

## EVIDENCE OF EOSINOPHIL GRANULE MAJOR BASIC PROTEIN IN HUMAN PLACENTA

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A protein immunologically similar to the major basic protein of eosinophil granules (gMBP)<sup>1</sup> is present in high concentration in the plasma of pregnant women (1). The blood level of this pregnancy-associated major basic protein (pMBP) increases early in gestation and plateaus by week 20 at concentrations more than 10 times normal. After another increase just before parturition, the peak level of pMBP in pregnancy approaches the levels seen in patients with the hypereosinophilic syndrome. pMBP returns to baseline within 6 wk post partum. Unlike hypereosinophilic syndrome plasma, however, pregnancy plasma does not contain increased amounts of other eosinophil granule-derived proteins such as eosinophil-derived neurotoxin, eosinophil cationic protein, or Charcot-Leyden crystal (1). In addition, there is no eosinophilia demonstrable in peripheral blood, uterus, or placenta during pregnancy (1, 2). These observations and the immunofluorescent localization of pMBP in fetally derived X cells and giant cells in human placentae (2) suggest that pMBP is not derived from the eosinophil.

Despite their apparent origin from different cells, pMBP and gMBP are identical in their reactivities with polyclonal rabbit antisera (1) and a panel of 14 mouse mAbs (3). The following study was undertaken to define the chemical basis for this immune crossreactivity. The results indicate that the pMBP is virtually identical chemically to gMBP.

### Materials and Methods

*Materials.* gMBP was prepared from human eosinophils as described (4, 5). Plasma samples were obtained from pregnant women (third trimester), from patients with eosinophilia, and from normal persons by centrifugation of venous blood (EDTA as an anticoagulant) and stored at -20°C. Mouse mAbs against gMBP were prepared as described (6) by using the FO myeloma fusion partner and purified from ascitic fluid by protein A affinity chromatography. PBS consisted of 0.003 M KCl, 0.001 M KH<sub>2</sub>PO<sub>4</sub>, 0.008 M Na<sub>2</sub>HPO<sub>4</sub>, and 0.15 M NaCl

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<sup>1</sup> *Abbreviations used in this paper:* gMBP, major basic protein of eosinophil granules; NEpHGE, non-equilibrium pH electrophoresis; pMBP, pregnancy gradient-associated major basic protein.

at pH 7.4. PPF-E buffer consisted of 0.1 M  $\text{PO}_4$  (pH 7.4), 0.5% FCS, 0.1%  $\text{NaN}_3$ , 0.01 M EDTA, and 1% protamine sulfate (salmon, grade X) at pH 7.4. Placental cyst fluid was aspirated from septal and subchorial cysts with a 3-ml syringe and stored at  $-20^\circ\text{C}$ . Molecular weight markers included transferrin, BSA, ovalbumin, trypsinogen,  $\beta$ -lactoglobulin, cytochrome *c*, blue dextran, and dinitrophenyllysine.

**Reduction and Alkylation of Placenta-derived Fluids.** 5 vol of placental extract, cyst fluid, or plasma was diluted in 13 vol of Tris buffer (0.33 M Tris, 0.15 M NaCl, and 0.01 M EDTA at pH 8.1); 2 vol of 0.075 M dithiothreitol (in Tris buffer) was added and the solution was incubated for 1 h at  $22^\circ\text{C}$ . Then, 2 vol of 0.15 M iodoacetamide was added and the solution was incubated for 20 min at  $22^\circ\text{C}$ . Samples were analyzed immediately or dialyzed against PBS.

**Radioimmunoassay for MBP.** A competitive binding RIA with mAb was used to measure MBP activity of both gMBP and pMBP. Briefly, FAST sticks (Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) were coated with protein A-purified J14-7A2 anti-gMBP mAb (10  $\mu\text{g}/\text{ml}$  in PBS) and blocked with PPF-E buffer. Reduced and alkylated unknowns or gMBP standards were diluted in PPF-E buffer; 100- $\mu\text{l}$  portions were placed in 96-well plates with 100  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled gMBP (5 ng/ml, 30  $\mu\text{Ci}/\mu\text{g}$ ) and incubated with mAb-coated sticks for 18 h at  $4^\circ\text{C}$ . The sticks were washed with PBS containing 0.05% Tween 20 and assayed in a gamma counter. MBP activity was quantitated from a gMBP standard curve with a Hewlett-Packard computer (HP 9845; program 15031). Radioiodination of gMBP (and other proteins) was performed by the chloramine T method (7).

**Purification of pMBP.** pMBP was purified from placental septal fluids and extracts of septa by a four-step procedure. First, placental septa were diced into 5-mm pieces and suspended in extraction buffer (0.15 M NaCl, 0.01 M EDTA, 0.5 mM PMSF, and 0.33 M Tris at pH 8.1) using 2 vol of buffer per gram of tissue. The suspension was homogenized in a Waring blender (3 min,  $4^\circ\text{C}$ ), digested with collagenase (0.1% collagenase, type IV, in 0.02 M  $\text{CaCl}_2$  for 5 h at  $37^\circ\text{C}$ ) and with deoxyribonuclease (400 U of DNase per milliliter of extract in 0.004 M  $\text{MgCl}_2$  for 5 h at  $37^\circ\text{C}$ ), and centrifuged at 10,000 *g* for 10 min. The extract was stored at  $-20^\circ\text{C}$ . Total protein was measured by the Lowry (8) or biuret assay (9).

Second, pMBP was purified by affinity chromatography. Protein A-purified mAb anti-gMBP (J14-7A2, or J6-8A4) was immobilized on cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions (5–10 mg of mAb per milliliter of gel). Reduced and alkylated placental septal extracts or cyst fluid were applied to the mAb columns (1  $\times$  10 cm), washed extensively with PBS (6 ml/h, upward flow) until the absorbance at 280 nm was  $<0.02$ , washed with 0.5 M NaCl, 0.3 M Tris at pH 8.0 (3 column volumes), and eluted with 0.1 M glycine-HCl at pH 3.0.

Third, gel filtration chromatography in the presence of a dissociating solvent was used to fractionate pMBP by molecular size. Eluates from the affinity purification were equilibrated with 6 M guanidine-HCl (GuCl), reduced and alkylated again, and fractionated on Sepharose CL-6B equilibrated with 6 M GuCl. The molecular weight of pMBP was calculated as described by Mann and Fish (10). pMBP-containing fractions were detected by dot blot assay or measured by RIA after dialysis against PBS. pMBP-containing pools were desalted over Sephadex G-15 (1  $\times$  15 cm column, 0.5 M acetate buffer, 5 ml/h).

Fourth, pMBP was purified by reversed-phase HPLC on a microBondpak  $\text{C}_{18}$  column (25 cm  $\times$  10  $\mu\text{m}$ ) (Waters Associates, Milford, MA). The starting buffer was 0.1% trifluoroacetic acid, and proteins were eluted by a linear gradient (0–100%) of 90%  $\text{CH}_3\text{CN}$  in 0.1% trifluoroacetic acid. Protein-containing fractions were detected by absorbance at 280 nm, pooled, and lyophilized.

**Gel Electrophoresis and Western Blot Assay.** One-dimensional SDS-PAGE and two-dimensional nonequilibrium pH gradient electrophoresis (NEpHGE)/SDS-PAGE were performed as described (11). Proteins were detected by silver staining (12). The Western blot assay (immunoassay) was performed by electrophoretic transfer of proteins to nitrocellulose (Transblot Cell; Bio-Rad Laboratories, Rockville Center, NY) with 15 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3 and 90 V and 0.26 A for 1 h. Filters were blocked with 3% BSA in PBS at pH 7.4 (1 h at  $22^\circ\text{C}$ ). MBP antigenic activity was detected by reaction of the filter with a mixture of four mAbs (J14-7A2, J13-6B6, J14-1C3, and J6-8A4), each at 1  $\mu\text{g}/\text{ml}$  in PPF-E buffer, for 2 h at  $22^\circ\text{C}$ . After it was washed, the filter was exposed to sheep anti-mouse  $^{125}\text{I}$ -

labeled IgG, 20 ng/ml in PPF-E buffer, for 18 h at 4°C. Paired filters were incubated with normal mouse serum in the first stage as a negative control. The filters were extensively washed with PBS containing 0.05% Tween 20 and autoradiographed at -70°C on XAR-5 film (Eastman Kodak, Rochester, NY). Dot blot assays were performed in the same manner except that 25  $\mu$ l of each column fraction was spotted directly onto the nitrocellulose.

**Peptide Mapping.** For trypsin and cyanogen bromide digestions,  $^{125}$ I-labeled gMBP and  $^{131}$ I-labeled pMBP were mixed, boiled in SDS sample buffer, and acetone precipitated in the presence of 200  $\mu$ g of BSA as a carrier. Trypsin digestion was performed in 200  $\mu$ l of 0.1 M  $\text{NH}_4\text{HCO}_3$  at pH 7.8; a total of 60  $\mu$ g of *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp., Freehold, NJ) added in three equal portions over 9 h of incubation at 37°C was used. Cyanogen bromide digestion at tryptophan residues was performed according to Huang et al. (13). In each case, the radiolabeled peptides were separated by reversed-phase HPLC as described above and detected by analysis of 1-ml fractions in a dual-channel gamma counter (TM 1191; TM Analytics, Elk Grove Village, IL). The results were analyzed with a Hewlett-Packard computer (HP 9845) and a statistical graphics program (No. 15021). gMBP or pMBP (10  $\mu$ g in 0.25% acetic acid) was cleaved at aspartic acid residues by treatment at 105°C for 6 h in sealed glass vials. Cleavage products were analyzed by SDS-PAGE.

**Amino Acid Analysis.**  $\text{NH}_2$ -terminal analysis of pMBP and sequence determination of the COOH-terminal peptide generated by cleavage in dilute acid were performed as described (11).

## Results

### *Purification of pMBP*

Initial gel filtration of placental extracts indicated a mass of >1,000 kD (elution in the void volume of Sepharose CL-4B columns) for pMBP (data not shown). Because this excessive size may have reflected polymerization or binding to a large carrier molecule, subsequent studies used DNase treatment to minimize complexes with DNA. However, even after nucleic acid removal, pMBP still eluted from Sepharose CL-4B columns in multiple high molecular weight peaks, a behavior similar to that of plasma gMBP which binds to plasma proteins through ionic and disulfide bonds (4); gMBP can be resolved to the monomer, ~14 kD, by reduction and alkylation and then acidification of plasma (4). Analogous interactions for pMBP are suggested by its requirement for reduction and alkylation in order to maximize immunoreactivity with polyclonal antisera (1) and mAbs (3) and by its low molecular weight, as judged by immunodiffusion after reduction under denaturing conditions (results not shown) and by Western blot analysis (see below).

After DNase digestion and reduction and alkylation of placental extracts, to minimize pMBP interactions with DNA and placental proteins, respectively, pMBP was partially purified from placental extracts or cyst fluid by affinity chromatography with mAb immobilized on Sepharose 4B. The resulting preparations were 300–700-fold enriched from placental extracts and 50–150-fold enriched from cyst fluid with 70% to almost 80% recoveries (Table I). Eluates were analyzed by SDS-PAGE and Western blot assay to detect immunoreactive MBP. Multiple proteins were present in these eluates even though the immunoprecipitates had been washed with buffers containing high salt concentration (Fig. 1 A). Two immunoreactive species with molecular masses of ~14 and 30 kD were present in affinity-purified cyst fluid and placental extract (Fig. 1 B). The 30-kD molecule was replaced by a 14-kD molecule when the eluates were reduced in 8 M urea and therefore it appears to be a SDS-stable polymer (data not shown). Purified eosinophil gMBP polymerizes on storage to yield a 30-kD SDS-stable species (Fig. 1 B).

TABLE I  
Purification of pMBP From Placental Extracts and Cyst Fluid

Purification step	Volume <i>ml</i>	Total protein	MBP* $\mu\text{g}$	MBP ( $\mu\text{g}/\text{mg}$ protein)	Recovery %
From placental extract					
Placental extract	150	7.4 g <sup>†</sup>	629	0.066	
Affinity chromatography	1	10.1 mg <sup>‡</sup>	492	48.7	78
Gel filtration in 6 M GuCl	1	2.6 mg <sup>§</sup>	450	173	72
Reversed-phase HPLC	1	300 $\mu\text{g}$ <sup>§</sup>	250	833	39
From cyst fluid					
Cyst fluid	19	1.5 g <sup>†</sup>	391	0.26	
Affinity chromatography	1	8.4 mg <sup>‡</sup>	300	36	77
Gel filtration in 6 M GuCl	1	3.8 mg <sup>§</sup>	300	79	77
Reversed-phase HPLC	1	250 $\mu\text{g}$ <sup>§</sup>	200	800	51

\* Measured by RIA.

† Measured by biuret method.

§ Measured by Lowry method.

On gel filtration of affinity-purified pMBP over Sephadex G-200 equilibrated with 0.01 M HCl and 0.15 M NaCl (a procedure that releases monomer gMBP from plasma protein complexes [1]), pMBP eluted in the void volume (>400 kD) (data not shown). This result is in contrast to the size of 14 kD shown on Western blots (Fig. 1 B) and suggests that the complexes of pMBP with placental proteins can be disrupted only by more stringent denaturing solvents. Therefore, the second purification step used gel filtration over Sepharose CL-6B in the presence of 6 M GuCl (Fig. 2). Column fractions were screened for pMBP by dot blot assay, and

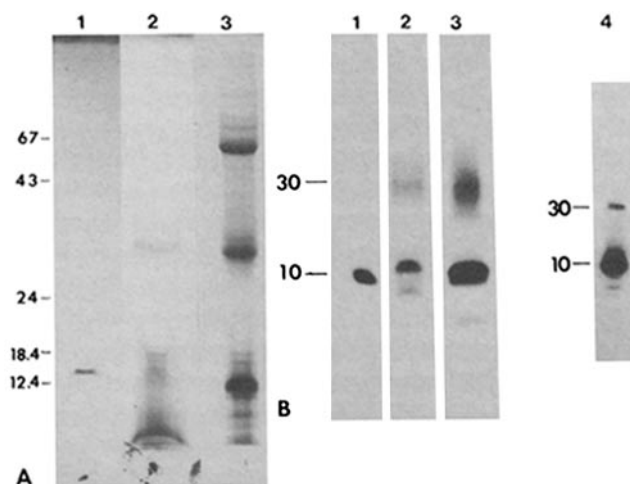


FIGURE 1. SDS-PAGE (A) and Western blot assay (B) of gMBP and affinity-purified pMBP. pMBP was purified from reduced and alkylated placental extracts or cyst fluid by affinity chromatography. Molecular mass markers (kilodaltons) are indicated to the left. (A) SDS-PAGE (under reducing conditions). Lanes: (1) purified eosinophil gMBP; (2) column eluate of cyst fluid; (3) column eluate of placental extract. gMBP (lane 1) migrated with mass 14.5 kD. (B) Western blot assay. Proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose. Immunoreactive MBP was detected by using mAb anti-gMBP followed by sheep anti-mouse <sup>125</sup>I-labeled IgG and autoradiography. A paired blot with normal mouse serum as the first-stage antibody was negative (not shown). Lanes 1, 2, and 3 correspond to lanes 1, 2, and 3 in A; (lane 4) eosinophil gMBP stored for 6 mo at -70°C in nonreduced state (note stable 30-kD polymer).

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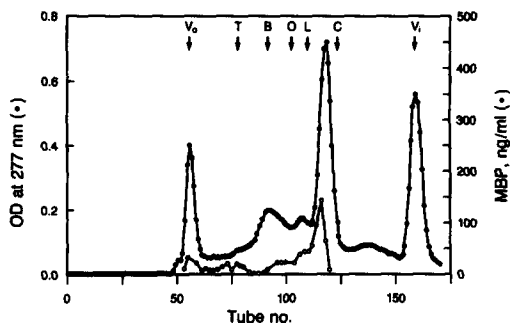


FIGURE 2. Sepharose CL-6B chromatography in the presence of 6 M GuCl of affinity-purified pMBP from a placental extract. Immunoreactive MBP was measured by RIA. Molecular mass markers: blue dextran (Vo), transferrin (T), BSA (B), ovalbumin (O),  $\beta$ -lactoglobulin (L), cytochrome *c* (C), and dinitrophenyllysine (Vi). pMBP mass = 14.7 kD.

positive fractions were dialyzed into PBS for MBP quantitation by RIA. The calculated mass for pMBP was 14.7 kD, in keeping with the results on Western blot assay (Fig. 1). In some cases, both the 14.7-kD molecule and the 30-kD polymer were detected in column fractions. gMBP eluted from this column with a mass of 13.8 kD (data not shown).

pMBP-containing fractions from Sepharose CL-6B columns were desalted, lyophilized, and subjected to reversed-phase HPLC (Fig. 3 A). Each protein peak was collected and subjected to SDS-PAGE followed by Western blot assay to detect MBP. Peak 5 gave a single band by SDS-PAGE ( $\sim$ 15 kD) and a single spot by two-dimensional gel electrophoresis (see below). Similar results were seen with purified pMBP from cyst fluid. Fig. 4 shows the purity of pMBP at each step of the purification.

#### *Physicochemical Comparison of pMBP and gMBP*

**Molecular Weight.** Eosinophil gMBP has a predicted molecular weight of 14,047 based on its amino acid sequence (14), close to the values of 14,500 by SDS-PAGE (Fig. 1) and 13,800 by elution from Sepharose CL-6B in the presence of 6 M GuCl (results not shown). The molecular weight of pMBP estimated by these procedures is also  $\sim$ 14,000 (Fig. 1, 2, and 3). Further, the size similarity between gMBP and pMBP is shown by their comigration in two-dimensional gels (see below).

**Isoelectric Point.** Eosinophil gMBP has an isoelectric point of 11.6 predicted by its amino acid sequence (14). The absence of ampholytes capable of creating a stable gradient in this pH range precludes the exact measurement of the pI of gMBP and pMBP. However, NEpHGE, the first dimension of two-dimensional gels, allows comparison of the relative migrations of gMBP and pMBP in a pH gradient. Both pMBP and gMBP migrated  $>2$  cm ahead of cytochrome *c* (pI = 10.6) when NEpHGE focusing was performed for 2 h (500 V), indicating a pI for each of  $>10.6$  (data not shown). Because gradient formation may vary in side-by-side gels, mixing experiments were performed to compare the relative migrations of gMBP and pMBP. When trace amounts of  $^{131}\text{I}$ -labeled pMBP were added to 5  $\mu\text{g}$  of purified gMBP, the mixture was subjected to two-dimensional gel electrophoresis, and the gel was silver stained to detect gMBP and autoradiographed to detect  $^{131}\text{I}$ -labeled pMBP, the pMBP spot superimposed over the gMBP spot (Fig. 5, A and B). Comigration also was found in the converse experiment with 5  $\mu\text{g}$  of purified pMBP and trace amounts of  $^{125}\text{I}$ -labeled gMBP (Fig. 5, C and D).

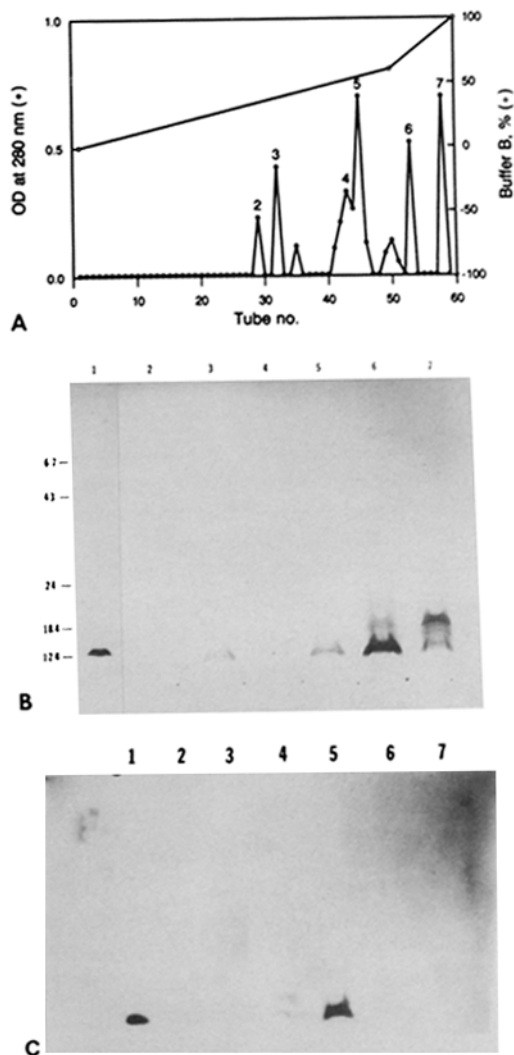


FIGURE 3. Reversed-phase HPLC of pMBP. (A) Partially purified pMBP from placental extracts was applied to a reversed-phase  $C_{18}$  column and eluted with a linear gradient of 90%  $CH_3CN$  in 0.1% trifluoroacetic acid (buffer B). Each peak was collected and analyzed by SDS-PAGE and Western blot assay. pMBP was found in peak 5, at 54% buffer B. (B) SDS-PAGE. Protein peaks from A were separated under reducing conditions and silver stained. Lanes: (1) purified eosinophil gMBP; (2-7) peaks 2-7 from column in A. Size markers (kilodaltons) are indicated at left. gMBP (lane 1) migrated at 14.5 kD. (C) Western blot assay. A duplicate of the SDS-PAGE in B was transferred to nitrocellulose and MBP was detected as described in Fig. 2 B. Lanes: (1) eosinophil gMBP; (2-7) peaks 2-7 from column in A. pMBP was in peak 5. Comparison of B and C indicates that pMBP migrated at  $\sim 15$  kD.

**Hydrophobicity.** The interaction of purified gMBP and pMBP with a  $C_{18}$  reversed-phase HPLC column was used to compare the relative hydrophobicities of these two proteins. When differentially radiolabeled forms of the proteins were analyzed simultaneously,  $^{125}I$ -labeled gMBP and  $^{131}I$ -labeled pMBP coeluted on reversed-phase HPLC (Fig. 6).

**Peptide Mapping.** When  $^{125}I$ -labeled gMBP and  $^{131}I$ -labeled pMBP were mixed and digested with trypsin, and the peptides generated were analyzed by reversed-phase HPLC, the major peptide peaks for gMBP and pMBP coincided (Fig. 7 A). An identical result was obtained when the proteins were treated with cyanogen bromide under conditions that cleave at tryptophan (Fig. 7 B).

The third peptide mapping protocol used dilute acid to cleave gMBP at its lone aspartic acid residue at position 71 to form two peptides that can be separated by

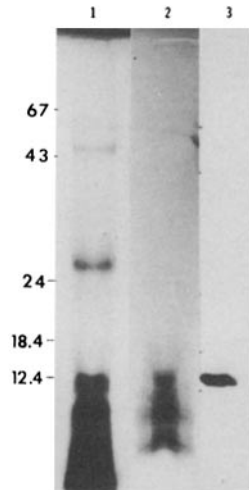


FIGURE 4. SDS-PAGE of pMBP at each purification step. Lanes: (1) eluate from anti-MBP affinity column of placental extract; (2) pMBP pool from Sepharose CL-6B column run in 6 M GuCl; (3) purified pMBP after reversed-phase HPLC. Size markers (kilodaltons) are indicated at left. pMBP (lane 3) had a calculated molecular mass of 14.5 kD.

SDS-PAGE (but not by reversed-phase HPLC) (14). Similar treatment of pMBP produced three bands on SDS-PAGE that represented native pMBP and two cleavage products that were identical in size to the peptides generated from gMBP (Fig. 8).

*Amino Acid Sequence.* NH<sub>2</sub>-terminal sequence analysis was attempted on three samples of pMBP without success. Appropriate cleavage of a radiolabeled control protein indicated that this was not due to problems with the sequencer and sug-

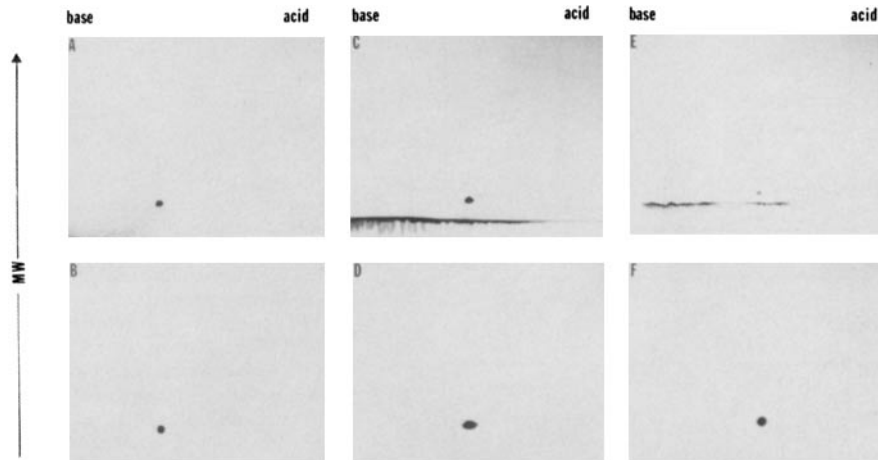


FIGURE 5. Comigration of gMBP and pMBP on two-dimensional gel electrophoresis. Mixtures of radiolabeled and unlabeled gMBP and pMBP were subjected to NEpHGE focusing in the first dimension followed by size separation by SDS-PAGE. (A and B) Mixture of 5  $\mu$ g of gMBP and 5 ng of <sup>131</sup>I-labeled pMBP (purified from placental extracts), with spots detected by silver staining (A) for gMBP and autoradiography (B) for <sup>131</sup>I-labeled pMBP. Note identity of positions. (C and D) Mixture of 5  $\mu$ g of pMBP purified from cyst fluid and 5 ng of <sup>125</sup>I-labeled gMBP with spots detected by silver staining (C) for pMBP and autoradiography (D) for <sup>125</sup>I-labeled gMBP, note identity of positions. (E and F) Same as C and D, except that pMBP was purified from cyst fluid.

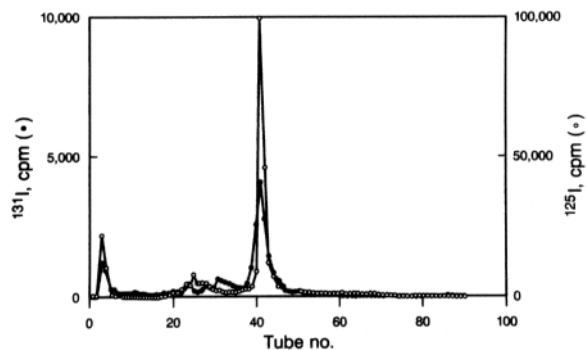


FIGURE 6. Coelution of gMBP and pMBP on reversed-phase HPLC. A mixture of  $^{125}\text{I}$ -labeled gMBP (O) and  $^{131}\text{I}$ -labeled pMBP (●) was subjected to reversed-phase HPLC on a  $\text{C}_{18}$  column with elution by a linear gradient of 0–100% of 90%  $\text{CH}_3\text{CN}$  in 0.1% trifluoroacetic acid.  $^{125}\text{I}$  and  $^{131}\text{I}$  in the 1-ml fractions were measured in a dual-channel gamma counter. pMBP and gMBP coeluted at 54%  $\text{CH}_3\text{CN}$ .

gested chemical blockage of the pMBP  $\text{NH}_2$  terminus. The peptide mapping studies outlined above suggested the possibility of determining the sequence of the COOH-terminal peptide generated by cleavage at aspartic acid. When pMBP was digested in dilute acid and the peptide mixture was analyzed directly, a single amino acid sequence was obtained that corresponded exactly with the sequence of residues 72–92 of gMBP (Fig. 9).

#### Discussion

Two sources of pMBP were used for purification. Fluid from placental subchorial and septal cysts is the richest source of pMBP (up to  $100\ \mu\text{g}/\text{ml}$ ) (1) but is not suitable as the sole source of pMBP because of the rarity of cysts containing large amounts

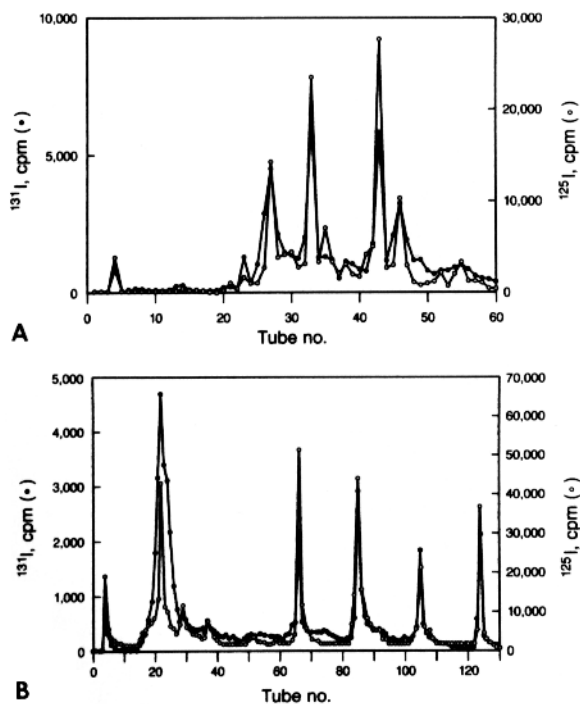


FIGURE 7. Peptide maps after digestion of mixture of  $^{125}\text{I}$ -labeled gMBP (O) and  $^{131}\text{I}$ -labeled pMBP (●). (A) Trypsin digestion. Radiolabeled peptides were separated by reversed-phase HPLC on a  $\text{C}_{18}$  column with a 0–70% gradient of 90%  $\text{CH}_3\text{CN}$  in 0.1% trifluoroacetic acid. 1-ml fractions were collected and analyzed in a dual-gamma counter. (B) Cyanogen bromide cleavage (at tryptophan). Radiolabeled peptides were separated and measured as in A.



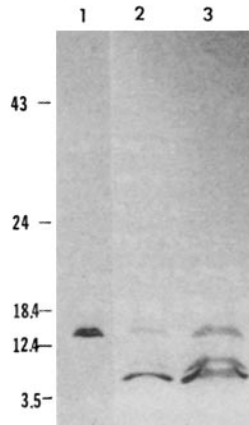


FIGURE 8. Cleavage of gMBP and pMBP at aspartic acid. gMBP and pMBP were dissolved separately in 0.25 M acetic acid and incubated for 6 h at 105°C. The peptides generated were separated by SDS-PAGE. Lanes: (1) undigested gMBP; (2) partial digestion of pMBP showing native protein (top band) and two digestion products (7.2 and 4.8 kD); (3) partial digestion of gMBP, showing native protein (top band) and two digestion products (8.25 and 4.80 kD). Size markers are indicated at left.

	70	75	80	85	90
gMBP	V-D-G-S-R-W-N-F-A-Y-W-A-A-H-Q-P-W-S-R-G-G-H-C				
pMBP		-G-S-R-W-N-F-A-Y-W-A-A-H-Q-P-W-S-R-G-G-H-			

FIGURE 9. Amino acid sequence of COOH-terminal peptide of pMBP after cleavage at aspartic acid. The single amino acid sequence of 20 residues aligned perfectly with the sequence of gMBP (residues 72–92) derived from dilute acid cleavage.

of fluid. Therefore, initial purification efforts focused on extracts of placental septal tissue.

Affinity chromatography was chosen as an early purification step because of its specificity, high yield, and ability to process large volumes. Attempts to purify pMBP by affinity chromatography before removal of DNA from placental extracts were unsuccessful, probably due to the inaccessibility of epitopes as a result of DNA-pMBP interaction. Such complexes were demonstrated by phase partitioning and hydroxyl-apatite adsorption that removed both nucleic acids and pMBP (3). These complexes could not be disrupted by 2 M NaCl, which removes lysine-rich histones from DNA, but they were separated by 2 M GuCl, which removes the more tightly bound arginine-rich histones from DNA (15). The results are consistent with the hypothesis that pMBP is an arginine-rich basic protein and so is chemically related to gMBP (14). Disruption of pMBP-DNA complexes with 2 M GuCl decreased antibody binding, presumably because it caused protein denaturation, and so extracts were treated only with DNase before affinity chromatography.

Although affinity chromatography resulted in significant purification of pMBP from both placental extracts and cyst fluid, several observations indicated that the isolated pMBP was polymerized or bound to carrier proteins. Therefore, gel filtration in the presence of a denaturing solvent (6 M GuCl) was used in order to disrupt pMBP interactions with other molecules. This procedure was successful in separating monomer pMBP (14 kD) from the other proteins present, analogous to results with eosinophil gMBP in the plasma of patients with hypereosinophilic syndrome, which has been shown to polymerize and bind to plasma proteins by ionic and disulfide bonds (4). However, those conditions (reduction, alkylation, and acidification) are inadequate to separate the pMBP found in pregnancy plasma (1). The more rig-

orous conditions necessary to disrupt pMBP-protein complexes may reflect chemical differences between gMBP and pMBP or, more probably, a higher affinity of binding of pMBP to pregnancy-associated proteins, the bulk of which are very acidic (16).

Another similarity between pMBP and gMBP is the ability to self-polymerize. Several lines of evidence suggest that the 30-kD pMBP species seen in some extracts and cyst fluid represents a polymer: (a) the 30-kD and 14-kD species coeluted on reversed-phase HPLC, (b) the isolated 30-kD protein was replaced by a 14-kD molecule when reduced in 8 M urea, and (c) peptide mapping studies performed on preparations of pMBP contaminated with the 30-kD molecule gave the same peptide maps as the pure 14-kD protein did (data not shown). On prolonged storage, gMBP also formed a SDS-stable 30-kD polymer.

The final purification step, reversed-phase HPLC, relied on hydrophobic interaction to separate pMBP from other proteins and resulted in a homogeneous product as determined by one- and two-dimensional gel electrophoresis. Because the placenta is such a vascular organ, the question can be raised whether the purification outlined above simply selects for contaminating gMBP from the plasma in this organ. This possibility is excluded by the yield of pMBP per milliliter of extract and by the recovery of >200  $\mu$ g of pMBP from only 19 ml of cyst fluid (Table I). Only nanogram quantities would be expected from similar volumes of plasma because eosinophil gMBP is present in the plasma of normal humans at 200–400 ng/ml (4). Another possibility is that the pMBP isolated from the placenta is derived from eosinophils in maternal or fetal blood. In the case of septa, this possibility is unlikely because very few eosinophils are present in these tissues; in the case of the cyst fluid, this possibility can be excluded because eosinophils are not present in the cyst fluid or in the septal tissues surrounding the cysts (2).

During pMBP purification, many characteristics of this protein were found to resemble those of gMBP. In addition, gMBP and pMBP have the same molecular mass (by both SDS-PAGE and gel filtration), isoelectric point (comigration in NEpHGE), and relative hydrophobicity (coelution on reversed-phase HPLC). Sequence similarities are indicated by indistinguishable peptide maps after three separate digestions. Thus, these studies provide strong evidence that gMBP and pMBP are identical except for the blocked NH<sub>2</sub> terminus on pMBP. Furthermore, the cloning of eosinophil MBP from a human placental cDNA library supports these findings (17).

The apparent identity of gMBP and pMBP raises important questions as to the cell source of pMBP and its biologic function in the placenta. Eosinophil gMBP is cytotoxic to mammalian cells (18, 19) and parasites (20). It also is known to affect clotting (21), to induce histamine release from rat mast cells and human basophils (22, 23), and to affect the response of tracheal muscle to contractile stimuli (24, 25). The chemical similarities between pMBP and gMBP suggest that the placental protein may have similar biologic effects. pMBP may be produced by fetally derived X cells (2). These cells are the most invasive form of trophoblasts (26), penetrating deep into the decidua and myometrium. Thus, the question arises as to whether a cytotoxin (like gMBP) could facilitate this invasion. In fact, early increases in plasma level of pMBP during pregnancy do correlate with implantation (27). There is another sharp increase in plasma pMBP concentration late in pregnancy that is as-

sociated with the onset of labor (27). The possibility that pMBP is involved in the initiation of labor is suggested by the known effects of gMBP on tracheal smooth muscle contractility (24, 25). The cynomolgus monkey, which also produces placental pMBP (28), may be a model system for investigation of the role of pMBP in primate reproduction.

### Summary

A protein immunochemically related to the eosinophil granule major basic protein (gMBP) is found in increased concentration in the plasma of pregnant women and has been localized to placental trophoblasts by immunofluorescence. Pregnancy MBP (pMBP) is indistinguishable from gMBP in its reactivity with polyclonal antisera and a panel of 14 mouse mAbs. We report the purification of pMBP from human placenta by: (a) affinity chromatography over mAb immobilized on Sepharose, (b) gel filtration in 6 M guanidine-HCl buffer, and (c) reversed-phase HPLC. Purified pMBP and gMBP are biochemically indistinguishable in that both: (a) bind to DNA, (b) polymerize and bind to carrier proteins via disulfide linkages, (c) have a molecular weight of 14,000, (d) have isoelectric points >10.6, (e) comigrate in two-dimensional gels, (f) coelute during reversed-phase HPLC on C<sub>18</sub> columns, (g) have identical peptide maps after three different digestions, and (h) have partial amino acid sequence identity. This physicochemical identity has important implications as to the role of pMBP in human placentation.

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