JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1986, p. 424–430
0095-1137/86/090424-07$02.00/0

Vol. 24, No. 3

Detection and Differentiation by Sandwich Enzyme-Linked Immunosorbent Assay of Human T-Cell Lymphotropic Virus Type III/Lymphadenopathy-Associated Virus- and Acquired Immunodeficiency Syndrome-Associated Retroviruslike Clinical Isolates

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Received 12 December 1985/Accepted 23 May 1986

Monoclonal antibodies can be used in sandwich enzyme-linked immunosorbent assays to measure viral antigens. Such an assay was developed to detect the core protein, p24, of human T-cell lymphotropic virus type III and lymphadenopathy-associated virus, etiologic agents of the acquired immunodeficiency syndrome (AIDS). Another AIDS-associated virus, AIDS-associated retrovirus type 2 (ARV-2) could not be detected in this assay because of the low affinity of one of the monoclonal antibodies to ARV-2 p24. Detection of ARV-2 was accomplished with a monoclonal antibody-rabbit polyclonal antibody sandwich enzyme-linked immunosorbent assay. These two assays were used to efficiently detect AIDS-related viruses in lymphocyte cell cultures and to distinguish strains of the viruses.

An immunoassay for the detection of lymphadenopathy-associated virus (LAV) by using immunoglobulin from seropositive humans was described (12). This assay was used to monitor virus production in cultures of lymphocytes experimentally infected with LAV and was found to be as sensitive for detecting the virus as the more cumbersome and costly reverse transcriptase assay. We also developed enzyme-linked immunosorbent assays (ELISAs) that use monoclonal antibodies to detect the major core antigen of human T-cell lymphotropic virus type III (HTLV-III), LAV, and acquired immunodeficiency syndrome (AIDS)-associated retrovirus type 2 (ARV-2). All these isolates have been associated with AIDS. Researchers working with HTLV-III refer to this protein as p24, while those working with LAV and ARV-2 refer to it as p25. As the monoclonal antibodies were prepared by using HTLV-III, the former designation will be used in this paper. The ELISAs use technology that we previously applied to the detection of the major core protein, p27, of feline leukemia virus (FeLV) (11). The FeLV p27 detection system is an antibody sandwich ELISA that uses a battery of monoclonal antibodies, each recognizing a different epitope of the core protein. One of these monoclonal antibodies was used to catch the antigen and fix it to the well, and two other noncompeting monoclonal antibodies conjugated to horseradish peroxidase were used as second antibodies. This assay detected as little as 50 ng of FeLV p27 per ml of body fluids or infected tissue culture supernatant.

We describe in this paper two antigen-capture sandwich ELISAs that detect human AIDS virus core proteins in cell culture supernatants. By using monoclonal antibodies to different epitopes of p24, we were able to differentiate field strains of viruses having an HTLV-III/LAV-like p24 epitope from field strains having a p24 epitope characteristic of ARV-2.

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MATERIALS AND METHODS

Virus purification. The H9 cell line infected with HTLV-III was provided by R. C. Gallo (4). The isolation and characterization of HTLV-III was previously described (14). LAV was provided by F. Barré-Sinoussi (7) and was grown in HUT-78 cells (6). ARV-2 grown in HUT-78 cells was provided by J. Levy (9).

Cell culture fluid was clarified by low-speed centrifugation (2,000 × *g* for 1 h) and then by filtration in a Pellicon cassette system (Millipore Corp., Bedford, Mass.) with a 1,000,000-dalton exclusion membrane. The retained filtrate was then concentrated in the Pellicon apparatus with a 100,000-dalton exclusion membrane, and the virus was pelleted in a rotor (type 21; Beckman Instruments, Inc., Palo Alto, Calif.) at 45,000 × *g* for 2 h. The pelleted virus was suspended in 0.01 M Tris–0.1 M NaCl (pH 7.2) and loaded onto 15 to 50% sucrose gradients. After centrifugation in a Beckman SW41 rotor at 200,000 × *g* for 3 h, fractions were collected by tube puncture. The fractions containing peak Mg^{2+}-dependent reverse transcriptase (3) activity were diluted with the Tris sodium chloride buffer, and the virus was pelleted by ultracentrifugation. The virus pellets were resuspended in the buffer, and the protein concentration was determined by the procedure of Bradford (2).

Production of monoclonal antibodies. Eight-week-old BALB/c mice were immunized with 50 μg of gradient-purified HTLV-III, disrupted in 0.05% sodium dodecyl sulfate (SDS), and injected intraperitoneally in complete Freund adjuvant. Booster injections were given every 3 to 8 weeks for several months. Seventy-two hours after the final immunizations, mouse spleen cells were isolated and fused with mouse myeloma cells. The fusion, screening, cloning,

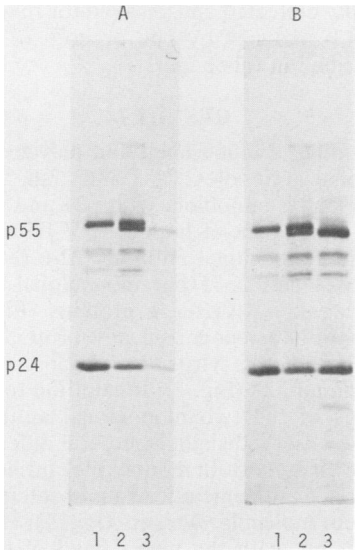

FIG. 1. Reactivity of anti-HTLV-III monoclonal antibodies with HTLV-III (lane 1), LAV (lane 2), and ARV-2 (lane 3). The viral proteins were electrophoresed on 8% Laemmli gels, transblotted to nitrocellulose paper, and reacted with monoclonal antibody 22-3 (A) and monoclonal antibody 22-6 (B) at dilutions of 1/400.

and ascites production procedures were identical to those described by Lutz and co-workers (11). The monoclonal antibodies were shown to react with p24 (see Results).

Production of polyclonal antibodies. Rabbits were immunized with 0.1 mg of 0.05% SDS-disrupted HTLV-III injected intramuscularly every 3 to 4 weeks until high-titered serum was obtained. The first dose was given in Freund complete adjuvant; subsequent doses were given in incomplete Freund adjuvant.

Preparation of immunoglobulins. Immunoglobulins from mouse ascites fluid or rabbit serum were purified by ammonium sulfate precipitation (5). Aliquots of these immunoglobulin preparations were conjugated to horseradish peroxidase by the method of Wilson and Nakane (17).

Sandwich ELISA. Microelisa plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 1 μg of immunoglobulin in 0.1 M $NaHCO_3$ buffer (pH 9.6) for 3 h at 37°C and stored at 4°C. Just before use the plates were rinsed three times with wash buffer (0.15 M NaCl, 0.05% Tween 20). Standard amounts of HTLV-III, LAV, or ARV-2 diluted in ELISA dilution buffer (0.15 NaCl, 1 mM EDTA, 0.05 M Tris hydrochloride [pH 7.4], 0.1% Tween 20, 0.1% bovine serum albumin) were added to wells in duplicate. The standards ranged from 0 to 200 ng of total viral protein, which corresponded to a range of 0 to 80 ng of p24, based on a comparison of our virus stock with immunoaffinity-purified p24. In tests of cell culture supernatants, 50 μl of supernatant was mixed in the wells with 50 μl of ELISA dilution buffer containing 0.2% Tween 20. The plates were allowed to incubate for 1 h at 37°C and were washed three times with wash buffer. Horseradish peroxidase-conjugated immunoglobulin diluted in ELISA dilution buffer was added to the appropriate wells, and the plates were incubated for 1 h at 37°C. After washing, a substrate solution of 2,2′-azino-bis(3-ethylbenzthiazolinesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) and 0.01% H_2O_2 in 0.05 M citric acid (pH 4.0) was added to each well. The enzymatic reaction was stopped

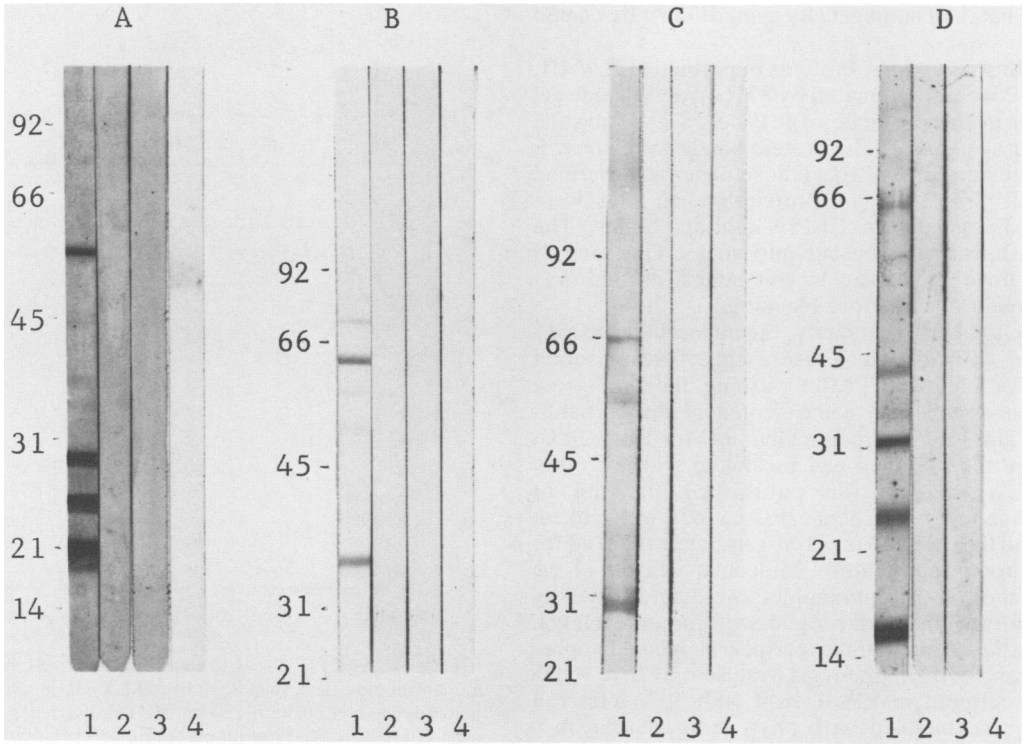

FIG. 2. Cross-reactivity of monoclonal and polyclonal antibodies with other retroviruses. Immunoblots of HTLV-I (A), FeLV (B), bovine leukemia virus (C), and simian AIDS retrovirus type 1 (D) proteins were prepared as described in the legend to Fig. 1. Strips from these blots were incubated with a positive control for each individual virus (lane 1), monoclonal antibody 22-3 (lane 2), monoclonal antibody 22-6 (lane 3), and rabbit polyclonal antibody (lane 4).

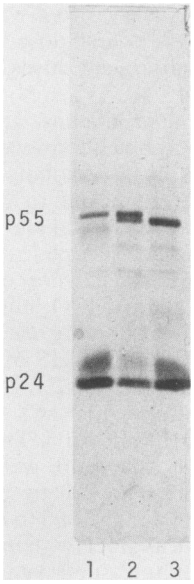

FIG. 3. Reactivity of rabbit anti-HTLV-III polyclonal serum with HTLV-III (lane 1), LAV (lane 2), and ARV-2 (lane 3). The immunoblots were prepared as described in the legend to Fig. 1. The rabbit serum was diluted 1/50.

with 0.1 M HF, and the plates were read on a Dynatech ELISA reader. All volumes were 100 μl per well.

Standard amounts of disrupted whole virus or purified p24 were run on each plate, so that the amount of reacting antigen in unknown samples could be determined. The optimal dilution of conjugated immunoglobulin was determined for each batch of conjugate by using HTLV-III-coated ELISA plates.

Immunoblotting procedure. Proteins of purified HTLV-III, LAV, or ARV-2 were fractionated by 8% polyacrylamide gel electrophoresis in the presence of 0.1% SDS by using the Laemmli buffer system (8). The protein bands were electrophoretically transferred to nitrocellulose paper as described by Tsang et al. (16). Unbound protein-binding sites were blocked with 3% gelatin in ELISA dilution buffer. The nitrocellulose sheet was then cut into strips. One lane of each gel contained molecular weight standards and was stained with amido black before blocking.

To determine antibody specificity, the nitrocellulose strips were incubated with diluted mouse ascites fluid or rabbit serum (1:100) for 2 h at 24°C. After washing, the strips were reacted with horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin G for 1 h at 24°C. The strips were then washed and incubated with a freshly prepared substrate solution containing 0.5 mg of diaminobenzidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml and 0.01% H_2O_2 in 0.1 M Tris buffer (pH 7.4) for 5 to 10 min at room temperature. Molecular weights of the viral antigen bands on the immunoblots were determined by comparison with the standard protein strip run on each gel.

Virus cultivation from human peripheral blood lymphocytes. Peripheral blood lymphocytes from seropositive AIDS patients were cultured in RPMI 1640 with 10% fetal calf serum and were stimulated with phytohemagglutinin in a manner similar to that used by Barré-Sinoussi et al. (1) and Levy and Shimabukuro (10). Uninfected peripheral blood lymphocytes from a healthy blood donor were added to the cultures every 10 to 14 days. Supernatant from the cultures was periodically collected and assayed for reverse transcriptase activity (3) and p24 by one or both of the sandwich ELISAs described in this paper.

RESULTS

Characterization of monoclonal and polyclonal antibodies. Two monoclonal antibodies, 22-3 and 22-6, were found to react strongly on immunoblots with p24 and the core polyprotein precursor, pp55, of both HTLV-III and LAV (Fig. 1). In contrast, monoclonal antibody 22-6 reacted strongly with ARV-2 p24 and pp55 but monoclonal antibody 22-3 reacted only weakly with these proteins (Fig. 1). Neither monoclonal antibody recognized any proteins of HTLV-I, FeLV, bovine leukemia virus, or simian AIDS retrovirus type 1 on immunoblots (Fig. 2). In addition to differences in the reactivities of the two monoclonal antibodies on immunoblots, we observed slight structural differences in pp55 from the three viruses. Both monoclonal antibodies resolved LAV pp55 as two antigenically related proteins (doublet) of slightly different molecular weights (Fig. 1). HTLV-III pp55 comigrated mainly with the upper band of the LAV pp55 doublet, whereas ARV-2 pp55 comigrated mainly with the lower band (Fig. 1).

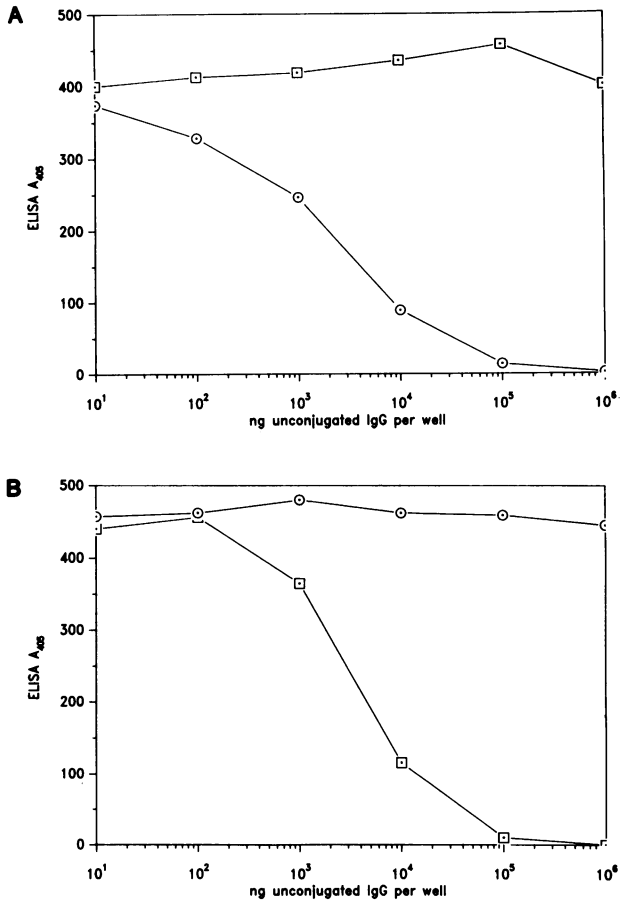

FIG. 4. Assay of the degree of competitive binding of monoclonal antibodies 22-3 and 22-6 to HTLV-III in an ELISA. ELISA plates were coated with 250 ng of HTLV-III per well. (A) Peroxidase-conjugated 22-3 immunoglobulin was preincubated with unconjugated 22-3 immunoglobulin (⊙) and unconjugated 22-6 immunoglobulin (⊡). (B) Peroxidase-conjugated 22-6 immunoglobulin was preincubated with unconjugated 22-3 immunoglobulin (⊙) and unconjugated 22-6 immunoglobulin (⊡).

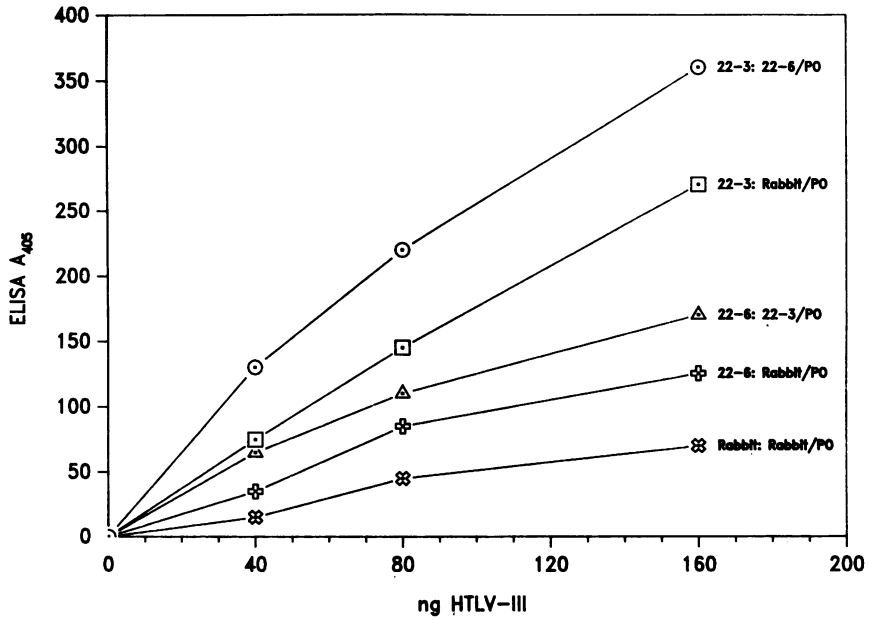

FIG. 5. Determination of the best combinations of antibodies to use in antigen detection assays. Monoclonal or polyclonal immunoglobulin was coated onto ELISA wells (1 μg per well). HTLV-III in the range of 0 to 160 ng per well was allowed to react with the antibodies in the wells and then incubated with peroxidase-conjugated (PO) monoclonal or polyclonal immunoglobulin G.

Hyperimmune rabbit anti-HTLV-III serum reacted strongly with several proteins of HTLV-III, LAV, and ARV-2, but the strongest reactions were against p24 and pp55 (Fig. 3). No reactivity was seen in immunoblots against HTLV-I, FeLV, bovine leukemia virus, or simian AIDS retrovirus type 1 (Fig. 2). Polyclonal rabbit antiserum also recognized the same characteristic differences in the pp55 of these viruses that were seen with the monoclonal antibodies (Fig. 3).

Competitive binding of monoclonal antibodies to HTLV-III p24. ELISA plates were coated with 250 ng of HTLV-III per well and washed. A constant amount of conjugated 22-3 or 22-6 immunoglobulin was mixed in the wells with various amounts of unconjugated 22-3 or 22-6 immunoglobulin. After 1 h of incubation at 37°C and a wash step, azino-bis(3-ethylbenzthiazolinesulfonic acid substrate solution was added. Increasing amounts of unconjugated 22-3 immunoglobulin progressively inhibited the binding of conjugated 22-3 immunoglobulin, whereas there was no inhibition up to 1 mg per well with increasing amounts of unconjugated 22-6 immunoglobulin (Fig. 4A). Increasing amounts of unconjugated 22-3 immunoglobulin did not inhibit the binding of conjugated 22-6 immunoglobulin, whereas increasing amounts of unconjugated 22-6 immunoglobulin were inhibi-

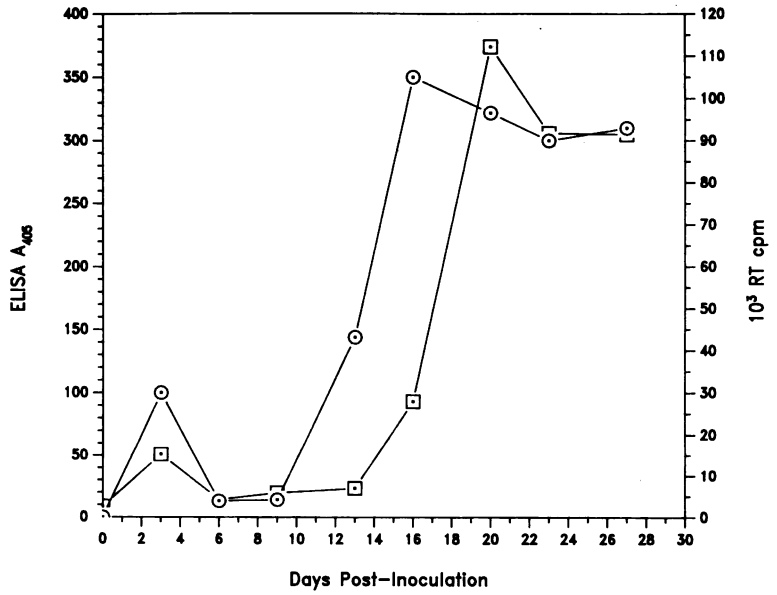

FIG. 6. Correlation of p24 ELISA with reverse transcriptase assay (RT). Uninfected HUT-78 cells were inoculated with HTLV-III-infected cell culture fluid at day 0 and kept in culture for 27 days. Equal volumes of cell culture fluid were tested for RT activity (⊡) and for p24 by the monoclonal-monoclonal ELISA (⊙).

tory (Fig. 4B). These competitive binding studies indicated that monoclonal antibodies 22-3 and 22-6 were directed against different epitopes of HTLV-III p24 and were suitable for use in a sandwich ELISA.

Selection of antibodies for use in antigen detection assays. To determine which combinations of immunoglobulins would work best in antibody sandwich ELISAs, combinations of anti-HTLV-III monoclonal and polyclonal immunoglobulins were used as primary and secondary antibodies. The conditions of the ELISAs were as described in Materials and Methods. For comparative purposes, all assays were run simultaneously. The reactions were linear in all assays in the range of 0 to 160 ng of whole HTLV-III protein per well (Fig. 5). 22-3 immunoglobulin appeared to be the best catching antibody, followed by 22-6 immunoglobulin and polyclonal rabbit immunoglobulin (Fig. 5). The combination of 22-3 immunoglobulin for catching and 22-6 immunoglobulin for the conjugated second antibody (designated the monoclonal-monoclonal assay) was the most sensitive for HTLV-III p24 detection. Although it was not as sensitive, the combination of 22-6 immunoglobulin as the catching antibody and polyclonal rabbit immunoglobulin as the second antibody (monoclonal-polyclonal assay) was also useful, as will be shown.

Correlation of p24 ELISA with reverse transcriptase assay. Uninfected HUT-78 cells were incubated in medium containing 20% cell-free culture fluid from HTLV-III-infected HUT-78 cells. Fresh medium was provided every 3 or 4 days, and equal portions of the spent medium were collected and frozen at −70°C. The portions were assayed at the same time for reverse transcriptase and by the p24 monoclonal-monoclonal assay (Fig. 6). Measurable p24 at day 3 was undoubtedly residual virus that remained from the original inoculum; the level of p24 rose after day 13 and peaked at day 16 (Fig. 6). Reverse transcriptase activity began rising at day 16 and peaked at day 20 (Fig. 6). To confirm that p24 and reverse transcriptase activity were associated with virions, cells harvested at days 14, 17, and 20 were probed for HTLV-III RNA by in situ hybridization and for retrovirus by electron microscopy. No viral RNA was detected at day 14, but both viral RNA and budding viruses were seen in cells on days 17 and 20.

Detection of p24 in LAV and ARV-2. Purified HLTV-III and LAV reacted equally well down to 25 ng per well in the monoclonal-monoclonal ELISA (Fig. 7A). Only a slight reaction was seen, however, with virus purified from ARV-2-infected cultures (Fig. 7A). Because monoclonal antibody 22-3 reacted so weakly with ARV-2 (Fig. 1), this result was not unexpected. At least seven times as much ARV-2 was needed to produce an optical density similar to that produced with HTLV-III and LAV. However, the monoclonal-polyclonal assay using 22-6 immunoglobulin as the catching antibody in combination with polyclonal rabbit immunoglobulin as the conjugate detected all three viruses with equal sensitivity (Figure 7B).

Detection of p24 in lymphocyte cultures. Lymphocytes from 11 HTLV-III-seropositive human subjects were cultured in the prescribed manner for up to 41 days, and supernatants were periodically assayed for reverse transcriptase and p24. Because of the apparent inability of the monoclonal-monoclonal assay to detect ARV-2 p24, culture supernatants which were reverse transcriptase positive but antigen negative by the monoclonal-monoclonal assay were also assayed with the monoclonal-polyclonal ELISA.

Reverse transcriptase activity appeared in all the culture supernatants from 7 to 24 days after culture initiation (Table 1). P24, detectable by the monoclonal-monoclonal assay, appeared simultaneously with reverse transcriptase in 8 of 11 cultures (Table 1). In cultures 9, 10, and 11, however, antigen was detected by the monoclonal-polyclonal assay but not by the monoclonal-monoclonal assay. It would seem, therefore, that the viruses in these three cultures possessed p24 and pp55 that were more closely related to ARV-2 than to HTLV-III or LAV.

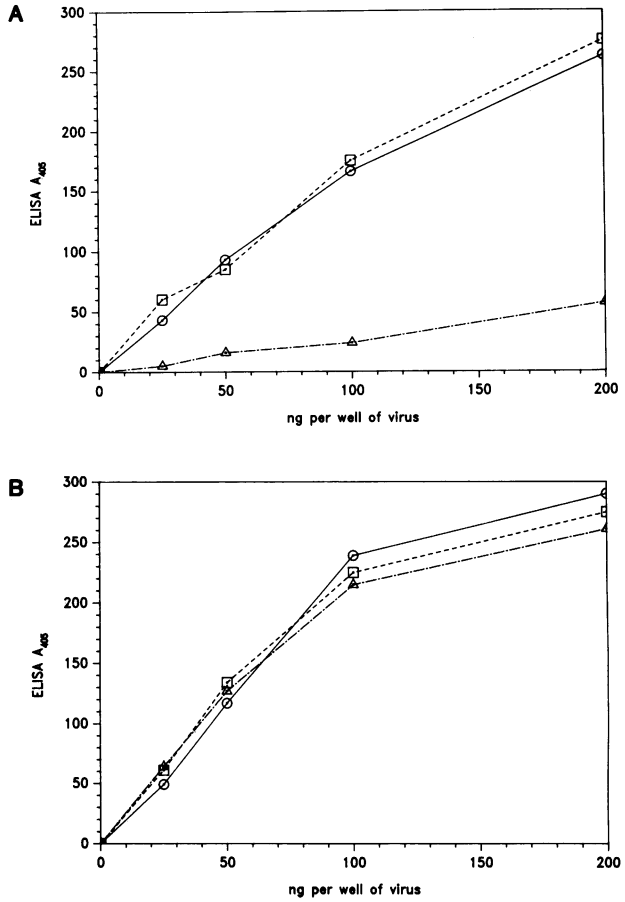

FIG. 7. Comparison of reactivity of HTLV-III, LAV, and ARV-2 by using two sandwich ELISAs. HTLV-III (⊙), LAV (⊡), and ARV-2 (△) were tested in the monoclonal-monoclonal ELISA (A) and the monoclonal-polyclonal ELISA (B).

The results of sandwich ELISAs on supernatants from culture 10 are shown in Fig. 8. Equal portions of cell culture fluid were collected from culture 10, frozen at −70°C, and tested by ELISA at the end of the experiment. One set of culture fluid samples was incubated on plates coated with 22-3 immunoglobulin, and another set was incubated with 22-6-immunoglobulin-coated plates. The conjugate used in both assays was peroxidase-labeled polyclonal rabbit immunoglobulin. Large amounts of p24 were detected on days 31, 34, 38, and 41 with 22-6 immunoglobulin as the catching antibody. No activity was seen when monoclonal antibody 22-3 was used as the catcher, except for a very small amount on day 38. Reactivity with standardized amounts of HTLV-III was the same in both assays, whereas purified ARV-2 from HUT-78 cells reacted strongly in the assay with 22-6 immunoglobulin and very weakly in the assay with 22-3 immunoglobulin (data not shown).

TABLE 1. Correlation between reverse transcriptase and p24 found in supernatants of human peripheral blood lymphocyte cultures[a]

Culture no.	Reverse transcriptase/p24 detected in culture at day:								
	7	13	17	20	24	27	31	34	41
1	+/+	+/+	+/+		+/+				
2			+/−		+/+	+/+			
3	+/+	+/+	+/+						
4	+/NT	+/NT					+/+	+/+	−/−
5		NT/+		+/+	+/+	+/+	+/+	NT/+	−/−
6		NT/+	NT/+		NT/+	+/+	−/+	+/+	+/+
7		+/+	NT/+	+/+	+/+	−/+	−/+		
8	−/−	+/+	+/+		−/+				
9		+/NT	+/NT	+/NT	+/−(+)	+/NT		+/−(+)	−/−(−)
10		−/NT	−/NT	−/NT	+/−(+)	+/−	NT/−(+)	+/−(+)	+/−(+)
11		−/−(−)	+/−(+)	+/−	+/−(+)	+/+(+)		+/+(+)	+/−(+)

[a] Cultures were from seropositive humans belonging to several high-risk groups. Antigen was measured by the monoclonal-monoclonal assay. Antigen measured by the monoclonal-polyclonal assay is given in parentheses. NT, Not tested.

DISCUSSION

We developed two antibody sandwich ELISAs for the detection of the major core protein, p24, of HTLV-III, LAV, and ARV-2. The first is a highly specific and sensitive assay using two monoclonal antibodies, each directed to a different epitope of p24. One monoclonal antibody is anchored to ELISA wells as a catching antibody, and the second is bound to horseradish peroxidase and used to detect any bound p24. The other assay uses a monoclonal antibody for catching and a rabbit polyclonal immunoglobulin for the labeled second antibody. The ability of these assays to detect virus in lymphocyte cultures from infected humans is comparable to that described by McDougal and co-workers (12) for a similar assay using polyclonal human antibodies.

The most important finding of this study is the striking difference in the abilities of the two assays to detect the p24 of ARV-2. The monoclonal-monoclonal ELISA was very insensitive in detecting ARV-2 p24 compared with its sensitivity to HTLV-III or LAV p24. This lack of sensitivity was apparently due to the poor binding of monoclonal antibody 22-3 to its epitope on ARV-2. Monoclonal antibody 22-6, however, reacted equally well with p24 from ARV-2, LAV, or HTLV-III. By using monoclonal antibody 22-6 as a catcher and polyclonal rabbit anti-HTLV-III immunoglobulin as the labeled second antibody, we were able to detect ARV-2 p24 with comparable sensitivity. By using both assays simultaneously, we were able to differentiate ARV-2-like from HTLV-III/LAV-like isolates.

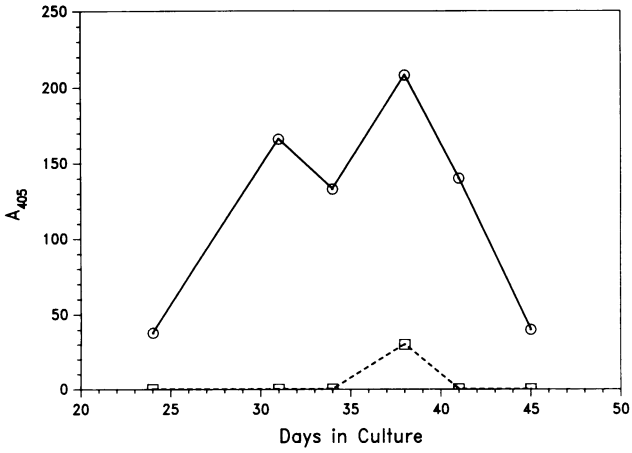

FIG. 8. Reactivity of clinical isolate supernatants in a sandwich ELISA with monoclonal antibody 22-3 (⊡) or 22-6 (⊙) as the coating antibody. Polyclonal rabbit immunoglobulin was used as the conjugate in both assays.

The reason monoclonal antibody 22-3 failed to react well with ARV-2 p24 is not known. The epitope to which monoclonal antibody 22-3 bound may be present in a slightly altered form on ARV-2 p24. This alteration would decrease the efficiency or avidity of binding of the antibody. This lowered efficiency of binding was seen on both immunoblots and in the ELISA. Comparing the sequences of HTLV-III (13) and ARV-2 (15) p24, we found only two minor differences. The first occurs at amino acid 144, where there is leucine in ARV-2 and isoleucine in HTLV-III. The second occurs at amino acid 318, where aspartic acid in ARV-2 is replaced by glutamic acid in HTLV-III. Because monoclonal antibodies can be directed to epitopes as small as 6 to 9 amino acids, it is possible that such changes altered the 22-3 epitope in ARV-2 enough to decrease the efficiency of antibody binding. There are also a number of amino acid differences in sequences downstream of p24. These differences could have affected the way that pp55 was cleaved to p24 or the tertiary structure of the p24 cleavage product. Indeed, we observed differences in pp55 from HTLV-III, LAV, and ARV-2. The LAV pp55 formed a distinct doublet, the ARV-2 pp55 comigrated with the lower protein band of the LAV pp55 doublet, and the HTLV-III pp55 comigrated with the upper protein band.

The fact that antigen-detecting ELISAs using monoclonal antibodies can recognize very small differences in a protein has obvious diagnostic implications. First, care must be taken to use assays with combinations of monoclonal and polyclonal antibodies that do not overlook positive specimens because of small changes in the antigen detected by the assay. Second, variable epitopes might be used as markers for certain field isolates. It is noteworthy that 3 of 11 of our local isolates reacted in a manner similar to that of ARV-2, which was originally isolated in the nearby San Francisco area. If different AIDS retrovirus isolates have subtle but detectable differences in their core proteins, it should be possible to use these differences as virus markers. Such markers would be valuable in tracing the spread of substrains of the AIDS virus from one individual or group to another within and across defined geographic areas.

ACKNOWLEDGMENTS

We thank Charlene Bush and Margaret Rodriquez for performing the in situ hybridization and Robert Munn for electron microscopy. The valuable technical assistance of Christa Fraser and Elise Keddie

in preparing monoclonal antibodies and viral antigens is greatly appreciated.

This research was supported in part by grants from the California Universitywide Task Force on Acquired Immune Deficiency Syndrome.

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