Quantitation of Antibody Reactivity to Human Immunodeficiency Virus (Type 1) Proteins and Glycoproteins on Western Immunoblots by Reflectance Densitometry

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The human serum antibody response to polypeptides of human immunodeficiency virus type 1 (HIV-1) was quantitated by reflectance densitometry of Western immunoblots by using two commercially available blotting systems. In one system, human antibodies were detected by an avidin-biotin method using peroxidase as the label, and in the other, human antibodies were detected by peroxidase-labeled conjugate against human immunoglobulins. When staining intensity was plotted against the log of the serum dilution, a shallow slope was evident, with a 50% change in staining intensity requiring as much as a 100-fold change in antibody content. The linear range of the staining intensity curves was frequently found in serum dilutions of 1:2,500 to 1:1,000,000, and a plateau was often observed at high antibody concentrations (1:80 to 1:640). When replicate strips were tested, staining intensities varied by ± 7 to 37%. Antibodies to p24^{gag} and gp160^{env} were readily detectable in several sera diluted 1:1,000,000, a result seen with both blotting systems. If Western blotting were to be used to observe increases or decreases in levels of antibodies to various polypeptides, several widely spaced serum dilutions would need to be tested.

The major laboratory method for identification of human immunodeficiency virus type 1 (HIV-1) infections is detection of antibodies by enzyme-linked immunosorbent assay (ELISA) using solubilized virus (12, 14, 15). Although the available ELISA kits show high sensitivity in detecting persons with antibodies to HIV-1, a small proportion of people in the general population test reactive even though they have no risk factors (23), indicating that the ELISAs presently available show some false-positive results. Because of the distinct staining pattern of polypeptides, the Western immunoblot has been established as the major confirmatory test for serum specimens exhibiting ELISA reactivity (3, 7, 8, 16, 21). Standardization of Western blots remains difficult because of inconsistent staining reproducibility from laboratory to laboratory (1, 5), as well as the problems of subjective visual interpretation. Application of reflectance densitometry in HIV-1 Western blot analyses in clinical laboratories could provide numerical values which, as with the ELISA, allows objective differentiation between positive and negative specimens.

Scanning densitometry in the reflectance mode was used in this study to analyze the human immune response to HIV-1 polypeptides on Western blots with two commercially available systems. Dilutions of human sera from 1:80 to 1:5,200,000 were tested. When strips with decreasing amounts of antibodies were viewed by eye, progressive small changes in apparent density was observed. Reflectance densitometry showed that the gradual changes observed by eye were also observed densitometrically when staining intensities decreased gradually with increasing serum dilutions.

MATERIALS AND METHODS

Human sera. Twelve HIV-1 antibody-positive serum specimens were evaluated in this study. These samples were

selected from specimens submitted to the Washington State Public Health Laboratories, Seattle, which were reactive twice on ELISA and confirmed as positive by Western blot. Positive sera showed at least two of the three major bands of diagnostic significance: gp160^{env} or gp120^{env}, gp41^{env}, and p24^{gag} (4). Sera were stored at -20°C .

Western blotting. Preblotted HIV-1 nitrocellulose strips and peroxidase-labeled goat anti-human immunoglobulin G were purchased from Organon Teknica Corp., Durham, N.C. (manufacturer, Epitope Inc., Beaverton, Ore.). Western blot kits also were purchased from E. I. du Pont de Nemours & Co. Inc., Biotech Research Laboratories, Rockville, Md. Strips and conjugate from Epitope will be referred to as the indirect system, and materials from du Pont will be referred to as the avidin-biotin system.

Indirect system. Epitope HIV-1 strips (with identical numbers) were stained by a modified procedure recommended by the manufacturer (personal communication). Briefly, human serum dilutions were made in twofold dilution steps (ranging from 1:80 to 1:5,200,000) in 0.15 M phosphate-buffered saline containing 5.0% nonfat dry milk, 0.4% Tween 20, and 0.005% thimerosal, pH 7.3 (milk buffer). Primary antiserum incubation was performed for 18 h at 4°C . Strips were washed for 15 min in three changes of 0.15 M phosphate-buffered saline containing 0.5% Tween 20, incubated with conjugate (peroxidase-labeled goat anti-human immunoglobulin G [heavy and light chain specific] diluted 1:500 in milk buffer) for 1 h at room temperature, and washed. Strips were incubated with a color development solution (0.05% diaminobenzidine and 0.02% hydrogen peroxide in phosphate-buffered saline) for 15 min, followed by a wash for 10 min in several changes of distilled water.

Avidin-biotin system. Human sera were diluted in twofold dilution steps (1:80 to 1:5,200,000) in 0.1 M NaCl containing 0.02 M Tris, a proprietary mixture of nonfat dry milk and heat-activated goat serum, and 0.005% thimerosal, pH 7.4 (working blotting buffer). The strips were incubated with

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FIG. 1. Western blots stained with dilutions of human serum. Left to right, twofold serum dilutions from 1:80 (strip 1) to 1:5,200,000 (strip 17). The strips used were from a single preblotted nitrocellulose sheet.

these dilutions overnight at room temperature; washed three times for 5 min each time in a solution containing 0.02 M Tris, 0.1 M NaCl, 0.3% Tween 20, and 0.005% thimerosal, pH 7.4 (wash buffer); incubated in working blotting buffer containing a 1:2,000 dilution of biotinylated goat anti-human immunoglobulin G (heavy and light chain specific) for 1 h at room temperature; washed with wash buffer; and incubated for 1 h in working blotting buffer containing a 1:2,000 dilution of avidin-conjugated peroxidase. Following a final wash with wash buffer, strips were allowed to react with a solution containing 0.02% hydrogen peroxide and 3.9 mM 4-chloro-1-naphthol in citrate buffer for 10 min, followed by two washes in distilled water.

Scanning densitometry. Densitometric evaluation of Western blots was performed with a CS-9000 Flying Spot scanning densitometer (Shimadzu Corp., Kyoto, Japan) using a reflected 0.16-mm² linear beam at 590 nm for the avidin-biotin system and 330 nm for the indirect system. The densitometer was blanked on an unstained area of a strip prepared with the most dilute antiserum strip for each dilution series to avoid signal distortions at high serum concentrations. Staining intensity for each peak was calculated by the integration facility of the densitometer (values for staining intensity are expressed in arbitrary units). Data were analyzed by using Excel software (Microsoft Inc., Bellevue, Wash.) on a Macintosh SE computer (Apple Computer, Inc., Cupertino, Calif.).

RESULTS

Relationship of staining intensity to serum dilution. To determine the relationship of staining intensity to human serum concentration, a wide range of serum dilutions (1:80 to 1:2,600,000) were tested against HIV-1 antigen strips by Western blot. The resulting stained strips showed only a gradual decrease in intensity with increasing serum dilutions when viewed by eye (Fig. 1). When strips were evaluated by scanning densitometry comparing serum diluted 1:80 (Fig. 2A) and 1:10,240 (Fig. 2B), the staining intensities of six antigen peaks (p66^{pol}, p55^{gag}, p51^{pol}, gp41^{env}, p31^{pol}, and p17^{gag}) were reduced at 1:10,240; however, these reductions in area were not proportional to the dilution for each of the polypeptides. The areas of the peaks for gp160^{env} and p24^{gag} showed little change, whereas the areas for gp41^{env} and p17^{gag} were greatly reduced at the 1:10,240 dilution. At

FIG. 2. Densitometer tracings of HIV-1 Western blots (the Western blots for this serum are shown in Fig. 1, strips 1 and 8) stained with two dilutions of a high-titered antiserum with the indirect Western blotting system. Human serum dilutions, 1:80 (A) and 1:10,240 (B).

either serum dilution (Fig. 2), the strongest staining was shown by antigen bands gp160^{env}, gp41^{env}, and p24^{gag}.

When band staining intensity was plotted against the log of the antiserum dilution (Fig. 3), the slope of the linear portion of each curve was shallow; a 50% reduction in area was seen when the sera were diluted 10- to 100-fold (depending on the specimen; Fig. 3 and 4). Plateau regions were commonly found between dilutions of 1:80 and 1:640. Prozone areas were found in titration curves for polypeptides toward which sera had the highest antibody titers (Fig. 3). The greatest intensity of staining was found for gp160^{env} or p24^{gag} in sera judged to contain the most antibodies because they stained all major viral bands (gp160^{env}, gp120^{env}, gp41^{env}, p31^{pol}, p24^{gag}, and p17^{gag}). Antibodies to gp160^{env} were detectable in some sera diluted 1:2,600,000 (data not shown), and one specimen stained the p24^{gag} band at a serum dilution of 1:5,200,000 (Fig. 1).

Comparison of blotting systems. A comparison of the avidin-biotin system with the indirect system showed that the strips supplied in the avidin-biotin kit stained best for gp41^{env} (transmembrane glycoprotein), whereas strips used in the indirect system stained best for gp120^{env} (outer membrane glycoprotein) (Fig. 4). In addition, the avidin-

FIG. 3. Staining intensities of p24^{gag} bands (expressed in arbitrary units) produced over twofold dilutions (1:80 to 1:2,600,000) of four different sera. The curve with the highest p24^{gag} titer (▲) illustrates a prozone area in addition to plateau and linear regions (indirect Western blotting system).

biotin kit gave comparatively better staining of p24^{gag} (core) and p17^{gag} (inner shell), whereas the indirect system gave better staining of gp160^{env} (precursor of envelope glycoproteins [data not shown]).

Reproducibility of strip staining intensity. The reproducibility of staining intensity was determined by evaluating the staining intensity of HIV-1 bands by using five strips for each of four primary antiserum dilutions. The reproducibilities for measurement of antibodies to p24^{gag} ranged from 6 to 26%

FIG. 4. Comparison of the staining intensities of a human serum for gp41^{env} and gp120^{env} using the indirect and avidin-biotin blotting systems. Panels: A, gp41^{env} antibodies measured by avidin-biotin (□) or indirect Western blotting (■); B, gp120^{env} antibodies measured by avidin-biotin (△) or indirect Western blotting (▲). Dilutions, 1:80 to 1:1,200,000.

(Table 1). Antibodies to gp160^{env} varied by 7 to 37% (Table 1; Fig. 5). The indirect test system showed increasing error with increasing antibody dilution, a result which was expected on the basis of the testing of five parallel serial dilutions (Table 1). In contrast, the variation in reproducibility of the avidin-biotin system was not related to serum dilution. Other than these differences, the reproducibilities of the two systems were equivalent.

DISCUSSION

Human serum antibodies to polypeptides of HIV-1 were quantitated by scanning densitometry of Western blots. Antibodies were detected in strongly positive sera at a dilution of 1:5,200,000. Shallow slopes were observed when staining intensities were plotted against the log of the serum dilution and the decrease in the intensity of staining of viral polypeptide bands varied depending on the serum specimen. A 50% reduction in staining required a 10- to 100-fold decrease in the serum concentration, provided that the assay was on the linear portion of the curve (Fig. 3 to 5). High-titered sera showed both prozones and plateau areas. The demonstration of shallow slopes and prozones in Western blots by scanning densitometry was unexpected and has been little studied. Similar and dissimilar results have been obtained by others. Ungar and Nash (22) measured the antibody response to *Cryptosporidium* antigens on Western blots by reflectance laser densitometry and found a shallow slope when the band density ratio of a 23-kilodalton protein was plotted against the log of the serum dilution from an individual with cryptosporidiosis. The data reported by Blomberg and Klasse (2, 3) for a study of HIV-1 Western blots by reflectance densitometry likewise showed shallow slopes falling twofold between serum dilutions of 1:2 and 1:512. They also showed absorbance plateaus for some polypeptide bands at high serum concentrations. Pinter and Honnen (13) demonstrated a 50% reduction in staining intensity of gp120 and p24 bands between serum dilutions of 1:100 and 1:250 on Western blots when they used densitometry to compare the sensitivities of Western blotting and a radioimmunoprecipitation assay. For measles virus polypeptides, the results were different because a steeply linear relationship was found between serum dilution and Western blot staining intensity, with no plateau or prozone areas, when the proportionalities of staining intensities of four virus polypeptides at five serum dilutions (1:20 to 1:320) were evaluated by densitometry (9).

The finding that the staining intensity decreased only gradually with the logs of increasing serum dilutions on commercially available Western blots differs from those of other systems which use reflectance scanning densitometry for quantitation. In thin-layer chromatography systems (20), staining is directly proportional to the concentration of the material stained. These color development systems use chromogens which bind specifically and stoichiometrically to the target molecules. In Western blotting, the color development process is much more complex and requires application of five to six successive reagents: antigen, primary antiserum, enzyme-linked conjugate or biotinylated antibody, avidin- or streptavidin-labeled enzyme, enzyme substrate, and chromogen. These same factors are present in ELISA, but all of the color product is retained in the homogeneous fluid system, whereas the colored product in the Western blotting system must be insoluble and form crystals which are retained within the paper. Furthermore, surface dye crystals affect the reflectance of light, whereas

TABLE 1. Reproducibility in measurements of staining intensities of p24^{gag} and gp160^{env} bands^a

Serum dilution	Mean staining intensity ($\pm$ % SD)			
	gp160 ^{env}		p24 ^{gag}	
	ID ^b	AB ^c	ID	AB
1:100	24,686 (7.41) ^d	96,060 (17.58)	22,075 (7.3)	94,537 (11.87)
1:1,000	37,537 (8.96)	112,970 (27.06)	35,948 (9.76)	114,260 (14.33)
1:10,000	25,694 (14.12)	89,429 (18.34)	37,240 (10.36)	71,175 (9.64)
1:100,000	7,473 (37.66)	36,878 (10.75)	21,894 (13.02)	29,176 (20.28)

^a Five replicates, each at four serum dilutions, were used, in a comparison of the avidin-biotin and indirect Western blotting systems.

^b ID, Indirect system—commercial blotting materials using horseradish peroxidase-labeled conjugate and diaminobenzidine as the chromogen.

^c AB, Avidin-biotin system—commercial blotting kit using biotinylated anti-human immunoglobulin G and avidin-labeled horseradish peroxidase as the conjugate and 4-chloro-1-naphthol as the chromogen.

^d Staining intensities are expressed in arbitrary units.

deeper crystals have little effect. Blomberg and Klasse (3) suggested that successive reagents had greater and greater difficulty penetrating into the interior of the paper. The finding that radiolabeled human antibodies from a person with acquired immunodeficiency syndrome show a steep slope (3) without a plateau at high serum concentrations suggests that the nonlinearity of Western blot staining results from steps subsequent to antibody binding.

The decrease in antibody levels to HIV-1 antigenic components in individuals infected with HIV-1, most notably to the viral core (p24^{gag}) and possibly inner shell (p17^{gag}) proteins, is considered an important prognostic sign (6, 10, 19). A decrease in core antibody levels is an earlier predictor of progression to clinical acquired immunodeficiency syndrome in some patients than is a decline in CD4⁺ lymphocytes (6). Progression to clinical acquired immunodeficiency syndrome has been monitored by densitometric analysis of Western blots. Schiavini et al. (16) showed that staining intensities of p24^{gag} and p17^{gag} bands on strips stained with sequential sera from infected individuals decreased as they moved from the asymptomatic phase to the clinical phase of acquired immunodeficiency syndrome. Schmidt et al. (17, 18) showed that the ratio of staining intensities of gp41^{env} to p24^{gag} bands increased in serial serum samples at a single antiserum dilution (1:100) in specimens from individuals progressing into symptomatic disease. If Western blots were to be used to measure changes in antibodies, such as those to the p24^{gag} polypeptide, a number of dilutions would need to be tested to determine significant changes. Since sera are commonly tested at a dilution of 1:100, it would take a 10- to

1,000-fold decrease in antibodies to p24^{gag} (depending on the specimen) before any change could be visually detected (Fig. 1). The plateau areas observed (Fig. 3 to 5) probably reflect saturation of the number of dye crystals which can adhere to a given band. Although xylene has been used to clarify nitrocellulose, allowing measurement by transmission densitometry (11), reflectance densitometry more closely parallels the naked eye.

Since commercially available preblotted nitrocellulose strips were used in our study, our data should be relevant to other laboratory workers using similar materials. Because of the proprietary nature of these strips, we did not know the actual amounts of polypeptides present on them; however, both test systems detected antibodies to all viral proteins but with differing signal magnitudes (Fig. 1; Table 1). We found the highest antibody levels to p24^{gag} and gp160^{env}, whereas Blomberg and Klasse, using their own blotted strips, found the highest antibody levels to p64 and p53 (reverse transcriptase).

Despite limitations, some conclusions can be drawn from these results. Evaluation of several dilutions of primary antiserum is necessary to establish levels of antibodies to individual HIV-1 polypeptides on Western blots. Each serum must be evaluated individually, because the proportions of antibodies to the different HIV polypeptides varied between specimens (levels of antibodies to some polypeptides fell more rapidly than others; Fig. 2 and 3). For measurement of changes in magnitude of antibody-antigen reactivity, dilutions of primary antiserum should be analyzed on linear portions of previously determined staining intensity curves. It is surprising that a technique as complex as Western blotting has turned out to be a useful clinical diagnostic tool. Reasons for the success of this assay are that (i) a broad range of antibody levels can be detected in a single dilution assay and (ii) the staining intensity changes only gradually with changes in serum antibody concentration. Scanning densitometry should provide a more objective method for distinguishing positive and negative sera.

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