Antigen-Antibody Reaction in Solution in Capture Competition Immunoassay for Human Immunodeficiency Virus Antibodies

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In the capture competition immunoassay, undiluted serum was reacted in solution with purified human immunodeficiency virus (HIV) antigen in wells of microtiter plates coated with anti-HIV immunoglobulin G antibodies (HIV capture antibodies). HIV antibodies present in the serum being tested combined with the HIV antigen and thus blocked (completely or partially) the fixation of the antigen to the capture layer. Unblocked antigenic activity was measured in subsequent steps by the use of biotinylated anti-HIV immunoglobulin G and peroxidase-conjugated avidin. The assay was evaluated in comparison with indirect enzyme-linked immunosorbent assay and Western (immuno-) blot (WB). A total of 180 serum samples which reacted repeatedly as positive in indirect enzyme-linked immunosorbent assay but negative in WB were found to be negative by the capture competition assay. Of 54 serum samples showing dubious reactions (single p24 bands in WB), 53 were clearly separated into positive or negative reactions, whereas 1 serum sample gave a borderline reaction. It was concluded that a characteristic feature of this kind of inhibition assay is a very low frequency of equivocal results.

Screening of blood donations for the presence of antibodies against human immunodeficiency virus (HIV) is now used routinely in order to minimize transfusion-associated HIV infections. The assay principle most widely applied for screening is the indirect immunoassay using solid-phase-bound purified HIV antigen (11, 12). The well-recognized occurrence of false-positive reactions in this kind of assay due to the presence of cell-specified contaminants in the antigen has made confirmatory examination of reactive specimens, preferably by using an alternative type of assay, a necessary part of the diagnostic routine (5, 13, 14).

Assays commonly used for confirmatory purposes include Western (immuno-) blot (WB), radioimmunoprecipitation, and indirect immunofluorescence. WB is presently the most widely applied method for confirmatory serology (4). Unlike the conventional indirect assay, WB is capable of differentiating the antibody response into reactivities against individual viral polypeptides, which allows positive sera with HIV-characteristic band patterns to be identified with high specificity. In contrast, the specificity of single-band responses (predominantly corresponding to the position of p24) cannot be judged conclusively by WB (1, 2). In such cases the serological diagnosis depends on the possible appearance of additional HIV-characteristic bands in WB performed on subsequent samples. The definitive serological answer may thus be delayed for several weeks to months.

There is therefore a need for alternative methods of high specificity to aid in the confirmatory diagnosis of HIV infection. Inhibition immunoassays might be of value in this respect because in this kind of assay the specificity depends on the indicator antibodies, the purity of which can be secured by simple immunochromic procedures, and not on the purity of the viral antigen.

This paper describes an inhibition enzyme-linked immunosorbent assay (ELISA) for detection of HIV-specific antibodies and presents data concerning its diagnostic value with sera giving equivocal reactions in indirect ELISA and/or WB.

MATERIALS AND METHODS

Cell and virus propagation. The CDC 451 strain of HIV-1 was propagated in cloned HUT-78 cells (Clone 6D5 from the Centers for Disease Control [3]). The persistently infected cells were cultured in screw-cap flasks (Catalog no. 156502; Nunc, Roskilde, Denmark) at 36°C in 5% CO2, using RPMI 1640 supplemented with antibiotics and 10% fetal calf serum. Half of the medium was changed twice weekly, and the doubling time of the cells was 1 to 2 weeks. The cell line was maintained at a density of approximately 5 × 105 cells per ml, with a ratio of live to dead cells of about 6:4.

The amount of virus released into the medium was monitored by a biotin-avidin-potentiated double-antibody sandwich ELISA for antigen detection (8). The titer of immunochromatically reactive viral antigen in harvested medium was approximately 10,000. This level of reactivity remained stable for months in most cultures and was not influenced by shorter or longer intervals between changes of medium.

Purification of HIV. Medium from the persistently HIV-infected cell cultures was clarified at 10,000 × g at 4°C for 30 min and made 7% (wt/vol) with polyethylene glycol 6000. After overnight incubation at 4°C with gentle stirring, the precipitate was collected by centrifugation at 10,000 × g for 15 min. The precipitate was suspended in NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.8) at 1:100 the original volume and clarified at 3,000 × g for 5 min. The supernatant was made 25% (wt/vol) with Nycodenz (Nyegaard, Oslo, Norway), transferred to TV850 tubes (Ivan Sorvall, Inc., Norwalk, Conn.) over a 60% (wt/vol) Nycodenz cushion, and centrifuged at 200,000 × g at 4°C for 24 h. Fractions were collected by bottom puncture and analyzed by refractometry and for HIV antigen by ELISA (8). The broad antigenic peak in the density range from 1.13 to 1.18 was pooled and recentrifuged as described above. The antigen from the second run was diluted 1:5 in NET buffer.
and centrifuged for 4 h in an AH629 rotor at 100,000 × g at 4°C through 5 ml of 20% (wt/vol) sucrose. The pellet was suspended in NET buffer with 1% (vol/vol) Triton X-100–1 mM phenylmethylsulfonyl fluoride–0.05% (wt/vol) sodium azide at 1:2,000 the original volume, incubated at 56°C for 1 h, and sonicated. The purified HIV antigen was kept at −80°C. WB analysis of purified antigen preparations using known anti-HIV-positive sera revealed the presence of all of the major antigens specified by the viral genome.

**Anti-HIV IgG.** Immunoglobulin G (IgG) was purified from a pool of four human serum samples with exceptionally high titers of IgG against HIV (above 100,000 by indirect ELISA) and with strong reactivity in WB against all major viral bands. Purification was done by ion-exchange chromatography on DEAE-Tris acryl M (LKB Instruments, Inc., Rockville, Md.) in 25 mM Tris, pH 8.8–35 mM NaCl; affinity chromatography was then performed on protein A-Sepharose CL 4B (Pharmacia-LKB, Uppsala, Sweden) as previously described (9). Purified IgG was concentrated by negative-pressure ultrafiltration followed by dialysis against phosphate-buffered saline, pH 7.4, and adjusted to a protein concentration of 5 mg per ml. A part of the purified IgG was labeled with biotin as previously described (9).

**Capture competition immunoassay.** Flat-bottomed irradiated polystyrene microtest plates (Immunoplate I; Nunc) were incubated overnight at room temperature with 100 μl of purified anti-HIV IgG diluted 1:2,000 in phosphate-buffered saline, pH 7.4, per well. The plates were washed five times in washing buffer (0.05 M phosphate, pH 7.2, 0.5 M NaCl, 1% [vol/vol] Triton X-100). All subsequent washing cycles were carried out as described above. A 25-μl volume of undiluted test serum was added to each of two adjoining wells, whereas 75 μl of HIV antigen diluted 1:60,000 in dilution buffer (wash buffer supplemented with 1% [wt/vol] bovine serum albumin) was added to one of the wells (the test well) and dilution buffer without antigen was added to the other (the control well). After overnight incubation at room temperature, the plate was washed and incubated for 1 h with 100 μl of biotinylated anti-HIV IgG added to each well.

After another wash, the plate was incubated for 30 min at room temperature with 100 μl of horseradish peroxidase-conjugated avidin (KemEnTec, Copenhagen, Denmark) per well diluted 1:1,000 in dilution buffer. After a final wash, 100 μl of enzyme substrate (0.05 M citrate buffer, pH 5.0, with ortho-phenylenediamine dihydrochloride [0.4 mg/ml] and 30% hydrogen peroxide [0.5 μl/ml]) was added per well. Color development was allowed to proceed for 30 min at room temperature, whereafter 150 μl of 1 M sulfuric acid was added. Plates were read in a single-channel micro ELISA reader (model MR 580; Dynatech Industries, Inc., McLean, Va.) at 492 nm, using a reference beam of 620 nm. The photometer was blanked with substrate plus sulfuric acid.

Results were expressed by calculation of an inhibition coefficient (IC) defined as: IC = [OD(NC)+Ag] × [OD(NC)+Ag]−1 × 100, where OD(NC)+Ag is the optical density at 492 nm (OD492) of the test well (test serum and antigen) and OD(NC)+Ag is the mean OD492 of five replicate wells with negative control serum and antigen. Sera displaying IC values of ≥50 were deemed positive (3). This cutoff level corresponded to the mean + 5 standard deviations of the IC values obtained with sera from 200 blood donor sera negative for anti-HIV by indirect ELISA. Test results accompanied by control well OD values of ≥0.1 × OD(NC)+Ag were considered influenced by nonspecific factors, and the sera were deemed unfit for analysis in this kind of assay.

**Indirect ELISA.** Technical details of the indirect ELISA have been published elsewhere (12). In the present study, 100 μl of purified HIV antigen diluted 1:2,000 in carbonate buffer, pH 9.6, was added to each well of a microtest plate (Immunoplate I; Nunc) and incubated at 4°C overnight. The washing procedure and composition of wash buffer and dilution buffer was identical to the inhibition assay described above.

Serum was added in 1:100 dilution in duplicate (100 μl in each well) and incubated for 2 h at room temperature. After a wash, 100 μl of horseradish peroxidase-conjugated rabbit anti-human IgG (catalog no. P 214; Dakopatts, Copenhagen, Denmark) was added to each well and incubated for 1 h at room temperature. After a final wash, enzyme activity was detected as described above. A panel of 10 negative serum samples was included in every plate, and serum samples with OD readings >3 times the average OD value of the negative panel were considered positive for HIV antibodies, while serum samples with OD values ≤3 times the mean negative OD value were deemed negative.

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A reagent combination with a low antigen dose was selected in order to provide optimal conditions for detection of the HIV antigen-combining activity elicited by antibody-positive sera.

The 180 serum samples which were positive in indirect ELISA and negative in WB all gave a negative result in the inhibition ELISA (Fig. 1). The 44 serum samples which were positive in indirect ELISA and dubious in WB (single p24 reaction) were separated into two groups by the inhibition ELISA (31 were negative and 12 were positive). One serum sample gave a reaction equal to the cutoff level. The 10 ELISA-negative serum samples giving dubious reactions in WB were all negative in the inhibition assay.

Table 1 shows that 7 of the 782 unselected donor serum samples reacted positively in indirect ELISA, and one of these serum samples was also positive in the inhibition test. This single specimen was confirmed positive in WB, while the remaining six ELISA-positive serum samples were found to be negative.

**TABLE 1. Results of 782 donor serum samples examined in indirect ELISA and capture competition ELISA**

<table>
<thead>
<tr>
<th>Capture competition ELISA results</th>
<th>No. of indirect ELISA results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>775</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
</tr>
</tbody>
</table>

* Negative in WB.
* Positive in WB.

**DISCUSSION**

It is well recognized that inhibition immunoassays are superior to indirect ELISA in terms of specificity. The reason is that the specificity of the inhibition assay is based on the subsequent detection of the antigen introduced into the assay, which means that only epitope specificities identical to those of the detecting antibody preparation are registered.

One of the drawbacks on the indirect ELISA is that antibodies to cellular contaminants in the purified HIV antigen preparation can cause false-positive reactions (5, 14). We have previously shown that serum antibodies to nonviral epitopes present in the antigen preparation used in an inhibition assay do not cause inhibition (9).

The inhibition assay might, at least in theory, generate false-negative results due to nonspecific binding of capture antibodies to the detecting antibodies. Rheumatoid factor in the serum being tested might cause this reaction. We have previously shown that the use of biotinylated detection antibodies in this kind of assay greatly reduces this possibility (9). The reason for this is that biotinylated antibodies are less denatured than antibodies coupled directly to enzyme molecules and because the biotin-avidin amplification makes it possible to use very small amounts of detecting antibodies.

The presence of HIV antigen in the serum sample under test likewise links the detecting antibodies to the capture antibodies. The inclusion of a control well without antigen in this kind of assay is therefore of paramount importance, as it reveals such reactions. In the present study, none of the 1,016 serum samples tested displayed control well reactions, but sera with a known high content of HIV antigen did give readings up to OD values of 0.5 in the control well. The same reaction occurred in the test well (but was unseen) and counteracted the inhibition caused by the coexisting HIV antibodies (unpublished observations).

It has previously been shown that inhibition assays based on extracellular HIV-antigen and using polyclonal capture and detecting antibodies of human origin first and foremost detect antibodies against the major core protein (p24) of HIV (7). In accordance with this, some of our sera giving single p24 reactions in WB were positive in the competitive assay with 100% inhibition. Conversely, sera containing little or no detectable antibody reactivity to p24, as is often seen in the later stages of HIV infection (10), may give false-negative results in this kind of assay (7).

Apart from specificity, the most important quality of the assay is therefore its ability to distinguish clearly between positive and negative sera. The 234 serum samples tested were "trouble serum samples" selected from the Danish blood bank screening of approximately 250,000 donations, and only one of these serum samples gave an indeterminate reaction in the inhibition assay.

Some of the WB-dubious sera (p24) exhibited complete inhibition, and some were clearly negative in the competitive assay. Most likely the positive sera contained antibodies against virus-specific p24. This was confirmed by demonstration of seroconversion in five cases from which serum samples drawn later could be obtained. The nature of the WB p24 reactions of sera giving negative reactions in the inhibition assay remains to be determined.

**LITERATURE CITED**


