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Infection and immunoregulation of T lymphocytes by parainfluenza virus type 3

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ABSTRACT **Human parainfluenza virus type 3 (HPIV3) is a major cause of disease in newborns and infants. It also has a striking potential to reinfect individuals throughout their lives, suggesting that HPIV3 does not induce lifelong immunity; however, the operative mechanism for the failure to prevent reinfection is not known. We have assessed the potential of the virus to infect nontransformed human T lymphocytes and have found that T cells are readily infected by the virus. Productive infection requires activation of the T cells and results in a marked inhibition of proliferation. Furthermore, our results indicate that exposure to the virus, even without overt expression of viral proteins as detected by immunohistology, profoundly alters the functional capacity of the T cells. The capacity of the virus to regulate T-lymphocyte function may play an important role in the failure of the virus to induce lifelong immunity.**

Human parainfluenza virus type 3 (HPIV3) causes severe respiratory tract infections in infants and children (1–5) and upper respiratory tract infections in adults (6–8). It is efficiently spread from person-to-person (5, 6); consequently, all adults possess anti-HPIV3 immunoglobulins (6).

Persistent infection of the virus has been documented in various clinical situations (8–10). Moreover, reinfection after a primary infection with HPIV3 is a common occurrence (1, 6). In one study the frequency of HPIV3 reinfection was found to be 58% in 1-year-old children and 34% in 2-year-old children (1). Even in the presence of circulating neutralizing antibodies, adults have been shown to be susceptible to reinfection (6). This clinical characteristic is distinct from infection with either measles or mumps viruses, which result in lifelong immunity against reinfection. It appears that infection with HPIV3 does not result in the establishment of a state of long-lasting immunity (11).

The mechanisms for persistent infection or for reinfection with HPIV3 have not been determined; however, it is likely that the equilibrium between the human host and the viral parasite arises as a result of the complex interactions between the host's immune system and the virus. One significant aspect of this interaction is infection of T lymphocytes by viruses. Viral infection of T lymphocytes has been demonstrated for many different viruses and has been shown to profoundly alter the immune response (12–15). Although T cells have the potential to lyse virally infected cells, help B lymphocytes produce immunoglobulins, and establish long-lived memory cells, the possibility that HPIV3-mediated immunoregulation of T lymphocytes plays a role in either persistence or reinfection has not been considered.

In this report we demonstrate that HPIV3 has a marked immunoregulatory effect on human T lymphocytes even in the absence of productive infection as detected by immunohistology.

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MATERIALS AND METHODS

Virus and Cells. CV-1 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% (vol/vol) fetal bovine serum. Viral stock HA-1, National Institutes of Health catalog no. 47884, was obtained from Mark Galinski (Cleveland Clinic Foundation). CV-1 cells were used for growing virus and for plaque assays.

Peripheral Blood Mononuclear Cells (PBMC). PBMC were isolated from healthy adult volunteers by discontinuous centrifugation on a histopaque cushion. PBMC were incubated with HPIV3 for 2 hr at 37°C at concentrations ranging from 1 to 30 plaque-forming units (pfu) per cell. After the initial 2-hr exposure to HPIV3, cells were washed and replated at either 0.5 or 1×10^6 cells per ml. The medium for infection and subsequent cultures was RPMI 1640 supplemented with streptomycin (5 μg/ml), penicillin (5 units/ml), glutamine (2 mM), and 10% fetal bovine serum. Culture stimuli were added immediately after the 2-hr incubation with HPIV3.

Experiments were also performed by infecting the PBMC with HPIV3 without washing out the virus. The results from these experiments were similar to the results from experiments that were performed with the washing step included (data not shown).

T cells from the PBMC were activated by adding anti-CD3 (OKT3; Ortho Diagnostics) at a concentration of 2 ng/ml. Activation was inhibited by adding cyclosporin A at a concentration of 500 ng/ml. In some cultures, recombinant interleukin 2 (IL-2) was added at a concentration of 10 units/ml. Restimulation of T cells was accomplished by adding anti-CD3 every 7 days, irradiated (5000 rads; 1 rad = 0.01 Gy) allogeneic PBMC at a density of 10^6 per ml and IL-2 at a concentration of 10 units/ml.

The effect of HPIV3 infection on [^{3}H]thymidine uptake by anti-CD3-stimulated PBMC was determined by plating 10^5 PBMC in quadruplicate flat-bottom wells of five replicate 96-well microtiter tissue culture plates. The culture medium included anti-CD3 at a concentration of 2 ng/ml, and various concentrations of HPIV3 were added. After a variable number of days of incubation, [^{3}H]thymidine at 0.5 μCi per well (1 μCi = 37 kBq) was added to each well of a single plate, and after 18 hr the contents of the wells were individually collected on glass fiber filters with an automated harvester and were processed for liquid scintillation counting on a Beckman LS6000IC counter.

T-Cell Clones. T-cell clones from three individuals were established and maintained as described (16, 17). The V1 clone is specific for influenza hemagglutinin H2. The Datt 30 and Datt 42 clones are specific for tetanus toxoid. 8L1 and

Abbreviations: HPIV3, human parainfluenza virus type 3; pfu, plaque-forming unit(s); PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2.

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DK355 were cloned by stimulating the cells with anti-CD3, so the antigen specificities of these lines are not known.

Infection of the clones was performed in a manner similar to the infection of PBMC except that T-cell clones were infected 3 days after stimulation at a time when the irradiated feeder cells were completely absent from the cultures. It should be noted that T-cell clones are devoid of macrophages as indicated by flow cytometric analysis and by their inability to proliferate in response to the plant lectin phytohemagglutinin without the addition of freshly irradiated PBMC (data not shown).

Immunohistology. To assess infection, we washed 0.5–2 × 10^6 cells twice in phosphate-buffered saline. The pellet was resuspended in 50 μl of phosphate-buffered saline, and 7 μl was added to wells on eight-well Teflon-mat screening slides. The slides were allowed to air-dry, and then they were fixed in acetone for 10 min. After drying a second time, the slides were stored at −70°C until stained in batches. Staining was performed with 7 μl of anti-HPIV3 murine monoclonal antibodies (Baxter Scientific Products, McGraw Park, IL) at 37°C for 30 min. Slides were then washed twice in phosphate-buffered saline and blotted dry. The same procedure was then carried out for secondary fluoresceinated goat anti-mouse antibody. Samples were coded and visually inspected under a fluorescent microscope without knowledge of the culture origin of the sample. Cells were scored positive if they demonstrated at least a patch of green fluorescence. Infection of CV-1 cells and HeLa cells resulted in 100% infection by day 5 of incubation.

Flow Cytometry. PBMC and T-cell clones either infected with HPIV3 or uninfected were stained sequentially with a 1:150 dilution of anti-HPIV3 rabbit antiserum (generously supplied by Mark Galinsky, Cleveland Clinic Foundation) and fluoresceinated goat anti-rabbit antibody (ICN). Dual-color reagents for CD4 and CD8 (Dako) were used. Double-staining for CD4 or CD8 (Coulter) and HPIV3 F protein (BioDesign, New York) was performed after the cells were fixed in acetone for 10 min at room temperature. For staining with single reagents, we used anti-CD6 (Dako) and anti-CD3 (Ortho Diagnostics). Analysis was performed on a FACScan flow cytometer (Becton Dickinson).

RESULTS

Requirement of Cellular Activation for HPIV3 to Infect Nontransformed Human T Lymphocytes. To determine whether HPIV3 is capable of infecting nontransformed human T cells, we exposed PBMC to HPIV3 and then stimulated the T lymphocytes with antibodies directed against CD3. Infection was assessed by immunohistology after 7 days, and a significant number of virally infected cells were found. Because these cultures were stimulated with anti-CD3 monoclonal antibodies, >90% of the cells analyzed were T lymphocytes as judged by flow cytometric analysis with monoclonal antibodies specific for CD3 and CD6. Moreover, double staining 10 days after stimulation for CD8 and HPIV3 (F protein) and for CD4 and HPIV3 (F protein) demonstrated that all of the infected cells were T lymphocytes. Of the cells infected with HPIV3, 45% were $CD8^+$ and 55% were $CD4^+$.

To test whether infection altered the ratio of $CD4^+$ to $CD8^+$ cells, we analyzed infected and uninfected PBMC 7 days after stimulation with anti-CD3. Double staining for CD4 (33% and 31% positive in uninfected and infected cultures, respectively) and CD8 (60% and 57% in uninfected and infected cultures, respectively) did not reveal any significant differences in the ratio of $CD4^+$ cells to $CD8^+$ cells between infected and uninfected cultures.

Infection of T lymphocytes by other viruses has been shown to require the activation of the T cells (18–23). The role of cellular activation in infection by HPIV3 was assessed by comparing the level of infection obtained in cultures of T cells left unactivated, stimulated with anti-CD3, exposed to anti-CD3 but inhibited with the immunosuppressive agent cyclosporin A (24), or exposed to anti-CD3 and cyclosporin A but given IL-2 to restore the capacity of the cells for activation. Infection was assessed by immunohistology on a daily basis for a week (Fig. 1). The results showed that infection occurred only in the cultures activated by anti-CD3 antibodies. Cyclosporin A inhibits the activation of T lymphocytes by anti-CD3 antibodies, and it effectively inhibited HPIV3 infection. Furthermore, the addition of IL-2 which reverses the inhibition of cyclosporin A on T-cell proliferation, did not allow for infection as determined by the presence of cytoplasmic viral antigen.

Production of Infective Virions by T Cells Infected by HPIV3. Infection of T lymphocytes by HPIV3 was further characterized by assessing the production of infective virions. Supernatants from either anti-CD3-stimulated or unstimulated PBMC infected with HPIV3 (1 pfu per cell) were collected after 5 and 7 days. The supernatants were tested for their titer in producing plaques on a monolayer of CV-1 cells. In cultures with anti-CD3-activated lymphocytes, viral titers obtained were between 10^6 and 10^7 pfu per ml. In cultures left without activation, no infectious virus was detected in the

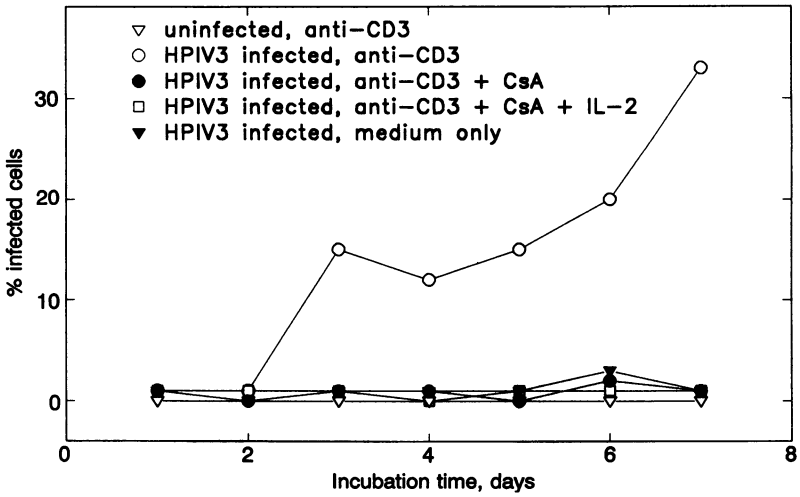

FIG. 1. Dependence of viral antigen expression on cellular activation. PBMC were infected with HPIV3 at 1 pfu per cell and cultured with stimuli as indicated. Cells were processed for immunohistology daily for a week.

Table 1. Infection of nontransformed human T-cell clones by HPIV3

T-cell clone	Infected cells on incubation days, %				
	4	7	11	14	18
V1	12	13	27	23	21
Datt 30	8	5	38	30	28
Datt 42	10	7	23	20	18
8L1	45	42	41	—	—

T-cell clones were infected with HPIV3 at 3 pfu per cell 3 days after stimulation with irradiated PBMC, anti-CD3 monoclonal antibodies, and recombinant IL-2. Fresh medium and IL-2 were added, and the cells were incubated at 37°C. On days 4 and 11, the cells were restimulated with irradiated PBMC, anti-CD3 monoclonal antibodies, and recombinant IL-2. On days 7 and 14, the cells were resuspended in fresh medium and recombinant IL-2.

plaque assay. Similar results were obtained with a $CD8^+$ human T-cell clone DK355 (data not shown).

Plateau Levels of HPIV3 Infection of T Lymphocytes. Although 30–40% of anti-CD3-activated PBMC were infected with HPIV3 (1–3 pfu per cell) after 7 days, exposure of HeLa or CV-1 cells to HPIV3 resulted in 100% infection in <5 days. Moreover, infection of PBMC with measles virus results in >80% infection within the same time (25). We assessed viral antigen expression for up to 10 days after infection, and the results show that from day 7 to day 10, the percentage of cells infected with HPIV3 did not exceed the 30–40% level. Furthermore, we restimulated cultures of infected PBMC weekly for three stimulations and assessed the level of infection twice weekly. Our results indicate that the cultures reached a plateau of approximately 35%, and this level of productive infection was maintained without increasing for 3 weeks, when the viability of the cultures was too low to analyze viral antigen expression.

We have attempted to enhance the plateau level of productive infection by increasing the dose of HPIV3 in the initial infection up to 30 pfu per cell. Our data show that the plateau level for infection was independent of the viral titer of the infecting inoculum up to 30 pfu per cell (data not shown).

To consider the possibility that the potential for T cells to be infected by HPIV3 is a clonally distributed property, we assessed the capability of HPIV3 to productively infect cloned human T cells. Infection of four nontransformed human T-cell clones with HPIV3, as assessed by the percentage of infected cells, failed to demonstrate any differences among the clonal population in terms of infection with HPIV3 (Table 1). Moreover, plateau levels of infection were observed for each of the clones. Flow cytometric analysis of the cell surface of infected PBMC and a T-cell clone with an antiserum specific for HPIV3 surface glycoproteins showed an apparently single predominant population of infected cells (Fig. 2). Infection did not appear to clonally discriminate among T cells.

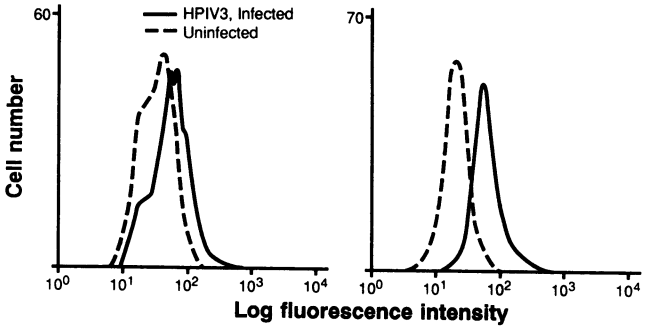

FIG. 2. Flow cytometric analysis of HPIV3-infected T cells. PBMC (*Left*) and a $CD8^+$ T-cell clone 8L1 (*Right*) were infected with HPIV3 at 3 pfu per cell and stimulated with anti-CD3 antibody. PBMC after 7 days of incubation and 8L1 after 4 days of incubation were processed for surface expression of HPIV3 envelope proteins by flow cytometric analysis.

Proliferation of HPIV3-Infected T Lymphocytes. Although only 30–40% of PBMC or cloned cells were infected, we noted that the viability of the cells in infected cultures gradually diminished over 3 weeks until the cultures no longer contained any viable cells. To more clearly assess the capacity of infected T cells to proliferate, we performed daily cell counts on the cultures shown in Fig. 1 (Fig. 3). These data show that HPIV3 infection markedly decreased the proliferation of T lymphocytes stimulated with anti-CD3.

Furthermore, although IL-2 reversed the growth inhibition mediated by cyclosporin A of anti-CD3-stimulated T cells in uninfected cultures, the addition of IL-2 along with cyclosporin A to anti-CD3-stimulated cultures infected with HPIV3 did not reverse the inhibition of cellular proliferation (Fig. 3). This inability to respond to IL-2 could not be explained by a failure in expression of the IL-2 receptor. Cells from infected and uninfected cultures expressed equivalent levels of the inducible chain of the IL-2 receptor complex (data not shown). Thus, HPIV3 infection alters the proliferative capacity of T lymphocytes and prevents IL-2 from inducing growth in receptor-positive cells.

A further indication of the virus' effect on T-cell proliferation was demonstrated with another experimental protocol. PBMC were infected with HPIV3 (1 pfu per cell), but activation with anti-CD3 was delayed for 4 days. In uninfected cultures, total cell numbers increased from 2.5×10^6 to 16×10^6 between 3 and 5 days after anti-CD3 activation; however, in the cultures infected with HPIV3, there was no increase in the cell number. The cells stimulated with anti-CD3 4 days after infection did not present any sign of infection, as indicated by immunohistology. Again the functional effect of the virus was not dependent on overt infection of the cells.

A functional alteration in the T cells infected with HPIV3 was also indicated by assessing the incorporation of tritiated thymidine into the T cells. PBMC were stimulated with anti-CD3 in the presence of various concentrations of HPIV3, and tritiated thymidine incorporation was assessed (Fig. 4). There was a dose-dependent inhibition of incorporation by the virus. Even a dose of 0.04 pfu per cell, which does not result in detectable viral infection within the time frame of the experiment (data not shown), mediated a profound inhibition of tritiated thymidine incorporation.

DISCUSSION

We have shown that nontransformed human T cells are readily infected by HPIV3 and that this infection leads to the production of infective virions. Our data show that infection is absolutely dependent on the activation of the T cells and, furthermore, that the proliferative capacity of the activated T cells is profoundly affected by exposure to the virus.

The effect of HPIV3 exposure on T-cell proliferation are reflected by (*i*) an abortive proliferation after an activating stimulus; (*ii*) the failure of IL-2 to restore the proliferation of T cells in the presence of the immunosuppressive agent cyclosporin A; and (*iii*) the failure of infected T cells to proliferate with activation 4 days after exposure to the virus. It is important to note in the latter two instances that the functional consequences of HPIV3 infection were not dependent on the production of viral proteins as indicated by immunohistology or on the production of infective virus as indicated by a plaque assay. Furthermore, the marked inhibition of T-cell proliferation seen with as little virus as 0.04 pfu per cell, a viral concentration that does not result in

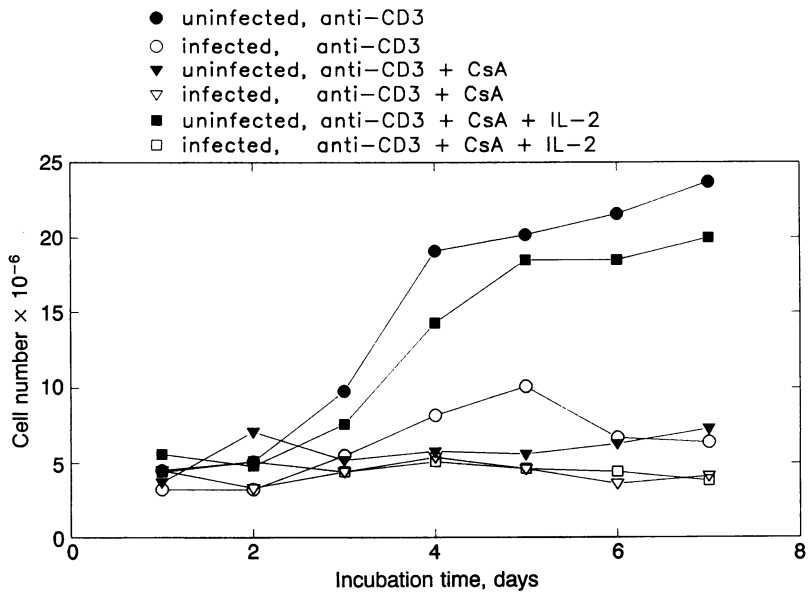

FIG. 3. Effect of HPIV3 infection on T-cell proliferation. PBMC were infected with HPIV3 at 1 pfu per cell and cultured with stimuli as indicated. Cells were counted daily for a week. CsA, cyclosporin A.

detectable infection within 1 to 2 weeks, is an indication of the potency of the immunoregulatory capacity of the virus.

Many different viruses have been shown to affect T lymphocytes with significant consequences to the immune response (12–15, 25, 26). For instance, the human immunodeficiency virus infects $CD4^+$ T cells, and this infection eventually leads to a state of profound immunodeficiency. Measles virus has been demonstrated to readily infect human T lymphocytes and natural killer cells (25). These cells do not generate cytotoxic function nor do they help in the synthesis of immunoglobulins and their proliferative capability is markedly compromised. Likewise, exposure of T cells to cytomegalovirus *in vitro* causes depressed T cell function (27, 28) and infection *in vivo* is immunosuppressive.

HPIV3 has the potential to both persist and to reinfect *in vivo* (1, 6–10); however, the mechanisms for these phenomena have not been described. It seems likely that the virus has the potential for manipulating the immune response. Our data suggest that HPIV3-mediated immunoregulation of T cells may play an important role in the immune response to the virus.

Generalized immunosuppression has not been associated with infection by HPIV3. Moreover, infected individuals make anti-HPIV3 antibodies and recover from the infection, indicating that the specific immune response is not totally obliterated. However, the capacity of the virus to reinfect and persist is distinctly different from the situation with measles virus infection, which results in lifelong immunity. It is possible that HPIV3 infection preferentially inhibits the establishment of specific anti-HPIV3 memory T cells. The role of specific T cells in the immune response to HPIV3 has not been investigated.

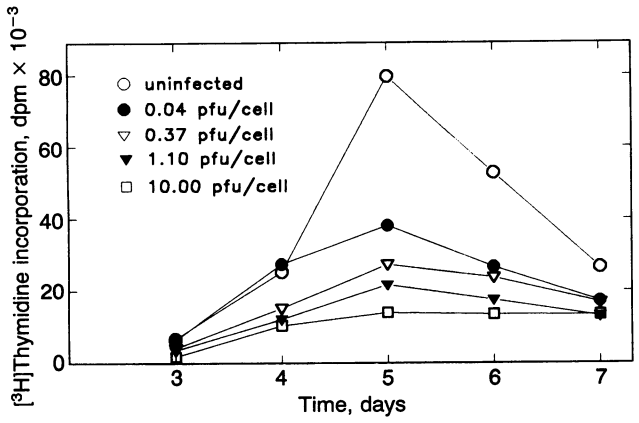

FIG. 4. Effect of HPIV3 infection on T cell [^{3}H]thymidine incorporation. PBMC were stimulated with 2 ng of anti-CD3 per ml in the presence of various concentrations of HPIV3. Tritiated thymidine incorporation was determined at the indicated times.

The requirement for cellular activation to obtain productive infection of T cells by HPIV3 is a requirement similar for several other viruses that infect T cells (18–23). It seems most likely that activation of the cell is necessary for the establishment of the cellular machinery needed for viral replication. In this regard it is interesting to note that HPIV3 infection reaches a plateau value between 30% and 40% of PBMC or T-cell clones. It appears that the plateau values are a reflection of the dynamics of the culture, which are clearly affected by the presence of viral particles.

Other viruses infect activated T cells to different levels *in vitro*. Measles virus infects >80% of PBMC (25) while another paramyxovirus, mumps virus, infects 95% (29). Cytomegalovirus infection of activated PBMC is limited to only 1–3% of the cells (27). A recent report indicates that cytomegalovirus viral antigens are preferentially expressed in a small subpopulation of $CD8^+$ T cells (28). The variation in the capacity to express viral antigens in activated PBMC probably represents significant differences in the viral mechanisms at work.

The mechanism of immunoregulation mediated by HPIV3 has not been elucidated. It is possible that viral proteins interact with cellular proteins that mediate proliferative responses, thereby inhibiting their function. Alternatively, the presence of the virus may induce the secretion of an inhibitory cytokine, such as transforming growth factor β.

The functional alterations in the measles virus-infected T cells can be explained by direct viral infection or cytopathic effects, since most of the cells are infected (25). In contrast, productive viral infection cannot explain the marked functional alterations in the HPIV3-exposed T cells, since detectable viral protein synthesis as detected by immunohistology was not required for profound inhibition of T-cell proliferation.

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