

Serological Cross-Reaction Between Lipids of *Mycoplasma pneumoniae* and *Mycoplasma neurolyticum*

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The complement-fixing activity of crude lipid extracts of 10 *Mycoplasma* species was compared with that of whole organism antigens employing immune rabbit serum. Five species (*M. pneumoniae*, *M. neurolyticum*, *M. granularum*, *M. laidlawii*, and *M. fermentans*) showed serological activity, whereas the remaining five species (*M. canis*, *M. felis*, *M. gallisepticum*, *M. hyorhinis*, and *M. pulmonis*) did not show significant activity in their lipid fractions. The lipid fractions of the five species which had serological activity in their lipid fractions showed three groups of serological specificity. *M. pneumoniae* cross-reacted with *M. neurolyticum*, *M. granularum* cross-reacted with *M. laidlawii*, and *M. fermentans* showed specific activity. Acute and convalescent sera from human pneumonia patients from whom *M. pneumoniae* had been isolated showed antibody increases which could be measured nearly as well by lipids of *M. neurolyticum* as by those of *M. pneumoniae*. Only a few human convalescent sera showed antibody measurable by lipids of *M. granularum*, *M. pneumoniae* did not cross-react with *M. neurolyticum* by other serological parameters such as growth inhibition on agar or double immunodiffusion, indicating that only the lipid antigens of these two species cross-react.

Studies of the antigenic structure of mycoplasmata have thus far revealed only a few characteristics of their antigens. The information available indicates that the organisms in the order *Mycoplasmatales* are surprisingly heterogeneous, not only in serology (11) but also in chemical class of the major antigens. The major antigen of *Mycoplasma pneumoniae* as measured by complement fixation against either rabbit or human convalescent sera is found in the lipid fraction (13). The serologically active lipids are glyceroglycolipids which contain both glucose and galactose (2) and probably represent three or more serological specificities (16). On the other hand, the major antigen of *M. mycoides* var. *mycoides* appears to be polysaccharide although serologically active glycolipids are present (14). Whereas *M. fermentans* contains specific serologically active lipids as its major complement-fixing antigen (10), another glycolytic species with a similar guanine plus cytosine content (% GC), *M. pulmonis*, does not contain serologically active lipids, but the major complement-fixing antigen appears to contain protein components (4, 10). A survey of some nonglycolytic, arginine-utilizing species has shown that these species [*M. hominis*, *M. pharyngis* (*orale I*), *M. orale II*, and *M. arthritis*] did not contain major

lipid antigens but that their major antigens are heat-labile (10). Hollingdale and Lemcke (9) have shown that *M. hominis* contains a serologically active lipid as a minor antigen which was specific in the sense that it reacted with homologous rabbit antiserum, but it did not react with antisera to *M. salivarium*, *M. orale I*, or *M. arthritis*. *M. laidlawii* contains serologically active glucolipids (15). [A new genus, *Acholeplasma*, has recently been proposed for *M. laidlawii* and *M. granularum* (5)].

This study is an investigation of the occurrence and specificity of serologically active lipids in 10 glycolytic species in the order *Mycoplasmatales*. Some of these data were presented in a preliminary communication (Abstr., Xth Int. Congr. Microbiol., 1970, p. 92).

MATERIALS AND METHODS

Antigens and antisera. The source, strains, and clonal status of the *Mycoplasma* species employed (Table 1) have been described previously (11). The serological test antigens, immunogens, and rabbit antisera were prepared as also described previously (11). Serological test antigens were propagated in soy-peptone-fresh yeast dialysate medium (10) supplemented with 10% agamma horse serum, whereas immunogens were cultivated in the same broth sup-

TABLE 1. Serological activity of lipid fractions compared to whole organisms as measured by complement fixation

Organism	Strain	Antigen titer		Organism	Strain	Antigen titer	
		Whole organism	Lipid			Whole organism	Lipid
<i>Mycoplasma pneumoniae</i>	AP-164	128	256	<i>M. hyorhinis</i> ^a	BTS-7	512	8
<i>M. neurolyticum</i> ^a	KSA	128	32	<i>M. felis</i> ^a	C ₀	128	8
<i>M. laidlawii</i>	A	128	128	<i>M. canis</i> ^a	PG-14	512	<8
<i>M. granularum</i>	BTS39	128	64	<i>M. gallisepticum</i>	S-6	64	<8
<i>M. fermentans</i> ^a	PG-18	64	32	<i>M. pulmonis</i> ^a	63	512	<8

^a Species which have a low per cent guanine plus cytosine content (25 to 29%; reference 21) and which appear to be loosely serologically related (11).

plemented with 10% agamma calf serum. Serological test antigens contained from 6 to 12 mg of mycoplasmic protein per ml.

Extraction of lipids. Lipids were extracted from 4 ml of serological test antigen with 150 ml of chloroform-methanol (2:1). The suspension was partitioned with 37.5 ml of 0.1 M KCl. The chloroform phase was evaporated and the lipid was solubilized in 4 ml of absolute ethanol.

Serological testing. Complement fixation was carried out in a microtitration system as described previously (13). Serial dilutions of alcoholic solutions of lipids were prepared in complement fixation diluent in 1- to 5-ml volumes, and samples were dropped into Microtiter cups. The alcohol did not interfere with complement fixation (2). Crude (whole organisms) antigens were similarly titrated, and protein concentrations of the antigens were identical to those previously published (11). Sera were serially diluted and also dropped into Microtiter cups. Antigens and antisera were assayed by block titration, and the end points reported here are expressed as the reciprocal of highest dilution of antigen or antibody which gave complete fixation of complement in an overnight complement fixation test employing two full units of complement.

RESULTS

Serologically active lipids. Crude lipid preparations from five species (*M. pneumoniae*, *M. neurolyticum*, *M. fermentans*, *M. laidlawii*, and *M. granularum*) were serologically active and showed complement-fixing titers of 1:32 or greater against homologous rabbit antisera (Table 1). The lipid antigen titer approximated that of the antigen in the whole organism for four organisms, but not for *M. neurolyticum* where the lipid antigen titer was fourfold lower than the titer for the whole organism. That difference was confirmed in a number of experiments. However, a study of four other lots of *M. fermentans* antigen showed that the titer of lipid was approximately equal to that of the whole organism antigen. No or minimal serological activity was

TABLE 2. Cross-reactions between mycoplasmic lipids by complement-fixing antibody titers

Lipid	Antiserum titer				
	1	2	3	4	5
<i>Mycoplasma pneumoniae</i> ...	256	128	<16 ^a	<16	<16
<i>M. neurolyticum</i>	128	64	<8	<64	<32
<i>M. fermentans</i> ..	<16	<8	64	<8	<8
<i>M. granularum</i> ..	<32	32	<32	128	128
<i>M. laidlawii</i>	<8	<8	<8	64	256

^a Value <16 indicates that either the preimmune serum or the anticomplementary control of the test serum had a titer of 1:16. Serum titers were begun at 1:8.

found in the lipid fractions of five species (*M. canis*, *M. felis*, *M. gallisepticum*, *M. hyorhinis*, and *M. pulmonis*). The presence or absence of lipid antigens did not necessarily correlate with % GC. Four of the six species in the low % GC group which appear to have some serological relationship (11) did not have serologically active lipids, whereas lipid was a minor antigen for *M. neurolyticum* and a major antigen for *M. fermentans*.

Serological specificity of lipids. The five organisms which had lipid antigens were cross-compared by block titration. The five species fell into three groups: *M. pneumoniae*-*M. neurolyticum*, *M. granularum*-*M. laidlawii*, and *M. fermentans* (Table 2, 3). The lipids of *M. pneumoniae* could not be distinguished from *M. neurolyticum* by antibody titer (Table 2); however, antigen titers were markedly different (Table 3). It appears likely that the glycolipids of *M. pneumoniae* contain multiple serological specificities (16). Although preliminary evidence (*data not shown*) suggests that *M. neurolyticum* has several glyco-

TABLE 3. Cross-reactions between mycoplasmic lipids by complement-fixing antigen titers

Lipid	Antiserum titer				
	1	2	3	4	5
<i>Mycoplasma pneumoniae</i>	256	16	<16 ^a	<16	<16
<i>M. neurolyticum</i>	64	64	8	<64	<32
<i>M. fermentans</i>	<16	<8	64	<8	<8
<i>M. granularum</i>	<32	32	<32	64	64
<i>M. laidlawii</i>	<8	<8	<8	64	128

^a Value <16 indicates that the antigen was either anticomplementary or showed that titer against the preimmune serum. Antigen titers were begun at 1:8.

lipids, some of which show similar migration patterns on thin-layer chromatography to those of the glycolipids of *M. pneumoniae*, quantitative data are not yet available on relative quantities of glycolipids. The fact that lipids of *M. laidlawii* cross-react with those of *M. granularum* is not surprising because of their close relationship by other serological parameters (11). *M. fermentans* lipids were clearly unrelated to the other organisms. It was more difficult to determine whether any cross-reactions were observed between *M. laidlawii*-*M. granularum* and *M. pneumoniae*-*M. neurolyticum*. Antigens prepared from *M. laidlawii* and *M. granularum* were frequently strongly anticomplementary. This anticomplementary effect resulted from the presence of antibody in some guinea pig sera because sera could be selected which did not react with these lipid antigens; also, heat-inactivated guinea pig serum (from lots against which lipids showed strong anticomplementary activity) fixed complement with lipid antigens when a nonreactive guinea pig serum was used as the complement source. Rabbit preimmune sera frequently had antibody to lipids of both *M. pneumoniae*-*M. neurolyticum* and *M. laidlawii*-*M. granularum* but not to *M. fermentans*. Thus antibody to lipids of four species appeared to be present in many normal sera from both rabbits and guinea pigs; this factor made detection of minor cross-reactions between those species difficult.

***M. pneumoniae*-*M. neurolyticum* cross-reaction.** Since the cross-reaction between *M. pneumoniae* and *M. neurolyticum* was quite unexpected because of their dissimilar % GC values [39.9 versus 26.2% (21), respectively], the cross-reaction was further investigated. Another strain of *M. neurolyticum* (Sabin type A, ATCC 19988) was tested and was also found to contain serologically active lipids which cross-reacted with those of *M.*

pneumoniae. Neither strain showed any common antigens with *M. pneumoniae* when tested by micro double immunodiffusion where six to seven lines were obtained in each homologous system (11). Cross-reactions were not observed when both organisms were cross-tested by disc inhibition on agar by using the method of Clyde (3).

Human serum antibody studies. The antibody response of humans known to be infected with *M. pneumoniae* as evidenced by isolation of the organism (6) was tested with lipids from three species: *M. pneumoniae*, *M. neurolyticum*, and *M. granularum*. Table 4 shows representative data for six pairs of acute and convalescent sera of the 20 tested which showed fourfold antibody rise to *M. pneumoniae*. Essentially the same antibody response attained with *M. pneumoniae* lipids was observed with *M. neurolyticum*. This close correlation is shown in more detail in Fig. 1 which shows comparative values for 42 sera. A tendency is shown towards slightly higher antibody titers with *M. pneumoniae* lipid in the experiments shown here. (The regression line is plotted at theoretical perfect correlation.) However, in another set of experiments, slightly higher titers were seen with *M. neurolyticum*, again with good correlation between the two tests. The variations are small (within one twofold dilution) and likely due to the fact that the serological activity is not a function of a single pure antigen but is determined by a mixture of glycolipids. When serum pairs from 10 pneumonia patients who did not show antibody rise against lipids of *M. pneumoniae* but had high stationary titers ($\geq 1:32$) were tested with *M. neurolyticum* lipids, all sera showed stationary titers and no pair showed an antibody

TABLE 4. Comparison of human serum antibody titers measured by lipids of *Mycoplasma pneumoniae*, *M. neurolyticum*, and *M. granularum*

Patient ^a	Antibody titer					
	<i>M. pneumoniae</i> lipid		<i>M. neurolyticum</i> lipid		<i>M. granularum</i> lipid	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
PN 3283	<2	128	2	32	<2	4
PN 3430	<2	64	<2	16	<2	<2
PN 3550	16	256	8	32	<2	<2
PN 3645	<2	128	2	64	<2	16
PN 3841	<2	32	<2	8	<2	<2
PN 4069	<2	16	4	32	<2	<2

^a Patients with pneumonia and *M. pneumoniae* isolates.

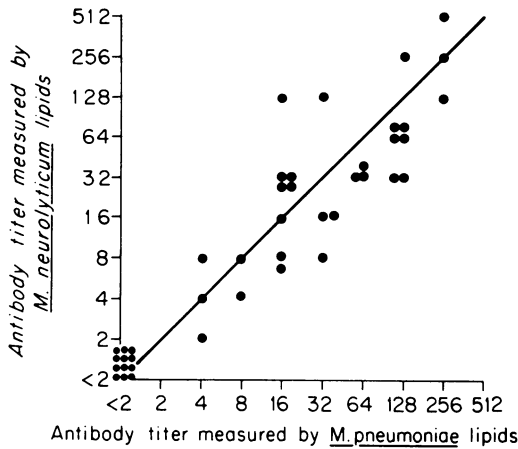


FIG. 1. Correlation of human serum antibody titers measured with lipids of *M. pneumoniae* with antibody titers determined with lipids of *M. neurolyticum*. Twenty-one pairs of human sera from pneumonia cases were tested.

increase. Similarly, 80 patients without antibody to *M. pneumoniae* also did not show antibody to *M. neurolyticum*. Only two of six patients showed antibody measurable with *M. granularum* lipids (Table 4). A survey of an additional 12 pairs of sera revealed only one other serum pair which showed an antibody increase to *M. granularum* lipids.

DISCUSSION

The fact that lipid preparations of *M. pneumoniae* cross-reacted strongly in the complement fixation test with those of *M. neurolyticum* suggests that although the lipid fraction contains the major complement-fixing activity of *M. pneumoniae* this antigen is not unique to *M. pneumoniae*. Since glyceroglycolipids are simple compounds with fairly broad distribution in nature, this is not surprising. For example, spinach glycolipids have been shown to cross-react with antiserum to *M. pneumoniae* (16). Similarly, diglycosyl diglyceride prepared from *Streptococcus* MG has been shown to cross-react with the diglycosyl diglyceride of *M. laidlawii* (15).

Substantial evidence exists to support the concept that lipid antigens are the determinants for both the metabolic-inhibition test (17, 19) and the complement-mediated killing reaction of *M. pneumoniae* (7). Preliminary results suggest that *M. neurolyticum* antibody will readily kill *M. pneumoniae* in the presence of complement (J. L. Gale, personal communication). However, the reciprocal experiment has not yet been carried out. No cross-reactivity between the two species was observed by disc inhibition on agar. This fact suggests that lipid is not the determinant for this

reaction. However, it is possible that a different antibody (either antibody against a different configuration of the lipids on the membrane surface or antibody of a different immunoglobulin class) against lipids might play a role in inhibition on agar. Rabbit antibodies which inhibit growth of *M. pneumoniae* on agar are produced later than antibodies measured by complement fixation with lipid antigen (12). However, it would seem rather difficult for lipid to be the determinant of growth inhibition on agar for both *M. pneumoniae* and *M. neurolyticum*.

The cross-reaction between *M. pneumoniae* and *M. neurolyticum* was much more evident when lipids were compared than when whole organism antigens were tested. In a previous study only two of four *M. pneumoniae* antisera reacted with intact *M. neurolyticum* whole organism antigen, and neither of the two *M. neurolyticum* antisera reacted with intact *M. pneumoniae* (11). One reason for this is that lipid was found to be a relatively minor antigen for *M. neurolyticum*, whereas it is the major antigen for *M. pneumoniae*. It is also possible that the glycolipids may be organized differently in the intact membrane or that the relative concentrations of the various glycolipids differ between the two organisms. In this regard, it has been demonstrated that when *M. pneumoniae* glycolipids are reaggregated with delipidized membranes of *M. laidlawii* and the reaggregated complex is injected into rabbits, the antisera produced will have substantial antibody against *M. pneumoniae* as measured by metabolic-inhibition testing and complement fixation against lipid antigen (19). The cross-reaction of *M. neurolyticum* with *M. pneumoniae* appears to be a natural model of this phenomenon since the cross-reaction appears based only on common lipid antigen(s).

The possession of lipid antigens does not appear to be the sine qua non for organisms of the order *Mycoplasmatales*. Thus, the organisms in the *Mycoplasmatales* are not only markedly heterogeneous physiologically (1), in deoxyribonucleic acid base composition (21), protein patterns on polyacrylamide electrophoresis (18), and comparative serology (11) but also they are heterogeneous in chemical class of major antigen.

The presence of common lipid antigens between *M. pneumoniae* and other mycoplasmic and bacterial species has significance for the interpretation of serological testing of human sera. Fourfold antibody rises between acute and convalescent sera from pneumonia patients correlate well with isolation of *M. pneumoniae* (8). However, the presence of cross-reacting or common lipids in other organisms suggests the need for caution in interpreting the results of serological surveys of human sera. Since lipid is necessarily the deter-

minant for complement-fixing antibody measured against lipid antigen and lipid appears to be the determinant for antibody measured by both metabolic-inhibition and the complement-mediated killing reaction, caution is advised in interpreting the presence of antibody as necessarily indicating prior infection with *M. pneumoniae* when using these serological tests. Other organisms could provide the antigenic stimulus for these antibodies. A good natural example of this is the frequent occurrence of both antilipid complement-fixing antibody and complement-mediated killing antibody to *M. pneumoniae* in normal rabbits (7, 13). Rabbits are not known to be infected with either *M. pneumoniae* or *M. neurolyticum*. Whether antibody induced in humans by other organisms which contain immunologically similar glyceroglycolipids would protect against infection is a provocative question.

The cross-reaction between *M. pneumoniae* and *M. neurolyticum* appears to be fortuitous and should not be taken to imply taxonomic relationships between these two species. *M. neurolyticum* appears to belong to that loosely related group of *Mycoplasma* species which have low % GC ranging from 25 to 28% (see Table 1 for the other species in this group). The low % GC group showed no relationship to *M. pneumoniae* when compared by double immunodiffusion tests where 6 to 11 lines were seen in the homologous system (11). Therefore, *M. pneumoniae* and *M. neurolyticum* appear to be species far more distinct phylogenetically than is indicated by their present classification since organisms in the same genus or family would be expected to contain a number of common antigens.

The presence of fortuitously cross-reacting agents does raise the possibility of serological diagnosis of infections due to uncultivable mycoplasmata. For example, *M. pneumoniae* infections could have been diagnosed with great specificity as early as 1940 by using the lipids of *M. neurolyticum*, an organism which was already known at that time (20). Although this discovery did not occur, another organism, *Streptococcus* MG, was found to have some serological utility for diagnosis of that portion of atypical pneumonia which was ultimately determined to be caused by *M. pneumoniae*.

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