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HTLV–III and HTLV–I Infection in Populations at Risk in the Veneto Region of Italy*

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Abstract—*A seroepidemiological survey has been carried out in the Veneto region to determine the prevalence of HTLV-*III *and HTLV-*I *antibodies in subjects at risk for development of AIDS and related conditions. Serum samples were tested by ELISA and, for confirmation, by radioimmunoassay (Western blot), using disrupted virus as antigen. The results show that* 22 *out of* 112 *hemophiliacs had antibodies against HTLV-*III*; however disaggregation of data resulted in* 22.6 *and* 77.8% *positivity for patients with severe forms of hemophilia A and B, respectively. Two patients with hemophilia A and two with hemophilia B were positive for antibodies to HTLV-*I.

*The prevalence of HTLV-*III *antibodies in the homosexual and intravenous drug abuser groups was* 52 *and* 33% *respectively. No positive cases for antibodies to HTLV-*I *were found in homosexuals, while* 4.3% *seropositivity to HTLV-*I *was observed in drug abusers. Among patients suffering from various pathologic conditions not strictly AIDS related, only 1 with generalized non-Hodgkin lymphoma was positive for HTLV-*I *antibodies.*

*In a further group of patients with clinical diagnosis of LAS and AIDS, antibodies to HTLV-*III *were found in* 90 *and* 100% *respectively, while seropositivity for HTLV-*I *was observed only in* 6.4% *of LAS patients.*

*The implications of these findings are discussed, particularly in view of the potential oncogenic effect possessed by HTLV-*I.

INTRODUCTION

SINCE THE first *status quo* report on acquired immunodeficiency syndrome (AIDS) in Europe [1], many other cases of AIDS and related conditions (persistent generalized lymphadenopathy, PGL; lymphadenopathy syndrome, LAS; AIDS related complex, ARC) have been described [2–9], indicating that the epidemiology of these syndromes in Western European countries is quite similar to that observed in the U.S.A. Thus, three main risk groups have been identified, namely hemophiliacs and subjects receiving repeated blood transfusions for various reasons, intravenous drug abusers, and homosexuals with high promiscuity habits.

Human retroviruses belonging to the lymphotropic retrovirus family have been isolated in France [10] and in U.S.A. [11] from people affected with LAS and AIDS, and they are now generally regarded as causative agents of these diseases. Although their definite nomenclature is still debated (e.g. lymphadenopathy-associated virus, LAV; human T-cell lymphotropic virus type III, HTLV-III; AIDS-related virus, ARV [12, 13]), it is clear that the different isolates represent variants of the same virus as shown by morphology, antigenicity, strict tropism for lymphocytes of T4 subset, and molecular analysis. Moreover, the marked T4 lymphocyte killing exerted by the LAV/HTLV-III isolates provides the basic mechanism for the pathogenesis of the disease [14, 15].

On the other hand, before the characterization of this novel retrovirus, a number of studies on AIDS etiology have been performed using as antigen the first isolate of the HTLV family, the HTLV-I, and a positive correlation between antibody occurrence and AIDS development was reported [16]. While these results could be due to some serological crossreactions of the not yet well purified reagents, Tedder *et al.* using a more specific serological test have recently found that about 5% of LAS patients have antibodies against HTLV-I [17]. This observation might be of relevance in view of the fact that some lymphomas have been described in AIDS [18] and HTLV-I, instead of being cytopathic, immortalizes T4 lymphocytes and is frequently associated with lymphoid malignancies [19].

Here we present data on a serological survey

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undertaken in the Veneto region (Italy) to determine the prevalence of HTLV-III and HTLV-I antibodies in subjects at risk. Besides high antibody prevalence to HTLV-III, we also found low but definite seropositivity to HTLV-I.

MATERIALS AND METHODS

Subjects

The subjects consisted of the following groups of people: (a) Hemophiliacs and patients with rare clotting disorders (deficiency of factor V, VII, X, XII), undergoing regular clotting factor replacement therapy or repeated blood transfusions; (b) intravenous drug abusers; (c) homosexuals; (d) patients with lymphomas involving T cell lineage; (e) controls consisting of patients with pathologic conditions not related to AIDS, and healthy blood donors and laboratory workers.

The subjects were drawn mainly from Hemophilia Centers and Infectious Disease Units of the General Hospitals in Padova, Verona, Vicenza, Castelfranco Veneto. Blood was collected from May 1984 to March 1985 and the aliquoted sera were stored at −20°C until testing.

Virus preparation

HTLV-III virus obtained from H9/HTLV-III infected cells was concentrated by zonal centrifugation (15–60% sucrose gradient); the pellet was disrupted by the addition of an equal volume of Tris–HCl (pH 7.9, 100 mM), Triton-X (0.5%), DTT (0.04 M), EDTA (2 mM), KCl (1 M) and PMSF (2 mM), clarified by centrifugation for 30 min at 35 K rpm and dialyzed against PBS overnight. HTLV-I virus obtained from HUT-102 cells as previously described [20] was disrupted and clarified as reported for HTLV-III virus.

Enzyme linked immunoassorbent assay (ELISA)

The wells of microtiter plates were filled with 100 μl of 50 mM sodium bicarbonate (pH 9.6) containing disrupted virus at a concentration of 5 μg/ml and incubated overnight at 4°C. The plates were washed with water and filled with 100 μl of 10% normal goat serum and 10% fetal calf serum in PBS, followed by 5 μl of the test sera. After incubation overnight at 4°C the plates were washed three times with PBS containing 0.05% Tween 20. The wells were incubated for 1 hr at room temperature with 100 μl of peroxidase-labeled goat anti-human IgG diluted 1 : 4000 in PBS containing 0.5% Tween 20 and 1% normal goat serum. The wells were washed three times with PBS–Tween and once with PBS and reacted for 30 min with 100 μl of a substrate mixture containing 0.05% ortophenylendiamine and 0.005% H_2O_2 in phosphate citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μl of 4 N sulfuric acid. The color yield was measured at 492 nm using Multiscan MC plate reader.

Assays were done in triplicate and the absorbance reading greater than two times the negative control, selected on the average of 100 normal negative controls, was taken as positive.

Virus strip radioimmunoassay (Western blot) technique

HTLV-I or HTLV-III viruses were prepared as reported for ELISA and 200 μg of viral protein was fractionated by electrophoresis on a 12% polyacrylamide slab gel in the presence of SDS. The protein bands on the gel were electrophoretically transferred to a nitrocellulose sheet as described [21]. The nitrocellulose sheet was incubated at room temperature for 2 hr with 1% bovine serum albumin (BSA) in PBS and cut into 0.5 cm strips. Each strip was incubated at room temperature overnight in a screw cap tube containing 2.5 ml of 1% BSA in PBS with 50 μl of test sera. The strips were washed once with a solution containing Tris–HCl (pH 7.4, 10 mM); 0.9% NaCl, two times with Tris–HCl, NaCl, 0.05% NP 40 and once with Tris–HCl, NaCl. The strips were incubated for 1 hr at room temperature with 1×10^6 cpm of affinity purified I^{-125} labeled goat anti-human immunoglobulins in 2.5 ml of mixture reaction (1% BSA and 1 1% NGS in PBS). The strips were washed as previously described, mounted and exposed to X-ray film.

RESULTS

The results on antibody prevalence to HTLV-III and HTLV-I among the asymptomatic individuals belonging to risk groups are shown in Table 1. Firstly, it should be noted that the concordance between the ELISA and Western blot assays was quite good for detection of HTLV-III antibodies, all but three sera positive in ELISA were also positive by Western blot. Less satisfactory was the comparison when HTLV-I was used as antigen, 12 serum samples positive in ELISA were found not reactive by Western blot.

In hemophiliacs antibodies to HTLV-III were detected in the serum of 22 out of 112 subjects; however disaggregation of data resulted in 22.6 and 77.8% positivity for patients with severe forms of hemophilia A and B, respectively. On the other hand, only two patients with hemophilia A and two with hemophilia B were positive for antibodies to HTLV-I.

Patients suffering from rare coagulation disorders (abnormalities of factors other than VIII and IX) showed 2.8% seropositivity (one case out of 35 subjects) for HTLV-III but none possessed antibodies to HTLV-I.

In the homosexual group the prevalence of

Table 1. HTLV-III and HTLV-I antibodies in asymptomatic subjects at risk

Subjects	No. tested	Antibodies against HTLV-III No. positive			HTLV-I No. positive		
		E*	Wb†	%‖	E*	Wb†	%‖
Hemophiliacs A							
mild‡	42	1	1	2.4	2	1	2.4
severe§	53	12	12	22.6	4	1	1.8
	95	13	13	13.7	6	2	2.1
Hemophiliacs B							
mild‡	8	2	2	25.0	1	0	0
severe	9	7	7	77.8	2	2	22.2
	17	9	9	52.9	3	2	11.8
Rare coagulation disorders	35	1	1	2.8	0	0	0
Homosexuals	23	12	12	52.2	1	0	0
Drug abusers	139	49	46	33.0	12	6	4.3
Healthy controls	105	0	0	0	0	0	0

*ELISA
†Western blot
‡Receiving less than 10.000 U/year
§Receiving more than 10.000 U/year
‖The percentages are referred to the Wb results

Table 2. HTLV-III and HTLV-I antibodies in patients with various pathologic conditions

Patients	No. tested	Antibodies against HTLV-III No. positive			HTLV-I No. positive		
		E	Wb	%	E	Wb	%
Non Hodgkin lymphomas	11	0	0	0	2	1	9.1
Hepatitis and other chronic liver diseases	21	1	0	0	1	0	0
Chronic kidney diseases (hemodialyzed)	9	1	0	0	1	1	11.1

HTLV-III antibodies was 52% (12 out of 23 tested) and no positive cases for antibodies to HTLV-I were found. In the intravenous drug abuser group 33% out of the 139 subjects tested had antibody to HTLV-III, whereas the seropositivity to HTLV-I was 4.3%.

Patients suffering from various pathologic conditions not strictly AIDS-related were also investigated (Table 2). The non-Hodgkin lymphomas were mostly of the T-cell types. The only patient who was positive for HTLV-I antibodies was a 22-yr-old male who was born in the Seychelles Islands and returned there frequently: he had a long story of opportunistic infections and a generalized lymphoma was found at death (details of this case will be reported elsewhere). None of the patients with liver diseases was positive but one patient undergoing periodic hemodialytic treatment was seropositive for HTLV-I.

A further group studied consisted of patients with clinical diagnosis of LAS or AIDS (Table 3); of 31 LAS patients 22 were drug addicts, three were drug abusers and homosexuals, one was homosexual, one had coagulation (Factor X) abnormality, one was a female partner of a male drug addict, one was a male born in Burundi and the other two were males with apparently no risk story. Of the five AIDS patients, one was suffering from severe hemophilia A, one was homosexual, one travelled frequently for professional reasons in countries endemic for HTLV-III infection and two were drug abusers. Antibodies to HTLV-III, (Table 3) were found in 90 and 100% of LAS and AIDS patients, respectively; in addition, 6.4%

Table 3. HTLV-III and HTLV-I antibodies in patients with LAS or AIDS

Serum donors	No. tested	HTLV-III Antibodies against HTLV-I No. positive			HTLV-I No. positive		
		E	Wb	%	E	Wb	%
LAS patients	31	29	28	91.2	4	2	6.4
AIDS patients	5	5	5	100	1	0	0

(two out of 31 tested) of LAS patients also showed antibodies to HTLV-I.

It should be noticed that among the three subject series examined above (Tables 1–3) all but three cases antibody positive against HTLV-I were also reactive against HTLV-III, the three exceptions being the T-cell lymphoma, a hemodialyzed and a severe hemophiliac A patient.

Representative results of the Western blot analysis on serum samples are shown in Fig. 1. It is clear that antibodies to HTLV-III reacted markedly with a 41,000 dalton protein which is likely to represent a viral envelope component [22]. Other HTLV-III antigens recognized by the majority of sera are 24,000 and 15,000 dalton proteins that may represent *gag* gene products [22]. In some cases also proteins of 31,000, 55,000 and 65,000 daltons are detected by the same sera.

HTLV-I strip radioimmunoassay showed a main pattern of reaction with viral antigens of 46,000, 32,000, 24,000, 19,000 daltons (Fig. 1, panel B).

DISCUSSION

The pattern of HTLV-III antibody prevalence found in the Veneto region both in asymptomatic people at risk and in LAS or AIDS patients is grossly similar to that reported in other European countries [2–9], indicating that the spread of HTLV-III infection for the time being is almost confined to the population belonging to classic risk groups. However, a few points merit discussion.

Firstly, subgrouping of hemophilia patients according to the severity of the disease and, consequently, to the amount of blood products administered resulted in a remarkable difference in the percentage of HTLV-III seropositivity. This could be explained as a result of both a statistical increase of risk for HTLV-III infection, and a higher antigenic burden that may contribute to the establishment of a persistent HTLV-III infection. Also of interest is the observation that patients with severe forms of hemophilia B had HTLV-III antibodies in 78% of cases. Although the number of patients is too limited to draw conclusions, it should be mentioned that in another study on hemophiliacs carried out in the south of Italy an HTLV-III antibody prevalence of 50% was found among hemophiliac B patients. (F. Lucivero, personal communication). Clearly, the source of commercial pooled blood products seems to have played a crucial role in determining the infection in these patients.

The HTLV-III antibody prevalence in homosexuals is somehow higher than that reported by others [2, 3]; these subjects represent however a rather selected group since the majority were partners of homosexuals already known to be positive for HTLV-III antibodies.

The 33% seropositivity found in the intravenous drug abusers is in line with other studies, the only discrepancy being the very low antibody prevalence (1.5%) observed in one British series [2]. From the public health point of view, however, the drug abusers deserve major consideration since, at least in our country, they are the principal category responsible for the emergence of a new group at risk as demonstrated by the frequent sero-conversion of their heterosexual partners and by the actual possibility of transplacental transmission of HTLV-III infection to the progeny (unpublished observation). Furthermore, as also shown by present results, the rate of LAS development in drug abusers is quite remarkable.

The fact that healthy as well as diseased controls were free from HTLV-III antibodies while patients with LAS or AIDS were seropositive in 90 and 100%, respectively, confirms once more the general idea that this retrovirus behaves not as an opportunist but should be regarded as the cause of AIDS and related syndromes.

In this connection the role of HTLV-I needs to be revised. Early studies on AIDS etiology suggested an involvement of HTLV-I since a significant number of patients contained in their sera antibodies reacting to membrane antigens of HTLV-I producing cells [16]. Moreover, opportunistic infections and other symptoms suggesting immunodeficiency were not seldom observed in leukemias and lymphomas associated with HTLV-I infection [19]. Following the discovery of the LAV/HTLV-III agent these data have been considered as due to some cross reactions between antigens of

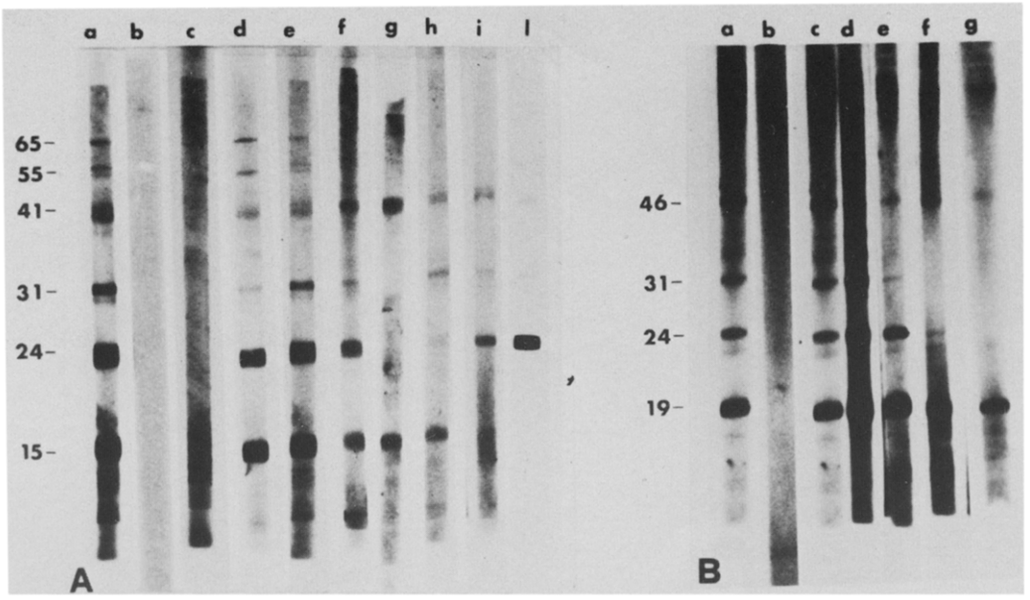

Fig. 1. *Representative samples of virus strip RIA (Western blot). Panel A. HTLV-*III *antigens reacting with sera from:* a, *representative positive control (US AIDS patient)*; b, *healthy negative control;* c, *diseased negative control (acute hepatitis B)*; d, *LAS patient;* e, *hemophiliac A patient;* f, *asymptomatic drug abuser;* g–h, *AIDS patients;* i, *asymptomatic homosexual;* l, *shows the reactivity found with cerebrospinal fluid from a thalassemic patient. Panel B. HTLV-*I *antigens reacting with sera from:* a, *representative positive control (U.S. adult T-cell lymphoma patient)*; b, *healthy negative control;* c–d, *lymphoma patient, with two different samples;* e, *hemophiliac A patient;* f–g *asymptomatic drug abusers.*

the two virus isolates. A more recent study by Tedder *et al.*, however, has shown that using different well defined assays antibodies to HTLV-I were detected in approx. 5% of LAS patients and in 1% of unselected homosexuals and drug abusers [17]. Robert-Guroff *et al.* similarly reported that antibodies to HTLV-I were detected in 7% of AIDS patients [23]. Our present results confirm that in asymptomatic individuals at risk for AIDS and in LAS patients, a low but definite (about 3.5%) antibody prevalence to HTLV-I is detectable. Particularly interesting was the finding that four out of 112 hemophiliacs were seropositive since it was at variance with negative results on HTLV-I infection reported in these subjects [17, 24]. Moreover, the absence of antibodies to HTLV-I p24 in the serum of Austrian and U.S. hemophiliacs with no symptoms of AIDS was interpreted by Chorba *et al.* as an indication of the lack of easy transmissibility of HTLV-I in lyophilized cell-free factor concentrates [24]. However, because the assay used detected only anti p24-antibody it could well be possible that, as also suggested by these authors, other HTLV-I antigens were missed. Actually, in our four cases, the strongest reaction seen in Western blots was directed against the p19 virus component, the p24 being detected in only one serum sample. On the other hand it is known that transmission of HTLV-I *in vitro* does not strictly require cell-to-cell contact but it may occur using cell-free materials [25, 26]. It has also been found that cloned lymphocytes can be doubly infected with HTLV-I and HTLV-III and that both viruses can be expressed in the same cell (A. De Rossi *et al.*, in preparation). Thus, while the possibility that a crossreactivity between antigens shared by HTLV-I and HTLV-III could not be ruled out we as others favoured the hypothesis that, albeit rarely, individuals might be infected by the two viruses.

This is also supported by our observation that the same serum sample from "doubly" seropositive donors recognized by Western blot, besides HTLV-III antigens, a p19 HTLV-I specific component. If the existence of a concomitant infection with the two HTLV isolates will be confirmed as a real possibility, increasing attention should be paid to these subjects in view of the fact that the oncogenic potential possessed by HTLV-I might be potentiated by a less efficient immune surveillance caused by HTLV-III.

A final point worthy of discussion is the usefulness of using another assay in addition to the ELISA to assess the specificity of serum diagnosis. Although in our study the percentage of samples positive for HTLV-III in ELISA but negative in Western blot (operationally defined as "false positive") were very low (4.2% in all cases examinated) with some of the presently available commercial kits it is reported to be much higher. Therefore the problem of confirming the specificity of a positive ELISA test must be seriously considered in view of the social, psychological and clinical problems inherent to the diagnosis of HTLV-III infection. Furthermore, the hypothesis that the reactivity pattern of sera revealed by the Western blot analysis may be of prognostic relevance for subjects at risk for AIDS (3) is interesting and must be further investigated.

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