

Specificities and Sensitivities of Three Systems for Determination of Antibodies to Human Immunodeficiency Virus by Electrophoretic Immunoblotting

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Electrophoretic immunoblotting (EIB [Western blotting]) has emerged as the major method for verification of seropositivity for human immunodeficiency virus (HIV) and therefore needs to be thoroughly characterized. The specificities of three EIB systems, our own and two commercial systems, were studied with antiviral sera and serial dilutions of human sera. We demonstrated that in one system, anti-HLA classes I and II gave bands comigrating with viral proteins, which can be controlled by EIB with uninfected H9 cells. In addition, animal antiserum, including anti-immunoglobulin enzyme conjugates, occasionally reacted with HIV gag proteins, necessitating appropriate controls. Whereas none of 10 blood donors reacted at the standard dilution in serum (1/100 or 1/400) in any of the three systems, 6, 1, and 2 of 10 donors reacted with p24, p55, or both at a dilution of 1/10 for the three systems tested. Thus, nonspecific reactions can arise in several ways and justify critical EIB interpretation. The sensitivity of the three systems was studied by comparative titrations and direct quantification of bound immunoglobulin G (IgG). In the titrations with all three, the minor anti-HIV bands p53 and p64, coded from pol, were often detectable in higher dilutions than were antibodies to any other HIV protein. The minimum visible amounts of IgG bound per HIV protein band estimated by extra- and interpolation in densitometric curves and liquid scintillation counting of radiolabeled patient IgG were approximately 0.1, 0.05, and 0.02 ng per band in the three systems. One of the commercial systems had both the highest sensitivity and highest specificity.

False-positive reactions in the sensitive present-day human immunodeficiency virus (HIV) antibody-screening tests occur (9–13, 16, 19), and at least one confirmatory test, usually the electrophoretic immunoblot (EIB [Western blot]), is therefore needed (7, 8, 17). Obviously, the EIB must itself be very specific but, in addition, highly sensitive.

False-positive results in HIV EIB could be due either to reactions with nonviral molecules or, theoretically, to cross-reacting antibodies to hitherto undescribed human retroviruses (3). Thus, sources of unspecific reactions should be traced. Sensitivity is also important. For example, we need the highest possible sensitivity to prevent transmission from blood donors, and in comparative studies we must find good ways to express sensitivity. In this study, we examined three different EIB methods for detecting antibodies to individual HIV proteins by comparative titrations and quantitative EIB, by the variation of the EIB pattern at different dilutions of serum, and with respect to some factors which can mislead in the interpretation of the EIB.

MATERIALS AND METHODS

Sera. There were 10 HIV antibody-positive and 10 antibody-negative control (blood donor) sera used from our diagnostic routine. The criteria of the Centers for Disease Control for classification of patients with acquired immunodeficiency syndrome and related disorders (5) were followed.

The following animal sera against human cellular components were used. Six rabbit anti-HLA class I and one preimmune control serum samples, as well as one rabbit anti-HLA class II serum, were kindly provided by Per Petterson, Department of Cell Biology, University of Uppsala, Uppsala, Sweden. Rabbit anti-B2-microglobulin was kindly provided by Lars Björck of our department. Rabbit antiactin, antiactinin, antitubulin, antidesmin, and antispectrin were purchased from BioYeda, Rehovoth, Israel. Rabbit antitubulin was also obtained from Bio-Genex Laboratories, Dublin, Calif. Mouse monoclonal antibodies included anti-interleukin-2 (IL2) receptor. OKT4 and OKT8 (Becton Dickinson and Co., Mountainview, Calif.), antitubulin (Bio-Genex), as well as antivinulin (BioYeda). For EIB, rabbit sera were diluted 1/200, with the exception of rabbit antitubulin (1/25) from Bio-Genex, and mouse monoclonals (1/50). For inhibition studies we used either rabbit anti-HLA (diluted 1/25)-human anti-HIV serum (diluted 1/5,000) or rabbit anti-HLA (diluted 1/5,000)–human anti-HIV (diluted 1/25).

Rabbit anti-human immunoglobulin G (IgG) peroxidase conjugate was from DAKO, Copenhagen, Denmark. Goat anti-human and goat anti-rabbit IgG peroxidase conjugated IgGs (affinity purified) were from Sigma Chemical Co., St. Louis, Mo.

EIB. System A is described in detail in the companion paper (2). Briefly, doubly sucrose-banded HIV was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose sheet. This was cut into strips, which were incubated overnight with patient sera, which, if not otherwise indicated, were diluted 1/400 and 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).

The commercial EIB HIV antibody confirmatory tests were used according to the instructions of the manufacturer (system B, HTLVIII immunoblot kit; Bio-Rad Laboratories, Richmond, Calif.; system C: DuPont Western Blot Test, manufactured by Biotech Research Laboratories, Inc.,

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Rockville, Md.). The latter test involved a biotin-avidin enhancement step. The normal serum dilution in these two systems was 1/100.

Quantification of EIB strips. Quantification methods are elaborated in the accompanying paper (2). Briefly, for densitometry, a Bio-Rad model 620 video densitometer was used in its reflectance mode. The integrated absorbances are thereafter referred to as color yields and are expressed in color yield units. Color yields were converted to nanograms of IgG by interpolation in a standard curve derived from nitrocellulose strips with dots containing known amounts of IgG or from measurements of strips concomitantly developed with known amounts of $^{125}$I-labeled anti-HIV IgG. Initially, we had problems quantifying p53 and p55 separately because occasionally the two molecules were insufficiently separated. The color yields of p53 and p55 were therefore expressed as p53-p55.

RESULTS

Comparison of three EIB systems. (i) Specificity. The specificity of the three EIB systems was tested with human negative control sera and with poly- or monoclonal animal antisera directed against abundant cellular proteins, belonging either to the cytoskeleton or to the cell surface. Table 1 depicts the degree of reactivity of different animal anticytoskeletal sera in the three systems. System A exhibited the strongest reaction with both cytoskeletal and cell surface proteins, although the virus antigen had been prepared by two successive ultracentrifugations in sucrose gradients. Especially notable were the reactions of anti-HLA class I and II sera to proteins migrating closely to HIV gp41 and HIV p31, respectively, as well as reactions of a commercial rabbit antitubulin serum to proteins inseparable from HIV p24 and p55 (Fig. 1).

Theoretically, the reactions of the anti-HLA sera could be due to a cross-reactivity with viral proteins. We therefore tried to inhibit either the binding of a limiting dilution of rabbit anti-HLA class I and II sera with a large excess of three different human anti-HIV sera or the binding of a limiting dilution of human anti-HIV serum with a large excess of the hyperimmune anti-HLA class I and II sera. In both cases, we controlled the experiments so that the anti-human and -rabbit conjugates did not react appreciably in heterologous crosses with human and rabbit IgG in the EIB and enzyme-linked immunosorbent assay. No inhibition was observed, however (data not shown).

The possibility that antitubulin antibodies could cross-react with HIV gag proteins was explored by use of two

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**TABLE 1. Reactions with anticellular sera in three EIB anti-HIV systems**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Type</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HLA class I</td>
<td>R</td>
<td>++</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Anti-HLA class II</td>
<td>R</td>
<td>++</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Anti-$\beta$-microglobulin</td>
<td>R</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-spectrin</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL2 receptor</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OKT4</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OKT8</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antivinculin</td>
<td>M</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antitubulin</td>
<td>R</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Antitubulin</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antitubulin</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antitubulin</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antidesmin</td>
<td>R</td>
<td></td>
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</tr>
</tbody>
</table>

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**TABLE 2. Titration of eight anti-HIV sera in three different EIB systems**

<table>
<thead>
<tr>
<th>Antibodies to protein(s)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg(^a)</td>
<td>Range</td>
<td>Avg(^b)</td>
</tr>
<tr>
<td>p17</td>
<td>1.2</td>
<td>0-2.6</td>
<td>1.7</td>
</tr>
<tr>
<td>p24</td>
<td>1.6</td>
<td>0-3.1</td>
<td>2.2</td>
</tr>
<tr>
<td>p31</td>
<td>1.0</td>
<td>0-2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>gp41</td>
<td>1.9</td>
<td>0.5-3.6</td>
<td>2.3</td>
</tr>
<tr>
<td>p53-p55(^d)</td>
<td>1.9</td>
<td>0.3-3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>p64</td>
<td>1.7</td>
<td>0-3.0</td>
<td>1.9</td>
</tr>
<tr>
<td>gp120</td>
<td>0.05</td>
<td>0-0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

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\(^a\) With the normal working dilution counted as 1/1. Titters outside of chosen dilutions were approximated by extrapolation.
\(^b\) Geometric mean.
\(^c\) Ratio of the average titer relative to that of system A.
\(^d\) Anti-p53 and -p55 antibodies were quantified together because in some gels they could not be measured separately.
additional antitubulin sera, one monoclonal antibody preparation, and one rabbit hyperimmune serum. None reacted with p24-p55, thus raising the possibility that the anti-p24 reaction was due to inadvertent immunization by infection of the rabbit with another retrovirus. During our EIB work we have occasionally encountered other rabbit and goat sera with anti-HIV p24 activity. Of 12 commercial anti-human IgG conjugates, 3 (2 rabbit and 1 goat) gave a relatively strong anti-p24 band at dilutions of 1/200 to 1/500.

We also tested the extent of false-positive reactivity at different dilutions of human serum. In samples of 10 blood donors, none reacted with any viral protein in concentrations of 1/400 (system A) and 1/100 (systems B and C). However, increasing the concentration in serum to 1/25 gave a weak staining of p24 with 3, 0, and 0 sera of systems A, B, and C, respectively. When we further increased the final dilution to 1/10, weak reactions with p24, p55, or both were seen in 6, 1, and 2 of 10 samples in systems A, B, and C, respectively.

(ii) Sensitivity. Eight anti-HIV-positive sera were diluted from the normal working dilution (1/1) to 1/256 in negative control serum and further treated as normal serum samples in EIB with three different systems (A, B, and C); densitometry followed (Table 2). A titer value was derived from the inspect of the dilution curve with a cutoff limit of 0.3 color yield units. Systems B and C proved to have a somewhat higher sensitivity than that of system A (average relative log10 titer: A; 1; B, 1.37; and C, 1.41). The densitometric results regarding three different anti-HIV bands are shown for one serum in Fig. 2, which illustrates that in individual sera, variations in the pattern of relative color yields could occur among the EIB systems and also among different dilutions of serum.

We were surprised to find that the minor bands arising from pol-encoded p53 and p64 often were faintly visible even at the highest dilution when the other bands had disappeared (Fig. 2). At the normal dilutions of 1/400 (system A) and 1/100 (systems B and C), the dominating bands were those of p24 and gp41. However, the intensity of these bands dropped steeply upon further dilution. In contrast, p53 and p64 exhibited a flatter curve. The changing relative intensities during titration of another anti-HIV-positive serum are shown photographically (Fig. 3). We also compared the sensitivity of IgG detection in the three EIB systems by incubating two strips dotted with known amounts of IgG per dot in the enzyme-anti-IgG conjugate of each EIB system, by washing and by developing the color. We found that the limit of visibility corresponded to 0.2, 0.1, and 0.04 ng of IgG per dot in EIB systems A, B, and C, respectively. This corresponded to approximately 0.1, 0.05, and 0.02 ng of IgG per band on the EIB strips. A similar value was obtained for system A with radiolabeled anti-HIV IgG (0.1 ng of IgG per band).

**DISCUSSION**

**Sensitivity.** The sensitivity of densitometry was somewhat lower (around 0.2 ng of IgG per band in system A) and the sensitivity of the radiolabeled IgG system was somewhat higher than that of the human eye (around 0.03 ng of IgG per band in system A). Judged by densitometric analysis of the comparative titrations, the sensitivity of the three routine EIB systems tested was highest for system C, somewhat lower for system B, and lowest for system A.

Surprisingly, in all three EIB systems, the endpoint dilutions of the antibodies reactive with p53 and p64 often were
pitfalls in HIV serology. In the present paper we have demonstrated a number of factors which can give rise to a false interpretation of HIV EIB. HLA proteins present in most HIV preparations can simulate HIV proteins because of electrophoretic comigration. Human and animal sera used in HIV EIB can give false-positive reactions with gag proteins. This phenomenon is disturbing because in the early stages of HIV infection, anti-gag can be the only visible EIB reaction. We are now studying the causes of this false-positive reaction.

The variations in EIB pattern with IgG concentration and EIB system create problems for studies of the correlation of EIB pattern with prognosis. This source of variation could, however, be at least partly controlled by the inclusion of a reference serum in each EIB run.

One of the three systems tested had both the highest sensitivity and specificity. We must use the most sensitive and specific verification systems available for maintaining the credibility of blood bank testing, for voluntary screening of concerned individuals, and for diagnostic testing.

**LITERATURE CITED**


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