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Immunogenicity of *Mycoplasma pneumoniae*

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The immunogenicity of *Mycoplasma pneumoniae* for New Zealand white female rabbits was studied by using an immunization scheme which employed initial intramuscular immunization with vaccine in incomplete Freund adjuvant followed 3 weeks later by a series of five intravenous injections of fluid vaccine. Small doses of immunogen (15 to 150 μg of mycoplasmic protein per rabbit) gave rise to sera which contained antilipid complement-fixing antibody, produced one to three precipitin lines, but gave poor growth-inhibition on agar. Larger doses of immunogen (1.5 to 15 mg per rabbit) gave rise to sera which gave higher antilipid complement-fixing titers, four to eight precipitin lines, and good growth-inhibition. Doses smaller than 15 μg per rabbit failed to give rise to detectable antibody. Growth-inhibiting antibody was produced later than the other antibodies. The number of precipitin lines was controlled more critically by the quality of the antisera, as determined by the amount of immunogen, than by the quantity of serological test antigen. All sera which gave any precipitin lines produced a common precipitin line which stained for lipid.

The results and interpretation of serological analyses of whole organisms (which are complex mixtures of antigens) are largely dependent upon the strength and breadth of the antisera used for the serological reactions studied. For example, the serological relationships observed between *Mycoplasma* species may be markedly different if the antisera employed happen to contain antibodies specific only to a few major components of each species in contrast to the results obtained with antisera which contain antibodies to many antigenic determinants of each agent. This problem becomes most marked when simple titration methods are employed to estimate antigenic relationships. When analytical methods, such as Ouchterlony double immunodiffusion (15), are employed, the narrow response of a given antiserum is usually betrayed by the paucity of precipitin lines.

The present study reports the effect of varying the immunogen dose of *Mycoplasma pneumoniae* for rabbits on antibody response as measured by a variety of serological tests.

MATERIALS AND METHODS

Organisms. *Mycoplasma pneumoniae* strain AP-164 (11) in the 11th to 18th passage on artificial medium from the patient was employed for the production of both immunizing and serological test antigens. A triply colony-cloned strain of AP-164(10) at similar passage levels was used for the second experiment.

Antigens. The details and rationale for the methods used to produce both immunizing and serological test antigens have been described (9, 10). Microorganisms used for preparing immunizing antigens were propagated in soy peptone-fresh yeast dialysate broth (8) supplemented with 10% agamma calf serum, whereas organisms used for preparing serological test antigens were propagated in the same medium supplemented with 10% agamma horse serum. Both agamma calf serum and horse serum were specially selected for clarity. (No deposit was obtained upon centrifugation of uninoculated but incubated medium.) Broth cultures in 800-ml volumes in 32-oz (ca. 960 ml) prescription bottles were incubated at 37 C with agitation from a magnetic spin bar at 60 to 100 rev/min. Incubation was for 4 to 6 days and was terminated when the culture contained numerous spherules and before the *p*H had fallen to 6.5. The final *p*H of the medium after propagation of the immunizing antigens was 6.5 to 6.7. Some lots of medium used to propagate serological test antigens reached a final *p*H of 6.5, whereas later lots were buffered with 10 mM TES [*n*-tris (hydroxymethyl)-methyl-2-aminoethane-sulfonic acid] or HEPES [*n*-2-hydroxyethyl piperazine-*n*-2 ethanesulfonic acid (17)] and reached a final *p*H of 6.8 from an initial *p*H of 7.3. Immunizing antigens were concentrated 400-fold from the original broth culture, washed three times in normal saline, and resuspended in normal saline (for experiment 1, shown on Table 1). For the second experiment, the immunogen was similarly concentrated, washed three times with TES saline (10), and resuspended in TES saline. Serological test antigens were concentrated 400- to 1,000-fold, washed three times with TES saline, and

resuspended in water. Protein was measured by the Lowry method (13). Whereas HEPES buffer has been reported to give substantial color with the Folin phenol reagent, TES buffer does not interfere at 10 mM (7). Since the antigens grown with HEPES buffer were washed three times with TES saline, no interference or false color with the Folin phenol reagent was observed in the present experiments.

Immunization schedule. The immunization schedule was adapted from that of Lemcke (12) and has been reported elsewhere (8). The schedule of injections was as follows: day 1, 2 ml of antigen emulsified with 2 ml of Freund incomplete adjuvant (Difco) was injected intramuscularly at four sites in the flanks of New Zealand white female rabbits (approximate weight, 3 kg); days 21 to 31, four intravenous doses of fluid antigen (without adjuvant) were injected intravenously into the marginal ear vein at 3 or 4 day intervals using increasing dosages (0.1, 0.2, 0.3, and 0.4 ml on each succeeding injection); day 39, animals were injected intravenously with 1 ml of fluid antigen. Thus, the total dosage of antigen for each rabbit was 4 ml. Animals were bled on days 0, 21, 39, and 46. Antisera, serological test antigens, and immunogens were stored at −20 C.

Serology. Details of the serological methods have been described elsewhere (10, 11). Complement-fixing titer values are reported as the reciprocal of the greatest dilution of serum which gave complete fixation of complement in an overnight complement fixation test against 4 units of lipid antigen (11). Precipitin lines are reported as the number of distinct lines observed on double immunodiffusion assays employing a plastic matrix on thin agarose films (10), except that slides were stained with Amido Schwarz after drying. Antigens were disrupted by sonic treatment immediately before use. Sudan black was employed for detecting lipid by the method described by Crowle (4). Growth-inhibition values were determined by the method of Clyde (1), and values reported represent the distance from the edge of the disc to the first appearance of colonies.

TABLE 1. *Effect of immunizing dose on rabbit antibody response to M. pneumoniae*

Immunizing dose/rabbit (in mg of protein)	Rabbit no.	Test system	Sequential rabbit sera			
			Pre-immune	1 (21)[a]	2 (39)	3 (46)
15[b]	330	Antilipid CF	<2	64	512	256
		Precipitin lines[c]	0	4	5	6
		Inhibition (mm)	0[d]	0	5.0	6.0
	331	Antilipid CF	<2	128	256	512
		Precipitin lines	0	2	4	4
		Inhibition (mm)	0	0	5.0	6.0
1.5	332	Antilipid CF	<2	32	512	512
		Precipitin lines	(1)[e]	3	4	3
		Inhibition (mm)	0	0	5.7	5.8
	333	Antilipid CF	<2	16	128	256
		Precipitin lines	0	1	2	2
		Inhibition (mm)	0	0	4.0	4.0
0.15	334	Antilipid CF	<2	2	64	64
		Precipitin lines	0	1	2	3
		Inhibition (mm)	0	0	1.8	2.0
	335	Antilipid CF	4	8	32	64
		Precipitin lines	(1)	1	3	2
		Inhibition (mm)	0	0	1.7	2.5
0.015	336	Antilipid CF	4	4	64	64
		Precipitin lines	0	0	1	1
		Inhibition (mm)	0	0	0	0
	337	Antilipid CF	<2	4	64	64
		Precipitin lines	0	(1)	2	2
		Inhibition (mm)	0	0	0	0

[a] Numbers in parentheses indicate day animal was bled after initial immunization.
[b] As measured by Lowry method.
[c] Number of clearly distinguishable precipitin lines in a microimmunodiffusion test employing a serological test antigen containing 5 mg of mycoplasmic protein/ml.
[d] 0 = Inhibition zone less than 1 mm.
[e] ( ) = Weak reaction.

RESULTS

Growth inhibition and complement fixation. Two experiments were performed to estimate the minimal antigenic dose of *M. pneumoniae* required for the induction of antibody in rabbits. (Table 1 shows the results from experiment one.) Antibody measurable by complement fixation with lipid antigen was induced equally as well by 1.5 mg as by 15 mg of mycoplasmic protein per rabbit. Low levels of antilipid complement-fixing antibody were induced by 15 to 150 μg of mycoplasmic protein per rabbit. In another experiment, doses of 0.6 or 6 μg failed to induce measurable antilipid complement-fixing antibody in any of six rabbits, whereas doses of 0.06, 0.6, and 6.0 mg per rabbit gave results comparable to those shown in Table 1 with 0.15, 1.5, and 15 mg, respectively. Antibody measurable by growth-inhibition methods was induced equally well by antigen doses of 1.5 to 15 mg, whereas a dose of 0.15 mg gave weak antisera. Growth-inhibition antibodies were found only in sera obtained after the intravenous immunization series.

Double immunodiffusion. A comparison of the intensities and numbers of precipitin lines observed in double immunodiffusion produced by antisera representing each of the four immunization levels is shown in Fig. 1A. When 8 mg of serological test antigen per ml was employed in the central well, one common precipitin line was seen with all the antisera, including that from the rabbit immunized with 15 μg of mycoplasmic protein. This line stained for lipid (arrow). Another lipid-staining line was observed close to the serum well of the rabbit immunized with the highest dose (arrow). Five common precipitin bands were produced by the sera immunized by the two highest immunogen doses (wells A and B, Fig. 1A). Two other lines were seen with serum A that were not seen with serum B. Serum C produced the strong lipid-containing line and a faint line between the lipid line and the antiserum well which was better resolved on Fig. 1B than on Fig. 1A. In general, some weak lines could be readily seen by eye on the stained immunodiffusion slide, but some of these were relatively poorly resolved on the photographic print. Only one line was seen with the serum produced with 15 μg of mycoplasmic protein as immunogen in this particular example (well D, Fig. 1A). No clear precipitin lines were seen with animals immunized with 6 μg of mycoplasmic protein in the second experiment although the common line did tend to bend toward the antiserum well as though some small amount of antibody were present. Table 1 shows a summary of the number of clearly distinguishable

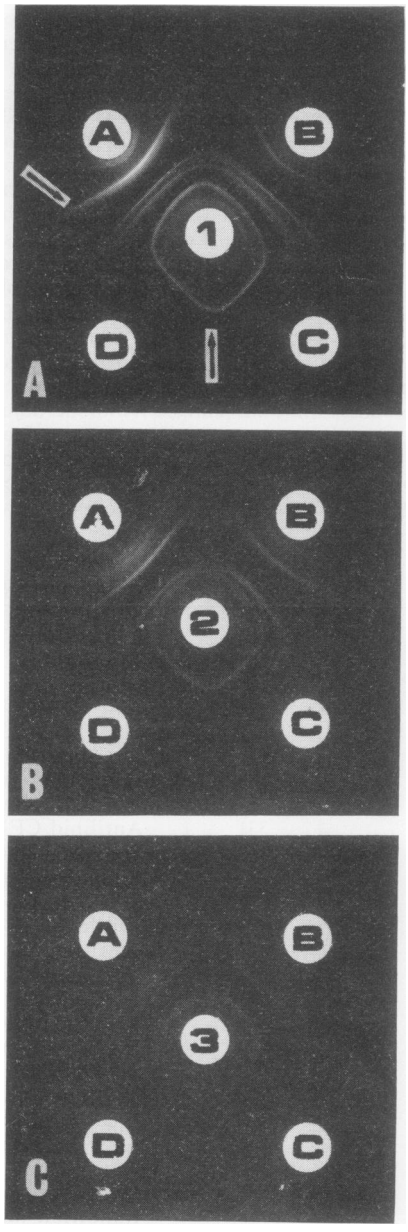

FIG. 1. *Effect of immunogen dose on the ability of rabbit antisera to produce precipitin lines with various concentrations of serological test antigens. Antisera were prepared in rabbits immunized with the following immunogen doses: (well A) 15 mg, (well B) 1.5 mg, (well C) 0.15 mg, and (well D) 0.015 mg of mycoplasmic protein per rabbit. Serological test antigen dosages were: (1) 8 mg, (2) 2 mg, and (3) 0.5 mg of mycoplasmic protein per ml. Arrow indicates sudan black (lipid)-staining lines. Photographic magnification ×4.6.*

precipitin lines observed with the antisera produced in the first experiment. The maximum number of clearly distinguishable precipitin lines observed in most experiments was six although as many as eight precipitin lines were observed on some individual immunodiffusion slides. The number of precipitin lines decreased as the amount of immunogen used to produce a given antiserum was reduced.

The results were fairly consistent throughout both experiments with the maximal number of precipitin lines being produced by animals immunized with 0.6 mg or more of mycoplasmic protein and only 1 to 3 lines produced by animals which had lesser immunogenic doses. In the second experiment, no precipitin lines or very faint precipitin lines were observed with immunogen doses of 6 μg per rabbit.

When serological test antigens containing 2 or 0.5 mg of mycoplasmic protein per ml were employed, the intensities of precipitin lines in general decreased progressively (Fig. 1B and 1C, respectively). However, some individual lines could be visualized better at lower serological test antigen concentrations than at higher levels (Fig. 1A and 1B, antiserum C as mentioned above). The common lipid-staining line was visible at all concentrations of antigen tested. Accordingly, changes in the amount of serological test antigen appeared to have less effect on the number of precipitin lines observed than did the capacity of the rabbit antisera to recognize antigenic components.

Controls. All rabbit antisera were tested for complement-fixing antibody titers against horse serum antigen. Agamma horse serum at optimum dilutions of 1:100 and 1:1,000 (as determined by block titration against agamma calf serum antibody) was employed as test antigen. All antisera had antibody titers of 1:4 or less, whereas antibody to agamma calf serum titered 1:64. No double immunodiffusion lines were observed when these antisera were tested against 1:1,000 agamma horse serum and only one to two faint lines were produced against 1:100 horse serum with some antisera. These were not common to the mycoplasmic precipitin lines. It should be noted that the horse serum contamination level of the serological test antigens prepared in the manner described corresponds to 1:1,000 horse serum or less (10).

DISCUSSION

Few reports of the actual immunogen doses in quantitative terms have appeared in the literature of mycoplasmic immunology. A reason for this is that most mycoplasmic medium formulations frequently give rise to substantial spontaneous precipitates which rendered pointless the reporting of the protein content of the resultant mixture of mycoplasmata and medium components. The medium employed in this study remained clear during incubation, and the resulting mycoplasmic antigens contained little contaminating material (10).

This study was undertaken because preliminary experiments with intravenous injections of concentrated antigens yielded antisera which gave good complement fixation titers, but these antisera had little potential as reagents for double immunodiffusion or growth-inhibition studies. The immunization system used in the present study was adapted from that of Lemcke (12). She showed that complement-fixing antibody titers obtained after intramuscular immunization of rabbits with mycoplasmic vaccines in Freund complete adjuvant could be markedly increased by subsequent intravenous administration of a fluid vaccine in a series of injections. The essential difference between the system described in this paper and that of Lemcke is the fact that Freund adjuvant without mycobacteria was used in this study. Quite likely, the antibody response would be enhanced by utilization of mycobacteria; but I felt that the results of a serological comparison of species might be difficult to interpret if mycobacterial antibodies were also present in the sera.

Morton and Roberts (14) studied the antibody response of rabbits to various mycoplasmic antigens. They confirmed Lemcke's observation of enhanced antibody response when intramuscular injections with adjuvant were followed by injections of fluid vaccine intravenously. Immunogen doses per rabbit of 4 to 40 mg of protein produced satisfactory antibody; however, the response to lower doses was not tested. Antibody response was measured by agglutination of sensitized latex particles and growth-inhibition on agar. Antibody measurable by agglutination appeared first, and growth-inhibition antibody appeared later. Similarly, in the present study, antibody measurable by growth-inhibition appeared later than antibodies measurable either by antilipid complement fixation or double immunodiffusion, a phenomenon also noted by Clyde (*personal communication*). Fernald et al. (5) have reported the divergence of antibody response to *M. pneumoniae* in both rabbits and man, as measured by different serological tests and related to both time and immunoglobulin class.

Pollack et al. (17) have shown that both the immunogenicity and serological activity of *M.*

pneumoniae organisms grown on glass are affected by the *p*H that the medium achieves during incubation of the culture. Apparently, final *p*H values of 6 or less resulted in weak antigens. Better serological test antigens (as measured by double immunodiffusion against human antisera) and better immunogens resulted if the *p*H was maintained near neutrality. The *p*H of cultures used to prepare both the serological test antigens and the immunogens did not decline below 6.5 in this study, most likely because the medium used, soy peptone-yeast dialysate, did not contain added glucose. The immunogen appeared to be potent by the serological parameters tested here. Although metabolic-inhibition antibody testing has not yet been carried out on these sera, lipid is the likely determinant for this antibody (18, 19); thus, the presence of lipid antibody may suggest the presence of metabolic-inhibition antibody. Studies are in progress to determine both the antibody classes in these antisera which are responsible for the observed serological reactions and the ability of these antisera to kill *M. pneumoniae* in the complement-mediated killing reaction (6).

Antilipid complement-fixing antibody was induced by minute antigenic stimuli. The fact that the glycolipids of *M. pneumoniae* are simple compounds and may be extensively distributed in nature [e.g., spinach glycolipids which cross-react with *M. pneumoniae* rabbit antisera (16)] suggests that rabbits may well be exposed to similar antigens in their environment. In previous studies (6, 11), both antilipid and complement-mediated killing antibodies were found frequently in unimmunized rabbits. The rabbits employed for the present experiments were selected for lack of antilipid antibody in their preimmunization sera. Possibly the antilipid antibody observed here resulted from exposure to the antigens in nature during immunization and thus did not result from the minute immunization dosage. However, this is unlikely since in one experiment low dosages of antigen (0.6 to 6 μg of protein per rabbit) did not induce significant antibody in the six rabbits tested. Another possibility is that the antibodies are secondary responses to similar or related antigens which the rabbits have contacted previously but to which they did not show detectable antibody.

The greatest intensity and number of precipitin lines were observed at serological test antigen levels of 8 mg of protein per ml. Higher concentrations have not yet been tested. As the antigen dose was progressively reduced, all precipitin lines became weaker; thus it appears that the immunogen dose as reflected in the strength and breadth of the antisera is more important in determining the number of observable precipitin lines than is the concentration of the serological test antigen. The antisera which produced any precipitin lines always produced a precipitin line which stained for lipid. Conant et al. (2) found that some human convalescent sera produced one to three precipitin lines against *M. pneumoniae*, thus the results with human antisera resemble those obtained with sera from rabbits immunized with small doses of antigen. The positive human antisera always gave rise to a common lipid-staining precipitin line. Pollack et al. (18) have further demonstrated that a membrane fraction of *M. pneumoniae* produces a lipid-staining line with human antisera which fuses with a line obtained with the whole organism. These results suggest that the common lipid-containing line observed in the present study contains membrane components. Since membranes are of large molecular weight, the relative ease of stimulation of antibody to the common lipid-staining component is not surprising. The same line of reasoning also helps to explain the ease of stimulation of antilipid complement-fixing antibody since it is likely that the lipid antigen(s) is contained in the membrane. The nature of the remaining antigens as demonstrated by immunodiffusion lines is presently unknown.

Although the immunization scheme described in this paper is arbitrary, the scheme has proven practical for the routine production of rabbit antisera to both mycoplasmata (10) and rhinoviruses (3). Considering all serological tests, the antibody response observed was fairly uniform between rabbits which had similar levels of immunogen. A similar result has been observed with the rhinoviruses (3). The immunogen for *M. pneumoniae* should contain at least 1 mg (4 mg per rabbit) of mycoplasmic protein per ml. For other species, concentrations of 1 to 4 mg/ml have proven satisfactory (10). Broth cultures concentrated 200- to 400-fold usually yield sufficient concentrations of immunogen. The immunization method has worked sufficiently well in practice that animals can be pre-bled, immunized, and bled-out without resorting to laborious trial bleeding efforts to determine whether satisfactory antibody has been produced. Since most rabbits have produced a good antibody response prior to the 1-ml booster injection, rabbits can be bled-out on day 39 with a good yield of antibody. Most antisera have proven excellent for both double immunodiffusion and growth-inhibition.

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