Coupling of Peroxidase to Poliovirus Antibody: Characteristics of the Conjugates and Their Use in Virus Detection

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Antibody to poliovirus type 1 (Po-1) was coupled to peroxidase by use of glutaraldehyde or 4,4'-difluoroo,3,3'-dinitro diphenyl sulfone. Glutaraldehyde was found to be the superior coupling agent, yielding conjugates that had up to 2.8 x 10^4 enzyme units/ml (75% of total enzyme input). Conjugates migrated as a single band when centrifuged in sucrose density gradients, demonstrating that the purification procedure used was effective in removing both noncoupled enzyme and heterogeneous antibody components. Conjugates were specific for Po-1 and did not adsorb to cells infected with unrelated enterovirus types. Adsorption of conjugates to Po-1-infected cells was demonstrable within 6 h postinfection.

The use of enzyme-labeled antibodies for detection of viral antigens has been described for a number of virus-host systems (1, 3-6, 9-12). Further, the method has been found to be as sensitive and reproducible as the fluorescent antibody technique (10, 11). In a previous report (J. E. Herrmann and S. A. Morse, in press), we found that enzyme-conjugated poliovirus antibody would bind to virus-infected cells only if the virus neutralization capacity (VNC) of purified conjugates was retained. VNC was found to be dependent on the molecular weight of the enzyme used for coupling: enzymes with molecular weights greater than that of immunoglobulin (Ig)G (β-galactosidase and β-glucuronidase) inactivated antibody; those with molecular weights less than or equal to IgG (ribonuclease, alkaline phosphatase, and peroxidase) retained VNC. Because peroxidase-conjugated antibody retained VNC and can be accurately assayed, it was used for the conjugation studies presented here. This paper compares methods currently in use for preparing enzyme-labeled antibodies and characterizes the conjugates obtained. Also, the use of peroxidase-conjugated poliovirus antibody for demonstrating specific enterovirus infection is described.

MATERIALS AND METHODS

Enzymes and reagents. Peroxidase (horseradish), code HPOFF, was obtained from Worthington Biochemical Corp., Freehold, N.J. Grade B peroxidase was from Calbiochem, La Jolla, Calif. o-Dianisidine dihydrochloride, 3,3'-diaminobenzidine dihydrochloride, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo. 4,4'-difluoro-3,3'-dinitro-diphenyl-sulfone (DFNS) was from K & K Laboratories, Inc., Plainview, N.Y. Glutaraldehyde, 8%, EM grade, was from Polysciences, Inc., Warrington, Pa.

Viruses and antisera. Poliovirus type 1 (Po-1) strain LSc-2ab was obtained from O. C. Liu, Northeastern Water Laboratory, Narragansett, R.I. Cox sackievirus type B4 (CB-4) and echovirus type 11 (EC-11) were obtained from D. O. Cliver, University of Wisconsin, Madison, Wis. Viruses were propagated in Vero tissue cultures, and stored at ±20 C. For immunization of rabbits with Po-1, the virus was partially purified by Sephadex G-25 column chromatography. A 1-ml amount of virus was inoculated intravenously at weekly intervals until sufficient virus neutralization titers were obtained (1:640-1:2560). The neutralization titer was the serum dilution which caused an 80% or greater Po-1 plaque reduction in Vero monolayers after incubation of the serum for 2 h at room temperature with 100 ± 20 plaque-forming units (PFU) of Po-1.

Conjugation of peroxidase to Po-1 antibody. Antibody to Po-1 was isolated from whole serum by precipitation with (NH4)2SO4 at 40% of saturation, followed by dialysis against 0.1 M phosphate buffer (pH 6.9) for 2 days at 4 C. The method for coupling antibody to peroxidase by use of glutaraldehyde was essentially that of Avrameas (2): 10 mg of peroxidase (specific activity, 460 units/mg) was added to 1 ml of dialyzed antibody (10 mg of protein) plus 0.3 ml of phosphate buffer (0.1 M, pH 6.9). To this was added 9 μg of 125I-labeled rabbit IgG in 0.05 ml of
Table 1. Effect of coupling agent on the conjugation of peroxidase to poliovirus antibody

<table>
<thead>
<tr>
<th>Coupling Agent</th>
<th>¹²⁵I (Counts/min/ml)</th>
<th>Peroxidase (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernat fluid</td>
<td>Precipitate</td>
</tr>
<tr>
<td>DFNS Glutaraldehyde</td>
<td>2,665</td>
<td>48,350</td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>57,970</td>
</tr>
</tbody>
</table>

Fig. 1. Density gradient centrifugation (5 to 40% sucrose) of 0.05 ml of crude peroxidase conjugate (middle) and 0.05 ml of purified peroxidase conjugate (bottom). Top: 0.05 ml of unconjugated enzyme preparation centrifuged under the same conditions as the conjugate.

saline (obtained from A. B. MacDonald, Harvard School of Public Health) to serve as an antibody marker. The mixture was stirred, and 0.15 ml of a 0.5% aqueous solution of glutaraldehyde was added in 25-µliter increments. After 4 h of incubation at room temperature (no stirring), the preparation was dialyzed at 4 C overnight against 2 liters of phosphate buffer. The opalescent preparation was clarified by centrifugation at 30,000 x g for 30 min at 4 C. The supernatant fluid was precipitated with (NH₄)₂SO₄ at 40% of saturation, dialyzed as above, and stored at -20 C.

An alternate method, which uses DFNS as the coupling agent (7, 9, 10), was tested for comparative purposes. The Po-1 antibody, ¹²⁵I-labeled antibody, and peroxidase were used in the amounts described above; the buffer was 0.35 ml of 0.1 M carbonate (pH 9.2). For coupling, 0.1 ml of a 0.5% solution of DFNS in acetone was added. The reaction time and subsequent treatment was the same as that used for the glutaraldehyde procedure. For both preparations, antibody content was determined by counting ¹²⁵I disintegration in a Nuclear-Chicago automatic gamma counter.

Enzyme assay. Peroxidase content in conjugate preparations was measured by a modified Worthington Biochemical Co. method: 0.1 ml of enzyme diluted in 0.05 M phosphate buffer (pH 6.0) was added to 2.9 ml of the same buffer containing 0.001 M H₂O₂, 0.004 M o-dianisidine dihydrochloride, and a 1:600 final concentration of Triton X-100. The change in absorbancy per minute was read at 460 nm. One unit of peroxidase is the amount of enzyme decomposing 1 µmol of H₂O₂ per min at room temperature under the conditions above.

Histochemical staining of infected cells. Monolayers of Vero cells grown on cover slips were incubated with selected enteroviruses at an input of 10 to 30 PFU/cell. The cultures were incubated (in Earle balanced salt solution plus 5% fetal calf serum) for 6 to 18 h at 37 C. After rinsing the cell sheets with phosphate-buffered saline (PBS), the cells were fixed in 95% ethanol for 15 min at room temperature and air-dried. The preparations were incubated with peroxidase-conjugated antibody (0.2 ml/cm² surface area) for 1 h at room temperature. The cells were rinsed three times with PBS to remove nonspecifically adsorbed conjugate, and a few drops of staining reagent were added (0.025% 3,3-diaminobenzidine dihydrochloride, 0.5% ethylenediaminetetraacetic acid, 0.1% H₂O₂ in phosphate buffer [0.02 M, pH 6.0]). After 3 to 5 min reaction time, excess reagent was removed, and the cover slips were mounted in glycerol on glass slides. Sites of peroxidase activity were seen as dark brown precipitates when viewed by phase-contrast microscopy.

Density gradient centrifugation. Gradients of 5 to 40% (wt/vol) sucrose were prepared in ultracentrifuge tubes (5 ml/tube) by use of an ISCO model 570 gradient former. Samples were added, and the tubes were centrifuged for 2 h at 40,000 rpm (192,000 x g maximum) in a Spinco model L2-65 B ultracentrifuge, SW50.1 rotor. An ISCO model 640 density gradient fractionator was used to collect fractions of 0.3 ml each. Sucrose concentration was measured by refractometry; enzyme and antibody content were assayed as described above.
FIG. 2. Binding of peroxidase-anti-PO-1-infected Vero cells. a, Cell control; b, cells infected with coxsackievirus type B-4; c, cells infected with echovirus type 11; d, cells infected for 6 h with PO-1; e, cells infected for 18 h with PO-1; f, same as e. Methodology was as described in text. The arrows indicate location of peroxidase conjugates as demonstrated by histochemical staining with 3,3’-diaminobenzidine and hydrogen peroxide. The nuclei are indicated by n. Magnification is 500×.
RESULTS
Efficiency of enzyme coupling. The relative efficiency of glutaraldehyde and DFNS coupling procedures was determined by measuring the amount of peroxidase and antibody contained in \((\text{NH}_4)_2\text{SO}_4\) precipitates of unpurified conjugates. At the salt concentration used (40% final concentration), free (unconjugated) enzyme does not precipitate. It can therefore be assumed that any peroxidase activity in the precipitates is antibody associated. The results presented in Table 1 show that glutaraldehyde was about 1.7 times as effective for coupling as was DFNS. In addition, the conjugate prepared with glutaraldehyde was less turbid, as DFNS is not soluble in aqueous solutions. The amount of antibody precipitated, as measured by \(^{125}\text{I}\) disintegrations, was approximately the same for both preparations.

The efficacy of the conjugate purification procedure was determined by comparing sedimentation patterns of purified and unpurified conjugates (glutaraldehyde coupled) in sucrose density gradients. It can be seen from Fig. 1 that the purification steps were effective in removing both noncoupled peroxidase and heterogeneous antibody components. The purified conjugate contained only one peak of both enzyme and antibody, which were coincident.

The enzyme activity of conjugated antibody was dependent on the purity of the initial enzyme preparation. The use of grade B peroxidase resulted in a maximum conjugate activity of \(3.2 \times 10^8\) units/ml; the more active enzyme (Worthington Biochemical Corp.) resulted in a maximum activity of \(2.8 \times 10^4\) units/ml. The virus neutralization capacity for both preparations was about the same: 1:320 in purified preparations.

Binding capacity of conjugates. The binding capacity and specificity of Po-1 antibody conjugates was tested by adsorbing conjugate to cells infected with EC-11, CB-4, or Po-1. The cells were assayed for adsorbed peroxidase activity by histochemical staining, as described above. The results in Fig. 2 demonstrate that the conjugate was specific for Po-1, as these were the only preparations with distinct peroxidase activity. The time of infection (6 or 18 h) did not appreciably influence conjugate binding; note the large amount of stained peroxidase surrounding lysed cells in the 18-h preparations (Fig. 2e and f).

DISCUSSION
The advantages of the enzyme-labeled antibody technique over fluorescent antibody technique have been cited by several authors (2, 4, 7, 11). These include the use of light microscopy, permanent mounting of preparations, continual reaction product formed, and the use of more than one enzyme and/or substrate. In addition, the method offers a theoretical advantage in that one virion can be detected, based on the fact that single molecules of enzyme can be assayed (8).

From the results presented in the present paper, it is apparent that the conjugates we used can detect enterovirus antigen specifically, with very little "background" peroxidase activity in cell or virus control preparations. The specificity of the method also allows for rapid typing of enteroviruses (J. E. Herrmann and S. A. Morse, in preparation). This would reduce the time required from 3 to 4 days for diagnosis by conventional neutralization tests to less than one day by use of enzyme-coupled antibody assays.

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LITERATURE CITED
