Quantification of Immunoglobulin on Electrophoretic Immunoblot Strips as a Tool for Human Immunodeficiency Virus Serodiagnosis

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Electrophoretic immunoblotting (EIB [Western blotting]), the main method for verification of human immunodeficiency virus (HIV) seropositivity, needs thorough characterization and standardization. We explored the possibilities of quantifying immunoglobulin G (IgG) bound to EIB strips by densitometry of the peroxidase-labeled bands and by measurement of radioactivity with labeled anti-HIV IgG. The radioactivity method is inherently more exact but was more cumbersome. However, despite saturation phenomena at high IgG densities, the densitometric method was more convenient and yielded reproducible estimates of the amount of bound IgG. We found it useful primarily for documentation of changes in the relative abundance of antibodies to different HIV proteins from individual patients over time. To explore the potential usefulness of the method, we studied a small set of HIV-seropositive persons. The average p24/gp41 color yield ratios and standard deviations in 3 persons with recent seroconversion, 15 healthy subjects, and 6 diseased HIV-seropositive persons were 6.6 ± 0.9, 2.3 ± 1.9, and 1.3 ± 0.5, respectively. These data are in accord with previous qualitative or semiquantitative observations but are too limited for any conclusions regarding the use of quantitative EIB for prognostic use, with individual patients. Quantitative EIB is a valuable tool for comparative methodological studies and for research on the protective role of anti-HIV antibodies in acquired immunodeficiency syndrome pathogenesis. Its possible use in prognostication for individual patients must be evaluated in long-term studies.

A multitude of more or less sensitive human immunodeficiency virus (HIV) antibody detection systems are now in use. So far, the individual features of each system have not been assessed in a systematic manner. However, the consequences of a false result can be devastating. We need an exact HIV serology.

The occurrence of false-positive reactions in the HIV antibody screening tests necessitates at least one confirmatory test, mainly the electrophoretic immunoblot (EIB [Western blot]) (9, 10, 17, 21). The EIB normally yields qualitative information. However, both for quality control and for laying a firmer foundation of our understanding of HIV pathogenesis and epidemiology, it is desirable to establish more precise, preferably quantitative, diagnostic criteria for the EIB. The sometimes divergent HIV serological results (e.g., 6, 18, 19, 27) and reports of false-positive EIB results (2, 7, 25) underscore the importance of increased diagnostic stringency. In the present paper, we describe two methods of measuring the absolute or relative amount of immunoglobulin bound to each HIV protein band: (i) direct measurement of radiolabeled immunoglobulin bound to each HIV protein band and (ii) densitometry of colored EIB bands and interpolation in a standard curve. By the densitometric method we demonstrated differing p24/gp41 color yield ratios among HIV-infected persons in different states of health.

MATERIALS AND METHODS

Sera. Thirty HIV-antibody-positive sera from patients with or without symptoms attributable to HIV were taken from our diagnostic routine. Three of them were the first clearly HIV-seropositive samples from three patients with recent primary HIV infections, as evidenced by symptoms of acute HIV disease and an anti-HIV activity rise 1 to 3 weeks later in the enzyme-linked immunosorbent assay and EIB. The criteria of the Centers for Disease Control for classification of patients with acquired immunodeficiency syndrome and related disorders (8) were followed. Rabbit anti-human immunoglobulin G (IgG) peroxidase conjugate was from DAKO, Copenhagen, Denmark.

Preparation of radiolabeled anti-HIV IgG from patient sera. For inactivation of HIV, 0.25 ml of a serum sample was mixed with an equal volume of 2% Triton X-100 in phosphate-buffered saline and incubated for 30 min at room temperature before further experimentation. This mixture was then added to a protein A Sepharose column (Pharmacia, Uppsala, Sweden) made from 0.22 g (dry weight) of matrix preswollen in 0.1 M phosphate buffer, preswashed with 6 ml of 0.2 M acetic acid, and again equilibrated with 0.1 M phosphate buffer. IgG was eluted with 6 ml of 0.2 M acetic acid and collected in a tube prefilled with 0.6 ml of 2.0 M sodium phosphate (pH 7.4) to minimize IgG denaturation. The IgG yield was determined by spectrophotometry at 280 and 310 nm. For quantification of anti-HIV IgG bound per HIV protein band, IgGs derived from three HIV-seropositive patients and prepared by affinity chromatography on protein A Sepharose were iodinated by the lactoperoxidase method (22) to a specific radioactivity of $5 \times 10^5$ to $10 \times 10^6$ cpn/μg. Briefly, 0.5 mCi of Na$^{125}$I-10 μg (10 μl) of IgG-10 μl of lactoperoxidase (0.5 mg/ml)-2 μl of hydrogen peroxide (1/20,000) were reacted for 1 min. After the addition of 0.5 ml of 10 mM sodium azide, macromolecules were purified by gel exclusion chromatography (PD10, Pharmacia) in a column equilibrated with 0.2% ovalbumin in phosphate-buffered saline. Commercial IgG (AB KABI, Stockholm, Sweden) was iodinated in the same way to a specific activity of $5 \times 10^4$ to $10 \times 10^5$ cpn/μg.

EIB. HIV (strain HTLVIII B) virions (a kind gift from Prem Sarin, National Institutes of Health, Bethesda, Md.) purified by being banded twice in sucrose gradients (400 μg
per gel of 24 strips), were mixed with an equal volume of 4% sodium dodecyl sulfate–0.1 M dithiothreitol–0.1% bromphenol blue–35% sucrose–1.5 M Tris hydrochloride (pH 8) and heated to 95°C for 3 min. For EIB with uninfected cells, 10^7 to 10^8 H9 cells were extracted in 2 ml of 0.1% (vol/vol) Triton X-100 in phosphate-buffered saline by vortexing them for 30 s. The extract was clarified at 1,000 × g for 10 min. Extract (0.5 ml) was mixed with an equal volume of sodium dodecyl sulfate–dithiothreitol–bromphenol blue–sucrose–Tris and further treated as outlined above. After treatment with iodacetamide at a final concentration of 0.05 M and 3 min of centrifugation in an Eppendorf centrifuge, the mixture was added to a polyacrylamide gel. The separation gel consisted of a 5 to 20% gradient of a mixture (29/2/0.8, respectively) of acrylamide-bisacrylamide. After separation overnight at 75 V, the proteins were blotted to nitrocellulose in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.); washed; cut; incubated overnight with patient sera diluted 1/400 in a washing-dilution buffer containing 10 mM Tris, 30 mM NaCl, 1 mM NaNO_3, and 0.1% (vol/vol) Tween 20; and again washed. Bound immunoglobulins were indicated after 2 h of incubation with peroxidase-conjugated anti-human IgG (DAKO), followed by washing and a 15-min incubation with 0.02% carbazole in 50 mM sodium acetate (pH 5.5).

Densitometry. The Bio-Rad model 620 video densitometer was used with the following settings: filter frequency, 4.0; enhancement frequency, 0.04; boost, 5; and aperture, 11. The readings were made in the reflectance mode. Absorbance values were integrated over each peak by means of the integration facility of the densitometer. The integrated absorbances are hereafter referred to as color yields and are expressed in color yield units.

Initially, we had problems in quantifying antibodies to p53 and p55 separately because of incomplete separation. The color yields of p53 and p55 were therefore expressed as p53-p55 in some experiments.

Quantification of IgG bound in EIB by interpolation in a standard curve. A series of 12 twofold dilutions (1,280 to 0.75 ng/0.2 ml) of commercial IgG, labeled by us with ^125I to a known specific radioactivity, was applied to nitrocellulose in a Minifold (Schleicher & Schuell, Inc., Keene, N.H.) dot-blot apparatus. These IgG standard strips were kept frozen until use. In each EIB experiment, one strip of 12 IgG dots was subjected to the same incubation with anti-human IgG peroxidase conjugate, washing, and color development as for the EIB strips. The amount of IgG bound per dot was determined by a scintillation counter.

After densitometry of both EIB strips and the IgG standard strip, the color yields were stored in a data base and processed with a program (BlotPlot) which transformed color yields to IgG equivalents by interpolation in a standard curve. The 0.2-mm path measured by the densitometer was 1/17.7 of the 4.5-mm diameter of the IgG-loaded dot. The same 0.2-mm path was 1/20 of the 4-mm-wide HIV protein bands. Therefore, the amount of IgG per dot was multiplied by 1.13 (20/17.7) to compensate for the difference in geometry.

Quantification of IgG bound in EIB by use of radiolabeled anti-HIV IgG. Radiolabeled IgG (1.25 μg/ml) from HIV-seropositive patients was diluted with and without the same unlabeled anti-HIV IgG to final concentrations of 80 to 0.156 μg/ml of dilution fluid in 10 twofold steps. The strips were developed with the carbazole substrate and evaluated by densitometry. Subsequently, they were autoradiographed (X-Omat AR5 film, Eastman Kodak Co., Rochester, N.Y.) overnight without an intensifying screen. The stained bands or corresponding positions on very weakly stained strips, and the areas between them, were cut into rectangles (1 by 4 to 4 by 4 mm). The nitrocellulose pieces were evaluated in a scintillation counter. The average radioactivity of interband pieces was subtracted from the radioactivity in a band, with a correction for their lengths. The amount of IgG, in nanograms, bound per band was calculated from the known specific activity of the IgG.

RESULTS

Basic parameters of densitometric evaluation of EIB. (i) Reproducibility. Six sera were quantified by densitometry two to six times (average 3.3) over a period of 1.5 months. The relative standard deviation of the color yields for all protein bands (p17, p24, p31, gp41, p53-p55, and p64) was ± 23%.

(ii) Effect of fading. Three EIB strips were developed with the carbazole stain. They were then quantified 3 days after color development and 23 days later. During this period, they were stored in a dark cupboard in a binder. The average loss of color yield was 18% (standard deviation of 4.5% [n = 12] for bands p17, p24, p31, gp41, p53-p55, and p64).

Titrations of radiolabeled anti-HIV IgG from patients. We looked for signs of saturation of binding with IgG prepared from three different anti-HIV-positive sera. As an example, in a log-log plot (Fig. 1), the binding was close to linear (r = 0.97 to 0.99) for antibodies binding to all proteins within the range 0.1 to 129 ng of IgG per band. In these experiments, p53 and p55 could be separated. The slopes for both anti-p53 and -p64 were less steep than the slopes of the other curves.
color yields to IgG equivalents (roughly equivalent to nanograms of bound IgG) and for presentation of binding data in histogram form was written. The program automatically controlled if the EIB color yield was within the linear range of the standard curve derived from a concomitantly processed strip of 12 different concentrations of passively adsorbed IgG.

Use of quantitative EIB for follow-up of anti-HIV-positive patients. To explore the possible prognostic usefulness of our densitometric method, we analyzed a series of sera from a 30-year-old homosexual male. A gradual decrease of the p24/gp41 ratio was clearly seen (Fig. 3A). This patient is still healthy. Subsequently, we analyzed sera from 3 acutely infected, 15 healthy, and 6 diseased (3 with acquired immunodeficiency syndrome and 3 with lymphadenopathy syndrome) HIV-seropositive persons by this method (Fig. 3B). The acutely infected all had high ratios (average, 6.6; standard deviation, ±0.9), whereas the chronically diseased generally had low ratios (1.3 ± 0.5). Sera from healthy seropositive patients exhibited wide variations (2.3 ± 1.9). Sera from all patients were unreactive with strips made with uninfected H9 cells.

**DISCUSSION**

Quantification of EIB by densitometry. Because of its discriminating power and high sensitivity, EIB has become the most common confirmatory test. It is, however, a complex system. Variations can occur in antigen, electrophoresis, blotting, nitrocellulose, buffers, immunoglobulin of patients, anti-immunoglobulin-enzyme conjugate, substrate, and development of the colored product. Densitometry itself is also influenced by many factors. Variables background levels create problems with evaluation of small peaks. Incompletely separated bands cannot be accurately evaluated. The color intensities may gradually fade. The instrument may give different readings at different occasions after recalibration.

Despite these sources of variation, densitometric values proved to be reproducible. Fading turned out to be a smaller problem than anticipated. The amount of IgG bound per band could be established with reasonable accuracy below the plateau which occurred at high IgG densities. Some of the interassay variations can be compensated for by adjustment according to the color yields of a standard serum.

Our experience with densitometric evaluation of EIB bands is similar to that of previous investigators (4, 12, 16, 24) in EIB systems not involving HIV antibodies. However, to our knowledge, an absorbance plateau at high IgG concentrations per band or dot has not been described before. Maybe the lattice of insoluble colored product which accumulates during color development prevents the substrate from reaching the enzyme. Possibly, the linear range could be extended by changes in the IgG detection system. In the enzyme-linked immunosorbent assay, nonlinearity has been observed with some, but not other, peroxidase-immunoglobulin conjugates (23), showing that it may have a multifactorial origin. For more exact determinations, we prefer the radiolabeled IgG method.

Quantification of EIB by radioactive anti-HIV IgG. With radiolabeling, the amount of IgG bound per EIB band can be directly and quantitatively measured for a certain patient. However, this requires that IgG from each serum sample be purified, quantified, and iodinated. Furthermore, each band and a suitable area for control of background binding had to be cut out and measured in a scintillation counter. We found

Signs of saturation were seen only in one experiment, in which the anti-p24 and -p17 antibody curves started to bend beyond 70 ng of IgG per band (data not shown).

**Relation between amount of IgG bound to nitrocellulose and color yield.** In contrast to the data for IgG binding, the plots of color yield versus bound nanograms of IgG all exhibited some nonlinearity above 20 ng per band (Fig. 2) for strips similar to those used for Fig. 1. The limit of visibility, around 0.1 ng per band, corresponded to a color yield of approximately 0.1, i.e., it was not accurately measurable. The compound regression line between color yield and nanograms of bound IgG was roughly linear (r = 0.92) below 20 ng of IgG for antibodies to all HIV proteins and in three experiments had slopes of 0.75, 1.2, and 1.3, approximating 1 color yield unit per ng of bound IgG. Further data regarding EIB sensitivity measured by the two quantification methods are given in the companion paper (5).

**Determination of bound IgG by interpolation in a standard curve.** Radioactive commercial IgG was adsorbed to nitrocellulose in strips of 12 dots with decreasing IgG content. After densitometry, the strip was cut and the amount of human IgG remaining in each spot was estimated by scintillation counting. The results (heavy line) are shown in Fig. 2. It can be seen that the color yield per IgG molecule decreased above an IgG concentration of 20 ng per dot. However, below this limit, relatively constant proportions between color yield and amount of bound IgG were seen. In five experiments, the mean and range were 0.9 and 0.7 to 1.2 color yield units per ng of bound IgG.

This technique produced ratios of IgG (nanograms)/color yield that were 10 to 20% lower than those obtained by measurement of IgG radioactivity in EIB (see above). However, the relationship was relatively constant up to color yields of 10 to 20 (Fig. 2). That this plateau effect was not due to a shortage of enzyme-conjugated secondary antibody was shown in an experiment in which we found only slightly increased color yields with doubled and quadrupled conjugate concentrations (data not shown).

**Standardized interpretation of EIB by densitometry.** A computer program (BlotPlot) for automatic conversion of

**FIG. 2.** Example of the relationship between color yield units (ABS) and absolute amount of IgG bound per HIV protein band in EIB for one human HIV-antibody-positive serum. Line one has been drawn for antibodies against each HIV protein. The color yields of various amounts of nonspecifically bound IgG are shown by the heavy line.
that the correction for nonspecifically adsorbed IgG had to be done with precision; otherwise, over- or undersubtraction occurred. Although this method is inherently more precise, it cannot be performed as a clinical routine.

Consequences of the differences in titration curve for antibodies to different viral proteins. The variable slopes of the reaction curves of eight different anti-HIV-positive patient sera with the major viral antigens seen with both IgG quantification methods proved that the relative distribution of IgG per HIV protein varies with concentration of serum. Thus, because of the somewhat variable slopes of the titration curves for antibodies to individual HIV proteins, there will always be a degree of ambiguity regarding the relative color yield in each band. To minimize this effect, comparisons between sera should be made at the same dilution and preferably with the same IgG concentration.

The reason for the more horizontal slopes of the titration curves, both with respect to color yield and nanograms of IgG, for antibodies reactive with the pol polypeptides p53 and p64 is not evident from the present work. Differences in affinity are the most likely explanation.

Feasibility and desirability of quantitative serology. Traditionally, serology has been performed in a semiquantitative way by titration to limiting dilution. This approach is adequate in many situations. However, if the diagnosis of an infection with far-reaching consequences (like infection with HIV) is solely dependent upon the demonstration of antibodies to the etiological agent, the development of more precise methods is justified. Research on acquired immunodeficiency syndrome pathogenesis, the exchange of results between laboratories, diagnosis of HIV carriage, and recognition and differentiation of aberrant serological patterns (1, 18, 19, 27) all would benefit from an accurate description of the anti-HIV antibody status. If in the future a protective role of some anti-HIV antibodies is described and they become used therapeutically, quantitative serology will be necessary for supervision of the levels of these antibodies. The methodology presented here also permits comparisons of sensitivity, based on quantification of bound IgG, among different EIB methods, as well as other direct-binding solid-phase immunoassays for HIV antibody.

Clinical usefulness of quantitative EIB. It is now widely accepted that there is a degree of correlation between health status and loss or persistence of certain anti-HIV antibodies in the HIV infected (see below). However, an interesting variation between African and other HIV-1-infected persons in this respect has recently been reported (1). Quantitative EIB is an objective method which is potentially useful for such serological follow-up. We have preferred calculating a ratio of color yields at a fixed concentration in serum, instead of using the absolute values, to diminish the dependence on overall anti-HIV IgG levels and methodological variations. The widely different p24/gp41 ratios we found for healthy seropositive patients could be due to individual tendencies to react with IgG to different HIV proteins, be a prognostic sign, or less likely, be due to methodological variation. Our observations of high p24/gp41 ratios in a few recently infected patients and low ratios in a few patients with HIV-related disease are, however, in accord with previous qualitative or semiquantitative descriptions (3, 11, 13–15, 20, 26). Ideally, a long-term prospective study with more patients, regular sampling, and several other indicators of disease progression is needed to establish the clinical validity of quantitative EIB, as well as to point out further possible improvements of the method. That is, however, beyond the scope of the present paper.

LITERATURE CITED

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FIG. 3. (A) Variation of the ratio of the p24 and gp41 color yields as determined by densitometry of EIB strips. Seven consecutive sera from a 30-year-old homosexual male who is still healthy were analyzed. (B) Ratio of the color yields of anti-p24 and -gp41 in EIB for 24 patients, including those with acute, primary HIV infection; those healthy with HIV-positive sera; and HIV-infected patients with acquired immunodeficiency syndrome or lymphadenopathy syndrome.

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