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Induction of Humoral Immune Responses following Vaccination with Envelope-Containing, Formaldehyde-Treated, Thermally Inactivated Human Immunodeficiency Virus Type 1

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The lack of success of subunit human immunodeficiency virus type 1 (HIV-1) vaccines to date suggests that multiple components or a complex virion structure may be required. We previously demonstrated retention of the major conformational epitopes of HIV-1 envelope following thermal treatment of virions. Moreover, antibody binding to some of these epitopes was significantly enhanced following thermal treatment. These included the neutralizing epitopes identified by monoclonal antibodies 1b12, 2G12, and 17b, some of which have been postulated to be partially occluded or cryptic in native virions. Based upon this finding, we hypothesized that a killed HIV vaccine could be derived to elicit protective humoral immune responses. Shedding of HIV-1 envelope has been described for some strains of HIV-1 and has been cited as one of the major impediments to developing an inactivated HIV-1 vaccine. In the present study, we demonstrate that treatment of virions with low-dose formaldehyde prior to thermal inactivation retains the association of viral envelope with virions. Moreover, mice and nonhuman primates vaccinated with formaldehyde-treated, thermally inactivated virions produce antibodies capable of neutralizing heterologous strains of HIV in peripheral blood mononuclear cell-, MAGI cell-, and U87-based infectivity assays. These data indicate that it is possible to create an immunogen by using formaldehyde-treated, thermally inactivated HIV-1 virions to induce neutralizing antibodies. These findings have broad implications for vaccine development.

Although it has been 20 years since human immunodeficiency virus type 1 (HIV-1) was first isolated, the virus remains an emerging pathogen worldwide, with 14,000 to 16,000 new infections occurring daily. Researchers have developed potent chemotherapeutic strategies to treat HIV infection, which have dramatically reduced the number of AIDS cases and progression to disease in the United States and Europe. Nonetheless, these regimens have not been uniformly successful. Therefore, it is clear that studies must be targeted at the identification and development of protective HIV vaccine immunogens. Cogent arguments exist for a variety of HIV-1 vaccine strategies, including one based on inactivated virions. This technology has worked successfully for a variety of viral vaccines, including retroviral vaccines (19, 31, 40).

To date, the majority of efforts directed towards developing a preventive HIV-1 vaccine have focused on recombinant subunit vaccines, such as those consisting of envelope proteins, and the use of vector-based delivery systems (1). The minimal success of subunit vaccines indicates that protective immunity is possible but that multiple components or a complex virion structure may be required. Recent work to model a killed HIV-1 vaccine using simian immunodeficiency virus (SIV) has demonstrated that covalent modification of nucleocapsid zinc fingers by 2,2′-dithiodipyridine can preserve antigenic structures on the surface of SIV_{Mne} and HIV_{MN} (3, 35). Moreover, SIV-specific antibodies were shown to be present following intravenous injection of 2′2-dithiodipyridine-treated SIV_{Mne} into a juvenile pig-tailed macaque (15). With few exceptions (11), the ability of vaccines to induce these types of responses has been poor (6). Studies using vaccination with immunogens containing V3 sequences have generally elicited antibodies that recognize linear clade-specific antibodies (20, 29). Attempts have also been made modify gp120, for instance, by deleting variable loops or glycan residues. These too have failed to generate potent heterologous antibody responses. For instance, vaccines based on HIV DH12- or 89.6-derived Env containing deletions in variable loops failed to induce heterologous neutralizing antibodies at all (22, 33), and similar constructs based on HXB2 generated low-level heterologous neutralizing antibodies in mice and rats (21, 38). Other approaches, such as gp120-CD4 cross-linked immunogens, have elicited neutralizing antibodies against a panel of primary viruses in macaques, but a clear determination as to whether these responses were against gp120 or CD4 (13) has not been made. Finally, studies attempting to use chimeric gp120 molecules with C3d elicited higher antibody titers than gp120 alone, but the antibodies were not able to neutralize heterologous viruses (16).

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Inactivated vaccines are theoretically advantageous, since they represent a complex mixture of viral antigens closely resembling native virions. Ideally, inactivation would result in conservation of linear and conformational epitopes required for both humoral and cellular immune responses. Furthermore, we have demonstrated inactivation protocols that result in the exposure of normally cryptic neutralization epitopes (18). In this manner, it might be possible to enhance the immunogenicity of the vaccine beyond that achieved by native virions.

Early efforts to model a killed HIV-1 vaccine using SIV in rhesus macaques were unsuccessful. Although protection against live challenge was conferred, it was the result of immune responses directed towards xenoantigens in the vaccine preparations rather than towards epitopes on SIV (2, 10). Despite the protection afforded by killed vaccines for other viral diseases, research devoted to developing a killed vaccine for HIV-1 has been minimal. This is primarily due to concerns regarding shedding of gp120 from virions, safety considerations surrounding vaccine preparation, and the failures of early SIV vaccine preparations. We previously addressed a number of these concerns in vitro (18). In those studies, we demonstrated that virus could be inactivated by at least 7 logs and not only maintain but enhance binding capacity to broadly reactive, conformation-dependent neutralizing antibodies. We now present data which demonstrate that these preparations are capable of inducing neutralizing antibody responses in small animals and nonhuman primates.

MATERIALS AND METHODS

HIV immunogens. Vaccine immunogens were prepared either by electroporation of primary peripheral blood mononuclear cells (PBMC) (18) or by transfection of 293T cells grown in Nunclon Triple Flasks as previously described (17). In either instance, cell-free supernatants were treated with Benzonase (Merck) for 2 h at 37°C to digest contaminating nucleic acids. Benzonase-treated virus stocks were subjected to ultrafiltration through a 300-kDa cutoff device using a Pall Mini-ultrasette tangential flow ultrafiltration device. These samples were subjected to ultracentrifugation in a fixed-angle rotor for 2 h at an average relative centrifugal force of 40,000 × *g*. Virus pellets were resuspended in serum-free medium (AIM V), and formaldehyde (10% ultrapure; Polysciences) was freshly diluted in phosphate-buffered saline (PBS) and added to the virus for a final concentration of 0.02%. After incubation, an equal volume of 0.2% bovine serum albumin in PBS was added to quench residual aldehyde. The buffer was removed by diafiltration in a 100-kDa ultrafiltration device (Millipore) by centrifugation at 10,000 rpm. The filtrate (approximately 95% of the volume) was removed by aspiration, and PBS was added to the upper cell to reconstitute the sample to the original volume. After mixing by inversion, the device was centrifuged at 10,000 rpm. This process was repeated a total of four times, resulting in a 160,000-fold dilution of the low-molecular-weight molecules, including residual aldehydes. Thermal treatment of HIV-1 was performed as described previously (18). In brief, samples were loaded into thin-wall 0.5-ml microtubes and subjected to three successive 10-min incubations at 62°C in a heat block filled with water. Inactivation was confirmed by infection of allogeneic pools of PBMC with undiluted supernatants as previously described (18).

Reagents used in capture enzyme-linked immunosorbent assays (ELISAs). Monoclonal antibody (MAb) 2G12 was a gift of H. Katinger, MAb IgG1b12 was a gift of D. Burton, MAb 17b was a gift of J. Robinson, MAb 447-52D was a gift of S. Zolla-Pazner, and sCD4 was obtained from the AIDS Reagent Repository (14). Plasmid CDM7-CD4Eγ1 coding for CD4-immunoglobulin G (IgG) (8) was a gift of D. Camerini and was transfected (25 μg) into 293T cells (5×10^6) by standard methods (37). Supernatant was collected at 48 h, titrated, and used at a 1:10 dilution for all assays.

gp120 capture ELISA. Capture of gp120 was performed as described previously (18, 26). In brief, 80 μl of clarified culture supernatant was incubated with 20 μl of human anti-gp120 MAb or with CD4-IgG (2 to 10 μg/ml) in a U-bottom microtiter plate. Where appropriate, the sample was preincubated with 2 ng of sCD4/well in PBS with 0.2% bovine serum albumin for 30 min at 37°C. Samples and antibodies were allowed to react in the liquid phase for 45 min at 37°C. Triton X-100 was added to a final concentration of 1% for 15 min at 37°C. This concentration of detergent will not disrupt the immune complex (39). At the end of the incubation period, the contents were transferred to an ELISA plate precoated with sheep anti-gp120 (International Enzyme). The gp120-Ab complex was captured onto the plate at 37°C for 60 min. After washing, the plate was incubated with goat anti-human IgG (horseradish peroxidase conjugated; Accurate Chemical) for 45 min at 37°C. Following a final wash, 200 μl of tetramethyl benzidine substrate was added to each well for 20 min. The reaction was terminated by addition of 4 N H_2SO_4 (final concentration of 0.8 N) and read at 450 nm (Molecular Dynamics). A standard serial dilution of concentrated HIV_{SX} was used as a standard to normalize gp120 binding in all assays.

Fractionation of virus on a Percoll gradient. After ultrafiltration, HIV_{SX} was formaldehyde treated and either held at 4°C or heated to 62°C for 30 min. Next, 200 μl of each preparation was layered onto 1.8 ml of undiluted Percoll (Pharmacia), and the samples were centrifuged at 56,000 × *g* for 60 min at 4°C. Fractions (100 μl) were removed from the top of the gradient. The samples which were previously held at 4°C were then heated to 62°C for 10 min to normalize optical density readings on the ELISA. HIV p24 was measured by capture ELISA (Coulter), and gp120 ELISA was as described above with either CD4-IgG or 2G12 as the capture antibody.

Mice. Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories. All mice were allowed a 1-week period of acclimatization prior to initiation of experiments. Mice were vaccinated subcutaneously with inactivated vaccines in the presence of either 20 μg of CpG ODN 1826 (Oligos Etc), 20 μg of QS 21 (kindly provided by C. Kensil, Antigenics, Inc), or 50 μg of Alhydrogel (Superfo AB) as an adjuvant as indicated. Vaccine dose contained either 1, 5, or 20 μg of p24, as indicated in the figure legends and tables. In these experiments, 20 μg of p24 corresponded to 1 μg of gp120 as measured by ELISA on unheated samples. Blood was obtained 2 weeks after the final vaccination by cardiac puncture. All protocols were approved by the Animal Research Committee at the University of California, Los Angeles (UCLA).

Nonhuman primates. Juvenile rhesus macaques were housed at the Yerkes Primate Center. Macaques were vaccinated intramuscularly with inactivated vaccines (20 μg of p24) in the presence of 10 μg of QS 21 as an adjuvant. In this experiment, 20 μg of p24 corresponded to 1 μg of gp120 as measured by ELISA on unheated samples. The macaques received a total of three vaccinations at 5-week intervals. Blood was drawn under anesthesia prior to each vaccination and 2 weeks after each vaccination. Animals were released from study upon completion. All protocols were approved by Animal Research Committees at both UCLA and Emory University.

Virus stocks for PBMC and MAGI neutralization assays. Virus stocks were propagated on allogeneic pools of PBMC. Infectious viral titers were determined on allogeneic pools of PBMC. Half-log dilutions of viral stocks were applied to the cells for 16 h. Supernatants were changed at day 7 and day 14 and harvested at day 21 to determine the 50% tissue culture infective dose ($TCID_{50}$) for each virus. $TCID_{50}$s were calculated by the method of Reed and Muench (34).

Virus stocks for the PhenoSense assay were prepared by cotransfection of an HIV genomic vector (pHIVlucΔU3) containing a luciferase reporter gene with a plasmid expressing one of a number of different HIV envelopes (pHIVenv).

Virus neutralization assays. All serum samples were heat treated at 55°C for 30 min prior to assay. All murine sera and nonhuman primate sera that were tested in the MAGI cell assay described below were subjected to preabsorption against the cell substrate, in which the virus stocks were made to remove any nonspecific anticellular antibodies that might be present. For this purpose, serum samples were subjected to three rounds of adsorption against an equal volume of packed cells at 4°C. The first incubation lasted 2 h (to remove high-affinity anti-cell substrate antibodies with slow on/off rates), the second incubation lasted 1 h (to remove intermediate-affinity antibodies), and the third incubation lasted 30 min (to remove low-affinity antibodies). Analysis of the serum by indirect immunofluorescence assay demonstrated removal of antibodies which bound to uninfected cells by the end of the third incubation (data not shown).

In some experiments, virus neutralization was measured in a standard HIV p24 neutralization assay. For this purpose, virus (200 $TCID_{50}$) was incubated with serum from each of the immunized mice for 30 min at 37°C. Virus and serum were added to phytohemagglutinin (PHA)-stimulated PBMC for 24 h, and the cultures were washed to remove input serum and virus. Ninety percent of the medium was removed and replaced with fresh medium at days 1, 2, 5, 7, and 8. HIV p24 production in the supernatant was measured by ELISA on day 9. In other experiments, virus neutralization was measured on MAGI CCR5 cells (National Institutes of Health [NIH] AIDS Reagent Repository) (9). In these assays, viral inocula (100 $TCID_{50}$) were incubated for 30 min at 37°C with an

equal volume of serum at various dilutions (as indicated). Neutralization was calculated as a percent reduction in β-galactosidase (β-Gal) production relative to the virus control 48 h after infection. Spots were quantitated by using an automated system (Immunospot; Cellular Technologies). Samples were run in triplicate. Control sera were obtained from the AIDS Reagent Repository (43).

Finally, the presence of virus-neutralizing antibodies in sera from the nonhuman primate studies was independently confirmed by the ViroLogic PhenoSense HIV neutralization assay. For this purpose, neutralizing antibody responses were measured following preincubation of recombinant viruses pseudotyped with selected viral Envs with serial dilutions of sera from the macaques. These samples were used to infect U87 cells expressing CD4 plus the CCR5 and CXCR4 coreceptors (U87/CD4/CCR5/CXCR4). Neutralizing activity was calculated as the percent inhibition of viral replication (luciferase activity) at each antibody dilution compared to a control culture without antibody. The 50% inhibition concentration (IC_{50}) was determined, and neutralization titers were expressed as the reciprocal of the plasma dilution conferring 50% inhibition. This high-throughput assay required larger volumes of serum than the previous studies, and therefore we were unable to subject the sera to preabsorption against the cell substrate in which the vaccine was produced due to the excessive numbers of cells this would have required. As such, neither the prevaccine sera nor the postvaccination sera were preabsorbed. The assay included a virus pseudotyped with a non-HIV Env (amphotropic murine leukemia virus [aMLV]) as a specificity control. Samples that score positive in this assay must demonstrate at least a threefold-higher IC_{50} than the same serum tested with the aMLV control.

RESULTS

HIV-1 envelope remains associated with virions following formaldehyde stabilization and thermal treatment. Shedding of HIV-1 envelope has been described for some strains of HIV-1 (24, 27, 28) and has been cited (reviewed in reference 7) as one of the major impediments to developing an inactivated HIV-1 vaccine. In order to decrease envelope shedding, we developed protocols that include pretreatment of vaccine immunogens with sublethal (0.02%) concentrations of formaldehyde prior to thermal inactivation. As previously shown (18), this treatment regimen does not disrupt the antigenic structure of viral Env, and in fact incubation with formaldehyde followed by thermal treatment resulted in binding of CD4 to gp120 that continued to rise from 3 to 30 min (111 to 162% of control). This result is in contrast to the kinetics of thermal treatment alone, where binding reaches a maximum between 3 and 10 min following treatment. Experiments conducted using formaldehyde at higher concentrations demonstrated that as little as 0.1% formaldehyde could reduce binding of Env to reagents which recognize neutralization epitopes by 90% (data not shown).

We have previously used velocity gradient sedimentation through Percoll to examine the association of viral envelope with virions after thermal inactivation. In these studies, the major gp120 and p24 peaks as measured by ELISA were identified in the same fraction when the virions were held at 4°C. In contrast, we observed that a substantial amount of envelope did not remain associated with intact virion cores after thermal inactivation (18), as evidenced by the presence of gp120 in multiple fractions.

In the present study, we examined preparations that were treated with 0.02% formaldehyde prior to thermal inactivation. Fractions were analyzed by ELISA for gp120 and for p24. We demonstrate here that a single, major gp120 peak was retained when the virions were treated with 0.02% formaldehyde prior to thermal inactivation (Fig. 1a). This was the same fraction in which the major p24 peak was identified. In contrast, as shown in Fig. 1a, virions that are subjected to thermal inactivation alone fail to retain a single gp120 peak. These data indicate that the majority of envelope remains associated with virions following thermal inactivation if the virions are first treated with a sublethal dose of formaldehyde. We further examined viral envelope following formaldehyde treatment and thermal inactivation by Western blotting. As can be seen in Fig. 1b, formaldehyde-treated, thermally inactivated virions (Fig. 1b, lane 2) do not appear to be significantly different from untreated virions that were held at 4°C (Fig. 1b, lane 1) with respect to levels of gp120 that can be detected by Western blotting, further supporting the use of these inactivation protocols.

Sera from mice vaccinated with envelope-containing, thermally inactivated virions can neutralize the vaccine strain. We previously reported an increase in binding to reagents and antibodies that recognize the CD4 and CD4-induced epitopes following thermal treatment (18). In the present work, we sought to demonstrate that this in vitro phenomenon of enhanced antigenicity translated into an ability to induce neutralizing antibodies in vivo. As such, BALB/c mice were vaccinated with formaldehyde-stabilized, thermally inactivated virions. Control mice were vaccinated with an irrelevant antigen (KLH). The studies described below utilized vaccine preparations prepared in primary PBMC. Mice received one prime and three boosts at 5-week intervals for a total of four immunizations. In order to aid in the induction of both Th1 and Th2 immune responses, we used an oligonucleotide containing CpG sequences as an adjuvant (ODN 1826) for these experiments (4). Anti-HIV-1 binding antibodies were evident following the first vaccination and reached sustained binding titers in excess of $1:10^6$ by 2 weeks after the second immunization (data not shown). In addition, strong antibody reactions against all major viral proteins were seen when these sera were analyzed by commercial Western blotting (data not shown).

Two weeks following the final immunization, the mice were sacrificed, and sera were collected by terminal cardiac puncture. These sera were used to perform virus neutralization assays in primary PBMC. For these studies, sera were preabsorbed against the cell substrate in which the vaccine was produced (primary PBMC). This step was added to address the potential for immune responses against human cellular (xenogeneic), as opposed to viral, antigens. As can be seen in Fig. 2, sera from mice vaccinated with formaldehyde-stabilized, thermally treated HIV_{SX} contained antibodies capable of neutralizing the vaccine strain at concentrations of 1:20 and 1:50. Importantly, a mean of at least 70% neutralization was observed in the groups receiving the highest dose of the vaccine at serum dilutions of 1:20 and 1:50. No neutralizing antibodies were observed in any of the control animals.

Sera from mice vaccinated with envelope-containing, thermally inactivated virions can neutralize primary isolates from various clades. The above studies demonstrated neutralization of the vaccine strain, HIV_{SX}. In order to examine the breadth of the antibody response in these mice, we measured neutralizing antibody responses against primary viral isolates in sera from the mice whose results are shown in Fig. 2. Using a panel of five primary non-clade B viral isolates, we demonstrated the ability of formaldehyde-stabilized, thermally inactivated virus preparations to induce cross-clade neutralizing antibodies in mice (Fig. 3). In these experiments, neutralizing antibody re-

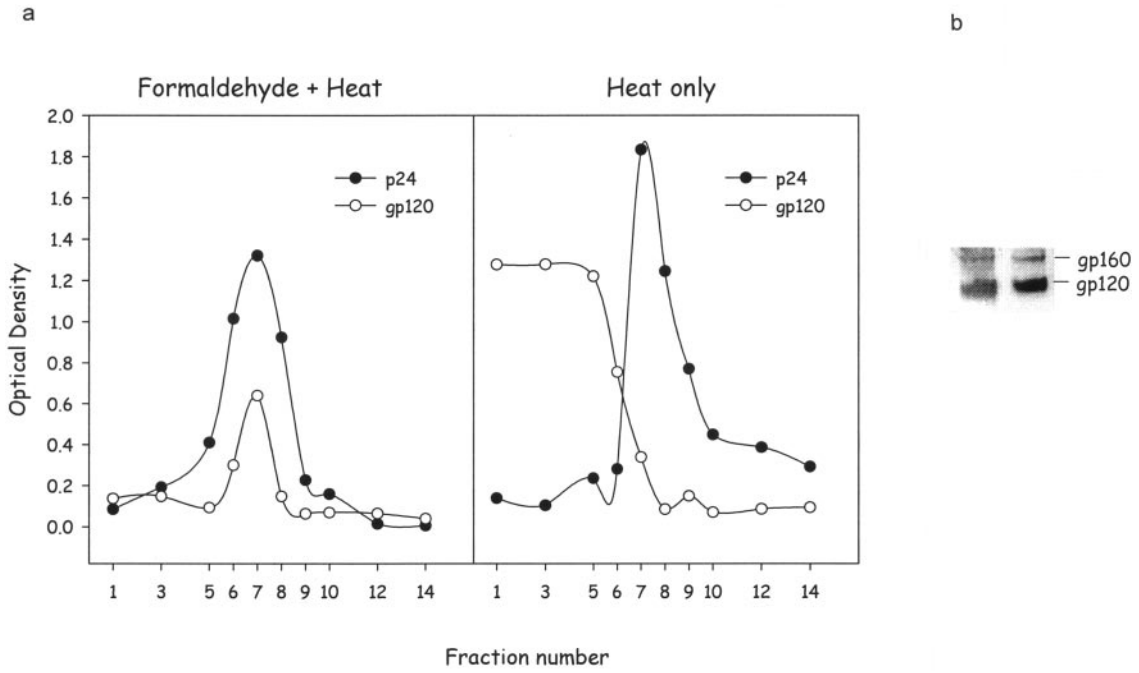

FIG. 1. Treatment of virions with formaldehyde prior to thermal inactivation decreases envelope shedding. (a) After ultrafiltration, HIV_{SX} was either heat inactivated or treated with formaldehyde and then heat inactivated as described in the text. Next, 200 μl of each virus preparation was layered onto 1.8 ml of undiluted Percoll (Pharmacia), and the samples were centrifuged at 56,000 × *g* for 60 min at 4°C. Fractions (100 μl) were removed from the top of the gradient. HIV-1 gp120 ELISA was done as described in the text using 2G12 as the capture antibody, and HIV-1 p24 assay was performed with a commercially available ELISA. Filled circles represent p24 (in nanograms per milliliter), and open circles represent gp120 (in nanograms per milliliter). (b) DNA was transfected into 293T cells by calcium phosphate as previously described. Samples were analyzed on an 8% gel and probed with a cocktail of anti-Env monoclonal antibodies. Left lane, untreated HIV_{SX}; right lane, formaldehyde-treated, heat-inactivated HIV_{SX}. These immunizations were repeated in three separate experiments. The data presented here are from one of these three trials.

sponses were the weakest against the two clade C viruses and were the strongest against the clade A and group O viruses. This result suggests that neutralizing antibody responses can be generated against viral strains that differ in envelope sequence from the vaccine strain.

Adjuvant choice can influence antibody titers. An inactivated HIV vaccine should primarily elicit humoral immune responses. The nature of the antibody response can be impacted by the inclusion of appropriate adjuvants. As such, we examined the ability of three different adjuvants to induce antibodies in mice of the IgGa (Th1-like) subtype. For these studies, BALB/c mice were vaccinated with formaldehyde-stabilized, thermally inactivated virions in the presence of either CpG ODN (as described above), the nontoxic saponin derived from the soapbark tree *Quillaja saponaria* (QS 21) (44), or alum. As can be seen in Fig. 4, vaccination in the presence of QS 21 resulted in the highest levels of IgG antibodies and in the greatest proportion of antibodies of the IgG2a subtype. Given these data, all subsequent vaccine trials were conducted in the presence of QS 21.

Vaccine preparations prepared in 293T cells remain immunogenic in vivo. Large-scale preparation of vaccines in primary PBMC is not likely to be a practical approach for a preventive HIV vaccine. Therefore, we examined whether we could prepare comparable vaccine immunogens by transfection of DNA into 293T cells. As we have previously observed with PBMC-prepared vaccine immunogens, these preparations demonstrated at least a 180% increase in binding to CD4-IgG after formaldehyde treatment and thermal inactivation (data not shown). This increase in binding indicated that increases in antigenicity seen with vaccine prepared in PBMC were also observed in vaccine preparations made in 293T cells. Next, BALB/c mice (n = 5/group) were vaccinated a total of four times at 5-week intervals in the presence of 10 μg of the adjuvant QS 21. We examined the ability of serum drawn 2 weeks after the final vaccination to neutralize three different clade B viruses, as well as the primary isolates from clades A, C, and E and group O described above. In addition, we tested these sera for their ability to neutralize infection mediated by a non-HIV envelope, that of $SIV_{mac1a11}$. Serum endpoint titers as determined by a MAGI CCR5 virus neutralization assay are reported in Table 1. As can be seen from the data in Table 1, these immunogens were capable of inducing mean 50% virus-neutralizing antibody (VNA) titers of ≥1:156 (range, 1:20 to ≥1:640) against all HIV isolates except for HIV_{JRCSF}, where neutralization was undetectable in two of five mice. In addition, no neutralizing antibodies were observed when MAGI cells were infected with virus bearing an SIV envelope. Of note, human control serum-neutralizing titers of the non-clade B viruses were in the 1:20 to 1:40 range in this assay. Therefore, these data reinforce the notion that a complex virion-based vaccine which demonstrates broad immunogenicity in vivo can be designed.

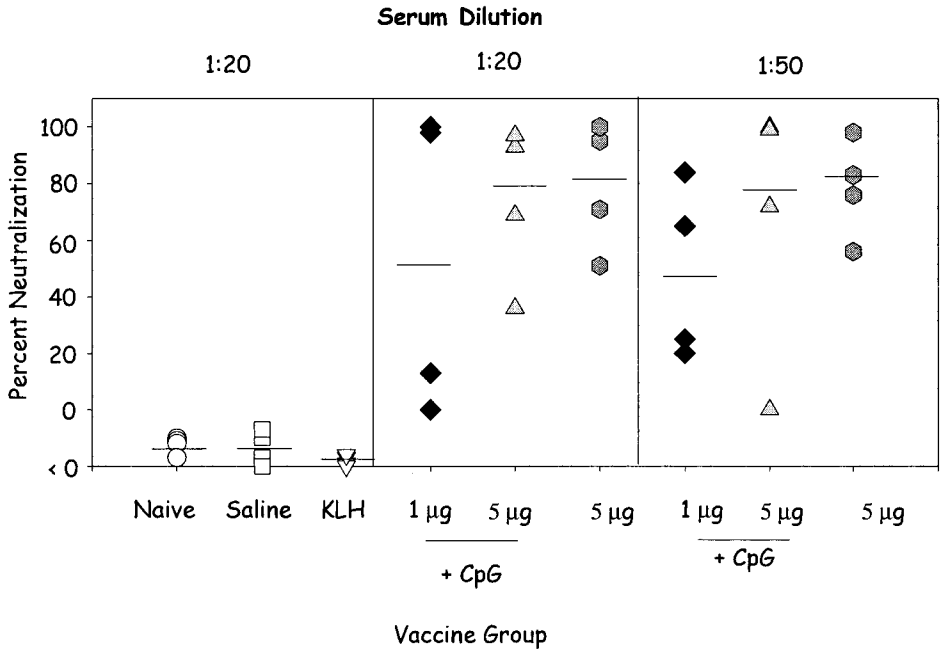

FIG. 2. Mice vaccinated with formaldehyde-treated, thermally inactivated virions produce antibodies capable of neutralizing infection with the vaccine strain in primary PBMC. Mice were vaccinated with vaccine containing either 1 or 5 μg of p24, as indicated in the figure. Virus neutralization was measured in a standard HIV-1 p24 neutralization assay. For this purpose, HIV_{SX} (200 $TCID_{50}$) was incubated with serum from each of the immunized mice for 30 min at 37°C. Virus and serum were added to PHA-stimulated PBMC for 24 h, and the cultures were washed to remove input serum and virus. At days 1, 2, 5, 7, and 8, 90% of the medium was removed and replaced with fresh medium. HIV-1 p24 production in the supernatant was measured by ELISA on day 9. Symbols represent individual mice. Bars represent mean percent neutralization for each group.

Thermal treatment of virus results in a preparation that is immunogenic in nonhuman primates. Having identified neutralizing antibody responses in a small-animal model, we next investigated whether these preparations would be immunogenic in nonhuman primates. For these studies, we vaccinated rhesus macaques (*Macaca mulatta*) with envelope-containing, formaldehyde-stabilized, thermally inactivated virions in the presence of the adjuvant QS 21. Six macaques received one vaccination and two boosts for a total of three vaccinations at 5-week intervals. Because previous vaccine studies using inac-

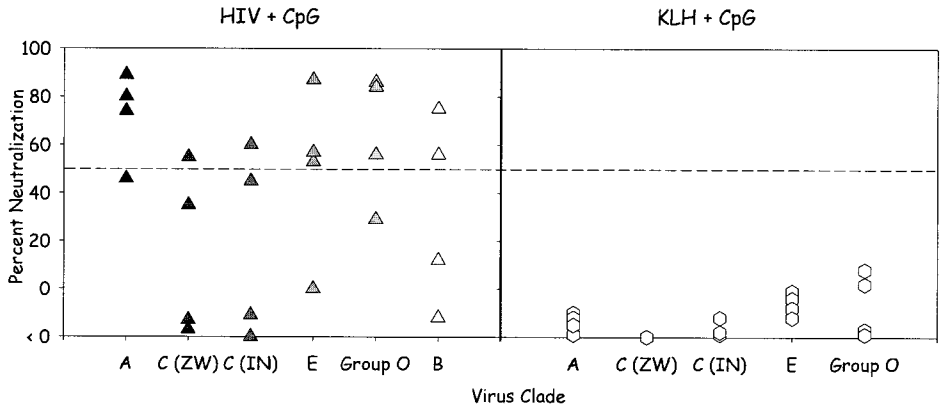

FIG. 3. Mice vaccinated with formaldehyde-treated, thermally inactivated virions produce antibodies capable of neutralizing infection with heterologous HIV-1 strains in primary PBMC. Virus neutralization was measured in a standard HIV-1 p24 neutralization assay. Serum (a 1:20 dilution) from each of the immunized mice was preincubated with cells. Next, virus (200 $TCID_{50}$) from clades A (TK1135), B (SX), C (92ZW101 and 93IN109), and E (93THHIVC), as well as virus from group O (305A9), was incubated with the pretreated serum from each of the immunized mice for 30 min at 37°C. Virus and serum were added to PHA-stimulated PBMC for 72 h, and then the cultures were washed to remove input serum and virus. At days 3 to 6, 90% of the medium was removed and replaced. HIV-1 p24 production in the supernatant was measured by ELISA on day 7. The dotted line represents 50% neutralization. The neutralizing antibody responses seen in animals vaccinated with formaldehyde-treated, heat-inactivated HIV-1 were significant ($P = 0.03$) for all non-clade B isolates except clade C (IN) by the Wilcoxon rank-sum test when compared to animals receiving KLH as the immunogen.

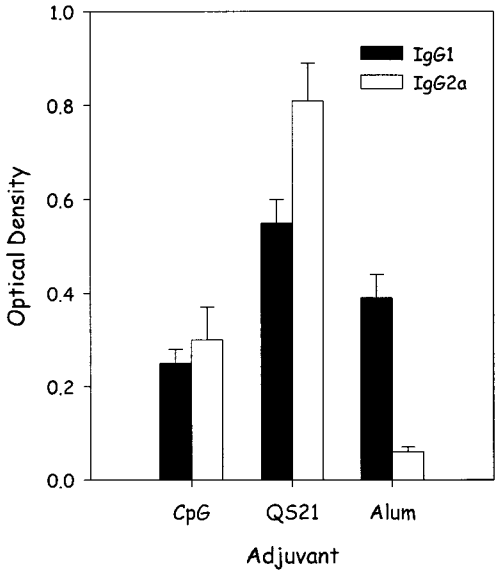

FIG. 4. Inclusion of different adjuvants in vaccine regimens results can influence serum IgG levels. Groups of four mice received four vaccinations at 5-week intervals. Twenty micrograms of CpG/vaccination was used in the group receiving CpG as an adjuvant. Twenty micrograms of QS 21/vaccination was used in the group receiving QS 21 as an adjuvant. Fifty micrograms of Alhydrogel/vaccination was used in the group receiving alum as an adjuvant. Mice were sacrificed 3 weeks after the final vaccination, and serum was assayed by ELISA using purified HIV-1-coated plates and goat anti-mouse IgG1 and IgG2a isotype-specific conjugates to measure antibody titers. Mice were vaccinated with preparations containing 20 μg of HIV p24, which corresponded to 1 μg of gp120 when measured by ELISA on unheated samples.

tivated immunogens prepared in human cells resulted in protective anti-human antibody responses in macaques, three macaques were vaccinated with immunogens that were prepared by deletion of Env-specific sequences from the plasmid DNA prior to transfection. After transfection of 293T cells, particles which were devoid of HIV Env as determined by Western blotting were formed (data not shown). Because cellular proteins are incorporated into the virion lipid bilayer during viral budding, these immunogens should contain all cellular proteins that are typically present in the thermally inactivated virions but would not contain functional gp120 against which virus-specific neutralizing antibody responses could be directed (2, 41).

Serum samples were collected prior to vaccination and 2 weeks after each vaccination. Envelope-specific binding antibodies could be detected after the first vaccination (data not shown), but neutralizing antibodies were detectable only after the third and final vaccination. As can be seen from Table 2, three of three sera from macaques vaccinated with envelope-containing, thermally inactivated virions were able to neutralize the vaccine strain at titers of 1:40 or 1:80 in a MAGI cell assay. None of the sera from macaques vaccinated with the thermally inactivated virions with envelope deletions were able to neutralize the vaccine strain in this assay.

We next examined the breadth of the immune response by independent assay of blinded serum samples by using the ViroLogic PhenoSense HIV neutralization assay. As can be seen in Table 3, macaques vaccinated with immunogens which contained HIV envelope generally demonstrated higher postvaccination neutralizing antibody responses. Although these sera scored positive in this assay only when tested against HIV_{NL4-3} and HIV_{SF162}, with the aMLV controls to establish assay cutoff criteria, meaningful data may still be discerned provided that the postvaccination sera demonstrate increases in titer relative to the prevaccination sera. In this case, antibody responses in macaques RRG8 and RIA8 against a number of heterologous viruses ranged from 1:35 to 1:568 after vaccination (Table 3, bold values). These responses were broadest, although primarily clade B restricted, in macaques RIA8 and RRG8. Of note, IC_{50}s of 1:61 and 1:79 were detected against HIV_{JRFL} in this assay in macaques RIA8 and RRG8. These titers are comparable to those detected in the MAGI assay against virus bearing the HIV_{JRFL} Env (HIV_{SX}).

In contrast, there were few to no HIV-specific neutralizing antibodies detected in macaques vaccinated with immunogens that did not contain HIV-1 envelope (data not shown). In these macaques, the only detectable response was against HIV_{NL4-3} in one of three macaques, with a titer of 1:94 being recorded in the postvaccination sera and an undetectable response being

TABLE 1. Neutralizing antibodies which block infection by virus-bearing heterologous envelopes can be raised in mice[a]

Mouse no.	Serum endpoint titer								
	HIV_{SXSL9}	HIV_{NL4-3}	HIV_{JRCSF}	TK1135 (clade A)	92ZW101 (clade C)	93IN109 (clade C)	93TH305 (clade E)	HIV-1CA9 (group O)	SIV
1	1:640	≥1:160	≥1:160	1:80	1:80	1:80	1:80	1:80	<1:20
2	1:80	≥1:160	1:80	1:160	1:80	1:80	1:80	1:160	<1:20
3	1:20	≥1:160	<1:20	1:160	1:80	1:80	1:160	1:160	<1:20
4	1:20	1:40	<1:20	1:160	1:80	1:80	1:160	1:80	<1:20
5	1:20	≥1:160	1:40	≥1:640	≥1:640	≥1:640	≥1:640	≥1:640	<1:20

[a] For these studies, sera were heat inactivated and subjected to three successive rounds of preadsorption against an equal volume of packed 293T cells. Viral inocula remained constant (at 100 $TCID_{50}$), and inocula were incubated for 30 min at 37°C with an equal volume of serial half-log dilutions of serum from 1:20 (the dilution we previously determined not to be toxic or to interfere with detection) through 1:640 to determine approximate 50% VNA. Virus without serum served as the marker for 100% infection. Virus-neutralizing and nonneutralizing polyclonal antibodies diluted in normal mouse serum were included as controls. Neutralization was calculated as a percent reduction in β-Gal production relative to the virus control 48 h after infection. Spots were quantitated by using an automated system (Immunospot; Cellular Technologies). When compared by Fisher's exact test to neutralization of virions bearing SIV envelope, the neutralization observed against the HIV-1 isolates was statistically significant ($P = 0.05$ when corrected for multiple comparisons). In addition, the non-clade B-neutralizing antibody titers induced by vaccination were significant by Fisher's exact test when compared to responses seen with clade B-specific human immune sera in the virus neutralization assay ($P = 0.05$ when corrected for multiple comparisons). Mice were vaccinated with preparations containing 20 μg of HIV p24, which corresponds to 1 μg of gp120 when measured by ELISA on unheated samples in the presence of 20 μg of QS 21.

TABLE 2. Pre- and postvaccination serum titers show that neutralizing antibodies that block infection by the vaccine strain can be raised in macaques[a]

Animal ID	Virion characteristic	Prevaccination serum titer	Postvaccination serum titer
RRG8	Env^+	<1:20	1:40
RFZ7	Env^+	<1:20	1:40
RIA8	Env^+	<1:20	1:80
ROG8	Env^-	<1:20	<1:20
RMG8	Env^-	<1:20	<1:20
RSG8	Env^-	<1:20	<1:20

[a] For these studies, sera were heat inactivated and subjected to three successive rounds of preadsorption against an equal volume of packed 293T cells. Viral inoculum size remained constant (at 100 $TCID_{50}$), and inocula were incubated for 30 minutes at 37°C with an equal volume of serial half-log dilutions of serum from 1:20 (the dilution we previously determined not to be toxic or to interfere with detection) through 1:80 to determine approximate 50% VNA. Virus without serum served as the marker for 100% infection. Virus-neutralizing and nonneutralizing polyclonal antibodies diluted in normal macaque serum were included as controls. Neutralization was calculated as a percent reduction in β-Gal production relative to the virus control 48 h after infection. Spots were quantitated with an automated system (Immunospot; Cellular Technologies).

observed in the prevaccination sera. Taken together with the titers observed in the MAGI cell neutralization assay, these data are consistent with our experiments in mice where neutralization of heterologous viral isolates could be demonstrated following vaccination with formaldehyde-treated, heat-inactivated virions.

DISCUSSION

We demonstrate here that vaccination with formaldehyde-treated, thermally inactivated HIV-1 virions can induce neutralizing antibodies in mice and nonhuman primates. The perceived difficulty of inactivating HIV-1 without losing or destroying viral envelope has been cited as a major stumbling block to the development of an inactivated vaccine (reviewed in reference 7). Retroviral envelopes can be easily shed, particularly following concentration by ultracentrifugation or sucrose gradient banding, common methods utilized by most investigators for concentration of HIV-1 (12). Our protocols include treatment of virions with a sublethal dose of formaldehyde prior to thermal treatment in order to maintain gp120 on the surface of virions. We report here for the first time that formaldehyde-treated, thermally inactivated virions retain the major gp120 peak in the same gradient fraction as viral p24 following sedimentation through Percoll as untreated virions. In contrast, sedimentation through Percoll of thermally inactivated virions that were not subjected to formaldehyde treatment resulted in the presence of gp120 in multiple fractions. Therefore, we have developed a novel method to produce virions which retain gp120 even in the face of thermal inactivation.

We previously demonstrated retention of several major neutralization epitopes on viral envelope following treatment with formaldehyde and heat. Moreover, we found that binding of gp120 to some of these epitopes was enhanced greater than 1.8-fold following thermal treatment. These induced sites include epitopes recognized by potent neutralizing antibodies, including that recognized by the MAb 17b, which has been postulated to be partially occluded or cryptic in native virions. Because we observed enhancement of binding to known epitopes, it is possible that thermal inactivation may also result in the exposure of other antigenic sites (18). Taken together, we have developed methods of inactivating HIV-1 virions that allow for retention of gp120 on virions and of major conformational epitopes proposed to be important for the induction of virus-neutralizing antibodies.

Early attempts to develop an inactivated SIV vaccine failed after it was ascertained that most, if not all, immune reactivity was directed at xenoantigens on the surface of the virions (2). It was later determined that these immune responses were observed because the budding virions capture a number of cellular antigens as they pass through the plasma membrane (2, 10, 32). With these data in mind, we addressed the possibility that the neutralizing antibody responses we observed in the present study might not be HIV-1 specific by two different methods. First, a control arm in the nonhuman primate studies included macaques that were vaccinated with formaldehyde-treated, thermally inactivated preparations that did not contain HIV-1 gp120. Since cellular molecules would still be present in the lipid bilayers of these virions with gp120 deletions, any antibody neutralization attributed to anticellular antibodies should be measured by our assays. In these studies, zero of three sera from these control animals were able to neutralize HIV_{SX} by MAGI assay, and low-level neutralization in the ViroLogic PhenoSense assay was detected only against HIV_{NL4-3} in one control macaque. This result suggests that anticellular neutralizing antibody responses from this vaccine were minimal in the present study. Second, except for the PhenoSense assays, all sera were preabsorbed against the cell

TABLE 3. Neutralizing antibody response following immunization with formaldehyde-treated, thermally inactivated virions

Virus	Clade	Receptor usage	Prevaccination titer			Postvaccination titer			$HIV\text{-}1^+$ plasma
			RRG8	RFZ7	RIA8	RRG8	RFZ7	RIA8	
92RW021	A	R5	<34	<34	<34	<34	<34	<34	27
92BR014	B	R5/X4	<34	<34	<34	<34	<34	<34	92
91US056	B	R5	<34	<34	40	<34	<34	<34	177
92BR021	B	R5/X4	<34	<34	<34	<34	<34	<34	187
301959	B	NA	<34	<34	<34	34	<34	**59**	192
92BR020	B	R5	<34	<34	<34	<34	<34	<34	222
92HT593	B	X4	<34	<34	39	**41**	<34	42	290
QZ4589	B	R5	46	<34	44	**68**	<34	**65**	377
93IN101	C	R5	<34	<34	<34	<34	<34	<34	75
92UG021	D	X4	<34	<34	<34	<34	<34	<34	61
92UG046	D	X4	<34	<34	<34	**35**	<34	**38**	92
JRFL	B	R5	<34	<34	<34	**61**	<34	**79**	66
JRCSF	B	R5	<34	<34	<34	<34	<34	**46**	440
BAL	B	R5	<34	<34	<34	**60**	<34	**77**	644
NL43	B	X4	<34	<34	39	**95**	**41**	**126**	2,154
SF162	B	R5	<34	<34	<34	**516**	**174**	**568**	7,351
aMLV			56	46	51	<34	<34	<34	<20

[a] For these studies, sera were heat inactivated. Neutralizing antibody responses were measured following preincubation of recombinant viruses pseudotyped with selected viral Envs with serial dilutions of sera from the macaques. These samples were used to infect U87 cells expressing CD4 plus the CCR5 and CXCR4 coreceptors (U87/CD4/CCR5/CXCR4). Neutralizing activity was calculated as the percent inhibition of viral replication (luciferase activity) at each antibody dilution compared to a control culture without antibody. The IC_{50} was determined, and neutralization titers were expressed as the reciprocal of the plasma dilution conferring 50% inhibition. Titers in bold were higher after vaccination than before vaccination. $HIV\text{-}1^+$ plasma titers are provided for comparison.

substrates in which the vaccine stocks were grown. This step was included to remove anticellular antibodies that might be present in the sera. Indirect immunofluorescence assays confirmed that binding to those cells was not detectable after three rounds of absorption. This may explain why neutralization of HIV_{NL4-3} was not observed in the MAGI assay using sera from the macaques vaccinated with preparations with envelope deletions.

In general, the neutralizing antibody response to HIV-1 during natural infection is not particularly robust. Nonetheless, we now recognize that there are a limited number of monoclonal antibodies developed from HIV-1-infected individuals that are broadly neutralizing in vitro (5, 23, 25, 42, 45, 46). More importantly, these antibodies can protect rhesus macaques from challenge with SIV-HIV chimeras (SHIVs) containing various HIV-1 envelopes in an SIV backbone when used in passive immunization strategies (30, 36). These studies provide evidence that immunogens that are capable of inducing these types of antibodies may prove to be protective. It is likely that that the failure of current vaccine strategies to induce protective antibodies is linked to the inability of native envelope structures to readily elicit these types of antibodies. The neutralizing antibody titers observed in the present study were also not likely to be high enough to prevent infection from challenge. This result may be due to the limited number of vaccinations ($n = 3$ in macaques; $n = 4$ in mice) or to a need to modify viral envelope structures beyond that which occurs following thermal inactivation. Future studies with thermally inactivated virions with modified envelopes may improve upon the responses seen here by providing immunogens better capable of stimulating potent antibody responses. This improvement would be necessary in order to make the use of an inactivated vaccine a viable option in vaccine regimens.

Despite the protection afforded by killed vaccines for other viral diseases, research devoted to developing a killed vaccine for HIV-1 has been minimal. This has been primarily due to concerns regarding shedding of gp120 from virions, safety considerations surrounding vaccine preparation, and the failures of early SIV vaccine preparations. In the present work, we reexamined the concept of a killed HIV-1 vaccine using thermally inactivated virus preparations that we demonstrated maintained and or had enhanced binding capacity to broadly reactive, conformation-dependent neutralizing antibodies. Together with other data from the field, the data presented here suggest that it may be possible to develop a killed HIV-1 vaccine that could elicit protective humoral immune responses.

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