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Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein

(epitope/serodiagnosis)

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ABSTRACT **A peptide of 21 amino acids with the sequence Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser encoded by a segment in the *env* gene of human T-lymphotropic virus type III (HTLV-III), corresponding to amino acids 586–606 of the precursor envelope glycoprotein, has been synthesized by the Merrifield solid-phase method. Combined serological and chemical analyses of this peptide and related peptides have revealed the importance of certain amino acid residues in the antigenic determinant of the relevant peptide. Enzyme immunoassay (EIA) employing this peptide as an antigen adsorbent was shown to reproducibly detect antibodies in sera of patients with HTLV-III infection. This assay provided positive results with all sera that were reactive with gp41 envelope protein of HTLV-III in electrophoretic immunoblot analysis. Thus far, no false-positive sera have been encountered in control populations. Our EIA with this peptide as the coating antigen is shown to have advantages over that with the whole HTLV-III virus as an immunoadsorbent.**

Since the identification of a human retrovirus, named human T-lymphotropic virus type III (HTLV-III), acquired immune deficiency syndrome (AIDS)-related virus (ARV), or lymphoadenopathy-associated virus (LAV) as the infectious agent for AIDS (1–4),† substantial progress has been made in the studies of the virus and in the development of diagnostic methods for the detection of HTLV-III infection. With the establishment of permissive T-cell lines for mass production of HTLV-III virus (1), it has been possible to elucidate the structure and gene organization of HTLV-III, to determine the complete nucleotide sequence of the HTLV-III genome (5–8), and to use heat-inactivated HTLV-III as the immunoadsorbent for detection of antibodies against HTLV-III in sera of patients with HTLV-III infection (9–18).

Efforts employing recombinant DNA technology to identify HTLV-III antigenic peptides reactive with sera from AIDS and AIDS-related complex (ARC) patients and asymptomatic HTLV-III-infected individuals have also been undertaken by many investigators (19–23). Success has been obtained by this approach. However, it has not been able to localize and identify the peptide sequences representing the antigenic epitope(s).

Synthetic peptides are used increasingly to map antigenic or immunogenic sites on the surface of proteins and, in turn, as possible vaccines (reviewed in ref. 24). We have synthesized numerous peptides corresponding to various regions of envelope protein of HTLV-III and analyzed their reactivity with sera from AIDS patients. Our initial emphasis was on the gp41 glycoprotein, the transmembrane portion of envelope protein gp160, which has previously been identified as the antigen most consistently recognized by antibodies in patients with AIDS and ARC (25, 26).

In this paper, we report (*i*) the identification of a peptide 21 residues in length with the sequence Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser, corresponding to amino acids 586–606 of the precursor envelope protein of HTLV-III, that is highly reactive with sera from AIDS patients; (*ii*) the characterization of this epitope at a single amino acid level; and (*iii*) the development of a sensitive and specific immunoassay for detecting HTLV-III antibodies by using this peptide.

MATERIALS AND METHODS

Synthesis of Peptides. All syntheses followed the stepwise solid-phase strategy developed by Merrifield (27) and were performed on automated peptide synthesizers (models Sam II and 9500, Biosearch, San Rafael, CA), with the acid-labile *tert*-butyloxycarbonyl (Boc) group for temporary amino-terminal protection and more acid-stable groups for protection of the side chains. Protected amino acids were obtained from Biosearch and Peninsula Laboratories (San Carlos, CA). Their purities were assessed by melting point and thin-layer chromatography. The following side-chain protecting groups were used: *O*-benzyl for Thr, Ser, Glu, and Tyr; N^{ω}-tosyl for Arg; *N*-tosyl for His; N^{ϵ}-2-chlorobenzyloxycarbonyl for Lys; *S*-4-methylbenzyl for Cys; and *O*-cyclohexyl for Asp. A 4-methylbenzhydrylamine resin (catalog no. AA-7547, 1% cross-linked; 100–200 mesh; 0.3 meq/g; Biosearch) was utilized throughout the study, resulting in a universal carboxyl-terminal amide group on each peptide synthesized. This served as a constant basis by which each synthesis could be evaluated. Successive amino acids were added as dictated by the sequence to be synthesized. In the case of peptide SM284, the resultant peptide on solid support was evaluated by solid-phase sequencing techniques (28) for sequence integrity. The synthetic protected peptide-resins were cleaved and extracted according to the method of Tam *et al.* (29). Analytical HPLC of the peptides was performed on a reverse-phase Vydac C_{18} column (4 × 300 mm) in a LKB instrument over a linear gradient from 5% to 100% solution B (0.05% trifluoroacetic acid in acetonitrile) into solution A (0.05% trifluoroacetic acid in water) in 17 min, at 1.7 ml/min.

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Abbreviations: AIDS, acquired immune deficiency syndrome; ARC, AIDS-related complex; HTLV-III, human T-lymphotropic virus type III; EIA, enzyme immunoassay.

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†The members of a subcommittee empowered by the International Committee on the Taxonomy of Viruses to propose an appropriate name for the retrovirus isolates recently implicated as the causative agents of AIDS have proposed the name human immunodeficiency virus, to be abbreviated HIV [(1986) *Science* **232**, 697, and *Nature (London)* **321**, 10].

Source and Characterization of Clinical Materials. Surplus serum/plasma samples, previously collected for use in other clinical/diagnostic tests, were generously provided by J. Gold, S. Cunningham-Rundles (Memorial Sloan–Kettering Cancer Center), S. Gupta (Department of Medicine, University of California at Irvine), D. M. Knowles (New York University Medical School), F. P. Siegal (Long Island Jewish Hospital), and N. Chiorazzi (The Rockefeller University Hospital). A total of 451 samples from patients diagnosed as having AIDS, ARC according to standard criteria of the Centers for Disease Control, and in the high-risk groups including asymptomatic healthy homosexual males, intravenous drug users, children and sexual partners of AIDS and ARC patients, and hemophiliacs were screened in our assay. In addition, a total of 387 control sera from patients with primary immunodeficiencies, leukemias and other malignancies unrelated to HTLV-III infection, and autoimmune diseases (including systemic lupus erythematosus, rheumatoid arthritis, and allergies), as well as from normal individuals, were also included in the test for specificity determination. Commercial whole-virus HTLV-III EIA kits, including those from Electro-Nucleonics, Abbott, and DuPont, were used according to the manufacturer's instructions for comparison with our test. All sera were coded prior to testing in the assay, and the origin of a serum was not known to the person performing a test. Electrophoretic immunoblot ("western blot") analysis was performed on 397 of 838 samples with inactivated HTLV-III virus as antigen as previously described (9).

Solid-Phase EIA for the Detection of Antibodies to HTLV-III. Synthetic peptides, or purified HTLV-III that had previously been inactivated by addition of 0.5% Nonidet P-40 and heating at 56°C for 30 min, both at 5 μg/ml in 0.01 M $NaHCO_3$ buffer (pH 9.5), were coated on 96-well microtiter plates (Nunc) as described (30). Patient serum or plasma specimens, which had previously been heat inactivated at 56°C for 30 min, along with the positive and negative controls were diluted 1:20 into 0.01 M phosphate-buffered saline containing 20% normal goat serum and 0.05% Tween 20 and incubated in coated microtiter wells for 15 min at 37°C. After six washes with phosphate-buffered saline containing 0.05% Tween 20, the plates were incubated with horseradish peroxidase conjugated with affinity-purified goat antibodies to human IgG (UBI-Olympus, Lake Success, NY) for 15 min at 37°C. Plates were again washed and developed with 100 μl of 0.067% *ortho*-phenylenediamine dihydrochloride in sodium citrate buffer (pH 5.5). The reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4 to each well, and color development was measured in a plate reader (Multiscan, Flow Laboratories) at 492 nm. The cutoff value used to determine seropositivity was calculated based on statistical analysis as: NC (negative control = mean value of 118 normal individuals + 1 SD) + 0.1 × PC (positive control obtained with pooled AIDS serum at a dilution of 1:300, previously adjusted to have an experimental EIA value of ≈1.0)—i.e., cutoff = NC + 0.1 × PC.

RESULTS

Identification of a 21-mer Peptide as a Major Antigenic Site on gp41 Envelope Protein of HTLV-III. A region located between the two hydrophobic areas of gp41 and spanning 102 amino acid residues (corresponding to amino acid residues 546–647 of the HTLV-III envelope protein) was initially chosen for study. Ten overlapping peptides in the 19- to 21-mer size range were constructed and synthesized on the basis of the deduced amino acid sequence (5–7). For all peptides used in further immunochemical studies, reverse-phase high-performance liquid chromatography over a Vydak C_{18} column revealed a major peak under reducing conditions in the presence of dithiothreitol. The peptides produced by solid-phase synthesis were examined by EIA under nonreducing conditions for reactivity with sera from AIDS patients. In each well, 100 μl of each peptide was coated onto 96-well microtiter plates as immunoadsorbent at 5 μg/ml in 0.01 M $NaHCO_3$ buffer (pH 9.5). Pooled AIDS serum known to have anti-HTLV-III reactivity and pooled normal human serum (as negative control) were then assayed, both at a 1:20 dilution.

Among the 10 peptides screened, only one, a 21-mer designated SM284 (Fig. 1), demonstrated strong reactivity with the pooled AIDS serum (with A_{492} being >2.0) but not the normal control. This chemically synthesized viral envelope 21-mer was further compared with inactivated HTLV-III for reactivity with HTLV-III antibody in the same solid-phase EIA. Both antigens were coated onto wells of polystyrene plates as immunoadsorbent. As shown in Fig. 2, the pooled AIDS patient serum gave positive signals at dilutions as high as 1:10,000 with both antigen forms. However, the signal-to-noise ratio exhibited with SM284 peptide, when coated at the same concentration as that of inactivated HTLV-III (5 μg/ml), was much higher than that with inactivated virus. To evaluate the usefulness of SM284 peptide as an antigen in screening sera for antibodies to HTLV-III, similar studies were performed over a wide dilution range with 30 patient sera, selected from three categories—AIDS, ARC, and healthy homosexuals—by their initial high reactivity to the 21-mer. Again, high sensitivity was observed with all 30 sera tested in our EIA, with high signal-to-noise ratio (Fig. 2 *Inset*).

Localization of Important Amino Acids in the 21-mer as a Relevant Antigenic Site on gp41 Envelope Protein of HTLV-III. To further localize the amino acid residues important in antibody binding, multiple peptide fragments with sequences within the 21-mer region were synthesized and used as immunoadsorbent for reactivity determination. The reactiv-

--------IEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLLGIWGCS--------

e.g. SM284:

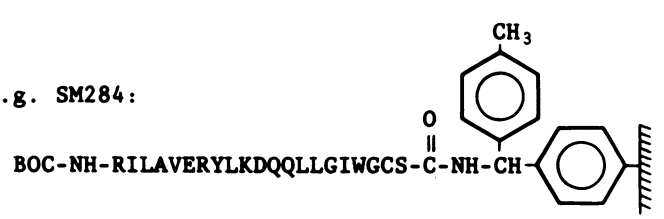

FIG. 1. One region encompassing a total of 102 amino acids derived from the deduced sequence of HTLV-III envelope protein gp41 as previously translated (1–3) was chosen for epitope analysis. Each of the 10 overlapping peptides covering this region in the 19- to 21-mer size range was synthesized on a separate 4-methylbenzhydrylamine support in the orientation shown. The one-letter code used to denote each amino acid is A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

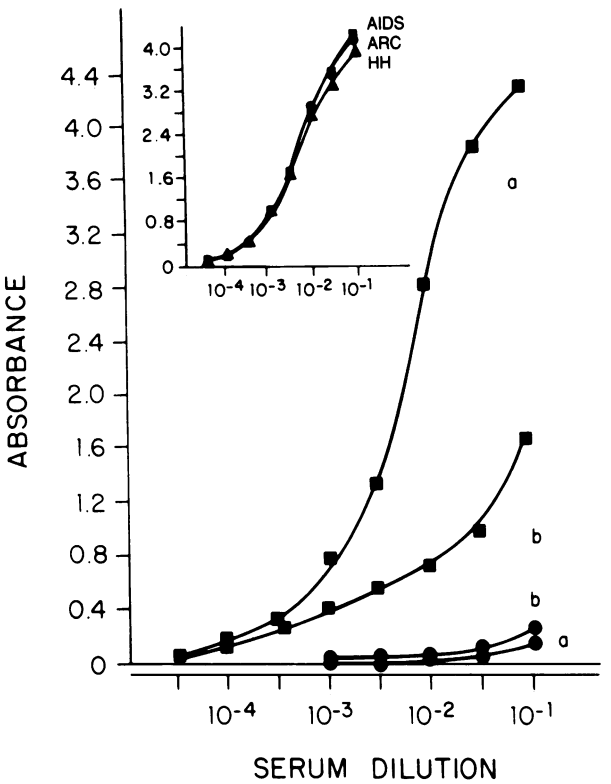

FIG. 2. Solid-phase EIA was employed to detect binding of human antibodies to HTLV-III by using SM284 21-mer (curves a) or inactivated HTLV-III virus (curves b) as immunoadsorbent. ■, Pooled AIDS serum; ●, pooled normal serum. (*Inset*) Three serotype dilution curves on SM284 21-mer immunoadsorbent. Sera were obtained from three patient groups (■, AIDS; ●, ARC; ▲, asymptomatic healthy homosexuals) each containing 10 individual samples giving an initial EIA A_{492} reading greater than 2.0 on the SM284 21-mer solid-phase immunoadsorbent. Each point on the curve represents the mean value of 10 data points. The mean ± SD of the first three serum dilutions for AIDS, ARC, and asymptomatic homosexual groups are 4.218 ± 0.279, 3.557 ± 0.817, and 2.887 ± 1.156; 4.134 ± 0.291, 3.520 ± 0.779, and 2.960 ± 1.053; and 3.886 ± 0.429, 3.243 ± 1.211, and 2.760 ± 1.406, respectively.

ity of each peptide was defined as percent of maximum antibody binding at half-saturating serum dilution (1:300) as detected by EIA when compared to that of the 21-mer peptide as shown in Table 1. Among all these peptides examined, 21-mer SM284 gave the highest binding reactivity. Residues Arg-1, Ile-2, and Lys-10 all played important roles in the antigen–antibody interaction, since the deletion or addition of one amino acid resulted in significant loss or restoration of the corresponding peptide's reactivity. Deletion of Trp-Gly-Cys-Ser residues at the carboxyl terminus also reduced the binding reactivity, although to a lesser extent. Cleaving the 21-mer in between Lys-10 and Asp-11 resulted in a total loss of binding reactivity. Extending amino acids from the amino terminus for peptides with carboxyl-terminal Lys-10 did not restore the peptides' antibody-binding reactivities. Conservative substitution of Ile-2 and Lys-10 by Val and Arg, respectively, as in certain virus isolates, did not much affect the human antibody binding to the corresponding 21-mer analogs (data not shown).

Table 2. Results of sera tested with the EIA employing a synthetic 21-mer peptide as immunoadsorbent antigen

Patient group	Immunoblot with HTLV-III		EIA with 21-mer	
	No. tested	No. reactive	No. tested	No. reactive
AIDS	122	118	228	224
ARC	97	88	145	136
Asymptomatic healthy homosexuals and high-risk groups	27	24	78	27
Control diseases	116	0	269	0
Normal blood donors	35	0	118	0

All samples tested by the immunoblot assay have been previously tested by EIA. All samples positive by immunoblot assay were positive in EIA.

Use of SM284 Peptide as an Antigen in EIA for Detection of Antibodies to HTLV-III. After the initial study, a broader panel of sera was tested. This panel consisted of serum samples from healthy normal individuals and from patients with (*i*) AIDS, (*ii*) ARC, (*iii*) high risk for HTLV-III infection (intravenous drug users, hemophiliacs, children and sexual partners of patients with AIDS or ARC, and healthy homosexual males) and, (*iv*) diseases including rheumatoid arthritis, systemic lupus erythematosus, allergies, leukemias and other malignancies unrelated to HTLV-III infection. As shown in Table 2 and Fig. 3, the assay clearly detected 224 of 228 (98.3%) patients with AIDS, 136 of 145 (93.6%) patients with ARC, and 27 of 78 (34.6%) of asymptomatic healthy homosexuals and individuals in high-risk groups as having antibodies to HTLV-III, with over 60% of the positive cases giving EIA values >2.0 in comparison to the cutoff value of 0.19. None of the 387 samples in the control groups

Table 1. Reactivity of anti-HTLV-III in pooled AIDS patient serum with SM284 peptide and related peptides

Peptide no.	Reactivity, %	Structure	No. residues
		(577) (586) (606)	
		1 21	
1	<5	V W G I K Q L Q A R I L A V E R Y L K	19
2	13.5	Q L Q A R I L A V E R Y L K	14
3	<5	R I L A V E R Y L K	10
4	28.3	I L A V E R Y L K D Q Q L L G I	16
5	30.3	R I L A V E R Y L K D Q Q L L G I	17
6	20	I L A V E R Y L K D Q Q L L G I W G C S	20
7	66.8	R L A V E R Y L K D Q Q L L G I W G C S	20
8	100	R I L A V E R Y L K D Q Q L L G I W G C S	21
9	53.9	V E R Y L K D Q Q L L G I W G C S	17
10	38.9	K D Q Q L L G I W G C S	12
11	8	D Q Q L L G I W G C S	11
3 + 11	8	R I L A V E R Y L K+D Q Q L L G I W G C S	10 + 11

Reactivity is defined as percent of maximum antibody binding, at half-saturating serum dilution, with the specified peptide in comparison to that with the SM284 21-mer (peptide 8). Numbers in parentheses indicate position in intact gp160.

gave EIA values higher than the cutoff value. Sera from all 118 of the AIDS patients, 88 of the ARC patients, and 24 healthy homosexuals previously found to be reactive in electrophoretic immunoblot analysis were shown to be reactive with this 21-mer (Table 2). Four sera from the AIDS group and nine sera from the ARC group were not reactive with this 21-mer in our EIA (Table 2 and Fig. 3). These sera also gave negative results by immunoblot analysis and commercial kits. However, three sera in the healthy homosexual and high-risk groups, which showed negative results for gp41 with immunoblot analysis, were found positive with this 21-mer in our EIA. This positivity should not be considered false-positive in view of the lack of false-positive serums in the control groups without high risks.

DISCUSSION

In this report, we have summarized our experiments using synthetic peptides to localize a highly antigenic determinant in HTLV-III gp41 envelope protein with the sequence Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser, which showed high reactivity with antibodies in sera of patients infected with HTLV-III. The rationale for the selection of this peptide and its usefulness for the screening of antibodies to HTLV-III will also be emphasized.

Selection of Regions of the HTLV-III Envelope Protein for Epitope Analysis. In selecting regions of the envelope protein for epitope analysis, three strategies were applied. First, regions that exhibited a relatively high conservation of amino acid sequence between the various isolates of HTLV-III were sought (5–8). Second, sequences rich in hydrophobic residues were avoided since, according to Hopp and Woods, such regions often do not appear to be antigenic (31). Third, since antibodies to HTLV-III were reactive with viral proteins denatured by sodium dodecyl sulfate, as demonstrated by immunoblot analysis, indicating a certain stability in the antigenic epitope, we therefore designed, synthesized, and characterized multiple, overlapping, linear peptides covering the selected regions of gp41. On the basis of these analyses, a region located in between the two hydrophobic areas of the gp41, spanning 102 amino acid residues, was chosen for study. Among the 10 overlapping peptides synthesized, one peptide of 21 amino acids, designated SM284, was found to be highly reactive with sera from patients with AIDS or ARC.

Further studies of the binding of human antibodies to this 21-mer in AIDS patient sera by using related peptides allowed the localization of antigen–antibody interaction at the level of individual amino acids as shown in Table 1. We found that amino acid residues Arg-1, Ile-2, and Lys-10 are of unique importance to the interaction, since a deletion of Arg/Ile or addition of Lys at the said position resulted in complete/significant loss or restoration of reactivity (in Table 1, compare peptides 6, 7, and 8; and 10 and 11, respectively). The peptide bond between Lys-10 and Asp-11 also appeared significant since, as immunoadsorbent, a mixture of two nonreactive peptides (3 and 11) covering the whole 21-mer sequence showed no reactivity. In addition, the peptide region covering Trp-Gly-Cys-Ser also contributes, to a certain extent, to the antigenic configuration (see peptides 4, 5, and 9), as does the Val-Glu-Arg-Tyr-Leu region. Little or no reactivity was observed with peptides 1, 2, and 3, although they share 10 consecutive amino acid residues, including those three that are important for antibody binding, thus indicating the supportive role played by the region defined as residues Asp-11 through Ser-21, to which the configuration of this antigenic epitope is ultimately attributed.

Improved Sensitivity and Specificity Using SM284 21-mer Peptide as an Antigen Adsorbent in EIA. Comparison of EIA results obtained by using this 21-mer peptide with those obtained by immunoblot analysis and commercial EIA kits demonstrated the high degree of sensitivity and specificity of our assay. In fact, 5 of 373 sera from patients at risk for AIDS or ARC that showed low reactivity with the whole virus were found to be clearly reactive with the SM284 peptide. It is indeed remarkable to find that one small peptide of 21-mer size can be used to detect nearly all patients with HTLV-III infection when considering the marked nucleotide sequence heterogeneity observed among different isolates of the virus. In this regard, our choice of a highly conserved region in the gp41 envelope protein for study proved correct. Another factor contributing to the high sensitivity of our assay was the fact that the peptide antigen density employed as immunoadsorbent was much higher, on a molar basis, than that with whole virus. Furthermore, since the peptide used for coating is chemically well defined, giving much lower non-

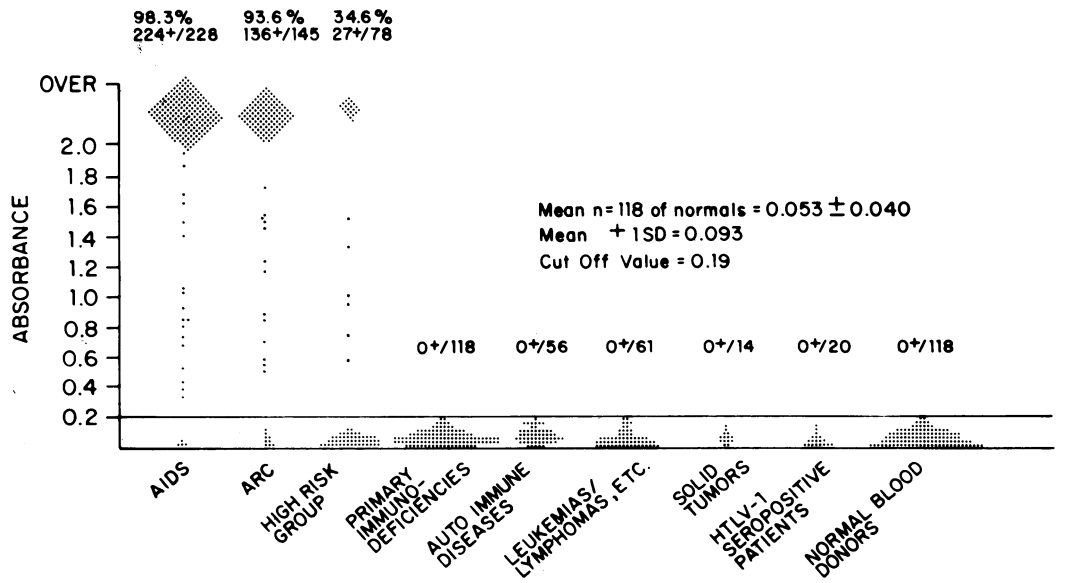

FIG. 3. Histogram showing EIA values obtained with sera from AIDS patients, ARC patients, asymptomatic healthy homosexuals and high-risk individuals, patients with control diseases unrelated to HTLV-III infection, and normal blood donors by using synthetic 21-mer SM284 as the solid-phase immunoadsorbent. Each dot represents the value for one individual.

specific background, we were able to perform the assay at a lower serum dilution than those tests assaying the whole virus (1:20 vs. 1:100–400), thus adding another dimension of sensitivity to our assay. Our ability to identify three samples in the high-risk group, probably from individuals at early onset of infection by HTLV-III, previously shown to be gp41 negative and gp24 positive by immunoblot analysis, further suggests a better sensitivity with our assay than immunoblot analysis. These results also underscore the need for revision of serological diagnostic criteria for past infection with HTLV-III as more sensitive assays become available.

Assays for antibodies to HTLV-III based upon chemically synthesized peptides show several advantages over assays utilizing whole disrupted virus or bacterially produced immunoadsorbents. The 21-mer can easily be synthesized in gram quantities by using automated solid-phase methods, thus providing a highly reproducible integrity of antigen and consistent yields. Isolation of antigens from biological systems precludes such reproducibility. More important, nonspecific reactivities seen in non-HTLV-III-infected individuals are likely due to the heterogeneity of the preparations used for assay. This is particularly true for assays using either whole virus or *Escherichia coli*-derived recombinant products as the immunoadsorbents, with which the host cells' major histocompatibility antigens or endogenous bacterial proteins are frequently copurified (19, 23). Since antibodies to these contaminating antigens are frequently found in normal individuals, false-positive results cannot be eliminated by using current antigen isolation processes. The presently described assay thus clearly eliminates those false-positive reactions encountered in the other methods and heightens sensitivity to true positives by increasing the signal-to-noise ratio, another characteristic of the assay conferred by the quality of immunoadsorbent.

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