Antibodies To *Mycoplasma pneumoniae*: Correlation of Complement Fixation and Tetrazolium Reduction Inhibition Tests[1] (36661)

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Complement fixation (CF) (1), immunodiffusion (ID) (2), immunofluorescence (IF) (3), and growth inhibition tests (4–6) are used to detect antibody response to infection or vaccination with *Mycoplasma pneumoniae*. The significance of these tests in measuring resistance to infection is not clear because they may be detecting more than one antigen–antibody system.

We were interested in the relationship between the tetrazolium reduction inhibition (TRI) and CF tests and in the nature of the antigens involved in these reactions. Utilizing antigens prepared in a variety of ways, we have shown a correlation between a neutralizing antibody as measured by TRI and antibody detected in CF with lipid antigens.

Materials and Methods. Organisms. M. pneumoniae strain FH, originally isolated by C. Liu was used between the 306th and 317th passage in artificial medium. Strain 65-2053 was reisolated from a clinical specimen supplied by Jose Canchola, Children's Hospital, DC, and used between the 5th and 7th passages. Strain PI-1428, isolated at Parris Island, SC, was used between the 3rd and 14th passage. All strains were grown on medium of Hayflick's formulation (7) or on media containing either 3 or 5% bovine serum fraction (8). To avoid cross reactivity due to common medium components, antigens for serologic tests were prepared with media of a different formulation than used in preparing antigens for immunization.

Concentration of mycoplasmas. Organisms were grown following the procedure of Somerson *et al.* (9) in either 200 or 500 ml of media contained in 2-liter Povitsky bottles. Cultures were incubated for 2 to 4 days at 37°. In all experiments, the supernatant fluids were discarded and the sheet of organisms adherent to the glass was washed three to four times with 0.15 *M* phosphate buffered saline (PBS), pH 7.3. A rubber policeman was used to scrape the organisms into PBS. The concentrations of organisms in the final suspensions were varied by adjusting the volume with PBS.

Enzyme and detergent treatments. Porcine pancreatic lipase (Worthington Biochemical, Freehold, NJ), trypsin and *Naja naja* venom (both from Sigma Chemical Co., St. Louis) were added to PBS suspensions of *M. pneumoniae* to give a final concentration of 1 mg enzyme/ml.

A neutral detergent, BRIJ58 (Atlas Chemical Ind., Wilmington, DE), was added to the *M. pneumoniae* PBS suspensions to a final concentration of 480 μg/ml.

The lipase reaction mixture was incubated at 56° for 25 min. All other reaction mixtures were incubated at 37° for 15–25 min.

The immunogenic potential of mycoplasma suspensions treated as above was determined in the following manner: *M. pneumoniae* strain 65-2053 was grown as previously described. A suspension of the organisms was divided into three aliquots; the aliquots were subjected to heat, detergent, or enzyme,

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inactivated with 1:4000 formalin, and used as described below.

Immunology and serology. Young adult male hamsters were pretested by examining their serum for the presence of nonspecific inhibitors and antibody detectable by the CF or TRI tests. Immunogenic analyses were performed by injecting seronegative hamsters with *M. pneumoniae* antigens and testing their sera for the development of antibody. Animals were immunized by a single intramuscular injection of 1.0 ml of a formalin (1:4000) inactivated suspension of *M. pneumoniae* containing 20 μg of nitrogen/ml. Sera were obtained from bleedings at 4 to 6 weeks. Complement fixation tests were done by the microtiter method, as described by Sever (10). Antigens for CF were prepared from, (i) untreated whole cells, (ii) mycoplasmas treated with 0.5% phenol, and (iii) the residue from a chloroform–methanol extract.

TRI tests were performed by the microtiter method of Senterfit and Jensen (4). The seed antigen was a suspension of *M. pneumoniae* strain PI-1328.

The double-diffusion gel precipitation technique of Conant, Somerson and Senterfit (2) was employed for assessment of the antigenicity of cellular fractions. Washed and dried immunodiffusion slides were stained with a modified Crowle's triple stain, or, for evidence of lipid, with 0.5% Sudan black B (D. F. 26150, National Aniline) in propylene glycol (w/v) for 1 hr. Excess dye was removed by washing for 15 min with 15% water in propylene glycol (v/v).

Results. Immunodiffusion reactions. With *M. pneumoniae* preparations of whole organisms serving as gel antigens (2), one or more precipitation bands were found in reactions with human sera from patients infected with *M. pneumoniae*. One precipitation band stainable with the lipid stain Sudan black was common to all positive sera.

A serum specimen which gave only a single lipophilic ID line was selected as a source of antibody. Preparations treated with lipase and trypsin did not form a precipitation band with this serum. The loss of reactivity from treatment with lipase was additional evidence of the lipid nature of the antigen involved. *M. pneumoniae* suspensions treated with acid phosphatase or snake venom were reactive in this ID system. Heating *M. pneumoniae* preparations at 56° for up to 5 hr did not destroy their reactivity. Reactivity was lost, however, on exposure to 63° or higher for 30 min.

Immunogenicity of M. pneumoniae *preparations.* Hamsters were immunized with mycoplasma suspensions which had been subjected to either lipase, neutral detergent, or heat at 56° for 30 min. Sera were tested in CF with three different antigen preparations (Table I). Similar results were obtained with immunogens prepared from 2 and 4 day harvests and were duplicated in two experiments.

Stimulation of growth inhibiting antibody as measured by TRI was eliminated by lipase treatment, and drastically reduced by neutral detergent. Heating the immunogen did not eliminate the capacity to stimulate TRI antibody production. Similar results were obtained in the CF test in which a chloroform–methanol (lipid) antigen was used. Lipase and detergent destroyed activity, while heating had a lesser effect.

In contrast when phenol-treated antigen was used in the CF test, antibody was detected in the sera of animals immunized with lipase, detergent, or heat-treated mycoplasma. Whole cell antigen in CF gave values intermediate between those found with phenol-treated and chloroform–methanol antigens.

A regression line, with X the dependent variable as the TRI titer and Y the CF titer with lipid antigen, gave a correlation coefficient of 0.95–0.96, thus demonstrating that with animal sera using chloroform–methanol antigen in the CF test the results are essentially similar to those obtained by TRI. Complement fixation using phenol-treated antigen did not correlate with antibody measured in growth inhibition tests.

Relationship between TRI and CF titers and ID reactions in human sera. Fifty sera with levels of TRI antibody ranging from 0 to 256 were tested in the CF test using the chloroform–methanol antigen. Figure 1 shows

TABLE I. Effect of Various Treatments on the Immunogenicity of *M. pneumoniae* Suspensions.[a]

ment of inogen	Expt[b]	2 Day growth				4 day growth		
		Metabolic inhibition	Complement fixation			Metabolic inhibition	Complement fixati	
		(TRI) seed PI 1428	Chloroform–methanol antigen	Phenol-treated antigen	Whole cell antigen	(TRI) seed PI 1428	Chloroform–methanol antigen	Phenol-treated antigen
	1	61 ± 10	52 ± 9	39 ± 9	24 ± 3	102 ± 21	109 ± 21	54 ± 10
	2	83 ± 10	64 ± 8	42 ± 5	30 ± 5	82 ± 12	78 ± 13	46 ± 6
e lipase	1	2 ± 0.5	4 ± 0.2	24 ± 4	6 ± 3	3 ± 0.5	<1	38 ± 6
	2	2 ± 0.5	2 ± 0.5	41 ± 5	20 ± 3	<1	0	31 ± 8
l detergent	1	8 ± 2	10 ± 2	36 ± 7	6 ± 3	9 ± 4	6 ± 2	25 ± 7
	2	13 ± 4	14 ± 4	36 ± 5	19 ± 3	4 ± 2	2 ± 1	21 ± 10
min	1	33 ± 6	64 ± 9	62 ± 13	40 ± 5	38 ± 6	44 ± 6	68 ± 15
	2	35 ± 8	36 ± 8	46 ± 6	24 ± 3	30 ± 6	43 ± 8	44 ± 11

h entry represents the mean titer of sera from 8 to 12 animals ± standard error of mean.

ılts of two experiments: In Expt. 1, suspensions were prepared from organisms grown in 500 ml of unbuffered media contained in ottles. For Expt. 2, organisms were grown in 200 ml of buffered media.

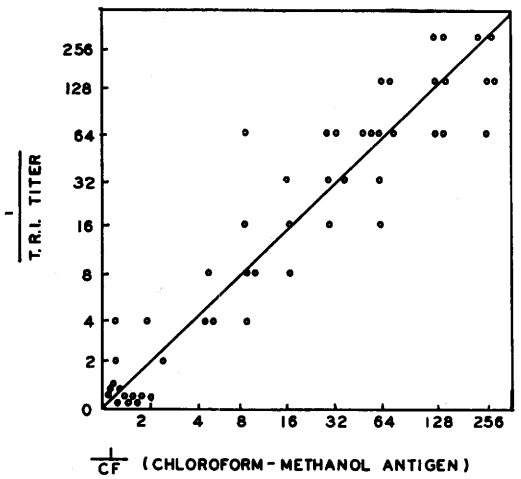

FIG. 1. Comparison of TRI and lipid CF titers in 50 human sera.

that the specificity and sensitivity of these two tests are similar, with a correlation coefficient of 0.78. ID reactions characterized by a lipophilic line of precipitation were positive in every serum with a titer of at least 8 by either TRI or CF.

Discussion. The reactivity of *M. pneumoniae* suspensions in immunodiffusion is affected by treatment with lipase and neutral detergent but less so with heat. These treatments also reduce the immunogenicity as detected in TRI and with CF using the residue from a chloroform–methanol extract as antigen. These studies emphasize the importance of lipoidal components as antigenic determinants which stimulate the production of, and react with a neutralizing antibody.

The degree of correlation between titers of the TRI and lipid CF tests with 50 human sera was high (correlation coefficient of 78%). This indicates that the lipid CF measures either the same or similar antibody as the TRI test. The presence of positive ID tests in sera with a TRI or CF titer of 8 or greater suggests that this procedure might be used as a rapid screening method for the presence of antibody to *M. pneumoniae*. Since correlation of TRI titers with resistance to febrile illness caused by *M. pneumoniae* has been demonstrated (11), the correlation we have shown suggests that the lipid CF titer also will serve as an indicator of immunity to primary atypical pneumonia caused by *M. pneumoniae*.

Summary. The role of lipoidal components of *M. pneumoniae* as antigenic determinants *in vitro* and *in vivo* is described.

Sera with TRI or CF titers with lipid antigen of 8 or greater are usually positive in the ID test, suggesting that this test might be used as a screening test for antibody to *M. pneumoniae*.

Since there is a high degree of correlation between TRI and CF titers when lipid extracts of *M. pneumoniae* are used as antigens, we feel that the lipid CF test will serve as an indicator of neutralizing antibody to *M. pneumoniae*.

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