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HTLV-II AMONG ITALIAN INTRAVENOUS DRUG USERS AND HEMOPHILIACS

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The seroprevalence of HTLV-I/II was evaluated in 1247 Italian individuals at high risk for HIV infection. The population studied consisted of 985 intravenous drug users (IVDUs), 474 of whom on methadone maintenance and 511 in a therapeutic community, 110 HIV-infected patients in various stages of HIV-related disease and 152 hemophiliacs. Sera were screened for antibody to HTLV-I/II by enzyme immunoassay (EIA) and confirmed by Western blot and radioimmunoprecipitation assay. Confirmed positive samples were further differentiated by EIA using HTLV-I and HTLV-II specific peptides. The overall prevalence of anti-HTLV-I/II was 4.0% in IVDUs, with the highest prevalence (8.2%) among HIV-infected symptomatic patients. None of the hemophiliacs was anti-HTLV-I/II positive, even though 63.1% tested positive for HIV antibodies. The trend of seroprevalence in drug users and the evaluation of possible risk factors demonstrated that HTLV-I/II infection has been present in Italy before the onset of HIV epidemic. The overall seroprevalence showed no significant changes during the 10 year period covered by this survey but correlated with HIV seropositivity, age and duration of drug use. Peptide testing showed that HTLV infection was mainly due to HTLV-II.

INTRODUCTION

Human T-cell lymphotropic virus type I (HTLV-I) is a type-C retrovirus which primarily infects T-cells (6, 16). This virus has been associated with adult T-cell leukemia, an aggressive form of cancer characterized by malignant T-cells with multilobed nuclei, lymphadenopathy, skin lesions due to leukemic infiltrations and hypercalcemia (9). HTLV-I has also been associated with a slowly progressive myelopathy which primarily affects the pyramidal tracts and to a lesser extent the sensory system. The

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syndrome is called tropical spastic paraparesis (TSP) or HTLV-I associated myelopathy (HAM) (5, 15).

Endemic regions for the HTLV-I virus were identified in Japan, Africa and part of the Caribbean (1, 7, 18). Relatively little is known for human T-lymphotropic virus type II (HTLV-II), an agent associated with a rare form of Leukemia (17). Unlike HTLV-I, for which an etiological role has been defined, any causal relationship to diseases has yet to be established for HTLV-II. With the exception of recent findings of HTLV-II in cohorts of IVDUs in the U.S. and Italy (2, 11) and in the native Indian population from the U.S. and Panama (8, 10), little is known concerning the epidemiological features of this virus. Although it is reasonable to anticipate that its mode of transmission would be similar to that of HTLV-I, its regions of endemicity are almost totally unknown.

Until recently, HTLV-II has been detected by cross reactivity to HTLV-I antigens. Established confirmation procedures such as Western blot (WB) and Radioimmunoprecipitation (RIPA) cannot distinguish between the two viruses. Differentiation has relied principally upon the use of polymerase chain reaction (PCR) which is not only labor intensive but requires lymphocytes which are often not available. In order to assess the seroprevalence of both HTLV-I and HTLV-II in different population groups we have developed an EIA based on synthetic peptides which can detected antibodies specific to HTLV-I or HTLV-II. We tested serum samples from 1247 subjects from Italy; the population was composed of patients with HIV-related disease, IVDUs, which represent the cathegory at highest risk for HIV-I infection in Italy accounting for more than 70% of AIDS cases, and whose sera were collected over a period of 10 years, and hemophiliacs.

MATERIALS AND METHODS

Subjects - Sera from a total of 1247 individuals belonging to three different groups were examined in order to estimate the annual HTLV prevalence rate. The study population included three groups at high-risk for HIV infection: a) 985 subjects with a history of intravenous drug use, consisting of 511 former drug users admitted to a therapeutic community for drug rehabilitation, which hosts mostly subjects from North-Central Italy, and 474 individuals on methadone maintenance treatment at different centers in the Lombardy region. Sera were collected between 1979 and 1988; b) 110 known HIV-positive IVDUs, hospitalized in an infectious diseases clinic in Milan during the period of 1987-88, consisting of 26 with AIDS, 28 with AIDS-related complex (ARC) and 56 with lymphadenophaty syndrome (LAS); c) 152 hemophiliacs, 123 with hemophilia A, 26 with hemophilia B and 3 with von Willebrand's disease, all attending a specialized hemophilia center in Milan from 1985-88. Gender was known for all subjects, age was known for 281 individuals and information on type and duration of drug use was available for 275 out of 511 subjects living in the therapeutic community. A single serum sample was collected from each individual and stored at -20°C until testing.

Serological testing - Antibodies to HIV were screened by EIA from different manufacturers. EIA positive samples were confirmed by WB and those with antibodies to at least two gene products were considered confirmed. Antibodies to HTLV-I/II were detected by EIA (Abbott Laboratories, North Chicago, Illinois) followed by WB and RIPA using a lysate from HTLV-I infected HUT-102 B2 cell line which was methabolically labelled with 35S cysteine and methionine (2).

A sample was considered confirmed by WB alone or by a combination of WB and RIPA according to the CDC criteria, that require the presence of antibodies to both *gag* (p 24 core or p 19 matrix protein), and *env* (gp-46/61) gene products (2, 21). Samples reacting with viral proteins from only one gene product or two gene products but without the presence of anti-p 24 were considered indeterminate.

Differentiation of HTLV-I from HTLV-II was carried out by an EIA using peptide sequences from the gp-46 regions as antigens on the solid phase. Briefly, amino acid sequences from the N terminal regions of gp 46 showing the least homology between the two viruses were synthesized and purified. Polystyrene beads were coated with the HTLV-I or HTLV-II specific peptides. Samples were incubated with the beads for 1 hour, washed and the solid phase probed with a goat anti-human IgG labeled with horseradish peroxidase. The substrate ortho-phenylenediamine was then added to reveal the bound antibodies. Known HTLV-I or HTLV-II positive sera validated by polymerase chain reaction (PCR) and viral isolation were used as positive controls (11, 19). Samples with an optical density greater than 3 times the negative control were considered positive.

Statistical analysis - The association between HIV and HTLV was evaluated by Chi-square test, and the odds ratio (O.R.) with 95% confidence limits was also calculated. The association between HTLV-seropositivity and sex, age and duration of intravenous drug use was assessed by the use of odds ratio (O.R.), and statistical significance was evaluated by Chi-square for trend.

To further evaluate the prevalence rates of HTLV and HIV antibodies in IVDUs over time, the subjects were stratified according to the year of sample collection for those attending methadone clinics or according to the year of entry for those who remained as residents of the rehabilitation community. Given that no further parenteral drug exposure could take place for those IVDUs who remained in the

TABLE 1. - Prevalence of anti-HIV and anti-HTLV among Italian intravenous drug users.

Group	N	Anti-HIV+ (%)	Anti-HTLV+ (%)
IVDUs (Methadone centers)	474	148 (31.2%)	14 (3.0%)
Former IVDUs (Community)	511	205 (40.1%)	21 (4.1%)
HIV-related disease	110	110 (100 %)	9 (8.2%)
Total	1095	463 (42.3%)	44* (4.0%)

* Differentiation between HTLV-I and HTLV-II by synthetic peptides showed that 40/44 (90.0%) anti-HTLV seropositive were due to HTLV-II. The remaining four gave a cross-reaction against both HTLV-I and HTLV-II peptides.

community, this stratification would give a good identication of the temporal evolution of both HIV and HTLV infections.

RESULTS

Seroprevalence of anti-HIV and anti-HTLV-I/II

None of the 152 hemophiliacs examined (of whom 63.1% were anti-HIV positive) was seropositive for HTLV-I/II, whereas 48 serum samples collected from IVDUs were repeat reactives by EIA. When tested by Western blot, one of the 48 EIA positive samples was negative and 47 gave indeterminate results, showing a strong reactivity against p24 and occasionally against p 19 core proteins. RIPA testing of the 47 WB indeterminates showed antibodies against both *env* and *gag* proteins in 28 samples, against *env* gp61 only in 16 samples and against "core" p 24 only in one sample. Additionally, 4 samples were reactives also against tax/rex p 40X. By combining WB and RIPA results, 44/48 EIA repeat reactives (91.7%) were confirmed as positive. Thus, among IVDUs, the overall prevalence of HTLV-I/II antibodies was 4.0% (44/1095) with a peak of positivity (8.2%) among those with HIV-related disease. The prevalence of HIV antibodies in the two groups of asymptomatic IVDUs, that included the subjects in methadone maintenance and the hosts of the therapeutic community, was 35.6 (Table 1).

HIV seropositive IVDUs were more likely to have anti-HTLV-I/II antibodies than HIV-negative subjects. Seropositivity rate was 6.4% in HIV-positives and 2.5% in HIV-negatives ($p < 0.002$); the O.R. was 2.75 (95% C.I. = 2.27-3.33). Information on age was available for 281 individuals and on duration of addiction for 275 subjects. The proportion of HTLV-seropositive subjects increased significantly with both age and duration of intravenous drug use (Table 2). No differences of HTLV infection was found between males (35/843, 4.1%) and females (9/252, 3.6%).

TABLE 2. - Association between HTLV seropositivity, age and duration of intravenous drug use.

Age (years)	N	Anti-HTLV+ (%)	O.R.
< 25	50	1 (2.0%)	1
26-30	143	9 (6.3%)	3.3
31-35	73	8 (10.9%)	6.0
> 36	15	3 (20.0%)	12.3
$p < 0.01$ (Chi-square for trend)			
Years of addiction	N	Anti-HTLV+ (%)	O.R.
1- 5	127	5 (3.9%)	1
6-10	126	7 (5.5%)	1.4
> 10	22	4 (18.2%)	5.4
$p < 0.05$ (Chi-square for trend)			

Temporal pattern of anti-HIV and anti-HTLV

As shown in figure 1, HTLV-I/II infection was already present in 1979-80 at rate of 8.2% (4/49). From 1981 to 1988 the rate of HTLV-I/II infection remained between 2.3% an 6.6%, showing no significant difference in seroprevalence rates among years. Infected individuals per number tested for each successive year were 2/79, 5/76, 3/100, 2/98, 3/131, 8/139 and 8/313, respectively. In contrast, HIV infection was absent in 1979-80, appeared in 1981 at 1.3% (1/79) and thereafter increased to 26.3% (20/76) in 1982, 36% (36/100) in 1983, 38.8% (37/98) in 1984 and reached a plateau of approximately 45% from 1985 onwards (60/131, 61/139 and 138/313 for 1985, 1986 and 1987-88 respectively) (Fig. 1).

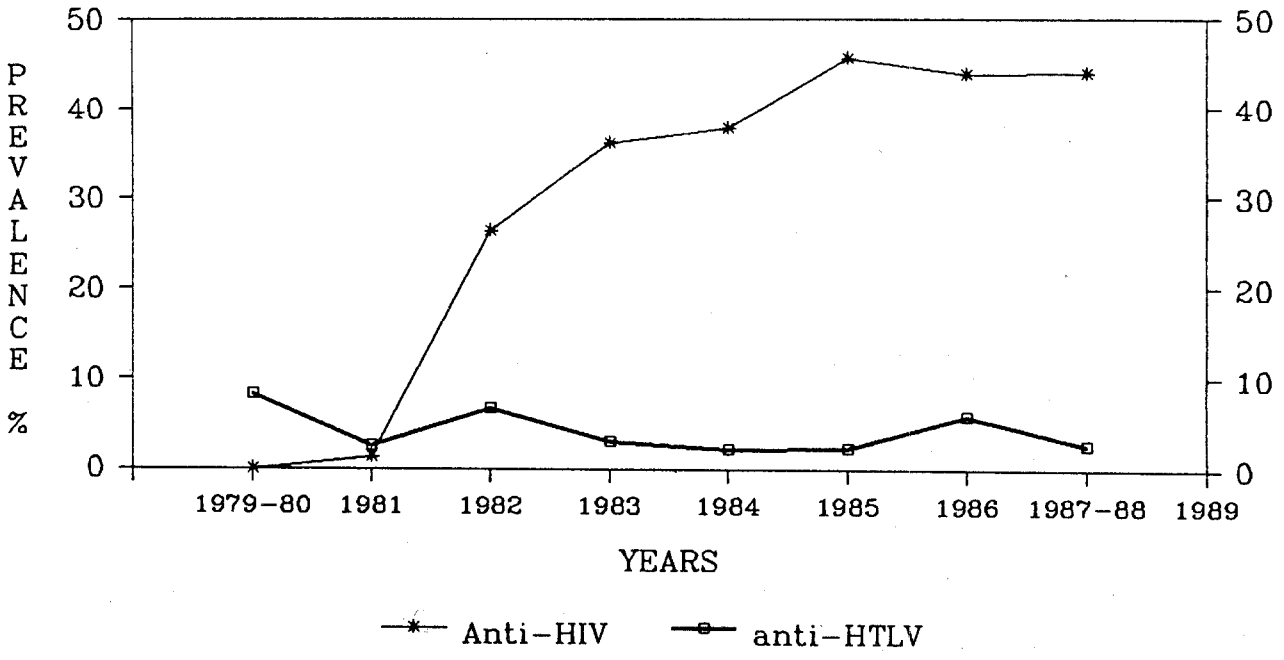

Figure 1. - HIV and HTLV seroprevalence by year among Italian IVDUs. Cumulative data from 474 IVDUs in methadone maintenance (y. of sampling) and 511 former IVDUs in community (y. of entry).

Discrimination of HTLV-I and HTLV-II by peptide assays.

None of the 44 samples collected from the HTLV seropositive subjects reacted against HTLV-I *env* peptide alone, while 40 (90.9%) gave a positive signal when tested against HTLV-II peptide and 4 cases cross-reacted against both HTLV-I and HTLV-II peptides.

DISCUSSION

Infections with human T lymphotropic viruses (HTLV-I and HTLV-II) have been well documented among intravenous drug users in several US cities as well as in Europe (14) and Italy (3, 13, 20). HTLV-I and II are closely related viruses, showing a 75% homology that results in a high level of cross-reactivity between antibodies to each virus. Consequently, current serological assays for anti-HTLV-I can also detect antibodies directed against HTLV-II. Thus, we may speculate that the HTLV-I seroprevalence reported in studies which did not differentiate between HTLV-I and HTLV-II may be overestimasted, since a number of the presumed HTLV-I infections might be due to HTLV-II. For example, by using the polymerase chain reaction (PCR) method, Lee *et al.* (11) showed that the majority of apparent HTLV-I seropositive IVDUs in New Orleans were indeed infected by HTLV-II. This finding has been confirmed in successive surveys and also signalled in Italy (22).

Our study shows that none of the 152 hemophiliacs and 4% (44/1095) of the examined IVDUs had antibodies to HTLV-I/II when screened by HTLV-I EIA and confirmed by WB and RIPA. In our hands, WB has been shown to be inadequate as supplemental assay for the confirmation of specific antibody reactivities to HTLV-I/II. The high number of WB indeterminate results (47 out of 48 EIA repeat reactives), consisting of patterns of antibody bands to *gag* (core and occasionally matrix) gene products alone, might be due to the relative low concentration sensitivity of *env* gp 46 proteins (12). However, using the additional test RIPA, a method which is known to be more sensitive than WB in detecting antibodies to *env* proteins, 44 out of 47 WB indeterminates were confirmed as HTLV-I/II positives.

Differentiation between anti-HTLV-I and anti-HTLV-II using EIAs with specific peptides for the two viruses indicates that at least 91% (40 out of 44) of anti-HTLV confirmed positives were due to HTLV-II. A clear differentiation was not achieved in the remaining four cases since they gave reactions against both HTLV-I and HTLV-II peptides. Interference due to a cross-reactivity between the two viruses or a dual infection might explain this finding (12).

On the whole these results indicate that, as seen in the US, the Italian HTLV seropositive IVDUs are primarily infected with HTLV type II. The availability of simple serological tests capable of differentiating between HTLV-I and HTLV-II is needed in order to better define the epidemiology and clinical relevance of these two retroviruses. Additionally, since, in

contrast to HTLV-I, HTLV-II has not yet been consistently associated with any disease, such tests will be helpful also for counseling purposes.

From our data it also emerges that HTLV-II infection was present among Italian IVDUs before the beginning of HIV epidemic. In this regard, it is interesting to note that the temporal analysis of the two retroviral infections among IVDUs over a period of ten years (1979-1988) showed a significant difference in the rate of infection between HIV and HTLV.

In fact, whereas the seroprevalence rate of anti-HTLV-II showed minor fluctuations by year, ranging between 2.3% and 8.2%, HIV antibodies first appeared in 1981 (1.3%) and rapidly increased thereafter to a peak of 45% in 1985-88, suggesting that HTLV-II is an older infection that is spread less efficiently than HIV. This probably reflects a lesser infectivity of HTLV-II, which is transmitted via infected lymphocytes rather than by both infected cells and freee virions as is the case in HIV (12). Demographic data showed that HTLV-II seroprevalence was found to increase with both age and duration of intravenous drug addiction while no difference was seen among genders. It is noteworthy that the HTLV-II seroprevalence was significantly higher in anti-HIV positive than in anti-HIV negative IVDUs (6.4% vs. 2.5%, $p < 0.002$).

Finally, the absence of HTLV antibodies among hemophiliacs, most of whom (63%) were anti-HIV positive, confirm and extends previous observations (2) indicating that cell free plasma is not able to transmit this infection. This is also consistent with the *in vitro* studies on HTLV, where efficient infection of target cells requires cocultivation of infected cells.

While HTLV-I has been reported to accelerate HIV-related disease (4), little is known concerning the effect of HTLV-II on the course of HIV infection. A prospective study is in progress in order to clarify this important issue.

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