Circulating and Localized Immune Complexes in Experimental Mycoplasma-Induced Arthritis-Associated Ocular Inflammation

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Ocular deposits of immune complexes are believed to contribute to the anterior segment inflammations observed in association with the human arthritides. Arthritis-related ocular inflammations may be reproduced in animals by infection with certain species of mycoplasma. To evaluate the role of immune complexes in the production of ocular lesions, we studied their involvement in the rodent model of experimental arthritis-associated ocular inflammation induced by Mycoplasma arthritidis. Sprague-Dawley rats were infected with viable concentrates of M. arthritidis and monitored for the production of related circulating and intraocular immune complexes. Circulating immune complexes were monitored by antigen capture systems, and localized intraocular complexes were identified by indirect immunohistochemistry. Polyacrylamide gel immunoblot analysis of captured complexes confirmed the antigen(s) involved as proteins derived from M. arthritidis. Indirect immunofluorescence revealed localized complexes containing mycoplasma antigens within the ciliary-iris vasculature. Concentrations of the generated complexes diminished rapidly over a 30-day period. While complex deposits within ocular tissues could represent a contributing cause to the localized anterior segment inflammation reported in this rodent model, secondary challenge with viable M. arthritidis, which reproduced high concentrations of intraocular and circulating immune complexes, failed to elicit any ocular response.

MATERIALS AND METHODS

Mycoplasma cultivation. M. arthritidis (strain 14152) (American Type Culture Collection) was cultured in GIBCO mycoplasma broth supplemented with 5% horse serum and 0.5% arginine hydrochloride, with the addition of 0.001% phenol red. Agar plates for colony counts and isolation of M. arthritidis from blood samples were prepared with the same additives. No antibiotics were used in these culture media. Lysed extracts of M. arthritidis, to be used as test antigens, were prepared by centrifugation of broth cultures. Concentrations were resuspended and washed in phosphate-buffered saline (pH 7.5) (PBS) and then briefly sonicated and stored for use at −20°C (31).

Rodent inoculation and tissue preparation. On day zero, 36 6-week-old female Sprague-Dawley rats were infected with M. arthritidis via a single tail-vein inoculation with 1.0 ml of a centrifuged culture concentrate containing approximately 10⁵ viable CFU, washed and resuspended in PBS as previously described (32). Animals were treated in accord with the Guiding Principles in the Care and Use of Animals (11a). A separate group of 12 rats each received a single tail-vein inoculation of 1.0 ml of complete, serum-supplemented mycoplasma culture medium to act as uninfected, negative comparison controls. Groups of one control and three test rats were exsanguinated at 5-day intervals following injection, up to day 30. Viable mycoplasmas from blood samples were isolated by the direct inoculation of 0.25 ml of whole blood from freshly killed animals onto plates and into 10-ml volumes of mycoplasma isolation broth. These culture media were incubated aerobically at 37°C for 1 week and examined for evidence of mycoplasma growth. Eyes from the test and control rats were promptly removed and fixed in 10% buffered formalin, dehydrated in ethanol, embedded in paraffin, and sectioned. Serum from each group of rats was stored at −20°C for immunological examination.

On day 35, the remaining 18 test rats, now recovered from the acute phase of the disease, were challenged with a...
second, identical intravenous dose of viable \textit{M. arthritidis}, and the observations continued. Reinfected rats were sacrificed in groups of three along with one control at 5-day intervals following challenge, and blood, eyes, and sera were collected for study as described above.

**Hyperimmune rabbit serum.** Two female New Zealand albino rabbits were immunized with \textit{M. arthritidis} washed and homogenized in PBS as previously described (31). Two weeks following the last injection, the rabbits were exsanguinated to provide antiserum which was quantified for immunoglobulin concentration by quantitative radial immunodiffusion and stored for use at $-20^\circ$C.

**ELISA antibody assay** (36). The antibody response of each pool of sera from infected rats to mycoplasma antigens was evaluated and compared with the antibody reactivity of uninfected control rats which had received a single injection of complete culture medium. Microplates were coated with 10 \textmu g/ml of \textit{M. arthritidis} test antigen per well, and serum samples from each group were titrated for antibody content by doubling dilutions, from 1:20 to 1:2,560, down individual columns. Antibody reactions with \textit{M. arthritidis} antigens were resolved through the application of rabbit anti-rat polyclonal immunoglobulins (Sigma) and then alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma) at a dilution of 1:1,000 each, using para-nitrophenyl phosphate as a substrate. Overall average antibody response throughout the study period was obtained from duplicate assays. The relative antibody activity of each pool of test serum at 1:20 was recorded and plotted when the 1:20 dilution of control serum reached an optical density of 0.3 at 405 nm.

**ELISA antigen capture system.** A modification of the enzyme-linked immunosorbent assay (ELISA) method described by Swenson and Kaplan (27) was applied to detect mycoplasma antigens within circulating immune complexes. Microplates were coated overnight at 4°C with rabbit anti-\textit{M. arthritidis} serum at a concentration of 10 \textmu g of IgG per well, using bicarbonate coating buffer (pH 9.6). Rat serum from both test and control animals was diluted double down separate columns of the microplates starting from 1:2 to 1:256 and allowed to react at room temperature for 1 h. Any available mycoplasma antigens were captured by the rabbit anti-\textit{M. arthritidis} serum bound to the plate. Following a thorough wash, rat immunoglobulins associated with captured antigens were detected through the application of a 1:1,000 dilution of goat anti-rat IgG–alkaline phosphatase conjugate reacting on para-nitrophenyl phosphate. Duplicate assays were performed from which the average relative absorption of the 1:2 dilution of serum from each test group was compared with that of the 1:2 dilution of control serum when it reached an optical density of 0.3 at 405 nm. The results were recorded and compared for variation over time.

**Immunohistochemistry.** Sections (6 \mu m) of rat eyes from each group were deparaffinized and placed in normal physiological saline, in which all antisera dilutions were prepared. Sections were probed with the following antisera to detect intraocular deposits of mycoplasma antigens, aggregates of rat complement, and immunoglobulins: (i) rabbit anti-\textit{M. arthritidis} serum produced in this laboratory; (ii) fluorescein isothiocyanate-conjugated goat anti-rabbit complement component C3 (U.S. Biochemicals) and (iii) fluorescein isothiocyanate-conjugated goat anti-rat polyclonal immunoglobulins (U.S. Biochemicals), all used at a dilution of 1:100. Each of these sera was allowed to react on separate sections of eyes from each group at room temperature for 30 min before washing in fresh saline. Antibody binding to localized mycoplasma antigens was resolved by indirect immunofluorescence with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Cappel). Sequential sections were examined for immunofluorescence, and positive findings were photographed. Results obtained from sensitized rats were compared with sections of the eyes of the uninfected control rats.

**Western immunoblot analysis of complexes.** Antigens entrapped within circulating immune complexes produced in the first set of rats were analyzed by using a polyacrylamide gel electrophoresis (PAGE) procedure based on those originally described by Matsuo et al. (21, 22) and Vinayak et al. (35). The antibody content of 500-\mu l samples of each set of test and control rat sera was absorbed onto Pansorbin (Calbiochem). The mixture was incubated at room temperature with agitation for 1 h, and the Pansorbin was collected by centrifugation. Bound immunoglobulins and their related antigens were eluted from the Pansorbin by boiling for 10 min in PAGE sample buffer (13). The supernatant was electrophoresed on a 1.5-mm-thick, 20-cm 12% polyacrylamide gel and blot transferred to nitrocellulose (34). The transfer was blocked in PBS containing 0.5% Tween and 0.05% nonfat milk before being probed with rabbit anti-\textit{M. arthritidis} serum at a dilution of 1:1,000. Mycoplasma antigens, present in serum samples obtained over the initial 30-day period prior to challenge, were subsequently resolved by using goat anti-rabbit IgG-horseradish peroxidase conjugate reacting on the substrate 3,3’-diaminobenzidine. This technique was confined to the first set of rats that experienced a single infective dose, since its application to those receiving a secondary challenge would reflect that of previously sensitized animals.

The competence of the rabbit anti-\textit{M. arthritidis} serum prepared in this laboratory to identify the antigens of \textit{M. arthritidis} in Western blot reactions was compared with the activity of pooled rat sera obtained from day 30 of the study. Both were evaluated at a dilution of 1:1,000 in side-by-side reactions on the same blot transfer of strain 14152 of \textit{M. arthritidis}.

**RESULTS**

Conjunctivitis appeared in all test animals in the first set within 24 h postinfection but was resolved completely by day 5. Intravitreal leukocyte infiltration, induding intraocular inflammation, was found histologically in only 5 of the first set of 18 test rats (3 on day 15 and 2 on day 20) and in none of the second set of challenged animals. Swollen joints appeared in all test rats on day 5 and continued throughout the first 30 days of the study, following the same pattern which we and others have previously described (28, 29). Viable mycoplasmas were present in blood samples obtained from the first group on day 5, but not thereafter. Secondary challenge on day 35 again produced a similar pattern of septicemia, with viable isolates present in blood only on day 40, 5 days postchallenge, and not thereafter (Fig. 1). No evidence of overt joint or eye involvement appeared in the secondary challenge group of rats. Uninfected control rats, injected with complete serum-supplemented culture medium, showed no response in any of the parameters examined throughout the study.

**ELISA antibody assay.** Overall antibody response to infection showed an elevation in antibody reactions to \textit{M. arthritidis} antigens which increased rapidly to peak and persist throughout the first phase of the study period. Serum from uninfected control rats assayed under the same conditions gave background readings against which the antibody reac-
A405 values were recorded when the control sera had an optical

erational activity was evaluated at a dilution of 1:20 for overall

ELISA antigen capture system. Since living mycoplasmas were demonstrable in the blood of test animals 5 days following both initial exposure and secondary challenge, complexes captured during the transient septicemia were not considered representative of true immune complexes. Circulating antibody complexed with non-viable M. arthritidis antigens was detected on day 10 and thereafter in decreasing concentrations in all test serum samples until day 30 following initial infection. Secondary challenge resulted in the reappearance of high concentrations of circulating immune complexes, which again declined progressively to the end of the study (Fig. 1).

Immunohistochemistry. Examination of sectioned eyes from infected rats, obtained on days 5 through 30, showed the early appearance of intense immunofluorescence indicating the present of mycoplasma antigens, aggregated C3, and rat immunoglobulins entrapped within the iris-ciliary mi-

crovasculature (Fig. 2). These reactions were most intense during the first 15 days following infection, but declined rapidly to day 30, when no immunofluorescence was detectable. The second set of rats, reinfected on day 35, exhibited a similar pattern of localized complexes. Reactions were most intense on day 40 and gradually declined to zero at the end of the study on day 65. After challenge, secondary deposits of mycoplasma antigens, complement, and immunoglobulins occurred and declined in the complete absence of arthritic and ocular signs. Eyes from uninfected control rats showed no abnormalities.

Western immunoblot analysis of complexes. M. arthritidis antigens present in captured immunoglobulin complexes were detected in decreasing quantities on days 10, 15, and 20. The principal antigen resolved by the rabbit anti-M. arthritidis serum exhibited a relative molecular mass of approximately 65 kDa and was present in serum samples from days 10 to 20, after which no further reactions were found by this method. Serum from uninfected control rats gave no reaction in this assay (Fig. 3a). Western blot assays of the rabbit anti-M. arthritidis sera and pooled rat sera obtained from test rats on day 30 showed similar immunoreactivity when compared on a blot transfer of M. arthritidis (Fig. 3b).

Discussion

Mycoplasmas produce an assortment of ocular inflammations in a variety of animals, but the pathogenic process is not fully understood. The M. arthritidis model of arthritis-associated ocular inflammation shares similarities with the human arthritides. Ocular lesions associated with rheumatoid arthritis may result from soluble circulating immune complexes which eventually deposit within the blood vessels of the eye and initiate localized inflammatory reaction (23). Char et al. (5) showed a correlation between the relative concentrations of circulating immune complexes and the severity of ocular inflammation in patients with chronic uveitis and iridocyclitis. Howes and McKay (15) demonstrated increased vascular permeability in rabbits in which they had produced immune complexes and associated the occurrence of localized lesions with intravascular deposits. However, the mere presence of intraocular immune complexes cannot be held solely responsible for the induction of uveitis since Hylkema et al. (16) produced immune complexes within the eyes of mice with no resultant inflammatory reaction. There are also examples of high concentrations of circulating immune complexes found in normal
healthy humans who exhibit none of the effects usually associated with immune complexes (11, 12, 20). Some predisposing events, such as trauma or infection of specific tissues, may be required to involve immune complexes in the production of the lesions found in immune complex diseases (24).

Kirchhoff et al. (19) described fluctuating levels of immune complexes circulating in the sera and localized in joints of M. arthritidis-infected rats which were resistant to secondary infection. As in our study, they reported that viable mycoplasmas circulating in the blood immediately after the tail vein inoculation were rapidly cleared, leaving only fragmented or dead mycoplasmas trapped with related antibodies detectable in serum samples. Although no longer present in the blood, viable mycoplasmas reportedly have been isolated from a variety of organs, with joint isolates evident up to 200 days following infection (14).

A complete understanding of the so-called immune complex disease requires identification of the antigen within the complex. In some cases of recognized autoimmune diseases, the antigens contained in associated immune complexes have been identified and incriminated in the disease process. For example, rheumatoid factors consist of antibodies reacting with other antibodies, and lupus erythematosus is associated with anti-DNA complexes. In recent years, antigen capture systems similar to those described here have identified some of the antigens involved in diseases with known and suspected immune complex involvement. These include the detection of retinal S-antigen–antibody complexes in the blood serum of patients with uveitis (22), specific DNA sequences in lupus erythematosus (3), hepatitis B antigens (26), and circulating herpes virus antigens (21). In the present study, Western blot analysis of circulating antibody-bound M. arthritidis antigens demonstrated the presence of several mycoplasma antigens in blood sera assayed 10 days postinfection. A rapid decline ensued to day 20, when only a single reaction with an antigen-antibody reaction approximating 65 kDa was resolved. After day 20, no further immunological reactions could be resolved by using the Western blot antigen capture system described here. The intensity of the 65-kDa reaction suggests that this mycoplasma protein represents a major antigenic component of M. arthritidis. It should be understood that antigenic processing in the infected rats may have resulted in modifications to this component which could exhibit a different size when identified in a fresh culture extract.

While all infected rats in the first group of 18 experienced transient conjunctivitis, only 5 exhibited indications of intraocular inflammation. Although immune complexes may be involved in the production of the recorded ocular lesions, they are not the only contributing factor since secondary ocular deposits incited in the challenged group of reinfected rats failed to provoke any pathological response. Overall antibody response rose only slightly following challenge, indicative of saturation titers or the limitations of the assay procedures used in this study. This anomaly may add to the controversy surrounding the humoral immune response of rats following infection with M. arthritidis (37).

We have previously described the isolation of viable mycoplasmas from enucleated eyes and ocular exudates of M. arthritidis-infected rodents (29, 33). It is therefore possible that intraocular complex deposits include viable mycoplasmas, capable of survival and reproduction. Localized modulation of the host’s immune response could accordingly occur since metabolic by-products of M. arthritidis are known to have both stimulatory and inhibitory effects on leukocyte behavior (1, 2, 6–10).

We conclude that in the Sprague-Dawley rat model of M. arthritidis-induced arthritis-associated ocular inflammations, eye reactions occur only in newly infected animals. Conjunctivitis is the most consistent ocular feature of the disease while intraocular inflammations occur with a much lower frequency. The presence of intraocular immune complexes cannot be held solely responsible for the associated eye changes since their reproduction in previously sensitized rats fails to incite any pathological response. The ocular events which we have shown to occur in naive animals may require predisposing factors, such as tissue damage induced by the initial exposure to some yet-to-be-defined cytotoxic product of the mycoplasma. However, once the rat is sensitized to the mycoplasma product, any subsequent exposure is neutralized by the protection ensuing from the primary response to infection. The rat model therefore fails to reflect the most significant feature of arthritis-associated ocular inflammations in humans, a chronic anterior segment involvement (iritocyclitis) characterized by progressive inflammation linked to immune complex deposits.

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