The genome of the HTLV-III/LAV retrovirus, the etiologic agent of the acquired immunodeficiency syndrome (AIDS), encodes the viral structural proteins (envelope and core proteins), the reverse transcriptase, a transactivation protein (tat-III), as well as two other proteins (3'orf, sor) of unknown function. We studied the prevalence of natural antibodies against envelope, gag, 3'orf, sor, and tat-III in the sera of HTLV-III infected individuals in an attempt to correlate clinical status with seropositivity to specific HTLV-III antigens. We selected 101 sera; 16 were obtained from normal donors with no known risk factors, and 85 were from patients with full-fledged AIDS (28 cases), AIDS-related complex (ARC, 22 cases), and healthy people at risk (homosexuals, intravenous [IV] drug users, relatives of AIDS patients; 35 cases). Seropositivity for antibodies against the envelope (gp41) and gag antigens (p15, p24) was determined by Western blot using disrupted HTLV-III virions. Of the 101 sera, all 16 from nonrisk donors and 3/35 from healthy at-risk donors were negative for antibodies against either the gp41 or p15 and p24. The remaining 82 sera were seropositive for either the gp41 and/or the p15 and p24. All sera were then tested against the three known HTLV-III antigens (3'orf, sor, and tat-III) that have been synthesized in bacteria. Our data indicate that all the HTLV-III antigens tested are immunogenic in vivo. No significant difference in antibody prevalence to gp41 (close to 100%) and to the 3'orf, sor, and tat-III proteins (approximately 50%) was observed with regard to stage of the disease. In contrast, the prevalence of antibodies against the core antigens decreased from approximately 100% in infected people with no clinical signs of disease to 50% in ARC and AIDS patients. The percentage of patients seropositive for all five antigens tested was increased in the AIDS group. These results indicate that the greatest antibody prevalence was obtained using viral envelope antigen and further suggest that screening with the newly identified 3'orf, sor, and tat-III proteins as antigens would confer no further diagnostic advantage. The pattern of natural antibodies observed during disease progression did not suggest any pathogenetic mechanism.

Spectrum of Natural Antibodies Against Five HTLV-III Antigens in Infected Individuals: Correlation of Antibody Prevalence With Clinical Status

By Genoveffa Franchini, Marjorie Robert-Guroff, Anna Aldovini, Nancy C. Kan, and Flossie Wong-Staal

The infused immunodeficiency syndrome (AIDS) was first described in the United States in 1981, 4 and since then the disease has reached epidemic proportions. 2 The syndrome consists of a series of clinical manifestations directly related to the severe immune dysfunction in the affected people, such as opportunistic infections and various disorders such as autoimmune diseases, malignancies, and neurologic manifestations, the pathogenetic origin of which is less obvious. Infection by HTLV-III/LAV is the common denominator among these patients. 3-5 HTLV-III infection correlates with depletion of the OKT4+ T cells in vivo and in vitro. 6,7 While the immunodeficiency observed in patients with AIDS could be a direct consequence of the cytopathic effect of HTLV-III exerted on T cells as it is observed in vitro, 7 the role played by HTLV-III in the pathogenesis of the various clinical manifestations of the syndrome is still not clear. The molecular cloning and the nucleotide sequencing of HTLV-III 8-10 allowed the identification of the envelope, polymerase and the core protein genes, and three additional open-reading frames, 3'orf, sor, and tat-III, that encode proteins of 27, 23,10-12 and 14Kd respectively. 13 The tat-III protein seems to enhance virus expression in the infected 14,15 cells and may play a key role in the mechanism of viral replication. The functions of the 3'orf and sor proteins relative to the viral life cycle or the cellular metabolism of infected cells remain to be uncovered. We studied the expression in vivo of these viral genes by analyzing the natural antibody response in infected individuals in an attempt to correlate the stage of the disease and the different clinical manifestations with the expression of specific viral proteins.

MATERIALS AND METHODS

Virus preparation. HTLV-III produced by H9 cells was harvested from 50 liters of cell-free tissue culture media using zonal ultracentrifugation on two sequential ribonuclease-free sucrose gra-

dients as previously described. 14 Purified virus was disrupted in 50 mmol/L Tris-HCl buffer, pH 7.9, containing 0.25% Triton X-100, 20 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.5 mol/L KCl, and 1 mmol/L phenylmethyl sulfonyl fluoride by stirring for one hour at 4 °C. The solubilized virus preparation was dialyzed extensively against phosphate-buffered saline (PBS) and centrifuged for one hour at 100,000 x g. The supernatant fraction was used in Western blot 7 assays following protein estimation by the method of Lowry. 18 Purification of the bacterially synthesized viral proteins. The bacterially synthesized HTLV-III proteins were purified by different methods. The 3'orf protein was obtained from the total cell lysate of E. coli bacteria after solubilization in 50 mmol/L Tris-HCl, pH 8, containing 1.5 mol/L sodium chloride and 8 mol/L urea and application of the extract to a Sephacryl S-300 column. The fractions containing the 3'orf protein were pooled and applied to a Sepharose 4B column coupled with an antibody directed against the amino terminus of the hybrid protein. 19 The 3'orf protein was recovered by elution with 2% formic acid and used in a Western blot assay. 19 The bacterially synthesized 27 Kd 3'orf protein is a hybrid protein that comprises 84% of the 3'orf gene and 36 amino acids derived from the prkorytokary vector. 20 By coincidence the molecular weight of the bacterial protein is the same as the molecular weight of the native protein (p27) detected in infected cells. 20 The sor protein was purified by application of the bacterial lysate to a cation-

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0006-4971/87/6902-0008$3.00/0

exchange Water Protein-Pak Sp-5PW HPLC column and elution with a 25-mL gradient of 0% to 80% 0.75 mol/L NaCl in the equilibration buffer. The bacterial sor (p18) is a hybrid protein representing 83% of the sor gene. The native protein product has been identified as a 23Kd protein. The tat-III protein was purified by applying the bacterial lysate to a 15% SDS-polyacrylamide gel. After electrophoresis the 14 Kd protein was eluted from the appropriate gel slice. The 14 Kd tat-III protein expressed in Escherichia coli is not a fusion protein but represents the complete protein product of the tat-III gene. It therefore has the same size as the native protein product detected in infected cells.

Western blot analysis of the viral proteins. Lysate of HTLV-III or purified recombinant proteins (Yorf, tat-III, and sor) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to nitrocellulose sheets as described, and the sheets were incubated for one hour at room temperature with a solution containing 5% nonfat dry milk and 0.01% Antifoam (Sigma, St Louis) in PBS to block nonspecific protein-binding sites. Strips were obtained from the nitrocellulose sheets and individually reacted with human sera diluted 1:100 for one hour at 4°C in the same solution containing 5% nonfat dry milk. After incubation the strips were washed in PBS containing 0.5% sodium deoxycholate and reacted for 20 minutes with 125I staphylococcal protein A (5 x 10^4 cpm/mL). The strips were washed again, and autoradiograms were obtained.

RESULTS

Location of the coding regions for the HTLV-III proteins. A summary of the genes identified in the different open-reading frames of the HTLV-III genome by computer analysis described in the upper part of Fig 1. To date the core proteins p24, p15, have been identified as well as the gag120 and gp4121,22 for the exterior and transmembrane portion of the envelope respectively. Three other HTLV-III genes have been uncovered by studying the viral c-DNAs, and the features of their spliced mRNAs are summarized in the lower part of Fig 1. The native products of the three genes, Yorf, sor, and tat-III have been identified in infected cells cultured in vitro. The three genes encode proteins p27, p23, and p14 respectively.

Correlation of antibody response and stage of the disease. We attempted to correlate the stage of the disease with the prevalence of antibodies against specific viral antigens. We employed 82 human sera seropositive for antibodies against the HTLV-III core proteins (p15 and/or p24) or the gp41 or both. The sera, all obtained from Americans, included 32 samples from healthy individuals at risk for HTLV-III infection; 28 samples from patients with full-blown AIDS, and 22 samples from American patients with ARC. The patients in the risk group defined 'healthy' included individuals with and without lymphadenopathy (LAS). Among them, three were IV drug users and one was a relative of a patient with AIDS. The rest were homosexuals.

ARC patients were staged according to the criteria of the Centers for Disease Control, and the group included patients with at least two laboratory findings of immunodeficiency and two clinical manifestations of disease. All the AIDS patients had serious manifestations of disease, including either Kaposi's sarcoma (50%) or opportunistic infections (50%). We also studied 16 human sera seronegative for antibodies against the gag and envelope proteins of HTLV-III obtained from a population not at risk for HTLV-III infection and three sera from previously identified virus-positive, antibody-negative healthy carriers. An example of a Western blot of some of these sera using disrupted HTLV-III is given in Fig 2, first panel. The sera were also reacted against the purified Yorf, sor, and tat-III protein products expressed in E. coli (Fig 2, panels 2, 3, and 4). Some sera from each group of patients (healthy at risk, ARC, AIDS) recognized the bacterially synthesized HTLV-III proteins. Sera of the normal donors (HN) lacking antibodies to the envelope and the core proteins of HTLV-III also did not react with the tat-III and the Yorf proteins; in contrast some of the normal sera recognized the sor protein. The three sera obtained from the healthy virus-positive individuals failed to recognize the Yorf, sor, and tat-III proteins. Later samples from these same individuals seroconverted and became positive for envelope and core antigens.

A histogram of the distribution of natural antibodies against the five HTLV-III proteins analyzed in this study is shown in Fig 3. The antibody prevalence against the envelope antigen gp41 was constant in all phases of the disease. Almost all patients tested were seropositive for this antigen, in agreement with other studies that have shown the viral envelope to be the major target antigen in HTLV-III exposed individuals. While minor fluctuations in antibody prevalence were observed with disease progression, overall a constant pattern occurred with the Yorf, sor, and tat-III proteins. In the case of these antigens, however, the percentage of seropositive individuals was in the intermediate range of 30% to 65% depending on the antigen. The antibody prevalence to the gag antigenic determinants was greatest among the healthy at risk group, as reported also by other investigators. When individuals in the early phases of disease were analyzed with regard to the presence or absence of lymphadenopathy, no differences in the antibody prevalence to any of the five antigens were detected.

Specificity of seroreactivity to the bacterially synthesized sor protein. As indicated in the histogram of Fig 3, 25% of
**IMMUNORESPONSE IN HTLV-III-INFECTED PATIENTS**

Fig 2. The first panel represents the autoradiograph of nitrocellulose strips containing HTLV-III viral proteins from the disrupted virion reacted with various human sera. Panels 2, 3, and 4 represent examples of the reactivity of human sera toward the 3'orf, sor, and tat-III purified proteins respectively. The molecular weight of the proteins was derived from molecular weight standard proteins electrophoresed on the same gel (Bethesda Research Laboratory, Bethesda, MD). HN, HH, and HM refer to human sera and represent a healthy, normal, not-at-risk patient; healthy homosexuals; and a healthy mother of a child with AIDS. AIDS and ARC stand for sera obtained from patients with the AIDS syndrome and from patients with AIDS-related complex.

Sera from uninfected individuals reacted with the recombinant 18Kd sor protein. This reactivity was specific for the recombinant protein, since a protein of similar size is not detected in the control bacterial lysate (Fig 4, lanes 1 and 2). Similarly, the specific antiserum obtained from a rabbit immunized with the purified protein reacted with the recombinant sor protein (lane 4), while no reaction was observed in the total bacterial lysate (lane 3). The sor protein used in these studies is a chimeric protein that contains at the amino terminus 13 amino acids derived from the wclI gene of the vector. To rule out that the reactivity of the human sera was directed against vector epitopes rather than sor epitopes, we tested the reactivity of the same sera against another chimeric protein expressed in the same vector containing 27% of the coding sequences of the HTLV type I envelope gene p21E. Figure 4, lanes 5 and 6, shows that a human serum positive for the recombinant sor protein does not recognize the p21 E recombinant HTLV-I chimeric protein, while a serum obtained from a patient with adult T cell leukemia does. Thus, the reactivity of human sera from uninfected individuals seems to be directed against the antigenic determinants of the recombinant sor protein.

**DISCUSSION**

All the known proteins encoded by HTLV-III are immunogenic in vivo, but the spectrum of antibodies that they

**Fig 3.** The spectrum of natural antibodies in patients' sera is expressed as a percentage of total patients analyzed for each antigen, which is represented on the Y axis. The legend for each antigen is reported in the right upper part of the figure.

**Fig 4.** Total cell lysate from bacteria expressing the sor protein (lanes 2 and 4) and from control bacteria (lanes 1 and 3) were analyzed by Western blot using a human serum from an uninfected individual (lanes 1 and 2) and a rabbit antiserum raised against the recombinant sor protein (lanes 3 and 4). A Western blot against the purified recombinant protein containing the antigenic determinants of the HTLV-I p21E (lanes 5 and 6) was obtained with the same human serum used in lanes 1 and 2 (lane 5) and with a serum from a patient with adult T cell leukemia (lane 6).
elicit in infected individuals does not seem to correlate with disease stages as they are currently described. We noticed that as infected individuals progressed toward AIDS, they tended to acquire antibodies to all the HTLV-III antigens studied (data not shown). This finding may simply reflect continual expression of the virus in vivo with a greater likelihood of exposure to each antigen with increasing time after infection. Fluctuations in antibody prevalence were observed with stage of disease, particularly with the \textit{SOR} and tat-III proteins, although the differences observed did not yield statistically significant correlations. These varying prevalences may result from different levels of circulating antigens with disease progression and immune complex formation, leading to lessened detection of serum antibodies. More extensive studies would be necessary to determine whether the observed fluctuations have underlying biologic significance. Direct analysis for circulating immune complexes or complexes trapped in lymph nodes would be informative. It is remarkable that the immune response against all HTLV-III antigens in AIDS patients seems to be comparable to that of the healthy at-risk patients, despite the high degree of immune system failure observed in the former group. Our data suggest that it may be necessary to investigate individuals very soon after viral infection to determine whether sequential exposure to HTLV-III antigens is correlated with laboratory or clinical manifestations of AIDS. In this regard we analyzed sera of three patients at risk who were virus positive, seronegative for antibodies against the HTLV-III envelope and \textit{gag} proteins and who subsequently seroconverted. Their sera were negative for the 3'\textit{orf}, \textit{gag}, and tat-III before the time of seroconversion, suggesting that these proteins might not be useful in the diagnosis of the early phase of infection. More serum samples from this kind of patient should be studied before a definitive conclusion on this point can be reached. Our analysis has shown that of the five HTLV-III antigens studied, the most immunogenic one appears to be the gp41 transmembrane protein. As the major envelope protein gp120 also appears to be highly immunogenic, it would be useful to include it in screening procedures when the purified protein becomes available. Obviously the use of a combination of bacterially synthesized antigens for large scale antibody testing not only could increase the sensitivity of antibody detection but could also eliminate the problem of obtaining false-positive results due to contamination of virus preparations with human antigens. In this regard it should be noticed that the \textit{SOR} protein should not be used in the antibody screening procedure because it could be the cause of false-positive results. In fact, we have shown that some human sera from not-at-risk, uninfected individuals have antibodies that react with the \textit{SOR} and \textit{gag} determinants. We believe that this reactivity may be due to crossreactive determinants in either endogenous or exogenous proteins that elicit an immune response in humans.

ACKNOWLEDGMENT

We are very grateful to Drs J. Ghrayeb, T. Papas, C. Debouch, and K. Samuel for providing purified proteins and to Dr Robert C. Gallo for his support. We also thank Dee Goodrich for editorial assistance.

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