Antigens of Mycobacterial Cell Walls

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Three antigenic fractions from the cell walls of eight strains of mycobacteria were studied. Isolation and purification of these antigens were effected by enzymatic digestions, differential and sucrose gradient centrifugations, dialyses, and column chromatography. Two of the fractions were termed cell wall tuberculins (CWT-1, solubilized with lipase; CWT-2, solubilized with lysozyme); the third was termed “C” (cross-reacting) antigen. All appeared to be lipopolysaccharides. The CWT antigens, as compared with purified protein derivatives (human), were relatively species (group)-specific in both double immunodiffusion and guinea pig skin tests; in the latter, the reactions resembled those of delayed hypersensitivity. The C antigens reacted heterologously in double immunodiffusion and skin tests; the latter were the “immediate” type of reaction.

The cell walls of mycobacteria are noted for their content of lipid and for their biological activities. Walls from the BCG strain of Mycobacterium were shown (9) to contain 61% lipid. The chloroform-soluble cellular lipids (waxes B, C, and D), as well as bound lipid and phosphatides, were shown to be localized almost exclusively in the walls. Injected intradermally into normal rabbits, mycobacterial cell walls produced skin lesions and elicited delayed type hypersensitivity (12). Walls from BCG cells disrupted in mineral oil were immunogenic for mice (10), the immunity being comparable to that effected with viable BCG (1). Wax D fractions acted as Freund-type adjuvants (15). Thus the immunological activity of mycobacterial cell walls is well documented. There are few published reports, however, concerning isolation and purification of immunologically active components of these walls. In particular, scant attention has been given to cell walls of the atypical mycobacteria, a group only recently recognized as important human pathogens.

It is now known that phagocytic cells of the reticuloendothelial system contain a variety of hydrolytic enzymes. It is plausible to assume that these enzymes may be important in the host’s response to foreign material. Hence, we elected to use hydrolytic enzymes to purify and solubilize cell walls obtained from both tubercle bacilli and also atypical mycobacteria. It is conceivable that enzymatic degradation may be less traumatic than the more classical chemical extractions.

MATERIALS AND METHODS

Bacteria. Eight strains of mycobacteria were used, namely: Mycobacterium kansasii strains P1 and P8, scotochromogenic strains P5 and P19, Battey strains P17 and P39, M. phlei, and M. tuberculosis H37Ra. The cells were grown in the dark on Sauton’s synthetic medium (18) at 37 C for 2 to 12 weeks, depending on the individual strains. Cultures were harvested, before showing signs of lysis, by centrifugation (1,465 x g; 4 C, 20 min) and washed three times with sterile distilled water containing 0.1% Tween 80 (Tween-water).

Preparation of cell walls. A homogeneous 25% suspension of heat-killed cells (65 C, 1 hr) in Tween-water was chilled and disrupted with a cold French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) six times at 18,000 psi. Unbroken cells were largely removed from the pressed material by differential centrifugation six times at 755 x g for 15 min. The cell wall fragments and remaining unbroken cells in the supernatant fraction were sedimented (23,500 x g, 1 hr), rehomogenized in Tween-water by sonic oscillation (Branson Sonifier; Branson Instruments, Inc., Stamford, Conn.), and separated by sucrose density gradient centrifugation (19) with a Sorvall HB-4 swinging bucket (2,520 x g, 1 hr). All the sucrose solutions and the 1 M KCl diluent contained 0.1% Tween 80.

One gram (wet weight) of cell wall fragments homogenized in 50 ml of 0.05 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.5) was treated with 20 μg of deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N.J.) and 200 μg of ribonuclease A (Worthington) at 37 C with constant stirring for 6
hr, after which 2 mg of trypsin (Calbiochem, Los Angeles, Calif.) was added and incubation was continued for another 3 hr. Cell walls were sedimented and washed thoroughly with distilled water.

Isolation of antigens from purified cell walls. One gram (wet weight) of purified cell walls was homogenized in 50 ml of 0.2 M Tris buffer (pH 8.1), to which 10 ml samples of the following were added: 2% sodium azide, 1% sodium deoxycholate (Difco), 5 × 10⁻⁵ M CaCl₂, 2 M NaCl, and lipase (PLI, Worthington) at 0.1 mg/ml in 5 × 10⁻⁴ M CaCl₂. After incubation at 40 C for 24 hr on a rotary shaker (210 rev/min), the cell wall residues were sedimented (23,500 × g, 1 hr) and digestion was repeated once with half of the original amounts of lipase, salts, and buffer. The supernatant fractions were pooled, filtered (RAWP04700, 1.2 μm; Millipore Corp., Bedford, Mass.), and dialyzed against 0.1 M Tris buffer (pH 8.1) at 4 C for 4 days and then for another 4 days against distilled water with daily changes of buffer or water. The dialyzed supernatant fraction was concentrated by evaporation. The precipitate which formed in the tubing during dialysis and evaporation was removed by centrifugation and washed thoroughly with sterile distilled water.

The lipase-treated cell wall residues were washed and digested with lysozyme as follows: 1 g (wet weight) of cell wall residues were homogenized in 50 ml of 0.2 M Tris buffer (pH 7.5) and 10 mg of lysozyme (3 × crystalline; Nutritional Biochemical Corp., Cleveland, Ohio) in 10 ml of sterile distilled water, 10 ml of 2% sodium azide, 10 ml of 0.1 M NaCl, and 20 ml of 2.5 × 10⁻⁴ M disodium (ethylene-dinitrilo) tetra-acetate. Incubation was at 37 C for 24 hr. As in the case of lipase treatment, digestion was repeated once; the supernatant fractions were separated from the cell wall residues, pooled, filtered, and dialyzed against distilled water at 4 C for 4 days. After concentration by evaporation, the precipitate was removed and washed.

Purification of cell wall antigens. The C antigens in the concentrated supernatant fractions were separated from the enzymes (lipase or lysozyme) with a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column (2.5 by 100 cm), and eluted with 0.05 M phosphate buffer, (pH 6.0) containing 0.025% sodium azide. Fractions were examined for carbohydrate (5), protein (7, 11), and antigenic reactivity (by double immunodiffusion).

The antigenic fractions were pooled, dialyzed, concentrated, and further purified with a Sepharose 4B (Pharmacia) column (1.7 by 85 cm). The eluent used and assays for carbohydrate and antigenic reactivity were the same as described above.

The washed precipitate from lipase or lysozyme supernatant fraction was divided into two portions. One portion was redissolved in 0.0625 M Tris buffer (pH 8.1) at a concentration of 30 mg (wet weight) of precipitate per 4 ml of buffer containing 1 mg sodium azide. The mixture was centrifuged at 100,000 × g at 4 C for 1 hr. The supernatant fraction was removed, and Tween 80 was added to it to a concentration of 0.005%. It was then chromatographed in a Sephadex G-50 column (1.7 by 85 cm) with Tris-Tween buffer as eluent. The elution volumes of the fractions were determined.

\[ \text{Optical Density} = \frac{1}{490} \times \text{Absorbance at 490 nm} \]

0.005% Tween 80 and 0.025% sodium azide. Antigenic fractions from the column were heated at 65 C for 1 hr and stored at 4 C.

The other portion was digested with Pronase. The reaction mixture contained: 30 mg (wet weight) of washed precipitate suspended in 3 ml of sterile distilled water, 0.25 ml of Pronase (Calbiochem) in 10 ml of buffer (pH 8.1) containing

FIG. 1. Separation of lysozyme from C antigen with a Sephadex G-50 column. Fractions were assayed for the presence of antigen by immunodiffusion tests. Carbohydrate was determined by the phenol-sulfuric acid method at 490 nm and protein by Lowry's method at 300 nm.

FIG. 2. Purification of C antigen with a Sepharose 4B column. The antigenic fractions of Fig. 1 were pooled, dialyzed, concentrated, and further purified on a Sepharose 4B column. The C antigen was assayed by immunodiffusion and carbohydrate was determined by the phenol-sulfuric acid method at 490 nm. All fractions were diluted 1/10 for optical density readings.
sterile distilled water (1 mg per ml), 0.25 ml of 1 m Tris buffer (pH 8.1), and 0.5 ml of 2% sodium azide. Incubation was at 45 C for 24 hr, followed by 2 hr at 60 C and 30 min at 80 C. The mixture was then purified by centrifugation and column chromatography as described above.

Antisera. Albino male rabbits weighing 2.5 to 3.5 kg were given 5 to 6 weekly or biweekly subcutaneous injections of purified cell wall at 5 mg (wt weight) per dose suspended in 0.5 ml of physiological saline and 0.5 ml of Freund's incomplete adjuvant (Difco). This was followed by four to five intravenous injections at weekly intervals with the same dose of antigen suspended in physiological saline. The rabbits were bled 7 to 10 days after the last injection. Anti-whole cell sera were prepared in the same way, except that heat-killed whole cells were used instead of purified cell walls.

Gel double-diffusion tests. Tests were done in plastic boxes filled with gel containing (per liter) 10 g of agar (Ionagar no. 2, Consolidated Laboratories, Inc., Chicago Heights, Ill.). 335 mg of NaH2PO4, 639 mg of Na2HPO4, 172 mg of NaCl, and 0.5 g of sodium azide. The boxes were incubated in a humidified chamber at 37 C for 24 to 48 hr and then at 4 C for 24 hr.

Hypersensitivity tests with guinea pigs. Albino guinea pigs weighing in excess of 450 g were used. Heat-killed cells were suspended in Freund's incomplete adjuvant, 12.5 mg (wt weight) per ml, and each of the two inguinal regions of the guinea pig received 1 ml by subcutaneous injection. Intracutaneous skin tests were made between the 5th and 10th week after sensitization. The diameter of skin induration was measured 24 and 48 hr after injection.

RESULTS

Isolation of cell walls. With live M. phlei, it was found that cell walls could be effectively separated from unbroken cells by the procedures described. After purification by sucrose gradient centrifugation, 0.5-ml samples of the cell wall fractions were plated on brucella agar (Albimi Laboratories, New York, N.Y.). Such samples gave colony counts ranging from 0 to 8 per 10 mg (wt weight) of cell walls. No whole cells were detected by acid-fast stains.

Isolation and purification of antigens from purified cell walls. Three antigenic fractions were obtained from the cell walls of each of the eight strains of mycobacteria. The C antigens were the "cross-reacting" fractions found in the lipase and lysozyme supernatant fractions after dialysis and concentration, the more hydrophobic cell wall tuberculin (CWT) antigens having precipitated out. For any given strain of Mycobacterium, the C antigen in the lipase supernatant fraction appeared to be identical, as determined by double immunodiffusion, with that in the lysozyme supernatant fluid. Results of purification of a C antigen with column chromatography are illustrated by Fig. 1 and 2.

Figure 3 shows the results of purification of the CWT fractions. A spectrum of a phenol-sulfuric acid test of a crude CWT fraction dissolved in Tris buffer showed at least four absorption maxima in addition to the peak around 480 nm for carbohydrate. After passing through a Sephadex G-50 column, the CWT antigen was found in peak "A", which showed a typical carbohydrate peak at 490 nm. The material that interfered with the phenol-sulfuric acid test was found in the second peak "I".

Double-gel diffusion tests. The results may be summarized as follows.

(i) Anti-whole cell sera and anti-cell wall sera were compared with purified antigens, and no difference was found in the immunodiffusion patterns. This is evidence that the purified antigens studied here were not artifacts resulting from the isolation and purification manipulations.

(ii) Heating at 65 C for 1 hr to kill the cells before their disruption in the French press did not significantly affect the antigens studied here. Rabbit sera against purified cell walls isolated from live M. phlei gave practically the same immunodiffusion patterns with either purified C or CWT antigens as the sera against purified cell walls from heat-killed M. phlei. Moreover, as judged by their immunodiffusion patterns and skin reactions in sensitized guinea pigs, antigenic fractions of cell walls obtained from heat-killed M. phlei appeared to be identical with those of cell walls obtained from live M. phlei.

(iii) Digestion with Pronase for 24 hr had no effect on the immunodiffusion patterns of the C and CWT antigens.

(iv) The C antigens reacted with both homologous and heterologous antisera with little or no specificity. However, they were apparently not a single molecular species as they did not form bands of identity in double-gel diffusion tests.

(v) In contrast to the C antigens, the CWT antigens were relatively species (group)-specific. They gave only trace reactions with some heterologous sera.

(vi) The two M. kansasii strains, P1 and P8, were found to be identical in respect to their CWT-2 antigens, even though P1 was a smooth strain and P8 a rough one. Similar lines of identity were observed with the CWT-2 antigens isolated from the two strains of scotochromogens, P5 and P19, and with those obtained from the two Battey strains, P17 and P39.

(vii) The purified CWT-2 antigens from the scotochromogens P19 and P5 were partially identical with those from the Battey strains P39 and P17. Close serological relationship between some scotochromogens and Battey strains has been reported (13).

Hypersensitivity skin tests with guinea pigs. The
purified C antigens elicited "immediate" type skin reactions with little or no specificity. Skin reactions were apparent 1 hr after injection and reached maximum intensity after about 6 hr. The reactions then faded rapidly, and, by the end of 48 hr, they were almost completely gone. In contrast, the time sequence of skin reactions caused by the CWT antigens resembled that for a delayed type reaction. Induration was maximal about 24 hr after injection and diminished slowly.

The effect of Pronase on the CWT antigens is shown in Table 1. Treatment with Pronase decreased the potency of the CWT-2 antigen from M. tuberculosis H37Ra by about 23% and that from M. kansasii P8 by about 21%. However, the specificity of the antigens was increased. No cross-reaction was observed with the treated preparations, but, with the untreated CWT-2 antigens, there were some cross-reactions with M. kansasii P8 and the Battey strains P17 and P39 in guinea pigs sensitized with M. tuberculosis H37Ra, and some cross-reactions with scotocromogen P5 and M. tuberculosis H37Ra in guinea pigs sensitized with M. kansasii P8. Pronase apparently removed the traces of peptide or protein on the cell walls of the treated preparations. It may be assumed that this peptide or protein caused the cross-reactions.

The specificity of the Pronase-treated cell wall tuberculins (CWT-2p) was compared with that of purified protein derivatives (PPD; human) from a commercial source in guinea pigs sensitized to various strains of mycobacteria (Fig. 4). In contrast to PPD (human), the CWT-2p fractions were quite specific, causing little or no cross-reactions in guinea pigs sensitized with heterologous strains. Their specificity in skin tests correlated well with the serological data obtained.
by double immunodiffusion. In guinea pigs sensitized with *M. kansasii* P1, only the CWT-2p fractions from the two *M. kansasii* strains P1 and P8 gave good positive reactions. Similar results were observed with guinea pigs sensitized with the other *M. kansasii* strain P8. As might be expected, there were some cross-reactions between the scotochromogens and the two Battey strains. The CWT-2p fraction from *M. tuberculosis* H37Ra was very specific; it did not cause noticeable reactions in guinea pigs sensitized with heterologous strains as did PPD (human) but gave a good positive reaction in guinea pigs sensitized with the homologous strain. This specificity, however, was relative rather than absolute. At a concentration of 4 μg of carbohydrate per 0.2-ml dose, the CWT-2p antigens from *M. kansasii* P1 and Battey strains P17 and P39 gave slight cross-reactions in guinea pigs sensitized with *M. tuberculosis* H37Ra (Table 2).

In general, the CWT-1p antigens were slightly less potent and specific than the CWT-2p fractions (Table 3). Furthermore, the CWT-1p antigens occasionally caused slight nonspecific reactions in normal guinea pigs; this was not observed with the CWT-2p fractions.

Crude precipitates from both concentrated lipase and lysozyme supernatants contained inert and nonspecific material. The "nonspecific factors" found in the pellets after centrifugation at 100,000 × g for 1 hr of the redissolved crude CWT fractions in Tris buffer elicited skin reactions in normal guinea pigs. Though insoluble in Tris buffer, they could be solubilized in 2% sodium deoxycholate and gave a typical carbohydrate peak at 490 nm in phenol-sulfuric acid tests. The material from peak "I" was nonreactive in gel diffusion tests, and neither elicited dermal reactions in guinea pigs nor acted synergistically when injected together with CWT-2p fractions. Finally, the residues from the cell walls, after digestion with lipase and lysozyme, retained little or no dermal reactivity.

**DISCUSSION**

Degradation with enzymes offers a way other than classical chemical extraction for the study of mycobacterial cell wall antigens. Although mycobacteria are relatively resistant to degradation by lysozyme, several investigators have reported alteration of isolated mycobacterial cell walls by this enzyme (8, 14, 16, 17). In the work reported here, we wished clearly to distinguish between wall and nonwall antigens. Accordingly, and prior to attempts to solubilize the cell walls, crude wall preparations were purified by repeated differential

*Table 1. Effect of Pronase on cell wall tuberculin fractions in dermal tests with sensitized guinea pigs*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean reaction diameter</th>
<th>H37Ra sensitization</th>
<th>M. kansasii P8 sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated CWT</td>
<td>Untreated CWT</td>
<td>Treated CWT</td>
</tr>
<tr>
<td><em>Mycobacterium kansasii</em> P1</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><em>M. kansasii</em> P8</td>
<td>0</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Scoto. P5*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scoto. P19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Battey 17</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Battey P39</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>H37Ra*</td>
<td>10</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Values in Tables 1-3 are the averages of readings from at least three groups of guinea pigs, expressed in millimeters. Reactions smaller than 2 mm in diameter were considered negative. Values in parentheses indicate ranges of skin test diameters observed. Each guinea pig was simultaneously tested with 2 μg of protein of purified protein derivatives (human). Reaction diameters ranged from 14 to 19 mm in *M. tuberculosis* H37Ra-sensitized guinea pigs and from 7 to 19 mm in other heterologously sensitized animals. Sensitizing dose (WT-2) was 2 μg of carbohydrate per 0.2-ml dose.

* b Scotochromogen.

* c *M. tuberculosis* H37Ra.
and sucrose gradient centrifugation, and by treatment with deoxyribonuclease, ribonuclease, and trypsin. Such purified walls were then treated with lipase. The reasoning here, in part, was that initial digestion with lipase would remove sufficient lipid to permit attack of residual wall by lysozyme. Two major kinds of antigens were obtained, and both appeared to be lipocarbohydrate in nature. The “C” (cross-reacting) antigens displayed neither strain nor species specificity in double immunodiffusion and guinea pig skin tests. In the latter tests, the reaction was that of the “immediate” as opposed to delayed type.

In skin tests, the CWT-2 antigens, as compared with PPD (human), were highly, though not absolutely, species (group)-specific, causing less or no cross-reactions in guinea pigs sensitized with heterologous strains. Though the time sequences and overt induration of the skin reactions caused by these CWT antigens resembled those of delayed hypersensitivity, these reactions could have been mixed “immediate” and delayed type re-

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**Fig. 4.** Comparison of specificity of CWT-2p antigens and purified protein derivatives (PPD; human) in skin tests with guinea pigs sensitized to various strains of mycobacteria. Each dose of CWT-2p antigen consisted of 2 µg of carbohydrate in 0.2 ml and PPD was in the amount of 2 µg of protein in 0.2 ml. The diluent was 0.85% saline containing 0.005% Tween 80. The values are the averages of readings from at least three guinea pigs. Reactions smaller than 2 mm in diameter were considered negative.
Table 2. Effect of concentration on specificity of CWT-2p fractions in dermal tests with sensitized guinea pigs

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean reaction diametera</th>
<th>H37Ra sensitization</th>
<th>M. kansasii P8 sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 µg of CHOb/dose</td>
<td>4 µg of CHO/dose</td>
<td>2 µg of CHO/dose</td>
</tr>
<tr>
<td>Mycobacterium kansasii P1</td>
<td>0</td>
<td>3 (2-5)</td>
<td>10</td>
</tr>
<tr>
<td>M. kansasii P8</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Scoto. P5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scoto. P19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Battey P17</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Battey P39</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>H37Ra</td>
<td>10</td>
<td>14 (9-12)</td>
<td>0</td>
</tr>
<tr>
<td>M. phlei</td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
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a Values and abbreviations are as in Table 1.
b Carbohydrate.

Table 3. Comparison of CWT-1p and CWT-2p fractions in dermal tests with sensitized guinea pigs

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean reaction diametera</th>
<th>M. kansasii P8 sensitization</th>
<th>Battey P39 sensitization</th>
</tr>
</thead>
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<tr>
<td></td>
<td>CWT-1p</td>
<td>CWT-2p</td>
<td>CWT-1p</td>
</tr>
<tr>
<td>Mycobacterium kansasii P1</td>
<td>9 (7-10)</td>
<td>10 (9-11)</td>
<td>0</td>
</tr>
<tr>
<td>M. kansasii P8</td>
<td>9 (7-10)</td>
<td>11 (9-12)</td>
<td>0</td>
</tr>
<tr>
<td>Scoto. P5</td>
<td>4 (3-6)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Scoto. P19</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Battey P17</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Battey P39</td>
<td>0</td>
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<td>10</td>
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<tr>
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<tr>
<td>M. phlei</td>
<td>0</td>
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a Sensitizing dose was 2 µg of carbohydrate per 0.2-ml dose.

Responses. Other investigators have also reported that cell components of mycobacteria other than protein may play a role in delayed type hypersensitivity (2, 4, 6), and that these nonprotein sensitins show some specificity (3).

In light of the increasing importance of atypical mycobacteria in both human and animal disease and the need for better means to detect the presence of these organisms, the cell wall tuberculin appears to merit further examination as diagnostic tools. The CWT-2 antigens appear particularly promising as skin test agents, since they are appreciably more species (group)-specific than the commonly used PPD (human), and also seem to be desirably free of strain specificity. Yet another role as diagnostic tools may well be in serological tests. We have found, for example, that CWT-2 preparations from the tubercle bacillus, when deposited on formalized group O erythrocytes, react specifically with antimycobacteria serum in passive hemagglutination and indirect Coomb’s tests. Finally, it may be noted
that the C antigen may be found useful as an antigen with broad generic reactivity in diagnostic serology.

ACKNOWLEDGMENTS

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