Immune Inhibition of *Mycoplasma pneumoniae* (36463)

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Since the discovery of *Mycoplasma pneumoniae* as a human pathogen, a wide variety of tests have been developed for detection of the immune response to this organism. Relative to the tests performed in broth, Taylor-Robinson *et al.* (1) and Fernald, Clyde, and Denny (2) reported that a heat labile accessory factor was required to demonstrate immune inhibition. It has been suggested that complement-mediated lysis is involved as the mechanism of this inhibition (3, 4). The results reported by Coleman and Lynn (5), however, indicate that the requirement for a heat labile accessory factor, presumably complement, is dependent upon the time which has elapsed following immunization. The present study was undertaken to provide insight into the multiple mechanisms of immune inhibition in broth which develop during immunization of rabbits with *M. pneumoniae*.

Materials and Methods. Mycoplasma pneumoniae, strain F.H., was grown in a basal medium prepared from Difco beef heart for infusion. Ten percent fresh yeast extract, 2% glucose, 0.02 mg/ml phenol red, and 10% agamma horse serum (inactivated at 56° for 1 hr) were added to the basal medium.

Immunization procedure. New Zealand white rabbits were immunized with a whole cell, glass-grown preparation of *M. pneumoniae*. Protein concentrations of the inoculum were calculated according to the method of Lowry *et al.* (6). Both the initial and booster inoculations contained 0.13 mg of protein. Rabbits were bled by cardiac puncture 5 and 17 days after the initial inoculation.

Growth of labeled cells. One milli-Curie of ^{3}H methyl thymidine (Calatomic) per 100 ml of medium was added with an initial inoculum of 10^6 cells. The organisms were allowed to sheet out on a glass surface and were harvested after 72 hr of growth. The nucleic acids from a portion of these cells were extracted according to the method of Marmur (7).

Sucrose gradient ultracentrifugation. A 5 to 20% continuous gradient was prepared in a buffer containing 0.01 *M* Tris and 0.1 *M* NaCl, pH 7.4. Centrifugation was carried out utilizing a SW-39 rotor at 38,000 rpm for 3.5 hr.

Exposure of labeled cells to immune serum. Equal volumes of isotope labeled *M. pneumoniae* (in basal medium without additives) and undiluted serum were incubated at 37° for 4 hr prior to layering on the sucrose gradient. In order to ascertain the effect of complement, nomal guinea pig serum at a final dilution of 1:200 was added.

Kinetics of neutralization. Determination of the kinetics of neutralization was made by a modification of the technique of Gale and Kenny (3). Reaction mixtures composed of 0.25 ml of the serum dilution, 0.25 ml of the basal medium, and 0.5 ml of a 72 hr culture of *M. pneumoniae* (approx 10^6 colony forming units/ml) were incubated at 37°. Guinea pig serum, at a final dilution of 1:200, was added to the reaction mixture where indicated. Samples were removed at specified time intervals and diluted 1:100 and 1:1000 before inoculating a PPLO agar plate with 0.01 ml of the dilution.

Metabolic inhibition test. Wasserman tubes with Morton closures were utilized in the metabolic inhibition test (MI). The test consisted of 0.2 ml of the serum dilution, 0.2 ml of complete growth medium, and 0.2 ml

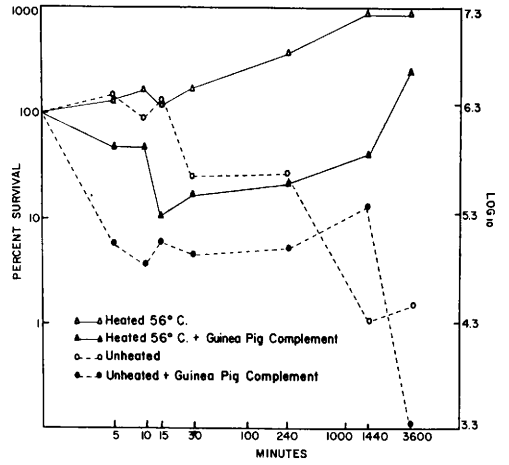

FIG. 1. Kinetics of neutralization of 1:20 dilution of serum collected 5 days after initial inoculation: Both axes of the figure are logarithmic.

of organisms (approx 10^6 cfu/ml) bringing the final volume in each tube to 0.6 ml. Tubes were incubated at 37° and examined at 3, 5, and 7 day intervals. The titer was calculated as the highest dilution of serum that prevented a pH change of 0.5 units.

Results. Preimmune rabbit sera, even in the presence of unheated guinea pig serum, had no demonstrable bactericidal effect on *M. pneumoniae* during an exposure period of 60 hr. However, sera collected as early as 5 days after the injection of 0.13 mg protein yielded such activity (Fig. 1). The unheated sera produced a 75% reduction in the number of viable colonies within the first 30 min. The addition of fresh guinea pig serum to the unheated immune sera increased the rate of killing. Sera heated at 56° for 1 hr lost its inhibitory effect. The addition of guinea pig serum to this heated sera yielded an initial decrease in viability followed by a significant increase in the number of viable organisms after 4 hr incubation. The addition of higher concentrations of normal guinea pig serum did not eliminate the late increase in viable organisms (Fig. 2).

A heat stable inhibitory activity was demonstrable with rabbit sera collected 17 days after primary inoculation; 7 days postbooster (Fig. 3). The heated sera showed a delayed

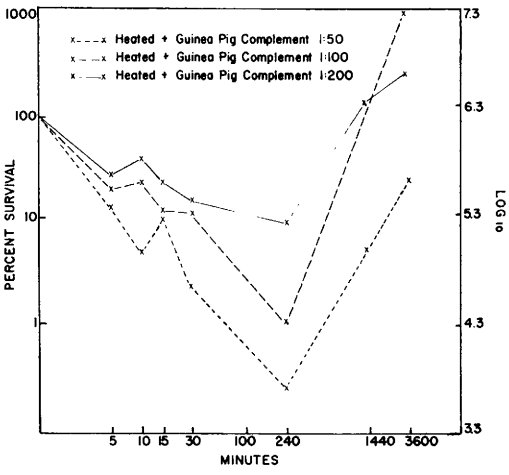

FIG. 2. Effect of guinea pig serum concentration on kinetics of neutralization of heat-inactivated serum collected 5 days after initial inoculation: Both axes of the figure are logarithmic.

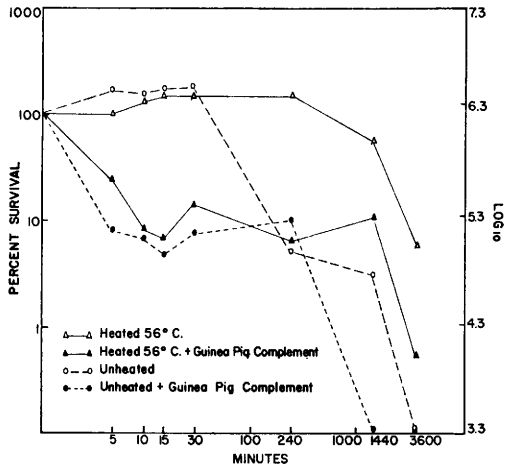

FIG. 3. Kinetics of neutralization of 1:20 dilution of serum collected 17 days after initial inoculation, and 7 days after a booster injection: Both axes of the figure are logarithmic.

but definite inhibitory effect (95% reduction of the number of viable organisms within 60 hr). The addition of unheated normal guinea pig serum to either heated or unheated sera produced an increased initial rate of inhibition followed by a second, slower rate leading to greater than 99% inactivation within 60 hr.

The role of lysis in the immune inhibition of *M. pneumoniae* was investigated by fol-

TABLE I. Distribution of Radioisotope Following Sucrose Density Centrifugation.[a]

Sucrose (%)	Whole cells[b]	Nucleic acids[c]	Sonicated cells[d]
5.0– 7	9.8 ± 5.0	2.4 ± 0.7	50.6 ± 7.0
7.1– 9	4.6 ± 1.1	2.0 ± 0.6	10.8 ± 9.8
9.1–11	3.6 ± 1.1	5.5 ± 1.8	9.9 ± 0.5
11.1–13	2.1 ± 1.3	2.3 ± 1.3	3.1 ± 0.6
13.1–15	1.2 ± 0.7	26.5 ± 10.8	0.5 ± 0.5
15.1–17	1.1 ± 0.1	31.5 ± 2.0	1.7 ± 0.7
17.1–19	1.8 ± 0.3	14.4 ± 7.0	1.7 ± 0.4
19.1–20	0.4 ± 0.4	—	—
>20	75.4 ± 8.4	15.1 ± 5.1	2.2 ± 1.8

[a] Results expressed as percentage recovery based on calculated input of ^{3}H-thymidine-labeled cells or nucleic acids that were layered on the sucrose gradient.

[b] Whole cell ^{3}H-thymidine-labeled *M. pneumoniae*.

[c] ^{3}H-Thymidine-labeled nucleic acids extracted from *M. pneumoniae* according to the method of Marmur (7).

[d] ^{3}H-Thymidine-labeled *M. pneumoniae* sonicated for 5 min at 10 kc.

lowing the release of labeled DNA from the organism during incubation with immune rabbit sera. Samples of whole cells, cells sonicated at 10 kc for 5 min, and isolated, highly polymerized DNA were subjected to sucrose density centrifugation. The results are compiled in Table I. The data suggested that release of labeled nucleic acids would result in an increase in counts in the 13 to 19% sucrose range if the nucleic acids were in the macromolecular configuration, or in the 5 to 11% sucrose range if smaller units were released.

In Table II, fresh undiluted preimmune sera (MI titer of 80) produced significant lysis, with the majority of the released label banding between 5 and 11% sucrose. Heat inactivation eliminates the nonspecific lysis and reduces the MI titer to less than 20. Sera collected 5 days after initial injection induced no significant lysis above that seen in the preimmune control, even though these sera had a MI titer of 320. The exposure of labeled cells to unheated late immune sera resulted in a significant increase in the release of labeled nucleic acids. Heat inactivation of the sera eliminates this effect, although the MI titer of these sera following heat inactivation is not significantly reduced.

Discussion. Reports in the literature relative to several mycoplasma strains suggest that more than one mechanism of immune inhibition may develop in animals for inhibition of mycoplasma growth in broth (8, 9). The data presented in this paper indicate that multiple mechanisms also develop in rabbits immunized with *M. pneumoniae*. Metabolic inhibition and the reduction of the num-

TABLE II. Release of Radioisotope from ^{3}H-Thymidine-Labeled *M. pneumoniae* Following Incubation with Immune Rabbit Sera.[a]

		Serum treatment[b]		
		Unheated		Heated
	Sucrose (%)	Undiluted	Diluted 1:2 + GPS[c]	Undiluted
PI[d]	5.0– 11	41 ± 12	ND[e]	16
	11.1–>19	6 ± 2	ND	6
	>19	53 ± 12	ND	78
MI[f] titer		80	ND	<20
EI[g]	5.0– 11	42 ± 2	44	26 ± 0.5
	11.1–>19	11 ± 5	2	13 ± 3
	>19	47 ± 3	54	61 ± 3
MI titer		320	ND	<20
LI[h]	5.0– 11	63 ± 2	ND	21
	11.1–>19	24 ± 11	ND	14
	>19	13 ± 10	ND	65
MI titer		640	ND	320

[a] Results expressed as percentage recovery based on number of counts in ^{3}H-thymidine-labeled cells put into reaction mixture.

[b] Serum collected by cardiac puncture, stored at —75° until use. Heated samples exposed to 56° for 45 min.

[c] Rabbit serum diluted 1:2 in PPLO broth. Guinea pig serum added at final dilution of 1:200.

[d] PI = serum collected 2 days before initial inoculation.

[e] ND = not done.

[f] MI = metabolic inhibition titer.

[g] EI = early immune serum collected 5 days after the initial inoculation.

[h] LI = late immune sera collected 17 days after the initial and 7 days after a booster inoculation.

ber of viable organisms were used as assays of inhibition in broth by rabbit immune sera. A heat labile accessory factor is required to demonstrate metabolic inhibitory and bactericidal activity in immune sera collected early in the course of immunization. In the absence of guinea pig serum, the early immune sera show second order kinetics in their bactericidal activity. Taken together with the lack of significant lysis, even in the presence of guinea pig serum, the results with early immune sera suggest the involvement of some form of complement mediated bacteriostasis.

Significant lysis was demonstrable with sera collected after a single booster injection. This activity was dependent upon a heat labile factor. Heating of the late immune sera eliminates the lytic effect but does not destroy the MI or bactericidal activity. This suggests that the MI and bactericidal activity of the heated late immune sera is not mediated by a complement dependent mechanism. The nature of the nonlytic mechanisms of inhibition and the class of globulins involved is currently being investigated.

Summary. Several immune mechanisms of inhibition develop during the course of immunization of rabbits with *Mycoplasma pneumoniae*. A nonlytic complement-mediated immune inhibition was demonstrable in sera collected early in the immune response. A lytic mechanism, dependent upon a heat labile factor, was demonstrable only with sera collected late in the immunization. The presence of an immune inhibitory activity not dependent on a heat labile accessory factor was also detected in the latter sera.

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