High prevalence of antibodies to acquired immune deficiency syndrome (AIDS)-associated retrovirus (ARV) in AIDS and related conditions but not in other disease states

(immunofluorescence assay/Epstein–Barr virus/T cells)

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ABSTRACT   A rapid, sensitive indirect immunofluorescence assay has been developed for detection of antibodies to the acquired immune deficiency syndrome (AIDS)-associated retrovirus (ARV). The human T-cell line HUT-78 was chronically infected with ARV-2 and used to detect antibodies to virus-specific cytoplasmic antigens. Because the helper T-cell marker Leu-3 is substantially reduced in this cell line after ARV infection, it appears to be an important receptor for virus infection. Nearly all patients with AIDS and most cases with related conditions showed antibodies against ARV. Some healthy individuals in risk groups for developing AIDS also had antibodies to the agent. In contrast, no antibodies to the virus were found in any individuals outside the risk groups for developing AIDS or with diseases other than those associated with AIDS. The titers of antibodies to ARV and to Epstein–Barr virus varied independently from each other. The level of anti-ARV antibodies in a patient's serum was found to reflect the severity of the disease; it was lower in individuals with more severe manifestations. Taken together, these data support the role of ARV in AIDS and its related disorders.

Acquired immune deficiency syndrome (AIDS), first described in 1981, is a multifaceted immunologic disorder which, after a long latency period, may ultimately lead to fatal opportunistic infections or malignancies. The syndrome is primarily found in certain segments of the population including homosexual and bisexual males, i.v. drug abusers, hemophiliacs, transfusion and blood-product recipients, Haitians, and Central Africans (1–5). The occurrence of this syndrome has increased dramatically over the last three years; more than 10,000 cases have been reported throughout the world, with over 1000 cases from San Francisco.

Our laboratory has isolated lymphocytopathic retroviruses called AIDS-associated retroviruses (ARV), which appear responsible for this disease (6). Similar viruses have been found in other laboratories and have been named lymphadenopathy-associated virus (LAV) (7), and human T-cell lymphotropic virus type III (HTLV-III) (8). These viruses have a distinct morphology by electron microscopy, possess a magnesium-dependent reverse transcriptase, cause fusion of T lymphocytes, and show preference for replication in T lymphocytes of the helper phenotype (6–9).

We report in this paper the development of a sensitive indirect immunofluorescence assay (IFA) for the detection of antibodies to ARV. Our results indicate a close correlation between AIDS or symptoms considered "pre-AIDS" and

detection of antibodies to this human retrovirus. ARV-specific antibodies are also prevalent in population segments that are prone to the development of AIDS. These antibodies have not been found thus far in individuals outside the known risk groups for AIDS. Moreover, many other disease states with immunologic dysfunctions are seemingly not associated with infection by this virus.

MATERIALS AND METHODS

Cells. The HUT-78 cell line, provided by A. Gazdar (National Institutes of Health), was originally derived from a patient with acute T-cell leukemia (10). It contains no detectable human retroviruses (11, 12). The line is grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells characteristically grow in clumps consisting of cells ranging in size from 9 to 15 μm.

Assessment of T-Cell Subsets. For determination of the major phenotypic markers of the cultured HUT-78 cells, they were stained with monoclonal antibodies with selected specificities (13). Cells were identified by use of simultaneous two-color immunofluorescence (13).

Virus and Sera. The AIDS-associated retrovirus, ARV-2, which was isolated from a homosexual man before he developed AIDS (6), was used for these experiments. Sera collected from patients were provided by the AIDS Clinic at San Francisco General Hospital (San Francisco) or by private physicians located throughout the United States. Sera from the Centers for Disease Control (Atlanta) were provided by H. Jaffe. All sera were heated at 56°C for 30 min prior to use.

IFA. For detection of antibodies to ARV, a HUT-78 cell line with 30–50% ARV-infected cells was used in an IFA (see below). HUT-78 cells were sedimented at 2000 rpm for 5 min at 4°C, washed in 10 ml of cold phosphate-buffered saline, and resuspended in phosphate-buffered saline at 10^6 cells per ml. Aliquots (10–20 μl) of the suspension, containing 5000–10,000 cells, were placed on 6-mm Hendley–Essex slides (Shandon-Southern, Sewickley, PA). They were allowed to air-dry and then were fixed in cold acetone for 15 min. They were used immediately after drying or stored at −20°C or −70°C for later use. The antigens in the cells are stable for 3 weeks at −20°C and 2 months at −70°C.

Abbreviations: AIDS, acquired immune deficiency syndrome; ARV, AIDS-associated retrovirus; IFA, indirect immunofluorescence assay; EBV, Epstein–Barr virus.
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Heat-inactivated sera, diluted 1:10 in commercial FA buffer (pH 7.2; Difco), were applied to the slides (20 μl per well). The slides were incubated at 37°C in a humidified chamber for 30 min. Excess serum then was aspirated off with a Pasteur pipette. Slides were washed in FA buffer for 5 min with slow stirring, patted dry, and overlayed with 20 μl of fluorescein-conjugated goat anti-human IgG (Cooper Biomedical, Malvern, PA) at a 1:50 dilution. Slides were incubated again for 15 min and then washed for 5 min in fresh FA buffer. They were dried at 37°C and examined immediately with a Leitz fluorescence microscope at ×400 magnification. A buffered (pH 8.0) glycerol or an elvanol solution was placed on the slides, followed by a coverglass. This procedure preserved the fluorescence for several days. Sera were considered positive for ARV antibodies when they stained 10–40% of the cells specifically for cytoplasmic ARV antigens. When a weak or negative reaction was obtained with a 1:10 serum dilution, a 1:5 dilution was tested.

RESULTS

Establishment of the Infected HUT-78 Cell Line. Filtered medium from a peripheral mononuclear cell culture releasing ARV-2 was used to infect HUT-78 cells in the presence of Polybrene (1 μg/ml) (6). Reverse transcriptase assays were conducted on the supernatants every 3–4 days; by day 15, 10^5 cpm were detected per ml of supernatant fluid (6). An IFA performed at that time, using an AIDS patient's serum, indicated that 1–3% of the cells were infected with ARV-2. These cells were mostly multinucleated giant cells with reticular cytoplasmic staining (Fig. 1A). The infected cell line was cocultured with fresh, uninfected HUT-78 cells every 7–10 days in the presence of Polybrene. Over a period of 2–3 weeks, the percentage of infected cells increased to 30% and was associated with substantial cellular destruction. Many cells showed characteristic cytopathology with large multinucleated forms and "ballooning." Without further addition of HUT-78 cells, a highly infected cell line, not showing any cytopathology, was gradually established, with

immunofluorescence-positive cells fluctuating between 80 and 100% (Fig. 1B). This cell line (called E) consists primarily of a large cell type 15 μm from which smaller cells (10 μm) are derived. Similar cell types are seen in the uninfected HUT-78 line.

Characteristics of the Infected E Line. For detection of ARV-2 in the E line, an IFA was performed with sera from AIDS patients (6). By this assay, the ARV-2 antigens stained in four distinct patterns: reticular or diffuse cytoplasmic staining (Fig. 1 A and C); crescent staining (Fig. 1A), which detected viral antigens in a localized region at the periphery of the cell; and a pattern in which highly concentrated foci of viral antigen were localized in the cytoplasm of multinucleated cells (Fig. 1B). The reticular cytoplasmic staining has been observed only during early viral infection. It has not been seen in the established E line.

On maintenance of the E line at 34°C or 39°C, viral protein synthesis decreased substantially so that an IFA-positive AIDS patient's serum stained the cells only weakly. Moreover, virus reverse transcriptase activity in the supernatant decreased by a factor of 10–30 (data not shown). Upon return to 37°C, both antigen and virus production resumed. We have also seen fluctuations in antigen-expression when the E line was kept in culture without a change of medium or when it was switched from one growth medium to another or to heated (56°C, 30 min) fetal calf serum. In brief, cells kept in log phase expressed the highest levels of viral antigen.

Establishment of the IFA for Detection of Anti-ARV Antibodies. To detect antibodies to ARV, we developed an IFA whose specificity was controlled by mixing (1:1) logarithmically growing E-line cells with uninfected HUT-78 cells. In this manner, nonspecific reactions became evident when both infected and uninfected cells were stained. Sera that stained ARV antigens in distinctive patterns (Fig. 1) in at least 10% of the cells were considered positive. With some sera, a prozone was noted: dilutions of 1:5 and sometimes 1:10 were negative by IFA, but further dilutions were positive. Such prozones could reflect antigen–antibody complexes. About 1% of sera stained all cells diffusely in a nonspecific pattern. This result indicated the presence of antibody to the T cells.
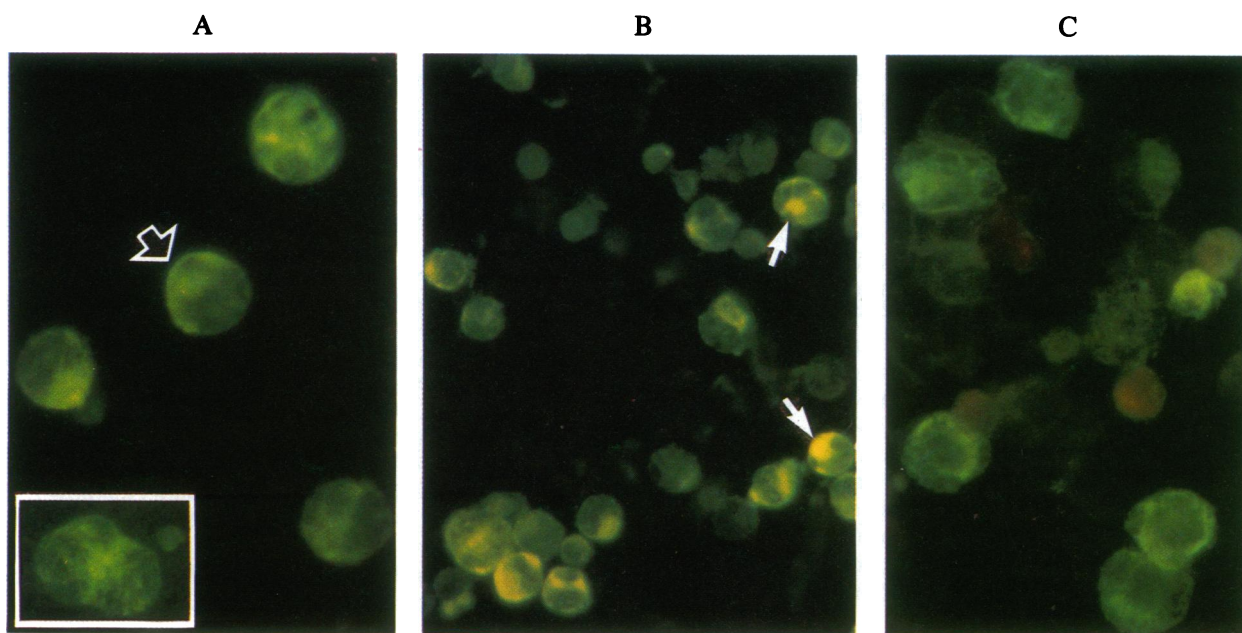
A   B   C

Fig. 1.   IFA using the HUT-78 cell line infected with ARV-2. A human serum with high antibody titer against ARV was used. (A) Note crescent staining in cell infected with ARV-2 (arrow). (×700.) (*Inset*) Multinucleated giant cell. Note reticular pattern in the cytoplasm. (×500.) (B) HUT-78 cell line 70% infected with ARV-2. Note the highly concentrated foci of viral antigen in multinucleated cells (arrows). (×525.) (C) The infected HUT-78 cell line mixed 1:1 with uninfected HUT-78 cells. Note the diffuse and reticular cytoplasmic staining in the ARV-2-infected cells. (×800.)

Table 1.   Phenotype of HUT-78 cells before and after infection with ARV

Immunological surface maker	Cellular subset detected	% positive cells	
		HUT-78	Line E
Leu-4	T cell	86	77
HLA/DR	Ia-like antigen	99	92
4/DR	Activated T cells	82	63
Leu-3	Helper/inducer T cells	67	23
Leu-8	Regulatory subpopulations of T cells (Leu-3$^+$ and Leu-2$^+$)	48	83
Leu-3/8	Inducer T cells for suppressors	43	20
Leu-2	Suppressor/cytotoxic T cells	4	7
Leu-15	Regulatory subpopulations of Leu-2$^+$ T cells	14	38
Leu-2/15	Suppressor T cells	3	7
Leu-9	T and null cells	17	42
Leu-M3	Monocytes/macrophages	2	
B1	B cells	16	

Such sera were adsorbed with uninfected HUT-78 cells to obtain interpretable results.

To determine whether infectious ARV can survive the acetone treatment used for the IFA, acetone-fixed E-line cells were scraped and cocultivated with normal human peripheral mononuclear cells for several weeks (6); no infectious virus was recovered.

Subset Analysis of Infected and Uninfected Cells. The HUT-78 cell line was examined for T-cell subsets by use of monoclonal antibodies (Table 1). They were nearly 100% Leu-4$^+$ and showed activation as demonstrated by the *HLA*-encoded DR antigen. No cells with the suppressor marker were present in the population, including the Leu-2$^+$/15$^-$ cytotoxic cell. The cells showed substantial staining with the Leu-3 monoclonal antibody. These results confirmed that HUT-78 is a T-cell line of helper subset. This phenotype has remained stable during over a year of continuous maintenance of the cell line in our laboratory.

After the HUT-78 cell line was 60% infected with ARV-2, the phenotype was again studied. It maintained its T-cell phenotype (Leu-4$^+$), but the number of cells staining for the helper subset (Leu-3$^+$) dropped substantially (Table 1). There was still no staining for the suppressor phenotype (Leu-2$^+$). The most likely explanation for this reduction in the phenotypic marker was that infection with ARV involved the helper T-cell marker. This observation, made also by others (9), agrees with recent studies indicating a receptor role of the helper T-cell marker in ARV virus infection (14–16).

Detection of Antibodies to ARV in Patients and Healthy Individuals. To determine the sensitivity and specificity of the IFA, we studied three separate groups of sera obtained from the West Coast (San Francisco), East Coast (Philadelphia), and the Centers for Disease Control (CDC). As shown in Table 2, we found a nearly 100% correlation between the

diagnosis of AIDS in an individual and the detection of antibodies to ARV. Antiviral antibodies were also found at high frequency in AIDS-related disease states and sexual contacts of AIDS patients. A lower, but substantial, number of clinically healthy homosexual men also had antibodies to ARV, as has been reported (6, 17–20). In the control population, none of 743 healthy individuals tested were positive for ARV antibody.

Comparison of ARV- and Epstein–Barr Virus (EBV)-Specific Antibodies. B-cell proliferation has been observed in many patients with AIDS and, occasionally, high titers of EBV-specific antibodies are found in these individuals. We therefore examined whether there was any correlation between the titers of antibodies to EBV and to ARV in individual cases. None was observed (Table 3).

Prevalence of Antibodies to ARV in Other Disease States. In examining whether the AIDS-associated retroviruses could be responsible for other diseases, we studied sera from a wide variety of patients with different clinical diagnoses (Table 4). These patients included those with systemic lupus erythematosus, rheumatoid arthritis, lymphoma, leukemias, and carcinomas and recipients of allografts. None of these were positive for antibodies to the virus. Antinuclear antibodies were detected in some sera from autoimmune patients but could be easily distinguished from antibodies to ARV.

Titers of Antibodies to ARV. Randomly selected sera from each of the four seropositive groups of individuals were titered to their end point by the IFA (Fig. 2). Clinically healthy homosexual men had the highest antibody titers (up to 1:10,000, with a reciprocal geometric mean of 4224), followed by patients with persistent lymphadenopathy syndrome (mean titer, 1694), Kaposi sarcoma (540), or opportunistic infections (*P. carinii* pneumonia) (364). In addition,

Table 2.   Prevalence of antibodies to ARV in the United States

Diagnosis	No. positive/no. tested			
	West Coast	East Coast	CDC*	Total (%)
AIDS with Kaposi sarcoma	147/147	NT	16/16	163/163 (100)
AIDS with opportunistic infections	62/62	9/10	22/22	91/92 (99)
Lymphadenopathy syndrome	55/59	19/21	20/20	75/79 (95)
Sexual contacts of AIDS patients	22/28	NT	NT	22/28 (78)
Clinically healthy homosexual men	133/290	NT	NT	133/290 (46)
Clinically healthy heterosexual individuals	0/93	0/192	0/58	0/343 (0)
Blood bank sera	0/200	0/200		0/400 (0)

NT, none tested.
*Centers for Disease Control, Atlanta.

Table 3. Relationship of serum antibodies against ARV and EBV in patients with AIDS and other diseases

Patient diagnosis	Antibody titer*			
		EBV		
	ARV	VCA (IgG)	D	EBNA
AIDS	1280	160	<10	80
AIDS	640	160	<10	40
AIDS	640	80	<10	5
AIDS	80	20	<10	5
AIDS	640	320	<10	20
LNS	2560	160	<10	80
LNS	2560	320	<10	>80
LNS	640	320	<10	80
Hepatosplenomegaly	–	5,120	1280	40
AML	–	10,240	1280	20
AML	–	1,280	640	320
Renal transplant	–	2,560	320	40
Lymphadenopathy	–	5,120	1280	40
Renal transplant	–	2,560	–	80
Mononucleosis	–	160	–	10
Ataxia	–	2,560	640	80

Antibodies to EBV were detected as described (21). Antibodies to ARV were detected by IFA. –, Not detectable. Abbreviations: VCA, viral capsid antigen; D, diffuse antigen; EBNA, nuclear antigen; LNS, lymphadenopathy syndrome; AML, acute myeloblastic leukemia.
*Numbers represent reciprocal of final positive serum dilution.

sera from those with early, moderate, or late-stage Kaposi sarcoma were titered. Individuals with more advanced Kaposi sarcoma had lower titers of ARV antibodies, but the results were not statistically significant.

DISCUSSION

The HUT-78 adult T-cell line is sensitive to infection by ARV (6). This susceptibility appears to be related to its high concentration of the helper T-cell marker on the cell surface. The virus most likely uses this receptor for entry into the cell and replication, since this marker is reduced substantially after infection (Table 1). Using the ARV-infected HUT-78 cell line E, we have developed an IFA that is very sensitive

Table 4. Absence of ARV antibodies in other disease states

Clinical symptoms or diagnosis	No. of cases
Renal transplant recipients	14
Recurrent infectious mononucleosis	14
Childhood leukemias/lymphomas	9
Lupus erythematosus	12
Rheumatoid arthritis	15
Fever of unknown origin	8
Generalized fatigue	8
Nasopharyngeal carcinoma	7
Leukopenia	7
Lymphadenopathy*	7
Hepatosplenomegaly	5
Others†	9

Of the 115 patients tested, none were positive for anti-ARV antibodies as assayed by IFA.
*Patients not homosexual.
†Acute infectious mononucleosis, ataxia telangiectasia, Gaucher disease, chronic liver disease, post-viral syndromes, and inflammatory bowel syndrome.
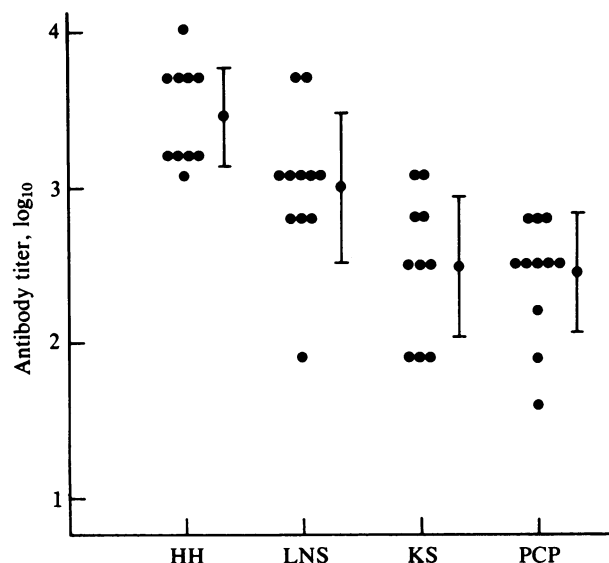

FIG. 2. Anti-ARV antibody titers in randomly selected sera from clinically healthy homosexual men (HH) and from patients with lymphadenopathy syndrome (LNS), Kaposi sarcoma (KS), or *Pneumocystis carinii* pneumonia (PCP). Mean ± SD is shown to the right of each group of individual data.

and specific for detection of antibodies to ARV. The IFA is as sensitive as immunoblot analysis for detection of ARV antibodies and is very rapid; it can be completed in less than 1 hr. Moreover, some sera evaluated in our laboratory have only been positive by IFA and not by immunoblot (unpublished data). This observation probably reflects the reaction of some sera with cell membrane-associated ARV antigens that may not always be present in sufficient quantities in purified virus preparations.

Our data confirm other reports on the close association of ARV antibodies with AIDS, the lymphadenopathy syndrome, and other pre-AIDS conditions (6, 17–20, 22). They demonstrate a high level of exposure to ARV in clinically healthy homosexual men. The ability of the IFA to identify specifically individuals who have had contact with ARV is contrasted by the absence of antibodies in individuals not belonging to an AIDS risk group and in patients with other diseases and with polyclonal activation following EBV infection (Tables 3 and 4).

Previous studies, using other retroviruses isolated from AIDS cases (human T-cell lymphotropic virus type III and lymphadenopathy-associated virus), have shown by ELISA that 37–97% of AIDS cases have antibodies to the virus (19, 20, 22–25). Moreover, recent work using ELISA and immunoblot analysis showed a predominance of borderline results by ELISA which had to be restudied by electroblotting (26, 27). These differences in efficiency of antibody detection reflect the variation in viral antigens present in the specific tests employed. IFA offers an excellent reproducible method for detecting antiviral antibodies with a very low level of false negatives. The sensitivity of this test can be assured by maintaining a continuous cell line with high viral antigen expression. The test has the advantage of being rapid and of showing distinct patterns of reactivity of the antibodies with the infected cells; this latter fact together with the presence of admixed uninfected cells serves to ensure the specificity of the observations. These built-in controls help distinguish between reactions due to autoantibodies in some patients' sera and those due to antibodies to the AIDS retrovirus.

The cytoplasmic reticular staining observed soon after ARV infection of HUT-78 cells may indicate a reaction due

to early viral antigens; it needs to be explored further as a possible parameter for clinical evaluation. Early antigens in EBV infection have been helpful in this regard (21). We did find as well that the antibody titers detected by IFA reflected to some extent the severity of the disease, since they are lowest in those individuals with the most extensive pathology.

These data indicate the strong relationship of ARV to AIDS and that the IFA described is sensitive, specific, and rapid for determination of antiviral antibody levels. The test can be employed easily for seroepidemiology and to monitor in part the immunologic status of infected individuals.

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