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Antimetabolic Antibodies to Mycoplasma Pneumoniae Measured by Tetrazolium Reduction Inhibition.* (31252)

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Antigenic experience with *Mycoplasma pneumoniae* results in the development of several kinds of antibody whose role in immunity is still being investigated. Recent reviews(1,2) describe the serological technics which have been applied to the study of this organism and to primary atypical pneumonia. Of particular interest in the development of vaccines are procedures which measure antibody capable of inhibiting replication of the organism. Because technics utilizing suppression of colonial growth are laborious and relatively insensitive(3,4,5) we have investigated more rapid and simpler procedures based upon the observation that broth cultures of *M. pneumoniae* reduced 2, 3, 5-triphenyl tetrazolium chloride to its red formozan(6). The presence of specific antiserum inhibits metabolism and thus stops tetrazolium reduction. The measurement of this phenomenon with standardized quantities of reagents provides a sensitive and reproducible means of quantitating metabolism-inhibiting antibody. Its application to the study of serological patterns in natural and vaccine-induced immunity are described below.

Materials and methods. Medium. The medium was that described by Chanock *et al* (7) consisting of 7 parts PPLO broth (Difco) supplemented with 1% dextrose, 1 part yeast extract (25%), 2 parts horse serum with 1,000 U/ml penicillin and 500 ppm thallium acetate. Stock aqueous solutions of 2, 3, 5-triphenyl tetrazolium chloride (Fisher or National Aniline), protected from light, were added at 0.05% final concentration to the broth immediately prior to use.

Mycoplasma. The 2 strains (PI-1428 and FH) of *M. pneumoniae* were obtained from R. M. Chanock, National Institute of Allergy and Infectious Diseases, N.I.H., and had been serially transferred on the standard agar or broth medium a total of 13 and 189 times, respectively.

Antisera. Pre- and post-vaccination sera were from rabbits and cynomolgus monkeys injected with formaldehyde-killed preparations of the FH strain grown in a serum-free medium(8). Hamster sera were obtained both post-vaccination and post-infection. Human sera were collected during an outbreak of mycoplasmal infections in children and adults. All sera were heated at 56°C for 30 minutes before testing.

Serologic methods. Complement fixation tests were performed as described by Sever with phenol treated or lipid antigens extracted by the chloroform-alcohol procedure of Kenny and Grayston(10). The IHA procedure was that of Dowdle and Robinson (11) as modified by Taylor-Robinson *et al* (12) for the preparation of sensitized eryth-

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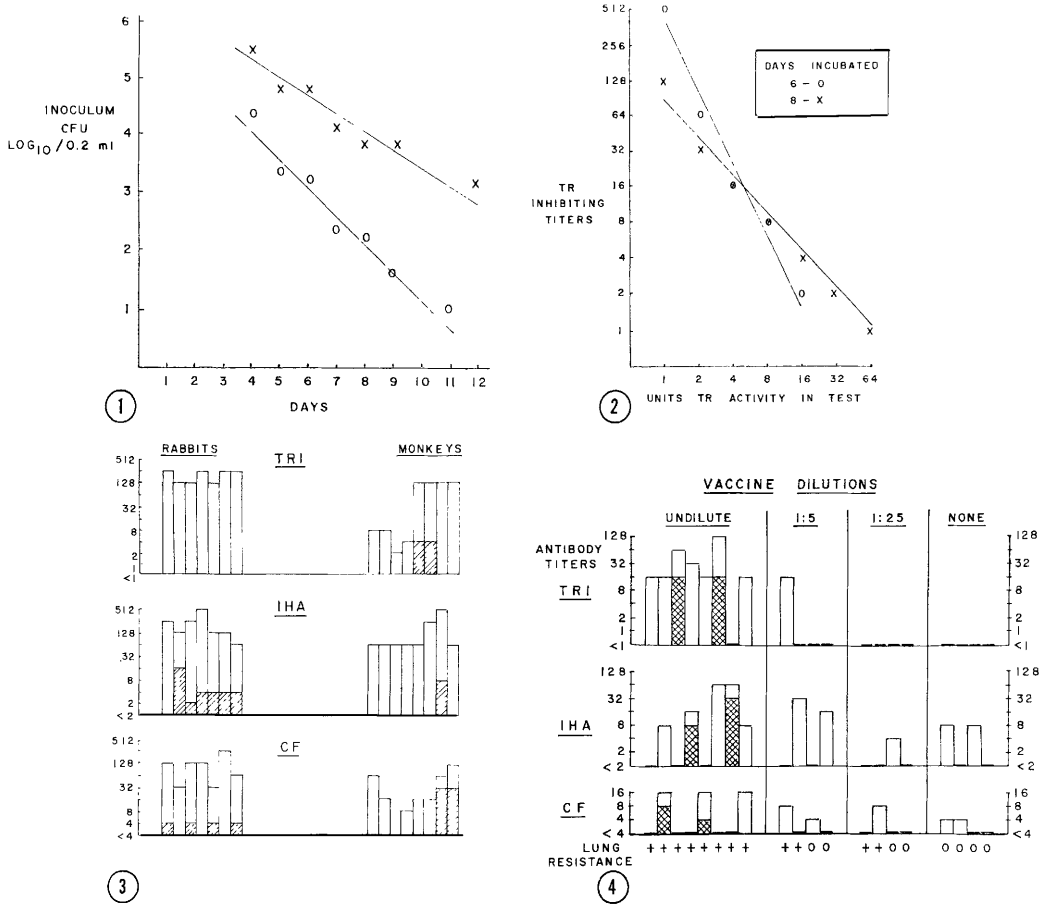

FIG. 1. Incubation time required for reduction of tetrazolium in broth cultures. Inocula were 2-fold serially diluted suspensions of *M. pneumoniae,* strains PI-1428 (x) or FH (o).

FIG. 2. Relation of antibody endpoint to concentration of metabolizing mycoplasma. Dilutions of rabbit antiserum mixed with dilutions of broth culture of strain PI-1428 and incubated for 6 (o) or 8 (x) days.

FIG. 3. Comparison of serologic responses of rabbits and monkeys to inactivated *M. pneumoniae* vaccine. Pre-vaccination—shaded, post-vaccination—not shaded.

FIG. 4. Antibody responses and resistance to *M. pneumoniae* in hamsters following injection of an inactivated vaccine. 2 weeks post-vaccination—not shaded, 9 weeks post-challenge—shaded.

rocytes and their agglutination by antisera. All tests were conducted in plastic microtitration depression plates using Takatsy wire loops.

Results. Reduction of tetrazolium. The relationship of the number of mycoplasma inoculated to the rate of tetrazolium reduction was investigated by making 2-fold serial dilutions of broth cultures (containing 10^6 to 10^7 viable organisms/ml) in 0.025 ml of the tetrazolium medium. After dilution, 0.175 ml of the medium was added to each cup to bring the final volume to 0.2 ml. The plates were sealed with plastic tape and incubated at 37°C in the dark to avoid photoreduction of the tetrazolium. Endpoints were recorded as the highest dilution of a culture giving a color change to orange or red.

The time required to reduce the indicator was directly related to the numbers of viable organisms in the inoculum (Fig. 1). With the FH strain, red formozan was first observed on the 4th day of incubation in cups receiving $10^{4.3}$ colony-forming units (CFU). With continued incubation, an initial inoculum of only 10 CFU resulted in a color change after 11

days. Using the PI-1428 strain required an inoculum of more than 10^5 CFU to change the indicator in 4 days and 10^3 CFU in 12 days. Clearly, different rates of multiplication in the broth medium could explain these differences. However, either strain could be used to titrate antibody provided that one TR unit (defined as the greatest dilution of culture causing color change after a standard incubation period) was employed as the inoculum in the test.

Specific antiserum inhibition of tetrazolium reduction. The addition of antiserum to broth cultures of *M. pneumoniae* inhibited metabolism of the organism and tetrazolium was not reduced. Fig. 2 illustrates the indirect relationship between the number of organisms in the test and the inhibition endpoint. In the experiment cited, a rabbit antiserum and varying concentrations of strain PI-1428 were incubated at 37° for 6 or 8 days. Simultaneous titrations of the mycoplasma suspension provided an estimate of the number of TR units employed in each portion of the test. These results led to the adoption of a 6-day incubation period. The standardized procedure subsequently employed was as follows:

Serial 2-fold dilutions of serum were made in 0.025 ml of standard medium. 0.175 ml of a dilution of broth culture, calculated to contain one TR unit, was then added. Readings were made of serum titer endpoints when the parallel titrations of inoculum indicated one TR unit was present in the test, usually at 6 days. A known positive and negative serum was included in each test.

Enhancing effect of normal sera. Fresh, unheated sera from normal guinea pigs, rabbits, hamsters, and monkeys were found to inhibit mycoplasmal reduction of tetrazolium, even when highly diluted. However, inhibition was greatly reduced or eliminated by prior heating of the serum to 56°C for 30 minutes. When such heated guinea pig serum was added to the medium in concentrations of 2 to 10%, the TRI antibody titers of various antisera were enhanced 2- to 16-fold as compared with those obtained when no guinea pig serum was present. This unexpected potentiating effect was observed with antisera from rabbits, hamsters, monkeys and some but not all humans. It was most pronounced in tests with PI-1428 strain. Based on these observations, 5% inactivated guinea pig serum was routinely added to the basal culture medium and the PI-1428 strain was used for the TRI antibody determinations cited below.

Antibody responses of vaccinated and infected animals. When sera from rabbits and monkeys immunized with formaldehyde-killed *M. pneumoniae* were titrated by 3 different serological procedures, there was poor correlation between the endpoints. Examples are shown in Fig. 3, where pre-vaccination rabbit sera frequently contained CF and/or IHA antibodies but rarely had antibodies measured by TRI. After vaccination, rabbit serum samples often had higher TRI than CF titers. Responses of monkeys to vaccination were also highly variable as measured by the 3 different techniques. These observations suggested that antibodies resulting from immunization with *M. pneumoniae* vaccines could be directed at more than one mycoplasmal antigen.

Such conclusions were further supported by measurement of antibodies in post-vaccination and post-infection sera of hamsters. Groups of hamsters were injected with vaccine in 5-fold serial dilutions and serum samples collected 2 weeks later. At that time, vaccinated and control animals were exposed to an intranasal inoculum of 10^3 CFU of live *M. pneumoniae*. Nine days later a second serum was obtained, the animals sacrificed and their lungs cultured for mycoplasma and scored for resistance to mycoplasma replication by procedures described elsewhere(13). The serum titers by TRI, IHA and CF shown in Fig. 4 exemplify the lack of correlation in antibody patterns. The undiluted vaccine stimulated antibodies in several hamsters, but in the selected examples, animals with antibody by one test were negative by the other assays. Nearly all of the post-infection sera from this group had TRI antibody while few were positive by CF and IHA. All hamsters with serum TRI antibody had pulmonary resistance to mycoplasmal growth. However, among the vaccinated animals which did not develop detectable serum TRI activity post-

TABLE I. Relation of Antibody to Illness in Patients with Positive Throat Culture for *M. pneumoniae*.

Clinical status	No. in group	Acute serum* positive at 1:2 or greater by:			Convalescent serum† with rises 4-fold or greater by:				
		TRI	CF	IHA	TRI	CF	IHA	TRI and CF or IHA	Any test
Ill	15	0	5	14	5	5	4	2	11
Healthy	7	7	3	7	2	1	2	0	5
Total	22	7	8	21	7	6	6	2	16

* Serum taken at time of throat swab.
† Serum specimen collected 2 to 3 wk after first serum.

infection, 4 of 8 were resistant. TRI antibody responses occurred in 7 of 8 animals given undiluted vaccine and challenged two weeks later by intranasal instillation of live mycoplasma. Apparently the vaccination experience sensitized and the intranasal inoculum stimulated a rapid, anamnestic production of antibody, particularly of the type which interferes with metabolism of mycoplasma. Among the non-vaccinated animals, none exhibited TRI antibody responses during this interval. Thus the specificity of this antibody seems to be confirmed.

Human serologic responses to infection. During an investigation of intrafamilial spread of *M. pneumoniae,* we studied the relationship of TRI to other serologic responses in subjects infected with this organism. Among a group of 22 subjects from whom *M. pneumoniae* had been isolated from the nasopharynx, 15 presented symptoms of respiratory disease while 7 remained well (Table I). It was of particular interest that none of the 5 clinically ill patients had demonstrable TRI antibody in their acute phase sera while all of those who remained symptom-free had this antibody at the time when *M. pneumoniae* was isolated from the throat. The presence of this antibody apparently modified the replication or spread of oral mycoplasma resulting in sub-clinical infections. The incidence of TRI antibody responses was approximately the same in the ill and healthy as 5 of 15 patients with symptoms and 2 of 7 of the subclinical cases had a significant rise in convalescence. Among the patients with clinical illness, 5 had acute phase serum CF antibody and 14 had IHA antibody in various titers. These findings strengthen the concept that the presence of TRI antibody is directly related to protection against illness and that the CF and IHA procedures do not furnish an adequate measurement of resistance, although the latter tests are useful for serologic diagnosis of infections.

Discussion. By using the TRI procedure, one can identify antibodies capable of interfering with the metabolism of *M. pneumoniae* and which may be different from those measured by the CF or IHA technics. Other tests for antimetabolic antibodies to species of mycoplasma which do not reduce tetrazolium are being developed(14,15). These are based on acid or base production resulting from the metabolism of glucose or arginine.

Among mycoplasma of human origin, only *M. pneumoniae* appears to reduce tetrazolium in sufficient activity to permit this dye to be used in serological testing. Whether this capacity is in some way related to pathogenicity or other characteristics of the organism is not known. In this connection, it is of interest that TR activity of the FH strain has been retained for many serial transfers on artificial media whereas Couch *et al*(16) found that its virulence for man had apparently waned during laboratory cultivation. As the hemolysin produced by *M. pneumoniae* is believed to be a peroxide(17), it is tempting to speculate that hemolysin production and tetrazolium reduction are in some manner related. At least their presence suggests a metabolism unique to *M. pneumoniae* among the mycoplasma found in man(2).

The role of guinea pig serum as an enhancer of TRI antibody titers has not been defined. Analogous results with certain mycoplasma strains have been reported in studies

of specific serum inhibition of arginine splitting(14) and glucose fermentation(15). Because heat-inactivated serum potentiates antibody, a complement effect is apparently ruled out. However, it is possible that certain heat-stable components have attached to the organism rendering it more susceptible to the action of metabolic-inhibitory antibodies. In this regard, Eaton *et al*(18) found that while neutralization of infectivity for tissue culture required fresh immune serum, in contrast, heated immune serum would prevent infection of cotton rats and suggested that accessory immunity mechanisms were involved. Clearly, more investigation of this phenomenon is required.

Following the observation that tetrazolium reducing activity could be inhibited by antibody(6), subsequent studies in animals(13) and man(19) have furnished evidence that the TRI antibody is associated with resistance to disease. It is absent in the acute sera from natural cases(1) and of pre-infection sera from experimentally-infected human volunteers who developed clinical illness(19). In this latter study, volunteers with TRI antibody at the time of challenge did not develop clinically recognizable pulmonary disease, although multiplication of mycoplasma in the oropharynx was demonstrated.

Although this technique has approximately the same sensitivity as the CF and IHA, the time required for the procedure makes it less useful for diagnosis of acute infection. A combination of CF, IHA and immunofluorescence will provide serologic diagnosis in a shorter time(1). Because the presence of TRI antibody is associated with resistance, its most important application is in the development and standardization of vaccines and in studies of the role of antibody in preventing or modifying mycoplasmal illness.

Summary. The reduction of tetrazolium by *M. pneumoniae* is inhibited by specific serum. Based on this, a method has been derived for measuring growth inhibiting antibody. This procedure was applied to post-vaccination and convalescent sera from animal and human subjects and compared with CF and IHA antibodies. The presence of TRI antibody correlated well with resistance to disease.

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