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Research Article

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ABSTRACT The concentration of multiplication stimulating activity (MSA), an insulinlike growth factor (IGF), is high in fetal rat serum. We now report that MSA is exclusively associated wth an albumin-size binding protein in fetal rat serum; the growth hormone-dependent, gamma globulin-size binding protein, which predominates in the older animal, is absent from fetal rat serum. When ^{125}I-MSA was incubated with fetal rat serum and then gel filtered on Sephadex G-200, specific radioactivity eluted in the void volume (peak I) and the albumin region (peak III); by contrast, specific radioactivity eluted mainly in the gamma globulin region (peak II) in adult rat serum. Pools of the Sephadex G-200 fractions were chromatographed on Sephadex G-50, in 1 M acetic acid, to separate the binding protein from IGF activity. Analysis of IGF activity by chick embryo fibroblast bioassay, competitive protein binding assay, and MSA by radioimmunoassay revealed that all the IGF activity and MSA in fetal rat serum resided in peak III. Measurement of MSA binding capacity of the stripped binding protein by Scatchard analysis demonstrated that the majority of binding capacity also was found in peak III in fetal rat serum; most of MSA binding capacity was in peak II in adult rat serum. In fetal rat sera, in addition to the peak III binding protein, which is the major carrier of endogenous MSA, there is a component in peak I capable of specifically binding ^{125}I-MSA. This component elutes as a single species from a Sepharose-6B column. As MSA associated with peak III gradually declined in early neonatal life, peak II-associated IGF activity measured by chick embryo fibroblast bioassay showed a rise of activity with a peak at 5 d of neonatal life, a nadir at 20 d, with an increase again to attain adult levels. These studies demonstrate that the MSA binding protein in the fetus is different from the growth hormone-dependent binding protein in adult life.

INTRODUCTION

Multiplication stimulating activity (MSA)[1] is a family of polypeptides purified from serum-free media conditioned by a rat liver cell line (BRL-3A) (1–3). MSA had been shown to be closely related to the human insulinlike growth factors, IGF-I/somatomedin C (SM-C), IGF-II, and somatomedin A (SM-A) in studies that examined competition for binding to receptors on cells and purified plasma membranes and for binding to serum binding proteins (4–6). Recently, Marquardt et al. (7) have reported that the amino acid sequence of one of the lower molecular weight species of MSA is identical to the primary structure of human IGF-II except for five amino acids and have proposed that MSA is rat IGF-II. Another rat IGF purified by Daughaday et al. (8, 9) shows amino acid sequence homology with human IGF-I. Using a radioimmunoassay for MSA (10), we have reported that levels of MSA are 20- to 100-fold higher in fetal rat serum than in maternal

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[1] *The abbreviations used in this paper*: BSA, bovine serum albumin; IGF-I, IGF-II, insulinlike growth factor I and II; MSA, multiplication stimulating activity; PBS, phosphate-buffered saline; SM-A, SM-C, somatomedin A and C.

serum and that MSA levels gradually decline following birth to reach low concentrations by 25 d of extrauterine life (11). Indeed, most of the IGF activity in fetal rat serum is accounted for by MSA. This developmental pattern suggests that MSA may be a fetal growth factor in the rat; presumably other rat IGF such as IGF-I assumes importance as MSA levels decline.

In adult rat serum, IGF activity is found associated with larger proteins (12). Most of the IGF activity is associated with a gamma globulin-size protein (peak II); the remainder of the IGF activity is complexed to a protein slightly smaller than albumin (peak III). The gamma globulin-size binding protein is growth hormone dependent (13, 14); in serum from hypophysectomized rats the gamma globulin-size binding protein is absent and the small amount of IGF activity is found complexed to an albumin-size binding protein. We now report that in fetal rat serum, MSA is exclusively associated with an albumin-size binding protein. The concentration of the gamma globulin-size binding protein is very low in fetal rat serum. The decline in serum MSA levels following birth is accompanied by the appearance of non-MSA rat somatomedin(s) and the growth hormone-dependent binding protein.

METHODS

Animals. Fetuses were removed from 19-d and 21-d gestation rats (Sprague-Dawley, Zivic Miller Laboratories, Allison Park, PA) under ether anesthesia, the fetuses were decapitated, and the blood collected. Blood was also collected from rats aged 1, 3, 5, 10, 15, 20, 25, 30, 40, and 120 d following decapitation. Blood was allowed to clot at 4°C and serum was collected after centrifugation.

MSA purification and radioiodination. MSA was purified from serum-free culture media conditioned by a line of rat liver cells (BRL-3A) as reported elsewhere (3). A mixture of MSA II polypeptides (MSA II-1, 2, 3, and 4) was used as standard in the competitive protein binding assay and the MSA radioimmunoassay. The mixture of MSA II polypeptides has been shown to be equipotent to MSA II-1 by bioassay, radioreceptor assay, and radioimmunoassay (3, 10). MSA II-1 (8,700 mol wt), isolated from the mixture of MSA II polypeptides by preparative disc gel electrophoresis, and MSA III-2 (7,100 mol wt) were used for radioiodination. MSA II-1 and MSA III-2 were iodinated with Na ^{125}I to a specific activity of 32–170 μCi/μg, using a modification of the chloramine-T procedure (15). Maximum binding of the tracer was determined using serial dilutions of normal rat serum in the presence and absence of unlabeled MSA. Maximum specific binding for these experiments ranged from 26 to 39% and was used in the calculations for Scatchard analysis of MSA binding.

Gel filtration of serum on Sephadex G-200. A 2.6 × 40-cm Sephadex G-200 column was equilibrated with 0.05 M NH_4HCO_3, pH 8.0, at 4°C. 1 ml of rat serum was incubated with ^{125}I-MSA for 3 h at 20°C and diluted with 1 ml of 0.05 M NH_4HCO_3 before application to the column. Column fractions (2.4 ml) were collected and radioactivity measured in a Beckman 310 gammacounter (Beckman Instruments, Inc., Fullerton, CA) with 85% efficiency. The protein elution profile was measured at 280 nm with a LKB detector, model 4580 (LKB Produkter AB, Bromma, Sweden). Column fractions were stored at 4°C until further study.

Gel filtration on Sepharose-6B. The peak I binding component in fetal rat serum was gel filtered on Sepharose-6B (35 × 2.6 cm) in Dulbecco's phosphate-buffered saline (PBS) (without calcium and magnesium) with 0.1% Triton X-100 at 4°C. The location of the ^{125}I-MSA binding component in the column fractions was determined both by incubation with ^{125}I-MSA for 3 h at 20°C before gel filtration and by measuring specific ^{125}I-MSA binding activity on 200-μl aliquots from each column fraction (2.4 ml) following gel filtration. The column fraction aliquot was incubated with ^{125}I-MSA-II-1 in a total volume of 0.4 ml in Dulbecco's PBS (without calcium and magnesium) with 0.25% bovine serum albumin (BSA) for 3 h at 20°C. Bound ^{125}I-MSA was separated from free by BSA-treated charcoal (12). Nonspecific binding was determined by incubation with 1 μg/ml MSA in a duplicate tube.

Dissociation and separation of somatomedin binding protein from IGF activity. On the basis of radioactivity profile following Sephadex G-200 gel filtration of serum and ^{125}I-MSA, pools of the void volume, peak II, peak III, and free MSA regions were made. Each pool was lyophilized, dissolved in 1 M acetic acid, and gel filtered on Sephadex G-50 (2.6 × 90 cm), in 1 M acetic acid to dissociate and separate the binding protein from the IGF activity (16). Stripped binding protein was found in the Sephadex G-50 void volume. The postvoid volume fractions that contained IGF activity were pooled and lyophilized.

Measurement of IGF activity by bioassay, competitive protein binding assay, and MSA by radioimmunoassay. IGF activity in the Sephadex G-50 postvoid volume was assessed by ability to stimulate the incorporation of [^{3}H]thymidine into the DNA of tertiary chick embryo fibroblasts as previously described (17). The [^{3}H]thymidine incorporation assay was performed in duplicate on three serial dilutions of the Sephadex G-50 postvoid volume pool. A competitive protein binding assay (12), using partially purified rat binding protein, was also used to measure IGF activity in the Sephadex G-50 postvoid volume pool. The assay was performed in duplicate on serial dilutions of the Sephadex G-50 postvoid pool, and the amount of MSA in the sample was determined from a standard curve generated by serial dilutions of MSA II. MSA was measured in the Sephadex G-50 postvoid volume pool by an MSA radioimmunoassay using ^{125}I-MSA-III-2 and MSA II standard (10, 11). The radioimmunoassay was performed in duplicate on serial dilutions of the sample.

Determination of binding capacity by Scatchard analysis. The Sephadex G-50 void volume fractions containing stripped binding protein were pooled, lyophilized, and redissolved in 0.05 M NH_4HCO_3, pH 8.0. The amount of stripped binding protein that produced half-maximal binding of ^{125}I-MSA was used for Scatchard analysis of MSA binding (18). An MSA dose-response curve was generated using a preparation of MSA II polypeptides. Incubation was at 4°C for 18 h. Bound MSA was separated from free MSA using charcoal that had been activated by incubation for 4–7 d at 4°C in PBS containing 20 mg of fatty acid-free BSA/ml (12). Scatchard plots were analyzed by linear regression (HP-65 Stat Pac 1, Hewlett-Packard Co., Palo Alto, CA).

RESULTS

^{125}I-MSA binding to fetal and 40-d-old rat sera. When ^{125}I-MSA was incubated with serum from 40-d-

old rats and gel filtered on Sephadex G-200 in 0.05 M NH_4HCO_3, pH 8.0, radioactivity was found in five peaks designated I, II, III, ^{125}I-MSA, and ^{125}I (Fig. 1, bottom panel). The addition of excess unlabeled MSA (1.25 μg/ml) to the incubation mixture before chromatography demonstrated that specific ^{125}I-MSA binding was confined to peak II, eluting in the gamma globulin-size region of the column, and peak III, eluting just after albumin as reported previously (13). The small amount of binding in peak I was nonspecific.

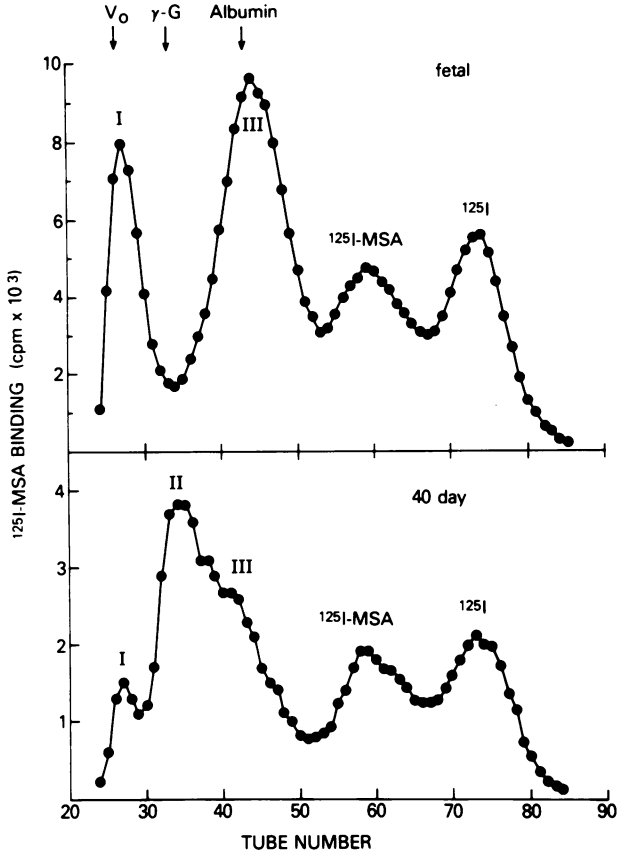

FIGURE 1 Chromatography of sera from fetal (19-d) and 40-d-old rats on Sephadex G-200, in 0.05 M NH_4HCO_3, pH 8.0. Top panel. ^{125}I-MSA-II-1 (2.5×10^5 cpm) was incubated with 1 ml of fetal rat serum for 3 h at 20°C before chromatography; the elution profile of radioactivity is shown. The exclusion volume (Vo) and the elution position of gamma globulin (γ-G), albumin, free MSA (^{125}I-MSA), and free iodide (^{125}I) are labeled. Identical elution profiles were obtained with three other fetal sera. Bottom panel. ^{125}I-MSA-II-1 (1.0×10^5 cpm) was incubated with 1 ml of serum from 40-d-old rats before chromatography; the elution profile of radioactivity is shown. The experiment was repeated two times with identical results. (When ^{125}I-MSA-II-1 was gel filtered without serum, the ^{125}I peak was of similar magnitude, that is, the ^{125}I does not represent degradation of ^{125}I-MSA-II-1 by serum.)

By contrast, when ^{125}I-MSA was incubated with 19-d fetal rat serum and gel filtered on Sephadex G-200 in 0.05 M NH_4HCO_3, pH 8.0, radioactivity was found in four peaks designated I, III, ^{125}I-MSA, and ^{125}I; ^{125}I-MSA binding was absent from the peak II region (Fig. 1, top panel). Addition of unlabeled MSA to the mixture before incubation and gel filtration demonstrated that specific ^{125}I-MSA binding was confined to peak I, eluting in the void volume and peak III, eluting just after albumin (Fig. 2).

The size distribution of IGF activity in fetal rat serum. To determine the location of endogenous IGF activity in the Sephadex G-200 column fractions, fractions comprising peak I, II (estimated from the ^{125}I-MSA binding profile for 40-d-old serum), III, and free IGF were pooled separately and gel filtered on Sephadex G-50 in 1 M acetic acid to dissociate and separate the binding protein from endogenous IGF activity. IGF activity was measured on the postvoid pool from Sephadex G-50 gel filtration by a bioassay ([^{3}H]-

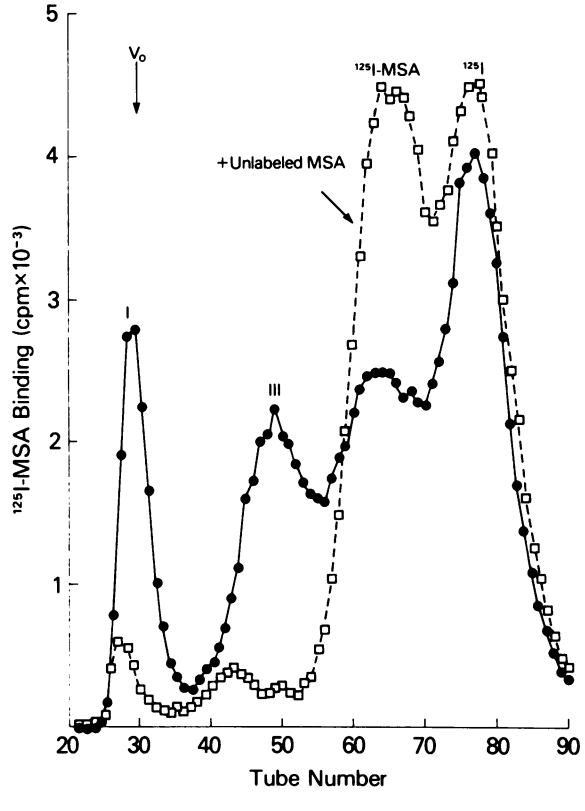

FIGURE 2 Chromatography of serum from fetal (21-d) rats on Sephadex G-200, in 0.05 M NH_4HCO_3, pH 8.0. ^{125}I-MSA-II-1 (1.25×10^5 cpm) was incubated with 0.1 ml of fetal serum with (□) or without (●) 5 μg of unlabeled MSA II, for 3 h at 20°C before chromatography; the elution profile of radioactivity is shown.

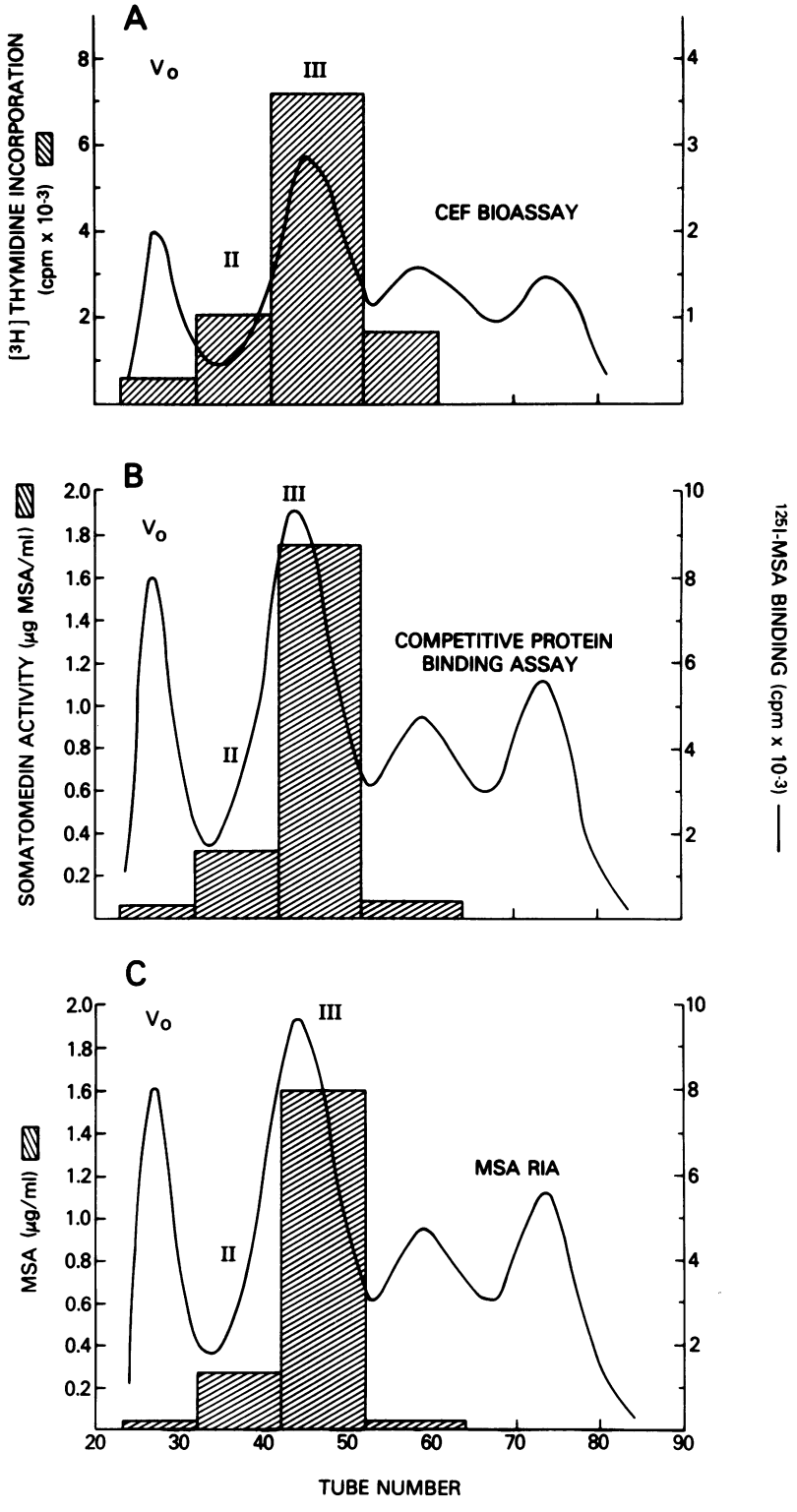

FIGURE 3 The size distribution of IGF activity in Sephadex G-200 pools of fetal rat serum (1 ml). In panels A, B, and C the elution profile of ^{125}I-MSA-II-1 binding (——) is reproduced. Pools representing the exclusion volume (Vo), peak II, peak III, and free MSA were made, and

thymidine incorporation into DNA in chick embryo fibroblasts [17]) and a competitive protein binding assay using rat serum binding protein (12). Human SM-C/IGF-I, IGF-II, and SM-A are active in the bioassay and competitive protein binding assay (4, 6, 19). Rat somatomedin is also measured by the bioassay (20) and a preparation of rat somatomedin (provided by W. H. Daughaday) having a biologic potency of 42 mU IGF-I/mg protein was 40% as potent as MSA II in the competitive protein binding assay.[2] Thus, these assays measure both MSA and other IGF; MSA was measured by a specific radioimmunoassay (10).

Fig. 3 illustrates the distribution of endogenous IGF activity and MSA determined by the three assays. IGF activity as measured by the chick embryo fibroblast bioassay is shown in panel A and activity measured by the competitive protein binding assay is shown in panel B. MSA levels by radioimmunoassay are shown in panel C. In contrast to the distribution of IGF activity in normal rat serum, which is predominantly in peak II (12), all the assays demonstrated that in fetal rat serum IGF activity was associated primarily with peak III, the albumin-sized binding protein. The total amount of IGF activity or MSA in the G-200 pools as measured by the competitive protein binding assay or the radioimmunoassay is the same as the amounts measured in whole serum that was gel filtered directly on Sephadex G-50 without prior Sephadex G-200 gel filtration (data not shown). In addition, the radioimmunoassay results in panel C indicate that the amount of free MSA in fetal rat serum is <2.5% of the total MSA.

Time dependence of MSA binding to peak III and peak II binding proteins. Preliminary to performing Scatchard analysis of MSA binding to stripped binding protein, the time-course of ^{125}I-MSA specific binding was examined to determine equilibrium conditions. Sephadex G-200 column pools from fetal peak III and 40-d peak II binding protein regions were chromatographed on Sephadex G-50, in 1 M acetic acid, to remove the endogenous IGF. The stripped binding proteins were assayed for specific ^{125}I-MSA binding activity after various periods of incubation at 4°C (Fig. 4). Equilibrium was reached for both peak III and peak II binding proteins after 12-h incubation.

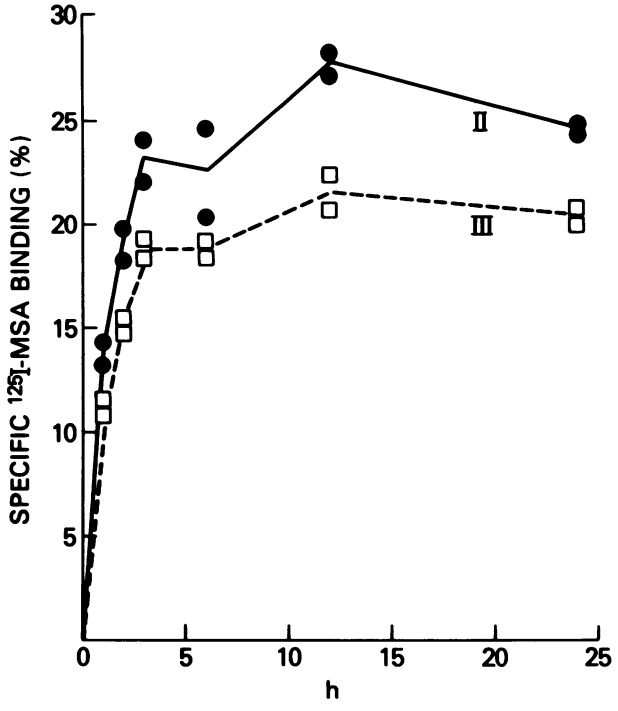

FIGURE 4 Time dependence of ^{125}I-MSA binding to peak III (□) and peak II (●) binding proteins. Sephadex G-200 column pools from fetal (21-d) peak III and 40-d peak II regions were gel filtered on Sephadex G-50, 1 M acetic acid to dissociate and separate endogenous IGF from binding protein. The Sephadex G-50 void volume pool was lyophilized and dissolved in 10 ml of 0.05 M NH_4HCO_3, pH 8.0, and 100-μl aliquots were incubated with ^{125}I-MSA-II-1 (20,000 cpm) for the indicated times at 4°C in a total volume of 0.4 ml with Dulbecco's PBS containing 0.25% BSA. Bound tracer was separated from free by BSA-treated charcoal. Nonspecific binding was determined by including unlabeled MSA II (1 μg/ml) in duplicate tubes.

The size distribution of MSA binding capacity in fetal and adult rat serum. To determine whether the

[2] White, R. M. Unpublished observations.

each pool was chromatographed on G-50 Sephadex in 1 M acetic acid to dissociate and separate the binding protein from the IGF activity. Stripped binding protein was found in the void volume of G-50 and IGF activity was in the postvoid region. Panel A. The distribution of IGF activity as measured by incorporation of [^{3}H]thymidine into DNA of chick embryo fibroblasts is shown. The data represent stimulation above control dishes containing medium alone. Panel B. The distribution of IGF activity as measured by a competitive protein binding assay, using partially purified rat serum binding protein, is shown. The data are expressed as micrograms MSA per milliliter based on the volume of serum chromatographed. Panel C. MSA levels were measured by a specific radioimmunoassay. The data are expressed in micrograms per milliliter based on the volume of serum chromatographed. The experiments in panels A, B, and C were repeated with another fetal serum sample, with identical results.

location of the binding protein in the Sephadex G-200 column fractions corresponded to the distribution of IGF activity (Fig. 3), binding capacity measurements were made on preparations of stripped binding protein that had been generated by gel filtration of Sephadex G-200 column pools on Sephadex G-50 in 1 M acetic acid. Before Scatchard analysis was performed, increasing amounts of the stripped binding protein preparations were incubated with a fixed amount of ^{125}I-MSA-II-1. This is shown for peak II (40-d) and peak III (21-d fetal) binding protein preparations in Fig. 5A. Based on these titration curves, aliquots of binding protein preparations that gave approximately half-maximal binding were chosen for generation of MSA dose-response curves shown in Fig. 5B. Scatchard analysis was performed on the dose-response data (Fig. 5C). Scatchard analysis of MSA binding to the stripped protein of fetal serum (Fig. 6) showed that MSA binding capacity was predominantly in the peak III region similar to the distribution of somatomedin activity shown in Fig. 3. In Fig. 7 we determined the location of the binding protein in adult rat serum (120 d). MSA binding capacity was located in peak II (lower panel). Location of IGF activity as measured by chick embryo fibroblast bioassay (top panel) confirmed the previous finding that somatomedin activity in adult rat serum was predominantly in peak II (12).

The age dependence of the size distribution of MSA and IGF activity in rat serum. Fetal, neonatal, and adult rat sera were incubated with ^{125}I-MSA and then chromatographed on Sephadex G-200, in 0.05 M NH_4HCO_3, pH 8 (Fig. 8). Radioactivity profiles for

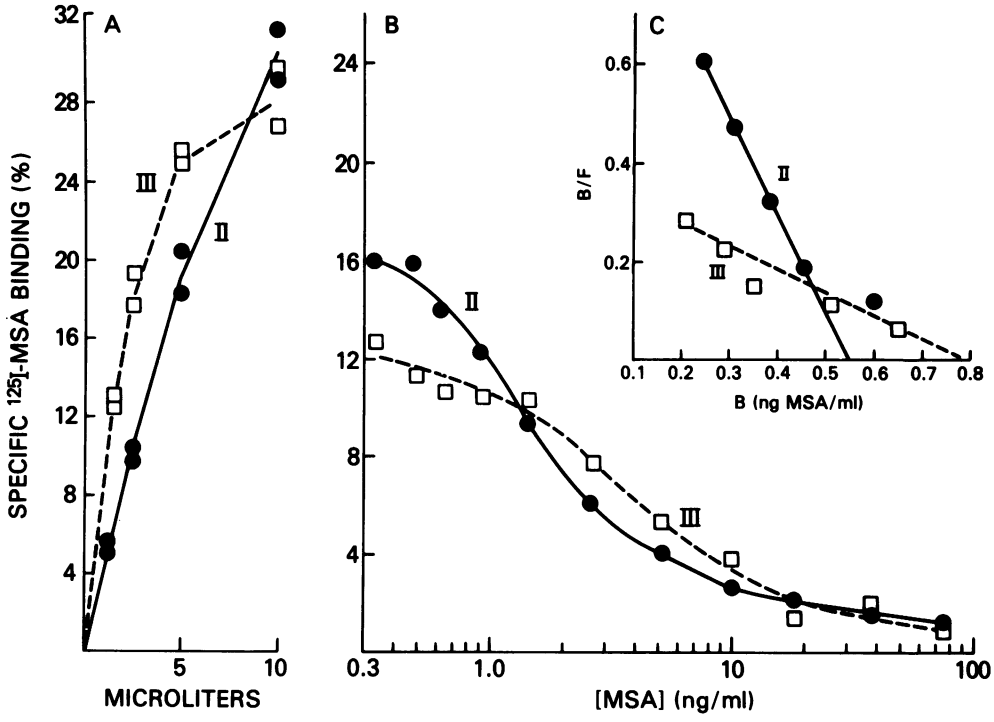

FIGURE 5 Scatchard analysis of ^{125}I-MSA binding to peak III (□) and peak II (●) binding proteins. A. Aliquots of the Sephadex G-50 void volume pools described in Fig. 4 were incubated with ^{125}I-MSA-II-1 (18,500 cpm) for 18 h at 4°C. The binding assay is described in Fig. 4. The amount of binding protein added (abscissa) is expressed as the volume of serum from which the binding protein preparation was derived (100 λ of the Sephadex G-50 void volume pool in NH_4HCO_3, pH 8.0 represents 10 μl of serum). B. Serial dilutions of MSA-II (abscissa) were incubated together with ^{125}I-MSA-II-1 (34,500 cpm) and peak II (2.5 μl serum equivalent) or peak III (1.25 μl serum equivalent) stripped binding protein prepared by Sephadex G-50 gel filtration described in Fig. 4. Incubation was for 18 h at 4°C. Details of the binding assay are described in Fig. 4. C. The MSA binding data from panel B was analyzed according to the method of Scatchard. Only the data between 20 and 80% maximum specific binding were analyzed. Maximum binding of the MSA tracer preparation was determined by incubating a constant amount of ^{125}I-MSA-II-1 (34,500 cpm) with increasing concentrations of rat serum. Maximum binding was 37% and was used in the calculation of B and B/F, that is, percent specific binding values (based on total tracer input) in panel B were multiplied by 100/37.

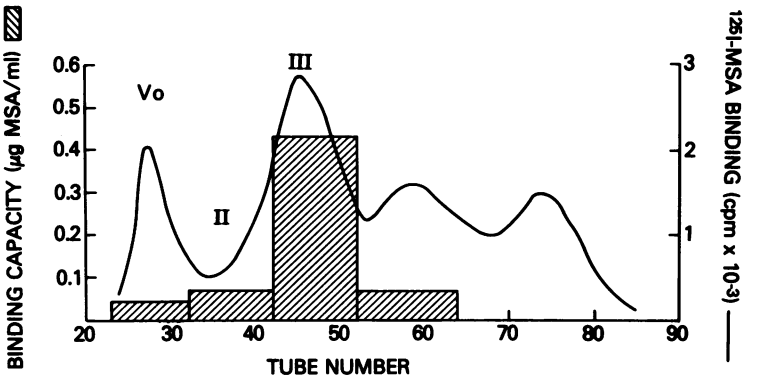

FIGURE 6 The size distribution of MSA binding capacity in fetal rat serum. The elution profile of ^{125}I-MSA-II-1 binding (——) is redrawn from Fig. 1 for fetal serum. Stripped binding protein preparations from the exclusion volumes of the Sephadex G-50 gel filtrations described in Fig. 2 were used. Binding capacity was determined by Scatchard analyses of MSA binding to stripped binding protein as illustrated in Fig. 5. Binding capacities are expressed as micrograms MSA per milliliter based on the volume of rat serum applied to the column. The experiment was repeated with another fetal serum sample, with identical results.

fetal, 3-, 5-, 10-, and 15-d rat sera were essentially identical to the profile in Fig. 1, top panel. The radioactivity profiles for 25-, 30-, and 40-d rat sera were similar to the profile in Fig. 1, bottom panel. The radioactivity profile for 20-d-old rat serum first showed the appearance of peak II binding with equivalent binding present in peak III.

Fractions corresponding to peak II and peak III were pooled separately and then chromatographed on Sephadex G-50, in 1 M acetic acid, to dissociate and separate the endogenous IGF activity and MSA from the binding proteins; three fractions between peak II and peak III were eliminated from the pools in order to decrease the chance of overlap of activity between peak II and peak III. The IGF activity in the Sephadex G-50 postvoid pools was assayed by the chick embryo fibroblast bioassay; MSA was measured by radioimmunoassay. MSA was located primarily in peak III, was high in fetal serum, and gradually declined after birth (Fig. 9, top panel). This pattern of MSA levels in peak III is similar to the previously reported pattern of MSA concentrations measured in rat sera that had not been separated into peak II and peak III by Sephadex G-200 gel filtration (11). Very low levels of MSA were detected in peak II (Fig. 9, top panel) of fetal and neonatal rat sera. The data show that MSA is essentially restricted to the peak III binding protein both in fetal rat serum and in the neonatal rat until the age of 20 d when MSA declines to very low levels.

The chick embryo fibroblast bioassay performed on the same samples (Fig. 9, bottom panel) showed that IGF activity in peak III followed a pattern similar to MSA by radioimmunoassay; high in fetal sera followed by a decline in activity in neonatal samples. By contrast, levels of bioactivity in peak II were low in early neonatal life, peaked at 5 d, declined to a nadir at 20 d, and then increased again to a plateau in the older animal. The increase in bioactivity in peak II after 20 d can be explained by the expected appearance of growth hormone-dependent somatomedins and binding protein in peak II (12, 13).

Characterization of the peak I binding activity in fetal rat serum. Although MSA in fetal rat serum is found associated with peak III, the experiment in Fig. 2 suggested that ^{125}I-MSA binding to peak I is also specific. This is in contrast to adult rat serum where peak I ^{125}I-MSA binding is nonspecific (13). To confirm that the ^{125}I-MSA binding in peak I was specific, we directly examined aliquots of the Sephadex G-200 column fractions in the peak I region for ability to specifically bind ^{125}I-MSA (Fig. 10). Peak I column fractions from gel filtrations of 19- and 21-d fetal sera showed specific binding of ^{125}I-MSA, confirming the result of Fig. 2 where specific binding had been assessed by incubation of ^{125}I-MSA with the serum sample before gel filtration. An MSA dose-response curve indicated high affinity binding with half-maximal competition for ^{125}I-MSA-II-1 binding at 6 ng/ml; insulin did not complete at 10 μg/ml (data not shown). Scatchard analysis indicated that the binding capacity of the peak I binding component was 130 ng MSA-II/ml of serum (data not shown). Apparently the peak I binding activity is inactivated by acid and/or lyophilization since very low binding capacity was measured in the peak I region in the experiment in Fig. 6. We gel filtered the peak I binding activity on Sepharose-

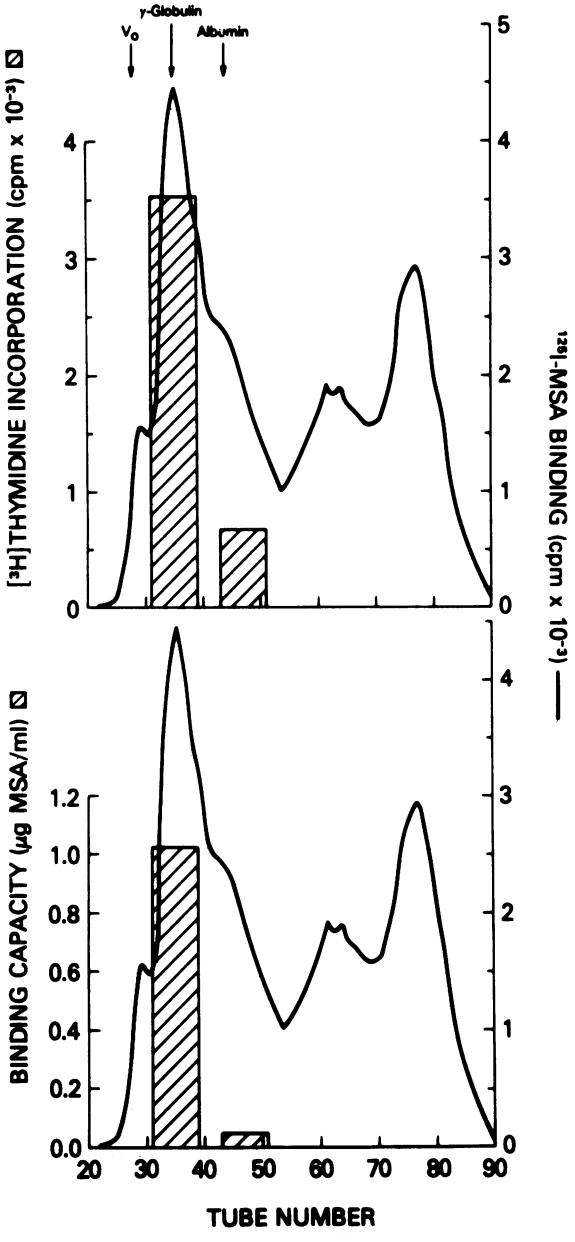

FIGURE 7 The size distribution of IGF activity and MSA binding capacity in 120-d-old rat serum. 1 ml of serum was gel filtered on Sephadex G-200. The elution profile of ^{125}I-MSA-II-1 binding is drawn for reference. Binding protein was freed of IGF activity by Sephadex G-50 chromatography in 1 M acetic acid as described in Fig. 3. Studies were only performed on peak II and peak III pools comprising a major area of the peaks. Top panel. The distribution of IGF activity as measured by incorporation of [^{3}H]thymidine in DNA of chick embryo fibroblasts is shown. The data represent the stimulation over control (counts per minute per dish). The void volume and elution volume of gamma globulin and albumin are labeled. Bottom panel. Binding capacity was determined by Scatchard analysis of MSA binding to stripped 6B in Dulbecco's PBS containing 0.1% Triton X-100 (Fig. 11). This gel filtration system has been used to characterize the size of receptors solubilized from membranes including the SM-C/IGF-I receptor from human placental membranes (21). We located the ^{125}I-MSA binding component in the column fractions by incubating ^{125}I-MSA with the Sephadex G-200 peak I pool before gel filtration and by directly measuring specific ^{125}I-MSA binding to aliquots from individual column fractions as was done in the experiment shown in Fig. 10. The peak I MSA binding activity behaves as a single component with a distribution coefficient (K_d) value of 0.43, compared with K_d = 0.37 for an MSA/IGF-II receptor purified from rat chondrosarcoma cells and analyzed on the same column.[3]

DISCUSSION

We recently reported that MSA levels determined by radioimmunoassay ranged from 1.8 to 4.4 μg/ml in fetal rat sera and were 20- to 100-fold higher than in maternal sera (11). Following birth, the MSA serum concentration gradually declined until by day 25 of extrauterine life the MSA concentration approached maternal levels. MSA levels in fetal serum measured by a rat liver membrane radioreceptor assay, a competitive protein binding assay, and a [^{3}H]thymidine incorporation bioassay using chick embryo fibroblasts all agreed with the high values obtained by the specific MSA radioimmunoassay (11), suggesting that most or all of the IGF activity in fetal rat serum is accounted for by MSA. Using a rat placenta radioreceptor assay specific for IGF-II, Daughaday et al. (22) have reported elevated levels of IGF-II in fetal rat serum. Since amino acid sequence data for one of the MSA species (7) shows extensive sequence homology with human IGF-II, the report of Daughaday et al. (22) agrees with our MSA radioimmunoassay results (11). These findings led us to propose that MSA is a fetal growth factor in the rat and that another IGF assumes importance as MSA levels decline (11).

The present study demonstrates that during late gestation and the first 10–15 d of extrauterine life, MSA is associated with a binding protein approximately the same size as albumin (peak III); the growth hormone-dependent, gamma globulin-size somatomedin binding protein (peak II) is absent from fetal

[3] August, G., and P. Nissley. Unpublished observations.

binding protein. Binding capacities are expressed as micrograms MSA per milliliter based on the volume of serum added to the column.

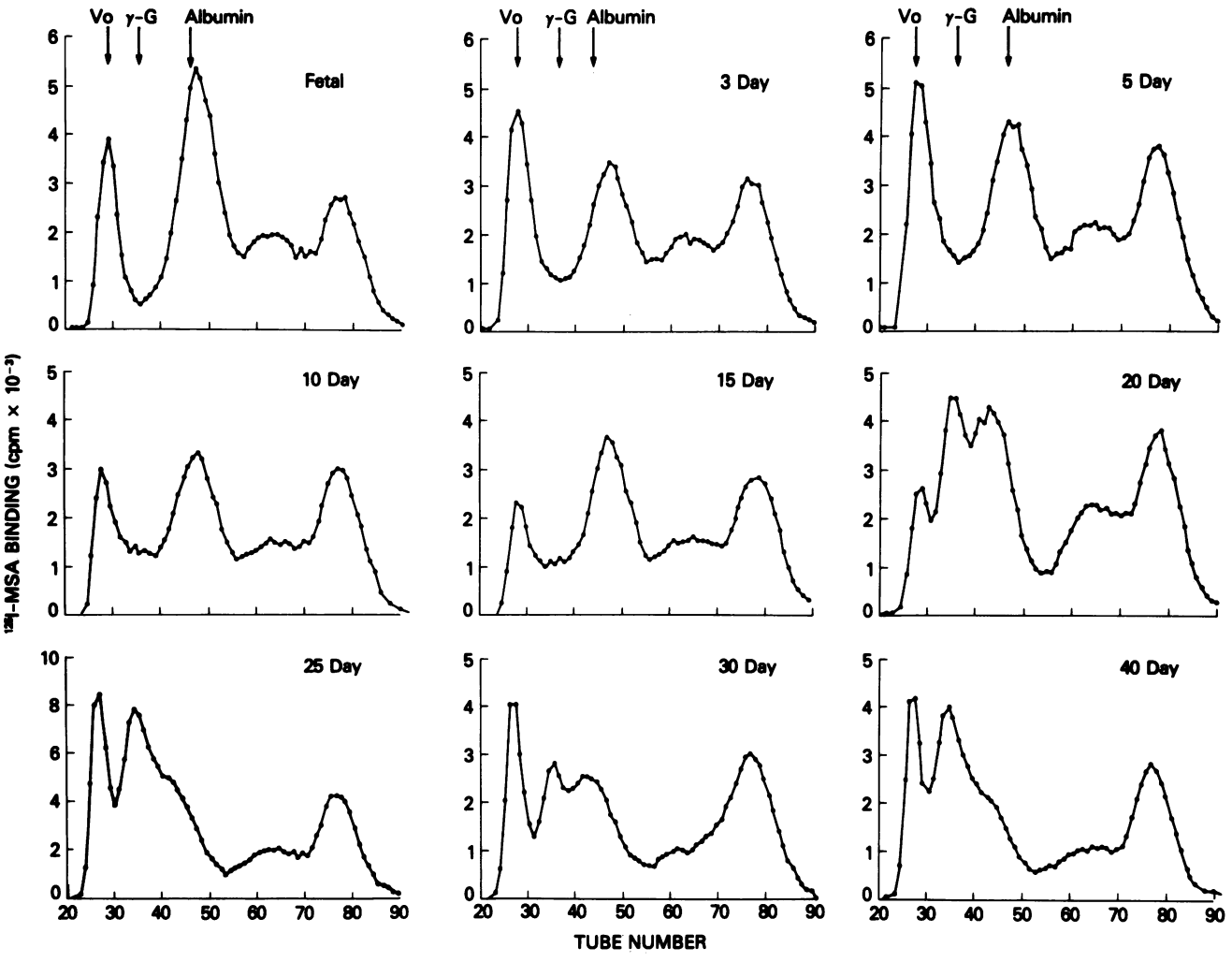

FIGURE 8 The age dependence of the Sephadex G-200 ^{125}I-MSA-II-1 binding profile. ^{125}I-MSA-II-1 was incubated with 1 ml of rat sera for 3 h at 20°C before chromatography on Sephadex G-200, in 0.05 M NH_4HCO_3, as in Fig. 1.

rat serum. Thus, MSA measured by radioimmunoassay was found near the albumin region of the Sephadex G-200 column; there was very little MSA in the column void volume, the gamma globulin region, or in the fractions corresponding to the elution position of free ^{125}I-MSA. The MSA radioimmunoassay results were confirmed by measurements of IGF activity using a competitive protein binding assay and a chick embryo fibroblast bioassay. These two assays would be expected to be less specific than the MSA radioimmunoassay when used on rat serum since all the human IGF (IGF-I/SM-C, IGF-II, and SM-A) as well as rat somatomedin are detected by both the competitive binding protein assay and the chick embryo fibroblast bioassay (4, 6, 19, 20).[2] Thus, confirmation of the MSA radioimmunoassay results with these two other assays shows that a rat IGF other than MSA is not present in the gamma globulin region following Sephadex G-200 gel filtration of fetal rat sera.

Scatchard analysis of MSA binding to fetal binding protein(s) that had been freed of endogenous IGF activity demonstrated that most of MSA binding capacity was also located in the pool of fractions eluting near albumin. The binding capacity in the peak III region was lower than the amount of MSA measured by radioimmunoassay, suggesting that there was some loss of binding protein during the Sephadex gel filtrations and lyophilization steps.

Although most of the MSA in fetal rat serum was found associated with the peak III binding protein,

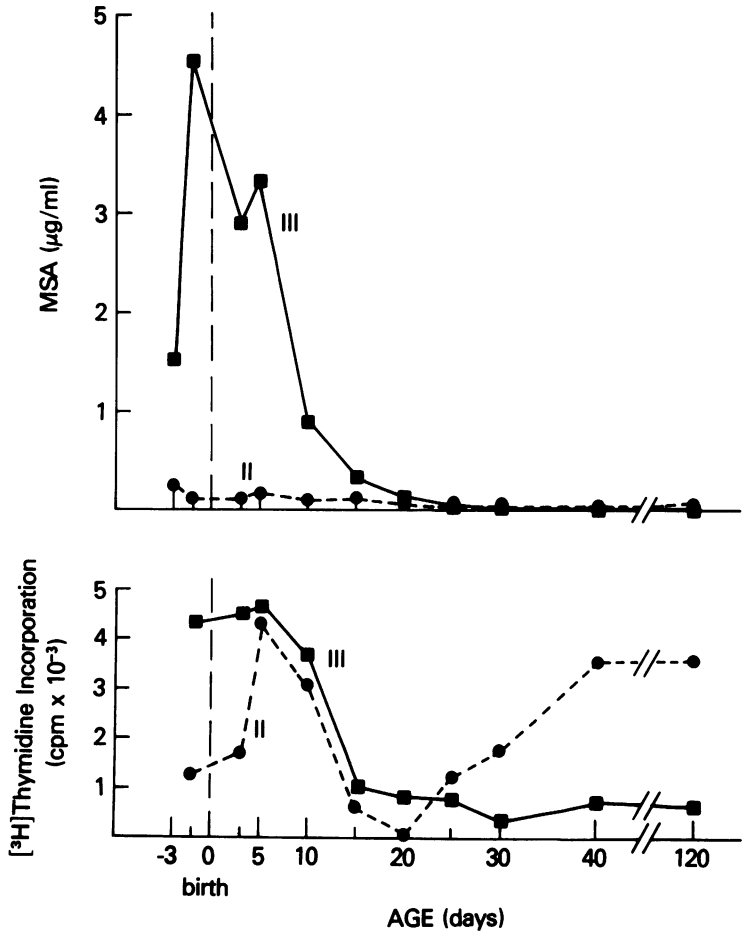

FIGURE 9 The age dependence and size distribution of MSA and IGF activity in rat serum. 1 ml serum samples of fetal, neonatal, and adult rats were chromatographed on Sephadex G-200, in 0.05 M NH_4HCO_3, pH 8. Peak II and peak III fractions were pooled based on the ^{125}I-MSA elution profile of each serum sample. The binding protein was stripped from the IGF activity by Sephadex G-50 gel filtration, in 1 M acetic acid. The postvoid volume pool from Sephadex G-50 was divided into two equal parts and used in the two assays. Top panel. Using a specific radioimmunoassay for MSA, peak II (●) and peak III (■) associated MSA were measured. MSA levels are expressed in micrograms per milliliter based on the volume of serum applied to the column. All samples were analyzed in the same assay. Bottom panel. IGF activity as measured by incorporation of [^{3}H]thymidine into DNA of chick embryo fibroblasts was examined in peak II (●) and peak III (■). The results represent stimulation above control dishes containing medium alone and are the average of duplicate determinations.

there was a component in the Sephadex G-200 void volume region (peak I) capable of specifically binding ^{125}I-MSA. This peak I binding activity behaved as a single component when gel filtered on Sepharose-6B. Interestingly, the elution volume on Sepharose-6B (K_d = 0.43) of the peak I binding component was only slightly greater than the elution volume (K_d = 0.37) of an MSA/IGF-II receptor purified from rat chondrosarcoma cells,[3] raising the possibility that the peak I component is a large fragment of the MSA/IGF-II plasma membrane receptor. Although the binding capacity of the peak I component (130 ng MSA/ml of serum) is lower than the binding capacity of the peak III binding protein (430 ng MSA/ml, Fig. 6), it is surprising that very little of the endogenous MSA is found associated with the peak I component. A possible explanation would be that if MSA were secreted from tissues of origin as an MSA-peak III complex, during

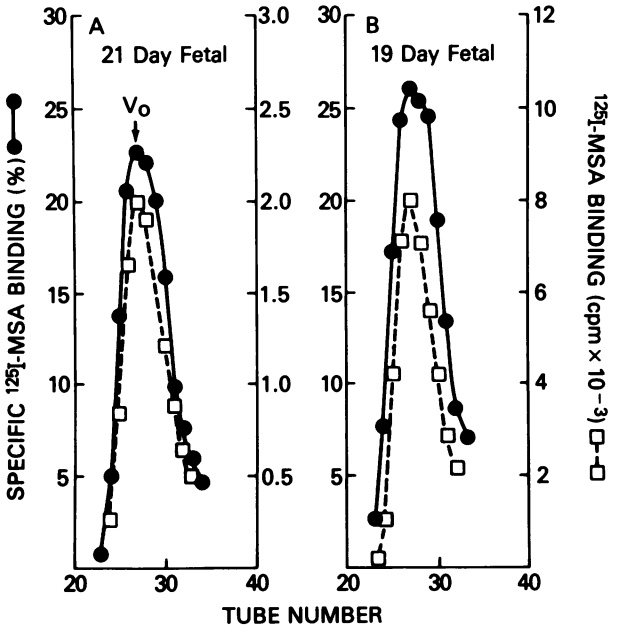

FIGURE 10 Measurement of specific ^{125}I-MSA binding activity of the fetal (19 and 21 d) peak I binding component. 1 ml of serum was incubated with ^{125}I-MSA-II-1 and gel filtered on Sephadex G-200 as described in Methods and as shown in Fig. 1. ^{125}I-MSA binding in the void volume fractions is shown (□). Specific ^{125}I-MSA binding activity (●) was also measured on 100-μl aliquots from each fraction following gel filtration. The binding assay is described in the legend of Fig. 4. Nonspecific binding was determined by including unlabeled MSA (1 μg/ml) in duplicate incubations.

the life time of MSA in the circulation there might be insufficient time for equilibration of MSA with the peak I component.

By contrast to the findings in fetal rat serum, when the size distribution of endogenous somatomedin activity and binding capacity were examined in adult rat serum, somatomedin activity and binding capacity were found in the gamma globulin region of the Sephadex G-200 column fractions. The gamma globulin-size binding protein was previously demonstrated to be growth hormone dependent (13). The size of the fetal binding protein is similar to the size of the peak III binding protein previously demonstrated in hypophysectomized adult rat serum and as a minor component in normal adult rat serum (13). Although the fetal binding protein and the peak III adult binding protein may be the same molecule, we have no direct evidence to support this possibility.

When we examined the developmental pattern of the size distribution of endogenous MSA and somatomedin activity in rat sera, we found that MSA by radioimmunoassay was always found associated with the albumin size, fetal binding protein (peak III). The very low levels of MSA detected in the pool from the gamma globulin-size region (peak II) of the Sephadex G-200 column fractions could have originated from peak III due to incomplete resolution of peak II and peak III by Sephadex G-200 gel filtrations.

The developmental pattern of the size distribution of IGF activity, as measured by the chick embryo fibroblast bioassay, showed increasing amounts of somatomedin activity associated with the gamma globulin-size binding protein following 20 d of extrauterine life. This finding agrees with the data obtained on whole rat serum using the rat costal cartilage sulfate incorporation assay (23) or the SM-A radioimmunoassay (24) where somatomedin levels were low in fetal blood and then gradually increased to higher levels in the adult. By contrast with these reports, Daughaday et al. (22) reported that IGF-I/SM-C levels are not as low in fetal rat serum using a specific IGF-I/SM-C radioimmunoassay performed on acid ethanol extracts of serum. There is general agreement, however, that IGF activity levels are substantial in the adult animal and our data show that this activity is associated with the gamma globulin-size binding protein. The MSA radioimmunoassay results clearly show that the IGF activity associated with peak II is not MSA. One candidate for this peak II-associated IGF, which is found in the older rat, is a rat somatomedin purified from sera of rats bearing a growth hormone-secreting tumor (8). This rat somatomedin has been shown not to cross-react in the MSA radioimmunoassay (10). In addition, the primary structure of one of the MSA species indicates extensive homology with IGF-II (7) whereas the sequence of 26 of the first 29 amino terminal residues of rat somatomedin is identical with IGF-I (9).

The increase of peak II-associated IGF activity noted at day 5 was unexpected. The previous reports by Stuart et al. (23) and Sara et al. (24) using the rat costal cartilage bioassay and the SM-A radioimmunoassay, respectively, failed to detect a significant increase in somatomedin activity at day 5. Similarly, we found that the peak II-associated IGF activity at day 5 was only weakly reactive in a competitive protein binding assay. Thus this peak II-associated material at day 5 may only be detected by the chick embryo fibroblast bioassay. The only other IGF-like property of this activity is that it can be generated from gamma globulin-size material by acid treatment and is the size of somatomedins by acid gel filtration on Sephadex G-50.

Draznin et al. (25) reported that IGF binding protein was very low in fetal rat serum compared with levels in the adult animal, whereas the results in this paper show that fetal peak III binding capacity (0.43 μg

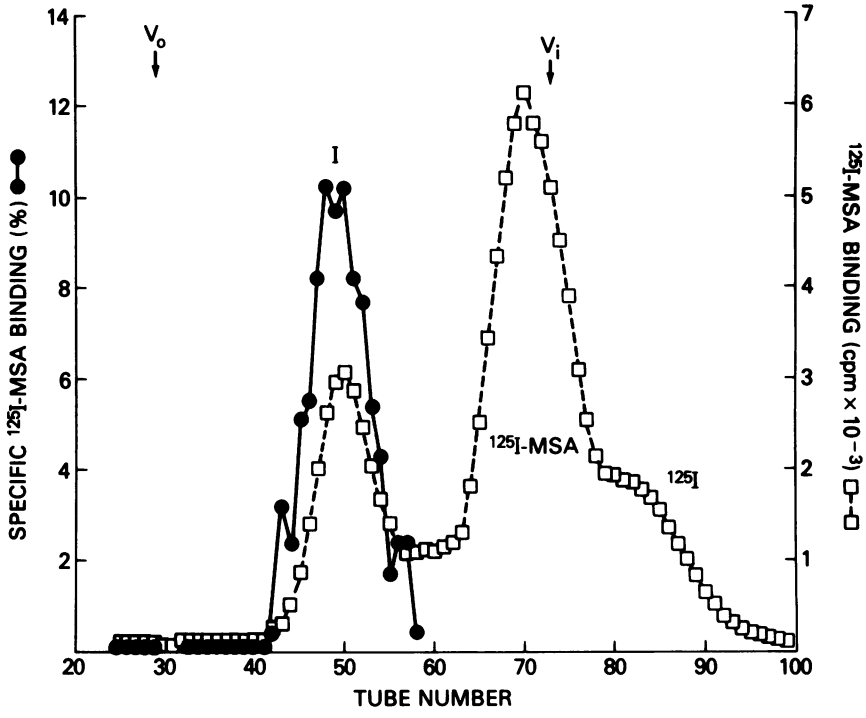

FIGURE 11 Gel filtration of the fetal peak I binding component on Sepharose-6B. Void volume fractions from Sephadex G-200 gel filtration of 1 ml of fetal (21-d) rat serum were pooled (total volume 12.5 ml). 1 ml of the pool was incubated with ^{125}I-MSA-II-1 (200,000 cpm) for 3 h at 22°C and gel filtered on Sepharose-6B in Dulbecco's PBS with 0.1% Triton X-100 (Methods). ^{125}I-MSA binding in each fraction is shown (□). 1 ml from the Sephadex G-200 void volume was also gel filtered without prior incubation with ^{125}I-MSA and specific ^{125}I-MSA binding activity was measured on a 200-μl aliquot from each column fraction as described in Methods (●). The exclusion volume (V_0) and inclusion volume (V_i) were determined with blue dextran and dinitrophenyl-alanine, respectively. The distribution coefficient (K_d) is calculated as follows: $K_d = (V_e - V_0)/V_i - V_0)$ where V_e is the elution volume of the binding component.

MSA/ml) is not much lower than the binding capacity of peak II in the adult animal (1.0 μg MSA/ml). Draznin et al. measured binding activity and expressed the results in terms of a partially purified human binding protein standard, whereas we measured binding capacity by Scatchard analysis. If the binding affinity of the fetal peak III binding protein is low, as suggested by the results in Fig. 5C, then the measurements of Draznin et al. on the fetal sera may be falsely low when expressed in terms of a binding protein standard with higher binding affinity.

It is of interest that the size of the MSA binding protein in fetal rat serum is the same as the size of the binding protein in serum-free medium conditioned by the BRL-3A2 and BRL-3A rat liver cell lines (12, 26). Also, Fennoy et al. (27) recently reported that the MSA binding protein synthesized by fetal rat liver explants in organ culture is also albumin sized. These fetal liver explants previously had been shown to produce polypeptides indistinguishable from MSA (28). D'Ercole and Underwood (29) recently demonstrated that in fetal mouse serum, SM activity, as measured by a radioimmunoassay for SM-C, was also exclusively associated with an albumin-size binding protein. However, in contrast to the developmental pattern of serum MSA in the rat, these workers found that the immunoreactive SM-C activity was low in fetal mouse blood and gradually increased during extrauterine life. Since none of the mouse somatomedins have been purified and characterized, it is not known which of the mouse somatomedins is being measured by this radioimmunoassay for human SM-C. Therefore, it is possible that in fetal mouse blood the SM-C radioimmunoassay is actually measuring mouse MSA.

D'Ercole et al. (30) recently examined the size distribution of SM-C in human fetal blood. Before 27-wk gestation SM-C by radioimmunoassay was found in the region of the Sephacryl G-200 column eluate corresponding to a size slightly smaller than that of albumin. We have confirmed these findings on four human sera

from premature infants using a different method (31), measurement of specific ^{125}I-MSA binding activity on individual Sephadex G-200 column fractions.[2] Thus, the findings in midgestation human serum agree closely with the results in the rat and mouse.

We previously proposed that because of the developmental pattern of serum MSA levels in the rat, MSA may be a fetal growth factor and that rat somatomedin(s) assume importance at a later age (11). The findings in this paper show that accompanying this transition from fetal MSA to adult somatomedin(s), there is a profound change in the binding protein; in fetal rat serum only an albumin-size binding protein is found, while in the older rat the growth hormone-dependent, gamma globulin-size binding protein predominates.

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