Production of Fetuin and Other Serum Proteins by Fetal Sheep Liver *in vitro*.* (32577)

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It is known that most serum proteins are produced by the liver(1-8). In adult animals the synthesis of albumin, transferrin, β_{1C}, and several other α and β globulins by the liver is stimulated by infection(2), endotoxin injection(3), partial hepatectomy(3), etc. Similar procedures also induce formation of C-reactive and C_x-reactive protein(3,4) as well as of other acute phase proteins(2-8) by the liver.

In most laboratory rodents, liver from newborn animals, or from animals in the latter part of the gestation period, is much more active in production of serum proteins than liver taken from normal adults(9). Since fetal animals of varying ages are more readily obtained from larger animals with long gestation periods, a study of the development of serum protein synthesis in fetal liver and in lymphoid tissues was made in the lamb. Particular attention was given to the production of fetuin—an α_1-globulin present in fetal, but not in adult sheep sera—believed to be analogous to the fetuin described in fetal calf serum(10).

Materials and methods. Sheep embryos were obtained by Caesarian section from random-bred sheep of known gestation time(11). The normal ovine gestation period is 150 days. Weighed amounts of minced tissue from liver, lymph node, spleen and thymus were cultured for 48 hours at 37°C in roller tubes, as described previously(12,2,3). A modified Eagle's culture medium was employed containing 1 μc per ml of each of two uniformly labeled amino acids (C^{14}-lysine and C^{14}-isoleucine, 100 to 200 μc/mMol) and 0.5% ovalbumin(12,2,3). Both amino acids are known to be present in bovine fetuin(13).

After the incubation period, tissue culture fluids containing labeled serum proteins were dialyzed, lyophilized, and reconstituted to 1/15 their original volume. Labeling of serum proteins was detected by means of autoradiography of immunoelectrophoretic (IE) patterns(12,2,3). Carrier IE patterns were prepared using adult or fetal sheep serum as the antigen and rabbit antisera to whole sheep serum, as well as specific rabbit antisera to sheep fetuin and sheep complement (C'). The antiserum to sheep fetuin was prepared by absorption of an antiserum to fetal sheep serum with adult sheep serum. Anti-sheep C' was prepared as described previously for antisera to mouse and rat C'(14). The antiserum primarily precipitated 2 β globulins, one of which appeared to be analogous to human β_{1C} and the other to β_{1E} globulin in their IE behavior upon hydrazine treatment and absorption by antigen-antibody complexes of fresh sheep serum. These proteins have therefore been provisionally designated β_{1C} and β_{1E} (Fig. 1B). Transferrin was identified by means of its ability to bind $Fe^{59}Cl_3$(15). Results obtained on synthesis of immunoglobulins by lymphoid tissues of these and other fetal sheep have been reported in a previous publication(11).

Results and discussion. The results of analyses of autoradiographs prepared from IE slides with similar carrier patterns and different concentrated culture fluids are given in Table I. The intensities of the autoradiographic images were graded, and the ranges given in the table. It can be seen that fetuin production occurred in the liver, and further that it was much stronger during the first half of the gestation period than during the second (Fig. 1A). Production of albumin and transferrin was high until day 120, but very low in livers from older fetuses (Fig.

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1C). None of these 3 proteins was synthesized by lymphoid tissue.

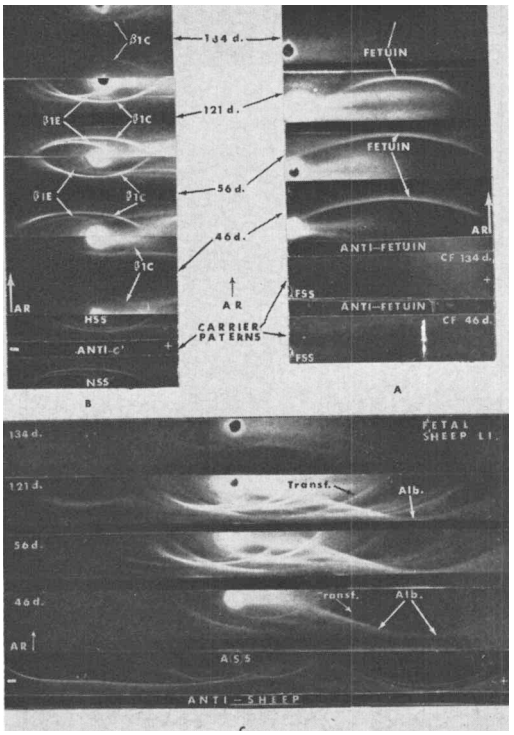

FIG. 1. Autoradiographs (AR) of immunoelectrophoretic (IE) patterns prepared with culture fluids from fetal sheep livers taken at various days of the gestation period. A. The carrier patterns used with the cultures were prepared from fetal sheep serum (FSS) with a rabbit antiserum specific for fetuin. 2 different IE patterns are represented: when culture fluid from a liver taken at the 46th day (CF 46d) was used, it evidently contained so much fetuin that it altered the appearance of the precipitation arc. Culture fluids from livers taken later in the gestation period (CF 134d) did not change the precipitin arc made with the carrier serum alone. Note that the autoradiographs also show a change in appearance and labeling intensity of the fetuin arc depending on the amount of fetuin present in and produced by the liver culture. At 134 days the fetuin production was no longer detectable.
B. Carrier patterns used with the cultures were prepared from fresh adult sheep serum (NSS) and hydrazine-treated sheep serum (HSS) with a rabbit anti-sheep C'. Note the faster mobility of the β_{1C} in the hydrazine-treated serum and the changed appearance in the β_{1E} line (more in antibody excess). Labeling of these 2 proteins was much more evident at 56 and 121 days than earlier or later in the gestation period.
C. The carrier patterns used with the cultures were prepared from adult sheep serum with a rabbit anti-whole sheep serum (bottom). Note that the labeling of albumin and transferrin is stronger with liver taken at the 46th to 121st day than with liver taken at the 134th day.

Synthesis of the proteins designated β_{1C} and β_{1E} globulin (presumably representing C'3(16) and C'4(17)), however, occurred both in liver and lymphoid tissue, as has been shown in all species studied thus far (2,9,18). The possibility that macrophages in lymphoid tissues are responsible for production of these proteins has been discussed extensively in other publications(19,20). The liver appeared more active in the synthesis of both these complement proteins during an intermediate gestation period than it did at early or late gestation times (Fig. 1B). This appears analogous to what was found to occur in mice and guinea pigs where a high albumin production preceded formation of significant amounts of β_{1C}, and production of all serum proteins by the liver considerably decreased during the first month after birth (9). Clear-cut variations with age were not found for β_{1C} and β_{1E} production by lymphoid tissue. Overall hemolytic complement activity is lacking in fetal sheep sera(21). Borsos (personal communication) has found that, in particular, C'4 activity is absent throughout the gestation period. The technique used for C'4 titration(22) is extremely sensitive, probably more sensitive than the one used here to demonstrate β_{1E} labeling. Thus, it seems possible that the designation β_{1E} (C'4) for the protein demonstration by the anti-C' serum is erroneous. Other reasons for the discrepancy could be the presence of a C'4 inhibitor in fetal lamb sera, or the production of a hemolytically inactive serum protein with similar electrophoretic and antigenic properties as the active complement component. It should also be realized that labeling of the β_{1E} protein could possibly be caused by production of a protein complexing with β_{1E} rather than by production of β_{1E} itself.

It should be noted that in the present studies, lymphoid tissues were not examined before the 88th day and liver not before the 31st gestation day (Table I) so that the earliest day of detectable serum protein production by these tissues was not established. Immunoglobulin production by these tissues was also studied. Labeling of these proteins was seen in many of the lymph node and

TABLE I. Serum Protein Production *in vitro* by Various Tissues from Fetal Sheep.

Tissue	Fetal age (days)	No. of animals	Protein synthesized*				
			Fetuin	Albumin	Transferrin	β_{1C}	β_{1E}†
Liver	31– 55	4	++	++	++	w+ to +	— to w+
	56– 62	3	++	++ to +++	++ to +++	++ to +++	w+ to +
	109–121	2	+	++ to +++	++ to +++	++ to +++	+ to ++
	133–134	3	— to w+	— to w+	— to w+	— to w+	— to w+
Lymph node	88–149	9	—	—	—	w+ to ++	w+ to +
Thymus	136–149	3	—	—	—	w+	w+
Spleen	90–149	6	—	—	—	w+ to +	— to w+

* Intensities of autoradiographic images were graded from — to +++.

† This protein was designated β_{1E} because of its similarity in immunoelectrophoretic patterns to human β_{1E}. It was shown particularly well by the anti-C′ antiserum, and was partially removed from fresh serum by absorption with antigen-antibody complexes.

spleen cultures, particularly in lymphoid tissue taken from immunized fetal lambs(11), but was never observed in liver or thymus cultures.

The general decrease in serum protein synthesis by the liver apparently occurs before birth in the sheep and after birth in rodents (9). It will be interesting in this respect to determine at which time, in relation to birth, production of the recently described fetal rat serum protein(23) discontinues. A recent study(24), employing a method similar to the one used here to determine where human and rat α-feto proteins are produced, also implicates the fetal liver—among a variety of organs studied—as their site of synthesis. In the rat the fetoprotein is apparently still produced by liver from 2-day-old animals, but no further comparisons between livers from different age groups were made. Synthesis of rat fetoprotein was also shown in the yolk sac lining of the placenta(24). The very low or undetectable fetuin production by fetal sheep liver around 20 days before birth agrees fairly well with the disappearance of this protein from the circulation just before the end of the gestation period.

Diminished production of fetuin, with continued high production of other serum proteins, by livers taken on days 109 through 121 may be caused by variations in the synthesizing activities of different cells within the liver. Another possibility is that regulation at an intracellular level determines the production rates for various proteins by individual cells. The enhanced production of certain acute phase proteins by the liver of adult animals after stimulation with endotoxin, turpentine, etc., may represent another example of the same phenomenon. Fluorescent antibody studies(25) indicate that production of fibrinogen and albumin in the liver usually occurs in different cells. The recent finding in this laboratory, however, that an established, cloned, rat hepatoma cell line (H4-II-Ec3) produces various serum proteins *in vitro*(26) suggests production of more than 1 serum protein by the same parenchymal liver cell.

Summary. A study was made of the incorporation of C^{14}-labeled amino acid into various serum proteins by fetal lamb tissues *in vitro*. The fetal liver was quite active in production of fetuin during the first half of the gestation period, diminished in activity after this and barely showed any labeling of fetuin when taken during the last weeks before birth. Production of other serum proteins by the liver was at its peak around days 100-120, but also diminished towards the end of the gestation period. Lymphoid tissues did not synthesize fetuin.

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An Automated Technique for the Clearing and Staining of Fetal Bone in the Autotechnicon. (32578)

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Since a large number of fetuses must be cleared and stained as part of the teratogenic studies required for complete safety evaluation of new drugs, it became necessary to find a method in which these processes could be conducted and controlled automatically. While several convenient clearing and staining techniques are presently available for the examination of fetuses(1,2,3) they are manual in their operation and are tedious and time consuming.

The Autotechnicon has been utilized for the automatic processing and staining of tissues for many years; therefore, it was decided to adapt the method of Crary(1) to this instrument. The results of this adaptation are reported herein.

Methods and materials. Rat and mouse fetuses, which are removed from the dam by Caesarian section, or newborn pups are put into the autopsy trays of the Autotechnicon. These trays are fitted with removable dividers so that each fetus is maintained and processed separately. Each tray can hold at least 6 mice or 4 rats. A paper label containing the dam and fetus number is placed in each tray compartment with the fetus. This identifies the fetus so one may correlate skeletal anomalies with weight, length, or any other distinguishing characteristic of that particular offspring. 6 trays are placed in the Autotechnicon basket in such a fashion that one acts as a lid for another.

The Autotechnicon beakers are arranged so that the baskets containing the fetuses or pups are sequentially processed in the following solutions: a) Acetone; b) 1% Potassium Hydroxide; c) a solution of 6 mg alizarin red S stain per liter of distilled water; and d)