

Serum antibodies to human T-cell leukaemia virus type I in different ethnic groups and in non-human primates in South Africa

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Summary

The prevalence of humoral antibodies to human T-cell leukaemia virus type I (HTLV-I) was investigated in different ethnic groups and in non-human primates in South Africa. Serum antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) using either disrupted whole HTLV-I or purified p24 core protein (p24 HTLV-I) as antigens. ELISA was complemented by direct radio-immunoprecipitation assays using either purified iodinated p24 HTLV-I or radiolabelled lysates of an HTLV-producing cell line as antigen followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis of the immunoprecipitates, and by immunofluorescence using the HTLV-I-producing cell line HUT-102 as antigen. Antibodies were demonstrated in 3,5% of Asians, 3,5% of blacks and 4,1% of coloureds, but not in whites, and also in 29% of vervet monkeys and 33% of baboons. We conclude that HTLV-I or closely related viruses cause widespread infection in non-human primates in South Africa and in a lower percentage of humans, including apparently healthy blood donors. We are currently isolating retroviruses from seropositive reactors and investigating the possible relevance to disease in South Africa.

S Afr Med J 1985; 67: 445-449.

Human T-cell leukaemia virus type I (HTLV-I) is a type C retrovirus which was first isolated by Poesz *et al.*¹ from a patient with cutaneous T-cell lymphoma. Meanwhile nationwide surveys in Japan had revealed a high incidence of adult T-cell leukaemia/lymphoma (ATL) in which a striking feature was the clustering of patients in south-western Japan.^{2,3} Hinuma *et al.*,³ Miyoshi *et al.*⁴ and Yoshida *et al.*⁵ showed a close association between ATL and a type C retrovirus which

productively infected cell lines derived from the peripheral blood lymphocytes of patients with ATL and which they characterized and designated adult T-cell leukaemia/lymphoma virus (ATLV). ATLV and HTLV-I are now regarded as identical and designated as HTLV-I.⁶

Miyoshi *et al.*⁷ first reported infection of monkeys (*Macaca fuscata*) with ATLV, and subsequent reports indicated that many but not all troops of Old World primates in Asia and Africa, both in the wild and in captivity, have antibodies to HTLV-I.⁸⁻¹⁰

We are investigating the incidence of HTLV-I infection and its possible relation to disease in South Africa and present the results of our survey for serum antibodies in humans and in non-human primates. Our preliminary findings were presented at a recent International Congress of Virology in Japan.¹¹

Subjects

The human sera were obtained from adults of both sexes in the Cape Province and Natal. Most were healthy donors and the remainder were patients, none of whom was suffering from leukaemia, lymphoma or immune deficiency. In all, 171 Asians, 543 blacks, 210 whites and 121 coloureds from South Africa and 485 whites from the Federal Republic of Germany were tested.

Non-human primate sera were obtained from vervet monkeys (*Cercopithecus aethiops pygerythrus*) captured in the Cape Province, Orange Free State and Natal, and from baboons (*Papio ursinus*) from the Cape Province. These primates were caged individually. Sera from 6 bushbabies (*Galago senegalensis*) bred in captivity were also tested. All sera were stored in aliquots at -20°C .

Material and methods

Cell cultures

The HTLV-I-producing cell line of T lymphoblasts, HUT-102, was provided by Dr R. C. Gallo (Bethesda, Md, USA) and cultured in RPMI-1640 medium supplemented with 2% glutamine, 20% heat-inactivated fetal calf serum (FCS), and antibiotics, but without added interleukin-2.

Peripheral blood lymphocytes (from freshly drawn venous or neonatal cord blood) were separated from heparinized blood by Histopaque gradient centrifugation and cultured in RPMI-1640 medium containing 2% glutamine, 20% heat-inactivated FCS, antibiotics and 10% interleukin-2. Phytohaemagglutinin (Wellcome; reagent grade) 0,2% was added for the first 3 days of culture. Cells were grown at a density of $0,5 \times 10^5/\text{ml}$ in Costar 24-well plastic plates or Falcon 25 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2 . The medium was replenished 3 times a week.

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HTLV-I antigen

Purified p24 core protein of HTLV-I (p24 HTLV-I) was kindly supplied by Dr Gallo and was iodinated using the chloramine T method described previously.¹² HTLV-I to be used for enzyme-linked immunosorbent assays (ELISA) was harvested from HUT-102 cell cultures and purified by density gradient centrifugation in sucrose according to standard procedures.

Anti-p24 HTLV-I sera

Hyperimmune goat anti-p24 HTLV-I serum was provided by Dr Gallo. We prepared hyperimmune rabbit anti-p24 HTLV-I serum by a series of intramuscular injections of antigen emulsified in Freund's complete adjuvant.

ELISA

The sera were tested for antibodies by one or more of the following three ELISA methods which yielded comparable results.

Firstly, we used commercially available kits (Biotech Research Laboratories, Rockville, Md, USA) employing detergent-disrupted HTLV-I antigens which were chemically fixed and coated onto 96-well microtitre plates. We generally followed the protocol supplied by Biotech, except that the final colour reaction was stopped by adding sodium hydroxide as soon as the positive control serum reached an optical density reading at a wavelength of 405 nm (OD₄₀₅ reading) of 0,6 (usually 20 minutes after adding the substrate). Sera were tested at 1/20 dilution.

Secondly, sucrose density gradient-purified HTLV-I was disrupted with 1% Nonidet P-40 and 0,2% desoxycholate in phosphate-buffered saline (PBS), pH 7,2, and allowed to absorb for 16 hours at 37°C to the wells of 96-well microtitre plates. Each well received 500 ng of viral antigens. After absorption the wells were emptied and allowed to dry and then potential nonspecific binding sites were blocked by incubating the wells filled with 1% gelatine in PBS for 1 hour at 37°C, after which they were emptied and allowed to dry. Sera were tested at a dilution of 1/20.

Thirdly, commercially available kits (Litton Bionetics, Charleston, SC, USA) employing purified p24 HTLV-I antigen were used. We followed the protocol supplied and tested sera at a dilution of 1/50 as recommended.

The OD₄₀₅ reading was obtained with a Titertek Multiscan.

Radio-immunoprecipitation assays (RIPA)

We followed the previously described procedure.¹² Briefly, iodinated p24 HTLV-I (10⁵ trichloroacetic acid-precipitable counts per minute per test tube) was incubated at 4°C for 16 hours with 10 µl antiserum or dilutions thereof in a total volume of 200 µl buffer (1% myoglobin, 0,5% Nonidet P-40, 2 mM phenylmethylsulphonyl fluoride and 1% Trasylol in PBS). Cross-linking of resultant antigen-antibody complexes was achieved by the addition of 50 µl of appropriate anti-IgG antiserum and incubation for 1 hour each at 37°C and 4°C. Immune complexes were sedimented at 1 500 g for 5 minutes at 4°C and washed by adding 400 µl RIPA buffer and incubating for 1 hour each at 37°C and at 4°C before sedimenting again. After a second wash the final sediments were counted in a scintillation counter.

Alternatively, high-titre sera (by ELISA) were tested for their ability to immunoprecipitate p24 from biosynthetically labelled lysates of the HTLV-producing cell line V-197 (W. B. Becker *et al.* — in preparation). Cells were labelled at a density of 2 x 10⁶/ml overnight in methionine-free Eagle's

MEM (Flow Laboratories, Irvine, Scotland) containing sulphur-35 methionine (Amersham, Bucks, UK) at 10 µCi/ml, then lysed in buffer containing 1% Nonidet P-40. The nuclei were removed by centrifugation at 2 000 g for 10 minutes, and the cytoplasmic fraction cleared by centrifugation at 100 000 g for 30 minutes. Sepharose-Protein A beads (Pharmacia, Uppsala, Sweden) precoated with normal human serum were added and the lysate was incubated for 15 minutes at 4°C after which the beads were removed by centrifugation. This pre-cleared supernatant was then incubated for 15 minutes at 4°C with Sepharose-Protein A beads precoated with test serum. The beads were washed according to the procedures of Lee *et al.*¹³ Labelled proteins were eluted by heating for 5 minutes at 100°C in buffer containing sodium dodecyl sulphate (SDS).

SDS-polyacrylamide gel electrophoresis

Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described previously¹² and based on the method of Laemmli¹⁴ to verify precipitation of p24 HTLV-I. When iodinated p24 HTLV-I had been used, the 5% stacking/10% separation gels were stained with Coomassie blue, dried and autoradiographed on Kodak X-omat X-ray films. When cell lysates had been used, the 5% stacking/12% separation gels were fluorographed with Amplify (Amersham, Bucks, UK), dried and autoradiographed on Fuji Rx film in X-ray cassettes fitted with Cronex Hi-plus enhancing screens (Dupont, Wilmington, De, USA) to identify the ³⁵S-labelled virus proteins.

Indirect fluorescent antibody (IFA) tests

The following methods were used. Firstly, serum antibodies against HTLV-I-associated antigens were determined using acetone-fixed HUT-102 cells as antigen. Cells grown in suspension were sedimented, resuspended in Difco FA buffer and spotted onto Hendley-Essex Multispot Teflon-coated glass slides at approximately 4 000 cells per spot, dried at room temperature for 1 hour, fixed in acetone at room temperature for 10 minutes, and stored at -90°C. In the test, cells were treated with sera diluted 1/10 for 30 minutes at 37°C, then washed and treated similarly with fluorescein-conjugated sheep anti-human IgG (Wellcome). After washing, the slides were mounted in Difco FA mounting fluid and examined in a fluorescence microscope using incident light illumination.

Secondly, antibodies to cell membrane antigens associated with HTLV-I were determined at the Harvard School of Public Health by the method described by Essex *et al.*¹⁵ In this method unfixed HTLV-I-infected cells are treated in suspension with sera as for the fixed cells before being mounted on slides for examination under the fluorescence microscope. Sera were tested at a dilution of 1/4 and judged positive on the basis of their ability to cause fluorescence in more than 40% of the HUT-102 and/or MT-2⁵ target cells used in the test. Two HTLV-I negative control human lymphoblastoid cell lines were used, namely 8402, a T-cell line, and NC-37, a B-cell line, which is positive for the Epstein-Barr virus (EBV) genome.

Thirdly, serum antibodies to Epstein-Barr virus capsid antigens (EBVCA) were detected using a commercial kit and following the recommended procedure (Zeus Scientific Inc., Raritan, NJ, USA).

Electron microscopy

Peripheral lymphocytes cultured in suspension were fixed by adding sufficient 25% glutaraldehyde to give a final concentration of 2,5% in the medium and holding at 4°C for 30

minutes. The cells were then washed twice in 0,1M cold Sörenson's buffer, pH 7,4 and osmicated for 30 minutes at room temperature in 1% OsO₄ in buffer. After being washed twice in buffer the cells were dehydrated to 70% ethyl alcohol and stained for 10 minutes in 2% uranyl acetate in 70% alcohol. Dehydration was then completed, the cells were transferred through acetone and embedded in Epon. Ultrathin sections were stained with 1% uranyl acetate for 15 minutes followed by Reynold's lead citrate for 7 minutes, and examined by transmission electron microscopy.

Results

The incidences of human seropositive reactors to HTLV-I as determined by ELISA and RIPA are presented in Table I. Most of the simian sera gave higher test readings and had fourfold or higher titres of antibodies than the human sera. However, some human sera had titres as high as those of simians, as shown in an experiment in which selected sera with high test readings were titrated by ELISA (Fig. 1). This was also shown in RIPA titrations; Figs 2 and 3 illustrate the results obtained with 3 human and 4 simian sera respectively. In these RIPA titrations the control goat and rabbit anti-p24 HTLV-I sera precipitated 90 - 100% of the homologous radiolabelled antigen. The control sera from Japanese patients with ATL¹⁶ also precipitated virtually all the p24 HTLV-I antigen (not shown), whereas the test sera individually have lower titres and give incomplete (40 - 80%) precipitation of the antigen. SDS-PAGE of the immunoprecipitates confirmed that p24 HTLV-I antigen was precipitated. These RIPA results suggest infection with retroviruses related to rather than identical with the prototype HTLV-I.¹⁶

TABLE I. PREVALENCE OF ANTI-HTLV-I ANTIBODIES IN SERA FROM HUMANS AND NON-HUMAN PRIMATES

	No. tested	Positive	
		No.	%
Humans			
Asians	171	6	3,5
Blacks	543	19	3,5
Whites	695*	2	0,3
Coloureds	121	5	4,1
Non-human primates			
Baboon	190	63	33,2
Vervet monkey	100	29	29,0

*485 from Federal Republic of Germany.

Immunoprecipitation was performed from ³⁵S-methionine-labelled lysates of the cell line V-197 using 4 human sera which had the highest anti-p24 titres on ELISA. The pattern of proteins precipitated was compared by SDS-PAGE with that produced by the homologous V-197 simian serum, by goat anti-p24 HTLV-I serum and by a negative control normal human serum. The latter produced no p24 precipitation while the goat anti-p24 HTLV-I antiserum produced the most dense band of p24 precipitation. A distinct though less dense band was obtained with the simian serum, while faint bands were obtained with 2 of the 4 human sera (Fig. 4).

Comparative tests for HTLV-I antibodies were done on 45 baboon sera and 49 vervet monkey sera with the ELISA and the two IFA tests. The results are correlated in Table II and show that 24 sera were positive in all three tests, that 5 were positive in two and 7 in one of the tests, whereas 58 sera were negative in all three test systems. One serum which reacted

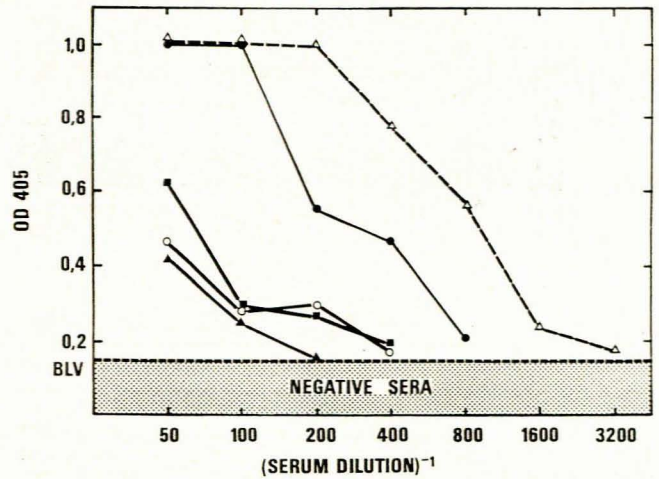


Fig. 1. ELISA using purified p24 HTLV-I antigen: titration of human and animal sera (BLV = baseline value; OD₄₀₅ > 1 recorded as 1).

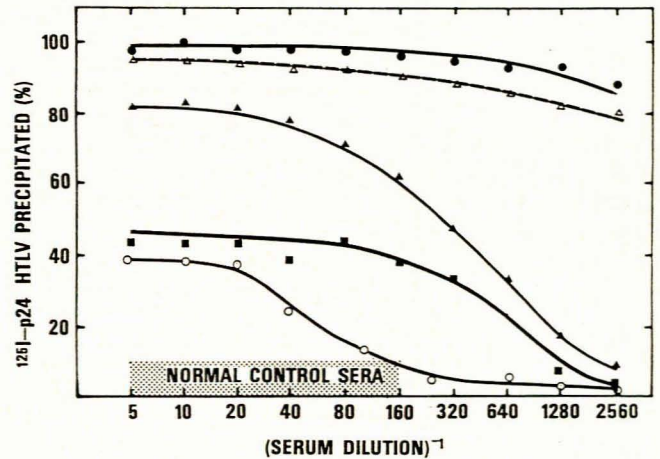


Fig. 2. RIPA of p24 HTLV-I antigen with human sera (100% = maximum immunoprecipitation).

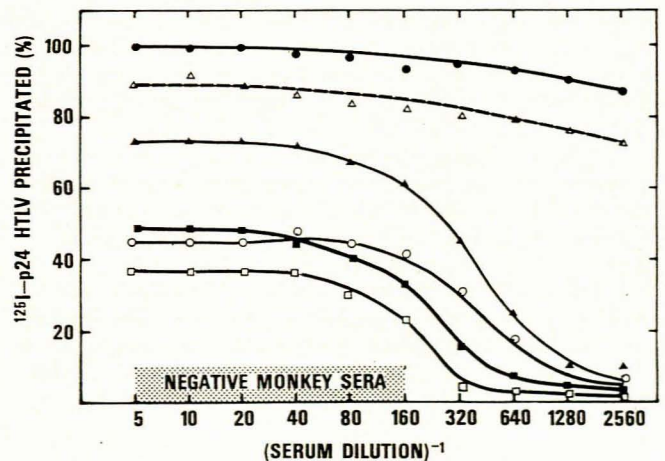


Fig. 3. RIPA of p24 HTLV-I antigen with simian sera (100% = maximum immunoprecipitation).

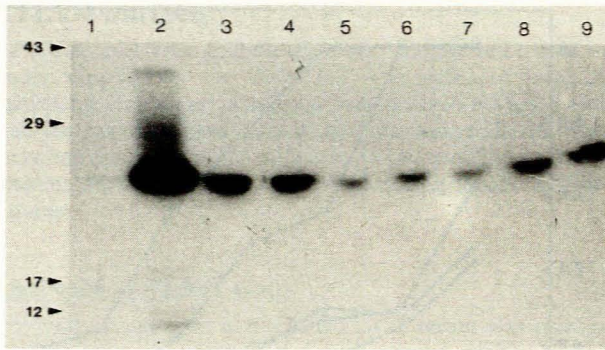


Fig. 4. SDS-PAGE of human and simian antibodies precipitated in RIPA with iodinated p24 core antigen of HTLV-I (serum dilutions 1/40). Lane 1 — normal goat serum; lane 2 — goat anti-p24 HTLV-I-positive control serum; lanes 3 and 4 — Japanese leukaemia sera No. 7 and No. 4; lanes 5, 6 and 7 — baboon sera P205, P21 and P277; lanes 8 and 9 — vervet monkey sera S38 and S40. The standard proteins were ovalbumin (M_R 43 000), carbonic anhydrase (M_R 29 000), horse myoglobin (M_R 17 000) and cytochrome c (M_R 12 000).

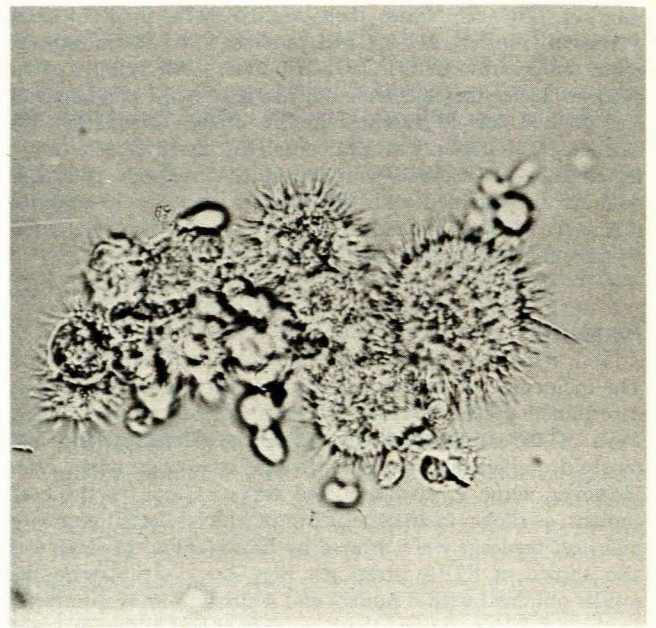


Fig. 5. Phase-contrast photomicrograph of the lymphoid cell line V-191 illustrating the hairy morphology of the cells (x 550).

TABLE II. COMPARATIVE TESTS FOR HTLV-I ANTIBODIES IN SIMIAN SERA

	ELISA 1/50	IFA	IFA
		(acetone- fixed cells) 1/10	(living HUT-102 and MT-2 cells) 1/4
No. of simian sera	24	+	+
with the indicated	3	+	—
spectrum of	1	—	+
reactivity	1	+	+
	3	+	—
	1	—	+
	3	—	—
	3	—	+
	58	—	—
No. positive/No. negative	94	31/63	29/65

strongly with the two control HTLV-I-negative cell lines in the IFA test was excluded from the results. In contrast to the uniformly negative reactions of the sera with the control 8402 T-cell line, most sera reacted to a limited extent with the control NC-37 B-cell line which carries the EBV genome. All the sera were also tested for EBVCA with the commercial kit and found to be positive, with the exception of 2 which were strongly positive for anti-HTLV-I antibodies with the ELISA and both IFA tests. This is added confirmation of the different specificities of the HTLV-I and EBVCA test systems.

To confirm infection of simians with an HTLV retrovirus, T cells from the peripheral blood lymphocytes of 2 seropositive vervet monkeys were co-cultured with human neonatal cord lymphocytes and a cell line established from each (W. B. Becker *et al.* — in preparation). The two lines, designated V-191 and V-197, appear 'hairy' and are productively infected with a morphologically typical type C retrovirus. These features are illustrated in the phase-contrast photomicrograph of the V-191 cells in culture (Fig. 5) and in the transmission electron micrograph of ultrathin sections of the same cell line (Fig. 6).

Discussion

We have used specific serum antibodies to HTLV-I as an

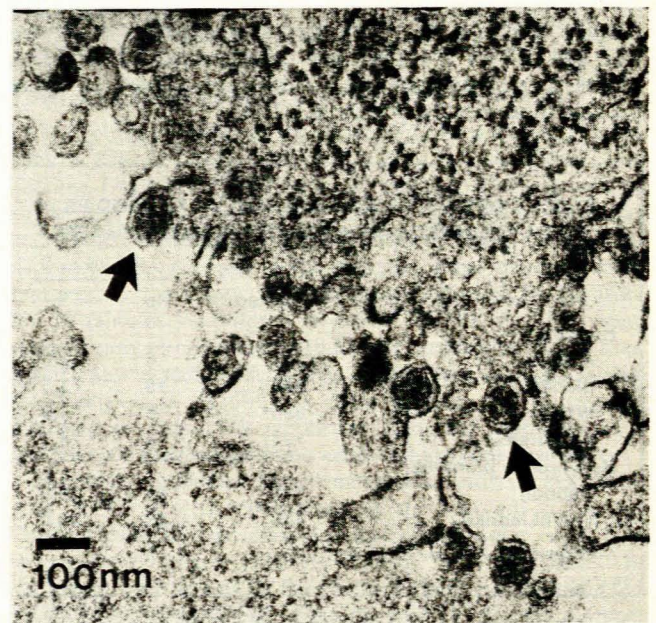


Fig. 6. Transmission electron micrograph of an ultrathin section of the 'hairy' lymphoid cell line V-191 showing morphologically typical type C retrovirus particles (arrows) in the intercellular spaces.

index of infection either with this leukaemia-associated retrovirus or with an antigenically closely related strain. It is clear from our results that HTLV-I-related infection occurs in South Africa, but its extent and significance need to be established. The human sera in our series were from adults who were either healthy donors or were suffering from an illness unrelated to leukaemia/lymphoma. They were otherwise unselected for age, sex or geographical region. We found that the incidence of antibodies in whites was a fraction of 1% but in other ethnic groups it was from 3,5% to 4,1%. Our survey was not extensive enough for conclusions to be drawn about

racial incidence or the possible regional clustering of seropositive reactors.

Our results differ from those of Saxinger *et al.*,¹⁷ who recently reported on HTLV-I antibodies in various parts of Africa. Their survey was carried out with the ELISA method but confirmatory tests were necessary to eliminate nonspecific positive reactions which were particularly troublesome when serum IgG levels were grossly elevated. This question of nonspecific positive reactors was also discussed by Hunsman *et al.*,¹⁸ who found that RIPA followed by SDS-PAGE appeared to be the most stringent of the three test systems they used. We could confirm the specific immunoprecipitation of p24 HTLV in this test system with some of our human sera which had high titres of HTLV-I antibodies on ELISA or RIPA.

As opposed to endogenous retroviruses which are spread vertically in the germ line, HTLV-I is an exogenous retrovirus which spreads horizontally. Therefore infection and consequently the incidence of antibodies can be expected to increase with age. This is well illustrated by the findings of Hino *et al.*¹⁹ in Japan.

It has not been established whether genetic factors influence susceptibility to infection or disease due to HTLV-I. If this were so, a different incidence could be expected not only in clearly different ethnic groups but also in genetically different subpopulations. The latter may be detected as geographical or regional differences in incidence, which is a feature of the disease in Japan.³ Interesting observations on differences in the prevalence of HTLV-I antibodies in the ethnic groups in French Guiana have been reported.²⁰

Approximately a third of the monkeys and baboons tested had serum antibodies and we confirmed viral infection in vervet monkeys by establishing two T-cell lines from the peripheral blood lymphocytes of 2 seropositive reactors and then demonstrating HTLV-I-associated antigens in these cell lines by IFA. Furthermore we showed by electron microscopy that these cells were productively infected with morphologically characteristic type C retrovirus particles. This high incidence of simian seropositive reactors probably reflects the situation in the wild. All the simians were apparently healthy but they were not kept long enough to observe the natural history of their retrovirus infection. A similar high incidence of serum antibodies has been reported by Hayami *et al.*¹⁰ in a number of species of Old World monkeys, whereas New World monkeys were apparently not infected. The same authors showed a clear-cut difference in geographical distribution between seropositive simians and humans in Japan, indicating the improbability of direct transmission from simians to humans. We have tested 40 animal handlers for HTLV-I serum antibodies, including 3 who had had prolonged close contact with vervet monkeys and especially baboons during experimental surgical procedures. Two were whites whose contact extended over periods of 12 and 17 years and 1 was coloured with a contact period of 17 years. None of the 40 had antibodies. This suggests that there may not be significant infectivity of simian HTLV-I-related viruses for man. However there is a lack of published information on this aspect which, together with the relation of simian retrovirus infection to simian

disease, warrants investigation. Such chronic infections of simians need to be excluded if their tissues are used for transplants. The same applies to latent infection of simians with herpesviruses, in which context our findings that all of 50 monkeys and 50 baboons tested were seropositive for EBVCA, suggesting a widespread infection with EBV or a closely related agent, are relevant.

We would like to thank S. Engelbrecht, U. Mikschy, C. Piek and B. Robson for expert technical assistance. This work was supported by grants from the University of Stellenbosch and the Poliomyelitis Research Foundation (to W.B.B.) and from the Homberger Foundation (to R.K.).

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