Use of peroxidase-conjugated antiglobulin as an alternative to immunofluorescence for the detection of antinuclear factor in serum

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SYNOPSIS A method of detecting serum antinuclear factor by the use of antiglobulin conjugated with horseradish peroxidase has been compared with a standard procedure using a fluorescent label. The horseradish peroxidase conjugate was evaluated by block titration against positive serum and by its performance as a test reagent for screening and titrating sera. It is concluded that the horseradish peroxidase method, in which an ordinary light microscope is employed, provides a satisfactory alternative to immunofluorescence for routine tests for antinuclear factor.

It has been shown (Nakane and Pierce, 1966) that horseradish peroxidase can be coupled to antibody by a simple procedure to give stable conjugates which retain immunological reactivity and can be used for immunotracing in a similar manner to fluorescein-labelled antibody. In tissues treated with peroxidase conjugate the sites of uptake are made visible by a simple histochemical procedure. Peroxidase catalyses the oxidation by hydrogen peroxide of a number of substrates, in some cases producing a highly coloured insoluble reaction product.

The work of Nakane and Pierce (1966) and others (eg, Davey and Busch, 1970) suggests that the horseradish peroxidase method is at least as sensitive as the immunofluorescence technique. Nakane and Pierce (1966) state that 'this might be expected because enzyme is not consumed in the reaction with substrate, and each molecule of enzyme-labelled antibody bound to the antigenic site deposits many molecules of reaction product'.

Antibodies combining specifically with components of cell nuclei are characteristically found in the serum of patients with certain 'autoimmune' conditions, notably systemic lupus erythematosus, and are usually detected by the indirect fluorescent antibody method (Coons and Kaplan, 1950). This involves treating preparations containing nuclei, eg, tissue sections, with the patient's serum followed by staining with antiserum to human γ -globulin conjugated with a fluorescent dye. Nuclear fluorescence indicates a positive reaction. Since immuno-

fluorescence was first used for detecting antinuclear factor (Holborow, Weir, and Johnson, 1957) the method has undergone considerable development (Johnson, Beutner, and Holborow, 1967; Holborow and Johnson, 1969) to improve its usefulness in diagnosis and is increasingly used in clinical laboratories. Difficulties, however, are still encountered, especially those associated with the special requirements of fluorescence microscopy. These would be avoided by the use of a label such as horseradish peroxidase which could be rendered visible by conventional microscopy. In this paper we compare the sensitivity of the two procedures.

Materials and Methods

PATIENTS' SERA

These were drawn from the serum bank of this Unit, and were maintained at -20°C .

FLUORESCENT CONJUGATE

The globulin fraction obtained by precipitation of monospecific rabbit antiserum to human IgG with 50% saturated ammonium sulphate was conjugated with fluorescein isothiocyanate isomer 1 (BDH) by a modification of the method described by Marshall, Eveland, and Smith (1958). After conjugation unreacted dye was removed by passing the solution through Sephadex G25. Analysis of the conjugate indicated a protein concentration of 8 mg/ml and a labelling ratio of 1.1:1 ($\text{OD}_{495\text{ nm}} : \text{OD}_{280\text{ nm}}$). It contained 4 units of precipitating activity (Beutner,

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Holborow, and Johnson, 1967) and was evaluated by block titration as previously described (Beutner *et al*, 1967) using the antinuclear factor system. A satisfactory working dilution was 1:40.

FLUORESCENCE MICROSCOPY

A Zetopan microscope (Reichert) fitted with HBO 200 high-pressure mercury vapour burner and cardioid condenser was used. The exciting filter was BG 12/3 mm (Schott and Genossen) and the barrier filter GG9 (Schott and Genossen).

HORSERADISH PEROXIDASE CONJUGATE (NAKANE AND PIERCE, 1966)

0.25 ml of 0.5% 4,4'-difluoro 3,3'-dinitro diphenyl sulfone¹ in acetone was added to 2 ml of 0.5 M carbonate buffer pH 10.0 containing 50 mg of horseradish peroxidase² and 50 mg of a globulin fraction prepared from the same rabbit antiserum used for the fluorescein isothiocyanate conjugate. The mixture was gently agitated at 4°C for six hours, dialysed against 0.15 M NaCl containing 0.01 M PO₄ pH 7.2 ('PBS') overnight and the small amount of precipitate removed by centrifugation. An equal amount of saturated ammonium sulphate in distilled water was added and mixed gently by inversion. After standing for 30 minutes at 4°C the mixture was centrifuged for 15 minutes at 3,500 rpm at 4°C and the supernatant containing free horseradish peroxidase was discarded. The precipitate was washed twice with 50% saturated ammonium sulphate in phosphate-buffered saline, resuspended in 0.5 ml of phosphate-buffered saline, and dialysed against phosphate-buffered saline to remove ammonium sulphate.

IMMUNOLOGICAL STAINING PROCEDURE

The method previously described (Holborow and Johnson, 1969) was followed. Cryostat sections, 6 μ , were cut from snap-frozen blocks of fresh liver from Black Hood rats. Multislide slides (O'Neill and Johnson, 1970) were used to facilitate handling. All dilutions were made in phosphate-buffered saline. Staining was carried out in a damp chamber at room temperature, and the slides were washed after treatment with serum and conjugate, with the aid of a magnetic stirrer. Duplicate sections treated in parallel were stained either with fluorescein isothiocyanate or horseradish peroxidase conjugate. After washing, sections treated with the fluorescein conjugate were mounted in glycerol and examined under the fluorescent microscope. Sections tested with the horseradish

peroxidase conjugate were washed, stained for horseradish peroxidase by the method described below, and examined under a light microscope.

In each system tests were randomized and read independently without knowledge of their identity.

HISTOCHEMICAL LOCALIZATION OF HORSERADISH PEROXIDASE (GRAHAM AND KARNOVSKY, 1966)

Preparation of substrate solution

3,3'-diaminobenzidine tetrahydrochloride (Sigma—97.99%)—25 mg

0.05 M Tris/HCl buffer pH 7.6—50 ml

3% hydrogen peroxide—0.15 ml

Substrate was freshly prepared for each batch of sections and the hydrogen peroxide was added immediately before use.

STAINING METHOD

Sections were stained for 10 minutes at room temperature in the substrate solution, washed briefly in 0.05 M Tris/HCl pH 7.6 buffer followed by distilled water, dehydrated in absolute alcohol, cleared in xylene, and mounted in DPX (BDH).

Results

APPEARANCE OF SECTIONS STAINED WITH HORSERADISH PEROXIDASE CONJUGATE

Sections that had been treated with antinuclear factor-positive sera showed deeply staining brown nuclei, the rest of the tissue being only very faintly stained (Fig. 1a). Nuclei in sections treated with antinuclear factor-negative sera on the other hand were indistinguishable from the background (Fig. 1b). Leucocytes present in some sections invariably showed strong cytoplasmic staining due to the presence of endogenous peroxidase.

EVALUATION OF HORSERADISH PEROXIDASE CONJUGATE

The performance of the horseradish peroxidase conjugate in the antinuclear factor system was evaluated by block titration as described for the fluorescein isothiocyanate conjugate (Holborow and Johnson, 1967). Dilutions of an antinuclear factor-positive serum were tested against dilutions of the horseradish peroxidase conjugate. A range of staining intensities resulted enabling titration end-points corresponding to the lowest level of reactivity to be recorded. The results are shown in Figure 2. It will be seen that a constant titre for the antinuclear factor serum was obtained over a range of dilutions of the horseradish peroxidase conjugate. The histogram shows a plateau similar to that obtained when fluorescent conjugates are tested in this way.

¹Obtained from K & K Laboratories Inc., U.K. Distributors Kodak Limited, Kirkby, Liverpool, England.

²Peroxidase (Horseradish) Grade II RZ Value 2.24. Seravac Ltd, Maidenhead, Berks.

Fig. 1 Detection of antinuclear factor by horseradish peroxidase method ($\times 500$). a Positive result. b Negative result.

Fig. 2 Block titration of a known antinuclear factor-positive serum using horseradish peroxidase conjugate.

ASSESSMENT OF HORSERADISH PEROXIDASE CONJUGATE FOR USE IN ROUTINE ANTINUCLEAR FACTOR TESTS

Detection of positive sera

Twenty-four sera were tested for antinuclear factor by both methods. The horseradish peroxidase conjugate was used at a dilution of 1:10. Ten sera were positive and 14 negative by both methods. Details of results are shown in Table I.

Degree of Staining	Number of Sera	
	FITC ¹ Conjugate	HRP ² Conjugate
Positive	4	5
Weak positive	3	2
Very weak positive	3	3
Negative	14	14

Table I Comparison of results with two conjugates

¹FITC = fluorescein isothiocyanate.

²HRP = horseradish peroxidase.

Titration of positive sera

Results of titrations by both procedures of six known antinuclear factor-positive sera were in close agreement (Table II).

Serum No.	Antinuclear Factor Titre	
	FITC Conjugate ^a	HRP Conjugate ^a
1	1 : 250	1 : 250
2	≥ 1 : 1250	≥ 1 : 1250
3	1 : 1250	1 : 250 ^b
4	≥ 1 : 1250	≥ 1 : 1250
5	1 : 250	1 : 250
6	1 : 250	1 : 250

Table II Results of titrations by both procedures

^aNot tested at higher dilution than 1 : 250.

^bFITC = fluorescein isothiocyanate.

^cHRP = horseradish peroxidase.

DETECTION OF VARIANTS OF THE NUCLEAR STAINING PATTERN

Sera giving homogeneous, speckled, or nucleolar staining with fluorescein isothiocyanate conjugate gave the same pattern when tested with the horseradish peroxidase conjugate.

DETECTION OF OTHER AUTOANTIBODIES

Preliminary tests indicated that horseradish peroxidase conjugates may also be used to detect antibodies reacting with gastric parietal cells, smooth muscle, mitochondria, 'bile canaliculi', and thyroid microsomes. In these tests the conjugate was absorbed with a suspension of rat liver as previously described (Holborow and Johnson, 1967).

Discussion

In this study the fluorescence readings were made by experienced observers using darkground illumination with which positively stained nuclei appear bright apple-green against a dark background. The appearance of horseradish peroxidase tests, however, was unfamiliar and their brown colour provided less intense contrast. Nevertheless, when bias was eliminated by randomization of tests, the results show a striking correlation of sensitivity between the two methods. This finding suggests that the horseradish peroxidase method may provide a useful alternative to immunofluorescence for the detection of antinuclear factor and perhaps also for detecting other tissue-reactive antibodies.

The advantages of the horseradish peroxidase procedure over immunofluorescence are: (1) a simple light microscope is required; (2) preparations are permanently mounted; (3) the reaction product

is not subject to bleaching during microscopy. Although an extra step is necessary in order to demonstrate uptake of horseradish peroxidase the increase in staining time is only about 20 minutes.

Counterstaining may be helpful in localizing the reaction product and has been successfully applied to tissues after histochemical demonstration of peroxidase (see especially Straus, 1970a and b; Lillie, 1965; Pearse, 1960). Thus eosin and light green have been used to stain cytoplasm and connective tissue; nuclear stains, including methyl green, methylene blue, basic fuchsin, and Mayer's haemalum and Romanovsky type stains, have also been employed. Faint staining of peroxidase might be obscured by counterstaining which should therefore be avoided when maximum sensitivity is required.

Other substrates for peroxidase give different coloured reaction products, eg, benzidine (blue) (Adler and Adler, 1904; Straus, 1964) and 3-amino-9 ethylcarbazole (red) (Graham, Lundholm, and Kanovsky, 1965). They should give better contrast with some counterstains and may also enable different specific immunological reactions to be demonstrated in contrasting colours in the same preparation. The reaction products formed, however, are less stable than that from diaminobenzidine.

The desirability of characterizing fluorescent conjugates physicochemically is now acknowledged (Holborow, 1970). The essential requirements are: (1) adequate antibody activity, (2) optimal fluorochrome: protein labelling ratio, and (3) absence of unconjugated antibody and free fluorochrome. A similar degree of characterization of horseradish peroxidase conjugates may be desirable.

The reaction products from diaminobenzidine after treatment with osmium tetroxide can be readily identified in electron micrographs (Graham and Karnovsky, 1966). Fixation of tissues is essential for ultrastructural studies and must precede staining. Hoedemaeker and Ito (1970) have described the ultrastructural localization of gastric parietal cell antigen by the use of horseradish peroxidase conjugates of IgG obtained from patients with pernicious anaemia. It was found during the present work that short fixation of tissue sections with formaldehyde did not prevent their subsequent reaction with the autoantibodies tested, and it seems likely therefore that ultrastructural localization of the antigens involved in these reactions may also be possible.

Many animal tissues normally show peroxidase activity. Although this presented no difficulty in this study its presence could make interpretation difficult. Methods are being sought for the irreversible inhibition of endogenous activity.

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