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Expression of the Superantigen *Mycoplasma arthritidis* Mitogen in *Escherichia coli* and Characterization of the Recombinant Protein

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***Mycoplasma arthritidis* mitogen (MAM), is a soluble protein with classical superantigenic properties and is produced by an organism that causes an acute and chronic proliferative arthritis. Unfortunately, the process of obtaining purified MAM from *M. arthritidis* culture supernatants is extremely time-consuming and costly, and very little material is recovered. Thus, our laboratory has expressed MAM in *Escherichia coli* by using a protein fusion expression system. The construction and expression of recombinant MAM (rMAM), as well as a comparison of the biological properties of rMAM to those of native MAM, are discussed. Briefly, conversion of the three UGA codons to UGG codons was required to obtain full-length expression and mitogenic activity of rMAM. Antisera to native MAM recognized both rMAM and the fusion protein. The T-cell receptor V_β and major histocompatibility complex class II receptor usages by rMAM and the fusion protein were identical to that of native MAM. In addition, the ability to induce suppression and form the superantigen bridge could also be demonstrated with rMAM. Importantly, dose-response experiments indicated that homogeneous native MAM and rMAM were of equal potency. Thus, MAM has been successfully expressed in *E. coli*, thereby creating a viable alternative to native MAM.**

Mycoplasma arthritidis, which induces a spontaneous polyarthritis in rodents, produces an immunomodulatory protein that demonstrates classical superantigenic properties (reviewed in reference 5). *M. arthritidis* mitogen (MAM) is a typical superantigen (SAg) in that it is presented to murine or human T cells by direct binding to major histocompatibility complex (MHC) molecules present on accessory cell surfaces. MAM is also recognized by specific V_β chain segments of the T-cell receptor (TCR) for antigen without MHC restriction (6, 9). MAM was first identified as a SAg in 1989 when it was shown that the T-cell mitogen contained in *M. arthritidis* culture supernatants specifically activated V_β8-bearing T cells (10). Despite its being one of the first SAgs described, studies utilizing MAM have been limited due to the high cost and difficulty in obtaining the pure protein (1, 2, 19).

Partially purified preparations of MAM from *M. arthritidis* culture supernatants were initially used to characterize MAM as a basic, heat- and acid-labile protein with a molecular mass of 15 to 30 kDa and a pI of ≥9.0 (1, 19). The high isoelectric point and hydrophobic nature of the protein made initial attempts at purification to homogeneity difficult because MAM could bind nucleic acids and other proteins in the culture medium as well as glass or plastic surfaces (2). Pure MAM was eventually obtained by using a series of cationic and hydrophobic interaction columns with an approximate yield of 20 to 50 μg of MAM per liter of culture (2). Analysis of this purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a single band with a molecular mass of 27 kDa which correlated with the calculated molecular mass of 25.3 kDa based on the predicted amino acid sequence (11). The mitogenic activity of this pure protein could be detected in the picogram range, and it possessed the same biological and physical properties previously described with the less pure preparations of MAM (2).

Highly and partially purified preparations of MAM have been used to show that this SAg preferentially binds to the conserved E_α chain of the murine H-2E or to the alpha chain of the human HLA-DR molecule (5). However, murine E_α is more efficient than human DR_α in presenting MAM to human peripheral T cells, suggesting that MAM has a greater affinity for E_α than DR_α (24). There is also evidence that MAM can be presented to T cells by some murine H-2A molecules, although at a greatly reduced efficiency (6). Recognition of the MHC-MAM complex is achieved by a number of V_β TCRs and depends on the V_β haplotype of the mouse strain used. For example, in $V_\beta{}^b$ haplotype mice, which include the majority of inbred strains, MAM engages V_β5.1 and -6 and the V_β8 family of TCRs (3, 6, 9). In $V_\beta{}^a$ and $V_\beta{}^c$ mice, which lack various combinations of these V_βs due to genomic and somatic deletions, MAM engages V_β1, -3.1, -7, and -16 (6). MAM also possesses an immunosuppressive property such that lymphocytes from mice injected intravenously (i.v.) with MAM demonstrate a marked decrease in their ability to respond in vitro to MAM, phytohemagglutinin, or concanavalin A (ConA) (12). In addition, MAM forms the SAg bridge (15, 26) in that B cells pulsed with MAM not only activate MAM-reactive T cells but also themselves undergo differentiation, resulting in polyclonal immunoglobulin (Ig) production (26).

Here we demonstrate that MAM can be successfully expressed in *Escherichia coli* and that the recombinant fusion protein and the cleaved recombinant MAM (rMAM) have identical superantigenic properties compared with those of native MAM.

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MATERIALS AND METHODS

Construction and cloning of rMAM. The MAM gene (*mam*) was isolated from a genomic library of *M. arthritidis* PG6 constructed in EMBL3. Screening of the genomic library and subsequent sequencing of *mam* have been described elsewhere (11). Previously, it was determined that *mam* was contained within a 1.4-kbp *Xba*I fragment in an EMBL3 clone (11). This *Xba*I fragment was ligated into plasmid pTZ18R (Pharmacia) to generate plasmid pZX1.4B. MAM contains three UGA codons encoding tryptophans at positions 132, 177, and 178 of the mature protein (11). The UGA codons at positions 177 and 178 were converted to UGG codons for tryptophans through PCR by using the mutagenic primer XP-1 (5′-GAAGAGATCTAACCACCAGAGCTC-3′) and primer MAM7 (5′-GATAAAGCTGTCAAACTAACCT-3′). The resulting 153-bp product was digested with *Bgl*II restriction enzyme and ligated into the similarly digested plasmid pZX1.4B to generate plasmid pWWII. As an aid to screening, the mutagenic primer XP-1 was also designed to introduce a unique *Sac*I site in *mam* by changing a thymine to a cytosine, resulting in a conserved CTT-to-CTC codon change of leucine at position 176. The UGA codon at amino acid position 132 was changed to UGG by site-directed mutagenesis with an overlap PCR technique similar to that of Ho et al. (17). Briefly, complementary mutagenic primers W1 (5′-CAAGAGTTATGGGATAAAGCTGTCAAAC-3′) and MAMP3 (5′-GTTTGACTGCT-TTATCCCATAACTCTTG-3′) were used in two separate PCRs with primers flanking the 3′ (MAMP2; 5′-AAAAAGCT TGCAAGGAATTTATTTAAAATCCCCCC-3′) and 5′ (MN3; 5′-TCAGAAT TCATGAAACTTAGAGTTGAA-3′) ends of *mam*, respectively, with pWWII as the template. The products of the two separate PCRs were used as the template in the final PCR with primers MN3 and MAMP2 to generate the rMAM gene. Following digestion with the restriction enzymes *Eco*RI and *Hin*dIII, the rMAM gene was directionally cloned into the protein fusion expression vector pMAL-c2 (New England Biolabs Inc., Beverly, Mass.) to generate plasmid pKK2. Plasmid pKK2 was then used to transform *E. coli* DH5α (Life Technologies, Gaithersburg, Md.). The change of the UGA to UGG codons was confirmed by sequence analysis by the Sanger et al. dideoxy chain termination method (23).

Expression of rMAM in *E. coli*. Expression of rMAM via the pMAL-c2 protein fusion expression system (New England Biolabs Inc.) was achieved by a modification of the manufacturer's suggested protocol. Briefly, an overnight culture of *E. coli* DH5α containing pKK2 was diluted 1:50 in Rich medium (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 2 g of glucose per liter of water) containing 100 μg of ampicillin per ml and grown at 37°C with agitation (250 rpm) until the A_{600} equaled 0.5. rMAM overexpression was then induced by addition of 1 mM (final concentration) isopropyl-β-D-thiogalactoside (IPTG; Gold Biotechnologies, Inc., St. Louis, Mo., or Sigma Chemical Co., St. Louis, Mo.). Following 3.5 h of induction, the cells were centrifuged at 4,000 × *g* for 10 min at 4°C and frozen overnight at −20°C. The frozen cells were resuspended in column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4) at 1/20 of the original culture volume and sonicated (Branson Sonic Power sonifier; Danbury, Conn.). Cellular debris was removed by centrifugation (9,000 × *g*, 30 min), and the supernatant was passed through a 0.45-μm-pore-size filter (Nalgene, Rochester, N.Y.).

Purification of MAM, maltose-binding protein–rMAM (MBP-rMAM), and rMAM. Purified MAM (i.e., MAM derived from *M. arthritidis*) was obtained by procedures described previously (2). The MBP-rMAM fusion protein and rMAM were purified by using affinity chromatography with amylose resin according to the manufacturer's protocols (New England Biolabs). The column eluent containing MBP-rMAM was concentrated to 1 to 5 mg/ml by centrifugation with Centricon-30 concentrators (Amicon, Inc., Beverly, Mass.). To cleave rMAM from the MBP, 5 μg of factor Xa protease was added per 100 μg of the fusion protein and allowed to digest overnight at 4°C in column buffer. The proteins in the digestion mixture were separated on an SDS–10% PAGE gel (20) with a Bio-Rad (Hercules, Calif.) Mini-Protean II electrophoresis cell and stained for 5 min with $CuCl_2$ as described previously (21). The rMAM protein band was excised from the gel with a razor blade and transferred to an electroelution chamber (Bio-Rad Model 422 Electro-Eluter). Pure rMAM was recovered from the gel by following the manufacturer's (Bio-Rad's) protocols. Protein concentration was determined with the bicinchoninic acid protein assay reagent by following the manufacturer's protocols (Pierce, Rockford, Ill.). The plasmid pMAL-c2 gene product, consisting of a protein fusion between MBP and the α subunit of the β-galactosidase protein (MBP–β-Gal), was used as a negative control in some experiments.

Mice. The following mouse strains were used in this study: BALB/c (*H-2*^{d} V$_\beta$^{b}), CBA/Ca (*H-2*^{k}V$_\beta$^{b}), (C3H (*H-2*^{k} V$_\beta$^{b}), C57BL/10 (*H-2*^{b} V$_\beta$^{b}), C57BR (*H-2*^{k} V$_\beta$^{a}), RIIIS (*H-2*^{r} V$_\beta$^{c}), SWR (*H-2*^{q} V$_\beta$^{a}), and B10.RIII (*H-2*^{r} V$_\beta$^{b}). These were purchased from the Jackson Laboratory (Bar Harbor, Maine).

Production of antibodies against MAM and MBP-rMAM. Murine anti-MAM antibody was raised by first injecting 10^9 live *M. arthritidis* organisms i.v. into CBA/Ca mice. After 6 weeks, a secondary injection (i.v.) of approximately 10 μg of purified MAM was administered. Serum was collected 13 days after the secondary injection. Rabbit anti-MBP-rMAM was raised by injecting 300 μg of MBP-rMAM suspended in RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, Mont.) into New Zealand White rabbits according to the manufacturer's protocol. Serum collected 10 days following the fourth booster injection was used for these studies.

Lymphocyte proliferation and neutralization of proliferation. The proliferative response of splenocytes to native MAM, rMAM, or MBP-rMAM was measured in a microtiter plate assay at 48 h with a 6-h pulse of [^{3}H]thymidine ([^{3}H]TdR). Lymphocyte proliferation procedures have been detailed previously (1). ConA at 5 μg/ml was used as positive control in all proliferation assays.

Blocking of lymphoproliferation was accomplished by first mixing 2.5 ng of MAM or 1 μg of ConA with varying dilutions of mouse anti-MAM or rabbit anti-MBP-rMAM antisera in a final volume of 125 μl. Following a 1-h incubation at 37°C, the mixture was added to 5 × 10^5 splenocytes (0.1 ml) in microtiter wells, and incubation proceeded as described above.

Recognition of rMAM by ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed by modifications of a previously described protocol (13). Briefly, 2.5 μg of rMAM, MBP-rMAM, MBP, or MBP–β-Gal resuspended in 0.15 M potassium phosphate (pH 7.6) was used to coat individual wells of a Linbro/Titertek 96-well microtiter plate. After overnight incubation at 4°C, blocking buffer (phosphate-buffered saline [PBS]) containing 1% bovine serum albumin [BSA], fraction V [Miles, Inc., Kankakee, Ill.]) was added to each well and incubation was continued for 5 h at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20, and 100 μl of a 1:500 or 1:1,000 dilution of murine MAM antiserum was then added. After overnight incubation at 4°C, the plates were washed again and 100 μl of a 1:2,000 dilution of biotinylated anti-mouse serum (Sigma) was added for 2 h at room temperature. After washing, 100 μl of horseradish peroxidase-avidin D (Vector Laboratories, Burlingame, Calif.) was added. After another wash, 400 μM substrate azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) resuspended in 0.1 M citric acid–0.2 M dibasic sodium phosphate (pH 5.2) was added. After 8 to 10 min, the colorimetric reaction was stopped by addition of 1% SDS in dimethyl formamide. The plates were read at 405 nm with a Bio-Rad Model 3550 UV microplate reader. All assays were performed in triplicate wells.

Determination of TCR V$_\beta$ usage by fluorescence-activated cell sorting. Fluorescein isothiocyanate (FITC)-conjugated antibodies to the murine V$_\beta$ TCRs were used at a final concentration of 2.5 μg/ml and were obtained from PharMingen (San Diego, Calif.). The V$_\beta$ TCRs recognized by these antibodies are as follows: KJ25, hamster anti-mouse V$_\beta$3 TCR; RR4-7, rat anti-mouse V$_\beta$6 TCR; TR310, rat anti-mouse V$_\beta$7 TCR; RR3-15, rat anti-mouse V$_\beta$11 TCR. Monoclonal antibodies F23.1 (recognizes V$_\beta$8.1, -8.2, and -8.3 TCRs) and F23.2 (recognizes V$_\beta$8.2 TCRs) (they were obtained from P. Marrack, University of Colorado Health Sciences Center) were biotinylated with biotin–disulfide–*N*-hydroxysuccinimide (Sigma) and diluted 1:100 or 1:10, respectively, for use in these studies. BALB/c splenocytes collected as described above were resuspended in 10 ml of complete RPMI 1640 with 10% fetal calf serum (1.25 × 10^6 cells/ml) and were cultured in T-25 tissue culture flasks (Sarsted) in the presence of 1 μg of MAM, rMAM, or MBP-rMAM per ml or 5 μg of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, Fla.) per ml for 2 days. The cells were washed once with RPMI 1640 medium and expanded with interleukin-2 for 2 days. The cells were washed once in RPMI 1640 medium and twice in 1% BSA in Hanks' balanced salt solution and counted, and the viable cells were resuspended in Hanks' balanced salt solution to give a concentration of 4 × 10^6 cells/ml. Aliquots of 0.5 ml containing 2 × 10^6 viable cells were then transferred to 1.5-ml microcentrifuge tubes. FITC-conjugated antibodies to murine V$_\beta$ TCRs were then added, incubated for 1 h on ice, and washed twice with 1% BSA in PBS. When a biotinylated monoclonal antibody was used, the cells were resuspended in avidin-FITC diluted with 1% BSA in PBS and incubated on ice for 30 min. The cells were resuspended in 1% BSA in PBS to a final volume of 1 ml. Samples were submitted to the Flow Cytometry Core Laboratory, Huntsman Cancer Center, University of Utah, for analysis on a Becton Dickinson FACScan flow cytometer with Becton Dickinson's CELLQUEST computer program for immunocytometry systems (San Jose, Calif.). Data are expressed as percentages of total viable splenocytes as defined by forward light scatter parameters and propidium iodide staining (0.5 μg/sample; Sigma).

In vivo inhibition of lymphocyte proliferation. BALB/c mice were injected i.v. with 0.5 to 0.005 μg of MBP-rMAM or PBS. Forty-eight hours later, the spleens were removed and the ability of the splenocytes to proliferate in response to 50 pg of MAM or 5 μg of SEB was measured as described above with a 72-h incubation with an 18-h [^{3}H]TdR pulse. Assays were performed in triplicate. The results were expressed as the percentages of [^{3}H]TdR uptake of splenocytes from MAM-induced mice compared to the PBS-injected controls.

Antibody production via formation of the SAg bridge. Two sources of T cells were used to generate the SAg bridge. The first consisted of BALB/c splenocytes clonally expanded in culture for 7 days in the presence of 50 pg of MAM and subsequently exposed to 2,000 rads of gamma irradiation (T-MAM). The second was a V$_\beta$8.2$^+$ T-hybridoma line (2Hd-11.2) that was exposed to 10,000 rads of gamma irradiation. A pure B-cell population was obtained by panning BALB/c splenocytes as described previously (13).

In duplicate 96-well microtiter plates, 2 × 10^5 B cells were mixed with 10^5 gamma irradiated T cells in the presence of one of the following inducers: 50 pg of MAM, MBP-rMAM, or rMAM; 5 μg of SEB per ml; or 20 μg of lipopolysaccharide (LPS) B from *E. coli* (Difco, Detroit, Mich.) per ml. Following a 3-day incubation at 37°C, the cells from one of the plates were pulsed with [^{3}H]TdR for 5 h, harvested, and counted. Controls consisted of B cells only, gamma-irradiated

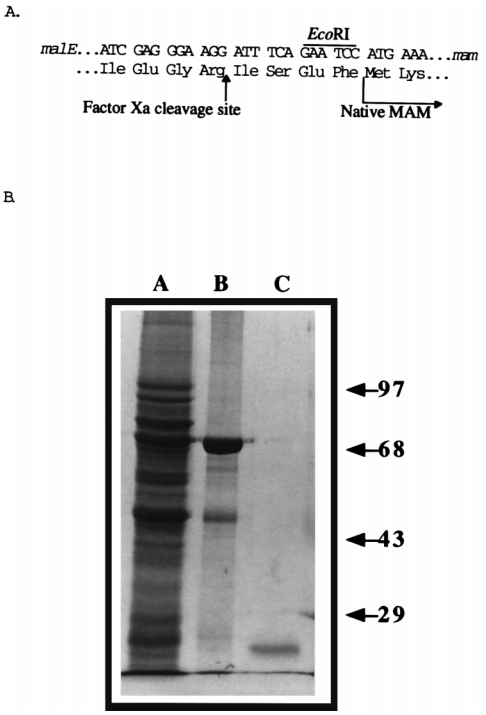

FIG. 1. (A) Diagram of the factor Xa cleavage site of MBP-rMAM. Nucleic acid and amino acid sequences of the region in which MBP and MAM are fused are shown. The factor Xa cleavage site and the start of the mature native MAM protein are indicated by arrows. (B) SDS–10% PAGE gel showing the IPTG-induced MBP-rMAM fusion protein in an *E. coli* lysate (lane A), purified MBP-rMAM fusion protein (lane B), and purified rMAM (lane C). Molecular weights (in thousands) are indicated to the right.

T-MAM or 2Hd-11.2 cells only, B cells and gamma-irradiated T-MAM or 2Hd-11.2 cells, B cells with each inducer, and gamma-irradiated T-MAM or 2Hd-11.2 cells with each inducer.

The other plate was incubated for 10 days, and supernatants were then harvested and assayed for immunoglobulin G (IgG) and IgM levels by the sandwich ELISA method described previously (13). Horse anti-mouse IgG, goat anti-mouse IgM, biotinylated horse anti-mouse IgG, biotinylated goat anti-mouse IgM, and horseradish peroxidase-avidin were obtained from Vector Laboratories, Inc.

RESULTS

Construction, expression, and purification of MBP-rMAM and rMAM. After successfully changing the UGA-encoded tryptophans to UGG-encoded tryptophans by site-directed mutagenesis, the rMAM gene was ligated into the pMAL-c2 plasmid and expressed in *E. coli* (see Materials and Methods). The resulting fusion protein, MBP-rMAM, was cleaved with factor Xa to yield rMAM as illustrated in Fig. 1A. Because the factor Xa cleavage site is not adjacent to the first codon of mature MAM, rMAM possesses an additional 4 amino acids located at the amino-terminal end of the protein (Fig. 1A). As shown in Fig. 1B, affinity-purified MBP-rMAM gave the appropriate 68-kDa protein (lane B). Factor Xa cleavage released a ca.-25-kDa protein (lane C) which corresponded to the calculated 25.3-kDa protein from the known amino acid sequence of MAM (11). rMAM derivatives that possessed the UGA-to-UGG codon changes at either MAM amino acid position 132 or positions 177 and 178 were also generated, to give plasmid pKK3 or pKK4, respectively. Expression of these genes with the pMAL fusion system gave proteins of ca. 64 or 59 kDa, respectively (data not shown). Typically, 15 mg of MBP-rMAM and 500 μg of rMAM were obtained from 1 liter of culture.

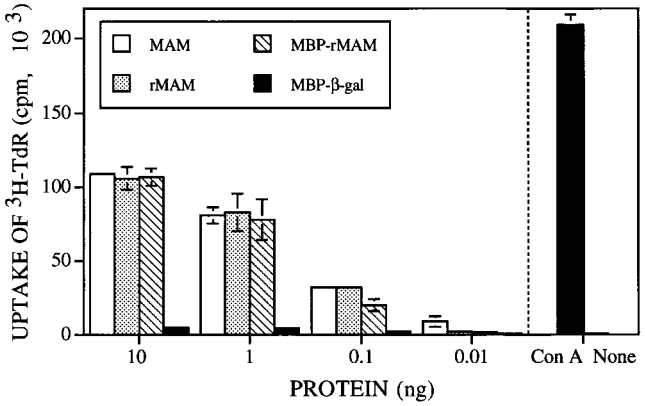

FIG. 2. Proliferation of B10.RIIIS ($V_\beta{}^b$) splenocytes stimulated with MAM and rMAM. A total of 5×10^5 splenocytes were cultured in the presence of various amounts of MAM, MBP-rMAM, rMAM, or MBP–β-Gal. Spontaneous uptake of [^{3}H]TdR was used to measure levels of lymphoproliferation. Values shown represent the means of triplicate measurements. Responses to 5 μg of ConA per ml and no protein (None) represent the positive and negative controls, respectively, for cell inducibility.

Lymphocyte proliferation by native MAM and rMAM. Using B10.RIIIS ($V_\beta{}^b$) splenocytes, which express all of the V_β TCRs used by MAM, we showed that the levels of lymphoproliferation induced by MAM, rMAM, and MBP-rMAM were equal at all concentrations examined (Fig. 2). Maximal mitogenic activity was achieved in the presence of 10 ng of each protein per ml, but activity could still be observed at levels as low as 10 pg/ml. The negative control, MBP–β-Gal, failed to induce proliferation, and the expressed fusion proteins from plasmids pKK3 and pKK4, which contained truncated MAM, were also nonmitogenic (data not shown).

Immunorecognition of MAM and rMAM. Antibody to native MAM raised in CBA/Ca mice was examined for its ability to recognize rMAM and MBP-rMAM by ELISA. rMAM and MBP-rMAM, but not MBP or MBP–β-Gal, were recognized by anti-MAM antisera while normal (prebleed) antisera did not recognize any of these proteins (Fig. 3). In a separate

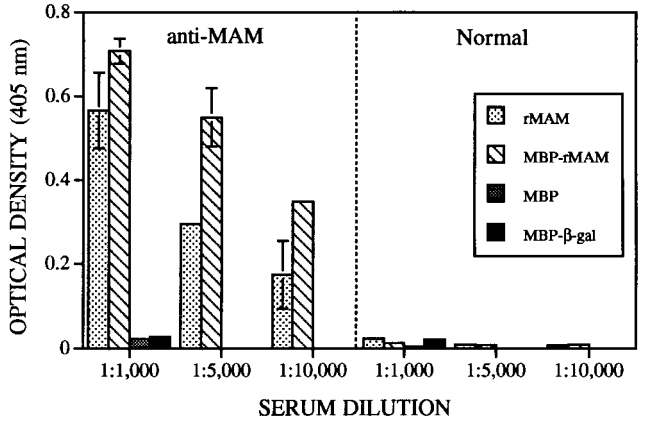

FIG. 3. Immunorecognition of rMAM by antibody raised against native MAM. Varying dilutions of murine anti-MAM and normal sera were tested for their ability to recognize 2.5 μg of rMAM, MBP-rMAM, MBP, or MBP–β-Gal by ELISA. Values represent the mean optical densities of triplicate measurements.

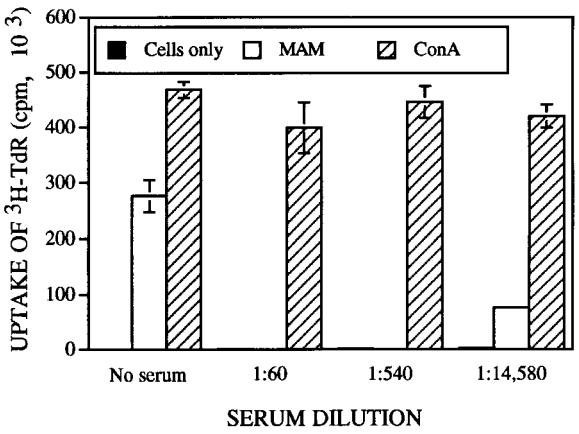

FIG. 4. Rabbit anti-MBP-rMAM blocks MAM-induced lymphoproliferation. Varying dilutions of antisera against MBP-rMAM were examined for their ability to block MAM-induced lymphoproliferation. Spontaneous uptake of [^{3}H]TdR was used to measure levels of lymphoproliferation. Values shown represent the means of triplicate measurements.

experiment, it was shown that the anti-MAM antisera specifically recognized MBP-rMAM and the fusion protein products from pKK3 and pKK4 equally as well (data not shown).

Due to the limited availability of native MAM, in place of an ELISA, we employed an assay which measured the ability of rabbit anti-MBP-rMAM to block MAM-induced proliferation. MAM-induced proliferation of BALB/c splenocytes was completely inhibited by the rabbit antibody, and proliferation could not be detected until the rabbit serum was diluted 1:14,580 (Fig. 4). Normal serum failed to inhibit proliferation. The antibody was not toxic to the cells, since ConA-induced proliferation was not affected.

TCR V_β usage by MAM or rMAM. BALB/c splenocytes were clonally expanded with 1 μg of MAM, MBP-rMAM, or rMAM per ml or 5 μg of SEB per ml and tested for percent expression of specific V_β chains. As outlined in Table 1, MAM, MBP-rMAM, and rMAM preferentially expanded T cells bearing the V_β8.1, -8.2, -8.3, and -6 chains but not those with V_β3, -7, or -11. MAM and the rMAM derivatives appeared to be especially effective in inducing T cells bearing the V_β8 lineage of TCRs compared to those with the V_β6 TCRs. MAM, rMAM, and MBP-rMAM were similarly effective in expanding T cells bearing the V_β6 or the V_β8 family of TCRs. SEB expanded the V_β8-bearing but not the V_β6-bearing T cells.

MHC and V_β haplotype influence MAM, rMAM, and MBP-rMAM reactivity. Splenocytes from mouse strains bearing different MHC or V_β haplotypes were examined for their ability to proliferate in response to MAM, rMAM, and MBP-rMAM. The proliferative ability of splenocytes in the presence of all MAM derivatives was similar with each mouse strain examined. All three inducers promoted high levels of proliferation of lymphocytes from BALB/c and C3H mice, which both bear the H-2E_α chain and express the $V_\beta{}^b$ haplotype (Table 2). There was at least a 10-fold reduction in the proliferative response by splenocytes from the C57BL/10 mouse line, which does not express E_α but which has the $V_\beta{}^b$ haplotype. There was a two- to fourfold reduction in proliferative response by splenocytes from the C57BR mouse strain, which lacks the V_β8 family of TCRs (i.e., $V_\beta{}^a$ haplotype) but continues to express E_α as well as V_β6. There was a 15- to 40-fold reduction in proliferation by splenocytes from RIIIS mice, which fail to express both the V_β6 and the V_β8 family (i.e., $V_\beta{}^c$ haplotype) of TCRs but continue to express E_α. Splenocytes from SWR mice, which do not express E_α or the V_β6 or -8 TCRs, fail to proliferate in the presence of MAM, rMAM, or MBP-rMAM.

In vivo MBP-rMAM inhibits SAg-induced lymphocyte proliferation. Inhibition was examined by measuring the proliferative ability of splenocytes obtained from mice challenged first with MBP-rMAM in vivo and then with MAM or SEB in vitro. As shown in Fig. 5, inhibition of the proliferative response was dose dependent relative to the amount of MBP-rMAM used to inject the mice. Moreover, MBP-rMAM also suppressed the responses to SEB, which also uses the V_β8 TCRs. Similar results were previously reported with native MAM (12).

Ig production via the SAg bridge. The ability of MAM, rMAM, MBP-rMAM, SEB, or LPS to induce B cells to proliferate and produce IgM or IgG through the formation of the SAg-MHC-TCR trimolecular complex was examined. The possible influence of T-cell proliferation and overgrowth of B cells was eliminated by gamma irradiation of the T cells prior to addition of the B cells and inducers. As shown in Fig. 6, B-cell proliferation occurred only in the presence of both the SAg and the gamma-irradiated T-cell population. Similar high levels of B-cell proliferation were observed when MAM, rMAM, or MBP-rMAM was used as the inducer in the presence of either MAM-expanded T cells (T-MAM) or the 2Hd-11.2 T-hybridoma line, which expresses the V_β8.2 TCR chain. The level of B-cell proliferation induced by SEB in the presence of 2Hd-11.2 cells was much less than the level induced by MAM or rMAM. In the presence of T-MAM, SEB was able to promote only 20% of the levels induced by MAM or rMAM. The level of B-cell proliferation induced by LPS was minimal and probably due to the fact that the B cells were harvested only 3 days after incubation with the B-cell mitogen.

MAM, MBP-rMAM, and rMAM were all able to induce IgM and IgG production by B cells when presented by either T-MAM or 2Hd-11.2 T-hybridoma cells (Fig. 6). There was a direct relationship between levels of IgM but not IgG production and levels of B-cell proliferation. rMAM induced the highest level (170 μg/ml) of IgM production, with MBP-MAM

TABLE 1. Percentage of SAg-induced BALB/c splenocytes recognized by FITC-labeled TCR V_β-specific antibody

Treatment	%[a] of cells responding to antibody (TCR V_β recognized):						
	None	KJ-25 (V_β3)	RR4-7 (V_β6)	TR310 (V_β7)	F23.1 (V_β8.1-3)	F23.2 (V_β8.2)	RR3-15 (V_β11)
Cells plus MAM	2.0	2.7	10.4[b]	2.9	80.9[b]	76.1[b]	1.4
Cells plus MBP-rMAM	3.0	2.1	15.4[b]	2.3	60.7[b]	55.1[b]	2.1
Cells plus rMAM	0.3	0.2	10.4[b]	0.1	62.9[b]	34.3[b]	0.5
Cells plus SEB	0.1	ND[c]	0.8	ND	84.8[b]	ND	ND

[a] Percentage of total cells reacting to antibody after expansion with various MAM and SEB preparations.
[b] Culture showing selective expansion of specific V_β TCR-bearing cells.
[c] ND, not determined.

TABLE 2. Similar levels of incorporation of [^{3}H]TdR[a] by MAM- or rMAM-induced splenocytes from different mouse strains

Inducer	Incorporation for mouse strain (H-2E_α presence and V_β haplotype[b])					
	BALB/c (E_α^+, V_β^b)	C3H (E_α^+, V_β^b)	C57BL/10 (E_α^-, V_β^b)	C57BR (E_α^+, V_β^a)	RIIIS (E_α^+, V_β^c)	SWR (E_α^-, V_β^a)
MAM	227.2	231.6	21.9	56.5	7.8	0.4
MBP-rMAM	310.4	308.4	11.2	119.2	8.4	0.6
rMAM	329.2	297.4	36.3	148.6	20.8	1.0

[a] Incorporation of [^{3}H]TdR is expressed as 10^3 cpm.
[b] V_β^b mice express all V_β TCR chains utilized by MAM. V_β^a mice possess deletions which include the V_β8 gene family. V_β^c mice possess deletions in the V_β8 and V_β6 genes.

and MAM inducing 90 and 73 μg/ml, respectively. Conversely, SEB induced higher levels of Ig production when presented by V_β8.2-bearing T cells. As expected, LPS induced IgM production in the presence or absence of a T-cell population.

DISCUSSION

MAM is a soluble protein that is found in culture supernatants and demonstrates classical superantigenic activity (4, 5). Unfortunately, the process of extracting pure MAM from culture supernatants is costly and time-consuming, and only 20 to 50 μg of MAM is recovered per liter of culture (2). In addition, the hydrophobic nature of the protein has made handling difficult because MAM can bind nucleic acids and other proteins in the culture medium as well as glass or plastic surfaces (2).

The expression of a recombinant form of MAM in *E. coli* was therefore pursued. Based on the alternative codon usage by mycoplasmas (22), the sequence of MAM was predicted to possess three UGA codons that would be read as tryptophans instead of as universal stop codons (11). In order to express MAM in *E. coli*, the three UGA codons were converted to UGG codons for tryptophan by site-directed mutagenesis to give rMAM. Conversion of all three UGA codons was necessary to achieve expression of the ca.-25-kDa full-length protein (Fig. 1). Plasmids containing derivatives of rMAM in which the UGA codons at amino acid position 132 (pKK3), or at positions 177 and 178 (pKK4), were changed failed to express the full-length protein in *E. coli* (data not shown).

Unfortunately, like purified MAM, rMAM also appeared to stick to glass and plastic surfaces and lose activity if stored at 4°C and was more stable if stored in the presence of a protein carrier. The MBP-rMAM fusion protein, however, was remarkably stable, since prolonged storage (at least 6 months) at 4°C in PBS did not appear to affect its ability to induce lymphocyte proliferation (data not shown). Because the fusion

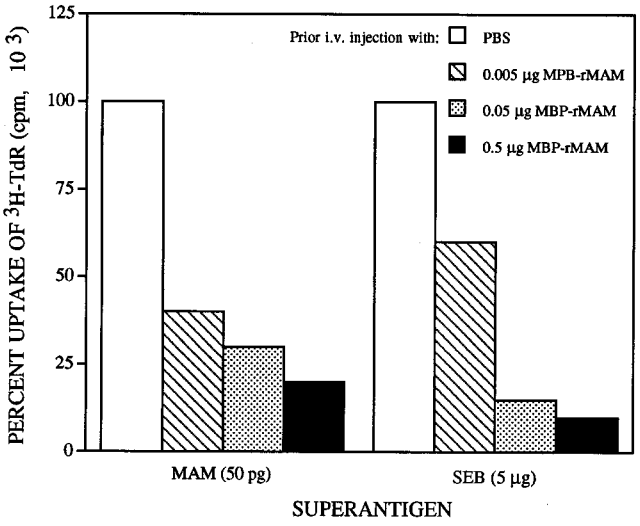

FIG. 5. Inhibition of lymphocyte responsiveness to MAM or SEB in vitro as induced by prior i.v. injection of MBP-rMAM. BALB/c mice were injected with PBS or 0.5, 0.05, or 0.005 μg of MBP-rMAM. After 72 h, splenocytes were tested for responsiveness to 50 pg of MAM or 5 μg of SEB. Levels of lymphoproliferation are expressed as the percentage of uptake of [^{3}H]TdR obtained with lymphocytes from mice injected with MAM versus the uptake obtained with lymphocytes from mice injected with PBS. Values shown represent the means of triplicate measurements.

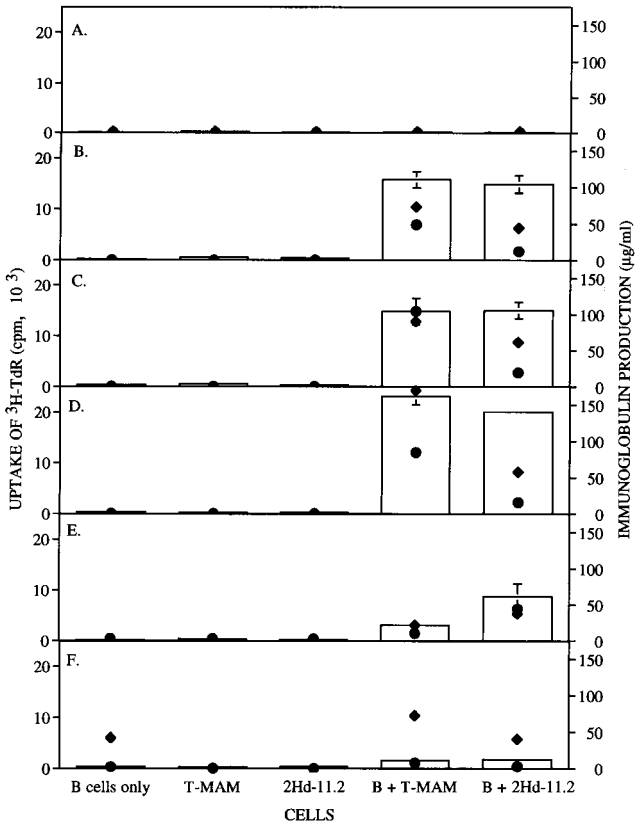

FIG. 6. B-cell proliferation and Ig production via SAg bridge formation. A total of 10^5 gamma-irradiated MAM-expanded T cells (T-MAM) or 2Hd-11.2 T-hybridoma cells bearing the V_β8.2 TCR were used to present 50 pg of MAM per ml (B), 50 pg of MBP-rMAM per ml (C), 50 pg of rMAM per ml (D), 5 μg of SEB per ml (E), or 20 μg of LPS per ml (F) to 2×10^5 panned B cells. Levels of proliferation in the absence of an inducer are indicated in panel A. The bar graph shows levels of lymphoproliferation as represented by spontaneous uptake of [^{3}H]TdR. Values shown are the means of triplicate measurements. Closed diamonds (◆) and circles (●) indicate levels of IgM and IgG, respectively.

protein appeared to be particularly stable, it was also included in our studies comparing the bioactivities of MAM and rMAM.

Approximately 15 mg of fusion protein was recovered per liter of culture. Because rMAM comprises about one-third of the fusion protein, it was predicted that 5 mg of rMAM would be obtained following cleavage with factor Xa. Unfortunately, we were able to recover only 1/10 of the predicted amount of rMAM. This low yield was probably due to the sticky nature of the molecule and the low efficiency of the electroelution purification process. Electroelution was used to obtain pure rMAM because column chromatography either failed to consistently yield rMAM or did not completely remove the contaminating MBP. Since the contents of the factor Xa digestion mixture were separated on a gel, contamination by factor Xa protease was also eliminated. It should be emphasized, however, that the yield of rMAM is 10-fold higher than that of native MAM derived from a liter of culture. In addition, the higher yield was achieved at a considerably lower cost and with much less labor.

While rMAM appeared to have the same physical properties as native MAM, comparisons of their immunobiological properties were also made to ensure that their bioactivities were also identical. Antibody to MBP-rMAM blocks MAM-induced lymphocyte proliferation, suggesting that antibodies to active domains were generated (Fig. 4). It should be noted that anti-MAM antibody recognized the protein fusion products generated from plasmids pKK3 and pKK4 (data not shown), indicating that MAM-like amino acid sequences were expressed in the truncated proteins. As previously demonstrated for MAM (2, 8), we also showed that both MBP-rMAM and rMAM are highly potent mitogens in which lymphoproliferative activity can still be demonstrated in the presence of picogram levels of the protein (Fig. 2). The protein fusion products generated from pKK3 and pKK4 were not mitogenic (data not shown), suggesting that the carboxy-terminal end of the protein is necessary for activity.

The ability to preferentially recognize and expand T-cell subsets bearing specific V_β TCRs is a defining characteristic of SAgs (for reviews, see references 16 and 18). We show here that rMAM preferentially expands the T-cell subsets bearing $V_\beta6$ and $V_\beta8$ TCRs (Table 1), which supports previous observations for MAM (6, 10) and results reported for rMAM obtained by measuring levels of interleukin-2 production (11).

Similar levels of mitogenic response to MAM and rMAM were observed for splenocytes from mouse strains that differed with respect to the presence of the H-$2E_\alpha$ chain and V_β haplotype (Table 2). As demonstrated previously with MAM (7) and here with rMAM, the H-$2E_\alpha$ chain is important for presentation because its absence results in a great reduction in mitogenic response, even though the Vβ TCR repertoire is the same. In addition, these data demonstrate the preferential use of TCRs bearing $V_\beta6$ and $V_\beta8$ by MAM and rMAM. Splenocytes from BALB/c and C3H mice, which express the E_α chain and both $V_\beta6$ and $V_\beta8$, exhibited high mitogenic activity in the presence of the SAgs, but the mitogenic response was lower from C57BR splenocytes and lower yet with RIIIS splenocytes, which have deletions of $V_\beta8$ or $V_\beta6$ and $V_\beta8$, respectively, while expressing the E_α chain.

MAM possesses a $CD4^+$-dependent suppressive property such that lymphocytes from mice injected i.v. with MAM demonstrate a marked decrease in their ability to respond in vitro to MAM (12). Here we show that MBP-rMAM fusion protein also possesses this property. Fusion protein-challenged, but not PBS-challenged, mice demonstrated splenomegaly, as observed in previous studies with MAM (12). In addition, splenocytes from fusion protein-challenged mice exhibit a dose-dependent inability to respond to MAM or SEB in vitro. These studies did not include mice challenged with rMAM because highly purified rMAM was not available at that time. It remains to be determined whether these inhibitory effects of MAM are due to active suppression, to anergy, or to apoptosis.

We show that rMAM and MBP-rMAM, like MAM (15, 26), are able promote SAg bridge formation, resulting in B-cell proliferation and Ig production (Fig. 6). The ability of B cells to proliferate is dependent upon the presence of both the SAg and the T cell. The low levels of B-cell response to LPS are probably due to the 3-day incubation period prior to harvesting, since maximal B-cell proliferation is usually observed after 5 days. Higher levels of B-cell proliferation were observed when the T-hybridoma cell line and T-MAM cells presented MAM or rMAM than when SEB was used, suggesting that MAM has a greater affinity or avidity for murine lymphocytes than SEB.

Interestingly, higher levels of Ig production were observed when T-MAM cells presented MAM or rMAM than when T-hybridoma cells were used (Fig. 6). The reason for this difference is not clear. One possible explanation is that levels of expression of Vβ TCRs that present MAM are higher in the T-MAM population. Another possible explanation is that some other factor may be involved in promoting Ig production. T-MAM cells are a heterogeneous population of splenocytes, while $V_\beta8.2$ cells are a pure population of T cells. It is possible that a cell type present in the T-MAM population is more effective in inducing Ig production. It should be noted that, in previous studies with MAM, polyclonal Ig production through SAg bridge formation used populations of SAg-presenting cells that were also heterogeneous (14, 25). The possible contribution of SAg bridge formation and subsequent polyclonal Ig production to clinical syndromes marked by immunostimulation and hypergammaglobulinemia is currently being addressed, and such study has been made possible by the availability of ample supplies of the recombinant form of MAM.

In conclusion, we have shown that a recombinant form of MAM has been expressed in *E. coli* and that this recombinant protein has the same physical properties and biological activities as the native protein. Since the recombinant protein can be generated at a low cost, rMAM can now be used in studies requiring large amounts of the protein, such as in those solving the crystal structure or involving monoclonal antibody production or in vivo immunomodulatory experiments.

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REFERENCES

1. **Atkin, C. L., B. C. Cole, G. J. Sullivan, L. R. Washburn, and B. B. Wiley.** 1986. Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis*. V. A small basic protein from culture supernatants is a potent T-cell mitogen. J. Immunol. **137:**1581–1589.
2. **Atkin, C. L., S. Wei, and B. C. Cole.** 1994. The *Mycoplasma arthritidis* superantigen MAM: purification and identification of an active peptide. Infect. Immun. **62:**5367–5375.
3. **Baccala, R., L. R. Smith, M. Vestberg, P. A. Peterson, B. C. Cole, and A. N. Theofilopoulos.** 1992. *Mycoplasma arthritidis* mitogen. Vβ engaged in mice, rats, and humans, and requirement of HLA-DRα for presentation. Arthritis Rheum. **35:**434–442.
4. **Cole, B. C.** 1991. The immunobiology of *Mycoplasma arthritidis* and its superantigen MAM. Curr. Top. Microbiol. Immunol. **174:**107–119.
5. **Cole, B. C., and C. L. Atkin.** 1991. The *Mycoplasma arthritidis* T-cell mitogen, MAM: a model superantigen. Immunol. Today. **12:**271–276.
6. **Cole, B. C., R. A. Balderas, E. A. Ahmed, D. Kono, and A. N. Theofilopoulos.** 1993. Genomic composition and allelic polymorphisms influence Vβ usage

by the *Mycoplasma arthritidis* superantigen. J. Immunol. **150:**3291–3299.
7. **Cole, B. C., C. S. David, D. H. Lynch, and D. R. Kartchner.** 1990. The use of transfected fibroblasts and transgenic mice establishes that stimulation of T cells by the *Mycoplasma arthritidis* mitogen is mediated by Eα. J. Immunol. **144:**420–424.
8. **Cole, B. C., R. A. Daynes, and J. R. Ward.** 1981. Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis*. I. Transformation is associated with an H-2-linked gene that maps to the I-E/I-C subregion. J. Immunol. **127:**1931–1936.
9. **Cole, B. C., D. R. Kartchner, and D. J. Wells.** 1990. Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis* (MAM). VIII. Selective activation of T cells expressing distinct Vβ T cell receptors from various strains of mice by the "superantigen" MAM. J. Immunol. **144:**425–431.
10. **Cole, B. C., D. R. Kartchner, and D. J. Wells.** 1989. Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis*. VII. Responsiveness is associated with expression of a product(s) of the Vβ8 gene family present on the T cell receptor α/β for antigen. J. Immunol. **142:**4131–4137.
11. **Cole, B. C., K. L. Knudtson, A. Oliphant, A. D. Sawitzke, A. Pole, M. Manohar, L. S. Benson, E. Ahmed, and C. L. Atkin.** 1996. The sequence of the *Mycoplasma arthritidis* superantigen, MAM: identification of functional domains with microbial superantigens and plant lectins. J. Exp. Med. **183:**1105–1110.
12. **Cole, B. C., and D. J. Wells.** 1990. Immunosuppressive properties of the *Mycoplasma arthritidis* T-cell mitogen in vivo: inhibition of proliferative responses to T-cell mitogens. Infect. Immun. **58:**228–236.
13. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.).** 1994. Current protocols in immunology, vol. 1. John Wiley & Sons, Inc., New York, N.Y.
14. **Crow, M. K., Z. Chu, B. Ravina, G. Zagon, J. R. Tumang, B. C. Cole, and S. M. Friedman.** 1992. Human B cell differentiation induced by microbial superantigens: unselected peripheral blood lymphocytes secrete polyclonal immunoglobulin in response to *Mycoplasma arthritidis* mitogen. Autoimmunity **14:**23–32.
15. **Friedman, S. M., D. N. Posnett, J. R. Tumang, B. C. Cole, and M. K. Crow.** 1991. A potential role for microbial superantigens in the pathogenesis of systemic autoimmune disease. Arthritis Rheum. **34:**468–480.
16. **Herman, A., J. W. Kappler, P. Marrack, and A. M. Pullen.** 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. Annu. Rev. Immunol. **9:**745–772.
17. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
18. **Irwin, M. J., K. R. Hudson, K. T. Ames, J. D. Fraser, and N. R. J. Gascoigne.** 1993. T-cell receptor β-chain binding to enterotoxin superantigens. Immunol. Rev. **131:**61–78.
19. **Kirchner, H., G. Brehm, R. Nicklas, R. Beck, and R. Herbert.** 1986. Biochemical characterization of the T cell mitogen derived from *Mycoplasma arthritidis*. Scand. J. Immunol. **24:**245–249.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **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.
22. **Muto, A., Y. Andachi, F. Yamao, R. Tanaka, and S. Osawa.** 1992. Transcription and translation, p. 331–347. *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.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Sawada, T., R. Pergolizzi, K. Ito, J. Silver, C. Atkin, B. C. Cole, and M.-D. Y. Chang.** 1995. Replacement of the DR alpha chain with the E alpha chain enhances presentation of *Mycoplasma arthritidis* superantigen by the human class II DR molecule. Infect. Immun. **63:**3367–3372.
25. **Tumang, J. R., E. P. Cherniack, D. M. Gietl, B. C. Cole, C. Russo, M. K. Crow, and S. M. Friedman.** 1991. T helper cell-dependent, microbial superantigen-induced murine B cell activation: polyclonal and antigen-specific antibody responses. J. Immunol. **147:**432–438.
26. **Tumang, J. R., D. N. Posnett, B. C. Cole, M. K. Crow, and S. M. Friedman.** 1990. Helper T cell-dependent human B cell differentiation mediated by a mycoplasmal superantigen bridge. J. Exp. Med. **171:**2153–2158.

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