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Immunology

Serological responses in chimpanzees inoculated with human immunodeficiency virus glycoprotein (gp120) subunit vaccine

(acquired immunodeficiency syndrome/envelope glycoprotein/viral neutralization)

LARRY O. ARTHUR*†, STEPHEN W. PYLE*, PETER L. NARA‡, JULIAN W. BESS, JR.*, MATTHEW A. GONDA*, JOHN C. KELLIHER§, RAYMOND V. GILDEN*, W. GERARD ROBEY‡¶, DANI P. BOLOGNESI‖, ROBERT C. GALLO**, AND PETER J. FISCHINGER‡

*Program Resources, Inc., National Cancer Institute–Frederick Cancer Research Facility (NCI–FCRF), Frederick, MD 21701; ‡Virus Control Unit, NCI Office of the Director, NCI–FCRF, Frederick, MD 21701; §Primate Research Institute, Holloman Air Force Base, Alamogordo, NM 88330-1027; ‖Duke University Medical Center, Durham, NC 27710; and **National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **The major envelope glycoprotein of a human immunodeficiency virus (HIV) has been purified and was utilized as a prototype vaccine in chimpanzees. The 120,000-dalton glycoprotein (gp120) was purified from membranes of human T-lymphotropic virus (HTLV)-IIIB-infected cells and the final preparation contained low levels to no detectable HTLV-IIIB core antigen (p24) and low levels of endotoxin. Chimpanzees inoculated with gp120 responded by developing antibodies that precipitated radiolabeled gp120 and neutralized *in vitro* infection of HTLV-IIIB. Antibodies to HTLV-IIIB p24 were not detected in the gp120-immunized chimpanzees. Peripheral blood leukocytes from the vaccinated animals were examined for $T4^+$ and $T8^+$ cells, and no decrease in the T4/T8 ratio was found, indicating that immunization with a ligand (gp120) that binds to T4 has no detectable adverse effect on the population of $T4^+$ cells. The only current animal model that can be reproducibly infected with HIV is the chimpanzee. Immunization of chimpanzees with HIV proteins will provide an experimental system for testing the effectiveness of prototype vaccines for preventing HIV infection *in vivo*.**

Infection of man by human immunodeficiency retrovirus (HIV) causes a progressive helper T-lymphocyte depletion manifested by a variety of prodromal symptoms that may lead to the fatal acquired immunodeficiency syndrome (AIDS) (1, 2). The virus has rapidly spread among individuals in high-risk groups in the United States, such as homosexuals, drug abusers, and recipients of blood products, and has been increasing among sexually active Africans so that several million people are estimated to have been infected worldwide. As in other human viral diseases, prevention by means of a safe and effective vaccine is a major goal. Based on other animal retroviral models, such as murine, feline, bovine, and simian, a vaccine to prevent primary infection with the causative virus may be difficult but feasible (3–10). The main target antigen was in all cases the envelope (*env*) gene-coded, major external glycoprotein (gp) and to a lesser degree the transmembrane protein (8).

It has been shown that the major envelope glycoprotein of 120,000 daltons (gp120) of HIV binds to the CD4 molecule on $T4^+$ cells (11–13) and this binding is considered the initial step in the infection process. Initial studies with HIV have suggested that the gp120 isolated from HIV-infected cell membranes or genetically engineered gp120 was highly immunoreactive in a number of species and could induce neutralizing antibodies (14–17). A major potential problem is the extensive heterogeneity of HIV since less related HIV variants were not neutralized by anti-gp120 antibodies that could type-specifically neutralize the homologous variant (18–22). On the other hand, chronically infected humans apparently could produce group-specific neutralizing antibody (16, 23).

The chimpanzee is essentially the only animal species readily infectable by HIV (24–27). Although an imperfect model because no significant immunodeficiency has been observed in HIV-infected chimpanzees, the chimpanzee could answer the question whether vaccination with HIV proteins could prevent the initial infection by HIV. Current observations detail an initial series of serological responses of eight chimpanzees inoculated with the human T-lymphotropic virus (HTLV)-IIIB isolated external glycoprotein to define the feasibility of challenge experiments with infectious HIV.

MATERIALS AND METHODS

Cells and Virus. The HIV used in this study was the HTLV-IIIB isolate propagated in H9 cells (28). Infected cells were grown in roller bottles and after 3–4 days of growth, culture medium was removed and clarified of cells, and virus was purified from the culture medium by sucrose-gradient centrifugation. A weekly production run yields 250 liters of culture fluid containing virus and ≈250 g of virus-infected cells.

Immunoaffinity Chromatography. The HTLV-IIIB gp120 was purified from HTLV-IIIB-infected cells as described (15). Membranes from ≈200 g of infected cells were prepared, extracted, and applied to an immunoaffinity resin. The resin was prepared by coupling immunoglobulins, purified from plasma of selected AIDS patients, to Sepharose 4B-CL. The adsorbed antigens were eluted from the resin with 4.0 M $MgCl_2$ and peak fractions were located by monitoring the eluate by refractive index and UV absorption at 280 nm. The eluate containing gp120 was extensively dialyzed against distilled water until a precipitate formed in the dialysis bag. The contents of the dialysis bag were clarified by centrifugation, and the supernatant fraction, containing the gp120, was lyophilized to dryness.

Radioimmunoprecipitation Assays. The proteins to be used in radioimmunoassays (RIAs) were radiolabeled with ^{125}I using chloramine-T. Antibodies to p24 and gp120 were detected by incubating 40,000 cpm of radiolabeled antigen with 50 μl of serum diluted in RIA buffer (0.05 M Tris, pH

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Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; gp, glycoprotein; SFU, syncytial-forming units.

†To whom reprint requests should be addressed.
¶Present address: Abbott Laboratories, AP10, Abbott Park, IL 60064.

7.8/0.15 M NaCl/0.4% Triton X-100/0.5% bovine serum albumin/300 mg of phenylmethylsulfonyl fluoride per liter/0.1% sodium azide) for 2 hr at 37°C and then overnight at 4°C. *Staphylococcus aureus* was added to each tube to facilitate precipitation of the antigen–antibody complexes. Pellets were collected, and, after washing, the radioactivity remaining in the pellet was determined with a γ-counter.

Competitive RIAs. Limiting dilutions of antiserum were incubated with appropriate dilutions of unlabeled antigen for 1 hr at 37°C. Approximately 15,000–20,000 cpm of radiolabeled antigen was added and incubation was continued for 1 hr at 37°C and then at 4°C overnight. *S. aureus* was then added to each tube to facilitate precipitation of immune complexes, pellets were collected and washed once, and radioactivity in the pellet was determined. Results were evaluated using a modification of the program by Rodbard *et al.* (29).

NaDodSO$_4$/PAGE. Electrophoretic separation of polypeptides was based on the methods of Maizel (30). Either continuous 10–20% polyacrylamide gel gradients or 10% homogenous gels were cast in a vertical slab format. The stacking gel was 4% acrylamide in 0.125 M Tris·HCl (pH 6.8). After electrophoresis the gels were then stained with 0.01% Coomassie blue to reveal the separated proteins.

Virus Neutralization Assay. Chimpanzee sera were evaluated for neutralizing antibody by a microtiter syncytium-forming assay described by Nara *et al.* (31). A clone of HIV-sensitive CEM cells (5×10^4 cells per well) was treated with 25 μg of diethylaminoethyl-dextran per ml and made to adhere to 96-well microtiter plates. Virus preincubated with medium controls or the appropriate serum dilution for 60 min at 22°C was added to the CEM cells. The virus/serum mixture on the CEM cells was incubated at 37°C in 5% CO_2 in air for 60 min, removed, and replaced with 100 μl of RPMI 1640 medium containing 10% fetal bovine serum. The cells were examined for the presence of adherent syncytia at 5 days after infection and the syncytia were counted using an inverted microscope. Approximately 250 syncytial-forming units (SFU) of various HIV isolates were incubated either with medium as a control or with chimpanzee sera. Uninfected CEM cells were plated under identical concentrations and served as a control. Viruses in the assay were the original HTLV-IIIB isolate and an isolate from a Haitian patient, HTLV-IIIRF (28). The virus stocks were kept frozen at −70°C at an *in vitro* infectivity titer of 2.5×10^3 SFU/ml for HTLV-IIIB and 4.5×10^4 SFU/ml for HTLV-IIIRF. These virus stocks were retitered in each assay. Also sera from several bleedings were assayed in one experiment to allow a direct comparison of neutralization titer using the same virus stock. Preinoculation sera of all animals were included as controls in these assays.

Preparation of the Immunogen. Dilutions of Alhydrogel and water were added to a constant amount of gp120 and incubated for 30 min at room temperature. After incubation, flocculation was noted and the precipitated alum was collected by centrifugation. The adjuvant selected was alum (Alhydrogel) because it is the only Food and Drug Administration-approved adjuvant for vaccine use in humans and because experiments using threonyl muramyl dipeptide and gp120 did not give responses greater than alum and gp120 in lower primates. All detectable gp120 was precipitated with a 50:50 ratio of gp120 and Alhydrogel (1:10).

Animals. Chimpanzees to be inoculated with the HIV subunit vaccine were maintained at the Primate Research Institute (Alamogordo, NM). This colony of chimpanzees has been utilized extensively in infectious disease studies, predominately hepatitis vaccine development and safety testing of blood products. Animals used in this vaccine study were housed individually. Prior to inoculation, all animals were tested for antibodies to HIV envelope and core antigens. These animals were given a physical examination every 2 wk, at which time blood was drawn and submitted for hematology, blood chemistry, T4/T8 ratio, and serum preparation.

RESULTS

Characterization of the Subunit Vaccine. The HTLV-IIIB envelope glycoprotein purified by immunoaffinity chromatography was examined for purity by NaDodSO$_4$/PAGE. Purity of two vaccine lots (V-002 and V-003) is shown in Fig. 1. Each vaccine lot was examined by amino acid analysis and competitive RIA for HTLV-IIIB gp120 (Table 1). Results of the amino acid analysis were used to determine protein concentration of the vaccine lots. The lots were examined for endotoxin levels and potential contamination by the most abundant HIV core antigen by competitive RIAs for HTLV-IIIB p24. Core antigen was detected in only vaccine lot V-002 and endotoxin levels ranged from 8 to 28 ng/ml.

Immunization of Chimpanzees with HTLV-IIIB gp120. Ten HIV seronegative chimpanzees were chosen for the vaccination experiments; eight of the animals received 50 μg of gp120 in alum per immunization and two served as controls receiving only alum. Larger quantities of gp120 were not available when these experiments were done. The inoculations were administered so that 33 μg of each inoculum was delivered by intramuscular injection with the remaining 17 μg being given intradermally. No adverse reactions were noted at the site of inoculation for either the intramuscular or the intradermal injections. Subsequent immunizations were given on days 35, 63, and 150 following the first injection. Blood was taken from the animals every 2 wk during the study and peripheral blood leukocytes were purified and examined for $T4^+$ and $T8^+$ lymphocytes. No significant change was found in either absolute numbers of $T4^+$ cells or T4/T8 ratios. The T4/T8 ratios were consistently >1.5 and, although the maximum variation of total $T4^+$ cells was from 266 $T4^+$ cells

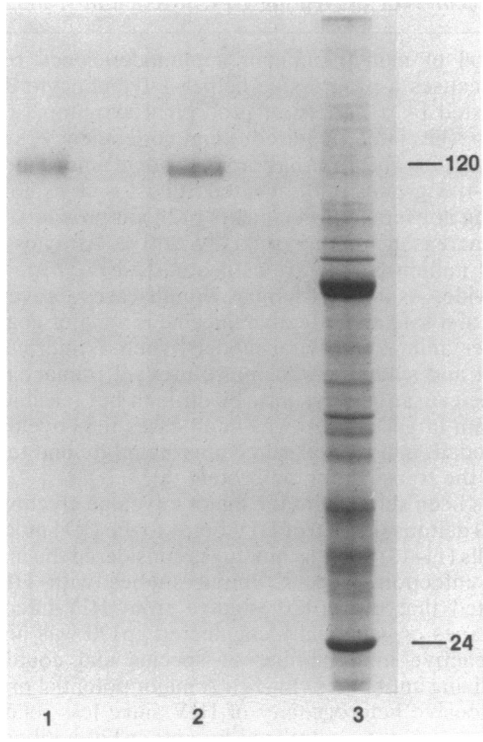

FIG. 1. NaDodSO$_4$/polyacrylamide gel of HTLV-IIIB gp120 pooled vaccine lots. The gel was stained with Coomassie blue. Lane 1, vaccine lot 2 (V-002); lane 2, vaccine lot 3 (V-003); and lane 3, lysed HTLV-IIIB. The positions of gp120 and p24 are indicated.

Table 1. Characterization of vaccine lots of HTLV-IIIB gp120 isolated from H9 cell membranes

Parameter	HTLV-IIIB vaccine lot number*				
	V-002	V-003	V-004	V-005	V-006
Volume (ml)	9.4	14.3	10.0	13.1	12.0
Protein (μg/ml)†	279.1	170.3	212.3	86.7	144.0
Total protein (μg)	2624	2435	2123	1136	1728
Endotoxin (ng/ml)	12	28	8	21	21
gp120 (μg/ml)‡	159	178	126	50	149
p24 level (ng/ml)§	7	<1	<1	<1	

*Vaccine lots were prepared by pooling sufficient purification lots to yield >1 mg of gp120.
†From amino acid analysis.
‡gp120 concentration was measured by competitive RIA using purified HTLV-IIIB gp120 lot EC-012 as a standard.
§p24 level was measured by competitive RIA using purified HTLV-IIIB p24 as a standard.

per μl of blood to 1136 $T4^+$ cells per μl of blood, no correlation was found between this variation and gp120 vaccinations. Typical variation of $T4^+$ cells per μl of whole blood in gp120 immunized animals was 210–826, which was comparable to alum-inoculated chimpanzees.

Serological Response of the Chimpanzees to gp120 Immunization. The sera taken from both gp120-immunized and alum-inoculated control chimpanzees were examined for antibodies to gp120 and p24 by radioimmunoprecipitation assays. Antibody to p24 was not detected throughout the study, demonstrating the lack of immunogenic levels of core antigen in the vaccine preparations. As shown in Fig. 2, the two alum-inoculated control animals were negative for antibodies to gp120 throughout the study. The sera from all animals vaccinated with HTLV-IIIB gp120 precipitated radiolabeled HTLV-IIIB gp120. Maximum response was 2 wk following each of the immunizations. Antibody levels increased following each vaccination, with the highest levels following vaccination no. 4. Though it appeared that most of the chimpanzee sera did not precipitate gp120 at the bleeding taken on day 150 (Fig. 2), the same sera were positive when examined by a more sensitive immunoassay for gp120 (Fig. 3). In this assay, each serum was incubated with ≈10^6 cpm of ^{125}I-labeled gp120 (^{125}I-gp120) and the resulting pellet was examined by $NaDodSO_4$/PAGE and autoradiography. All

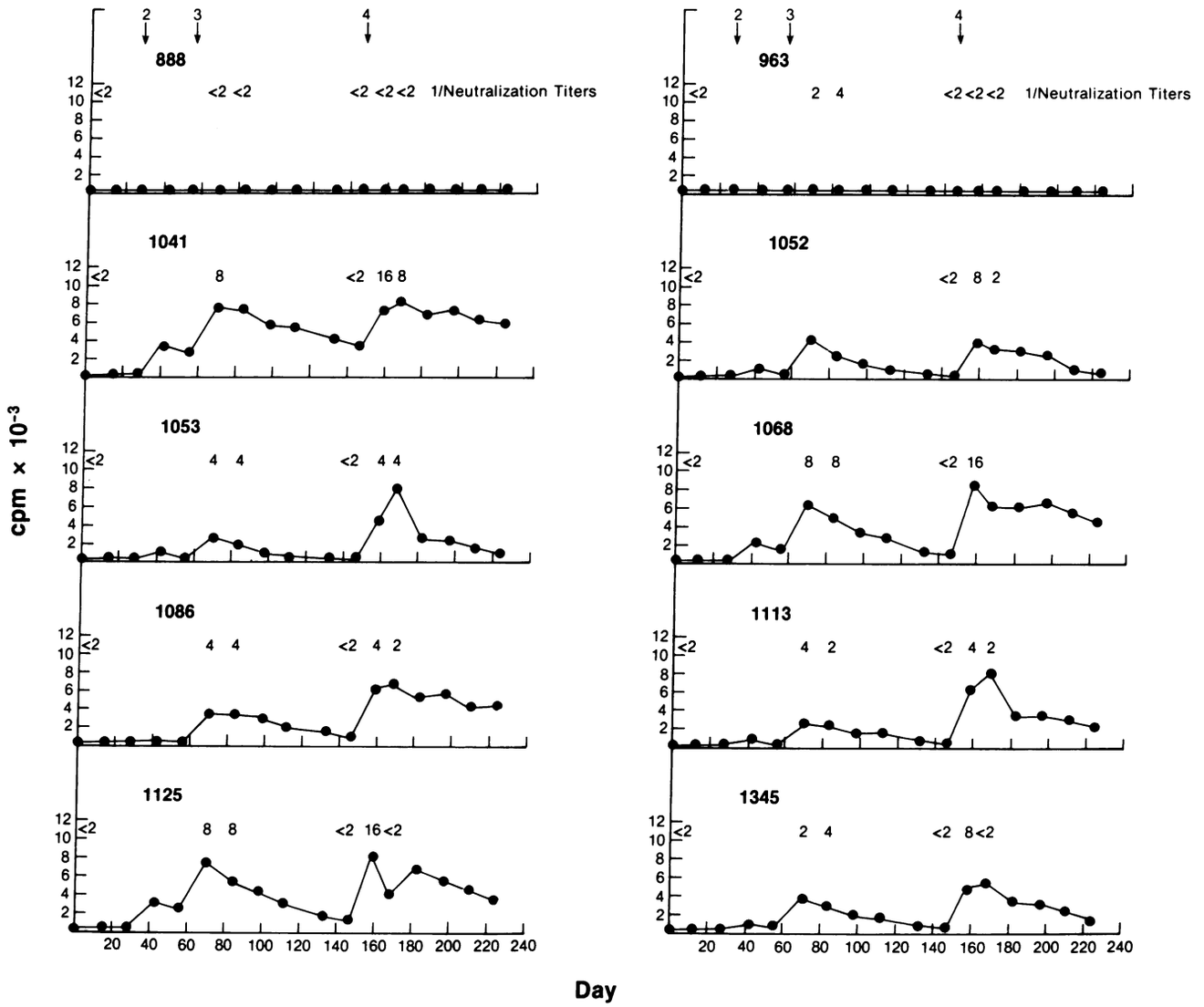

FIG. 2. HTLV-IIIB gp120 radioimmunoprecipitation assay analysis of sera from chimpanzees in the immunization study. Data are expressed in cpm of ^{125}I-labeled HTLV-IIIB gp120 specifically precipitated by a 1:50 dilution of the serum. Animals were immunized with 50 μg of alum-precipitated gp120 on day 7, day 35, day 63, and day 150. Control chimpanzees (nos. 888 and 963) received only alum injections. Neutralization titers are listed at the appropriate time point for each animal.

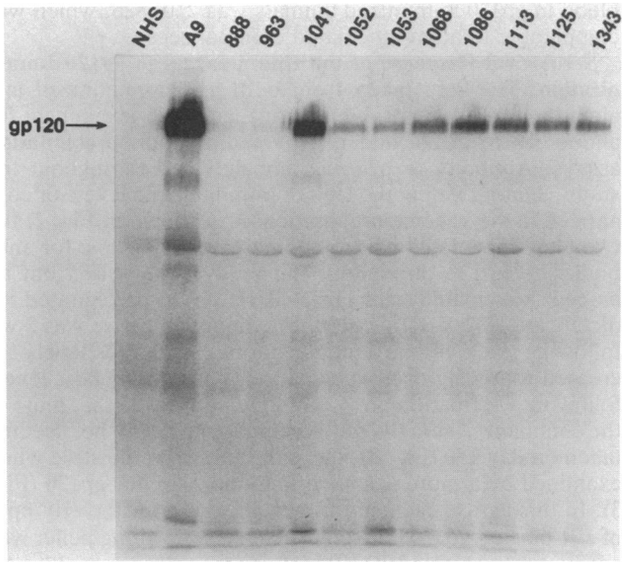

FIG. 3. Autoradiogram of immunoprecipitations. Serum taken from study animals on day 150 after immunization was used to precipitate ^{125}I-gp120 ($\approx 10^6$ cpm), and the immunoprecipitates were examined by NaDodSO$_4$/PAGE and autoradiography. The first two lanes on the left are precipitates by normal and AIDS patient sera, respectively. Numbers above the remaining wells indicate serum from each individual chimpanzee. Control chimpanzees (nos. 888 and 963) received only alum injections.

animals immunized with gp120 were found to have antibodies to gp120 at the bleeding taken on day 150 (Fig. 3). Maximum relative response in both assays was obtained with serum from animal 1041. Serum from an AIDS patient served as a positive control and precipitated ^{125}I-gp120 (lane 2), whereas ^{125}I-gp120 was not precipitated with sera from the control chimpanzees immunized with alum (nos. 888 and 963). The faint bands observed with the control sera were due to small quantities of radiolabeled proteins nonspecifically adhering to the *S. aureus* during the immunoprecipitations.

Neutralizing Antibodies. A prerequisite for *in vivo* challenge experiments is the demonstration of homologous neutralizing antibodies in vaccinated animals. The sera from all animals inoculated with gp120 were found to neutralize the HTLV-IIIB infection. Virus-neutralizing titers paralleled the precipitation titers with maximum neutralizing antibody levels detected 2 wk after the fourth vaccination. Sera from the control animals and from the preinoculation blood samples of the vaccinated animals had no neutralizing antibodies.

To determine the extent of neutralization, the sera from certain gp120-immunized chimpanzees were tested for neutralization against the homologous virus, HTLV-IIIB, and a HIV variant, HTLV-IIIRF. As shown in Table 2, HTLV-IIIB gp120 postinoculation samples from animals 1041, 1068, and 1125 effectively neutralized HTLV-IIIB. This is demonstrated by a 98–99% reduction of syncytial-forming virus as compared to preimmune sera from the same animals. However, none of the sera neutralized the HTLV-IIIRF. Nonspecific neutralization of HTLV-IIIB and HTLV-IIIRF averaged 30–40% of the total virus SFU used at low serum dilution (1:2 and 1:4) and does not appear to be affected by heat inactivation (Table 2).

Table 2. Neutralization of HIV variants by sera from HTLV-IIIB immunized chimpanzees

Animal	Serum collection, days after inoculation	HIV variant: HTLV-IIIB/H9 No. of syncytia*	HTLV-IIIB/H9 % reduction of SFU	HTLV-IIIRF/H9 No. of syncytia*	HTLV-IIIRF/H9 % reduction of SFU
1041	†	125	—	110	—
	70	48	62	105	4.5
	84	50	60	115	0
	147	1	99	98	10
1068	†	100	—	140	—
	70	5	95	150	0
	84	3	97	135	3
	147	2	98	133	5
1125	†	130	—	160	—
	70	40	69	159	<1
	84	38	70	158	<1
	147	1	99	148	8

*Number of syncytia remaining after treatment of the virus with serum at a 1:4 dilution. Approximately 250 and 300 SFU of HTLV-IIIB and HTLV-IIIRF, respectively, were added to each microtiter well.
†Preinoculation.

DISCUSSION

Because of previous favorable responses in lower species (14, 15), the HTLV-IIIB gp120 was the choice for a prototype subunit vaccine to be tested in chimpanzees. To conserve this species, only animals previously used for human hepatitis studies were considered. By analogy, the majority of individuals in the highest human risk group, the male homosexuals, have also been previously exposed to human hepatitis viruses. The HTLV-III gp120 inocula were free of infectious virus and contained no other detectable viral antigens. Low levels of the gp120 antigen vaccine induced moderate levels of antibody specific only to the gp120. The adjuvant alum was purposely used because it is the only agent currently acceptable for human use. The key question of whether the isolated gp120 had, *per se*, an adverse effect on T4 population or other obviously negative health effects was answered by the facts that analyses by fluorescent-activated cell sorting techniques revealed no loss of T4 lymphocytes and that all eight chimpanzees showed no untoward effects after four immunizations of gp120. In all animals, an immune response was rapid and progressive but tended to decay with time. A clear booster effect was observed after the fourth immunization, which implied adequate recall function. The latter was confirmed by recent studies defining at least two conserved T-cell-specific epitopes on the gp120 defined by immunogenic peptides of the amphopathic regions (32).

On the humoral level, it has been observed that the gp120 antibody was group-specific for precipitation of several distantly related HIV gp120s (15). In contrast, analyses of all time points in this communication revealed only type-specific neutralizing antibody that is analogous to that seen after gp120 immunization of lower species and rhesus monkeys. Chronically infected humans could apparently mount a variable group-specific neutralizing antibody response (16, 23). The evidence was inferential because of the uncertain exposure status to HIV in man. However, more direct evidence of HIV infection leading to a group-specific neutralization immune response was obtained by studies of chronically infected chimpanzees exposed to a single HIV variant. These animals developed type-specific and, after several months, group-specific neutralizing antibodies under conditions where the input virus underwent no significant change in its gp120 structure and remained susceptible to homologous type-specific neutralizing antibody (33). A major question under test is whether with time, or after sequential or concurrent exposure with distantly related HIV gp120s, the less immunodominant epitopes defining the group-specific

neutralizing site are indeed on the gp120 and also could elicit strong group-specific neutralizing antibodies. Other aspects of humoral and cell-mediated immunity may be analyzed in vaccinated chimpanzees as well.

From a practical perspective, the difficulty of producing large amounts of HIV gp120 from human tumor cells suggests that the experiments with isolated gp120 be considered as benchmarks with which to compare genetically engineered gp120 proteins in bacteria, yeasts, or mammalian cells, which are accessible in large quantities. Segments of gp120 were recently expressed by means of bacterial vectors (17). These were not glycosylated but at least one, PB-1, could induce adequate type-specific neutralizing antibody in several species without being able to bind to the natural T4 viral binding site (17). This same PB-1 molecule can be tested as a vaccine in chimpanzees.

Although the chimpanzee may not be a perfect test system, prevention of primary infection could be attempted even if no disease occurs. A test for disease prevention can be performed in parallel with subunits or other approaches in several simian acquired immunodeficiency virus models (34–38). The spectrum of response can be explored in the chimpanzee with multiple, distantly related HIV gp120s in various combinations.

A commonly expressed concern is the feasibility of distinguishing between vaccinated and infected susceptible species. At present, two indices of infection are reliable: (*i*) HIV isolation was possible on every occasion from infected chimpanzees and (*ii*) infected animals developed antibodies to p24 (25, 26), neither of which is seen in vaccine recipients. Current data thus set the stage for a HIV challenge in gp120-vaccinated chimpanzees. Challenge of the gp120 immunized animals with homologous *in vivo* chimpanzee-titered virus has been initiated and the final complex consequence will be discussed elsewhere. Additional studies using heterogeneous HIV-variant systems have to be undertaken and the roles of the many structural and nonstructural HIV proteins have to be explored.

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