DIRECT HAEMAGGLUTINATION BY *MYCOPLASMA PNEUMONIAE* AND ITS INHIBITION BY IMMUNE SERUM

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ALTHOUGH haemadsorption by colonies of *Mycoplasma pneumoniae* has been reported, direct haemagglutination by these organisms has not been previously recognised (Del Guidice and Pavia, 1964). Feldman and Suhs (1966) recently showed that a combination of 30 per cent. horse serum and *M. pneumoniae* agglutinated vervet monkey red cells, but *M. pneumoniae* alone did not. An explanation for this phenomenon has been suggested in a previous communication (John, Stahl and Fulginiti, 1966). The present paper describes the direct agglutination of guinea-pig red cells by *M. pneumoniae* and the detection and quantitation of haemagglutination-inhibiting antibodies in immune sera.

MATERIALS AND METHODS

M. pneumoniae was obtained from Dr R. M. Chanock who had passed it 127 times on agar medium. The diphasic and agar media used were described by Grayston *et al.* (1965) and Chanock, Hayflick and Barile (1962). A pool was made after five passages in the diphasic medium and the organisms were stored frozen at -70°C . This pool provided the organisms for the present studies.

Stationary and shaker cultures (incubator-shaker, Model G26, New Brunswick Co.) were sampled daily and serial ten-fold dilutions were plated on agar medium for colony counts. Both the diphasic medium and its broth phase alone were used for stationary cultures, and only the broth phase was used for shaker cultures. Convenient volumes were also taken daily and centrifuged at 59,000*g* for 45 min. on a Spinco model L ultracentrifuge; the centrifuged deposits were resuspended in a tenth volume of fresh medium and checked for haemagglutinating (HA) activity. Equal volumes of serial two-fold dilutions of the above suspension in saline (0.85 per cent. NaCl) and a 0.2 per cent. suspension of guinea-pig erythrocytes in saline, were mixed well and incubated at room temperature for 2 to 2½ hr; the highest dilution that produced complete or nearly complete agglutination was recorded as the HA titre of the test suspension.

Sera. Normal sera from four adult rabbits, pooled sera from 2 rabbits immunised with *M. pneumoniae* (Klieneberger-Nobel, 1962) and sera from several patients with suspected or proven *M. pneumoniae* infection were studied for the presence of haemagglutination-inhibiting (HAI) antibodies. Serial two-fold dilutions of sera and 4 haemagglutinating doses of *M. pneumoniae* were mixed in equal volumes and incubated at room temperature for 1 hr before red cells were added. The highest dilution of serum that completely or almost completely inhibited HA was recorded as the HAI titre of the test serum.

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RESULTS

The results of parallel determinations of viable counts and HA activity are shown in figs. 1 and 2. The shaker cultures yielded the highest numbers of

FIG. 1.—The growth of *M. pneumoniae* in stationary and shaker-cultures.

FIG. 2.—Daily haemagglutination (HA) titres of culture-concentrates of *M. pneumoniae*.

colony-forming units on the 4th day with HA activity demonstrable in dilutions up to 1 in 64. Three separate experiments gave identical results for HA titres. In these experiments the inoculum was 5 ml of the stored pool of *M. pneumoniae*

per 100 ml of fresh medium. When the inoculum was reduced to 0.5 per cent., the peak was reached on the 5th day. The HA titre of the suspension was not reduced by freezing at -70°C for up to 8 wk. Sheep red cells and red cells of the African green monkey were less sensitive than guinea-pig red cells to the haemagglutinin. Phosphate-buffered saline (Dulbecco and Vogt, 1954) or veronal-buffered saline (Mayer *et al.*, 1946) at pH 7.4, with or without added gelatin or glycerol, was less suitable as diluent than simple saline (0.85 per cent. NaCl). HA activity was equally good at room temperature and 37°C , but poor at 4°C .

TABLE I

Comparison of complement-fixation (CF) and haemagglutination-inhibition (HAI) titres of 24 sera

Serum no.	CF titre *	HAI titre *
1	< 8	< 2
2	< 8	< 2
3	< 8	< 2
4	< 8	< 2
5	< 8	< 2
6	< 8	< 2
7	< 8	< 2
8	8	< 2
9	8	< 2
10	8	2
11	8	2
12	8	8
13	8	16
14	8	16
15	16	4
16	16	4
17	16	8
18	16	16
19	16	< 2
20	32	2
21	128	16
22	64	4
23	> 128	32
24	< 8	4

* Expressed as reciprocals.

Non-specific inhibitors were detected in both rabbit and human sera. They were inactivated by heat at 56°C within 30 min. Receptor-destroying enzyme did not remove these non-specific inhibitors. The HAI titres reported in tables I and II were determined with heat-inactivated sera. All sera were titrated for *M. pneumoniae* complement-fixing (CF) antibody according to standard techniques (Chanock, James *et al.*, 1962).

The CF antibody content and HAI titres of 24 sera are compared in table I. Serum no. 23 was rabbit antiserum. The sera numbered 20, 21 and 22 were the acute-phase, convalescent- and late convalescent-phase (8 wk after illness) sera from one patient ("A" in table II). The HAI titres of paired sera from 4 patients with cold-agglutinin-positive primary atypical pneumonia are shown

in table II. Throat swabs from all these patients had yielded *M. pneumoniae* in culture. Patients C and D had their first serum samples taken 2 wk after the onset of illness.

TABLE II
HAI titres in paired sera

Patient	Reciprocal of HAI titre of	
	first serum sample	second serum sample
A	2	16
B	< 2	16
C	8	16
D	16	32

DISCUSSION

It is not surprising that an organism that produces haemadsorbing colonies when grown on agar medium should produce haemagglutinin when grown in fluid medium. The efficiency of the haemagglutinin seems to be poor since a suspension that contained approximately 10^9 colony-forming units per ml exhibited a titre of only 1 in 64. Moreover, the rise and fall of the HA titre are very rapid, thereby making the day of harvest rather crucial.

Shaker-cultures have previously been shown to produce larger yields of viable organisms than stationary cultures (Low and Eaton, 1965). The day of the peak varied according to the number of organisms inoculated and their level of adaptation to artificial medium. The higher yield in shaker cultures may mean an absolute increase in the number of organisms, or may merely reflect better dispersion of organisms that may aggregate in stationary cultures to form a smaller number of larger colonies.

The diphasic medium offered no advantage over the broth phase alone in stationary cultures.

Haemagglutination-inhibiting antibodies were demonstrated in the sera of immunised rabbits and of patients infected with *M. pneumoniae*. The HAI titres were in general lower than the complement-fixing (CF) antibody titres. Further studies are required to assess the specificity and sensitivity of the HAI test in comparison with other serological tests.

SUMMARY

Direct haemagglutination of guinea-pig red cells by *Mycoplasma pneumoniae* has been demonstrated. Optimal haemagglutinin yields were obtained when the organisms were grown in shaker cultures, harvested on the day when the viable count was highest and concentrated ten-fold. Haemagglutination-inhibiting antibodies were demonstrated and quantitated in sera of immunised rabbits and of patients infected with *M. pneumoniae*.

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