Evaluation of atypical human immunodeficiency virus immunoblot reactivity in blood donors

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Blood donors reactive by enzyme-linked immunosorbent assay for antibody to the human immunodeficiency virus (HIV) who showed atypical patterns of viral core protein reactivity on Western blot were monitored for several months. Characterization of their antibodies was performed by 1) use of recombinant HIV proteins; 2) determination of cross-reactivity to HTLV-I, HTLV-II, and HTLV-IV: 3) assessment of immune status; and 4) identification of potentially interfering autoantibodies. Nineteen of 20 donors maintained the same HIV antibody reactivity throughout the follow-up period; the other donor became fully antibody-positive. Eighteen of 20 donors' sera showed clear reactivity with HIV recombinant core proteins. Ten of 19 donor samples demonstrated cross-reactivity to HTLV-IV; 3 of these 10 also cross-reacted with HTLV-I. The immune status of all donors was normal, although the medical histories and HLA antibody screens suggested possible autoimmune reactivity in 9 of 18 donors. During follow-up interviews, three donors reported possible risk factors for HIV infection that had not been acknowledged at the time of blood donation. We conclude that exclusion of donors with these atypical serologic test results is warranted while further studies to determine significance are being conducted. **TRANSFUSION** 1988;28:412-418.

SINCE THE LICENSURE of reagents to screen donated blood for the presence of antibody to human immunodeficiency virus (HIV), it has become apparent that these assays have varying degrees of sensitivity and specificity. In addition, some widely used enzyme-linked immunosorbent assays (ELISA) identify a group of donors whose Western blot (WB) patterns are neither reactive nor nonreactive but have "indeterminate" or "atypical" patterns characterized by reactivity with group-specific (core) viral protein antigens. Possible explanations for this reactivity include the early stage of the donor's seroconversion to HIV positivity, the biologic variability of the response to HIV, a cross-reaction with other retroviral antibody or autoantibodies, and technical artifact. To reconcile equivocal test results, it is often prudent to recall the donor and test an additional blood sample. In our experience, notification of donors with ambiguous HIV test results is fraught with difficulty and evokes considerable anxiety in the donor. Our investigations are aimed at understanding the significance of a repeatedly reactive ELISA screening test associated with atypical WB patterns and implementing this knowledge in follow-up testing interviews and donor notification.

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Materials and Methods

Study design

The Syracuse Regional Blood Services of the American Red Cross participated with the Red Cross National Headquarters Laboratories in comparing and evaluating several HIV antibody screening assays for use in blood collection centers. During the winter of 1985-1986 and again in June 1986, prospective comparisons of two ELISA; (Abbott Laboratories, North Chicago, IL) and (DuPont, Wilmington, DE) were performed. WB assays of all repeatedly reactive samples were performed at Abbott Laboratories and at Biotech Research Laboratories (Rockville, MD). Samples that tested Abbott-reactive and DuPont-negative were WB negative. When the Abbott ELISA was negative and the DuPont ELISA was reactive, several samples had "indeterminate" or "atypical" WB patterns. To verify the WB reactivity and to investigate its origin, we selected the 20 most strongly reactive samples for further evaluation. These donors agreed to be retested and studied, and they were enrolled in an ongoing study of healthy blood donors reactive for anti-HTLV-III, conducted by the American Red Cross (ARC) Transmissible Diseases Laboratory, Rockville, MD, and the ARC Los Angeles/Orange Counties, Northeast (Boston), and Syracuse Blood Services Regions. Follow-up visits occurred at 6-month intervals; the donor filled out an extensive lifestyle questionnaire, a general health assessment was performed, and a blood sample was collected. Laboratory assays were performed to determine if antibody detected in these donors' samples was directed specifically toward HIV or was due to cross-reacting antibody from other retroviruses or to interference by autoantibody.

HIV serologic assays

HIV antibody screening was performed using Abbott and DuPont ELISA, according to each manufacturer's established procedures. Immunoblotting was performed by Bio-

tech Research Laboratories, Genetic Systems Corp. (Seattle, WA), Abbott Laboratories, and the Applied Biotechnology Group at DuPont, with each laboratory using whole-virus (HIV) lysate as the antigen and performing the immunoblotting according to its own standard procedures.

Recombinant protein assays

Three assays using viral proteins derived from DNA recombination techniques were applied to donor samples. The recombinant protein "GAG 55" (DuPont) was derived from a construct of the entire HIV core protein-encoding region (*gag*) expressed in *Escherichia coli*.[1] Since antibody is induced in response to the *gag*-encoded precursor protein p55, as well as to its cleavage products p17 and p24, "GAG 55" is a useful tool for detecting antibody to any or all of these core proteins.

"GAG 55" was used in two types of studies: 1) direct immunoblotting using "GAG 55" as the antigen and 2) inhibition of the whole-virus lysate immunoblot by preincubation of sera with "GAG 55." For direct immunoblotting, a test antigen strip of "GAG 55" and a control antigen strip of *E. coli* protein without "GAG 55" were tested against donor sera. Reactivity on the test strip without interference in the same region(s) on the control strip was read as positive. For inhibition experiments, donor sera were incubated with "GAG 55" to absorb a specific antibody, before being added to the whole-virus lysate antigen strip. As controls, untreated serum and serum incubated with *E. coli* protein extracts lacking "GAG 55" were assayed. The absence or weak evidence of a WB band in the test strip, as compared to that in the control strips, indicated that inhibition had occurred. Previous inhibition experiments using HIV antibody-positive samples from acquired immune deficiency syndrome (AIDS) patients have demonstrated that preincubation with "GAG 55" blocks *gag*-specific reactions, but leaves *env* and *pol* reactions unaffected.[2]

The recombinant envelope protein "ENV 9" (DuPont), which includes the immunodominant regions of gp41 and a short portion of gp120, served as antigen for an ELISA on which donor sera were tested as described by Kenealy et al.[3] In detection of HIV seropositivity, "ENV 9" compared favorably with radioimmunoprecipitation.[4]

Other retroviral assays

Three assays were used to detect antibody to other human retroviruses. WBs were performed to detect HTLV-I and HTLV-IV, and an ELISA using sucrose gradient-purified HTLV-II produced by the cell line Mo-T (a gift of Dr. David Golde, Department of Medicine-UCLA School of Medicine, University of California, Los Angeles Center for Health Sciences, Los Angeles, CA)[5] was performed to detect HTLV-II.

Immunologic assays

Lymphocyte counts were performed using a cell counter (Model 660, Coulter Electronics, Hialeah, FL) for the white cell count and a manual reading for the cell differential. An absolute lymphocyte count of greater than 750 per mm^3 was considered normal. Ratios of CD4 to CD8 lymphocytes were performed using monoclonal antibodies T4 and T8 (Coulter Immunology, Hialeah, FL) to label mononuclear cells. A ratio greater than 1.0 was considered normal.

To assess the in vitro mononuclear cell response to mitogens, optimal proliferative doses of pokeweed mitogen (PWM) and phytohemagglutinin (PHA) were added to mononuclear cells, incubated, pulsed with tritiated thymidine, and harvested to measure thymidine incorporation by scintillation counting. The mean counts per minute (cpm) of quadruplicate cultures divided by the mean cpm of quadruplicate control cultures yielded a stimulation index (SI) for each mitogen. Normal values for these assays were SI >1.5 for PWM and SI >2.0 for PHA. Assays for rheumatoid factor (Organon Teknika Corp., Durham, NC) and antinuclear antibody (Electro-Nucleonics, Inc., Columbia, MD) were performed according to manufacturer's instructions.

Table 1. *Comparison of WB patterns*

	Initial sample		Follow-up sample		
Donor number	Biotech Research Laboratories	Months between samples	Biotech Research Laboratories	Genetic Systems	Abbott Laboratories
S-029	p17	2	p17	p18	Neg
S-080	p17	10	p17	p18	Neg
S-088	p17, p55, p > 100	2	p17	p18, p25	Neg
S-098	p17, gp41, p > 100	2	p17	p18	Neg
S-137	p17, gp41, p > 100	2	p17	p18	Neg
S-139	p17	2	p17	p18	Neg
S-182	p17, p24	2	p17	p18	Neg
S-035	p17	2	p17, p55	Neg	Neg
S-116	gp41, p53, p64	2	p17, p55	p25	Neg
S-195	p17	2	p17, p55	p18	Neg
S-198	p17, p55	10	p17	p18	Neg
S-016	p24	10	p24	p25	Neg
S-031	p24, p55	9	Neg	p25	Neg
S-086	p24, p55	9	p24, p55	p18, p25	Neg
S-112	p24, p55	10	p24, p55	p25	Neg
S-167	p24, p55	10	p24, p55	p25	Neg
S-095	p17, p24	10	p24	Neg	Neg
S-106	p17	2	p17	p18, p25	Neg
S-191	p24	2	p24	p18, p25	Neg
S-162	p24	2	All bands	All bands	Reactive

Table 2. *Reactivity with HIV recombinant proteins and other retroviral antigens*

Test Results	HIV ENV-9 ELISA	HIV GAG 55: Direct WB using GAG 55	HIV GAG 55: GAG 55 inhibition of HIV WB	Other retroviral antigens: HTLV-I WB*	Other retroviral antigens: HTLV-II ELISA	Other retroviral antigens: HTLV-IV WB*
Reactive	1	15	18	3	0	10
Nonreactive	19	5†	2	16	20	9

* Nineteen of 20 samples tested.
† Includes three indeterminate samples.

Donor sera were screened for antibody to HLA antigens. Antibody to Class I HLA antigens was assayed by a standard complement-mediated microcytotoxicity assay using a panel of mononuclear cells from 20 normal individuals who were previously HLA-typed and selected to represent the known A, B, and C specificities. Antibody to Class II HLA antigens was assayed by the same microcytotoxicity technique using a panel of mononuclear cells depleted of T lymphocytes. "DR" types (used in the Results section) indicated the HLA-DR specificity of the panel cells used in the test.

Results

HIV core protein reactivity of each initial sample was confirmed on each follow-up sample by at least one assay (Table 1). Four donors (S-088, S-098, S-116, and S-137) were selected for repeat testing because of their unusual WB patterns. When subsequent samples from these donors were tested, only core protein reactivity was observed. These differences are probably due to the antibodies present in the serum samples, although they could represent variation in immunoblots that were performed at different times. In gen-

Table 3. *Assays using recombinant GAG 55*

Donor number	GAG 55 immunoblot: Control*	GAG 55 immunoblot: Test†	Whole virus immunoblot inhibition using GAG 55 (bands observed): Control‡	Control§	Test‖
S-029	0	++	p17	p17	None
S-080	0	+	p17	p17	None
S-088	0	+++	p17	p17	p17
S-098	0	+	p17	p17	None
S-137	0	++	p17	p17	None
S-139	0	++	p17	p17	p17
S-182	0	+	p17	p17	None
S-035	BKGD	BKGD	p17, p55	p17, p55	p17 (weak)
S-116	0	+	p17, p55	p17, p55	p17
S-195	BKGD	BKGD	p17, p55	p17, p55	p17 (weak)
S-198	0	+++	p17, p55	p17, p55	p17 (weak)
S-016	0	0	p24	p24	None
S-031	0	+	p24, p55	p24, p55	None
S-086	0	0	p24, p55	p24, p55	p55
S-112	0	+++	p24, p55	p24, p55	p24 (weak)
S-167	0	+	p24, p55	p24, p55	None
S-095	0	++	p17, p24	p17, p24	None
S-106	0	+++	p17, p24	p17, p24	p17
S-191	BKGD	BKGD	p17, p24	p17, p24	None
S-162	0	++	All bands	All bands	Only core bands blocked
Negative control	0	0	None	None	None
Positive control (AIDS serum)	0	+++	All bands	All bands	Only core bands blocked

* Immunoblot strip with *E. coli* proteins reacted directly only with serum (0 = negative; BKGD = interfering background reactivity).
† Immunoblot strip with GAG 55 and *E. coli* proteins reacted directly with serum (0 = negative; + = weakly positive; ++ = positive; +++ = strongly positive; BKGD = uninterpretable).
‡ No incubation of sera with *E. coli* proteins or GAG 55.
§ Sera incubated with *E. coli* protein prior to performance of assay.
‖ Sera incubated with GAG 55 and *E. coli* proteins prior to performance assay.

eral, agreement between the Biotech and Genetic Systems WBs was seen, although the antigen was prepared from different cell lines (Biotech from H9, Genetic Systems from CEM). We had anticipated that immunoblot core protein reactivity would not be seen when the Abbott WB was used, because the study subjects were selected from a group of donors whose sera were positive on the DuPont ELISA and negative on the Abbott ELISA, and the specificity of each manufacturer's WB is usually set to match the reactivity of its ELISA.

Although minor changes were observed in the intensity of core protein bands and/or the detection of some core and/or glycoprotein bands, the initial and follow-up samples remained atypical in all but one donor (S-162). The initial sample from this donor showed only p24 on WB, but the sample collected 2 months later showed full reactivity with core, envelope, and polymerase bands on WBs from all laboratories. The fortuitous detection of this donor's HIV seroconversion provided an HIV-reactive sample for comparison with the other 19 atypical samples. Retrospective examination of the initial sample from donor S-162 found it to be positive on the DuPont ELISA and the Genetic Systems ELISA and negative on the Abbott ELISA; to show p24 alone on the Biotech and Abbott WBs; to show p18, p25, and p40 on the Genetic Systems WB; and to be reactive on the "ENV 9" ELISA. The follow-up sample from donor S-162 was positive on all of these assays.

The nature of the protein reactivity seen on the WBs was examined using *E. coli*-expressed proteins "GAG 55" and "ENV 9." The results of this testing are summarized in Table 2, and the details of "GAG 55" testing are given in Table 3. When recombinant HIV core protein "GAG 55" was used as antigen for direct immunoblotting, four strongly positive, five positive, and six weakly positive reactions were obtained. Two samples were nonreactive. Three samples showed background reactivity with *E. coli* proteins, which obscured evaluation. Previous control experiments for "GAG 55" direct immunoblotting showed that none of 100 ELISA HIV-negative sera had background reactivity with *E. coli* proteins.[1] The inhibition experiment, performed by incubating "GAG 55" protein with each donor serum prior to reacting the donor serum with HIV whole-virus protein on immunoblot, showed full blocking in 10 samples, partial blocking in 8, and no blocking in 2. These data suggest that antibody detected by ELISA and WB is indeed reactive with HIV retroviral core protein. Our data also show that only the initial sample from the donor who seroconverted (S-162) was reactive with the "ENV 9" ELISA, a reaction that has been shown to be a sensitive detector of the early stages of HIV seroconversion.[6]

Results of the testing for potential cross-reactivity with HTLV-I, HTLV-II, and HTLV-IV are summarized in Table 2. Three of 19 samples had weak reactivity to HTLV-I on WB: 2 with a p26 band and 1 with a p53 band. In these three

Table 4. *Medical history, lifestyle assessment, and laboratory indications for unusual immunoreactivity*

Donor number	Age	Gender	Donor exclusion criteria	Animal exposure	Autoimmune health history indicators	HLA Class I antibody	HLA Class II antibody	Rheumatoid factor	Anti-nuclear antibody
S-029	47	M	no	Cats, dogs; drank unpasteurized milk	None	–*	–	–	–
S-080	37	F	no	Unknown	Multiparous female, transfusion within 2 years	–	–	–	–
S-088	46	M	no	Cats, fish, small animals	History arthritis with rheumatoid factor in past	–	–	–	–
S-098	38	F	no	Cats, dogs, pigs, dairy farmer; drinks unpasteurized milk	Multiparous female, ? discoid lupus	–	+† DR3	–	–
S-137	27	M	no	None	None	–	–	–	–
S-139	34	M	no	Cats, dogs, goats, pig farmer	None	–	+ DR3,5	–	–
S-182	53	F	no	Cats, dogs, small/large animals; drank unpasteurized milk	Multiparous female	–	+ DR5,7	–	–
S-035	18	F	no	Cows; worked dairy farm; drinks unpasteurized milk	None	–	–	–	–
S-116	23	M	no	Cats, dogs, monkey; worked dairy farm; drank unpasteurized milk	None	–	–	–	–
S-195	71	M	no	Unknown	None	–	+ DR5	–	–
S-198	27	F	no	Dogs	None	–	–	–	–
S-016	27	M	no	Pigs, cows; worked dairy farm	None	–	+ DR1,5	–	–
S-031	36	M	no	Dogs	?transfusion 10 years ago	–	+ DR1	–	–
S-086	58	F	no	None	Multiparous female, Rheumatoid arthritis	–	–	+	–
S-112	21	F	no	Unknown	None	–	+ DR4,5	–	–
S-167	39	M	yes‡	Cats, dogs, rabbits, reptiles	None	–	–	–	–
S-095	24	M	no	Dogs	None	ND§	ND	–	–
S-106	33	M	no	Lived/worked dairy farm; hunts game; drinks unpasteurized milk	None	–	–	–	–
S-191	40	M	yes‡	Worked dairy farm; drank unpasteurized milk	None	–	+ DR5	–	–
S-162	26	M	yes‡	Unknown	None	ND	ND	–	–

* – = negative.
† + = positive.
‡ Had had sex with another man since 1977.
§ Not done.

samples, the relative molecular weights of the HTLV-I bands were analogous to the HIV core protein bands. All of the samples were nonreactive with HTLV-II on ELISA. WBs showed 10 of 19 samples to be reactive with HTLV-IV: 7 samples with p26 alone, 1 with p55 alone, 1 with p26 and gp32, and 1 with p26, p51, p55, and p66. The single follow-up sample (S-162) that showed envelope reactivity to HIV in the gp41 region of the WB also showed a gp32 band of HTLV-IV in the WB. Similarities in proteins between HIV and HTLV-IV have been described by Kanki et al.,[7] who showed that p18, p26, and gp32 of HTLV-IV were comparable to the p17, p24, and gp41 of HIV, respectively. In 8 of 10 HTLV-IV-reactive samples, the bands seen had relative molecular weights comparable to the bands of HIV seen on WB, strongly suggesting that the reactivity was against similar proteins of HIV and HTLV-IV.

The immune status of all donors including the seroconverter (S-162) was normal as measured by absolute lymphocyte count, CD4/CD8 lymphocyte ratio, and mononuclear cell response to mitogens. No antibody to Class I HLA antigens was detected in any donor. However, sera from 8 of 18 donors exhibited complement-mediated cytotoxicity to T-lymphocyte-depleted mononuclear cell panels, suggesting the presence of antibody to Class II HLA antigens or other lymphocyte or monocyte antigens (Table 4). Only 5 of 20 donors reported a history (pregnancy, transfusion) that could have resulted in alloimmunization leading to cross-reacting antibody. The presence of rheumatoid factor was confirmed in one donor. All donor sera were negative for antinuclear antibody.

At the time of blood donation, all donors had denied risk factors for HIV infection and, therefore, completed the donation process. The extensive histories obtained in conjunction with notification of their atypical WB results indicated that 3 of 20 individuals should have excluded themselves as blood donors (Table 4).

Donor interview evaluation also yielded information regarding animal exposure. Specific questions were asked to determine if exposure to animals with the known ability to harbor retroviruses could have been the source of antigens that induced the cross-reacting antibody. Bovine viruses were of primary interest, because a significant portion of our donor population is based in a rural, dairy farming region. Of 16 donors who responded to animal exposure questions, 6 had a history of or were presently living or working on a dairy farm, and 7 drank unpasteurized cow's milk (Table 4).

Discussion

Atypical WB patterns in 19 of 20 of our donors remained substantially the same over time, as others have reported in their donors.[8-10] We agree with Courouċé[9] that the significance of this finding is unclear and that blood products from such individuals, when they have been identified, should not be transfused. In addition, our data show that the presence of p24 alone in WB should not be regarded as a "false positive" without subsequent testing of the individual. Data from a multicenter AIDS cohort study[4] and a case report of HIV seroconversion with only p24 antibody reactivity[11] support this view. Our study donor, initially similar serologically to the other 19 donors, developed antibody to HIV envelope glycoproteins and to additional core proteins when retested 2 months after the initial HIV-positive (ELISA) blood donation. The detection of this seroconversion in our donor base, which has a very low prevalence of HIV seropositivity (<0.01%), emphasizes that any group of healthy blood donors with repeatedly reactive screening results and atypical WB patterns may include HIV-infected individuals who are in the early phase of seroconversion. The recent report by Ranki et al.[12] also raises the possibility that atypical reactivity, indistinguishable from that described in this report, can persist for prolonged periods (7–14 months) in individuals whose exposure to HIV was sexual.

Our data suggest that antibody to core proteins alone represents exposure to a retrovirus or to retrovirus-like antigens, the identity of which is unclear. Antibody reactivity to HIV core protein is substantiated by direct reaction and inhibition by recombinant "GAG 55," as well as by reactivity on WB employing viral antigens derived from two different cell lines (H9 and CEM). Antibody reactivity to HIV envelope glycoproteins was limited to the seroconverter. Although HTLV-I and HTLV-IV antibody detected on WB may be responsible for some of the reactivity of these samples, additional reactivity due to infection with more than one retrovirus[13,14] or with as yet unknown retroviruses cannot be ruled out.

All study donors had normal immune status as measured by selected in vitro tests. In search of some non-HIV-induced antibody that might be responsible for the ELISA and WB HIV reactivity, we were surprised to find that 8 of 18 donors had antibody to Class II HLA antigens or other monocyte or lymphocyte antigens on the test cells. Two of these eight donors were multiparous females, and one other probably had received a blood transfusion (hospital chart review was not performed), but five had no readily identifiable reason for this finding. Unlike the previous reports that HLA DR4 antibodies may interfere with ELISA HIV tests,[15-17] only one of our eight donors had antibody reactive to HLA DR4-positive cells. The only other antibody we identified was rheumatoid factor in one donor who did not have HLA Class II antibody.

Upon close evaluation of lifestyle and historical data, we observed a large proportion of individuals who had either lived or worked on dairy farms (6/16) and frequently drank unpasteurized cow's milk (7/16). We have found no documentation of a relationship between dairy farm employment and retroviral illness, although this association has been hypothesized.[18] However, immunologic cross-reactivity between the core antigens of bovine leukemia virus (BLV) and HTLV-I has been reported,[19] and a possibility of HIV and BLV cross-reactivity has been suggested.[20] Most interesting is a recent report[21] of the characterization

of a bovine lentivirus, bovine immunodeficiency virus (BIV), which has immunologic cross-reactivity with the core proteins of HIV. Also of interest are reports of cross-reactions between human thymic and placental antigens with HIV on p17[22] and with the related *gag*-encoded proteins of HTLV-I and BLV.[23,24] Retroviral culture studies of individuals with consistently atypical HIV WB patterns will be necessary to establish a basis for determining which donors in the subset with equivocal findings are infected with retroviruses and which donors have nonretroviral proteins that interfere with the HIV assays. One report[9] has shown six such cultures to be negative. Viral culture studies on our donors are in progress and will be the subject of a later report.

The significance of data generated from a non-case-controlled study of this type is difficult to determine. Nevertheless, these observations suggest that undefined autoimmune phenomena, bovine exposure, or cross-reactivity with other human retroviruses could be possible causes for consistently reactive HIV immunologic assays performed for routine blood donor evaluation. Transfusion recipients of previous blood donations from our study donors have not been evaluated; however, this type of "look-back" testing of a similar donor group was found to be negative by van der Poel et al.[9] The significance to the donor of an HIV-positive ELISA and an atypical WB with antibody reactivity to core proteins remains to be determined. Follow-up testing of blood donors will be necessary to answer these questions and to refine donor notification strategies.

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BIOTECH-SPONSORED AABB FOUNDATION GRANT

The American Association of Blood Banks Foundation is pleased to announce a special 1-year grant sponsored by the Biotech Division of Ortho Pharmaceutical Corporation to be awarded July 1, 1989. Applications are open to institutions with the recommendations of a "visiting fellow." Projects dealing with any aspects of autologous transfusion, alternatives to transfusion or potential applications of Hemopoetic growth factors as applied to transfusion medicine will be considered.

The grant will be awarded for a 1-year period. Funding will be limited to a maximum of $15,000 and may not cover indirect costs, travel, or publication. Application forms can be obtained by contacting:

AABB Foundation
1117 North 19th Street, Suite 600
Arlington, VA 22209
Telephone: (703) 528-8200

Completed applications must be received at the Foundation Office by December 1, 1989.

The Board of Trustees of the Foundation will award the grant based on the recommendations of an independent review committee. Recommendations for funding will be based solely on merit.