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Safety and immunogenicity of HIV-1 DNA constructs in chimpanzees

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A global effort to control the HIV epidemic is likely to rely heavily on immunization strategies. As our closest genetic relative, the chimpanzee provides the most important model for preclinical safety and immunogenicity studies. We have immunized adult, pregnant and infant chimpanzees with our plasmid vaccines. We have found these vaccines to be safe and well tolerated in all of these groups. The same vaccines have induced both humoral and cellular immunity in each instance. © 1998 Elsevier Science Ltd. All rights reserved

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The spread of HIV-1 has resulted in significant morbidity and mortality both here in the United States and worldwide. Vaccines are likely to play an integral role in any strategy designed to control HIV-1 and HIV-2 in the world's population. Unfortunately, the effort to design effective vaccines for HIV-1 has encountered a number of significant obstacles. In a 1993 poll by the journal Science[1], 150 top AIDS researchers agreed that the most significant obstacle has been the lack of consistent correlates of protection from natural or experimental infection. Vaccine developers have been left with the task of assessing vaccine performance with a less than ideal set of standards. As a result, a consensus has begun to emerge, supporting the somewhat simplified yet logical hypothesis that a successful vaccine will have to induce substantial humoral and cellular immune responses. Of the traditional approaches to vaccination against pathogenic human viruses, only live attenuated virus preparations active all arms of the immune system in a manner similar to native infection. However, as an attenuated product, rare yet measurable instances of disease do occur when the vaccine virus mutates and reverts toa more pathogenic isolate. This problem is accentuated when live vaccine is given to individuals either transiently (e.g. infants) or permanently immunosuppressed. This obvious drawback has limited current investigations to animal models and driven the development of alternative vaccine strategies, including the use of plasmid DNA.

In theory, direct genetic immunization mimics aspects of live attenuated vaccines in that both humoral and cellular immunity are induced while avoiding the risks of infection with live virus. The theory has now been verified by several independent investigators[2–6] studying several viruses in different animal models. Genetic immunization is dependent upon injection of a nucleic acid sequence directly into a host target tissue. The synthesis of specific foreign proteins occurs in the host as in natural infection, with either attenuated or wild type virus. These host-synthesized viral proteins then become the subject of immune surveillance via both humoral and cellular pathways[7]. The development of HIV vaccines has relied upon a number different animal models. Our laboratory has reported on the development of immune responses to HIV-1 antigens in both small mammal (murine)[6] and small primates (macaques)[8] after genetic immunization. In order to further develop this technology into preclinical studies we have immunized a number of chimpanzees with HIV-1 DNA-based vaccines. As our closest genetic relative, the chimpanzee provides an important model for preclinical safety and immunogenecity studies. Challenge studies are also possible given the ability to infect chimpanzees with HIV-1, although these experiments are ultimately limited by HIV-1's relative lack of pathogenicity in these animals. We present here the accumulated safety and immunogenicity data that has been gathered in our effort to develop DNA vaccines for use against HIV-1 in the chimpanzee model.

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Table 1 Description of subject characteristics and experimental parameters in immunization experiments with DNA plasmid vaccines encoding HIV-1 proteins

Parameter				
Sex	5 male	8 female		
Age at first immunization	2 neonates	4 infants	5 adults	2 pregnant
Number of constructs	9 env/rev & gal/pol	2 gal/pol alone	2 env/rev alone	
Number of immunizations	3–8			
Individual dose range	50–1000 μg			
Immunization period	12–52 weeks			
Immunization interval	3–27 weeks			
Route	13 intramuscular	5 intravaginal	1 intrarectal	
Intramuscular injection method	5 needle and syringe	8 needleless biojector		

MATERIALS AND METHODS

Immunization of chimpanzees (pan troglodytes) with HIV-1 genetic constructs

Table 1 describes 13 chimpanzees that have been immunized with DNA plasmid vaccines encoding proteins of HIV-1. The animals have ranged from 1 month old to adult at the time of their first immunization, with six of the animals considered either neonates or infants. Both males and pregnant (2) and non-pregnant females have been immunized. Individual animals have been vaccinated a total of three to eight times with *env* and *gag/pol* constructs either alone, or in combination. The plasmids were constructed as previously described[9,6]. The dose of individual constructs has ranged from 50 μg to 1 μg and dosing intervals have ranged from 3 to 27 weeks. Vaccines have been delivered via both systemic and mucosal routes, with all intramuscular injections formualted in 0.25% bupivaccine-HCl, while preparations for mucosal delivery were suspended in phosphate buffered saline (PBS) alone. Intramuscular injections were administered into the quadriceps muscle with subsequent injections delivered to the contralateral leg, to maximize the ability to differentiate adverse local reactions. Separate immunization sites were used for each construct when both were given concurrently to facilitate the identification of local reactions. Intravaginal administration was performed by visualizing the vaginal opening and inserting the liquid preparation using a needleless syringe, after washing the vaginal vault with 5–20 ml of PBS prior to vaccine delivery. All experiments were performed in the spirit of the Good Laboratory Practice Regulations currently in effect, [i.e. United States FDA (21 CFR, Part 58 of June 1979 and as modified by the final rule effective October 5, 1987)], and the OECD Guidelines and Standard Operating Procedures of White Sands Research Center (WSRC) of the Coulston Foundation in Alamogordo, New Mexico. These studies were conducted in accordance with requirements as specifically stated in paragraph 2–3(d)(1) of the Animal Welfare Act (9 CFR) regulations at WSRC.

Chimpanzee safety monitoring after immunization with HIV-1 genetic constructs

Safety was assessed by monitoring hematological, serum chemistry and physical observations according to *Table 2*. Animals were observed daily for the duration of the studies beginning at least 2 weeks prior to inoculation with the constructs. Ech animal was observed at least once each morning and each afternoon throughout the studies for pharmacotoxic signs and/or signs of AIDS, particularly for signs of diarrhea. Any abnormal observations (e.g. lethargy, emesis, leukoplakia) were reported. Vital signs included body weights, rectal temperatures, heart and respiration rate, and systolic and diastolic blood pressures. The physical examination consisted of the aforementioned vital signs and examination of the following systems: integument, respiration, cardiovascular, gastrointest-

Table 2 Blood collection and physical assessment schema for safety monitoring

Specimen/assay	Frequency
Serum for chemistry (see *Table 3*)	every 2–4 weeks
Heparinized whole blood for hematology (see Table 4)	every 2–4 weeeks
Lymphocyte subsets (CD3, CD4, CD8, CD20)	every 4 weeks
Veterinary assessment	daily
Physical observation/vital signs	daily
Chest X-ray	once at study inception
Electrocardiogram	once at study inception
Weight monitoring	evcery 2–4 weeks
Complete physical examination	every 2–4 weeks
Urinalysis	every 2–4 weeks

Table 3 The following serum chemistry indices were measured using a Hitachi 737

Alanine aminotransferase (ALT)	Cholesterol (Chol)	Lipemic factor
Albumin (Alb)	Chloride (Cl)	Osmolality (derived value; Osmol)
Alb/Glob ratio (derived value; A/G)	Creatine kinase total (CK)	Phospholipids
Alkaline phosphatase	Creatinine (Creat)	Phosphorus (P)
Aspartate aminotransferase (AST)	γ-glutamyl-transpeptidase (GGT)	Potassium (K)
Bicarbonate (HCO3)	Globulin (derived value; Glob)	Protein, total (T Prot)
Bilirubin, direct (D Bili)	Glucose (Glu)	Sodium (Na)
Bilirubin, total (T Bili)	Hemolytic factor	Triglyceride (Trig)
Blood urea nitrogen (BUN)	Icteric factor	Uric acid (Uric)
BUN/creatine ratio (B/C)	Iron (Iron)	
Calcium (Ca)	Lactic acid dehydrogenase (LDH)	

Table 4 The following hematological indices were measured using a Sequoia-Turner Celldyn 1600 on whole blood collected prior to vaccine administration. Two blood smears were made from droplets taken from the sample

Reticulocyte count	Hematocrit (derived value; Hct)
Hemoglobin concentration (Hgb)	Leukocyte counts, total (WBC)
Leukocyte count, differential (diff; microscopic exam)	Mean corpuscular volume (MCV)
Mean corpuscular hemoglobin (MCH)	Mean corpuscular hemoglobin concentration (derived value, MCHC
Platelet count (Plt)	Erythrocyte count (RBC) and morphology (microscopic exam)

inal, urogenital, nervous system, musculoskeletal, lymphatics, endocrine and special senses. Special attention was paid to examination of the eyes and the inoculation site, which were graded with regard to the absence of presence of a local reaction.

Assessment of vaccine immunogenicity in chimpanzees

The immunogenicity of each construct was assessed for each animal listed in *Table 1*. Serum from each animal was assayed using ELISA for the presence of specific antibodies produced through vaccination as described previously[10]. In addition, serum was assayed for the capacity to neutralize homologous and heterologous virus in a subset of the animals, according to the procedure described previously[11]. Cellular immune responses were also measured, including lymphocyte proliferation to specific vaccine antigens, as well as cytotoxic T-lymphocyte Lysis (CTL) of targets displaying specific vaccine antigens, according to previously described methods[11,12]. The lymphocyte subset analyses described below were performed using the antibodies listed in *Table 2*, as described previously[13].

RESULTS AND DISCUSSION

Physical safety data

No evidence of significant pharmacotoxicity or AIDS-like illness have been observed in any of the 13 animals during any of the studies. All animals appeared to be in good overall health throughout the studies, as assessed by physical observation and vital signs. No signs of viral infection (lethargy, lymphadenopathy, hepatosplenomegaly) has been observed and weight gain was consistent for both pregnant animals and all six growing chimpanzees. No anaphylactoid reactions suggesting immediate hypersensitivity or behvioral changes, including appetite, were noted. Daily observation of the animals revealed only minor abnormalities (*Table 5*). The reactions noted at the injection site have been minimal, with only transient erythema and no induration or edema. We have previously reported a lack of muscle histopathology observed at the site of DNA injection[8].

Table 5 Physical observation of the chimpanzees revealed the following minor abnormalities which were not associated with vaccination

Loose stools of 1 day's duration in three animals
Transient (1–9 days) slight irritation at the injection site in four animals
Self-resolving penile rash in one animal
Transient (2 days) eye irritation in one animal
Lymph node biopsy sites healed without incident in three animals
Unrelated rectal abscess in one animal

Serum chemistry, hematology and serology determinations from blood

All 13 chimpanzees were monitored for any untoward effects on serum chemistry measurements. The results were unremarkable with two exceptions. Seven of the 13 animals had transient elevations in creatine kinase levels ($1000 < CK < 2000$) which all returned to normal by the next measurement. These measurements were not temporally associated with immunization and may represent unrecognized (due to heavy coat) muscle bruising resulting from daily contact with other animals. One animal also had a transient drop in serum bicarbonate, which also resolved spontaneously. There was no evidence of alterations in renal or hepatic function in any of the animals as a result of immunization. Assays to determine the presence of anti-nuclear antibodies in the serum of three animals were negative. These assays were performed to address the theoretical possibility that non-specific antibodies will arise in response to plasmid DNA similar to what has been seen in autoimmune diseases. The results of complete blood counts and smears have also been largely unremarkable with exceptions listed in *Table 6*. None of the abnormalities persisted or occurred in more than one animal suggesting a pattern of pharmacotoxicity.

Immune responses

Lymphocyte subset analyses were performed by flow cytometry in each animal immunized with HIV-1 genetic constructs. There were no consistent patterns of increasing or decreasing individual markers of T lymphocytes (CD_3^+), T helper (CD_4^+), T suppressor/killer (CD_8^+), or B lymphocytes (CD_{20}^+) noted in relation to immunization. *Figure 1* shows the percentage of lymphocytes bearing the CD_4 and CD_8 surface markers for a representative animal. In this particular case, one can see changes in both CD_4 and CD_8 early in the experiment, with values leveling off despite repeated immunizations. The variation early on

Table 6 Hematological measurements performed on heparinized chimpanzee blood revealed the following minor abnormalities

Transient elevated total WBC count which returned to normal by the next measurement in one animal
Elevated total WBC count in the course of rectal abscess
Bandemia in one animal, which returned to normal by the next measurement 4 days later
Erythrocyte indices (total RBC, hgb, hct) were transiently elevated in one animal
Elevated (> 1000/μl) total eosinophil counts on three occasions in one animal, returning to normal in the intervening weeks

may or may not be related to vaccination, as only a single pre-vaccination sample is available and the variability demonstrated in the first few weeks may also have been observed without immunization in this animal.

Antibodies were measured in the serum of every chimpanzee vaccinated with DNA plasmids encoding genes of HIV-1, as shown in *Table 7*. Titers varied from 1:4096, to undetectable in response to individual antigens. Both the envelope and *gag/pol* DNA vaccines elicited humoral responses, but not in each animal. Both vaccines elicited antibody responses when administered intramuscularly, although responses were more variable (3/6 responders) when the *env* vaccine was given via the mucosal route. Neither the *gag/pol* nor the *env*-based vaccines were clearly superior over the other construct in eliciting humoral responses. Antibody responses were evident as early as four weeks after the first immunization. Data regarding the ability of these antibodies to neutralize the virus is incomplete at present, as only three of the 13 animals have been tested for the ability to neutralize HIV-1_{MN}. These animals demonstrated neutralizing antibody titers ranging from 1:20 to 1:320 after immunization with both *env* and *gag/pol* constructs.

DNA-based vaccines have been shown to induce antibody responses in primates, although the significance of these responses remains unclear. Although humoral immunity in the form of antibody production provides a significant contribution to protective responses in a number of successful viral vaccines, this may not prove to be the case for HIV. One can cite studies (including our own[11]) which support[15] and refute[16,17] the contribution of humoral responses in the control and/or prevention of HIV infection. At present, one can only postulate that a robust humoral response

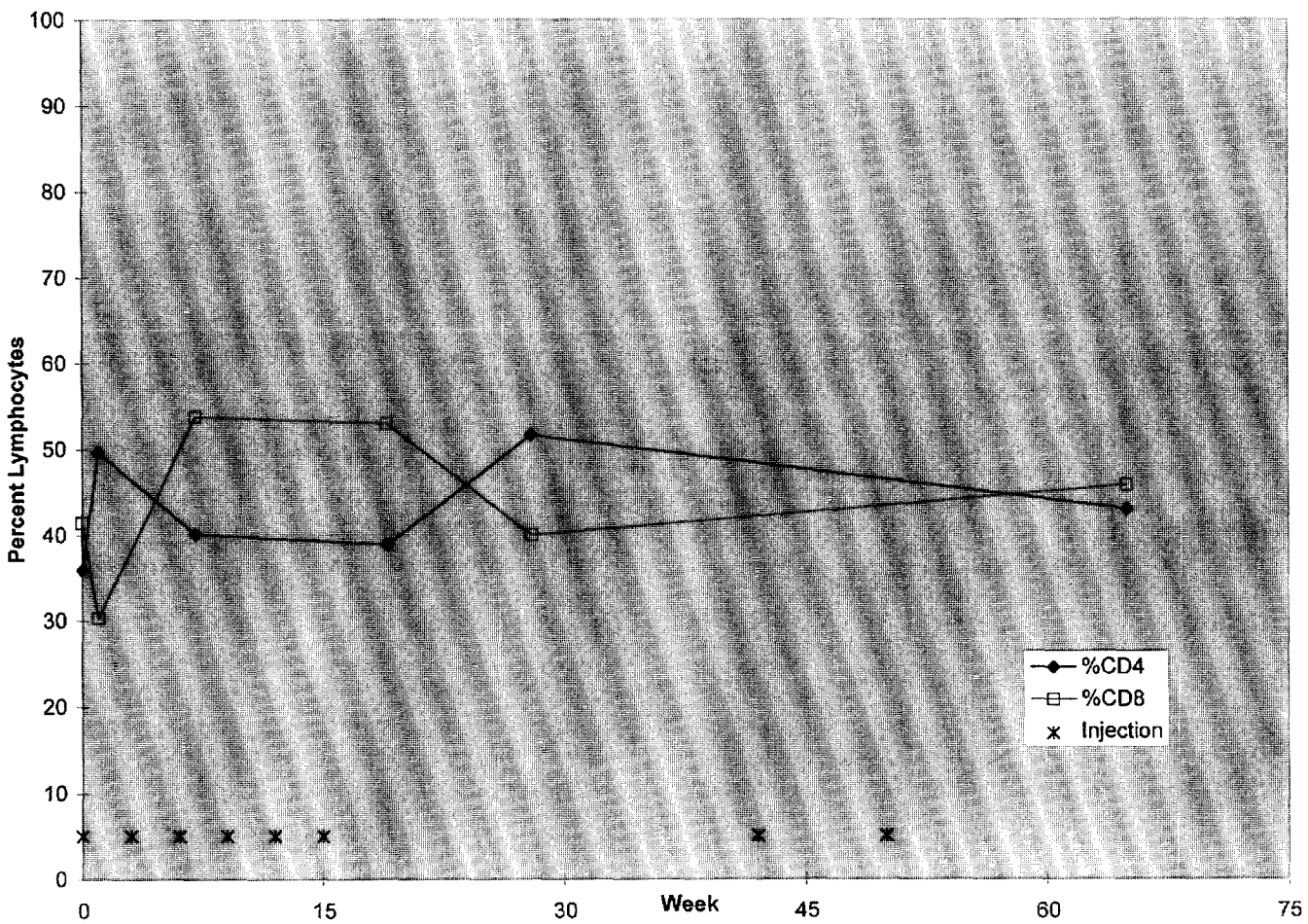

Figure 1

Table 7 Humoral immune responses measured in the serum of chimpanzees vaccinated with DNA plasmid vaccines encoding HIV-1 proteins

Antigen	Animals positive	Animals tested	Maximum titer	Minimum titer
gp160	2	2	4096	1024
gp41	5	8	100	100
gp120	8	12	400	50
V3 loop	4	4	N/A	N/A
Pr55	11	11	400	20
p24	2	2	400	400
Neutralization of HIV-1(MN)	3	3	320	20

Table 8 Cellular immune responses of chimpanzees vaccinated with DNA plasmid vaccines encoding HIV-1 proteins

	Lymphoproliferative responses	Cytotoxic T-lymphocyte responses		
Antigen/target	Pr55	*env* (B)	*env* (E)	*gag/pol*
Animals positive	4	5	2	2
Animals tested	9	11	3	9

may contribute to protective responses, without being sufficient in all cases.

Cellular responses were also measured in the various experiments in which chimpanzees were immunized with HIV-1 DNA constructs (*Table 8*). A number of the animals have demonstrated lymphoproliferative responses to vaccine encoded antigens, most notably after intramuscular administration of *gag/pol* constructs. Although the lymphoproliferative responses of these animals has not been thoroughly characterized, the data demonstrating the ability of DNA constructs to elicit cytotoxic T-lymphocyte (CTL) responses in the chimpanzees is more complete. To date, half of the animals tested have shown significant CTL responses for specific vaccine encoded antigens (*gag/pol* and *env*). The responses appear to be MHC Class-I restricted and mediated by CD_8 lymphocytes. We have elicited CTLs after both systemic and mucosal delivery of the plasmid. We have demonstrated cross-reactive CTLs to targets displaying E clade *env* antigen after administration of plasmids encoding B clade *env*. We have also demonstrated lysis of targets encoding *env* from so-called macrophage-tropic strains of HIV-1 after immunization with $HIV\text{-}1_{MN}$ based constructs (data not shown). These responses are as inconsistent as they are promising, leading one to believe that host factors may be influencing the results in this outbred population. As is the case with humoral responses, the importance of these responses in the ultimate development of an effective vaccine is unknown at present. Similarly, evidence supporting the role of CTLs[18,19] in conferring immunity to infection is contradicted by evidence to the contrary[20]. We are currently left with the task of assessing vaccine performance with a less than ideal set of measuring devices, while the search for additional correlates of protection continues. The situation is similar to the lack of an ideal animal model, in that no single measure appears to consistently predict protection. Consequently, it is important to continue to pursue an approch that induces the broadest humoral and cellular immunity, in an attempt to provide all or perhaps any of the potential correlates of protection.

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