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Toxicity But Not Arthritogenicity of *Mycoplasma arthritidis* for Mice Associates with the Haplotype Expressed at the Major Histocompatibility Complex

BARRY C. COLE,* RUTH N. THORPE, LEWIS A. HASSELL, LEIGH R. WASHBURN, AND JOHN R. WARD

Division of Rheumatology, Department of Internal Medicine, University of Utah, College of Medicine, Salt Lake City, Utah 84132

The use of inbred and congenic mouse strains established that toxicity and death induced by *Mycoplasma arthritidis* associates with the haplotype expressed at the murine major histocompatibility complex. Mice bearing $H\text{-}2^k$ and $H\text{-}2^d$ are susceptible, whereas those bearing $H\text{-}2^b$ are much more resistant. Mice susceptible to toxicity exhibited massive peritoneal adhesions and a decreased ability to clear organisms from the peripheral circulation. However, the severity of acute arthritis developing over a 3-month period was not statistically related to the haplotype expressed at the major histocompatibility complex. Lymphocyte activation in vitro by a soluble T-cell mitogen is also dependent on a similar haplotype expression.

Mycoplasma arthritidis is a causative agent of spontaneous arthritis in rats and will induce experimental chronic arthritis in mice and rabbits (7, 8, 20). Previous studies indicated differences in the susceptibility of different outbred and inbred mouse strains to both the arthritogenic and toxic effects of *M. arthritidis* for mice (6). Arthritogenicity and toxicity did not necessarily correlate among the strains. Thomas (18) has also referred to the toxicity of *M. arthritidis*, and recently Thirkill and Gregerson (17) reported on ocular lesions associated with a toxic syndrome in mice.

M. arthritidis generates a soluble T-cell mitogen (3) which is active only in the presence of Ia-bearing, adherent, accessory cells (5). The mitogen activates lymphocytes from mouse strains expressing the $H\text{-}2^k$ or $H\text{-}2^d$ haplotypes but is inactive for lymphocytes from strains expressing $H\text{-}2^b$, $H\text{-}2^s$, and $H\text{-}2^q$ (2, 3). These studies also showed that the I-E/I-C subregion of the mouse major histocompatibility complex (MHC) is responsible for controlling lymphocyte reactivity to the *M. arthritidis* mitogen, and we have recently obtained evidence that this Ir subregion functions by coding for a molecule which acts as the mitogen receptor (4).

The present study was initiated to determine whether the toxic or arthritogenic properties of *M. arthritidis* might relate to the haplotype-associated specificity of the mycoplasma mitogen. We demonstrate here that susceptibility to the toxic effects of *M. arthritidis* in vivo is associated with the MHC as is seen for lymphocyte activation in vitro. In contrast, the differences seen in the degree of arthritis obtained with the various mouse strains did not correlate with the MHC haplotype.

MATERIALS AND METHODS

Mycoplasma cultures. The source of *M. arthritidis* strains 158P10P9 and 14124P10 was as previously described (10). The organisms were grown in modified Hayflick broth consisting of pleuropneumonia-like organism broth (Difco Laboratories, Detroit, Mich.) supplemented with 15% (vol/vol) heat-inactivated horse serum, 5% (vol/vol) fresh yeast extract, 0.5% (wt/vol) L-arginine monohydrochloride, and 500 U of penicillin G per ml (1, 11). Logarithm phase cultures were harvested by centrifugation at 27,000 × *g* for 30 min. The organisms were suspended in fresh medium, distributed in samples, and stored at −70°C. Samples were thawed and assayed for CFU. Identity of the organisms was established by immunofluorescent staining of colonies (9) with reagents prepared in our laboratories which were standardized against reference National Institutes of Health antisera.

Animals and induction of disease. Mice of either sex between the ages of 2.5 and 3.5 months were used. The breeding stocks for B10.D2 and C3H.SW mice were generously provided by Jack Stimpfling, Great Falls, Mont., and D. Schreffler, Department of Genetics, Washington University, St. Louis, Mo., provided the stock for C3H.B10 mice. Germfree barrier-maintained C3H mice (screened for the absence of mycoplasma and 11 common viruses) were obtained from Clarence Reeder of the Animal Genetics Reproduction Branch of the National Cancer Institute and were used to establish a breeding colony. The mice were maintained in the laboratories of R. A. Daynes, Department of

Pathology, University of Utah College of Medicine, Salt Lake City, Utah. Additional C3H and C3H.SW mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Mice were injected intravenously (i.v.) or intraperitoneally (i.p.) with doses of 1×10^9 to 6×10^9 CFU of *M. arthritidis*. Mice were examined at intervals through a 3-month period and scored for arthritis as previously described (7a).

Antibody studies. Representative mice of each strain were screened by enzyme-linked immunosorbent assay (ELISA) to ensure absence of preinfection with *M. arthritidis*. The method was based on that previously described (21), except that the ELISA antigen was prepared from mycoplasmas grown in a dialysate medium containing 10% agamma serum (13). Sera were titrated twofold beginning at 1:20. Twenty-six known negative mouse serum samples were tested at 1:20 to establish the standard deviation of the mean absorbance at 492 nm. Test sera were considered positive only when the mean absorbance was greater than 2 standard deviations higher than the control sera. The uninjected control mice used in these studies all exhibited titers of <1:20 against *M. arthritidis* antigens.

Mice injected with *M. arthritidis* grown in Hayflick broth or injected with Hayflick broth alone were also tested for antibodies to *M. arthritidis* by ELISA. Importantly, the sera from mice injected with Hayflick broth alone did not react with the ELISA antigen of *M. arthritidis*.

Peripheral blood mycoplasma counts. C3H and C3H.SW female mice, 7 to 8 weeks of age, were injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9. Blood (0.05 ml) for mycoplasma CFU counts was withdrawn from the tail by using calibrated heparinized capillary tubes, serially diluted 10-fold in mycoplasma medium, and transferred to mycoplasma agar. Mycoplasma counts were expressed as CFU per milliliter of blood.

Statistical analysis. Incidences of toxicity, death, paralysis, abscesses, or arthritis were compared in inbred versus congenic mice by means of chi-square analysis with Yates correction for continuity (χ^2_c). Data regarding mean day of death, mean day of onset and peak arthritis, and mean maximum score of arthritis were assessed nonparametrically by the Mann-Whitney rank sum analysis (MWRSA). The Student *t* test (*t* test, one tailed-testing) was utilized in comparing C3H and C3H.SW peripheral blood CFU levels of *M. arthritidis* at various time periods (22).

RESULTS

Haplotype-associated toxicity. A short term experiment established that C3H (*H-2*^{k}) mice developed pronounced toxic symptoms in response to injection with *M. arthritidis* 158P10P9, whereas C57BL/10 (*H-2*^{b}) mice did not. Toxicity was manifested by ruffled fur, lethargy, and conjunctivitis. The latter was associated with a purulent discharge and developed in approximately 50% of the animals within 8 h of injection of the organisms. Fecal impaction was seen in some animals. Death resulted in many C3H mice but not in C57BL/10 mice. In another preliminary experiment, 5×10^9 CFU of strain 158P10P9 resulted in 100% deaths in C3H mice and 73% deaths in BALB/c mice. A second strain of *M. arthritidis* (14124P10) produced 100% deaths in both C3H and BALB/c mice with an i.p. inoculum of 6×10^9 CFU.

To determine the possible influence of the MHC in the expression of *M. arthritidis*-induced disease, each subsequent experiment included an inbred mouse strain and a congenic strain which differed only in the haplotype present at the *H-2* gene complex.

In the first experiment, C3H mice (*H-2*^{k}) and congenic C3H.SW mice (*H-2*^{b}) were injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9. In the second experiment, C3H and congenic C3H.B10 (*H-2*^{b}) mice received an identical injection. Mice were examined over a 12-week period and assessed for the development of both toxic symptoms and joint swelling (Table 1). The incidence of toxicity, defined as one or usually a combination of ruffled fur, lethargy, or conjunctivitis, was significantly higher in C3H mice than in their *H-2*^{b} congenic counterparts (experiment 1, $\chi^2_c = 22.634$, $P < 0.001$; experiment 2, $\chi^2_c = 4.655$, $P < 0.05$). Likewise, the incidence of death in both experimental groups was markedly higher for C3H compared with C3H.SW or C3H.B10 mice ($\chi^2_c = 9.186$, $P < 0.005$; $\chi^2_c = 8.330$, $P < 0.005$, respectively). In the two experiments, C3H mice exhibited a mean day of death of 6.6 and 5.5, respectively, whereas the only death within the congenic mouse strains occurred at 10 days. The results suggest that the toxic death induced by *M. arthritidis* is associated with the haplotype expressed at the MHC.

Chi-square analysis of the incidence of arthritis revealed no difference between inbred C3H mice and the two congenic strains. The limited number of arthritic animals in the first experimental group due to early deaths of C3H mice prevented a comparison of other parameters of arthritis (average day of onset and peak, average maximum score per arthritic animal). Analysis (MWRSA) of the second group (C3H versus C3H.B10) indicates no appreciable difference between the two haplotypes. Figure 1 summarizes data which demonstrate the similar course of arthritis per surviving animal seen in C3H and congenic mice. Although experiments described in this paper were not designed to evaluate a differential response due to sex, the difference in magnitude of the average arthritic responses in the male versus female groups (Fig. 1) led to a comparison of the mutual C3H strain in the two groups. No statistically significant differences in incidence of arthritis (χ^2_c), death or toxicity (χ^2_c), or average day of death (MWRSA) were seen.

A third experiment was carried out with both

TABLE 1. Toxicity and acute arthritis in C3H and congenic C3H.SW and C3H.B10 mice in response to *M. arthritidis*

No. and sex of mouse strain[a]	Toxicity[b]			Arthritis				
	MHC haplotype	% Incidence	% Death (mean day)	% Incidence[c]	Onset day	Peak day	Mean Maximum score[d]	Maximum score per arthritic mouse[e]
Expt 1								
15 C3H females	*H-2*^{k}	93.3	66.7 (6.6)	13.3	6.0	7.5	0.33	2.5
15 C3H.SW females	*H-2*^{b}	0	6.7 (10.0)	33.3	7.8	9.5	0.67	2.0
Expt 2								
8 C3H males	*H-2*^{k}	100.0	50.0 (5.5)	50.0	5.0	8.5	2.8	5.5
8 C3H.B10 males	*H-2*^{b}	37.5	0	62.5	5.0	8.6	4.4	8.8

[a] All animals were injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9.
[b] Paralysis and abscess development were not routinely assessed in these initial experiments. See Table 2. Toxicity was assessed as presence of one or more of ruffled fur, lethargy, or conjunctivitis.
[c] Percentage of arthritis which developed in all injected mice.
[d] Maximum scores of each mouse were totalled and divided by the total number of mice.
[e] Maximum scores were totalled and divided by the number of arthritic mice.

male and female mice of the inbred C57BL/10 (*H-2*^{b}) strain and the congenic B10.D2 (*H-2*^{d}) strain. Animals were injected i.v. with 10^9 CFU of *M. arthritidis* 158P10P9 and were again examined through a 12-week period (Table 2). Toxicity occurred at a greater frequency with the congenic strain B10.D2 (*H-2*^{d}) than with the background C57BL/10 strain ($\chi^2_c = 3.172$. $P < 0.10$). The incidence of death was also higher in B10.D2 mice ($\chi^2_c = 7.3000$, $P < 0.01$). Although the mean day of death in the B10.D2 mice occurred earlier (15.8 days) than with the C57BL/10 mice (34.0 days), the difference was not statistically significant (MWRSA). However, paralysis, usually of the hind limbs, was significantly higher in the B10.D2 mice (68%) as compared with the C57BL/10 mice (35.7%; $\chi^2_c = 4.290$, $P < 0.05$). Interscapular abscesses, which developed 10 to 42 days postinjection, occurred more than twice as frequently in B10.D2 mice. The results, however, were not statistically different, probably due to the lesser survival of B10.D2 mice. The purulent contents were cultured on mycoplasma and nutrient agar and were found to contain pure cultures of *M. arthritidis* as identified by immunofluorescence. The data from this experiment support that from previous experiments and indicate a role for the MHC in expression of *M. arthritidis*-induced toxicity and death (Tables 1 and 2).

The results from experiment 3 (Table 2 and Fig. 2) also support the previous findings on arthritis in that there were no statistical differences between C57BL/10 and B10.D2 mice regarding the incidence of arthritis (χ^2_c), average day of onset and peak, or mean maximum score per arthritic animal (MWRSA). Although males of both strains exhibited slightly higher mean maximum arthritis scores (10.0 for C57BL/10 and 7.2 for B10.D2) than did females (6.9 and 5.2, respectively), the differences were not statistically significant. Likewise there was no significant difference between males and females in incidence of arthritis, mean day of onset, or day of peak of arthritis.

Mechanisms of toxic death. Female C3H and C3H.SW mice, 7 to 9 weeks of age, were injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9 and were sacrificed in groups of three

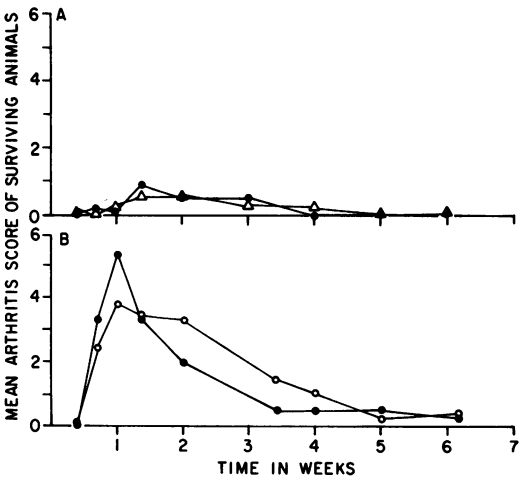

FIG. 1. (A) Development of arthritis in surviving female C3H (*H-2*^{k}) mice (—●—) or congenic (*H-2*^{b}) C3H.SW mice (—△—) after injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9. (B) Development of arthritis in surviving male C3H (*H-2*^{k}) (—●—) or congenic C3H.B10 (*H-2*^{b}) (—○—) mice injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9.

at intervals through 11 days. The C3H mice exhibited marked abdominal distension by day 3 and were found to have dilated bowel loops and extensive peritoneal lesions. The abdominal wall was thickened and opalescent. The congenic C3H.SW mice showed none of these changes and appeared normal. No major differences in the gross appearance of other organs were seen. Histological studies are being conducted.

The clearance of *M. arthritidis* from the blood stream of 10 C3H and 5 C3H.SW mice was next examined. Blood samples (0.05 ml) were collected 1, 2, 3, and 4 days after i.p. injection of 5×10^9 CFU of *M. arthritidis* and assayed for CFU. The data (Fig. 3) demonstrate that through the 2-day time period C3H mice exhibited an apparent marked inability to clear the injected organisms as compared with C3H.SW mice. The results could alternatively indicate that more organisms reached the peripheral circulation of C3H mice. Differences in CFU/ml of blood between the groups were significant at 24 h ($P < 0.0005$), at 48 h ($P < 0.005$), at 72 h ($P < 0.0025$), but not at 96 h ($P < 0.10$) (Student's *t* test, one tailed).

Since the previous observation on altered clearance might indicate suppression of host defenses in susceptible animals, serum samples collected from the mice used in experiments 1 and 3 were assayed by ELISA for antibodies to *M. arthritidis* antigens. Preinjection serum samples were all <1:20. Mice bearing *H-2*^{k}, *H-2*^{d}, and *H-2*^{b} all developed a prolonged antibody response to *M. arthritidis*. For example, in experiment 1 (Table 1) mean antibody titers 2 months postinjection were 1,440 for C3H mice and 937 for C3H.SW mice. There were no significant differences (MWRSA) in titers between mice susceptible or resistant to *M. arthritidis*-mediated toxicity. Mice injected with modified Hayflick medium failed to exhibit antibodies (<1:20) to the ELISA antigen.

TABLE 2. Toxicity and acute arthritis in C57BL/10 and congenic B10.D2 mice in response to *M. arthritidis*

No. and sex of mouse strain[a]	MHC haplotype	Toxicity				Arthritis				
		% Incidence	% Death (mean day)	% Incidence of Paralysis	% Incidence of Abscess	% Incidence[b]	Onset day	Peak day	Mean maximum score	Maximum score per arthritic mouse
C57BL/10 (19 female, 9 male)	*H-2*^{b}	35.7	10.7 (34.0)	35.7	14.3	89.3	3.5	5.6	7.0	7.8
B10.D2 (15 female, 10 male)	*H-2*^{d}	64.0	48.0 (15.8)	68.0	32.0	92.0	3.4	6.0	5.6	6.1

[a] All animals were injected i.v. with 10^9 CFU of *M. arthritidis* 158P10P9.
[b] Percentage of arthritis which developed in all injected mice.

DISCUSSION

We have confirmed previous work (6, 17, 18) which indicated that *M. arthritidis* is toxic for mice after systemic administration. In addition, the present studies have shown pronounced differences in the susceptibility of different mouse strains to the toxic syndrome. With inbred and congenic mice, toxicity was shown to be associated with the haplotype expressed at the murine MHC, since mice bearing *H-2*^{k} or *H-2*^{d} were susceptible, whereas those expressing *H-2*^{b} were much more resistant.

Of considerable interest was our finding that the haplotype-associated specificity of in vivo toxicity was identical to that seen for lymphocyte activation in vitro by a T-cell mitogen derived from *M. arthritidis*. The mitogen, which has now been demonstrated to be a polyclonal

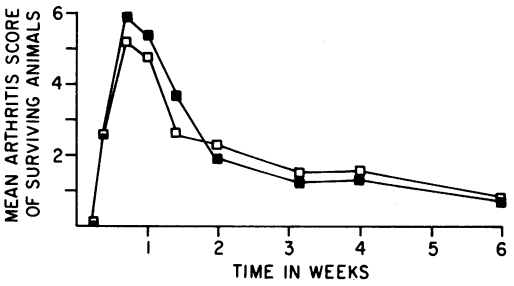

FIG. 2. Development of arthritis in surviving mice of both sexes injected i.v. with 10^9 CFU of *M. arthritidis* 158P10P9 C57BL/10 (*H-2*^{b}) mice (—■—) and B10.D2 (*H-2*^{d}) mice (—□—).

T-cell activator (R. L. Yowell, B. C. Cole, and R. A. Daynes, J. Immunol., in press), stimulates murine splenic cells from strains which express the *H-2*^{d} or *H-2*^{k} haplotypes at the I-E/I-C subregion of the *H-2* gene complex (2, 3, 4). Although it remains to be established whether in fact the two phenomena are related, the results suggest that the mitogen may be responsible for in vivo toxicity in mice responsive to the mitogen.

The mechanism(s) of toxicity induced by *M. arthritidis* is not yet clear. Evidence was obtained that the organisms were eliminated more slowly from the peripheral circulation of susceptible mice than from resistant mice, implying an impairment of host defenses. However, this apparent lack of clearance could have been due to continued entry of organisms from the peritoneal cavity of responder mice. Preliminary experiments suggested that the susceptible C3H mice exhibited a more prolonged leukopenia after injection with *M. arthritidis* than did the resistant C3H.SW mice (unpublished data). These data support the concept of an impairment of host defenses. Although increased replication of organisms in responsive mice may have played a role in the peritoneal adhesions observed after i.p. injection of organisms, fibroplasia and collagen synthesis induced by local lymphokine release (12, 16, 19) are more likely explanations. Although eye cultures were not performed in our studies, Thirkill and Gregerson (17) reported that only 2 of 20 involved eyes yielded mycoplasmas by culture, suggesting that ocular involvement was not dependent on a direct infectious process. Preliminary attempts to protect responsive (C3H) mice against the toxic effects of *M. arthritidis* by immunizing them with mitogen-containing supernatants of *M. arthritidis* failed (unpublished data). These results might suggest either that insufficient mitogen is present in supernatants, that the mitogen does not participate in the toxic syndrome, or that the mitogen may be nonimmunogenic in responsive mice. The lack of immunogenicity could be due to the ability of the mitogen to nonspecifically activate the immune system, thus preempting the development of a specific immune response.

The presence of antibodies to *M. arthritidis* antigens as measured by ELISA in both susceptible and resistant mice suggests that a generalized humoral immunosuppression does not occur. The effect of the T-cell mitogen on cell-mediated immune responses remains to be studied. Experiments are now in progress to determine whether mitogen-induced T-cell activation in vivo alters host defense mechanisms and whether lymphokine production plays a role in the toxic effects observed. The availability of susceptible and nonsusceptible mouse strains provides an ideal model system to answer these questions.

Another conclusion to be drawn from our studies is that the development of acute arthritis is not associated with susceptibility to toxicity and is not dependent on lymphocyte reactivity to the T-cell mitogen. It remains to be determined whether the T-cell mitogen contributes to the chronic phase of arthritis seen previously in Swiss Webster mice (7). Differences in the sus-

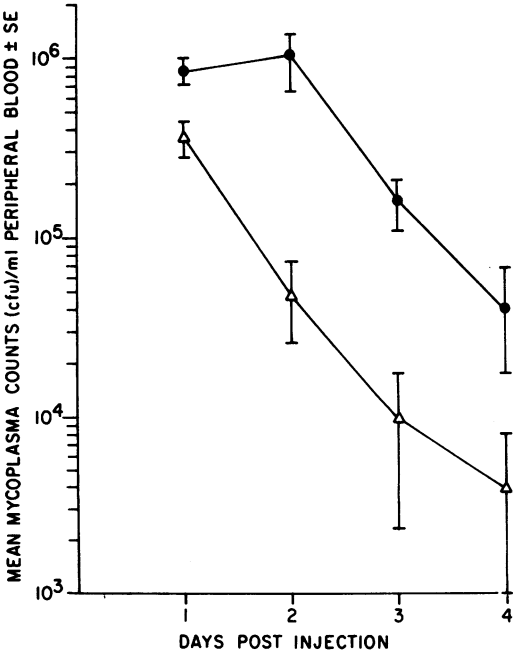

FIG. 3. Clearance of *M. arthritidis* from the peripheral circulation of mice. Female C3H (—●—) or congenic C3H.SW (—△—) mice were injected i.p. with 5×10^9 CFU of *M. arthritidis* 158P10P9. Heparinized blood samples were assayed for viable organisms (CFU) at days 1 through 4. Bars = standard error.

ceptibility of different mouse strains to both *M. arthritidis* and *Mycoplasma pulmonis* have been noted (6, 14, 15). Recent studies by Keystone et al. indicated that although the lymphocytes of both C3H and C57BL/10 mice were equally stimulated by the *M. pulmonis* mitogen(s), arthritis was much more severe in C3H mice (14). Since mycoplasmal arthritis of rodents is initially an infectious process which progresses to chronic inflammation (7), it is likely that disease susceptibility is under multigene control. It is clear that much work will be required to elucidate the mechanisms and controlling genes responsible for this inflammatory disease.

Our previous work, which established the linkage between lymphocyte activation induced by an *M. arthritidis* mitogen and MHC gene expression, was obtained by in vitro systems. The present studies provide evidence that viable *M. arthritidis* induces a toxic syndrome in vivo, which exhibits a similar haplotype-associated specificity as seen for the mitogen in vitro. These observations may have relevance to other infectious diseases. Many pathogenic microbial agents generate mitogenic substances, but the precise role of these lymphocyte activators in disease processes remains poorly understood. Our unique finding of resistant and nonresistant mouse strains provides an opportunity to examine the role of polyclonal T-lymphocyte activation in disease and its relationship with the expression of specific host gene products.

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