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Induction of anti-HIV neutralizing antibodies by synthetic peptides

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Two synthetic peptides containing amino acid sequences analogous to the envelope glycoprotein of human T-lymphotropic virus (HTLV) type III (HTLV-III) and lymphadenopathy associated virus (LAV) were produced and used to immunize rabbits. The subsequent rabbit antisera neutralized HTLV-III infectivity *in vitro*. The two synthetic peptides corresponded to regions associated with the gp120 or gp41 subunits respectively, of human immunodeficiency virus (HIV). This data indicates that at least two neutralizing epitopes are present on the envelope glycoprotein of HIV and these epitopes are associated with two distinct virus envelope glycoproteins. Antisera generated against these peptides neutralized infectivity of two different isolates of HTLV-III. The data is discussed in terms of possible strategy for developing an effective vaccine against the etiologic agents of acquired immune deficiency syndrome (AIDS).

Key words: HTLV-III/HIV/neutralizing antibodies/synthetic peptides

Introduction

The acquired immune deficiency syndrome (AIDS) was first described in 1981 (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981). The etiologic viral agents associated with AIDS have been isolated (Barre-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984; Gallo *et al.*, 1984; Levy *et al.*, 1984), cloned (Shaw *et al.*, 1984), and the nucleotide sequences determined (Rattner *et al.*, 1985; Wain-Hobson *et al.*, 1985; Sanchez-Pescador *et al.*, 1985; Muesing *et al.*, 1985). These viral agents have been referred to as human T-lymphotropic virus (HTLV) type III (HTLV-III), lymphadenopathy associated virus (LAV), or AIDS associated retrovirus (ARV) by numerous investigators. Recently, it was proposed that these viruses be referred to as human immunodeficiency virus (HIV). With retroviruses, as with other enveloped viruses, the antigenic determinants or epitopes associated with the induction of neutralizing antibodies appear to reside within the surface envelope glycoproteins. Indeed, it has been demonstrated that the most immunogenic component of AIDS-associated viruses in viral exposed individuals is the envelope glycoprotein (Allan *et al.*, 1985; Barin *et al.*, 1985; Montagnier *et al.*, 1985).

The envelope gene product is synthesized as a polyprotein precursor and is subsequently glycosylated within infected cells. This glycosylated polyprotein with a mol. wt of 160 kd (gp160) is processed into an amino terminus subunit, gp120, and a carboxyl transmembrane subunit, gp41 (Muesing *et al.*, 1985; Robey *et al.*, 1985; for a review see Wong-Staal and Gallo, 1985). The amino-terminal subunit of LAV-1 has been designated gp110 (Montagnier *et al.*, 1985; Montagnier and Klatzman, 1985); however, the predicted amino acid sequences deduced by cloning the envelope proteins of HTLV-III and LAV indicates that gp120 and gp110 are composed of the same protein backbone (Rattner *et al.*, 1985; Wain-Hobson *et al.*, 1985) and will be referred to throughout the rest of this manuscript as gp120.

Several published reports have demonstrated that antibodies from AIDS patients or patients with AIDS related complex (ARC) have specific viral neutralizing activity *in vitro* (Weiss *et al.*, 1984; Robert-Guroff *et al.*, 1984; McDougal *et al.*, 1985; Ho *et al.*, 1985; Clavel *et al.*, 1985). In these patients, infection had presumably occurred before the development of neutralizing antibody and whether or not the induction of neutralizing antibody prior to infection would result in protective immunity is unknown. To date, the structures and precise location of AIDS-associated viral neutralizing epitope have not been identified.

Synthetic peptides corresponding in amino acid sequence to segments of the capsid proteins of tobacco mosaic virus (Anderer and Schlumberger, 1965, 1966; Fearney *et al.*, 1971) foot-and-mouth disease virus (Bittle *et al.*, 1982; Pfaff *et al.*, 1982) and poliovirus (Emini *et al.*, 1983) have been used to determine neutralizing epitopes associated with the intact virion. Recently, our laboratories have chemically synthesized a synthetic peptide corresponding to amino acid sequence 735-752 of HTLV-III, gp160 and antisera produced in rabbits against this peptide specifically recognized HTLV-III, gp160 by radioimmunoprecipitation (Kennedy *et al.*, 1986). This data indicated that we had identified a native epitope on HTLV-III gp41 by utilizing synthetic peptides. However, whether or not the rabbit antisera could neutralize HTLV-III infectivity *in vitro*, a prerequisite for examining putative synthetic peptide vaccine candidates, was not determined.

The studies reported herein describe the selection and synthesis of two synthetic peptides associated with the gp120 or gp41 subunits respectively from the deduced amino acid sequence of HTLV-IIIB (Rattner *et al.*, 1985). Antibodies produced in rabbits was capable of neutralizing HTLV-IIIB and NY-5 infectivity *in vitro*. The peptides were either linked to a carrier protein or immobilized on the solid phase support using an acid-stable linking group for immunization of rabbits. This data indicates that there are at least two neutralizing epitopes on the envelope glycoprotein of HIV and these epitopes are present in different subunits.

Results

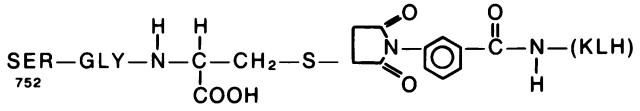
Peptide sequence selection

The amino acid sequences of two synthetic peptides that correspond to the residues associated with envelope glycoprotein of HTLV-IIIB are shown in Figure 1. These two peptides are analogous to amino acid sequences 503-532 and 735-752 from

gp 41 735-752

a) H_2N—TYR—ASP—ARG—PRO—GLU—GLY—ILE—GLU—GLU—
735

GLU—GLY—GLY—GLU—ARG—ASP—ARG—ASP—ARG—



(CYS)

gp 120 503-532

b) H_2N—VAL—ALA—PRO—THR—LYS—ALA—LYS—ARG—ARG—
503

VAL—VAL—GLN—ARG—GLU—LYS—ARG—ALA—VAL—GLY—

ILE—GLY—ALA—LEU—PHE—LEU—GLY—PHE—LEU—

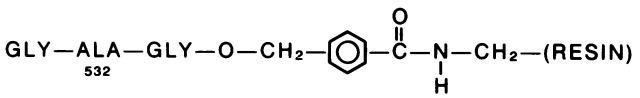


Fig. 1. Amino acid sequence of the gp41 synthetic peptide 735–752 (**A**) and gp120 synthetic peptide 503–532 (**B**). The conjugation of peptide 735–752 through a C-terminal cysteine SH group to amino groups on KLH via maleimidobenzoyl N-hydroxysuccinimide ester is depicted. Attachment of a C-terminal glycine added to peptide 503–532 by *p*-(oxymethyl)benzoic acid to the solid phase polyamide support is also shown.

Table I. Amino acid analyses of gp120 503–532 peptide resin

Residues	Before HF	After HF
Thr	0.75 (1)[a]	0.85 (1)
Glu/Gln	2.30 (2)	2.15 (2)
Pro	ND (1)	1.07 (1)
Gly	4.85 (5)	5.35 (5)
Ala	4.70 (5)	5.19 (5)
Val	3.60 (4)	3.59 (4)
Ile	0.94 (1)	1.04 (1)
Leu	2.70 (3)	3.12 (3)
Phe	2.00 (2)	2.00 (2)
Lys	2.73 (3)	4.10 (3)
Arg	4.00 (4)	3.90 (4)
μmol/g	71	50

[a]Values are uncorrected for destruction during hydrolysis. The number in parentheses represents the theoretical yield for each amino acid based on the particular sequence.
ND = not determined.

gp160 of HTLV-IIIB or correspond to sequences 508–537 and 740–757 from gp160 of LAV-1 (Pauletti *et al.*, 1985). The peptide associated with sequences 503–532 corresponds to the carboxyl terminus of gp120 and the amino terminus of gp41, whereas 735–752 comprises the gp41 transmembrane subunit. Our selection of sequences chosen for preparation of HTLV-IIIB synthetic peptides was based on predictions of a computer program modified by our laboratories (Pauletti *et al.*, 1985). This program utilizes the parameters and the hydrophilic values arrived at by Hopp and Woods (1981) to predict possible antigenic determinants present on the envelope glycoprotein of three AIDS-associated viral isolates as deduced from the published nucleotide sequence (Rattner *et al.*, 1985; Wain-Hobson *et al.*, 1985; Sanchez-Pescador *et al.*, 1985). In addition to hydrophilicity, regions of the envelope glycoprotein were also selected for synthesis based on predicted beta turn secondary structure, and were composed of relatively invariant stretches of amino acids when comparing the predicted sequences of HTLV-IIIB, LAV-1 and ARV-2. In particular, the amino acid sequences of these two peptides were identical between HTLV-IIIB and LAV-1. The two peptides in Figure 1 fulfill all three of the above criteria.

The induction of an anti-native response with synthetic peptides often required the conjugation of the peptide to a carrier protein (reviewed in Arnon *et al.*, 1983; Sutcliffe *et al.*, 1983). This was the strategy we selected for producing an anti-native response to gp41 with synthetic peptide 735–752 (Kennedy *et al.*, 1986). In addition to peptide conjugated to keyhole limpet hemocyanin (KLH) we have also utilized resin-bound peptides to produce an anti-native response to hepatitis B virus synthetic peptides (Kanda *et al.*, in preparation). By utilizing the *p*-(oxymethyl benzamide) handle to attach the C-terminal amino acid to the solid-phase resin support (Figure 1), anhydrous hydrogen fluoride (HF) treatment of the completed peptide will remove the side-chain protecting groups without considerable loss of the peptide from resin. Therefore, the resin-bound peptide can serve directly as the immunogen and one can bypass peptide cleavage, purification and coupling to carrier proteins prior to immunization. This approach was used to generate antibody to peptide 503–532. The amino acid composition of peptide 503–532 before and after HF treatment is shown in Table I. The data are consistent with the expected amino acid composition of the peptide with an additional glycine at the C terminus. Deprotection of the peptide with HF resulted in a 28% loss of resin-bound peptide chains when compared with the untreated resin (50 μmol/g versus 71 μmol/g). The amino acid composition of peptide 735–752 has been previously shown to correspond to that expected for this peptide (Kennedy *et al.*, 1986).

Specificity of the rabbit anti-peptide antisera

Previously it was demonstrated that the two rabbits immunized with peptide 735–752 coupled to KLH produced an anti-peptide and anti-gp160 response (Kennedy *et al.*, 1986). Two rabbits immunized with resin-bound peptide 503–532 produced a specific anti-peptide response as determined by an enzyme-linked immunosorbent assay (ELISA) (Figure 2). Antisera obtained from the two rabbits prior to immunization demonstrated little or no reactivity to either the AIDS or a control-resin-bound peptide ($OD_{410} < 0.15$ at a 1:50 dilution). Both anti-peptide rabbit antisera bound peptide 503–532 at comparable levels, whereas little binding to a control-resin–peptide containing sequences analogous to hepatitis B surface antigen was observed. Subsequent studies have shown that only antiserum (no. 1) produced a detectable anti-HTLV-III response specific for gp120 based on radioimmunoprecipitation (RIP) (Allan and Essex, personal communication). The other anti-peptide antiserum (no. 2) did not develop a detectable anti-HTLV-III response as assessed by RIP. Based on these data it is possible that peptide 503–532 may possess at least two epitopes, one of which is associated with a native gp120 determinant and is recognized by rabbit no. 1 anti-peptide antiserum. The other epitope(s) fail to identify native gp120 determinants and are therefore associated only with the peptide. These epitopes are predominantly recognized by rabbit no. 2 antiserum which fails to immunoprecipitate native gp120.

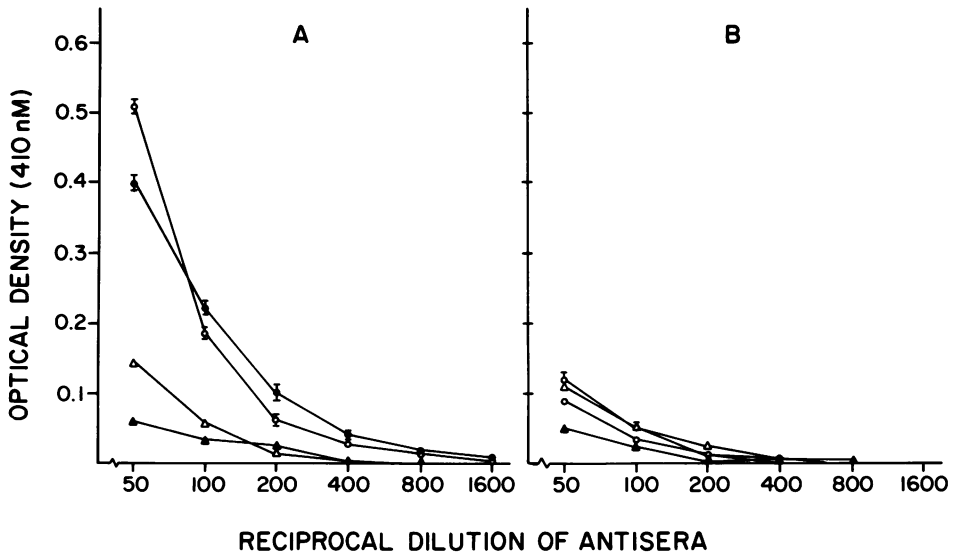


Fig. 2. Anti-peptide binding curves of antisera obtained from rabbits immunized with gp120 peptide 503–532 resin preparation. Rabbit antisera were obtained 30 days after the fourth immunization. The following resin-bound peptides were used to coat the solid phase [gp120 peptide 503–532 (**A**); hepatitis B surface antigen peptide (**B**)]. Rabbit anti-peptide antisera were obtained from: rabbit no. 1 (●); rabbit no. 2 (○). Pre-immune sera from each rabbit binding to the two peptide resins are shown for rabbit no. 1 (▲) and rabbit no. 2 (△). Each point represents the mean of triplicate values. The brackets refer to the range of values.

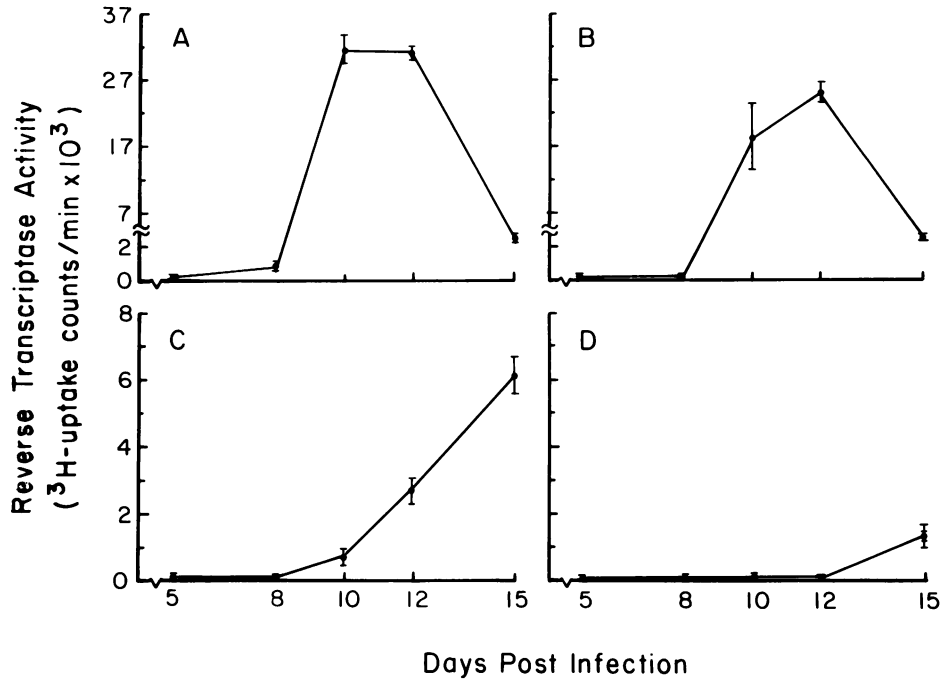


Fig. 3. The kinetics of *in vitro* HTLV-III replication was determined by assaying reverse transcriptase activity (uptake of [^{3}H]TTP). The virus dilutions were as follows: (**A**) 10^{-1}; (**B**) 10^{-2}; (**C**) 10^{-3}; (**D**) 10^{-4}. Each point represents the mean ^{3}H c.p.m. of five determinations performed in duplicate. The brackets refer to ±SEM.

Similar results utilizing peptide antigens and differential immune responses in experimental animals have been reported previously with other protein systems (for a review see Atassi, 1985).

Kinetics of HTLV-III replication in vitro

Prior to examining the ability of the rabbit anti-peptide antisera to neutralize HTLV-III isolate NY-5 infectivity *in vitro*, it was necessary to determine the kinetics of HTLV-III replication in a susceptible human T-cell line. Various dilutions of an HTLV-III viral stock were incubated with normal human and pre-human rabbit sera similar to the methods utilized to examine viral neutralization with the anti-peptide reagents. Following a 1 h incubation with the sera, the HTLV-III viral dilutions were added to cultures of susceptible A3.01 cells. These cultures infected with HTLV-III were maintained for 15 days and supernatant fluid from the cultures was removed on days 5, 8, 10, 12 and 15. HTLV-III replication was assessed by the presence of reverse transcriptase (RT) activity in the culture supernatant fluids. RT activity was determined by the c.p.m. of [^{3}H]thymidine

Table II. Neutralization of HTLV-III isolate NY-5 infectivity *in vitro*

	Human	Rabbits			
	AIDS serum	Anti-503–532 peptide		Anti-735–752 peptide	
Virus dilution		1	2	3	4
	Percent reduction of RT activity (10 days)				
10^{-1}	98	96	0	0	0
10^{-2}	97	94	0	90	97
10^{-3}	ND	ND	ND	ND	ND
	Percent reduction of RT activity (12 days)				
10^{-1}	91	23	0	0	0
10^{-2}	97	45	0	67	67
10^{-3}	100	70	0	100	ND[a]
	Percent reduction of RT activity (15 days)				
10^{-1}	0	0	0	0	0
10^{-2}	0	0	0	0	0
10^{-3}	90	24	0	95	0

[a]The number of c.p.m. of ^{3}H uptake in that particular culture was < 2000 c.p.m. and the percent reduction of RT activity was not determined.

triphosphate ([^{3}H]TTP) incorporated. The kinetics of four dilutions of HTLV-III (A, 10^{-1}; B, 10^{-2}; C, 10^{-3}; D, 10^{-4}) based on RT activity at days 5, 8, 10, 12 and 15 following infection are shown in Figure 3. The points on the curve reflect the mean c.p.m. of [^{3}H]TTP uptake based on five different determinations performed in duplicate. The range bars signify the SEM. Significant replication of HTLV-III, NY-5 isolate diluted 10^{-1} and 10^{-2} did not occur until 10 days after infection. At 12 days the virus was also actively replicating and by 15 days the decrease in RT activity indicated the cytolytic action of these dilutions of HTLV-III for the susceptible target cells (Zagury *et al.*, 1986). Replication of the 10^{-3} dilution of HTLV-III was observed at days 12 and 15, whereas little or no replication was demonstrated with 10^{-4} dilution of virus even by day 15. We selected >2000 c.p.m. of ^{3}H uptake as an indication of HTLV-III replication, based on the fact that 11 out of 12 determinations (days 5 and 8 with all dilutions of virus, day 10 for 10^{-3} and 10^{-4} dilutions and days 12 and 15 for 10^{-4} dilution) with <2000 c.p.m. had SDs and SEMs greater than or equal to the mean c.p.m.

This indicated that the individual c.p.m. values were extremely variable and it would be difficult at best to determine whether low RT activity resulted from specific neutralization or just random variation of the RT assay. Indeed, when examining neutralization of plaque-forming virus, such as herpes simplex or cytomegalovirus, one must titrate the viral stock to yield >80 plaques per assay in order to determine whether a reduction in plaques represents significant neutralization of the virus (Dreesman and Benyesh-Melnick, 1967). Antibodies may not efficiently neutralize virus if overwhelming quantities of infectious virions are present. Based on the kinetic studies and our particular HTLV-III viral stock, we determined that neutralization of HTLV-III infectivity by human and rabbit anti-peptide antisera would be examined at days 10, 12 and 15 for the 10^{-1} and 10^{-2} dilution of HTLV-III, NY-5 isolate.

Neutralization of anti-peptide antisera

If a synthetic peptide preparation is to represent a putative vaccine candidate for HTLV-III, antibodies produced in experimental animals to the peptide should neutralize that infectivity of several HTLV-III isolates *in vitro*. We assessed the ability of rabbit antisera generated to peptides 503–532 and 735–752 to neutralize HTLV-III, NY-5 isolate infectivity based on a reduction of RT activity. In Table II, a single rabbit anti-peptide 503–532 antiserum efficiently reduced NY-5 replication at day 10 when compared with pooled human sera from AIDS patients at both 10^{-1} and 10^{-2} dilutions of virus. A second rabbit antiserum to this peptide failed to reduce HTLV-III replication and served as a control antiserum throughout the RT assay. No anti-HTLV-III activity was detected in this particular antiserum based on radioimmunoprecipitation; even though the rabbit received a similar immunogen and produced a detectable anti-peptide response. The antiserum that neutralizes HTLV-III detects both gp120 and gp160 envelope glycoproteins (Allan and Essex, personal communication). Rabbit no. 1 antiserum was found to be less efficient in neutralizing HTLV-III when compared with human AIDS serum on day 12 and 15. The percent reduction of RT activity had decreased by day 12 from >90% (day 10) to 23 and 45% for 10^{-1} and 10^{-2} dilution of virus respectively. The more dilute the virus, the greater the reduction of RT activity at day 12 indicating that the ability of the antisera to neutralize is dependent on the amount of virus. Both rabbit antisera to peptide 735–752 neutralized a 10^{-2} virus dilution at day 10 but were not as efficient at neutralizing higher concentration of virus (10^{-1}) when compared with the serum from a human AIDS patient and the rabbit anti-peptide 503–532. Rabbit no. 3 neutralized efficiently (>95%) a 10^{-3} virus dilution at both 12 and 15 days. No reduction in RT activity was obtained with antiserum from rabbit no. 4 on day 15. This may be reflected in the fact that rabbit no. 3 has a higher antibody titer to HTLV-III when compared with rabbit no. 4 (Kennedy *et al.*, 1986). No reduction of RT activity was observed on day 15 with any of the antisera at the high concentrations of virus indicating that infectious virus was present in the culture and the antisera were not effective in inhibiting viral replication at this time point. Both antisera from the human AIDS patient and rabbit no. 3 inhibited RT activity at day 15 with a 10^{-3} dilution of virus. Control rabbit antisera produced against non-HTLV-III envelope glycoproteins coupled to KLH and a hepatitis B surface antigen control-resin-bound peptide preparation at similar dilutions employed in the *in vitro* neutralization test demonstrated no significant inhibition of RT activity (<25%).

In addition, neutralization of the HTLV-IIIB isolate by the anti-peptide antisera was also examined by utilizing a constant concentration of the virus with various dilutions of the antisera. The HTLV-IIIB and LAV-1 amino acid sequences are identical for the two peptides selected for synthesis. Neutralization in this assay was defined as the inhibition of syncytia formation and <90% reduction of RT activity compared with the control cultures. Rabbit antisera versus peptide 503–532 and peptide 735–752 demonstrated neutralizing activity both at a titer of 1:32. The pre-immune rabbit serum either fail to show any neutralization or neutralized at a titer of 1:4. Thus, the anti-peptide antisera was between 8- and 32-fold more efficient at neutralizing HTLV-IIIB infectivity *in vitro* when compared with the pre-immune rabbit sera. Together these data suggest that at least two neutralizing epitopes are present on the gp160 from two different isolates HTLV-III, one of these subunits is associated with the gp120 subunit (peptide 503–532) and the other associated with the gp41 subunit (peptide 735–752).

Discussion

Synthetic peptides corresponding in sequence to a segment of HTLV-III envelope glycoproteins are useful in identifying viral neutralizing epitopes. Numerous studies have implicated the use

of synthetic peptides as possible candidates to produce inexpensive and reproducible vaccines against infectious agents (reviewed in Arnon *et al.*, 1983; Sutcliffe *et al.*, 1983). Although the role of neutralizing antibodies in the induction of protective immunity against AIDS-associated viruses is not clear, synthetic peptides have been implicated as putative vaccine candidates for HIV (Fishinger *et al.*, 1985; Francis and Petricciani, 1985). Our laboratories have previously demonstrated that a synthetic peptide analogous to amino acid sequences associated with gp41 when coupled to a carrier protein can induce an anti-HTLV-III specific response in rabbits (Kennedy *et al.*, 1986). However, it was not determined whether this rabbit anti-peptide antisera could neutralize HTLV-III infectivity *in vitro*. This would be a prerequisite prior to the expense of testing putative synthetic peptide vaccine candidates in a relevant *in vivo* animal model, such as chimpanzees (Alter *et al.*, 1984; Francis *et al.*, 1984; Gajdusek *et al.*, 1985. Eichberg *et al.*, 1986; Fultz *et al.*, 1986). We tested the neutralizing capacity of this rabbit antiserum (735–752) along with another rabbit anti-peptide (503–532) preparation, with two different HTLV-III isolates by examining the reduction of RT activity *in vitro* and inhibition of syncytia formation using an HTLV-III susceptible cell line. Several published reports have examined the presence of neutralizing antibodies in AIDS and ARC patients (Weiss *et al.*, 1984; Robert-Guroff *et al.*, 1984; McDougal *et al.*, 1985; Ho *et al.*, 1985; Clavel *et al.*, 1985). In one study HTLV-III replication was monitored in target cells by indirect immunofluorescence and RT activity using an anti-HTLV-III monoclonal antibody that recognizes a core protein, p24 (Robert-Guroff *et al.*, 1984). In the second study neutralization was measured by inhibition of syncytium formation, neutralization of envelope pseudotypes of vesicular stomatitis virus and reduction of viral infectivity by immunofluorescence (Weiss *et al.*, 1984). Based on these and other studies, we feel that anti-peptide antisera demonstrated significant neutralization of two HTLV-III isolates *in vitro*. We elected to titrate the viral stock when examining neutralization of the NY-5 isolate. A correlation was obtained with the percent reduction of RT activity and the concentration of the virus suggesting that the ability to neutralize a given virus stock was dependent on the concentration of the neutralizing antibodies in the particular antisera. Alternatively, we titrated the antisera relative to a constant concentration of virus when examining HTLV-IIIB neutralization.

The levels of virus-specific RT activity differed significantly after incubation of virus with serum obtained from individual rabbits prior to inoculation with each respective peptide (Figure 2). In addition, one of the pre-immune rabbit sera neutralized HTLV-IIIB infectivity at a 1:4 dilution. This most likely indicated the presence of cross-reactive antibody to HTLV-III and/or non-specific factors in normal rabbit sera. In this light, it becomes imperative to determine the presence of virus neutralizing antibody by simultaneous testing of both the pre-inoculation (normal) serum as well as the respective immune sera obtained from the same animal.

It is noteworthy that these two peptides from HTLV-IIIB are identical in sequence to regions 508–539 and 740–757 from gp160 of LAV-1. In the ARV-2 sequence, aspartic acid is substituted for a glutamic acid in the 735–752 region and a valine is inserted along with a methionine for leucine substitution in the 503–532 region. These insertions or substitutions do not alter either the hydrophilicity or secondary structure predictions when comparing the three viral amino acid sequences (Pauletti *et al.*, 1985). Due to the variability of the gp160 nucleotide sequences from different AIDS-associated viral isolates (Shaw *et al.*, 1984; Benn *et al.*, 1985), peptides of conserved epitopes are critical when evaluating vaccine candidates for immunoprotective purposes in the control of the HIV infection. Also, it becomes important to examine neutralization with several diverse HIV isolates.

The identification of neutralizing viral epitopes on HIV gp160 may be helpful when designing putative vaccine candidates, especially if neutralizing antibody is associated with the induction of protective immunity. This report indicates that at least two neutralizing epitopes are present on gp160. One epitope is present on the gp120 subunit and is identified by a rabbit antiserum to peptide 503–532, whereas the other neutralization site is on the gp41 subunit and is identified by rabbit antisera to peptide 735–752. Together these data indicate that it might be more efficient to incorporate both gp120 and gp41 subunits into potential HIV vaccine formulations.

Materials and methods

Synthesis of peptides

Peptide 503–532 was synthesized on a polyamide-based support. The polyamide resin was generated by the free radical co-polymerization of dimethylacrylamide and N,N'-bisacryloyl-1,3-diaminopropane (Helpern and Sparrow, 1980). An aqueous solution of these monomers was suspended in a carbon tetrachloride–hexane medium, and an emulsion created by addition of sorbitan sesquioleate. This method is a modification of that described previously (Arshady *et al.*, 1981; Smith *et al.*, 1983), and will be described in detail elsewhere (Kanda *et al.*, in preparation). The amino methyl function was introduced during polymerization as an N-protected allylamine. The degree of functionalization of the beaded polyamide resin was checked by coupling N-α-Boc-alanine to the resin using diisopropylcarbodiimide as activator. Amino acid analysis revealed a substitution of ~0.15 mmol/g resin. The peptide was synthesized by first coupling the C-terminal Boc-glycine to the amino methyl resin via its 4-(oxymethyl) benzoic acid derivative. This handle confers acid stability on the peptidyl–benzyl ester linkage. The Boc-glycyl-4-(oxymethyl) benzoic acid was synthesized by a procedure similar to that used to prepare the phenylacetic acid derivative described by Mitchell *et al.* (1978). It was coupled to the resin using dicyclohexylcarbodiimide as activator and 4-dimethylaminopyridine as catalyst. The loading was checked by amino acid analysis and found to be 0.15 mmol/g resin. The peptide was assembled on this glycyl resin using a Biosearch Sam II automated synthesizer, Boc-protected amino acids, and *in situ* diisopropylcarbodiimide activation.

Side-chain protecting groups were as follows: benzyl ether for threonine hydroxyl; benzyl ester for carboxyl of glutamic acid; *p*-tosyl for the guanidine of arginine; and 2-chlorobenzyloxycarbonyl for the ε-amino group of lysine. Trifluoroacetic acid (40% in CH_2Cl_2) was used to remove Boc and the TFA salt was neutralized with N,N-diisopropylethylamine (10% in CH_2Cl_2). 1-Hydroxybenzotriazole was added during glutamine coupling to prevent dehydration. The specific steps of the synthesis have been published elsewhere (Sparrow, 1976).

The protecting groups were removed following synthesis by treatment with anhydrous HF at 0°C in the presence of scavengers according to the method previously described (Bhatnagar *et al.*, 1983). Because the peptide is attached to the resin by an (oxymethyl) benzoic acid that is resistant to cleavage by HF, the peptide will remain attached to the resin while side chains of amino acids are completely deprotected. The polyamide nature of the resin allows swelling in both polar, aprotic organic solvents and neutral pH buffers.

Peptide 735–752 was synthesized on N-*t*-Boc-*S*-4 methylbenzyl cysteine polystyrene. The synthesis of the peptide has been described elsewhere (Kennedy *et al.*, 1986). The cysteine residue at the C terminus was added to provide a functional SH group for coupling to carrier proteins. Glycine was included in both syntheses to provide a spacer amino acid prior to the HTLV-III amino acid sequences. A tyrosine residue was added to the amino terminus of peptide 735–752 for radioactive labeling with ^{125}I to determine peptide to carrier coupling efficiency.

Amino acid composition

Peptide–resin was hydrolyzed with 12 N HCl, propionic acid (1:1, v/v) containing 0.05% phenol for 2 h at 135°C. Analyses were performed on a Beckman 7300 amino acid analyser.

Generation of rabbit anti-peptide antisera

Peptide 735–752 was coupled to KLH and used to immunize rabbits. The specificity of the rabbit antisera for HTLV-III gp160 and the methods for peptide to carrier coupling have been described previously (Kennedy *et al.*, 1986). In addition, two rabbits were each immunized with peptide 503–532 containing resin (200

μg of peptide per dose) in Freunds complete adjuvant. Rabbits received an intramuscular injection every 2 weeks for a total of three injections. Subsequent immunizations were given at monthly intervals. Serum was obtained 30 days after the fourth injection. Control anti-peptide antisera was produced in rabbits immunized with either an SV40 tumor antigen peptide coupled to KLH similar to the methods described for the generation of anti-735−752 peptide antisera or a hepatitis B surface antigen resin-bound peptide similar to the methods described in producing anti-503−532 peptide antisera.

Anti-peptide activity

The polyamide resin−peptide conjugate was crushed with a mortar and pestle and a suspension of the resin was made in borate buffered saline, pH 8.0. 100 μl of this emulsion containing ~10 μg of peptide (weight basis as calculated by amino acid composition) was adsorbed to the solid phase of microtiter plates (Immulon II, Dynatech, Alexandria, VA, USA) for 8 h at 4°C. Non-specific sites were blocked with 10% normal goat serum and the assay was performed as previously described (Kennedy *et al.*, 1986). Briefly the binding of rabbit antibodies to the peptide was detected using biotin-goat antibody to rabbit IgG and avidin-conjugated horseradish peroxidase. Peroxidase activity was determined with 1,2′-amino-di(3-ethylbenzthiazoline-sulfonic acid) and H_2O_2 as the substrate. A resin-bound peptide corresponding to a hepatitis B surface antigen sequence served as a control. The binding of the rabbit anti-peptide was quantitated spectrophotometrically at 410 nm with a plate reader. Each value represents the mean of triplicate determinations.

Kinetics of HTLV-III replication in vitro

One normal human serum and the four pre-immune rabbit sera were heat inactivated at 56°C for 1 h and filter sterilized through a 0.2 μm filter. 150 μl of a 1:5 dilution were incubated with an equal volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions of an HTLV-III isolate, termed NY-5 (Benn *et al.*, 1985) for 1 h at 37°C. Following the incubation, the antibody-treated virus mixture was added to 10^6 A3.01 cells (Folks *et al.*, 1985). The mixture was incubated for 2 h at 37°C in the presence of 1 μg/ml of polybrene. The infected A3.01 cells were washed and resuspended in 1 ml of RPMI media containing 10% heat-inactivated FCS and dispersed into 24-well microtiter plates. 500μl of spent media supernatant was removed at days 5, 8, 10, 12 and 15 after infection and frozen at −135°C until reverse transcriptase activity was determined. Following the removal of supernatant, the individual cultures were fed with 500 μl of RPMI plus FCS. Each culture was performed in duplicate.

Neutralization assays

Pooled human AIDS serum that test positive by ELISA and Western blot was the gift of Dr Thomas Folks, Laboratory of Immunoregulation, NIAID, Bethesda, MD. The human sera and rabbit anti-peptide antisera were treated as described above. In each instance the pre-immune sera of that particular rabbit served as the negative control indicative of no neutralization of HTLV-III infectivity as determined by RT activity c.p.m. when compared with the individual rabbit anti-peptide preparation. The pre-immune and rabbit anti-peptide antisera were incubated with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions of the NY-5 isolate as described above. Each culture was performed in duplicate. Supernatants from infected A3.01 cells were removed at days 5, 8, 10, 12 and 15, frozen at −135°C, and assayed for RT activity. The percent inhibition of RT activity was determined by the following formula.

$$1 - \left[\frac{\text{c.p.m. RT assay of anti-peptide antisera cultures}}{\text{c.p.m. RT assay of pre-immune sera cultures}}\right] \times 100$$

Background counts ranging from 200 to 750 c.p.m. were subtracted from each determination prior to calculating percent inhibition.

Viral neutralization assays were also performed using the H9 clone as the target cells and the HTLV-IIIB isolate as the virus inoculum (Popovic *et al.*, 1984). Cell-free virus was harvested from a chronically infected H9 culture, titrated on uninfected H9 cells and the titer expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml. Each virus inoculum (100 μl containing 50 $TCID_{50}$). was pre-incubated with 100 μl of 2-fold serial dilutions of the rabbit serum for 1 h at 37°C. This was inoculated onto $1.5-2.0 \times 10^6$ H9 cells. Seven to 10 days after inoculation each culture was examined for characteristic cytopathic effects with syncytia formation and for RT activity in the supernatant fluid as previously described (Ho *et al.*, 1984). Neutralization was defined as inhibition of syncytia formation and > 90% reduction in RT activity when compared with control cultures, which were established using pre-immune serum to pre-incubate the virus inoculum.

Reverse transcriptase activity

The methods for performing RT activity assays have been described in detail elsewhere (Barre-Sinoussi *et al.*, 1983). Briefly, 15 μl of each supernatant from A3.01 infected cultures were added to 96-well microtiter plates that contained 50 μl of virus dilution buffer [0.05 M Tris−HCl, pH 7.8, 0.1 M NaCl, 0.15 mg/ml dithiothreitol (DTT), 0.1% Triton X-100]. 50 μl of the reaction mixture (1 M Tris−HCl, 3 M KCl, 0.15 M $MgCl_2$, 10% Triton, poly(A), oligo(dT) and [^{3}H]TTP) was added and incubated 1 h at 37°C. Following the incubation, 50 μl of the mixture was dotted on nitrocellulose filter papers. The filter papers were washed successively in beakers containing: (i) 5% trichloroacetic acid (TCA) and 5% sodium pryophosphate; (ii) 5% TCA; and (iii) 50% ethanol. The filter papers were counted in an automatic scintillation counter and the c.p.m. of [^{3}H]TTP were determined.

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