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Antibody in Host Defense Against Mouse Pneumonitis Agent (Murine *Chlamydia trachomatis*)

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In a murine model of chlamydial pneumonia employing murine *Chlamydia trachomatis*, immune serum given intranasally delayed death in nude (*nu/nu*) mice and prevented death in *nu*/+ mice. Serum-derived immunoglobulin G and A fractions and immune lavage fluid fractions containing immunoglobulins G and A were effective in producing protection when used to opsonize the murine *C. trachomatis* inoculum. In hyperclean mice (previously made germfree and then colonized with a limited flora nonpathogenic to mice) antibody given intravenously was ineffective. The effects of antibody and nonspecific stimulation of cell-mediated immunity (after previous infection with *Histoplasma capsulatum*) were additive in increasing host resistance to murine *C. trachomatis*.

The role of antibody in host defense against *Chlamydia trachomatis* is unclear. In experimental infections of the eye with *C. trachomatis*, secretory immunoglobulin A (IgA) in eye secretions has been thought to be involved in host defense (9, 10). In genital infections in female guinea pigs, humoral immunity, probably secretory IgA, has been shown to be an important factor (12). In addition, local antibody has been implicated as a host defense factor in endocervical infections in humans as well (2).

Much less is known about host defense against *C. trachomatis* at nonmucosal sites and about the role of other immunoglobulins. It is known, however, that IgG antibodies neutralize the infectivity of *C. trachomatis* in vitro for HeLa-229 cells by a process which does not involve interference with attachment to cells (3).

We have investigated the role of antibody in murine host defense against *Chlamydia* pneumonia, an invasive, rather than a purely mucosal, infection. The infectious agent used was the mouse pneumonitis agent (MoPn), which is iodine positive and sensitive to sulfadiazine but which differs from human *C. trachomatis* in DNA homology studies (18).

Previous work demonstrated that nude athymic (*nu/nu*) mice are more susceptible to MoPn than are their furred heterozygous (*nu*/+) littermates (18, 19). *nu/nu* mice, in contrast to *nu*/+ mice, do not make antibody against MoPn (18) and are also impaired in their cell-mediated immunity (CMI) response to this infection (17). It has also been shown previously that immune *nu*/+ serum given intravenously (IV) delays death from MoPn in *nu/nu* mice and prevents it in *nu*/+ mice (20). Because of bacterial contamination in our mouse colony, we converted our mice to germfree mice (by using germfree foster mothers) and then repopulated the mice with a defined, primarily anaerobic flora nonpathogenic to mice. This procedure is known to reduce the level of background nonspecific CMI in mice (6). To our surprise, IV antibody was much less effective in these hyperclean mice than it had been previously. This suggested that a background of stimulated CMI might be needed for antibody to be maximally effective in host defense against MoPn. At the very least, it suggested that the efficacy of antibody should be evaluated in a setting of various levels of background CMI to look for additive or antagonistic immunological effects of antibody and CMI in host defense against MoPn. As intranasally (IN) delivered antibody was effective even in our hyperclean model, the current studies were designed to examine the effect of antibody given by that route.

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The studies reported here were designed to answer the following questions. (i) What is the role of antibody given IN in a model of invasive (rather than purely mucosal) infection? (ii) Can IgG and non-IgG fractions both provide protection when given by this route? (iii) Is there an additive effect of CMI and antibody?

MATERIALS AND METHODS

Mice. Specific-pathogen-free *nu/nu*, *nu*/+, and +/+ (homozygous BALB/c) mice originally derived from germfree animals were used in these studies. They were determined to be free from *Chlamydia* spp., mycoplasmas, and viruses by serological testing and culturing and had only a carefully defined nonpathogenic bacterial flora consisting primarily of anaerobes. They were maintained as previously described (17, 18).

MoPn. MoPn was obtained and maintained in yolk sac as previously described (18, 20). Mice were infected by the IN route (18, 20) with 0.05 ml of MoPn. Mortality was followed daily.

***Histoplasma capsulatum*.** Isolate G217B of *H. capsulatum*, which was obtained from Ram Tewari (Southern Illinois University School of Medicine, Springfield) and maintained in the log phase (17), was given to mice IN at the sublethal dose of 5×10^3 organisms per mouse.

Indirect immunofluorescence. Antibodies to MoPn were measured as described previously by the microimmunofluorescence method of Wang and Grayston (16) with homologous antigen. Titers of IgA, IgM, and IgG specific antibodies were determined for each sample with fluorescence-labeled antimouse reagents (Cappel Laboratories, Cochranville, Pa.).

Passive transfer of antibody. *nu/nu* or *nu*/+ mice were given 0.05 ml of antibody IN 5 min before IN infection with MoPn; alternatively, organisms were opsonized by incubation in normal or immune *nu*/+ serum or trachael lavage fluid containing antibody to MoPn. *nu/nu* mice do not produce their own antibody to MoPn (18, 20) and are therefore a good

model for studying exogenous antibody over the total course of infection.

Opsonization studies. For IN opsonization-neutralization studies, 5×10^5 inclusion-forming units of MoPn in McCoy modified medium were incubated with (i) 50% (by volume) normal mouse serum or normal tracheal lavage fluid, (ii) 50% immune whole mouse serum or immune tracheal lavage fluid, or (iii) 50% immune serum-derived or immune tracheal lavage fluid-derived IgA and IgG fractions. The inoculum was incubated for 45 min at 37°C and then given IN (0.05 ml) to recipient *nu/nu* or *nu/+* mice. The mixture was constantly blended with a Vortex mixer during incubation to guard against sedimentation. In initial experiments, incubation was actually performed in separate 0.1-ml total volume amounts to guard against individual mice getting unequal inocula because of agglutination, but results obtained were the same as when the incubation was done with a larger volume (0.5 ml) and blending with a Vortex mixer.

Antibody fractionation. Mouse sera or tracheal lavage fluids were obtained from *nu/+* mice infected with MoPn for 6 weeks (serum) or 3 weeks (lavage) and determined to have positive titers of IgG and IgA to MoPn by microimmunofluorescence. Sera and lavage fluids were fractionated in a protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column by elution with buffers of decreasing pH, as described by Prowse and Jenkin (11).

Production of antibody to MOMP. *nu/+* mice were immunized subcutaneously with 10 μg of MoPn major outer membrane protein (MOMP) in complete Freund adjuvant. Over the next 6 weeks two booster doses of MOMP were given intraperitoneally in saline. The mice were then bled, and the serum was titrated as described above. MOMP was prepared as previously described (3).

Measurement of interferon. Immune serum, tracheal lavage fluid, and fractions therefrom were assayed for murine interferon with a microtest assay which quantifies interferon by measurement of the protective effect against cytocidal infection with vesicular stomatitis virus (1). Samples were coassayed with National Institutes of Health reference standards. All assays were negative for interferon (<3 U/ml), whereas the mouse standard was positive at 23,000 U/ml.

Tracheal lavage. This antibody-containing material was obtained by the method of Ryning and Remington (13) by inserting an 18-gauge needle with a three-way stopcock into the trachea and lavaging the lungs three times with 2 ml of phosphate-buffered saline. The mice had previously been exsanguinated (13).

Electron microscopy. To test whether immune serum agglutinated MoPn, a 0.05-ml sample of MoPn plus immune mouse serum (IgA, 1:4,096; IgG, 1:1,024) and a similar sample of MoPn plus normal mouse serum were allowed to dry on a 300-mesh grid and prepared for transmission electron microscopy with shadow casting by using gold palladium (21). With this method the number of organisms in different 3,400× fields on each grid could be counted and aggregation looked for. To exclude false-negative results caused by an excess of antibody, we repeated the procedure with 1:10 and 1:100 dilutions of the initial serum.

Statistics. Mouse mortality was analyzed with the generalized Wilcoxon test and the Fisher exact test (4).

RESULTS

Table 1 shows mortality in *nu/+* and *nu/nu* mice given normal or immune *nu/+* serum (IgG, 1:2,048; IgA, 1:512; IgM, 0) IN followed by 10^6 MoPn IN. At this high dose both *nu/+* and *nu/nu* mice given normal serum IN experienced 100% mortality by day 10. Immune serum prevented death in *nu/+* mice and significantly delayed death in *nu/nu* mice ($P < 0.05$ for both as compared with controls). By day 30 significant protection was seen only in *nu/+* mice ($P < 0.05$), which have intact CMI and produce their own antibody to MoPn (7, 17, 18, 20). A repeat experiment showed similar results, with 2 of 10 *nu/+* mice given immune serum dead by day 30, compared with 9 of 10 *nu/nu* mice given immune serum dead by day 30; however, both groups of mice given immune serum survived significantly longer than the appropriate controls given normal serum.

TABLE 1. Mortality in *nu/+* and *nu/nu* mice given 0.05 ml of immune *nu/+* serum IN before IN infection with MoPn

Recipent	Serum	No. of mice dead[a] by day:			
		10	15	20	30
nu/+	Normal	10			
nu/+	Immune[b]	1	2	3	3
nu/nu	Normal	10			
nu/nu	Immune[b]	0	8	9	10

[a] Out of a total of 10 mice.
[b] IgG, 1:2,048; IgA, 1:512; IgM, 0. $P < 0.05$ as compared with normal serum.

To better assure contact of antibody and MoPn and thus permit the testing of lower-titer-antibody fractions, further experiments were performed with opsonized MoPn. MoPn was opsonized with immune serum, IgG- or non-IgG-containing immune serum fractions, immune lavage fluid, or immune lavage fluid fractions. All samples used in these studies were free of interferon activity. To counter the possibility of antibody leading to unequal distribution of organisms through clumping, resulting in some mice receiving more organisms than others, we carried out initial studies with 100-μl samples, with each mouse receiving the whole sample. Although this would not prevent clumping, it would tend to assure that each mouse would receive the same amount of MoPn. In these studies antibody protected at least as well as in the studies in which a larger volume was used. For example, at day 20, 100% mortality was seen in 10 *nu/nu* mice given organisms incubated with 50% normal mouse serum, and no mortality was seen in 10 *nu/nu* mice inoculated with organisms opsonized with immune serum (IgG, 1:1,024; IgA, 1:512; IgM, 0); in each case a 100-μl mixture was used ($P < 0.05$). As it is technically easier, the remaining studies were performed by opsonizing organisms in a volume of 0.5 ml, with each mouse receiving 0.05 ml.

As a further test for antibody-induced clumping, transmission electron microscopy with shadow casting was performed on samples of MoPn incubated with normal and immune sera. Undiluted serum and 1:10 and 1:100 dilutions of serum were used, and the number of organisms per field at 3,400× was determined. The experiment was repeated three times. For technical reasons, the method was only semiquantitative, and random differences in the number of organisms occurred from grid to grid. However, in all slides at all serum dilutions, the organisms were evenly distributed over each grid, with no significant differences from field to field on each grid. For example, with undiluted immune serum, five randomly chosen fields had 17, 18, 16, 14, and 16 organisms, whereas with undiluted normal serum, counts were 9, 12, 12, 10, and 9, respectively. Thus, no evidence of clumping (characterized by uneven distribution over the fields) was seen with undiluted, 1:10 diluted, or 1:100 diluted immune or normal mouse serum.

Table 2 shows the results of opsonization experiments

TABLE 2. Mortality in *nu*/+ and *nu*/*nu* mice given MoPn opsonized with whole *nu*/+ mouse serum

Recipent	Serum	No. of mice dead[a] by day:			
		10	15	20	30
nu/+	Normal	4	8	8	10
nu/+	Immune[b]	0	0	0	0
nu/*nu*	Normal	10			
nu/*nu*	Immune[b]	0	7	10	

[a] Out of a total of 10 mice.
[b] IgG, 1:1,024; IgA, 1:4,096; IgM, 0. $P < 0.05$ as compared with normal serum.

with whole immune sera and either *nu*/+ or *nu*/*nu* recipients. As was shown in Table 1, immune serum prevented death in *nu*/+ mice but only delayed it in *nu*/*nu* mice, which lack CMI and are unable to produce their own antibody. A repeat experiment showed similar results.

Experiments were then performed with purified IgG and non-IgM–non-IgG serum fractions obtained by separation in a protein A-Sepharose column. Eight experiments were performed with different samples. A representative group of experiments is shown in Table 3. Both IgG and non-IgM-non-IgG fractions significantly delayed death in *nu*/*nu* mice. *nu*/*nu* rather than *nu*/+ mice were used in all these experiments, as *nu*/*nu* mice do not produce their own antibody to MoPn (17, 18, 20), and thus any effect seen was very likely due to the opsonizing antibody alone.

Table 4 shows that whole immune lavage fluid also significantly delayed death, as did a lavage fluid fraction (IgA, 1:128; IgG, 1:32; IgM, 0). In two separate experiments, however, one fraction (IgA, 1:64; IgG, 0; IgM, 0) (the highest IgA titer obtained without IgG contamination) was not protective, perhaps because the titer was too low. We deliberately used lavage specimens collected 3 weeks after infection because preliminary studies have shown that lavage (but not serum) samples collected after this time may have lymphokine activity (D. M. Williams, G. I. Byrne, and J. Schachter, unpublished data).

It is recognized that although our IgA fractions did not contain IgM or IgG antibody detectable by microimmunofluorescence, IgE antibody was not excluded. Therefore, the term "IgA fractions" as used in these studies could include IgE in addition to IgA.

In a final experiment in this series, MoPn was opsonized with *nu*/+ mouse sera containing antibody to the MoPn MOMP. As shown in Table 5, this produced significant protection.

Thus, whole immune serum, IgG and IgA serum fractions, whole immune lavage fluid, and combined IgG and IgA lavage fractions were all capable of delaying death in *nu*/*nu* mice.

Experiments were then performed to test the interaction of antibody given locally and CMI. Half of the *nu*/+ mice used in these experiments were infected IN with *H. capsulatum*, which activates CMI and partially protects against subsequent infection with MoPn, as has been demonstrated previously (17).

In our initial experiment with high-titer immune serum (IgG, 1:2,048; IgA, 1:256; IgM, 0) given IN before MoPn was given IN, significant protection was afforded both by prior *H. capsulatum* infection and by immune serum, as compared with controls given only normal serum ($P < 0.05$) (Table 6, experiment 1). Because this high-titer serum alone protected so well, it was not possible to assess the effect of *H. capsulatum* plus immune serum optimally except to say that

TABLE 3. Mortality in *nu*/*nu* mice given MoPn opsonized with *nu*/+ mouse serum fractions

Expt	Serum	No. of mice dead/no. tested by day:			
		10	15	20	30
1[a]	Whole normal	8/10	10/10		
	IgA, 1:128; IgG, 0; IgM, 0 fraction	0/10	4/10	8/10	
	IgG, 1:256; IgA, 0; IgM, 0 fraction	1/10	8/10	9/10	
2[b]	Whole normal	6/10	9/10	10/10	
	IgA, 1:128; IgG, 0; IgM, 0 fraction	0/10	0/10	5/10	8/10
	IgG, 1:128; IgA, 0; IgM, 0 fraction	0/10	0/10	3/10	8/10
3[c]	Whole normal	9/13	13/13		
	IgG, 1:64; IgA, 0; IgM, 0 fraction	2/13	9/13	12/13	

[a] $P < 0.002$ for fractions.
[b] $P < 0.05$ for fractions. No control, uninfected *nu*/*nu* mice died.
[c] $P < 0.05$ for fraction.

no antagonism was seen and that mice treated with the combination had the lowest mortality. To better show the effect of *H. capsulatum* plus immune serum, we repeated the experiment with two changes. We used immune serum with a lower titer (IgG, 1:256; IgA, 1:256), and *H. capsulatum*-infected mice were tested at 2 weeks postinfection, when the CMI stimulation is much less effective (17).

In this experiment (Table 6, experiment 2), normal *nu*/+ mice were rendered significantly but transiently more resistant than control mice when given low-titer immune serum ($P < 0.05$). However, mice given *H. capsulatum* plus immune serum were significantly more resistant than any other mice ($P < 0.05$). Thus, the effect of the combination of CMI and IN antibody was additive.

In contrast to earlier results (20), in these hyperclean mice, immune *nu*/+ serum (IgG, 1:2,048; IgA, 1:256) given in three IV doses of 0.1 ml each in repeated experiments with *nu*/+ recipient mice did not provide consistent protection.

DISCUSSION

These studies have demonstrated the following concerning antibody in host defense against MoPn pneumonia. (i) Immune serum given locally protects hyperclean *nu*/+ and *nu*/

TABLE 4. Mortality in *nu*/*nu* mice given MoPn opsonized with whole *nu*/+ trachael lavage fluid or trachael lavage fluid immunoglobulin fractions

Expt	Lavage fluid	No. of mice dead[a] by day:		
		10	13	20
1	Whole normal	5	9	10
	IgG, 1:128; IgA, 1:128; IgM, 0 whole immune[b]	2	4	8
2	Whole normal	6	8	9
	IgA, 1:128, IgG, 1:32; IgM, 0 fraction[c]	1	5	9
	IgA, 1:64; IgG, 0; IgM, 0 fraction[d]	3	6	8

[a] Out of a total of 10 mice.
[b] $P < 0.05$.
[c] $P < 0.05$.
[d] P not significant.

TABLE 5. Mortality in *nu/nu* mice given MoPn opsonized with *nu/+* mouse sera with specific antibody titers against MOMP

Serum	No. of mice dead[a] by day:			
	5	10	15	20
Normal *nu/+*	0	0	3	9
MOMP *nu/+* (IgG, 1:1,024; IgM, 1:64; IgA, 0)[b]	0	0	0	3

[a] Out of a total of 10 mice.
[b] $P < 0.05$ as compared with normal serum.

nu mice against MoPn. (ii) Protection is better in *nu/+* recipients. (iii) Direct opsonization with either serum-derived IgG or IgA or lavage fractions containing both IgG and IgA affords protection against MoPn. (iv) The mechanism of action of antibody does not seem to be simple aggregation or clumping, at least in vitro. (v) Antibody and activated CMI can be additive in effect (although the immunological mechanisms involved may or may not involve a direct interaction of antibody and CMI).

However, as antibody given IV was effective before our mice were rendered hyperclean and as our mice presumably had higher levels of nonspecific CMI before being rendered hyperclean (6), it is tempting to speculate that an immunological mechanism involving both antibody and activated CMI may be important in host defense against MoPn pneumonia.

Previous studies have examined antibody in host defense against mucosal infections with *Chlamydia* spp. Studies by Rank and Barron (12) have implicated secretory immunoglobulins as an important host defense mechanism in resistance to mucosal genital chlamydial infections in guinea pigs, and others (9, 10) have found a similar phenomenon in mucosal eye infections. Thus, the effect of local antibody at a mucosal surface perhaps in preventing attachment, causing direct cytotoxicity, or agglutinating MoPn in vivo may be important. Clearly, however, the mechanism of action of antibody is not understood. Caldwell and Perry have shown that IgG antibody neutralizes the infectivity of *C. trachomatis* in vitro by a method not involving aggregation or inhibition of attachment but by interfering with the infectious process after the organism is internalized in HeLa-229 cells (3). The immunological mechanism involved was not further identified. Of perhaps more relevance to our model of interaction of antibody and CMI, however, are studies by Wyrick and Brownridge (22) and Wyrick et al. (23). They have shown that whereas nonopsonized *C. psittaci* prevents phagolysosomal fusion in macrophages and multiplies, opsonized organisms are taken up more readily by macrophages and are rapidly destroyed in macrophage phagolysosomes. These data demonstrate an immunological interaction of antibody and CMI. Antibody-dependent cell-mediated cytotoxicity is another immunological mechanism in which antibody and CMI could interact. Lammert and Wyrick have provided evidence for specific cellular cytotoxicity during infection of mice with *C. psittaci* (8) (although whether antibody was involved in the specificity of the toxicity was not clear). In addition, evidence for cytotoxic host defense mechanisms against *C. trachomatis* does not exist. Thus, this mechanism is purely speculative at this time.

TABLE 6. Mortality in *nu/+* mice given a nonlethal dose of *H. capsulatum* to stimulate CMI or immune serum IN or both before MoPn infection

Expt	Treatment[a]	No. of mice dead/no. tested by day:		
		5	10	20
1[b]	IS + *H. capsulatum*	0/10	0/10	1/10
	NS + *H. capsulatum*	0/9	5/9	9/9
	IS	0/10	1/10	3/10
	NS	5/10	10/10	
2[c]	IS + *H. capsulatum*	0/9	2/9	4/9
	NS + *H. capsulatum*	5/10	8/10	9/10
	IS	3/10	8/10	8/10
	NS	9/10	9/10	9/10

[a] IS, Immune serum; NS, normal serum.
[b] IgG, 1:2,048; IgA, 1:256; IgM, 0 IS; IgG, 0; IgA, 0; IgM, 0 NS.
[c] IgG, 1:256; IgA, 1:256; IgM, 0 IS; IgG, 0; IgA, 0; IgM, 0 NS.

Our studies have shown that both IgG and non-IgG fractions (containing IgA but not IgM or IgG against MoPn) are protective in our model. This is consistent with previous studies which showed protection by IgA against both *Klebsiella* pneumonia (5) and *Mycoplasma* pneumonia (15) in mice. The mechanism of action of IgA was unclear. Cooper et al. concluded that IgA extended its protective effect in the upper respiratory tract perhaps by inhibiting attachment or enhancing mucociliary clearance (5). It is also of interest that the secretory form of IgA has recently been shown to exert antibody-dependent cell-mediated cytotoxicity against *Shigella* spp. at the mucosal level (14). The mechanism of action of IgA needs further evaluation.

We have not investigated further the role of complement in host defense against MoPn because of the following discouraging early data. Mice deficient in the fifth component of complement (DBA2J, male OSN$^-$J) were no more sensitive to MoPn than the appropriate control mice (DBA1J, NSN$^+$J). In addition, up to eight five-unit doses of *Naja haje* cobra venom factor given IV during infection did not increase susceptibility to MoPn (D. Williams and J. Schachter, unpublished observations). It is still possible that early complement components or complement plus antibody already present at the time of infection may play a role, however, and this deserves further investigation.

In summary, these studies show that local antibody present at the time of infection is effective in providing protection against MoPn pneumonia in hyperclean mice, a setting in which IV antibody is ineffective. Protection is better in a setting of intact (*nu/+*) or stimulated CMI or both.

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