INFECTION OF MONOCYTES BY HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 BLOCKED BY INHIBITORS OF CD4-gp120 BINDING, EVEN IN THE PRESENCE OF ENHANCING ANTIBODIES

By CARLO-FEDERICO PERNO,* MICHAEL W. BASELER,‡ SAMUEL BRODER,* AND ROBERT YARCHOAN*

*From the *Clinical Oncology Program, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and ‡Program Resources Inc., National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland 21701*

It is well established that CD4 acts as the principal receptor for the binding of HIV to T cells (1–4). However, as the number of identified cellular targets for HIV infection has expanded, it has been hypothesized that other receptors for HIV may exist (5–7). There is recent evidence, for example, that some muscle or glioma cell lines that lack detectable surface CD4 may be infected by HIV (5). Moreover, it has been observed that infection of human lymphocytes or the monocytoid cell line U937 by HIV can be enhanced by low titers of anti-HIV antibodies, and it has been hypothesized that Fc receptors or complement receptors may act as an entry site for HIV under these conditions (6–8).

Human peripheral blood monocyte/macrophages (M/M)[1] can be easily infected by certain strains of HIV, and there is increasing evidence that infection of such cells plays a crucial role in the pathogenesis and progression of AIDS and related disorders (9, 10). M/M are phagocytic cells, and it seemed possible that this could provide a route for viral entry. In addition, M/M bear Fc and complement receptors and, in the case of certain other viruses (e.g., flaviviruses), are prime targets for enhancement of infection by antiviral antibodies (11–13). It therefore seemed conceivable that a CD4-independent pathway for infection may exist for M/M, either in the presence or absence of low titers of anti-HIV antibodies. This question is potentially important from a therapeutic viewpoint as strategies to inhibit HIV-CD4 binding are actively being investigated as therapies for HIV infection (14–19). The present set of experiments were designed to study whether infection of M/M could be en-

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Address correspondence to Robert Yarchoan, Clinical Oncology Program, National Cancer Institute, Building 10, Room 13N248, National Institutes of Health, Bethesda, MD 20892. Carlo-Federico Preno's present address is the Dipartmento di Medicina Sperimentale e Scienze Biochimiche, II Univerita' di Roma, Italy.

[1] *Abbreviations used in this paper:* CML, chronic myelogenous leukemia; GM-CSF, granulocyte-macrophage colony-stimulating factor; MID, minimum infectious dose; MM, monocyte/macrophage; sCD4, soluble CD4.

hanced by low titers of anti-HIV antibodies, and in addition, whether a second entry mechanism for HIV infection of M/M might be identified, particularly in the presence of such antibodies.

Materials and Methods

Cells. Monocyte-enriched PBMC were obtained from healthy, HIV-seronegative donors using a cell separator (Fenwall C3000; Travenol Inc., Deerfield, IL). Fresh M/M were subsequently purified from this population by elutriation as described by Gerrard et al. (20). In certain experiments, the fresh elutriated M/M were cultivated for 5 d in the presence or absence of 100 chronic myelogenous leukemia (CML) U/ml of human recombinant granulocyte-macrophage CSF (GM-CSF) (Sandoz Research Institute, East Hanover, NJ) as described (21). Also, in certain experiments, mature adherent M/M populations (called 5-d adherent M/M) were obtained by incubating 10^6 PBMC/well in 48-well plates (Costar, Cambridge, MA) for 5 d, followed by extensive washing to remove nonadherent cells (22). Using this method, the yield after removal of the nonadherent cells was $\sim 10^5$ M/M per well. The viability of cells obtained with each of these methods was consistently >95%. M/M obtained with each of these methods were <1% E-rosette positive and >95% nonspecific esterase positive (Technicon Instruments, Tarrytown, NY), and >95% morphologically resembled monocytes when examined after Giemsa staining. Other characteristics of these cells have been previously described (21, 22). In certain experiments, U937, a $CD4^+$ monocytoid cell line, or ATH8, an HTLV-1-infected human T cell line (21, 22) (gift of Dr. H. Mitsuya, National Cancer Institute, Bethesda, MD), were used.

The expression of surface antigens on the cells was assessed by flow cytometry using the following fluorescein-conjugated mAbs: CD4, Leu-3a (Becton Dickinson & Co., Mountain View, CA); Cd11b, OKM1 (Ortho Diagnostic Systems Inc., Westwood, MA); CD36, OKM5 (Ortho Diagnostic Systems Inc.); HLA-DR, OKIa (Ortho Diagnostic Systems Inc.). Paired isotype-specific control antibodies were run with each sample. Dead cells were excluded from analysis using propidium iodide, and the percentage of antigen-positive cells was calculated by straight channel integration, with the integration channel set so that <1% of the isotype control cells appeared positive. The density of CD4 on the surface of the cells was compared by staining cells with fluorescein-labeled Leu-3a mAb (Becton Dickinson & Co.), followed by analysis on a profile flow cytometer (Coulter Electronics Inc., Hialeah, FL). Fluorescence was then compared with appropriate fluorescein-labeled isotype controls (IgG1-FITC; Becton Dickinson & Co.) using logarithmic-to-linear conversion tables supplied by Coulter Electronics Inc. The mean channel of positive fluorescence was then calculated by the following formula: mean channel antibody peak minus mean channel isotype control peak.

Virus. A monocytotropic strain of HIV-1, $HTLV\text{-}III_{Ba\text{-}L}$ (gift of Drs. S. Gartner, R. C. Gallo, and M. Popovic, National Cancer Institute) (9), and a lymphocytotropic strain of HIV-1, $HTLV\text{-}III_B$ (Electro-Nucleonics Laboratory, Inc., Silver Spring, MD), were used as previously described (22). These will be referred to as $HIV\text{-}1_{Ba\text{-}L}$ and HTLV-IIIB, respectively. Supernatants from infected cultures of fresh M/M were used as the source of $HIV_{Ba\text{-}L}$; these were filtered and stored in liquid nitrogen before use. Titration to determine infectivity was performed in a primary M/M system; the minimum infectious dose (MID) was defined as the minimum amount of virus that induced viral replication in at least two of four M/M cultures. Pelleted supernates of infected H9 cells that were concentrated by centrifugation were used as the source of HTLV-IIIB. The MID of this strain was assessed in the AHT8 cell line (23, 24).

Anti-HIV Antibodies. Preparations of human antibodies to HIV (called HIV-Ab1 and HIV-Ab2) were the gifts of Dr. Larry Cummins, Abbott Laboratories, Irving, TX. These antibodies were prepared from the plasma of asymptomatic, HIV-infected individuals who lacked detectable serum HIV p24 antigen. HIV-Ab1 was prepared from donors who had high titers of antibody to p24 and gp120 but low titers of antibody to gp41. HIV-Ab2 was prepared from a donor who had high titers of antibody to gp120 and gp41, but no detectable anti-p24 antibody. Plasma from these donors was inactivated by treatment with 1% tri-*N*-butyl-phosphate and 1% Tween 80 for 4 h at 30°C, and fractionated by the Cohn-Oncley cold ethanol proce-

dure (25, 26). Additional purification was obtained using QAE Sephadex gel (27). The final preparation contained >95% monomeric IgG as determined by HPLC. These preparations were then diluted at 50 mg/ml in saline, sterile filtered, and stored at 4°C until used. A control preparation of human IgG, obtained from HIV-seronegative donors, was purified in the same manner.

Preliminary experiments showed that HIV-Ab1, but not HIV-Ab2, interfered with the RIA used to measure HIV p24 antigen (Dupont Co., Wilmington, DE). However, even at the highest concentration used (5,000 μg/ml), the washing procedure performed 48 h after viral challenge (see below) reduced the concentration of antibody by $\sim 10^{-8}$, so that it no longer had any detectable effect on the assay. Neither antibody preparation interfered with the ELISA used to measure HIV p24 antigen (Dupont Co.), even at the highest concentrations used.

Anti-HIV Agents and Controls. Recombinant soluble CD4 (sCD4) containing the four extracellular domains was provided by Dr. Dan Capon of Genentech, Inc. (So. San Francisco, CA) (14). OKT4A, an IgG2a murine mAb against CD4 (Ortho Diagnostic Systems, Inc.), OKT4C (Ortho Diagnostic Systems Inc.), another IgG2a anti-CD4 murine mAb that binds to a different determinant, and D3-2H2-9-21 antidengue antibody (American Type Culture Collection, Rockville, MD), an IgG2a murine mAb used as an isotype control, were diluted in PBS, extensively dialyzed against PBS, and passed through a 0.4-μm filter before use. The final protein concentration in these preparations was evaluated by spectrophotometric measurement. All reagents were stored at 4°C until used.

Infection and Culture of M/M Populations. 10^5 M/M prepared by the various techniques described above (fresh elutriated cells, elutriated cells cultured 5 d with or without GM-CSF, or 5-d adherent M/M) were suspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% heat-inactivated low-endotoxin FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco Laboratories) (complete medium). The cells were exposed to various concentrations of sCD4, OKT4A, OKT4C, or D3-2H2-9-21 for 30 min to 2 h at 37°C, challenged with 100–500 MID/well of HIV-1_{Ba-L}, and incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. In some experiments, the sCD4 or antibodies were added to the cultures 1 or 6 d after the viral challenge. 2 d after viral exposure, the M/M were extensively washed to remove excess virus and then cultured under the same conditions and drug concentrations as before. The supernatant was partially replaced with fresh complete medium every 5 d. Viral production into the supernatant was evaluated every 4–5 d starting from day 6 by RIA or ELISA for HIV p24 antigen (Dupont Co.). In some experiments, inhibition of syncytia formation by these agents was evaluated by visual inspection; this was only done in cultures of mature or GM-CSF-exposed M/M, as fresh M/M usually do not form syncytia in our hands.

Evaluation of the Effects of Anti-HIV Antibody. To evaluate the enhancement of HIV infection by low concentrations of anti-HIV antibody, 3 MID/well of HIV-1_{Ba-L} was mixed with serial 10-fold dilutions of HIV-Ab1 or HIV-Ab2 in 200 μl of complete medium and incubated at 4°C for 2 h. The virus-antibody mixtures were then added to fresh or 5-d adherent M/M that were previously incubated for 30 min at 4°C with sCD4, OKT4A, D3-2H2-9-21, or control medium in 48-well plates. The cells were cultured for 2 d at 37°C in a humidified atmosphere supplemented with 5% CO_2 as described above. The M/M were then washed extensively to remove excess virus, sCD4, or antibodies, resuspended in 1 ml of complete medium (without either anti-HIV antibody or other agents), and continued in culture. They were fed every 5 d, as above, and viral production into the supernatants was periodically assessed as described above.

Toxicity Assessments. The toxic effects of antibodies or sCD4 were assessed by the trypan blue dye exclusion method, by evaluation of phagocytosis of 0.8-μm latex beads, and by measure of [^{3}H]thymidine incorporation in the U937 cell line. For the latter, 2×10^4 U937 cells/well were suspended in 0.2 ml of complete medium in wells of a 96-well plate (Costar) and then cultured for 5 d in the presence or absence of different concentrations of sCD4, OKT4A, D3-2H2-9-21 antibody, HIV-Ab1, or HIV-Ab2. Cells were then pulsed for 6 h with 1 μCu of [^{3}H]thymidine, harvested, and counted in a liquid scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, CA).

Statistics. The statistical significance of the effects of OKT4A and sCD4 was assessed with the two-sided Wilcoxon signed rank test for paired values.

Results

The expression of surface antigens on the various M/M populations is shown in Table I. Most of the M/M expressed CD11b and HLA-DR. Fresh elutriated M/M also expressed CD36; however, consistent with previous results (22), we found that expression of this antigen declined somewhat in mature, cultured M/M. The expression of CD4 was quite variable in the M/M populations, with the mean expression ranging between 39% in M/M cultured with GM-CSF and 56% on elutriated M/M cultured without GM-CSF. In each instance, however, the percentage of M/M expressing CD4 was less than either Th cell clones (such as ATH8) or the U937 monocytoid cell line (Table I, and results not shown). In addition, those M/M that expressed CD4 generally had a low density of antigen as compared with ATH8 T cells (fivefold less) or U937 cells (twofold less) (Fig. 1). These results indicated that while some M/M expressed CD4, overall, the expression of this antigen was less in this population than in prototype $CD4^+$ T cells.

With this background, we asked whether OKT4A or sCD4, two agents that block the binding of HIV *env* gp120 to CD4, inhibited HIV-1 infection of the various M/M populations. As seen in Fig. 2 *A*, fresh M/M exposed to 100–500 MID/well of HIV_{Ba-L} produced substantial amounts of HIV p24 antigen starting after 2 wk and continuing at least up to day 35. However, HIV replication was inhibited throughout this period of observation by the addition of 1 μg/ml of OKT4A or 5 μg/ml of sCD4 (Fig. 2 *A*); these concentrations are equal to or less than the concentrations of these agents required to inhibit T cells. Similar results were observed with 5-d adherent M/M and with elutriated M/M precultured for 5 d with or without GM-CSF (Fig. 2 *B*, and results not shown). It is worth stressing that while GM-CSF-stimulated M/M had a relatively low expression of surface CD4, and in the absence of inhibi-

TABLE I

Expression of Surface Antigens on Various Monocyte Populations Used in these Studies

Monocyte population	Percent positive cells using: CD4	CD11b	CD36	HLA-DR
Fresh elutriated M/M	13–80 (45 ± 28)	95–99 (97 ± 1)	93–98 (96 ± 3)	90–97 (94 ± 5)
Elutriated M/M cultured 5 d	27–83 (56 ± 19)	74–98 (84 ± 11)	39–99 (64 ± 25)	87–99 (91.5 ± 1)
Elutriated M/M cultured 5 d with GM-CSF	11–62 (39 ± 26)	87–96 (92 ± 5)	3–67 (39 ± 32)	68–93 (81 ± 18)
5-d adherent M/M	33–49 (40 ± 8)	68–92 (77 ± 13)	12–51 (29 ± 20)	77–96 (87 ± 13)
U937	97–99 (98 ± 1)	72–75 (74 ± 3)	74–75 (74.5 ± 1)	1–5 (3 ± 2.8)

Expression of various surface antigens of the various M/M populations and of the U939 monocytoid cell line, as assessed by flow cytometry using the following fluorescein-conjugated mAbs: CD4, Leu-3a; CD11b, OKM1; CD36, OKM5; HLA-DR, OKla. Data reported represent the range and arithmetic mean (± SD) of cells prepared from four to eight different donors.

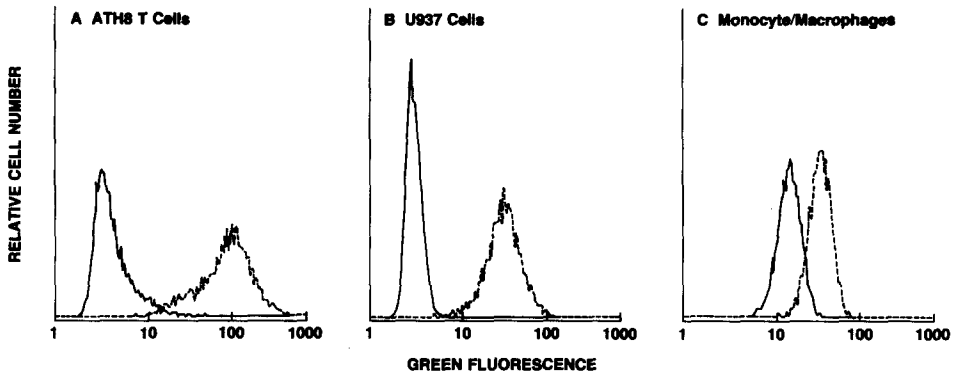

FIGURE 1. Density of CD4 on the cell surface of ATH8 T cells, and fresh M/M. Shown here is a typical experiment in which the above cells were stained with fluorescein isothiocyanate-labeled Leu-3a. The percentages of cells expressing CD4 in this experiment were: ATH8 T cells, 96%; U937 cells, 99%; fresh M/M, 70%. The mean positive channels (log scale) for ATH8 T cells, U937 cells, and fresh M/M were 103.3, 40.1, and 20.6, respectively.

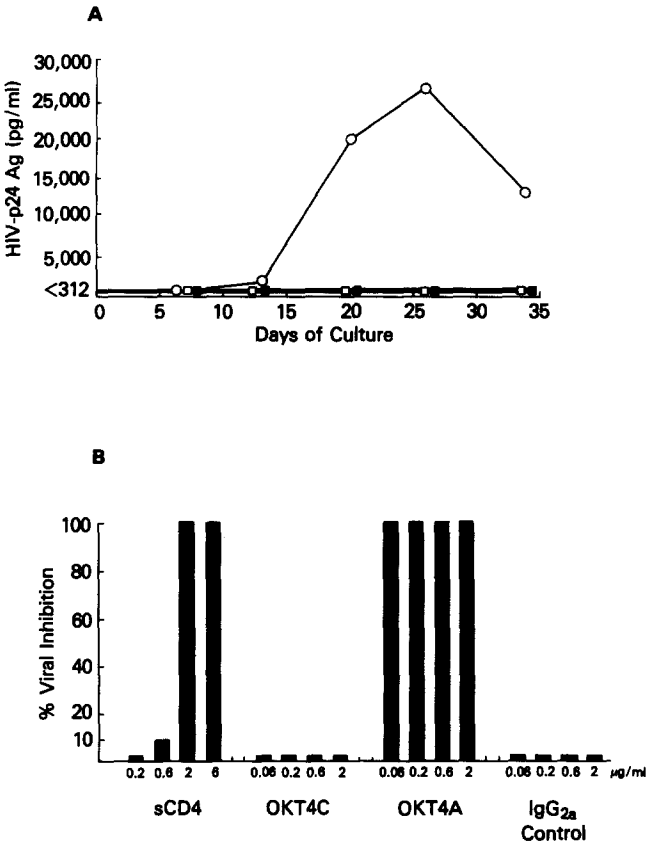

FIGURE 2. Inhibition of HIV_{Ba-L} replication in M/M by agents that block *env* gp120-CD4 binding. M/M were pre-exposed to sCD4, OKT4A, or D3-2H2-9-21 (IgG2a isotype control antibody) and then challenged with 300 MID/well of HIV_{Ba-L}. (*A*) Kinetics of HIV replication in fresh elutriated M/M. Results shown here represent the average of two different experiments. (○) M/M without inhibiting agents; (□) M/M with 1 μg/ml OKT4A; (■) M/M with 5 μg/ml sCD4. In six additional experiments not shown here, similar suppression of viral replication by comparable concentrations of sCD4 or OKT4A was obtained using M/M cultured for 5 d with or without GM-CSF and with 5-d adherent M/M. (*B*) Inhibition of viral replication at day 17 in 5-d adherent M/M by OKT4A or sCD4. OKT4C (a murine mAb that binds to a CD4 epitope different than OKT4A) and D3-2H2-9-21 (a control IgG2a murine mAb) were ineffective in inhibiting HIV replication. Antigen production in the absence of antibodies was 9,563 pg/ml HIV p24.

tors produced substantially more HIV p24 antigen than the other populations (21), the level of inhibition on this population induced by OKT4A or sCD4 was comparable with that of the other M/M populations.

In contrast to the above results, HIV infection of the M/M was not inhibited by comparable concentrations of OKT4C, a murine mAb that binds to a different domain on CD4 than does OKT4A, and that does not either inhibit gp120-CD binding or block HIV infection of T cells (Fig. 2 *B*). Also, the IgG2a control mAb, D3-2H2-9-21, had no inhibitory activity. Thus, agents that inhibit CD4-gp120 binding, but not control antibodies, consistently blocked productive HIV infection in the various M/M populations.

We next asked whether these agents might be effective if added to the cultures after the time of initial viral challenge. As seen in Table II, partial inhibition was still observed when OKT4A or sCD4 were added 24 h after the exposure of fresh M/M to $HIV_{Ba\text{-}L}$. This may be the result of these agents blocking secondary spread of HIV. Alternatively, the partial activity of OKT4A added at 24 h may have resulted from an effect on a post-binding step of viral replication (e.g., fusion or entry). In contrast, if either OKT4A or sCD4 was added at day 6 and the supernatants harvested at day 10, no inhibitory effect was observed (Table II). As expected, no inhibitory effect was observed if the IgG2a control antibody D3-2H2-9-21 was added either at 24 h or at day 6 (Table II). Taken together, these results suggest that in the M/M populations studied, none of the substances tested act at late stages of HIV replication (e.g., viral budding). In addition, they provide evidence that neither substance was toxic for M/M per se.

More direct evidence of a lack of toxicity of these substances was provided by the

TABLE II

Effect of the Time of Addition of sCD4 and OKT4A on Inhibition of Infection of M/M Populations by $HIV_{Ba\text{-}L}$

Inhibitor	Day added	HIV-p24 Ag at Day 10
		pg/ml
None		3,676
sCD4 (20 μg/ml)	0	<312
	1	320
	6	3,570
OKT4A (2 μg/ml)	0	<312
	1	1,395
	6	4,705
D3-2H2-9-21 (2 μg/ml)	0	4,320
	1	5,350
	6	4,360

300 MID/well of $HIV_{Ba\text{-}L}$ was added to fresh adherent M/M at day 0. sCD4, OKT4A, or antidengue control IgG2a murine mAb (D3-2H2-9-21) were added either .5 h before infection (day 0), at 24 h (day 1), or at day 6, as noted. The cultures were washed and fed on days 1 and 6, and the sCD4 or antibodies were replenished (if previously added) at that time. On day 10, the supernatants were harvested and assayed for HIV-p24 antigen by RIA. Neither sCD4 nor the antibodies at the concentrations used interfered with the RIA for HIV-p24 Ag.

fact that neither caused cell death, as evidenced by trypan blue exclusion, and that neither affected the phagocytosis of latex beads up to the highest concentrations tested (10 μg/ml OKT4A or D3-2H2-9-21 and 50 μg/ml sCD4) (results not shown). To further assess the possible toxicity of these agents, we examined their effects on the proliferation of the U937 monocytoid cell line and found no toxic effects at the concentrations tested (up to 50 μg/ml sCD4, 10 μg/ml OKT4A, 5,000 μg/ml HIV-Ab1, or 500 μg/ml HIV-Ab1 plus 10 μg/ml OKT4A; results not shown).

We next asked whether infection of the M/M populations could be enhanced by anti-HIV antibodies and, if so, whether this might occur by a CD4-independent pathway. To assess this, we used purified IgG anti-HIV preparations as described above. In preliminary experiments, we found that HIV-Ab1 had neutralizing activity against HIV infection of ATH8 cells even at a high multiplicity of infection of virus (1,000 MID/well): 50% inhibition of the cytopathic effect of HTLV-IIIB was achieved with 500 μg/ml HIV-Ab1, while >95% inhibition was achieved with 5,000 μg/ml HIV-Ab1. This preparation did not cause appreciable toxicity at those concentrations. In contrast, HIV-Ab2 had no discernable anti-HIV activity at equivalent concentrations in this T cell system. Also, a control preparation of IgG prepared from HIV-seronegative donors had no activity at up to 5,000 μg/ml.

HIV-Ab1, likewise, had substantial anti-HIV activity in M/M exposed to HIV_{Ba-L} (3 MID/well) (Figs. 3 and 4). Interestingly, HIV-Ab2 also had activity against HIV_{Ba-L} in M/M, in spite of its not being active against HTLV-IIIB in ATH8 cells

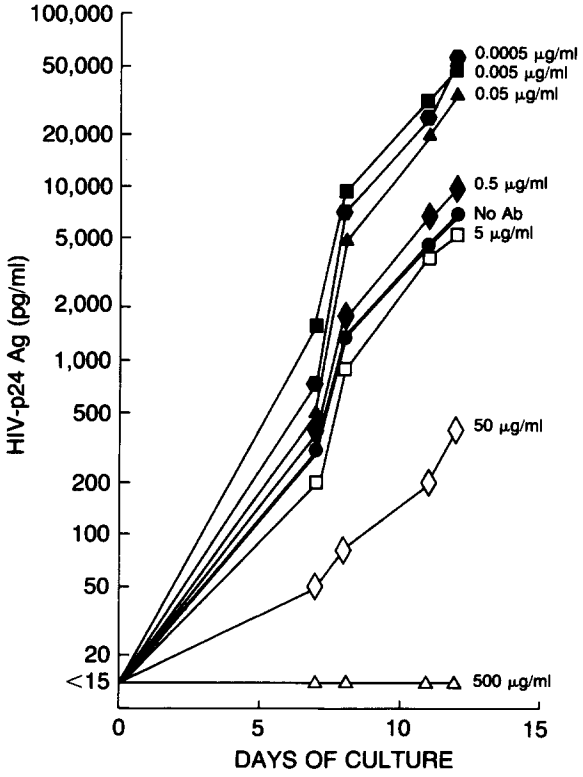

FIGURE 3. Enhancement of HIV replication in mature (5-d adherent) M/M by HIV-Ab1 and HIV-Ab2. 3 MID/well of HIV_{Ba-L} was mixed with serial 10-fold dilutions of HIV-Ab1 or HIV-Ab2, incubated at 4°C for 2 h, and then added to the M/M. After a 2-d incubation, the M/M were washed extensively in order to remove free virus and antibodies, and subsequently cultured in 1 ml of complete medium without antibodies. Open symbols denote neutralizing concentrations of HIV-Ab1, while closed symbols denote concentrations with either no effect or enhancing activity. Represented here is a typical experiment; three other experiments using fresh or 5-d adherent M/M yielded similar results, while in one experiment no enhancement was seen. In no case was enhancement observed with comparable concentrations of a normal control IgG antibody purified in the same way.

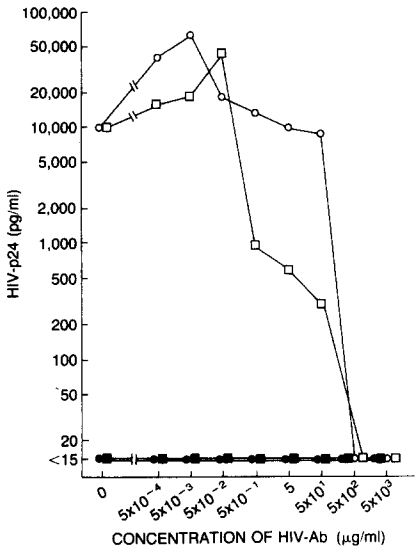

FIGURE 4. Enhancement of HIV replication in mature (5-d adherent) M/M by different concentrations of HIV-Ab1 and HIV-Ab2, and its inhibition by OKT4A. M/M were preincubated with OKT4A before challenge with a mixture of HIV and anti-HIV antibody. After a 2-d culture, M/M were extensively washed and were cultivated in 1 ml of complete medium; neither HIV-antibodies nor OKT4A were added after washing. Closed symbols represent M/M exposed to HIV in the presence of 1 μg/ml of OKT4A. Open symbols represent M/M exposed to HIV in the absence of OKT4A. (○, ●) HIV-Ab1; (□, ■) HIV-Ab2. Represented here is a typical experiment of six using either fresh or 5-d adherent M/M. Inhibition of syncytia formation by OKT4A was also observed in these cultures.

(Fig. 4). This differential activity may have been due in part to the lower multiplicity of infection used in the M/M cultures. However, 50 μg/ml of either HIV-Ab1 or HIV-Ab2 inhibited infection of both fresh and mature M/M exposed to 300 MID/well of HIV_{Ba-L} (data not shown), suggesting that these antibodies were indeed more potent inhibitors of HIV in the M/M than in T cells. It is possible that differences between the virus preparations used, or alternatively intrinsic differences between the target cells, may have contributed to these effects.

It has previously been reported that low levels of anti-HIV antibodies can paradoxically enhance HIV infection of lymphocytes or the U937 monocytoid cell line (6, 8, 28). We next sought to determine whether HIV-Ab1 or HIV-Ab2 had enhancing activity and, if so, whether this was inhibited by OKT4A or sCD4. In cultures of M/M using a high multiplicity of infection (300 MID/well of HIV_{Ba-L}), we found no or inconsistent evidence of enhancement (data not shown). However, when we used a lower multiplicity of infection (3–10 MID/well), an increase in HIV infection (enhancement) was observed in mature (5-d adherent) M/M with extremely low concentrations of HIV-Ab1 or HIV-Ab2 (5×10^{-2} to 5×10^{-4} μg/ml) in four of five experiments (Fig. 3). The degree of enhancement averaged four- to fivefold. Also, in five additional experiments using 3 MID/well of HIV_{Ba-L}, productive infection of fresh or 5-d adherent M/M was attained only in the presence of 5×10^{-4} to 5×10^{-6} μg/ml of HIV-Ab1 or HIV-Ab2 (Fig. 5). In no case was enhancement observed using a control IgG preparation. The addition of complement had no consistent effect on enhancement in this system. In this regard, it should be remembered that these antibody preparations contained >95% monomeric IgG, and these results do not exclude the possibility that complement may affect the results seen with other antibody preparations. Enhancement of infection in M/M was not restricted to HIV_{Ba-L}; in the one experiment where this was examined, 100 MID/well of HTLV-IIIB (a lymphocytotropic strain) failed to infect fresh M/M in the absence of antibody, while productive infection was observed in the presence of 5×10^{-3}

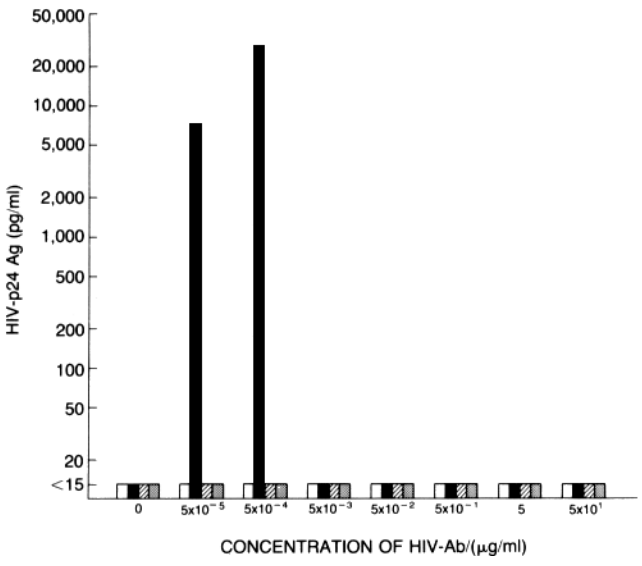

FIGURE 5. Enhancement of HIV replication in fresh M/M exposed to 3 MID of HIV-1 with or without HIV-Ab1 in the presence or absence of OKT4A or sCD4. OKT4A or sCD4 were added to the cultures before viral exposure and not replaced after extensive washing at day 2. (*Open bars*) M/M + HIV; (*filled bars*) M/M + HIV + HIV-Ab1; (*hatched bars*) M/M + HIV + HIV-Ab1 + OKT4A (1 μg/ml); (*stippled bars*) M/M + HIV + HIV-Ab1 + sCD4 (5 μg/ml). Shown here is one representative experiment of three in which we detected consistent infection only when M/M were exposed to HIV in the presence of HIV-Ab1. No detectable infection of M/M was obtained in two additional experiments (total five) performed using a low MID of HIV_{Ba-L}.

μg/ml of HIV-Ab1 (data not shown). Overall, with HIV_{Ba-L}, there was variation in the optimal concentration of antibodies yielding enhancement in M/M populations from various donors (range from 5×10^{-2} to 5×10^{-6} μg/ml). In addition, there was variation in the degree of enhancement. However, although the effect was sometimes moderate and was observed only with a low viral inoculum and very low concentrations of antibody, this was a reproducible phenomenon under these experimental conditions.

It has been hypothesized that antibody-mediated enhancement of HIV infection might involve viral binding and entry via a CD4-independent mechanism (6–8, 28). To explore this possibility, we studied the effect of OKT4A and sCD4 on M/M infection that was enhanced by anti-HIV antibodies. The results obtained in representative experiments are shown in Figs. 4 and 5. Infection of either fresh or mature M/M under conditions of enhancement (and with a low MID/well of HIV_{Ba-L}) was consistently inhibited by >95% by 1–5 μg/ml of OKT4A or by 5 μg/ml of sCD4. Inhibition was observed even when these agents were removed from the cultures 2 d after viral exposure. Inhibition was seen both in experiments where there was infection of the M/M in the absence of anti-HIV antibody (Fig. 4) and in experiments where infection was only observed in the presence of anti-HIV antibody (Fig. 5). Overall, inhibition by OKT4A and by sCD4 was seen in each of six experiments where this was examined ($p < 0.05$).

We also examined the activity of OKT4A in U937 cells exposed to HTLV-IIIB in the presence of low concentrations of anti-HIV antibodies. We found that viral production was increased in the presence of 5×10^{-1} to 5×10^{-4} μg/ml of HIV-Ab1 or HIV-Ab2. At the same time, complete inhibition of infection of these monocytoid cells was observed with 5 μg/ml of OKT4A, even under conditions of enhancement (data not shown). It is worth stressing that our system used a measurement

of HIV p24 antigen produced directly by the M/M or the U937 cells and did not involve a second "indicator cell" population. Also, M/M were subjected to extensive washing to remove excess virus after viral challenge, as previously described (22), and additional controls were established in each experiment to verify this point. Finally, neither the anti-HIV antibodies, nor the OKT4A or sCD4 at the concentrations in the supernatants, had any appreciable effect on the assays used to measure HIV p24 antigen.

One concern in these experiments was that by binding to HIV, sCD4 might somehow interfere with subsequent viral fusion or entry, and that inhibition might occur even if HIV entered by a CD4-independent pathway. However, this concern would not apply for OKT4A. On the other hand, the effect of OKT4A could conceivably have resulted from competitive inhibition of OKT4A with the anti-HIV antibodies for binding to the Fc receptor on the M/M. However, 5 μg/ml of D3-2H2-9-21 (an irrelevant murine mAb of the same isotype as OKT4A) failed to inhibit HIV infection of 5-d adherent M/M that was enhanced by low concentrations of HIV-Ab1 (data not shown). Thus, the overall evidence suggests that binding of gp120 to CD4 is an essential step in the infection of these M/M populations by HIV_{Ba-L}, even in the presence of enhancing antibodies.

Discussion

The results of this study demonstrate that agents that block the binding of *env* gp120 to CD4 inhibit the infection of both fresh and precultured (mature) human peripheral blood M/M. In addition, they demonstrate that infection of HIV in M/M can be enhanced by very low concentrations of anti-HIV antibodies, but that even under such conditions, infection is still blocked by inhibitors of gp120-CD4 binding. Overall, these results suggest that even under conditions of "enhancement," CD4 binding is an essential component of infection of M/M by HIV.

The ability of low concentrations of antiviral antibodies to enhance viral infection in vitro is a well described phenomenon for a number of viruses (especially flaviviruses), and may in certain cases be clinically important (11–13, 29–32). Dengue, for example, may be more severe in individuals with low levels of antidengue antibodies than in seronegative individuals (33, 34). There is evidence that for flaviviruses two mechanisms may contribute to this phenomenon. First, antibodies may serve to attach the virus to either Fc receptors or (indirectly) to complement receptors; the viruses then enter by receptor-mediated endocytosis (35, 36). Second, the antibodies may increase the specific infectivity of bound virus; flaviviruses enter the cytoplasm by an acid-dependent uncoating process and antibodies appear to make this process more efficient by altering the pH of virus-containing endosomes (37–39). In contrast to the flaviviruses, HIV can enter cells both via acid-dependent and acid-independent pathways (40), and the latter mechanism may not apply. Also, while a cellular receptor for flaviviruses has not been clearly identified (in the absence of antibodies), CD4 is a defined receptor for HIV. Indeed, in the present experiments, CD4 appears to act as an essential receptor for HIV even in the presence of enhancing antibodies. It is possible that anti-HIV antibodies anchor HIV to the cell surface (via binding of the antibody to the Fc receptor), and thus increase the likelihood that the virus will come into contact with CD4. It is conceivable that this mech-

anism might exert a greater effect in cells (such as M/M) where there is a relatively low expression of surface CD4. Alternatively, it is possible that anti-HIV antibodies may enhance viral fusion or some other step that follows binding to CD4. Finally, binding of antibody-virus complexes might increase expression of surface CD4 on target cells. Whatever the mechanism(s), the available evidence suggests that under the conditions studied, antibody-mediated enhancement of M/M still requires viral binding via CD4 as an essential step.

One must consider whether the present results might have been caused by toxicity of sCD4 or OKT4A. This is unlikely to be the case, however, as late addition of these agents had no effect on HIV production, and there was no other evidence of toxicity. The lack of suppression of HIV replication from late addition of sCD4 also argues against the possibility that this agent might have an effect on virus budding or other late stages in replication in M/M, as has been proposed in other cell systems (41). However, as noted above, it is still possible that upon binding to HIV, sCD4 might interfere with cell fusion or viral entry (as opposed to viral binding). Such a mechanism, however, could not account for the suppression observed with OKT4A, and taken in toto, the results suggest that CD4 binding is an essential step for HIV infection of M/M in the presence of enhancing antibodies.

It should be noted that the results presented here are somewhat in contrast to these of Homsy et al. (7), who reported that antibody-induced enhancement of HIV infection was not always blocked by inhibitors of gp120-CD4 binding. Variations in the M/M populations, the source or strain of HIV, or anti-HIV antibodies might account for the differing results, and these issues will have to be explored further. Also, the experiments reported here do not involve coculture of M/M with indicator cells that might simply be infected by HIV absorbed onto the surface of M/M (by antibodies) and thus not appear to be inhibited by anti-CD4 antibodies or by sCD4. Additionally, certain M/M preparations may release proteases that may destroy proteins that inhibit gp120-CD4 binding.

It has been proposed that antibody-induced enhancement of HIV infection might have clinical implications in disease pathogenesis, the development of a vaccine against HIV, or certain therapeutic modalities. However, we could elicit enhancement only under very stringent conditions (low multiplicity of infection and very low concentrations of antibodies), and even then, the effect seen was only moderate. It would thus appear that clinically significant effects would be observed only under very limited conditions, if at all. During a narrow window of time early in the course of HIV infection, for example, enhancing antibodies might cause a burst of HIV infection (28). Subsequently, however, antibody titers would be higher than those associated with enhancement. In regard to therapeutic modalities, enhancement has been proposed to be a theoretical concern in the administration of hyperimmune anti-HIV gamma globulin. However, the titers attained in patients with HIV-Ab1 would be at least 10,000-fold higher than those associated with enhancing activity. Lack of activity against M/M or enhancement may also be a theoretical concern in the use of CD4-Ig fusion proteins (19). However, such proteins are effective inhibitors of HIV infection of M/M (19), and in preliminary experiments, we have not been able to detect enhancement with this agent (C. F. Perno, S. Broder, and R. Yarchoan, unpublished observation). Additional studies, however, will be needed to further assess the potential clinical implication of these phenomena.

Summary

Infection of monocyte/macrophages (M/M) by a variety of viruses (including HIV-1) has been shown to be enhanced in the presence of low concentrations of antiviral antibodies. This process has been hypothesized as occurring through binding of the virus-antibody complex to Fc or complement receptors followed by endocytosis. In the current study, we explored whether such a mechanism might provide a CD4-independent route of infection by HIV-1 for any of several populations of M/M. In the absence of anti-HIV antibodies, replication of HIV-1 in M/M was blocked by viral binding inhibitors such as soluble CD4 or OKT4A mAb. Furthermore, while infection of the M/M populations by a low multiplicity of infection of HIV-1 was found to be somewhat enhanced by the presence of very low concentrations of anti-HIV antibodies, this process was also consistently inhibited by recombinant soluble CD4 and by OKT4A antibody. These results suggest that under the variety of conditions studied, CD4 binding was an essential step in the infection of M/M by HIV. Moreover, they are consistent with the notion that "enhancing" antibodies may serve to concentrate HIV onto CD4 receptors or, alternately, may act at other steps in the process of viral entry and replication.

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References

1. Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature (Lond.).* 312:763.
2. Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature (Lond.).* 312:767.
3. McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson. 1986. Binding of HTLV-III/LAV to $T4^+$ T cells by a complex of the 100K viral protein and the T4 molecule. *Science (Wash. DC).* 231:382.
4. Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell.* 47:333.
5. Clapham, P. R., J. N. Weber, D. Whitby, K. McIntosh, A. G. Dalgleish, P. J. Maddon, K. C. Deen, R. W. Sweet, and R. A. Weiss. 1989. Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T-cells and monocytes, but not for brain and muscle cells. *Nature (Lond.).* 337:368.
6. Takeda, A., C. Tuazon, and F. A. Ennis. 1988. Antibody-enhanced infection of HIV-1 via Fc receptor-mediated entry. *Science (Wash. DC).* 242:580.
7. Homsy, J., M. Meyer, M. Tateno, S. Clarkson, and J. A. Levy. 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. *Science (Wash. DC).* 244:1357.
8. Robinson, W. E., Jr., D. C. Montefiori, and W. M. Mitchell. 1988. Antibody-dependent enhancement of human immunodeficiency virus type I infection. *Lancet.* i:790.
9. Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, and R. C. Gallo. 1986.

The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science (Wash. DC).* 233:215.

10. Ho, D. D., T. R. Rota, and M. S. Hirsch. 1986. Infection of monocyte/macrophages by human T lymphotropic virus type III. *J. Clin. Invest.* 77:1712.
11. Halstead, S. B., and E. G. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes: infection enhancement by non-neutralizing antibody. *J. Exp. Med.* 146:201.
12. Peiris, J. S. M., and J. S. Porterfield. 1979. Antibody-mediated enhancement of flavivirus replication in macrophage-like cell lines. *Nature (Lond.).* 282:509.
13. Porterfield, J. S. 1986. Antibody-dependent enhancement of viral infectivity. *Adv. Virus. Res.* 31:335.
14. Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman, and D. J. Capon. 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science (Wash. DC).* 238:1704.
15. Fisher, R. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Constopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsch, R. T. Schooley, and R. A. Flavell. 1988. HIV infection is blocked *in vitro* by recombinant soluble CD4. *Nature (Lond.).* 331:76.
16. Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H.-S. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski, and E. L. Reinherz. 1988. A soluble CD4 protein selectively inhibits HIV replication. *Nature (Lond.).* 331:78.
17. Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet. 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature (Lond.).* 331:82.
18. Traunecker, A., W. Luke, and K. Karjalainen. 1988. Soluble CD4 molecules neutralize human immunodeficiency virus type I. *Nature (Lond.).* 331:84.
19. Capon, D. J., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith. 1989. Designing CD4 immunoadhesins for AIDS therapy. *Nature (Lond.).* 337:525.
20. Gerrard, T. L., C. H. Jurgenson, and A. S. Fauci. 1983. Differential effect of monoclonal anti-DR antibody on monocytes in antigen- or mitogen-stimulated response: mechanism of inhibition and relationship to interleukin-1 secretion. *Cell. Immunol.* 82:394.
21. Perno, C.-F., R. Yarchoan, D. A. Cooney, N. R. Hartman, D. S. A. Webb, Z. Hao, H. Mitsuya, D. G. Johns, and S. Broder. 1989. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. *J. Exp. Med.* 169:933.
22. Perno, C. F., R. Yarchoan, D. A. Cooney, N. R. Hartman, S. Gartner, M. Popovic, Z. Hao, T. L. Gerrard, Y. A. Wilson, D. G. Johns, and S. Broder. 1988. Inhibition of human immunodeficiency virus (HIV-1/HTLV-III$_{Ba-L}$) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2',3'-dideoxynucleosides. *J. Exp. Med.* 168:1111.
23. Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 1985. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro. Proc. Natl. Acad. Sci. USA.* 83:7096.
24. Mitsuya, H., and S. Broder. 1986. Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy virus-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA.* 83:1911.
25. Cohn, E. J., L. E. Strong, W. L. Hughes, D. J. Mulford, J. N. Ashworth, M. Melin,

and H. L. Taylor. 1946. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* 68:459.

26. Oncley, J. L., M. Melin, D. A. Richert, J. W. Cameron, and P. M. Gross, Jr. 1949. Separation of the antibodies, isoagglutinins, prothrombins, plasminogen, and beta-lipoproteins into subfractions of human plasma. *J. Am. Chem. Soc.* 71:541.
27. Condie, R. M. 1980. Preparations and intravenous use of undenatured human IgG. *In* Immunoglobulins: Characteristics and Uses of Intravenous Preparations. B. M. Alving and J. S. Finlayson, editors. DHHS Publication No. (FDA)-80-9005. U. S. Government Printing Office, Washington, DC. 179–194.
28. Robinson, W. E., Jr., D. C. Montefiori, W. M. Mitchell, A. M. Prince, H. J. Alter, G. R. Dreesman, and J. W. Eichberg. 1989. The antibody-dependent enhancement of human immunodeficiency virus type 1 (HIV-1) infection in vitro by serum from HIV-infected and passively immunized chimpanzees. *Proc. Natl. Acad. Sci. USA.* 86:4710.
29. Traavik, T., L. Uhlin-Hansen, T. Flaegstad, and K. E. Christie. 1988. Antibody-mediated enhancement of BK virus infection in human monocytes and a human macrophage-like cell line. *J. Med. Virol.* 24:283.
30. Fagbami, A. H., S. B. Halstead, N. Marchette, and K. Larsen. 1987. Potiskum virus: enhancement of replication in a macrophage-like cell line. *Acta Virol. (Prague) (Engl. Ed.).* 31:463.
31. Lewis, R. M., T. M. Cosgriff, B. Y. Griffin, J. Rhoderick, and P. B. Jahrling. 1988. Immune serum increases arenavirus replication in monocytes. *J. Gen. Virol.* 69:1735.
32. Gould, E. A., A. Buckley, B. K. Groeger, P. A. Cane, and M. Doenhoff. 1987. Immune enhancement of yellow fever virus neurovirulence for mice: studies of mechanisms involved. *J. Gen. Virol.* 68:3105.
33. Halstead, S. B., N. J. Marchette, and J. S. S. Chow. 1973. Enhancement of dengue virus replication in immune leukocytes as a mechanism in the immunopathogenesis of dengue shock syndrome. *Adv. Biosci.* 12:401.
34. Halstead, S. B., H. Shortwell, and J. Casals. 1979. *In vivo* enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* 140:527.
35. Helinius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* 84:404.
36. Gollins, S. W., and J. S. Porterfield. 1984. Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. *J. Gen. Virol.* 65:1261.
37. Gollins, S. W., and J. S. Porterfield. 1985. Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. *J. Gen. Virol.* 66:1969.
38. Gollins, S. W., and J. S. Porterfield. 1986. A new mechanism for the neutralization of enveloped viruses by antiviral antibody. *Nature (Lond.).* 321:244.
39. Gollins, S. W., and J. S. Porterfield. 1986. The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. *J. Gen. Virol.* 67:1941.
40. Stein, B. S., S. D. Cowda, J. D. Lifson, R. C. Denhallow, K. G. Bensch, and E. G. Engleman. 1987. pH-Independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell.* 49:659.
41. Nara, P. L., K. M. Hwang, D. M. Rausch, J. D. Lifson, and L. E. Eiden. 1989. CD4 antigen-based antireceptor peptides inhibit infectivity of human immunodeficiency virus *in vitro* at multiple stages of the viral life cycle. *Proc. Natl. Acad. Sci. USA.* 86:7139.