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Observations after human immunodeficiency virus immunization and challenge of human immunodeficiency virus seropositive and seronegative chimpanzees

(AIDS/vaccine/immunotherapeutic)

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ABSTRACT **Two human immunodeficiency virus (HIV)-seropositive chimpanzees (A-3 and A-86c) infected 4 yr earlier with HIV, along with one uninfected animal (A-36), were inoculated intramuscularly three times in a year with a γ-irradiated gp120-depleted HIV immunogen in incomplete Freund's adjuvant. Both previously infected animals promptly developed an anamnestic humoral antibody response after the first dose, and the uninfected animal developed a primary humoral response to the first dose and then an anamnestic response to the second dose. Although HIV had been recovered repeatedly from the seropositive animals, they became persistently virus-culture negative at the time of or just before the first inoculation of the immunogen. Intravenous challenge with 40 chimpanzee-infectious-doses of a heterologous HIV strain (HIV_{IIIB}) was done 4 mo after the third inoculation in the three treated chimpanzees and in an untreated control animal (A-189a). The immunized naive animal (A-36) and the unimmunized control (A-189a) became infected, and virus has been isolated from their peripheral blood mononuclear cells for >2 yr after challenge. However, the two previously infected chimpanzees (A-3 and A-86c) resisted challenge and have remained virus negative by peripheral blood mononuclear cell cocultivation for >2 yr of observation after challenge; moreover, no evidence of reinfection was detectable by PCR. Despite the *in vivo* resistance, however, peripheral blood mononuclear cells from the resistant animals (A-3, A-86c) remained susceptible to infection by HIV *in vitro*. These findings reveal that a state of immunity can develop and/or be induced to control and/or prevent HIV infection in the chimpanzees. In the absence of any detectable level of neutralizing antibody in A-3 and a low level in A-86c, the patterns of the responses to challenge seen in the four animals suggest that the cell-mediated immune mechanism must have played a significant role in the resistant chimpanzees both in control of their HIV infection and in their resistance to challenge.**

In anticipation of studies on exploring the prospects for the control of AIDS by immunization of HIV-seropositive human subjects (1), the present study was undertaken to determine the immunogenicity and safety of a γ-irradiated human immunodeficiency virus (HIV) preparation administered in incomplete Freund's adjuvant to two HIV-seropositive chimpanzees and one HIV-seronegative chimpanzee. This communication reports the observations made during this study, which indicate that an immunity to HIV infection in the chimpanzee can develop and/or be induced and suggest that cell-mediated immunity (CMI) in some form plays an essential role in the immunological control of HIV infection.

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MATERIALS AND METHODS

Animals. The use of chimpanzees (*Pan troglodytes*) in this study was approved by the Public Health Service Committee on the Use of Chimpanzees in the Study of AIDS. The four animals selected were from the closed colony established in 1962 for the study of slow, latent, and temperate viruses of the nervous system. Their pertinent clinical histories are summarized in Table 1. At time of entry into this study, the animals were healthy by clinical and laboratory criteria.

Immunogen. HIV strain HZ-321 isolated by Getchell *et al.* (2) from serum obtained in 1976 from a patient in Zaire was used for preparation of the immunogen. The method used for purification was as follows: Supernatant fluid from a 7-day culture of infected $HUT\text{-}78_{HZ321}$ cells grown in RPMI 1640 medium/5% calf serum was passed through a Prostack 0.45 μm filter (Millipore) to which β-propiolactone (1:4000 for 5 hr at 37°C) was added. The HIV immunogen was then concentrated 50- to 100-fold by Pellicon (Millipore). The concentrate was frozen at −70°C and then thawed at room temperature for 1 hr followed by 37°C in a water bath; it was then pelleted by ultracentrifugation for 18 hr at 28,000 rpm with a SW 28 rotor in a L5-50 ultracentrifuge (Beckman). The virus pellet was resuspended in STE buffer (10 mM Tris chloride, pH 8.0/100 mM NaCl/1 mM EDTA), layered over a 27–45% sucrose gradient, and centrifuged for 4 hr at 28,000 rpm in an SW 28 rotor. Virus bands were resuspended in STE buffer, frozen at −70°C, and exposed in frozen state to ^{60}Co radiation (Radiation Sterilizers, Tustin, CA). The irradiated antigen was partially thawed at room temperature for 1 hr and then at 37°C until completely thawed; it was then layered on a preformed 15–45% sucrose gradient and ultracentrifuged overnight (12–18 hr) at 28,000 rpm in an SW 28 rotor in a Beckman L5-50 ultracentrifuge. The virus bands were collected, resuspended in 10–15 volumes of phosphate-buffered saline (PBS), and pelleted by ultracentrifugation in a SW 28 rotor at 28,000 rpm for 2–2.5 hr. The pellet was suspended in sterile saline, and samples were removed for protein determination [by bicinchoninic acid (BCA) assay] for pyrogenicity, safety, inocuity, sterility, and for Western (immuno)blot analysis. The purified antigen was then diluted to a stock concentration of 2.0 mg/ml, aliquoted, and frozen at −70°C. Before use, 100 μg of HIV antigen in 0.5 ml of saline was emulsified with 0.5 ml of incomplete Freund's adjuvant in a Spex 2000 emulsifier (Spex Industries, Edison, NJ). Each 1.0-ml dose was administered i.m.

Abbreviations: CMI, cell-mediated immunity; EIA, enzyme immunoassay; gp120, 120-kDa external glycoprotein of HIV-1; HIV-1, human immunodeficiency virus type 1; p24, 24-kDa core protein of HIV-1; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutin; PBL, peripheral blood lymphocyte.

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Table 1. Pertinent chimpanzee histories

	Chimpanzee			
	A-86c	A-3	A-36	A-189a
Sex	Female	Female	Female	Male
Prior exposure to HIV	7/84: i.v. 30 ml of HIV culture supernate (A-26/173P) Per rectum/vagina: 9/85: HIV A26 10/85: HIV A26 11/85: HIV A26 1/86: HIV A26 4/86: HIV A26 HIV Ph1/H9 HIV RF11/H9	10/83: 10% (wt/vol) suspension of brain from AIDS patient, intracerebrally and by multiple peripheral routes	10/83:10% (wt/vol) suspension of brain from AIDS patient, intracerebrally and by multiple peripheral routes 1/86: HIV-infected brain and cell-culture supernate i.v.	8/84: pool of 10% homogenate of liver, spleen, and lymph node from AIDS patient i.v.
Result of exposure	Became HIV-antibody and PBL-culture positive (1984) Increase in antibody titer after one reexposure; virus repeatedly isolated from PBL	Became HIV-antibody and PBL-culture positive	No evidence of infection by ELISA, Western blot, culture, or PCR	No evidence of infection by ELISA, Western blot, culture, or PCR
Age at entry into study	7 yr	24 yr	4 yr	6 yr
HIV immunogen	4/15/87 2/2/88 4/5/88	6/12/87 2/2/88 4/5/88	4/15/87 12/22/87 4/5/88	None
HIV challenge	7/26/88	7/26/88	7/26/88	7/26/88

PBL, peripheral blood lymphocyte.

Treatment for Virus Inactivation. The first batch of immunogen used for the first dose administered to chimpanzees A-36 and A-86c was exposed to 2.2 megarads (1 rad = 0.01 Gy) of ^{60}cobalt radiation. The subsequent doses for these animals and for chimpanzee A-3 were made from immunogen treated with β-propiolactone at a 1:4000 concentration at 37°C for 5 hr, as described, followed by exposure to 4.5 megarads of ^{60}cobalt radiation (3). The β-propiolactone was added to the filtered supernatant for safety in processing. The material was then irradiated with ^{60}cobalt for injection safety; the quantity of radiation used was increased from 2.2 to 4.5 megarads to increase margin of safety. The material that had been exposed to 2.2 megarads was also treated by sonication before emulsification with incomplete Freund's adjuvant and was shown to be free of infectivity by *in vitro* coculture assay, as well as *in vivo*, in the seronegative chimpanzee (A-36), from which virus was not recovered; no HIV-DNA PCR signals were observed either. The absence of antibody to gp160/120 after immunization of A-36 with the gp120-depleted immunogen also indicates an absence of *in vivo* HIV replication of infectious virus after immunization.

Anti-HIV Enzyme Immunoassay (EIA). The standard anti-HIV ELISA used whole-virus lysate (Genetic Systems; Organon Bionetics) as antigen according to the manufacturers' instructions.

Anti-p24 EIA. The anti-p24 immunoassay used purified recombinant HIV p24 antigen (Pharmacia Genetic Engineering) as the reagent for antibody capture. Wells were coated with 500 ng of p24 antigen suspended in 100 μl of PBS and dried overnight at 37°C. Nonspecific protein binding was blocked by the addition to each well of 200 μl of PBS/5% bovine serum albumin. After 2-hr incubation at room temperature, the plates were washed, and serum samples serially diluted in 5% bovine serum albumin/PBS were added at 100 μl per well. After 1-hr incubation, the wells were washed three times with wash buffer (PBS/0.05% Tween 20), and 100 μl of horseradish peroxidase-conjugated goat anti-human immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD), at 1:12000 in 5% bovine serum albumin/PBS, was added per well. After a 1-hr incubation, plates were rinsed three times with wash buffer. HIV-specific antibody was detected by the addition of 2,2′-azino-di[3-ethylbenzthiazolinesulfonate (6)] substrate at 100 μl per well. After 30 min the reaction was stopped with addition of 1% SDS. Optical density was read at 405 nm on a Nippon 2000 immunoreader (Intermed, NJ). Background absorption was determined by using wells to which only conjugate and substrate had been added, and this value was subtracted from experimental readings.

Western (Immuno)Blot Assay. Assays were conducted using Novopath Immunoblot Assay kits (Bio-Rad) following the manufacturer's instructions.

Isolation of HIV from Chimpanzee Peripheral Blood Mononuclear Cells (PBMCs). Attempts to isolate infectious HIV-1 were performed by cocultivating phytohemagglutin (PHA)-stimulated chimpanzee PBMCs with PHA-stimulated PBMCs from seronegative human donors. Chimpanzee and human PBMCs were purified by Ficoll/Hypaque (Pharmacia), then stimulated with PHA at 5 μg per 10^6 cells for 2–4 days. Ten million (1×10^7) chimpanzee PBMCs were cocultivated with 5×10^6 human PBMCs in RPMI 1640 medium/10% fetal bovine serum/antibiotics/interleukin 2. Cultures were fed on days 3, 7, 10, 14, 17, 21, 24, 27, and 31, and every 7 days the cultures were supplemented with 3×10^6 PHA-stimulated human PBMCs of the same donor used for the initial cocultivation. Filtered supernatant fluids were concentrated by ultracentrifugation at 28,000 rpm for 1 hr and assayed for RNA-dependent DNA polymerase activity or p24 antigen by EIA (Coulter Immunology) every 7 days.

***In Vitro* Infection of Chimpanzee PBMC.** After stimulation for 4 days with 5 μg of PHA, 10^7 purified chimpanzee PBMCs were infected at a multiplicity of infection of 0.1 syncytia-forming units of HIV_{IIIB}. At 1 hr after absorption, cells were washed and seeded into T-25 Falcon flasks in RPMI 1640

medium/10% fetal bovine serum/interleukin 2. At 24 hr after infection cells were centrifuged, washed with PBS, and seeded into new tissue-culture flasks in growth medium. Samples were removed at intervals for p24 antigen EIA.

RESULTS

Humoral Response to Immunization. Antibody responses after each of the three doses were measured by a whole-virus EIA (Fig. 1) and Western blot (Fig. 2). The two previously seropositive chimpanzees, A-3 and A-86c, had a marked anamnestic response detectable at 7 days and more strikingly at 14 days after the first dose; a second dose induced a further response. The seronegative chimpanzee (A-36) had a primary antibody response after the first dose; a second dose administered at 8 mo induced an anamnestic response evident at 7 days. The third dose of immunogen did not induce any appreciable rise in the antibody titer in any of the three animals. Neutralizing antibody was detected only in animal A-86c; preimmunization titers of 1:32–1:64 did not increase after immunization (data not shown).

Western blot analysis (Fig. 2) revealed that A-3 and A-86c had antibodies to both envelope and core proteins before immunization, and a single dose of immunogen induced a marked anamnestic response. The seronegative chimpanzee A-36 responded to the first dose with a primary-type antibody response to core proteins (p55, p39, p24, and p17) and to the transmembrane protein gp41. However, there was no detectable response to gp160/120 because of the loss of gp120 from the immunogen during the purification and concentration. The second dose induced a prompt anamnestic response similar to that seen after the first dose in the seropositive animals A-3 and A-86c and, after the third dose, the Western blot patterns increased further in intensity, and a weak response appeared to p66. The absence of anti-gp160/120 antibody in A-36 made it possible to distinguish HIV seropositivity induced by the immunogen from that induced by infection.

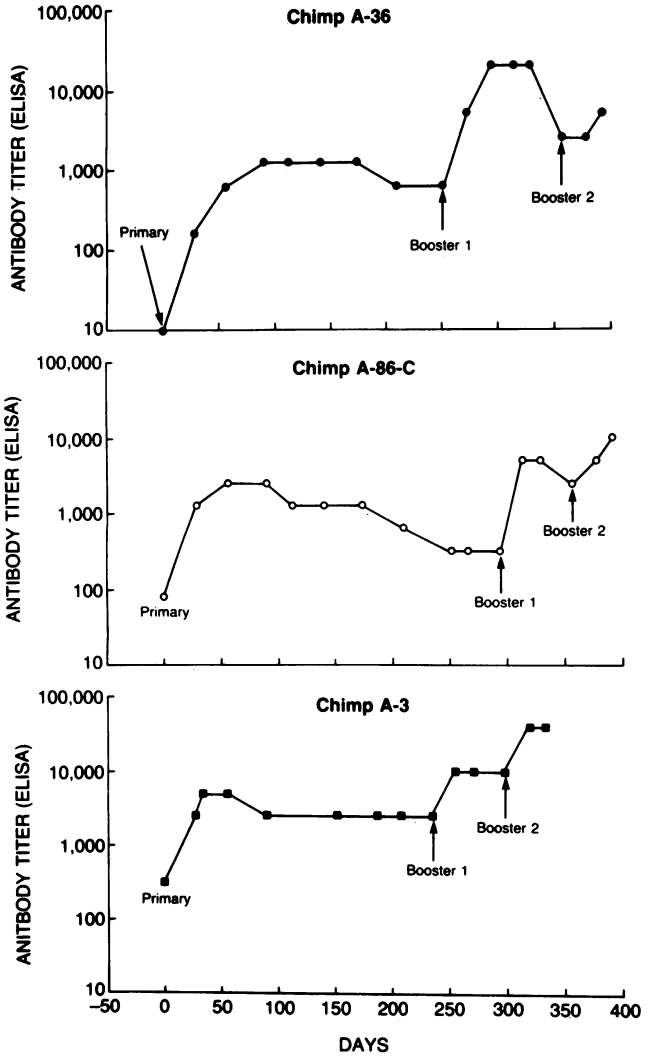

FIG. 1. Antibody responses after each of three doses of immunogen in one seronegative (A-36) and two seropositive chimpanzees (A-3 and A-86c) as measured by EIA using whole HIV virus lysate. The standard anti-HIV EIA that uses whole virus lysate (Genetic Systems, Seattle, or Organon Bionetics) as antigen was done according to the manufacturers' instructions.

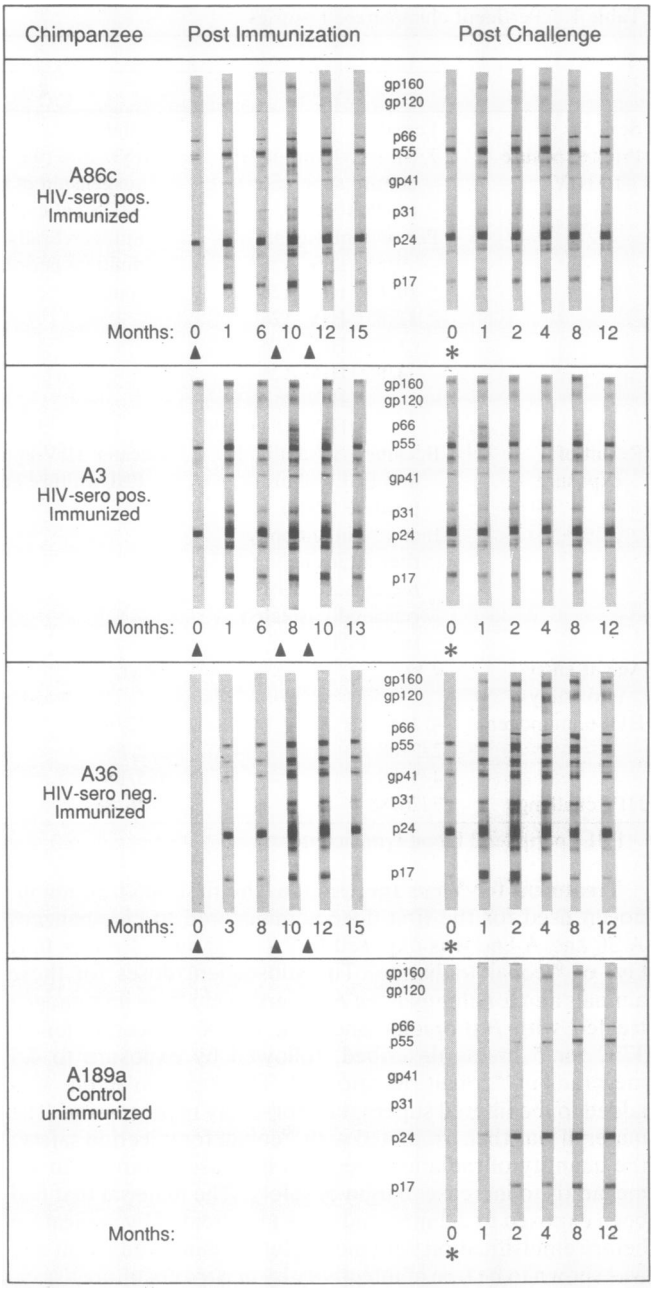

FIG. 2. Western (immuno)blot patterns before and after HIV immunization and before and after live virus challenge in immunized and control chimpanzees. All specimens were tested at the same time using the Bio-Rad HIV Novapath Immunoblot assay (Bio-Rad) according to manufacturers' instructions. ▲, Time of immunization; *, time of challenge.

Table 2. Chimpanzee PBMC cultures for HIV before entry into study (1983–1987), and before and after immunization

	Chimpanzee		
	A-86c seropositive	A-3 seropositive	A-36 seronegative
Year			
1983		+	−−
1984	+++	+	−−−−
	++		−−−−
			−−−−
1985	+++	+−	−−−
	+++		−−−
1986	++	++−	−−−−
	++		−−−−
			−−−−
1987	++−	−−	−
Mo before immunization			
4		−	
3	+		
2			−
1	+		−
Mo after immunization	−	−	−
1–6	−−	−−	−−
7–12	−−−−	−−−	−−−−
13–16	−	−	−

+, a positive culture; −, a negative culture.

HIV Infection Before Virus Challenge. The attempts to isolate HIV from PBMCs of chimpanzees A-3 and A-86c before entry into this study, and in relation to the initiation of immunization, are summarized in Table 2. Table 2 shows that PBMC cultures for HIV in animals A-3 and A-86c were positive over a period of 4 yr after initial infection, but at the time of immunization PBMC cultures derived from both chimpanzees were negative for HIV when using both PHA-stimulated and -unstimulated chimpanzee cells for cocultivation. The seronegative chimpanzee A-36 showed no evidence of HIV infection by either type of PBMC culture (Table 2).

Immunological and Virological Observations After Challenge. The three immunized chimpanzees and an unimmunized control (A-189a) were each challenged i.v. with 1 ml of cell-culture fluid containing 40 chimpanzee infectious doses of the standardized HIV_{IIIB} pool (from L. Arthur and P. Nara, National Cancer Institute, Frederick, MD). Subsequent to challenge, immunized seropositive chimpanzees A-3 and A-86c showed no evidence of HIV in PBMCs by cocultivation (Table 3), no increase in their low level of HIV DNA by PCR with the lysate of 400,000 PBMCs, showing that viral genomic DNA has been <10 copies (data not shown), and no increase in prechallenge antibody levels (Fig. 2 and Table 3). However, the immunized seronegative chimpanzee A-36 became infected after challenge, as did the control animal A-189a, as revealed by the detection of HIV by cocultivation of PBMC (Table 3) and by PCR. Indeed, virus continues to be isolated intermittently from their PBMCs during the >2 yr after challenge. An anamnestic antibody response to infection was seen in chimpanzee A-36, and a primary response was seen in chimpanzee A-189a.

***In Vitro* Susceptibility to HIV Infection.** One possible explanation for the resistance of chimpanzees A-3 and A-86c to infection after viral challenge is that their PBMCs were, for some reason, unable to internalize and/or replicate HIV. However, as shown in Table 4, PBMCs from chimpanzees A-3 and A-86c after challenge were as susceptible to HIV infection *in vitro* as PBMCs from chimpanzees A-36 and A-189a and from a naive control animal, C-345. In all cases, p24 antigen peaked at or around day 8.

DISCUSSION

Several unexpected observations were made in the course of these studies, which together suggest that immunologic con-

Table 3. Comparison of serologic and virologic response patterns in chimpanzees after HIV challenge with 40 chimpanzee-infectious doses administered i.v.

	Anti-p24, Ab titer × 10^{-3}				HIV culture (PBL)			
	Immunized			Control	Immunized			Control
	A-86c	A-3	A-36	A-189a	A-86c	A-3	A-36	A-189a
Status at immunization	+	+	−		−*	−*	−	
Mo after challenge								
0	8	64	32	<0.1	−	−	−	−
1	16	64	256	1.6	−−−	−−−	−++	−++
2	16	128	512	6.4	−−−	−−−	++++	++++
3	16	128	512	1.6	−−	−−	++	++
4	16	64	512	1.6	−−	−−	−+	++
5	16	64	256	1.6	NT	NT	NT	NT
6	16	64	128	1.6	−	−	−	+
7	16	64	128	1.6	−	−	−−	−
8	8	64	128	1.6	−−	−−	−−	−+
9	8	32	64	1.6	−−−	−−−	−−+	−++
10	8	32	32	1.6	−	−	−−	+−
11	8	32	32	1.6	−	−	−+	+−
12	8	32	32	1.6	−	−	−+	+−
13	16	64	32	1.6	−−	−−	−−	+
14	8	16	16	3.2	−−	−−	−−	−
15	16	64	NT	3.2	−−	−−	−−	−+
18	8	32	16	12.8	−−	−−	−−	−+
23	16	32	32	3.2	−−	−−	−+	−+
24	16	32	32	3.2	−−	−−	−−	++

Each symbol (− or +) for HIV culture after challenge represents results of tests of separate specimens through 9 mo and duplicates on the same specimen thereafter. NT, not tested. Ab, antibody.
*Previously virus culture was positive.

Table 4. Infection of chimpanzee PBMCs with HIV-1 *in vitro*

Chimpanzee	p24 antigen in supernatant fluid after infection, pg × 10^{-3}			
	1 day	8 days	15 days	22 days
A-3	0.000	120	13	3.0
A-86C	0.017	108	32	5.7
A-36	0.028	56	19	11
A-189a	0.017	140	62	11
C-345*	0.032	62	46	8.5

*Control chimpanzee C-345 was not previously exposed to HIV and was seronegative.

trol of an established HIV infection in the chimpanzee may occur and/or be induced along with a state of resistance to reinfection. The first of these unexpected observations was that both previously infected seropositive chimpanzees, A-3 and A-86c, were able to mount an anamnestic humoral response to the HIV immunogen. It had been anticipated that chronic stimulation of B cells by endogenously released HIV antigens would preclude responsiveness to an exogenous source of HIV antigens. The ability of chimpanzees A-3 and A-86c to augment their anti-HIV antibody titers in response to immunization indicates that endogenous virus and antigen production in these animals were insufficient to saturate the capacity for the induction of a further humoral response.

Because animals A-3 and A-86c had been virus-culture positive over a period of >4 yr before entry into this study, it was unexpected that they were found to be virus-culture negative before, or at the time of, immunization and remained consistently negative in the year after immunization, as well as during the >2 yr after challenge with a heterologous virus. The negative HIV cultures seen in A-3 and A-86c preimmunization, like their anamnestic humoral response to immunization, suggest that, before the time of immunization, control of the course of endogenous virus was already underway. The persistence of culture negativity after immunization may have ensued as a natural course of events in these animals, or immunization may have enhanced the immune mechanism(s) necessary not only for controlling HIV viral antigens but also for resistance to subsequent viral challenge.

Because neutralizing antibody to the challenge virus was undetectable in one of the two resistant chimpanzees (A-3) and of low level in the other (A-86c), the presence of neutralizing antibody appears not to have been critical for developing culture negativity or for resistance to reinfection. With respect to other antibodies, all three immunized animals had comparably high anti-p24 antibody titers by EIA and similar antibody profiles by Western blot, except for the (expected) absence of anti-gp160/120 in chimpanzee A-36. Because animal A-36 did not resist infection when challenged, it is evident that the anti-HIV antibodies present did not have the capacity to protect against infection. Nevertheless, since PBMCs from A-3 and A-86c remained susceptible to HIV infection *in vitro*, it appears likely that some immunological mechanism was operative *in vivo* to account for their conversion to consistent HIV culture negativity and for their resistance to viral challenge. It is thus reasonable to consider that some form of CMI was operating in animals A-3 and A-86c; this result was induced as a result of their prior infection and was possibly reinforced by subsequent immunization.

The hypothesis regarding the role of CMI in protecting chimpanzees A-3 and A-86c against HIV challenge is consistent with observations others have made for CMI in modulating and/or preventing simian immunodeficiency virus infection. Miller *et al.* (4) have shown that monkeys that develop a major histocompatibility complex-restricted cytotoxic T-cell response to simian immunodeficiency virus *gag* appear to produce less virus and live longer than animals that do not. Desrosiers *et al.* (5), Murphey-Corb *et al.* (6), and Gardner *et al.*¶ were able to prevent simian immunodeficiency virus infection in rhesus monkeys by immunization with inactivated whole virus immunogens administered in a CMI-inducing adjuvant (SAF-1) (7).

To extrapolate these findings to the prevention and/or control of human infection with HIV, it will be necessary to establish and induce the respective *necessary and sufficient* conditions for immunological resistance to the virus. It is clear from these studies that the induction of HIV immunity is far more complex than simply inducing neutralizing antibody, an approach that has been successful in the control of infectious diseases attributable to *cell-free virus*. However, induction of the appropriate cellular immune mechanism(s), directed at eliminating virus-infected cells, the producers and primary disseminators of HIV, will also need to be addressed to develop an effective means for controlling HIV infection in seropositive and/or seronegative human subjects.

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