Blood donations in the United States have been screened for antibody to human T-lymphotropic virus type I (HTLV-I) by HTLV-I enzyme immunoassay (EIA) since November 1988. Specimens repeatedly found to be reactive by EIA undergo confirmation by supplementary serologic tests. We assessed the accuracy of blood center testing of 994 HTLV-I EIA repeat-reactive specimens in five US blood centers between November 1988 and December 1991. Of 410 confirmed HTLV-I/II donations, 407 (99.3%) were infected with HTLV-I/II, as determined by polymerase chain reaction (PCR) (403 cases) and by repeat serologic testing (4 cases). The three false-positive results occurred in the first year of testing. Of 425 HTLV-indeterminate specimens, 6 (1.4%) were found to be infected by PCR (5 with HTLV-II and 1 with HTLV-I). None of 159 confirmatory test-negative donations was PCR positive. Of HTLV-I/II-seropositive specimens, 80.2% to 95.4% could be typed as HTLV-I or HTLV-II by type-specific serologic assays. These results support recommendations that HTLV-I/II-seropositive donors should be advised that they are infected with HTLV-I, HTLV-II, or HTLV-I/II (depending on results of type-specific assays). HTLV-indeterminate donors should be advised that their results only rarely indicate HTLV infection. HTLV confirmatory test-negative donors should be reassured that they are not infected with HTLV-I or HTLV-II.

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establish the rates of HTLV-I and HTLV-II infection among serologically confirmed positive donors, as well as the rate of infection among donors classified as serologically indeterminate. In addition, we evaluated the performance of three widely used HTLV-I and HTLV-II peptide EIA typing systems relative to virus typing by PCR.

MATERIALS AND METHODS

Sample selection and routine HTLV supplemental test procedures. Eligible participants were persons who voluntarily donated blood from November 1988 to December 1991 at five regional blood centers in the United States: American Red Cross (ARC) Blood Services of Greater Chesapeake/Potomac (Baltimore, MD/Washington, DC), Southern California (Los Angeles), and Southeastern Michigan (Detroit) Regions; Irwin Memorial Blood Centers (San Francisco, CA); and Oklahoma Blood Institute (Oklahoma City). All blood samples were screened at the respective blood centers for antibody to HTLV-I/II by a licensed HTLV-I viral lysate EIA (Abbott Laboratories, Abbott, IL). Repeatedly reactive samples underwent anti-HTLV-I supplemental testing according to the centers' standard operating procedures. For samples donated to the two non-ARC blood centers, confirmatory testing consisted of HTLV-I whole virus lysate WB and, if not confirmed as antibody-positive, RIPA, both performed at the Abbott Virology Reference Laboratory, Abbott Laboratories. Criteria for HTLV-I/II seropositivity included gag p24 and env gp46 or gp61/68 reactivity in WB or RIPA. The three ARC Blood Services Regions used a modified confirmatory testing algorithm consisting of WB testing at ARC National Reference Laboratory for Infectious Diseases (ARC-NRLID) with HTLV-I whole virus lysate immunoblot strips (Abbott Laboratories) and a recombinant p21e EIA (Cambridge Biotech, Worcester, MA) for detection of envelope antibody. Samples were classified as HTLV-I/II seropositive if both p24 and gp46 could be identified by WB, or if p24 was identified by WB and the p21e EIA was positive with a signal-to-cutoff (S/C) ratio greater than 6.0. If the WB was positive for p24 and negative for gp46 and the S/C ratio of the p21e EIA test was between 0.8 and 6.0, the sample was tested further by RIPA (Abbott Laboratories). This modified confirmatory testing algorithm has been shown to have equal sensitivity and specificity to the standard algorithm of WB and RIPA, while reducing the number of RPIAs required. At all five blood centers, samples with reactivity to HTLV were separated by Ficoll-Hypaque from enrollment blood samples that tested positive (seropositive) before October 1990, which is when the WB was positive for p24 and negative for gp46 and the S/C ratio identified by WB, or if p24 was identified by WB and the p21e EIA showed no bands on WB were classified as confirmatory test negative.

REDs evaluation protocol and procedures. The REDs evaluation had three elements. The first consisted of HTLV-I and HTLV-II PCR analysis of peripheral blood mononuclear cells (PBMCs) from 295 donors classified as HTLV-I/II antibody supplemental test positive (seropositive) before October 1990, which is when the prospective laboratory component of REDs began. These donors were identified from blood center test records and were recalled and enrolled into the REDs ongoing prospective cohort study. PBMCs were separated by Ficoll-Hypaque from enrollment blood samples from these subjects within 24 to 48 hours of phlebotomy and frozen in vapor-phase liquid nitrogen at the REDs central laboratory (SRA Collection, Rockville, MD). Serum or plasma was stored at −70°C. For the present study, PBMCs were thawed and tested using HTLV-I and HTLV-II PCR assays directed at the pol and tax/rex regions of the virus (see below). If PCR results were negative, both PCR and confirmatory serologic assays were repeated. In this instance, we used a recombinant p21e spiked WB and the recombinant p21e EIA (Cambridge Biotech, Rockville, MD) and, if necessary, RIPA. If repeat PCR and supplemental serologic results remained discrepant, donors were recalled and all testing was repeated using fresh blood samples.

The second component of the study began in October 1990 and continued through December 31, 1991. During this period, aliquots of blood from anti-HTLV-I repeat reactive donor units were shipped to SRA Technologies, where PBMCs and serum or plasma specimens were separated and frozen. For logistic reasons, blood shipment and cell separation were not performed until 2 to 7 days postcollection, and a portion of reactive units, including the majority of reactive autologous units, was not available for study. HTLV-I and HTLV-II PCR analysis was performed on coded PBMCs from these units without knowledge of supplemental serologic results from ARC-NRLID and Abbott. After serologic and PCR results were obtained and compared by the coordinating center, the central laboratory was instructed to perform serologic and repeat PCR assays on all samples with discrepant results (ie, positive-serology/negative-PCR; negative-serology/indeterminate-serology/positive-PCR). If results remained discrepant, donors were recalled and all studies were repeated on freshly collected samples, which were processed within 24 to 48 hours of phlebotomy. In addition, we performed further supplemental serologic studies on all samples classified as HTLV-indeterminate by Abbott or ARC-NRLID, irrespective of PCR results.

Lastly, to evaluate the performance of synthetic peptide typing ELISAs on HTLV-I/II-seropositive sera compared with PCR typing, we obtained peptide EIA results from Abbott and ARC-NRLID for all serologically and/or PCR-confirmed positive donors evaluated in the previous studies. For these determinations, Abbott used an in-house peptide EIA system, whereas ARC-NRLID used a commercially available test kit (United Biomedical, Inc [UBI], Lake Success, NY). The REDs central laboratory also tested sera from all positive donors using a third peptide EIA system (Coulter Diagnostics, Hialeah, FL). Results of each peptide typing system were compared with PCR typing results and with one another. All samples with discrepancies in type designations between assays were resubmitted for repeat PCR and serologic typing at the source reference laboratory.

PCR methodologies. PCR testing was performed by SRA without knowledge of the serologic test results. Appropriate procedural controls were used to prevent "carryover" DNA extraction was performed according to previously described procedures. Each DNA extract was amplified as duplicate reactions with one negative control per sample, with each of two primer sets, SK110/111 (pol) and SK43/44 (tax/rex), for 40 cycles as follows: 94°C for 90 seconds; 94°C for 75 seconds; 55°C (for SK43/44) or 58°C (for SK110/111) for 75 seconds; and 72°C for 60 seconds. Routine PCR contained DNA from 3 × 10⁴ cells/reaction. 1.25 mmol/L MgCl₂, 1.0 mmol/L each dNTP, and either 1.5 U (nol) or 2.5 U (tax/rex) of AmpliTaq (Perkin Elmer, Norwalk, CT) per reaction. Detection of products was by solution hybridization to 32P-labeled oligo probes and electrophoresis on 10% polyacrylamide gels by procedures that have been previously described in detail. Each SK43/44 product, a generic probe was used; for SK110/111, type-specific probes were used. An assignment of I or II was made based on the observed pol reactivity, with generic tax reactivity being used for confirmation of HTLV-I/II positivity. The MT-2 cell line obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD) was included as an HTLV-I-positive control, and MOT (American Type Culture Collection, Rockville, MD) was used for an HTLV-II control. On selected samples, other PCR procedures were used. High-in-
put PCR was performed using DNA extracted from $9 \times 10^6$ PBMCs per reaction. Hot-start PCR\textsuperscript{23} was performed using AmpliWax (Perkin Elmer) according to standard procedures recommended by the manufacturer. In each case, detection of amplified products was according to the standard solution hybridization procedure.

**RESULTS**

Of the 295 recalled donors who had been classified as HTLV-I/II seropositive by blood center reference laboratories before October 1990, PBMCs from 292 (99%) tested PCR positive. Eighty-eight (30%) cases typed as HTLV-I and 204 (70%) as HTLV-II; no nontypable or dual virus-positive infections were found. The 3 PCR-negative donors were recalled for additional follow-up testing at 2 to 5 months after the initial sample date. Repeat PCR analyses of new samples were again negative. Additional serologic testing of follow-up serum samples from these 3 donors was performed in both the blood center reference laboratory that initially classified them as HTLV-I/II seropositive and in the REDS central laboratory. WBs in both labs showed gag antibodies, but no env antibodies were detected by either recombinant or native virus antigen-based assays, including RIPAs, in both laboratories. We concluded that these 3 donors were misclassified as seropositive (on the basis of purported env reactivity detected by RIPA) after their original blood donations, all of which occurred during 1989 within the first year of routine HTLV antibody screening of donors.

From October 1990 through December 1991, the five participating blood centers tested 1,107,270 donations and identified 854 HTLV repeat-reactive donations. Of these, 699 (82%) had specimens submitted to the REDS central laboratory for parallel PCR analysis. The majority (96 of 155) of the reactive samples that were not submitted were from autologous donors; indeed, only 3 of 99 EIA-reactive autologous units were submitted to the study, with the remainder released for autologous transfusion. The supplemental test-positive rate of nonautologous donations tested by PCR was similar to the positive rate for those not submitted for evaluation (113 of 696 [16.2%] v 11 of 59 [18.6%]), indicating a representative study population.

The results of PCR analysis of the 699 HTLV-I EIA repeat-reactive donations are summarized in Table 1 according to their classification by supplemental serologic testing at the source reference laboratory. None of 159 donations classified as HTLV negative by supplemental assays was PCR positive when retested. In contrast, 103 of 115 (91.3%) supplemental test-positive donations tested PCR positive. Further serologic evaluation of the 12 PCR-negative samples using p21e WB and RIPA corroborated the presence of gag and env antibodies in all 12 cases. Repeat PCR analysis was performed for all additional aliquots of PBMCs from these 12 specimens and was again negative. We suspected that the lower PCR-positivity rate observed with these donations, compared with the 99% PCR-positivity rate observed with the 295 recalled donors discussed above, might be attributable to the fact that the cells from these 115 donors were obtained from the original donation bags up to 1 week after phlebotomy. Ten of the 12 donors consented to study enrollment, an average of 5.8 months (range, 2 to 15 months) after their index donations. (For the 2 non-enrolled donors, 1 typed as HTLV-I and 1 as HTLV-II by type-specific EIA.) All 10 follow-up sera were again confirmed as serologically positive. PCR analysis of freshly isolated PBMCs was positive for HTLV-II DNA in 7 of the 10 donors. The 3 remaining cases that were repeatedly negative by standard PCR were further studied using two enhanced sensitivity PCR techniques (hot-start and high-input PCR). One of these cases was positive for HTLV-I DNA by high-input PCR, whereas the other 2 remained negative. These 3 donors were recalled a third time, and again tested serologically positive, with the 1 case PCR positive for HTLV-I by high-input analysis and the other 2 cases PCR negative. By HTLV type-specific peptide EIA analysis, 1 of the 2 PCR-negative cases was typed as HTLV-I and 1 as HTLV-II. (The 1 HTLV-I PCR-positive case was also peptide-typed as HTLV-I.)

Of 425 serologically indeterminate samples, 6 tested PCR positive (Table 1). None of 183 donations with isolated p19 reactivity by WB was PCR positive (Table 2). In contrast, 4 (3%) of 132 donations with p24-only by WB were positive by PCR for HTLV-II. The positive PCR results were confirmed by testing a second aliquot of PBMCs from the donation sample in all 4 cases, and by PCR evaluation of follow-up samples obtained from 3 of the 4 donors (1 refused enrollment), an average of 14 months postdonation. Of 108 indeterminate donations with p19 and p24 reactivity by WB, 1 was PCR positive for HTLV-I and 1 for HTLV-II (Table 2). These PCR results were also confirmed by reanalysis of index donation-derived PBMCs, as well as on an 8-month follow-up specimen from 1 of the 2 donors (the other

<p>| <strong>Table 1. Results of HTLV-I and HTLV-II PCR Assays on 699 HTLV-EIA Repeat Reactive Donations According to Serologic Classification of Specimens by Supplemental Antibody Assays at Blood Center Laboratories</strong> |</p>
<table>
<thead>
<tr>
<th><strong>Anti-HTLV-I/II</strong></th>
<th><strong>Supplemental Serology</strong></th>
<th><strong>PCR Results</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(WB/RIPA/p21e EIA)</td>
<td>N</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td>Indeterminate</td>
<td></td>
<td>425</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>159</td>
</tr>
</tbody>
</table>

* Ten of 12 HTLV-I/II-seropositive, PCR-negative donors enrolled in follow-up studies; 7 were positive for HTLV-II and 1 for HTLV-I by PCR on freshly separated PBMCs.

<p>| <strong>Table 2. Results of HTLV-I- and HTLV-II-Specific PCR Assays on 425 Indeterminate Donations</strong> |</p>
<table>
<thead>
<tr>
<th><strong>HTLV-I Western Blot Reactivity</strong></th>
<th><strong>PCR Results</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>p19 only</td>
<td>183</td>
</tr>
<tr>
<td>p24 only</td>
<td>132</td>
</tr>
<tr>
<td>p19 + p24</td>
<td>108</td>
</tr>
<tr>
<td>Nonviral</td>
<td>2</td>
</tr>
</tbody>
</table>

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to be seropositive in the previous studies). Whereas only determinate, PCR-positive specimens subsequently shown greater than 99% of cases. Moreover, if peptide typing was generally successful with another manufacturer's kit. The overall frequency of HTLV infection detectable by PCR among donors classified as HTLV indeterminate was 1.4% (6 of 425). Among donors with a p24 band (±p19 band) by WB, 2.5% (6 of 240) were PCR positive, whereas no donors with p19 only were infected. Five of 6 PCR-positive, HTLV-indeterminate donors were determined to be infected with HTLV-I1. Similar rates of detection of HTLV-I1-positive sera (6.7% [5 of 74] in this study). Using recombinant envelope p24e-based supplemental assays that use envelope antigenic determinants common to both viruses, we detected envelope reactivity in all 6 of these donors' sera.

Table 3. Performance of HTLV-I and HTLV-II Serologic Typing Systems Compared With Virus Type Determined by PCR

<table>
<thead>
<tr>
<th>Typing System (Lab)</th>
<th>No. of HTLV-I/II-Seropositive Donors Tested</th>
<th>HTLV-I</th>
<th>HTLV-II</th>
<th>Nonreactive</th>
<th>Reactive But Untypable</th>
<th>Type* Conflict With PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR (REDS)</td>
<td>395</td>
<td>120</td>
<td>30</td>
<td>271</td>
<td>69</td>
<td>4</td>
</tr>
<tr>
<td>Peptide EIAs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbott (Abbott)</td>
<td>110</td>
<td>40</td>
<td>36.4</td>
<td>55</td>
<td>50.0</td>
<td>14†</td>
</tr>
<tr>
<td>UBI/Olympus (ARC)</td>
<td>288</td>
<td>76</td>
<td>26.4</td>
<td>155</td>
<td>53.8</td>
<td>49†</td>
</tr>
<tr>
<td>Coulter (REDS)</td>
<td>395</td>
<td>116</td>
<td>29.4</td>
<td>261</td>
<td>66.0</td>
<td>15§</td>
</tr>
</tbody>
</table>

* Number of specimens for which HTLV type determined by PCR and peptide EIA were discrepant, relative to number for which both PCR and peptide type designations were obtained.
† Of the 15 specimens untypable by the Abbott test, 1 was PCR positive for HTLV-I and 14 and HTLV-II.
‡ Of the 57 specimens untypable by the UBI/Olympus test, 6 were PCR positive for HTLV-I and 49 for HTLV-II, and 2 were PCR-negative.
§ The 2 specimens with repeatable discrepant peptide EIA/PCR typing results were from different donors. In both cases, the results of an alternative peptide EIA typing system were not inconsistent with PCR, ie, the UBI/PCR discrepancy typed consistent with PCR (HTLV-II) by Coulter, whereas the Coulter/PCR discrepancy was nonreactive by UBI typing.
¶ Of the 18 specimens untypable by the Coulter test, 5 were PCR positive for HTLV-I and 12 for HTLV-II, and 1 was PCR negative.

DISCUSSION

Our independent laboratory study of 994 HTLV-I EIA-reactive blood donor specimens, using HTLV-I and HTLV-II PCR and additional serologic techniques, indicates that HTLV supplemental antibody test procedures and algorithms used by blood bank reference laboratories are highly accurate. Of 410 confirmed positive donations investigated, only 3 (0.7%) false-positive classifications were identified. These misclassifications all occurred during the first year of HTLV testing of blood donors, and likely represented over-interpretation of RIPA results during the early experience with this assay, because resubmission of these sera for evaluation by contemporary RIPA did not show envelope reactivity. Four additional seropositive donors were PCR negative, but in 2 of these cases PCR was performed on specimens collected under suboptimal conditions. All 4 of these donors were confirmed as seropositive on repeat testing, and all could be typed serologically as HTLV-I (2 cases) or HTLV-II (2 cases). Therefore, these 4 donors were believed to be infected with HTLV-I or HTLV-II.

Similarly, supplemental test-negative donors appear to be truly negative for HTLV-I/II. We detected no false-negative results (ie, PCR positivity) among the 159 supplemental test-negative donors evaluated. However, it is important to note that we cannot exclude the possibility that some HTLV-II-infected samples might be missed by HTLV-I-based screening assays usually used, and would therefore escape detection by the blood center testing algorithms. Such an insensitivity for HTLV-II-infected samples has been documented.22

The overall frequency of HTLV infection detectable by PCR among donors classified as HTLV indeterminate was 1.4% (6 of 425). Among donors with a p24 band (±p19 band) by WB, 2.5% (6 of 240) were PCR positive, whereas no donors with p19 only were infected. Five of 6 PCR-positive, HTLV-indeterminate donors were determined to be infected with HTLV-II. Similar rates of detection of HTLV-II and, rarely, HTLV-I infections among indeterminate donors have been reported by others.23-25 It is not surprising that HTLV-I viral lysate-based WB and RIPA fail to detect envelope antibody in a small proportion of anti-HTLV-II-positive sera (6.7% [5 of 74] in this study). Using recombinant envelope p24e-based supplemental assays that use envelope antigenic determinants common to both viruses, we detected envelope reactivity in all 6 of these donors' sera. Although they are unlicensed, HTLV-supplemental anti-
SUPPLEMENTAL TESTS FOR ANTI-HTLV-I AND -II

body assays that incorporate p21e and other recombinant HTLV-I- and HTLV-II-specific envelope antigens are already available.11-13,16 Use of these assays in place of, or in conjunction with, HTLV-I viral lysate-based reagents should result in a reduction in the already low rate at which infected donors are misclassified as indeterminate.

Our study establishes that virtually all persons with serologic evidence of HTLV-I or HTLV-II antibodies harbor persistent viral infections. Of 407 donors with confirmed supplemental serologic results tested by PCR, 403 (99%) were positive (123 for HTLV-I and 280 for HTLV-II). If we accept the peptide EIA type-specific results for the 4 PCR-negative donors, the rate of persistent viremia for HTLV-I is at least 98.4% (123 of 125) and the rate for HTLV-II is at least 99.3% (280 of 282). We are continuing to study and observe the 2 enrolled PCR-negative, confirmed antibody-positive donors to determine whether they have evidence of intermittent detection of low concentrations of HTLV-I- or HTLV-II-positive cells using enhanced-sensitivity PCR techniques.

With respect to differentiation of HTLV-I and HTLV-II type discriminations by peptide EIAs, it was reassuring that misclassifications by peptide EIA relative to PCR were rare. We identified only 2 such cases among the 395 HTLV-positives tested. The more common failure of the peptide EIA typing systems relates to an inability to assign a viral type, which we observed in 5% to 20% of cases, depending on the assay system. In our experience, more than 80% of confirmed-positive sera not typed by one peptide EIA system could be typed using peptide EIAs from another manufacturer, leaving fewer than 5% of donors untyped by serologic methods. For those donors, PCR could be performed on PBMCs obtained at the time of notification of their confirmed positive HTLV results.

Overall, the present data support the following counseling guidelines recently published by a CDC/USPHS working group.3 (1) Donors classified as seropositive are infected and should be advised accordingly. (2) Donors classified as indeterminate are rarely infected, especially if they show evidence of p19 only on WB, or are determined to be indeterminate on two occasions and therefore not seroconverting. (3) EIA-reactive donors who test negative by supplemental assays are uninfected and should be reassured accordingly. (4) HTLV-I versus HTLV-II typing is practical and accurate using peptide EIA kits and/or PCR, and should be a routine element of supplemental testing for HTLV seropositive subjects.

ACKNOWLEDGMENT

The authors thank Drs Chyang Fang (ARCNNRLID) and Steve Delaney (Abbott) for providing peptide typing data and assistance in resolution testing. We also thank the REDS nurses and technicians for collection and submission of samples, and the staff at SRA Laboratories and Westat. Finally, we acknowledge the blood donors who consented to participate in this study.

APPENDIX

The Retrovirus Epidemiology Donor Study (REDS) is presently the responsibility of the following persons:

| --- |

REFERENCES


Accuracy of supplementary serologic testing for human T-lymphotropic virus types I and II in US blood donors. Retrovirus Epidemiology Donor Study

MP Busch, M Laycock, SH Kleinman, JW Jr Wages, M Calabro, JE Kaplan, RF Khabbaz and CG Hollingsworth