The *Mycoplasma arthritidis* Superantigen MAM: Purification and Identification of an Active Peptide

Curtis L. Atkin,1,2 Suhua Wei,1 and Barry C. Cole1

Rheumatology Division, Department of Internal Medicine,1 and Department of Biochemistry,2 University of Utah
School of Medicine, Salt Lake City, Utah 84132

Received 6 May 1994/Returned for modification 14 June 1994/Accepted 16 September 1994

The prototypical superantigen MAM is an extracellular T-cell mitogen produced by *Mycoplasma arthritidis*, an organism which causes chronic proliferative arthritis of rodents. We here describe purification of MAM to homogeneity. Pure MAM exhibits all of the major properties previously described for partially purified MAM, including preference for H-2E molecules in presentation to T cells, Vβ T-cell receptor specificity for T-cell activation, and in vivo inhibition of T-cell functions but enhancement of B-cell activity as mediated by the superantigen bridge. Edman degradation of pure MAM gave a 54-residue partial amino-terminal sequence. The oligopeptide MAM15-31, synthesized according to the Edman sequence, blocked mitogenicity of MAM and supported assignment of the amino acid sequence.

The newly recognized class of superantigens is composed of immunomodulatory protein molecules that have been hypothesized to induce autoimmune diseases (10, 17, 22, 30, 32). Bacterial exotoxins (4, 24, 29, 30) and cell wall components (34, 35) as well as endogenous mouse tumor provirus and retroviral proteins (1, 23) and rabies virus (27) have been shown to possess superantigenic properties. *Mycoplasma arthritidis*, an agent of chronic rodent arthritis, releases a soluble extracellular T-cell mitogen (9). Subsequent work (reviewed in reference 7) established that the mitogen called MAM activates murine and human lymphocytes and exhibits all of the characteristic properties of superantigens, including presentation to T cells by major histocompatibility complex (MHC) molecules (preferentially, but not restricted to, H-2E) without the need of processing by accessory cells, and recognition of MAM in a non-MHC-restricted manner by specific Vβ chains of the αβ T-cell receptor for antigen (TCR) (6, 11).

It was previously found that MAM is a basic, heat- and acid-labile protein with a molecular weight of 15,000 to 30,000 (2, 25). We hypothesized that the high pI (>9) and hydrophobicity of MAM, and consequent binding of MAM to nucleic acids and other culture macromolecules as well as to surfaces, prevented extensive purification. Considering these properties, we devised a new purification scheme that resulted in homogeneous MAM and the subsequent derivation of its N-terminal sequence.

### MATERIALS AND METHODS

**Animals.** Animal care was in accordance with institutional guidelines. For use in assays to quantify mitogen, female CBA/J mice 8 to 12 weeks of age were obtained from Jackson Laboratory (Bar Harbor, Maine).

For comparative studies, the activity of MAM was also tested by using lymphocytes from the following strains of mice that were also obtained from Jackson Laboratory: BALB/c (*H-2^b^, V_β^b^), C57BL/10 (*H-2^b^, V_β^b^), C57BR (*H-2^b^, V_β^-), SWR (*H-2^b^, V_β^-), RIIS (*H-2^b^, V_β^-), B10.BR (*H-2^b^, V_β^-), and B10.RIII (*H-2^b^, V_β^-).

**Quantitative lymphocyte proliferation assay.** The assays were performed as previously described (2). Aliquots of fractions were diluted with complete RPMI 1640 medium (GIBCO, Chagrin Falls, Ohio) and sterilized by triple ultrafiltration. Proliferation of CBA/J mouse splenocytes by serial dilutions of MAM-containing samples was determined by uptake of [3H]Thymidine (90 Ci/mmol). Filter mats were counted on a Skatron beta counter. As before, unknown samples were compared with a standard reference preparation of MAM by calculating the dilutions which gave 50% of the optimal [3H]Thymidine uptake in log-dose–response curves (2). MAM at 1 U/ml is defined as giving half-maximal proliferation in this standardized assay (2).

**Induction and assay of interleukin-2.** The hybridomas expressing Vβ1 (KSEA18/4-9.2), Vβ3 (5KC-73-8), Vβ7 (KOX7-66), Vβ8.1 (KJ16), and Vβ11 (1BV11-17.7) were kindly supplied by P. Marrack (National Jewish Center, Denver, Colo.). Vβ6 (BG16) was kindly supplied by B. Huber (Tufts University, Boston, Mass.). The origins of the line bearing Vβ8.2 (2Hd-11.2) and the 2PK-3 B lymphoma line, used as accessory cells, were described previously (6).

T cells (2.5 x 10^5 per well) and 2PK-3 accessory cells (10^5 per well) were cultured in the presence of inducers as previously described (6), and supernatants were collected after 24 to 28 h and assayed for interleukin-2, using reduction of [3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as a measure of cell growth (31).

**AMEH growth medium.** Autoclaved modified Edward–Hayflick (AMEH) medium consisted of 84 g of Bacto PPLO broth without crystal violet (Difco Laboratories, Detroit, Mich.), 62.5 g of Fleischmann's dry baker's yeast, 750 ml of defined equine serum (HyClone, Logan, Utah), and 4,250 ml of Millipore deionized water (Millipore Corp., Bedford, Mass.). After autoclaving at 15 lb/in^2^ (132°C) for 40 min, the mixture was cooled in an ice bath overnight and was then centrifuged in polycarbonate centrifuge bottles at 11,000 relative centrifugal force (i.e., at 9,000 rpm in a Beckman JA-10 rotor) for 30 min at 4°C without braking. Floating residues were discarded; supernatants were decanted through cheesecloth layers, and if the supernatant was not particle free, it was...
again centrifuged. N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid (HEPES; 1% [wt/vol]; Sigma Chemical Co., St. Louis, Mo.) and 0.5% arginine · HCl (Sigma) were then added. After being adjusted to pH 7.0 with NH₄OH, the medium was autoclaved in two 4-liter flasks for 15 min with nonabsorbent cotton stoppers. If necessary, further clarification was performed by pressure ultrafiltration on large-diameter 0.22-μm-pore-size MF filters (Millipore); alternatively, ultrafiltration supplanted the second autoclaving. The sterile medium was supplemented with 1,000 U of penicillin G (Apotone, Pinceta, N.J.) per ml.

Culture procedures and turbidimetry. For inocula, 1-ml aliquots of mid-exponential-phase, 37°C cultures of various strains of M. arthritidis in Edward-Hayflick medium (20) or modified Edward-Hayflick medium (2) were frozen at −70°C. For screening strains, cultures in 5 ml of modified Edward-Hayflick medium were grown for 7 days at 37°C in very slowly rotated cotton-stoppered tubes.

To produce MAM for purification, M. arthritidis PG6 (ATCC 19611) was adapted to grow in AMEH broth by six serial passages, and 5-ml aliquots were stored at −70°C. For inoculation, two vials of M. arthritidis culture were rapidly thawed at ≤37°C, and each was added to 21 of AMEH medium preheated to 37°C. Incubation at 37°C was continued without stirring for 24 h, after which gentle stirring at 25 rpm was maintained on an orbital table with 2-cm-diameter motion. Hourly, starting at 48 h, aliquots were aseptically withdrawn from the flasks and cooled to room temperature, and growth was estimated turbidimetrically from A₅₅₀ versus an absorbance reference of sterile growth medium. Static or diminishing turbidity, usually with maximum A₅₅₀ of ~0.2/cm at about 52 h in both flasks, indicated the transition from growth maximum to a lytic phase, whereupon the two cultures were combined and immediately subjected to purification.

Protein assay. Lowry and dye methods were inadequate for protein measurement because of high concentrations of amino acids and salts (especially ammonium ions) in many fractions. Extinction coefficients of protein mixtures or of MAM are unknown; however, A₂₈₀, averages 10/cm for 1% (wt/vol) stock solutions of a large list of proteins (26). Total protein in solution was therefore measured spectrophotometrically at A₂₈₀; aliquots of fractions with high optical densities were diluted with phosphate-buffered saline (PBS) such that the A₂₈₀ became ≤1/cm. Values of A₂₈₀ per centimeter · dilution were, for the interim, equated to milligrams of protein per milliliter.

Buffer solutions. PBS consisted of 0.15 M NaCl and 10 mM Na₂HPO₄, adjusted to pH 7.2 with NaOH. Buffer 1 consisted of 1 M (NH₄)₂SO₄ and 10 mM Tris·HCl, adjusted to pH 8.3 with aqueous ammonia. Buffer 2 consisted of 10 mM KH₂PO₄, adjusted to pH 7.2 with KOH. Buffer 3 consisted of 0.5 M KH₂PO₄, adjusted to pH 7.2 with KOH. Buffer 4 consisted of 2 M (NH₄)₂SO₄ and 50 mM KH₂PO₄, adjusted to pH 7.2 with KOH. Buffer 5 consisted of 50 mM KH₂PO₄, adjusted to pH 7.2 with KOH.

PAGE. Sodium dodecyl sulfate (SDS)-gradient polyacrylamide gel electrophoresis (PAGE) was performed as described by Lambin et al. (28). Gels were stained overnight with Coomassie blue R250.

Purification of MAM. The entire scheme was scaled to process about 4 liters (accurately measured) of senescent whole culture. Aliquots centrifugally clarified or sterilized by ultrafiltration were prepared for the absorbance protein assay or for the subsequent proliferation assay, respectively. Similar aliquots from each of the subsequent purification steps were also stored prior to activity assays.

The whole warm senescent culture was subjected to (NH₄)₂SO₄ (Schwarz-Mann ultrapure) fractionation as described by Green and Hughes (18). The pellet resulting from the 50 to 80% fraction was dissolved in 60 ml of H₂O, and insoluble residue was removed by centrifugation. The clarified extract was subjected to gel filtration chromatography with eluent buffer 2 at 40 ml/h on a 2-liter column (diameter/height = 1.5 to 1:10) of medium-grade Sephacryl G-50 (Pharmacia-LKB, Piscataway, N.J.) that had previously been equilibrated with buffer 1. One fraction of 200 ml was collected according to elution volumes previously determined for 30-kDa molecular size standard carbonic anhydrase (Sigma). Conductivity and pH of the collected fraction were decreased to the values of buffer 2 by dialysis or by repeated dilution with buffer 2 and centrifugal concentration and ultrafiltration in Centricon-10 or -30 tubes (Amicon, Danvers, Mass.). The material was then adsorbed onto a 30-ml column (diameter/length = 1.5 to 1:10) of Fast Flow Sepharose-S cation-exchange resin (Pharmacia-LKB) that had been equilibrated with buffer 2. After washing, the column was then subjected to frontal elution with buffer 3. The eluted 280-nm-absorbing peak was equilibrated with buffer 2 as before.

A second cation exchange was performed at 1 ml/min by adsorbing the adjusted fraction onto an HR 5/10 fast protein liquid chromatography (FPLC) column of 1 ml of Mono S resin (Pharmacia-LKB) also preequilibrated with buffer 2. After unabsorbed proteins were washed away, the column was eluted with a 20-ml linear gradient to 40% (vol/vol) buffer 3, with use of a flow cuvette for continuous monitoring at A₂₈₀ and 0.5-ml fractions were collected.

The final step consisted of hydrophobic interaction chromatography using an HR 5/10 FPLC column containing 1 ml of alkyl-Superose (Pharmacia-LKB) previously equilibrated with buffer 4. Active fractions from the Mono S column were adjusted to contain 2 M (NH₄)₂SO₄ and were then passed over the alkyl-Superose resin. After being washed with buffer 4, absorbed proteins were eluted, at 1 ml/min, with a 20-ml inverse linear gradient to the final conditions of buffer 5. For eventual desalting to physiological buffer conditions, fractions were collected directly into Centricon-10 or -30 tubes.

Edman degradation. The high (NH₄)₂SO₄-containing solutions in final fractions 13 and 14 of preparation 1 (Fig. 2) were converted to 100 and 10 mM sodium acetate solutions, respectively, by dilution and centrifugal ultrafiltration-concentration using the Centricon tubes. Edman degradation (15) of each fraction was performed with a model 477A protein sequencer (Applied Biosystems, Inc., Foster City, Calif.), using an Applied Biosystems proprietary pulsed liquid-phase method with a BioBreen-treated fiber glass filter sample support.

RESULTS

Strains for production of MAM. Thirty-one strains of M. arthritidis were screened. Soluble T-cell mitogen in supernatant ultrafiltrates of mature cultures ranged from 1/1,000 to 10 times that from strain 14124p10, used in our earlier study (2). Productive strains included E1 (isolated in this laboratory); PG6 (ATCC 19611); and 9102E, 9102L, 9103A, and 9113E (kindly supplied by J. G. Tully). The type strain PG6 was chosen for all further studies because it grew rapidly and consistently produced several- to 10-fold more mitogen than did strain 14124p10. (After completion of initial studies, we learned that Homfeld et al. [21] effected a fourfold increase of MAM production for strain ATCC 14124 by growing it at 30°C rather than at 37°C.)

Production and decay of MAM in cultures. M. arthritidis
PG6 readily adapted to growth in AMEH medium, and aliquots were frozen after serial passages. In many experiments with osmotic, alkaline, or detergent lysis and salt extraction, we failed to obtain significant amounts of mitogen from exponentially growing cells. The appearance of mitogen in culture supernatants corresponded to the transition from growth to alkaline lysis (33), which was identified by a precipitous decline in viability as well as, conveniently, by a fractional drop in turbidity ($A_{490}$) of the cultures. Mitogenicity of cultures, culture supernatants, or crude fractions decayed with a half-life of tens of hours, presumably because of the action of mycoplasmal proteases.

**Purification of MAM.** We used the quantitative mitogenicity assay (2) to separately optimize each step of the present procedure, such that elution volumes and absorbance profiles could be used directly for rapid selection of active fractions. Since the yield of MAM from fractionation with (NH$_4$)$_2$SO$_4$ was indifferent to the presence of cells, we added (NH$_4$)$_2$SO$_4$ to 50% saturation directly to the warm, senescent culture and thus eliminated cellular debris with the first (NH$_4$)$_2$SO$_4$ precipitate. Precipitate from 80% saturated (NH$_4$)$_2$SO$_4$ mixture was effectively back extracted with 1 M (NH$_4$)$_2$SO$_4$, which was calculated to give dissolved MAM in a volume appropriate for the next step of gel filtration chromatography. Sephadex G-50 used for the latter also contained 1 M (NH$_4$)$_2$SO$_4$, giving an ionic strength six times that of 0.5 M buffer A that was previously used (2) for gel filtration on the much slower Sephacryl S-200 Superfine resin (Pharmacia-LKB). Collection of one active fraction (not shown) from G-50 depended upon standardization of the chromatography using a 30-kDa standard protein.

These modifications to our previous procedure were required before impure MAM could be efficiently adsorbed to any cation exchanger. Even then it was critical to adjust the pH and ionic strength of the applied specimens. Serial chromatography on two sulfonate-based cation-exchange resins proved effective. On the first of these (Fast Flow Sepharose-S; Pharmacia-LKB), washing and frontal elution effected separation of a large unabsorbed fraction from a single absorbance peak of basic proteins (not shown) that was collected in its entirety.

Figure 1 illustrates the resolution afforded by gradient elution of the second cation exchanger (Mono S; Pharmacia-LKB); in this case, fractions 6 to 8 of preparation 1 were pooled and immediately carried on to the final chromatography. In other runs, this approach sometimes failed when, for unknown reasons, the absorbance profiles of consecutive and supposedly identical preparations were slightly different. Since Mono S fractions were stable at -70°C, most purifications were conducted using fractions that exhibited the highest mitogenic activity by biological assay. These fractions were then run individually over the final column. Active Mono S fractions typically contained ≤10% pure MAM.

The last chromatographic step, hydrophobic interaction on alkyl-Superose (Pharmacia-LKB), removed unabsorbed proteins in the wash (not shown) and reliably gave elution profiles such as shown in Fig. 2, where a complex of contaminant proteins was followed ultimately by a small, discrete absorbance band (fractions 13 and 14) corresponding to about 1 ml of 50 mM potassium phosphate with roughly 1.4 M (NH$_4$)$_2$SO$_4$ (pH 7.2) and containing MAM that proved to be homogeneous.

Table 1 characterizes three serial purifications. The final fractions of about 20 μg of MAM represented about 10$^6$-fold purification from culture supernatant proteins, giving an overall yield of about 20%. As there were seven fractionations (three salt steps and four chromatographies) in each preparation, a typical product of 20 μg of homogeneous MAM and about 20% overall yield corresponded to a mean yield of 80% per fractionating step.

The final peak fractions of all three preparations showed specific activities that varied in proportion to the mitogenic activities of the starting material (1 × 10$^4$ to 7 × 10$^5$ U/mg) and ranged to about 1 × 10$^8$ to 10 × 10$^9$ U/mg of homogeneous MAM. Lability of MAM and variability in bioassays presumably each contributed variance to the specific activity results. Furthermore, interruptions during the purification scheme markedly reduced these yields, sometimes in excess of an order of magnitude.

Fractions corresponding to the peak that contained maximum MAM activity after alkyl-Superose chromatography were run in denaturing SDS-polyacrylamide gels (Fig. 3). Single bands that corresponded to a molecular weight of approximately 27,000, as estimated by comparison with standard markers, were obtained.

Unlike Mono S fractions, alkyl-Superose fractions containing (NH$_4$)$_2$SO$_4$ lost activity upon freezing. Furthermore, desalting the fractions against PBS or water by using dialysis or centrifugal ultrafiltration resulted in loss of all activity. Homogeneous MAM was found to be destroyed by exposure to glass and was also lost by absorption or denaturation on charge-neutral plastics, especially polystyrene. Homogeneous MAM could be stabilized by the addition of protein such as bovine.

![FIG. 1. Elution of MAM preparation 1 from a Mono S Sepharose column. The unadsorbed protein washed out with starting buffer (buffer 2) is not shown. The elution time and volume commenced with the start of the salt gradient. SALT indicates the programmed molarity of eluent potassium phosphate, pH 7.2. Salt concentration in simultaneously eluting solution would be shown by the same gradient shifted approximately 2 ml (or 2 min) to the right. The profile of eluted protein is given by the $A_{280}$/cm curve. Specific mitogenic activity (S.A.) of 0.5-ml fractions, using CBA mouse splenocytes, is shown in terms of units per milliliter divided by $A_{280}$ per centimeter, or, approximately, units per milligram of protein. fr6, fr7, and fr8, fractions 6, 7, and 8, respectively.](http://iai.asm.org/Downloaded_from/yyyy-mm-dd)
vitro studies.\(^5\) (vol/vol) solutions to albumin.

SALT indicates desalting of the starting material.\(^1\) Prepn 2.

Culture supernatant was precipitated by ammonium sulfate (NH4)\(_2\)SO\(_4\) at a final concentration of 50% (NH4)\(_2\)SO\(_4\) and 2.5% (NH4)\(_2\)SO\(_4\), followed by centrifugation at 4,000 x g for 1 h. The supernatant was reprecipitated by additional ammonium sulfate at 2.5% (NH4)\(_2\)SO\(_4\) to give a precipitate of about 1 mg/ml. This precipitate was then dissolved in sterile 0.1 M NaCl and sterile culture medium.

Amino-terminal sequence of MAM and identification of an active peptide. Automated Edman degradation was conducted on fractions 13 and 14, which corresponded to the leading and trailing regions, respectively, of the MAM peak of preparation 1 (Fig. 2). The first 53 of 54 residues of the N-terminal region of MAM were successfully sequenced (Table 2). Material from

![FIG. 2. Hydrophobic interaction chromatography of preparation 1 on an alkyl-Superose column. Parameters are as for Fig. 1 except that SALT indicates programmed molarity of (NH4)\(_2\)SO\(_4\) eluent.](image)

![FIG. 3. SDS-polyacrylamide gradient gel electrophoretogram (28), stained with Coomassie blue R250, showing electrophoretic homogeneity of purified MAM. The concentrations and nominal molecular weights of the standards were according to the suppliers. Lanes: A, 4 \(\mu\)g of bovine pancreatic trypsin inhibitor (Sigma); B, 3 \(\mu\)g each of Pharmacia-LKB standard proteins (bottom to top, bovine \(\alpha\)-lactalbumin [14.4 kDa], bovine pancreatic trypsin inhibitor [20.1 kDa], bovine erythrocyte carbonic anhydrase [30 kDa], hen egg white ovalbumin [43 kDa], bovine serum albumin [67 kDa], and rabbit muscle phosphorylase \(b\) [94 kDa]); C, 5 \(\mu\)g of bovine erythrocyte carbonic anhydrase (Sigma); D, 60 \(\mu\)l of final hydrophobic interaction chromatographic fraction 14, preparation 1 (see Fig. 2), containing about 1 \(\mu\)g of MAM; E, 1 \(\mu\)g each of Bio-Rad (Hercules, Calif.) standard proteins, similar to lane B. M.wt., molecular weight.)

### TABLE 1. Purification of three consecutive preparations of MAM to homogeneity\(^a\)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Protein (mg/ml)</th>
<th>Mitogen (U/ml)(^b)</th>
<th>Mitogen yield (%)</th>
<th>Specific mitogenic activity (U/mg)</th>
<th>Net purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepn 1</td>
<td>Culture supernatant</td>
<td>4,000</td>
<td>17.2</td>
<td>2.1 (\times) 10^3</td>
<td>100</td>
<td>1.2 (\times) 10^2</td>
</tr>
<tr>
<td></td>
<td>Alkyl-Superose fractions</td>
<td>12</td>
<td>0.5</td>
<td>0.015</td>
<td>0.2 (\times) 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>0.5</td>
<td>0.030</td>
<td>1.1 (\times) 10^6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0.5</td>
<td>0.020</td>
<td>1.9 (\times) 10^6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>0.5</td>
<td>0.010</td>
<td>0.1 (\times) 10^6</td>
<td>1</td>
</tr>
<tr>
<td>Prepn 2</td>
<td>Culture supernatant</td>
<td>4,000</td>
<td>17.4</td>
<td>2.9 (\times) 10^3</td>
<td>100</td>
<td>1.7 (\times) 10^2</td>
</tr>
<tr>
<td></td>
<td>Alkyl-Superose fractions</td>
<td>10</td>
<td>0.5</td>
<td>0.010</td>
<td>0.6 (\times) 10^6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0.5</td>
<td>0.008</td>
<td>2.5 (\times) 10^6</td>
<td>11</td>
</tr>
<tr>
<td>Prepn 3</td>
<td>Culture supernatant</td>
<td>8,300</td>
<td>14.5</td>
<td>1.0 (\times) 10^4</td>
<td>100</td>
<td>7.1 (\times) 10^2</td>
</tr>
<tr>
<td></td>
<td>Alkyl-Superose fractions</td>
<td>10</td>
<td>0.5</td>
<td>0.035</td>
<td>0.2 (\times) 10^6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0.5</td>
<td>0.026</td>
<td>2.0 (\times) 10^7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0.5</td>
<td>0.020</td>
<td>1.8 (\times) 10^7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>0.5</td>
<td>0.014</td>
<td>0.4 (\times) 10^7</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) MAM was purified as described in Materials and Methods from strain PG6 (ATCC 19611) grown in AMEH medium. Absorbance and mitogenicity of whole culture starting material were actually determined on ultrafiltered, sterile culture supernatants; cells and particulate debris were irrelevant because they were discarded in the 50% saturated (NH4)\(_2\)SO\(_4\) precipitate.

\(^b\) MAM at a concentration of 1 U/ml gives half-maximal proliferation of CBA lymphocytes in a defined culture system (see Materials and Methods and reference 2).
both fractions gave identical sequences, thus confirming homogeneity.

On the basis of the N-terminal sequence, we synthesized three peptides which corresponded to MAM residues 1 to 13 (peptide 1), 27 to 44 (peptide 2), and 15 to 31 (MAM15-31; peptide 3), each with an added cysteine (designated by -C) (Table 2). None of the peptides tested were mitogenic for BALB/c splenocytes, nor were they inhibitory to concanavalin A (ConA)-mediated lymphocyte proliferation. However, peptide 3 at concentrations ranging from 0.1 to 2 mg/ml blocked proliferation of lymphocytes induced by the intact MAM molecule (Fig. 4). Peptides 1 and 2 did not affect MAM-induced proliferation. A similar method was used by Griggs et al. (19) to identify active fractions of staphylococcal enterotoxin A (SEA).

To ensure that the inhibition mediated by MAM15-31 was not due to a nonspecific or toxic effect on lymphocyte functions, we compared the inhibitory effects of this peptide on proliferation induced by MAM, ConA, or MAM plus ConA. The results (Fig. 5) demonstrated that MAM15-31 failed to inhibit proliferation induced by ConA or by ConA plus MAM yet markedly inhibited proliferation induced by MAM alone.

**Biological properties of homogeneous MAM.** Homogeneous MAM was tested for its ability to activate lymphocytes from strains of mice that differ in expression of $E_a$ and in the TCR $V_b$ chains (Table 3), BALB/c mice ($V_b^b$ haplotype), which express an intact H-2E molecule and all of the major $V_b$ TCRs that are used by MAM, i.e., $V_b^b$, $V_b^s$, and $V_b^8$, showed very high lymphocyte proliferation when stimulated with MAM, and significant activity was still present at the lowest MAM concentration tested (0.1 ng/ml). Lymphocytes from other $E_a^+$, $V_b^b$ haplotype mice (B10.BR and B10.RIII) also responded to all concentrations of MAM. Although lymphocytes from the C57BR, $V_b^8$ mouse (which lacks the MAM-reactive $V_b^8$ TCRs) responded less well to high concentrations of MAM, they still responded to lower MAM doses. Lymphocytes

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Amino acid N-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAM1-4</td>
<td>MKLRVENFKK AQKHFVQNL NVVFTNKELE DYNLSEKEE TKVLCITTERK ?VNQ</td>
</tr>
<tr>
<td>Oligopeptide 1, C-MAM1-13</td>
<td>C MKLRVENFKK AQK</td>
</tr>
<tr>
<td>Oligopeptide 2, MAM27-44-C</td>
<td>KELE DYNLSEKEE TKVLCITTERK</td>
</tr>
<tr>
<td>Oligopeptide 3, MAM15-31-C</td>
<td>PVQNLN NVVFTNKELE DC</td>
</tr>
</tbody>
</table>

**FIG. 4.** Effects of synthetic oligopeptides on MAM-induced lymphocyte proliferation. Each of the peptides was incubated at the indicated concentrations with BALB/c splenocytes for 1 h at 37°C prior to the addition of MAM. Peptide 3 (MAM15-31-C) inhibited the proliferative activity of MAM.
from RIIS mice (V \textsubscript{\beta}\textsuperscript{e} haplotype), which express E\textsubscript{\alpha} but which, because of an additional genomic deletion, lack all of the TCR V\textsubscript{\beta} chains that are preferentially used by MAM in V\textsubscript{\beta}\textsuperscript{a} mice, gave very low responses to MAM.

Lymphocytes from some mice which failed to express E\textsubscript{\alpha} but which expressed a full complement of MAM-reactive V\textsubscript{\beta} TCRs (i.e., C57BL/10) responded only to high concentrations of MAM. Lymphocytes from other mice (i.e., SWR) which also fail to express E\textsubscript{\alpha} totally failed to respond to MAM. ConA gave high lymphocyte proliferation with all strains of mice (data not shown). The pattern of reactivity seen by homogeneous MAM for lymphocytes from different mouse strains is thus identical to that previously noted for crude or partially purified MAM (8, 12), suggesting that only one mitogenic moiety was present in these earlier MAM preparations (2).

T-cell hybridomas derived from V\textsubscript{\beta}\textsuperscript{a} mice were also tested for the ability to produce interleukin-2 in response to homogeneous MAM in the presence of \gamma-irradiated 2-PK3 lymphoma accessory cells. Hybridomas expressing V\textsubscript{\beta}6 and V\textsubscript{\beta}8.2 produced interleukin-2, whereas those expressing V\textsubscript{\beta}1, V\textsubscript{\beta}3, V\textsubscript{\beta}7, and V\textsubscript{\beta}11 did not. As shown by others, SEA activated V\textsubscript{\beta}1, V\textsubscript{\beta}3, and V\textsubscript{\beta}11 hybridomas, whereas SEB activated those expressing V\textsubscript{\beta}3 and V\textsubscript{\beta}8.2 (data not shown).

Homogeneous MAM was also tested for its ability to induce a V\textsubscript{\beta}\textsuperscript{a}-specific suppression of T-cell functions in vivo. BALB/c mice were injected intravenously with approximately 500, 100, and 20 ng of MAM or with PBS, and 2 days later, splenocytes were collected and challenged in vitro with MAM, SEA, SEB, or lipopolysaccharide (LPS) (Fig. 6). All concentrations of MAM suppressed responses to in vitro challenge with MAM. Responses to SEB were partially suppressed, but there was no suppression of responses to SEA. Responses to LPS were markedly enhanced by a prior injection of MAM.

**DISCUSSION**

Our success in purifying MAM to homogeneity was largely dependent upon five main considerations. First, we screened a total of 32 strains of *M. arthritidis* and selected PG6 on the basis of ease of growth and amount of MAM secreted. Second, we developed a simpler culture medium by autoclaving modified Edward-Hayflick medium and removing the proteinaceous sediment that resulted. In previous studies, we had demonstrated that MAM readily bound to serum proteins and to nucleic acids (2). The autoclaved medium was largely free of these entities. Third, we used a quantitative assay for measurement of MAM activity which enabled us to identify the most active fractions and to calculate the percent recovery of MAM at each stage of the purification process. Fourth, because standard approaches to cation-exchange chromatography of proteins failed to work with MAM, we empirically determined the order and nature of every step of purification. Guided by evidence for extreme stickiness of MAM that we originally attributed to both charge and hydrophobicity factors (2), we found that unusually high ionic strength in Sephadex G-50 gel filtration yielded a MAM-containing fraction that could successfully be further fractionated by cation-exchange chromatography. The high ionic strength would suppress ionic interaction or binding of MAM to other macromolecules but would enhance hydrophobic interactions. We also empirically determined that 30-kDa carbonic anhydrase defined the elution volumes of MAM in this gel filtration system. It should be noted that our gel filtration methods are specific for MAM and may give difficulties with other proteins.

The homogeneity of MAM prepared in the manner described above was indicated by a single band present on SDS-polyacrylamide gels of the MAM-containing peak resulting from alkyl-Superose chromatography. Further evidence of homogeneity was obtained by sequence analysis. Edman degradation (15) was used to obtain the N-terminal sequence of the leading and trailing portions of the active alkyl-Superose peak. In both cases, an identical sequence of the first 53 of 54 amino acids was obtained, thus confirming homogeneity. Fur-

**TABLE 3. Lymphocyte responses to homogeneous MAM**

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>Presence of E\textsubscript{\alpha}</th>
<th>V\textsubscript{\beta} haplotype(^{a})</th>
<th>Uptake of [\textsuperscript{3}H]Tdr (10\textsuperscript{3} cpm)(^{b}) in response to MAM concen(^{c}) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 ng/ml</td>
</tr>
<tr>
<td>BALB/c</td>
<td>+</td>
<td>b</td>
<td>346.6 ± 19</td>
</tr>
<tr>
<td>C57BL/10</td>
<td>b</td>
<td>b</td>
<td>54.5 ± 3.2</td>
</tr>
<tr>
<td>B10.BR</td>
<td>+</td>
<td>a</td>
<td>50.8 ± 6.7</td>
</tr>
<tr>
<td>B10.RIII</td>
<td>b</td>
<td>a</td>
<td>65.2 ± 4.0</td>
</tr>
<tr>
<td>C57BR</td>
<td>+</td>
<td>a</td>
<td>27.9 ± 1.7</td>
</tr>
<tr>
<td>SWR</td>
<td>+</td>
<td>a</td>
<td>10.2 ± 5.8</td>
</tr>
<tr>
<td>RIIS</td>
<td></td>
<td></td>
<td>10.2 ± 5.8</td>
</tr>
</tbody>
</table>

\(^{a}\) The V\textsubscript{\beta}\textsuperscript{a} haplotype mouse strains listed express all of the V\textsubscript{\beta} chains that are used by MAM. V\textsubscript{\beta}\textsuperscript{e} mice lack the MAM-reactive V\textsubscript{\beta}6, V\textsubscript{\beta}8.2, and V\textsubscript{\beta}8.3 as a result of genomic deletions. V\textsubscript{\beta}\textsuperscript{a} mice also lack the MAM-reactive V\textsubscript{\beta}6 in addition to the deletions seen in V\textsubscript{\beta}\textsuperscript{e} mice.

\(^{b}\) Background [\textsuperscript{3}H]Tdr uptake for all strains did not exceed 10\textsuperscript{3} cpm.

\(^{c}\) The MAM concentrations were only approximate since they were based upon A\textsubscript{280} values of the MAM-containing peak from alkyl-Superose chromatography.
The results further suggest that the derived sequence was in fact that of MAM. In a preliminary search of databases containing over 50,000 protein sequences, including those of the bacterial and retroviral superantigens, we failed to find any that contained regions structurally similar to the N-terminal amino acid sequence of MAM. Thus, MAM appears to represent a previously undescribed mitogenic protein.

The peptide blocking data suggest that the N-terminal region of MAM contains a biologically active region. Thus, the inhibition of MAM-induced proliferation by the peptide MAM(15-31)-C appeared to be specific for MAM, since no effects were seen on proliferation induced by ConA. Furthermore, the inhibition was not due to a toxic effect, since splenocytes treated with MAM(15-31)-C still responded normally to ConA. Relatively high concentrations of peptide were required to block even low doses of MAM, as has also been shown for other superantigens (19). These observations might suggest a poor avidity of linear peptides for MHC or TCR molecules in comparison with motifs based upon the three-dimensional superantigen structure.

Homogeneous MAM was found to possess the properties previously reported for impure preparations. Thus, the use of inbred and congenic mouse strains showed that MAM reacted preferentially with lymphocytes from strains that expressed Eα. Also, lymphocytes from mouse strains which lack most of the MAM-reactive Vβ chains of the TCR gave poor responses to MAM. In addition, using T-cell hybridomas, we demonstrated that activation was dependent upon accessory cells and that the pattern of MAM-reactive Vβ chains was the same for homogeneous and impure MAM.

Homogeneous MAM was also shown to have marked immunomodulatory responses in vivo. The suppressive effect on lymphocyte proliferation appeared to be Vβ specific, since responses to MAM and SEB, which share usage of the Vβ8 TCR family, were both suppressed, whereas responses to SEA, which does not share Vβ TCR usage with MAM, were unaltered. Previous studies established that the apparent suppression induced by MAM was not due to a deletion or anergy of MAM-reactive clones, since CD4+ splenocytes from MAM-injected mice actively suppressed the response of splenocytes from control mice to MAM (13).

Pure MAM in vivo also induced a marked increase in responses to bacterial LPS, thus confirming previous findings on more crude preparations in which activation of B-cell functions was found (5). This is an important observation since it demonstrates that the superantigen bridge which cross links Tβ cells with resting B cells, leading to B-cell differentiation (36), is also mediated by MAM and not by a different contaminating protein.
Although the estimate of the concentration of homogeneous MAM used was only approximate, it is clear that MAM is much more active for H-2-E-bearing murine lymphocytes than is SEA or SEB. Thus, whereas optimal concentrations of the staphylococcal superantigens are 5 to 10 μg/ml for BALB/c splenocytes (3, 4a, 16), the optimal concentration of MAM for these cells was found to be approximately 5 ng/ml (Table 3), i.e., 1,000-fold less. The differences in the degree of proliferation seen with mycoplasma or staphylococcal superantigens could reflect differences in the avidity of these proteins for murine MHC molecules or in the transduction of signals across the T-cell membrane by superantigen-MHC complexes. Human lymphocytes also differ in the degree of response to various superantigens. In this case, the staphylococcal superantigens are more potent in inducing proliferation of human T cells than is MAM (14, 16).

The availability of homogeneous MAM is now allowing sequence and structural studies to be undertaken. Future work will compare the structure of MAM with that of other superantigens and will determine the molecular basis for the interaction of MAM with MHC and TCR molecules.

ACKNOWLEDGMENTS

This work was supported by grants AI12103 and AM02285 from the National Institutes of Health and by a grant from the Nora Eccles Treadwell Foundation. Peptide synthesis was supported by the Huntsman Cancer Center, University of Utah. We thank D. Kartchner, G. J. Sullivan, S. McCall, and E. A. Ahmed for excellent technical assistance.

REFERENCES