DETECTION OF IgG ANTIBODIES TO LYMPHADENOPATHY-ASSOCIATED VIRUS IN PATIENTS WITH AIDS OR LYMPHADENOPATHY SYNDROME

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Summary
IgG antibodies to lymphadenopathy-associated virus (LAV) were found by a specific enzyme-linked immunosorbent assay in 18/48 (37.5%), patients with acquired immunodeficiency syndrome, 38/51 (74.5%) patients with lymphadenopathy syndrome, 8/44 (18%) homosexual men without lymphadenopathy, and 1/100 unselected blood donors.

Introduction
Epidemiological studies of acquired immunodeficiency syndrome (AIDS) have suggested that an infectious agent is involved in the transmission and pathogenesis of this newly recognised disease.1

Human T cell leukaemia virus (HTLV-I) has been isolated from three patients with AIDS,2 proviral DNA sequences have been found in peripheral-blood T lymphocytes from 2 of 33 AIDS patients,3 and antibodies to HTLV-associated-membrane antigens (HTLV-MA) have been detected in 25–36% of patients with AIDS.4

We have isolated a new human retrovirus from cultures of T lymphocytes from the lymph-node of a homosexual man with lymphadenopathy syndrome (LAS).5 The virus has been named lymphadenopathy associated virus (LAV). Other viruses, similar or identical to LAV, have been isolated from several patients with frank AIDS or at risk of AIDS. They include a virus isolated from a homosexual man with Kaposi’s sarcoma (KS) and viruses isolated from two siblings with haemophilia B, one of whom had AIDS.6,7 All these viruses had common characteristics—ie, magnesium-dependent reverse transcriptase, similar appearances by electron microscopy,6 a selective tropism for the OKT4 lymphocyte subset6 (D. Klatzmann et al, unpublished) and an antigenically related major protein p25. Differences between these viruses may be revealed by further characterisation. However, they clearly differ from HTLV-I morphologically and because their p25 protein and that of HTLV-I (p24) are antigenically unrelated.

We set up an enzyme-linked immunosorbent assay (ELISA) to determine the presence of specific IgG antibodies to the first isolate of LAV in patients with AIDS and LAS. The results were compared with those obtained in a radioimmune precipitation assay (RIPA) of LAV p25. In addition, HTLV-I antibodies and cytomegalovirus (CMV) IgG were measured in the same patients to determine whether there were correlations between these three factors.

Materials
Serum samples were obtained from 4 groups—patients with LAS, patients with AIDS, healthy homosexual men, and controls.

LAS—51 patients with LAS were selected. They met the following criteria9—unexplained lymphadenopathy for at least 3 months, involving two or more extra-inguinal sites; no current illness or drug use known to cause lymphadenopathy; reactive hyperplasia in a lymph-node established by biopsy. 40 patients were homosexual men, 4 also being heroin addicts; 8 patients were intravenous drug abusers only (2 women and 6 men); and 3 were Haitian patients who had been living in France for about two years (1 woman and 2 men). They were not homosexuals or drug abusers. Patients with LAS were aged from 23 to 54 years (mean 31 years). Biopsy specimens of lymph-nodes from 15 patients showed the typical follicular hyperplasia found in LAS.9 Serum samples were usually collected at the first clinical examination; all patients had
had LAS for more than 3 months and some had also had fever or weight-loss.

AIDS.—Sera from 48 patients with AIDS were collected. They included 1 with β-thaemophilia, 35 homosexual men, 4 Haitians, and 8 Africans (6 from Zaïre, 1 from Congo, and 1 from Cameroon). The Haitian and African patients denied any homosexual activity or drug abuse. 30 of the AIDS patients had opportunistic infections, 12 presented with Kaposis's sarcoma (KS), 5 had both opportunistic infections and KS, and 1 had a brain lymphoma. The sera tested were not always collected at an early stage of the disease; in some cases, they were taken at a very late stage. Whenever possible, sera taken at different times were assayed. All sera were stored at −70°C and some were thawed and frozen several times before being tested for LAV by ELISA.

Homosexual men.—Serum samples were taken from 44 homosexual men without LAS who visited a venereal disease clinic in Paris. Their mean age was 30 years.

Controls.—Sera were also taken from 100 unselected blood donors (mean age 38 years) and from 30 healthy laboratory workers.

### Methods

**Antigen preparation.**—Activated T lymphocytes from a healthy donor were infected with LAV. The culture conditions have been described elsewhere.\(^6\) Cell-free supernatant was precipitated with 10% polyethylene glycol, then the virus was banded twice to equilibrium in a 20–60% sucrose gradient. After concentration by high-speed centrifugation, the viral pellet was resuspended and lysed in a RIPA buffer containing sodium dodecyl sulphate (SDS) (0–5%) for 15 min at 37°C. A control preparation was made from uninfected, cultured T lymphocytes from the same donor. Cells were lysed by a RIPA buffer containing 0–5% SDS. After centrifugation of the cell extract at 10 000 g for 10 min, the supernatant (crude cytoplasmic extract) was used as control antigen.

The protein concentrations of the viral and control antigens were measured by the method of Lowry et al.\(^10\)

**LAV ELISA procedure.**—The assay was carried out on Nunc ELISA microtitre plates. The amount of coated antigen was determined by block titration with several dilutions of antigens and anti-IgG, against dilutions of a control positive serum from the patient in whom LAV was isolated. We used a protein concentration of approximately 2–5 µg/ml to coat the plates. Viral or control antigens (100 µl) diluted in carbonate buffer (pH 9.6) were incubated in wells overnight at 4°C. After one wash with deionised water, 300 µl of formalin (5%) was added for 10 min at room temperature. Plates were rinsed once with phosphate buffered saline (PBS) and filled with 1% bovine serum albumin (BSA) for 2 h at 37°C. After one wash with PBS—Tween 20 (0.05%) plates were stored at −80°C. 100 µl of a 1/40 dilution of each serum (in PBS—Tween 20/0.5% BSA) was added per well and duplicate dilutions were incubated on viral and control antigens for 90 min at 37°C. After four washes, 100 µl of an appropriate dilution of antihuman-IgG conjugated with peroxidase was added to each well for 1 h at 37°C. After five washes 100 µl of a substrate solution was added (0–3% orthophenylene-diamine and 0–03% hydrogen peroxide in citrate buffer [pH 5.6]). The reaction was allowed to proceed for 20 min in the dark at room temperature and then was stopped with concentrated sulphuric acid. The absorbances (optical density, OD) were measured at 492 nm. Sera were regarded as positive when \(\Delta\ OD\geq 0.30\) (\(\Delta\ OD = OD\ virus - OD\ control\ antigen\)). The control positive serum at four dilutions and a control negative serum were tested on each plate, to evaluate the reproducibility of the test. The standard curve was used to calculate the antibody titre.

**LAV RIPA.**—The method is described elsewhere in detail.\(^5\)

### Results

#### Lymphadenopathy Syndrome Patients

38 of the 51 LAS patients (74.5%) (see table) had IgG antibodies to LAV. Sera from 29 of the 40 homosexual men, 6 of the 8 drug addicts, and all 3 Haitian patients were positive. ELISA (Bionetics) demonstrated p24 HTLV-I antibodies in the sera of 5 of the 51 LAS patients—ie, 4 homosexual men and 1 Haitian (4 of them also had antibodies against LAV). Only 1 homosexual man was positive for p24 HTLV-I by RIA (L. Schaffar, personal communication). 12 (24%) patients (10 homosexuals and 2 heroin addicts) did not have antibody to LAV or to HTLV-I.

In 12 patients blood samples collected at different stages of the disease were available. Except for patient 19 who only had antibodies to LAV 2 years after the onset of LAS, all sera remained consistently positive or negative according to the result at the first determination.

The virus used for antigen preparation was isolated from patient 1. Viruses identical to LAV were isolated from cultured T lymphocytes derived from lymph nodes of patients 49 and 51. Both were positive for LAV antibodies. AIDS subsequently developed in patient 51. He is a French homosexual who had lived for 2 years in Haiti (1980–81). After his return to France a persistent fever developed in January, 1982, LAS developed in March 1983, and KS (mucosal and cutaneous) developed in June, 1983. He had had antibodies to LAV since January, 1982, and presumably was infected before any sign of disease appeared.

<table>
<thead>
<tr>
<th><strong>SEROLOGICAL TESTS</strong></th>
<th><strong>LAV IgG ELISA</strong></th>
<th><strong>p24 HTLV-I ELISA</strong> (^*)</th>
<th><strong>CMV IgG ELISA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AIDS</strong></td>
<td>18/48 (37.5%)</td>
<td>6/46 (12.5%)</td>
<td>45/46 (98%)</td>
</tr>
<tr>
<td>opportunistic infection (OI)</td>
<td>12/30</td>
<td>5/30</td>
<td></td>
</tr>
<tr>
<td>Kaposis's sarcoma (KS)</td>
<td>3/12</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>OI + KS</td>
<td>2/5</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Brain lymphoma</td>
<td>1/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Haemophilia B</td>
<td>1/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Homosexual men</td>
<td>10/35</td>
<td>4/35</td>
<td></td>
</tr>
<tr>
<td>Haitians</td>
<td>3/4</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>Africans</td>
<td>4/6</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td><strong>LAS</strong></td>
<td>38/51 (74.5%)</td>
<td>5/51 (94%)</td>
<td>46/50 (92%)</td>
</tr>
<tr>
<td>homosexual men</td>
<td>29/40</td>
<td>4/40</td>
<td>37/40</td>
</tr>
<tr>
<td>Drug addicts</td>
<td>6/8</td>
<td>0/8</td>
<td>6/7</td>
</tr>
<tr>
<td>Haitians</td>
<td>3/3</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Homosexual controls</td>
<td>8/44 (18%)</td>
<td>0/44 (&lt;1%)</td>
<td>41/44 (93%)</td>
</tr>
<tr>
<td>Blood donors</td>
<td>1/100 (1%)</td>
<td>0/100</td>
<td>45/100 ND</td>
</tr>
<tr>
<td>Laboratory workers</td>
<td>0/30</td>
<td>0/30</td>
<td></td>
</tr>
</tbody>
</table>

\* When all sera were tested by p24 HTLV RIA (L. Schaffar) only one (from a homosexual man with LAS) remained positive.

\(\dagger\) 3/6 were also LAV positive.

\(\#\) 4/5 were also LAV positive.

92% of patients were positive for CMV IgG. There was no correlation between LAV seropositivity and CMV seropositivity and no correlation between the decrease of T4/T8 ratio and the presence of LAV antibodies. In general the OKT8 peripheral-blood lymphocyte subset was significantly increased, and positivity studies in 15 patients demonstrated a homogeneous increase of OKT8 cells in the germinal centre and perifollicular areas of lymph-nodes.

In 18 cases we also determined antibodies to LAV p25 by RIPA. In 13 of these cases ELISA and RIPA results were in
accord. In the 5 other cases, antibodies to LAV p25 were detected by RIPA but not by ELISA.

AIDS Patients

18 (37.5%) of the 48 patients with AIDS diagnosed in France were positive for LAV antibodies by ELISA and 6 (12.5%) had HTLV-I antibodies. However, none of these sera was positive for p24 HTLV; antibodies when tested by RIA (L. Schaffar, personal communication). 12 (40%) of the 30 patients with opportunistic infections had antibodies to LAV, 5 (13%) had antibodies to HTLV-I (Bionetics), and 1 was positive for both. Of 12 patients presenting with Kaposi’s sarcoma (KS), 3 (25%) were positive for LAV and none for HTLV-I (Bionetics). Of 5 patients with opportunistic infections and KS, 2 had LAV antibodies and 1 of them also had HTLV-I antibodies (Bionetics). The patient with brain lymphoma had antibodies to LAV only.

The following had LAV antibodies: the haemophiliac, 10/35 homosexual men, 3/4 Haitians, and 4/8 Africans. In France no cases of AIDS have been reported in drug addicts. In 2 patients (1 and 39) serological investigations were conducted before the onset of AIDS. Both had LAV antibodies in the first serum tested. In patient 1 antibody titre decreased (from 1/160 to 1/40) at the onset of AIDS, whereas it remained the same (1/60) in patient 39. In a few other cases where sera taken during the development of AIDS were analysed antibody titre remained the same (34) or declined (29). HTLV-I antibodies (ELISA) were also negative in the last serum sample from patient 29, while CMV IgG titre remained unchanged.

Retroviruses similar to LAV were isolated from several AIDS patients. They were named immuno deficiency associated virus (IDAV). IDAV-I was isolated from patient 38 (KS). He was seronegative by ELISA against LAV but positive when his own virus was used as the antigen for ELISA. IDAV-II was repeatedly isolated from peripheral lymphocytes of a patient with haemophilia B (1). This patient’s healthy brother (also a haemophiliac) had LAV IgG antibodies and a virus similar to IDAV-II was isolated from his peripheral-blood lymphocytes. Their parents were negative for LAV IgG. IDAV-III was isolated from peripheral-blood lymphocytes of a Zairian woman (6) who had emigrated to France (C. Leport, et al, unpublished). Antibodies to LAV were not detected by ELISA, but they were detected by RIPA. Patient 6 was positive for HTLV-I antibodies by ELISA (Bionetics) but negative by RIPA and RIA (L. Schaffar, personal communication).

Sera of 25 patients were also tested for LAV by RIPA. In 18 cases results coincided with those obtained by ELISA. In 5 cases antibodies to p25 LAV were detected by RIPA and not by ELISA as in patients 38 and 6 mentioned above. However, in 2/25 cases LAV RIPA was negative and LAV ELISA was positive.

Control Groups

8 (18%) out of 44 healthy homosexual controls were LAV positive. All but one of the LAV-positive homosexuals had had more than 50 partners in a year. All were negative for HTLV-I (ELISA), 41/44 (93%) had CMV IgG. Among blood donors and healthy laboratory workers only one had LAV antibodies by ELISA and was negative by LAV RIPA. In this last group, 45% were seropositive for CMV IgG, as expected for a 30–40 year old North European population.

Discussion

LAV and IDAV isolates I, II, and III appear to belong to a new group of viruses which have the usual characteristics of retrovirus—ie, release by budding at the plasma membrane; reverse transcriptase able to make DNA from both endogenous and exogenous templates, and high molecular weight RNA. All the isolates have a similar morphology by electron microscopy and an antigenically related major core protein (p25). We set up an ELISA with purified virus disrupted by SDS. It is likely that the core proteins are the main antigens detected by this test. The p25 protein is the only protein labelled with 35S methionine which is recognised by sera of patients in RIPA. No antibodies against an envelope protein could be detected in RIPA after metabolic labelling of the virus with 35S methionine or 35S cysteine. A few sera were positive by ELISA and negative by p25 RIPA, suggesting that such sera contained antibodies against viral proteins which have not yet been identified.

Sera from some AIDS patients bound a lot of cellular protein. In ELISA this problem was overcome by comparing the serum binding to the viral antigen with binding to a lysate of uninfected lymphocytes. This binding was apparent in the RIPA and only sera which specifically precipitated the p25 were regarded as positive. Such control was not possible when a commercial ELISA was used to detect p24 HTLV, and false-positive results could only be eliminated by RIPA or RIA. Only one serum from an LAS patient (2) was p24 antibody positive by RIA (L. Schaffar, personal communication). p25 antibodies were detected by LAV RIPA in some sera which were negative by ELISA. In ELISA sera were tested at quite a high dilution (1/40).

The fact that an AIDS patient (38) had no antibody to LAV by ELISA but showed a positive antibody titre when his own virus was used as antigen may indicate antigenic differences between LAV isolates.

A high proportion of patients with LAS (74.5%) had IgG to LAV and therefore were, or had been, infected with this virus or a related virus. Most LAS patients have no outward signs of immunosuppression. Our LAS patients consistently showed an increase of OKT8 cells in peripheral blood and lymph-nodes. This increase of suppressor and cytotoxic cells suggests an immune reaction against a viral antigen. The high proportion of LAS patients who were LAV seropositive also showed antibodies to the LAV being involved in the aetiology of LAS. LAS antibodies were also present in a considerable number (18%) of healthy homosexual men who had multiple partners and venereal diseases—a group at high risk of AIDS.

Less than 1% of blood donors and laboratory workers were LAV seropositive. Unlike CMV, LAV is not ubiquitous in the French population.

AIDS patients were significantly more likely than the control groups to be LAV-positive (37.5%) but they were less likely than the LAS group to be LAV-positive. One explanation for this may be that LAV is more closely related to lymphadenopathy syndrome than to AIDS. It may be regarded as one of the opportunistic viral agents found in AIDS. Although HTLV-I is one of the viruses implicated as having a role at the onset of AIDS none of our AIDS sera had antibodies to HTLV major core protein (p24) by RIA. Reports from the USA indicate that antibodies to antigens expressed on the cell surface of HTLV-I transformed lymphocytes (HTLV-MA) were detected in the sera of 25–36% of AIDS patients, 25–30% of patients with LAS, and 1% of matched homosexual controls or blood donors.

Another explanation is that the severe immune impairment at the late stage of AIDS affects B lymphocytes and that this may make the humoral response against viral proteins undetectable. Several examples support this hypothesis. Patient 29 was LAV positive at the beginning of AIDS and...
then became negative at a later stage. Similarly the haemophilic had a decreased titre of IgG LAV antibodies at the onset of AIDS although the retrovirus was repeatedly isolated from his peripheral T lymphocytes. A high proportion of the sera which had been collected at quite a late stage of AIDS.

There are indications in this study that LAV infection is present in AIDS patients living in the USA and in equatorial Africa. Prospective seroepidemiological studies are needed to establish the involvement of LAV in AIDS. Comparative studies in groups at high risk of AIDS and in control populations are underway in various countries.

Addendum

Since submission of this paper, we have introduced the following technical modifications to the ELISA—virus was purified three times in sucrose gradient and disrupted with 1% Triton X 100 and 0.1% sodium deoxycholate. These modifications have increased the sensitivity of the test. When tested by this method 75% of the AIDS patients and over 90% of the LS patients were LAV seropositive. Only 1 serum from 330 blood donors was LAV positive.

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This study was supported by grants B3C1411 and B3C1414 from the Ministry of Research and Industry. Tables showing individual results for LAV IgG, p24, HTLV-I antibodies, CMV IgG, and T4/T8 ratios in 51 LS patients and 48 patients with AIDS are available from F. B-V.

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REFERENCES


RANDOMISED TRIAL OF CHEMO-ENDOCRINE THERAPY, ENDOCRINE THERAPY, AND MASTECTOMY ALONE IN POSTMENOPAUSAL PATIENTS WITH OPERABLE BREAST CANCER AND AXILLARY NODE METASTASIS

Ludwig Breast Cancer Study Group*

Summary Adjuvant therapy after total mastectomy and axillary clearance in postmenopausal women with breast cancer and axillary node metastasis was assessed; chemo-endocrine therapy (cyclophosphamide, methotrexate, fluorouracil, prednisone, and tamoxifen; CMF+p+T) was compared with endocrine therapy (prednisone and tamoxifen; p+T) and with no adjuvant treatment in 463 patients aged 65 years. Endocrine therapy was compared with no adjuvant therapy in 320 patients aged 66-80 years. At median follow-up of 36 months, disease-free survival was significantly longer in CMF+p+T patients than in p+T or control patients; p+T also significantly increased disease-free survival. There were no significant differences in overall survival between the randomised groups. In analyses of patterns of first failure, chemo-endocrine therapy reduced local, regional, and distant relapses, whereas endocrine therapy reduced local and regional recurrences only.

Introduction

Postoperative adjuvant drug treatment in operable breast cancer can significantly reduce the rate of disease relapse.1-5 Multiple drugs are apparently superior to a single agent.6-7 Adjuvant endocrine therapy delayed recurrence and...