

Protection of Rats against *Mycoplasma arthritidis*-Induced Arthritis by Active and Passive Immunizations with Two Surface Antigens

LEIGH RICE WASHBURN* AND ELIZABETH J. WEAVER

Department of Microbiology, University of South Dakota School of Medicine, Vermillion, South Dakota 57069-2390

Received 28 October 1996/Returned for modification 8 January 1997/Accepted 4 February 1997

We previously identified two surface-exposed *Mycoplasma arthritidis* protein antigens, designated MAA1 and MAA2, that may be involved in cytoadherence. Since adherence to host tissues is an important first step in most bacterial infections, we suggest that MAA1 and MAA2 may be virulence factors for *M. arthritidis*. In order to provide evidence for such a role, we conducted a series of experiments in which rats were actively immunized with each of these proteins purified from sodium dodecyl sulfate-polyacrylamide gels or passively immunized with poly- or monoclonal antibodies against MAA1 and MAA2. In each case, immunity against MAA1 and MAA2 conferred at least partial protection against *M. arthritidis*-induced disease. The greatest protection was achieved by passive immunization with monoclonal antibody A9a, directed against a surface-exposed epitope of putative adhesin MAA1. Because protective immunity in most bacterial infections is directed against major virulence factors, these results suggest that MAA1 and MAA2 may play a role in the pathogenesis of *M. arthritidis*-induced arthritis of rats, possibly by mediating initial colonization of joint tissues.

Mycoplasma arthritidis causes an acute, self-limiting, but often severe polyarthritis of rats under experimental conditions (10). It has also been reported to occur in wild rats under natural conditions (11, 31), but little is known about transmission or progression of disease in the wild. *M. arthritidis* pathogenesis is not well understood, although several potential virulence factors have been identified in recent years, including the *M. arthritidis* superantigen MAM (3), an as-yet-unidentified factor carried by virulence-associated bacteriophage MAV1 (17), and two surface proteins thought to be involved in cytoadherence (24). So far, there is no direct evidence for a role for MAM in the rat disease, although its involvement in a toxic shock-like condition in mice is well established (6), and the possibility that it plays no role in rats is highly unlikely. The association of MAV1 lysogeny with virulence of *M. arthritidis* is also well established (17), and although the virulence factor it carries has not been identified, several candidate open reading frames have been found (16). In addition to these, a number of mechanisms have been suggested for other *Mycoplasma* species that may play a role in *M. arthritidis*-induced disease as well, and current thinking concerning mycoplasmal pathogenesis is the subject of recent reviews by Tryon and Baseman (15) and Simecka et al. (14).

Thus, there are many possible mechanisms by which mycoplasmas can cause tissue damage, both directly and indirectly via immunological pathways. One thing these mechanisms all have in common is the requirement for attachment of the microbe to host tissues, and cytoadherence is understood to be the first and often most critical step in many bacterial infections. We have identified two surface antigens, designated MAA1 and MAA2, that may be involved in this process (24), although the original study was conducted primarily with an in vitro tissue culture adherence assay system, and little evidence

of a role for these proteins in pathogenesis was available at the time. One way of gathering evidence for such a role is based on the observation that protective immunity in many bacterial infections is directed against those cell components that function as major virulence factors. Therefore, if we could demonstrate that MAA1 and/or MAA2 could elicit protective immunity in rats, this would be a major step toward proving their importance in *M. arthritidis* pathogenesis. This paper describes experiments in which rats were actively and passively immunized against MAA1 and MAA2 and which show that both antigens were in fact able to elicit at least partial protection against arthritis induced by intravenous challenge with viable mycoplasmas.

(Parts of this study were presented at the 96th General Meeting of the American Society for Microbiology, New Orleans, La., 1996 [30], and at the 11th International Congress of the International Organization for Mycoplasmaology, Orlando, Fla., 1996 [29]).

MATERIALS AND METHODS

Mycoplasmas and mycoplasmal antigens. Immunizing antigens and challenge injections were prepared from *M. arthritidis* 158p10p9 (9). Viable mycoplasmas for challenge injection were derived from frozen stocks prepared as follows. Mycoplasmas were grown overnight in 50 ml of a modified Edward-type broth medium supplemented with 7.5% (vol/vol) heat-inactivated horse serum and 5% (vol/vol) fresh yeast extract (20), transferred to 1 liter of fresh medium, incubated for an additional 18 h, concentrated by centrifugation, suspended in 20 ml of suspending medium containing 15% (wt/vol) sucrose in place of horse serum, and frozen in 1-ml aliquots at -70°C . These stock cultures usually contained approximately 10^{11} CFU/ml. Immediately prior to injection, one vial was thawed and the contents were diluted in suspending medium to a concentration of 3.0×10^9 CFU/ml. Each rat was injected with 0.3 ml of this suspension (10^9 CFU).

Immunizing antigens were prepared from *M. arthritidis* membrane proteins sliced from polyacrylamide gels as follows. An *M. arthritidis* suspension containing 10 mg of protein was extracted with Triton X-114 (Boehringer GmbH, Mannheim, Germany) as described by Bordier (1). The hydrophobic phase was diluted to 1 ml with phosphate-buffered saline (PBS), treated with 1 ml of electrophoresis solubilizing solution (0.05 M Tris [pH 6.8] containing 2% [vol/vol] glycerol, 0.5% [vol/vol] 2-mercaptoethanol, and 2% [wt/vol] sodium dodecyl sulfate [SDS]), boiled for 3 min, and electrophoresed on a 6% (wt/vol) acrylamide SDS-polyacrylamide slab gel. The gel was stained with sterile 0.3 M CuCl_2 (13); bands corresponding to the two putative adhesins MAA1 and MAA2

* Corresponding author. Mailing address: Department of Microbiology, University of South Dakota School of Medicine, 237 Lee Building, Vermillion, SD 57069-2390. Phone: (605) 677-5170. Fax: (605) 677-5658. E-mail: LWASHBUR@SUNBIRD.USD.EDU.

were sliced from the gel with sterile razor blades, destained with sterile 0.25 M EDTA-0.25 M Tris (pH 9.0), and stored at -70°C until use.

Western immunoblot and antibody assays. Enzyme-linked immunosorbent assays (ELISAs) were performed on rat and rabbit sera and mouse monoclonal antibodies (MAbs) against a whole-cell lysate of *M. arthritidis* 158p10p9 as previously described (21). Secondary antibodies were peroxidase-conjugated goat anti-rat, goat anti-rabbit, and rabbit anti-mouse immunoglobulin G (IgG) (whole-molecule-specific; Cappel, Organon Teknika, Durham, N.C.) diluted 1:1,600, 1:800, and 1:1,600, respectively. Metabolism inhibition (MI) antibody assays were performed as previously described (19). Titers are expressed as \log_2 of the endpoint dilution factor.

Western immunoblotting was performed as previously described, using the same peroxidase-conjugated antisera at the same dilutions as were used for ELISA and a substrate consisting of H_2O_2 -4-chloro-1-naphthol (28). Rat and rabbit sera were tested at a dilution of 1:40; MAbs were usually used at 1:800.

Mono- and polyclonal antisera. MAbs A9a and 7a against MAA1 and MAA2, respectively, were described previously (24); MAb A9a was derived from the same clone as the MAb G9a described in reference 24. Both MAbs were classified as IgG2a. They were purified from mouse ascites fluids by protein A-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) affinity chromatography according to the manufacturer's instructions, concentrated, dialyzed against PBS, and stored at -20°C until use.

For preparation of polyclonal monospecific antisera against MAA1 and MAA2, one rabbit was immunized with each protein as follows. Polyacrylamide gel slices containing MAA1 or MAA2, prepared from 10 mg of total mycoplasma protein as described above, were minced with scalpel blades and emulsified in sterile 0.15 M NaCl (saline) by forcing the gel suspension several times through first an 18- and then a 20-gauge needle. A sample from each preparation was reelectrophoresed on a 6% SDS-polyacrylamide gel and tested by Western immunoblotting against rabbit antiserum against intact *M. arthritidis* 158p10p9 (see below) and MAbs A9a and 7a to confirm the presence of the appropriate antigens. For the primary immunizing injections, gel fragments were suspended in 1 ml of saline and further emulsified in an additional 1 ml of incomplete Freund's adjuvant; each rabbit received three 0.5-ml subcutaneous (s.c.) injections of this preparation. Rabbits were boosted twice at 4-month intervals with s.c. injections of the same amount of gel emulsified in saline but without adjuvant. Antibody responses were assessed by ELISA and MI assay as described above. Two weeks after the second booster injection, rabbits were sacrificed and exsanguinated by cardiac puncture under ketamine (Fort Dodge Laboratories, Fort Dodge, Iowa) anesthesia.

Polyclonal antiserum against intact *M. arthritidis* 158p10p9 was prepared as previously described (26, 27).

Immunizations and induction and assessment of arthritis. Male LEW/SsNHsd rats purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) were used in all experiments. Rats were housed in wire cages and provided standard food and water *ad libitum*.

In the first experiment, rats were actively immunized with MAA1 and MAA2 sliced from SDS-polyacrylamide gels. Rats were divided into three groups of eight each. Antigens for immunization were prepared from emulsified gel slices as described above. For each set of injections, gel slices containing single proteins were pooled from two SDS-polyacrylamide gels (each set of protein bands was derived from a total of 20 mg of mycoplasma protein), minced, emulsified in the appropriate diluent, and divided evenly among the eight rats. For the primary injection, gel fragments were emulsified in saline and incomplete Freund's adjuvant (1:1) and injected s.c.; for booster injections, gel fragments were emulsified in saline only and injected intraperitoneally. Booster injections were given at weekly intervals beginning 1 week after the primary injection. Antibody titers were measured by ELISA versus a whole-cell lysate of *M. arthritidis* 1 week after each booster injection. Rats immunized with MAA1 received one primary and six booster injections; rats immunized with MAA2 received one primary and four booster injections. A third group was immunized with emulsified polyacrylamide gel not containing any mycoplasma protein; this group also received one primary and four booster injections. A previous study indicated that injection with polyacrylamide alone had no adverse effects over the time frame used here (23). One week after the final booster injection, rats were challenged by intravenous (i.v.) injection of 10^9 CFU of *M. arthritidis* 158p10p9 into a caudal vein. At the time of challenge, rats weighed an average of 295.9 ± 28.0 (standard deviation [SD]) g.

In the second experiment, rats were passively immunized with polyclonal monospecific antisera against MAA1 and MAA2. Rats weighing an average of 108.8 ± 5.2 g were divided into four groups of six each. Group 1 received 0.5 ml of rabbit anti-MAA1 by i.v. injection into a caudal vein, group 2 received 0.5 ml of rabbit anti-MAA2, group 3 received 0.5 ml of rabbit anti-*M. arthritidis* 158p10p9, and group 4 received 0.5 ml of nonimmune rabbit serum. ELISA antibody titers of these sera were 8.32, 8.32, 12.32, and <3.32 , respectively (corresponding to serum endpoint dilution factors of 320, 320, 5,120, and <10 , respectively; titers of <3.32 indicate no detectable ELISA antibody), and MI titers were 1.00, <1.00 , 8.00, and <1.00 , respectively (corresponding to serum endpoint dilution factors of 2, <2 , 256, and <2 , respectively; titers of <1.00 indicate no detectable MI antibody). Two hours after the immunizing injection, each rat was challenged with 10^9 CFU of viable *M. arthritidis*.

In the third experiment, rats were passively immunized with MAbs A9a and 7a. Rats weighing an average of 128.0 ± 27.6 g were divided into three groups of six

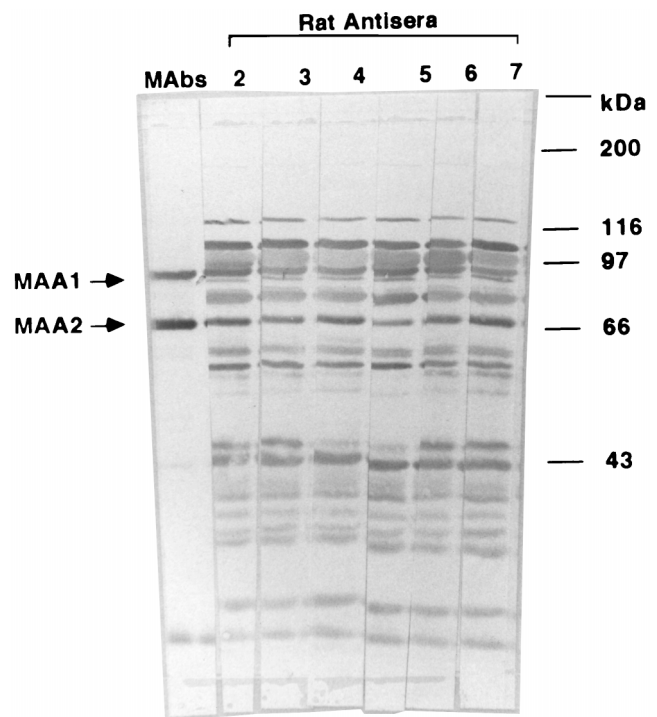


FIG. 1. Recognition of putative adhesins MAA1 and MAA2 by rats convalescing from *M. arthritidis*-induced arthritis. Triton X-114-extracted membrane proteins of *M. arthritidis* 158p10p9 were electrophoresed on a 10% (wt/vol) SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to pooled MAbs A9a and 7a (lane 1) and sera from six rats injected 3 weeks previously with *M. arthritidis* 158p10 (lanes 2 through 7). Rat sera reacted with both MAA1 and MAA2.

each. Group 1 received 0.3 ml of sterile PBS (the diluent in which the MAB solutions were prepared) by i.v. injection into a caudal vein; group 2 received 0.3 ml of MAb A9a (3 mg of IgG protein, ELISA antibody titer of 14.32 [serum endpoint dilution factor of 20,480], no detectable MI antibody), and group 3 received 0.3 ml of MAb 7a (3 mg of IgG protein, ELISA titer of 14.32, MI titer of 1.00 [serum endpoint dilution factor of 2]). Two hours after the immunizing injection, rats were challenged with 10^9 CFU of viable *M. arthritidis* by i.v. injection.

Rats were weighed and scored for arthritis daily for the first 7 days after injection and thereafter on days 10, 12, 14, 17, 19, and 21. Numbers of arthritic joints were also recorded at each time point. Arthritis scores were determined by methods established by Cole et al. (4) and Hannan and Hughes (12) and used by us in previous studies (17, 18, 23-25). On day 21, all rats were killed by CO_2 asphyxiation and exsanguinated by cardiac puncture.

Statistical analysis. For comparison of data from multiple groups, one-factorial analysis of variance (ANOVA) was used, with Fisher's protected least-significant difference (PLSD) test. For comparison of multiple group means with a single control, the Dunnett *t* test was used. Differences were considered significant for values of $P < 0.05$.

RESULTS

Recognition by rats of putative adhesins MAA1 and MAA2. Before attempting any immunization experiments, it was necessary to confirm that proteins MAA1 and MAA2 were in fact immunogenic for rats. Sera from six rats injected 3 weeks previously with *M. arthritidis* 158p10 were tested by Western immunoblotting against Triton X-114-extracted hydrophobic membrane proteins of *M. arthritidis* 158p10p9 (Fig. 1). Strain 158p10 differs from 158p10p9 only in that the latter has been passed nine consecutive times through mice (9); 158p10 and 158p10p9 are otherwise identical in antigenic and chromosomal DNA restriction profiles and in virulence for rats (8, 17, 28). The rats from which these sera were collected composed an unimmunized control group in a previous vaccine study

(25). Sera from all six rats reacted strongly with peptide antigens migrating identically with MAb-identified MAA1 and MAA2, suggesting that both were immunogenic for experimentally infected animals.

Active immunization with MAA1 and MAA2. In the first experiment, rats were actively immunized with MAA1 and MAA2 sliced from polyacrylamide gels as described in Materials and Methods. Great care was taken to isolate single protein bands from these gels and to keep them completely separate so that they did not become cross-contaminated. The bands were assessed for purity by Western immunoblotting against their respective MAbs and rabbit anti-*M. arthritidis* 158p10p9. When stained with both antisera, the isolated MAA2 band showed a single major peptide migrating in the 66-kDa range plus a faint lower-molecular-mass ladder pattern. This MAA2 ladder pattern is often seen on Western immunoblots overloaded with mycoplasmal protein on staining with MAb 7a and suggests a subunit composition for this protein (not shown). Western immunoblots of the isolated MAA1 band stained with MAb A9a showed a single peptide migrating at 91 kDa. Blots stained with polyclonal rabbit antiserum against *M. arthritidis* showed an additional peptide comigrating with MAA2; the second band also reacted with MAb 7a (not shown). Several different antigen preparations were similarly analyzed with similar results. Previous studies indicated that all *M. arthritidis* strains expressing the epitope recognized by MAb 7a also express the epitope recognized by MAb A9a but not vice versa (28). This does not necessarily rule out an association between the proteins bearing these epitopes but may simply reflect variation among surface-exposed epitopes in membrane proteins that are otherwise conserved among different strains. The present study suggests that a relationship may exist between these two proteins, although its nature is as yet unknown. Nevertheless, it must be pointed out that in the experiment described below, rats immunized with MAA1 also apparently received a low dose of MAA2 or a peptide containing a cross-reacting epitope. However, in a later experiment in which rats were passively immunized with MAbs recognizing epitopes unique to each protein, it was possible to separate the roles of each in eliciting protective antibodies.

The mean antibody titer against a whole-cell lysate of *M. arthritidis* 1 week after the fourth booster injection was 1.87 ± 2.65 for MAA1-immunized rats and 10.20 ± 0.64 for MAA2-immunized rats. The MAA1 group was given two additional booster injections, the final one consisting of twice the amount of antigen used in the previous injections; antibody titers increased to 3.87 ± 2.47 and 7.45 ± 1.64 after the fifth and sixth injections, respectively. A preliminary experiment indicated that additional booster injections of MAA1 were unlikely to result in a further increase in antibody titer (data not shown). Rats receiving blank gel developed no detectable antibody to *M. arthritidis* prior to the challenge injection.

Prechallenge sera were also tested by Western immunoblotting against a whole-cell lysate of *M. arthritidis* to confirm recognition of the appropriate antigens (Fig. 2). Sera from all rats recognized the antigen with which they had been immunized. In addition, sera from four of the rats receiving MAA1 also reacted with an antigen comigrating with MAA2, consistent with the observation described at the beginning of this section. Sera from both groups also reacted with a low-molecular-mass band migrating with the dye front, possibly representing degradation products.

Rats receiving MAA2 and blank gel (control group) were challenged with viable mycoplasmas 1 week after the fourth booster injection; rats receiving MAA1 were challenged with an identical dose from the same frozen stock 1 week after the

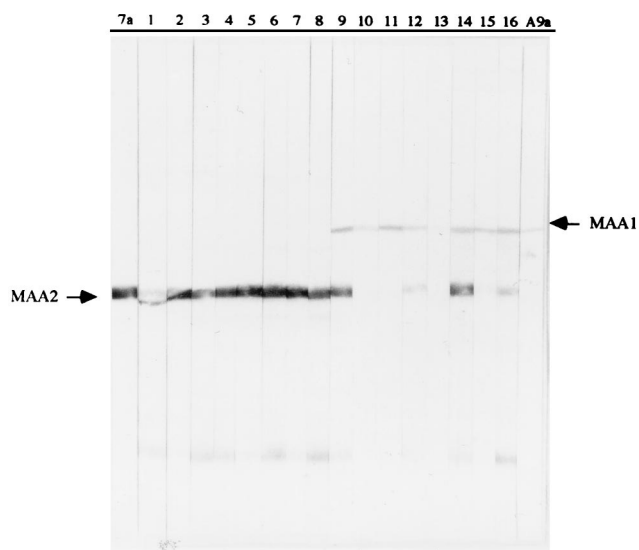


FIG. 2. Recognition of mycoplasma antigens by rats actively immunized with putative adhesins MAA1 and MAA2 just prior to the challenge injection. A whole-cell lysate of *M. arthritidis* 158p10p9 was electrophoresed on a 7.5% (wt/vol) SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed to MAb 7a (lane 7a), MAb A9a (lane A9a), sera from rats immunized with MAA2 (lanes 2 through 8), and sera from rats immunized with MAA1 (lanes 9 through 16). Rats immunized with MAA2 recognized only MAA2. All rats immunized with MAA1 recognized MAA1, but four of the eight also recognized MAA2.

sixth booster injection. Development of arthritis over the 3-week observation period and maximum scores and numbers of arthritic joints are shown in Fig. 3. MAA2-immunized rats developed significantly less arthritis (Fig. 3B; $P < 0.05$ by ANOVA/PLSD) in significantly fewer joints ($P < 0.01$ by ANOVA/PLSD) than did MAA1-immunized rats, although maximum scores for both groups were significantly less than for the blank gel-immunized control group ($P < 0.025$ for MAA1-immunized rats and $P < 0.005$ for MAA2-immunized rats by Dunnett *t* test). In addition, the maximum numbers of arthritic joints were significantly lower for the MAA2-immunized rats than for the blank gel-immunized control group ($P < 0.005$ by Dunnett *t* test).

There was greater variability in antibody response to active immunization with MAA1 than with MAA2 (see above). Regression analysis showed a significant indirect correlation between maximum arthritis scores and antibody titer prior to challenge in the MAA1-immunized group ($r = 0.837$, $P < 0.01$). Antibody titers prior to challenge were uniformly high in MAA2-immunized rats, and no such relationship was seen in this group. By the end of the 3-week observation period, average antibody titers against *M. arthritidis* in MAA1-, MAA2-, and blank gel-immunized rats were 10.04 ± 0.49 , 10.92 ± 0.53 , and 9.95 ± 0.52 , respectively. Titers in MAA2-immunized group were significantly higher than in the other two groups; there was no significant difference between titers from the MAA1- and blank gel-immunized rats.

Passive immunization of rats with polyclonal monospecific antisera against MAA1 and MAA2. Rabbits from which these polyclonal antisera were derived responded to immunization with gel-purified proteins in a manner similar to the rats in the experiment described above, in that rabbit anti-MAA1 antiserum also recognized an antigen comigrating with MAA2 but not vice versa (not shown).

Development of arthritis, maximum arthritis scores, and maximum numbers of arthritic joints in rats passively immu-

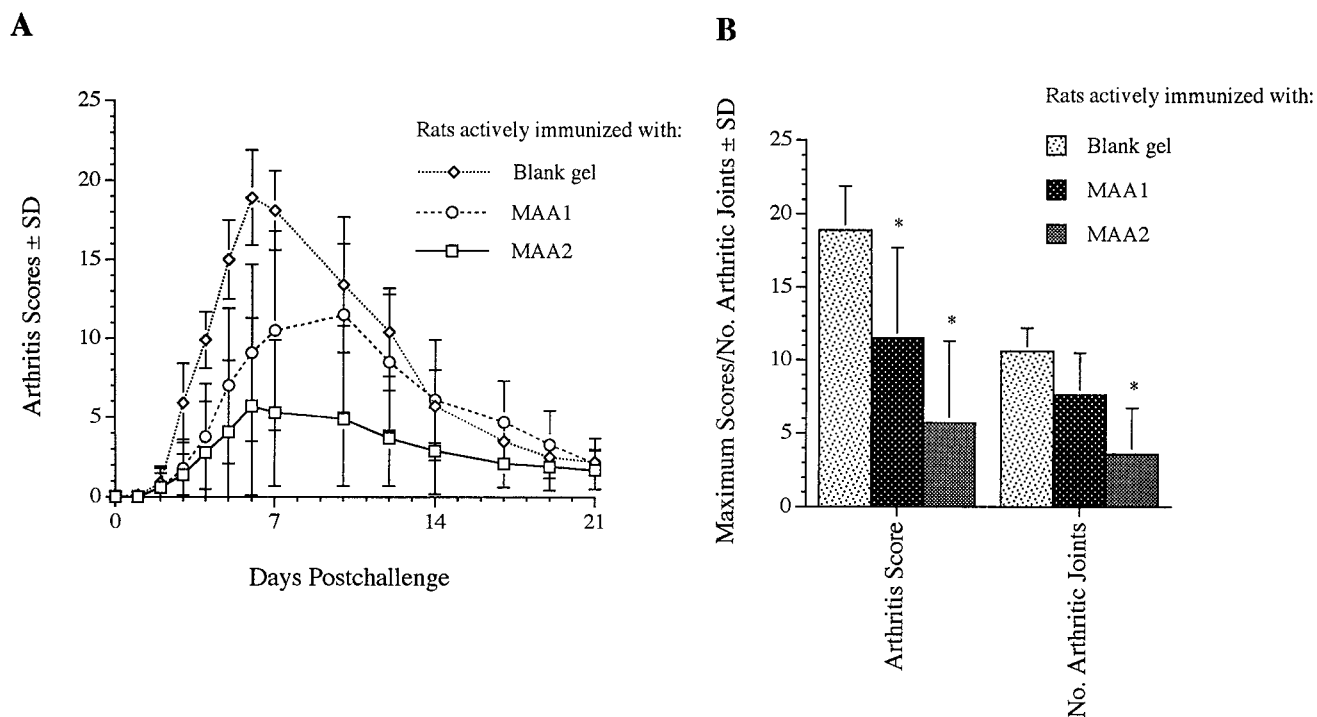


FIG. 3. Protective effect of active immunization with MAA1 and MAA2 in rats challenged with 10^9 CFU of *M. arthritidis* 158p10p9. (A) Development of arthritis over the 21-day observation period in rats actively immunized with putative adhesin MAA1 or MAA2 or with blank gel containing no mycoplasmal protein. Each point represents the mean \pm SD for eight animals. (B) Maximum scores and maximum numbers of affected joints in rats actively immunized with MAA1, MAA2, or blank gel. Each bar is the mean \pm SD for eight animals. *, significantly different ($P < 0.025$) from control group values by Dunnett *t* test.

nized with rabbit anti-MAA1 and -MAA2 were compared with those of rats passively immunized with rabbit anti-*M. arthritidis* 158p10p9 and nonimmune rabbit serum (Fig. 4). Rats receiving anti-*M. arthritidis* 158p10p9 were completely protected against infection. Rats receiving nonimmune rabbit serum developed severe arthritis, indicating that nonimmune serum afforded little or no protection. Rats receiving anti-MAA1 and -MAA2 were partially but significantly protected; that is, they developed significantly less arthritis in significantly fewer joints than rats receiving nonimmune serum, even when disease was at a maximum (Fig. 4B; $P < 0.01$ by Dunnett *t* test). In addition, by 1 week after challenge, rats receiving anti-MAA1 and anti-MAA2 had gained significantly more weight than rats receiving nonimmune serum ($P < 0.01$ and 0.005 for anti-MAA1 and anti-MAA2 rats, respectively, by Dunnett *t* test; data not shown). This was still true for rats receiving anti-MAA2 by the end of 2 weeks ($P < 0.05$), although weight gains were no longer significantly different by the end of the 3-week observation period. At no time was there any significant difference in arthritis scores, numbers of affected joints, or weight loss between groups receiving anti-MAA1 and anti-MAA2 except on day 21 postchallenge, when rats receiving anti-MAA1 had slightly but significantly lower arthritis scores ($P < 0.025$ by ANOVA/PLSD).

Antibody response was assessed by ELISA against a whole-cell lysate of *M. arthritidis* 3 weeks after challenge. Rats receiving anti-*M. arthritidis* 158p10p9 developed essentially no detectable antibody against *M. arthritidis*. Titers for the other three groups were as follows: anti-MAA1 group, 11.32 ± 1.67 ; anti-MAA2 group, 10.99 ± 1.03 ; nonimmune serum, 11.49 ± 0.75 . These figures are not significantly different.

Passive immunization with MAbs against MAA1 and MAA2.

Development of arthritis, maximum arthritis scores, and max-

imum numbers of arthritic joints in rats passively immunized with MAbs A9a and 7a versus MAA1 and MAA2, respectively, were compared with those of rats receiving PBS (Fig. 5). Protection was nearly complete in rats receiving A9a; only two of the six rats in this group developed signs of illness, consisting of low-grade arthritis involving only one or two joints in each animal. Maximum arthritis and numbers of arthritic joints were significantly less than in the unimmunized rats (PBS group) (significance level was $P < 0.005$ for both measurements by Dunnett *t* test). Rats receiving MAb 7a were also protected, although to a lesser extent. While maximum arthritis scores and numbers of affected joints were significantly less than in the control group ($P < 0.01$ and $P < 0.025$, respectively, by Dunnett *t* test), they were also significantly greater than those in the A9a-immunized group ($P < 0.05$ and $P < 0.0025$ for scores and numbers of arthritic joints, respectively, by ANOVA/PLSD). By 1 week postchallenge, rats receiving MAb A9a had gained significantly more weight than rats in both the PBS control group and the 7a-immunized group ($P < 0.01$ by ANOVA/PLSD; data not shown). This trend continued through 2 weeks postchallenge, although by 3 weeks, there was no longer a significant difference between the A9a and 7a groups; this was the only time point at which rats receiving MAb 7a showed significantly greater weight gain than did control rats ($P < 0.05$ by ANOVA/PLSD).

Rats passively immunized with MAb A9a had developed significantly less antibody against *M. arthritidis* by 3 weeks after challenge than rats receiving PBS (8.99 ± 0.82 for A9a rats versus 10.16 ± 0.41 for PBS rats; $P < 0.01$ by ANOVA/PLSD). Titers in rats receiving MAb 7a (9.49 ± 0.75) were not significantly different from those in either control or MAb A9a-immunized rats.

A second experiment was performed under similar condi-

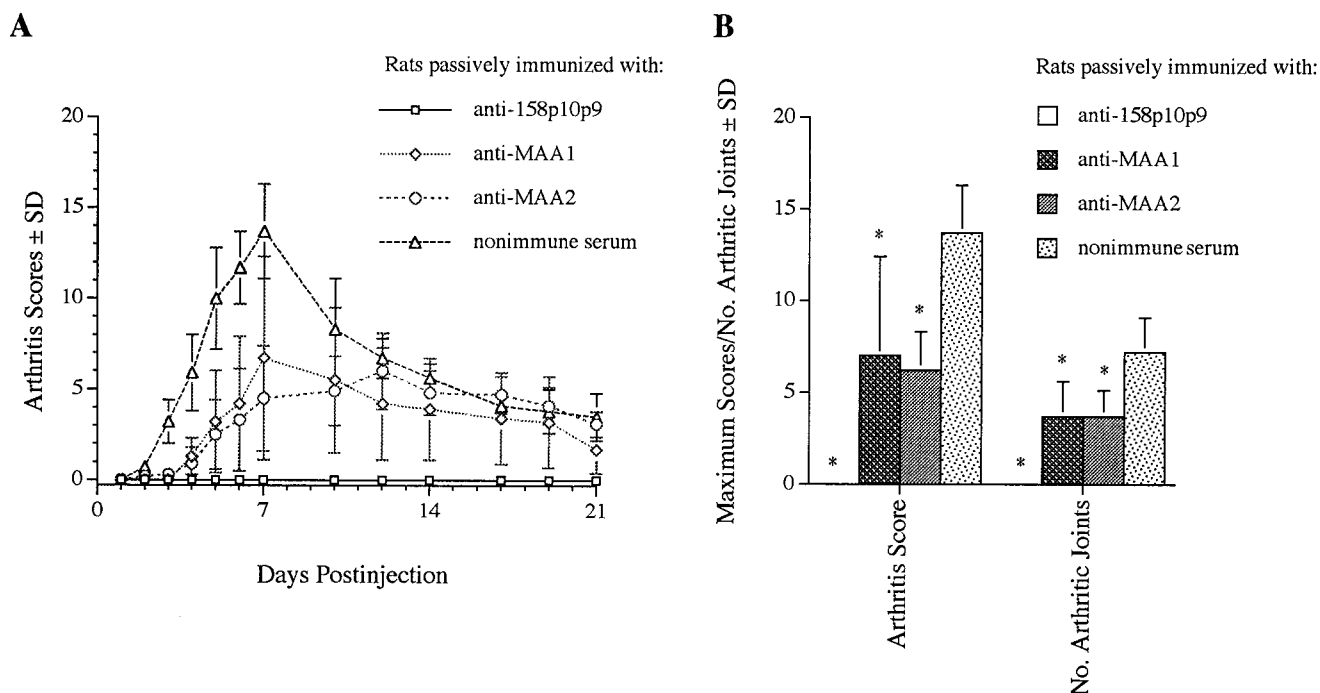


FIG. 4. Protective effect of passive immunization with polyclonal rabbit antisera in rats challenged with 10^9 CFU of viable *M. arthritis* 158p10p9. (A) Development of arthritis over the 21-day observation period in rats passively immunized with polyclonal rabbit anti-*M. arthritis* 158p10p9, anti-MAA1, anti-MAA2, or nonimmune rabbit serum. Each point represents the mean \pm SD for six animals. (B) Maximum scores and maximum numbers of affected joints in rats passively immunized with rabbit anti-*M. arthritis* 158p10p9, anti-MAA1, anti-MAA2, or nonimmune rabbit serum. Each bar is the mean \pm SD for six animals. *, values significantly different ($P < 0.01$) from those of the control group (nonimmune serum) by Dunnett t test.

tions with similar results, although this time, four of the six rats passively immunized with A9a developed low-grade arthritis involving one or two joints each (data not shown). In this experiment, an additional group was included in which rats were passively immunized with equal volumes of both MAbs. Arthritis scores, numbers of affected joints, and weight gain were not significantly different from those of rats receiving A9a alone.

DISCUSSION

Putative adhesins MAA1 and MAA2 were identified and partially characterized in a previous study in which MAbs against both proteins inhibited cytoadherence in an in vitro assay system. In addition, a spontaneously appearing stable mutant, designated LC1 and possessing a truncated version of MAA1, showed greatly reduced adherence compared to that of the wild-type parent strain 158p10p9 (24). Both proteins are exposed on the surface of the cell (24), and MAA2 is phase variable (28). Further characterization of both proteins at the molecular and genetic levels is proceeding in our laboratory. The present study was undertaken to identify a possible role for these proteins in pathogenesis of *M. arthritis*-induced arthritis of rats. One way to gather evidence for such a role was to determine whether they were able to elicit a protective immune response against experimental infection with *M. arthritis*, since protective immunity in most infectious diseases is directed against major microbial virulence factors.

In a previous immunization study (23), we showed that rats vaccinated with *M. arthritis* antigens that had been heated (100°C for 10 min), formalized, or sonicated were almost completely protected against disease, as were rats immunized with polyacrylamide gel fractions containing protein antigens in five different molecular mass ranges. In the latter experiment, the

fraction containing the highest-molecular-mass proteins elicited slightly greater protection than the other four; MAA1 would have been included in this fraction (23).

In the present study, passive immunization with polyclonal antibodies and MAbs against MAA1 and MAA2 as well as active immunization with SDS-polyacrylamide gel electrophoresis (PAGE)-derived proteins provided partial but significant protection against *M. arthritis*-induced arthritis. The active-immunization experiment and the passive-immunization experiment using polyclonal rabbit antisera provided an indication that immunity to MAA2 alone could protect, although a role for other proteins migrating in the same molecular mass range that might have been copurified with MAA1 and MAA2 could not be ruled out on the basis of these two experiments. Moreover, in both cases, the MAA1 preparations used contained small amounts of a component comigrating with MAA2 by SDS-PAGE, so on the basis of these two experiments alone, a role for MAA2 in the protective effect observed with MAA1 also could not be ruled out. However, use of MAbs in the second passive-immunization experiment did permit individual assessment of epitopes belonging to each protein, and this experiment demonstrated unequivocally that MAA1 and MAA2 alone are indeed capable of eliciting a protective response.

Evidence for the biological activity of these proteins is indirect, but as discussed above, previous work strongly suggests that they play a role in cytoadherence (24). The protective effect of adherence-inhibiting MAbs A9a and 7a provides further evidence that protective immunity is directed at least partly against cytoadherence-associated epitopes. The dramatic protective effect of passive immunization with MAb A9a compared to that achieved with equal amounts of MAb 7a further suggests that MAA1 may be more important in pathogenesis than MAA2. A role for MAA1 in *M. arthritis* virulence was first

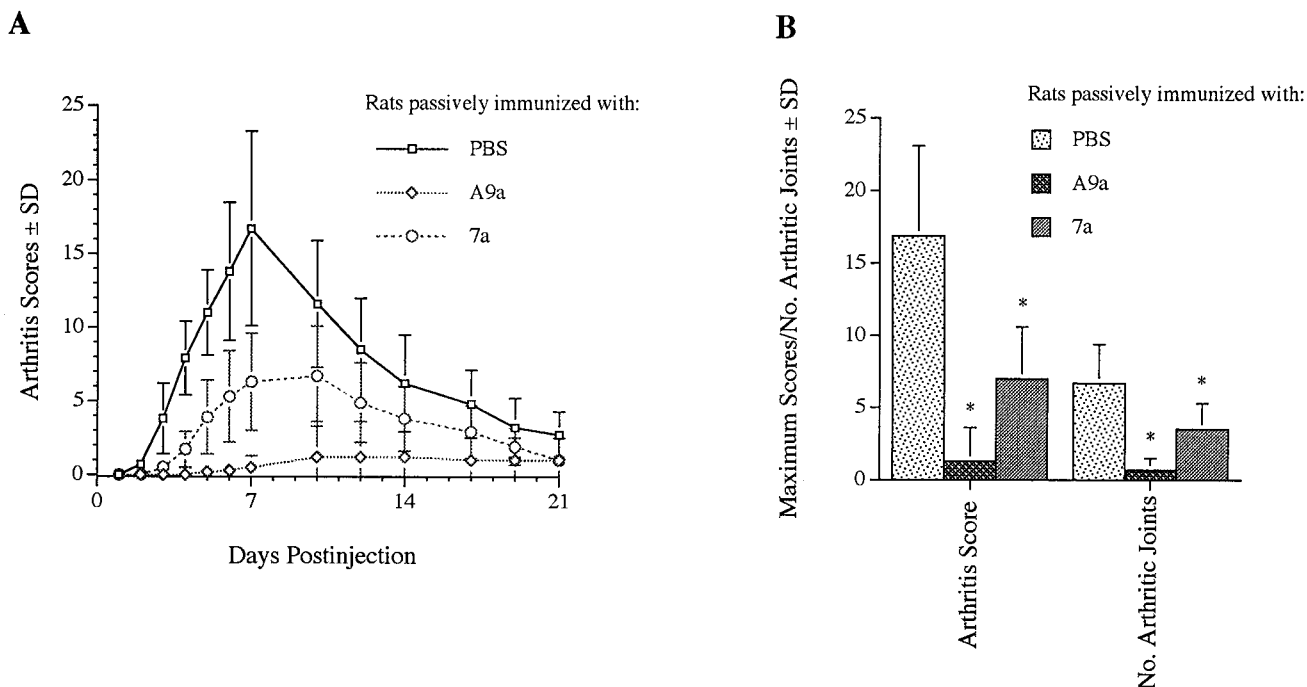


FIG. 5. Protective effect of passive immunization with MABs against MAA1 and MAA2 in rats challenged with 10^9 CFU of viable *M. arthritis* 158p10p9. (A) Development of arthritis over the 21-day observation period in rats passively immunized with MAB A9a, MAB 7a, or PBS. Each point represents the mean \pm SD for six animals. (B) Maximum scores and maximum numbers of affected joints in rats passively immunized with MAB A9a, MAB 7a, or PBS. Each bar is the mean \pm SD for six animals. *, values significantly different ($P < 0.01$) from those of the control group (PBS) by Dunnett t test.

suggested by our observation that rats injected with the LC1 mutant developed slightly but significantly less arthritis than did rats injected with the wild-type parent strain (24). The moderately low antibody response against *M. arthritis* detected 3 weeks after challenge in rats passively immunized with MAB A9a suggests that mycoplasmas did not persist long enough or in sufficient numbers in circulation after the challenge injection to stimulate a stronger response. This could have resulted from attachment inhibition alone, in that an inability to adhere to tissues after i.v. challenge may have rendered the mycoplasmas more susceptible to nonspecific clearance mechanisms. Previous work has suggested the importance of early, nonspecific clearance factors in resistance of rats to infection with this organism (25).

Other than inhibition of adherence, no specific biological activity against *M. arthritis* has been attributed to poly- and monoclonal antisera against MAA1 and MAA2; all four of the monospecific antisera used for passive immunizations showed minimal or undetectable MI antibody activity. Opsonizing antibody activity was not assessed, although it was previously reported that neither rats nor mice are capable of producing opsonizing antibodies against *M. arthritis* (8); therefore, opsonizing activity is unlikely to be present in the mouse-derived MABs, although it cannot be ruled out for the rabbit-derived polyclonal monospecific antisera. Rabbit antisera against intact *M. arthritis* typically show both strong MI and opsonizing antibody activities (5, 7, 21, 22). Thus, rats passively immunized with anti-*M. arthritis* 158p10p9 would have cleared the challenge dose very rapidly from circulation, possibly before they had the opportunity to produce a specific immune response of their own, and in fact, we detected no rat antibodies to *M. arthritis* in this group 3 weeks after the challenge injection.

Oddly, the protective response elicited on active immunization of rats with MAA1 was much diminished compared to that

achieved by passive immunization with MAB A9a. This may have been because MAA1 is not a particularly abundant protein in the *M. arthritis* cell; this is more apparent on protein-stained polyacrylamide gels (not shown) than on Western immunoblots. It means that rats actively immunized with MAA1 most likely received a lower antigen dose than did rats immunized with MAA2. It is also possible that the protective epitope on MAA1 was partially denatured by conditions preparatory to SDS-PAGE. Either of these possibilities may also explain why development of antibody was delayed in these rats and why antibody levels remained low in comparison to those in MAA2-immunized animals. Transfer of the protein to nitrocellulose may have resulted in its partial renaturation; this is suggested by its strong reaction with specific antisera on Western immunoblotting, although that signal still remains weaker than the one generated by MAA2 (Fig. 1 and 2; see also references 24 and 28). Rabbit polyclonal anti-MAA1 also was less protective than A9a, possibly because antibodies against the protective epitope were less abundant. In that regard, rabbits were also immunized with antigens subjected to denaturing prior to SDS-PAGE.

The inability, for the most part, of immunity against these proteins to confer complete protection indicates that other mycoplasma factors are also involved in pathogenesis. This is not surprising, since bacterial virulence is often a multifactorial process. Indeed, at least two additional potential virulence factors have been identified for *M. arthritis*, neither of which is known to be associated with expression of MAA1 or MAA2. These are the *M. arthritis* superantigen MAM (3) and an as-yet-uncharacterized factor carried by *M. arthritis* temperate bacteriophage MAV1, whose association with virulence was recently demonstrated by Voelker et al. (17). All *M. arthritis* strains tested so far produce MAM regardless of their virulence (2), while epitopes recognized by MABs A9a and 7a

are not expressed in all strains (28). Similarly, MAV1 is present only in virulent *M. arthritis* strains (17), while expression of these epitopes is distributed among both virulent and avirulent strains (17, 28). These observations argue against a relationship among adherence, MAM, and MAV1. A final complicating factor is the observation that all *M. arthritis* strains tested so far have the ability to adhere to rat cells in vitro regardless of their virulence (24; unpublished data), further indicating that adherence is but one step in a complex process that only virulent strains can carry to completion.

In summary, their ability to elicit protective antibodies strongly suggests that MAA1 and MAA2 are involved in the pathogenesis of *M. arthritis*-induced arthritis of rats, and previous work indicates that this role may involve cytoadherence (24). Further analysis of both of these proteins is under way.

ACKNOWLEDGMENTS

We thank Denise Riley for technical assistance and Richard Duman for preparation of many of the media and reagents used in this study.

This work was supported in part by grant R01 37924 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, by National Science Foundation grant OSR-9108773, and by the South Dakota Future Fund.

REFERENCES

- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604-1606.
- Cole, B. C. Personal communication.
- Cole, B. C., and C. L. Atkin. 1991. The *Mycoplasma arthritis* T-cell mitogen, MAM: a model superantigen. *Immunol. Today* **12**:271-276.
- Cole, B. C., J. F. Cahill, B. B. Wiley, and J. R. Ward. 1969. Immunological responses of the rat to *Mycoplasma arthritis*. *J. Bacteriol.* **98**:930-937.
- Cole, B. C., L. Golightly-Rowland, J. R. Ward, and B. B. Wiley. 1970. Immunological response of rodents to murine mycoplasmas. *Infect. Immun.* **2**:419-425.
- Cole, B. C., R. N. Thorpe, L. A. Hassell, L. R. Washburn, and J. R. Ward. 1983. Toxicity but not arthritogenicity of *Mycoplasma arthritis* associates with the haplotype expressed at the major histocompatibility complex. *Infect. Immun.* **41**:1010-1015.
- Cole, B. C., and J. R. Ward. 1973. Fate of intravenously injected *Mycoplasma arthritis* in rodents and effect of vaccines. *Infect. Immun.* **7**:416-425.
- Cole, B. C., and J. R. Ward. 1973. Interaction of *Mycoplasma arthritis* and other mycoplasmas with murine peritoneal macrophages. *Infect. Immun.* **7**:691-699.
- Cole, B. C., J. R. Ward, R. S. Jones, and J. F. Cahill. 1971. Chronic proliferative arthritis of mice induced by *Mycoplasma arthritis*. I. Induction of disease and histopathological characteristics. *Infect. Immun.* **4**:344-355.
- Cole, B. C., L. R. Washburn, and D. Taylor-Robinson. 1985. Mycoplasma-induced arthritis, p. 108-160. *In* S. Razin and M. F. Barile (ed.), *The mycoplasmas*, vol. IV. Mycoplasma pathogenicity. Academic Press, Inc., New York.
- Collier, W. A. 1939. Infectious polyarthritis of rats. *J. Pathol. Bacteriol.* **48**:579-589.
- Hannan, P. C. T., and B. O. Hughes. 1971. Reproducible polyarthritis in rats caused by *Mycoplasma arthritis*. *Ann. Rheum. Dis.* **30**:316-321.
- Lee, C., A. Levin, and D. Branton. 1987. Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* **166**:308-312.
- Simecka, J. W., J. K. Davis, M. K. Davidson, S. E. Ross, C. T. K.-H. Stadländer, and G. H. Cassell. 1992. Mycoplasma diseases of animals, p. 391-416. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
- Tryon, V. V., and J. B. Baseman. 1992. Pathogenic determinants and mechanisms, p. 457-471. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C.
- Voelker, L. L., L. R. Washburn, and K. Dybvig. 1996. Characterization and sequencing of *Mycoplasma arthritis* bacteriophage MAV1. *IOM Lett.* **4**:352.
- Voelker, L. L., K. E. Weaver, L. J. Ehle, and L. R. Washburn. 1995. Association of lysogenic bacteriophage MAV1 with virulence of *Mycoplasma arthritis*. *Infect. Immun.* **63**:4016-4023.
- Washburn, L. R. 1996. Experimental models of arthritis, p. 349-359. *In* J. G. Tully and S. Razin (ed.), *Molecular and diagnostic procedures in mycoplasmaology*, vol. II. Academic Press, Inc., New York.
- Washburn, L. R. 1983. Spectrophotometric method for determining titers of antimycoplasma metabolism inhibition antibody. *Appl. Environ. Microbiol.* **45**:343-346.
- Washburn, L. R., B. C. Cole, M. I. Gelman, and J. R. Ward. 1980. Chronic arthritis of rabbits induced by mycoplasmas. I. Clinical, microbiologic, and histologic features. *Arthritis Rheum.* **23**:825-836.
- Washburn, L. R., B. C. Cole, and J. R. Ward. 1980. Chronic arthritis of rabbits induced by mycoplasmas. II. Antibody response and the deposition of immune complexes. *Arthritis Rheum.* **23**:837-845.
- Washburn, L. R., and S. Hirsch. 1990. Comparison of four *Mycoplasma arthritis* strains by enzyme immunoassay, metabolism inhibition, one- and two-dimensional electrophoresis, and immunoblotting. *J. Clin. Microbiol.* **28**:1974-1981.
- Washburn, L. R., S. Hirsch, M. McKenzie, and L. L. Voelker. 1992. Vaccination of Lewis rats against *Mycoplasma arthritis*-induced arthritis. *Am. J. Vet. Res.* **53**:52-58.
- Washburn, L. R., S. Hirsch, and L. L. Voelker. 1993. Mechanisms of attachment of *Mycoplasma arthritis* to host cells in vitro. *Infect. Immun.* **61**:2670-2680.
- Washburn, L. R., and J. R. Ramsay. 1989. Experimental induction of arthritis in LEW rats and antibody response to four *Mycoplasma arthritis* strains. *Vet. Microbiol.* **21**:41-55.
- Washburn, L. R., J. R. Ramsay, and M. B. Andrews. 1988. Recognition of *Mycoplasma arthritis* membrane antigens by rats and rabbits: comparison by immunoblotting and radioimmunoprecipitation. *Vet. Microbiol.* **17**:45-57.
- Washburn, L. R., J. R. Ramsay, and L. K. Roberts. 1985. Characterization of the metabolism inhibition antigen of *Mycoplasma arthritis*. *Infect. Immun.* **49**:357-364.
- Washburn, L. R., L. L. Voelker, L. J. Ehle, S. Hirsch, C. Dutenhofer, K. Olson, and B. Beck. 1995. Comparison of *Mycoplasma arthritis* strains by enzyme-linked immunosorbent assay, immunoblotting, and DNA restriction analysis. *J. Clin. Microbiol.* **33**:2271-2279.
- Washburn, L. R., and E. Weaver. 1996. Protective effect of active and passive immunization of rats against two surface antigens of *Mycoplasma arthritis*. *IOM Lett.* **4**:31.
- Washburn, L. R., E. Weaver, and L. Vail. 1996. Passive and active immunization of rats against two *Mycoplasma arthritis* surface antigens, abstr. G-16, p. 284. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
- Woglom, W. H., and J. Warren. 1938. A pyogenic filterable agent in the albino rat. *J. Exp. Med.* **68**:513-528.