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Specificity of human immunodeficiency virus (LAV/HTLV-III) – reactive antibodies in African sera from southeastern Tanzania

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Summary

The prevalence of antibodies to human immunodeficiency virus (HIV = LAV/HTLV-III) in a rural population from the Ifakara area in southeastern Tanzania was investigated. Sera from 286 individuals collected from 1982 to 1984 in connection with a study on liver disorders were tested by an ELISA. Fifty-two (18.2%) of the sera were found positive. While the positives were largely confirmed by one commercial ELISA, they were completely negative by two others. Confirmatory testing by Western blot and competition Western blot showed that the reactivity detected by more sensitive of these assays was largely due to IgG antibodies binding to the HIV core (*gag*) proteins p17, its precursor p55 and, in some cases, p24. These tests also indicated, however, that the reactive antibodies could not have been elicited by HIV, but possibly by an unknown retrovirus or another cross-reactive agent. Thus, by 1984, the area investigated was largely free of HIV infection, but a significant proportion of its population may harbor another retrovirus of unknown pathogenicity.

Key words: human immunodeficiency virus; serology; cross-reactivity; retrovirus; Africa.

Introduction

Cases of acquired immune deficiency syndrome (AIDS) are occurring in many countries of tropical Africa (Biggar, 1986). Though the clinical manifestations are somewhat different from those of AIDS in other areas, they are based

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on the same type of immune deficiency. The disease is caused by infection with viruses of a family of human lentiviruses now called human immunodeficiency viruses (HIV) (Coffin et al., 1986). The same viruses are also known as lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983), human T-lymphotropic retrovirus Type III (HTLV-III) (Popovic et al., 1984; Gallo et al., 1984; Schüpbach et al., 1984; Sarngadharan et al., 1984), and AIDS-related retrovirus (ARV) (Levy et al., 1984). Recently, new members of this virus family called LAV-II (Clavel et al., 1986) and HTLV-IV (Kanki et al., 1986) were isolated from AIDS patients and healthy individuals from West African countries. It is not clear yet whether these new isolates are indeed different from each other, but their relatedness to previous isolates of HIV is clearly limited.

Preliminary serological studies based on enzyme-linked immunosorbent assays (ELISA) suggested a wide and early presence of HIV in many regions of central Africa, sometimes even in regions where AIDS cases have so far not been observed (Biggar et al., 1985a, b; Brun-Vézinet et al., 1984; Bayley et al., 1985; Epstein et al., 1985; Saxinger et al., 1984; Van de Perre et al., 1985). However, investigations based on more specific tests such as radioimmunoprecipitation, competition radioimmunoassays, or competition ELISAs, could not confirm some of these early results (Carswell et al., 1986; Fleury et al., 1986; Hunsmann et al., 1985; Okpara et al., 1986). Some of the interesting findings of those more detailed studies were the observation that reactivity to HIV antibody tests was in some populations correlated with malaria or other parasitic infections and with the presence of circulating immune complexes in serum (Biggar et al., 1985c). This suggested some type of reactivity not specific for HIV. However, the reasons for this reactivity have never been investigated in detail and may, in addition, be different for the various populations tested and the various assays used. It emerges from these studies that much of the serology done so far in Africa is unreliable and must be done again with a more careful approach. In the present study we have attempted this with ELISA-reactive serum samples from southeastern Tanzania. AIDS has so far not been observed in that area, but is present in the northern and western parts of the country (Forthal et al., 1986).

Materials and Methods

Study area

The present study was undertaken with sera from the Ifakara division (Kilombero District, Morogoro Region) in southeastern Tanzania. The area is situated in the Kilombero River plain at approximately 250 m elevation and 320 km inland from the Indian ocean coast. The Tanzania–Zambia railway passes the district capital, Ifakara, an important trading place. Details about the study area have been published (Lukmanji and Tanner, 1985).

Sera

The sera came from a hospital-based investigation on the etiology of liver disorders in the Ifakara area undertaken between 1982 and 1984. They were collected from 139 adults with at least one sign of liver disorders including hepatomegaly (greater than 12 cm in the medioclavicular line), jaundice, ascites, abdominal vein distension, liver tenderness, or tumors in the right upper abdomen. All these patients came from the Ifakara area and were admitted to the St. Francis Designated District Hospital. In addition, 140 control sera (none of the signs of liver disease mentioned) were obtained from patients attending the outpatient department of the hospital for minor complaints or admitted for elective surgery. The details of the liver study have been published elsewhere (Robyn, 1986; Stahel et al., 1984). The serum of a clinically confirmed Tanzanian AIDS patient (a 25-year-old male) from Shirati Hospital (North Mara Region, Tanzania) was kindly provided by Dr. A. Levin (Clinical Research Centre, Harrow, U.K.) and Dr. G. Brubacker (Shirati Hospital).

Serologic assays

ELISAs for antibodies to HIV were performed using standard methods (Sarngadharan et al., 1984), or according to the procedures described by the manufacturers of commercial products. Commercial tests used were the Abbott HTLV-III enzyme immunoassay (Abbott EIA), the Abbott confirmatory enzyme immunoassay (Abbott C-EIA) and the Du Pont HTLV-III/LAV ELISA. The Abbott tests were kindly performed by Dr. H. Joller, Institute of Clinical Immunology, University Hospital, Zurich.

Western blots for antibodies to HIV were done with a modification of procedures described (Schüpbach et al., 1985). In short, density banded virus from the prototype isolate HTLV-III (Popovic et al., 1984) (5000 or 10,000 fold concentrated, a gift from Dr. R. C. Gallo, NIH, Bethesda, USA) was electrophoresed on preparative 12% sodium dodecylsulfate polyacrylamide slab gels (Lämmli, 1970). The separated proteins were electroblotted to nitrocellulose sheets (Towbin et al., 1979) and these saturated for 3 h at 37°C with a 1:1 mixture of fat-free milk from a local supermarket and phosphate buffered saline (PBS). The sheets were cut to strips then incubated overnight at 4°C with test serum in dilutions of 1:100 or 1:50. The strips were washed 3 times with PBS containing 0.05% Tween 20, incubated for 30 min with 1:1000 dilutions of biotinylated goat antibodies to human IgG and IgM (Vector Laboratories, Inc., USA) in milk/PBS and washed again. Horse radish peroxidase-conjugated avidin (Vector) was added for 30 min at a dilution of 1:1000 in milk/PBS and the strips were washed again. Finally, binding of antibodies to their respective antigens was made visible by the addition of a substrate consisting of 0.05% 4-chloro-1-naphthol and 0.01% H_2O_2 in PBS. In some initial blots, a solution based on gelatine (LB Solution, Inotech AG, Switzerland) was used instead of the milk/PBS mixture. Commercial Western blots (Bio Rad, Du Pont) were performed according to the manufacturer's instructions.

Competition assays. For competition WB, serum samples were incubated for 3 h at 37°C with appropriate concentrations of the competing material in a volume of 500 μl. The mixture was then added to HIV strips, and the WB procedure was performed as indicated above. The strips were then scanned with a densitometer to document the extent of competition. Competition ELISAs were done accordingly with the exception that the total volume of the mixture was 100 μl.

Cellular lysates for competition assays were produced by resuspending washed cell pellets in a buffer consisting of 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% desoxycholate, 0.02% NaN_3 and 1 mM phenylmethylsulfonyl fluoride. The cells were homogenized in a Dounce homogenizer and insoluble material was pelleted at 10,000 *g* for 30 min.

Malaria soluble antigen. The soluble *P. falciparum* antigen was prepared from cultured infected red blood cells (RBC). The infected RBC were kindly provided by Dr. H. Matile (Hoffmann-La Roche Ltd, Basel). The RBC were lysed in a citrate buffer (150 mM NaCl and 15 mM Na-citrate, pH 7.0) containing 0.01% saponin. After lysis and centrifugation, the pellet was washed twice with saponin-free citrate buffer, sonicated, and centrifuged at 23,000 *g* for 30 min. The supernatant (soluble antigen) was either used for the preparation of ELISA plates using standard procedures, or for competition assays.

Results

Serological reactivity with proteins of HIV

Sera of 286 individuals (male/female ratio = 1.4) from Ifakara, Tanzania, were screened at NIH by ELISA for antibodies to the human retrovirus HIV (courtesy of Dr. W. A. Blattner). Fifty-two of the sera were positive (18.2%; prevalence ratio male/female = 1.75). The median age of the seropositives was 40 years, compared to 26 of those seronegative ($2p < 0.01$, U-test). Forty-nine of these positives as well as 21 ELISA-negative matched controls were further tested in Zurich by the Abbott EIA, the Abbott C-EIA, the Du Pont ELISA, and various WB. The results are summarized in Table 1.

None of the sera positive in the NIH ELISA was positive in the Abbott EIA or in the Abbott C-EIA. By WB, however, antibody reactivity was detected against distinct protein bands corresponding in size to HIV p17, p24, and their precursor, p55. No antibodies to other viral proteins were detected. Representative examples of WB results obtained under various test conditions are shown in Fig. 1, along with Swiss positive reference sera for HIV antibodies and the serum of a Tanzanian AIDS patient (Fig. 1C) which shows reactivity similar to that of European or American AIDS patients. Strips saturated with milk/PBS (Fig. 1B) had much less background than those saturated with LB solution (Fig. 1A). Detection of p55 varied greatly depending on the amount of this protein in the virus batch used for preparation of the strips. The Bio Rad strips used in these experiments were especially rich in p55 (Fig. 1D), but the amount of this protein was greatly reduced in later batches.

Antibodies to p17 were detected in 43 (88%) of the 49 ELISA-positive samples tested. Antibodies reactive with the Mr 55,000 protein were detectable in 40 of them (93% of the p17-positives), but only 4 (9%) of them were also

Table 1. Summary of confirmatory testing in 70 Tanzanian sera

	Tests					
	WB home-made	WB Bio Rad	WB Du Pont	Du Pont ELISA	Abbott EIA	Abbott C-EIA
Reactive in	43/49	8/8*	12/49*	42/49*	0/49	0/49
NIH screening	43× p17	7× p17	12× p17			
(n = 49)	40× p55	8× p55	2× p55			
	4× p24	2× p24	2× p24			
Non-reactive in	5/21	n. d.	4/21	4/21*	0/21	1/21**
NIH screening	5× p17		1× p17			
(n = 21)	(very weak)		3× p24			

* all of them positive in the home-made WB, as described under Methods

** positive for core antibodies in a sample, negative by all other tests

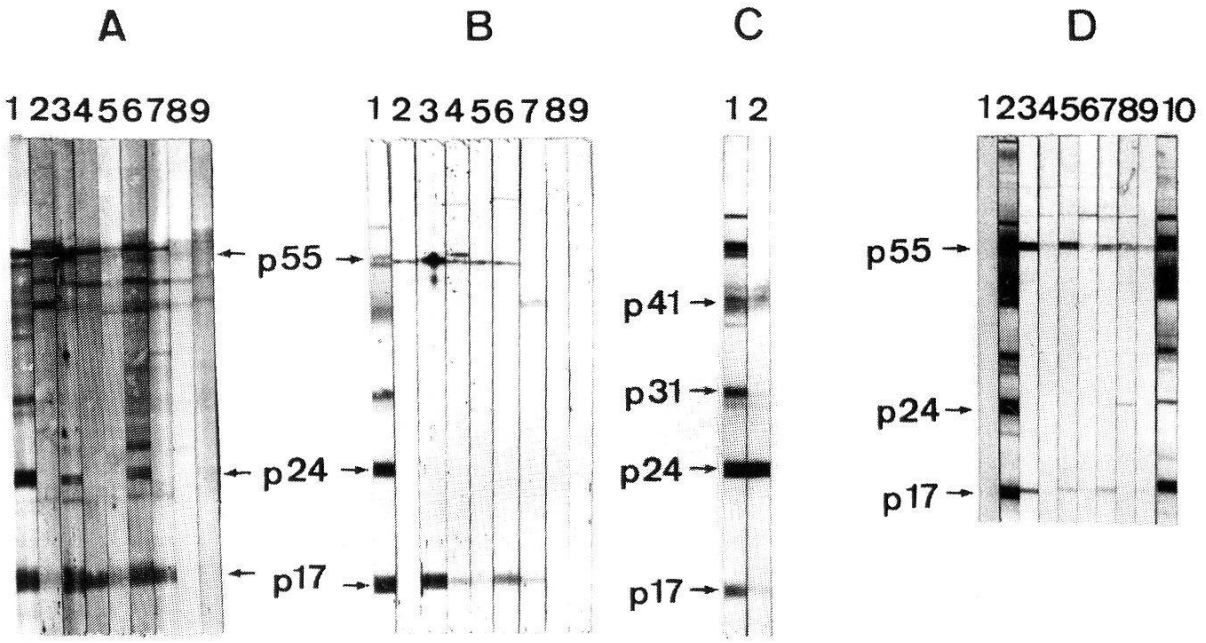

Fig. 1. Representative Western blots of ELISA-reactive sera from Ifakara, Tanzania. The WB were done under various assay conditions using a gelatine-based incubation buffer in A, a milk-based buffer in B and C, and the Bio Rad assay in D. P55, p24, p17, etc., designate viral proteins of 55 kD, 24 kD, or 17 kD, resp. Positive reference samples from Swiss patients with HIV infection are shown on strip 1 of panels A, B, and C, and on strips 2 and 10 of D. Negative Tanzanian controls are shown on strips 8 and 9 of A and B, and on strip 1 of D. Serum of a Tanzanian AIDS patient is shown on C2. Note the exclusive recognition of *gag* proteins p17, p24, and p55 by the Ifakara sera, p55 being best visible on Bio Rad strips.

positive for p24. Among the 21 sera initially negative by ELISA, WB revealed p17 bands in 5 cases (24%). When all 70 samples were retested in Zurich by Du Pont ELISA, concordance between the WB and the Du Pont ELISA result was found in 61 cases (87%), discordance in 5 (7%), and in 4 instances (6%) the comparison was equivocal. The highest reactivity by ELISA was detected in the samples with the strongest p17 bands in WB.

Similar results were obtained when the sera were tested with 2 commercial WB kits (Bio Rad and Du Pont). Eight sera positive in our WB were also positive in the Bio Rad WB with very distinct bands at the p55 and, usually much weaker, at the p17 and/or p24 sites. Results with Du Pont WB were positive in only 16 of the 70 sera, mostly in those having the strongest bands in our WB.

Specificity assays

Competition WB were then done in order to further exclude the possibility that the reactivity observed could be directed against cellular proteins copurified with the virus (Schüpbach et al., 1984). Aliquots of 25 μl of the serum with the strongest reactivity against the p17 band were preincubated with cellular extract from H9 cells (the uninfected, cloned cell line used for propagation of HTLV-III$_B$; Popovic et al., 1984) or from the infected H9/HTLV-III$_B$ cell line.

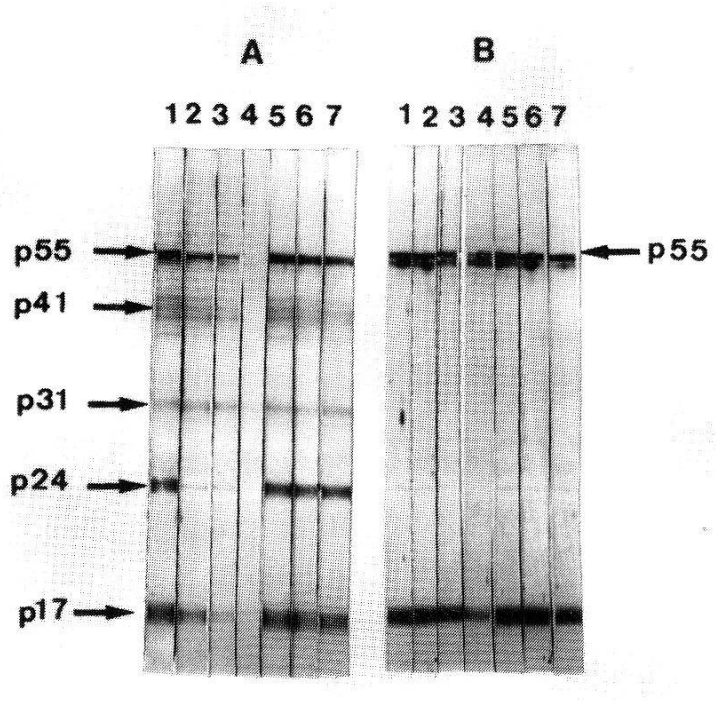

Fig. 2. Competition Western blots. A Swiss positive reference serum is shown in A and a strongly positive Tanzanian serum in B. Strips 1 in both panels show the reaction of uncompeted serum. With strips 2, 3, and 4 the sera were preincubated with 0.1 mg, 0.5 mg or 2.5 mg of H9/HTLV-III extract, while in 5, 6, and 7 they were preincubated with the respective amounts of uninfected H9 extract. Panel A shows virtually complete competition of all viral bands. In panel B, partial but significant competition of both p17 and p55 (upper band of the doublet) is visible on strip 4.

A control of the same serum was incubated with extraction buffer alone. The mixtures were then added to HTLV-III_B virus strips, and the normal procedure for WB was completed. Competition of the p17, a very weak p24, and the p55 band occurred with the H9/HTLV-III_B-competed, but not with the H9-competed serum probes (Figs. 2, 3). Similar results were obtained when the competition was done in the ELISA system (not shown). This indicates that the p17, p24, and p55 bands recognized by this serum represent viral and not cellular proteins. However, considerably more competing H9/HTLV-III_B protein was needed with the Tanzanian serum than with the Swiss reference serum to achieve the same degree of competition, and even at lower serum concentrations the reactivity could not be competed out completely. These results show that the reactive antibodies in the Tanzanian serum have a much lower affinity for HTLV-III_B *gag* proteins than those from an individual with known HIV infection.

Some authors have noted a correlation of HIV antibody reactivity and malaria (Biggar et al., 1985c). We determined the presence of antibodies to *P. falciparum* in our test population. Indirect immunofluorescence assays (Ambroise-Thomas, 1974) and ELISA based on circumsporozoite antigen of *P. falciparum* (Del Giudice et al., 1986) revealed more than 90% antibody-positives and similar levels of antibody titers in both the HIV-seropositive and -sero-

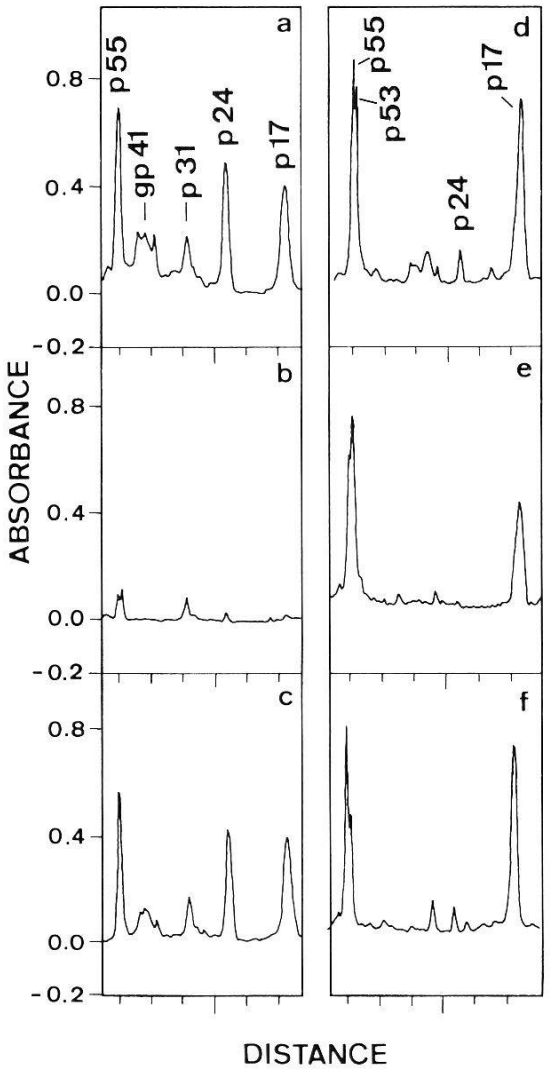

Fig. 3. Densitometric evaluation of competition Western blots. The strips shown in Fig. 2 were scanned with a densitometer. Panels a–c show the Swiss positive reference serum, panels d–f show the Tanzanian serum. Uncompeted sera: a and d; competition by 2.5 mg of H9/HTLV-III: b and e; competition by 2.5 mg of H9: c and f. Note the significant but incomplete competition of p55 and p17 in d, e, and f. P53 is a cellular contaminant recognized by the Tanzanian but not the Swiss serum.

negative individuals (not shown). In order to directly address the possibility of a cross-reactivity between *P. falciparum* and HIV we set up a series of competition ELISAs. On plates coated with *P. falciparum* antigen, the reaction of a sample from a malaria patient was not competed by H9/HTLV-III$_B$ or H9 extracts, but was significantly competed by the *P. falciparum* antigen itself (Fig. 4). Similarly, the reactivity of HIV-positive reference serum on an HIV-coated plate was not competed by *P. falciparum* antigen (not shown). Therefore, no direct cross-reactivity between HIV and *P. falciparum* was detected.

The same authors have also noted a correlation of ELISA reactivity and the presence of circulating immune complexes (CIC) (Biggar et al., 1985c). We addressed this possibility by setting up comparative WB with native sera and sera in which the CIC were removed by precipitation with 2.5% polyethylene-

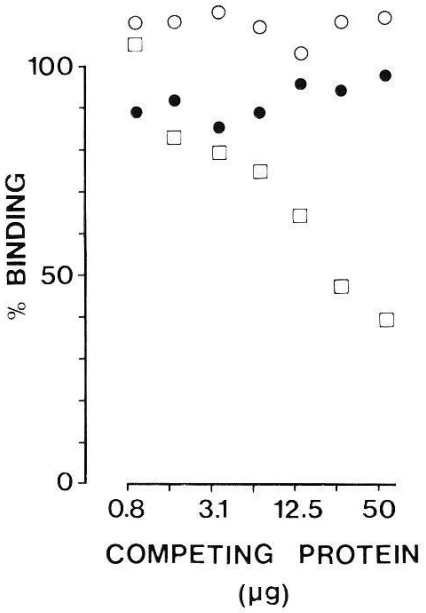

Fig. 4. Competition assays on ELISA plates coated with *P. falciparum* soluble antigen. A strongly HIV p17-reactive Tanzanian serum also positive for antibodies to *P. falciparum* was preincubated with serial dilutions of soluble *P. falciparum* antigen (squares), extracts from H9 (open circles), or H9/HTLV-III$_B$ cells (full circles). The mixtures were added to the wells of *P. falciparum* soluble antigen-coated ELISA plates and the binding of the competed samples was determined in relation to uncompeted controls (100%). No competition was observed with either uninfected or infected H9 cells.

glycol. The results showed that the removal of the CIC did not significantly affect the recognition of the *gag* proteins. Neither was significant p17 reactivity detectable in the resuspended CIC precipitates (not shown). Thus, the reactivity to *gag* proteins was not caused by material present in CIC.

Discussion

Our investigations show the presence of antibodies reactive with distinct proteins on WB prepared from purified HIV, isolate HTLV-III$_B$, in a population from Ifakara in southeastern Tanzania (Table 1, Fig. 1). The proteins recognized are predominantly p17, p55 and, in some cases, p24. The proteins recognized are of viral origin, as shown by their typical mobility on SDS-PAGE and the competition experiments illustrated in Figs. 2 and 3.

However, the WB reactivity pattern of these individuals is completely different from that of European or American HIV-infected individuals, or a Tanzanian AIDS patient from the North of the country (Fig. 1). HIV-infected individuals usually have antibodies to viral *gag, env,* and *pol* gene products, the most prominent reactivities by WB usually being directed against p24 and p17 (*gag*), gp120 and gp41 (*env*), and p31, p66 and p51 (*pol*). When examined by WB, antibodies to p24 are usually detected first in the course of infection, soon followed by those to p55 and, more gradually, by those to p17, gp41, gp120, p65, p51, and p31 (Lange et al., 1986). In contrast, reactivity to *env* and *pol*

products was completely absent from the Tanzanian sera, and p17 was much more reactive than p24. No reactive IgM antibodies were detectable (not shown) and fresh infection by HIV is therefore unlikely. The serum samples were collected between 1982 and 1984. If an HIV epidemic started in 1982, the infected individuals were to have the full range of antibody reactivity at least by 1984. In addition, there was no increase in the prevalence of antibodies between 1982 and 1984, thus further excluding an HIV epidemic. The most likely explanation for the presence of these antibodies is that they were elicited by a different but cross-reactive immunogen.

Our experiments, as well as the findings of others (Biggar et al., 1985c; Facet et al., 1986) rule out a direct cross-reactivity of p17 and *P. falciparum* antigen (Fig. 4), as well as nonspecific reactivity due to circulating immune complexes. Though most of these individuals have antibodies reactive with a wide variety of cellular proteins, as detected by WB on strips made from H9 cell lysates (not shown), the HIV reactivity is not due to these anticellular antibodies, as it was not inhibited by H9 extracts (Figs. 2 and 3). Stretches of sequence homology resulting in immunologic cross-reactivity of HIV p17 and the thymic hormone thymosin-$\mathbf{a_1}$ were recently reported (Sarin et al., 1986). This explanation would however not account for the presence of antibodies to p24 in 9% of the p17-reactive sera. A more likely possibility is the presence of infection with a different retrovirus.

Infection with HTLV-IV, STLV-III, or LAV-2 is unlikely, as individuals infected with these viruses usually have antibodies that cross-react with p24 of HIV, but less with p17 (Clavel et al., 1986; Kanki et al., 1986; and M. Essex, P. Kanki, and L. Montagnier: communications at the International Conference on AIDS, Paris, June 23–25, 1986). HTLV-I infection can also be ruled out, as HTLV-I ELISA results (kindly provided by Dr. W.A. Blattner) did not correlate with the HIV serology (not shown). HTLV-II is also unlikely, as there is a high degree of cross-reactivity between HTLV-I and -II with respect to both *gag* and *env* antigens (Kalyanaraman et al., 1982; and our own observations). By exclusion of these known human retroviruses, the most likely interpretation of the presence of these antibodies is the existence of a so far unrecognized retrovirus with distant relationship to HIV. This putative virus appears to be present in a substantial segment of the population of the area investigated and may or may not be associated with human disease. It is probably not involved in the causation of AIDS or related diseases, as these have so far not been observed in that area.

The investigations also allow some insight into the general issue of sensitivity and specificity of antibody tests. The Du Pont ELISA detected a large percentage of the sera showing reactivity to HIV proteins. This result is not specific, however, as the reactivity is most likely not due to HIV infection. On the other hand, the Abbott tests were HIV-specific, but clearly missed all cases of HIV-reactive antibodies and thus possible infection by a cross-reactive retrovi-

rus. It follows that some tests are more suitable than others for a particular purpose of testing. Epidemiologic studies on the prevalence of the virus, e.g., should use a test of high stringency. For blood banking, it would be more advisable to chose a test with broad reactivity, as blood from individuals possibly infected with other retroviruses should not be used for transfusion. This type of screening test, however, needs to be combined with a confirmatory test of high specificity, in order to protect the individual from a false diagnosis of HIV infection. Competition WB is not only very sensitive, but also permits to differentiate between true reactivity and cross-reactivity. It thus may serve not only as a confirmatory assay, but also as a powerful tool for the detection of new retroviruses.

Acknowledgments

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