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Characterization of exocoelomic fluid protein from rat conceptuses cultured in rat and human sera: a measure of yolk sac activity during organogenesis

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SUMMARY

Fluid from the extraembryonic coelom of 11·5-day rat embryos cultured in 100% rat serum, 100% human serum and 90% human serum supplemented with 10% rat serum between days 9·5 and 11·5 postconception were compared using polyacrylamide gel electrophoresis and crossed immunoelectrophoresis. The protein composition of the exocoelomic fluids differed considerably from one another and from each of their respective culture sera. The majority of proteins in the exocoelom were derived from macromolecular transport but some contribution was made from protein synthesis by the conceptus. Eighteen proteins normally found in rat serum were found in the exocoelom of conceptuses cultured in 100% rat serum. Eighteen proteins were found in the exocoelom of rat conceptuses cultured in 100% human serum, of which ten were derived from human serum and eight were proteins normally found in rat serum. Analysis of fluid from conceptuses cultured in 90% human serum supplemented with 10% rat serum showed eleven human serum proteins and ten rat serum proteins. Differences in the composition of both human and rat proteins between the latter two fluids were also evident.

INTRODUCTION

Before the establishment of a chorioallantoic placenta the postimplantation mammalian embryo develops because of its ability to utilize biopolymers available in its immediate surroundings (Freeman, Beck & Lloyd, 1981). This material (the histiotroph) is available to the rat embryo as sluggishly circulating maternal blood contained in a sinus around Reichert's membrane (Merker & Villegas, 1970). Biopolymers are broken down in order to provide the embryo with amino acids (Freeman & Lloyd, 1981). It has been shown that even at this early stage of development maternal IgG is transferred from the culture medium across the yolk sac into the exocoelom without being subject to proteolytic digestion (Huxham & Beck, 1981), but it is not known to what extent other macromolecules are similarly transferred. Renfree, Hensleigh & McLaren (1975) have demonstrated the presence of albumen, transferrin and alphafoeto-protein in the amniotic fluid of mice between gestation days 11 and 18 but the origin of these proteins was not determined.

In a recent study by Reti, Beck & Bulman (1981) it was shown that rat embryos cultured in 100% human serum did not grow normally. However, supplementation of human serum with 10% rat serum resulted in restoration of its ability to support growth. It is conceivable that supplementation with 10% rat serum provided the developing rat embryo with species-specific macromolecules necessary for its growth. Alternatively, factors present in rat serum might serve to stimulate the extraembryonic membranes to transport, or synthesize, specific materials necessary for embryonic growth.

The contents of the exocoelomic fluid are not entirely dependent on yolk sac endoderm function since they may be added to by secretions of the embryo or of the extraembryonic mesoderm. Bearing these reservations in mind, we have nevertheless assumed that its constitution is largely determined by the visceral yolk sac.

In the present study we have attempted i) to determine the extent to which the macromolecular composition of exocoelomic fluid differed from maternal serum, ii) to determine the relative proportions of transported and of synthesized proteins in the exocoelomic fluid of conceptuses cultured in rat and human sera and iii) to see if a comparison of the macromolecular contents of the exocoelom could provide an indication of why 100% human serum is unable to support normal rat embryonic growth. Utilizing crossed immunoelectrophoresis and polyacrylamide gel electrophoresis we have used antisera to pregnant rat serum, human serum, IgG and transferrin to visualize human and rat serum proteins of exocoelomic fluid in an attempt to understand the nature of yolk sac activity and its relationship to the developing rodent embryo.

MATERIALS AND METHODS

Culture of rat embryos in rat and human sera

Wistar rat conceptuses were explanted at 9·5 days of pregnancy (timed from midnight preceding the morning on which vaginal plugs were observed) according to the standard methods of postimplantation rodent embryo culture (New, 1978). All human sera were supplemented with 1 mg/ml D-glucose. Three main cultures were performed. Rat conceptuses from pooled litters were cultured from 9·5 to 11·5 days of gestation in 100% rat serum, 100% human serum or 90% human serum supplemented with 10% rat serum.

Culture of rat embryos in radioactive medium

A fourth culture was performed in medium containing a mixture of radioactive amino acids. In this way the major protein components secreted into the exocoelom were distinguished from other proteins in the exocoelom.

Rat conceptuses were explanted at 9·5 days as above and cultured in rat serum until the embryo had been fully enveloped by the yolk sac (approxi-

mately 10·75 days of gestation). The conceptuses were then washed in 2 × 20 ml of Hanks' saline and rinsed in Medium 199 (M199, Gibco biocult), both at 37 °C and placed in a medium comprising 25% rat serum and 75% M199 together with 187 KBq/ml of a [^{14}C] amino acid mixture plus 37·5 KBq/ml [^{14}C] L-cysteine, 187 KBq/ml [^{14}C] L-glutamine and 374 KBq/ml [^{14}C] D-L-tryptophan (Amersham International, UK). The bottle was gassed with 40% 0_2, 5% CO_2, 55% N_2 and the conceptuses cultured for a further 24 h at 37 °C. At the end of culture embryos possessed a heart beat and were morphologically similar to those cultured in 100% rat serum.

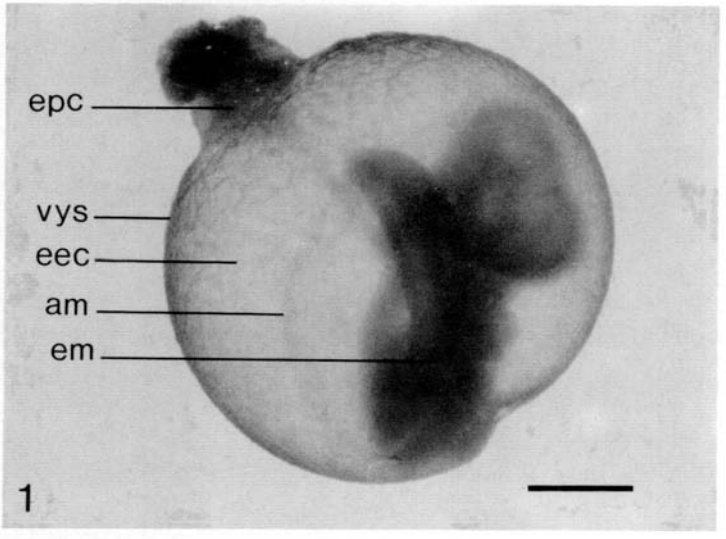

Fig. 1. The appearance of an 11·5-day rat conceptus after 48 h of culture in serum. The exocoelomic fluid was collected by micropuncture of the visceral yolk sac. epc, ectoplacental cone; eec, extraembryonic coelom; em, embryo; vys, visceral yolk sac; am, amnion. Bar = 1 mm.

Collection of the exocoelomic fluid

At the end of culture (11·5 days) all conceptuses (Fig. 1) were washed with 2 × 20 mls of Hanks' saline. The yolk sac membrane was punctured with a finely drawn glass pipette in an area of few vitelline vessels and the fluid in the extraembryonic coelom drawn out by suction. The yolk sac membrane collapsed onto the intact amnion filled with fluid and the embryo. In this way up to 10 μl of exocoelomic fluid was collected, free from contamination by serum and amniotic fluid.

Preparation of anti-sera

All preparations were carried out in New Zealand white rabbits. Rabbit anti-rat serum antiserum was collected by cardiac puncture after three bi-

weekly subcutaneous injections of 1 mg (in 0·5 ml) of pregnant rat serum emulsified with an equal volume of Freunds complete adjuvant. Rabbit anti-human serum was collected by cardiac puncture after three bi-weekly subcutaneous injections of 1 mg (0·5 ml) of human (female) serum similarly emulsified with Freunds complete adjuvant.

Pure rat IgG was prepared by protein A sepharose-4B (Pharmacia) affinity chromatography of rat serum. Rat IgG bound to the beads and was removed by elution with 1M-acetic acid. Rabbit anti-rat IgG anti-serum was collected by cardiac puncture after three bi-weekly subcutaneous injections of 0·5 mg (0·25 ml) of IgG emulsified with Freunds complete adjuvant.

Rat transferrin was prepared according to the methods of Schreiber *et al.* (1979). Rabbit anti-serum to rat transferrin was collected by cardiac puncture after three bi-weekly subcutaneous injections of 1 mg (0·5 ml) of rat transferrin emulsified with an equal volume of Freunds complete adjuvant.

Goat anti-human IgG was purchased from Miles, Teda Ltd. (Israel) and goat anti-human transferrin was purchased from Antibodies Inc. (U.S.A.)

Exocoelomic fluid analysis

The approximate relative molecular mass of proteins contained within the fluid was determined using Pharmacia 4–30% gradient polyacrylamide gels. Fluid samples (50 μl) were electrophoresed into the gel for 16 h and stained with 0·115% Coomassie brilliant blue R-250.

Crossed immunoelectropohoresis (Axelsen, Kroll & Weeke, 1973) was carried out in 1% agarose (Sigma, low endosmosis) in a 0·1M-Tris-borate-EDTA buffer at pH 8·6. Fluid samples (20 μl) were electrophoresed into 1% plain agarose gels for 1½ h at 2V/cm^2. The gel strip containing the sample was then electrophoresed at right angles into a second agarose gel or gels containing specific antisera. After electrophoresis at 2V/cm^2 overnight the gel was washed for 24 h in phosphate-buffered saline, pressed, washed again and dried. Immunocomplexes were visualized with 0·115% Coomassie brilliant blue R-250 stain.

Autoradiographic analysis of the major synthetic protein product within the fluid from conceptuses cultured in rat serum was achieved by crossed immuno-electrophoresis. The stained gel was then exposed to Kodak E.M. film for 6 weeks under vacuum.

The total protein content of fluids was determined by a modficiation of the method of Lowry, Rosenbrough, Farr & Randall (1951). A sample of pooled exocoelomic fluid from 15 conceptuses was used for each duplicate analysis. Protein values of fluid from conceptuses cultured in 100% rat serum, 100% human serum and 90% human serum/10% rat serum were obtained.

RESULTS

Culture of rat embryos in 100% rat serum and human serum supplemented with 10% rat serum resulted in the growth of normal embryos. Culture in 100% human serum, however, resulted in suboptimal development (Fig. 2). All such conceptuses were anaemic. The corpuscular elements were fewer in number and were colourless.

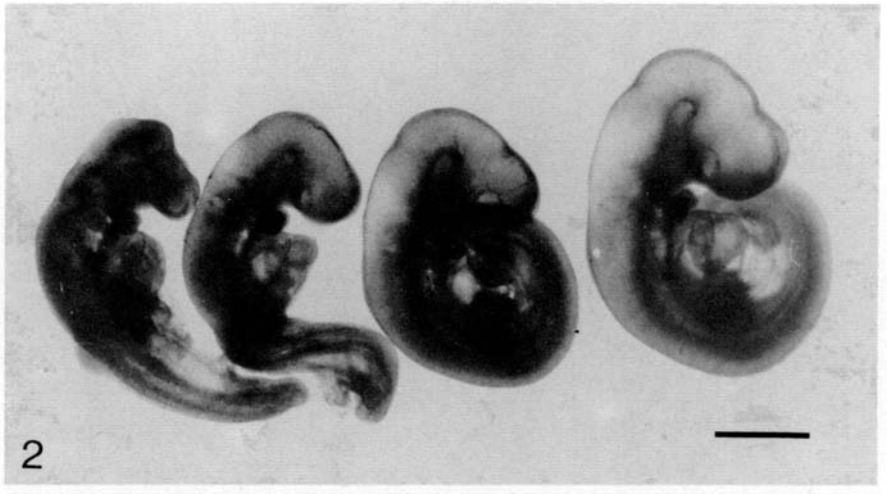

Fig. 2. The appearance of four 11·5 day rat embryo littermates after 48 h of culture in 100% human serum. All four conceptuses from which these embryos were obtained were anaemic. Whilst the two embryos shown on the left were growth retarded, the two shown on the right were morphologically similar to embryos cultured in 100% rat serum. Bar = 1 mm.

Analysis by polyacrylamide gel electrophoresis

Fig. 3 shows that exocoelomic fluid from conceptuses cultured in 100% rat serum (RS ECF, Fig. 3B) contained 15 major protein components and was very different from rat serum (Fig. 3A). Exocoelomic flluid from conceptuses cultured in 100% human serum (HS ECF, FIG. 3C) contained 13 major protein components and was very different from human serum (Fig. 3D). The two exocoelomic fluids also differed, each from the other.

Using Pharmacia high relative molecular mass marker proteins as a guide, the approximate relative molecular mass of each protein band was estimated and the results are summarized in Table 1. Four proteins present in RS ECF were not detected in HS ECF and a number of other proteins common to both fluids were present in different concentrations (see legend to Table 1).

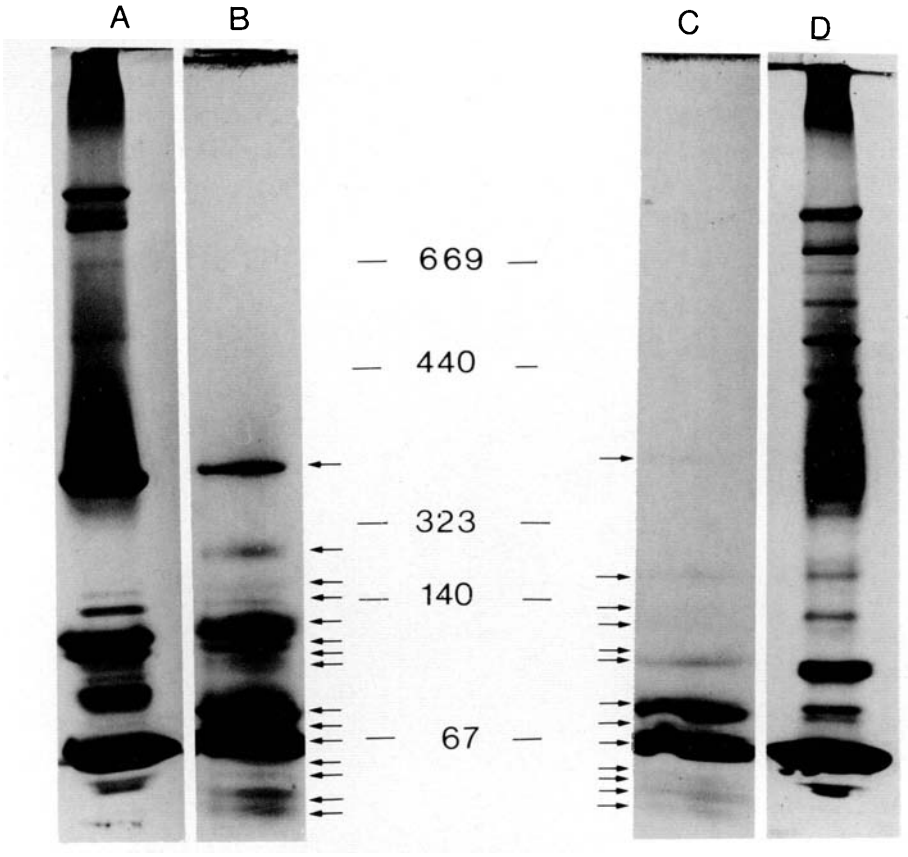

Fig. 3. Polyacrylamide gel electrophoresis in a 4–30% acrylamide gradient of rat serum (A) exocoelomic fluid from conceptuses cultured in 100% rat serum (B) and in 100% human serum (C) and human serum (D). An approximate relative molecular mass scale is given ($\times 10^{-3}$). Arrows indicate positions of the major proteins.

Crossed immunoelectrophoresis of exocoelomic fluids

Using pregnant rat anti-serum, 17 major rat serum proteins could easily be distinguished in serum (Fig. 4) and 18 in exocoelomic fluid from conceptuses cultured in 100% rat serum ($\simeq$ 2·5 mg/ml total protein) (Fig. 5). The distribution of peaks from exocoelomic fluid collected from 11·5 day conceptuses allowed to develop *in vivo* was the same as in Fig. 5 (data not shown). The distribution of peaks from exocoelomic fluid differed from that obtained with pregnant rat serum.

In order to detect both human and rat serum proteins in the same sample of exocoelomic fluid, electrophoresis of human-serum-derived exocoelomic fluid (HS ECF: 1·6–1·8 mg/ml total protein) into a second dimension agarose gel containing anti-rat and anti-human sera (corresponding to the lower end upper

Table 1. *The approximate relative molecular masses of protein within the exocoelomic fluids of rat conceptuses cultured in 100% rat and 100% human serum*

RS ECF	(SIDPA)†	HS ECF	(SIDPA)†
285,000	4·1096	285,000[e]	0·0197
200,000*	1·4219	–	
–		††160,000[a]	0·0228
††158,000*	0·0132	–	
142,000	0·0166	142,000[c]	0·0077
122,000	3·8928	122,000[e]	0·0039
108,000*	0·0803	–	
95,000	0·0177	95,000[d]	0·0022
82,000	0·0133	82,000[f]	0·0827
70,000	21·7832	70,000[b]	18·7823
69,000	0·0133	69,000[b]	0·0121
68,000	(off scale)	68,000	(off scale)
64,000*	0·0123	–	
62,000	0·0156	62,000[b]	0·0657
–		60,000[a]	0·0013
58,000	0·1337	58,000[c]	0·1084
56,000	0·0817	56,000[c]	0·0566
	Total number of bands detected		
	15		13

Polyacrylamide gel electrophoresis in 4–30% gradient gels.
RS ECF – Exocoelomic fluid from conceptuses cultured in 100% rat serum.
HS ECF – Exocoelomic fluid from conceptuses cultured in 100% human serum.
†Polyacrylamide gels were scanned with an LKB 2202 ultrascan laser densitometer and the staining intensity determined as unit area using an LKB 2220 recording integrator. This provided an indication of protein quantity for a comparative analysis of fluid as shown by SIDPA (staining intensity as determined by peak area: arbitrary units) and letters.

a; not detected in RS ECF.
b; present in equal amounts in RS and HS ECF.
c; less than in RS ECF.
d; very much less than in RS ECF.
e; considerably less than (×10) in RS ECF.
f; very much more than in RS ECF.
*; not detected in HS ECF.
††; the approximate relative molecular masses of these two proteins are too similar to each other to be certain of a distinct difference between them.

halves of the gels shown in Figs 6 and 7) revealed 18 major peaks (Fig. 6). By this method ten of the observed peaks were identified as human proteins (numbers 9 to 18) and eight were identified as rat proteins (numbers 1 to 8). A similar analysis of fluid derived from conceptuses cultured in 90% human serum supplemented with 10% rat serum (Fig. 7) revealed 21 major peaks. Eleven proteins were of human origin (numbers 11 to 21) and 10 were rat proteins (numbers 1 to 10).

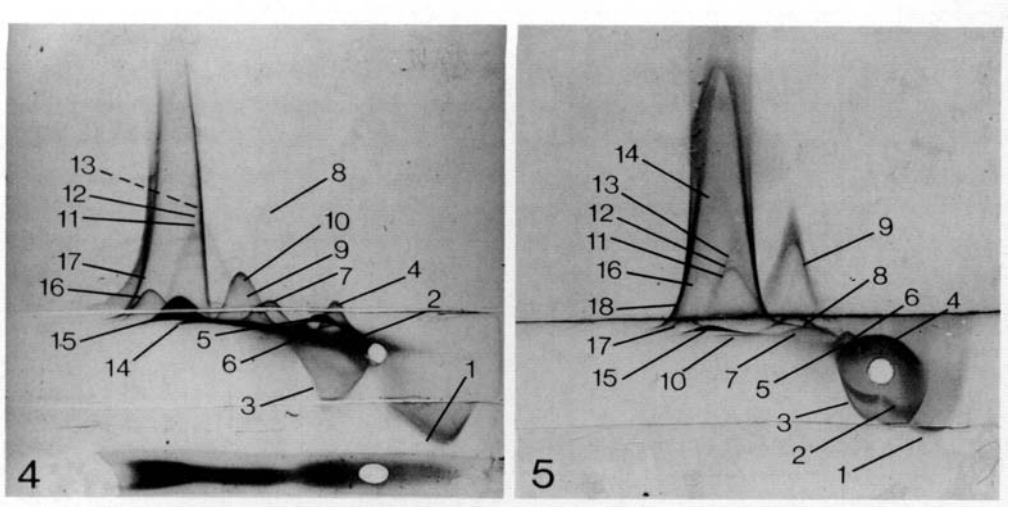

Fig. 4. Crossed immunoelectrophoresis of rat serum into an agarose gel containing 60 μl anti-rat serum/ml gel. The peaks numbered from 1 to 17 are in order of increasing electrophoretic mobility.

Fig. 5. Crossed immunoelectrophoresis of exocoelomic fluid from conceptuses cultured for 48 h in 100% rat serum into an agarose gel containing 60 μl anti-rat serum/ml gel. The peaks are numbered from 1 to 18 in order of increasing electrophoretic mobility. Number 4 = rat IgG; 11, 12 and 13 = rat transferrin.

Many of the proteins present in the exocoelom of conceptuses cultured in 90% or 100% human sera were of human origin. These proteins were, therefore, transported across the visceral yolk sac to the exocoelom. Five major proteins (numbers 18 in Fig. 6 and 12, 18, 19 and 20 in Fig. 7 marked with a star) represented the major differences in transport of human serum proteins into the exocoelom between conceptuses cultured in 100% human serum (Fig. 6) and in 90% human serum (Fig. 7).

Rat macromolecules evident in Fig. 6 represent the major products of protein synthesis and secretion by the conceptus during culture. A comparison of Fig. 6 with the lower half of Fig. 7 showed that culture in 100% human serum resulted in the synthesis and secretion of two additional rat proteins (numbers 5 and 6 in Fig. 6) into the exocoelom which were not found from cultures containing rat sera.

Specific antisera to transferrin and IgG demonstrated the presence of rat IgG (number 4 in Fig. 5) and transferrin (numbers 11, 12 and 13 in Fig. 5) in the exocoelomic fluid from conceptuses cultured in 100% rat serum. Human IgG (number 9 in Fig. 6) and human transferrin (number 13 in Fig. 6) were similarly found in the fluid of conceptuses cultured in 100% human serum. Exocoelomic fluid from conceptuses cultured in 90% human sera supplemented with 10% rat serum (Fig. 7) contained both human IgG (number 21),

rat IgG (number 1), human transferrin (number 13) and rat transferrin (number 7).

Autoradiographic analysis of the exocoelomic fluid from conceptuses cultured in medium containing radioactive amino acids showed only one major radioactive protein (arrows Fig. 8A, B). This peak possessed an identical electrophoretic mobility to the major rat protein (peak number 3) shown in Fig. 6 and 7.

At serum antigen concentrations of 2–3 mg/ml, interspecies cross reaction

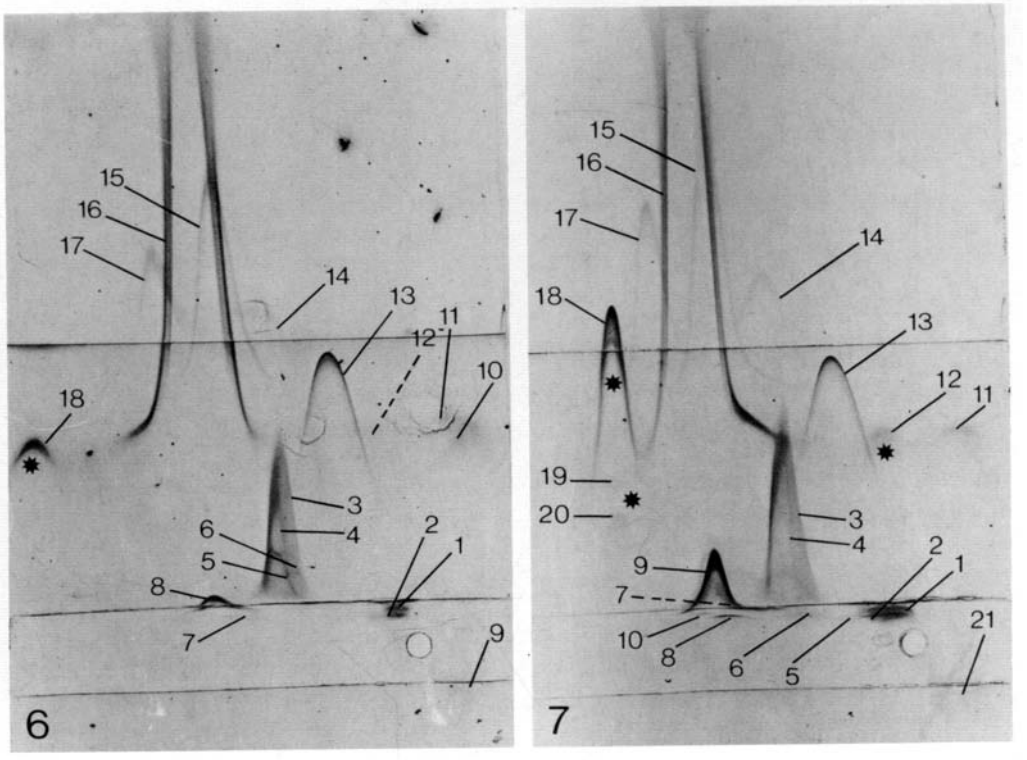

Fig. 6. Crossed immunoelectrophoresis of exocoelomic fluid from conceptuses cultured for 48 h in 100% human serum into agarose gels containing 60 μl anti-rat serum/ml (lower) gel and 40 μl anti-human serum/ml (upper) gel. Peaks 1 to 8 are rat proteins. Peaks 9 to 18 are human proteins. Number 9 = human IgG; 13 = human transferrin.

Fig. 7. Crossed immunoelectrophoresis of exocoelomic fluid from conceptuses cultured for 48 h in 90% human serum/10% rat serum as for Fig. 6. Peaks 1 to 10 are rat proteins. Peaks 11 to 21 are human proteins. Peak number 1 is rat IgG. Peak number 7 is rat transferrin. Peak number 13 is human transferrin. Peak number 21 is human IgG.

Stars indicate unknown human proteins that represent the main differences between Fig. 6 and 7. During electrophoresis some cathodic migration of human serum antibodies was observed. The small strip of agarose shown at the bottom of Fig. 6 and 7 contained anti-human serum.

between antisera was determined by performing crossed immunoelectrophoresis of rat serum against anti/human serum and human serum against rat serum antiserum. In both cases no precipitin bands were observed. A similar analysis of rat and human transferrin, rat and human IgG with each respective antisera of the opposite species by Ouchterlony immunodiffusion showed no cross reaction (data now shown).

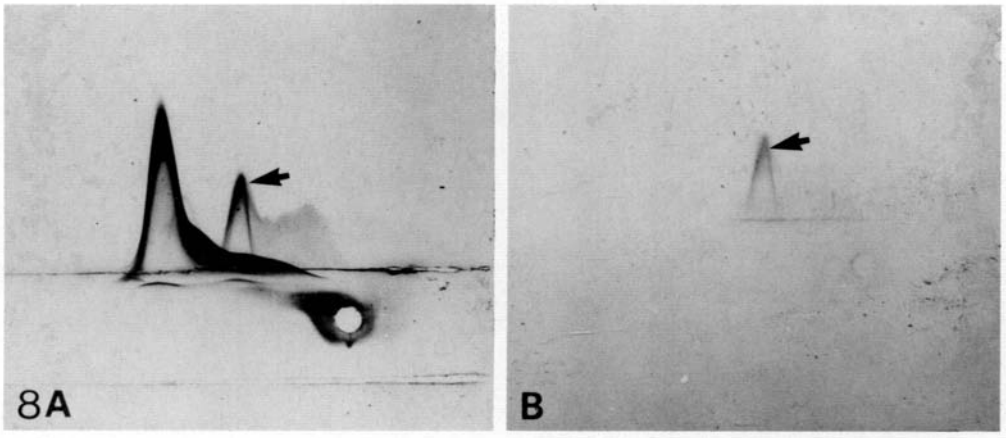

Fig. 8. Crossed immunoelectrophoresis of rat-serum-derived exocoelomic fluid after culture in [^{14}C]amino acids (A) into an agarose gel containing 80 μl anti-rat serum/ml gel. Exposure of Kodak EM film to the dried gel for 6 weeks under vacuum revealed only one major radioactive protein component (arrows) (B).

DISCUSSION

We present evidence for the presence of macromolecules in the exocoelomic fluid of rat conceptuses which are normally found in pregnant rat or human (male) sera. Our results allow a distinction to be made between serum proteins in the exocoelom which derive from the culture medium and proteins which are synthesized by the conceptus. If the culture medium is composed of 100% human serum, then proteins in the exocoelom of human origin can only result from passage of human serum proteins from the culture medium to the exocoelom. By contrast, rat serum proteins in the exocoelom of conceptuses cultured in 100% human serum have arisen from synthesis by the conceptus itself during culture. Having (as a result of culture in 100% human serum) ellucidated the extent of protein synthesis by the conceptus we find that we are able to detect the same proteins among those present in the exocoelom of embryos cultured in rat serum. It is likely that by difference, any other rat macromolecules which are visualized with pregnant rat antiserum have resulted from passage out of the culture medium into the exocoelom. The majority of the proteins found in the exocoelom were the products of protein transport and not of protein synthesis and were identical to proteins normally found in

pregnant rat or human sera. The protein concentration in the exocoelom was found to be an order of magnitude less than serum, but many of the proteins in the fluid were present at a different relative concentration to that found in serum. Thus, the visceral yolk sac appeared to act as a filter for specific maternal macromolecules. Indeed, Priscott, Gough & Barnes (1983) have shown that four serum proteins are selectively depleted from the culture serum of rat embryos during organogenesis.

During culture in 90% human serum the rat visceral yolk sac transported 11 human serum proteins to the exocoelom. Human IgG was identified as being present. It is known that all four classes of human IgG are transported across the neonatal rat intestine by a receptor mediated process (Walderman & Jones, 1976; Mackenzie, Morris & Morris, 1983) but, to date, no other serum proteins have been shown to cross in important quantities. In contrast, rat visceral yolk sac was observed to transport human IgG, transferrin and nine other human serum proteins. During culture in 100% human serum, however, three of the eight unknown human serum proteins mentioned above were not transported and another protein with an electrophoretic mobility greater than albumen was found to be transported. Thus, the characteristics of human serum protein transport were dependent upon the presence, or absence, of rat serum in the culture medium.

Since human transferrin was present in the exocoelom in approximately equal amounts from both 90% and 100% human serum cultures (as judged by peak area) the concentration of transferrin could not alone account for the observed anaemic appearance of the conceptuses after 2 days of culture. The binding sites for iron and its release from human and rat transferrin are known to be different (Morgan, Huebers & Finch, 1978). It is, therefore, likely that whilst human transferrin is taken up by the yolk sac and subsequently transported to the exocoelom, its contained iron is not utilized by the conceptus. Supplementation of human serum with 10% rat serum might thus have provided the rat conceptus with sufficient rat transferrin for iron capture by selective uptake and recycling of rat transferrin to and from the plasma membrane (Hopkins, 1983).

The yolk sac of various species is known to synthesize a variety of proteins. These include albumen, pre-albumen, alphafoetoprotein, alpha-antitrypsin, transferrin, embryo-specific alpha globulin and conalbumen (Gitlin & Kitzes, 1967; Gitlin & Pericelli, 1970). Using ^{35}S-met in a short-term culture, Janzen, Andrews & Tamaoki (1982) showed that 60% of the protein synthesized by the 11·5-day mouse yolk sac was alphafoetoprotein. Thus, the major synthesized protein secreted into the exocoelom of the much earlier rat embryo shown in this study is most likely to be alphafoetoprotein. It is interesting to note that two proteins (numbers 4 and 5 in Fig. 6) normally found in the serum of pregnant rats were synthesized in appreciable quantities by the rat conceptus when culture was performed in 100% human serum. Both proteins had

electrophoretic mobilities very close to what is probably alphafoetoprotein, but their identities are as yet unknown.

The selective uptake of specific macromolecules in endodoerm cells is thought to be mediated by plasma membrane receptors which are subsequently internalized via coated pits and receptosomes into the cell (Willingham & Pastan, 1980). Since many human serum proteins were found in the exocoelom bounded by the visceral yolk sac, it is possible that rat plasma membrane receptors of the visceral endoderm possessed cross specificity for many human serum proteins. Alternatively, the presence of human serum proteins in the ecocoelom could have arisen from passive transfer across the visceral yolk sac (Brambell, 1966). The concentration of protein in the exocoelom of conceptuses cultured in 100% human serum (1·6–1·8 mg/ml) was less than from conceptuses cultured in 100% rat serum (2·5 mg/ml). This would suggest inefficient uptake and transport of human serum proteins. However, the total protein content of exocoelomic fluid from conceptuses cultured in 90% human serum/10% rat serum was the same as from conceptuses cultured in 100% human serum. Since embryos belonging to the former category are well developed but embryos of the latter show characteristics of growth retardation and anaemia (Gupta & Beck, 1982), inefficient selective uptake of bulk human serum proteins by the visceral yolk sac is unlikely to be the only factor responsible for embryonic growth retardation. Secondly, the nature of human serum protein transport to the exocoelom was determined by the presence, or absence, of 10% rat serum in the culture medium. This observation would suggest that macromolecular processing by rat yolk sac is dependent upon the presence of specific molecules in rat serum. Similarly, the synthesis of rat serum proteins by the conceptus appeared to be dependent upon the composition of the serum used for culture. These observations suggest that the visceral yolk sac is more than a passive filter for the transport of maternal macromolecules and that its function partly relies on the nature of its immediate environment.

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