

Maturation of the Human Complement System

I. ONSET TIME AND SITES OF FETAL C1q, C4, C3, AND C5 SYNTHESIS

PETER F. KOHLER

*From the Division of Clinical Immunology, Department of Medicine,
University of Colorado Medical Center, Denver, Colorado 80220*

ABSTRACT The onset times and sites of human C1q, C4, C3, and C5 synthesis were determined by culturing tissues from 23 fetuses, 8–25 wk old, in the presence of [14 C]lysine and isoleucine. In parallel, IgG and IgM production was followed. Liver, spleen, placenta, peritoneal and bone marrow cells, thymus, and colon were cultured for 48 h and the concentrated media studied by immunoelectrophoresis and subsequent autoradiography using adult human serum as carrier and specific antisera. The quantitative synthesis was approximated by scoring the intensity of the labeled precipitin lines using uniform conditions. C5 production was detected earliest at 8 wk gestation and by 11 wk and thereafter, C3, C4, and C5 synthesis was uniformly present in multiple tissues. C1q synthesis, however, was limited almost exclusively to the spleen, began at 14 wk, and was not uniformly present. In contrast IgG and IgM production did not occur in three fetuses synthesizing complement and while detected as early as 11 wk, was inconstant, occurred predominantly in the spleen, and was quantitatively much less compared to C3, C4, and C5. These findings suggest that developmentally the complement system is a more primitive biological defense mechanism than antibody.

INTRODUCTION

The fundamental importance of the complement system as an effector mechanism modulating the diverse bio-

logical consequences of antigen-antibody interactions has been delineated over the past decade (reviewed in reference 1). Recently, evidence for complement activation independent of antibody (2, 3) and for its participation in blood coagulation (4) have also been reported.

Relatively limited data are available on the developmental aspects and tissue origins of the proteins which comprise the human complement system (5–8). Such information has potential relevance to human pathophysiological processes in general.

The objective of this study was to determine when fetal synthesis of complement proteins participating in initial, mid-, and terminal stages of the reaction sequence, e.g., C1q, C4, C3, and C5, begins. In parallel, the sites and onset time for synthesis of these proteins were compared and the relative synthesis of each protein was estimated. In addition, the temporal relationship between immunoglobulins G and M and complement synthesis was determined. To these ends, the technique of *in vitro* tissue culture in the presence of carbon- 14 -labeled amino acids followed by immunoelectrophoresis (IEP)¹ using specific antisera and subsequent autoradiography, was employed (9).

The results indicate that complement synthesis precedes immunoglobulin synthesis in the human fetus suggesting that it constitutes a more primitive, albeit less specific, defense system. In addition, complement production occurs in a wider range of tissues and is quantitatively greater than immunoglobulin synthesis.

METHODS

Fetal tissues. Tissues were cultured from 37 fetuses obtained after spontaneous or therapeutic abortion at the University of Colorado Medical Center and the General Rose

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¹Abbreviations used in this paper: autoradiograph, ARG; C3 PA, C3 proactivator; IEP, immunoelectrophoresis; PBS, phosphate-buffered saline; WHS, goat anti-whole human sera.

Hospital, Denver. The fetuses were aborted spontaneously in two instances, by amniocentesis in 12 and by hysterotomy or hysterectomy in the remainder. Fetal gestational age ranged from 8 to 25 wk as estimated from measurement of crown-heel length according to Scammon and Calkins (10).

Cultures. In vitro culture of various tissues was carried out in media containing carbon-14 amino acids. This was followed by cellulose acetate electrophoresis and IEP of the concentrated culture fluid with subsequent autoradiography as modified by van Furth (11) from the original technique of Hochwald, Thorbecke, and Asofsky (9). From 60 to 325 mg (wet weight) of tissue from (a) liver, (b) spleen, (c) placenta, (d) thymus, and (e) colon, were minced with a scalpel blade and washed at 4°C in 2 ml special McCoy's media 5-A lacking lysine and isoleucine (Microbiological Associates, Inc., Bethesda, Md.), then cultured in 2-ml portions of media to which high specific activity [¹⁴C] lysine and isoleucine (302–312 mCi/mmol, Amersham/Searle Corp., Arlington Heights, Ill.) were added at concentrations of 1 µCi/ml each. In addition, suspensions of (f) peritoneal cells obtained by injecting 2–4 ml of the special media into the peritoneal cavity followed by gentle kneading and recovery of the fluid, and (g) bone marrow cells, recovered from a single femur by injecting 2 ml of special media through the marrow cavity, were similarly studied. The 8 wk fetus which measured 22 mm was cultured *in toto*.

Glass culture tubes (125 × 15 mm) were gassed with a mixture of 5% CO₂ and 95% air, capped, and incubated at 37°C in a rotating drum for 48 h. The contents were frozen at -70°C, then thawed and dialyzed against 2 liter volumes of 0.015 M phosphate-buffered saline (PBS) × 6 for 72 h. After centrifugation at 4°C to remove insoluble constituents, the supernatant media were lyophilized and reconstituted with 0.3 ml distilled H₂O, and thereafter stored at -70°C. Cellulose acetate electrophoresis of the concentrated culture fluids without carrier serum was done with a Beckman Microzone Cell (Beckman Instruments, Inc., Fullerton, Calif.) using a 25 µl sample applicator. Electrophoresis was carried out for 20 min at 25 V/cm in 0.075 M Veronal buffer pH 8.6. Serum from the same fetus, when available, was included for comparison with the protein and autoradiograph (ARG) patterns of the various tissues.

Antisera. Monospecific rabbit antisera to the complement components, C1q, C4, C3, and C5 were either gifts from Dr. H. J. Müller-Eberhard (12) or prepared in this laboratory. The rabbit antiserum to IgG and subgroup G3 was raised by immunization with DEAE-cellulose-purified IgG from a patient with IgG3 myeloma. This antiserum did not react with purified kappa or lambda light chains. In addition, goat anti-whole human serum (WHS) and anti-IgM (Hyland Div., Travenol Labs, Inc., Costa Mesa, Calif.) were used. The specificity of each antiserum was verified by Ouchterlony analysis and IEP.

Immunoelectrophoresis. The micromethod of Scheidegger (13) was used with 2% Difco purified agar (Difco Laboratories, Detroit, Mich.) in 0.04 M Veronal buffer pH 8.2 at a potential of 5 V/cm for 45 min. The antigen wells, capacity 4 µl, were first filled with fresh carrier WHS followed by three applications of culture fluid. The precipitin patterns were developed over 48 h; the slides were washed first in 0.15 M NaCl, then distilled H₂O with frequent changes for 72 h, dried under filter paper, and stained with amido black. For the analysis of C1q, 1% agarose in 0.05 M PBS was used and the carrier was the H-chain disease plasma Matt., which contains an elevated C1q concentration of 0.410 mg/ml (14). Electrophoresis was for 90 min at 5 V/cm and the

washing and staining as described above. To prevent complexing of C1q to C1r and C1s, 0.01 M trisodium EDTA was included in the buffer.

Autoradiography and synthesis scoring. The stained slides were exposed in sealed boxes for 6 wk to Kodak KK or No Screen X-ray film (Eastman Kodak Co., Rochester, N.Y.) using foam rubber to compress the film firmly to the slides and then developed with Kodak Rapid X-Ray Developer. The intensity of the ARG precipitin lines was scored: ±, questionable; 1+, weak but definite; 2+, moderate; and 3+, intense. Only 1+ or greater ARG lines are considered in the results. To verify that the ARG lines were a direct consequence of in vitro protein synthesis, liver, placenta, and colon from a 25 wk fetus were cultured as shown in the presence of 12.5, 25.0, and 50 µg/ml of puromycin.

Fetal sera. Blood was obtained from 15 of the fetuses by cardiac puncture and/or from the umbilical vessels after careful cleansing of the cord. After clotting for up to 2 h at room temperature, the sera were separated by centrifugation in the cold and stored at -70°C. Studies of total hemolytic complement and component protein concentrations in these sera will be reported in detail later.²

RESULTS

Evidence that the positive ARG's were a direct consequence of in vitro protein synthesis was provided by cultures of liver, placenta, and colon in the presence of puromycin. A progressive decrease in ARG intensity, on both cellulose acetate and IEP with anti-WHS, occurred at concentration of 12.5 and 25 µg/ml puromycin and were completely inhibited at 50 µg/ml. Results with cellulose acetate electrophoresis and IEP using anti-WHS were concordant except for occasional 1 or 2+ single lines in peritoneal and bone marrow cell cultures corresponding to haptoglobin or alpha₂ macroglobulin, whereas the cellulose acetate ARG's were negative.

In each experiment, the culture was judged viable if fetal tissue, other than placenta, demonstrated a positive cellulose acetate ARG and a 1+ or greater ARG precipitin line against anti-WHS on IEP. The viability of the seven individual tissues was similarly considered.

By these criteria, 23 of the 37 cultures were successful. In the 14 fetuses obtained from spontaneous abortions or amniocenteses, placental protein synthesis occurred in 11 but only 2 had synthesis by another tissue, i.e., only 2 were viable. In contrast, 21 of 25 fetuses obtained by hysterotomy or hysterectomy were viable in culture. Viability was 100% for liver (22/22), spleen (21/21), and placenta (19/19) cultures, 93% for colon (18/19), 91% for thymus (20/22), and 74% for bone marrow (15/21) and peritoneal (15/21) cells (Table I).

² Kohler, P. F. Maturation of the human complement system. II. Component levels in fetal and neonatal sera. Manuscript in preparation.

TABLE I
Mean Synthesis Scores of Viable Tissues from 22 Fetal Cultures, Number of Tissues Synthesizing Each Protein, and Protein's Carbon-14 Specific Activity

Tissue	Viable cultures*		C1q	C4	C3	C5	IgG	IgM
	%	Number						
Liver	100	22	0.1 (2)‡	1.2 (16)	2.7 (22)	1.8 (17)	0.2 (4)	0.2 (4)
Spleen	100	21	1.3 (12)	0.9 (10)	0.7 (10)	1.2 (12)	0.8 (9)	0.6 (9)
Placenta	100	19	0	0.9 (11)	0.9 (11)	1.1 (12)	0.6 (9)	0.2 (2)
Peritoneal cells	74	15	0.1 (1)	0.2 (2)	0.2 (2)	0.5 (6)	0	0
Bone marrow cells	74	15	0	0.6 (4)	0.2 (2)	1.1 (6)	0.1 (2)	0.1 (1)
Thymus	91	20	0.1 (1)	1.4 (16)	1.9 (18)	1.6 (15)	0.5 (8)	0.4 (6)
Colon	93	18	0.2 (2)	1.3 (16)	1.4 (15)	1.5 (15)	0.4 (7)	0.4 (6)
Specific activity§			230	172	164	286	117	620
Specific activity relative to C3			1.38	1.03	1.00	1.61	0.70	3.72

* Producing positive ($\geq 1+$) ARG's on cellulose acetate electrophoresis and with anti-WHS on IEP.

‡ Numbers in parentheses indicate number of cultures synthesizing protein.

§ Moles lysine and isoleucine per mole protein.

SITES OF SYNTHESIS

Figs. 1 and 2 illustrate typical ARG results in the different tissues cultured from the same 16 wk fetus. The

C1q precipitins (Fig. 2, left) are diffuse, thereby decreasing the intensity of the ARG produced by the spleen (Fig. 2, right). This occurred frequently with

Immunoelectrophoresis

Autoradiographs

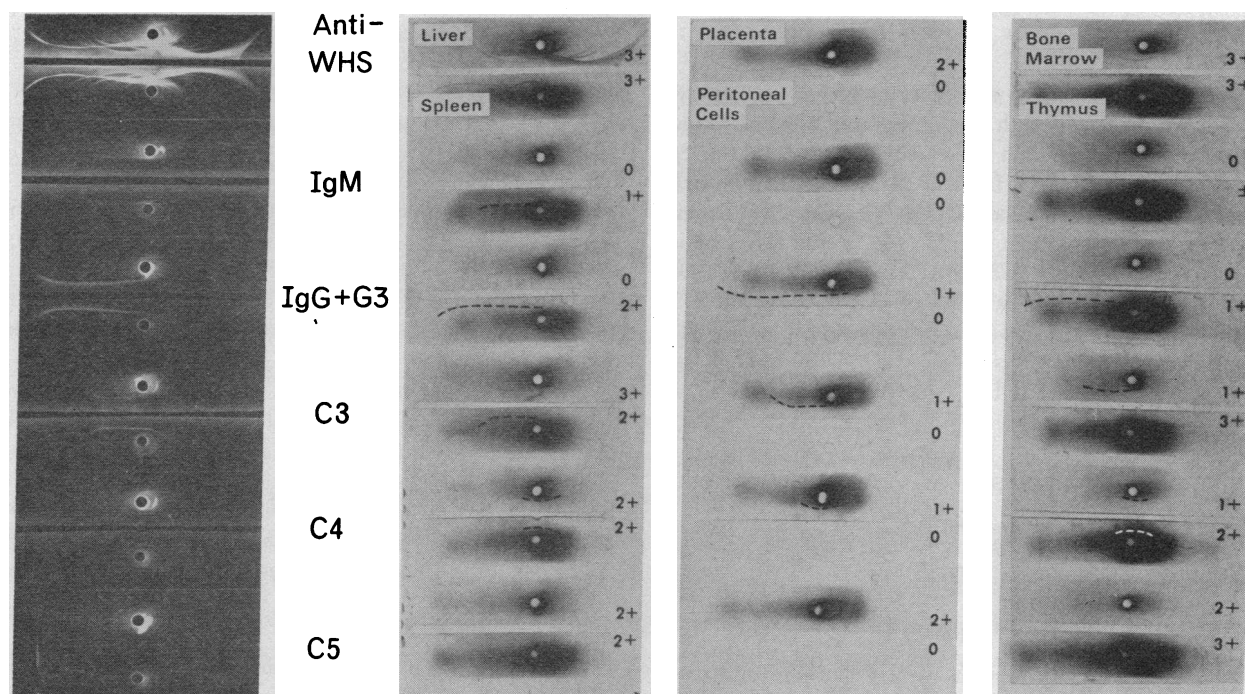


FIGURE 1 Synthesis of C3, C4, C5, and IgG by tissues from a 16 wk fetus. Left: IEP patterns produced by carrier serum and specific antisera. Right: ARG's resulting from in vitro synthesis of protein by liver, spleen, placenta and peritoneal cells, bone marrow, and thymus. Scoring from 0 to 3+ is indicated on the ARG's. Line tracings are provided for specific labeled precipitin lines which are indistinct on reproduction. The peritoneal culture is not viable.

Immunoelectrophoresis Autoradiographs

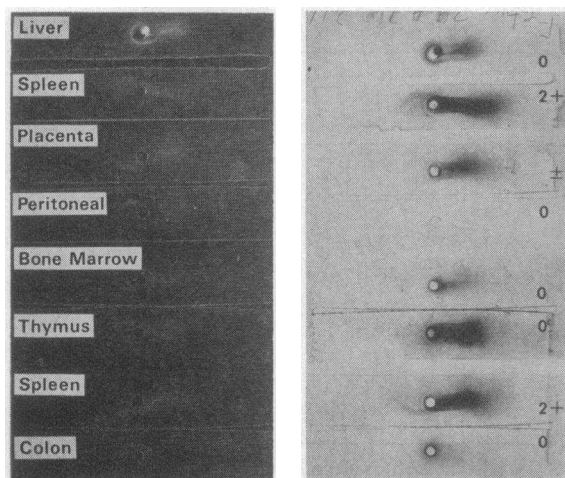


FIGURE 2 Synthesis of C1q by the same 16 wk fetal tissues as shown in Fig. 1. Carrier anti-C1q IEP pattern (left) and ARG (right). Definite (2+) synthesis is present in spleen only while a questionable (±) ARG is present in the placenta. Scoring is indicated on the ARG's.

C1q; however, the other precipitins were consistently sharp, making their ARG's more comparable. Table I is a summary of the mean protein synthesis scores, viability, and the number of each tissue producing the protein.

Liver. The primary site of complement protein synthesis was in liver with 1+ or greater labeling of two or more components in all viable cultures. In the culture of the total body from an 8-wk old fetus, a ±C3 ARG occurred while labeling of C5 was 1+. Definite synthesis was apparent by 11 wk in the two earliest fetal livers studied; C3 synthesis occurred in 100%, C4 in 73%, and C5 in 77% of the livers (Table I). Immunoglobulin G and M synthesis (1+) occurred in four livers from fetuses 11–22 wk old. Weak C1q synthesis was also detected in two livers at 16 and 17 wk.

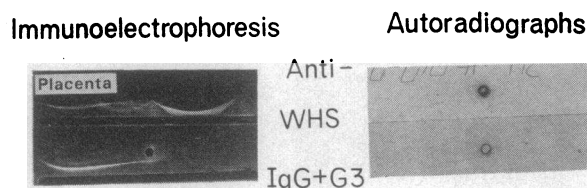


FIGURE 3 Restricted labeling of IEP lines by IgG synthesized by a 14 wk placenta. Carrier IEP patterns vs. anti-WHS and anti IgG-G₃ (left) and ARG's (right). Only the anodal portion of the IgG precipitins are labeled indicating synthesis of an electrophoretically restricted IgG population. This finding was also frequently present in spleen, thymus, and colon.

Spleen. The splenic cultures were of particular interest in that C1q synthesis was limited almost exclusively to their tissue (Fig. 2) and was of much greater magnitude compared to other sites (Table I). C1q synthesis first occurred at 14 wk, considerably later than the other components. Splenic C5 synthesis was essentially equal to C1q followed by C4 and C3. In addition, IgG and IgM production occurred primarily in the spleen and was first detected at 11 wk, increasing in parallel with fetal age.

Placenta. C5, C3, and C4 were the major products of the placenta, but occurred only in 58–63% of cultures. Placental IgG synthesis was present in approximately half of the cultures beginning at 14 wk. In many instances, only the anodal portion of the precipitin arch was labeled as seen in Fig. 3. IgM synthesis was present in a single culture from a 14 wk fetus. No evidence for placental C1q production was found aside from a single ±ARG from one 16 wk placenta (Table I and Fig. 2).

Peritoneal cells. C5 was the primary complement protein synthesized but was produced in only 40% of the viable cultures. Labeled protein with a fast beta mobility corresponding to haptoglobin was uniformly present with 2–3+ scores. A single culture at 20 wk demonstrated 2+ C1q synthesis while none showed IgG or IgM synthesis.

Bone marrow cells. C5 was the major product of the bone marrow cells occurring in 40% of viable cultures followed by C4 in 27%. Minimal synthesis of C3, IgG, and IgM occurred and there was no evidence for production of C1q (Table I). As in the peritoneal cell cultures, labeling of haptoglobin was frequently present.

Thymus. Significant labeling of C3, C4, and C5 occurred in 75–90% of the viable cultures. In addition, IgG and IgM production occurred as early as 14 wk, but was much less in comparison with the complement proteins. Thymic C1q synthesis occurred in one 16 wk fetus.

Colon. Significant synthesis of C3, C4, and C5 in 83–89% of the cultures was apparent as early as 11 wk and was essentially equal for all three components. Weak synthesis of both IgG and IgM was also present at 11 wk increasing in parallel with fetal age to 2+ at 17 wk. C1q synthesis (2+) occurred in a single culture obtained from a 16 wk fetus.

DISCUSSION

The technique of tissue culture in the presence of [¹⁴C]amino acids allows identification of individual protein synthesis by subsequent immunoautoradiography (6–9, 11). In theory, a positive ARG results only from *de novo* protein synthesis. Several precautions are essential for valid interpretations of the ARG results,

however. The use of algal mixtures of [14 C]amino acids may result in nonspecific ARG's secondary to binding of labeled cysteine (9, 11). Nonspecific labeling of alpha₂ macroglobulin has been attributed to its carrier properties and labeling of haptoglobin may occur via its complexing with [14 C]hemoglobin (11). These explanations probably account for the occasional appearance of label in these precipitin lines in cultures which failed to show any other labeled lines when tested against anti-WHS or the various monospecific antisera.

The relative synthetic rates of individual proteins were approximated by using standardized cultures, IEP, and ARG conditions. Scoring from 0 to 3+ was done by the author on several occasions on all ARG's with minor variation. van Furth (11) has reported that under standardized experimental conditions, as little as 0.0002 μ g of labeled immunoglobulins in a precipitin line is detectable by autoradiography and that quantitative comparisons of protein synthesis are possible.

The proportion of lysine and isoleucine (moles lysine and isoleucine per mole protein) in the complement proteins (1) is relatively low but constant: C1q, 230; C4, 172; C3, 164; and C5, 268. In contrast, the proportion is much higher in IgM, 620, and slightly lower in IgG, 117 (15). The 14 C specific activities of these proteins, relative to C3, are C1q, 1.38; C4, 1.03; C3, 1.00; C5, 1.61; IgG, 0.70; and IgM, 3.72 (Table I). Therefore detection of IgM, C5, and C1q was favored in comparison with the other proteins in these experiments. In addition, the fivefold variation in the wet weight of the solid tissues cultured and their great excess compared to the bone marrow and peritoneal cells influenced the quantity of labeled protein recovered as did several indeterminate variables such as amino acid membrane transport and pool size, which may differ in various tissues. Thus, comparisons of synthesis scores in different tissues are gross approximations. More meaningful comparisons can be made between proteins synthesized by the same tissue.

Complement components. Synthesis of at least one component was seen beginning with the youngest fetus. C5 synthesis was the first to occur at 8 wk. In this instance, the entire fetus was cultured. A questionable (\pm) C3 ARG was also produced but no evidence of C1q or C4 production was found. Thereafter, every fetus produced C5 in two or more of the seven tissues cultured. C5 synthesis was greatest, and essentially equal, in liver, thymus, and colon cultures but also occurred in spleen, placenta and peritoneal cells, and bone marrow (Table I). These are the first studies of human C5 synthesis. In the mouse C5 production has been reported to occur in liver and spleen (16).

Both C3 and C4 synthesis began at 11 wk gestation and were present in every culture from this age on-

wards. The liver was the main site of C3 and C4 production but thymus and colon were nearly as active (Table I).

C3 and/or C4 synthesis by human fetal tissues has been studied previously by others with the technique used herein. In 10 fetuses from 10 to 20 wk gestation studied by Adinolfi, Gardner, and Wood (7), hepatic C3 production first occurred in one of three cultures at 10 wk and C4 at 14 wk. Thorbecke, Hochwald, van Furth, Müller-Eberhard, and Jacobsen (6) had earlier observed C3 and C4 synthesis by liver from four fetuses 20-25 wk old. Gitlin and Biasucci (8) have provided evidence for C3 as well as C1 inhibitor production by fetal yolk sac cultures as early as 29 days gestation. In 7 of their 15 fetal cultures, algal hydrolysate mixtures of [14 C]amino acids were used introducing the possibility of nonspecific binding of labeled cysteine to carrier protein.

A distinctive finding was that C1q synthesis was almost exclusively limited to the spleen and began later in gestation than the other components which were produced in essentially all tissues cultured (Figs. 1 and 2, Table I).

Since C1q has receptors for sites on the Fc fragments of IgG and IgM, the possibility of interaction of carrier C1q with labeled IgG and IgM produced in vitro must be considered. No correlation between immunoglobulin and C1q synthesis existed in the present study. In one culture C1q synthesis occurred in the absence of immunoglobulin and in seven cultures IgG and/or IgM synthesis occurred but not C1q (Fig. 4). Therefore, the positive C1q ARG's are not felt to be due to binding of labeled immunoglobulins to carrier C1q.

C1q synthesis is clearly not limited to the spleen in adults as evidenced by serial quantitation of serum C1q concentrations in five adults before and up to 1 mo after splenectomy. In no instance was there a significantly prolonged decreased C1q compared to C4, C3, or C5 after splenectomy.^a

In a previous report of in vitro C1q synthesis (17), employing the same technique used in this study, splenic production occurred in two of two human fetal cultures and three of five cultures from adults. Peritoneal and lung macrophages, however, were more active in C1q as well as C3 and C4 synthesis. After purification of the macrophages by adherence to glass, synthesis of C1q was decreased. These investigators concluded that macrophages were the primary site of C1q, C3, and C4 synthesis. Synthesis of C1q by skin fibroblasts has also been reported (18).

In sharp contrast, Colten, Gordon, Borsos, and Rapp (19) using a hemolytic assay have presented evidence

^a Kohler, P. F. Unpublished observations.

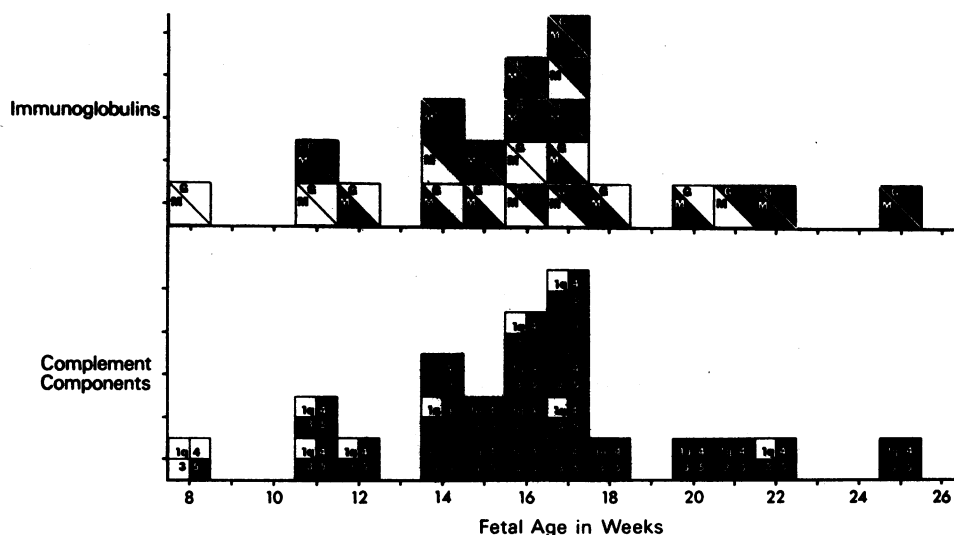


FIGURE 4 Composite summary of protein synthesis by the individual fetuses. Blackened squares indicate synthesis of the complement protein (below) and blackened triangles synthesis of IgG and/or IgM (above). While synthesis of C3, C4, and C5 was uniformly present from 11 wk gestation onward, immunoglobulin synthesis was inconstant and quantitatively much less (see text and Table I). In three fetuses, ages 8, 11, and 16 wk, complement but not IgG or IgM synthesis occurred.

that the entire human trimolecular first component complex, C1q,r,s, is formed primarily, if not exclusively, by colon and ileum *in vitro*. In a single experiment with 19 wk fetal tissue, ileum and colon synthesized hemolytically active C1 but not spleen, stomach, thymus, liver, lung, or kidney. These investigators had reported previously that in the guinea pig, the intestinal epithelial cell was not responsible for biologically active C1 production. A specific cell was not identified in the hemolytically active human colon and ileal cultures.

Immunoglobulins. Synthesis of both IgG and IgM began at 11 wk, similar to the findings of Gitlin and Biasucci (8), but was inconstant in comparison with the regular synthesis of C4, C3, and C5. In three fetuses complement synthesis occurred but not IgG or IgM (Fig. 4). Spleen and colon were the main sites of IgM production while spleen and placenta were for IgG (Table I). Often synthesis of one immunoglobulin occurred without production of the other (Fig. 4). Quantitatively, immunoglobulin production was significantly less than that of C4, C3, and C5 (Table I). This point is further strengthened by the fact that the experimental conditions strongly favored the detection of IgM, specific activity 3.72 relative to C3 (Table I).

Of interest was the frequent confinement of the ARG to the anodal end of the IgG precipitin lines (Fig. 3) suggesting synthesis of an electrophoretically distinct population of gamma heavy chains by fetal tissues. Stecher and Thorbecke (20) have reported a similar occurrence in rodent tissues but attributed it to free

light chain synthesis. In no instance was a ARG present in the IgG3 precipitin line except for two placental cultures. This indicates that IgG3 synthesis by the fetus may begin subsequent to the other subgroup(s).

GENERAL COMMENTS

These findings indicate that during maturation of the human fetus, complement production precedes and is quantitatively greater than that of IgG or IgM. Related studies of serum concentrations of complement proteins in fetuses from this study² and another report (21) of fetal serum levels also support this conclusion. Recent evidence that complement system activation can occur independent of antibody-antigen interactions is consonant with the hypothesis that complement may be a more primitive biologic defense system than specific antibody (2, 3, 22). This hypothesis is also supported by the observations that cobra factor can activate the complement sequence from C3 onwards, via a bypass mechanism involving C3 proactivator (C3 PA), in invertebrates which lack specific antibody protein (23). Investigation of fetal C3 PA synthesis, the protein responsible for the bypass (3) would provide additional evidence bearing on the postulate that ontogenetically complement is a more primitive biologic system than immunoglobulin.

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