Immunoelectrophoresis for Detection of Polysaccharides in Immune Complexes

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A procedure for detecting pneumococcal capsular polysaccharides in immune complexes is described. Separation of antigen from immune complexes is achieved by electrophoresis at 56°C.

Pneumococcal polysaccharide is present in the circulatory system of some patients with pneumococcal infection (1). By use of radioimmunoassay, G. Schiffman, J. E. Summerville, R. Castagna, R. Douglas, M. J. Bonner, and R. Austrian (Fed. Proc. **33**:758, 1974) showed that the polysaccharide in some sera is bound to circulating antibody (6) and is detectable only after acid-pepsin digestion of the sera. We have been interested in devising a simple method which could detect both free and complexed polysaccharide in clinical samples from patients with pneumococcal infection.

Milgrom et al. (3, 4) used immunoelectrophoresis to detect protein and nucleic acid antigens in complexes in serum and tissues of animals with immune complex nephritis. We applied their method to the problem of detecting polysaccharides in immune complexes. The method is based on electrophoresis in a gel medium at 56°C to facilitate dissociation of complexes. Free antigen is then detected by use of either counterimmunoelectrophoresis (CIE) or double immunodiffusion, the former technique providing greater sensitivity.

To evaluate the method, we prepared insoluble immune complexes by precipitating type-specific pneumococcal polysaccharides (obtained from Eli Lilly & Co.) in an excess of pneumococcal antibody at 4°C. We evaluated both type-specific rabbit antibody (Statens Seruminstitut, Copenhagen, Denmark) and human antibody (obtained from a patient who was convalescing from pneumococcal pneumonia). Gel plates were prepared with 1% agarose in barbital-saline buffer (pH 8.2; ionic strength, 0.05) (1). Buffer was added to the reservoirs of each of two electrophoresis trays, and one tray and a gel plate were warmed to 56°C in an incubator. Complexes were washed three times in cold saline, and the supernatant from the third washing was checked with CIE (1) to document that any free antigen had been eliminated from the com-

plexes. The washed complexes (25 μ l) were then transferred to 3-mm wells in the plates. Electrophoresis was performed for 30 min with a potential gradient of 2.5 V/cm of gel, with one plate at 56°C and a control plate at room temperature. CIE was used to detect the free antigen. For this step, type-specific antiserum (Statens Seruminstitut) was added to a longitudinal trough, the plate was rotated 90°, and electrophoresis was conducted for 45 min at room temperature. Polysaccharides of types 1, 3, and 8 were evaluated. Results of a typical experiment with type 3 are shown in Fig. 1.

Whereas antigen was readily separated from complexes at 56°C, antigen did not separate during electrophoresis of the control sample at room temperature. It has been suggested that certain immune complexes (e.g., human DNA-anti-DNA) may be dissociated by electrophoresis at room temperature or even at 4°C (2, 6), but this did not appear to be true for complexes of polysaccharide antigens under present conditions. Many factors may influence the stability of complexes, including the quantity and avidity of antibody attached to the antigen. We observed, for example, that prolonged incubation of polysaccharide with excess antibody increased the stability of the resultant complexes. Polysaccharide was readily separated at 56°C from complexes which were prepared by incubating type 3 antigen and rabbit antibody for 1 or 2 days, whereas no antigen could be separated from complexes when the incubation period was prolonged to 6 days. This observation was repeated in two separate studies.

The conditions needed for optimal sensitivity with electrophoresis at 56°C were evaluated with individual soluble polysaccharides (types 1, 3, 4, and 8). When antigen was subjected to electrophoresis for 30 min at 56°C, followed by CIE for 45 min at room temperature, we could detect 0.05 to 0.10 μ g of polysaccharide per ml. When the initial electrophoresis was prolonged

FIG. 1. Use of electrophoresis at 56°C to separate type 3 pneumococcal polysaccharide from immune complexes. Washed complexes prepared with rabbit (A) or human (B) anticapsular antibody were added to wells, and electrophoresis was performed at either 56°C (plate I) or at room temperature (plate II). Dissociated antigen was detected by adding type 3 antibody to the troughs and performing CIE. The precipitin line in plate I indicates that polysaccharide was dissociated from complexes at 56°C.

to 45 to 60 min, the antigen migrated an excessive distance through the gel and was less readily detected by CIE. With insoluble complexes, an initial electrophoresis of 30 min at 56°C also appeared optimal; more prolonged electrophoresis at 56°C resulted in excessive migration of the free antigen.

Using the technique worked out with insoluble complexes, we evaluated clinical samples from patients with pneumococcal infection. Twenty-one sera (stored at -20°C) from 14 patients with bacteremic pneumococcal pneumonia were available for study. The serotypes of the blood isolates in these cases were as follows: type 1 (one case), type 3 (three cases), type 4 (four cases), types 8 and 9 (two cases each), and types 11 and 12 (one case each). Sera were subjected to electrophoresis at 56°C, along with a control aliquot at room temperature, and free antigen was detected with polyvalent pneumococcal antiserum (Omniserum, Statens Seruminstitut). In addition, all sera were tested by a conventional method of CIE consisting of a 60-min period of electrophoresis at room temperature with the test serum and polyvalent antibody placed in opposing wells (1). Results are listed in Table 1. Four sera contained antigen which was detectable only by use of electrophoresis at 56°C. Further tests showed that the serotypes of antigen in these four sera were type 3 (two sera), type 4 (one serum), and type 11 (one serum). The antigen type corresponded with the serotype of the pneumococcal isolate from the blood in each case. The results obtained with electrophoresis at 56°C suggested that the four sera contained immune complexes of capsular polysaccharide.

TABLE 1. Detection of circulating capsular polysaccharide in bacteremic pneumococcal pneumonia^a

Source	Electrophoresis (no. positive/ no. tested)		Conventional CIE (no. positive/no. tested)
	56°C	Room temp	
Patients	9/14	7/14	7/14
Sera	12/21	8/21	8/21

^a Electrophoresis was for 30 min at indicated temperature, followed by CIE at room temperature.

In support of this possibility, we found that two of the sera also contained type-specific hemagglutinating antibody (titer of 32 in the case of the type 4 infection and titer of 16 in one of the cases of type 3 pneumonia). The antibody titer was less than 4 (i.e., negative) in the remaining case of type 3 infection, and we could not check for antibodies in the patient with type 11 infection because we lacked the necessary purified polysaccharide of type 11. The clinical aspects of these four cases were not unusual, except that two of the patients had pleural effusions which contained capsular polysaccharide and which could have readily entered the circulation to form complexes.

Our studies suggest that capsular polysaccharides can be separated from complexes by electrophoresis at 56°C and that this approach can give positive results in some cases where CIE at room temperature is negative. The number of additional positive cases which can be expected by electrophoresis at 56°C is not certain from the present small study. O'Reilly et

al., using radioimmunoassay, detected complexes of capsular polysaccharide in 17 out of 45 cases of hemophilus infection (5). However, the quantity of complexed antigen in many of their cases was quite low ($<0.01 \mu\text{g/ml}$), which is below the sensitivity of immunoprecipitin methods, including CIE.

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