NOTES

The Hemagglutination-Positive Phenotype of *Mycoplasma synoviae* Induces Experimental Infectious Synovitis in Chickens More Frequently than Does the Hemagglutination-Negative Phenotype

M. NARAT,1 D. BENČINA,1* S. H. KLEVEN,2 AND F. HABE1

Department of Animal Science, Biotechnical Faculty, University of Ljubljana, 1230 Domžale, Slovenia,1 and Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602-48752

Received 21 May 1998/Returned for modification 22 July 1998/Accepted 24 September 1998

Inoculation with hemagglutination-positive (HA⁺) cultures of *Mycoplasma synoviae* AAY-4 induced acute synovitis significantly more frequently (P = 0.001) in chicken tibiotarsal-tarsometatarsal joints than did inoculation with HA-negative (HA⁻) cultures derived from the same clone of AAY-4. Immunoblotting analyses showed that HA⁺ cultures abundantly expressed two phase-variable hemadherence-associated surface membrane proteins of 53 kDa and 48 to 50 kDa defined by monoclonal antibodies. HA⁻ cultures lacked the 53-kDa proteins and synthesized truncated 27- to 30-kDa forms of the 48- to 50-kDa proteins. Inoculation of cyclosporin A (CsA) into infected joints significantly decreased the frequency of acute synovitis (P = 0.001). Moreover, repeated intra-articular inoculation of CsA (three doses of 1 mg at 2-day intervals) significantly reduced the local antibody response to *M. synoviae* in the joints treated with CsA.

Although some viruses and bacteria can induce chronic arthritis, the mycoplasmas as a group are the most common etiologic agent of naturally occurring chronic joint inflammation (12). Mycoplasmas are well known arthritogenic agents in humans, cattle, sheep, goats, swine, rodents and poultry. Mycoplasma-induced arthritis of poultry under field conditions is almost entirely caused by *Mycoplasma synoviae* infection (18). *M. synoviae* infection most frequently occurs as a subclinical upper respiratory infection but may become systemic, resulting in arthritis, termed commonly infectious synovitis. Infectious synovitis is an acute to chronic disease of chickens and turkeys, involving primarily the synovial membranes of joints and tendon sheaths and producing an exudative synovitis, tendovaginitis, or bursitis (22). Following *M. synoviae* egg transmission, infectious synovitis has been observed in 6-day-old chickens. In flocks with clinical synovitis, morbidity varies from 2 to 75%, with 5 to 15% morbidity being most usual (22). The leg joints are most consistently involved, particularly the tibiotarsal-tarsometatarsal joints (hock joints) and tarsometatarso-phalangeal joints. Some of the arthritic and systemic forms of *M. synoviae* disease of chickens resemble an immune complex disease (12, 18). Whether autoimmune or other immunologic mechanisms are involved in the pathogenesis is not clear (12).

In experimentally induced *M. synoviae* arthritis in chickens, granular immunoglobulin G (IgG) deposits along with vascular and glomerulonephritis have been observed (19–21). Rheumatoid factors have been reported in both natural and experimental mycoplasmal infections (26, 37), but they have been seen in uninfected birds as well (12). Cold agglutinins may appear in *M. synoviae*-infected turkeys (32), but the significance of this is unclear.

Resistance to lesions induced by *M. synoviae* is bursal lymphocyte dependent (20, 23, 36), while thymus-dependent lymphocytes may be needed for the development of macroscopic synovial lesions (19, 20, 23). While B and particularly T lymphocytes seem to play an important role in the pathogenesis of *M. synoviae*-induced arthritis, there is no available information about the involvement of lymphocyte subpopulations. Moreover, data about cellular immunity in *M. synoviae* infection in chickens are lacking, and the cell-mediated response has been demonstrated only by leukocyte migration inhibition and skin testing (12, 35). A local antibody response to *M. synoviae* in synovial fluid from chickens has been reported (3) but without evidence of which proteins are targets of the antibody response. The synovial fluid synovitis synthesizes several major membrane antigens which undergo phase-variable expression associated with hemagglutination (HA) and hemadsorption (HAD) to chicken erythrocytes (4, 24, 25). *M. synoviae* (type strain WVU 1853) putative hemagglutinins MSPA (50 kDa) and the coexpressed MSPB (45 to 47 kDa) have recently been described (24). Because a single gene from a multigene family encodes *M. synoviae* hemagglutinin, MSPA and MSPB proteins are products of posttranslational cleavage (25). Adhesins play a crucial role in the initial stage of infection with pathogenic *Mycoplasma species* (28); this is probably also the case with *M. synoviae*. In *M. synoviae* AAY-4 isolated from a chicken in Slovenia (15), we identified groups of phase-variable surface membrane proteins with molecular masses from 45 to 80 kDa associated with HA and HAD (4, 6). A number of isogenic lineages with HAp-positive (HA⁺) and HA-negative (HA⁻) phenotypes were established. Proteins relevant to the HA⁺ phenotype were defined with monoclonal antibodies (MAbs) raised against *M. synoviae* AAY-4 hemagglutinin (4, 6).

The present study was undertaken to investigate the influence of the hemadherent phenotype of *M. synoviae* in experimentally induced arthritis. Our previous pilot experiments showed that inoculation of chicken hock joints with 10⁶ to 10⁷
CFU of *M. synoviae* induced infectious synovitis in the majority of inoculated birds and that cyclosporin A (CsA) could reduce clinical signs of synovitis if it was injected into the infected joint. Frey's broth medium used to grow *M. synoviae* (22) or heat-inactivated broth cultures (2 × 10^8 CFU; heated at 60°C for 30 min) did not induce synovitis even if they were inoculated into hock joints three times at 2- or 3-week intervals. For further studies, groups of 4- to 5-week-old broiler-type chickens from mycoplasma-free flocks were used. Before each experiment they were examined for mycoplasma infection by culture of tracheas and choanal clefts on Frey's agar and broth cultures of a low-passage (≤10 passages) arthrogenic *M. synoviae* strain, AAY-4, were used. To obtain HA^- and HA^+ clones, AAY-4 colonies (derived from a single colony) were examined for HAD of chicken erythrocytes. Individual HAD-positive (HAD^+) and HAD-negative (HAD^-) colonies were grown in broth to expand isogenic lineages, and aliquots of washed pelleted cells were assayed for HA titers to confirm their HA^- or HA^+ phenotype (22, 30). In experiments 1, 2, and 3, HA^- clones designated P1 (3 × 10^7 CFU; HA titer, 2 × 10^6), P2 (2.8 × 10^7 CFU; HA titer, 4 × 10^6), and P3 (1.1 × 10^6 CFU; HA titer, 4 × 10^6), respectively, were used. In experiments 1 and 2, HA^- clones N1 (3 × 10^7 CFU; hemagglutinin titer, <10^2) and N2 (3.1 × 10^6 CFU; hemagglutinin titer, <10^3) were used. Groups of 10 chickens were inoculated in the hock joint cavity with 0.2 ml of broth cultures in the log phase of growth. The design of experiments 1, 2, and 3 is shown in Table 1. Following inoculation with *M. synoviae*, joint lesions (swelling, edema) were observed daily and evaluated macroscopically. Lesions were scored from 0 to 3 (a score of 2 or higher was counted as positive) as described elsewhere (23). Synovial fluid was collected at necropsy 2 or 3 weeks post inoculation (p.i.) by syringe. From the majority of swollen hock joints a small sample of synovial fluid was collected during an acute phase when the first signs of inflammation became apparent. Synovial fluid samples were tested by IIPA using native *M. synoviae* colonies on agar blocks as antigen (see reference 3). Detection of specific IgG in samples diluted 1:100 is shown.

### Table 1. Synovitis, *M. synoviae* isolation, and synovial fluid antibodies in chickens after intrajoint inoculation with different cultures of *M. synoviae* AAY-4 and/or CsA

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Group</th>
<th>Joint</th>
<th>Inoculation with:</th>
<th>Acid-fast stains</th>
<th>CFU (10^8)</th>
<th>HAD^+</th>
<th>Positive (n)</th>
<th>Mean vol of syn. fluid (ml)</th>
<th>Antibody detection (no. positive/no. examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Right</td>
<td>HA^+</td>
<td>No</td>
<td>30</td>
<td>No</td>
<td>10</td>
<td>9</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>None</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>Right</td>
<td>HA^+</td>
<td>No</td>
<td>28</td>
<td>No</td>
<td>10</td>
<td>7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>10</td>
<td>1</td>
<td>0.3</td>
<td>0.13</td>
<td>6</td>
<td>1.4</td>
<td>5/7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Right</td>
<td>HA^-</td>
<td>Yes</td>
<td>31</td>
<td>No</td>
<td>10</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>None</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>&lt;0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>Right</td>
<td>HA^+</td>
<td>No</td>
<td>1.1</td>
<td>No</td>
<td>10</td>
<td>9</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>10</td>
<td>0</td>
<td>0.2</td>
<td>0.12</td>
<td>7</td>
<td>1.1</td>
<td>6/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Right</td>
<td>HA^-</td>
<td>No</td>
<td>1.1</td>
<td>No</td>
<td>10</td>
<td>9</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Right</td>
<td>—</td>
<td>No</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>&lt;0.10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>—</td>
<td>Yes</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>&lt;0.10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>None</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>&lt;0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a HA^- clones P1, P2, and P3 were used in experiments 1, 2, and 3, respectively. In addition, HA^- clones N1 and N2 were used in experiments 1 and 2, respectively.

b In experiment 2, the left joints of five chickens (groups A and B) were inoculated with 1 mg of CsA once, while in five other birds the left joints were inoculated with 1 mg of CsA three times at 2-day intervals. In experiment 3, joints were inoculated with 1 mg of CsA three times at 2-day intervals.

c Twenty microliters of synovial fluid from joints was plated on agar plates at 2 or 3 weeks p.i.

d Specific antibodies in the synovial fluid samples were detected by IIPA using native *M. synoviae* colonies on agar blocks as antigen (see reference 3). Detection of specific IgG in samples diluted 1:100 is shown.

e Inoculated with 0.2 ml of a vehicle for CsA (castor oil and ethanol were diluted in PBS [pH 7.4]) to an appropriate concentration.

f Specific IgG was not detected in joints which were treated with CsA three times.

g Uninoculated control.

h →, un inoculated.
scores were 2.8 and 0.5 for groups of birds inoculated with HA^+ and HA^- clones, respectively. The difference between the two groups in the frequency of clinical signs of acute synovitis is highly significant (P = 0.001; chi-square test [FREQ, Statistical package SAS/STAT, SAS 96]). M. synoviae isolation from synovial fluid 2 weeks p.i. did not correlate with signs of synovitis (Table 1, experiment 1). Articular lesions were not correlated with recovery of mycoplasmas, and this has also been reported previously (23).

In experiment 2 in which CsA was also used (see below and Table 1), HA^- clone P2 ((2.8 × 10^7 CFU) induced synovitis in seven chickens (mean group lesion score, 2.4) while a similar number of CFU of HA^- clone N2 (3.1 × 10^7 CFU) caused only one synovitis lesion (mean group lesion score, 0.4). A high frequency of acute synovitis was also induced in experiment 3 with the third HA^- clone (P3) of strain AAY-4, even though the inoculum was only 1.1 × 10^6 CFU. In two groups of chickens, 90% of joints inoculated with M. synoviae showed signs of acute synovitis, with mean lesion scores of 2.8 and 2.7 for groups A and B, respectively. A direct comparison between two HA^- clones and two HA^- clones derived from the same M. synoviae strain (AAY-4) (experiments 1 and 2) and using similar numbers of viable cells showed that HA^- populations are significantly (P = 0.001) more efficient in inducing experimental infectious synovitis.

**Differences between proteins of M. synoviae HA^+ and HA^- clones.** An interesting finding was that two HA^- clones caused a significantly higher frequency (P = 0.001) of acute synovitis than did the same number of viable cells of two HA^+ clones derived from the same M. synoviae population. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the whole-cell proteins of HA^+ and HA^- clones of the AAY-4 strain was done, and proteins were stained with Coomassie blue (1, 5). It was apparent that HA^- clones (including clones P1, P2, and P3 used to induce synovitis) synthesized proteins of 48 to 50, 53, and 67 to 70 kDa more abundantly than the corresponding HA^- clones (including clones N1 and N2). Further analyses using Triton X-114 partitioning to obtain integral membrane proteins of M. synoviae (1) and immunoblotting with the Phast-System (Pharmacia LKB) (5) indicated that the 48- to 50-kDa proteins defined by MAb 125 (17) are homologous of the MSPB proteins in M. synoviae WVU 1853 (17, 24). Furthermore, the 53-kDa protein defined by MAb 5E10/C9 (which inhibits binding of M. synoviae to erythrocytes) is homologous to MSPA hemagglutinin (4, 6, 24). The N-terminal amino acid sequences determined for the proteins defined by MAb 3E10/C9 in the AAY-4 HA^- clone (KF9) indicate the cleavage site in M. synoviae hemagglutinin which cleaves into MSPB and MSPA (6, 25). The 67- to 70-kDa proteins have the capability to hemagglutinate chicken erythrocytes and seem to be size and antigenic variants of M. synoviae hemagglutinin (6). HA^- and HAD^- phenotypes of the AAY-4 strain are thus associated with the expression of proteins detected by MAb 125 and 3E10/C9. HA^- and HAD^- clones lack the 53-kDa protein defined by MAb 3E10/C9 and synthesize truncated (27- to 30-kDa) proteins recognized by MAb 125. About 1% of the HAD^- population reverts in vitro to an HAD^- subpopulation, and the reversion from HAD^- to HAD^- occurs at similar rate (4, 6). M. synoviae attachment to erythrocytes (via sialic acid receptors) may be not exactly the same as its attachment to the cells on the surface of the hock joint cavity, but HA^- clones are more capable of inducing acute synovitis than are HA^- clones. We found this to be the case also for HA^- and HA^- clones of the type strain WVU 1853 of M. synoviae (unknown, high level of passages; obtained from Janet M. Bradbury, University of Liverpool). Cells of an HA^- clone (2 × 10^7 CFU; HA titer, 2 × 10^8) induced acute infectious synovitis in 50% of inoculated hock joints (n = 8), while a similar number of cells of an HA^- clone (2.2 × 10^7 CFU; HA titer, <10^5) did not induce synovitis. It has been previously reported that in strain WVU 1853, the hemadherent population expresses MSPA and MSPB, which are not expressed in the nonhemadherent population (24, 25). Our data also indicate a lack of MSPA-like protein in HA^- clones of WVU 1853. However, in HA^- clones, MSPB defined by MAb125 appears in a truncated form of 27 to 30 kDa, which might be equivalent to the 25- to 30-kDa MSPC protein which shares antigenic determinants with MSPB (6, 24, 25). It is possible that other proteins besides hemagglutinins are also involved in the induction of acute synovitis with M. synoviae, but this remains to be investigated.

**Suppression of synovitis by CsA.** CsA is a well-known immunosuppressor which, bound to its receptor (cyclophilin), prevents synthesis of interleukin-2 by blocking a late stage of the signalling pathway initiated by the T-cell receptor (33). In experimentally induced M. synoviae synovitis in chickens, T lymphocytes are necessary for development of macroscopic lesions (23). We attempted to suppress M. synoviae-induced synovitis with CsA. When hock joints were inoculated with M. synoviae, the left joints were also inoculated with 1 mg of CsA. Joints of half of the birds in experiment 2 and 10 birds in experiment 3 (groups A and C) were inoculated with 1 mg of CsA three times at 2-day intervals (Table 1). In experiment 2, in chickens inoculated with the HA^- culture, the frequency of synovitis was reduced from 70% in the CsA-untreated joints to 10% in the CsA-treated joints, and there was no swelling of hock joints treated with CsA three times. In addition, CsA completely prevented synovitis induced by the HA^- culture which induced mild swelling of a hock joint of only one chicken (Table 1). In experiment 3 with M. synoviae HA^- clone P3 (10^6 CFU), CsA given in multiple doses (three doses of 1 mg) completely prevented acute hock joint swelling (7 to 14 days p.i.), which otherwise appeared in 90% of control joints which were not treated with CsA. Altogether, CsA treatment significantly suppressed the induction of acute joint swelling with M. synoviae (P = 0.001). In addition, CsA reduced the mean lesion score of joints from 2.60 (induced with HA^- cultures) to 0.25 (Table 1, experiments 2 and 3). Inoculation with CsA only did not have any adverse effect on joints. Moreover, inoculation of the vehicle used to dissolve CsA did not induce any effect on synovitis or local antibody response (Table 1). The mechanism of CsA activity in suppressing M. synoviae-induced synovitis is at present unclear. It may be due in part to a mycoplastamastatic effect which we observed in vitro and has been also reported for M. pneumoniae (29). On the other hand, CsA activity in vivo was not mycoplasmal since it did not significantly reduce isolation rates and the number of CFU of M. synoviae from joints (Table 1). At least a part of CsA suppressor activity seems to be associated with the influence of CsA on immune response cells rather than with its mycoplastamastatic activity since CsA also prevented synovitis induced with M. synoviae membranes (data not shown).

**Examination of synovial fluid.** Marked edema and large amounts of synovial fluid of the inflamed joint cavity are characteristic of M. synoviae infectious synovitis (22, 23). In experiment 2, an HA^- culture caused synovitis lesions in which (3 weeks p.i.) the mean volume of synovial fluid was 0.43 ml, while with the HA^- culture the corresponding value was 0.15 ml. As shown in Table 1, the clinical signs of synovitis as well as the volume of synovial fluid were suppressed in the CsA-treated joints. The mean volume of synovial fluid was approximately three to four times lower than the corresponding values from joints inoculated with HA^- cultures but not treated
with CsA. On the other hand, large differences in the volume of synovial fluid within the same group—i.e., in experiment 2 in group A, exudate volumes in joints inoculated with the HA$^+$ culture varied from 0.2 to 0.75 ml—indicate that synovial exudate volume may not provide an accurate assessment of the degree of joint inflammation. However, volumes of synovial fluid collected at 3 weeks p.i. probably were not comparable with those during the acute stage of synovitis (e.g., 7 to 10 days p.i.) since at 3 weeks p.i. the joint inflammation could be regarded as an early chronic stage.

Unfortunately, systematic examination of populations of cells in synovial fluids was not done in the present study. Microscopic examination performed on only a few samples showed numbers of leukocytes per microliter of synovial fluids from joints inoculated with the HA$^+$ cultures several times higher than those from corresponding samples from joints also treated with CsA. Samples of synovial fluid obtained 2 or 3 weeks p.i. from joints infected with the HA$^+$ clones contained predominantly T cells, since up to 70% of cells had T-cell markers detected by polyclonal antibodies to chicken T cells. Examination of T-cell subpopulations with MoAbs CT4 and CT8, which define chicken T cells as T-cell-helper or -inducer and T-cell-cytotoxic or -suppressor cells, respectively (8), indicated the prevalence of T-cytotoxic cells in synovial fluids 2 weeks p.i. in birds inoculated with the HA$^+$ clone. However, only five samples obtained in experiment 2 were examined, and whether T cells with cytotoxic activity play a significant role in infectious synovitis remains to be seen. As presented in Table 1, M. synoviae recovery rates as well as counts of isolated colonies did not correlate with clinical signs of synovitis. The highest recovery rate was 80%, and colony counts varied considerably within groups. Examination of synovial fluid samples by direct immunofluorescence using fluorescein-labeled specific polyclonal antibodies showed M. synoviae in many samples from which culture was negative. It may be that in those samples M. synoviae cells were not viable, but low isolation rates of M. synoviae may also be associated with other factors (22).

**Antibodies to M. synoviae.** It has been suggested that specific antibodies may inhibit M. synoviae replication in the joints (23). We observed that in experimental infections of chickens with M. synoviae strains, inoculation into the upper respiratory tract might fail to induce a detectable systemic antibody response, while intrajoint inoculation with the same strains and numbers of organisms consistently induced a vigorous antibody response (data not shown). In this study all chickens inoculated with M. synoviae developed specific antibodies detectable in serum by rapid plate agglutination, IIPA, and immunoblotting. Immunoblotting using the PhastSystem (Pharmacia LKB) and M. synoviae AAY-4 HA$^+$ and HA$^-$ clones were conducted as described elsewhere (5). Briefly, following transfer of M. synoviae proteins onto an Immobilon P membrane (Millipore), the membrane was blocked in phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Tween 20 (0.5% PBS-T) for 30 min and cut into strips of appropriate width. The strips were incubated with chicken sera diluted at least 1:100 for 45 to 60 min. Following three consecutive washings for 15 min in 0.05% PBS-T, strips were reacted for 45 min with MoAbs (to chicken IgA, IgG, or IgM) conjugated with horseradish peroxidase (5, 14). Following washing in 0.05% PBS-T (twice for 15 min) and PBS (15 min), reacting proteins were visualized by incubation in a solution containing 3,3'-diaminobenzidine (0.25 mg/ml; Sigma) in 0.1 M Tris (pH 7.5) and 0.03% (vol/vol) H$_2$O$_2$. Examination of sera from chickens in experiment 2 (group A) showed that when acute synovitis became apparent (7 to 10 days p.i.), IgA, IgM, and IgG antibodies to several M. synoviae proteins were present. Examination of serum samples obtained 2 and 3 weeks p.i. indicated a tendency toward disappearance of specific IgA and IgM antibodies, while the titers of specific IgG antibodies in the IIPA with M. synoviae AAY-4 increased. In immunoblots, specific IgG antibodies recognized at least 10 M. synoviae proteins, with molecular masses from <25 to >200 kDa. They reacted with the greatest intensity with 48- to 50-kDa proteins of an HA$^+$ clone of strain AAY-4. Antibodies recognized the MSPB protein (48 to 50 kDa) defined by MoAb 125. In the IIPA those sera at low dilutions (1:2 to 1:8) blocked binding of MoAb 125 to M. synoviae colonies. Sera obtained from the same birds before inoculation of M. synoviae into joints did not show this activity.

In synovial fluids, IgA, IgM, and IgG antibodies to M. synoviae surface membrane proteins were detected by IIPA using intact colonies of strain AAY-4 in samples obtained from swollen hock joints about 1 week p.i. in experiments 2 (group A) and 3 (groups A and B). Selected samples (experiment 2 [n = 6]) with a strong IIPA reaction (at a dilution of 1:500) were assayed at the same dilution with immunoblotting using AAY-4 HA$^+$ and HA$^-$ clone proteins as antigens. Immunoblots revealed that in the early stage of acute synovitis, antibodies of the IgA, IgM, and IgG classes were produced against M. synoviae membrane proteins extracted with Triton X-114. The strongest antibody response was directed against proteins recognized by MoAb 125. Thus, in the HA$^+$ clone synovial fluid antibodies reacted strongly with 48- to 50-kDa proteins while in the HA$^-$ clone the reaction of 27- to 30-kDa proteins (a truncated MSPB protein form) was apparent (shown in Fig. 1A). As with serum antibodies, a trend toward disappearance of specific IgA and IgM class antibodies was found by the IIPA.

![Fig. 1](image-url)
for paired corresponding synovial fluids obtained 2 and 3 weeks p.i. On the other hand, levels of specific IgG antibodies were increasing and in immunoblotting they reacted with greater intensity and with more membrane proteins than acute-phase synovial fluid samples (Fig. 1B). All synovial fluid samples containing IgG antibodies gave intensive staining of MSPB proteins defined by MAb 125 (appearing as a 48- to 50-kDa protein in the HA1 clone and as a truncated 27- to 30-kDa protein in the HA2 clone of strain AAY-4). Since AAY-4 HA1 clones (in contrast to HA2 clones) lack the 53-kDa protein defined by MAb 3E10/C9, we looked for differences in the antibody response to the 53-kDa protein which might reflect differences between HA1 and HA2 clones used for infection. Less than half of the synovial fluids from joints inoculated with the HA1 culture (experiment 2, group A) right joint, three of seven samples tested) revealed a reaction of antibodies with the 53-kDa protein defined by MAb 3E10/C9. This finding and our observations from other experiments indicate that at least during early stages of the infection with M. synoviae (1 to 3 weeks p.i.) antibody response against the N-terminal part of hemagglutinating protein (this part is MSPB) is stronger and more consistent than against its C-terminal part, represented by the MSPA protein defined by MAb 3E10/C9. This is consistent with the general observation that chickens infected with M. synoviae raise HI antibodies later than 3 weeks after infection (22) and relevant to the fact that antibodies to MSPA have HI activity (6, 24).

Since CsA is a well-known immunosuppressive drug, we were interested to see if it would reduce the antibody response, especially the antibody response in treated joints. The IIPA, which is a very specific and sensitive assay for detection of antibodies to mycoplasmas in chickens (3, 5), was used to detect specific IgG antibodies 2 and/or 3 weeks p.i. Since sera and synovial fluid containing high titers of specific IgG antibodies in the IIPA intensively stained M. synoviae colonies at dilutions of 1:500 or even higher (score, 3+), unstrained colonies at a dilution of 1:100 were regarded as negative for specific IgG (3). In addition, a titer of IgG in a synovial fluid sample that was 10-fold lower than that in the paired synovial fluid sample taken from the other joint of the same bird at the same time was considered to show reduced antibody production. As shown in Table 1, some birds from three groups (experiments 2 and 3) did not have specific IgG in joints treated three times with CsA. Altogether, seven joints treated with CsA three times lacked specific IgG, while the corresponding CsA-untreated joints had IgG antibodies with the 53-kDa protein defined by MAb 3E10/C9. This could not be attributed to animal variation but was attributed to the suppression of the local antibody production. Additional cases (n = 5) of joints treated with CsA and with 10-fold-reduced levels of specific IgG antibodies confirm that suppression of local production by CsA is significant (P = 0.001). The suppression of the local antibody production by CsA is dose dependent. Indeed, in an experiment not shown here, the inoculation of 5 mg of CsA (given in 0.5- and 1-mg doses within 10 days) suppressed the production of local antibodies to M. synoviae as well as the appearance of synovitis in all joints (n = 5) treated with CsA.

Association of T lymphocytes with synovitis. Several studies have suggested that T lymphocytes are associated with the pathogenic mechanisms of M. synoviae-induced arthritis in chickens (20, 25). We conducted a few analyses of subpopulations of chicken CD4+ and CD8+ positive T lymphocytes, of cells expressing major histocompatibility complex II homologue molecules, of B cells expressing IgM, and of T cells expressing CD3 and T-cell receptor 1 (TCR1) (γδ) or TCR2 (αβ). MAbS recognizing these molecular markers were kindly provided by M. Cooper and N. Le Douarin and have been previously used in studies of others (7–10, 16).

For analysis the synovial fluids were resuspended in Hanks’ balanced salt solution, and then cells were washed three times in Dulbecco minimal essential medium (Sigma). Cells were then centrifuged over Ficoll Histopaque (Sigma) to remove dead cells and erythrocytes and washed three times with PBS (pH 7.4). Synovial fluid samples from uninoculated joints or joints treated only with CsA could not be analyzed by fluorescence-activated cell sorter (FACS) because of the small volume of synovial fluid, which contained very few cells. Cells examined by FACS (Becton Dickinson) were first treated with an appropriate MAb and then with goat IgG (to mouse IgG)-fluorescein isothiocyanate conjugate (Sigma) as described elsewhere (10). Examination of synovial fluids from chickens with M. synoviae naturally occurring arthritis by FACS revealed that almost 70% of cells had CD3, CD4, and CD8 markers, but the CD4/CD8 ratio was 1.01. On the other hand, during the acute stage of synovitis experimentally induced with an HA1 clone of M. synoviae (10 days p.i. with about 3 × 107 CFU), in synovial fluid samples the proportions of CD3, CD4, and CD8 were lower than in naturally infected chickens, while the CD4/CD8 ratio was 0.45. In synovial fluid samples from joints also inoculated with CsA (once with 1 mg) the percentage of cells with CD3, CD4, CD8, major histocompatibility complex II, and IgM markers was reduced 3.3, 1.6, 2.6, 6.0, and 2.9 times, respectively, in comparison with the samples from the corresponding CsA-untreated joints. In addition, the CD4/CD8 ratio was 0.75 in samples from CsA-treated joints. The small number of samples tested (eight joints inoculated in each group) does not allow the conclusion that CsA had a significant influence on cell population differences in M. synoviae-infected joints. However, cells from the synovial fluids of joints experimentally infected with M. synoviae (WVU 1853 HA2 clone) which were extensively washed were capable of inducing acute synovitis when inoculated into hock joints of chickens. Synovial fluids from joints in which inflammation was induced by washed cells (predominately T cells) contained similar proportions of CD3, CD4, and CD8 T cells but rather a high prevalence of cells with TCR2 (αβ) in comparison with TCR1 (γδ) cells (data not shown). Studies of expanded subpopulations of cells derived from synovial fluid were not possible because the cells replicated very slowly in vitro and died within 1 week. However, in vitro experiments with cells derived from chicken thymus indicated that M. synoviae AAY-4 more efficiently activated CD8+ cells than CD4+ cells and that CsA (final concentration, 0.8 mg of CsA/ml) downregulated CD8+ cell activation (from 68% of CD8+ cells detected after incubation with M. synoviae to 33% of CD8+ cells in the presence of CsA). It is well documented that mycoplasmas have mitogenic effects on immunocompetent cells and may produce superantigens, e.g., M. arthritidis (31, 34). Mycoplasma membrane lipoproteins may induce the production of proinflammatory cytokines; M. fermentans lipoproteins induce them by stimulation of tyrosine phosphorylation in human monocytes (27). M. synoviae has strong mitogenic activity on mouse lymphocytes and stimulates human lymphocytes to produce interferon (11, 13), but it is not known by which component(s). We found that M. synoviae membranes as well as proteins secreted into growth medium have a considerable mitogenic activity in vitro for mouse lymphocytes and chicken thymocytes. The significantly higher capability of strain AAY-4 HA1 cultures to induce synovitis suggests that hemagglutinating proteins, particularly MSPA, may be associated with mitogenic activity. It is possible that proteins associated with the HA1 phenotype stimulate
cytotoxic T cells (CD8\(^+\) cells), but this assumption needs further experimental work for confirmation.

*M. synoviae* induced synovitis is probably a complex multifactorial process, and this study did not identify all of the factors involved. Nevertheless, our data indicate that the phenotype of *M. synoviae* (HA\(^+\)) is an important factor and supports previous findings about involvement of immunocompetent cells, particularly T cells, in the development of infectious synovitis (18, 20, 23).

This work was supported by a grant of the U.S.-Slovene Science & Technology Program (21-A-05-11, USA/SLO 95/10-07) and by grant J4-7443 from the Ministry of Science & Technology of the Republic of Slovenia.

We thank J. M. Bradbury for WVU 1853 strain cultures and M. D. Cooper, N. Le Douarin, and D. H. Ley for MAbs.

REFERENCES


Editor: V. A. Fischetti