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Comparison of Indirect Immunofluorescence and Western Blot for Detection of Anti-Human Immunodeficiency Virus Antibodies

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There was 100% agreement between the results of indirect immunofluorescence (IF) and Western blot testing when these methods were used to detect antibodies to the human immunodeficiency virus in sera from 25 patients with acquired immune deficiency syndrome (AIDS), 20 patients with AIDS-related complex, 186 subjects at high risk for AIDS, and 40 healthy heterosexuals. However, there was only an 88.7% correlation between IF and Western blot results for 728 sera from blood and plasma donor centers that were selected on the basis of screening enzyme immunoassay reactivity. IF tests yielded nine false-negatives and were equivocal, yielding a nonspecific pattern of reactivity for both infected and uninfected cells for 73 of these specimens. The IF and Western blot methods were equal in performance for the detection of anti-human immunodeficiency virus antibodies in the high-risk and unselected low-risk groups, proving to be a practical approach for testing specimens from these subjects. However, the Western blot was the most acceptable method for the validation of specimens from groups at low risk for AIDS that were selected based on enzyme immunoassay reactivity.

The enzyme immunoassay (EIA) has now been widely used as a screening serology method for the detection of anti-human immunodeficiency virus (HIV) antibodies (2, 3, 11). The EIAs that are currently commercially available are very specific and sensitive. However, in the general population the predictive positive values with EIAs are relatively low because of the low overall prevalence of HIV infections outside known risk groups (4, 11). One explanation for false-positive reactions obtained with the anti-HIV screening EIAs is the presence of cross-reacting antibodies to histocompatibility locus antigens (HLA) that are known to contaminate the viral antigens used to coat the solid phase of the EIA test kits (8). This nonspecificity has required that alternate, more specific serological techniques be used to validate EIA-reactive sera, especially in population groups at low risk for acquired immune deficiency syndrome (AIDS) (1).

The Western blot method has been recommended and is currently the method of choice for this purpose (3, 11). Western blotting provides a very specific approach for HIV serology. Furthermore, Western blot analysis has been shown to predict infectivity of blood units that were validated by this method (5). However, despite these proven advantages the use of the Western blot has been limited to only a few reference laboratories in the United States. The reasons for the restricted use of the Western blot include the technical complexity of the procedure and the requirement for specialized equipment and reagents.

In contrast to the Western blot, indirect immunofluorescence (IF) is a well-accepted, widely used serological method for clinical laboratory diagnosis of a broad range of infectious diseases. Although IF has been used for detection of anti-HIV antibodies in high-risk groups (1, 2, 6, 7), it has not been thoroughly evaluated as an alternative to the Western blot for validation of anti-HIV EIA screening test results for serum specimens from blood and plasma donor centers. As a validating test for EIAs, IF offers many practical advantages over Western blot analysis. IF is a routine test format that is familiar to trained clinical laboratory personnel. The test can be performed in less than 2 h, whereas Western blotting requires overnight processing. In addition, IF is a potentially less expensive method for HIV serology. HIV purification and the immunoblotting steps necessary for the preparation of Western blot strips are very labor intensive and expensive compared with the growth of infected cell cultures and cell fixation to glass slides used for IF. Therefore, in this paper we report our evaluation of IF and Western blot methods after testing 999 patient sera, including 728 sera that were reactive as determined by EIA at blood and plasma collection centers.

MATERIALS AND METHODS

Serum samples. A total of 999 serum samples were obtained from 728 selected blood donors, 25 patients with AIDS, 20 patients with AIDS-related complex (ARC), 186 subjects at high risk for AIDS (including 104 homosexual men, 56 hemophiliacs, and 26 intravenous drug abusers), and 40 healthy heterosexuals. The blood donor sera were selected because of repeated reactivity as determined by commercial, Food and Drug Administration-approved EIAs performed on site at blood and plasma collection centers in the United States.

HIV. The H9 cell line productively infected with HIV was provided by R. C. Gallo (National Cancer Institute). Concentrated cell-free medium from infected cell cultures was centrifuged at 100,000 × *g* for 2 h. The pelleted virus was then purified by equilibrium banding in a 20 to 50% continuous sucrose gradient.

IF assay. H9 cells productively infected with HIV and uninfected H9 control cells were suspended (10^6 cells per ml) in phosphate-buffered saline, dropped (5 μl) onto microscope slides, air dried, and fixed in acetone. Serum samples were diluted 1:16 in phosphate-buffered saline, and 20 μl of each diluted sample was added to a smear for 30 min at 37°C in a moist chamber and then washed in phosphate-buffered saline at room temperature with stirring. Fluorescein isothio-

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cyanate-labeled goat anti-human immunoglobulin G was added (20 μl) for 30 min at 37°C. After washing in phosphate-buffered saline, the slides were mounted in buffered glycerol and read by fluorescence microscopy. Typical cytoplasmic fluorescence of infected cells was regarded as a positive reaction (7, 10). For absorption studies 100 μl of a 1:16 dilution of a selected specimen that appeared to react nonspecifically (NS), as evidenced by fluorescence staining of uninfected cells, was added to 10^7 uninfected H9 cells, and the preparation was incubated at room temperature for 1 h. The cells were removed by centrifugation, and the absorption procedure was repeated before the specimen was tested by the IF assay.

Western blot analysis. Purified disrupted HIV was fractionated by electrophoresis for 2 h on a preparative 10% polyacrylamide slab gel in the presence of sodium dodecyl sulfate. The protein bands were transferred by electrophoresis for 1.5 h at 1 A to a nitrocellulose sheet, which was then treated in buffer (0.15 M sodium chloride, 0.001 M edetic acid, 0.05 M Tris base, 0.05% Tween 20, 0.1% bovine serum albumin, 3% gelatin) and cut into strips of equal size (4 mm by 10 cm), each containing 3 μg of HIV antigen. One lane of each gel contained molecular weight standards and was stained with amido black.

Portions (1 ml) of diluted serum samples (1:100) were added to the strip blots, and the preparations were incubated overnight at room temperature. After three washes (5 min each) in 0.05% Tween 20 in normal saline, 1 ml of diluted peroxidase-conjugated goat anti-human immunoglobulin G was added to each strip, and the preparation was incubated at room temperature for 60 min. A 1-ml portion of fresh substrate mixture containing diaminobenzidine and H_2O_2 (0.01%) was added at room temperature for 5 to 10 min for color development. The molecular weights of viral antigen bands on the immunoblots were determined by comparison with the migration distances of protein standards in the original slab gel. For diagnostic purposes, interpretation of test results was by comparison of test sera with positive and negative controls, with specific attention paid to the presence of antibodies to p24 (core polypeptide) or gp41 (envelope glycoprotein) or both (3).

RESULTS

Specific IF patterns of reactivity to HIV antigens were clearly demonstrated in positive sera and were absent in negative sera (Fig. 1A and B, respectively). However, a NS pattern of reactivity (Fig. 1C) was observed in a significant number of serum samples that were selected by their reactivity during anti-HIV EIA screening performed at blood and plasma collection centers. This NS reaction occurred in both infected and uninfected cells as a dull, flat green fluorescence.

IF serology results and Western blot serology results were both positive for 118 (16.2%) sera from the selected blood donor group, and both methods produced negative results for 528 (72.5%) of these sera (Table 1). Nine blood donor sera were positive as determined by Western blot analysis but were IF negative. Eight of these nine discrepant sera were positive as determined by Western blot analysis to only the major core protein (p24) and core polyprotein precursor p55, whereas Western blot reactivity to the transmembrane glycoprotein (gp41) was absent.

A total of 73 blood donor sera yielded NS, uninterpretable IF results. One of the NS sera was Western blot positive, whereas the other 72 specimens were Western blot negative. Eight NS sera were retested after absorption by using uninfected H9 cells, and, although the intensity of the NS reaction was reduced by this approach, an unacceptable level of background staining remained. When NS sera were diluted (Table 2, specimens 1 through 4), their NS titers ranged from 1:128 to 1:256. For the one Western blot-positive sample that was also NS as determined by IF (Table 2, specimen 5), the specific anti-HIV IF pattern was revealed at a dilution of 1:64. Specimens 6 through 9 were Western blot positive without NS reactivity and yielded IF titers ranging from 1:64 to 1:512.

There was complete agreement between IF and Western blot results for the 271 sera from patients with AIDS, patients with ARC, subjects at high risk for AIDS, and

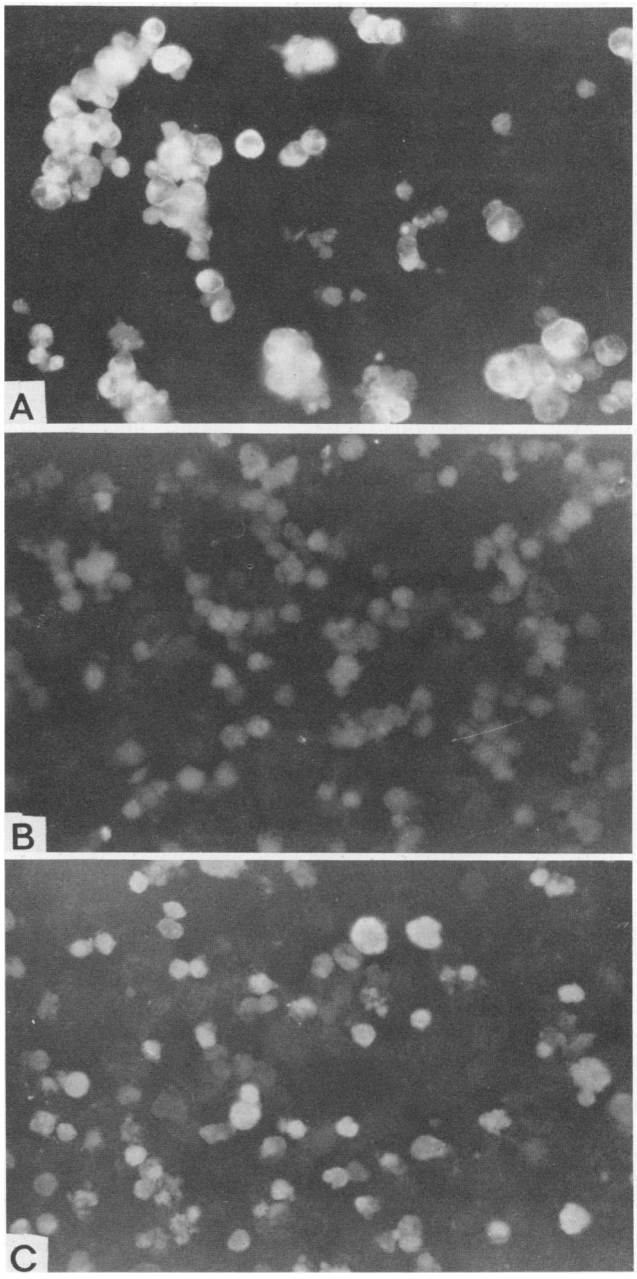

FIG. 1. IF staining of HIV-infected H9 cells. ×296. (A) Positive sera. (B) Negative sera. (C) NS reactive sera.

healthy heterosexual subjects (Table 1). In these groups 166 sera were positive by both methods, and 105 sera were negative. The results for none of these sera were equivocal as determined by IF.

DISCUSSION

IF and Western blot tests performed equally well in this study for the detection of anti-HIV antibodies for the 271 patients with AIDS, patients with ARC, subjects at high risk for AIDS, and unselected healthy heterosexual subjects. Similar results for these two serological methods have been reported previously; correlations of more than 99% were achieved when high-risk and unselected low-risk groups were tested (1, 2, 6, 7, 10). However, in our study IF performed less well when it was used to test blood donor sera that had been selected based on reactivity during previous EIA screening. In this group of 728 serum specimens, 73 (9.9%) were NS as determined by IF (including 1 Western blot-positive serum specimen), and 9 (1.2%) were falsely negative.

The NS sera reacted as previously reported (1, 2, 10) to both infected and uninfected cells with an atypical fluorescence pattern, which, although not characteristic of the specific pattern of antigen staining for positive sera, interfered with routine interpretations. In fact, for specimen 5 (Table 2), which was Western blot positive, the NS IF reaction completely occluded the specific IF pattern at the screening dilution. NS activity could be eliminated by titrating sera in doubling dilutions; however, for the examples shown in Table 2 (specimens 1 through 4) NS reactions were still evident at dilutions of 1:128 or greater. Since in some cases the NS titers were higher than the specific antibody titers (e.g., Table 2, specimen 9), dilution as a method for circumventing the problems presented by NS reactions does not seem practical since sensitivity would be reduced. We also found that extensive absorption of NS sera was not very useful in eliminating the NS IF reactions that we observed, although this approach has been successfully used by other workers for some NS specimens (6, 7).

IF failed to detect eight sera that were Western blot positive for the major core proteins (p24 and p55) but lacked reactivity to the transmembrane glycoprotein (gp41) of HIV. From the original recommendation of the Public Health Service (3) this Western blot pattern warrants a positive interpretation, and according to a recent report sera of this category reflect a very early stage of infection (5). Relevant clinical data on the subjects in our study were not available to clarify the true status of this uniquely reactive group of sera, and for the purposes of this study these specimens were categorized as IF false-negative.

TABLE 1. Comparison of Western blot and fixed-cell IF tests for detection of anti-HIV antibodies

Subject group	No. tested	Western blot results	IF results		
			No. positive	No. negative	No. equivocal
AIDS	25	Positive	25	0	0
		Negative	0	0	0
ARC	20	Positive	20	0	0
		Negative	0	0	0
High risk[a]	186	Positive	121	0	0
		Negative	0	65	0
Healthy heterosexuals	40	Positive	0	0	0
		Negative	0	40	0
Selected blood donors[b]	728	Positive	118	9[c]	1
		Negative	0	528	72

[a] This group consisted of 104 homosexual men, 56 hemophiliacs, and 26 intravenous drug abusers.
[b] Repeatedly (twice) positive as determined by anti-HIV screening EIAs.
[c] Eight of nine sera were Western blot positive for p24 but not gp41.

TABLE 2. Titration results for sera with specific IF staining patterns or NS IF staining patterns or both

Specimen no.	Reciprocal of serum antibody titer						
	16	32	64	128	256	512	≥1,024
1	NS[a]	NS	NS	NS	NS	NS	−
2	NS	NS	NS	NS	−	−	−
3	NS	NS	NS	NS	NS	NS	−
4	NS	NS	NS	NS	−	−	−
5	NS	NS	++[b]	++	++	++	++
6	++	++	++	++	++	++	−
7	++	++	++	++	++	++	−
8	++	++	++	++	++	++	−
9	++	++	++	−	−	−	−

[a] NS, NS reactivity for both infected and uninfected cells.
[b] ++, Specific IF pattern of reactivity for infected cells.

We feel that it is imperative to validate anti-HIV EIA screening serological results with tests that are more specific. This is shown by the false-positive rate demonstrated in repeat EIA-positive specimens from low-risk groups (82.4% in our study and 77.1% recently reported by the American Red Cross [11]). It is essential to minimize the reporting of false-positive results to avoid the psychological trauma created for individuals. IF has proven to be an acceptable test for the detection of anti-HIV antibodies in high-risk and unselected low-risk groups (1, 2, 6, 7, 10). However, the use of IF to validate sera that have been selected because of repeated EIA reactivity in groups at low risk for AIDS (e.g., blood and plasma donors) would present the problems that we describe above (i.e., reduced sensitivity and equivocal results). The NS reactivity of IF in this population group probably is a result of anti-HLA antibodies directed to the H9 T-cell line. The presence of anti-HLA DR4 antibodies has been reported previously as a cause of false-positive reactions in screening EIAs (8). Therefore, we feel that for this purpose, the Western blot technique, although technically demanding, should remain the test of choice for EIA validation. It must be noted that even though we feel that the Western blot technique is presently the most acceptable method for anti-HIV EIA validation, Western blot analysis is a subjective method with quality control limitations; the possibility of false-positive results still exists (9). Therefore, we hope that new, more specific and objective approaches will soon be available to provide definitive results for HIV serology.

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