

Mycoplasma-Associated Immunosuppression: Effect on Hemagglutinin Response to Common Antigens in Rabbits

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A *Mycoplasma*-associated immunosuppression of the primary and secondary hemagglutinin response to a common gram-negative antigen of *Escherichia coli* (014) was demonstrated in the rabbit when preincubated mixtures of common gram-negative antigen and *Mycoplasma arthritidis* membranes were injected intravenously. A similar immunosuppression was demonstrated only for the secondary hemagglutinin response to a common gram-positive antigen of *Staphylococcus aureus* when preincubated mixtures of common gram-positive antigen and *M. arthritidis* membranes were employed as immunizing materials. The immunosuppressive effect occurred with small quantities of *M. arthritidis* membranes and appeared not to be limited to the host in which arthritogenic properties of the organism were manifested.

Various strains of *Mycoplasma arthritidis* have been shown by a number of investigators (5, 10, 17) to be pathogenic to rats and mice, producing joint inflammation resembling in some ways human rheumatoid disease. Experimental production of arthritis in rats or mice has been useful in elucidating immunological and histopathological characteristics of joint disease (7-9). An immunosuppressive effect of *M. arthritidis* (PN) on humoral antibody response of infected rats to the Pseudomonas phage ϕ has been demonstrated by Kaklamanis and Pavlatos (13). Simultaneous injection of *M. arthritidis* and phage produced partial or complete suppression of phage-neutralizing antibody. However, there seems to be no information on immune responses to other stimuli in the presence of mycoplasmas in animals other than rodent hosts.

A unique type of suppression of the immune response in rabbits by materials associated with antigens has been described in the past few years by Whang and Neter (19-22). Alpha protein, lipopolysaccharide, endotoxin and its lipid A component, cardiolipin, normal serum, and chlorphenesin inhibited formation of antibody to common gram-negative (CA[-]) or gram-positive (CA[+]) antigen (16, 21, 24). The suppression of antibody response depended on incubation of the inhibitor with the antigen prior to immunization. The present study was undertaken to determine the effect of membrane components of *M. arthritidis* (158P10) on hemagglutinin (HA) response of the rabbit to

the CA(-) described by Kunin et al. (14) and the CA(+) demonstrated by Rantz et al. (18).

MATERIALS AND METHODS

Organisms. *M. arthritidis* (158P10) obtained from B. C. Cole, University of Utah College of Medicine, Salt Lake City, Utah, was used throughout the study. *Escherichia coli* (014) obtained from the American Type Culture Collection of Bethesda, Md. and a strain of coagulase-positive *Staphylococcus aureus* isolated from a clinical specimen at Loma Linda University Hospital, Loma Linda, Calif., were employed for the preparation of CA(-) and CA(+), respectively.

Preparation of *M. arthritidis* membranes. *M. arthritidis* (158P10) was cultured three successive times in rabbit-infusion broth enriched with rabbit serum, fresh yeast extract, and arginine hydrochloride (6, 20). The rabbit-infusion broth was prepared by heating to boiling 10 g of rabbit meat ground in a Waring blender in 80 ml of distilled water. After standing for 1 h, the mixture was filtered through gauze and Whatman no. 1 filter paper. Peptone (0.8 g), sodium chloride (0.4 g), and arginine hydrochloride (0.45 g) were added, and the mixture was heated to dissolve the ingredients. The broth was adjusted to a pH of 7.8 and autoclaved.

Immediately before use, 5% rabbit serum (vol/vol) and 5% fresh yeast extract (vol/vol) were added. A 10-ml amount of enriched rabbit-infusion broth was inoculated with 1 ml of stock culture containing 10^6 *M. arthritidis* (158P10) cells, and was incubated at 37 C for 48 h. This 10-ml culture was transferred aseptically to 90 ml of enriched rabbit-infusion broth and incubated at 37 C for 48 h, and the number of organisms (colony-forming units) was estimated by plate counts. Cultured cells were harvested by centrif-

ugation at $10,000 \times g$ for 30 min in a model RC-2 refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The pellet was washed in 30 ml of phosphate hemagglutination buffer (pH 7.3; BBL) and re-centrifuged at the same speed for 30 min. The cells were ruptured by osmotic shock by placing them in 4.0 ml of sterile distilled water at 37 C for 30 min. Lysis of cells was demonstrated by failure to obtain colony-forming units in appropriate cultural medium. The mixtures were centrifuged at $10,000 \times g$ for 30 min, washed with another 4.0 ml of sterile distilled water, sedimented again by centrifugation, and stored at -65 C. The membrane preparation was thawed and diluted 1:10 in phosphate buffer immediately prior to mixing with the common antigens.

Preparation of CA(-) and CA(+). *E. coli* (014) or *S. aureus* cells were individually grown on brain veal agar (Difco) in Kolle flasks for 18 h at 37 C, and were washed with phosphate buffer to remove medium constituents according to the technique of Suzuki et al. (19). Suspensions of the gram-negative organisms were transferred to sterile centrifuge tubes, heated in boiling water for 1 h, and centrifuged at $10,000 \times g$ for 20 min. The supernatants were mixed with ethanol to a final ethanol concentration of 85% and allowed to stand at room temperature for 18 h. After centrifugation at the same speed for another 20 min, the supernatants were allowed to evaporate to dryness at 37 C, and the residue was redissolved in phosphate buffer to the original volume of 25 ml.

Suspensions of the gram-positive organisms were treated similarly except that, since the CA(+) is ethanol insoluble, the sediment obtained after ethanol treatment and centrifugation at $10,000 \times g$ for 20 min was redissolved in phosphate buffer to the original volume of 25 ml. Both antigens were stored at 5 C and diluted 1:10 in phosphate buffer prior to use.

Immunization of rabbits. Male New Zealand rabbits weighing between 2 and 3 kg were given three intravenous (i.v.) injections of 1-ml mixtures of the CA(-) or the CA(+) with mycoplasmal membranes at 7-day intervals. Mixtures consisted either of 0.1 ml of the CA(-) or the CA(+) and 0.9 ml of membranes (1:10) or 0.5 ml of the CA(-) or the CA(+) and 0.5 ml of membranes (1:1). All mixtures were incubated in a water bath at 37 C for 30 min prior to injection into the marginal ear vein. Control rabbits were immunized in an identical manner with the mixtures of the CA(-) or the CA(+) with phosphate buffer. Single booster injections of the same materials were given to animals after an appropriate rest period, during which time their antibody titers had fallen to the pre-immunization levels.

Passive HA test. Titers of HA antibodies against CA(-) or CA(+) were determined on the rabbit sera obtained prior to immunization, 3, 7, 14, and 21 days after the initial immunization, and again at 7 and 14 days after the booster injections, according to the technique of Whang and Neter (21). Rabbit erythrocytes (2.5%) were washed three times in phosphate HA buffer (pH 7.3; BBL). The CA(-) or CA(+) diluted 1:10 in buffer was added to packed erythrocytes to restore original volumes. Mixtures were incubated at 37 C for 30 min and washed three times

in buffer to remove unattached antigen. Serum samples were inactivated at 56 C for 30 min, and serial twofold dilutions (0.2 ml) were mixed with equal volumes of antigen-coated or unaltered erythrocytes (2.5%). The mixtures were incubated in a water bath at 37 C for 30 min. HA titers were determined after centrifugation for 2 min at $1,300 \times g$ and by examining the pattern by using a concave mirror. Sera of known titer for CA(-) and CA(+) for control purposes were graciously supplied by E. Neter (Children's Hospital, Buffalo, N.Y.).

MI test. Titers of metabolic inhibition (MI) antibody against *M. arthritidis* were determined with rabbit sera by using the technique described by Cole et al. (5).

RESULTS

Primary HA response to CA(-). Groups of four New Zealand rabbits were inoculated i.v. three times with 1-ml preincubated mixtures of CA(-)-*M. arthritidis* membranes (1:10 or 1:1) at 7-day intervals. Control rabbits were inoculated with preincubated CA(-) buffer mixtures (1:10 or 1:1) at the same time intervals. On all sampling days, the mean HA titers were lower for animals receiving CA(-) membrane mixtures (Table 1). Three animals in the test group yielded titers of 20 or less on day 21, but three animals of the control group had titers of 80 or more on the same day.

Secondary HA response to CA(-). The control and the test rabbits from each group were given single booster injections of the same materials used for primary immunization after a 2-month period during which HA titers had returned to pre-immunization levels. Serum samples were obtained prior to administration of the booster injections and 7 and 14 days later. The CA(-)-membrane mixtures of both dilutions produced a diminution of antibody response to CA(-) when the HA titers of rabbits receiving mycoplasmal membranes were compared with those of animals receiving CA(-) in buffer (Table 2). The highest individual HA titer of a rabbit receiving *M. arthritidis* membranes was 80.

Primary HA response to CA(+). Groups of four New Zealand rabbits were inoculated i.v. three times with 1-ml of preincubated mixtures of CA(+)-*M. arthritidis* membranes (1:10 or 1:1) at 7-day intervals. Control rabbits were inoculated with preincubated CA(+)-buffer mixtures (1:10 or 1:1) at the same time intervals. Antibody response to CA(+) was measured by means of passive hemagglutination of rabbit cells coated with CA(+) from *S. aureus* as the indicator antigen. Mycoplasmal membranes in the 1:10 dilutions did not appear to substantially affect the titer or the time of

TABLE 1. Effect of *M. arthritis* membranes on primary HA response of rabbits to CA(-)

Immunizing materials	Mean HA titers on day				
	0	3	7	14	21
CA(-) + buffer (1:10)	<10	325 (10-640) ^a	360 (80-640)	200 (80-320)	200 (80-320)
CA(-) + <i>M. arthritis</i> membranes (1:10) . . .	<10	55 (10-160)	35 (10-80)	35 (10-80)	25 (10-40)
CA(-) + buffer (1:1)	<10	15 (10-20)	180 (40-320)	340 (40-640)	480 (320-640)
CA(-) + <i>M. arthritis</i> membranes (1:1)	<10	13 (10-20)	65 (20-80)	60 (20-160)	18 (10-40)

^a Range of HA titers.

TABLE 2. Effect of *M. arthritis* membranes on secondary HA response of rabbits to CA(-)

Immunizing materials	Mean HA titers on day		
	0	7	14
CA(-) + buffer (1:10) . . .	<10	320	400 (160-640) ^a
CA(-) + <i>M. arthritis</i> membranes (1:10)	<10	25 (10-40)	<10
CA(-) + buffer (1:1)	<10	170 (20-320)	180 (40-320)
CA(-) + <i>M. arthritis</i> membranes (1:1)	<10	80	60 (40-80)

^a Range of HA titers.

peaking (Table 3). A fivefold increase in CA(+) appeared to paralyze the immune response. However, mixing the CA(+) with membranes in the 1:1 ratio seemed to restore the ability of rabbits to respond to CA(+). The mean maximal antibody titer of 280 was obtained for test animals on day 3, whereas the mean antibody titer for the control group was only 15 on the same day.

Secondary HA response to CA(+). The control and the test rabbits from each group were given single booster injections of the same materials used for primary immunization after a 2-month period during which HA titers had returned to pre-immunization levels. Serum samples were obtained prior to administration of the booster injections and 7 and 14 days later. Antigen-membrane mixtures (1:10) appeared to inhibit the secondary response (Table 4). Titers of <10 were obtained for all animals receiving CA(+)-membrane mixtures (1:10), but animals of the control group had a mean maximal titer of 240 on day 14.

MI antibody response to *M. arthritis* membranes. To determine the effect of simultaneous injection of the gram-negative or gram-

positive antigen with *M. arthritis* membrane antigen on the antibody response to *M. arthritis*, the MI titers of sera taken at different intervals were measured. MI antibody against *M. arthritis* was not detected in any of the rabbits immunized with mixtures of either CA(-) or CA(+) and phosphate buffer. The MI antibody response of rabbits immunized with mixtures of CA(-) or CA(+) with *M. arthritis* membranes is shown in Table 5. In most instances, the mean MI titers of rabbits immunized with CA(-)-membrane mixtures were significantly higher than those of rabbits immunized with CA(+)-membrane mixtures.

DISCUSSION

Preincubated mixtures of membranes isolated from *M. arthritis* (158P10) and the CA(-) from *E. coli* (014) suppressed the HA response of rabbits against CA(-). Even increasing the amount of the CA(-) fivefold, it did not overcome the immunosuppressive effect of membranes. The interference in the secondary response to the CA(-) by mycoplasmal membranes paralleled the observed immunosuppressive effect of the primary response. When the ratio of the CA(-) to the membranes was 1:10, inhibition of the secondary response was greater than with the 1:1 mixtures, thereby indicating a dependence on proportion. The extent of inhibition by cardiolipin was also demonstrated by Whang and Neter (22) to be related to antigen dose.

The effect of preincubated mixtures of *M. arthritis* (158P10) membranes and CA(+) from *S. aureus* on primary HA antibody response of rabbits to the CA(+) also depended on membrane concentrations. The lack of significant interference with antibody synthesis in the primary response by using high concentrations of membranes may reflect differences in CA(-) and CA(+) determinants or paralysis by the larger quantity of CA(+). Restoration of im-

TABLE 3. Effect of *M. arthritis* membranes on primary HA response of rabbits to CA(+)

Immunizing materials	Mean HA titers on day				
	0	3	7	14	21
CA(+) + buffer (1:10)	<10	160	80	60 (40-80) ^a	50 (20-80)
CA(+) <i>M. arthritis</i> membranes (1:10)	<10	105 (10-320)	85 (10-160)	85 (10-160)	45 (10-80)
CA(+) + buffer (1:1)	<10	15 (10-20)	<10	<10	<10
CA(+) + <i>M. arthritis</i> membranes (1:1)	<10	280 (160-640)	160	80	80

^a Range of HA titers.

TABLE 4. Effect of *M. arthritis* membranes on secondary HA response of rabbits to CA(+)

Immunizing materials	Mean HA titers on day		
	0	7	14
CA(+) + buffer (1:10) ..	<10	240 (80-320) ^a	240 (160-320)
CA(+) + <i>M. arthritis</i> membranes (1:10) ...	<10	<10	<10
CA(+) + buffer (1:1) ..	<10	<10	<10
CA(+) + <i>M. arthritis</i> membranes (1:1)	<10	15 (10-20)	10

^a Range of HA titers.

munogenicity by the 1:1 CA(+)-membrane mixtures may have been associated with less competition for sites on antibody-producing cells if membranes complexed with or masked CA(+) determinants.

The secondary responses to antigen-buffer mixtures (1:10) were typically of greater magnitude than those exhibited after primary immunization. The lack of demonstrable antibodies in animals receiving membranes and CA(+) in a 1:10 proportion suggests that in at least two animals priming had not occurred. The secondary responses of animals receiving fivefold increases in common antigen with or without membranes remained effectively paralyzed.

It could be expected that various components of *M. arthritis* membranes might affect antibody synthesis to bacterial antigens in different ways since the physiochemical characteristics of the CA(-) and of the CA(+) are not the same. Hammarström (11) reported the immunogenic portion of CA of *E. coli* (014) to be part of the core structure. Chorprenning and Dodd (4) postulated that two different CA(+)'s occur, at least one of which is present in many foods. An antigen from streptococci studied by Moskowitz (15) and Jackson and Moskowitz (12) revealed

the presence of a polymer of glycerophosphate and D-alanine which reacted with antibodies. The possibility that different mechanisms may be involved in the immunosuppression of antigenic response to specific antigens must be considered. Further studies are needed to identify the specific determinants of the CA(-) and the CA(+).

All animals injected simultaneously with the CA(-)-membrane mixtures produced significantly high titers of MI antibodies against *M. arthritis* both after primary immunization and in response to the booster injections. It was noted that, although HA response against CA(-) was suppressed when CA(-)-membrane mixtures were injected, the production of MI against *M. arthritis* was apparently not affected (Tables 1, 2, and 5). Competitive inhibition may account for the lower HA titers if antibody-producing cells had different affinities for the immunogenic determinants of the CA(-) and of the mycoplasmal membranes. A comparison of the HA and the MI antibody responses induced by CA(+)-membrane mixtures revealed that neither the primary nor the secondary responses were associated with high HA or MI antibody titers (Tables 3-5). With the CA(+)-membrane mixtures, the primary MI antibody titers were lower than those obtained with the CA(-)-membrane mixtures. The low MI titers may indicate that responses of animals to stimulation by immunogenic determinants for MI antibodies may also be subject to the effect of competition by the alcohol soluble CA(+).

Antigens can act as effective competitors if animals are injected simultaneously with heterologous antigen (1). Williams et al. (25) demonstrated the propensity of mycoplasmal membranes to incorporate environmental components. Growth of mycoplasmas in rabbit-infusion medium could provide the opportunity for *M. arthritis* to act as a vehicle for heterolo-

TABLE 5. Mean MI titers of rabbits after primary and secondary immunizations

Immunizing materials	MI on days after primary immunization					MI on days after secondary immunization		
	0	3	7	14	21	0	7	14
CA(-) + <i>M. arthritis</i> membranes (1:10)	<10	<10	88	960	960	<10	1,280	1,920
CA(-) + <i>M. arthritis</i> membranes (1:1)	<10	25	415	760	720	<10	1,280	1,280
CA(+) + <i>M. arthritis</i> membranes (1:10)	<10	13	<10	<10	28	<10	45	80
CA(+) + <i>M. arthritis</i> membranes (1:1)	<10	85	88	250	150	<10	50	1,300

gous antigens. Conversely, a specific lipid or protein membrane moiety of *M. arthritis* could act as an immunosuppressive agent. The suppression of hemagglutinin responses to a CA(-) or a CA(+) demonstrated by Whang and Neter (19-22) suggests that a variety of materials can promote immunosuppression.

Competitive inhibition by membrane components may explain, in part, the failure to demonstrate MI antibodies in *M. arthritis*-induced arthritis in rats. Furthermore, it could explain Bartholomew's (2) inability to demonstrate growth-inhibiting antibodies in sera of patients with rheumatoid arthritis from whose joints mycoplasmas have been isolated.

Studies by Cahill et al. (3) indicated that the presence of a heterogenetic antigen common to membranes of *M. arthritis* and rat tissue may explain the lack of MI antibody production in the rodent host. However, the chemical nature of the antigen promoting this biological mimicry has not been identified.

The current study suggests that the immunosuppressive effects are not host specific and that they may be effective in very low concentrations. Infection does not appear to be a prerequisite for immunosuppression. Furthermore, the effect could be rather widespread since responses to both CA(-) and CA(+) were inhibited.

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