ABSTRACT Based on recent reports of antibody-dependent enhancement of human immunodeficiency virus type 1 (HIV-1) infection in vitro by serum from HIV-1-infected humans, sera from HIV-1 antibody-positive chimpanzees (Pan troglodytes) was evaluated for enhancing activity in an in vitro infection assay that uses MT-2 cells (a human lymphoblastoid cell line). Although fresh chimpanzee serum was found to have pronounced infection-enhancing properties in the absence of antibody to HIV-1, this effect was abolished by heat inactivation (57°C, 1 hr) or treatment with cobra venom anticomplementary protein. Heat-inactivated HIV-1 antibody-positive chimpanzee serum could enhance HIV-1 infection of MT-2 cells in vitro when combined with fresh, normal human serum. By serial serum samples from three HIV-1-infected chimpanzees, HIV-1 antibody-positive chimpanzees are shown to develop enhancing antibodies early in infection (2 mo postchallenge), whereas neutralizing antibodies develop later. Over the course of HIV-1 infection, this enhancing activity decreases while neutralizing activity increases, suggesting a possible role for enhancing and neutralizing activities in HIV-1 pathogenesis. The enhancing activity of an IgG fraction used to passively immunize chimpanzees against HIV-1 infection is shown to be present at dilutions as high as 1:65,000, offering an interesting possible reason for the failure of passive immunization to protect chimpanzees from HIV infection. These results suggest that serum from HIV-1-immunized chimpanzees might be tested to determine whether current HIV-1 candidate vaccines induce production of antibodies that mediate antibody-dependent enhancement of HIV-1 infection in this in vitro assay.

Most individuals (95% of 90 patients) whose sera have been tested and who are infected with human immunodeficiency virus type 1 (HIV-1) exhibit antibodies, which, in conjunction with the alternate complement pathway, accelerate HIV-1 infection of MT-2 target cells (a CD4+ lymphoblastoid cell line) in vitro (1, 2). These enhanced infections are characterized by an accelerated appearance of HIV-induced cytopathic effect (CPE) (1) and increased levels of HIV-1 antigen-positive cells, reverse transcriptase (RT) release, HIV RNA, and progeny virus synthesis at all time points measured after infection of target cells by HIV-1 (2). A similar phenomenon, known as antibody-dependent enhancement (ADE), has been documented in other viral infectious processes (3, 4) and has been confirmed recently for complement-mediated ADE of HIV-1 infection in peripheral blood monocytes (5). Subsequently, the definition of ADE of HIV-1 infection was broadened to include an apparent Fc receptor-mediated, complement-independent mechanism for ADE of HIV-1 infection (6, 7). The Fc receptor-mediated mechanism was demonstrated not only in sera obtained from HIV-1-infected individuals but also in antisera to HIV-1 obtained from guinea pigs and chimpanzees (6). Of special concern to vaccine development is the observation that complement-mediated ADE can reduce or completely abrogate the in vitro protective effects of neutralizing antibody within both homologous and heterologous human sera (1, 8). Because the only surrogate animal currently available for HIV-1 infectivity studies is the chimpanzee (Pan troglodytes) (9), the demonstration of complement-mediated ADE in the chimpanzee is critical to the evaluation of candidate vaccines for their capacity to induce ADE. This report describes the induction of ADE of HIV-1 infection in the chimpanzee and the comparative roles of chimpanzee versus human complement in mediation of this response by serum from both HIV-1 antibody-negative and HIV-1 antibody-positive subjects.

MATERIALS AND METHODS

Sera. Sera from chimpanzees X35 and X95 were from HIV-1 antibody-negative chimpanzees. Sera from chimpanzees X91, X118, X119, and X139 were HIV-1 antibody positive. Chimpanzees X91, X118, and X139 were all directly inoculated with tissue-culture-derived HIV-1. Chimpanzee X119 was inoculated with HIV-1 by serial transfer from another infected chimpanzee. All antibody-positive chimpanzee sera were confirmed to be HIV-1 antibody-positive by Western immunoblot. Chimpanzee complement-containing sera were fresh-frozen and stored at -70°C. Human complement-containing sera were purchased lyophilized from Sigma and were reconstituted immediately before use. When heat-inactivated sera were used, chimpanzee serum was heated for 60 min at 57°C, whereas human serum was heated for 50 min at 57°C.

Cells and Virus. H9/HTLV-III/lg, U937/HTLV-III/lg, and CEM/HTLV-III/lg (human T-lymphotropic virus type III/lg isolates of HIV-1 produced by H9, U937, and CEM cells, respectively), H9/HTLV-III/Rf, and U937/HTLV-III/Rf (human T-lymphotropic virus type III/lg isolates of HIV-1 produced by H9 and U937 cells, respectively), and MT-2 cells were cultivated in RPMI 1640 medium with 12% heat-inactivated fetal calf serum and 50 μg of gentamicin per ml.

Abbreviations: ADE, antibody-dependent enhancement; CPE, cytopathic effect; HIV-1, human immunodeficiency virus type 1; HTLV-III/lg, human T-lymphotropic virus type III/lg; moI, multiplicity of infection; RT, reverse transcriptase; IFA, immunofluorescent antibody.

†To whom reprint requests should be addressed.
Analysis of Enhanced HIV-1 Infections by RT Release and Indirect Immunofluorescence. In culture flasks, 5 ml of filtered HIV-1 from an H9/HTLV-IIIB culture were incubated for 1 hr at 37°C with (i) no serum, (ii) 250 μl of human complement-containing serum, (iii) 100 μl of chimpanzee complement-containing serum, (iv) 50 μl of human heat-inactivated chimpanzee serum X91 (HIV-1 antibody-positive), (v) 50 μl of human heat-inactivated chimpanzee serum plus 100 μl of human complement-containing serum, or (vi) 50 μl of heat-inactivated chimpanzee serum X91 plus 100 μl of chimpanzee complement-containing serum. To each flask 15 × 10³ MT-2 cells in 15 ml of growth medium were added and then incubated in sealed flasks at 37°C for 12 hr. Next, cells were washed once with RPMI 1640 medium, then suspended in 20 ml of growth medium and incubated for 30 hr at 37°C. At the end of the incubation, 5 ml of cell suspension was removed. After low-speed centrifugation, supernatant fluids were assayed for RT activity by the method of Poiesz et al. (11). Cell pellets were washed three times in 5 ml of phosphate-buffered saline, and indirect immunofluorescence was performed. Percent immunofluorescent antibody (IFA)-positive cells were determined by using polyclonal human anti-HIV serum followed by fluorescein-conjugated goat anti-human IgG as described (12).

RESULTS AND DISCUSSION

ADE of HIV-1 Infection in Vitro by HIV-1 Antibody-Positive Chimpanzee Serum. Preliminary attempts to show complement-mediated ADE by using heat-inactivated serum from an HIV-1-infected chimpanzee X91 plus fresh-frozen normal chimpanzee serum at 1:20 dilution was difficult to measure due to high background CPE in the virus-plus-normal chimpanzee serum control. Fresh, normal human serum was subsequently used as a complement source. Serum from chimpanzee X91 was serially 2-fold diluted from 1:4–1:8192 into growth medium containing 1:20 human complement-containing serum, and HIV-1 was added. Serum X91 accelerated the appearance of HIV-1-induced CPE at dilutions from 1:4–1:2048 in MT-2 cells (data not shown). These results were similar to those previously reported for HIV-1 antibody-positive human serum (1, 8). To test whether this enhanced CPE was truly ADE of HIV-1 infection or merely complement-mediated cytolysis of infected target cells, heat-inactivated serum from chimpanzee X91 was combined with either human complement-containing serum or HIV-1 antibody-negative chimpanzee complement-containing serum and then added directly to HIV-1-infected cells. H9/HTLV-IIIb, H9/HTLV-IIIb, CEM/HTLV-IIIb, U937/HTLV-IIIb, and U937/HTLV-IIIb. There was no increased CPE, a result signifying that complement-mediated cytolysis of infected cells had not occurred (data not shown). In other experiments, 2-fold dilutions of HIV-1 antibody-negative chimpanzee serum plus 1:20 human or chimpanzee complement were added to previously infected MT-2 cells; no enhanced CPE was observed (data not shown). These results do not agree with the findings of Nara et al. (13), who demonstrated that chimpanzee antibody against HIV plus complement could cause ⁵¹Cr release from chromium labeled HIV-1-infected cells. Why chimpanzee antisera to HIV fails to lyse HIV-1-infected target cells in this study is presently unclear (13), although our findings do agree with other reports for human serum against HIV-1 (1, 8). Perhaps the ⁵¹Cr release assay used by Nara et al. (13) detects nonlethal pore formation initiated by complement; the vital-dye uptake method used here would only detect lethal complement-mediated lesions. Therefore, chimpanzee antibody may mediate nonlethal complement activation. There is also no evidence that HIV-1 is inactivated by fresh, normal sera as occurs with many other enveloped viruses (14, 15).

Medical Sciences: Robinson et al.

Enhancement of HIV-1 infection by HIV-1 antibody-negative chimpanzee and human sera. HIV-1 antibody-negative chimpanzee sera were fresh-frozen to ensure that complement activity was retained. Human serum containing normal complement activity was purchased lyophilized (Sigma) and reconstituted immediately before use. Complement sera were 2-fold diluted into growth medium and assays were performed as described. Results are expressed as percent viable cells relative to HIV-1 infection control (no serum, eight replicates). ■ Chimpanzee X35; ● chimpanzee X95; ●, human complement serum (Sigma). Points represent a mean of three replicates ± 1 SD.

HIV-1 Antibody-Negative Chimpanzee Serum Accelerates HIV-1 Infection in Vitro. To investigate whether fresh, antibody-negative chimpanzee serum could, indeed, enhance HIV-1 infection, thereby leading to the high background CPE seen when chimpanzee serum was used as complement source, 2-fold dilutions of fresh, antibody-negative chimpanzee serum were done. As Fig. 1 shows, human complement-containing serum could increase HIV-1-induced CPE slightly but only to a dilution of 1:16; therefore, at a dilution of 1:20 no enhancement of infection could occur without the additional presence of antibody to HIV-1. Serum from control, antibody-negative chimpanzee X95 could cause an increased rate of viral-induced cytolysis to a dilution of 1:256, whereas serum from control, antibody-negative chimpanzee X35 could enhance viral-induced CPE to a dilution >1:512.

To determine whether antibody-negative chimpanzee serum X35 was enhancing HIV-1 infection by means of a complement-mediated mechanism, antibody-negative chimpanzee serum was heated for 1 hr at 57°C and then added to HIV-1. This heat-inactivation destroyed the ability of chimpanzee serum to enhance HIV-1 infection in vitro. The ability of cobra venom anticomplementary protein to abrogate the enhancing activity of the chimpanzee serum was tested also. A 1-hr preincubation of chimpanzee serum with cobra venom factor completely blocked the enhancement of HIV-1 infection by serum X35 (Fig. 2). Similar results have been reported for ADE of HIV-1 infection by human serum (1).

![Fig. 1. Enhancement of HIV-1 infection by HIV-1 antibody-negative chimpanzee and human sera. HIV-1 antibody-negative chimpanzee sera were fresh-frozen to ensure that complement activity was retained. Human serum containing normal complement activity was purchased lyophilized (Sigma) and reconstituted immediately before use. Complement sera were 2-fold diluted into growth medium and assays were performed as described. Results are expressed as percent viable cells relative to HIV-1 infection control (no serum, eight replicates). ■ Chimpanzee X35; ● chimpanzee X95; ●, human complement serum (Sigma). Points represent a mean of three replicates ± 1 SD.](image1)

![Fig. 2. Enhancing activity of antibody-negative chimpanzee serum is abrogated by cobra venom anticomplementary protein. HIV-1 antibody-negative chimpanzee serum (50 μl) from chimpanzee X35 was incubated at 37°C with 12.5 μg of cobra venom factor for 1 hr (●); 0 hr (●); or without cobra venom factor (■). Serum was then serially diluted in triplicate and HIV-1 was added. After 1 hr at 37°C, 2 x 10⁶ MT-2 cells were added to each well. Viable cells were determined 48 hr later as described (10).](image2)

Table 1. Enhanced HIV-1 infections are characterized by increased RT release and antigen synthesis

<table>
<thead>
<tr>
<th>Complement</th>
<th>IFA†</th>
<th>RT‡</th>
<th>IFA</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Human</td>
<td>17,600</td>
<td>60</td>
<td>122,400</td>
<td>1,408,000</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>2,352,000</td>
<td>100</td>
<td>1,408,000</td>
<td>1,408,000</td>
</tr>
</tbody>
</table>

†Serum X91 was HIV-1 antibody positive as confirmed by ELISA and Western blot. Serum was heat-inactivated and enhancing assays were performed in flasks as described.

‡IFA is given as percent HIV-1 antigen-positive cells as determined by indirect immunofluorescence and as described in text.

‡RT is given as cpm/ml of culture fluid and was determined as indicated in text. SDs of samples in our laboratory are ±10%.

Accelerated CPE Is Correlated with Increased Viral Antigen Synthesis. To confirm that increased CPE was associated with increased HIV-1 production, the effect of chimpanzee complement-containing serum on expression of RT activity and IFA-positive cells was determined (Table 1). Chimpanzee complement-containing serum with and without heat-inactivated serum from antibody-positive chimpanzee X91 greatly increased both RT activity and the percentage of IFA-positive cells at 42 hr post-virus challenge. Heat-inactivated serum from chimpanzee X91 alone could not increase these parameters. Heat-inactivated serum X91 at a 1:100 dilution in the presence of chimpanzee complement showed decreased RT activity compared to fresh, normal chimpanzee serum alone because serum X91 was slightly neutralizing at this concentration. Human complement-containing serum alone minimally increased both percent IFA-positive cells and RT activity; this increase was greatly enhanced by the addition of heat-inactivated serum from chimpanzee X91. Thus, enhanced HIV-1 infection by chimpanzee complement-containing serum as well as chimpanzee antibody plus human complement were correlated with enhanced HIV-1-induced CPE. Paradoxically, human antibody against HIV-1 in combination with chimpanzee complement was unable to accelerate infection above the complement-induced virus control (see Table 3). Because chimpanzee complement alone enhances infection, it is possible that human antibody cannot further increase this already accelerated rate of infection. Alternatively, the pronounced acceleration of HIV-1 infection in vitro by chimpanzee complement may be secondary to a partial species specificity manifest by complement receptors of the human target cell MT-2 in a human antibody/chimpanzee complement-mediated reaction.

Relative Levels of HIV-1 Neutralizing and Enhancing Antibody Activities Are Related to Duration of HIV-1 Infection. Because enhancing activity had been detected in HIV-1 antibody-positive chimpanzee X91, the sera from three other HIV-1 antibody-positive chimpanzees were evaluated for enhancing activity (Table 2). Chimpanzees X118 and X139 were tested both before and after HIV infection for enhancing antibodies. Chimpanzee X119 was analyzed only after virus challenge. Chimpanzees X118 and X139 had no enhancing-antibody activity before virus challenge, whereas all three chimpanzees had demonstrable enhancing-antibody activity after virus challenge. These antibody titers were greatest.
Table 2. Reciprocal neutralizing and enhancing antibody titers of chimpanzee serum after infection by HIV-1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Post-HIV-1 infection, mo</th>
<th>Neutralizing antibody</th>
<th>Complement depleted</th>
<th>Complement restored&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Neutralizing antibody</th>
<th>Enhancing antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>X118</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>216</td>
<td>0</td>
<td>&gt;17,496</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>648</td>
<td>24</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>648</td>
<td>24</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>648</td>
<td>72</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td>X139</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>216</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>216</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>648</td>
<td>24</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>648</td>
<td>72</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>648</td>
<td>72</td>
<td>648</td>
<td>648</td>
<td>648</td>
</tr>
</tbody>
</table>

Enhancing titers were done as described in text. Neutralizing titers were defined as the highest serum dilution giving 50% protection from viral-induced CPE as described (8). All sera were tested in a blinded fashion.

*Animals X118 and X139 were infected with the tissue culture-derived HTLV-III<sub>H</sub> isolate of HIV-1. Animal X119 was infected by serial transfer of HIV-infected chimpanzee serum. All animals seroconverted according to Western blot after infection.

†Measured in a constant amount (1:20) of fresh human serum. When both neutralizing and enhancing antibody activities are present, then enhancing activity occurs after neutralizing activity has been diluted out.

Early in infection and decreased during infection. Chimpanzee X118 had high enhancing-antibody activity even in the complete absence of neutralizing-antibody activity as early as 1 mo post-virus challenge. Neutralizing-antibody titers (as defined by 50% protection from virus-induced CPE) for all three chimpanzees rose during infection, although chimpanzee X119 fell from a high at 12 mo to a low at 21 mo post challenge. When the enhancing profiles of serum from chimpanzee X118 1 mo after infection (Fig. 3A), no neutralizing-antibody activity with and without complement, enhancing titer of 1:5832) is compared with serum from the same chimpanzee 3.7 mo after infection (Fig. 3B); no neutralizing activity with complement, weak neutralization without complement, enhancing activity >1:17,496, and 21 mo after infection (Fig. 3C); relatively high neutralizing activities with and without complement, weak enhancing activity), it is apparent that far greater enhancing and lower neutralizing antibody activities were present early in HIV-1 infection. In Fig. 3B, no neutralization in the presence of complement occurred at dilutions of 1:8 or 1:24, whereas in Fig. 3C neutralization in the presence of complement was seen at dilutions of 1:8, 1:24, and 1:72. Thus, the 100% viability at dilutions 1:8 and 1:24 in Fig. 3B was not from any neutralizing antibodies but may have been from a lack of enhancing antibody activity or, more likely, a balance between enhancing and neutralizing activities canceled both activities. These data are similar to those reported for ADE of West Nile virus (16). These values also compared well with values found in infected humans (1, 2). Also similar to humans was the ability of fresh complement serum to greatly reduce the neutralizing activity of heat-inactivated chimpanzee serum.

HIV-1 Enhancing Antibody Activity of HIV-1 Antibody-Positive Human IgG Fraction (HIVIG). Passive immunization against HIV-1 infection in chimpanzees has been attempted but yielded no protection of the immunized animal (17). Table 3 illustrates the reciprocal HIV-1 neutralizing and enhancing titers of the pooled HIV immune globulin fractions (HIVIG) used in this passive immunization experiment in chimpanzees. The 32-fold decrease in neutralization titer by human complement and the 4-fold inhibition seen for chimpanzee complement may indicate that the failure of passive immunization is secondary to complement-mediated ADE. Likewise, the acceleration in HIV-1 infection by chimpanzee complement alone may be significant in the failure of passive immunization to protect chimpanzees from HIV-1 infection because chimpanzee complement might override even minimal virus neutralization. Finally, since this assay uses human cells with human complement receptors (2), the high background antibody-independent enhancement by antibody-negative chim-

Table 3. Reciprocal HIV-1 neutralizing and enhancing antibody titers of pooled HIV-1 seropositive IgG fraction

<table>
<thead>
<tr>
<th>IgG fraction</th>
<th>Neutralizing antibody</th>
<th>Enhancing antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG fraction and human complement</td>
<td>64</td>
<td>65,536&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>IgG fraction and chimpanzee complement</td>
<td>512</td>
<td>0&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Enhancing and neutralizing assays were done as described.

*Enhancing titer relative to HIV-1 control infection (no serum).

†Enhancing titer relative to HIV-1 plus human complement (1:20) control infection.

‡Enhancing titer relative to HIV-1 plus chimpanzee complement (1:50) control infection.
panzee serum may be an artifact. It is possible that if a chimpanzee cell line were used, antibody-independent enhancement by fresh, normal chimpanzee complement would not occur, whereas ADE in the presence of chimpanzee complement and human antibody might occur.

CONCLUSIONS

Our data suggest that the production of enhancing antibodies may be important in HIV-1 infection of chimpanzees. Although HIV-1 infection induces neutralizing antibodies, the protection achieved is relatively ineffective in vitro in the presence of overwhelming enhancement of infection by serum factors. These factors in the human system and, apparently, in the chimpanzee system are antibody and complement (1). Detection of complement-mediated ADE of HIV-1 infection appears to require target cells bearing receptors for complement (2) as was reported for complement-mediated ADE of West Nile virus infection in vitro (18).

Although ADE of HIV-1 infection has been demonstrated in multiple laboratories (1, 2, 4–6), it must be emphasized that its significance in the natural pathogenesis of HIV-1 infection and importance in vaccine development have not been established. Nevertheless, the accelerated virus infection in vitro coupled with current vaccine failure in chimpanzees (17, 19, 20) is consistent with an in vivo role for enhancing antibodies in HIV-1 pathogenesis. That these two functional groups of antibodies could be fractionated on an affinity column containing a nonglycosylated fractional part of the envelope-precursor polyprotein (21) suggests that antigenic domains responsible for enhancing-antibody induction could be eliminated from future candidate vaccines. Such findings are consistent with ADE in other virus systems where virus-neutralizing and virus-enhancing activities of serum were separable (22, 23). Such results may mean that current attempts to formulate HIV-1 vaccines by using various recombinant gp160 and gp120 strategies could be improved by eliminating regions that induce enhancing antibodies while retaining regions that generate the desired neutralizing antibodies. Although the biological significance of ADE has yet to be determined, these results establish the validity of the chimpanzee as a model for the induction of ADE of HIV-1 infection, which can be used in the evaluation of candidate vaccines. Such evaluations in vivo in chimpanzees may not be useful due to high-background enhancement by fresh, normal chimpanzee serum, although such accelerated infection in vitro may be an artifact of human target cells used in the assay. Nevertheless, serum from chimpanzees vaccinated with various HIV-1 vaccine preparations can be heat-inactivated and tested with normal human serum to determine whether the vaccine elicited enhancing antibodies.

The authors thank Drs. David Karzon, William Schaffner, Dan Colley, and Barney Graham for critical review of the manuscript. We are also grateful to Mr. Pete Parrish and Mrs. Jean Kolodziej for aid in typing the manuscript. This work was supported, in part, by contracts with the National Institute of Allergy and Infectious Diseases (AI52576) and the National Heart, Lung and Blood Institute (N01-HB-7-7029).