Anti-bodies against Three Purified Proteins of the Human Type C Retrovirus, Human T-Cell Leukemia-Lymphoma Virus, in Adult T-Cell Leukemia-Lymphoma Patients and Healthy Blacks from the Caribbean

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ABSTRACT

Six black patients of Caribbean origin with adult T-cell leukemia-lymphoma, 18 of their healthy family members and relatives, and 337 healthy black individuals from the Caribbean were investigated for the presence of serum antibodies against human T-cell leukemia-lymphoma virus (HTLV). Three distinct structural proteins of this virus with molecular weights of 24,000, 19,000, and 15,000 were purified, radiolabeled, and used in radioimmune precipitation assays. Five of the patients, three of the family members (two of them spouses), and 11 of the normals had specific antibodies against at least the proteins with molecular weights of 24,000 and 19,000. High antibody titers against these proteins were often associated with antibodies against the protein with a molecular weight of 15,000. In all cases, antibody titers against this protein were considerably lower than those against the proteins with molecular weights of 24,000 and 19,000. HTLV is highly associated with Caribbean adult T-cell leukemia-lymphoma and is also endemic among the normal Caribbean population. By comparison of the frequencies of anti-HTLV positives among family members of patients and the normal population, we conclude that infection by HTLV occurs in a horizontal way and at least in the West Indian black and Japanese population probably without a requirement for uncommon genetic factors.

INTRODUCTION

Exogenous retroviruses (RNA tumor viruses) are known to cause leukemia and lymphoma in chickens, turkeys, cats, cows, certain wild mice, and gibbon apes (for reviews, see Refs. 5, 8, 10, and 13). These cancers often involve T-cells (10). The identification of human T-cell growth factor in this study (19, 20, 29) made long-term cultivation of human T-cells possible and finally led to the isolation of human retroviruses from patients with T-cell leukaemias-lymphomas (22–24). These first virus isolates were designated HTLV2 strain CR and strain MB. They were shown to be not closely related to other retroviruses. HTLV from one Japanese ATL patient, is immunologically indistinguishable from several HTLV isolates made in the United States (24). In the United States and Europe, sera positive against HTLV-2 have been found only sporadically and only in T-cell proliferative disorders. Another region where HTLV-associated T-cell cancers have been found in cluster is the Caribbean basin. The T-cell proliferative disorder, termed T-lymphosarcoma cell leukemia (3), is morphologically indistinguishable from Japanese ATL (6, 9, 30). It is typically found in blacks of Caribbean origin and has a high incidence of hypercalcemia (2). The first of these Caribbean ATL cases that were investigated for antibodies against HTLV were all positive (1, 2) and HTLV was identified and isolated in the neoplastic T-cells of 2 of 3 cell samples received for analysis (24). We now present a more detailed study of these and other cases, investigating the antibodies against 3 purified HTLV proteins, p24, p19, and p15.

MATERIALS AND METHODS

Sera

Sera of 6 ATL and ATL-like patients of Caribbean origin and their 18 family members were collected in the United States and Great Britain, transported either on dry ice or freeze-dried, sampled upon arrival, and stored at −70°C. Sera of 337 normal donors had been collected as part of an anthropological study of genetic markers in residents of St. Vincent Island (British West Indies) (12). These sera had been used for other purposes before testing for anti-HTLV antibodies and therefore had repeatedly been thawed and refrozen.

1 Fogarty International Fellow of the National Cancer Institute. He is also supported by the Swiss National Fund for the Advancement of Science.
2 The abbreviations used are: HTLV, human T-cell leukemia-lymphoma virus; BLV, bovine leukemia virus; ATL, adult T-cell leukemia/lymphoma; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.
3 S. Oroszlan, T. D. Copeland, V. S. Kalyanaraman, M. G. Sarngadharan, and R. C. Gallo, manuscript submitted for publication.
Viruses and Cells

HUT102 cells producing HTLVcr and HUT78 cells were grown in Roswell Park Memorial Institute Medium 1640 containing 20% fetal calf serum. HTLVcr was concentrated from cell culture supernatant fluids by continuous-flow centrifugation, and the virus was purified further by equilibrium density banding in a 25 to 60% sucrose gradient. Normal human T-cells were grown in medium supplemented with T-cell growth factor as described previously (20, 29). Other viruses and viral antigens used in this study were the following. Baboon endogenous virus (BaEV-M7) grown in human rhabdomyosarcoma cells (A204) and Rauscher murine leukemia virus grown in JLSV-9 cells were obtained through the Resources and Logistics, National Cancer Institute. BLV, grown in fetal lamb kidney cells, was purchased from Electronucleonics, Ltd. Feline leukemia virus was grown in FL74 cells; Mason-Pfizer monkey virus in dog thymus cells; and simian sarcoma virus-simian sarcoma-associated virus was grown in marmoset cells (71AP1).

Purification of Viral Proteins

p24 and p15. HTLV p24 was purified as described earlier (16). HTLV p15 was purified as follows. A nucleic acid-free extract of density gradient purified HTLV was prepared as described in Ref. 16. This material was dialyzed against Buffer A (10 mM N,N-bis-hydroxyethylaminoethylsulfonic acid, 1 mM EDTA, and 0.1 mM PMSF (pH 6.5) and chromatographed on a 5-ml column of phosphocellulose equilibrated with the same buffer. The column was then developed with a 100-ml 0 to 0.6 M NaCl linear gradient in Buffer A collecting 1.5-ml fractions. The proteins of the gradient fractions were analyzed by electrophoresis in a 12% polyacrylamide gel in the presence of SDS (17). p15 eluted at a concentration of 200 to 350 mM NaCl. The fractions containing p15 were pooled, concentrated by ammonium sulfate precipitation, and further purified by gel filtration in Bio-Gel P60. The column was developed in a buffer containing 10 mM sodium phosphate (pH 7.5), 1 mM NaCl, and 0.1 mM PMSF.

p19. HTLV was solubilized and made free of nucleic acid as described (16). The nucleic acid-free viral proteins were dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5), 20% glycerol, 1 mM dithiothreitol, and 0.5 mM PMSF (Buffer B) and passed through a column of phosphocellulose equilibrated with Buffer B. The column was washed with Buffer B containing 0.6 M NaCl and eluted with a gradient of 0.6 to 1.2 M NaCl. The fractions containing p19, as identified by SDS:polyacrylamide gel electrophoresis, were pooled, concentrated by ammonium sulfate precipitation, and further purified by gel filtration in Bio-Gel P60. The column was developed in a buffer containing 10 mM sodium phosphate (pH 7.5), 1 mM NaCl, and 0.1 mM PMSF.

Iodination of Viral Proteins

Purified proteins of HTLVcr were labeled with 125I to specific activities of 5 to 20 μCi/μg by the chloramine-T method (7). Ten μg of protein were labeled in a 100-μl reaction mixture containing 10 μg of chloramine-T and 500 μCi of Na125I.

Immunoprecipitation and Radioimmunoassay

125I-labeled HTLV p24, p19, or p15 (5,000 to 10,000 cpm corresponding to 1 to 2 ng) was incubated with serial 2-fold dilutions of human sera in a volume of 200 μl of a buffer consisting of 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, 0.1 mM PMSF, and bovine serum albumin (2 mg/ml). After 2 hr at 37°, the incubation was continued overnight at 4°. A 30-fold excess of goat anti-human IgG was then added, and the reaction mixture was diluted to 1 ml with the above buffer. After further incubation for 1 hr at 37° followed by 2 hr at 4°, the precipitates were collected by centrifugation at 2,500 rpm for 15 min in a Beckman centrifuge, and the radioactivity in the precipitates was determined. For competition radioimmunoassays, limiting dilutions of the appropriate serum, resulting in 20 to 35% precipitation of the labeled antigen in the absence of competition, were preincubated for 1 hr at 37° with serial 2-fold dilutions of the unlabeled competing antigens, starting with 100 ng of protein for viral extracts and with 50 μg of protein for cellular extracts. 125I-labeled p24, p19, or p15 (8,000 to 10,000 cpm) was then added, and the reaction mixture was incubated and processed as described above.

RESULTS

Purification of Viral Proteins. The SDS:polyacrylamide gel electrophoretic profile of 3H-leucine-labeled HTLV purified from the medium of HUT102 predominantly showed proteins of 46,000, 24,000, 19,000, 15,000, and 12,000 molecular weight (16). p24 has previously been shown to be the major structural core protein of the virus (16, 21). p19 and p15 have been identified by SDS:polyacrylamide gel electrophoresis, were pooled, concentrated, and chromatographed on a 5-ml column of phosphocellulose equilibrated with Buffer B. The column was then developed with a 100-ml 0 to 0.6 M NaCl linear gradient in Buffer A collecting 1.5-ml fractions. The proteins of the gradient fractions were analyzed by electrophoresis in a 12% polyacrylamide gel in the presence of SDS (17). p15 eluted at a concentration of 200 to 350 mM NaCl. The fractions containing p15 were pooled, concentrated by ammonium sulfate precipitation, and further purified by gel filtration in Bio-Gel P60. The column was then developed in a buffer containing 10 mM sodium phosphate (pH 7.5), 1 mM NaCl, and 0.1 mM PMSF.

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Table 1
Antibodies against HTLV proteins in the Caribbean sera

<table>
<thead>
<tr>
<th>Serum class</th>
<th>Serum identification</th>
<th>Date of serum</th>
<th>Titters $^a$</th>
<th>Comment</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>p19</td>
</tr>
<tr>
<td>I. ATL</td>
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<td>9/80</td>
<td>6,400</td>
<td>1,950</td>
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<td></td>
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<td></td>
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<td></td>
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<td>J</td>
<td>1/81</td>
<td>200</td>
<td>66</td>
</tr>
<tr>
<td>III. Positive normals, unrelated to known ATL patients</td>
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<td>-</td>
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<td>V-4</td>
<td>11/81</td>
<td>42,000</td>
<td>34,900</td>
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</table>

$^a$ Titters were determined as described under "Materials and Methods" and are expressed as the reciprocal of 1 ml serum dilution precipitating 20% of the labeled proteins.

$^b$ Patient A was a patient with mycosis fungoides from the United States whose T-cells gave rise to the first HTLV isolate. His serum is used here as a control.

$^c$ HTLV was isolated from the patient’s T-cells.

also been shown to be viral and probably the internal proteins of HTLV (28).$^4$

p24 and p15 are purified from the disrupted virus by a combination of chromatography on phosphocellulose and gel filtration in Bio-Gel P60. p19 was purified by sequential chromatographic steps on phosphocellulose and oligodeoxythymidylate-cellulose. The $^{125}$I-labeled p19 was further purified by SDS:polyacrylamide gel electrophoresis. Chart 1 shows the SDS:polyacrylamide gel electrophoretic profiles of $^{125}$I-labeled p24, p19, and p15. All 3 labeled proteins are >80% immuno-precipitable with a rabbit antibody to disrupted HTLV (data not shown). These proteins are used in the radioimmunoprecipitation studies with the human sera described below.

Antibodies to HTLV Proteins p24, p19, and p15 in the Sera of ATL Patients from the Caribbean. Single or multiple sera of 6 black patients with ATL were investigated for antibodies against HTLV proteins, as described under "Materials and Methods." The data are shown in Table 1 (Section I) and Chart 2. All sera had been positive in a solid-phase radioimmunoassay using disrupted HTLV (1).$^5$ All but one had antibodies against p24 and p19. One patient (Patient A), an American black, whose first diagnosis had been mycosis fungoides but whose case history is also consistent with a diagnosis of ATL, had in addition a low titer of antibodies against p15. Sufficient serum was no longer available from Patient G. This patient had a diagnosis of T-cell leukemia with cutaneous involvement also compatible with ATL, and HTLV (strain MB) was isolated from a T-cell line derived from her peripheral blood leukemia cells (23). The cases of all ATL patients investigated thus far showed a clear association with HTLV.

Antibodies in Sera of Family Members of ATL Patients. Among the sera of 18 family members, relatives, and friends of 4 patients, we found 3 (16.7%) with antibodies against HTLV proteins (Table 1, Section II; Chart 2). These positives were found in the families of 3 different patients. All have antibodies against p24 and p19, and one of them is also positive against p15. The negative samples were from 4 sons, one husband, one adopted daughter, 5 grandchildren (children of the antibody-positive daughter and an antibody-negative son), one friend and coworker, one aunt, one half-sister, and one daughter-in-law (1).

Antibodies in the Sera of Normals Originating from the Caribbean. Sera from healthy adults not known to have had contacts to ATL patients were also investigated. Among 337 sera, 11 had antibodies against p24 and 10 also against p19, and 5 were positive against p15 (Table 1, Section III; Chart 2). From one person, 4 sera were available at intervals over a decade. All were highly positive for antibodies against all 3 proteins and with even a slight but repeatedly confirmed increase in both anti-p24 and anti-p15 antibodies since 1973.

$^4$ V. S. Kalyanaraman et al., unpublished results.

$^5$ M. Robert-Guroff, personal communication.
Chart 2. Representative radioimmunoprecipitations of purified and labeled HTLV proteins p24 (A), p19 (B), and p15 (C) by serum samples of ATL patients (C), B-2; C-1; D-1; +; F), their family members (H), and healthy individuals from the Caribbean (I). V-4; V; M; N; O). Serial dilutions of serum were incubated with 8,000 to 10,000 cpm of the labeled protein. A 30-fold excess of goat anti-human IgG antibody was added, and the percentage of labeled antigen bound in the precipitate was determined.

Specificity of Immune Precipitations of HTLV Proteins by Human Sera. To test the specificity of the immune precipitations of purified HTLV proteins by antibodies in human sera, various competition studies were done. Proteins used for competition included extracts of type C retroviruses (simian sarcoma virus, BaEV-M7, BLV, Mason-Pfizer monkey virus, feline leukemia virus, and Rauscher murine leukemia virus) as well as lysates from HTLV-positive and -negative cells. Cell lysates were from phytohemagglutinin-stimulated, normal human T-cell cultures, HTLV-producing neoplastic T-cells (HUT102), and neoplastic human T-cells not producing HTLV (HUT78) (22). Precipitation of HTLV proteins p24, p19, and p15 by Serum V-4 was inhibited only by HTLV and cells producing HTLV (HUT102) (Chart 3). BLV, the p12 of which under certain conditions shows some cross-reactivity to HTLV p15, did not compete with p15 even at a 50,000:1 protein ratio. Further evidence for the specificity of these antigen-antibody reactions is presented in Chart 4. A mixture of labeled p24, p19, and p15 was precipitated by Serum V-4 and the washed precipitate was run on a 12% SDS:gel (Chart 4A). Competition by purified unlabeled p24, p19, or p15, respectively, is restricted to the homologous protein (Chart 4, B, C, and D). Since it is clear from Table 1 and Chart 2 that the antibody titer against p15 is significantly lower than those against p19 and p24 in any positive serum, the amount of p15 precipitated by the serum is much lower than those of p19 and p24. To confirm the p15 peak, twice the amount of Serum V-4 was used for the precipitation of 100,000 cpm of p15. The SDS:polyacrylamide gel electrophoresis analysis (Chart 4E) shows that this peak, although small in comparison to those of p24 and p19, is real. As a consequence of the high antibody levels of this serum against p24 and p19, small amounts of p24 and p19 present as minor contaminants (less than 1% each) in p15 preparation are also precipitated. Similarly, Serum V-4 recognizes and precipitates 2 other contaminants with relative mobilities of 0.5 (Mr, 35,000) and 0.85 (Mr, 13,000, respectively (Chart 4A). The first is removed from the precipitate by preincubation of the serum with p24 (Chart 4B), but not by p19, and p15, and is thus confined to the p24 preparation. The second is removed by preincubation with p19 (Chart 4C) and thus a contaminant of p19. The amount of these contaminants in the purified protein preparations is also less than 2%.

DISCUSSION

It has been shown that HTLV (22-24) is an exogenously acquired human retrovirus (4, 25). By immunological tests and by nucleic acid sequence analysis, HTLV is not closely related to any other animal retrovirus. This virus is strongly associated with the ATL found in Japan (15, 18). In the United States and
Japan led to the search for similar clusters of HTLV-associated cancers in other parts of the world.

Recently, a new entity within the T-cell malignancies of adults, T-lymphosarcoma cell leukemia, has been described (3), which is morphologically and clinically indistinguishable from the adult T-cell leukemia-lymphoma seen in Japan and has a prevalence in blacks originating from the Caribbean (2). The first 3 of these patients tested for involvement of HTLV showed high serum antibody titers against HTLV core protein p24 (1, 2). This prompted us to undertake a more detailed study of these and other ATL cases from the Caribbean, involving 3 distinct HTLV proteins, p24, p19, and p15. These proteins were purified (Chart 1), radiolabeled, and used for immune precipitations by human sera (Chart 2; Table 1). We show that only HTLV itself or lysates from cells known to produce HTLV are competitive in these assays (Chart 3) and that the 3 proteins do not compete with each other (Chart 4), thus demonstrating the specificity of these human antibodies for HTLV p24, p19, or p15.

All of the 7 ATL or ATL-like patients included in our study had an association to HTLV, either by their immunological response to this virus in 6 cases, by the demonstration of the virus alone in one case, or by both in 2 cases. In 5 of 6 cases, humoral responses were against both p24 and p19. Antibodies against p24, p19, and p15 were found in one case. These results show that HTLV is associated with Caribbean ATL, p24 and p19 appear to be more immunogenic than p15 under natural conditions of virus infection. Therefore, in cases where titers against p24 and p19 are low, we do not detect antibodies against p15. To date, in a survey of 337 sera of the normal healthy Caribbean population, a frequency of 3.6% antibody positives was found. It is of interest that the bulk of positives were over 40 years of age, which suggests that the prevalence of virus infection increases with age. In contrast, 3 out of 18 family members of ATL patients (16.7%) were positive. This higher prevalence may indicate that HTLV infection is acquired horizontally, by close contact to an HTLV source. This is further supported by the finding that 2 of the 3 positive family cases involve spouses. As such, they may be regarded as not genetically related, thus rendering the involvement of uncommon genetic factors in the processes of HTLV infection and the antibody response regarding the proteins investigated here rather improbable. Once positive, individuals may maintain or even increase their antibody titers over years (cf. Table 1, Case V), suggesting reexposure to the virus or persistence of the viral infection. At the present time, we do not have sufficient basis to determine whether the wide range of antibody titers we have observed among normal individuals or the persistence of antibody titers over extended periods has any relevance to a later development of leukemia. Further systematic immunological and epidemiological studies will be needed to determine the contribution of viral, host, and environmental factors in the pathogenesis of this disease.

ACKNOWLEDGMENTS

We thank L. Bruch and M. Jarvis for excellent technical assistance.

REFERENCES

1. Blattner, W. A., Kalyanaraman, V. S., Robert-Guroff, M., Lister, T. A., Galton, D. A. G., Saria, P., Crawford, M. H., Califsky, D., Greaves, M., and Gallo, R. C. The human type-C retrovirus, HTLV, in blacks from the Caribbean, Europe, where adult leukemias and lymphomas of T-cell origin are much less common (18), only rare cases of T-cell malignancies involving HTLV have been found. The fact that Japanese ATL occurs geographically clustered in certain regions.

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Chart 4. SDS-polyacrylamide gel electrophoretic analysis of purified and labeled HTLV proteins precipitated by Serum V-4. p24, p19, and p15 (100,000 cpm each) were mixed and incubated with 10 μl of Serum V-4, and precipitations were made as described under "Materials and Methods." The precipitate was washed twice in 5 ml of Buffer C, and analyzed by 12% SDS-polyacrylamide gel electrophoresis (A). To determine the specificity of individual human antibodies for either p24, p19, or p15, the serum was also preabsorbed during 3 hr at 37°C with 100 ng of purified unlabeled p24 (B). 500 ng of p19 (C), or 500 ng of p15 (D) before the mixture of the labeled proteins was added, and precipitations were performed and analyzed as above. A separate precipitate of labeled p15 by 20 μl of Serum V-4 was analyzed in E to give additional proof for the existence of the p15 peak in A to D. These data show that the purified protein preparations are free of major contaminants and that p24, p19, and p15 do not cross-react.

6 R. C. Gallo et al., submitted for publication.
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