Use of Horseradish Peroxidase in Identification of Serum Antibodies and Immune Complexes

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We made 202 examinations on specimens from 78 patients, to evaluate the application of horseradish peroxidase-labeled antisera as compared to fluorescein-labeled material in a clinical immunology laboratory. Various circulating and tissue-fixed antibodies were identified by both techniques. The horseradish peroxidase technique can be substituted for the fluorescein isothiocyanate method for the evaluation of anti-nuclear antibody and anti-mitochondrial antibody without loss of specificity or sensitivity. Although the results are comparable, fluorescein isothiocyanate appears to be slightly more sensitive in the detection of the other circulating and tissue-fixed human antibodies examined. Further refinements in preparation and technique could well lead to the fluorescein method being replaced by horseradish peroxidase staining in the future.

Additional Keyphrases: diagnosis of autoimmune diseases • fluorescein isothiocyanate method compared

The fluorescein-labeled antihuman immunoglobulin technique is widely accepted for identifying and measuring antibodies in patients with various autoimmune diseases. The method is specific and sensitive, and the materials are readily available from commercial sources. Despite these advantages, the use of FITC3 has major shortcomings, such as:

- a dark-field microscope equipped with a camera is needed to record results
- the fluorescence is transitory
- antibodies cannot be localized at the subcellular level
- sections cannot be reviewed in the future in the perspective of new knowledge

A labeling technique has been introduced in which HRP is used to overcome these disadvantages (1, 2). HRP-labeled preparations can be read by light microscopy. The sections are permanent and can be stored for prolonged periods for future review. Subcellular immune complexes can be localized by electron microscopy. The HRP technique is specific and sensitive in a variety of laboratory procedures but has not gained widespread acceptance (3–5), perhaps because the coupling procedure is tedious and the yield often low. With the availability of commercially prepared HRP-labeled antisera, this problem has been eliminated for the routine hospital laboratory. Here, we report the application of HRP-labeled antiserum and compare the results with those obtained for FITC preparations in various test procedures in a large clinical immunology laboratory.

Materials and Methods

Samples. The specimens tested were tissues and sera selected from those received in the immunology laboratory of the Cleveland Clinic. All had been previously examined by the fluorescent technique. Most examinations were performed on recently acquired material, a few4 on specimens that had been stored at −70 °C for as long as several months. Both indirect and direct examinations were performed. Indirect testing is performed with patients’ sera, to detect circulating antibodies and (or) immune complexes. Direct testing is done on patients’ tissues (kidney, skin) to detect fixed antibodies and (or) complexes. Patients’ sera that were positive were routinely diluted 160-fold, and in a few special cases as much as 5120-fold.

We did a total of 169 examinations with the use of HRP-labeled antiserum by the indirect technique, on sera from 48 patients (Table 1) and compared the results to those obtained when FITC was used.

Rat kidney was used as the source of antigen in indirect tests for antinuclear and antimitochondrial antibodies, human uterus for anti-smooth muscle antibody, and rhesus monkey esophagus for antibodies to intercellular substance and basement membrane. We also examined 15 kidney biopsies from 15 patients and 18 skin biopsies from 15 patients (Table 2). Concurrently, positive and negative control sera and tissue were incubated with both labeled and unlabeled antibody in each group of examinations.

FITC procedure. The fluorescent method has been described by many authors. In essence, our procedure for the direct technique was as follows. FITC-labeled rabbit antihuman antisera specific for human immunoglobulins (Hyland Laboratories,

4 Especially high-titered antinuclear antisera and positive skin and kidney tissue.
Costa Mesa, Calif. 92626 and Baltimore Biological Laboratories, Baltimore, Md. 21030), which we absorbed with mouse liver powder, were layered on appropriately prepared air-fixed sections of frozen tissue (the sections, 2-4 μm in thickness, were cut at -20 °C). The sections were incubated in a humidity chamber at room temperature for 20-30 min, washed in three changes of PBS, covered with coverslips, and examined under a Zeiss fluorescence microscope. The indirect technique differed only in the inclusion of a step in which the patient’s serum was layered on the appropriate tissue (e.g., rat kidney) and washed in three changes of PBS.

**HRP procedure.** The HRP procedure is very similar. Sections of frozen tissue, 2-4 μm thick, were made at -20 °C and placed on clean glass slides, air fixed, and washed briefly with PBS. Patients’ sera (in indirect tests) were incubated with the antigen source at room temperature for 30 min. The sections were washed with three 3-min changes of PBS, and incubated with a thin covering of HRP-labeled antisemum for 30 min. After three additional washes in PBS, the stain was developed according to the method of Graham and Karnovsky (6) by placing the slides for 30 min in a freshly prepared solution of 7.5 mg of 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo. 63178) in 10 ml of Tris-HCl buffer (50 mmol/liter, pH 7.6) containing 0.15 ml of a 30 g/liter solution of hydrogen peroxide. (Staining for 60 min did not significantly affect the results.) The sections were then washed in distilled water, successively dehydrated in 95% and absolute ethanol, cleared in xylene, and mounted with “Permount” (Fisher Scientific Co., Fair Lawn, N. J. 07410).

We did not fix the specimens in osmium tetroxide. Antisera (now available commercially from Bioware, Inc., Wichita, Kan. 67201) were developed by one of us (L. P. C.). The method used was that of Avrameas (7), in which HRP VI (Sigma) is coupled to goat antihuman IgG with glutaraldehyde. The goat antibody was purified by ammonium sulfate precipitation and chromatography on diethylaminoethyl-Sephadex (Pharmacia Co., Upsala, Sweden), then incubated with glutaraldehyde and horseradish peroxidase, precipitated with ammonium sulfate, and dialyzed against PBS. The reconstituted enzyme-labeled antibody was analyzed by immunoelectrophoresis and calibrated with World Health Organization antinuclear reagent (Division of Biological Standards, National Institute for Medical Research, Mill Hill, London N.W. 7, Great Britain). The enzyme/antibody ratio was determined with a Beckman Model DU Spectrophotometer set at 280 nm (for total protein) and 403 nm (for peroxidase). The results indicated a molar peroxidase/protein ratio of 0.98, or about one molecule of peroxidase per molecule of antibody.

**Results**

Tables 1 and 2 summarize our results. The results for antinuclear antibody were identical by both techniques in 66 of 70 examinations performed (Figure 1). In two tests, we could not observe HRP staining at dilutions greater than 160-fold, in contrast to FITC. However, in two other preparations HRP stained at a higher dilution than FITC. Staining patterns (diffuse, speckled, nucleolar) seen with FITC were the same as those observed in the HRP

![Fig. 1. Antinuclear antibody as demonstrated by HRP](image-url)
stained material (Figure 2). Similarly, antimitochondrial antibody could be identified in every case where the specimen was positive with FITC (Figure 3). In three instances the reaction was less intense with HRP than with FITC at higher dilutions of patients' sera. Sera positive for anti-smooth muscle antibody with FITC failed to exhibit antibodies when HRP-labeled antisera were used. Indirect staining for intercellular antibodies was similar with both techniques, but HRP did not appear to be as sensitive as FITC (Figure 4). In four of nine cases, HRP staining was very weak at higher dilutions of patient sera. Basement membrane antibodies were variably identified, especially when the serum was highly diluted.

The results of direct staining of renal biopsies with HRP were somewhat variable. Although the overall correlation between the techniques was favorable, the contrast tended to be suboptimal and localization difficult with HRP. Renal biopsies known to have positive glomerular and small vessel fluorescence were processed by the HRP technique (Figure 5). Nine of 10 specimens with positive immunofluorescence also had positive HRP staining. However, the HRP stain and fluorescence were occasionally distributed differently. Of three cases with diffuse immunofluorescence, only two exhibited diffuse HRP staining, the other showing a more discrete, localized pattern. Linear HRP staining was not observed in specimens known to have linear immunofluorescence. Small-vessel HRP staining was difficult to identify with certainty, largely because of inadequate contrast.
Results of direct HRP staining of skin specimens were disappointing. Specimens from only two of eight patients with known intercellular antibodies showed positive staining with the HRP technique. One individual known to have antibodies to basement membrane antibodies exhibited similar results. We saw no HRP staining in patients with the dermal-epidermal deposits seen by immunofluorescence in cases of lupus erythematosus.

Discussion

Our results are essentially in harmony with those of others, especially as to the value of HRP-labeled antisera in identifying circulating antibodies (8–11). The HRP label has been demonstrated to be as specific and as sensitive as FITC in identifying antinuclear and antimitochondrial antibodies. Indirect examinations for other circulating antibodies by the HRP method reveal close correlations, but the technique appears to lack the desired sensitivity at present. Anti-smooth muscle antibodies could not be localized by the HRP technique, but because the titers of anti-smooth muscle antibody were low even with FITC staining, this discrepancy could be accounted for on a sensitivity basis. Three of the five specimens tested were over a year old when examined by use of HRP and were only very weakly positive with FITC at that time. The other two sera were only positive by FITC when a 20-fold dilution was used. Possibly, with the low contrasts seen in the sections, a weak staining reaction might not be discernible. Similarly, with intercellular and basement membrane antibody localization (indirect), results were positive by both methods in almost every case but with serial dilutions greater than 80-fold, HRP staining was often lost. We omitted osmium tetroxide fixation in an effort to shorten the procedure and eliminate the need for additional equipment (hood and fan) and safety measures. However, the greater contrast offered by osmium tetroxide fixation might have improved results of indirect testing for certain circulating proteins.

In contrast to the polyclonal antisera used in FITC examinations, HRP was coupled only to antIgG. Human antibodies of other classes (M,A,D,E) would therefore not be detected, further decreasing the sensitivity of the method. Localization of HRP staining to glomeruli in patients with glomerular disease has been demonstrated by Davey and Busch (10). Their procedure, in which osmium tetroxide fixation is used, apparently resulted in a much higher background staining than in the cases reported here. Even so, they reported that a completely linear pattern was difficult to identify and that HRP staining in areas other than the glomeruli was variable.

We cannot explain our poor results in direct tests of skin for intercellular, basement membrane, and dermal-epidermal antibodies, especially in light of the highly satisfactory results of Fukuyama et al. (11). Their techniques differed in no essential way from those we used. Further examinations on additional patients are necessary to resolve this problem.

Despite its many advantages, the routine clinical laboratory use of HRP labeled antisera to detect circulating and tissue-fixed autoantibodies remains somewhat limited. The HRP method appears to be excellent for determining antinuclear and antimitochondrial antibodies, but lacks sensitivity for others. Nevertheless, results of the FITC and HRP technique do correlate, and future refinements in the latter procedure will in all probability, lead to its more widespread use. Such refinements might include efforts to couple antisera specific for additional human immunoglobulins, to increase the contrast (perhaps by counterstaining) and to increase the enzyme/antibody ratio.

References