tryptophan metabolism into the kynurenine pathway. Although the basis for such a shunt in rheumatoid subjects is not known, it is unlikely that adrenocortical steroids are responsible.

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An Indirect Hemagglutination Test for Diagnosis of *Mycoplasma pneumoniae* Infections. (29416)

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Serologic diagnosis of *Mycoplasma pneumoniae* (Eaton Agent) infection has not been a routine service of many diagnostic laboratories probably because no practical, relatively simple procedure has been available. Introduction of the indirect immunofluorescence test by Liu(1), whereby infected chick embryo lung sections are treated with serial dilutions of acute and convalescent sera and stained with fluorescein labeled gamma globulin, made possible a number of epidemiologic studies(2,3,4,5). However, this procedure is not likely to be adopted for routine diagnostic use as it is slow and requires elaborate equipment.

Chanock *et al* demonstrated that Eaton Agent could be grown on artificial media and suggested that colony impression smears might be substituted for chick embryo sections(6) but they later concluded that this method would not prove to be as satisfactory (7). More recently Chanock *et al* reported the use of a complement fixation test and found it to be at least 80% as sensitive as chick embryo immunofluorescence procedures (7). Other investigators have reported similar findings(8).

This paper describes a serologic test for *M. pneumoniae* which is based on Boyden's (9) findings that tannic acid-treated erythrocytes adsorb antigens and agglutinate when mixed with specific antiserum.

Materials and methods. Organism. The FH strain of *M. pneumoniae,* obtained on agar culture from Dr. R. M. Chanock, was used throughout the study.

Media. The culture media used were essentially those recommended by Chanock *et al*(6).

Antigen production and preparation. The entire contents of a 3-4 day old 60 mm agar plate were inoculated into 100 ml of broth per plate. Flasks were incubated for 4-5 days at 35°C and a second passage was made into fresh broth with a 10% inoculum. PPLO plate counts on the inoculum ranged from 10^3 to 10^4 colonies/ml. The second broth passage was incubated for 15 days and reduced to approximately 1/5 its original volume by dialysis against polyethylene glycol. The resulting concentrate was centrifuged for 1 hour at 30,000 rpm in a Spinco ultracentrifuge and the pellet was resuspended in a volume of phosphate buffered saline (pH 7.2) equal to ½ that of the original broth. Centrifugation was repeated under the same

conditions and the sediment was resuspended in buffered saline to give a final volume equal to 1/20 that of the original broth. Antigens for use in both complement fixation and hemagglutination tests were treated in a 9 KC Raytheon sonic oscillator for 10 minutes.

Sera consisted of: 1) acute and convalescent specimens from children and adults showing a diagnostic rise to *M. pneumoniae* by the indirect fluorescent antibody and/or complement fixation tests in this laboratory, 2) paired serum specimens furnished by Dr. R. M. Chanock from volunteers experimentally infected with *M. pneumoniae,* 3) paired sera from a variety of viral and bacterial illnesses in both children and adults, and 4) specific antiserum prepared in a horse by multiple intravenous injections of the washed *M. pneumoniae* antigen.

Hemagglutination (HA) test. The HA test was based, with minor modifications, on the procedures recommended by Stavitsky(10). Phosphate buffered saline (PBS) solutions at pH 7.2 and pH 6.4 were prepared by mixing 0.3 M NaCl with equal volumes of the proper 0.15 M Na_2HPO_3-KH_2PO_4 buffers prepared according to Stavitsky(10). PBS of pH 7.2 was used except where noted. The final suspensions of sensitized cells and all test serum dilutions were made in heat inactivated normal rabbit serum which had been adsorbed with 50% sheep erythrocytes and diluted 1:150 in PBS.

Sheep erythrocytes in Alsever's solution were washed 3 times with PBS, adjusted to a final concentration of 2.5%, and mixed with an equal volume of a freshly prepared solution of 1:20,000 tannic acid in PBS. After 10 minutes' incubation at 37°C the cells were centrifuged, washed once in PBS, and resuspended to 2.5% in PBS at pH 6.4.

One volume of antigen was mixed with one volume of the 2.5% suspension of tannic acid-treated cells in 4 volumes of PBS, pH 6.4, and the mixture was incubated for 30 minutes at room temperature. The cells were washed twice in rabbit serum diluent and adjusted to a 0.5% suspension in the same diluent.

All sera to be tested were inactivated at 56°C for 30 minutes, diluted 1:10 in rabbit serum diluent, and adsorbed at 4°C for 30 minutes using 0.1 ml of 50% erythrocytes per ml of diluted serum.

Treated sera were titrated in 2-fold dilutions using 0.4 ml rabbit serum diluent in 13 × 100 mm tubes and an equal volume of a 0.5% suspension of sensitized erythrocytes was added. The test was incubated at 35°C and read for hemagglutination when controls showed well formed buttons, usually after 2 to 3 hours. The reciprocal of the highest initial dilution yielding a clearly positive agglutination of the sensitized erythrocytes was considered the end-point. Controls for all *M. pneumoniae* serology consisted of 1) the first 3 dilutions of each serum plus tannic acid treated erythrocytes, 2) sensitized erythrocytes plus diluent, and 3) a positive control consisting of a complete titration of specific *M. pneumoniae* horse antiserum.

The proper concentration of antigen for sensitization of erythrocytes was determined by block titrations against immune *M. pneumoniae* horse serum. The highest concentration of antigen yielding the highest specific immune serum titer was considered the optimum, usually an antigen dilution of 1:32 to 1:64.

Hemagglutination-inhibition (HI) test. To determine the specificity of the HA reaction, antisera were treated and diluted in duplicate as indicated for the HA test. To one set of tubes was added 0.1 ml of antigen and to a second set was added 0.1 ml rabbit serum diluent for control. Four-tenths ml sensitized sheep erythrocytes were added and tubes were read for inhibition of hemagglutination.

Complement fixation (CF) test. The method used here for performance of the CF test has been described(11). Antigens employed were the same as those used for hemagglutination tests but were routinely heated at 56°C for 30 minutes to diminish anticomplementary activity.

Fluorescent antibody (FA) test. The indirect immunofluorescence procedure was essentially that described by Chanock *et al*(6) for staining agar colonies.

Results. Sensitivity of HA reaction. Paired sera from natural infections and from ex-

TABLE I. HA, CF, and FA Titers of Acute and Convalescent Sera from *M. pneumoniae* Infections.*

Patient‡	Titers† HA	CF	FA	Patient‖	Titers HA	CF
912	§0 – 640	§0 – 256	§0–20	§B	640 –1280	0 – 64
965	0 – 320	0 – 64	0–10	C	0 – 320	0 –128
990	0 – 160	0 – 64	0– 0	Ta	20 – 640	0 – 64
993	15 – 640	8 – 128	0–10	Bl	0 – 320	0 –128
1005	0 – 160	0 – 64	0– 0	Bu	0 – 10	0 – 8
1008	0 –1280	0 – 128	0–10	Ha	0 –2560	0 –256
D–1261	0 – 160	0 – 32	0–20	MC	0 –1280	0 –256
D–1296	20 – 640	0 – 64	0–10	Wi	20 – 320	0 – 32
BL	0 –1280	0 –>256	0–40	Fi	0 – 320	0 – 32
D–0117	160 –1280	128 –>256	40–80	Fe	0 – 160	0 – 8
D–0118	40 – 640	0 –>256	0–40	Va	0 – 320	0 – 32
				Jo	2560 –2560	0 – 64

* Convalescent sera collected 3-6 wk after infection. † Titer is expressed as the reciprocal of the dilution. ‡ Naturally occurring illnesses. § 0 = <1:10 for FA and HA, <1:8 for CF. ‖ From experimental *M. pneumoniae* infections (Dr. Chanock).

perimentally infected volunteers were used in the CF, HA, and FA tests. As shown in Table I, FA tests using colony impression smears appeared to be the least sensitive method for detection of antibody whereas the CF and HA tests showed higher antibody titers. The latter tests were generally in agreement, with 2 exceptions. Sera from patients "B" and "Jo" from the volunteer group showed antibody rises by CF from <8 to 64. These same patients were found to have high stationary titers by HA tests. The HA titers appeared to be specific. Hemagglutination was completely inhibited by *M. pneumoniae* antigen in HI tests and the HA titers were not reduced by 3 adsorptions with sheep erythrocytes.

The detection of these pre-infection titers by HA but not by CF, and the finding that only 1 of 5 control patients with specific high stationary HA titers showed an appreciable CF titer, suggested that HA antibody may persist for longer periods. In an attempt to demonstrate a differential loss in titer with time between CF and HA antibodies, sera were collected at various intervals from 5 children up to 10 months after natural infection and tested by both methods. The results are shown in Table II. The length of the study period apparently was not sufficient. There was no significant difference in rate of decrease of antibody detected by either test.

Specificity of the HA reaction. The specificity of the HA reaction was demonstrated by HI tests with homologous horse serum and high-titered human sera. Hemagglutination could be inhibited by varying dilutions of *M. pneumoniae* antigen but not by antigens from 2 different wild human PPLO strains prepared in the same manner as *M. pneumoniae*.

To exclude the possibility that a diagnostic HA reaction could also be associated with other respiratory diseases, HA tests were performed on paired sera collected from 67 children and adults with 1) illnesses diagnosed by isolation of the etiologic agent and/or serologic tests as being associated with parainfluenza virus types 1 and 3, influenza types A and B, mumps, adenoviruses, respiratory syncytial virus, herpesvirus, Coxsackie B-4, rubeloa, infectious mononucleosis, and B-hemolytic streptococci and 2) illnesses diag-

TABLE II. CF and HA *M. pneumoniae* Antibody Titers at Various Intervals Following Natural Infection.

Illness	Titers at indicated times: Acute CF	Acute HA	3-6 wk CF	3-6 wk HA	5-7 mo CF	5-7 mo HA	9-10 mo CF	9-10 mo HA
912	0*	0	256	640	64	80	—†	—
965	0	0	64	320	32	80	32	80
990	0	0	64	160	16	10	8	20
993	8	15	—	640	128	80	32	80
1005	0	0	64	160	32	40	16	40
1008	0	0	128	1280	32	40	16	40

* 0 = <1:8 for CF, <1:10 for HA.
† Not available.

TABLE III. Stationary *M. pneumoniae* HA Antibody Titers of Acute and Convalescent Sera from Respiratory Illnesses Associated with Agents Other than *M. pneumoniae*.

Titer range	No. of patients	%
<10	44	65
10– 20	11	17
40– 80	7	10
160–320	3	3
640	3	3

nosed clinically as respiratory diseases and not found to be associated with *M. pneumoniae* by CF. Of the paired sera examined, none showed a diagnostic rise to *M. pneumoniae* by HA tests but stationary titers to this agent were not uncommon. Table III lists the number and per cent of patients yielding antibody titers at levels indicated. At least ⅓ of the patients had some antibody titer. This finding is in agreement with earlier studies undertaken with immunofluorescence tests(2,4).

Discussion. The *M. pneumoniae* HA test appears to be as sensitive as the CF test for diagnosis of recent infections, perhaps more sensitive than CF for detection of pre-existing antibody, and far more sensitive than the indirect FA method using colony impression smears. The test would seem to be potentially useful. However, more complete evaluation awaits additional usage in the diagnostic laboratory and further characterization of the sensitizing antigen.

The technical advantages of the test are significant. Unlike the CF tests in general use, the HA test can be completed in one day and, unlike the time-consuming fluorescent antibody test, it does not require expensive equipment or preparation of tissue sections. For laboratories where requests for atypical pneumonia serology are few, the test has another advantage. Sensitized and tanned erythrocytes may be frozen in sealed ampoules according to the technique of Hubert *et al*(12), stored at —70°C, and thawed when required, thus avoiding the necessity of sensitizing erythrocytes for testing only a few sera at a time. This technique has been found to work well in limited usage in this laboratory.

Summary. An indirect HA test for detection of *M. pneumoniae* antibodies is described. The test appears to be immunologically specific, at least as sensitive as the CF test, and far more sensitive than immunofluorescence with colony impression smears.

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