vealed it to have been a fusion of two CNBr cleavage fragments, one beginning at amino acid 97 and the other beginning at amino acid 120 and put together in the following order: Ala-120 to Gly-127, Asn-105 to Glu-108, Arg-132, Val-110 to Lys-118, and Tyr-142. All of the amino acids are in the proper cycle of Edman degradation chemistry, with Met-96 and Met-119 as the points of chemical cleavage. A sequence with 100% identity to amino acids 96 to 104 was seen on sequencing but was interpreted as a minor sequence.
15. A cDNA library constructed from the murine helper cell line 2B4 in λgt10 was a gift from David Cohen. Screening was carried out with the two complementary 72-base oligonucleotides. Nitrocellulose filters containing phage DNA were prehybridized overnight and then hybridized in 30% formamide (Fluka), 5× SSPE (1× SSPE is 0.18*M* NaCl, 0.01*M* sodium phosphate, *p*H 7.7, and 0.01*M* EDTA), 5× Denhardt's solution, yeast RNA (200 μg/ml) (Calbiochem), salmon sperm DNA (100 μg/ml) (Sigma), and 10% dextran sulfate (Pharmacia) at 42°C. Synthetic probes were radiolabeled by addition of ^{32}P to the 5′ end with the use of T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham). Filters were subjected to serial washings in 2× SSC at 22°C, then at 0.2× SSC and 0.1% SDS at 42°C. Screening was repeated until plaque purification was obtained. Clonal phage DNA was prepared with Lambdasorb (Promega) according to the manufacturer's instructions. Inserts were purified out of agarose gels with DEAE membrane (Schleicher and Schuell) according to manufacturer's instructions. Purified insert was cloned into pGEM3Z (Promega).
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Second Conserved Domain of gp120 Is Important for HIV Infectivity and Antibody Neutralization

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Rabbit antisera were raised against three overlapping synthetic peptides with sequence homology to the second conserved domain of the external envelope glycoprotein (gp120) of the human immunodeficiency virus (HIV). All of the antisera immunoprecipitated the envelope glycoprotein. In particular, an antiserum directed against amino acids 254 to 274 of *env* was efficient in neutralizing three different isolates of HIV in vitro, without affecting the binding of the virus to CD4-positive cells. Therefore, this conserved region of gp120 appears to be critical in a postbinding event during virus penetration and may represent a target for antibody neutralization of HIV. These findings may be applicable in the design of a vaccine for the acquired immunodeficiency syndrome.

THE SEQUENCE OF THE *env* GENE varies greatly among different isolates of the human immunodeficiency virus (HIV), but this variation is not randomly distributed throughout the gene (*1*). Instead, a pattern of alternating variable and constant regions is observed (*2*). In the external envelope glycoprotein (gp120), several constant and variable domains have been identified. In the constant domains, sequence conservation is greater than 80% among different isolates of HIV, whereas in the most variable domains, sequence conservation is between 20 and 30%. Conservation of sequence within the constant domains presumably indicates selective pressure to maintain those sequences, although their functional importance is not understood.

The second conserved domain of gp120 is partially homologous to neuroleukin, a factor that has both neurotrophic and lymphokine activities (*3*). We found earlier that gp120 inhibits the neurotrophic action of neuroleukin on cultured neurons, and this finding may be important for understanding the pathogenesis of dementia observed in some patients with acquired immunodeficiency syndrome (AIDS) (*4*). To determine whether the second conserved domain is important for HIV infectivity, we prepared antisera against synthetic peptides with sequences homologous to that region of gp120. We now report that one of these antisera is strongly immunoreactive with gp120 and is also highly neutralizing for diverse isolates of HIV. However, this antiserum does not block the binding of HIV to CD4$^+$ cells. These findings suggest that one particular region, comprising amino acids 254 to 274 within the second conserved domain of gp120, takes part in a postbinding event during virus penetration and is an important target for antibody neutralization of HIV.

Three overlapping oligopeptides—designated T19V, C21E, and S19C—were synthesized in accordance with the amino acid sequence of the HTLV-III$_B$ isolate (*1, 5*) of HIV (Fig. 1). Each peptide was coupled to keyhole-limpet hemocyanin (KLH) and used to prepare antisera in rabbits. The titer and specificity of the three antisera were determined by enzyme-linked immunosorbent assay (ELISA). Each of the rabbits had a strong antibody response to its immunizing peptide but not to a control peptide, M20L (Table 1). The antiserum to C21E (anti-C21E) also reacted with the peptide that overlaps C21E at its amino terminus (T19V), but not with the peptide that overlaps at its carboxyl terminus (S19C). Anti-T19V showed little cross-reactivity with C21E; anti-S19C showed no cross-reactivity with C21E (Table 1).

All three of the antisera immunoprecipitated proteins of 120 or 160 kD, or both, from Molt-III cells infected with the HTLV-III$_B$ isolate (Fig. 2, lanes b). These proteins were not immunoprecipitated from uninfected

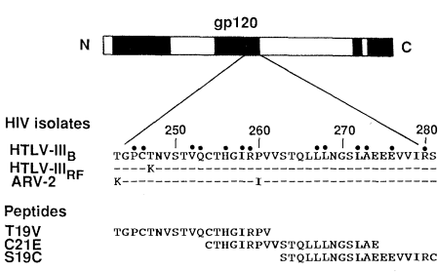

Fig. 1. The amino acid sequences of HTLV-III$_B$, HTLV-III$_{RF}$, and ARV-2 were obtained (*1, 2*), and the numbering system was based on that of Ratner *et al.* (*1*). The conserved regions of gp120 are shown in black. A dash indicates identity with the HTLV-III$_B$ sequence, and a dot denotes the amino acids that are homologous to neuroleukin (*3, 4*). The three peptides—T19V, C21E, and S19C—were synthesized by Peninsula Laboratories, Inc. (Belmont, California), and correspond to the sequence of HTLV-III$_B$; their composition was confirmed by amino acid analysis.

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Molt-III cells (Fig. 2, lanes a), and thus are likely to be gp120 and its precursor, gp160. In addition, sera from the rabbits before immunization (preimmune sera) did not react with either gp120 or gp160.

Sera from persons infected with HIV or an animal immunized with gp120 were tested by ELISA for immunoreactivity with T19V, C21E, and S19C (Fig. 3). Normal human sera did not react with these peptides. Most of the HIV-positive human sera were unreactive, and none were strongly reactive. Furthermore, serum from a rabbit immunized with purified gp120 (*6*) did not react significantly with the peptides. This antiserum had no immunoreactivity with T19V or C21E in ELISA; it had a titer of 1:250 with S19C.

Table 1. Titer and specificity of rabbit antisera raised against synthetic peptides with partial sequence homology to HIV. The peptides were conjugated to KLH through cysteine residues with the use of *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester. The amount of peptide bound to the carrier was determined by amino acid analysis and was generally 0.2 to 0.4 mg of peptide per milligram of KLH. Female New Zealand White rabbits were immunized three times at 2-week intervals with 500 μg of the conjugate emulsified in Freund's complete adjuvant for the primary immunization and in Freund's incomplete adjuvant for the secondary immunizations. For the ELISA, flexible polyvinyl chloride microtiter plates were coated overnight at 8°C with 100 μl of peptide per well at a concentration of 5 μg/ml in 150 m*M* sodium borate buffered at *p*H 9.6. The wells were then blocked by incubation with 3% bovine serum albumin in phosphate-buffered saline for 1 hour at room temperature and then incubated overnight with serial dilutions of the rabbit sera diluted in 10% horse serum, 20% goat serum, and 0.1% Triton X-100 in phosphate-buffered saline. Bound rabbit immunoglobulin was detected with a Vectastain kit (Vector Laboratories) that contained biotinylated goat antibody to rabbit immunoglobulin, avidin, and biotinylated horseradish peroxidase. Color development was achieved with *o*-phenylenediamine and hydrogen peroxide, and the ELISA was read on a dual-wavelength microplate spectrophotometer at 450 nm. The titers shown are the dilution of rabbit antiserum that gave half-maximal color development (maximum optical density usually 1.3). A control peptide, M20L, has the sequence MIPCDFLIPVQTQHPIRKGL (*11*).

Rabbit sera	T19V	C21E	S19C	M20L
R_1 preimmune	0	0	0	0
R_1 anti-T19V	1:5600	1:630	0	0
R_2 preimmune	0	0	0	0
R_2 anti-C21E	1:1400	1:5000	0	0
R_3 preimmune	0	0	0	0
R_3 anti-S19C	0	0	1:900	0

Table 2. HIV neutralizing titers and HIV binding inhibitory activities of rabbit antisera raised against synthetic peptides with partial sequence homology to HIV. Neutralization assays were performed against three diverse HIV isolates—HTLV-III_B, HTLV-III_{RF}, and ARV-2—as described (*7*), except the supernatant p24 antigen was assayed instead of particulate reverse transcriptase activity. Neutralization was defined as >90% reduction in both syncytia formation and supernatant p24 antigen compared to control cultures. HIV binding inhibition studies were performed according to the protocol of McDougal *et al.* (*9*). The virus stock used in the assays was prepared as follows. Supernatant fluid from HTLV-III_B–infected Molt-III cells were precleared by sequential centrifugation (300*g* for 7 minutes, followed by 1500*g* for 20 minutes) and then concentrated 1000-fold by ultracentrifugation (90,000*g* for 90 minutes). Ten microliters of this HIV preparation were treated with 10 μl of test sera for 30 minutes at room temperature before incubation with C8166 cells (5×10^5, 30 minutes at 37°C). Subsequently, the cells were washed and resuspended in 25 μl of a 1:50 dilution of human anti-HIV conjugated to fluorescein. After 30 minutes at 4°C, the cells were then washed, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Sera	Reciprocal of HIV neutralizing titer			Inhibition of HIV binding (%)
	HTLV-III_B	HTLV-III_{RF}	ARV-2	HTLV-III_B
Rabbit sera				
R_1 preimmune	<4	<4	<4	0
R_1 anti-T19V	8	8	16	0
R_2 preimmune	<4	<4	<4	1
R_2 anti-C21E	128	64	256	2
R_3 preimmune	<4	<4	<4	3
R_3 anti-S19C	16	16	32	0
R_4 anti-1-110	32	32	64	3
Control sera				
Human, seronegative (n = 6)	<4	<4	<4	0–5
Human, seropositive (n = 18)	20	18	25	93–100
Human, P982	32	32	64	99
Goat, anti-gp120	32	<4	4	94

We then tested the rabbit preimmune and immune sera for HIV neutralizing activity by using an assay described earlier (*7*). Briefly, 50 $TCID_{50}$ (50% tissue culture infective doses) of HIV were incubated with serial dilutions of the test serum for 1 hour at 37°C before inoculation onto 2×10^6 H9 (*5*) cells. Viral expression was subsequently determined on day 7 of culture, and neutralization was defined as >90% reduction both in syncytia formation and in the amount of p24 antigen in supernatant, as compared to controls (Table 2). The preimmune sera had no HIV-neutralizing activity. Anti-T19V and anti-S19C showed low neutralizing titers of 1:8 and 1:16, respectively, against HTLV-III_B, the isolate whose sequence was used in preparing the peptides. Similar titers (1:8 to 1:32) were obtained against isolates HTLV-III_{RF} (*2*) and ARV-2 (*8*). Strongest neutralization was observed with anti-C21E, which neutralized the three diverse isolates at dilutions ranging from 1:64 to 1:256. At 1:64 dilution, this antiserum completely neutralized all three HIV isolates based on the supernatant p24 antigen determinations performed on days 7, 10, and 14 of culture. The neutralizing titer of anti-C21E was severalfold higher than the mean titer of sera from HIV seropositive persons (Table 2). In particular, although human serum P982 was more reactive with gp160 and gp120 by radioimmunoprecipitation (Fig. 2), anti-C21E was fourfold more neutralizing than P982 against HTLV-III_B and ARV-2. Furthermore, anti-C21E also compared favorably with sera from goats,

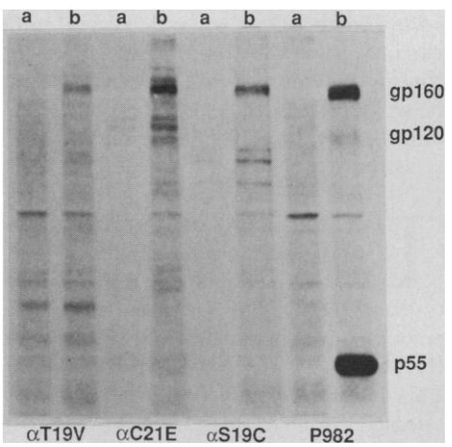

Fig. 2. Immunoprecipitation of extracts from HTLV-III_B–infected cells with antisera to synthetic peptides (αT19V, αC21E, and αS19C) from HIV *env*. Uninfected (a lanes) and infected (b lanes) Molt-III cells were metabolically labeled for 4 hours with [^{35}S]cysteine and [^{35}S]methionine (100 μCi/ml). The radioimmunoprecipitation and SDS–polyacrylamide (10%) gel electrophoresis were performed as described (*20*). P982 is a serum sample from an asymptomatic homosexual man, and p55 is the *gag* precursor protein.

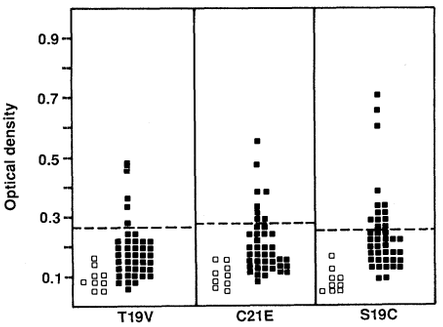

Fig. 3. Immunoreactivity of human sera with T19V, C21E, and S19C as determined by ELISA. Details of the ELISA were as described in the legend to Table 1, except that the human sera were diluted 1:200 in a mixture of 20% goat serum, 10% fetal calf serum, and 0.5% Tween 20 in 20 m*M* tris-buffered saline (*p*H 7.5), and the ELISA was developed with a Vectastain kit for human immunoglobulin. The mean optical density for HIV seronegative controls (□; $n = 9$) did not differ significantly with the three peptides. Samples from HIV seropositive persons (■; $n = 42$) were scored as positive in the ELISA if the optical density was greater than three times background (dashed lines) for the controls.

horses, or rabbits immunized with gp120 purified from the HTLV-III$_B$ isolate. In contrast to the broad neutralizing activity of anti-C21E, Matthews *et al.* (*6*) and Robey *et al.* (*6*) reported that these animal antisera to gp120 neutralized only the HTLV-III$_B$ isolate (titers of 1:20 to 1:60) but not diverse isolates (Table 2).

To explore the mechanism of neutralization, we also assayed the rabbit antisera, by the method of McDougal *et al.* (*9*), for their ability to inhibit the binding of HIV to CD4$^+$ cells. Test sera were incubated with a concentrated preparation of HIV for 30 minutes at room temperature before exposure to C8166 cells (*10*), a cell line that is highly positive for the CD4 molecule. Then, HIV bound to the T cells was detected and quantitated by fluorescein isothiocyanate–conjugated human antibody to HIV and flow cytometry. Neither preimmune nor immune rabbit sera inhibited HIV binding. In particular, anti-C21E, despite its high HIV-neutralizing activity, showed no inhibition of binding (Table 2). In contrast, human seropositive sera and goat antisera to gp120 were very efficient in blocking HIV binding (*9*) (Table 2).

Taken together, our findings show that the second conserved domain of gp120, particularly the region partially homologous to neuroleukin and represented by C21E, is important for HIV infectivity but not HIV binding. This conclusion is supported by our studies of a rabbit antiserum directed against a synthetic peptide designated 1-110 (amino acids 249 to 262; VSTVQCTHGIRPVV) (*11*) that overlaps with C21E. This antiserum reacted strongly with both peptide 1-110 and C21E by ELISA and also recognized gp120 and gp160 by radioimmunoprecipitation. It too neutralized diverse isolates of HIV without affecting their binding to CD4$^+$ cells (Table 2). In addition, our results are consistent with those obtained in mutagenesis studies by Willey *et al.* (*12*), who found that substituting a glutamine residue for asparagine at amino acid 269 (contained in C21E) produced a noninfectious mutant, although gp120 of this mutant still bound to CD4 normally. This mutation removed a potential *N*-linked glycosylation site; however, two other mutants (Leu^{268} → Asp and Gly^{270} → Asp), which left the potential glycosylation site intact, were also noninfectious. Thus, our studies with an antiserum specific for amino acids 254 to 274 of gp120 and the analysis of Willey *et al.* (*12*) on mutations of amino acids 268 to 270 reached the same conclusion—namely, the second conserved domain of gp120 is important for HIV infectivity but is not required for HIV binding to the CD4 receptor. This finding is consistent with recent demonstrations that the CD4-binding domain is located within the carboxyl portion of gp120 (*13*).

After the specific interaction of gp120 with CD4, the C21E domain may mediate an appropriate conformational change within the gp120-gp41 complex. In analogy to the situation observed with the influenza virus hemagglutinin (*14*), this structural alteration may result in the unveiling of the putative fusion domain on the amino terminus of gp41 (*13, 15*), which would then mediate fusion and thus allow virus penetration. Alternatively, the C21E domain may bind to or interact with another molecule on the cell surface to facilitate HIV entry.

Our results also suggest that the C21E domain may be an important site to consider when designing an AIDS vaccine. This region is well conserved among HIV isolates (*1, 2*) and induces high titers of neutralizing antibodies against strains that differ significantly in their gp120 amino acid sequence. Anti-C21E blocks a critical step in HIV infection that occurs after virus binding. This action is similar to the major mechanism of antibody neutralization for several enveloped viruses (*16*). The potential importance of the C21E domain in vaccine design is strengthened by our finding that anti-C21E can also mediate antibody-dependent cellular cytotoxicity against HIV-coated T cells (*17*).

Our results suggest that when the second conserved domain is presented to the immune system in the context of a larger molecule (such as gp120), it is minimally immunogenic. In contrast, peptides containing the carboxyl terminus of gp120 (*18*) or one specific domain of gp41 (amino acids 600 to 611) (*19*) are almost universally recognized in immunoassays of sera from HIV-infected individuals. The C21E domain is relatively "immunosilent" when it is part of gp120; yet by itself it is immunogenic and induces an antibody response capable of efficiently neutralizing a number of HIV isolates. This result suggests the need to reconsider vaccine strategies that are solely based on the use of gp120 or gp160. Studies to define precisely the *env* domains important for antibody neutralization should be pursued. A small well-defined oligopeptide may be a more effective vaccine than a large all-inclusive polypeptide if it can specifically direct the immune response against a biologically important domain that is conserved in all strains of HIV.

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