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Specific Inhibition of Granzyme B by Parainfluenza Virus Type 3

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T-cell-mediated cytotoxicity is an important means of defense against viral pathogens; however, several viruses possess mechanisms to disrupt cytotoxicity, thereby allowing them to avoid immune clearance. These viruses have been shown to inhibit cytotoxicity by interfering with the capacity of T lymphocytes to specifically recognize infected cells. An alternative mechanism for virally induced cytotoxic dysfunction is identified in this report. We show that parainfluenza virus type 3, a negative-stranded RNA virus, can inhibit cytotoxicity by causing a defect in the cytotoxic effector apparatus. This defect is identified as a selective inhibition of granzyme B mRNA.

Parainfluenza virus type 3 (PIV3) is a paramyxovirus that causes severe lower respiratory tract infections in children and infants (8, 13, 26). PIV3 infects essentially everyone in infancy, but initial exposure does not result in long-lasting immunity. Reinfection readily occurs, even in the presence of neutralizing antibodies (5, 8, 26). In addition, several reports have shown that PIV3 can cause persistent infections (3, 9, 11, 20).

Viruses have evolved diverse strategies that allow persistence and reinfection. One strategy involves virally mediated disruption of the immune response. Because cytotoxicity has been demonstrated to be an important effector mechanism for recovery from viral infections, it is not surprising that several viruses have evolved mechanisms to disrupt this function (1, 2, 4, 6, 17, 18, 25).

Adenovirus (1, 2), herpes simplex virus (17), and human cytomegalovirus (25) disrupt antigen-specific cytotoxicity by inhibiting the expression of major histocompatibility complex class I molecules in target cells (21). Recently, the variant cytotoxic T-lymphocyte (CTL) epitopes produced by human immunodeficiency virus (18) and hepatitis B virus (4) have been implicated as antagonists of antigen-specific cytotoxicity. For all of these viruses, disruption of the cytotoxic function has been shown to be mediated by prohibiting the specific recognition of target cells.

In this report we show that PIV3 can also inhibit cytotoxicity. Furthermore, we demonstrate that PIV3 directly perturbs the cytotoxic effector cell apparatus by selectively inhibiting the expression of granzyme B message. Thus, in contrast to other viral mechanisms for mediating cytotoxic dysfunction, which rely on interference with target cell recognition, PIV3 disrupts effector cells directly by disrupting the machinery of cytotoxicity.

MATERIALS AND METHODS

Virus and cells. Viral stock HA-1, National Institutes of Health catalog no. 47884, was obtained from Mark Galinski (Cleveland Clinic Foundation). Virus was grown on cv-1 monolayers, and titers of the virus were determined by plaque assays. cv-1 cells and PEER cells were maintained in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum.

Cytotoxicity tests for allogeneic responses. Peripheral blood mononuclear cells (PBMC) from healthy adult volunteers were isolated by centrifugation over a Histopaque cushion (Sigma, St. Louis, Mo.). Isolated PBMC were put into 24-well plates at 4×10^6 per well in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were stimulated with an allogeneic JY lymphoblastoid cell line at 2×10^6 cells per well. Prior to being used as stimulators, JY cells were grown for 2 days with either PIV3 at 3 PFU per cell or with an equivalent volume of control cv-1 supernatant. Immunohistology indicated that JY cells were consistently 70 to 80% infected by this protocol. After the 2-day incubation period, infected and uninfected JY cells were split into two groups, one which was irradiated (10,000 rads) and one which was fixed with 1% paraformaldehyde at room temperature for 10 min. Irradiation had no effect on viral infectivity, while fixation with paraformaldehyde prevented the release of live virions from infected cells. This finding was verified by the inability of culture supernatants from infected and fixed JY cells to induce cell death or syncytia in cv-1 monolayers. PBMC were cultured with the JY stimulators for 6 days, and then the cells were recovered and cytotoxicity was measured by the Jam test. Jam tests were performed as previously described (19). Targets for the Jam test were prepared by incubating JY or DK cells for 4 h with [^{3}H]thymidine at 2 μCi/ml. These labeled cells were then washed and added to 96-well round-bottom plates at 10^4 per well. Recovered effector cells were added at various effector-to-target cell ratios in quadruplicate wells. After a 4-h incubation, cells were harvested on a TOMTEC automated harvester system and analyzed with a liquid scintillation counter.

Further cytotoxicity tests involved cells which were activated with irradiated, uninfected JY 6 days prior to being exposed to either PIV3 at 3 PFU per cell or cv-1 supernatant. After 3 additional days of culture, these cells were tested for cytotoxic activity against JY targets by either the Jam test or chromium release assays. The Jam test measures DNA fragmentation, while chromium release is a reflection of membrane integrity.

Cytotoxicity tests for NK activity. PBMC were isolated as described above and suspended in RPMI 1640 with 10% fetal bovine serum. PBMC were put into culture at 2×10^6 cells per well in 24-well plates and either infected with PIV3 at 3 PFU per cell or left uninfected. Three days later, these cells were tested for their ability to kill natural killer (NK) cell-sensitive K562 targets in a chromium release assay.

Proliferation assays. PBMC suspended in RPMI 1640 with 10% fetal bovine serum were put into 96-well flat-bottom plates at 10^5 cells per well. Infected and uninfected JY stimulators were added to quadruplicate wells at 2×10^4 cells per well. Cells were pulsed with tritiated thymidine at a concentration of 0.5 μCi per well 18 h prior to being harvested onto glass fiber mats and subsequently analyzed with a liquid scintillation counter.

Flow cytometry. For flow cytometric analysis, cells were stained with dual-color CD4-CD8 (Coulter) antibodies. Murine immunoglobulin G antibodies were used as negative isotype controls. Cells were washed twice in phosphate-buffered saline (PBS)-bovine serum albumin-azide and then stained with antibody for 30 min on ice. Stained cells were washed three times with PBS and analyzed by flow cytometry. Dead cells were gated out on the basis of forward- and side-scatter characteristics. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

Immunohistology. Immunohistology for the detection of productively infected cells was performed as previously described (23).

RT-PCR analysis. RNA isolation for PCR analysis was performed according to a previously described method (10). Reverse transcriptase PCR (RT-PCR) analysis was done with the GeneAmp^R Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer, Norwalk, Conn.) and three pairs of oligonucleotide primers: beta-actin amplimers (Clontech, Palo Alto, Calif.), granzyme B primers (forward, 5′-AGAAGTCTCTGAAGAGGTGC-3′; reverse, 5′-TTGAG ACTTTGGTGCAGGCT-3′), and granzyme A primers (forward, 5′-GTCCTAC TTAGTCTTGACAG-3′; reverse, 5′-CCAGAATCTCCATTGCACGA-3′). Reverse transcription was carried out at 70°C for 15 min. Thermal conditions for PCR were 94°C for 1 min followed by 94°C for 1 min, 55°C for 30s, and 72°C for

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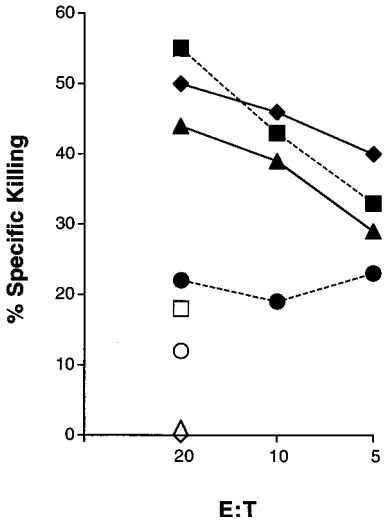

FIG. 1. Infective PIV3 inhibits cytotoxic activity of primary allospecific CTLs. The Jam test was used to assess killing of JY targets by PBMC responders that were cultured for 6 days prior to the assay with uninfected, irradiated JY (closed triangles); infected, irradiated JY (closed circles); uninfected, fixed JY (closed diamonds); or infected, fixed JY (closed squares). Killing of third-party allogeneic DK cells at an effector-to-target cell ratio (E:T) of 20:1 is indicated by the respective open symbols.

1 min for 30 cycles. The final extension was done at 72°C for 10 min. These conditions were used for all PCRs except for those done with the granzyme A primers, in which the annealing temperature was changed to 40°C. An equivalent volume of each reaction mixture was analyzed on a 1.5% agarose gel.

RESULTS

Effect of PIV3 on generation of allogeneic killers. To examine the effect of PIV3 infection on allogeneic cytotoxic responses, we cocultured PBMC with irradiated or paraformaldehyde-fixed allogeneic JY lymphoblastoid cells that had or had not been infected with PIV3. Irradiation did not inactivate the virus, while fixation with paraformaldehyde destroyed viral infectivity. After 6 days of incubation, measurements of cytotoxicity against JY and third-party DK lymphoblastoid targets were obtained. As can be seen in Fig. 1, uninfected irradiated and paraformaldehyde-fixed JY stimulators were able to induce allospecific CTL killing against JY targets. This response was specific since there was poor killing against third-party DK targets. In contrast, cultures from infected JY stimulators which were irradiated but not fixed with paraformaldehyde showed a marked defect in cytotoxic activity. Infected JY cells which were fixed in paraformaldehyde to prevent virus from spreading to responder populations stimulated the generation of allospecific CTLs. These results suggest that the infection did not prevent JY cells from processing or presenting antigen for the stimulation of T cells. Instead, it is likely that the inhibition of CTL generation was a reflection of an effect of the virus on the responding T cells.

Inhibition of proliferation mediated by PIV3 in allogeneic cultures. To determine if the infected stimulators could interfere with T-cell activation processes and could thereby contribute to the observed defect in cytotoxic activity, we measured the proliferative responses of PBMC when stimulated with infected allogeneic JY cells (Table 1). Virally induced inhibition of proliferation was similar to that noted in the cytotoxicity experiments. Infection of irradiated JY stimulators markedly inhibited proliferative responses of PBMC, and most of the virally induced inhibition was abrogated by fixation of the infected JY stimulators in paraformaldehyde. Since the infection did not inhibit the capacity of JY cells to process or present antigen, the finding that intact virus impeded activation suggests that the virus mediated this effect by acting on the responding T cells.

TABLE 1. Proliferative responses of PBMC stimulated with infected allogeneic JY cells[a]

Treatment of JY stimulators			PBMC proliferation (cpm)	
Irradiation (10^4 rads)	Fixation	PIV3 infection	Day 4	Day 6
+	−	−	81,799	165,374
+	−	+	2,423	916
−	+	−	63,871	161,319
−	+	+	34,884	126,305

[a] PBMC were put into 96-well flat-bottom plates at 10^5 cells per well. Stimulator JY populations were treated as indicated and placed into wells at 2×10^4 cells per well. Plates were pulsed with tritiated thymidine at a concentration of 0.5 μCi per well 18 h prior to being harvested onto glass fiber mats and subsequently analyzed with a liquid scintillation counter.

Effect of PIV3 on differentiated cytotoxic T cells. Besides the explanation that PIV3 has an effect on activation, an additional, and not necessarily incompatible, explanation for cytotoxic dysfunction is that PIV3 disrupts the cytotoxic machinery in the effector cells. To determine whether viral regulation of cytotoxicity could be mediated on cells that have already differentiated their capacity to effect cytotoxicity, we exposed CTLs to PIV3 or control supernatant 6 days after stimulation with uninfected JY cells. After 3 additional days of incubation, the cells were tested for their ability to kill JY targets (Fig. 2). CTLs infected 6 days after their original activation showed a significant reduction in cytotoxicity compared with controls. This result demonstrates that inhibition of cytotoxicity can occur at stages beyond the initial T-cell activation event.

Cell recoveries from infected and uninfected cultures were both 80% of the original cell counts, and cell viabilities, as measured by trypan blue dye exclusion, were 81 and 80% for uninfected and infected cells, respectively. Flow cytometric analysis indicated that uninfected responder populations were

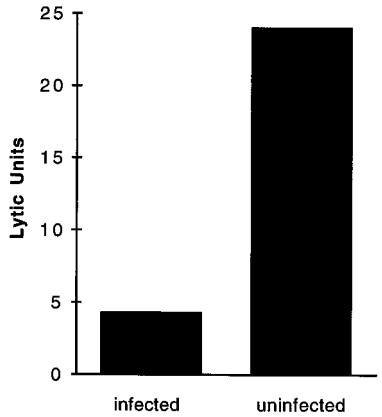

FIG. 2. Primed CTLs have reduced cytotoxic ability after infection with PIV3. CTLs to be infected with PIV3 were taken from cultures which were stimulated for 6 days with uninfected, irradiated JY (Fig. 1). These cells were given either PIV3 at 3 PFU per cell or equivalent amounts of cv-1 virus-free supernatant and cultured for another 3 days. Killing efficiency was measured by a Jam test for infected and uninfected responders. Lytic units were calculated per 10^6 cells at 45% killing.

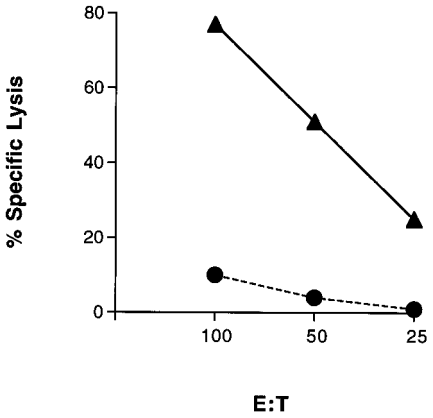

FIG. 3. NK cells lose the ability to kill K562 targets after 3 days of infection with PIV3. PBMC were put into culture at 2×10^6 cells per well and were either infected with PIV3 at 3 PFU per cell (●) or left uninfected (▲). Three days later, the cells were tested for their ability to kill NK cell-sensitive K562 targets in a chromium release assay. Spontaneous release was <20%. E:T, effector-to-target cell ratio.

46% $CD4^+$ and 36% $CD8^+$, while infected populations were 56% $CD4^+$ and 32% $CD8^+$.

PIV3 inhibition of NK activity. The effect of PIV3 infection on NK cell activity was also assessed. PBMC were placed in culture without any activating stimuli. PIV3 was added to some of the cultures. After 1 or 3 days of incubation, the cultures were tested for NK activity. After 1 day of incubation, there was no difference between infected and uninfected cultures (data not shown); however, there was a marked decrease in NK activity in infected cells after 3 days of culture (Fig. 3). Interestingly, cultures set up in this fashion do not demonstrate detectable levels of productive infection as judged by immunohistology (23). This result suggests that cytotoxicity may be influenced by the presence of PIV3 even in the absence of productive infection.

RT-PCR analysis for granzyme B message. Because granzyme B is an important component of the effector T-cell cytotoxic apparatus (15), we evaluated message levels in infected and uninfected allogeneic cultures prepared as before (see the legend to Fig. 2). These cells were infected 6 days after activation and subsequently incubated an additional 3 days. Again, infected cells demonstrated poor killing in a chromium release assay (49% specific lysis of JY targets by uninfected CTLs compared with 9% specific lysis by infected CTLs at an effector-to-target cell ratio of 25:1). RNA samples were recovered from both infected and uninfected responder populations for RT-PCR analysis (10) to identify beta-actin, granzyme A, and granzyme B mRNAs. We found that message levels for granzyme A and beta-actin were unaffected by infection (Fig. 4). In contrast, granzyme B mRNA levels were undetectable in CTLs infected with PIV3, while uninfected cells expressed significant levels of granzyme B mRNA. By using various amounts (100 to 1,000 ng) of RNA template in a semiquantitative analysis, we found that granzyme B message could be detected with 250 ng of RNA template derived from uninfected cells, whereas message was undetectable with as much as 1,000 ng of RNA template derived from infected cells. Detection of granzyme A message was independent of infection, and granzyme A was found at all RNA template levels examined.

We conducted additional experiments to determine if insults other than viral infection would result in the selective inhibition of granzyme B message. PBMC that were activated with

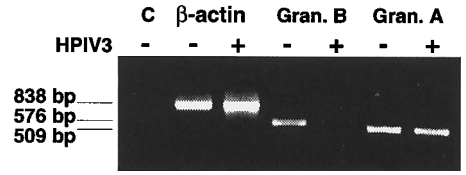

FIG. 4. Differential regulation of granzymes A and B in CTLs infected with PIV3. RT-PCR analysis of beta-actin, granzyme B (Gran. B), and granzyme A (Gran. A) mRNAs from human CTLs (prepared as described in the legend to Fig. 2) that were either infected with PIV3 (+) or uninfected (−) is shown. Total RNA templates (1 μg each) were analyzed, and a blank (C) without RNA template was included. RT-PCRs were performed with oligonucleotide primers specific for each transcript. The sizes of RT-PCR products are indicated. The 831-, 579-, and 509-bp amplified products are shown for the beta-actin, granzyme B, and granzyme A transcripts, respectively. A digitalized scanning image of the gel is shown.

phytohemagglutinin and subsequently treated with gamma irradiation, UV irradiation, heat shock, rapamycin, cycloheximide, or emetine failed to reproduce the corresponding pattern of selective loss of granzyme B message. This observation suggests that selective inhibition of granzyme B message is not associated with nonspecific cellular damage but is related uniquely to PIV3 infection. This conclusion is further supported by our failure to detect any virally mediated alteration in global protein synthesis (data not shown).

Because of the polyclonal nature of PBMC cultures, it was difficult to identify PIV3 as a direct mediator of granzyme B mRNA inhibition in T cells. To address this issue, we studied the effects of PIV3 infection in cells from a homogeneous leukemia cell line (PEER) that can be induced to express both granzyme A and granzyme B mRNAs upon activation with phorbol myristate acetate and dibutyryl cyclic AMP (12). In the presence of PIV3, activated PEER cells, like CTLs, demonstrated a loss in granzyme B message but not granzyme A message (Fig. 5). In contrast, both granzyme A and B messages were detected in uninfected cells. These findings suggest that PIV3 can act directly on exposed cells to mediate selective granzyme B mRNA inhibition.

DISCUSSION

Cytotoxicity involves several distinct events, including differentiation of the cytotoxic effector granules, recognition of the target, and exocytosis of granular contents on the target cell surface. Cytotoxic granules contain several different proteins involved in mediating cytotoxicity, including perforin, which is a pore-forming protein, and granzymes, which are serine esterases. cDNA clones encoding distinct human granzymes designated A and B have been reported (7, 24). The importance of granzyme A in cytotoxic activity is somewhat controversial

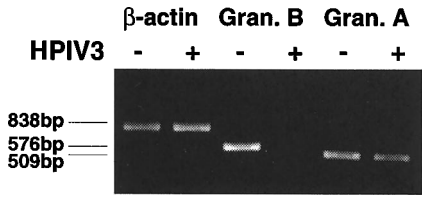

FIG. 5. PEER cell expression of granzyme B mRNA is inhibited by PIV3. PEER cells were activated with phorbol myristate acetate (16 mM) and dibutyryl cyclic AMP (0.5 mg/ml) in the presence of either PIV3 (3 PFU per cell) (+) or cv-1 control supernatant (−). Two days later, RNA was harvested and RT-PCR analysis was done to detect message for beta-actin, granzyme A (Gran. A), and granzyme B (Gran. B). A digitalized scanning image of the gel is shown.

since treatment of CTL granules with a granzyme A inhibitor did not significantly reduce lytic activity (14). Granzyme B, however, has been clearly implicated in CTL activity since granzyme B knockout mice have marked defects in cytotoxic function, particularly involving DNA fragmentation of the target cell (15). The role of granzyme B in cytotoxicity is further supported by the inhibitory activity of serine protease inhibitors on cytotoxicity (16, 22).

Our results are the first demonstration of virally mediated disruption of the machinery of cytotoxicity. The lack of granzyme B message in cells exposed to infective virus provides an explanation for the observed inhibition of cytotoxicity. These results demonstrate a direct effect of PIV3 on the cytotoxic machinery, which produces results similar to the inhibition of antigen recognition mediated by other viruses such as adenovirus, herpesviruses, human immunodeficiency virus, and hepatitis B virus. Interruption of recognition events involving the T-cell antigen receptor would presumably not suppress killing by NK cells. Conversely, inhibition of cytotoxicity by PIV3 occurs at the level of the effector apparatus and, thereby, is operative for both CTLs and NK cells.

We have previously demonstrated that PIV3 is able to infect T cells in vitro (23). Decreased thymidine incorporation in response to anti-CD3 antibody stimulation was observed in T cells exposed to virus in comparison with unexposed controls. Furthermore, we showed that the virus could mediate inhibition in the absence of productive infection. The data presented in this report further demonstrate the profound T-cell-inhibitory capacity of PIV3. It is likely that inhibition of T-cell function, particularly cytotoxicity, is important in viral pathogenesis. The inhibition of cytotoxicity mediated by PIV3 may play a role in the clinical viral persistence and reinfection observed with this virus.

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