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DEVELOPMENT AND EVALUATION OF ADENO-HTLV-III HYBRID VIRUS AND NON-CYTOPATHIC HTLV-III MUTANT FOR VACCINE USE

Annual Report

by

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Acquired immunodeficiency disease syndrome (AIDS) was initially recognized as a separate disease in 1981. Results from research groups in France and the United States determined that a previously unknown virus called HIV is the primary aetiological agent of AIDS.

Two HIV vaccines, a recombinant Adeno-HIV hybrid virus and a recombinant vaccinia HIV will be tested. The recombinant Adeno-HIV virus is being developed as part of this proposal. The vaccines will be tested in two species of monkeys, chimpanzees and African green monkeys. Vaccinated animals will be challenged with a defined dose of HIV virus. Assessment of vaccine efficacy against the virus challenge will include T4/T8 ratios, Interleukin-2 production, HTLV-III serology and ability to detect infectious HTLV-III virus in peripheral blood cells. T-cell mediated immunity will be assessed by monitoring cytotoxic T-cell activity and antigen-induced PBL proliferation.

In Phase II of this proposal, we will focus on whether generation and cloning of HIV specific killer T cells could be used to protect humans from AIDS.

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SUMMARY

The immune response of African Green monkeys, baboons and chimpanzees to HIV has been monitored in infected and vaccinated animals. Anti-HIV antibody was characterized for its ability to neutralize HIV virus and its reactivity in the HIV Western blot assay. Antigen-induced cellular proliferation was also studied. Preliminary experiments to establish protocols for measuring cytotoxic T cell responses in primates were conducted. Standardization of the conditions leading to reproducible infection of African Green monkeys with HIV established that this species could be infected most reproducibly by intravenous administration of HIV 2. 283 primates of various species were tested for "natural" infection with HIV. Seropositive animals of 5 species of monkeys have been demonstrated. Animals who are anti-HIV-2 positive are more frequent than HIV-1 positive animals.

Recombinant HIV-1 gp120/Adenovirus 2 isolates have been obtained by screening the mixture of wild type and recombinant virus progeny from human cells transfected with adenovirus DNA and plasmid constructs containing HIV-1 gp120 sequences. Preliminary characterization of one of these isolates demonstrates the presence of gp120 DNA and RNA sequences in cells infected with this recombinant virus as well as an intracellular accumulation of protein that reacts with monoclonal antibody directed against HIV-1 gp120.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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BODY OF PROPOSAL

A. Statement of Problem:

Acquired immunodeficiency disease syndrome (AIDS) was initially recognized as a separate disease in 1981. Results from research groups in France and the United States determined that a previously unknown virus called LAV in France and HTLV-III in the United States is the primary etiological agent of AIDS. Recently this virus has been renamed HIV-1. Two types of retrovirus have been isolated, HIV-1 in AIDS patients in United States, Europe and Central Africa (1,2) and HIV-2 in AIDS patients from West Africa (3,4).

AIDS is a highly lethal disease and presents a serious medical, social and economic problem of global dimensions. The World Health Organization estimates the total number of AIDS cases worldwide at 100,000 with 5 to 10 million asymptomatic HIV-infected individuals (5). In the United States alone, more than 71,000 cases had been reported by mid-1987 with a mortality rate of greater than 50% (6). James Curron of the U.S. Center for Disease Control, Atlanta, Georgia, estimates that, within 5 years, AIDS will be the leading cause of death among young and middle-aged men (7). In the past, the major mode of AIDS transmission was considered to be through homophilic sexual practices and via contaminated needles associated with drug abuse. However, evidence is accumulating that heterosexual transmission with attendant maternal-fetal infection is, likewise, an important mode of HIV transmission, thus, putting the entire population "at risk". The spread of AIDS into the general population underscores the urgency of developing an effective vaccine against HIV as immunoprophylaxis appears to be the most rational and effective means to control the spread of this disease.

Up to 100% of patients with AIDS and pre-AIDS syndrome have anti-HIV antibody in their sera (8), suggesting that anti-HIV antibody is not sufficient to protect patients from development of AIDS. The aim of this proposal is to study the role which T cell mediated immunity and anti-HIV antibody play in preventing or ameloriating HIV infection and the development of an effective HIV vaccine.

Monkey models studied include chimpanzees and Cercopithecus (Cercos). Chimpanzees are the only nonhuman primate that can be reproducibly infected with HIV (7-11). Preliminary experiments by one of us (D.Zagury) indicate that the Cercopithecus monkeys can

be used as an animal model to study HIV infection. Studies in Cercos will establish the strain and dose of HIV virus and the route of infection. Chimpanzees and Cercos will be vaccinated with a recombinant adeno-HIV virus and vaccinia-HIV hybrid virus. Assessment of vaccine efficacy against vaccine virus challenge includes HIV serology and the ability to detect infectious HIV virus in peripheral blood cells by virus isolation and and polymerase chain reaction (PCR). T cell mediated immune responses will be monitored by cytotoxic T cell activity and antigen-induced cellular proliferation. The occurrence of retrovirus infection of primates in remote regions of Zaire will be investigated and partially characterized.

B. Background:

AIDS is a recently identified disease characterized by increasing deficiencies in the body's cell mediated immune response in previously normal patients (8). A reduction in the number of helper T lymphocytes (OKT4+) is usually involved and is accompanied by multiple opportunistic infectious and/or malignancies (12,13). A syndrome designated AIDS related complex (ARC) has been identified in groups at risk. The dominant clinical expression in ARC is unexplained chronic lymphadenopathy or leukopenia involving a reduction in OKT4+ cells. Minor cutaneous infections, diarrhea, weight loss and fever may be associated with ARC (8). Results from research scientists in France and the United States strongly suggest that a previously unknown virus now called HIV 1 is the primary aetiological agent of AIDS (1,2). Western Blot analysis reveals that AIDS and ARC patients serum contains antibodies to all or some of HIV proteins (14).

Chimpanzee is the only non-human primate known to be susceptible to infection with HIV (9-11). Infected chimpanzees develop a transient severe lymphoadenopathy. Virus can be recovered from peripheral blood lymphocytes and bone marrow but not the plasma, saliva or cerebralspinal fluid (15). Although all HIV inoculated chimps remain persistently infected, a persistent decrease in T4+ cells does not develop and the chimpanzees remain clinically well (16).

Preliminary experiments by Dr. Daniel Zagury indicated that African Green monkeys (Cercopithecus) infected with HIV virus by intraperitoneal (IP) injection develop viral infection. Some animals showed evidence of infection as detected by reverse transcriptase activity, ability to transmit HIV infection to peripheral blood lymphocytes and the presence of HIV

genome in their peripheral blood lymphocytes. Some monkeys had a serum antibody response to HIV. Because not all of infected animals could be shown to be infected, an alternate model, HIV-2 infection of Cercos, has been developed.

When a foreign body invades the body, a variety of immune reponses, including antibody and cytotoxic T cells, can be induced. In order to be successful, a vaccine must elicit a immune response that is neutralizing or protective. Although anti-HIV antibodies may neutralize at the intial stages of viremia, they do not appear to be protective since patients with high titers of anti-HIV antibodies develop AIDS (17). Cytotoxic T cells can play an important role in host defense against viral infectious (18) and vaccination leading to cell mediated immunity to HIV may be desirable because cytotoxic T cells could protect from further cell to cell viral spread. HIV specific T lymphocytes in seropositive individuals have been demonstrated (19-22) 15). However, the role they play in AIDS is not known. T cell receptors present a heterodimeric reactivity which is directed against both self major histocompatibility antigens and processed antigenic determinants. In contrast to antibodies, which may be serotype specific, cytotoxic T cells exhibit broader antigenic specificities. This accounts for cross reactions observed by cytotoxic T cells of autologous targets infected by different strains of virus with serologically distinct influenza virus (23,24). Thus a vaccine inducing cytotoxic T cells against one subtype of HIV may destroy cells infected with other HIV subtypes.

The objective in the development of a HIV vaccine is to produce, with minimal side effects, an immune response of long duration in the host that will provide effective protection against viral infection and the subsequent onset of clinical AIDS. The worldwide prevalance of AIDS also makes it important that such a vaccine be easily administered and relatively inexpensive to produce.

The majority of viral vaccines now in use are preparations of either live, attenuated or killed virus. Concerns for reversion to virulence and the risk of reactivation of intact virions or proviral DNA make these approaches to a HIV vaccine untenable. At present, subunit vaccines utilizing immunogenic virus peptides or proteins derived from purified virions or by recombinant DNA technology appear to provide the best approach to development of a safe and effective vaccine. These subunit components may be administered in association with adjuvants or carriers, incorporated into artifical "membranes" or delivered by means of recombinant viruses. Several recombinant vaccinia-HIV

viruses have been developed and tested in non-human primates (25-29) and humans (30). Chimpanzees vaccinated with vaccinia-HIV demonstrate very low titers of neutralizing antibodies (25). Therefore the development of an alternate HIV recombinant vaccine may prove valuable. For a variety of reasons outlined below, we have proposed to utilize human recombinant (r) adenovirus (Ad) as a vector for expression of the HIV glycoprotein, gp120.

Bivalent immunization with live, attenuated Ad type 4 and type 7 has proven to be both safe and effective in military recruits over a period of 20 years (31-33). This vaccine, which is administered orally in enteric-coated capsules, liberates virus into the intestine where a subclinical infection is established that confers a high degree of immunity. Successful immunization with Ad types 1, 2 and 5 by this route have also been demonstrated (34). The fact that administration of live vaccines by the gastrointestinal route may facilitate spread of the Ad (35) may be advantageous in immunizing against HIV if a suitable, relatively non-pathogenic strain of adenovirus is used as vector. The majority of Ad infections result in self-limiting and short-lived clinical manifestations although prolonged asymptomatic or latent infections may occur (36). It is possible that reactivation of latent adenovirus, as has been proposed to occasionally occur in rubella infections (37), may provide a mechanism for a natural HIV "booster" immunization in the case of the rAd vaccine.

Ad structural proteins are synthesized in large quantities during infection and at least 80% of viral hexon, penton and fiber are not incorporated into progeny virus but remain in the infected cell in the form of readily soluble multimers (38). Theoretically, exogenous DNA sequences stably integrated into the Ad genome under the control of the major late promoter might also be expressed at a high level to provide a source of immunogen.

Ad is very stable and can tolerate temperatures of 4-36°C and pH 5-9 with minimal loss of infectivity, thus, alleviating many problems associated with vaccine transport and storage especially in under-developed countries.

The technology necessary to propagate large quantities of Ad to produce an enteric-coated vaccine is presently available so that production of a rAd vaccine should not require the development of new manufacturing processes.

This project is designed to investigate the feasibility of using infectious Ad to produce gp120 or other HIV immunogen and to determine whether these HIV antigens would be presented in such a way as to elicit protective immunity in the host.

C. Rationale

Since chimpanzees are the only non-human primate whose ability to be infected with HIV is well documented, our vaccination protocols have been changed to include chimpanzees. However we feel that further characterizing the Cercopithecus (Cercos) monkey as an alternative model is important since the chimpanzee model has several limitations. Chimpanzees are difficult to obtain in large enough numbers to conduct a vaccination protocol. They are expensive to maintain. Although chimpanzees can be chronically infected with HIV, they remain clinically well (15). Cercos may prove to be a useful model since enough animals to conduct a vaccination trial can be obtained and maintained at a relatively low cost. Preliminary experiments with Cercos established that this species can be infected with HIV. Future experimentation revealed that using the original protocol, not all monkeys responded with full evidence of infection and an anti-HIV immune response. Full evidence of infection was defined as reverse transcriptase (RT) activity in supernatants of peripheral blood lymphocytes (PBL), immunofluorescent staining of PHA activated PBL, HIV transmission to T cells and an intergrated HIV genomic pattern southern blot DNA hybridization. Experiments to determine the protocol necessary to infect 100% of African green monkeys were begun. Parameters studied included:

a) Varying the strain of African green monkey. Three strains, Cercoithecus aethiops, C. ascarius and C. cerebus were tested.

b) Varying the HIV strain HIV 1 and HIV 2 were tested.

c) Varying the route of infection. Intravenous (IV) and intraperitoneal (IP) routes were investigated.

d) Varying the number and dose of virus.

We have not conducted any studies with HTLV-III-X10-1 mutant virus since vaccination with live mutant HIV virus is not practical due to possibility of reversion to virulence. Therefore, the immune response to recombinant vaccinia-HIV virus containing gene sequen-

ces to HIV gp160 has been studied. Because T cells can play an important role in host defense against viral infections (18), it is important to investigate T cell mediated immunity in vaccinated or infected monkeys. Both T cell mediated lysis of infected targets and antigen induced cellular proliferation have been investigated. Serological studies have also been conducted.

Immunotherapies of AIDS and ARC patients require that an efferent stage in which viral proteins are expressed of cell surface occurs prior to release of infectious virus. Therefore, the stages of HIV infection were investigated to determine the mechanisms of HIV cytopathology. Human and chimpanzee HIV infected T-cells were isolated by a specific rosetting technique to determine if chimpanzee infected cells differ from human cells infected with HIV.

Since Cercos are more reproducibly infected with HIV-2, this model will be investigated. HIV-2 specific reagents including DNA probes, polymerase chain reaction (PCR) proteins and antibodies (monoclonal and polyclonal) will be developed and characterized. Similar to HIV-chimpanzees model, HIV-2 infected Cercos can be shown to be persistently infected with HIV-2 in the absence of clinical symptoms. HIV-2 infected Cercos will be coinfected with STLV to determine if an AIDS-like syndrome will result.

The rAd for vaccine purposes is obtained through recombination between homologous regions of viral DNA and a plasmid construct containing HIV sequences. Ad type 2 (Ad2) was selected as the vector because this serotype has been extensively characterized on the DNA, RNA and protein level and is easily propagated in the laboratory. The viral early region 3 (E3), which is not essential for Ad2 replication *in vitro*, is replaced with the desired HIV sequences. Nucleotide sequences encoding gp120, the major external glycoprotein exposed on the surface of HIV, were used in the initial construct of the rAd plasmid vector. Gp120 was selected because surface glycoprotein are the major retroviral antigen (39) and are essential for infectivity. Precedence has shown that immunization with retroviral glycoprotein(s) elicit both neutralizing and cytotoxic antibodies. The HIV gp120 DNA fragment used to construct the rAd vector extends from a *SspI* site 65 base pairs (bp) upstream of the initial ATG of the envelope coding sequence to a second *SspI* site at nucleotide 7567 of HIV strain HXB2. This fragment includes 87% of the gp120 coding sequence plus the 30 amino acid (aa) leader. Because the HIV transmembrane protein, gp41, may also be a potential immunogen, a second vector which includes the entire HIV *env* gp160 sequence has also been constructed. In

all constructs, the gp120 or gp160 coding sequences are flanked 5' by the Ad major late promoter including the tripartite leader and 3' by an Ad polyadenylation signal and VA RNA coding region.

Chimpanzees have been the sole animal model in which to conduct valid tests of potential vaccines because these animals are susceptible to HIV infection, react immunologically, and simulate the lymphoadenopathy syndrome. However, the proposed rAd vaccine cannot be evaluated in chimpanzees because human Ad do not replicate well, if at all, in monkey cells, and, virus replication is essential for expression of the HIV sequences. This block to Ad replication in simian cells appears to occur at the RNA splicing level (40) and is overcome by the carboxy terminus of the simian virus (SV) 40 T antigen (Ag)(41). Therefore, the initial rAd vaccine construct contains approximately 500 bp of 3' SV40 T Ag coding sequence plus 200 bp of early SV40 DNA driven by the SV40 promoter. This fragment with SalI sites on either end can easily be removed and the plasmid religated to provide a construct minus SV40 sequence for the human vaccine. The presence of SV40 sequences also provides a means to select recombinant Ad2 progeny virus based upon plaque size. Theoretically, rAd containing the SV40 fragment should replicate more efficiently and rapidly on the CV-1 African green monkey kidney cell line and produce larger plaques.

To facilitate homologous recombination into the Ad E3 region, the vaccine plasmid construct contains flanking Ad sequences with 1051 bp of E3 and protein pVIII coding region upstream and 1513 bp of E3 and fiber coding region downstream of the gp120-SV40 sequences.

Ad2 and vaccine plasmid DNA are cotransfected into the 293 Ad EIA-transformed human embryonic kidney cell line and the progeny virus plaqued on CV-1 cells. The largest plaque isolates are selected and plaque-purified prior to preparation of virus stocks in HeLa cells. Virus-infected cells are analyzed for the presence of HIV-specific DNA and RNA and for expression of gp120. The rAd is administered in enteric-coated capsules to Cercos which are monitored for evidence of viral infection, pathogenicity and virus shedding as well as for a rise in complement fixing (CF) and neutralizing antibody (Ab) titer directed against both Ad2 and HIV gp120.

Similar methods will be used to construct a human form of the rAd lacking SV40 T Ag. The deletion of this viral sequence will necessitate the selection of the desired rAd progeny on the basis of HIV gp120

sequences or protein detected in infected cells. Because Ad2 is endemic with neutralizing antibody found in 60% of the U.S. population (42), it is essential to change the Ad serotype in order to develop an effective human vaccine. Two approaches can be taken: 1) use a more rare and relatively nonpathogenic Ad serotype to construct the rAd vaccine virus or 2) exchange the Ad2 sequences encoding the fiber, which is primarily responsible for type-specific neutralizing antibody (43), with that of a rare strain. Therefore, the final live rAd vaccine virus will consist of HIV gp120 sequences expressed from the Ad major late promoter inserted in the E3 region of a modified Ad2 or non-Ad2 virus vector.

D. Experimental Methods:

Monkey units:

Two units of animals are maintained, the first at the Primatology Center of CNS in Villejuir, Paris (directed by Dr. Pierre Dubouch) and the second in Kinshaw, Zaire (directed by Mrs. Delphi Messinger, an American Zoologist).

Newly acquired primates are anesthetized with ketamine, T-B tested in the left eyefold and given an overall physical exam. Periodical checks are made for parasites and animals are wormed as needed. Animals are provided access to water and are fed a cake of wheat, corn and soy flour with peanuts, milk, millet, bananas, and other fruits, nuts and various greens.

A field laboratory at Lubutu, Zaire has been established in July 1988. Serum and cell samples from monkeys killed or trapped in this area are analyzed for evidence of retrovirus infection: In addition to Ms. Messinger and Dr. Daniel Zagury, the Lubutu Laboratory is staffed by 2 technicians, a Zairian doctor (Dr. Ngoi), his nurse, 1 Zairian veterinarian and 1 driver. Plasma, sera, spleen and PBL are frozen in liquid nitrogen the same day collected. Samples are taken from monkeys killed by inhabitants if it can be established that the monkeys are less than 6 hours dead.

Reverse Transcriptase

Culture supernatant (1 ml) was centrifuged at 1300 x g for 10 minutes. The supernatant was removed and centrifuged at 40,000 x g for 2 hours to pellet virus. The virus pellet was resuspended on ice in 20 ul of buffer containing 0.05M Tris-HCl pH 7.5, 0.3M KCl, 0.3% Triton X-100. To this was added 40 ul of buffer containing 0.07M Tris-HCl pH 8.3, 0.014M $MgCl_2$, 0.03M KCl, 0.0014 M DDT, 15 ug/ml polyadenylic acid, 15 ug/ml oligothymidylic acid (dT) 12-18, and 3 uCi

^{3}H-TTP (46 Ci/mmol). Samples were incubated at 37°C during 1 hour and then the reaction was stopped on ice by addition of 0.1 ml of 0.1M sodium pyrophosphate solution in TCA 5%, 250 ul of 0.5 mg/ml of yeast nucleic acid, and 3.5 ml of TCA 20%. Samples were kept 15 minutes at 4°C and then spotted onto a Whatman No. 3 disk. The radioactivity incorporated in trichloracetic-precipitable material was measured in a scintillation counter; and the results were expressed in counts per minute per ml of culture medium.

Virus Inoculation

Virus inoculation protocols varied and are described in results. The inoculata were cell-free virus or autologous infected cells. The virus strain used was HIV-2 NIH-DZ which was generously provided by R.C. Gallo. The inoculation of autologous infected cells involves *in vitro* infection of 3 day PHA-activated PBL, treated for 20 min with polybrene (2 mg/ml). The virus was added to the cell-pellet containing 10^{7} cells for 1 hour at ambient temperature and then 5 ml of RPMI 1640 15% FCS (fetal calf serum) containing 0.2% of interleukine 2 (recombinant), 10% hydrocortisone and 1% sheep anti-human interferon serum were added slowly above the cell-virus mixture. Reverse transcriptase activity was measured twice weekly until a peak of reverse transcriptase activity was obtained. The cells were washed, counted in trypan blue solution and resuspended in 2 ml of RPMI 5% FCS for inoculation. Generally, the highest RT level was obtained after 7-10 days (44).

Virus Isolation

Heparinized blood samples were centrifuged to remove the plasma. Ficoll-hypaque gradient purified PBLs were stimulated with Phythohemagglutinin (PHA) for 3 days in coculture with normal human PBL, in RPMI 1640 15% FCS supplemented with 0,2% IL-2, 10% hydrocortisone and 1% anti-human alpha interferon sheep serum. The medium was renewed twice a week and the reverse transcriptase activity measured. Positive supernatants were used to infect activated human PBLs by transmission (45), in order to obtain a high number of infected cells and to have sufficient material for DNA extraction.

Immunoassays

Anti-HIV antibody was measured using commercially available ELISA assays (Abbot, Chicago, IL). Positive sera were confirmed by Western Blot assay using HIV-II strips (Pasteur) or HIV-I strips (DuPont, Wilmington DE).

Commercial ELISA assay (Abbott) was used to detect HIV-I p24 antigen. Neutralization antibody assays were performed according to methods of Robert-Guroff (46). H9 cells were cultured with a titered suspension of HIV that had been preincubated with the test serum for one hour at 4° and then 30 mn at room temperature. HIV infection was measured by determining the percentage of positive cells 5 or 6 days later with indirect immunofluoresence (IFA). As controls for viral infection, virus was incubated with medium alone in the absence of serum and/or incubated with monkeys serum negative for anti HIV-II antibodies. Both these controls gave similar results: a range of 50 to 60 % of infected cells by IFA. The control for positive neutralization was a human serum positive for HIV-II antibody which blocked all infection (<1% infection by IFA).

IL2 Production Assay

Production of IL2 from PBL was assayed in vitro as previously reported (47). Mononuclear cells (2×10^5) were obtained by Ficoll-Hypaque technique from peripheral blood, then activated for 48 hours with 0.1% PHA. Supernatants of these activated cells (20 ul) were added to 14 day old T cell culture (CTC)(2×10^4 cells per culture). Positive donors consisted of CTC cultured in the presence of PHA only, a reference supernatant rich in IL2 and medium containing cloned IL2 (Transgene lot 11). T cell growth was evaluated 5 days later by 3H-Thymidine incorporation.

Indirect Immunofluorescence Assay (IFA)

Indirect immunofluorescence Assay was performed as already described (48). Cells were washed in 1X phosphate buffered saline, spotted on a slide, dried, and fixed acetone-methanol (1:1) for 15 minutes at room temperature. Ten microliters of human polyclonal antiserum to HIV-1 (diluted 1:50 with phosphate buffered saline) were applied and the cells were incubated for 55 minutes at 37° C. After washing (20 minutes), the fluorescein conjugated anti-human serum (1:100) was added, and the cells were incubated for 30 minutes at room temperature and then washed. A blank control contained no polyclonal human serum. H9 cells and HTLV-III infected H9 cells (H9-HTLV-III) were used as the negative and positive controls, respectively.

Lymphocyte Phenotyping

CD_4 and CD_8 T cell antigens were detected respectively by OKT_4 and OKT_8 monoclonal antibodies using the IFA or specific rosetting technique.

Specific Rosettes

T4 cells were incubated with AIDS patient sera at room temperature, washed and treated with bovine red cells linked to anti-human immunoglobulin antibodies with parabenzoquinone. Those cells that express viral antigens on their surface form rosettes and can be isolated with a micropipette under stereomicroscopy.

T-Cell Responses

Antigen dependent cell mediated proliferation was measured according to the methods of Zarling *et al.* (49), PBL were isolated by Ficoll Hypaque and suspended in RPMI supplemented with 10% heat-inactivated normal human serum. Medium (0.1 ml) containing various dilutions (10^{-1}-10^{-3}) of UV-irradiated (2500 joules) HIV-I (strains HTLV-III_B and HTLV-III_{RF}) or HIV-2 (strain NIH-DZ)[18], derived from stock suspensions of virus (1 ug ml^{-1} centrifuged from supernatants of infected H9 cells and exhibiting reverse transcriptase activity of 5-8 x 10^5 c.p.m. per ml) was added to 0.1 ml aliquots of 3 x 10^4 cells. The cells were cultured at 37°C in a 5% CO_2 incubator. Six days after stimulation, ^3H-thymidine was added for 18 hours and its incorporation into DNA was measured into a beta-counter. Cell mediated cytotoxicity assays were conducted according to the methods of Cerrotini and Brunner (50). Assays using vaccina-HIV recombinant virus (RV) infected targets were done as described by Plata et al. (51). The results are expressed as a percentage of the difference between lysing HIV antigen targets and uninfected targets.

Electron Microscopy

PBL were pelleted and fixed in a 2% glutaraldehyde solution of 0.2 M cacodylate buffer pH 7.2 for 30 minutes at room temperature. After washing, the cells were treated with 2% Os04 in cacodylate buffer. Dehydrated samples were embedded in Epoxy resin and ultrathin sections, stained with uranyl acetate in ethanol, were viewed in a Philips EM 300 and EM 201. HIV infected chimpanzee and human PBL for electron microscopy studied were obtained 4-6 days after T_4+ cells are infected with a high multiplicity of infection (MOI) of HIV. Only 15 - 30% of the cells expressed viral antigen as detected by immunofluorescence (IF) assay. Cells expressing HIV antigen were obtained by incubating the cells with human anti-HIV sera which has a high titer of anti-envelope antibodies and then rosetting the cells as described above.

Southern Blot Analysis

DNA was extracted from cultured cells after treatment of cells with a lysis buffer containing 10 mM Tris HCL pH 8, 10 mM EDTA, 10 mM NaCl 0,5% SDS and 100 ug/ml proteinase K for 2 hours at 60°C. Afterwards, a phenol-chloroform extraction was performed. The DNA was digested using HindIII and BamHI restriction endonucleases, electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Filters were hybridized using ^{32}P labeled probes (specific activity: $3X10^8$ cpm/ug of DNA). Hybridization was carried out at 42°C overnight in buffer containing 50% formamide, 5X SSC (Standard Saline Citrate), Ix Denhart's solution, 10% Dextran sulfate, 20 mM Na2HP04 pH 6.5, and salmon sperm DNA. Filters were washed under high stringent conditions (2X SSC, 0.1% SDS) for 3 hours at 65°C and autoradiographed (30,37). The specificity of these results was supported by the absence of any hybridization using DNA samples obtained from HIV-I infected cells.

Plasmid DNA Manipulations

Bacterial plasmid DNA preparation, enzyme digestions and agarose gel electrophoresis were carried out by standard techniques (52). Plasmid DNA was used to transform Escherichia coli (E. coli) DH5 cells. Ampicillin resistant transformants were screened by colony hybridization with a ^{32}P-gp120 probe (pAD.ENV(160)ND1) or on the basis of size and restriction endonuclease digestion pattern (pAD.ENV(160)). Restriction endonucleases were used according to the manufacturer's recommendations.

The plasmid, β-actin 2000, containing 2108 bp of β-actin gene sequence cloned into the HindIII site of pBR322 was kindly provided by Steve Hughes (NCI-FCRF, Frederick, MD). Ad2 DNA was purchased from BRL (Gaithersburg, MD).

Cell Culture and DNA Transfection

HeLa and COS-1 cells were obtained from G. Crouse (Emory University, Atlanta, GA); 293 cells were provided by T. Shenk (Princeton University, Princeton, NJ); and CV-1 cells were obtained from G. Pavlakis (NCI-FCRF, Frederick, MD). All cell lines were maintained as monolayers in Dulbecco's modified minimal essential medium (DMEM) with 1 gm/L glucose (GIBCO Laboratories) supplemented with 10% fetal calf serum (GIBCO) and were passaged by disassociation with trypsin-EDTA (GIBCO).

Cell monolayers at approximately two-thirds confluency were used for transfection. Culture medium was changed 3 hours before the addition of DNA. Calcium

phosphate-DNA precipitates were prepared according to the methods of Graham and Van der Eb (53) and Wigler et al.(54) and used at a concentration of 2 ug DNA/35 mm dish unless otherwise noted. The precipitated DNA was allowed to remain in contact with the cells for 6 hours, after which time the DNA-containing medium was removed and replaced with fresh culture medium. For transient expression experiments, expression time was 48-72 hours unless otherwise stated.

Viruses and Virus Infection

Ad2 (VR 846) and Ad 31 (VR 1109) were obtained from the American Type Culture Collection and propogated in HeLa cell monolayers. Infected cells and culture medium was frozen and thawed three times and the cell-free supernatant from a 3000 rpm centrifugation was used as virus stocks. Wild type (wt) Ad2 and rAd2 were plaqued on 293 and CV-1 cell monolayers. Virus dilutions were pepared in phosphate buffere saline (PBS) with 2% heat-inactivated fetal calf serum and absorbed to PBS-washed cell monolayers for 90-120 minutes at 37°C. Infected cells were overlayed with modified 2X Eagle medium (GIBCO) containing 4% heat-inactivated fetal calf serum and 0.7% Noble agar (Difco Laboratories) supplemented with 10% tryptose phosphate (GIBCO) and 25 mM $MgCl_2$. Additional plaque assay medium was added at 3 day intervals post-infection (PI) with 0.02% neutral red (GIBCO) incorporated into the final overlay for plaque visualization. Plaques were scored at 7-8 days PI on 293 cells and at 10-14 days PI on CV-1 cells.

DNA Isolation and Analysis

Low molecular weight viral DNA was isolated from Ad-infected HeLa cells using a modification of the method of Hirt(55) in which 0.5 mg/ml pronase was added to the sodium dodecyl sulfate (SDS) lysis solution. Total DNA was isolated (56) by lysis of virus-infected cells with 0.25 mg/ml proteinase K and 1% SDS in 0.15 M sodium chloride/0.015 M sodium citrate (1X SSC). Dilutions of the isolated DNA were applied to Zeta-Probe nylon membranes (Bio-Rad Labs.) using a Schleicher & Schuell dot blot manifold. For Southern blotting (57), the DNA was digested with appropriate restriction enzymes, electrophoresed through 0.7% agarose gels and transferred to Zeta-Probe membranes using an alkaline blotting procedure with 0.4M sodium hydroxide (58). Blots were probed with DNA fragments labeled with ^{32}P by nick translation. High stringency hybridizations were initially carried out in 50% formamide at 43° and, subsequently, in 0.27M sodium chloride/0.015M sodium phosphate/1.5mM ethylenediamine tetraacetic acid (1.5X SSPE), 1% SDS, 0.5% nonfat powdered milk with 10%

dextran sulfate at 68°C. The filters were washed at room temperature with solutions of increasing stringency concluding with a 55°C wash in 0.1X SSC-1% SDS. Autoradiograms of these filters were exposed on X-ray film (type XAR, Eastman Kodak Co.) with a DuPont Cronex intensifying screen for 4-18 hours at -70°C.

Screening by Dot Blot Hybridization

HeLa or 293 cells growing in 96 1/2-well plates (Corning Glass Works) were infected with dilutions of wild type and recombinant virus mixtures or with plugs removed from the agar overlaying cell monolayers on which these mixtures had been plaqued. The plates were monitored microscopically for the development of viral cytopathology. When the majority of wells showed viral cytopathology at a level of $\geq$ 75% infected cells, the culture medium was removed and stored at -20°C as a source of virus. Plates containing the infected cell monolayers were held at -70°C until processed by the addition of 0.2ml 0.3M NaOH, heating at 100°C for 10 minutes, and chilling on ice prior to transfer to Zeta-Probe membranes using the dot blot manifold. Hybridization with ^{32}P-labeled probes was carried out as described in the previous section.

RNA Isolation and Analysis

Total cellular RNA was isolated using the guanidine thiocyanate procedure (4) followed by centrifugation through a CsCl cushion (4) or by phenol-chloroform extraction and ethanol precipitation (personal communication, S. Hughes, NCI-FCRF, Frederick, MD). Poly(A)$^+$ RNA was purified by passage over oligo(dT)-cellulose. RNA dilutions were applied to Zeta-Probe membranes using the dot blot manifold. For Northern blotting, RNA samples were electrophoresed on 1% agarose formaldehyde gels(52), transferred to Zeta-Probe membranes and hybridized to nick-translated DNA fragments by standard techiques.

DNA Probes

The DNA probe corresponding to HXB2 HIV gp120 nucleotides 6159-7568 was derived by BamHI digestion of plasmid pAD.MLPgp120. The probe corresponding to SV40 T Ag sequences as obtained by digesting plasmid pAD.ENV.ND1 with SalI. Adenovirus fragments used as probes were derived from EcoRI digestion of Ad2 DNA. The fragments were separated on 1% agarose gels and recovered by electroelection using a Schleicher & Schuell elutrap. All fragments were uniformly labeled by nick translation with β-^{32}P-ATP (3000Ci/mmol, DuPont) by standard procedures (52).

Immunocytochemical Staining

Cells were grown in disposable 8-chamber glass tissue culture slides (Lab-Tek) pretreated with 50% fetal calf serum. Virus infections were carried out as described above. Cells were fixed by the addition of one-half volume freshly prepared 3:1 methanol-glacial acetic acid to the culture medium; and, after aspiration of the medium-fixative, by immersion in -20°C acetone for 30 minutes. For alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining, the slides were reacted with a 1:50 dilution of mouse monoclonal anti-gp120 (Biotech Research Labs, Inc.) and, subsequently, with second and third antibodies and substrate solution from the Biotech Research Labs, Inc. immunocytochemical kit for HIV-I p24 detection. Either polyclonal goat anti-gp160 (DuPont) or the mouse monoclonal anti-gp120 were used as primary antibodies at 1:500 and 1:50 dilutions, respectively, for horseradish peroxidase (HRP staining) with peroxidase conjugated rabbit anti-goat or anti-mouse immunoglobulins (Dako Corp.) as second antibody. Color was developed with freshly prepared 0.01M o-dianisidine and hydrogen peroxide. When necessary to increase contrast, cells were counter-stained with 5% fast green.

E. Results and Discussion

1. Persistent HIV-2 Infection of Rhesus Macaque, Baboon and Mangabeys

Prior to inoculating monkeys with HIV-2, the monkeys were tested for the presence of retrovirus by co-cultivating with Hut 78 and human normal PBL. No virus was detected and none of these monkeys were seropositive to HIV as determined by Western Blot. Six monkeys were challenged with $HIV\text{-}2_{NIH\text{-}DZ}$ as shown in Table 1. All monkeys remain persistently infected as determined by virus isolation (Table 2). Southern blot analysis of DNA extracted from PBL and digested with HindIII or BamHI restriction enzymes shows that all viral DNA is integrated into cellular DNA (results not shown).

The restriction patterns were different in each case. After BamHI digestion, the pattern of two bands around 23 and 11 Kb are detected for M2, three fragments of 23 Kb, 11.4 Kb, 1.8 Kb for B21 and 13 Kb, 8 Kb, 5 Kb for C39 and five bands of about 23 Kb, 8.5 Kb, 4 Kb, 2.3 Kb, 1.8 Kb for C34 (data not shown). Also, HindIII digestion yielded different restriction DNA patterns showing the presence of one band at 11 Kb for M2, three fragments around 13 Kb, 7.8 Kb, 7.8 Kb, 5 Kb for C25, C34 and C39 or 13 Kb, 5.8 Kb, 2.4 Kb for B21. The samples generated a smear of high

molecular weight DNA representing polyclonally integratred proviral DNA plus one band around 10 Kb corresponding to non-integrated viral DNA.

C24, C34, C39m developed a strong antibody response (Table 2). Peak antibody response in C24, C34 was ninth month of infection. Antibody response of C39 was bimodal, peaking at 2 months and rising again at 8 months. B21 and C25 developed a modest anti-HIV response peaking at the fifth month past inoculation. By Western Blot analysis, antibodies to gp105, and gp140 appear earliest followed by antibodies to other protein (p56, p41, p26, p16). No anti-HIV was detected in M2. p24 HIV antigen can be detected in sera of M2 and C24 at 11 month and 5 and 6 months, respectively (Table 2).

Infected animals have not exhibited any immunodeficiency.

2. Vaccination of Chimpanzees With Vaccinia-HIV$_{gp}$160 Candidate Vaccine

Five chimpanzees have been vaccinated with a vaccinia-HIV $_{gp}$160 recombinant virus (Table 3). All the chimps responded with anti-envelope antibody in Western Blot Test. In the cell mediated cytotoxicity (chromium release) assay, target cells were EBV transformed autologous B cells infected with vaccinia-HIV $_{gp}$160 recombinant virus. HIV specific cytotoxic T cells were demonstrated in C1013, C1019, C1017 and C1131. All the chimpanzees' PBL's responded to HIV antigen in antigen induced cell proliferation assay. Thus, all the vaccinated chimpanzees had a good humoral and cellular immune response to HIV.

The vaccinated chimpanzees will be challenged with HIV when development of PCR technology in Dr. Zagury's lab is completed. This will allow quantitation of HIV in PBL of chimps challenged with HIV.

3. Immunoelectron Microscopic Study of HIV-infected T4 Cells

When T4+ enriched cells are infected with HIV, approximately 80% of T4 cells had HIV antigens on their cell surface during the first hour after infection. During the efferent phase (days 2-6) of infection, only 10-30% of T4 cells were positive for viral proteins. When cells expressing HIV viral antigens were isolated by rosette formation and examined by electron microscopy, the cells do not show ultrastructural degeneration. To determine

if HIV antigens can be detected at the surface of cells not exhibiting viral particles, human and simian cells were incubated with AIDS patient serum (containing anti-gp160). After washing 3-4 times the cells were incubated with autoprobe EM reagent. The cells were fixed and sections examined by EM. A gold labeling showed viral antigen at the cell surface in human PBL, simian PBL and H-9 HTLV-III cells not expressing viral particles in the cytoplasm or nucleus. Thus cells expressing HIV antigens and their cell surface prior to viral particle formation exist and could theoretically be destroyed by cellular or humoral immune response in vivo. This would suggest that a successfuly vaccine does not have to totally prevent infection with HIV and that HIV seropositive individuals could theoretically be cured of their infection through appropriate chemo- and immunotherapies.

4. Origin and Relationship of Primate Retrovirus

A field laboratory and a virgin forest near Lubutu, Zaire has been established. Serum samples from 283 primates were collected. Results are shown in Table 4. Five species of primate, Cercopithecus (Wolfi) denti, Cercopithecus hamalyni, Cercopithecus hoesti, Cercopithecus mitis stholammoni and Cercopithecus phosesti, phoesti, have been shown to be infected with retrovirus. In some cases, PBL and/or spleen were collected and frozen in liquid nitrogen. The cells have been shipped to France where they will be cocultured with PHA-stimulated PBL to determine if retroviruses can be isolated. 18 monkeys were captured alive and shipped to Kinoshasa. PBL from these monkeys will be EBV transformed so that they can be used as targets in cellular cytotoxicity assays.

5. Selection of Recombinant HIV-1 env/adenovirus 2 on the Basis of Plaque Size on Monkey Cells

Putative HIV-1 gp120/Ad2 virus isolates derived from contransfection of pAD.ENV.ND1 and Ad2 DNA into 293 cells during Year 1 (1987 Annual Report) were purified by three plaquings on CV-1 cells. At each passage, the largest plaques ($\geq$ 3 mm diameter) were selected based upon the premise that SV40 sequences in the plasmid pAD.ENV.ND1, which was used in the original transfection, would enhance the replication of the recombinant virus in monkey cells. Following plaque purification, virus stocks to be used for analyzing the molecular biology and growth characteristics of 11 isolates were grown in HeLa cells.

Viral DNA was (55) extracted from infected HeLa cells and blotted onto nitrocellulose membranes. The DNA blots were hybridized at 42°C in 50% formamide with nick-translated ^{32}P-gp120 and ^{32}P-OB.ND1 probes to detect the presence of HIV and SV40 sequences, respectively. Of 9 viral isolates analyzed in this manner, 3 hybridized with the OB.ND1 probe but there was no specific hybridization of the viral DNA to the gp120 probe.

Viral DNA was digested with SmaI and separated by electrophoresis through a 0.8% agarose gel. The DNA fragments were transferred by blotting (57) to nylon membranes and hybridized with ^{32}P-gp120 and -OB.ND1 probes at 42°C in 50% formamide. The three virus isolates that were positive in the dot blot assay contained an approximately 9000 bp SmaI fragment which hybridized to the OB.ND1 probe. No other viral isolates or wt Ad2 SmaI fragments hybridized to OB.ND1. No hybridization to the gp120 probe was observed.

Digestion of all viral DNA isolates with BamHI, EcoRI, KpnI, and HindIII as well as SmaI resulted in fragments indistinguishable from those generated from wt Ad2 DNA in ethidium bromide-stained agarose gels. Fragment sizes corresponded to published values for Ad2 restriction enzyme digests. Southern blotting of DNA from two positive viral isolates and wt Ad2 digested with 4 restriction enzymes is shown in Figure 1. The ^{32}P-OB.ND1 probe reacted with fragments of approximately 9000 bp in SmaI and BamHI digests, 8000 bp in HindIII digests and 4000 bp in KpnI digests of the recombinant viral DNA isolates. All three positive viral isolates were derived from 293 cells transfected with pAD.ENV.ND1 plus Ad2 DNA and enriched by passage through CV-1 cells prior to plaque purification. We conclude that during passage on CV-1 cells, the putative recombinant virus lost all sequences except those necessary for growth in monkey cells. This essential SV40 fragment may be as small as 14 bp (60) and, thus, would not be detectable by restriction enzyme digests of crude DNA samples. Further restriction enzyme analysis is necessary to determine the site and extent of recombination of these SV40 sequences into the Ad2 genome.

A subsequent attempt was made to isolate recombinant HIV-1 gp120/Ad2 using a screening procedure rather than selection based upon the presence of SV40 sequences. Cell-free virus supernatants from the original transfection before and after passage through CV-1 cells for enrichment

(1987 Annual Report) were plaqued on CV-1 and 293 cells. Dot blot hybridization analysis of HeLa cells infected with 92 plaque picks yielded no positive isolates with the gp120 probe and 42 positive isolates with the OB.ND1 probe. Approximately 40% (17/44) of the virus isolates derived from CV-1 cell passage contained sequences which hybridized with the SV40 probe compared to 10% (5/48) of the plaque isolates from virus that had not been passaged. These results reinforced our conclusion that continuous passage through CV-1 cells exerts a selective pressure for recombinant Ad2 that contain SV40 T Ag sequences but not the HIV gp120 sequences.

6. Construction of Recombinant HIV-1 env/ Adenovirus 2

When it became apparent that selection based upon plaque size on monkey cells was not an effective means to isolate recombinant HIV-1 env/Ad2, a decision was made to repeat the transfection and screen the resulting progeny virus with a probe specific for the HIV-1 gp120 sequence. Ad2 DNA and plasmids pAD.ENV.ND1 or pAD.ENV, from which the SV40 sequences were deleted, were cotransfected into 293 and HeLa cells. Alternatively, 293 and HeLa cells were transfected with one of the plasmids and 18 hours later were infected with wt Ad2 or Ad31. At 5 days (293) or 6 days (HeLa) after transfection, when 75-100% of the cells showed evidence of viral cytopathology, the cells and culture medium were harvested.

DNA dot blot analysis with nick-translated ^{32}P-gp120 and -OB.ND1 probes demonstrated that all cells transfected with either pAD.ENV.ND1 or pAD.ENV contained DNA which hybridized with the gp120 probe. Only those cells that had been transfected with pAD.ENV.ND1 hybridized with the OB.ND1 probe. These results, indicative of the presence of the plasmids in these cells, demonstrated that the transfection had been successful.

Virus-containing cell-free supernatants from the transfected 293 cells were used to infect CV-1 and HeLa cells. When the majority of these cells showed viral cytopathology, the cells were harvested and a portion used for DNA dot blot analysis. The DNA from both HeLa and CV-1 cells infected with virus stocks derived from the transfection with pAD.ENV.ND1 or pAD.ENV hybridized with the ^{32}P-gp120 probe. The ^{32}P-OB.ND1 probe hybridized only to DNA from HeLa cells infected with virus stocks derived from 293

cells transfected with pAD.ENV.ND1. On the basis of these results, we conclude that the gp120-containing plasmids were successfully introduced into 293 cells and that this transfection resulted in Ad2 virus stocks which contained the desired HIV env recombinant. Interestingly, DNA from CV-1 cells infected with virus stocks derived from 293 cells transfected with either plasmid hybridized to the OB.ND1 probe raising the question as to the origin of the hybridizing SV40 sequences.

To verify these observations, HeLa cells were infected with virus-containing cell-free supernatant from the 293 cells used in the original transfection as well as the secondary infection of HeLa and CV-1 cells. Total DNA was isolated from the infected cells and digested with BamH1 prior to electrophoresis through a 1% agarose gel. Ethidium bromide staining revealed the expected 14.3, 10.7, 6.2 and 4.7 kb BamH1 fragments of wt Ad2 (Fig. 2B). The DNA fragments were transferred to a nylon membrane by blottin- and hybridized with a ^{32}P-gp120 probe (Fig. 2A). A BamH1 band of approximately 1400 bp was detected in the DNA from cells infected with virus from 4 of the original 293 cell transfectants, 4 of the secondarily infected HeLa cells and 3 of the secondarily infected CV-1 cells. This band corresponds in size to the 1410 bp BamH1 gp120 fragment in the original plasmids used to derive the recombinant HIV env/Ad2. A larger band of approximtely 2500 bp length which was present in several of the DNA samples also hybridized to the gp120 probe. The identity of this band has not been determined. We conclude that the virus progeny derived from the transfection of 293 cells with gp120-containing plasmid sequences and Ad2 DNA yielded an Ad2 stock in which some virus contained gp120 sequences. In addition, the gp120 sequence was retained through one passage in both HeLa and CV-1 cells.

7. Selection of Recombinant HIV-1 env/Adenovirus 2 by Screening for the Presence of gp120 Sequences

In order to isolate the recombinant HIV env/Ad2 from wt Ad2, the cell-free culture supernatant from transfected 293 cells was plaqued on CV-1 and 293 cells (Table 5). Plaque titers were approximately 2-2.5 logs higher in 293 cells; however, average plaque size on both cell lines was similar ranging from 1-2.5mm diameter. For selection, HeLa cells grown in multi-well plates were infected with plaque picks and monitored for development of CPE. Hybridization of the infected HeLa cells with a ^{32}P-gp120

probe was used as a means to select Ad2 plaque isolates that contained the gp120 sequence. Screening by dot blot hybridization of 792 plaques picked from 293 cells and 336 CV-1 plaque picks yielded two isolates which reacted positively with the ^{32}P-gp120 probe. One isolate was derived from 293 cells transfected with the plasmid pAD.ENV.ND1 and subsequently infected with wt Ad31. A Southern blot of DNA isolated from HeLa cells infected with a stock of this isolate did not hybridize to the ^{32}P-gp120 probe. Several attempts to reisolate this putative Ad31 recombinant virus have been unsuccessful. The second isolate, G3-8, was obtained from CV-1 cell plaquing of the virus stock derived from 293 cells cotransfected with pAd.ENV.ND1 and Ad2 DNA. The characterization and enrichment of this isolate is discussed in the next section.

As an alternative to screening individual plaques as a means to isolate recombinant viruses, an attempt was made to select by dilution for virus mixtures enriched for the desired recombinant. The cell-free culture supernatant from transfected 293 cells was diluted so that 10 ul inoculum containing approximately 1000 PFU was added to each well of a 96-well plate of HeLa cells. Six positive isolates out of 360 were detected by hybridization with ^{32}P-gp120. Three of these isolates were derived by recombination of Ad2 DNA with pAD.ENV.ND1 and three by recombination with pAD.ENV. An initial attempt to purify the recombinant virus from these isolates by screening plaque picks was unsuccessful. Therefore, dilutions of the isolates ranging from undiluted to 10^{-4} were used to infect 96-well plates of HeLa cells for screening. Three possible isolates have been obtained by this method; one of which was derived from 293 cells transfected with pAD.ENV and the others from transfection with pAD.ENV.ND1. Southern blot analysis of DNA isolated from HeLa cells infected with these isolates is being used to verify the identity of these putative recombinant HIV gp120/Ad2.

8. Characterization of Recombinant HIV-1 env/Adenovirus 2

A stock of the putative recombinant G3-8 isolated from a CV-1 cell plaque was grown in HeLa cells and titered at 3.7×10^9 PFU/ml by plaquing on 293 cells.

HeLa cells were infected with approximately 200 PFU/cell of G3-8 stock. At 4 days PI when all cells showed maximum cytopathology, the infected cells were harvested and total DNA extracted with SDS-pronase. The DNA was digested with BamHl and

the resultant fragments separated by electrophoresis through a 1% agarose gel. After transfer to a nylon membrane, the restriction enzyme-digested DNA fragments were hybridized first to a ^{32}P-gp120 probe and, subsequently, after washing the membrane, to a ^{32}P-OB.ND1 probe. Both the 1400 base pair BamH1 gp120 fragment as well as a larger fragment (approximately 9500 bp) containing SV40 sequences which reacted with the OB.ND1 probe were detected (Fig. 3).

HeLa cells infected with 25 PFU/cell of G3-8 were analyzed for the presence of gp120-specific RNA over a period of 30 hours PI. RNA was extracted from the infected cells at 6 hour intervals using 4M guanidinium and was pelleted through a CsCl step gradient to remove contaminating DNA and protein. Dilutions of total RNA were blotted onto nylon membranes and hybridized to a ^{32}P-gp120 probe (Fig. 4). No gp120-specific RNA was detected early in infection (0-12 hr), but could be detected in as little as 1 ug of RNA isolated from cells at 24 and 30 hr PI. The 18 hr RNA sample was lost, so the point at which accumulation of gp120 RNA first occured is not known. Northern blot analysis of this RNA showed a faintly hybridizing band of approximately 3400 bp size in the 24 and 30 hr RNA samples.

AdG3-8-infected HeLa cells grown in chamber slides were analyzed for the presence of gp120 protein by immunocytochemical staining procedures using either a mouse monoclonal antibody directed against HIV gp120 (Fig. 5) or a polyclonal antibody directed against gp160 with an alkaline phosphatase-anti-alkaline phosphatase or horseradish peroxidase detection system. HeLa cells infected with the putative HIV env/Ad2 recombinant virus reacted positively with both primary antibodies, whereas, HeLa cells similarly infected with wt Ad2 did not demonstrate this staining reaction. Uninfected control cells were also unstained.

Western blot analysis of HeLa cells infected with G3-8 was carried out in the laboratory of Dr. Steve Alexander, Biotech Research Labs, Inc. The initial attempt to demonstrate gp120 in AdG3-8-infected HeLa cell extracts was impossible to evaluate because the human and goat antibody used to detect HIV-specific proteins cross-reacted with a large number of proteins in both G3-8 and wt Ad2-infected cells.

We conclude that Ad2 isolate G3-8 represents a recombinant HIV env/Ad2 virus on the basis of the folowing results: 1, the presence of gp120 and SV40 OB.ND1 sequences in DNA isolated from HeLa cells infected with this virus; 2, the accumulation of gp120-specific RNA sequences late in the infection consistent with the use of the Ad major late promoter for initiation of transcription; and 3, the immunocytochemical detection in AdG3-8-infected cells of proteins that react with both a monoclonal and a polyclonal antibody directed against the HIV-1 major envelope protein. However, this virus isolate is not pure but is, rather, a mixture of wt and recombinant at an apparent ratio $\geq$ 100:1.

This estimate is based upon results from two attempts to recover recombinant virus isolate G3-8 from a second plaque purification on 293 or CV-1 cells (Table 6). Dot blots of HeLa cells infected with isolated G3-8 plaques did not hybridize strongly with either a gp120 or OB.ND1 probe; however, intense hybridization was observed with a probe derived from the EcoRI fragment E of Ad2 which includes the Ad E3 region to be replaced with gp120 sequences in the recombinant virus. Therefore, it must be concluded that the majority of viruses which comprise the G3-8 stock do not contain either HIV gp120 or SV40 T antigen sequences.

9. Construction of HIV-1 gp160 Plasmids

The plasmid pENV(160)ND1 constructed during Year 1 (1987 Annual Report) was digested with PvuI, NruI and SacII to yield a 5400 bp fragment containing the Ad late promoter driving the HIV gp160 gene and SV40 sequences (OB.NDI) under control of the SV40 promoter. pUC-AD2 was digested with BglII to remove 2635 bp of Ad sequence leaving 1051 bp at the 5' end and 1523 bp at the 3' end to facilitate recombination. After treatment of both with Mung bean nuclease, the 5400 bp fragment and BglII-digested pUC-Ad2 vector were blunt end-ligated to yield pAD.ENV.(160) ND1.

To obtain a plasmid construct free of SV40 sequences, pAD.ENV.(160) ND1 was digested with SalI to remove the OB.ND1 fragment. After ligation to regenerate an unique SalI site, the resultant plasmid, pAD.ENV(160), contained the HIV gp160 gene under the control of the Ad major late promoter flanked at the 5' and 3' ends by Ad sequences necessary to facilitate recombination into wt Ad2.

The plasmid DNA was used to transform E. coli DH 5 cells. Ampicillin-resistant transformants were screened by colony hybridization with a ^{32}P-gp120 probe [pAD.ENV.(160)ND1] or on the basis of size [pAD.ENV(160)]. The plasmid constructs were verified by mapping with diagnostic restriction enzymes (Fig. 7).

10. Transient Expression of HIV-I gp160 Plasmids

To determine if gp160 sequences contained in the vaccine vectors were expressed as RNA and protein, experiments analyzing transient expression of these plasmids were carried out. Two human (293, HeLa) and two monkey (COS-1, CV-1) cell lines were transfected with the plasmids pAD.ENV(160)ND1 and pAD.ENV(160) containing HIV gp160 sequences under the control of the Ad major late promoter. Dot blots of total RNA as well as oligo dT-selected poly(A)$^+$ RNA isolated from these cells after a 48 hour period of transient expression were positive when hybridized to a ^{32}P-gp120 probe. However, no specific gp160 protein expression could be detected by immunocytochemical staining of the transfected cells using polyclonal antibody directed against gp160 and a horseradish peroxidase-conjugated second antibody.

In a subsequent experiment, 293 and COS-1 cells were cotransfected with the vaccine plasmid pAD.ENV(160) and the plasmid pCV-1 that contains HIV-1 regulatory genes tat and rev under transcriptional control of the HIV-I LTR. Dot blot hybridization of total and poly(A)$^+$ RNA with a ^{32}P-gp120 probe demonstrated higher levels of gp160 RNA in the cotransfected cells than in cells transfected with pAD.ENV(160) alone. This result is of interest because the coding sequence for these regulatory genes are also present in pAD.ENV(160) where they are under the control of the Ad major late promoter. Analysis of these samples for the presence of gp160 protein has not been completed; however we conclude that the gp160 sequences contained in these plasmids are expressed as RNA in mammalian cells.

F. Conclusions

The immune response of Cercos, baboons and chimpanzees to HIV has been monitored in infected and vaccinated animals. Anti-HIV antibody was characterized for its ability to neutralize HIV virus and its reactivity in HIV Western blot assay. Antigen induced cellular proliferation was also studied. Preliminary experiments to establish protocols for measuring cytotoxic T cell

responses in primates were conducted. Standardization of the conditions leading to reproducible infection of Cercos monkeys with HIV established that this species could be infected most reproducibly by intravenous administration of HIV 2. 283 primates of various species were tested for "natural" infection with HIV. Seropositive animals of 5 species of monkeys were demonstrated.

Isolation of recombinant HIV-1 gp120/Ad2 by selection of large plaque isolates on CV-1 cells, as originally proposed, has resulted in the recovery of recombinant viruses that have retained the SV40 T Ag sequences necessary for replication on CV-1 cells but have lost (or never contained) the gp120 sequences. For this reason, it has been necessary to replace this selection procedure with screening procedures involving analysis of plaques derived from, or cells infected with, dilutions of the mixture of wt and recombinant progeny virus resulting from the transfection of cells with the vaccine plasmid constructs and Ad2 DNA. This approach, although tedious, has proven to be useful, as four putative recombinant HIV-1 gp120/Ad2 isolates have been obtained. One of these isolates, G3-8, has undergone preliminary characterization which supports the premise that G3-8 is a true recombinant HIV-1 gp120/Ad2. This conclusion is based upon the viral DNA content and the presence of gp120-specific RNA and protein in infected cells. However, stocks of these virus isolates are not pure but consist of a mixture of wt and recombinant adenoviruses. Additional purification is essential before valid characterization of the various HIV-1 gp120/Ad2 isolates can be carried out.

G. Recommendations

1. Testing of recombinant HIV-1 gp120/Ad2 in non-human primates

Serious consideration should be given to establishing protocols for initial testing of recombinant HIV-1 gp120/Ad2 in monkeys. This must include decisions as to the genus (*Cercopithecus, Macaca*), the number of animals to be tested, the route of administration (respiratory, enteric), the quantity of virus inoculum, and the appropriate controls (wt Ad2 virus, recombinant SV40 T Ag/Ad2 virus). In addition to monitoring the animals for immunity to HIV-1 and protection from subsequent infection as described in the original proposal, the fact that the animals are infected with the recombinant virus must also be verified. Therefore, these animals should be monitored to establish the occurrence and duration of the HIV-1 gp120/Ad2 infection, to determine whether shedding of the recombinant Ad occurs, and to analyze the immune response

to the antigenic components of the adenovirus itself.

2. Modified Ad2 serotype for human vaccine purposes

Ad2 hexon induces primarily group-specific CF antibody which usually disappears rapidly after acute infection (61). In contrast, Ad2 fibers are largely responsible for type-specific neutralizing antibody (43) which is long lasting (61) and may be of greater concern in the development of a live Ad2 vaccine. We recommend that the feasibility of exchanging Ad2 fiber coding sequences with that of a less common Ad serotype be considered. If the decision is made to continue with the proposed exchange of hexon coding sequence, we recommend the use of a serotype other than "oncogenic" Ad31 because of possible regulatory complications.

3. Simian immunodeficiency virus (SIV) and HIV-2 model system for AIDS

Proposed studies in which HIV-1 gp120/Ad2 is administered to Cercos monkeys are designed to demonstrate that subclinical infection with the recombinant Ad is established with attendant development of humoral and cell-mediated immunity. However, these animals cannot be reproducibly infected with HIV-1 to determine if this immune response might be protective. We recommend that similar recombinant Ad constructs using SIV *env* gene sequences be considered for use in macaques, which develop AIDS-like disease after infection with virulent SIV strains. This would provide a valid and much less expensive animal model system in which to test the efficacy of live Ad vaccines. Similarly, when the HIV-2 Cercos monkey model is established, rAd containing HIV-2 *env* genes can be administered and the monkeys challenged with HIV-2.

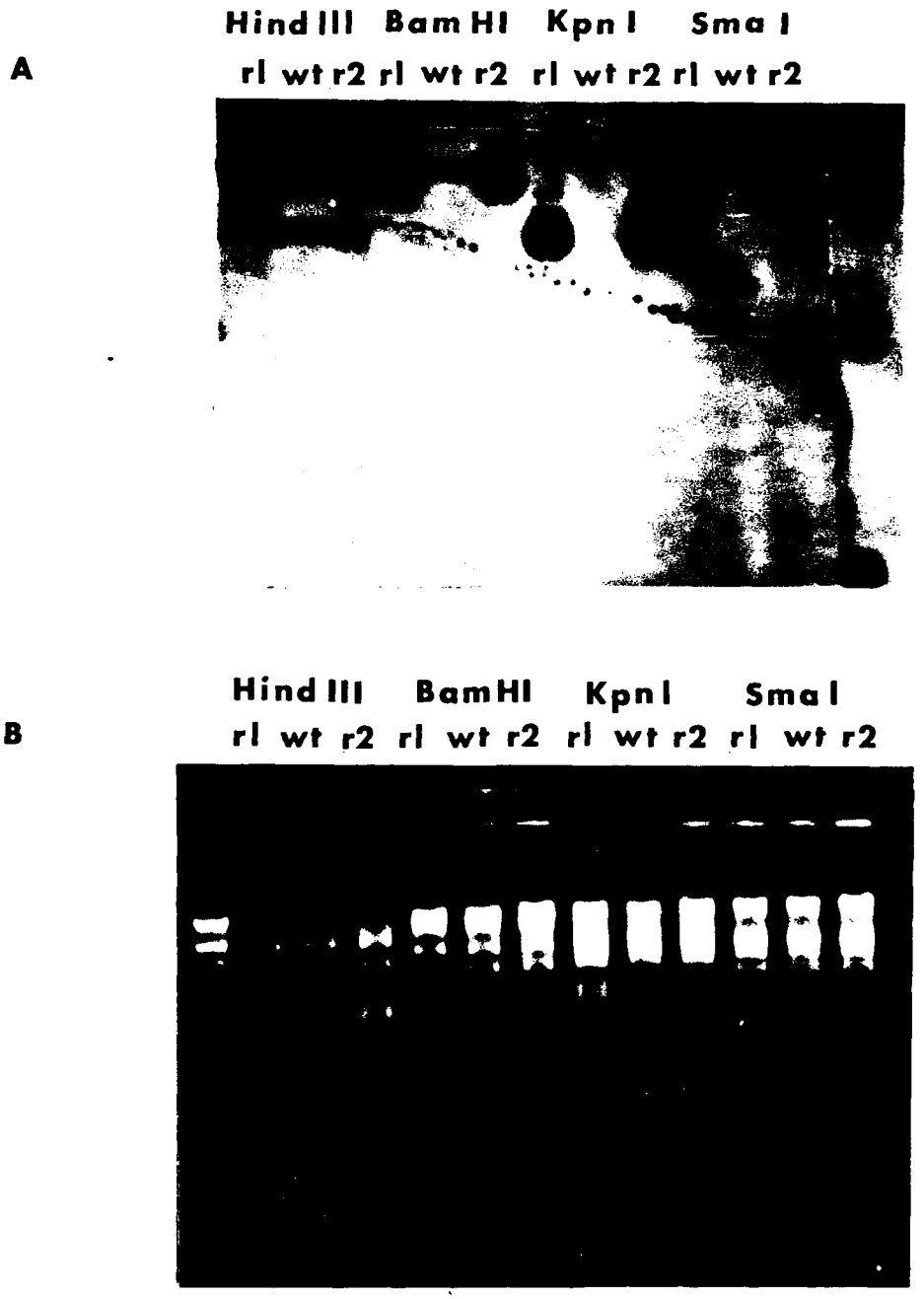


Fig. 1. Southern blot hybridization of recombinant adenoviruses isolated on the basis of plaque size on CV-1 cells. Viral DNA isolated from HeLa cells infected with wt Ad2 (wt) or with two recombinant adenovirus (r1,r2) was digested with HindIII, BamHI, KpnI and SmaI, electrophoresed in an agarose gel, transferred to a nylon membrane, and probed with nick-translated ^{32}P-OB.ND1 to detect SV40 sequences. A. Autoradiogram of blotted and hybridized DNA. B. Ethidium bromide stained gel used for the Southern blot. DNA fragment sizes corresponded to published values for Ad2 DNA digested with these restriction enzymes.

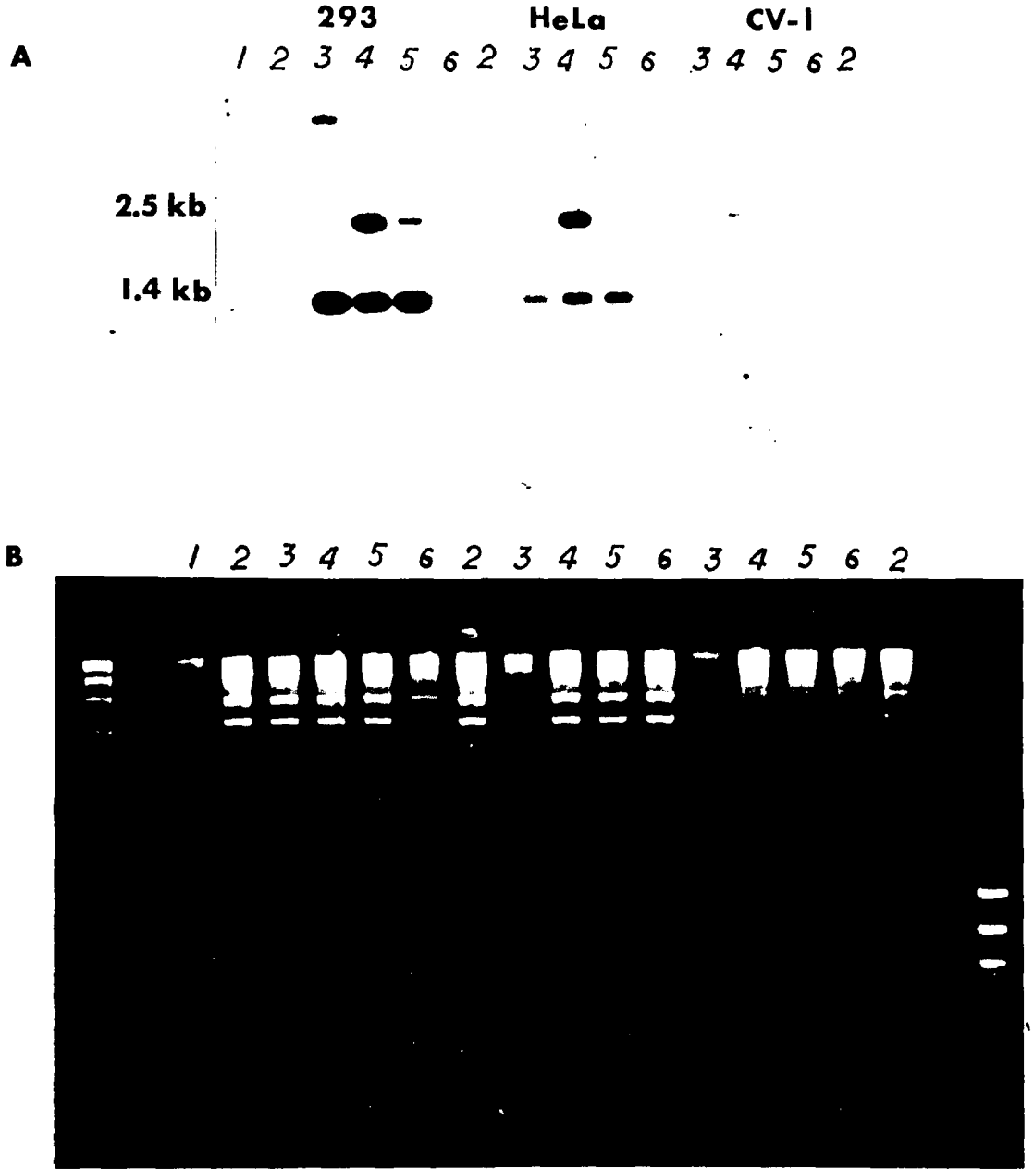


Fig. 2. Southern blot hybridization of DNA from cells infected with putative recombinant HIV-1 env/Ad2. DNA isolated from HeLa cells infected with virus stocks derived from the original transfection (293) and secondary infection of HeLa and CV-1 cells was digested with BamHI, electrophoresed in an agarose gel, transferred to a nylon membrane, and hybridized with a nick-translated ^{32}P-gp120 probe. The derivation of samples are: 1, pAD.ENV control; 2, Ad2 virus; 3,5, pAD.ENV.ND1 + Ad2 DNA (2 ug) and (1 ug); 4, 6, pAD.ENV + Ad2 DNA (2 ug) and (1 ug). A. Autoradiogram of blotted DNA hybridized to ^{32}P-gp120. B. Ethidium bromide stained gel used for Southern blot.

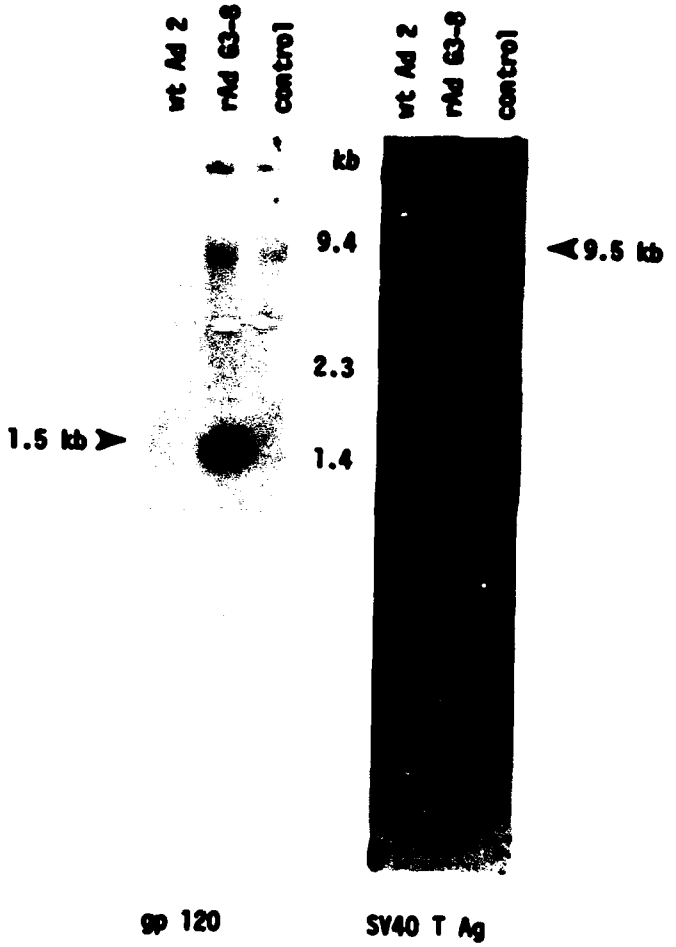


Fig. 3. Southern blot of DNA from HeLa cells infected with wild type and recombinant adenoviruses. DNA was isolated from uninfected HeLa cells (control) and HeLa cells infected with wt Ad2 or putative recombinant HIV-1 gp120/Ad2 (AdG3-8). After digestion with BamHI, the DNA fragments were electrophoresed in an agarose gel, transferred to a nylon membrane, and hybridized with nick-translated ^{32}P-gp120 and -OB.ND1 probes. Both the 1.4 kb BamHI gp120 fragment as well as a larger fragment (approximately 9.5 kb) that hybridized to the SV40 probe were detected.

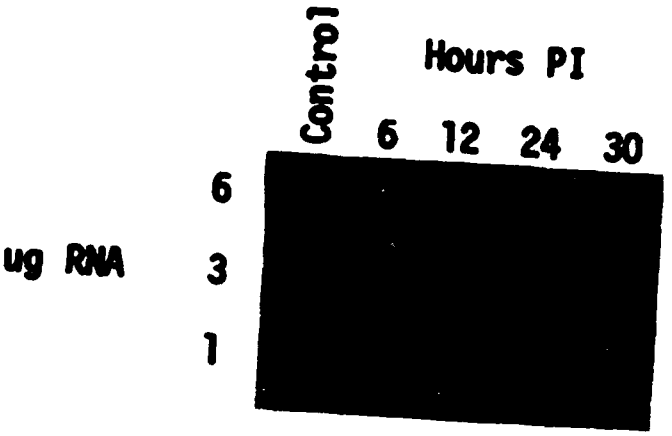


Fig. 4. Dot blots of RNA from HeLa cells at various times following infection with recombinant HIV-1 gp120/Ad2. Dilutions (6, 3 and 1 ug) of total cellular RNA derived from HeLa cells at 6 hr (2), 12 hr (3), 24 hr (4) and 30 hr (5) following infection with AdG3-8 were applied to a nylon membrane and hybridized to a nick-translated ^{32}P-gp120 probe. Lane 1 contains dilutions of total RNA extracted from uninfected HeLa cells. No gp120-specific RNA was detected early in infection but could be detected in as little as 1 ug RNA isolated from cells at 24 and 30 hr PI.

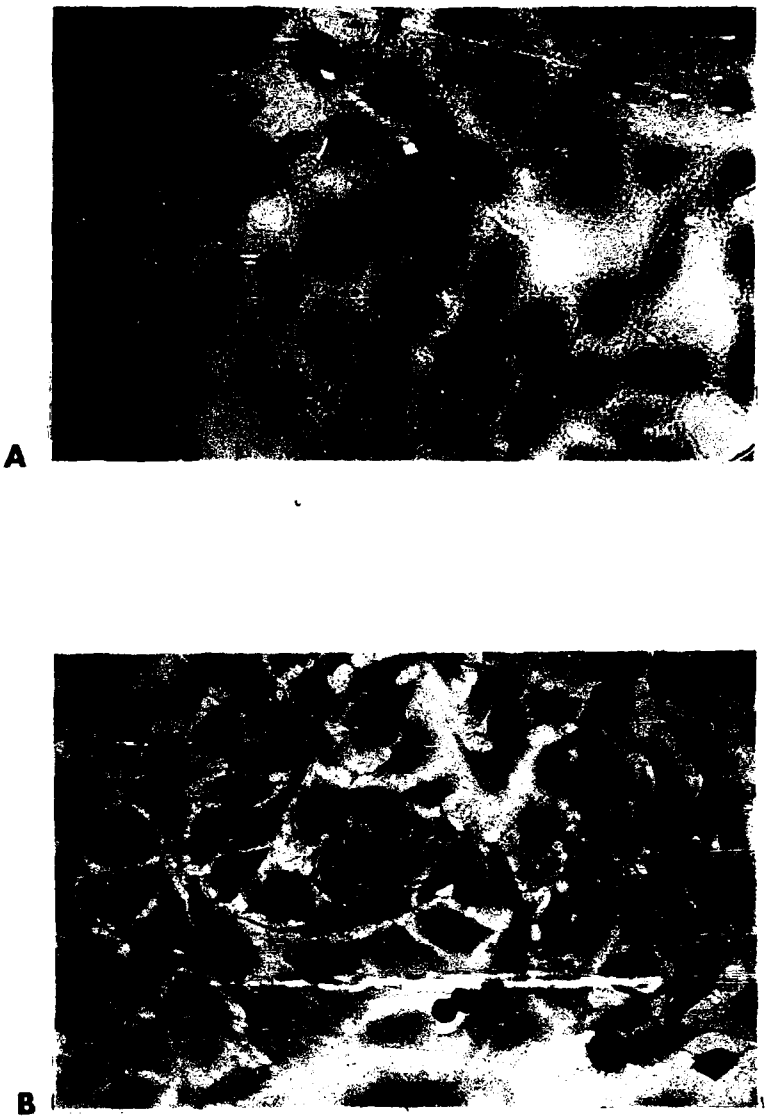
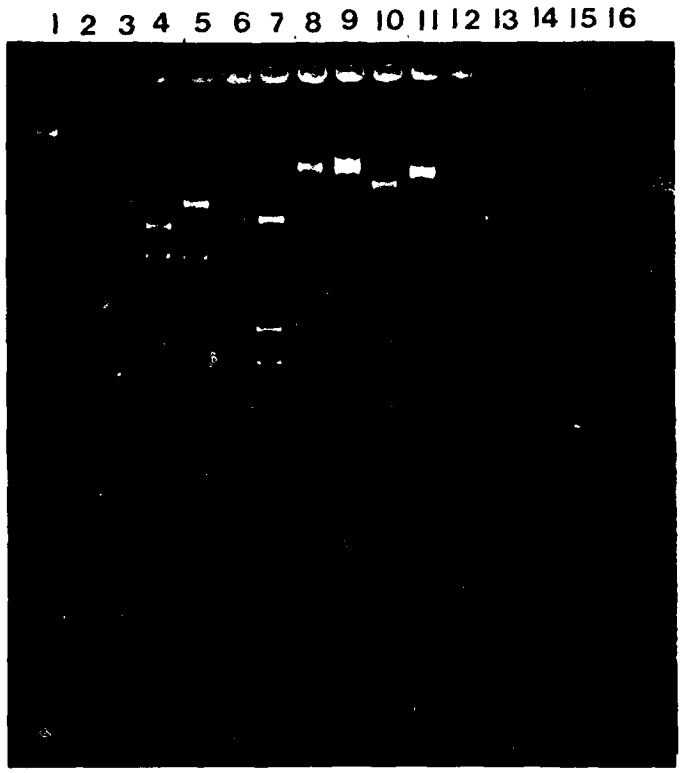
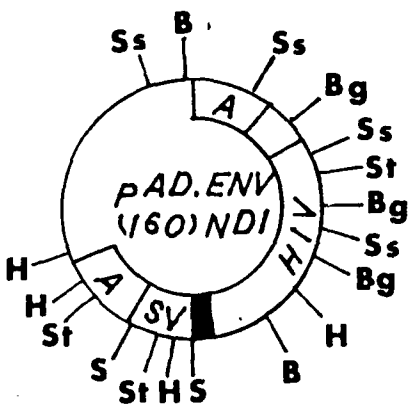


Fig. 5. Accumulation of HIV-1 gp120 in HeLa cells infected with recombinant HIV-1 gp120/Ad2. HeLa cells infected with Ad G3-8 (A) or wt Ad2 (B) were fixed in acetone at 30 hr PI and stained by using mouse monoclonal anti-gp120 as primary antibody and peroxidase-conjugated rabbit anti-mouse immunoglobulins as secondary antibody. Binding was visualized with hydrogen peroxide and o-dianisidine as an intense orange-brown color localized to the cytoplasm. Cells were counterstained with fast green.





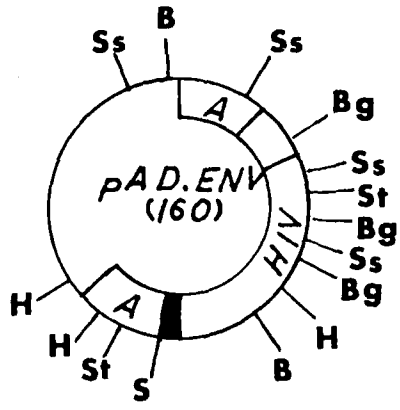


Fig. 6. Restriction enzyme analysis of plasmids pAD.ENV(160)ND1 and pAD.ENV(160). A. Plasmid DNA was digested with restriction endonucleases and fractionated on a 0.8% agarose gel. Lanes: λ-HindIII + ϕx-HaeIII standard. 1 and 16; uncut pAD.ENV(160)ND1, 2 and pAD.ENV(160), 3; pAD.ENV(160)ND1 and pAD.ENV(160), respectively, digested with SspI, 4, 5; BglII, 6, 7; SalI, 8, 9; StuI, 10, 11; BamHI, 12, 13; HindIII, 14, 15. B. Structure of pAD.ENV(160)ND1; and C. Structure of pAD.ENV(160) with relevant restriction endonuclease cleavage sites: Ss, SspI; Bg, BglII; S, SalI, St, StuI; B, BamHI; H, HindIII. Symbols: *HIV*, HIV gp160 fragment; ▭, Ad major late promoter and tripartite leader; ■, Ad VA sequences; *A*, Ad E3 flanking sequences; _____, pUC18 vector; *SV*, SV40 promoter and T antigen sequences.

TABLE 1

Infection of Monkeys with HIV-2_{NIH-DZ}
Various Inoculation Protocols

N'	Species	Inoculum	Route	Dose
C24	Cercocebus galeritus chrysogaster	Cells	IP	1
C25	Cercocebus galeritus chrysogaster	Cells	IV	2
C34	Cercocebus atterimus	VP	IV	1
C39	Cercocebus agilis	VP	IP + IV	2
B21	Baboon papio anubis	VP	IV	1
M2	Macaque rhesus	VP	IV	1

VP = $3.3\ 10^5$ TCID50 of viral particles
IV = Intravenous route
IP = Intraperitoneal route
Dose = Number of injections
Cells = $12\ 10^6$ of in vitro infected cells/inoculum

TABLE 2

HIV Infects Non-Human Primates

Animal	C24						C25					C34				
Time	1	2	4	7	9	11	1	2	5	7	9	2	4	6	8	10
PC (RT)	-	-	36	55	12	92	252	4,1	20	1,7	64	350	175	3,2	11,2	43
Trans (RT)	ND	ND	140	105	275	++	446	88	304	274	166	31	212	130	27,2	431
SB	ND	ND	ND	ND	ND	ND	+	+	ND	ND	ND	++	+	ND	ND	ND
WB-HIV-1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
WB-HIV-2	ND	ND	ND	ND	+	+	ND	ND	+	+	+	-	ND	+	+	+
Elisa Ab	-	-	+	++	+++	++	-	+/-	++	++	++	-	+	+	+++	+++
Elisa Ag	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-

Animal	C39				B21				M2			
Time	2	5	8	9	1	4	5	6	1	4	5	6
PC	220	479	4	61	25	231	106	137	52	-	44	104
Trans	438	453	122	427	215	145	228	ND	208	ND	245	355
SB	+	+	ND	ND	+	+	ND	ND	+	ND	ND	ND
WB-HIV-1	+	+	+	+	+	+	+	+	+	+	+	+
WB-HIV-2	ND	+	+	+	-	+	+	+	-	+	+	+
Elisa Ab	++	+	+++	+++	-	+	++	++	-	-	-	-
Elisa Ag	+	-	-	-	-	-	-	-	-	-	+	+

Time = months after last inoculation
PC = Primoculture (RT activity expressed in cpm x 10^3)
Trans = Transmission (RT activity expressed in CPM x 10^3)
SB = Southern Blot Analysis
WB = Western Blot using HIV-1 or HIV-2 strips
Elisa Ab = Detection of the presence of anti-antibodies in the sera by HTLV-III EIA Kit (Abbott)
Elisa Ag = Detection of the presence of p24 Ag in the sera by Abbott kit

TABLE 3

VACCINATION OF CHIMPANZEES WITH VACCINIA-HIV gp160 CANDIDATE VACCINE

Chimpanzee	Vaccination	Boost	Date of Testing	Boost	Date of Testing	Boost	Date of Testing	Date of Testing
C1013	05/11/87 10^5PFU V_{25} ID	08/4/87 Cell 2/3 SQ V_{25} 1/3 IV	08/20/87		11/27/87	12/15/87 BCG ID	02/11/88	03/4/88
			WB 180?		WB 160+		WB41+ 160+	WB41+ 160+
			CMC = ND		CMC 2%		CMC 34%	CMC
			CMI = ND		CMI i_s = 3		CMI i_s-2,4	CMI i_s-8,9
C1019	6/11/87 10^8PFU V_{25} ID	04/08/87 08/04/87 Cell V_{25} SQ/IV		08/16/87 10/08/87 gp160 1 ml iM		12/15/87 gp160 + Aracel iM		
			WB 180 +		WB 41+ 160+		WB41+ 160+	WB24+41+160
			CMC 57%		CMC 20%		CMC 75%	CMC = ND
			CMI = ND		CMI i_s = 3		CMI i_s = 1	CMI i_s=7,8
B1101	06/11/87 10^8PFU V_{25} ID	08/04/87 Cell V_{25} SQ/ID				12/15/87 BCG ID		
			WB 180 +		WB41+, 160+		WB41+ 160+	WB = ND
					CMC = ND		CMC = ND	CMC = ND
					CMI i_s = 2		CMI i_s = 5, 8,4	CMI i_s=4,3

WB = Western Blot
ND = Not determined

CMC= Cell Mediated Cytotoxicity is chromium ^{51} release assay. Targets were antologous EBV transformed B cells infected with vaccinia-HIV gp160 recombinant virus.

CMI= Cell Mediated Immunity is an antigen induced cell proliferation assay.

TABLE 3 (Cont'd)

VACCINATION OF CHIMPANZEES WITH VACCINIA-HIV gp160 CANDIDATE VACCINE

Chimpanzee	Vaccination	Boost	Date of Testing	Boost	Date of Testing	Boost	Date of Testing	Date of Testing
C1107	05/11/87 10^8PFU V_{25} ID	08/4/87 Cell V_{25}SQ/ID	08/20/87	9/16/88 gp160 iM	11/27/87	12/15/87 gp160(500)+ AC iM	02/11/88	03/4/88
			CMC = 49%				WB=41+160+ CMC= 18% CMI = 2,8	WB=41+160+ CMC = ND CMI = 18
C1131	05/29/87 10^8PFU V_{25}ID	08/04/87 Cell V_{25}SQ/ID	09/15/87	gp160 1 ml iM	11/27/87	12/15/87 Aracl 100		
			WB 160?		WB 41+160+		WB41+, 160+	WB41+ 160+
							CMI = 6,3	CMI = 46

TABLE 4

Preliminary Seroprevalence Sera Collected in Second Field Trip

SPECIES & NUMBERS TAKEN	HIV-1/HIV-2 Neg				HIV-2+/HIV-1 Neg				HTLV-I (STLV-1)(HIV 1,2)Neg			
	POS.	NEG.	BORDER LINE	UN-TESTED	POS.	NEG.	BORDER LINE	UN-TESTED	POS.	NEG.	BORDER LINE	UN-TESTED
Cercopithecus (wolfi) denti	1	48	0	43	7	41	1	43	1	40	1	50
Cercopithecus phoesti phoesti	1	29	1	18	6	25	0	18	2	19	0	28
Cercopithecus hamlyni	1	18	1	13	6	14	0	13	0	20	0	13
Cercopithecus mitis stholammani	0	22	0	26	2	20	0	26	1	17	0	30
Cercopithecus ascanius schmidti	1	22	0	4	1	21	0	5	0	20	0	7
Cercocebus albigena johnstoni	0	11	0	0	0	11	0	0	1	7	0	3
Papio Anubis	0	0	1	1	0	0	1	1	0	0	1	1
Cercopithecus l'hoesti l'hoesti	1	20	0	0	4	17	0	0	1	20	0	0

TABLE 5

PLAQUE TITRATION OF RECOMBINANT HIV env/ADENOVIRUS 2

SAMPLE	PFU/ml	
	CV-1 cells	293 cells
$CaPO_4$ Control	0	0
pAD.ENV.ND1	0	0
pAD.ENV	0	0
Ad2 DNA (2 ug)	8.6×10^4	1.3×10^7
Ad2 Virus	1.2×10^6	2.3×10^8
pAD.ENV.ND1 + Ad 31 Virus	0	1.2×10^4
pAD.ENV.ND1 + Ad2 DNA (2 ug)	7.3×10^5	2.1×10^8
pAD.ENV + Ad2 DNA (2 ug)	1.2×10^5	9.1×10^6
pAD.ENV + Ad2 Virus	1.4×10^6	2.0×10^8
pAD.ENV.ND1 + Ad2 DNA (1 ug)	3.2×10^5	3.0×10^7
pAD.ENV + Ad2 DNA (1 ug)	2.8×10^4	3.4×10^6
pAD.ENV.ND1 + Ad2 Virus	1.3×10^6	1.7×10^8

Plaque Titration of Recombinant HIV env/Adenovirus 2. Lysates of cotransfected or transfected-infected 293 cells were plaqued on CV-1 and 293 cells. Plaques were counted 8 days post infection of 293 cells and 12 days post infection of CV-1 cells. Total virus titer is reported in plaque forming units (PFU) per ml.

TABLE 6

PLAQUE PURIFICATION OF RECOMBINANT HIV-1 env/ADENOVIRUS 2 ISOLATE G3-8

Plaque Purification	Cell Line	Titer (PFU/ml)	Plaques Analyzed/ Total Plaques Counted	Positive Plaque Isolates
1	293	3.7×10^9	92/528	0
2	293	5.8×10^8	184/195	0
	CV-1	1.2×10^6	184/258	0

Plaque purification of recombinant HIV-1 env/Adenovirus 2 Isolate G3-8. Dilutions of G3-8 stock were plaqued on CV-1 and 293 cells. Plaques were counted and picked to infect HeLa cells grown in multi-well plates. Plaque isolates which hybridize to a ^{32}P-gp120 probe are considered to be positive.

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