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A Major Mechanism of Human Immunodeficiency Virus-Induced Cell Killing Does Not Involve Cell Fusion

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In vitro studies indicate that human immunodeficiency virus (HIV) infections are cytopathic for $T4^+$ peripheral blood lymphocytes and for most continuous lines of $T4^+$ lymphocytes. These cytopathic effects have been largely attributed to the formation of syncytia by HIV-infected cells. We report that HIV infections killed cultured peripheral blood lymphocytes and a line of $T4^+$-lymphoid cells (CEM cells) without causing cell fusion. We also report that the occurrence of syncytia is an early and transitory phenomenon following infection of a fusion-susceptible line of $T4^+$-cells (H9 cells). Mixing experiments and flow cytometry have been used to demonstrate that susceptibility to HIV-induced fusion is not determined by differences in presentation of viral envelope antigens or the surface levels of T4 receptor antigens on fusion-susceptible and -resistant cells. We conclude that a major mechanism of HIV-induced cell killing does not involve cell fusion and that HIV-induced cell fusion, when it does occur, requires factors in addition to viral envelope antigens and host T4 receptors.

The induction of acquired immunodeficiency syndrome (AIDS) in humans by human immunodeficiency virus (HIV) (1, 4, 6, 12) is associated with a marked loss in the number of T-helper/inducer cells (7, 16). The loss of this population of cells does not take place at a specified time after infection and appears to be a gradual process accompanied by the presence of remarkably low numbers of actively infected cells (in situ hybridization studies suggest that 1 in 100,000 peripheral blood lymphocytes [PBLs] is actively infected in pre-AIDS and AIDS patients) (8). In contrast to in vivo infections in which relatively few cells are actively infected with HIV, in vitro infections of mitogen-stimulated PBLs or certain established cell lines of lymphoid origin result in the infection and death of the culture within days of exposure to HIV. Studies with these and other culture systems indicate that the T4 (CD4) differentiation antigen of T-helper/inducer cells (21) serves as a receptor for HIV (3, 11, 15, 17, 22). Work with cell cultures has also suggested that the marked cytopathic effects of HIV for T4 cells are caused by cell fusion, with syncytia arising between cells expressing viral envelope glycoproteins (13, 23) and cells expressing the T4 receptor antigen (14, 22). In this report, we present findings which indicate that HIV infections frequently kill cultured PBLs as well as certain lines of $T4^+$ cells by mechanisms other than the induction of cell fusion.

MATERIALS AND METHODS

Cells and cell culture. H9 cells (a subline of HUT78 [19]; ATCC TIB 161), CEM cells (ATCC CCL 119), Jurkat cells (18), C8166 cells (15), U937 cells (ATCC CRL 1593), and Raji cells (ATCC CCL 86) were grown at densities between 0.5×10^6 and 1.5×10^6 cells per ml in RPMI 1640 supplemented with 10 to 15% fetal bovine serum. Each of these cell lines was obtained from the laboratories of Carel Mulder or John L. Sullivan at the University of Massachusetts Medical School. To have identifying phenotypes for these lines, histocompatibility types were determined and, where available, compared with reference human lymphocyte antigen (HLA) phenotypes. The histocompatibility types of the H9 cells and Jurkat cells agreed with the reference HLA type determined by Jeffrey Laurence for H9 cells (A_1, B_{15}, Bw_-, and C_-) and Jurkat cells ($A_{3,9}$, B_{27}, Bw_-, C_-). Reference HLA types were not available for CEM cells or U937 cells. Our CEM cells have an HLA type of $A_{1,w19}$, B_{40}, Bw_6, and C_-. The HLA type of our U937 cells is A_3, $B_{18,51}$, $Bw_{4,6}$, and $C_{1,3}$. PBLs were obtained from normal human donors. PBLs were mitogen stimulated (2 μg of phytohemagglutinin per ml) for 3 days in RPMI 1640 supplemented with 15% fetal bovine serum and 10% (vol/vol) interleukin 2 (Cellular Products Inc., New York, N.Y.) and then grown in the same medium. Cultures of PBLs were maintained at cell densities from 0.5×10^6 to 2.0×10^6 cells per ml.

Virus and virus infections. A stock of human T-cell lymphotropic virus type III (HTLV-III [HTLV-IIIb]) (6) was obtained from Carel Mulder at the University of Massachusetts Medical School. The stock of HTLV-III had been grown on H9 cells and had a reverse transcriptase titer of 1.5×10^6 cpm/ml and $\sim 5 \times 10^4$ IU/ml (determined by endpoint dilution on CEM cells). A recent HIV isolate from a patient, HIV(UMA-CB), was obtained from John L. Sullivan at the University of Massachusetts Medical School. HIV(UMA-CB) was isolated by cocultivation of mitogen-stimulated PBLs from a HIV-seropositive hemophiliac with mitogen-stimulated PBLs from normal donors. The HIV(UMA-CB) stock had undergone one passage on CEM cells and had a reverse transcriptase titer of 0.5×10^6 cpm/ml. All infections were accomplished by suspending cells in undiluted virus stocks. H9 and CEM cells were infected in the presence of 10 μg of Polybrene per ml for 4 h and then grown in the presence of 2 μg of Polybrene per ml. Mitogen-stimulated PBLs were infected and grown in the presence of 2 μg of Polybrene per ml.

Assays for HIV infection and cytopathic effects. HIV infections were monitored for HIV antigen-positive cells by using indirect immunofluorescence. Cells to be tested for HIV antigens were air dried onto microscope slides and fixed for

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10 min with methanol. Fixed cells were washed twice with phosphate-buffered saline (PBS) and incubated 30 min at 37°C with 50 μl of a 1:20 dilution of the first antiserum (either pooled sera from AIDS patients or normal controls). Cells were then washed with PBS, incubated 30 min at 37°C with 50 μl of a 1:20 dilution of the second antiserum, fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-human immunoglobulin G (Cooper Biomedical Inc., West Chester, Pa.), and washed with PBS before being mounted for viewing with a fluorescence microscope. Cytopathic effects were determined by microscopically counting trypan blue-staining (dead) and -excluding (viable) cells with a hemacytometer.

Assays for T4. The amount of the differentiation antigen T4 (CD4) (21) on the surfaces of cells was quantitated by using indirect immunofluorescence and flow cytometry. Cells were maintained in logarithmic growth for several days before analyses for T4. Cells were then washed with ice-cold PBS and incubated on ice for 30 min with saturating levels of the first antibody, OKT4A (a monoclonal antibody to T4; Ortho Diagnostic Systems, Inc., Raritan, N.J.). Cells were then washed with ice-cold PBS and incubated with saturating levels of the second antibody, fluorescein-conjugated goat anti-mouse immunoglobulin G (Ortho Diagnostic Systems). After being washed with ice-cold PBS, cells were fixed in 2% paraformaldehyde for 10 min at room temperature, washed with PBS, and suspended in growth medium for analyses by using a FACS IV flow cytometer (Becton Dickinson and Co., Paramus, N.J.). The FACS IV reported fluorescence intensity in logarithms, with 1 logarithm equal to 96 channels. These values are reported as linear numbers, with the reported number equal to $10^{\text{measured value}/96}$.

RESULTS

Occurrence of infected cells, dead cells, and syncytia in various HIV-infected cultures. As background information for studies on the cytopathic effects of HIV infections for $T4^+$ lymphoid cells, we monitored HIV infections in a line of $T4^+$ lymphoid cells which had been reported to be relatively resistant to HIV-induced cell killing (H9 cells) (19), a line of $T4^+$ lymphoid cells that had been reported to be highly susceptible to HIV-induced killing (CEM cells) (5), and cultures containing mitogen-stimulated PBLs from seronegative donors (~30 to 50% of the lymphocytes in such cultures are $T4^+$). These infections were done by using a laboratory stock of HIV, HTLV-III, as well as a recent isolate from a patient, HIV(UMA-CB). The spread of HTLV-III and UMA-CB in each of the cultures was monitored by testing for HIV antigen-positive cells using indirect immunofluorescence. Cytopathic effects were quantitated by scoring cells that failed to exclude the vital dye, trypan blue. The induction of syncytia was monitored by quantitating the fraction of infected cells containing at least four nuclei.

By 12 days after infection, HTLV-III had spread through and killed the vast majority of $T4^+$ cells in cultures of CEM cells, H9 cells, and mitogen-stimulated PBLs (Fig. 1). The spread of the infection and the appearance of cytopathic effects in the CEM and H9 cells were remarkably similar, with the appearance of infected cells slightly preceding the appearance of dead cells. By 12 days after infection, surviving populations of both CEM cells and H9 cells had begun to appear. The majority of these appeared to be uninfected (Fig. 1) and negative for surface T4 antigens (data not shown). The cultures of HTLV-III-infected PBLs exhibited more dead than infected cells, with cell death peaking at the same time as cell infection. One possibility is that this reflected the rapid death of PBLs after infection, since only living cells can be scored for viral antigens by indirect immunofluorescence. Another possibility is that infected PBLs produce toxic factors which kill uninfected PBLs.

Similar phenomena were observed with the recent isolate from a patient, UMA-CB (Fig. 1). Within 18 days of infection, UMA-CB had spread through and killed the vast majority of $T4^+$ cells in cultures of CEM and H9 cells. As might be expected, UMA-CB appeared to grow better in PBLs than in the cell lines, spreading through and killing $T4^+$ cells in these cultures within 12 days of infection. In the cell lines, infection appeared to slightly precede cell death, whereas in the lymphocyte cultures, infection appeared to be essentially simultaneous with cytopathic effects. Thus, all of the cultures were highly susceptible to HIV-induced cytopathic effects with reported differences in susceptibility to cytopathic effects not repeating with the H9 and CEM cells used in our experiments under our conditions of infection and culture.

Unexpectedly, the cytopathic effects of both HTLV-III and UMA-CB infections were not necessarily associated with syncytium formation (Fig. 1 and 2; Table 1). Syncytia appeared soon after infection of H9 cells with HTLV-III or UMA-CB. However, the fraction of infected H9 cells that were present in syncytia peaked while the number of dead cells in cultures were still rising (Fig. 1; Table 1). By the time infected H9 cells were undergoing massive cytopathic effects (12 to 18 days postinfection), less than 10% of the infected cells were present in syncytia. The failure of syncytium formation to correlate with cytopathic effects was even more dramatic in the cultures of CEM cells and PBLs. In these infected cultures, not a single syncytium was observed in more than 2,000 infected cells (Table 1). The photomicrographs in Fig. 2 illustrate the presence of mononuclear cells as well as syncytia among HTLV-III-infected H9 cells and the occurrence of only mononuclear cells among HTLV-III-infected CEM cells and PBLs.

Use of mixing experiments to test for the ability of HIV-infected cells to induce syncytia. To test whether differences

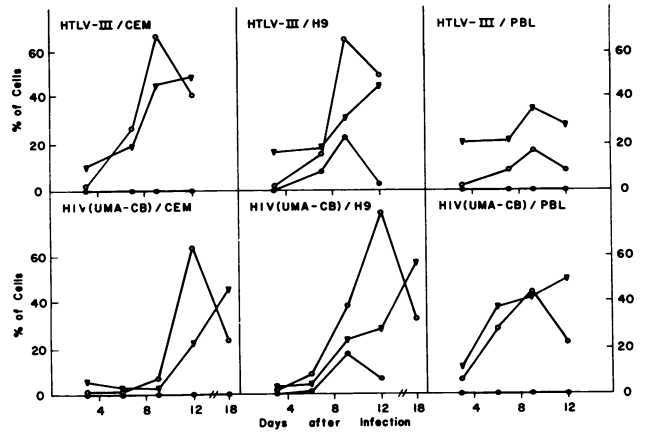

FIG. 1. Fate of CEM cells, H9 cells, and mitogen-stimulated PBLs infected with HTLV-III or HIV(UMA-CB). Symbols: O, HIV antigen-positive cells; ▼, dead (trypan blue-staining) cells; ●, syncytia. The number of dead cells are normalized for the number of dead cells in control cultures. All syncytia were HIV antigen positive and scored as one cell.

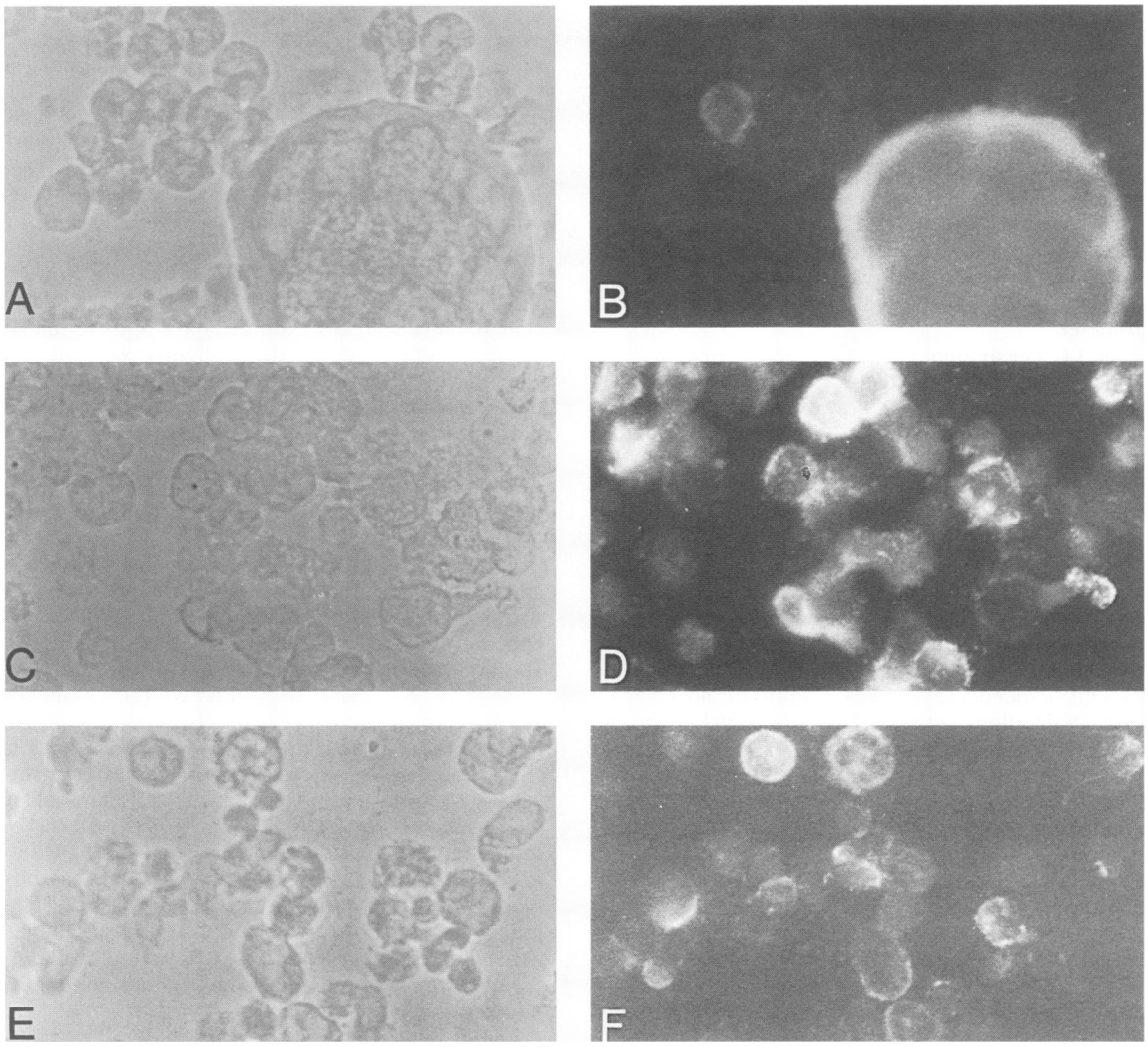

FIG. 2. HIV-infected H9 cells, CEM cells, and mitogen-stimulated PBLs. HTLV-III-infected H9 cells, CEM cells, and PBLs were tested for HIV infection by indirect immunofluorescence at 9 days postinfection. Photomicrographs (magnification, ×400) were made by using phase (panels A, C, and E) and fluorescence (panels B, D, and F) microscopy of the same fields of H9 cells (panels A, B), CEM cells (panels C, D) and PBLs (panels E, F).

in the occurrence of syncytia in HIV-infected cultures were determined by differences in viral envelope glycoproteins expressed by the different infected cells, various infected cells were mixed with various uninfected cells at a ratio of 1:10 (Table 2). HTLV-III-infected cultures at 9 to 10 days postinfection were used as the source of infected cells since, under our conditions, such cultures exhibit maximum numbers of infected yet living cells (Fig. 1). In the infections used for these experiments, ~10% of the infected H9 cells were in syncytia. When infected H9 cells were mixed with uninfected H9 cells, ~60% of the infected cells were found in syncytia. Thus, the infected H9 cells were more efficient at initiating syncytia with uninfected H9 cells than with infected H9 cells, a phenomenon which is consistent with the disappearance of the T4 antigen from the surface of HIV-infected H9 cells (3, 9; data not shown).

TABLE 1. Occurrence of syncytia in HTLV-III- and HIV(UMA-CB)-infected CEM cells, H9 cells, and mitogen-stimulated PBLs[a]

HIV isolate	Infected cell	HTLV-III$^+$ syncytia/HTLV-III$^+$ cells at days after infection			
		2–4	6–7	9–10	12–13
HTLV-III	CEM	0/779	0/614	0/642	0/588
	H9	70/564	375/609	165/603	49/616
	PBL	0/793	0/606	0/578	0/567
HIV(UMA-CB)	CEM	0/178	0/192	0/202	0/198
	H9	16/179	51/184	102/212	17/206
	PBL	0/183	0/186	0/205	0/173

[a] Infection and assay of infected cultures for HIV-infected cells is described in Materials and Methods. Data for HTLV-III are from three experiments. Data for HIV(UMA-CB) are from a single experiment.

Interestingly, infected H9 cells were unable to initiate syncytium formation when mixed with uninfected CEM cells or uninfected PBLs, whereas infected CEM cells and PBLs were able to initiate syncytia when mixed with uninfected H9 cells. Infected CEM cells and PBLs were also able to initiate syncytia when mixed with T4$^+$ Jurkat or C8166 cells. Both Jurkat and C8166 cells have been widely used in tests for HIV-induced syncytium formation. None of the infected cells were able to induce syncytia when mixed with

TABLE 2. Induction of syncytia by HTLV-III-infected cells mixed with uninfected cells[a]

Infected cell	Uninfected cell	HTLV-III$^+$ syncytia/HTLV-III$^+$ mononuclear cells	
		Actual count	Reduced ratio
H9	None	25/206	0.1
	H9	61/102	0.6
	CEM	5/105	<0.1
	PBL	3/97	<0.1
CEM	None	0/198	0
	H9	102/101	1.0
	Jurkat	74/101	0.7
	C8166	65/97	0.7
	U937	0/106	0
PBL	None	0/178	0
	H9	24/92	0.3

[a] Cells used to initiate syncytia were taken from cultures at 9 to 10 days after infection with HTLV-III. Infected cells were mixed with uninfected cells at a ratio of 1:10. Syncytia, which began to form within 4 h of mixing, were scored by indirect immunofluorescence at 20 h after mixing.

uninfected cells of a T4$^+$ line of monocytes, U937 cells, or Raji cells, a B-lymphoid line which does not express T4.

These results suggested that HTLV-III-infected H9 cells, CEM cells, and PBLs each expressed viral envelope antigens that could initiate fusion with a fusion-susceptible T4$^+$ cell. To verify that comparable levels of similarly processed envelope glycoproteins were expressed by infected H9 and CEM cells, [^{35}S]methionine-labeled proteins were immunoprecipitated from HTLV-III-infected H9 and CEM cells by using pooled sera from HIV-infected or normal humans. These analyses revealed no differences in the amount of processing of HTLV-III envelope glycoproteins in infected H9 and CEM cells (data not shown).

Tests for the amount of surface T4 on cells that do or do not undergo HIV-induced syncytium formation. Because our results indicated that T4$^+$ cells differ in their susceptibility to HIV-induced fusion, flow cytometry was used to determine whether surface densities of T4 correlated with the ability of a lymphoid cell to undergo HIV-induced fusion (Table 3). In these experiments, live cells were mixed with saturating levels of OKT4A, a monoclonal antibody to T4 which blocks both HIV infection and HIV-induced cell fusion (3, 14, 17, 23). Saturating levels of fluoresceinated goat anti-mouse immunoglobulin G were then added to the cells, and the cells were fixed and analyzed for the relative amounts of T4 as measured by fluorescence intensity. Rough estimates of the relative sizes of fluorescing cells were determined by forward-angle light scatter.

Flow cytometric analyses of H9 cells revealed two peaks of fluorescence intensity. Cells representing the populations in each peak were aseptically sorted and cultured. The two populations of cells were then reanalyzed for the amount of surface T4 by flow cytometry (both cell populations had retained their characteristic fluorescence intensity) and were tested for susceptibility to HIV-induced fusion in mixing experiments. These cells are presented as peak 1 and peak 2 in Table 3.

The highest surface densities of T4 antigens were observed on mitogen-stimulated PBLs which did not undergo HIV-induced fusion and on H9 cells (peak 2) and C8166 cells which did undergo cell fusion. Intermediate densities were observed on cells that both did (Jurkat) or did not (CEM) undergo HIV-induced fusion. Low densities of T4 antigens also were observed on cells that did (peak 1 H9 cells and Jurkat cells) or did not (U937) undergo cell fusion. Thus, the apparent surface densities of T4 antigens on different lines of lymphoid cells did not correlate with susceptibility to HIV-induced cell fusion.

DISCUSSION

Our results indicate that a major mechanism of HIV-induced cell death does not involve cell fusion. They also demonstrate that HIV-induced fusion of lymphoid cells requires a factor(s) in addition to the surface expression of viral envelope glycoproteins and host T4 receptor antigens.

Cytopathic effects of HIV infections and HIV-induced cell fusion. By far the most dramatic consequence of HIV infection of certain established lines of T4$^+$ cells is the formation of giant syncytia. Although the occurrence of HIV-induced fusions has not always correlated with the onset of cytopathic effects (an in vitro-generated mutant has been reported to induce syncytia in the absence of cytopathic effects [4]), the induction of syncytia has been considered to be a major mechanism of HIV-induced cell killing.

Our studies clearly indicate that HIV-induced cytopathic effects do not necessarily involve syncytium formation and suggest that HIV-induced syncytia are a phenomenon peculiar to certain lines or sublines of T4$^+$ cells. A number of laboratories have reported differences in susceptibility to HIV-induced fusion of T4$^+$ cells (2, 3, 10, 15). However, for the most part, these differences were not correlated with actual numbers of HIV-infected cells or dead cells. In our first experiment with HIV, we were surprised to observe cultures dying in the absence of syncytium formation. We therefore undertook quantitation of infected cells, dead cells, and syncytia with time after infection. The results of these analyses demonstrated that all of the HIV-associated cell death in our cultures of PBLs and CEM cells and much of the cell death in H9 cells occurred independently of syncytium formation. Thus, the major mechanism of HIV-induced cell death in our cultures did not involve syncytium formation.

We do not know the mechanism of HIV-induced cell death in mononuclear cells. Our bias is that such cell death is not caused by a specific viral product but rather by active HIV infections usurping a sufficient portion of the synthetic capacity of cells to tax viability.

Interference inhibits syncytium formation. Interestingly, syncytium formation was an early and transitory phenomenon after infection of H9 cells (Fig. 1; Table 1). We think this

TABLE 3. Relative densities of T4 on cell lines which do or do not undergo HIV-induced syncytium formation[a]

Cell	HIV-induced fusion	% Positive	MCF	FALS	MCF/FALS
PBL	−	31	30	44	0.70
CEM	−	90	15	59	0.25
U937	−	88	12	67	0.17
C8166	+	93	45	81	0.56
H9					
Peak 2	+	47	51	87	0.59
Peak 1	+	52	11	87	0.13
Jurkat	+	97	7	46	0.15
		(18)	(16)		(0.35)

[a] Indirect immunofluorescence and flow cytometry were used to quantitate T4 (see Materials and Methods for details). MCF, mean channel fluorescence ($10^{[\text{measured value}/96]}$); FALS, relative forward-angle light scatter. Parentheses indicate data for cells in a broad shoulder of fluorescence intensity.

was caused by the binding of newly synthesized viral envelope glycoproteins with host T4 receptors (9) interfering with the availability of T4 antigens for syncytium induction. This is in agreement with the finding that infected cells initiate syncytia more efficiently with uninfected than with other infected cells (Table 2). The masking of host receptors by newly synthesized envelope glycoproteins is a well-known phenomenon in retrovirology which is termed interference and has been classically associated with the establishment of superinfection resistance (24). In HIV-infected cells, interference appears to be able to establish resistance to syncytium formation as well as superinfection resistance (Tables 2 and 3).

The fact that interference affects the induction of syncytia by HIV needs to be taken into account when syncytia are used to determine titers of HIV stocks. Indeed, we have found that syncytia give accurate titers only under conditions of low multiplicity of infection. The ability to establish relatively continuous lines of infected mononuclear cells from fusion-susceptible cells is also in accord with the establishment of interference. Presumably the mononuclear, virus-producing cells in such lines are cells which have established interference (resistance to syncytium induction as well as superinfection) and have a viable balance between the metabolic activities of the infecting virus and host cell.

Host factors and susceptibility to HIV-induced fusion. Because syncytium formation has been shown to occur in response to the expression of only one viral product, viral envelope glycoprotein (13, 23), and to require the presence of only one host product, the T4 receptor antigen (14, 22), we tested whether differences in susceptibility of $T4^+$ cells to HIV-induced fusions correlated with differences in the expression of viral envelope or host T4 antigens. The results of these studies clearly indicate that infected cells which did not undergo syncytium formation expressed envelope antigens which could initiate cell fusions (Table 2). They also indicate that the surface density of T4 on cells did not determine whether a cell would be susceptible to HIV-induced fusion (Table 3). Thus, the susceptibility of a $T4^+$ cell to HIV-induced fusion requires a host factor(s) in addition to T4. Interestingly, one circumstance which can influence the susceptibility of PBLs to HIV-induced fusion is immortalization with HTLV-I (R. A. Koup and J. L. Sullivan, personal communication).

Role of HIV-induced cell fusions in the induction of AIDS. The finding that the cytopathic effects of HIV infections for cultures of mitogen-stimulated PBLs do not necessarily involve cell fusion suggests that the marked loss of $T4^+$-helper/inducer cells in AIDS patients also may not involve cell fusion. This suggestion is consistent with histopathologic data of lymph nodes from patients with AIDS and pre-AIDS persistent generalized lymphadenopathy (for a review, see reference 20). These nodes, whether undergoing hypertrophy or atrophy, reveal relatively low numbers of T-helper cells, atypically high numbers of T-suppressor cells, and disordered and "moth-eaten" growths of follicular dendritic cells. Some nodes display occasional syncytia similar to those observed in Epstein-Barr virus, cytomegalovirus, and measles virus infections. Whether the occasional giant cell is induced by HIV, one of the many opportunistic infections of AIDS patients, or by coinfection of a cell with HIV and an opportunistic agent is not known. It seems likely to us, however, that (i) the primary cause of T-cell loss in HIV-infected patients is not the result of HIV-induced cell fusions and (ii) T-cell lines which undergo HIV-induced cell fusion may not be appropriate model systems for the study of the loss of $T4^+$ cells in HIV-induced AIDS.

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