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Cytopathic effect of human immunodeficiency virus in T4 cells is linked to the last stage of virus infection

(human retrovirus/lymphocytes/acquired immunodeficiency syndrome/vaccine)

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ABSTRACT **A principal feature of acquired immunodeficiency syndrome is depletion of T4 lymphocytes, which is partly due to a direct cytopathic effect of the virus. Both syncytial formation (viral-induced cell fusion) and premature cell death have been cited as the major cause for this phenomenon. By kinetic analysis of cell proliferation and cell lysis we show that the cytopathic effect correlates chiefly with virus production from infected cells, including giant syncytial cells. Most T4 cells were, at least transiently, infected by human immunodeficiency virus (human T-lymphotropic virus type III_B strain); however, after phytohemagglutinin activation, only 10–30% of infected cells express virus (and die) at any one time, indicating that virus production, followed by cell killing, is linked to immune activation and cell differentiation. We also show that an interval exists before viral release, in which expression of viral antigens occurs on the cell surface, suggesting that infected cells are immunogenic before viral production. If so, they may induce a cell-mediated immune response that could minimize dissemination of human immunodeficiency virus, a possibility that has influenced our approaches to the development of a vaccine for prevention of acquired immunodeficiency syndrome.**

One major abnormality in acquired immunodeficiency syndrome (AIDS) is T4 lymphocyte depletion (1) and functional T4 cell impairment, the latter evidenced by the reduction of interleukin 2 (IL-2) production by the remaining T4 cells (2). T4 lymphocytes are targets of human immunodeficiency virus (HIV) infection because the viral envelope uses the T4 molecule as receptor (3, 4). Infection ultimately results in a massive degeneration of the cell population, referred to as the cytopathic effect (CPE) (5), which follows immune activation of the cells (6). Other HIV-infected and virus-producing cell types such as macrophages (7), HIV-transfected human T-lymphotropic virus type 1 (HTLV-I)-transformed T8 cells (8), or monkey epithelial cells (9) do not show the CPE. Because these cells contain few or no T4 molecules, T4 protein may not only be a receptor for HIV binding but also may be involved in T4 lymphocyte death when virus is expressed. This CPE may include an interaction of the viral envelope with T4 protein upon viral release because some envelope mutants of one strain of HIV (HTLV-III_B, X10-1), lead to an infectious but noncytopathic virus (10) and because death occurs only after virus release. We report on further studies of the mechanisms involved in the HIV CPE.

MATERIALS AND METHODS

Cells. Peripheral blood lymphocytes (PBL) were collected after Ficoll/Hypaque separation from heparinized blood derived from healthy seronegative donors or from AIDS patients. After phytohemagglutinin (PHA) activation T cells were cultured in RPMI-1640/10% fetal calf serum with IL-2 (Roussel; 200 units per ml of medium) at a cell concentration of 0.6–1.2 $\times$ 10^6 cells per ml under different physical conditions. T4 cell-enriched subpopulations ($T4^+$ cells) were obtained from PBL by removal of adherent cells and by removal of B and T8 cells by complement dependent cytotoxicity in the presence of OKB1 and OKT8 monoclonal antibodies (mAbs). This yielded cell cultures of 80 $\pm$ 10% T4 cells, as shown by indirect immunofluorescence assay (IFA).

Infection of Cells. Filtered supernatant from the HTLV-III_B-infected H9 cell line was added to primary cultured PBL or $T4^+$ cells (1:10). Supernatants yielded about 10^9 viral particles per ml with reverse transcriptase (RT) activity of 5–8 $\times$ 10^5 cpm per ml. In some experiments, cells expressing HIV antigens at the cell membrane were removed as follows. Cultures containing 10–25% of cells expressing HIV antigens (detected by IFA using anti-HIV mAbs) were incubated 2 hr with serum from an AIDS patient rich in antibodies against HIV gp160 (serum F91 provided by M. Sarngadharan, Bionetics Research, Rockville, MD) in the presence of complement. After this procedure the cultures were depleted of all virus-positive cells as verified by IFA assays using anti-HIV mAbs.

Assays. (*i*) RT was measured in culture supernatants as previously described (11), (*ii*) cell lysis was measured by the trypan blue exclusion test, and (*iii*) cell growth was measured by incubating aliquots of 2 $\times$ 10^4 cells for 12 hr in RPMI-1640/10% fetal calf serum containing 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine and measuring [^{3}H]thymidine incorporation into cellular DNA. The latter was expressed as the proliferation index, which is the ratio of cpm of test samples to cpm of standard 12-day-old T4 cell culture in which cells grew in the presence of IL-2. (*iv*) IL-2 production from PBL was assayed *in vitro* as reported (2) and expressed as ratio of test sample to a standard derived from a reference PBL sample. (*v*) Cell surface antigens, T4 and T8 cell antigens, were assayed by IFA using OKT4 and OKT8 mAb followed by rabbit anti-murine immunoglobulin antibodies coupled with fluorescein. (*vi*) HIV antigens—IFA and rosette-forming cells: HIV antigens were detected either by IFA with human sera (F91, see above) followed by goat anti-human immunoglobulin antibodies coupled with fluorescein (11) or by a specific rosette assay. In the latter procedure, HTLV-III_B-infected T4 cells, expressing viral antigens at the cell surface, were incubated with F91 serum at room temperature,

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Abbreviations: CPE, cytopathic effect; HIV, human immunodeficiency virus; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; AIDS, acquired immunodeficiency syndrome; RT, reverse transcriptase; IFA, immunofluorescence assay; HTLV, human T-lymphotropic virus; IL-2, interleukin 2; mAb, monoclonal antibody.

washed, and treated with bovine red cells linked to anti-human immunoglobulin antibodies with parabenzoquinone. Those cells that express viral antigens on their surface form rosettes and were isolated with a micropipette under stereomicroscopy. The cells were then studied by electron microscopy to evaluate for virus.

RESULTS AND DISCUSSION

Most T4 Cells Can Be Infected. Single-labeling experiments for HIV cell-surface and T4 antigens of a PHA-activated $T4^+$ cell-enriched population of infected PBL showed that ≈60–80% of total cells were $T4^+$, and 40–60% of the total were HIV^+ within the first few hours of infection (Fig. 1*A*). Moreover, double labeling confirmed that ≈80% of the T4 cells and <10% of the T4 negative cells were HIV-envelope antigen positive during these first hours. We refer to this as the "afferent" phase, and it measures only those cells to which virus has bound or entered. We conclude that HIV is recognized and binds to most T4 cells rather than being limited to a small fraction of the T4 cell population as has been suggested. The number of T4 antigen-expressing cells remained normal (>50%) until the third day, when they progressively decreased. Virus expression occurred between days 3 and 4 and peaked 6–8 days after infection (Fig. 1*B*). In this "efferent" phase of infection viral antigens were found on only 10–30% of cells, posing the question whether only these cells were truly infected or whether infected cells exhibited viral envelope antigen only at a certain stage of T4 cell differentiation. To address this problem, a series of

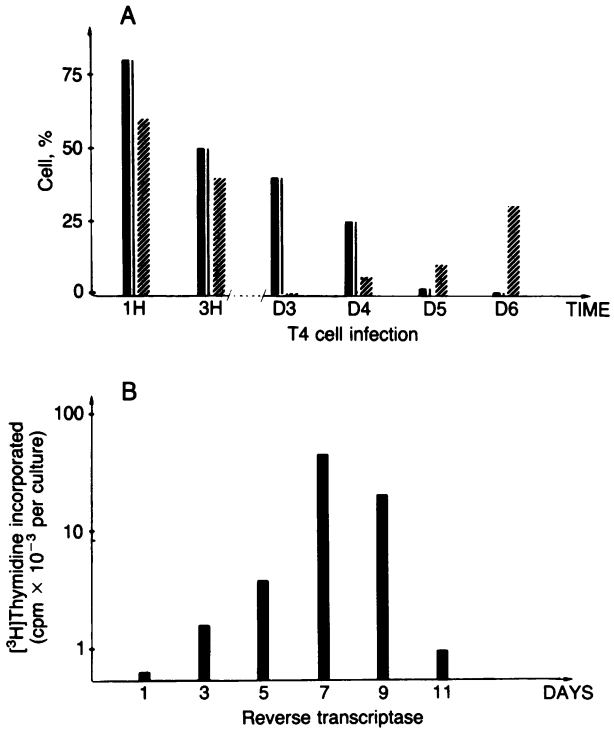

FIG. 1. Correlation of expression of T4 and HIV. (*A*) Expression of T4 and HIV cell-surface antigens. PHA-stimulated cells from a T4-enriched fraction of normal PBLs were infected by $HTLV\text{-}III_B$, and T4 antigens (solid bars) were measured with OKT4 mAb; HIV antigens (hatched bars) were measured with an AIDS serum rich in envelope antigen as described (12). Abscissa, time after infection (H = hr; D = day); ordinate, % positive cells. (*B*) Kinetics of viral production by cultured T4 cells infected with $HTLV\text{-}III_B$ ($>10^8$ particles per ml of culture medium). Abscissa, time after infection; ordinate, RT activity of 0.5-ml supernatant aliquots done as described (12).

experiments was done on 10- to 15-day-old cultured T cells derived from peripheral blood of AIDS patients and from uninfected and *in vitro*-infected normal T4 cells. Although <1% of T cells from PBL of AIDS patients express HIV (13), after 10–15 days of culture this amount increases to 10–20%. *In vitro*-infected T4 cells from normal donors express viral envelope antigen to about the same extent after this period. We removed virus-expressing cells by treating them with complement-dependent cytotoxic antibodies obtained from sera of selected AIDS patients. That these antibodies were cytotoxic was verified by trypan blue staining. Although virus-positive cells were not found by IFA immediately after removal of the virus-expressing cells, after 2–5 days of culture 8–25% of the remaining cells expressed envelope antigens (Table 1). Moreover, when these cells were removed by a second round of complement-dependent cytotoxic antibody treatment, cells again appeared that expressed HIV envelope antigens. That reinfection of a minor population of the cells in culture occurred—thereby generating new virus-expressing cells—was ruled out by the following approaches. (*i*) The cultured cells were washed in each case before removal of the antigen-expressing cells to remove free virions from the supernatant. (*ii*) Immediately after removal of the viral envelope-expressing cells, tests of the remaining cells for virus by IFA were uniformly negative. If virus were absorbed to even a few of the cells undergoing reinfection, these virus would have been readily detectable. (*iii*) The time of appearance (2–3 days) of virus-expressing cells is shorter than in a newly infected culture (5–6 days). (*iv*) Virus was not found in supernatants collected 1–3 hr after removal of virus-expressing cells, assayed by virus transmission on susceptible target T4 cells. (*v*) The 10- to 15-day-old infected cultures were depleted of T4 cells. Thus, the target cells for reinfection after removal of antigen-expressing cells were not present. We conclude that most T4 cells may be infected *in vitro* and these infected T4 cells express virus only at a certain stage of differentiation.

Inverse Correlation Between Expression of T4 Antigen and HIV Envelope. Activated, HIV-infected T4 cells showed a complete loss of $T4^+$ cells by day 6, while the viral antigen-expressing cells increased from 0 to 30% (Fig. 1). A negative correlation between T4 and virus expression is thus seen during the efferent phase of virus infection. From previous (6) and current results, the sequential events occurring after HIV infection appear to include the following: (*i*) an afferent phase, where T cells carrying T4 antigens are infected; an eclipse phase (where cells do not express viral antigens), occurring as long as the cells are not immune activated; and an efferent phase, initiated by immune activation (6) and characterized by viral RNA and protein synthesis and virus release. (*ii*) At the beginning of the efferent phase (just after immune stimulation), T4, but not viral, antigens are detected. Newly synthesized envelope molecules may form complexes with T4 receptors (14), but T4 antigen is present in excess and found on the cell surface without viral expression. Indeed, T4 mRNA continue to be expressed in infected T4 cells, and T4 viral envelope complexes have been identified in the cytoplasm of infected cells (14). (*iii*) Viral maturation occurs at a more advanced stage, perhaps after saturation of free T4 molecules, and is characterized by loss of T4 antigens (Fig. 1*A*), expression of HIV envelope antigens at the cell membrane (Fig. 1*A*), release of virions (Fig. 1*B*), and is followed immediately by cell death (see Fig. 2 and below).

CPE Is Due to Cell Lysis Not Compensated by Proliferation. Activated peripheral blood T4 lymphocytes infected by HIV produce virus and degenerate within 2–3 weeks in culture. To better define this phenomenon we studied the kinetics of cell growth, cell lysis, and virus expression and compared them to uninfected cells. Results (Fig. 3) indicate that the CPE occurs only after virus release. The peak of [^{3}H]thymidine

Table 1. HIV antigenic expression in differentiating infected T cells

Sample origin	Massive infection with HTLV-III$_B$	Specific cell lysis by complement-dependent cytotoxicity, %*	Viral antigenic expression by IFA after cytotoxicity test, % positive cells†			
			0	1 hr	3 day	5 day
N	–	0	0	0	0	0
N-1	+	18	11	0	8	20
N-2‡	+	23	20	<1	12	13
V-1	–	14	16	2	13	18
V-2	+	20	18	0	6	15
H9/HTLV-III§		66	77	0	—	—

*Aliquots from (10- to 15-day-old) cultured T4 cell-enriched fractions of normal PBL (N) or a HIV seropositive (V) individual either massively (10^8 particles per ml of culture) infected by HTLV-III$_B$ (rows 2, 3, and 5) or uninfected (rows 1 and 4) were incubated (1:10) with anti-HIV serum (F91 serum, see *Materials and Methods*) for 30 min at room temperature and then treated with rabbit serum (1:5) at 37°C for 1 hr (as complement source), after which the samples were washed three times. In assays for cell lysis, cells were also treated with complement or human antiserum alone as technical controls, and these cells showed only background lysis (<3%).

†HIV envelope antigens in cell samples were detected by IFA at different times after removal of cells by complement-dependent cytotoxicity. Mean results from duplicate experiments are expressed as % of antigen-positive cells.

‡Cell samples from rows 3 (N-2) and 5 (V-2) originated from cultures in which virus-expressing cells had previously been removed by complement cytotoxicity.

§H9/HTLV-III cultures were positive controls.

incorporation (day 3) occurs while cell lysis is minimal in both uninfected and infected cells (Fig. 3*A*). However, although some reduction in growth compared with 3-day cultures occurs, lysis of uninfected cells (grown with IL-2) remains low, and proliferation is maintained at rates compatible with cell survival for at least 1 mo. By contrast, [^{3}H]thymidine incorporation into DNA of infected cells rapidly declines; lysis is abundant 1 to 2 weeks after the cultures are initiated; lysis is not compensated by cell growth after 10–20 days; and culture degeneration—i.e., the CPE—occurs (Fig. 3*A*).

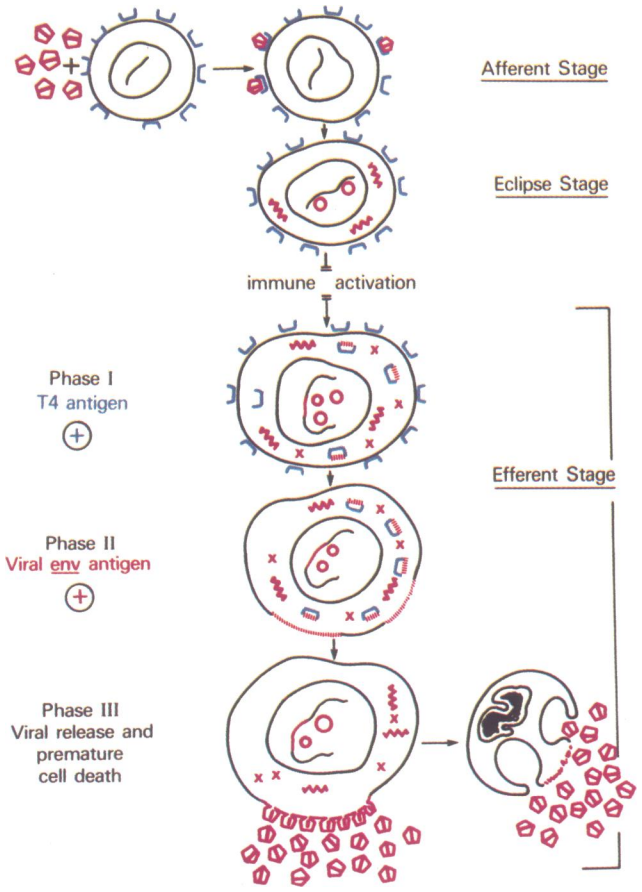

FIG. 2. Diagrammatic illustration of different stages of HIV infection of T4 cells leading to viral production and cell death.

Cell Lysis Is Associated with Viral Release. That lysis correlates with virus release (as monitored by both RT activity of culture supernatants and IFA of viral antigens in infected cells) is also shown in Fig. 1. HIV envelope cell surface antigens were found in up to 30% of cells. Although they parallel RT activity, cells expressing HIV envelope in the absence of virus release were readily detectable, as indicated by finding many rosette-forming cells (see *Materials and Methods*), which could be isolated and examined by electron microscopy (Fig. 4*C*). Note that these cells did not show ultrastructural degeneration at this stage. By contrast, cells producing virus have degenerative ultrastructural changes, including large external vacuoles surrounded by viral particles (Fig. 4*D*) or complete cell lysis as in Fig. 4*E*. Analogous to mechanisms known to lead to target cell lysis by cell-mediated cytoxicity (12), we propose that the premature death of infected T4 cells may be due to molecular perturbations of the cell membrane lipid bilayer generated by viral-envelope fusion proteins. When integrated in the plasma membrane, these proteins upon intense virus budding and release may produce cell membrane microholes, leading to lethal ionic alterations similar to those described for cell-mediated cytotoxicity (15). This could account for the lower rate of lysis seen when infected T4 cells are cultured at a lower temperature (Fig. 3*B*) where cell membrane fluidity is decreased. In fact, cells grown at 35°C instead of 37°C and/or in the absence of air proliferate for over 2 mo (Fig. 4*A*), even though they express viral antigens (Fig. 4*B*). In addition, although the T4 depletion was markedly diminished under these conditions, this was not accompanied by a marked decrease in syncytia, once again indicating that this phenomenon is not a major factor for the CPE.

Death of Syncytial Cells Is Also Associated with Viral Release. Like mononuclear infected cells, syncytial cells, which form by the interaction of CD4 with viral envelope (16), first express viral proteins, then release virus (Fig. 4*G*), which is followed by cell death. Also, like infected mononuclear cells, the syncytia exhibit normal ultrastructural patterns before virus production (Fig. 4*F*). These multinucleated giant cells also show synchronous mitosis (Fig. 4*H*). Moreover, degenerative syncytial cells are far less numerous

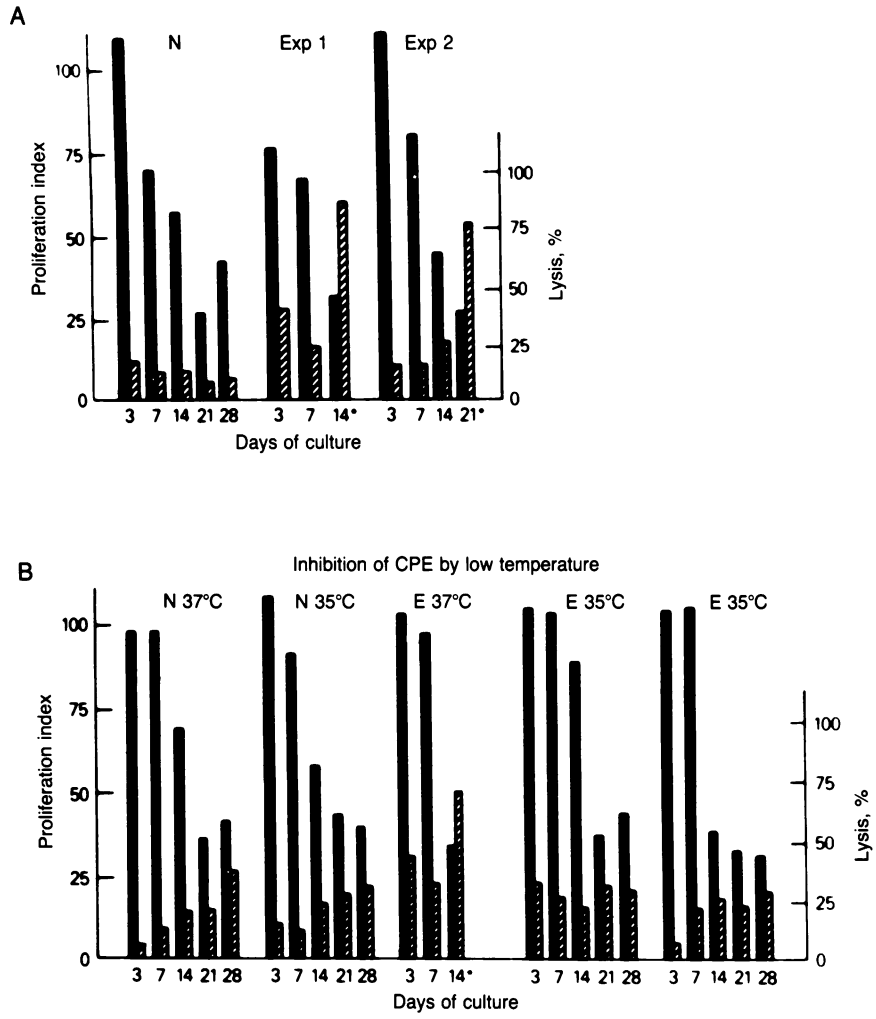

FIG. 3. Kinetics of proliferation, lysis, and expression of virus in HTLV-III_B-infected, T4-enriched, PBL from normal individuals. (*A*) Proliferation of T4 cells under standard conditions (N). Aliquots of $T4^+$ cells (8×10^5 cells per ml) were cultured in RPMI-1640/20% fetal calf serum with 10^4 irradiated macrophages, B cells, and recombinant IL-2 (Roussel Uclab, Paris) in 5% CO_2/95% air at 37°C. Experimental samples (Exp 1 and 2) consisted of HTLV-III_B-infected T4 cells cultured under the same conditions. Cell proliferation and cell lysis were then assayed. *, Culture was abandoned at this stage because >90% of cells were dead, and no incorporation of [^{3}H]thymidine was detected. Abscissa, age of the culture; ordinate, proliferation (solid) and lysis (hatched). (*B*) Proliferation of T4 cells at 35°C and deprived of air/CO_2. Normal (N) and infected (E) cultures were treated and monitored for cell proliferation (solid) and cell lysis (hatched) as in Fig. 1*A*. In these cultures samples were either cultured under standard conditions at 37°C with a 5% CO_2/95% air supply (N and E 37°C) or at 35°C deprived of air/CO_2 supply (N and E 35°C).

(<5%) than degenerative mononuclear cells. Thus, our results do not support previous ideas that syncytial formation is the major cause of the CPE (T4 depletion) induced by HIV (5), even considering the large number of T4 cells that may fuse. In fact, several additional lines of evidence also suggested to us that this explanation was far from sufficient. Giant cell formation due to viral cell fusion factors is found with many other viruses such as Sendai virus, HTLV-I, and HTLV-II, which do not cause a profound depletion of their target cell as HIV does in AIDS. Some cell lines destroyed by HIV infection (such as K-562 and ATH8) do not show significant syncytia (14), whereas other infected cell lines (such as H9/HTLV-III_B) may exhibit giant cell formation without significant CPE (17). Also, T4 cells infected by a deletion mutant of HTLV-III_B, X10-1 (10), still show substantial syncytial formation but little T4 killing. Finally, we find that under low temperature and/or low O_2/CO_2, infected cells form syncytia with little T4 killing. Therefore, we think that syncytial cell formation *per se* has little to do with T4 depletion. Sodroski *et al.* (18) suggested that a cell membrane fusion process similar to the one seen in giant cell formation might also occur in individual cells through endocytosis after reinfection by HIV.

Role of the CPE in the Pathogenesis of AIDS. An infected T4 cell first responds to signals for immune activation no differently than uninfected T4 cells—proliferating and differentiating into cells producing IL-2 and IL-2 receptors (6), but (as shown here) virus expression and virus release also occur and are immediately followed by premature cell death. Consequently, clonal expansion of the infected cells is reduced to only a few daughter cells. While each activated uninfected T cell generates many hundreds of progeny cells (19), thereby expanding the pool of memory cells in the peripheral lymphoid organs, the abortive clonal expansion of infected T4 cells may reduce the number of memory T4 cells. This phenomenon should contribute to the decrease in T4 cells but may not solely explain the depletion of infected memory cells. Other experiments have, indeed, shown that the number of PHA-activated cells is markedly diminished after HIV infection (20). Moreover, IL-2 production by T4 cells from AIDS patients' PBL is also dramatically reduced on a per cell basis (2). Thus, qualitative abnormalities of T4 cells from AIDS patients may also contribute to the T4 depletion and the subsequent immune deficiency. Consistent with these qualitative abnormalities are the indications that HIV may exert indirect effects, such as induction of suppressive factors that inhibit T-cell proliferation (21–23). Also, antibody-dependent cell-mediated cytotoxicity (24) could play a role in T4 depletion seen in AIDS (24).

In conclusion, (*i*) the CPE directly induced by HIV in T-cell cultures occurs when lysis is not overcome by cell proliferation, and it results from premature cell death after viral release of either single (the majority) or multinucleated (the minority) cells expressing HIV. (*ii*) Viral expression and release occurs only when infected cells are immune activated. The mechanism of this phenomenon is not fully understood, but it has been shown (25) that activation of T cells correlates with the production of a transcription factor NF-κB that has binding sites in the HIV-1 enhancer. It was suggested (25) that NF-κB may act in synergy with the HIV-1 *tat* gene product to enhance virus expression in HIV-infected T cells. (*iii*) Viral antigens are found at the cell surface before

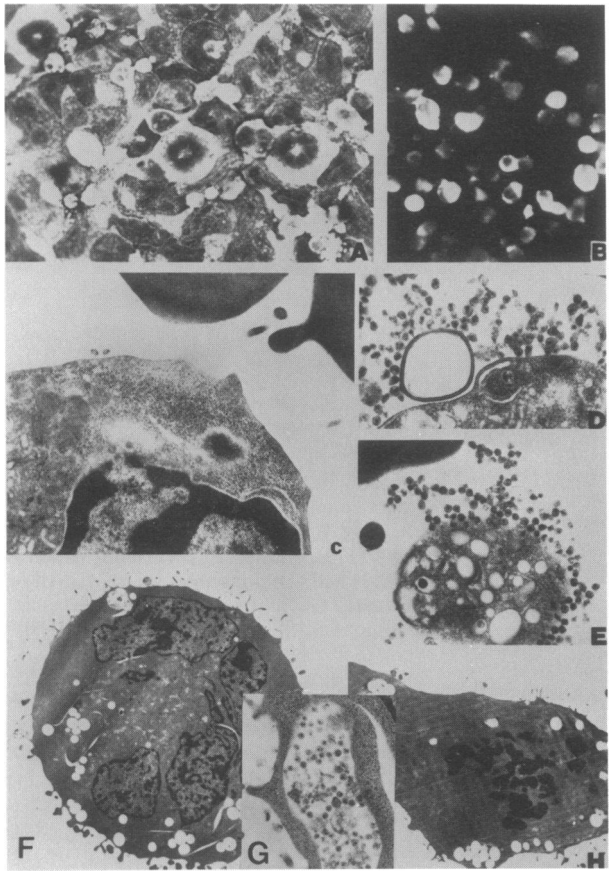

FIG. 4. Light and electron microscopy of HTLV-III$_B$-infected PBL. (*A*) Light microscopy of long-term cultured (60-day-old) HTLV-III$_B$-infected PBL. Cells were grown with IL-2 at 35°C and deprived of air/CO_2, smeared, and treated with Wright's stain. Cell proliferation is indicated by mitotic figures. (*B*) IFA for HIV antigens. HIV antigens were detected by IFA in 30-day-old primary cultured PBL (AIDS patient) grown with IL-2 at 35°C. The culture was reactivated on day 20 by treatment with PHA for 24 hr at 37°C in the presence of macrophages and B cells. Cells were mounted on slides, dried, acetone fixed, incubated with AIDS serum (F91), washed, and treated with goat anti-human immunoglobulin coupled with fluorescein. (*C–E*) Electron micrograph of HTLV-III$_B$-infected T4 cells expressing viral antigens. Cells that form rosettes after incubation with serum containing high levels of antibody to gp160 envelope glycoprotein were treated with bovine red cells coated with anti-human immunoglobulin antibody, isolated, and studied by electron microscopy as indicated. (*C*) Cell expressing viral antigens (forming rosette) without evident free extracellular viral particles; this cell has a well-preserved ultrastructure. Note along the outerpart of the cell membrane sections of microdigitations (diameter, 30 nm) without evident viral core formation, corresponding to the lymphocyte–red cell junction. (*D* and *E*) Cells actively producing virus. Note degenerative ultrastructure that follows virus release. (*C*) ×22,800; (*D*) ×12,000; and (*E*) ×10,200. (*F–H*) Electron micrograph of multinucleated giant cells isolated by micromanipulation under a stereomicroscope. (*F*) A multinucleated cell which shows no ultrastructural abnormalities. (*G*) Viral particles inducing cell fusion are shown in a syncytial cell. (*H*) Multiple nuclei undergo a synchronous mitotic process (nuclear membrane has disappeared, and chromosomal structures from different nuclei are spread within the cytoplasm). Typical representations were selected from the many such multinucleated cells that were isolated by micromanipulation and analyzed. (*F*) ×3000; (*G*) ×7800; and (*H*) ×3000.

viral release, a result important to our vaccine development strategies because it shows that infected cells may be immunogenic before viral release. These cells may trigger a cellular immune response by viral-modified self cells—i.e., generation of specific cytotoxic T lymphocytes directed against HIV-infected autologous cells. (*iv*) Finally, the CPE and consequent clonal abortion of infected T-cell progenies combined with indirect mechanisms, such as qualitatively altered T cells, suppressive factors, and antibody-dependent cell-mediated cytotoxicity may be sufficient to account for the T4 depletion leading to AIDS.

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