

THE IMMUNOLOGICAL DEVELOPMENT OF THE HUMAN FETUS

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The normal human fetus is said not to be engaged in the formation of immunoglobulins; the immunoglobulins present in the serum of the fetus and the newborn are considered to be of maternal origin. In favor of this opinion is the observation that directly after birth the serum of the newborn contains an appreciable amount of IgG, with Gm groups identical with those of the mother (68-70). The level of the IgG of the neonate decreases gradually during the first 3 months (3, 27, 71), which is generally explained by the catabolism of the maternal IgG, whereas the formation by the newborn infant is still inadequate to maintain the original level (16, 27). The best evidence for the transplacental passage is the almost complete absence of IgG in the serum of newborn infants from mothers with agammaglobulinemia (3, 27).

The transfer of the immunoglobulins appears to be a selective process (13, 15). While IgG is readily transferred, there is little, if any, transfer of IgA and IgM, as has been shown by ultracentrifugation and immunoelectrophoretic studies of the serum (12, 16, 19, 20) and the analysis of maternally-transferred antibodies (31-33). With more sensitive techniques such as the double diffusion test in agar or quantitative immunochemical techniques, however, minute amounts of both IgM and IgA have been detected in fetal blood as well as in cord blood from mature and immature neonates (12, 19, 20, 23, 26). Since large molecules do not pass the placenta, this may indicate that the trace amounts of IgM originate in the fetus itself, but transplacental passage has not been completely excluded.

The absence of immunoglobulin formation has been associated with the absence of plasma cells in the lymphoid tissues and bone marrow of the normal human fetus and the newborn. It has been reported that these cells do not appear until a few weeks after birth (2, 3, 14, 25, 34). However, in pathological conditions such as congenital syphilis and toxoplasmosis the human fetus has been found to respond to the antigenic stimulus with intrauterine formation of plasma cells after about the sixth month of gestation (24, 25).

The aim of the present study was to investigate whether the human fetus is capable of producing immunoglobulins and if so, to determine which cells are

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engaged in the synthesis and at which stage of the development this formation starts.

The methods applied were the technique for the synthesis of serum proteins *in vitro* (17), the direct immunofluorescent staining method, the morphological study of fetal tissues, and the analysis of the immunoglobulins in the serum.

Material and Methods

Tissues.—The investigated fetuses fulfilled the condition of having been born alive; the majority died immediately after delivery. The cause of abortion and the age of the fetus calculated according to Scammon and Calkins (35), are given in Table I. In the majority of the fetuses, the liver, spleen, and thymus were obtained directly after death and incubation was started within 2 to 3 hours after birth. A part of the organs was used for immunofluorescent and histological staining prior to incubation. Three exceptions are to be mentioned: fetus 4 was born dead but had probably died immediately before birth because it showed no maceration; for technical reasons the incubation of the tissues from fetus 12 could only be started between 5 and 6 hours after death; fetus 16 died 5 days after premature birth from a septic peritonitis and pneumonia acquired intrauterinely. These 3 fetuses were included because they fulfilled the criterion for the viability of the tissues; *i.e.*, the production of serum proteins by the liver cultures.

Fetal Serum.—Blood was obtained by cardiac puncture. The serum was analyzed by paper electrophoresis, microimmunoelectrophoresis (36), and double diffusion tests in agar (37) in which the antigen hole was filled three or more times with fetal serum. The IgM content of the serum was determined quantitatively with the single radial diffusion method described by Mancini *et al.* (21) and Rümke (62). In this assay an antiserum reacting specifically with IgM was used.

Cultures.—The incubation studies were performed according to Hochwald *et al.*, (17). The medium consisted of Hanks' balanced salt solution (Oxoid), 0.5 per cent ovalbumin (Nutritional Biochemicals Corporation, Cleveland, Ohio), glucose (up to 22 mM), 1 per cent BME vitamin mixture (Microbiological Association Inc., Bethesda, Maryland), 100 U/ml penicillin G, 2 ml of the amino acid mixture (38) from which lysine and isoleucine were omitted and to which was added 1 μ c/ml C¹⁴ L-lysine (UL, 124 μ c/mM, Schwarz Bio Research, Inc., Orangeburg, New York), and 1 μ c/ml C¹⁴ L-isoleucine (UL, 110 μ c mM, Schwarz Bio Research, Inc.). The pH was adjusted to 7.4 with a 3.75 per cent solution of sodium carbonate. The tissues were minced and a capillary pipette was used to place the fragments against the wall of a standard roller tube. The wet weight of the cultured tissue fragments varied from 68 to 375 mg. For each culture 1 ml culture medium containing 2 μ c/ml was used. The cultures were incubated for 48 hours at 37°C under continuous rotation and then frozen at -20°C.

After thawing, the cell-free culture fluid was dialyzed against 0.015 M phosphate buffer (pH 7.6) for 72 hours at 4°C, concentrated by lyophilization, and then dissolved in 0.1 ml bidistilled water.

Microimmunoelectrophoretic analysis was carried out with the culture fluid as described by Hochwald *et al.* (17). Normal human serum was used as the carrier serum. All the culture fluids were analyzed by autoradiography of the immunoelectrophoretic patterns obtained with an equine anti-human antiserum and with a rabbit anti-IgA or anti-IgM serum (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) and anti-IgD antiserum (kindly donated by Dr. D. S. Rowe). Autoradiography was performed at room temperature with a sheet film (Kodak RS pan, 650 ASA). The exposure time was 21 days. The films were developed with a 10 per cent Rodinal solution (Agfa) for 10 minutes at 20°C.

Immunofluorescence.—Immunofluorescent staining was performed on frozen sections and cell suspensions. The sections of an indicated thickness of 2 to 4 μ were cut with a rotary

microtome in a cryostat at -20°C from tissue samples quick frozen with CO_2 and stored at -90°C . The slides were fixed with 5 per cent acetic acid in 96 per cent ethanol for 15 minutes at -20°C , and then washed in phosphate-buffered saline (pH 7.2) at 4°C for 1 hour with four changes. Cell suspensions were prepared by means of a Borel mincer (4, 63), and subsequently washed in a solution containing 5 per cent bovine albumin, 1 per cent EDTA, and 100 U/ml penicillin G in phosphate-buffered saline (pH adjusted to 6.8). The cell suspensions were centrifuged for 10 minutes at 175 g at 4°C . From the sedimented cells suspended in 0.5 ml of the washing solution, slides were made with a sedimentation apparatus (4, 39, 40).

Peripheral blood preparations were made from blood collected in 5 per cent EDTA solution in phosphate-buffered saline. The sample was twice centrifuged for 3 minutes at 175 g at 4°C . The plasma layers were collected and centrifuged again for 10 minutes at 175 g. The sediment was then suspended in the bovine albumin washing solution and further prepared as described for tissue cell suspensions.

The conjugated antisera used for immunofluorescence were specific for only one immunoglobulin. The anti-IgG and anti-IgA antisera were obtained by immunizing rabbits with purified immunoglobulin preparations with Freund's adjuvants according to Cohn (41), followed by a series of intravenous injections with alum precipitates (42). The anti-IgM conjugate was obtained commercially (Roboz, lot 3112). After appropriate adsorption with the different immunoglobulin fractions and Bence-Jones proteins of both types, the antisera were tested for specificity by the immunodiffusion test (43). A combination of the antiserum conjugated with fluoresceine isothiocyanate and a counter stain of lissamine-rhodamine-labeled 2 per cent bovine albumin solution was used (22). After appropriate dilution both conjugates were mixed immediately before use. The slides were stained for 30 minutes, then washed in phosphate-buffered saline for 15 minutes, mounted in buffered glycerol, and sealed with paraffin. Further details have been published elsewhere (5, 6).

Histology.—For histological studies the tissues were fixed for maximally 5 hours in a mixture of Zencker's solution, 10 per cent formaldehyde, and 2 per cent trichloroacetic acid (90:5:5). The sections were stained with methyl green-pyronin according to Brachet (44). Cytological preparations made from the cell suspensions were stained with Giemsa stain.

RESULTS

Liver.—The *histological sections* showed the characteristic fetal liver structure with a considerable number of pyroninophylic cells, mainly hemopoetic blast cells. The presence of lymphoid blast cells could not be established with any degree of certainty, and no plasma cells were observed.

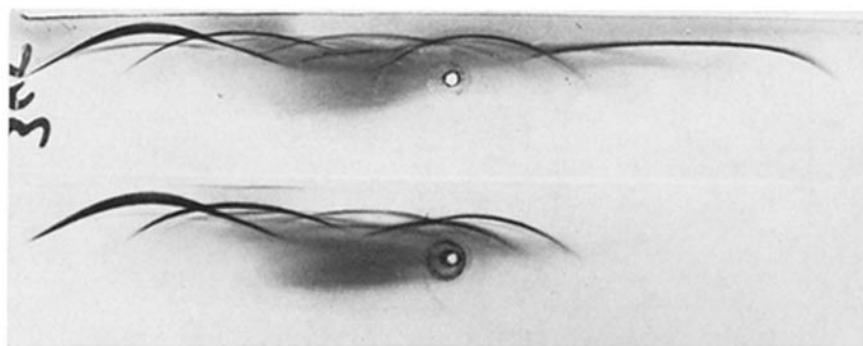
Samples of the liver were cultured from all the fetuses to serve as control for the viability of the tissues. These *cultures* showed an intense labeling of the albumin, α_{1A} -globulin, α_2 -globulin, Gc-globulin, α_2 -macroglobulin, siderophilin, and hemoglobin-haptoglobin or β_{1A} -globulin lines (Text-Fig. 1). None of the liver samples showed synthesis of immunoglobulins. A labeled spot observed on the autoradiographs on the cathodic side of the antigen reservoir is probably due to radioactive fetal hemoglobin. The cultures of fetuses 12, 16, and 19 showed only a weak labeling of the albumin, α - and β -globulin lines for the reasons mentioned in Table I, but were nevertheless included in this study.

The immunofluorescent staining showed for the majority of the liver samples only a few scattered weakly IgM-positive cells with a small rim of cytoplasm, most probably small lymphocytes.

Spleen.—The development of the white pulp could be observed in the *histo-*

logical sections. In the younger fetuses the white pulp showed only small aggregates of large cells with a small rim of weakly pyroninophylic cytoplasm and a large, chromatin-poor nucleus often containing a nucleolus, probably lymphoid blast cells. Only a few lymphocytes were present. The older fetuses showed an increase in both the amount of white pulp and the number of lymphocytes (Fig. 1). Germinal centers were not observed. A few cells scattered in the red pulp showed a more distinctly pyroninophylic cytoplasm and a round chromatin-rich nucleus, resembling cells of the plasma-cell family.

The autoradiographic image of the *cultures* showed that before the nineteenth week of gestation all spleen cultures were negative, but thereafter



TEXT-FIG. 1. *Fetal liver.* Above: Immunoelectrophoretic pattern of carrier serum and culture fluid of fetus 3, developed with horse anti-human antiserum. Below: Autoradiograph with labeling of albumin, prealbumin, α_{1A} -globulin, α_2 -globulin, Gc-globulin, α_2 -macroglobulin, siderophilin, and β_{1A} -globulin or hemoglobin-haptoglobin lines.

fourteen out of sixteen samples showed the synthesis of immunoglobulins. In fourteen cultures the IgG line and in thirteen cultures the IgM line was found to be labeled (Table I, Text-figs. 2 and 3). In the majority of the samples the intensity of the IgM line was equal to that of the IgG line and in two samples it was distinctly greater. No synthesis of IgA and IgD could be detected in any of the cases.

The weights of the culture could not be correlated with the results of the autoradiography, but it must be kept in mind that these weights are certainly only approximate because of the differences to be expected in the water content of these fetal tissues.

Immunofluorescent staining was carried out on frozen sections and cell suspensions. The cell suspensions from fetuses older than 20 weeks showed a moderate amount of cells positive for IgG. These cells were either medium sized cells or cells with an eccentric nucleus, probably cells of the plasma-cell family (Fig. 2). The single spleen-cell suspension investigated by immunofluorescence before

this stage was found to be negative for IgG. The staining for IgA was consistently negative in all the sections and cell suspensions, regardless of age.

Cells containing IgM were found in all cases. To the extent that the morphology could be judged in the frozen sections, the positive cells were large, medium

TABLE I
Results of the Autoradiography of Human Fetal Spleen Cultures and the IgM Content in the Fetal Serum

Fetus No.	Causes of abortions*	Crown-heel length	Fetal age†	Culture weight	Autoradiography‡		Serum IgM
					IgG	IgM	
		<i>cm</i>	<i>wks</i>	<i>mg</i>			<i>mg/ml</i>
1	U	9	13	68	—	—	¶
2	U	12	14	79	—	—	¶
3	U	17	17	143	—	—	—
4	P	18	18	114	—	—	—**
5	U	20	19	117	(+)	(+)	—
6	P.C.	22.5	20	158	—	—	0.010
7	C.S.	23.5	21	206	+	+	—
8	U.	24	21	151	(+)	(+)	—
9	C.S.	24.5	21	173	+	+	0.016
10	M.P.	26	22	113	+	+	0.022
11	M.P.	27	22	181	+	++	—**
12	H.A.	30	24	175‡‡	—	—	0.017
13	C.I.	30	24	154	+	+	0.081
14	I.A.	30.5	25	310	+	+	0.027
15	I.A.	31	25	182	+	+	—**
16	S.I.	33	27	373§§	+	+	0.146
17	P.C.	33	27	241	+	+	0.013
18	C.I.	34.5	28	171	+	++	0.031
19	C.I.	38	30	324	(+)	—	0.012
20	U.	39.5	31	262	+	+	0.013

The intensity of the autoradiographic image is graded from — = negative; (+) = just visible; + = clearly visible; to ++++ = very dark.

* U = unknown; P = pneumonia of the mother; P.C. = placenta circumvallata; I.A. = self-induced abortion; C.S. = cesarean section; M.P. = multiple pregnancy, twins; H.A. = habitual abortion; C.I. = cervical incompetence; S.I. = septic infection, peritonitis and pneumonia of the fetus which died 5 days after birth.

† Calculated from the crown-heel length (L) in cm with the formula: $T = (L/28 + 1.25)^2 + 0.74$ in which T = fetal age in months (35).

‡ IgA and IgD were always negative and are therefore not tabulated.

|| The figures represent the average of two determinations done with the single radial diffusion test (21).

¶ No serum available.

** Only determined in the double diffusion test in agar gel.

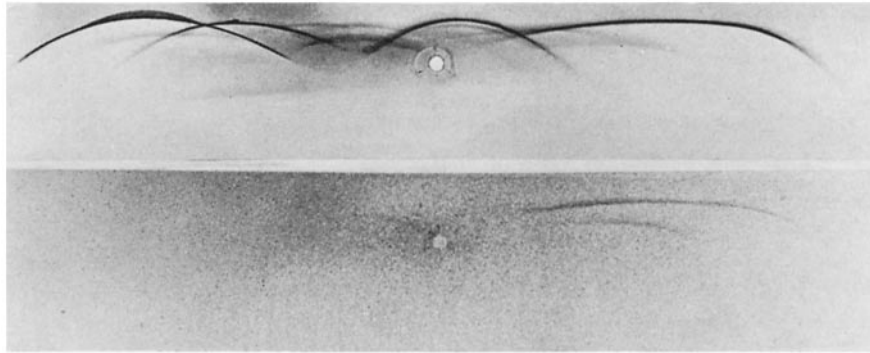
‡‡ Cultured 5 to 6 hours after death;

§§ Culture infected;

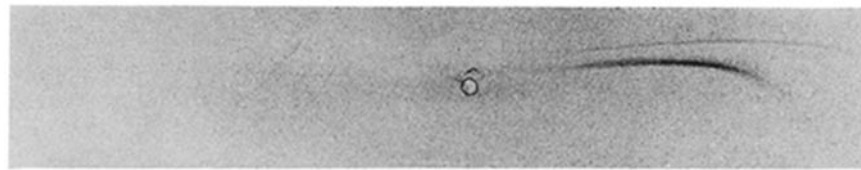
||| Unknown reason;

} resulting in weak labeling of the liver cultures.

sized, and small lying in aggregates in the white pulp region. In the youngest fetuses only a few weakly positive cells were seen; the fetuses aged from 22 to about 27 weeks showed small aggregates of cells with weak to clearly fluorescent cytoplasm; and in the older group of fetuses large aggregates of brilliantly fluorescent cells were observed (Fig. 1). Scattered in the red pulp, positive cells resembling cells of the plasma-cell family were seen. The same type of cells,



TEXT-FIG. 2. *Fetal spleen*. Above: Immunoelectrophoretic pattern of carrier serum and culture fluid of fetus 15, developed with horse anti-human antiserum. Below: Autoradiograph with labeling of IgG and IgM line.



TEXT-FIG. 3. *Fetal spleen*. Autoradiograph with labeling of IgG and IgM line (culture fluid of fetus 11).

and even an occasional Russell body, were found in the cell suspensions (Text-fig. 2). In addition, the suspensions showed numerous cells with a small rim of weakly fluorescent cytoplasm, most probably small lymphocytes (Fig. 2).

Thymus.—The *histological sections* of the fetal thymus showed a cortex and a medulla containing Hassall's bodies. The younger fetuses were characterized by a smaller cortical area. The fetal thymus showed a larger number of reticular cells than the adult thymus; these appear as cells with a large polygonal chromatin-poor nucleus and a distinct rim of pyroninophilic cytoplasm. The majority of the lymphoid cells are small lymphocytes, but scattered in the cor-

tex and to a greater extent in the medulla, medium sized cells with a clear rim of pyroninophilic cytoplasm were seen. Cells of the plasma-cell family could not be demonstrated with certainty.

Samples obtained from 17 fetuses were cultured. Fifteen *cultures* showed no formation of immunoglobulins. One thymus culture (fetus 10) demonstrated a very weakly labeled IgG line, and the other sample, obtained from the intrauterinely infected fetus (No. 16), showed a clearly labeled IgG line.

The *immunofluorescent staining* showed an occasional IgG-positive medium sized cell. No IgA-positive cells could be detected. In most of the sections a few IgM positive cells were seen. These cells lay scattered in the tissue and for the most part resembled small lymphocytes, but occasionally a medium sized cell and once even a cell of the plasma-cell family were observed.

Peripheral Blood.—The total *protein content* of the sera ranged from 2.6 to 6.0 g per cent with a gamma globulin content of 0 to 7.5 per cent. As the fetal age advanced, the total serum protein and gamma globulin content increased. The immunoelectrophoretic pattern of these sera showed precipitation lines for albumin, several α - and β -globulins, and IgG. The sera of the younger fetuses were distinguished by fewer α -globulin lines. No IgA or IgM lines could be detected.

With the double diffusion test in agar no IgM was detected in sera from fetuses younger than 22 weeks. However, after this stage 8 out of 11 sera were found positive for IgM.

With the single radial diffusion test the first positive serum sample was found in a 20-week-old fetus. Eleven out of the 15 fetuses aged 20 weeks and older were positive in this test, which proved to be slightly more sensitive than the double diffusion test. The IgM content varied from 0.010 to 0.146 mg/ml (Table I). The highest value was found for the serum of the intrauterinely infected fetus (No. 16).

In four cases peripheral blood from fetuses aged 21, 24, 27, and 28 weeks was investigated by *immunofluorescent staining*. The three older ones showed an occasional IgG-positive medium sized cell, but the staining for IgM showed in all four samples several positive medium sized lymphoid cells and a considerable number of cells with a small rim of weakly positive cytoplasm, most probably small lymphocytes (Fig. 3).

DISCUSSION

From the results of these investigations it may be concluded that the human fetus is capable of synthesizing IgG and IgM from about the twentieth week of gestation onwards. The localization of this production is mainly in the spleen; this organ showed the synthesis of immunoglobulins in tissue cultures from fourteen out of sixteen samples taken after the nineteenth week of gestation. All four samples from younger fetuses failed to show labeling of immunoglobu-

lins. These results were substantiated by the immunofluorescent staining and analysis of the circulating immunoglobulins.

The immunofluorescent staining of the spleen indicates that lymphoid cells in the white pulp and also cells of the plasma-cell family are engaged in this immunoglobulin production. The extent and intensity of the fluorescence agrees well with the developmental enlargement of the white pulp.

Analysis of the immunoglobulins in the serum demonstrates that a small amount of IgM is present from the twentieth week onwards. It was found that IgM could be demonstrated in the serum at about the same stage of gestation as in the spleen. The IgM content of the fetal sera is on the order of one tenth of the IgM content of the serum of normal adults (64).

The question arises whether these fetuses can be taken as representative for the normal situation. The similar results from cases of confessed self-induced abortion, therapeutically induced abortion by cesarean section, as well as other causes, and the detailed histories taken from the mothers justify the assumption that our fetuses had not been excessively stimulated by bacterial, viral, auto- or isoantigens, except in the two cases with infection. The first (No. 4) in which the mother suffered from pneumonia, was entirely negative. This result agrees with those obtained in other fetuses of the same age group. The other case (No. 16) failed to show the increased synthesis of immunoglobulins expected because this 27-week-old fetus had suffered from an intrauterine septic peritonitis and pneumonia and had survived for 5 days. The serum already contained an appreciable level of IgM. Bacterial contamination of the culture fluid may be held responsible for the absence of increased synthesis as judged autoradiographically.

The control experiments demonstrating the specificity of the applied techniques, the formation of the immunoglobulins *in vitro*, and the immunofluorescent staining have been published and discussed elsewhere (5, 6).

Liver cultures were made for each fetus to check the viability of the tissues. All fetuses showing no labeling of serum proteins were excluded. The three cultures which showed a weak labeling, due to the causes indicated in Table I, are included in the results. It is likely that the viability of these fetal tissues was not optimal. This may explain the almost complete absence of immunoglobulin synthesis *in vitro* of fetuses 12 and 19, which could be expected to have given positive results.

In the majority of the samples the autoradiographic lines of the spleen cultures showed an equal intensity for IgG and IgM, and in two samples a distinctly greater synthesis of IgM. In cultures of the adult spleen, however, IgG was often labeled to a greater extent than IgM (5, 7). It may therefore be concluded that, in contrast to the adult situation, the fetal spleen synthesizes relatively more IgM than IgG. This result closely resembles the picture found in the primary response postnatally, which is also associated with a greater

IgM production as compared to the synthesis of IgG antibodies (34, 45-49). This resemblance supports the view that the antigenic stimulus in the fetus is predominantly primary.

This formation of IgM during the fetal period agrees well with the minute amounts of IgM detected in the fetal blood and cord blood from immature and mature neonates (12, 19, 20, 23, 26). The present study also demonstrated a small amount of IgM in fetal sera after the twentieth week of gestation. However, the presence of minute amounts IgG produced by the fetus cannot be determined by immunodiffusion techniques because of the presence of the IgG from the mother.

Although the fetus *in utero* has been considered to be protected against antigenic stimuli, the intense maternal fetal communication makes it conceivable that antigenic substances may pass to the fetus and provoke a very slight immune response which results in immunoglobulin production. It may be supposed, for instance, that the maternal IgG in the fetal circulation acts as an antigen because it has been demonstrated recently that antigenic determinants of the foreign IgG molecules may induce formation of agglutinating antibodies after multiple blood transfusions (1). Recently, Fudenberg and Fudenberg (9) demonstrated the synthesis of IgG in the human fetus at the seventh month of gestation by the presence of anti-Gm(a) agglutinins in the serum of the mother in a case of maternal fetal incompatibility for Gm(a). Using the same approach, Mårtensson and Fudenberg (67) have extended this finding by establishing the presence of IgG in the cord blood synthesized by the human fetus.

The immunoglobulins are generally regarded as carriers of antibody activity (50), although the possibility of immunoglobulins lacking antibody activity has been raised (28, 29, 51). The possibility must therefore be envisaged that the immunoglobulins synthesized in the human fetus are not associated with antibody activity but are so-called normal immunoglobulins. This problem was not investigated in this study.

Since transplacental passage of small numbers of lymphocytes has been reported (52), the possibility that the immunoglobulin synthesizing cells of the fetus are of maternal origin must be kept in mind. However, the correlation between the histological development of the lymphoid system of the spleen, its immunofluorescent patterns, and the onset of the immunoglobulin synthesis favors a fetal origin of the immunoglobulin synthesizing cells.

The immunoglobulin-positive lymphoid cells of the peripheral blood, which consist predominantly of IgM-positive small lymphocytes and an occasional IgG- and IgM-positive medium sized cell, very probably originate in the fetal spleen. In other cases of primary immunization such as infectious mononucleosis and rubella, the peripheral blood from adults has been found to contain a large number of IgM-positive small lymphocytes, in contrast to blood from normal donors in which only a small number of small lymphocytes have been

found to be IgM-positive (5, 8). Therefore, only a small number of these cells could be expected in the fetal peripheral blood in case of transplacental passage of the IgM-positive small lymphocytes from the mother. The possible significance of these IgM-positive small lymphocytes in relation to primary antibody response has been discussed elsewhere (5, 8).

Two of the seventeen thymus samples showed a slight synthesis of only IgG. Both samples were obtained from fetuses whose spleen also synthesized immunoglobulins. One positive thymus culture, obtained from a fetus (No. 16) which died 5 days postnatally from a septic infection, gave results analogous to the neonatal findings (7). The other thymus was obtained from one of a twin birth (No. 11). The immunofluorescent staining revealed an occasional IgG- or IgM-positive medium sized cell and a few IgM-positive small lymphocytes. Mellors and Korngold (53) have also described an occasional IgM-positive cell in the thymus of 30-week-old twin fetuses which succumbed 2 days after delivery. The results obtained in the fetal thymus differed from those deriving from the postnatal situation in which the normal infant and adult thymus showed the production of IgG and IgA and the presence of immunoglobulin-positive medium sized lymphocytes and plasma cells. These findings have been discussed in detail in another publication where the hypothesis was put forward that the cells responsible for the immunoglobulin synthesis in the thymus originate not from this organ but from other lymphoid organs such as the spleen, and are transported by the blood to the thymus (5, 7). It seems probable that in the fetal thymus, too, the immunoglobulin-positive cells derive from the peripheral blood and that the synthesis of IgG in two of our samples might be explained in the same way. However, the absence of IgM formation in both the adult and the fetal thymus requires further elucidation.

The present observations demonstrate that the lymphoid spleen cells of a 20-week-old human fetus are already immunologically competent. Although a quantitative evaluation of the activity by means of the applied techniques is hazardous, it seems that under normal conditions the production of IgG and IgM is low. No synthesis of IgA was found in the fetal spleen. The small amount which can be found in the serum (5, 19, 20, 33, 26) is probably of maternal origin. Our studies also failed to reveal the formation of IgD, which is in agreement with its absence in the cord blood (66).

Our findings accord with the observation that in congenital syphilis the human fetus may respond with plasma-cell proliferation after 6 months of gestation (24, 25). In contrast to our observations in human fetal tissues of occasional cells of the plasma-cell family, which are easily seen not only with immunofluorescent technique but also in the preparations stained with methyl green-pyronin, are the reports of several authors who state that these cells are not present in the normal human fetus (2, 3, 25). It is possible that the techniques used in this study were more suitable for the recognition of these cell structures.

The present results are comparable to those obtained in fetal tissues from animals for which the production of immunoglobulins has also been reported recently. Culture experiments with lymph node and spleen tissues of unstimulated fetal lamb and guinea pigs have shown that spleen tissue synthesizes predominantly IgM, whereas lymph nodes show some production of IgG and IgM (54, 55). Thymus tissue from fetal and newborn animals generally show no immunoglobulin synthesis *in vitro* (56, 57, 65). Antigenic stimulation of fetal animals may result in an increased production of IgM *in vitro* (55), the formation of antibodies *in vitro* (58-60), and a proliferation of plasma cells (61).

In view of Haeckel's law (18) which states that ontogeny repeats phylogeny, it would be interesting to see whether the human fetus, which starts to synthesize IgG and IgM after the twentieth week of gestation, could be compared to a specific stage of the phylogenetic development of adaptive immunity (30). It is tempting to speculate that during the second trimester of gestation the human fetus reaches a situation comparable to the one found in the adult lamprey, this animal being the most primitive of the vertebrates to show the presence of immunoglobulins, antibody production, and proliferation of lymphoid cells after antigenic stimulation, lymphoid cells in the peripheral blood, and small lymphoid foci in the spleen (10, 11).

SUMMARY

The immunogenesis of the human fetus has been investigated by means of the formation of immunoglobulins *in vitro*, immunofluorescence, morphological studies, and analysis of the immunoglobulins in the serum. Twenty fetuses which were born alive but died soon after delivery, were studied; their ages ranged from 13 to 31 weeks.

The results of the spleen cultures demonstrated the synthesis of IgG and IgM, which starts at about the twentieth week of gestation. In the serum, IgM could be detected at about the same period. The immunofluorescent staining of the spleen tissue showed that medium sized and large lymphoid cells as well as plasma cells, even with Russell bodies, were positive for either IgG or IgM. The peripheral blood was also found to contain a small number of medium sized IgG and IgM-positive cells. Both the spleen and the peripheral blood showed a considerable number of fluorescent small lymphocytes which exclusively contained IgM. The relatively high ratio of IgM to IgG production prenatally as compared to the postnatal situation, agrees with a predominantly primary antibody response in fetal life.

In general, the fetal thymus did not synthesize immunoglobulins. No indications for the synthesis of IgA and IgD during fetal life were found.

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BIBLIOGRAPHY

1. Allen, J. C., and Kunkel, H. G., Antibodies to genetic types of gamma-globulin after multiple transfusions, *Science*, 1963, **139**, 418.
2. Black, M. M., and Speer, F. D., Lymph node reactivity. II. Fetal lymph nodes, *Blood*, 1959, **14**, 848.
3. Bridges, R. A., Condie, R. M., Zak, S. J., and Good, R. A., The morphological basis of antibody formation development during the neonatal period, *J. Lab. and Clin. Med.*, 1959, **153**, 331.
4. Balfour, B. M., Cooper, E. H., and Alpen, E. L., Morphological and kinetic studies on antibody-producing cells in rat lymph nodes, *Immunology*, 1965, **8**, 230.
5. van Furth, R., The formation of immunoglobulins by human tissues *in vitro*, Doctorate Thesis, Leiden, 1964.
6. van Furth, R., Schuit, H. R. E., and Hijmans, W., The formation of immunoglobulins by human tissues *in vitro*. I. The methods and their specificity, in preparation.
7. van Furth, R., Schuit, H. R. E., and Hijmans, W., The formation of immunoglobulins by human tissues *in vitro*. III. Spleen, lymph nodes, bone marrow and thymus, in preparation.
8. van Furth, R., Schuit, H. R. E., and Hijmans, W., The formation of immunoglobulins by human tissues *in vitro*. IV. Circulating lymphoid cells, in press.
9. Fudenberg, H. H., and Fudenberg, B. R., Antibody to hereditary human gamma-globulin (Gm) factor resulting from maternal-fetal incompatibility, *Science*, 1964, **145**, 170.
10. Finstad, J., and Good, R. A., The evolution of the immune response. III. Immunological responses in the lamprey, *J. Exp. Med.*, 1964, **120**, 1151.
11. Finstad, J., Papermaster, B. W., and Good, R. A., Evolution of immune response. II. Morphological studies on the origin of the thymus and organized lymphoid tissue, *Lab. Invest.*, 1964, **13**, 490.
12. Franklin, E. C., and Kunkel, H. G., Comparative levels of high molecular weight (19 S) gamma-globulin in maternal and umbilical cord sera, *J. Lab. and Clin. Med.*, 1958, **52**, 724.
13. Brambell, F. W. R., The passive immunity of the young mammal, *Biol. Rev.*, 1958, **33**, 488.
14. Good, R. A., Studies on agammaglobulinemia. II. Failure of plasma cell formation in the bone marrow and lymph nodes of patients with agammaglobulinemia, *J. Lab. and Clin. Med.*, 1955, **46**, 167.
15. Hemmings, W. A., and Brambell, F. W. R., Protein transfer across the foetal membranes, *Brit. Med. Bull.*, 1961, **17**, 96.

16. Hitzig, W. H., *Die Plasmaproteine in der klinischen Medizin*, Berlin, Springer-Verlag, 1963.
17. Hochwald, G. M., Thorbecke, G. J., and Asofsky, R., A new technique for the demonstration of the synthesis of individual serum proteins by tissues in vitro, *J. Exp. Med.*, 1961, **114**, 459.
18. Heackel, E., Die Gastraea-Theorie, die Phylogenetische Classification des Tierreichs und die Homologie der Keimblätter, *Jenaische Z. Med. und Naturwissensch.*, 1874, **8**, 1.
19. de Mural, G., and Roulet, D. L. A., Étude immunologique des protéines sériques foetales humaines, *Helv. Paediat. Acta*, 1961, **16**, 517.
20. de Mural, G., La maturation de l'immunité humorale chez l'homme, *Helv. Med. Acta*, 1962, **29**, suppl. 42.
21. Mancini, R., Vaerman, J. P., Carbonara, A. O., and Heremans, J. F., A single-radial-diffusion method for the immunological quantitation of proteins, in *Protides of the Biological Fluids*, Amsterdam, Elsevier Publishing Company, 1963, 370.
22. Nairn, R. C., *Fluorescent protein tracing*, (R. C. Nairn, editor), Edinburgh, E. and S. Livingstone, Ltd., 1964.
23. Roth, N., Zur semiquantitativen Erfassung der beiden Serum-Immunglobuline β_{2A} und β_{2M} im Neugeborenen- und Kindesalter, *Ann. Paediat.*, 1962, **199**, 548.
24. Silverstein, A. M., Congenital syphilis and the timing of immunogenesis in the human foetus, *Nature*, 1962, **194**, 196.
25. Silverstein, A. M., and Lukes, R. J., Fetal response to antigenic stimulus. I. Plasmacellular and lymphoid reactions in the human fetus to intrauterine infection, *Lab. Invest.*, 1962, **11**, 918.
26. West, C. D., Hong, R., and Holland, N. H., Immunoglobulin levels from the newborn period to adulthood and in immunoglobulin deficiency states, *J. Clin. Invest.*, 1962, **41**, 2054.
27. Zak, S. J., and Good, R. A., Immunochemical studies of human gamma globulins, *J. Clin. Invest.*, 1959, **38**, 579.
28. Pernis, B., Cohen, M. W., and Thorbecke, G. J., Specificity of reaction to antigen stimulation in lymph nodes of immature rabbits. I. Morphological changes and γ -globulin production following stimulation with diphtheria toxoid and silica, *J. Immunol.*, 1963, **91**, 541.
29. Sterzl, J., Kostka, J., Rina, I., and Mandel, L., Attempts to determine the formation and character of gammaglobulin and of natural and immune antibodies in young pigs reared without colostrum, *Folia Microbiol.*, 1960, **5**, 29.
30. Good, R. A., and Papermaster, B. W., Ontogeny and phylogeny of adaptive immunity, *Advances Immunol.*, 1964, **4**, 1.
31. Freda, V. J., Placental transfer of antibodies in man, *Am. J. Obstet. Gynecol.*, 1962, **84**, 1535.
32. Vahlquist, B., The transfer of antibodies from mother to offspring, *Advances Pediat.*, 1958, **10**, 305.
33. Hitzig, W. H., Über die transplacentare Übertragung von Antikörpern, *Schweiz. Med. Woch.*, 1959, **89**, 1249.

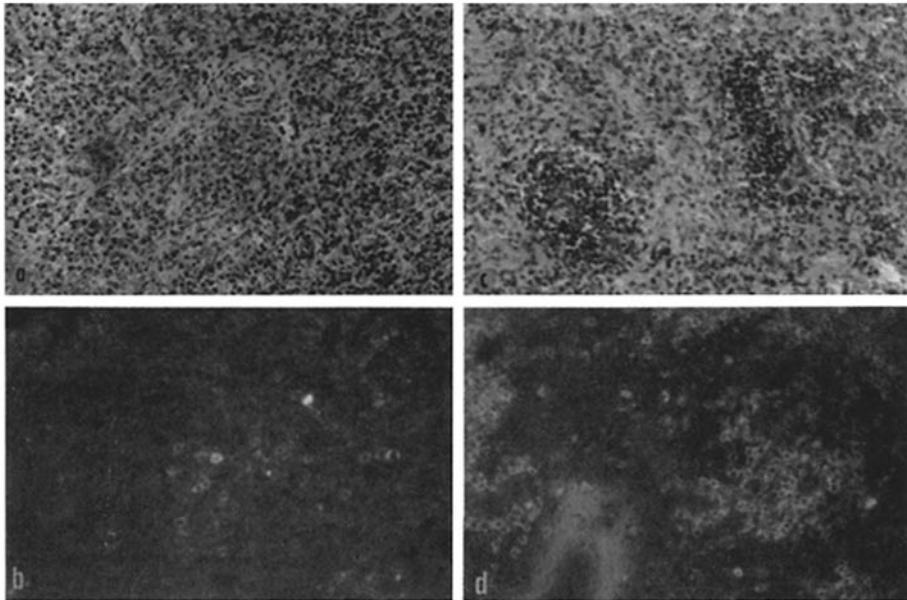
34. Smith, R. T., Response to active immunization of human infants during the neonatal period. Cellular aspects of immunity, *Ciba Foundation Symposium*, (G. E. W. Wolstenholme, and M. O'Connor, editors), London, J. and A. Churchill, Ltd., 1960, 348.
35. Scammon, R. E., and Calkins, L. A., The development and growth of the external dimensions of the human body in the fetal period, Minneapolis, The University of Minnesota Press, 1929.
36. Scheidegger, A., Une microméthode de l'immuno-électrophorese, *Int. Arch. Allergy*, 1955, **7**, 103.
37. Ouchterlony, Ö., Diffusion-in-gel methods for immunological analysis. II, *Progr. Allergy*, (P. Kallós, and B. H. Waksman, editors), Basel, S. Karger, 1962.
38. Neuman, R. E., and McCoy, Th. A., Growth-promoting properties of pyruvate, oxalate and α -ketoglutarate for isolated Walker carcinosarcoma 256 cells, *Proc. Soc. Exp. Med.*, 1956, **98**, 304.
39. Sayk, J., Cytologie der cerebro-spinal Flüssigkeit, Jena, Fischer Verlag, 1960.
40. Bots, G., Went, L. N., and Schaberg, A., Een toestel voor cytologisch onderzoek van de liquor cerebro-spinalis, *Ned. T. Geneesk.*, 1963, **107**, 445.
41. Cohn, M., Production of antibodies in experimental animals, *Methods Med. Research*, 1952, **5**, 271.
42. Kabat, E. A., and Mayer, M. M., Experimental immunochemistry, Springfield, Charles C Thomas, 1961.
43. Fahey, J. L., and McLaughlin, C., Preparation of antisera specific for 6.6 S gamma-globulins, β_{2A} -globulins, gamma₁-macroglobulins and for type I and II common gamma-globulin determinants, *J. Immunol.*, 1963, **91**, 484.
44. Brachet, J., The use of basic dyes and ribonucleose for the cytological detection of ribonucleic acid, *Quart. J. Micr. Sc.*, 1953, **94**, 1.
45. Bauer, D. C., and Stavitsky, A. B., On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens, *Proc. Nat. Acad. Sc.*, 1961, **47**, 1667.
46. Fink, C. W., Miller, W. E., Dorward, B., and LoSpalluto, J., The formation of macroglobulin antibodies. II. Studies on neonatal infants and older children, *J. Clin. Invest.*, 1962, **7**, 1422.
47. Uhr, J. W., and Finkelstein, M. S., Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage Φ X 174, *J. Exp. Med.*, 1963, **117**, 457.
48. Svehag, S. E., and Mandel, B., The formation and properties of poliovirus-neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction, *J. Exp. Med.*, 1964, **119**, 1.
49. Svehag, S. E., and Mandel, B., The formation and properties of poliovirus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis, and sensitivity to x-irradiation, *J. Exp. Med.*, 1964, **119**, 21.
50. Heremans, J. F., Immunochemical studies on protein pathology, the immunoglobulin concept, *Clin. Chim. Acta*, 1959, **4**, 639.
51. Thorbecke, G. J., and Keuning, F. J., Antibody and gamma-globulin formation in vitro in haemopoetic organs, *J. Infect. Dis.*, 1956, **63**, 157.

52. Desai, R. G., and Creger, W. P., Maternofetal passage of leukocytes and platelets in man, *Blood*, 1963, **21**, 665.
53. Mellors, R. C., and Korngold, L., The cellular origin of human immunoglobulins (γ_2 , γ_{1M} , γ_{1A}), *J. Exp. Med.*, 1963, **118**, 387.
54. Thorbecke, G. J., Development of immune globulin formation in fetal, newborn and immature guinea pigs, *Fed. Proc.*, 1964, **23**, 346.
55. Silverstein, A. M., Thorbecke, G. J., Kraner, K. L., and Lukes, R. J., Fetal response to antigenic stimulus. III. Gamma-globulin production in normal and stimulated fetal lambs, *J. Immunol.*, 1963, **91**, 384.
56. Thorbecke, G. J., Gamma globulin and antibody formation *in vitro*. I. Gamma globulin formation in tissues from immature and normal adult rabbits, *J. Exp. Med.*, 1960, **112**, 279.
57. Thorbecke, G. J., 1964, personal communication.
58. Silverstein, A. M., Uhr, J. W., Kraner, K. L., and Lukes, R. J., Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb, *J. Exp. Med.*, 1963, **117**, 799.
59. La Via, M. F., Uriu, S. A., and Warren, A. E., Antibody production by rat spleen explants in vitro, *Arch. Path.*, 1961, **71**, 28.
60. Kalmutz, S. E., Antibody production in the opossum embryo, *Nature*, 1962, **193**, 851.
61. Fennestad, K. L., and Borg-Petersen, C., Antibody and plasma cells in bovine fetuses infected with *Leptospira saxkoebing*, *J. Infect. Dis.*, 1962, **110**, 63.
62. Rümke, Ph., 1963, personal communication.
63. Fagraeus, A., 1963, personal communication.
64. Kalff, M. W., 1963, personal communication.
65. Thorbecke, G. J., and Cohen, M. W., Immunological competence and responsiveness of the thymus, in *The Thymus*, (Vittorio Defendi and Donald Metcalf, editors), Philadelphia, The Wistar Institute Press, 1964, 33.
66. Rowe, D. S., 1965, personal communication.
67. Mårtensson, L., and Fudenberg, H. H., Gm genes and γ_a -globulins synthesis in the human fetus, *J. Immunol.*, 1965, **94**, 514.
68. Bronnestam, R., and Nilsson, S. B., Gammaglobulin groups (Gm) of mothers and their newborn infants, *Vox Sanguinis*, 1957, **2**, 316.
69. Moellec, J., Kherumian, R., Stutton, E., and Espagnon, P., Contribution à l'étude du facteur de groupe Gm^a du plasma humain, *Rev. Hématol.*, 1956, **11**, 512.
70. Linnet-Jepsen, P., Galatius-Jensen, F., and Hauge, M., On the inheritance of the Gm serum group, *Acta Genet.*, 1958, **8**, 164.
71. Orlandini, T. O., Sass-Kortsak, A., and Ebbs, J. H., Serum gamma globulin levels in normal infants, *Pediatrics*, 1955, **16**, 575.

EXPLANATION OF PLATES

PLATE 77

FIG. 1. *Fetal spleen*. Seventeen-week-old fetus: (a) poorly developed white pulp, hematoxylin and eosin stained; (b) weakly IgM-positive fluorescence in a small number of lymphoid cells and in one plasma cell. Thirty-one-week old fetus: (c) well developed white pulp, hematoxylin and eosin stained; (d) IgM-positive, bright; fluorescence in a large number of lymphoid cells in the white pulp and in a few plasma cells. $\times 100$.



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PLATE 78

FIG. 2. *Fetal spleen*. IgG-positive fluorescence in: (a) plasma cell; (b) Russell body; (c) medium sized lymphocyte; (d) large lymphocyte. IgM-positive fluorescence in: (e) plasma cell; (f) small lymphocytes; (g) medium sized lymphocyte; (h) large lymphocyte. $\times 500$.

FIG. 3. *Fetal peripheral blood lymphocytes*. (a) Giemsa stained medium sized and small lymphocytes; (b) IgG-positive medium sized lymphocyte; (c) IgM-positive medium sized lymphocyte; (d) IgM-positive small lymphocytes. $\times 500$.

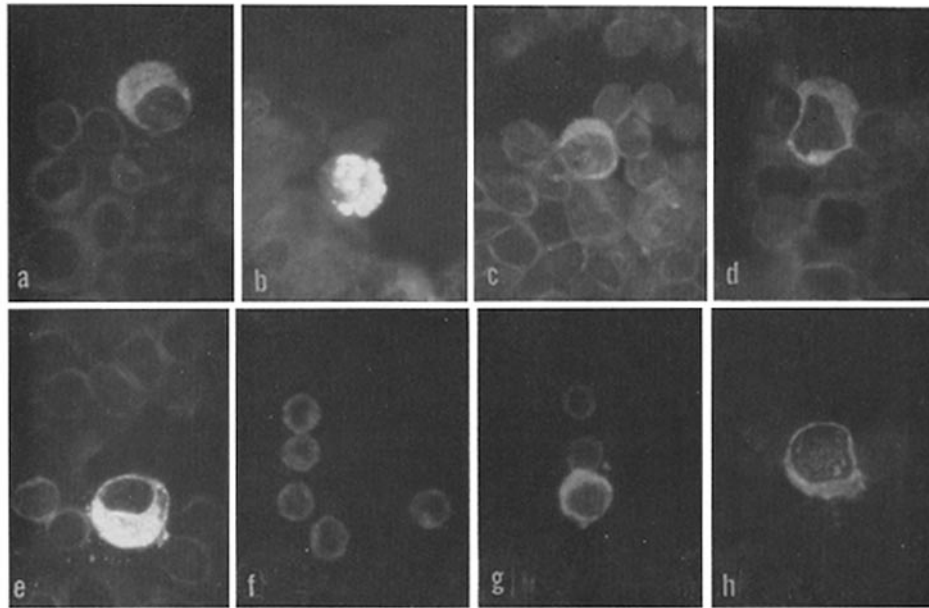


FIG. 2

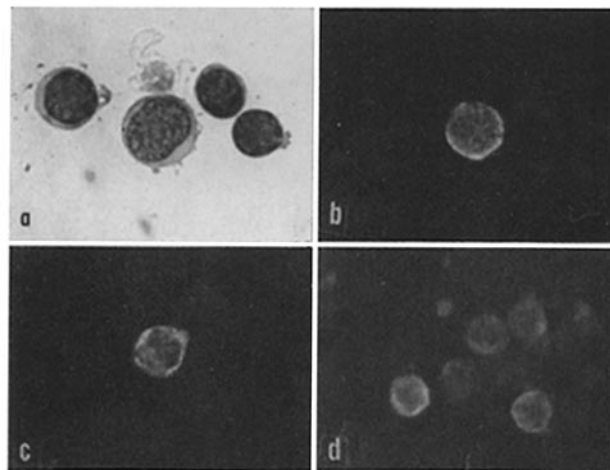


FIG. 3

(van Furth *et al.*: Development of human fetus)