstrated that wild-type Ad5 infects 1 to 5% of the cells in the dendritic cell preparations (data not shown). To determine whether this rate of infection would be sufficient for T-cell activation, normal donor MNCs were cultured with irradiated dendritic cells that had been infected with either wild-type Ad5 or Ad5 strain *dl*312, an Ad5 mutant with the E1A region deleted and consequently defective in early and late viral gene expression. On day 10, the cultures were pulsed with [^{3}H]thymidine for 5 h and analyzed for radiolabel incorporation. As shown in Fig. 1, mock-infected dendritic cells caused little or no proliferation of the MNCs. In contrast, both wild-type- and strain *dl*312-pulsed cells induced [^{3}H]thymidine incorporation at all of the ratios tested. Immunophenotyping of the proliferating cells demonstrated both $CD4^+$ and $CD8^+$ T cells in a ratio of about 2:1. A significant minority were $CD56^+$ (Fig. 1).

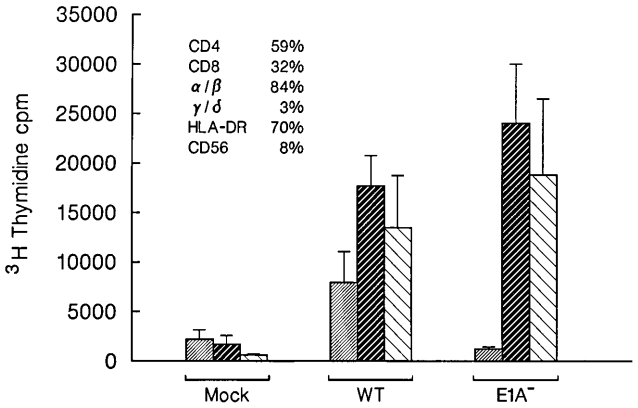

FIG. 1. Proliferation of MNCs in response to irradiated dendritic cells pulsed with medium alone (Mock), wild-type Ad5 (WT), or strain *dl*312 ($E1A^-$), an Ad5 mutant with the E1A region deleted. Responder-stimulator ratios: 10:1 (▨); 2:1 (▩); and 0.4:1 (▧).

Adenovirus-infected dendritic cells activate virus-specific CTLs. The functional capability of the T cells responding to adenovirus-pulsed dendritic cells was tested by determining whether they could specifically lyse virus-infected cells. T-cell lines were generated from two different donors by using two rounds of stimulation. Their cell surface phenotypes are presented in Fig. 2. The majority of responding cells were $CD4^+$ T cells. The cytotoxic activity of the lines was assayed against autologous or HLA-mismatched dermal fibroblasts infected with either wild-type Ad5, strain *dl*312 ($E1A^-$), or strain *dl*7001 ($E3^-$). The $E1A^-$ virus was included to determine whether expression of early viral genes is required to sensitize infected cells to lysis. The E3 region of the viral genome is responsible for downregulating expression of the major histocompatibility complex on the infected cell surface (reviewed in references 11 and 40), and its absence in *dl*7001 would be expected to allow higher levels of class I expression. The results are shown in Fig. 2. Neither of the donor-derived T-cell lines killed uninfected fibroblasts. In contrast, both T-cell lines killed infected cells, regardless of whether the targets were infected with wild-type, $E1A^-$, or $E3^-$ Ad5. However, the susceptibilities of the targets varied markedly. For donor A, $E3^-$-infected targets were more susceptible than $E1A^-$-infected targets, which, in turn, were more susceptible than wild-type-infected targets. Killing of the autologous infected targets was consistently greater than killing of the mismatched infected targets over the entire range of effector-to-target ratios tested. This suggested that much of the cytolytic activity of the T cells was HLA restricted, a hallmark of CTLs which recognize antigen presented via class I or II molecules.

Figure 2 shows that a higher-than-background level of killing of the HLA-mismatched, infected targets was present, particularly with T cells generated from donor A when assayed against $E3^-$ virus-infected cells. This suggested that adenovirus-pulsed dendritic cells also stimulated the proliferation of HLA-unrestricted killers. To test this hypothesis, we depleted $CD56^+$ cells from an aliquot of CTLs from donor B. CD56 is a cell surface molecule expressed by HLA-unrestricted killer cells. Depleted and undepleted aliquots were then assayed against a variety of infected and uninfected targets. As shown in Fig. 3, the undepleted T cells killed K562, an erythroleukemia cell line particularly sensitive to HLA-unrestricted killers.

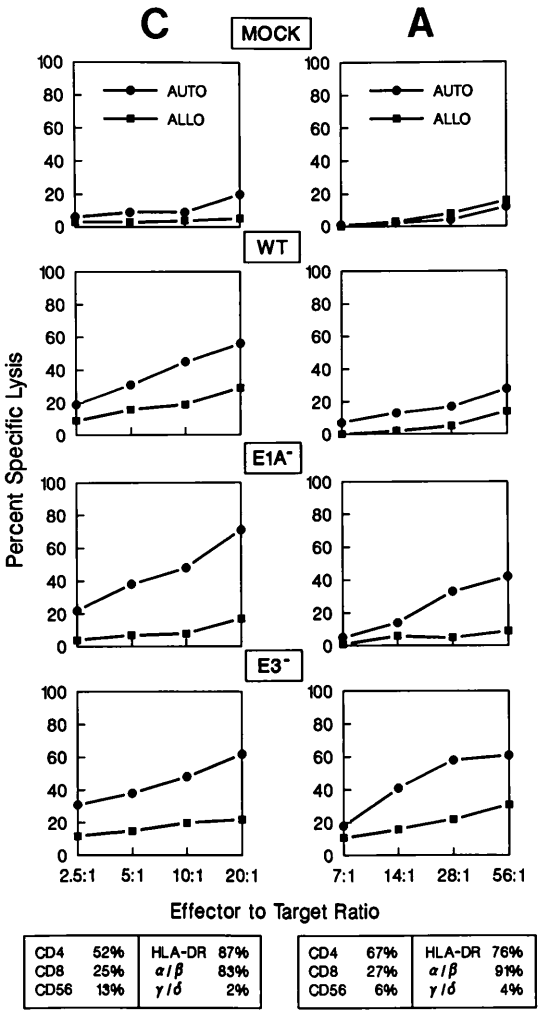

FIG. 2. Cytotoxicity and cell surface phenotype of T cells that grow in response to virus-pulsed dendritic cells. T-cell lines from donors A and C were prepared and assayed against mock-, wild-type Ad5 (WT)-, strain *dl*312 (E1A$^-$)-, or strain *dl*7001 (E3$^-$)-infected fibroblasts. Both autologous (AUTO) and HLA-mismatched (ALLO) fibroblasts were used.

In contrast, the depleted aliquot failed to lyse this target, thus confirming that the unrestricted killing was mediated by an NK-type cell. When assayed against virus-infected fibroblasts, the CD56$^-$ cells did not kill HLA-mismatched targets. However, the depleted aliquot retained its ability to lyse the matched, infected targets. Thus, adenovirus-infected dendritic cells produced both HLA-restricted and HLA-unrestricted T-cell populations.

The CTL preparations consisted of both CD4$^+$ and CD8$^+$ cells, and killing could potentially be mediated through either class I or II antigens. CD56$^+$-depleted, adenovirus-specific CTLs were assayed against virus-infected targets in the presence or absence of blocking antibodies directed against monomorphic determinants of either HLA-ABC or HLA-DR. As shown in Fig. 4, both antibodies inhibited the CTL-mediated killing. Importantly, when the percentages of suppression observed with the antibodies were summed, the result was nearly 100%. To confirm these results, CTL preparations were sorted into CD4$^+$ and CD8$^+$ populations and examined for the ability to kill virus-infected fibroblasts. The results demonstrate that pure populations of both class I-restricted CD8$^+$ cells and class II-restricted CD4$^+$ cells could kill autologous infected fibroblasts (Fig. 5). Collectively, these results indicate that both class I and II molecules present antigen to adenovirus-specific CTLs.

Expression of virus proteins in fibroblasts infected with adenovirus mutants. The killing of the E1A$^-$ virus-infected fibroblasts was unexpected, since this mutant should not express virus genes de novo. However, at high multiplicities of infection or in certain cell types, E1A$^-$ viruses can replicate (13), so we examined E1A$^-$ virus-infected fibroblasts to determine if, under the conditions used, virus proteins were being synthesized. Both the wild-type and E3$^-$ viruses produced a broad array of viral proteins following infection of primary fibroblasts, including the hexon molecule, a prototypical late viral protein. This result was not surprising, since the E3 region is not essential for any aspect of viral gene expression in tissue culture (13). In contrast, no viral protein synthesis was detected in fibroblasts infected with the E1A$^-$ virus (Fig. 6). Northern (RNA) blot analysis of RNA extracted from these cells did not reveal any detectable early viral RNAs (data not shown). The E1A region deleted in strain *dl*312 is required for early and late viral gene expression (29), and cells infected with this virus would not be expected to synthesize viral RNAs. Recognition of fibroblasts infected with the E1A$^-$ virus indicates that de novo viral gene expression is not required for CTL killing.

Endogenous viral gene expression is not required to sensitize fibroblasts to lysis by adenovirus-specific, HLA-restricted T cells. The sensitivity of the E1A$^-$ virus-infected cells to lysis by CTLs suggested that the inoculum itself rendered cells susceptible to killing. To further test this hypothesis, adenovirus-specific CTLs were assayed for the ability to kill E1A$^-$ virus-infected fibroblasts in the presence of actinomycin D. Such treated targets are doubly blocked for de novo viral gene expression, as actinomycin D inhibits mRNA synthesis at the level of transcription. As shown in Fig. 7, the drug had no effect on infected target cell lysis mediated by the virus-specific CTLs. This result suggested that fibroblasts process absorbed virion protein in such a way that they become targets for CTL-mediated lysis without the need for de novo viral gene expression.

Intracellular processing of adenovirus antigens is necessary for presentation to CTLs. The previous experiments demonstrated that adenovirus protein does not have to be synthesized de novo for the cell to present viral antigens, but the site of viral antigen processing cannot be discriminated on the basis of the data presented thus far. Brefeldin A (BFA) interrupts transport of molecules from the endoplasmic reticulum through the Golgi apparatus, effectively preventing presentation of intracellularly processed peptides on the cell surface. If BFA interferes with lysis of infected cells by CTLs, it is likely that antigens are processed inside the cell and transported to the surface; if it does not, then processing probably occurs outside of the cell. Fibroblasts were pretreated with BFA, infected with wild-type adenovirus, and then used as targets in a standard cytotoxicity assay. The results, shown in Fig. 8, demonstrate that in the absence of BFA, lysis of over 40% of the target cells was achieved, while the BFA-treated targets were not lysed. These results suggest that intracellular processing of antigens must occur for presentation of viral peptides on the cell surface.

CTLs generated against Ad5 lyse targets infected with Ad11. There are around 50 adenovirus serotypes classified into six subgroups, and there is evidence from work with rodent systems that cellular immunity against one subgroup does not protect against infection by another. To determine if this is

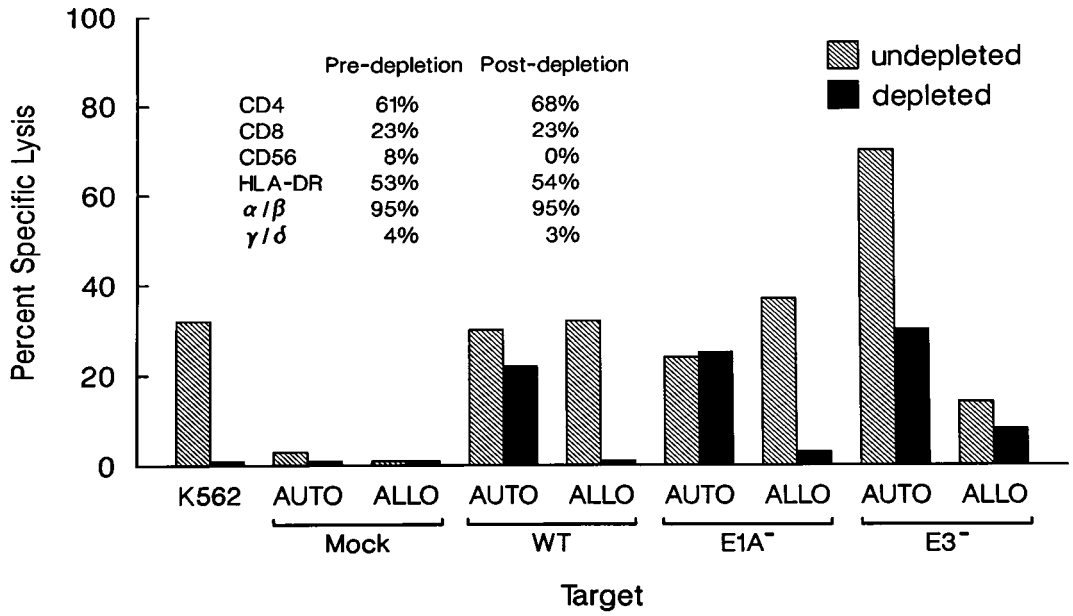

FIG. 3. Cytotoxicity of $CD56^+$-depleted versus nondepleted CTLs. $CD56^+$-depleted and nondepleted CTLs from donor C were prepared and assayed against either uninfected K562 cells or autologous (AUTO) or allogeneic (ALLO) fibroblasts either mock infected or infected with the indicated viruses. WT, wild type.

true for humans also, CTLs against Ad5 (subgroup C) were assayed against fibroblasts infected with Ad11 (subgroup B). These CTLs readily killed fibroblasts infected with either the homologous virus or Ad11 (Fig. 9). The almost complete absence of killing of the HLA-mismatched fibroblasts indicated that this killing was not due to NK cell activity. Thus, at least some of the antigenic epitopes recognized by adenovirus-specific human CTLs appear to be conserved among subgroups.

DISCUSSION

Our work has shown that adenovirus-specific cytotoxic T lymphocytes from normal adults can be grown in vitro. T cells from all of the donors tested mounted strong cellular responses to adenovirus-pulsed dendritic cells. This likely represents a secondary immune response, as almost all adults have experienced a group C adenovirus infection during childhood

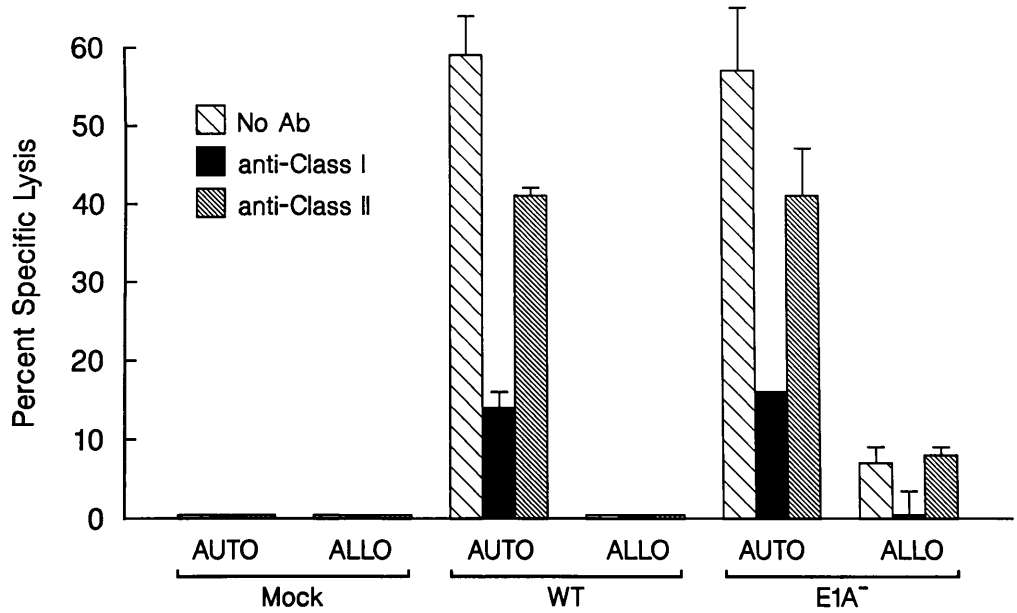

FIG. 4. Effects of blocking antibodies (Ab) specific for HLA-ABC or HLA-DR on lysis of infected fibroblasts mediated by $CD56^+$-depleted CTLs. As indicated, fibroblasts were either left uninfected (mock) or infected with either wild-type Ad5 (WT) or strain *dl*312 ($E1A^-$). Both autologous (AUTO) and HLA-mismatched (ALLO) fibroblasts were used as targets.

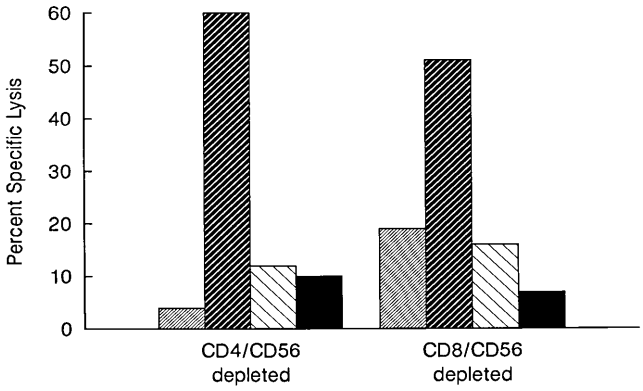

FIG. 5. Cytotoxicity of flow-sorted populations of $CD4^+$ and $CD8^+$ cells. CTLs were generated against Ad5-infected dendritic cells and stained with either CD4 and CD56 or CD8 and CD56 antibodies, and the nonstaining cells were purified. Purified $CD4^+$ (CD8/CD56-depleted) or $CD8^+$ (CD4/CD56-depleted) cells were used in a standard cytotoxicity assay with wild-type Ad5-infected autologous or allogeneic cells as the targets. Patterns: ▨, autologous, mock-infected cells; ▨, autologous wild-type Ad5-infected cells; ▧, allogeneic, mock-infected cells; ■, allogeneic, wild-type Ad5-infected cells.

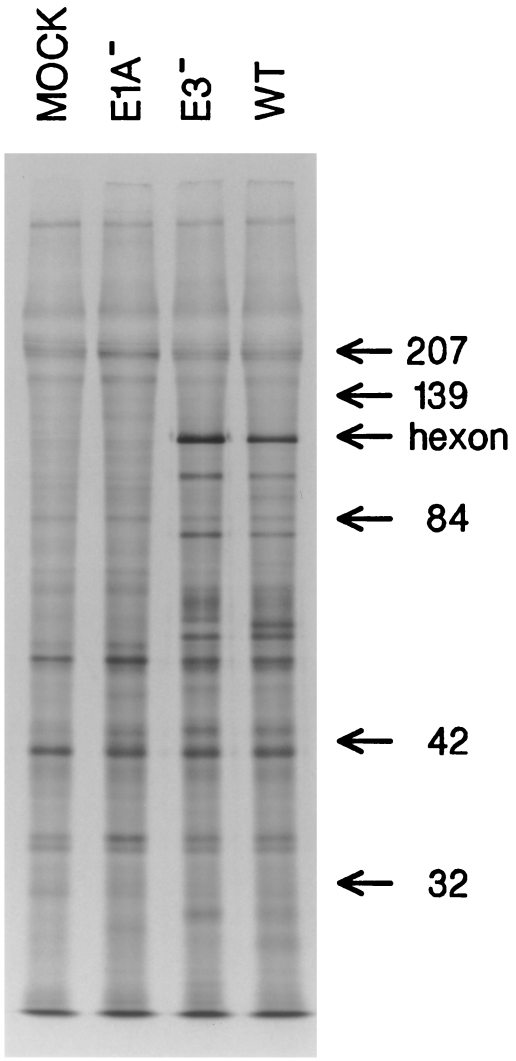

FIG. 6. Analysis of proteins made by fibroblasts infected with either wild-type (WT) Ad5, strain *dl*7001 ($E3^-$), or strain *dl*312 ($E1A^-$). Fibroblasts were infected at a multiplicity of infection of 100 to 200, pulsed at 18 h postinfection with 100 μCi of ^{35}S-labeled methionine and cysteine per ml for 1 h, and harvested for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel consisted of 12.5% polyacrylamide and 0.1% bisacrylamide. MOCK, mock-infected fibroblasts.

and therefore have memory B and T cells (32). Both HLA-restricted and HLA-unrestricted CTLs responded to the pulsed APCs. Removal of the unrestricted component by depleting cells positive for CD56 (Fig. 3) suggested that they were NK or lymphokine-activated killer cells. This dual response has been observed previously when human polyclonal CTLs were generated in vitro against viral antigens (33). We also demonstrated cross-reactivity of the adenovirus-specific CTLs, as CTLs generated against a subgroup C adenovirus recognized and killed cells infected with a subgroup B adenovirus.

Dendritic cells were chosen as adenovirus APCs because they are efficient APCs and are relatively easily enriched (25, 26). Our results suggest that dendritic cells process and present antigen derived from the input virus inoculum itself without a requirement for viral gene expression. The finding that dendritic cells are able to process virus capsid proteins is not unexpected, as previous studies showed that these cells can process and present exogenously provided whole proteins, as well as small peptides, to $CD8^+$ T cells on HLA class I molecules (26). Our results showing that de novo viral protein synthesis in the target fibroblasts was not required for recognition by CTLs was similar to recently published results of Riddell et al., who showed that de novo viral protein synthesis was not required for efficient antigen presentation in cytomegalovirus-infected fibroblasts (23). Those researchers also demonstrated that BFA could block killing of infected cells, presumably by preventing presentation of viral peptides (23). Bender et al. (4) demonstrated that inactivated influenza virus could elicit $CD8^+$ CTL responses when dendritic cells were used for antigen presentation. Collectively, these data provide strong support for the hypothesis that virus capsid proteins from the inoculum serve as a source of antigen for presentation and that the antigens were processed inside the cell.

The CTLs generated in vitro recognize the same class of antigen as CTLs taken from immune individuals, that is, capsid antigens. One concern we had about using an in vitro system to generate a cellular immune response against adenovirus is that it would not mimic the in vivo response to a normal infection. In vivo, fibroblasts and epithelial cells are the targets for adenovirus infection, while we used dendritic cells, which are not efficiently infected by adenovirus as APCs in vitro. Recently, Flomenberg and colleagues (10) demonstrated that either adenovirus antigens or whole virus stimulated human peripheral blood MNCs in 29 of 30 healthy donors and that the majority of the responding cells were $CD4^+$. This result is consistent with the hypothesis that viral capsid proteins are the targets of CTLs. These results were confirmed and extended by Souberbielle and Russell (34), who showed that there was specific recognition of late adenovirus proteins by T cells from normal donors by using purified viral antigens bound to nitrocellulose. The most significant responses were directed against the fiber and protein VI and, to a lesser extent, against hexon (34). One difference between our results and those of Flomenberg et al. (10) is that while in both studies the phenotype of the majority of the responding cells was $CD4^+$, we also show that most of the cytotoxic activity is found in the $CD8^+$ fraction (Fig. 4). This result implies that the antigen processing-associated transporter transport system plays a role in viral antigen processing and presentation (12). Collectively, the human data for

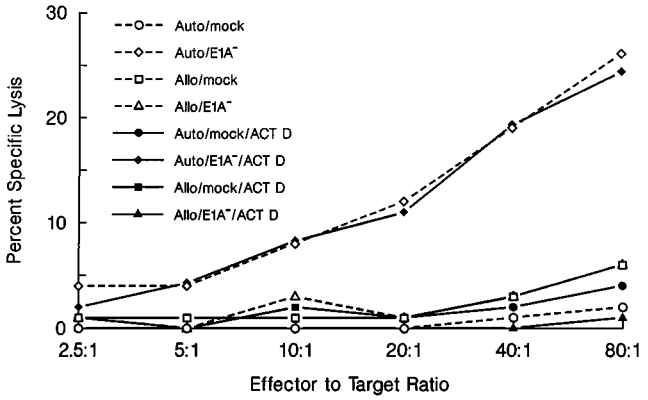

FIG. 7. Cytotoxicity of CTLs assayed against strain *dl*312 (E1A$^-$)-infected fibroblasts in the presence or absence of actinomycin D. CTLs were generated against Ad5 and assayed against mock- or strain *dl*312-infected autologous (Auto) or allogeneic (Allo) fibroblasts in the presence or absence of 20 μg of actinomycin D (ACT D) per ml.

adenovirus indicate strongly that there is a considerable response by T cells to the viral capsid proteins, a result that clearly contrasts with the work done to date with mice. However, the results are consistent with those from similar studies with other human DNA viruses, such as cytomegalovirus (24) and herpes simplex virus (36). In these cases, the cellular immune system can present viral capsid antigens without the need for productive infection. Recognition of processed capsid antigen potentially would allow rapid cytolysis of infected cells before the assembly of virions could occur.

The differences between the immune responses of humans and rodents to adenovirus infection may reflect either the differences in the in vitro and in vivo systems employed or true differences in the pathology of infections in a natural versus unnatural host. The major CTL response against adenovirus-infected rodent cells is directed against early viral proteins (21, 28, 31), while in humans it is directed against capsid antigens (10, 34). The form in which the adenovirus proteins are administered to rodents does not seem to make a difference, as early proteins are the targets for CTLs when animals are immunized either with adenovirus-transformed, syngeneic cells (31) or with replication-competent virus (28). Some similarities

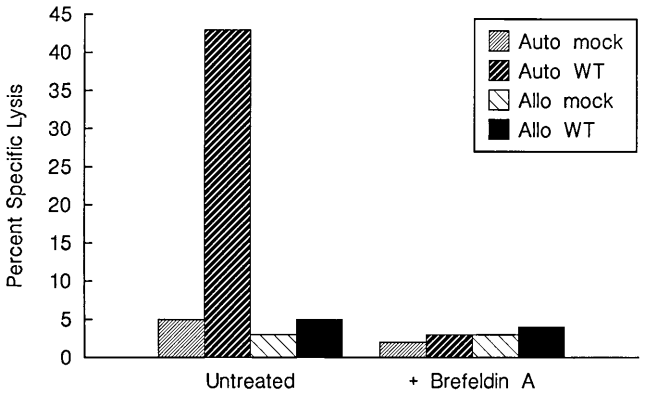

FIG. 8. BFA blocks killing of Ad5-infected target cells. CTLs were generated against wild-type (WT) Ad5 by using dendritic cells for antigen presentation. Cytotoxicity was assayed on Ad5- or mock-infected autologous (Auto) or allogeneic (Allo) fibroblasts that were either untreated or pretreated with 1 μg of BFA per ml.

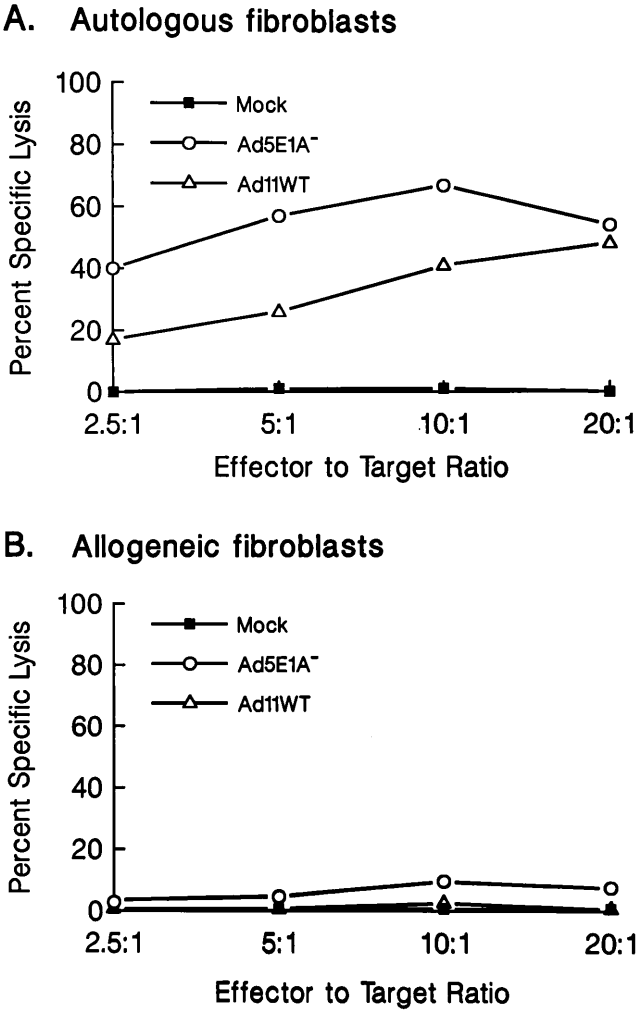

FIG. 9. CTLs raised against Ad5 lyse targets infected with Ad11. CTLs were generated against Ad5 and assayed against fibroblasts infected with either Ad5 or Ad11 at the indicated effector-to-target ratios. Mock, mock infection. WT, wild type.

do exist. Class I-restricted CTLs play a major role in eliminating adenovirus-infected cells in rodents, as shown by using transgenic mice lacking components of this pathway (42), and our results show that most of the killing of adenovirus-infected human cells is mediated by CD8$^+$ cells (Fig. 4). NK activity against adenovirus-infected cells in rodents is high (29), and while others have found little response by NK cells to adenovirus-infected human cells (29), our results show significant levels of HLA-unrestricted killing which is due to the CD56$^+$ cells in the population (Fig. 2). We do not know whether the target of the immune response in humans differs depending on the HLA type, as it does in mice, in which some targets respond to E1A while others respond principally to E2A (18, 22).

The E3-deleted adenovirus was included in this study because it lacks a region of the viral genome that encodes a variety of proteins that decrease the infected cell's susceptibility to lysis by CTLs and tumor necrosis factor (11, 40). Predictably, strain *dl*7001-infected fibroblasts were more susceptible to CTL-mediated killing with some donors (Fig. 2 and 3), a result consistent with the fact that the virus lacks a gene encoding a glycoprotein that interferes with class I antigen

expression on the cell surface (40). Cells from donor C showed no increased susceptibility when $E3^-$ virus-infected fibroblasts were used as targets, whereas large differences were found for infected fibroblasts from donor B (Fig. 3). Cells from donor A showed an intermediate effect (Fig. 2). This is potentially due to the difference in binding affinity of various class I molecules for the E3/gp19K protein (3). HLA-A2.1 and HLA-B7 bind very well to gp19K, and donors A and B have at least one of these alleles. HLA-A1 binds gp19K less tightly than do HLA-A2.1 and HLA-B7, and donor C, whose fibroblasts showed no difference in susceptibility to killing when infected with the virus with E3 deleted, is homozygous for this allele. Our data are consistent with the known binding affinities of gp19K and HLA alleles, but it may be premature to ascribe the differences in sensitization we found with and without E3 solely to this interaction. We do not know which viral peptides are presented to CTLs and which HLA proteins do the presenting. Further work in this area is necessary.

CTLs raised against Ad5 lyse fibroblasts infected with Ad11. Ad5 and Ad11 belong to subgroups C and B, respectively (2). By definition, antisera raised against members of one subgroup do not neutralize the infectivity of members of any other subgroup. Consequently, the finding that CTLs raised against one serological subgroup can cross-react with viruses of another was surprising (Fig. 7). However, similar conclusions were reached by Flomenberg et al. (10), who found that individuals who were seronegative for previous exposure to subgroup B Ad35 were nevertheless able to mount a vigorous proliferative response to the virus in vitro. All of these individuals were seropositive for antibodies to Ad2, and the researchers concluded that there were cross-reactive epitopes in the Ad2 and Ad35 capsids (10). These results stand in contrast to the finding that murine Ad5-specific CTLs do not kill cells infected with viruses of different subgroups (28). The reason for this difference is not clear, but it could be related to the possible difference in identity of the virus proteins recognized by human or mouse CTLs. The fact that human CTLs are cross-reactive has important practical consequences, especially in regard to the development of adenovirus-specific CTLs for human therapy. Unselected donor lymphocytes have been used successfully in a single case to treat an adenovirus infection following bone marrow transplantation (14), and infusions of either donor leukocytes (40) or virus-specific clones (27, 38) have been used to treat infections of immunocompromised individuals with other viruses. Our adenovirus results suggest that treatment and/or prophylaxis may be achievable with CTLs prepared against a single subgroup, an important point given that adenoviruses from most subgroups can cause infection in transplant patients (9).

The fact that input virus generates an immune response is significant, especially in view of the increasing use of replication-incompetent adenovirus for human gene therapy. The strong reaction of the immune system observed following the use of the current generation of adenovirus-based gene therapy vectors may not be significantly diminished by using either viral vectors with additional mutations (41) or vectors derived from different subgroups. Our data suggest that such vectors will be immunogenic even if they are entirely transcriptionally silent. Identification and characterization of the viral proteins recognized by human CTLs and definition of the epitopes may lead to the design of vectors capable of escaping a memory T-cell response.

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