Purification, Composition, and Structure of Macrophage Adhesion Molecule†

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ABSTRACT: Macrophage adhesion molecule (MAM) is a surface heterodimer consisting of the trypsin- and plasmin-sensitive glycopeptide gp160 (MAM-α) and the glycopeptide gp93 (MAM-β). MAM, which is the guinea pig analogue of Mo1 and Mac-1, was purified from detergent lysates of peritoneal neutrophils by lentil lectin chromatography and M2-antibody chromatography. The pure heterodimer molecule was dissociated by acidic conditions (pH 3.5), and MAM-α and MAM-β were separated by M7-antibody chromatography. MAM-β is an ~640 amino acid residue polypeptide with exceptionally high cysteine content. At 7.2 residues per 100 amino acids, Cys/2 of MAM-β is more than 3 times the mean for 200 purified proteins. Reactivity with six β-subunit-specific monoclonal antibodies recognizing at least four epitopes demonstrated that intrapeptide disulfide bonds are required to maintain the structure of MAM-β. All six antibodies failed to react when MAM-β was treated with reducing agents. MAM-β is 18% carbohydrate; the major monosaccharides are mannose, *N*-acetylglucosamine, galactose, and sialic acid. MAM-β is estimated to contain five to six N-linked carbohydrate units. MAM-α is an ~1100-residue polypeptide with lower Cys/2 content (2.0 residues per 100 amino acid residues). MAM-α is 21% carbohydrate. The major monosaccharides are mannose, *N*-acetylglucosamine, galactose, and sialic acid; the mannose content is higher in MAM-α than MAM-β. MAM-α is estimated to contain 12 N-linked carbohydrate units.

Macrophage adhesion molecule (MAM)[1] is a prevalent surface molecule consisting of gp160, which is sensitive to trypsin and plasmin, and gp93 (Remold-O'Donnell & Savage, 1988). gp160 and gp93, also called MAM-α and MAM-β, respectively, exist in the macrophage membrane as a heterodimer molecule; it is thought that ionic interactions are primarily responsible for their association. The name MAM (macrophage adhesion molecule) acknowledges the function of this molecule in the adhesion and spreading of macrophages on surfaces as demonstrated by specific and significant inhibition of adhesion by several anti-MAM monoclonal antibodies.[2] Multiple common properties indicate that MAM, which is found in guinea pig macrophages and neutrophils, is analogous to human Mo1 and mouse Mac-1 [compared in Remold-O'Donnell and Savage (1988)]. The synthesis of MAM is dramatically down-regulated in macrophages activated in vivo (Remold-O'Donnell & Lewandrowski, 1982; Remold-O'Donnell, 1987).

As a contribution to understanding its structure and function, MAM was purified to homogeneity from guinea pig peritoneal neutrophils. The pure heterodimer molecule was dissociated by acidic conditions, and MAM-α and MAM-β were isolated. Amino acid and carbohydrate composition is presented for both glycopeptides. A role for intrapeptide disulfide bonding in maintaining the structure of the cysteine-rich subunit MAM-β is demonstrated.

Materials and Methods

Peritoneal Neutrophils. Guinea pig peritoneal neutrophils were collected 16 h after intraperitoneal injection of 25 mL of 10% sodium caseinate (Oren et al., 1963); they were washed by pelleting in cold HBSS. The cells were ≥98% neutrophils (Wright's stain) and ≥98% intact (trypan blue exclusion).

Monoclonal Antibodies. The anti-MAM mouse mAbs have been described (Remold-O'Donnell & Savage, 1988). M2, a mouse IgG2b anti-MAM-α antibody, and M7, an IgG_1 anti-MAM-β antibody, were purified from ascites; other mAbs were used as culture supernatants.

Immunoprecipitation. Immunoprecipitation of lysates of ^{125}I-labeled macrophages with mAb was as described (Remold-O'Donnell & Savage, 1988).

Antibody Affinity Resins. Lyophilized CNBr-activated Sepharose 4B (Pharmacia) was rehydrated in 1 mM HCl, washed with 200 mM $NaHCO_3$, pH 8.5, and 500 mM NaCl (pH 8.5 buffer), and reacted with purified antibody at 2 mg/mL in pH 8.5 buffer (4 mg of antibody/mL of resin; 80-95% coupling efficiency) for 3 h at ~22 °C. The resin was incubated in 100 mM Tris-HCl, pH 8.0, for 2 h at ~22 °C; it was washed by three cycles of 100 mM sodium acetate buffer, pH 4.0, followed by pH 8.5 buffer and was stored in PBS-0.05% NaN_3.

Purification of MAM: Cell Lysis. Batches consisted of 1.5×10^{10} neutrophils. ^{125}I was introduced into 10% of the cells in 30 mL of HBSS at ~22 °C with 7 μM NaI, 750 μg of lactoperoxidase, 9 mCi of $Na^{125}I$, and 15 additions over 15 min of 0.5 mL of 0.03% H_2O_2. The neutrophils were lysed with 19 volumes of 0.5% NP-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DFP, and 3 mM iodoacetamide by pipetting for 6 min at 22 °C and for 15 min on ice; the lysates

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[1] Abbreviations: mAb, monoclonal antibody; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate; NP-40, the detergent Nonidet P-40; ramIgG, affinity-purified IgG fraction of rabbit anti-mouse IgG antiserum; MAM, macrophage adhesion molecule; GLC, gas-liquid chromatography; PBS, phosphate-buffered saline; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DFP, diisopropyl fluorophosphate; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] E. Remold-O'Donnell, manuscript in preparation.

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were clarified at 18 000 rpm in a Sorvall SS34 rotor for 30 min at 4 °C.

Lentil Lectin Chromatography. The combined NP-40 extract (500 mL) was applied to a lentil lectin–Sepharose column (Remold-O'Donnell, 1980; 4.0-cm diameter; 100 mL of resin; 2.5 mg of lectin/mL of resin) equilibrated against 0.3% NP-40, 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl (0.3% NTS) at ~22 °C. The column was washed with 1.3 volume of 0.3% NTS and eluted with 150 mL of 100 mM methyl α-mannoside in 0.3% NTS.

M2 mAb Affinity Chromatography. The lentil lectin eluate was incubated with 19 mL of the mAb affinity resin M2–Sepharose equilibrated against 0.3% NTS for 2 h at ~22 °C in a polystyrene bottle on a roller apparatus. The mixture was transferred to a column (2.5-cm diameter); the resin was washed with 3 volumes of 150 mM NaCl and eluted with 20–30 mL of 0.5% NP-40 and 200 mM sodium citrate, pH 3.5. After 5 min, the eluate was neutralized with 3 M Tris base and dialyzed against 0.3% NTS.

M7 mAb Affinity Chromatography. The M2 eluate was incubated with 20 mL of the mAb affinity resin M7–Sepharose equilibrated against 0.3% NTS for 2 h at ~22 °C on a rotary mixer. The mixture was transferred to a column (2.5 cm); the resin was washed with 4 mL of 0.3% NTS and with 2.5 volumes of 150 mM NaCl and was eluted with 25 mL of 0.3% NP-40 and 50 mM triethylamine, pH 10.5. The eluate was neutralized with 1 M Tris-HCl, pH 7.2; the nonadherent and eluate fractions were dialyzed against 0.3% NP-40 and 50 mM NH_4HCO_3.

Ethanol Precipitation. The M7 nonadherent fraction and the M7 eluate were lyophilized (two cycles), reconstituted with water to 4.0 and 2.0 mL, respectively, and precipitated by adding ethanol at −20 °C to 70% and 85%, respectively. After ≥24 h at −20 °C, the glycoproteins were pelleted at 15 000 rpm for 30 min at −2 °C in a Sorvall SS34 rotor.

Compositional Analyses. Amino acid composition was determined on a Dionex D-500 analyzer on samples hydrolyzed in 6 N HCl at 110 °C under reduced pressure for 24 h. Protein concentration was calculated by integrating amino acid determination. The carbohydrate content was determined by methanolysis followed by gas–liquid chromatography (GLC) of the per(trimethylsilyl) derivatives (Reinhold, 1972).

Electrophoresis and Immunoblots. SDS–polyacrylamide gel electrophoresis, a modification of the Laemmli procedure (Laemmli, 1970), double-detergent electrophoresis, and immunoblots were as described (Remold-O'Donnell & Savage, 1988).

Results

Isolation of MAM. Peritoneal neutrophils (1.5×10^{10}), a portion of which were radioiodinated, were lysed with the nonionic detergent NP-40. On lentil lectin–Sepharose chromatography, the bulk of proteins passed unretarded through the column. MAM adhered and was recovered quantitatively on elution with methyl α-mannoside (not shown), providing an effective purification step as previously described for MAM from macrophages (Remold-O'Donnell, 1980).

Antibody Affinity Purification. On M2 mAb chromatography, the remaining ^{125}I-labeled proteins passed unretarded through the column (Figure 1, compare lanes A and B). MAM adhered and was quantitatively eluted with citrate buffer, pH 3.5 (Figure 1, lane D). The M2 eluate fraction was pure MAM-α and MAM-β as judged by Coomassie blue stained SDS electrophoresis gels (shown below).

Dissociation of the Heterodimer by Acidic Buffers. The technique of double-detergent electrophoresis (used in Figure 1) does not disrupt the heterodimer bond of MAM (Remold-O'Donnell & Savage, 1988). Thus, this analysis demonstrates that the acidic elution buffer (0.2 M sodium citrate buffer and 0.5% NP-40, pH 3.5) converts the heterodimer into monomeric MAM-α and MAM-β (compare Figure 1, lanes A and D). Monomeric MAM-α in the eluate did not adhere to M2–Sepharose on rechromatography (not shown). Elution of MAM from other antibody resins (M7, M8, M11, M12, and M13) with acidic buffers (the pH 3.5 citrate buffer or 0.1 M glycine–glycine hydrochloride and 0.5% NP-40, pH 2.5) converts the heterodimer to monomeric glycopeptides. In contrast, elution of MAM from mAb resins (M7, M8, M11, and M13) with the alkaline buffers 0.1 M glycine, 0.5% NP-40, pH 10, or 0.05 M triethylamine and 0.5% NP-40, pH 10.5, does not dissociate the heterodimer (not shown).

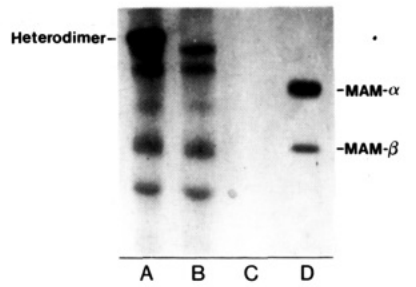

FIGURE 1: Purification of MAM on M2–Sepharose. Shown is an autoradiograph (^{125}I-labeled surface proteins) of a double-detergent electrophoresis gel of (A) the lectin eluate applied to M2–Sepharose, (B) the nonadherent fraction, (C) the wash fraction, and (D) the pH 3.5 eluate. Note the presence of the MAM heterodimer in the starting fraction (lane A) and dissociated MAM-α and MAM-β in the eluate (lane D).

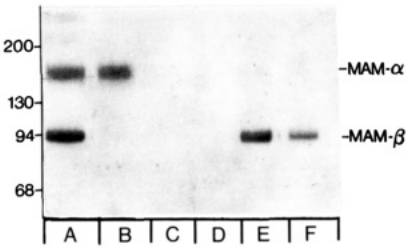

FIGURE 2: Coomassie blue stained SDS electrophoresis gel showing the separation of MAM-α and MAM-β on M7-Sepharose. Shown are (A) the neutralized M2 eluate applied to the M7 column, (B) the nonadherent fraction, (C and D) wash fractions, and (E and F) the eluate (pH 10.5 buffer).

Separation of MAM-α and MAM-β by Antibody Chromatography. Chromatography of the M2 eluate on M7 mAb–Sepharose served to isolate MAM-α (the nonadherent fraction) (Figure 2, lane B). MAM-β was eluted with pH 10.5 buffer (Figure 2, lane D). The glycopeptides were freed of salts and detergent by dialysis, lyophilization, and ethanol precipitation. The MAM-α fraction was homogeneous on Coomassie blue stained SDS gels. MAM-β preparations were contaminated to varying degrees (0–10%) with MAM-α, which could be reduced or eliminated by repeat M2–Sepharose chromatography.

Amino Acid Composition. With one notable exception, the amino acid compositions of purified MAM-α and MAM-β differ only slightly from each other and from the mean of >200 purified proteins (Table I, columns 2–4). Small differences were noted; i.e., the lysine content of MAM-α and alanine of MAM-β are slightly lower than the composite mean, and serine of MAM-α and glycine of MAM-β are slightly higher. The single striking compositional datum is the very high Cys/2 content of MAM-β. At 7.2 residues per 100 amino acids,

Table I: Amino Acid Compositions[a]

residue	no. of residues/100 amino acids: >200 proteins	MAM-α	MAM-β	no. of residues/molecule: MAM-α	MAM-β
Asx	10.7	8.2	8.3	90	53
Glx	10.6	11.1	10.9	122	70
His	2.2	1.7	1.5	19	10
Lys	6.5	4.3	5.7	47	36
Arg	4.4	4.9	5.6	54	36
Ser	6.3	8.7	6.5	95	42
Thr	5.7	6.6	5.5	73	35
Pro	4.8	3.9	4.5	43	29
Ala	8.5	6.1	5.0	67	32
Cys	2.3	2.0	7.2	22	46
Gly	8.1	10.0	12.0	110	77
Tyr	3.3	3.2	2.7	35	17
Val	6.8	6.8	6.2	75	40
Ile	5.0	4.8	3.4	53	22
Leu	8.1	9.9	8.3	109	53
Phe	3.7	5.0	3.8	55	24
Met	1.9	1.6	1.3	18	8
Trp	1.3	ND[b]	ND	ND	ND
				total 1100	640

[a] The mean amino acid composition of >200 proteins is from Reeck and Fisher (1973). Values for MAM-α and MAM-β are averages of two preparations. The MAM-β values were corrected for contaminating MAM-α which was estimated at 5% based on analysis of Coomassie blue stained electrophoresis gels. [b] Not determined.

Table II: Carbohydrate Compositions[a]

residue	no. of residues/100 amino acids: MAM-α	MAM-β	no. of residues/molecule: MAM-α	MAM-β
fucose	0.7	0.6	7	4
xylose	0.7	0.9	8	6
mannose	6.6	3.9	73	25
GlcNAc	3.8	3.1	41	20
galactose	4.4	4.0	49	25
GalNAc	0	0	0	0
sialic acid	0.7	1.1	7	7

[a] Values are averages of two preparations; MAM-β values are corrected as described in Table I.

Cys/2 of MAM-β is more than 3 times the mean of 200 purified proteins (Table I). MAM-α differs from MAM-β in its much lower Cys/2 content (2.0 residues per 100 amino acids). The apparent molecular weights of the carbohydrate-free polypeptides (Remold-O'Donnell, 1987) were used to express these data on a "per molecule" basis (Table I, columns 5 and 6). The MAM-α polypeptide, M_r 130 000, corresponds to an ~1100 amino acid molecule and the MAM-β polypeptide, M_r 75 000, to an ~640 amino acid polypeptide of which 46 residues are Cys/2.

Carbohydrate Compositions. The carbohydrate contents of purified MAM-α and MAM-β are 21% and 18%, respectively, equivalent to 34 000 daltons for MAM-α and 16 000 daltons for MAM-β. The major monosaccharides of both are mannose, galactose, *N*-acetylglucosamine, and sialic acid (Table II, columns 2 and 3). The mannose content is higher in MAM-α than in MAM-β.

Role of Intrapeptide Disulfide Bonds in MAM-β. Six monoclonal antibodies, which react on immunoblots with MAM-β, were used to probe the structure of this subunit. The six antibodies recognize at least four independent epitopes as follows. M14 mAb is unique in its inability to precipitate the MAM heterodimer; this antibody reacts with dissociated or monomeric β-chains and has been classified as "anti-monomeric-β" (Remold-O'Donnell, 1987). Among the remaining antibodies, M7 is unique because of its inability to bind to intact cells (nonsurface epitope).[2] The other four antibodies (surface epitopes) fall into two groups, M15 which inhibits cell adhesion and M11, M12, and M13 which do not.[2]

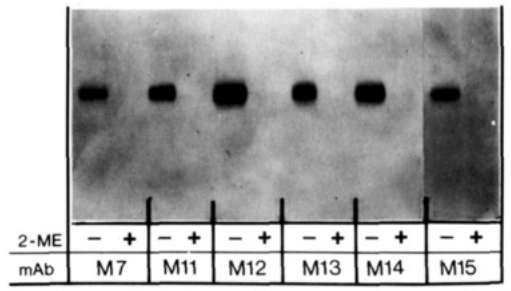

FIGURE 3: Immunoblot of macrophage proteins stained with anti-MAM-β mAb. Macrophage lysate was fractionated by SDS electrophoresis under nonreducing conditions or after reduction with mercaptoethanol (2-ME), transferred to nitrocellulose, and treated with the indicated mAb and ^{125}I-ramIgG. Autoradiography exposures were 3 h for the first five antibodies and 8 h for M15.

When disulfide bonds of macrophage proteins were disrupted by mercaptoethanol, all six antibodies failed to react with MAM-β (Figure 3), indicating that multiple epitopes of MAM-β depend on intrapeptide disulfide bonding. These findings suggest an important role for intrapeptide disulfide bonds in establishing and/or maintaining the overall conformation of MAM-β.

Discussion

MAM, the surface heterodimer molecule involved in the adhesion of macrophages and neutrophils to surfaces, was purified from peritoneal neutrophils by detergent extraction, lectin chromatography, and M2–antibody affinity chromatography. The pure glycopeptides, MAM-α and MAM-β, were separated by M7–antibody chromatography. No significant losses occurred during purification as judged by autoradiography and Coomassie blue staining of SDS gels.

With one exception, the amino acid compositions of MAM-α and MAM-β do not differ significantly from each other or from the composite mean of 200 purified proteins. The exception is the extremely high cysteine content of MAM-β (7.2 residues per 100 amino acid residues), which is more than 3-fold the mean for multiple purified proteins. The compositional data, together with the apparent molecular weights, indicate the MAM-β is an ~640 amino acid polypeptide of which 46 residues are Cys/2. MAM-α, which is estimated to be an 1100 amino acid polypeptide, does not have unusually high cysteine content.

Pure MAM-α consists of 21% carbohydrate or ~34 000 daltons. MAM-β consists of 18% carbohydrate or 16 000 daltons. The major monosaccharides of both are mannose, *N*-acetylglucosamine, galactose, and sialic acid, consistent with the presence of multiple N-linked carbohydrate units. No *N*-acetylgalactosamine was found, suggesting the absence of O-linked units. MAM-α is partially sensitive to endoglycosidase H, and MAM-β is insensitive, indicating that MAM-α contains both "high mannose" and "complex" units and MAM-β contains only "complex" units (Remold-O'Donnell, 1987). The higher content of mannose in MAM-α is consistent with the presence of "high mannose" units in MAM-α. The number of carbohydrate units is estimated at 12 per molecule in MAM-α and 5–6 in MAM-β, assuming 2400 daltons per "high mannose" unit ($Glc_3Man_9GlcNAc_2$; Robbins et al., 1977) and 3000 daltons per "complex unit". Both MAM-α and MAM-β were found as a series of discrete spots on isoelectrofocusing (Remold-O'Donnell & Savage, 1988), suggesting variation in glycosylation.[3]

Comparisons of MAM, a guinea pig molecule, with the analogous human and mouse molecules may reveal shared (conserved) features likely to be critical for function. Total amino acid sequence has been determined via cDNA cloning for the common β-subunit of the analogous human heterodimer family [LFA-1 by Law et al. (1987) and p150,95 by Kishimoto et al. (1987)]. The human β-subunit, like MAM-β, has a very high Cys/2 content (7.4 residues per 100 amino acid residues). With the exception of small differences in Asx and Gly content, the amino acid compositions of the human and guinea pig β-subunits are quite close. Whereas the human β-glycopeptide has 748 amino acid residues, the current study indicates that MAM-β has ~640 residues; this difference cannot presently be evaluated because the latter value is an estimate. The number of candidate sites for N-glycosylation in the human β-subunit is reported as five (Law et al., 1987) and six (Kishimoto et al., 1987). On the basis of carbohydrate composition, MAM-β contains five to six N-linked carbohydrate units, indicating that most candidate sites are glycosylated.

Amino acid compositions of the α-M and β-subunits of the "cellular adherence receptor" purified from human neutrophils by HPLC (Hickstein et al., 1987) are also similar to the amino acid compositions of MAM-α and MAM-β, respectively, with the exception of low methionine content in both human glycopeptides. On the other hand, the carbohydrate composition values for the purified human α-M and β-subunits are severalfold lower than the values reported here for MAM-α and MAM-β.

The importance of cystine residues to the structure of the β-subunit is indicated by the finding that six monoclonal antibodies, which recognize at least four independent epitopes on MAM-β, show dramatically decreased binding when disulfide bonds are disrupted. These findings indicate that intrapeptide disulfide bonds are important in establishing and maintaining the conformation of MAM-β.

Information has accumulated on the forces involved in forming and maintaining the heterodimer structure of MAM. The two glycopeptides are synthesized as separated monomeric precursors (Remold-O'Donnell, 1987). It appears that ionic forces are important in their association since the heterodimer is dissociated by acid and SDS but not by nonionic detergent, EDTA, or EGTA or by treatment at pH 10 or 10.5. The conformation of at least MAM-α differs in the monomer and heterodimer states since dissociated (monomeric) MAM-α does not adhere on attempted rechromatography to the anti-α antibody used in its purification. Thus, the conformation of MAM-α is altered when it forms the heterodimer molecule with MAM-β.

[3] An unidentified carbohydrate component was also detected in high quantities in pure MAM-α and MAM-β; after methanolysis, this material migrated at the position of glucose on GLC. The ratio of "glucose" to protein differed by a factor of 2.5 in two preparations (450 and 1200 "glucose" residues per molecule), indicating that the unknown carbohydrate is a copurifying component rather than an integral part of the MAM molecule.

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REFERENCES

Hickstein, D. D., Ozols, J., Williams, S. A., Baenziger, J. U., Locksley, R. M., & Roth, G. J. (1987) *J. Biol. Chem. 262*, 5576–5580.

Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M., & Springer, T. A. (1987) *Cell (Cambridge, Mass.) 48*, 681–690.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Law, S. K. A., Gagnon, J., Hildreth, J. E. K., Wells, C. E., Willis, A. C., & Wong, A. J. (1987) *EMBO J. 6*, 915–919.

Oren, R., Farnham, A. E., Saito, K., Milofsky, E., & Karnovsky, M. (1963) *J. Cell Biol. 17*, 487–501.

Reeck, G. R., & Fisher, L. (1973) *Int. J. Pept. Protein Res. 5*, 109–117.

Reinhold, V. N. (1972) *Methods Enzymol. 25*, 244–249.

Remold-O'Donnell, E. (1980) *J. Exp. Med. 152*, 1699–1708.

Remold-O'Donnell, E. (1987) *J. Immunol.* (in press).

Remold-O'Donnell, E., & Lewandrowski, K. (1982) *Cell. Immunol. 70*, 85–94.

Remold-O'Donnell, E., & Savage, B. (1988) *Biochemistry* (preceding paper in this issue).

Robbins, P. W., Hubbard, S. C., Turco, S. J., & Wirth, D. F. (1977) *Cell (Cambridge, Mass.) 12*, 893–900.