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Abstract

Two fetus-specific globulins, al and a2·fetoglobulins, were detected in rat fetal serum, and the former was detected in amniotic fluid, using respective monospecific rabbit immune sera. Immunochemical distinction of these two proteins was demonstrated. By polyacrylamide gel disc electrophoresis, al-fetoglobulin was further resolved into fast and slow migrating subcomponents having a similar reactivity against the specific immune serum. The concentrations of these globulins in the serum of adult rat with experimentally induced hepatic lesions were determined by quantitative immunoprecipitin method or the Ouchterlony test using the specific antisera.

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IMMUNOLOGICAL PROPERTIES OF TWO FETUS-SPECIFIC GLOBULINS OF RAT IN EXPERIMENTALLY INDUCED HEPATIC LESIONS

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Abstract: Two fetus-specific globulins, α_1 and α_2-fetoglobulins, were detected in rat fetal serum, and the former was detected in amniotic fluid, using respective monospecific rabbit immune sera. Immunochemical distinction of these two proteins was demonstrated. By polyacrylamide gel disc electrophoresis, α_1-fetoglobulin was further resolved into fast and slow migrating subcomponents having a similar reactivity against the specific immune serum. The concentrations of these globulins in the serum of adult rat with experimentally induced hepatic lesions were determined by quantitative immunoprecipitin method or the Ouchterlony test using the specific antisera.

Our studies on isozyme patterns of key glycolytic and gluconeogenic enzymes in injured livers of both animal and man have provided evidences of a reversion of differentiated liver cells upon liver injury towards those of regenerating and fetal livers and of hepatoma (1). In addition, the hepatitis and cirrhosis patients with increased faster migrating components of liver G6PD (glucose-6-phosphate dehydrogenase, EC 1. 1. 1. 49), which were closely associated with the increased ratio of G6PD/GSH in liver (2, 3), have been shown to be characterized by relatively high levels of serum α_1-fetoprotein (2). These observations prompted us to investigate the appearance of fetus-specific proteins in sera of adult rats with experimentally induced hepatic lesions.

The present report describes the nature two fetus-specific proteins of rat as well as quantitative or semi-quantitative data of their concentrations in fetal fluids and sera from adult rats with various hepatic lesions. A method of purification of these proteins and the microheterogeneity of purified α_1-fetoglobulin will be described elsewhere (4).

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats, weighing 200-300 g at the age of at least three months and being maintained on Oriental Laboratory Chow MF, were used. Fetal rat serum was collected by decapitation of fetuses, which were obtained by cesarean section of female rats of the same strain at 15-20th

days of pregnancy. Amniotic fluids were similarly obtained from 15-18 day-old fetuses. Hepatic tumors were induced with an azo dye carcinogen, 3'-Me-DAB (3'-methyl-4-dimethylamino-azobenzene), which was included in the diet at a concentration of 0.06% (5). When the tumors became palpable following 7 months of the dye feeding, the animals were sacrificed for experiment and referred to below as DAB rats. Cadmium-poisoned rats were prepared by giving a single intraperitoneal injection of 0.1 mg of cadmium chloride ($CdCl_2$) per 100 g body weight daily for two days and served for experiment 48 hr after the first treatmert. Carbon tetrachloride (CCl_4) and thioacetamide-treated, partially hepatectomized and AH-130-transplanted rats (referred to as AH-130 rats) were prepared as described previously (6, 7). The sera obtained from at least two animals were pooled and stored at −20°C until they were used.

Disc electrophoresis: Vertical disc electrophoresis on polyacrylamide gels was performed at 4°C by the method of DAVIS (8). Proteins were stained with Amido Schwarz (9).

Immunoelectrophoresis and double-diffusion test: The immunoelectrophoresis was carried out according to the standard procedure (10) with agar and/or agarose and the agar double-diffusion test was performed as outlined by OUCHTERLONY (11).

Antisera: Three male rabbits were immunized three times at an interval of two weeks each by subcutaneous injection of 0.1 ml of pooled fetal serum (1.5 mg protein) emulsified with an equal volume of Freund's complete adjuvant. The animals were bled 8 weeks after the first injection and the serum was separated. A specific antifetal-serum-immune serum was obtained by absorbing 3 ml of the whole antifetal-serum-immune serum with 1 ml of pooled adult serum. Specific anti-α_1 and α_2-fetoglobulin sera were prepared by absorbing 4 ml of the specific antifetal-serum-immune serum with 1.3 ml of $CdCl_2$-treated rat serum and with 2 ml of amniotic fluid (see Results under "Immunological properties of antiserum against fetal serum"). Absorptions were made in a water bath at 37°C for one hour.

Titration: The concentration of α_1-fetoglobulin in the serum was quantitatively determined by the single radial immunodiffusion method of MANCINI *et al.* (12). A pooled serum collected from a litter of fetuses was used as a reference standard; the concentration of α_1-fetoglobulin in the standard serum as estimated by disc electrophoresis was 7.7 mg/ml. The concentration of α_2-fetoglobulin was determined by the Ouchterlony method, and the titer was expressed as the reciprocal of the highest dilution of the antigen giving a visible line of precipitation with the antiserum.

Materials: Freund's complete adjuvant and Noble agar were purchased from Difco Laboratories Inc. Other reagents used were purchased from the sources reported previously (6, 7, 13).

RESULTS

Disc electrophoresis of proteins in sera, ascites and amniotic fluids obtained under various experimental conditions: The most outstanding feature in protein

profiles of fetal serum and amniotic fluid was the presence of two bands located between albumin and α_1-globulin of adult serum (Fig. 1). Fast and

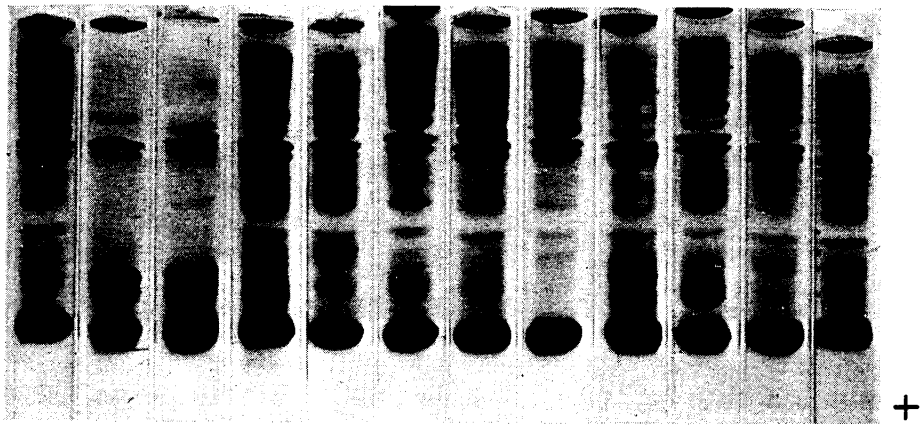

Fig. 1. Disc electrophoretic patterns of proteins in ascites, amniotic fluid and serum of experimentally treated rats. Each 2-3 μl of undiluted protein samples were put on standard gel columns and electrophoresed for 60 min at 4°C and 4 mA/tube. Samples applied on columns were as follows: 1 and 7, adult pooled serum; 2, fetal serum; 3, amniotic fluid; 4, ascites fluid obtained from AH-130 rats by peritoneal puncture; 5, $CdCl_2$-treated rat serum; 6, CCl_4-treated rat serum; 8, thioacetamide-treated rat serum; 9, DAB rat serum; 10, AH-130 rat serum; 11, partially hepatectomized rat serum (48 hr after hepatectomy); and 12, serum from sham-operated rats (48 hr after operation).

slow migrating components of the two fetal globulins had relative mobilities against bromophenol blue (BPB) as a marker, R_{BPB}, 0.58 and 0.55 and a ratio in the protein distribution of 35 : 65, respectively. These fetus-specific globulins were also demonstrated in the serum of DAB rats (R_{BPB}, 0.58 and 0.55; and protein distribution, 40 : 60, respectively). The similar globulin band in ascites and serum of AH-130 rats had slightly less R_{BPB} value of 0.57. The other one or two protein bands between albumin and α_1-globulin, which appeared to be distinct from the above described components and are present in normal adult serum, were also noted in the sera of $CdCl_2$-treated, hepatectomized, sham-operated and CCl_4-treated rats.

Immunological properties of antiserum against fetal serum: When whole antifetal-serum-immune serume was absorbed by adult serum, the resulting antiserum revealed two precipitin lines against fetal serum but not normal adult serum in the regions of α^1 and α_2-globulins (Fig. 2). The two arcs of precipitation did not merge into one another, showing a different reactivity of the globulins against the specific antifetal-serum-immune serum. These

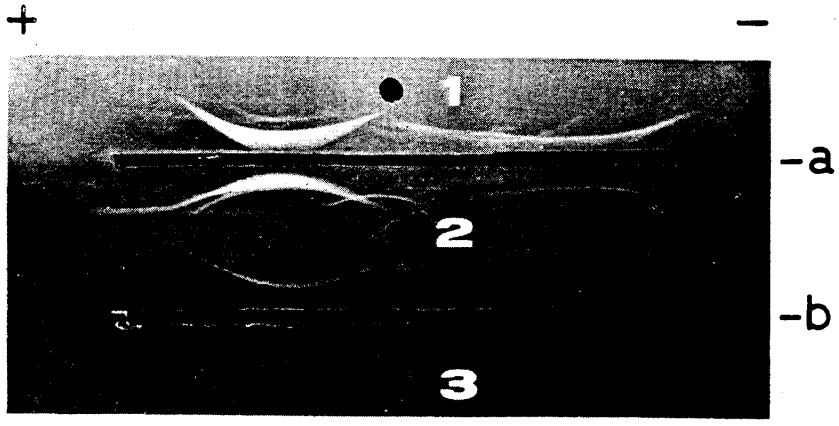

Fig. 2. Immunoelectrophoresis of α_1 and α_2-fetoglobulins from fetal serum. Wells 1 and 3 contained adult serum and Well 2 fetal serum. Electrophoresis was performed at room temperature for 100 min at 6 mA/cm and a constant voltage of 60 V on a mixture of 0.6% agarose and 0.6% Difco-Noble agar buffered at pH 8.6 with 12.5 mM barbital buffer. Trough a, whole antifetal-serum-immune serum; Trough b, specific antifetal-serum-immune serum.

fetus-specific proteins are referred to as α_1 and α_2-fetoglobulin, respectively. The arc of precipitation of α_1-fetoglobulin was located near the through containing the antiserum as compared to that of α_2-fetoglobulin (see also

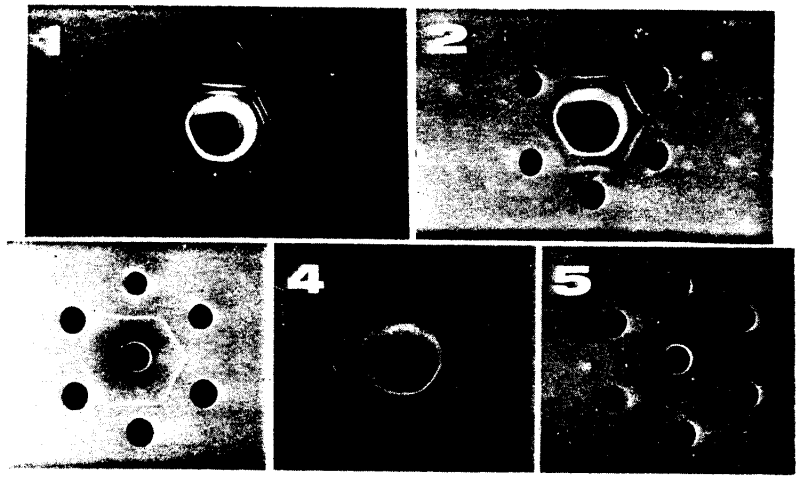

Fig. 3. Double-diffusion analyses of α_1 and α_2-fetoglobulins in fetal serum. Center wells in each plate contained fetal serum (Plates 1-4) or adult serum as control (Plate 5). Outer wells contained antisera indicated as follows (clockwise from top, undiluted, 2, 4, 8, 16 and 32 times diluted serum): 1, whole antifetal-serum-immune serum; 2 and 5, specific antifetal-serum-immune serum; 3, specific anti-α_2-fetoglobulin serum; and 4, specific anti-α_1-fetoglobulin serum.

Figs. 4 and 5). Similar observations were made on the Ouchterlony plate, in which α_1-fetoglobulin was demonstrated to be much closer to the central well containing the antiserum as compared to α_2-fetoglobulin (Fig. 3). α_2-Fetoglobulin gave a positive, specific periodic acid-Schiff reaction (14) and is therefore an α_2-glycoprotein.

Immunoelectrophoretic analyses of amniotic fluid and serum from $CdCl_2$-treated rat revealed single precipitin lines of α_1 and α_2-fetoglobulins, respectively (Fig. 4). This observation proves that the anti-α_1 and α_2-fetoglobulin

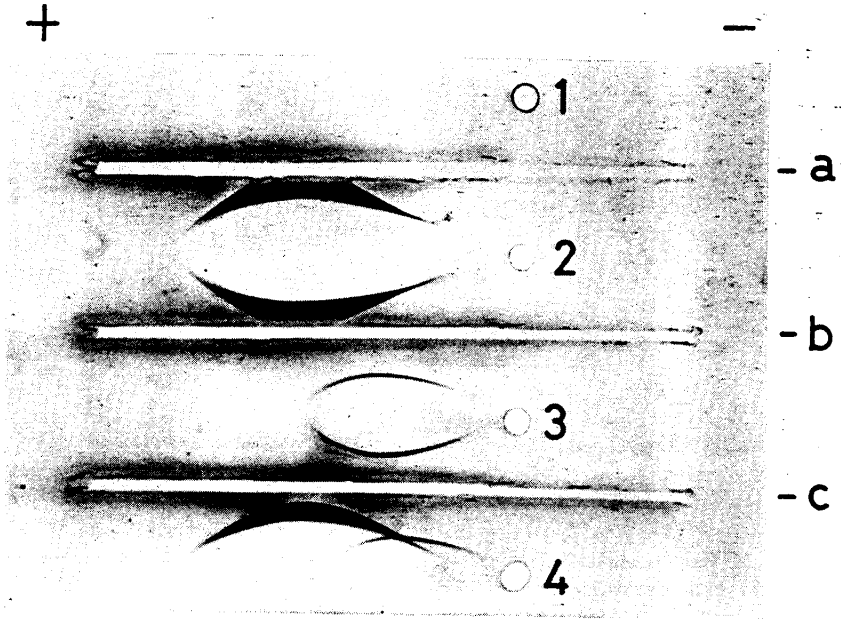

Fig. 4. Immunoelectrophoresis of α_1 and α_2-fetoglobulins in fetal serum, amniotic fluid and adult serum from $CdCl_2$-treated rat. Fifteen μl aliquots of normal adult serum (Well 1) amniotic fluid (Well 2), $CdCl_2$-treated adult rat serum (Well 3) and fetal serum (Well 4) were placed in the indicated wells and electrophoresed at room temperature for 90 min on 1.5% agarose buffered at pH 8.6 with 12.5 mM barbital buffer as described in Fig. 2. After electrophoresis, specific antifetal-serum-immune serum was poured into the upper, middle and lower troughs (a, b and c). The double-diffusion was performed at room temperature for 24 hr. The plate was repeatedly washed with saline and stained with Amido Schwarz.

sera prepared as described under Materials and Methods are monospecific. This was also demonstrated in the double-diffusion test with several antigens as indicated in Fig. 5. The identity of α_1-fetoglobulins in fetal serum, amniotic fluid and serum from DAB rat was also demonstrated by the double-diffusion analysis (Fig. 5). The serum of AH-130 rats did not react with the specific anti-α_1-fetoglobulin serum, although the protein band with an R_{BPB}

value similar to α_1-fetoglobulin was observed. In all the cases of experimental conditions tested, the sera of treated animals had α_2-fetoglobulins, which are immunologically identical as revealed by the formation of single precipitin lines against the antiserum (Fig. 5).

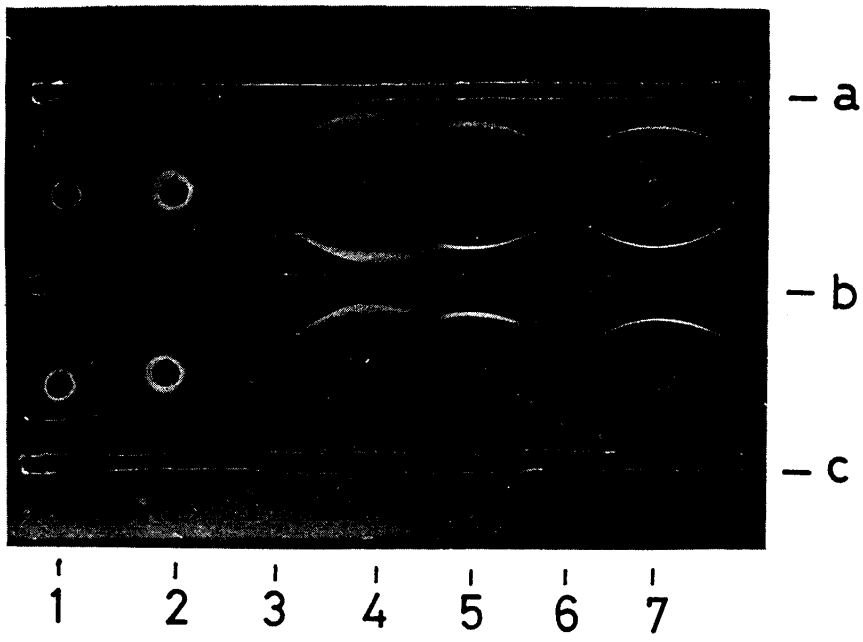

Fig. 5. Immunological identity of α_1 or α_2-fetoglobulin appeared in serum of rats under different experimental conditions. Serum or amniotic fluid applied in the upper and lower wells was diffused on a agar gel against specific anti-α_1-fetoglobulin serum (a), specific antifetal-serum-immune serum (b) and specific anti-α_2-fetoglobulin serum (c). Upper and lower rows of wells contained; 1, AH-130 rat serum; 2, partially hepatectomized rat serum; 3, $CdCl_2$-treated rat serum; 4, fetal serum; 5, amniotic fluid; 6, CCl_4-treated rat serum; and 7, DAB rat serum.

Immunological identity of two α_1-fetoglobulins on disc electrophoresis of serum from fetal and DAB rats: Densitometric tracings of protein bands separated by disc electrophoresis on sera from adult, fetal, DAB and AH-130 rats are shown in Fig. 6. Portions of gel corresponding to bands of fetus-specific protein were cut out separately and used for the double-diffusion analysis. Fused lines of fast and slow components of α_1-fetoglobulin were found in sera from both fetal and DAB rats (Fig. 7). However, the postalbumin peak in AH-130 rat serum failed to react with the antiserum as described previously. In all three preparations tested, another precipitin line of α_2-fetoglobulin, which did not fuse with the precipitin lines of α_1-feto-

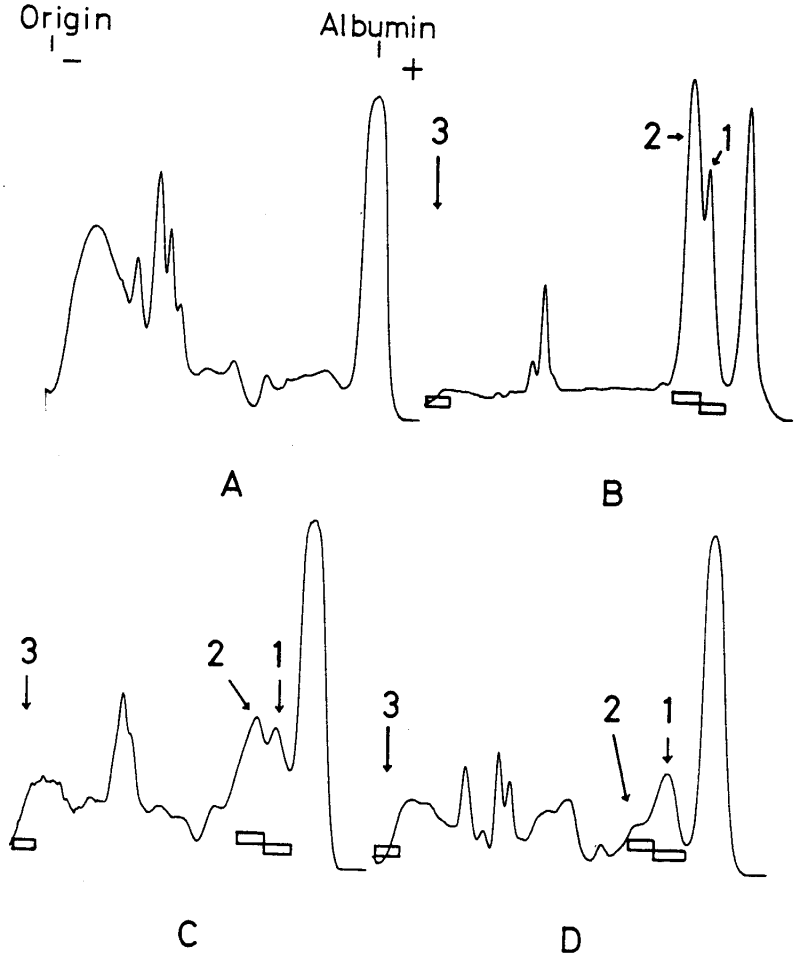

Fig. 6. Densitometric profiles of rat serum proteins under various experimental conditions on polyacrylamide disc electrophoresis. Two to three μl aliquots of serum were applied on gel columns and electrophoresed. Proteins were stained with Amido Schwarz. The stained gels were scanned from left (top) to right (bottom) by a densitometer. The large peak located at the extreme right corresponds to albumin. A, normal adult serum; B, fetal serum; C, DAB rat serum; and D, AH-130 rat serum. Numbers indicate portions of unstained gel as shown by ▭ at the bottom of figures. These gel portions were separately cut for double-diffusion analysis and put into outer large wells as indicated dy corresponding numbers (1, 2 and 3) in Fig. 7.

globulin, was formed between the wells containing a disc from the origin and the antiserum. Similar results were obtained with fetal serum by acrylamide gel immunoelectrophoresis (Fig. 8).

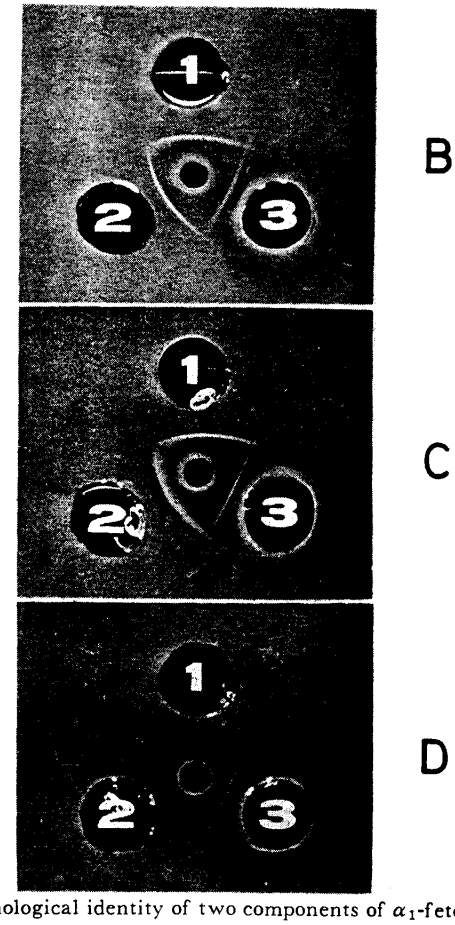

Fig. 7. Immunological identity of two components of α_1-fetoglobulin separated by disc electrophoresis. B, fetal serum; C, DAB rat serum; and D, AH-130 rat serum. Portions of electrophoresed gels were cut in 4-5 mm thick (1, 2 and 3 as illustrated in Fig. 6) according to $R_{B^{pB}}$ value of each component. Center wells in each plate contained specific antifetal-serum-immune serum.

Concentration of α_1 and α_2-fetoglobulin in sera of rats with experimentally induced hepatic lesions: Table 1 gives the concentrations of α_1 and α_2-fetoglobulins of ascites, amniotic fluid and sera of experimentally treated animals. Among sera of the treated adult rats, α_1-fetoglobulin was detected only in the serum from DAB rats; however, its concentration did not exceed that in fetus. α_2-Fetoglobulin was detected in all the sera of treated animals except amniotic fluid and adult serum. The highest level of α_2-fetoglobulin found was 256 for the serum of DAB rats.

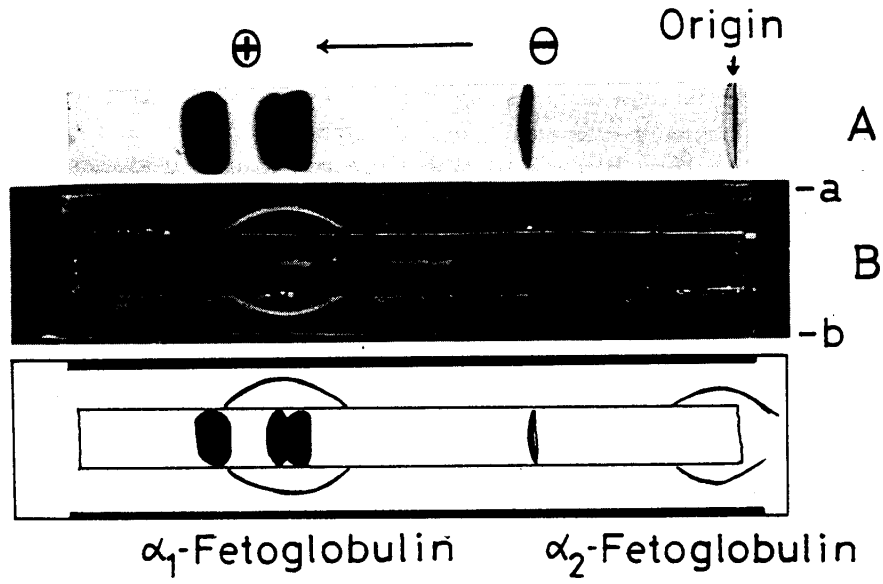

Fig. 8. Disc electrophoretic localization of α_1 and α_2-fetoglobulins in fetal serum on polyacrylamide gels. Three μl aliquots of fetal serum were applied on polyacrylamide gels and electrophoresed as described in legend to Fig. 1. An electrophoresed gel was stained for protein with Amido Schwarz (A). Another electrophoresed gel was placed on a glass plate and covered with 1.5% Difco-Noble agar at 50°C (B). Double-diffusion test was performed after specific antifetal-serum-immune serum was put into upper and lower throughs (a and b). A schematic illustration is shown by combining A and B.

TABLE 1 α_1 AND α_2-FETOGLOBULINS IN EXPERIMENTALLY INDUCED HEPATIC LESIONS OF RAT

Treatments of rat	Materials	α_1-Fetoglobulin (mg/ml)	α_2-Fetoglobulin*
Adult, untreated	Serum	ND**	ND
Fetus	Serum	7.2	128
	Amniotic fluid	1.3	ND
Adult, CCl_4-treated	Serum	ND	32
thioacetamide-treated	Serum	ND	16
$CdCl_2$-treated	Serum	ND	128
partially hepatectomized	Serum	ND	16
sham-operated	Serum	ND	8
with DAB-induced hepatoma	Serum	0.9	256
with ascites hepatoma (AH-130)	Serum	ND	32
	Ascites	ND	16

* Expressed as the reciprocal of the dilution (see the text).

** ND, not detected.

DISCUSSION

Three specific fetal antigens in fetal rat serum, antigen LA, α_2-glycoprotein and lipoprotein-esterase, have been already separated and characterized by STANISLAWSKI-BIRENCWAJG (15). The results of our studies on two fetoglobulins, α_1 and α_2-fetoglobulins, in fetal serum or amniotic fluid and sera or ascites fluid from adult rats with experimentally induced hepatic lesions agree well with those of STANISLAWSKI-BIRENCWAJG on two antigens, antigen LA and α_2-glycoprotein, although no lipoprotein-esterase was found in our studies. A single band of α_1-fetoprotein in the region of postalbumin found in Cellogel electrophoresis was further separated into two immunologically identical components by disc electrophoresis. α_1-Fetoprotein purified from fetal serum was also demonstrated to have electrophoretically two distinct components, details of this observation being published elsewhere (4).

Detection of α_1-fetoglobulin by Ouchterlony test in the sera of normal adult mice upon CCl_4 intoxication and partial hepatectomy has been reported (16). The concentrations of serum α_1-fetoglobulin in adult rats appeared to be much lower even at its maximum level (60–170 ng/ml), which may be attained at fourth day after CCl_4 administration and could be measured only after employing radioimmunoassay (17).

α_2-Fetoglobulin, a carbohydrate-containing macroglobulin, is known to occur in fetal and neonatal rats and in partially hepatectomized, pregnant, tumor-bearing and injured adult rats, but not in normal adult rats (18–20). This α_2-globulin has been reported to migrate more slowly than β-globulin in vertical starch-gel electrophoresis (21) and antigenically unrelated to any protein components of normal adult serum (20). The protein has been termed as slow α_2-globulin (19), α_2-AP (acute phase) globulin (20) or abnormal serum component (ASC) (22). The liver is shown to synthesize this protein (23, 24); *de novo* synthesis and secretion of α_2-(acute phase) globulin in turpentine-injured rat liver have been cleary demonstrated using the isolated perfused liver (25). Synthesis of this acute phase globulin have been chosen as a model system to investigate the mechanisms involved in regulation of mammalian gene expression and protein synthesis (25). The synthesis of this protein could be enhanced by administration of turpentine (20), bacterial endotoxin (26) or trypan blue (27). Although α_2-fetoglobulin is physicochemically and immunologically unrelated to C reactive protein (20), the results of these studies suggest that the presence of α_2-fetoglobulin is a sensitive index of tissue injury and cell death. α_2H protein frequentry found in sera of patients with neoplastic diseases has been assumed to be similar to α_2-fetoprotein in rat (28), while the α-fetoprotein found in primary hepatoma

patients (29) corresponds to α_1-fetoglobulin as may be appearently seen in the present studies and in those of Hirai's group (30, 31).

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