Haematology and Blood Transfusion Vol. 31
Modern Trends in Human Leukemia VII
Edited by Neth, Gallo, Greaves, and Kabisch

Restricted Neutralization of Divergent HTLV-III/LAV Isolates by Antibodies to the Major Envelope Glycoprotein

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A. Summary

By analogy to other retroviruses, the major envelope glycoprotein – gp120 – of HTLV-III/LAV is a probable target for neutralizing antibody. This antigen has been purified from H9 cells chronically infected with the HTLV-III_B prototype strain. Several goats immunized with the gp120 produced antibodies that neutralized infection of H9 by the homologous virus isolate. These same sera failed to neutralize the divergent HTLV-III_{RF} isolate. Individuals infected with HTLV-III/LAV commonly develop antibodies to gp120 which could be isolated using the gp120 antigen coupled to an immunoadsorbent resin. The antibody fraction that bound tightly to such a resin was found to neutralize the III_B but not the RF isolate in a fashion similar to that of the goat anti-gp120 sera. However, the nonbinding fraction (effluent) from the resin also contained neutralizing activity which was able to block infection by both virus isolates with similar efficacy. Human antibodies to the other virus envelope gene product, the transmembrane gp41, were also affinity-purified utilizing the recombinant peptide 121, but they failed to influence infection by either virus isolate.

B. Introduction

Human T-cell lymphotropic virus type III (HTLV-III) is a pathogenic human retrovirus that is structurally and genetically related to the subfamily Lentivirinae [1]. Epidemiological studies combined with virus isolation and antibody detection in acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) cases, especially in blood donors and recipients, have defined the association of HTLV-III infection with AIDS [2–5]. The virus contains an RNA genome capable of coding for at least six gene products [6–9]. The envelope (env) gene products and the internal structural gene (*gag*) products are the most antigenic in man because antibodies to them are readily detectable in patients by a variety of tests [4, 10–12]. We [15] and others [10, 12] have recently reported some of the characteristics of the HTLV-III envelope gene products. The primary gene product of this gene is a 160000 dalton glycosylated protein (gp160) that is processed by proteolysis into a gp120 external glycoprotein and a gp41 transmembrane protein [10, 12, 15].

Purification of HTLV-III gp120 to homogeneity was possible through the use of infected cells or cell culture fluids as the source of the glycoprotein [16]. The purified material is immunogenic in goats, horses, and rhesus monkeys in that antibody to gp120

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both precipitated gp120 and neutralized the infectivity of HTLV-III in cell culture. These results suggest that the establishment of protective humoral immunity to HTLV-III may be theoretically possible in man [16].

Neutralization of HTLV-III/LAV by naturally occurring human antibodies has been observed by several investigators with varying degrees of efficiency depending on the type of assay utilized [13, 14, 17]. However, two major issues concerning the specificity of virus neutralization require resolution. First, it has not been demonstrated that the antibodies in HTLV-III/LAV-infected individuals neutralize virus through reactivity with a virus-encoded gene product. Second, it is not known to what extent the neutralizing activity relates to the genetic diversity of the various isolates which is found in large part in the envelope gene of the virus [18, 19]. The exterior-envelope-encoded product of HTLV-III/LAV has been identified as gp120 [10, 11, 15, 20] and, by analogy to other retrovirus systems, is likely to represent the major target of neutralizing antibodies [21]. Interspersed within this gene are both hypervariable and conserved regions [19], both of which may contribute to formation of epitopes involved in virus neutralization. Retroviruses can also incorporate cellular antigens into their envelope structure, and it is known that antibodies to leukocyte alloantigens can neutralize virus infectivity [22]. Recent studies suggest that HLA-DR molecules can be found in association with HTLV-III/LAV and that some AIDS-related sera contain antibodies to these determinants [23].

In this study it was our aim to examine the role of the HTLV-III envelope gene product, gp120, as a target for neutralizing antibody and to assess the impact of gp120 polymorphism on such antibodies. We have compared the ability of heterologous antisera raised against purified gp120 and gp120 affinity-purified human antibodies to block infection by the homologous virus isolate, HTLV-III_B, as well as by the widely divergent Haitian HTLV-III_{RF} isolate [18]. The nucleotide sequences of these two isolates predict a 21% difference in amino acid residues in the corresponding gp120 polypeptides [19]. Both isolates readily infect the H9 cell line [18, 24], and it was thus possible to compare directly the activity of the various antibodies on these two dissimilar genotypes under the same assay conditions.

C. Materials and Methods

I. Cells

Uninfected H9 cells, H9 cells chronically infected with HTLV-III_B, and H9 chronically infected with the Haitian isolate HTLV-III_{RF} have been described [18, 24]. Cells were maintained in RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

II. Virus

Virus stocks were prepared from 100 ml cultures of chronically infected H9 cells that had been passed into fresh medium 24 h before harvesting. Cells were removed by centrifugation, and the supernatants were filtered through a 0.45 μm Millipore filter and frozen at −70 °C in 1 ml aliquots. The infectious titer of the virus stocks was measured in the following manner. Tenfold dilutions were made in growth medium, and 0.1 ml of each dilution was added to six well plates (Falco Labware) containing 1×10^5 H9 cells in 0.2 ml growth medium. The medium volume was doubled daily for 6 days. On the 7th day, cell clumps were disrupted and the culture volume was reduced to 0.5 ml. Fresh medium was added as before for 2 more days, after which growth of virus in the cultures was monitored on the basis of sedimentable reverse transcriptase (RT) activity (see below). The infectious titer of the virus stocks was taken as the highest dilution that yielded RT activity at least 20-fold that of background activity. Both the HTLV-III_B and RF isolates yielded virus stocks ranging in titer from 10^4 to 10^5 infectious units/ml.

III. Neutralization Assay

The virus stocks were diluted with growth medium to give 1000 infectious units per 0.1 ml, and that volume was added to indi-

vidual wells of the six Falcon well plates. Fourfold dilutions of test sera were made in growth medium, filtered (0.45 μm Millipore), and 100 μl of each dilution was added in duplicate to the virus-containing wells. The plates were incubated at 37 °C for 30 min, after which 1×10^5 H9 cells were added to each well in a volume of 0.1 ml. Fresh medium was added each day as described above, and the cultures were tested for virus release 10 days later. For that purpose, cells were removed by centrifugation and the supernatants assayed for RT activity. Briefly, virus was concentrated from 3 ml of culture fluid by precipitation with polyethylene glycol (PEG) and resuspended in 0.12 ml of Triton X-100 containing lysis buffer as described [25]. Duplicate 10 μl samples were tested for RT activity using poly (rA) p(dT) 12–18 as template primer [24, 25]. The results are expressed as average counts per minute per 10 μl sample assayed. The neutralizing titer of individual sera was defined as the reciprocal of the serum dilution required to inhibit RT activity by 50% in relation to no serum controls.

IV. Antigens

The purification and characterization of gp120 from H9 cells chronically infected with HTLV-III_B is described elsewhere [26]. Briefly, the antigen was isolated by affinity chromatography from cell lysates. The sequence of the N-terminal 18 amino acid residues matched that predicted by the corresponding *env* gene nucleotide sequence of the BH10 clone of HTLV-III_B [7]. A similar procedure was used to isolate gp120 from HTLV-III_{RF}. The recombinant peptide 121 is encoded by sequences in the transmembrane gp41 *env* gene and is specifically reactive with most, if not all, HTLV-III antibody-positive human sera [27].

V. Affinity Chromatography

Immunoadsorbent resins were prepared by coupling the purified gp120 (about 0.5 mg) or the peptide 121 (about 5 mg) to cyanogen-bromide-activated Sepharose 4B (Pharmacia) [28]. Two ml of test serum was incubated with these resins for about 2 h at room temperature. Nonbinding protein was washed from the resins with phosphate-buffered solution (PBS) until no further A280 absorbing material was detected. This flow through fraction was collected as effluent (usually 20–30 ml). The resins were further washed with 10 bed volumes of PBS (discarded). The bound fraction was eluted with 4 *M* magnesium chloride and dialyzed against PBS. The effluent was concentrated to about 2 ml and rechromatographed over the resins as before. This process was repeated so that each serum was passed over the indicated resins a total of three times. The eluate fractions were pooled together, and both the final effluent fraction and the eluate pool were concentrated to 2 ml through an Amicon PM10 filter.

D. Results

I. Neutralization by Unfractionated Human Serum

We have examined the ability of human serum to block infection of H9 cells in an assay system similar to that described by Robert-Guroff et al. [14], except that the end-point measurements for virus infection were based on development of sedimentable RT or levels of viral p24 antigen in the supernatant of challenged cells. A comparison of these tests using a human AIDS serum is shown in Table 1. Over 100 HTLV-III antibody-positive sera have been tested, about 90% of which were found to prevent infection completely at low-serum dilutions (1 : 8). This effect was titratable such that it was possible to assign to individual sera a specific neutralization end point based on the reciprocal of the serum dilution required to block RT activity by 50%. The most active of the sera tested yielded neutralization titers of about 500–1000 when assayed in this manner. Patient sera positive for antiviral antibodies with no neutralizing activity in these experiments were from individuals who either had recently seroconverted (2–6 months) or were in late stage of disease. At a serum dilution of 1–8 or higher, none of 55 antibody-negative sera showed any inhibi-

Table 1. Neutralization of HTLV-III as measured by RT and viral p24 antigen

Serum dilution[a] (Reciprocal)	RT[b] (cpm)	p24[c] (ng/ml)
8	2500	< 1
32	1700	< 1
128	70000	100
2048	82000	140
No serum	68000	130

[a] Serum from an AIDS patient was tested for neutralization of HTLV-III_B, as described in Sect. B.

[b] Ten days after infection, virus was concentrated from a 3 ml portion of cell-free supernatant for measurement of RT.

[c] A second portion of cell-free supernatant at 10 days was made in 0.5% Triton X-100 and tested for viral p24 antigen, using a competition radioimmunoassay developed by E.I. DuPont de Nemours and Company, Wilmington, Delaware. The lower limit of detection with this assay is about 1 ng/ml of culture supernatant.

tory activity. A compendium of these results will be reported elsewhere and in general are in agreement with those of Robert-Guroff et al. [14].

An example of a titration of a strongly neutralizing serum taken from an individual with PGL (persistent generalized lymphadenopathy) is shown in Fig. 1. Each dilution of serum was tested on 1000 infectious units of both the HTLV-III_B and HTLV-III_{RF} isolates. Both viruses were effectively neutralized with 50% neutralization titers of 700 for the III_B and 600 for the RF isolates – not a significant difference in this assay. To date, we have titrated 15 antibody-positive human sera against these two virus prototypes. Of the sera tested, seven failed to neutralize either virus, while the remaining sera blocked infection by both viruses with no more than a twofold difference in titer.

II. Neutralization by Goat Anti-HTLV-III_B gp120 Sera

As described elsewhere [26], purified gp120 from HTLV-III_B was used to obtain heterologous sera from two goats. One of these animals (NATIVE) received antigen exposed only to 4 *M* magnesium chloride and 0.1% Triton X-100. The other animal (PAGE) received the gp120 following sodium dodecyl sulfate (SDS) denaturation and electrophoresis as a final purification step. The antigens (50 μg per immunization) were administered intradermally in Freund's complete adjuvant twice over a 5-week period, after which the sera utilized for these studies were taken. We have reported elsewhere [26] that both of these animals developed antibodies that were capable of neutralizing the homologous HTLV-III_B virus isolate. Thus, the

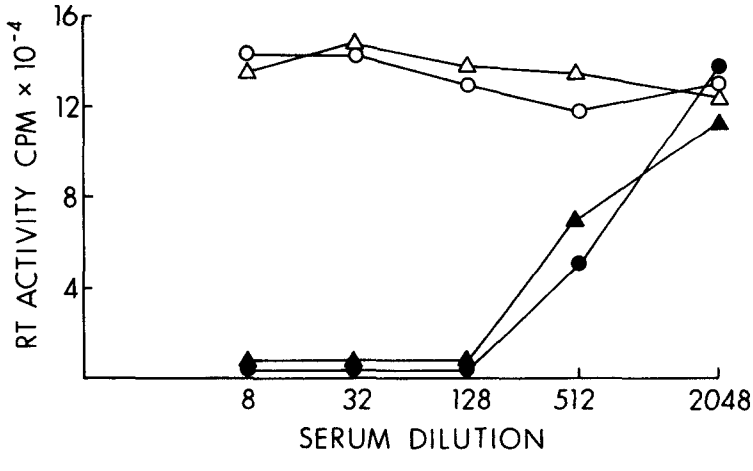

Fig. 1. Titration of human serum for neutralization of HTLV-III_B and HTLV-III_{RF}. Sera were obtained from a seronegative laboratory worker (*open*) and a seropositive bisexual with persistent generalized lymphadenopathy (*closed*). Serial fourfold dilutions of each sera were incubated for 30 min with 1000 infectious units of each HTLV-III_B (*circles*) and HTLV-III_{RF} (*triangles*). The virus-sera mixtures were added to H9 cells, and virus propagation was monitored 10 days later on the basis of sedimentable RT activity in the supernatant of the cultured cells

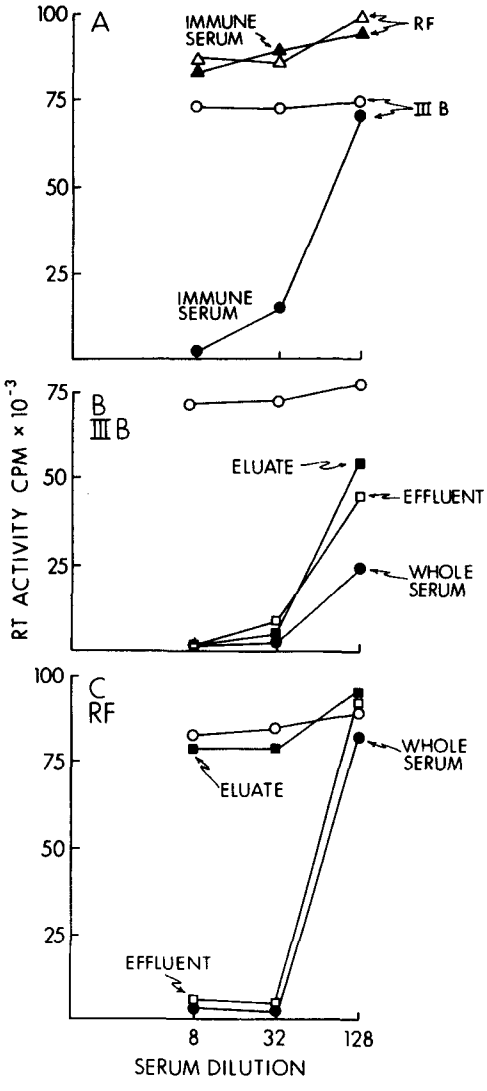

Fig. 2a–c. Titration of goat anti-HTLV-III$_B$ gp120 serum (**a**) and human serum fractions (**b** and **c**) for neutralization of HTLV-III$_B$ and HTLV-III$_{RF}$. In **a**, dilutions of preimmune goat serum (*open*) and immune goat serum (*closed*) were tested for their ability to block infection of the HTLV-III$_B$ isolate and the HTLV-III$_{RF}$ isolate. Panels **b** and **c** a 2 ml sample of serum from a seropositive homosexual with PGL was fractionated on an immunoadsorbent resin containing HTLV-III$_B$ gp120. Untreated serum, flow-through or effluent fraction, and eluate fraction were titrated for neutralizing activity against the III$_B$ isolate (**b**) and the RF isolate (**c**). Virus production in the presence of normal human serum is indicated by the *open circles* in **b** and **c**

SDS treatment did not irreversibly destroy epitopes that can induce neutralizing antibodies. The relative activity of the PAGE serum on the divergent HTLV-III$_{RF}$ isolate in comparison to the homologous HTLV-III$_B$ is shown in Fig. 2a. Again, this serum completely blocked infection by HTLV-III$_B$ at a dilution of 1–8 and yielded a titer of about 50. In contrast, there was no effect on the RF isolate, and the immune and preimmune bleeds from this animal were essentially identical. Each serum has been tested on both isolates three times, and the results of each experiment were the same as shown in Fig. 2.

Binding studies indicate that the inability to neutralize the RF isolate is not related to an overall lack of recognition by these goat sera. For example, in the solid-phase binding experiment shown in Table 2, the PAGE goat serum gave essentially equivalent levels of binding to the gp120 of HTLV-III$_B$ and HTLV-III$_{RF}$. It failed to react with recombinant peptide 121, and this is consistent with the apparent specificity of that serum for the gp120 portion of the envelope gene. Other radioimmunoprecipitation (RIP) and Western blot experiments (not shown) confirmed the results set out in Table 2. We conclude from these studies (a) that the neutralizing epitopes on the glycoprotein are only a subset of a larger number of potentially immunogenic sites on the molecule, and (b) that the neutralizing epitopes recognized by the goat sera are not conserved on the two divergent isolates studied here.

III. Neutralization by Human Antibodies Directed to gp120

The availability of the purified viral glycoprotein made it possible to fractionate human serum on immunoadsorbent resins and to estimate the proportion of neutralizing activity which can be attributed to the HTLV-III$_B$ gp120. Moreover, it was possible to analyze further the specificity of such antibodies with respect to neutralization of divergent isolates. An example of a serum fractionated in this manner is shown in Figs. 2b and 2c. When the homologous HTLV-III$_B$ isolate was used as the target virus (panel B), neutralizing activity could be detected in both the antibody-binding (eluate) and nonbinding (effluent) fractions. Even after repeated passes over the gp120 resin, we were unable to remove all of the neutralizing activity from the effluent. When serum fractions were tested in Western blot

Table 2. Antibody binding to gp120 of divergent isolates

Serum[b]	HTLV-III$_B$ gp120 cpm	HTLV-III$_{RF}$ gp120 cpm	Recombinant p121 cpm
Preimmune goat (1/100)	2600±100	2800±200	3800±200
Goat anti-gp120 (1/100)	35700±700	33900±800	4400±200
Normal human (1/10)	1800±200	1600±200	2900±300
Human PGL (1/10)	15200±400	17500±600	31000±600

[a] Solid-phase immunoassay was performed as described [17], except that binding antibody was monitored with ^{125}I-SpA (200000 cpm) per well. About 200 ng of each antigen was added per well of Immulon 1 microtiter strips (Dynatech Lab. Inc., Alexandria, Virginia), and sera were tested in triplicate.

[b] Sera were tested at the dilutions indicated in a final volume of 200 µl. The goat sera containing wells were incubated with 100 µl of rabbit anti-goat IgG (Cappel) at a 1–300 dilution before addition of ^{125}I-SpA.

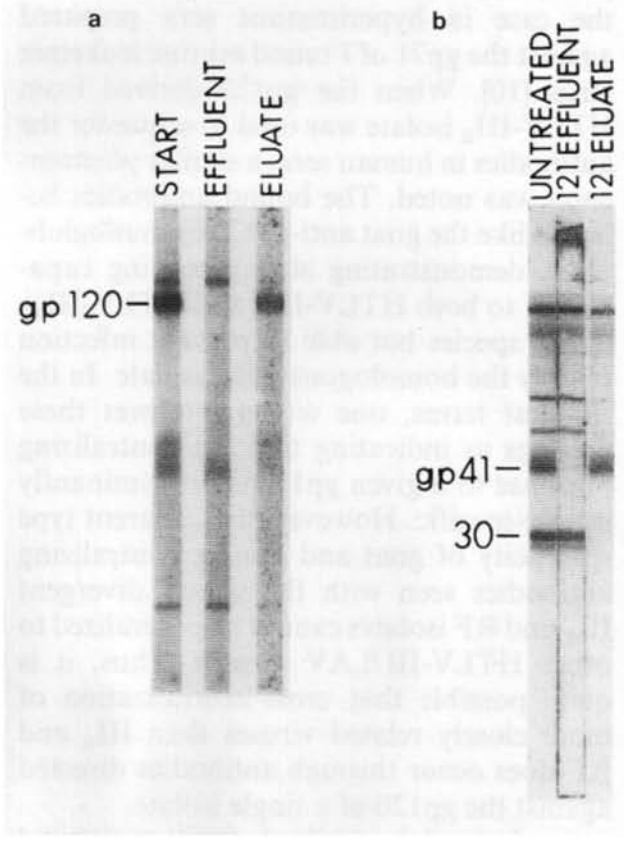

Fig. 3 a, b. Western blot analysis of human sera fractionated on affinity resins bearing gp120 (**a**) and p121 (**b**). In **a**, the serum fractions from the gp120 resin as shown in Figs. 2b and 2c were tested for binding to partially purified HTLV-III$_B$ gp120 by the Western blot technique, using ^{125}I-SpA to localize antibody. In **b**, serum from an AIDS patient was fractionated on the p121 affinity resin and tested for binding to disrupted HTLV-III$_B$ virus proteins by the Western blot procedure. Serum and serum fractions were incubated with appropriate nitrocellulose strips at a dilution of 1–100

(Fig. 3a) and plate-binding assays, we found no evidence of anti-gp120 antibodies remaining in the effluent fraction, and essentially all of the reactivity in whole serum (Table 2) was found in the tightly binding antibody fraction which was subsequently eluted from the resin. However, when measured by immunoprecipitation of radioiodinated gp120, considerable activity was also noted in the effluent fraction (not shown). This confirms earlier conclusions that RIP analyses are the most sensitive and accurate measure of antibodies to the gp120 [29] (see Discussion for further elaboration of this point).

The results when HTLV-III$_{RF}$ was used as a target for neutralization were qualitatively different (Fig. 2c). Of note is the fact that the activity against RF in the effluent fraction is essentially indistinguishable from that in untreated serum. In contrast, the material in the eluate had no detectable activity against the divergent RF strain. Although the absolute titers differed, three other human sera fractionated in this manner gave the same qualitative results shown in Figs. 2b and 2c. In all cases, the eluate fractions neutralized the III$_B$ but not the RF isolate, while the effluent fractions blocked infection by both viruses. Thus, we conclude that human serum commonly contains anti-gp120 antibodies that neutralize infection by HTLV-III in a fashion that is restricted with respect to divergent isolates. The identity of the more broadly neutralizing antibodies represented in the effluent is uncertain at this

time. It is possible that these antibodies are also directed against the gp120 but bind with insufficient avidity, to be totally removed by antigen isolated from a single virus genotype. Alternatively, other viral or cellular antigens may be involved as suggested earlier.

IV. Human Serum Fractionated by Recombinant Peptide 121

All known retroviruses contain a second *env*-gene-encoded product, a transmembrane polypeptide, that under certain conditions can also serve as a target antigen for neutralizing antibody [30]. The analogous polypeptide in the case of HTLV-III has been identified as gp41 [12]. Since almost all antibody-positive human sera react with gp41, as with gp120, it was therefore a good candidate for an additional target for neutralizing antibodies. We thus performed similar analysis, as shown in Figs. 2b and 2c, using an affinity column containing the recombinant peptide 121 [27]. This molecule contains only about one-half the gp41 sequences, but a major portion of the immunodominant epitopes of gp41 [27]. In three of the six sera tested, it was possible to remove all gp41 reactivity by Western blot analysis with the p121 resin, as shown in Fig. 3b. Sensitive plate-binding assays using the 121 recombinant peptide also failed to detect antibodies in the effluent fraction. Both these measures are more effective than RIP assays for measuring antibodies to gp41, unlike the situation with gp120 [10].

Analysis in the neutralization assay revealed that the bound fractions failed to neutralize either the III_B or RF isolates, while the effluent fractions showed only a modest reduction in titer, probably resulting from manipulative losses rather than from any specific effects. Rabbit serum raised against p121 also failed to neutralize the virus, even though it reacted strongly with gp41 (not shown). These results do not rule out the possibility that neutralization targets exist on gp41 which are not contained in the recombinant peptide 121. Nevertheless, on the basis of those sera from which we were able to remove all detectable gp41 reactivity, we feel this antigen does not play a major role in neutralization under the conditions used.

E. Discussion

The experiments described here focus attention on the polymorphism in the gp120 envelope region of HTLV-III/LAV. On the one hand, polyclonal sera raised in goats (and in other animal species; unpublished results) bound with similar efficacy to widely divergent gp120 species, but their ability to neutralize the infectivity of the respective viruses was much more restricted. It is possible that further immunization may overcome the apparent type specificity, as has proven to be the case in hyperimmune sera prepared against the gp71 of Freund murine leukemia virus [10]. When the gp120 derived from HTLV-III_B isolate was used to sequester the antibodies in human sera, a similar phenomenon was noted. The bound antibodies behaved like the goat anti-gp120 immunoglobulins, demonstrating strong binding capabilities to both HTLV-III_B and HTLV-III_{RF} gp120 species but able to prevent infection of only the homologous (III_B) isolate. In the simplest terms, one would interpret these findings as indicating that the neutralizing response to a given gp120 is predominantly isolate-specific. However, the apparent type specificity of goat and human neutralizing antibodies seen with the widely divergent III_B and RF isolates cannot be generalized to other HTLV-III/LAV viruses. Thus, it is quite possible that cross-neutralization of more closely related viruses than III_B and RF does occur through antibodies directed against the gp120 of a single isolate.

Analysis of the antibody fraction that did not bind to the HTLV-III_B gp120 resin raises a number of additional questions. First, antibodies are present in this fraction which, in spite of their lack of binding activity to the immobilized gp120, neutralize not only the HTLV-III_B but also the highly divergent HTLV-III_{RF} isolate. It is possible that these antibodies represent low-affinity immunoglobulins with significant neutralizing potential. An argument in favor of this is the fact that in RIP assays, one can readily detect anti-HTLV-III_B gp120 antibodies in the

effluent fraction. It is not clear why these antibodies do not bind the immobilized antigen well, but it is possible that there is a better opportunity for multivalent binding and resultant stabilization of the complex when the antigen is in solution.

Other possibilities exist, however, which could explain the presence of neutralizing activities in human sera which are not related to gp120. Potential targets for neutralization include other viral antigens such as the gp41 transmembrane envelope component, which is highly immunogenic in man and known to represent a target for neutralization in animal retroviruses, albeit in the presence of complement [30]. Our studies demonstrate that at least in the absence of complement, anti-gp41 antibodies do not play a detectable role in virus neutralization and do not account for the neutralizing activity in the gp120 unbound fractions. In addition, antibodies directed against leukocyte alloantigens which can associate with the budding virion may also be targets for neutralization, as demonstrated with feline leukemia virus [22]. The presence of antibodies to HLA in HTLV-III/LAV-infected individuals has also been documented, as has the association of HLA chains with HTLV-III_B [23]. Therefore, it is conceivable that at least some portion of the unbound fraction may contain such antibodies, and studies are in progress to determine this and the antibodies' potential role in virus neutralization.

Our studies represent a first step in defining the epitopes of the HTLV-III/LAV gp120 that are associated with biologically important functions and the degree to which these epitopes are immunogenic in man. The present results clearly demonstrate that this viral antigen can serve as a target for neutralizing antibodies. It is not yet known what portions of the gp120 are involved, but our results demonstrate that there is at least one such epitope that is polymorphic, i.e. not conserved in the HTLV-III_B and HTLV-III_{RF} isolates. If conserved sequences that can also serve as neutralizing epitopes are present, the goats immunized with gp120 have yet to recognize these regions. Likewise, it was not possible to isolate broadly reactive human neutralizing antibodies by immunoaffinity chromatography, using gp120 obtained from a single isolate. The latter result, however, could be due to other limitations of the technique, including avidity and/or steric accessibility to the epitopes in question. Because of these considerations, we cannot conclusively eliminate the possibility that conserved sites serve as targets for neutralizing antibody. The broad reactivity commonly observed with antibody-positive human sera might be due to such conserved sites or, alternatively, these might reflect a response by the individuals exposed to multiple virus genotypes. A more definitive answer to this question must await studies similar to those described here, ones utilizing purified gp120 from other prototypic strains (e.g., RF) as well as field isolates. Our results, however, raise the possibility that multiple gp120 immunogens might be required to generate antibodies that would neutralize the full spectrum of divergent viruses which exist in the population. Even if this were achievable, either naturally or through vaccination, much more needs to be learned about the role of virus neutralization in protection against HTLV-III/LAV infection.

Acknowledgements. This research was supported by NIH Program Project CA5862-06. We wish to thank Natalie Cates, Carl Stone, and Charlene McDanal for their expert technical assistance.

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