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Bacterial expression of the acquired immunodeficiency syndrome retrovirus p24 gag protein and its use as a diagnostic reagent

(p24 core antigen)

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ABSTRACT **A retrovirus [lymphoadenopathy-associated virus, human T-cell leukemia virus type III, acquired immunodeficiency syndrome (AIDS)-related virus] suspected of causing AIDS has been isolated recently. The detection of exposure to this retrovirus in donors of various blood products is important to prevent transmission of the disease from these donors to recipients. In the majority of cases, the detection of antibodies directed against either the viral core protein, a M_r ≈24,000 protein termed p24 gag, or the viral envelope antigen is proof of previous viral infection. Thus, we have expressed the p24 gag antigen in *Escherichia coli* in order to produce a diagnostic reagent for the detection of virus exposure. The bacterially synthesized antigen reacts with human and rabbit antisera directed against the native p24 gag protein in both electrophoretic transfer blot assay and ELISA. In addition, the use of bacterially produced antigens for ELISAs gave results that were comparable to those obtained by using antigens isolated from the virus.**

Acquired immunodeficiency syndrome (AIDS) is associated with a retrovirus [lymphoadenopathy-associated virus (1), human T-cell leukemia virus type III (HTLV-III) (2), AIDS-related virus (3)] that is cytotoxic for the $OKT4^+$ subset (helper subset) of T cells and that apparently can result in a devastating series of opportunistic infections that are frequently fatal (4–6). The AIDS retrovirus can be transmitted in blood products such as donated whole blood (7) and factor VIII concentrates (8), so that methods to determine previous exposure of potential donors to the retrovirus are essential. A M_r 24,000 protein, the p24 gag core protein of the AIDS retrovirus, has been found to induce antibody formation in a large percentage of individuals who have been previously exposed to the virus (refs. 9–13; unpublished results). This protein may thus be utilized as a diagnostic reagent to determine whether individuals have been exposed to the AIDS retrovirus. Although the protein may be obtained from virion particles, the problems associated with growth of the retrovirus and isolation of viral proteins suggest that a more readily obtainable source of the core protein would make a diagnostic reagent useful for detection of core protein antibodies safer and more practical. To this end, we have expressed p24 gag in bacterial cells utilizing the AIDS retroviral DNA sequence determined by Muesing *et al.* (14). A panel of 25 serum samples that had been characterized previously for their degree of reactivity with the disrupted virus was tested by using the bacterially produced p24 gag ELISA. All positively reacting serum samples were correctly detected by using the recombinant-derived ELISA with no false positives or false negatives.

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MATERIALS AND METHODS

NH_2-Terminal Sequence Determination of the p24 Core Protein. Purified virus was electrophoresed on a polyacrylamide gel (15) with 1 mM sodium thioglycolate in the upper reservoir buffer. The gel was stained with Coomassie blue and the p24 band was excised. The protein was recovered from the gel by electroelution (16). The purified protein was applied to a vapor-phase protein sequencer (model 470A; Applied Biosystems, Foster City, CA) as described (16). The amino acid derivatives released were determined by reverse-phase HPLC on a Microsorb C8 column.

Construction of Plasmids for the Bacterial Expression of the p24 Core Protein. The plasmid pH9c7 was a cDNA clone that contained a 2.2-kilobase insert derived from the 5′ region of the viral genome. DNA sequence analysis of this clone (14) revealed a region homologous to the NH_2-terminal p24 gag sequence (shown as * in Fig. 2). The entire p24 gene was reconstructed by ligating an incomplete p24 gene restriction fragment [beginning at the *Rsa* I site at nucleotide position 763 of Muesing *et al.* (14) and ending at a synthetic *Eco*RI site at position 2215] with a synthetic 39-mer DNA fragment, which corresponded to the first 10 amino acids of native p24 protein preceded by a *Bam*HI restriction site, into the *Bam*HI and *Eco*RI sites of plasmid pUC9. The resultant plasmid was digested with *Bam*HI, filled in with Klenow DNA polymerase I, digested with *Eco*RI, and ligated to the plasmid pFSL-2 (17). pFSL-2 is a bacterial expression plasmid that expresses inserted sequences under the control of a tryptophan operon promoter (18). The plasmid contains an initiator methionine to begin translation of downstream genes and a linker [frameshift linker 2 (17)] that allows for the correct reading frame. A truncated form of p24 gag was produced by digesting p24DE with *Hin*dIII, isolating the large fragment, and self-ligating.

Electrophoretic Transfer Blot Analysis of Bacterially Produced p24 Core Antigen. For the derepression of the truncated p24 (p24DEΔ*Hin*dIII), bacteria (*Escherichia coli* strain 294) containing the plasmid were grown in Luria broth containing 5 μg of tetracycline per ml to an A_{550} of 0.6 and diluted 1:20 into M-9 minimal medium containing 5 μg of tetracycline per ml and 25 μg of indoleacrylic acid per ml. Cells were grown for 2 hr. For production of the full-length precursor (p24DE), bacteria containing the plasmid were grown from a freshly streaked plate in Luria broth for 5 hr. Bacterial proteins were isolated according to the methods of Kleid *et al.* (19). Proteins were electrophoretically analyzed by using the $NaDodSO_4$/polyacrylamide system of Laemmli

Abbreviations: AIDS, acquired immunodeficiency syndrome; HTLV, human T-cell leukemia virus; ARC, AIDS-related complex; HHM, healthy homosexual male.

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(15). Proteins were analyzed by electrophoretic transfer blot analysis essentially as described (20).

For production of an immunoaffinity column, antiserum was obtained from a rabbit immunized by subcutaneous injection and booster injections with whole virus. The serum IgG fraction was purified by using protein A-Sepharose chromatography. To prepare an immunoaffinity column, 30 mg of purified antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described (21). Twenty grams of *E. coli* paste containing retroviral protein was suspended in 200 ml of 0.1 M Tris/0.2 M NaCl/50 mM EDTA, pH 7.5, and then sonicated for 20 min. Insoluble material was removed by centrifugation. Twenty milliliters of *E. coli* supernatant was applied to the immunoaffinity column. The column was washed and bound protein was eluted with 20 mM Tris/2 M KSCN, pH 7.5, and dialyzed against phosphate-buffered saline (P_i/NaCl).

Serum ELISAs Using the Bacterially Produced p24 Core Antigen. Immunoaffinity-purified recombinant p28 was bound to wells in 96-well Dynatech trays at ≈250 ng per well as described (22). Human serum samples were incubated in P_i/NaCl/bovine serum albumin buffer containing 0.65 mg of sonicated *E. coli* protein extracts per ml for 45 min to block background *E. coli* antibody reactions. The samples were then diluted onto the test wells and incubated for 1 hr at room temperature, after which the wells were washed by aspiration with P_i/NaCl containing 0.05% Tween 20. Antibody–enzyme conjugate (murine monoclonal anti-human IgG–horseradish peroxidase) was added at a 1:9000 dilution in P_i/NaCl/bovine serum albumin/0.05% Tween 20 and incubated for 1 hr and 45 min at room temperature. The wells were washed with P_i/NaCl containing 0.05% Tween 20. Color development was initiated by addition of 50 μl of 4.4 M *o*-phenylenediamine in pH 5.0 citrate/phosphate buffer containing 0.12% peroxide. The reaction was terminated by addition of 25 μl of 2.5 M H_2SO_4 and color development was measured by using a Dynatech plate reader at an absorbance of 495 nm (22).

RESULTS

Expression Results of the AIDS Retrovirus p24 gag Protein in *E. coli*. The DNA sequence determined for the AIDS retrovirus genome revealed an open reading frame in the 5′ region following the long terminal repeat (14). This region has been found to encode the gag precursor polypeptide in all other retroviruses examined. However, because the viral core protein is normally cleaved from the central region of a higher molecular weight gag precursor (14, 23), the location of the p24 core protein NH_2 terminus could not be determined from the nucleotide sequence data. Thus, the viral protein was isolated from $NaDodSO_4$-disrupted retrovirus virions by preparative gel electrophoresis and subjected to NH_2-terminal sequence analysis by using vapor-phase microsequencing (16). Fig. 1 shows the sequence, derived from both the protein and DNA sequence (14), for the first 25 amino acids of the AIDS retrovirus p24 gag protein. This region of the AIDS retrovirus gag sequence shows few amino acids in common with either the two other known human retrovirus core proteins (24, 25) or the core proteins from two other animal retroviruses (26, 27), although the homology to the bovine leukemia virus p24 NH_2 terminus (27) appears to be somewhat greater. This NH_2-terminal sequence enabled construction of an expression system for the specific production of the p24 gag core protein in bacteria.

Fig. 2 illustrates the plasmid constructions used for the production of the AIDS retrovirus p24 gag protein in the bacterium *E. coli*. Since most individuals have been exposed to bacterial antigens, particularly those found in the intestinal tract, the inclusion of *E. coli* sequences fused to the retroviral protein would likely lead to frequent false-positive antibody

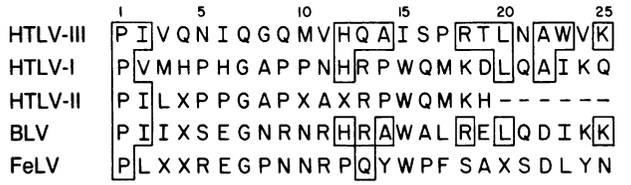

FIG. 1. NH_2-terminal sequence of the AIDS retrovirus (HTLV III) p24 protein. The NH_2 terminus of the p24 gag protein of the AIDS retrovirus (HTLV III) was determined by using gas-phase sequencing (16). The sequence is shown compared with two other human retroviruses, HTLV-I (24) and HTLV-II (25), and two animal retroviruses, bovine leukemia virus (BLV) (26) and feline leukemia virus (FeLV) (27). Boxed regions show amino acids in common between the HTLV-III sequence and the other retroviral core protein NH_2 termini. The single-letter amino acid code was used, with X referring to amino acids that were not determined.

reactivity. Thus, the p24 gag gene sequence was expressed in *E. coli* in the absence of fused bacterial protein sequences. The plasmids described in Fig. 2 contained either the p24 gag precursor gene (p24DE), which encodes an open reading frame that corresponds to a protein of M_r ≈44,000 in size and that consists of the p24 core protein plus a COOH-terminal polypeptide (14), or a truncated p24 protein reading frame (p24DEΔ*Hin*dIII) that contains only the first 178 amino acids of the viral core polypeptide. In both cases, the viral sequences are transcribed under the control of an inducible tryptophan operator and promoter (18).

Bacteria containing these plasmids were grown in minimal medium with indoleacrylic acid to derepress the synthesis of the p24 gag analogues (19). Though the bacteria containing the truncated construction grew at a relatively normal rate,

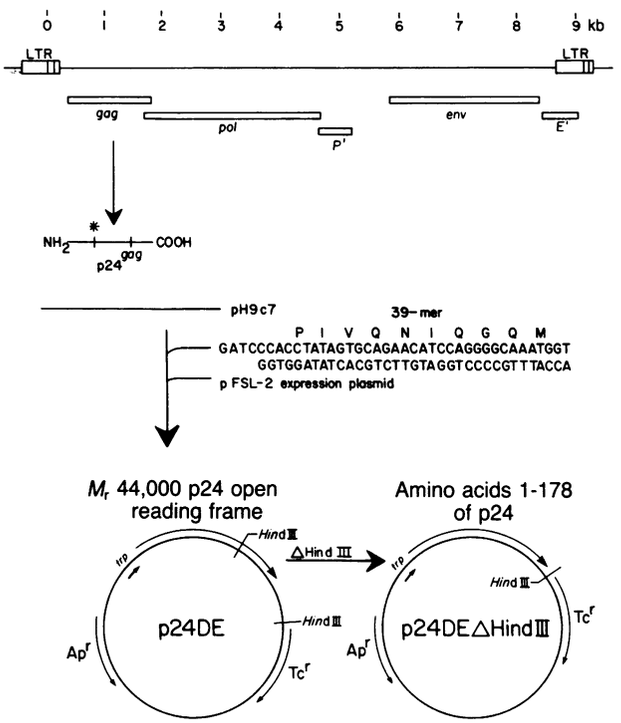

FIG. 2. Plasmids for bacterial expression of the AIDS retrovirus p24 gag antigen. The region marked with an asterisk was found to contain a sequence that encoded the p24 NH_2 terminus shown in Fig. 1. The plasmid p24DE directs the expression of the M_r 44,000 precursor protein containing the p24 gag antigen and a COOH-terminal polypeptide. The p24DEΔ*Hin*dIII plasmid is a truncated version of the p24DE plasmid that contains the first 178 amino acids of the p24 gag antigen. Tc^r, tetracycline resistant; Ap^r, ampicillin resistant; kb, kilobases; LTR, long terminal repeat.

those containing the full-length construction grew very slowly. Nonetheless, significant quantities of the protein expressed from the p24DE plasmid could be obtained from cells grown in standard L broth, probably due to transcriptional leakiness of the bacterial promoter (18). Fig. 3 shows $NaDodSO_4$ gel electrophoretic analysis of the viral proteins produced by these bacteria. Bacteria containing p24DEΔ-*Hin*dIII expressed significant quantities of a M_r ≈23,000 protein (Fig. 3, lane c), a size that is somewhat larger than that predicted from the amino acid sequence of this abbreviated gene, whereas bacteria that contained p24DE produced a major diffuse band at M_r ≈28,000 (Fig. 3, lane a).

Protein Blot Analysis of the Bacterially Synthesized p24 gag. To determine whether the proteins produced by *E. coli* bearing the viral gene constructions are recognized by antiserum directed against the native p24 gag protein, they were subjected to electrophoretic transfer protein blot analysis (20). Fig. 3, lane f, shows that the truncated form of the protein (p24DEΔ*Hin*dIII) reacts with rabbit antiserum directed against the native p24 gag protein. A major and a minor reacting species, possibly due to protein processing by the bacteria, can be seen. A more complex pattern was observed in the protein blot of the proteins produced in bacteria carrying p24DE (Fig. 3, lane d). This blot revealed a weak immunoreactive band at a M_r of 44,000 and two highly reactive, closely migrating bands at a M_r of ≈28,000 (referred to subsequently as recombinant p28). In addition, two less reactive bands are seen at M_r ≈18,000 and M_r ≈19,000. One interpretation of this result is that the bacteria proteolytically process the M_r 44,000 p24 precursor to give the smaller polypeptides. Although the processing events that produce the mature gag polypeptides have not yet been elucidated for the AIDS/lymphoadenopathy retrovirus, the gag precursors of other retroviruses are known to undergo proteolytic processing in the infected cell (28–31) and this type of processing may indeed be occurring in the bacteria. However, the major conclusion of the protein blot analysis is that the retrovirus-specific proteins produced in these bacteria react with antibodies directed against the native, viral p24 gag protein.

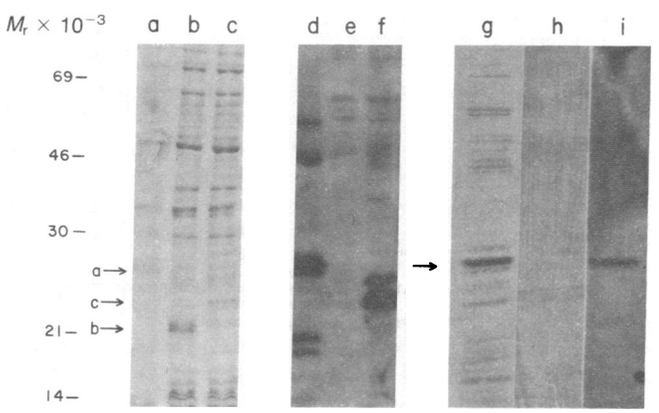

FIG. 3. Bacterial production of the AIDS retrovirus p24 protein and antibody reactivity on electrophoretic transfer blots. Lanes a–c, Coomassie-stained gels of proteins prepared from bacteria producing full-length p24 precursor analogue (recombinant p28) (lane a), human growth hormone (18) (lane b), and truncated p24 analogue (lane c). The a, b, and c arrows show the bacterial bands corresponding to the p28 protein, human growth hormone, and the truncated p24 protein, respectively. Lanes d–f, electrophoretic transfer blot analysis of gels in lanes a–c using rabbit antibodies produced against the AIDS retrovirus: full-length p24 precursor analogue (recombinant p28) (lane d), human growth hormone (lane e), and truncated p24 analogue (lane f). Lane g, Coomassie-stained gel of the immunoaffinity-purified p24 precursor analogue (recombinant p28). Lanes h and i, electrophoretic transfer blot analysis of proteins shown in lane g using normal human serum (lane h) and AIDS retrovirus-seropositive human serum (lane i). The arrow shows the major p28 doublet bands. Bacteria expressing the p24DE plasmid (lane a) grew very slowly in this experiment, resulting in a lower amount of protein loaded in this lane.

Although reaction of *E. coli* extracts containing p24 gag analogues with rabbit antiserum gave clear protein blot results, reactions with human antiserum gave high levels of binding to *E. coli* proteins. To avoid this problem, the processed form of recombinant p28 was partially purified from bacterial extracts by immunoaffinity chromatography using polyclonal rabbit antiserum. Fig. 3, lane g, shows that this procedure results in the purification predominately of the processed recombinant p28 doublet bands, which appeared to be ≈80% pure after a single pass over the immunoaffinity column. Electrophoretic transfer blot reactivity of the affinity-purified protein with human antiserum is shown in Fig. 3, lanes h and i. The protein blot shows no reactivity with normal human serum, whereas an obvious antibody-reactive band corresponding to the major recombinant p28 doublet bands in Fig. 3, lane g, is detected with serum from an AIDS patient, suggesting that the affinity-purified retroviral protein can be recognized by human antiserum directed against the AIDS retrovirus.

Use of the Bacterially Produced p24 gag Antigen in an ELISA. Although the electrophoretic transfer blot results with the immunoaffinity-purified recombinant p28 material demonstrated the usefulness of the partially purified protein in this type of diagnostic assay, a more convenient assay for the clinical diagnosis of AIDS retrovirus exposure would be preferable. To accomplish this, the partially purified recombinant p28 antigens were adsorbed onto plastic microtiter wells and utilized in a standard ELISA (21). Initial experiments demonstrated that, though the background levels of *E. coli* antibodies were considerably lower when using the partially purified recombinant p28 protein, they were still sufficiently high to decrease the sensitivity of the ELISA. Serum samples were thus preincubated with *E. coli* protein extracts before use in the recombinant p28 ELISA. Fig. 4 shows the results obtained by using this assay as compared with those obtained by using the whole-virus ELISA and electrophoretic transfer blot assay. The serum from control

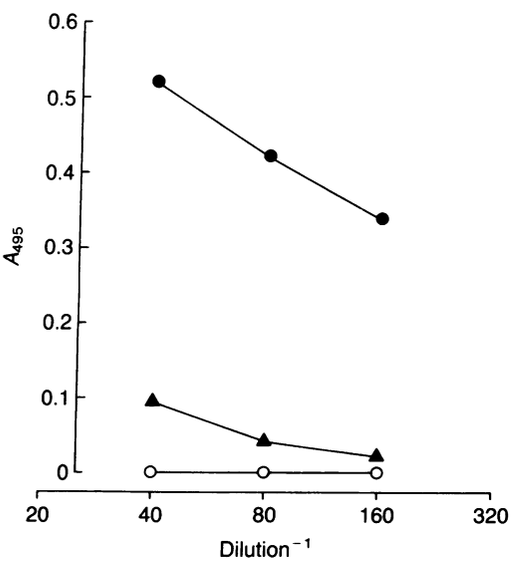

FIG. 4. ELISA for AIDS retrovirus p24 gag-specific antibodies using *E. coli*-derived p24 antigen analogues. The graph illustrates negative (patient 5, ○), weak positive (patient 13, ▲), and strong positive (patient 6, ●) titers. Standard ELISA techniques were used to detect AIDS retrovirus p24 gag antibodies (22).

patient 5 gave absolutely no reaction, indicating that background binding was eliminated. Patient 6, who had a high anti-AIDS retrovirus antibody titer in both whole-virus ELISA and whole-virus electrophoretic transfer blot assay, also showed a highly significant ELISA signal in the recombinant p28 assay. A more stringent test, however, was performed with patient 13, whose anti-p24 gag antibody level was low in the whole-virus electrophoretic transfer blot assay. Fig. 4 demonstrates that a positive signal was obtained by using the recombinant p28 ELISA, suggesting that the bacterially produced recombinant p28 ELISA was sensitive enough to detect low levels of anti-AIDS retrovirus p24 gag antibodies. These initial experiments suggested that the bacterially produced p28 protein could be used in an ELISA for the detection of antibodies directed against the retroviral p24 gag protein.

To more fully evaluate the accuracy of the recombinant p28 ELISA, a coded panel of 25 serum samples was tested. As shown in Table 1, the results obtained for the recombinant p28 ELISA correlated with the results obtained by using the whole-virus ELISA. For example, sera 1 and 4 were obtained from HHMs who were found to be positive for exposure to the AIDS retrovirus by using the recombinant p28 core antigen assay as well as the whole-virus ELISA and electrophoretic transfer blot assay. In the case of serum 4, the patient was a blood donor who transmitted AIDS to a recipient of his packed erythrocytes. Assuming that this patient was infected with the AIDS retrovirus at some time prior to his blood donation, testing of his serum with the recombinant p28 assay would have detected anti-p24 antibody and prevented blood donation and transmission of the disease. Additionally, several low antibody titer serum samples, such as sera from patients 11, 14, 15, 17, and 19, were detected by using the recombinant assay, although samples 15 and 17, both from AIDS patients, were only slightly above background. In the case of both the ARC and AIDS patients, the recombinant p28 ELISA was able to detect antibody responses in all cases, with the exception of patients 18, 21, and 22. Patient 18 was a case of advanced AIDS, a situation in which the donation of blood or blood-derived products would be highly unlikely and in which antibodies often decrease due to immune system dysfunction. Patients 21 and 22 were unusual patients in that they were positive for the AIDS retrovirus by virus isolation but were negative for antibody by using all current whole-virus assays. The recombinant p28 assay did not detect antibody in this serum, suggesting that assays based on antigen detection will have to be developed to determine the presence of AIDS retrovirus in such rare patients. Additionally, none of the negative control patient serum showed reactivity in the recombinant p28 assay. The negative controls included serum that may have large amounts of nonspecific antibodies, such as patient 23, a melanoma patient, and patient 24, a patient with systemic lupus erythematosus.

DISCUSSION

Our results demonstrate that a bacterially synthesized AIDS retrovirus p24 gag antigen analogue can be used in an ELISA to detect an immune response and, thus, previous exposure

Table 1. AIDS retrovirus antibody detection in patients

Patient code	Recombinant p28 ELISA*†	Electrophoretic transfer blot‡	Whole-virus ELISA§†	Patient diagnosis
1	+ (0.317)	+ (19, 24, 41, 65)	+	HHM
2	− (0.004)	−	−	HHM
3	− (0.005)	−	−	HHM
4	+ (0.392)	+ (19, 24, 41, 65)	+	HHM
5	− (0.004)	−	−	HHM
6	+ (0.527)	+ (13, 19, 24, 41, 65)	+	ARC
7	+ (0.647)	+ (19, 24, 41, 65)	+	ARC
8	+ (0.615)	+ (19, 24, 41, 65)	+	ARC
9	+ (0.585)	+ (19, 24, 41, 65)	+	ARC
10	+ (0.602)	+ (19, 24, 41, 65)	+	ARC
11	+ (0.067)	+ (weak; 19, 24, 41, 65)	+	ARC
12	+ (0.180)	+ (19, 24, 41, 65)	+	ARC
13	+ (0.096)	+ (19, 24, 41, 65)	+	AIDS
14	+ (0.054)	+ (weak; 19, 24, 41, 65)	+	AIDS
15	+/− (0.022)	+ (weak; 24, 41, 65)	+	AIDS
16	+ (0.152)	+ (19, 24, 41, 65)	+	AIDS
17	+ (0.031)	+ (weak; 19, 24, 41, 65)	+	AIDS
18	− (0.009)	+ (weak; 24, 41, 65)	−	Advanced AIDS
19	+/− (0.025)	+ (weak; 24, 41, 65)	−	Advanced AIDS
20	+ (0.048)	+ (weak; 41)	+	AIDS hemophiliac
21	− (0.017)	−	−	AIDS virus positive
22	− (0.018)	−	−	AIDS virus positive Antibody negative
23	− (0.016)	−	−	Melanoma
24	− (0.003)	−	−	Lupus
25	− (0.000)	−	−	Ewing sarcoma

HHM, healthy homosexual male; ARC, AIDS-related complex.

*ELISA performed as described in the legend to Fig. 4 using bacterially derived recombinant p28 as antigen. Numbers in parentheses represent the A_{495} readings at a serum dilution of 1:40. An arbitrary A_{495} value of 0.020 was chosen for the cut-off point for a positively reacting sample.

†+ = positive A_{495} ELISA color development; − = negative A_{495} ELISA color development.

‡Electrophoretic transfer blot analysis using isolated disrupted AIDS retrovirus. Numbers in parentheses denote the AIDS retroviral antibody-reactive protein molecular weights (given as $M_r \times 10^{-3}$).

§Whole-virus ELISA using isolated AIDS retrovirus configured in the Travenol–Genentech HTLV-III diagnostic kit.

to the AIDS retrovirus. To our knowledge, use of a bacterially produced antigen configured in a clinically relevant system (ELISA) for the diagnosis of an infectious disease has not been reported previously. One issue that still remains, however, is whether the core protein alone is sufficient for the diagnosis of all AIDS retrovirus exposures or whether the viral envelope protein will be needed for definitive identification of all AIDS virus infections. Although extensive seroepidemiological studies have not, as yet, been published, whole-virus electrophoretic transfer blot analysis of 252 patient serum samples, including those from AIDS and ARC patients as well as from sexually active, HTLV-III seropositive homosexual men, has demonstrated that serum from ≈98% of these individuals reacts with the AIDS retrovirus p24 core protein (unpublished results). Thus, the recombinant p28 assay would appear to be useful for the detection of AIDS antibodies in a high percentage of the positively reacting AIDS, ARC, and seropositive HHM serum. The addition of the envelope antigen to this assay may allow a more exact test for any potential carrier of the AIDS retrovirus. However, due to heterogeneity in the sequence of the envelope gene from various AIDS retrovirus isolates (14, 32–34), it is possible that a diagnostic assay containing the envelope gene product from a single AIDS retrovirus isolate may not detect all of the samples that are positive for retroviral envelope protein antibodies. The degree of heterogeneity found thus far in the core antigen is far less than that found for envelope (14, 32–34), suggesting that the p24 gag protein may be more useful to detect antibodies from individuals infected with different strains of the virus. The resolution of this issue must await further testing with different combinations of antigens.

Another important, and highly controversial, issue is whether the anti-p24 antibody levels detected by using any AIDS retrovirus diagnostic reagent actually mean that the patient has an active viral infection. Though tests to detect the presence of the retrovirus itself are possibly more useful in this context, they are usually cumbersome and expensive. The resolution of this issue will undoubtedly be attained only from a careful epidemiological study that relates the ability to culture active virus with the levels of antibody directed against the various viral structural components.

In conclusion, a bacterially produced AIDS retrovirus p24 gag protein analogue can be efficiently utilized in a diagnostic ELISA for the detection of viral core protein antibodies and, thus, prior infection with the AIDS/lymphoadenopathy retrovirus. The protein can be produced in large quantities and, unlike the production of the protein from virions, in a completely safe manner. In addition, the small-scale efficacy study shown here demonstrates that the protein gives results that are comparable to a prototype commercial ELISA produced using the whole AIDS retrovirus as an antigen source.

This paper is dedicated to the memory of Frances J. Stusser. We thank Becky Cazares for preparation of the manuscript and Drs. Dennis Kleid and Jack Obijeski for their careful reading of the manuscript. The whole-virus ELISAs in Table 1 were kindly performed by Francis Boches using a prototype of the Travenol–Genentech AIDS retrovirus diagnostic kit.

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