Laboratory and Epidemiologic Evaluation of an Enzyme Immunoassay for Antibodies to HTLV-III

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The enzyme immunoassays (EIAs) for antibody to human T-cell lymphotropic virus type III (HTLV-III) were rapidly adopted for screening donated blood and plasma. To evaluate the significance of a positive EIA reaction, test performance was examined in a bank blood screening program. Specimens were tested by EIA, Western blot assay, and HTLV-III/lymphadenopathy-associated virus (LAV) culture. The EIA was positive in 0.25% of 67-190 blood donations. Specimens were categorized and 57.3% had low (weak) reactivity, 12.7% had moderate reactivity, and 30.0% had high reactivity. Highly reactive specimens were strongly associated with a positive Western blot or culture (86.7%) in contrast to moderately and weakly reactive specimens (1.9%). Twenty-five of 29 donors interviewed with a highly reactive EIA had risk factors for HTLV-III/LAV infection. Risk factors were not identified for 74 of 75 interviewed donors with specimens of lower reactivity. The minimum calculated specificity was 99.82%. The use of the HTLV-III EIA has virtually eliminated the use of blood and plasma from HTLV-III/LAV infected donors.

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BLOOD transfusions have been implicated as the source of infection for 1.7% of all cases of acquired immunodeficiency syndrome (AIDS) reported to the Centers for Disease Control (CDC).1,4 Investigations of 22 of these cases identified at least one donor who was infected with human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), the cause of AIDS.5 The documentation of asymptomatic HTLV-III/LAV infection in these donors, often years after their donation, underscored the need for a reliable serologic screening procedure to supplement existing self-deferral recommendations.1,5,7

Enzyme immunoassays (EIAs) to detect antibody to HTLV-III were developed in 1984 and become commercially available in March 1985 for screening donated blood and plasma.2,8 The tests had demonstrated high sensitivity and specificity in limited trials before licensure.2,9,10 but information was not available to assess the significance of positive test results in blood donors, a population with a low prevalence of HTLV-III/LAV infection. The absence of this information created concern that a large number of donated units might be falsely positive, resulting in many donations being needlessly discarded and donors being notified of these false-positive results.11,17

After licensure of the first anti-HTLV-III EIA test kit, the CDC and the American Red Cross Blood Services (ARCBS), Atlanta Region, established a cooperative study to evaluate the specificity and predictive value of the antibody test in a blood bank screening program.

SUBJECTS AND METHODS

Blood Donor Study

From March 25 through July 31, 1985, all potential donors at the ARCBS continued to be provided with information about self-deferral of donors with a risk for HTLV-III/LAV infection or AIDS and were advised about the availability of alternative tests sites where the anti-HTLV-III test could be obtained inexpensively and anonymously. In addition, as part of the consent procedure for donation, donors were informed that their blood would be tested for antibody to HTLV-III, that additional tests might be performed at the CDC to evaluate test performance, and that they would be contacted if any of the tests suggested they had been exposed to HTLV-III/LAV.
At the time of donation, an additional 20 mL of sterile heparinized blood and 7 mL of clotted blood were collected for the study and stored at room temperature at the ARCBS. Those specimens to be studied were transferred to the CDC within 48 hours after donation; the remainder were discarded.

All units of blood collected in the ARCBS region were processed at a central facility and screened in a routine manner for antibody to HTLV-III, using an EIA. Tests were conducted as recommended by the package insert. Units with an initially negative EIA reaction were not retested at the ARCBS, but specimens from ten of these units each day were selected by systematic random sample and sent to the CDC as controls. Units reactive on the initial EIA test at the ARCBS were tested two additional times. If both subsequent tests were nonreactive, the EIA reaction was interpreted as negative. Units reactive on one or both subsequent EIA tests were interpreted as positive. Specimens from all units initially reactive on EIA irrespective of subsequent results at the ARCBS were sent to the CDC for additional testing. Specimens from approximately 12% of the donors with a positive EIA reaction and from 21% of the control donors were not available for testing at the CDC if the unit was donated on a weekend or if technical difficulties developed in obtaining the specimens from the donor.

Laboratory testing at the CDC included the Western blot assay, using disrupted whole-virus antigen in a microgel assay system with molecular-weight standards and positive and negative controls, and cocultivation of peripheral blood lymphocytes for isolation of HTLV-III/LAV.35 The Western blot assay results were reported as positive if either the p24 and/or gp120 precipitation bands were identified. The Western blot was repeated on specimens with only a p24 or gp120 band, and results were reported as positive only if the single band was consistently identified. Lymphocyte cultures for HTLV-III/LAV were reported as positive on the basis of elevated reverse transcriptase levels and electron microscopy examination. Cultures were not done on approximately 11% of the specimens sent to the CDC because the lymphocytes were not viable by the time the specimen was received. Laboratory personnel conducting the various tests were blinded to results of tests performed at the ARCBS and to other tests performed at the CDC.

Reactivity of the anti-HTLV-III EIA-positive specimens was assessed semiquantitatively by determining a ratio of the test absorbance value to the cutoff value obtained, as outlined in the package insert. The mean of the ratios for all tests performed at the ARCBS for each EIA-positive specimen was calculated and used as the basis for determining the level of reactivity. Categories for classifying the positive EIA results were arbitrarily defined by inspecting the distribution of the EIA mean ratios and distribution of the Western blot and HTLV-III/LAV lymphocyte culture results. The upper limits of the EIA ratios were dictated by the maximum absorbance read by the spectrophotometer, and the variable cutoff value was calculated for each test run. Three categories of EIA test reactivity were defined: low, with a mean ratio from 1.0 to 2.9; moderate, with a mean ratio from 3.0 to 5.9; and high, with a mean ratio of 6.0 or greater.

Statistical Methods Tests for statistical significance included the Spearman rank correlation for testing associations between ordinal variables, the nonparametric Wilcoxon rank-sum test for comparing the median values of two distributions, the Student t test for comparing the mean values of two distributions, and the chi-squared test for comparing two proportions. Fisher’s exact test was used instead of the chi-squared test when expected frequencies were small. Exact confidence bounds for proportions were calculated by the Clopper-Pearson method.

Epidemiologic Investigation Blood donors were notified of test results by the ARCBS if the EIA reaction was positive or if other test results at the CDC indicated possible HTLV-III/LAV infection. The donor was told about the CDC follow-up study by Red Cross personnel as part of the notification procedure. Those who gave informed consent were interviewed by a CDC investigator to identify risks for HTLV-III/LAV infection. A sample of donors from among those with only an initially reactive EIA and from those with specimens selected as controls were also interviewed. The CDC investigator was blinded to the donors’ test results until after completion of the interview.

RESULTS Blood Donor Study During the 18-week study period, 67 190 units of blood were collected by the ARCBS. The HTLV-III EIA test was initially reactive in 569 (0.85%) of the units, but was repeatedly reactive (positive) in only 171 units (0.25% of all donations). The mean number of EIA-positive specimens per week was 9.5, but this varied widely (range, two to 22). No significant change was found in the rate of EIA-positive specimens as the study progressed (P > .15, Spearman’s rank correlation).

Specimens from 150 positive units
Table 1.—Results of Western Blot Assay and HTLV-III Culture of Specimens Found to Be HTLV-III EIA−Positive at American Red Cross Blood Services, Atlanta Region

<table>
<thead>
<tr>
<th>EIA Reactivity</th>
<th>Western Blot Results</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total (%)</td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture positive</td>
<td>1</td>
<td>1</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Culture negative</td>
<td>0</td>
<td>78</td>
<td>78 (90.7)</td>
</tr>
<tr>
<td>Culture not done/contaminated</td>
<td>0</td>
<td>6</td>
<td>6 (7.0)</td>
</tr>
<tr>
<td>Total</td>
<td>1 (1.2)</td>
<td>85 (96.8)</td>
<td>86 (100.0)</td>
</tr>
<tr>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture positive</td>
<td>0</td>
<td>0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Culture negative</td>
<td>14</td>
<td>14</td>
<td>14 (73.3)</td>
</tr>
<tr>
<td>Culture not done/contaminated</td>
<td>5</td>
<td>5</td>
<td>5 (26.3)</td>
</tr>
<tr>
<td>Total</td>
<td>19 (100.0)</td>
<td>19 (100.0)</td>
<td>19 (100.0)</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture positive</td>
<td>22</td>
<td>1</td>
<td>23 (51.1)</td>
</tr>
<tr>
<td>Culture negative</td>
<td>13</td>
<td>4</td>
<td>17 (37.8)</td>
</tr>
<tr>
<td>Culture not done/contaminated</td>
<td>3</td>
<td>2</td>
<td>5 (11.1)</td>
</tr>
<tr>
<td>Total</td>
<td>38 (84.4)</td>
<td>7 (15.6)</td>
<td>45 (100.0)</td>
</tr>
</tbody>
</table>

*HTLV-III indicates human T-cell lymphotropic virus type III.
†EIA indicates enzyme immunoassay.

Table 2.—Comparison of Blood Donors by Sex, Age, and Donation History

<table>
<thead>
<tr>
<th>EIA Reactivity (Ratio)</th>
<th>Men</th>
<th>Women</th>
<th>No. (%) Who Donated Blood After March 1983</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean Age, y</td>
<td>%</td>
</tr>
<tr>
<td>Negative (&lt;1.0)</td>
<td>624</td>
<td>58</td>
<td>31.7</td>
</tr>
<tr>
<td>Low/moderate (1.0–5.9)</td>
<td>95</td>
<td>46</td>
<td>37.4</td>
</tr>
<tr>
<td>High (≥6.0)</td>
<td>43</td>
<td>88</td>
<td>29.1</td>
</tr>
</tbody>
</table>

*EIA indicates enzyme immunoassay.
†Of those with complete blood donation history on file with American Red Cross Blood Services, Atlanta Region.
‡Record search for vital statistics completed by American Red Cross Blood Services, Atlanta Region.

(87.7%) were sent to the CDC for additional testing; only these specimens are included in our analysis. The distribution of EIA-positive units by degree of test reactivity showed that over half clustered just above the cut-off point (ratio of test absorbance to cutoff value, <3.0). The number of positive blood units with any given reactivity ratio above this value decreased markedly, but the value of the ratios ranged up to 24.0. When test results were categorized by degree of reactivity, they showed a bimodal distribution: 57.3% of the specimens had low reactivity, 12.7% had moderate reactivity, and 30.0% had high reactivity (Figure).

Results of Western blot assay of the EIA-positive specimens correlated highly with degree of EIA test reactivity. Thirty-nine specimens (28.0%) had a positive Western blot result; 38 (84.4%) of the 45 specimens highly reactive on EIA were Western blot positive (Table 1). Only one (1.2%) of the 86 low reactive specimens and none of the 19 moderately reactive specimens had a positive Western blot result (P < .00001, Fisher's exact test). Of the Western blot–positive specimens, both the p24 and gp41 bands were present in 28 specimens (71.8%); the p24 band was present alone in 11 specimens (28.2%). The gp41 band did not occur as an isolated finding in any of these specimens. Lymphocyte cultures for HTLV-III/LAV were completed on 134 (89.3%) of the EIA-positive specimens; HTLV-III/LAV was isolated from 25 (17.2%) of the 143 specimens that could be cultured (Table 1). Specimens highly reactive on EIA were most likely to be culture positive, with HTLV-III/LAV isolated from 23 (57.5%) of 40 cultured specimens in this group. In contrast, none of 14 specimens with moderate EIA reactivity and only two (2.5%) of 80 specimens with low EIA reactivity were culture positive (P < .0001, Fisher's exact test).

The Western blot was positive in 23 (92.0%) of the 25 culture-positive specimens (Table 1). Conversely, 23 (83.9%) of the 28 Western blot-positive specimens cultured for HTLV-III/LAV were positive. Virus was isolated from 55.6% of the cultured specimens with only a p24 band on Western blot assay and from 62.9% of those cultured with both p24 and gp41 bands (P was not significant; Fisher's exact test).

Specimens from 628 EIA-negative control donors and from 306 donors whose specimens were initially EIA reactive but subsequently nonreactive on testing at the ARCBS also were evaluated at the CDC. The Western blot assay gave negative results on all specimens in the EIA-negative group, but gave positive results with a p24 band from one specimen that was initially reactive on EIA. Repeated Western blot testing of the same specimen and of a separate specimen obtained at a later date gave negative results when performed by laboratory personnel blinded to the results of the initial test. Lymphocytes from 50 of the seronegative specimens and from 227 of those that were EIA reactive only on the initial test were cultured for HTLV-III/LAV; all results were negative.

Of the 150 EIA-positive specimens from blood donors tested at the CDC, 40 (26.7%) had a Western blot or culture positive for HTLV-III/LAV. If we assume that these are the only true-positives, no more than 125 of the 67,190 units tested were falsely positive. This gives a calculated test specificity of 99.82%.

Donor Investigation

Demographic characteristics of donors with positive EIA reactions varied by degree of test reactivity and were different from those with negative EIA reactions (Table 1). Thirty-nine of the 45 moderately reactive donors were between the ages of 20 and 59, and 80 of the 139 low reactive donors were between the ages of 20 and 39. Of the donors whose specimens were initially reactive on EIA and who had EIA-reactive antibodies, 100% (P < .0001, Student's t test) were from 20 to 39 years of age (P < .05, x2). Donors of highly reactive specimens were almost exclusively male (P < .0001, x2), with an age distribution similar to that of the seronegative controls. The proportion of donors who had EIA-reactive antibodies and a history of blood donation since March 31, 1983, was not significantly different than for the seronegative control group (x2 test) (Table 2).

Interviews have been completed with 104 (69.3%) of the 150 EIA-positive donors. Twenty-six of these donors had a risk factor for HTLV-III/LAV infection; all but one had highly reactive...
EIA tests (Table 3). Twenty-four were men who were homosexual or bisexual during the five years before donation. The remaining man and the only woman had been heterosexual partners of an intravenous drug user and a bisexual man, respectively.

Seventy-eight of the 104 donors who were interviewed had no identified risk factor for HTLV-III/LAV infection. Only 26 of the 78 donors had highly reactive specimens on EIA; two of the four were Western blot and HTLV-III/LAV culture positive. Seventy-four of the 78 donors without an identified risk factor had moderate or low test reactivity on EIA; one of these was a woman who had a negative Western blot reaction but HTLV-III/LAV isolated on culture.

**COMMENT**

The anti-HTLV-III EIA test was successfully implemented into the routine screening procedure of the voluntary blood collection program at the ARCBS. The use of the test resulted in only 0.25% of donations being withheld from transfusion. The proportion of HTLV-III antibody-positive donors at the ARCBS is consistent with findings of a national survey conducted by the Food and Drug Administration.\(^2\)

Evaluation of a new test requires an established or known standard for comparison. At this point, however, no established standard exists for identifying HTLV-III infection in asymptomatic people. Current culture methods for HTLV-III identify virus in only 36% to 85% of persons with AIDS or related conditions\(^3\) and cannot be used as an absolute standard for HTLV-III/LAV infection. For this reason, we defined specimens positive on Western blot or culture as positive for infection with HTLV-III/LAV.

In a population with a defined prevalence of infection, positive predictive value for test results varies depending on the sensitivity and specificity of each test; for a given test with an established sensitivity and specificity, positive predictive value will vary with the prevalence of infection in the populations studied. In blood donors in our study, the proportion of all EIA-positive specimens infected with HTLV-III/LAV, or the positive predictive value, was 27.3%. The positive predictive value for the EIA test increased to 86.3% (95% confidence limits, 73.2% to 95.5%; binomial distribution) if the mean EIA ratio was 6.0 or greater. Conversely, the predictive value for test specimens with a mean EIA ratio of 1.0 to 5.9 was only 1.9% (95% confidence limits, 0.02% to 5.2%; binomial distribution).

Donors of EIA-positive specimens negative on Western blot and culture were more likely to be older and female than were seronegative donors. Others have found a similar relationship.\(^4\) Antibody to the HLA-DR4 or other cellular antigens may be the cause of some false-positive reactions.\(^5\)

Only the p24 band was identified in 28.2% of blood donors with a positive Western blot reaction. The presence of only a single band was verified by repeated testing and did not significantly affect the ability to culture virus. Persons recently infected with HTLV-III/LAV have been found on Western blot to initially have antibodies to p24 alone, followed by the later development of antibodies to gp41.\(^6,7\)

Our findings indicate that the anti-HTLV-III EIA is specific. We calculated the test specificity in this study to be 99.82%, which is almost identical with the 99.8% specificity estimated from studies conducted before licensure of the test.\(^8\)

Of 628 anti-HTLV-III EIA control specimens tested by Western blot and culture, none were positive for HTLV-III/LAV infection. This size and the low prevalence of HTLV-III/LAV infection of the control group do not allow for an accurate estimate of sensitivity. A study of similar design in a group of homosexual men at high risk for HTLV-III/LAV infection found none of 70 HTLV-III EIA-negative specimens positive on viral culture, while 43 (60%) of 72 EIA-positive specimens were culture positive.\(^9\)

Trials of test performance in symptomatic HTLV-III/LAV-infected persons conducted before licensure obtained a sensitivity of 95%\(^10\); as persons with AIDS may lose antibody, however, this may represent a low estimate.\(^11\) In selected populations, a small number of seronegative persons without AIDS have been found positive on HTLV-III/LAV culture.\(^2,5,10\)

Our study supports the need to discard units of blood that have a positive EIA reaction, as defined by the test manufacturer. Although we found no units of blood that were Western blot or culture positive if they had had only an initially reactive (nonrepeatable) EIA test, we had 2 units that had a positive EIA reaction and a negative Western blot result that were culture positive for HTLV-III/LAV. It is therefore important for persons at risk of infection to continue to refrain from donating blood.

Our study found little evidence that persons were donating blood primarily to have their antibody status determined. Most of the donors who had Western blot or HTLV-III/LAV culture-positive specimens had donated previously, and the proportion who were new donors was not different from those who were seronegative.

The study identified 26 HTLV-III/LAV-infected persons who donated blood after being provided with recommendations for self-deferral of donors with risks for HTLV-III/LAV infection. The reason why these persons continued to donate blood is unclear, although anecdotal information obtained on interview indicated that some donors did not perceive themselves at risk for HTLV-III/LAV infection. The recommendations for donor deferral have been rewarded to better inform persons of the reason for not donating blood.\(^12\)

The number of units of blood donated by HTLV-III/LAV-infected people is a small fraction of all donations, but the HTLV-III EIA screening program should have significant impact on potential virus transmission to transfusion recipients. The ARCBS collects more than 194,000 units of blood each year in Georgia, and an average of two blood components are derived from each unit. If the 0.07% prevalence rate of HTLV-III/LAV infection among blood donors remains constant, an estimated 136 seropositive units will be identified and 272 HTLV-III/LAV infections among transfusion recipients potentially averted in the Atlanta Region alone.

New cases of transfusion-associated AIDS will continue to occur among people exposed before the screening program was initiated, but the current
use of the EIA test to screen donated blood will markedly reduce the risk of exposure by this route in the future and ultimately the number of transfusion-associated AIDS cases.

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