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The development of lymphocytes with T- or B-membrane determinants in the human foetus

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SUMMARY

The development of T- or B-membrane determinants on human foetal lymphoid cells was studied by the direct immunofluorescence technique, using a tetramethyl rhodamine isothiocyanate (TRITC) labelled horse antihuman T-cell conjugate (ATC) for the detection of T lymphocytes and a fluorescein isothiocyanate (FITC) labelled goat antihuman Fab conjugate for the demonstration of Ig-bearing B lymphocytes. Human foetal lymphocytes were also tested for spontaneous rosette formation with sheep red blood cells (SRBC).

Cell suspensions of liver, spleen, thymus, bone marrow and blood of twenty-five human foetuses of 5·5–26 weeks of gestational age have been investigated. ATC-positive lymphoid cells were first seen in the liver at 5·5 weeks; E rosette-forming cells (ERFC) and Ig-bearing lymphoid cells were first found at 9 weeks. ERFC were also present in the thymus at 9 weeks. By 12 weeks, fluorescent B and T lymphocytes were found in bone marrow and spleen. ERFC were also found in bone marrow at this age, but not in spleen. At 15 weeks, more than 80% of blood lymphoid cells had T or B determinants.

A difference in the reactivity of lymphoid cells with the ATC and their capacity to form E rosettes was observed. In liver and spleen, the ATC determinant was detectable before the SRBC receptor. In bone marrow, blood and thymus the ATC determinant was found on a higher percentage of lymphoid cells than was the SRBC receptor when those organs were first investigated. During the entire investigated period of gestation, the majority of lymphoid cells in liver and bone marrow did not react with either of the conjugates, nor did they form E rosettes. In all organs investigated, except in the thymus, lymphoid cells were occasionally seen which reacted with both conjugates. By the 16th week of foetal age, more than 90% of lymphoid cells in thymus, spleen and blood had acquired T- or B-membrane determinants.

INTRODUCTION

Lymphocytes can be first found in the liver of the human foetus at 5·5 weeks of gestation (Carr, Stites & Fudenberg, 1975). At 7–8 weeks, they appear in the blood (Arey, 1966; Playfair, Wolfendale & Kay, 1963); they are found in the thymus at 8–9 weeks (Solomon, 1971; Prindull, 1974) and in the spleen and bone marrow at 12 weeks (Arey, 1966; Valdes-Dapena, 1957; August *et al.*, 1971; Yoffey & Thomas, 1964).

Investigations concerning the ontogeny of surface determinants on the lymphocytes have been concentrated mainly on the Ig-bearing B-cell system (Lawton *et al.*, 1972; Gathings *et al.*, 1976; van Furth,

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Schuit & Hijmans, 1965; Vossen & Hijmans, 1975). Lymphocytes showing surface immunoglobulin are first found in the human foetal liver at 9·3 weeks of gestation, in spleen and thymus at 11·5 weeks and in blood and bone marrow at 12 weeks. During foetal development, the different Ig classes are detectable on the B-lymphocyte membrane in the following sequence: μ, δ, γ, α. After the 14th week of gestation, the relative number of Ig-bearing lymphocytes in foetal blood and spleen equals that in blood of newborns and children (Lawton *et al.*, 1972; Gathings *et al.*, 1976; Vossen *et al.*, 1975).

Data concerning the ontogeny and distribution pattern of human lymphocytes carrying T-membrane determinants, namely, the receptor for SRBC (Wybran, Carr & Fudenberg, 1972; Jondal, Holm & Wigzell, 1972) and the specific human T antigen(s) (Aiuti & Wigzell, 1973; Owen & Fanger, 1974; Touraine *et al.*, 1974; Gattringer & Wick, 1976; Owen & Fanger, 1976) are more limited.

Cell suspensions from foetal organs have been examined only for the presence of ERFC after the 10th week of gestation (Hayward & Ezer, 1974; Wybran *et al.*, 1972). In thymus and bone marrow, ERFC are first detectable at 11 weeks; they are detected in blood at 12 weeks, spleen at 14 weeks and liver at 17 weeks. After the 16th gestational week, more than 90% of thymocytes form E rosettes.

In a previous paper, we reported on the preparation of a specific antihuman T-cell conjugate and its application in the direct immunofluorescence technique (Asma, Schuit & Hijmans, 1977). The application of the two-wavelength method in immunofluorescence enabled us to test lymphocytes in the same preparation for surface Ig and reactivity with the antihuman T-cell conjugate. In the present study, this technique, together with the E-rosette test, was used for the investigation of cell suspensions from different foetal organs. In order to obtain a better understanding of the ontogeny of the human T-lymphocyte system, we sought to answer the following questions: (1) is there a difference in the time of appearance of the two T-surface determinants? (2) What is the timing and sequence of T-lymphocyte appearance in the different organs? (3) Which relationship can be found between T- and B-lymphocyte development?

MATERIALS AND METHODS

Twenty-four human foetuses from 5·5 to 26 weeks gestational age were obtained by interruption of pregnancy, either on medical or psycho-social grounds. Permission was given by the parents to use this material for research purposes. In case the mother had been acutely ill during the pregnancy, or the foetus showed gross abnormalities no investigation was performed. Gestational age was calculated from crown–rump length (Hertig, 1968) and the date of onset of the last menstrual period of the mother. Foetuses were kept on melting ice from delivery until dissection; this period never exceeded 12 hr. Cell suspensions were made from thymus, spleen, liver, bone marrow and peripheral blood. Foetal thymus, spleen and liver were minced with scissors in a 1% bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS), pH 7·4 and pressed through several layers of nylon gauze to obtain single-cell suspensions. Foetal bone marrow was sampled by flushing both femurs and, if necessary, both tibiae with 1% BSA solution. Peripheral blood was obtained by cardiac puncture or by gently squeezing out the umbilical cord. The blood was mixed with a 0·5% disodium EDTA solution in a ratio of 10:1. Mononuclear cells were isolated by the Isopaque–Ficoll method (Lymphoprep [density 1·077 g/ml], Nyegaard & Company, Oslo, Norway) described by Böyum (1968) with slight modifications. All cell suspensions were washed once with the 1% BSA solution. After being washed, the cells were resuspended in the 1% BSA solution.

Antisera. An IgG fraction of a horse antihuman thymocyte antiserum supplied by the Upjohn Company, Kalamazoo, Michigan, U.S.A., was heat-inactivated for 30 min at 56°C and dialysed for several days against PBS to remove the glycine which had been added for stabilization. The antithymocyte IgG was extensively absorbed with human ABO erythrocytes and insolubilized normal human serum. An ATC was prepared from this fraction by conjugation with TRITC. This conjugate was rendered specific for human T lymphocytes by further absorption with lymphocytes isolated from the blood of patients suffering from a B-type chronic lymphatic leukaemia and cells of the myeloid line as previously described (Asma *et al.*, 1977). An FITC-labelled GaHu–Fab conjugate purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands, was used to detect the B lymphocytes.

Immunofluorescent assay. Cell staining for the direct immunofluorescence technique was performed as described previously (Asma *et al.*, 1977). In brief, cell suspensions were first incubated for 30 min at room temperature with the ATC in a dilution of 1:32 in PBS (pH 7·8) and, after being washed twice with the 1% BSA solution, incubated again with the GaHu–Fab conjugate in a dilution of 1:8. Slides were prepared and cells which satisfied the criteria of lymphoid cells in phase-contrast microscopy (Vossen *et al.*, 1975) were examined for the presence of surface fluorescence, using a Zeiss Standard Junior microscope, equipped with a Ploem-type vertical illuminator IV F1 and containing the combination of filters suitable for discriminating between TRITC and FITC fluorescence. The light source was a high-pressure mercury HBO 50 Watt.

E-rosette formation. This was carried out according to Jondal *et al.* (1972) with slight modifications (Asma *et al.*, 1977).

TABLE 1. Expression of T- and B-lymphocyte markers on liver and thymus cells in human foetuses aged 5·5–13 weeks

Gestational age (weeks)	No.	Liver*									Thymus†								
		Direct immunofluorescence						E rosettes			Direct immunofluorescence						F. rosettes		
		T‡			B§						T			B					
5–6	2	+	+		n.d.	n.d.		0	0										
6–7	2	+	+		0	n.d.		0	0										
7–8	2	+	0		0	n.d.		0	0										
8–9	2	+	+		+	0		+	n.d.		n.d.	n.d.		n.d.	n.d.		50%	n.d.	
9–10	3	+	0	n.d.	+	0	n.d.	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	64%	20%	n.d.
10–11	2	+	+		0	0		+	n.d.		96%	n.d.		0	n.d.		36%	n.d.	
11–12	3	+	+	n.d.	+	+	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12–13	2	+	+		+	n.d.		+	0		98%	n.d.		n.d.	n.d.		67%	n.d.	

+ = Positive lymphoid cells present; 0 = no reactive lymphoid cells present; n.d. = not done.

* Until the 12th week of gestation the liver yielded insufficient lymphoid cells to calculate percentages.

† Thymus tissue was not detectable before the 9th week of gestation.

‡ TRITC-labelled antihuman T-cell conjugate.

§ FITC-labelled GaHu–Fab conjugate.

RESULTS

In foetuses younger than 9 weeks of gestational age only the liver could be obtained. Thymus and spleen were, respectively, available from foetuses 9·5 and 12 weeks of age and older. Bone marrow did not contain lymphocytes before the foetal age of 12 weeks. Peripheral blood was first obtained from 15-week-old foetuses.

The relative number of cells with a lymphoid morphology *vs* all nucleated haemopoietic cells in those organs was as follows: less than 1% in the livers of the younger foetuses and between 1 and 4% after the 12th week; at 12 weeks, also less than 1% in spleen and bone marrow. In those organs, however, the relative number of lymphoid cells rose to more than 50% during foetal development. At 9·5 weeks, more than 90% of thymus cells were already lymphoid.

Immunofluorescent assay and E-rosette test

Table 1 gives the results obtained in liver and thymus suspensions from 5·5–13-week-old foetuses. In Table 2 data obtained in foetuses of 12 weeks of age and older are summarized.

Immunofluorescent assay. ATC-positive lymphoid cells were already found in liver preparations of the

TABLE 2. T- and B-lymphoid cells in organ suspensions of human foetuses aged 12 to 26 weeks

		Gestational age (weeks)						
		12	15	15·5	16	24	24	26
Thymus	T*	93	94	95	98	99	100	89
	B†	0	1	0	0	0	0	0
	D‡	0	0	0	0	0	0	0
	N§	7	5	5	2	1	0	11
	E-r¶	82	84	74	88	97	95	89
Spleen	T	4	24	30	22	31	21	56
	B	12	14	4	70	55	64	22
	D	0	1	10	4	2	1	11
	N	84	61	56	4	2	14	11
	E-r	0	32	45	23	22	16	61
Liver	T	2	5	2	8	16	2	n.d.
	B	4	4	0	12	21	14	n.d.
	D	0	1	4	0	6	0	n.d.
	N	92	90	94	80	57	84	n.d.
	E-r	2	1	4	6	9	1	n.d.
Bone marrow	T	10	30	18	17	10	13	10
	B	8	6	0	16	13	7	19
	D	1	8	2	8	0	0	0
	N	81	56	80	59	77	71	71
	E-r	4	0	8	4	2	5	5
Blood	T	n.d.	62	65	54	n.d.	32	70
	B	n.d.	16	7	35	n.d.	57	27
	D	n.d.	10	11	5	n.d.	1	0
	N	n.d.	12	17	6	n.d.	10	3
	E-r	n.d.	35	54	30	n.d.	30	53

n.d. = Not done.

* Percentage of lymphoid cells positive with a TRITC-labelled antihuman T-cell conjugate.

† Percentage of lymphoid cells positive with a FITC-labelled GaHu–Fab conjugate.

‡ Percentage of lymphoid cells positive with both conjugates.

§ Percentage of lymphoid cells negative with both conjugates.

¶ Percentage of lymphcid cells forming E rosettes.

5·5-week-old foetuses. Immunoglobulin-bearing lymphocytes were first observed at 9 weeks in foetal liver. The majority of liver lymphoid cells did not stain with the anti-Fab conjugate or the ATC at any time of gestation.

By 10 weeks, more than 90% of thymocytes were ATC-positive. Few Ig-bearing lymphocytes were found in the thymus preparations at any time of gestation. In 12-week-old foetuses, low numbers of both B- and T-lymphocytes were seen in spleen and bone marrow. With advancing gestational age, the majority of spleen lymphocytes acquired surface-Ig or the ATC determinant. As in the liver, the majority of bone marrow lymphocytes did not stain with the anti-Fab conjugate or the ATC at any age, although, in the older foetuses, B lymphocytes showed a trend towards higher values. A peak value for ATC-positive lymphoid cells was observed at 15 weeks.

In the blood 62% of the lymphocytes already reacted with the ATC and 16% with the anti-Fab conjugate at 15 weeks.

In liver, spleen, bone marrow and blood, lymphocytes were occasionally seen which did react with both conjugates. Highest values were observed in those organs at 15 and 15·5 weeks.

E-rosette test. In 9-week-old foetuses, E rosettes were first found in the liver and thymus preparations, followed by bone marrow at 12 weeks and spleen and blood at 15 weeks.

A difference in the reactivity of lymphocytes with the ATC and their capacity to form E rosettes was observed. In liver and spleen, the ATC determinant was detectable before the SRBC receptor. In bone marrow and blood, the ATC determinant was found in a higher percentage of lymphoid cells than was the SRBC receptor when those organs were first investigated. Moreover, the increase in the percentage of ATC-positive lymphoid cells observed in the bone marrow at 15 weeks was not reflected in the percentage of E rosettes.

Thymocytes of the 9- and 9·5-week-old foetuses were tested only for E-rosette formation. At 10 weeks, however, 96% of thymocytes was found to react with the ATC, whereas only 36% E rosettes were seen. As the discrepancy existing between the two markers in the younger foetuses disappeared during foetal development, the relative E-rosette values also rose to more than 90% (Fig. 1).

In all foetal material investigated, except in the spleen at 15 and 15·5 weeks, relative values for ATC-positive lymphoid cells were higher than, or sometimes equal to, E-rosette numbers.

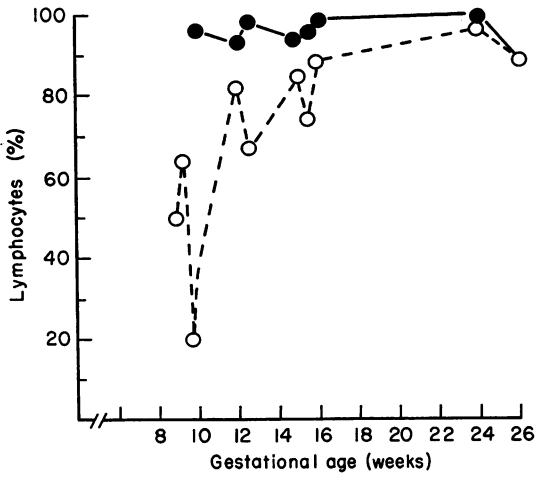

FIG. 1. Expression of T lymphocyte markers on thymus cells in human foetuses aged 9–26 weeks. ATC (———); E rosettes (– – –).

DISCUSSION

In the present study concerning the ontogeny of human lymphocytes with T- or B-membrane determinants, a striking difference was observed in the time of appearance of the ATC determinant and the receptor for SRBC on human foetal lymphoid cells. In all organs investigated, the ATC determinant was detectable before, or in a higher percentage of lymphoid cells than was the SRBC receptor, when those organs were first investigated. Moreover, ATC-positive lymphoid cells were already seen in the liver of 5·5-week-old foetuses. This is well before the anlage of the thymus appears. In man, the thymus primordium is first recognizable towards the end of the 6th week (Arey, 1966; Valdes-Dapena, 1957) and remains epithelial until the 9th week, when it becomes populated with lymphocytes (Solomon, 1971; Prindull, 1974).

We first found ERFC in the liver suspensions at 9 weeks of gestation. A similar divergence was observed by other investigators (Stites, Carr & Fudenberg, 1974) who studied the responsiveness of human foetal lymphocytes to phytohaemagglutinin (PHA) and allogeneic lymphocytes in the mixed lymphocyte reaction (MLR). Liver suspensions of 7·5-week-old foetuses already showed a significant response in the one-way MLR, whereas PHA responsiveness was almost never significant. This dichotomy of PHA-unresponsive, MLR-responsive cells is also observed in some mouse systems and in patients with thymic aplasia or dysplasia (Meuwissen *et al.*, 1968; Gatti, Garrioch & Good, 1970). A possible explanation for those and our own findings may be found in data obtained in the mouse system. Roelants *et al.* (1976) have demonstrated that, in surgically or congenitally athymic mice and in the thymus at early stages of embryogenesis, a population of lymphocytes belonging to the T lineage can be found, which differ in several aspects from normal T lymphocytes. Those cells are produced in the bone marrow, have a life span of 1–2 days, a slow electrophoretic mobility and do not circulate through the thoracic duct. On those cells, however, a low density of theta antigen is already present and they also express the TL antigen. These cells disappear after thymus grafting. The results of those experiments are in agreement with the postulation of Boyse & Abbott (1975) that the commitment of stem cells to the T pathway takes place outside the thymus and does not even require the presence of the thymus. The thymus is needed, however, for further differentiation. Recently, Sato, Waksal & Herzenberg (1976) using the brain-associated T antigen as a marker for committed T cells came to the same conclusion.

Although other interpretations of our data are certainly possible, we think that this hypothesis may also be applied to the human system and that the ATC-positive cells in the 5·5-week-old livers are committed T cells. This commitment process may be taken over by the bone marrow at a later stage of embryogenesis, explaining the much higher percentage of ATC-positive lymphoid cells than of E rosettes, especially around the 15th week of gestation. The finding that only after the 9th week of gestation E rosettes are detectable makes it highly probable that the expression of the SRBC receptor is under thymic influence.

The percentage of lymphocytes capable of E-rosette formation in thymus and spleen observed by us is in agreement with the percentage found by Hayward *et al.* (1974), but much higher than that reported by Wybran *et al.* (1972) for thymus, spleen, bone marrow, liver and blood. A difference in technique may explain this discrepancy, as it is well known that optimal E-rosette formation is highly dependent on experimental conditions (Bach, 1973; Jønsson, 1974).

Our findings on the first appearance of surface-Ig on lymphoid cells in the different organs are in agreement with those reported by Lawton *et al.* (1972). The relative number of Ig-bearing lymphocytes found by those investigators in spleen and blood, and also by Hayward *et al.* (1974) in spleen, are slightly higher in foetuses up to 16 weeks of age than was observed by us and Vossen *et al.* (1975). This may be due to the biological variation in the foetal material or to a difference in the methods used to detect fluorescent B lymphocytes. In view of the proposed maturation process of the T lymphocytes, correlation with the ontogeny of the B-cell system gives rise to some difficulties. It may be questioned if the expression of surface-Ig on B lymphocytes may not be a relatively late event in the maturation process of B lymphocytes, comparable with the expression of a SRBC receptor on T lymphocytes. Comparison of our findings on the first appearance of cells with surface-Ig and E rosettes shows that they are detectable

at the same time of gestation. They are first found in the liver at 9 weeks, in bone marrow and spleen at 12 weeks and in blood at 15 weeks. The commitment of stem cells to the B pathway may be expressed by membrane determinants other than immunoglobulin. Analogous to the mouse system (Lamelin *et al.*, 1972), an antihuman B-cell antiserum may be of use in the further study of the early stages of the development of the human B-cell system. Furthermore, the fact that the location of the equivalent of the avian bursa of Fabricius in mammals is not known makes it impossible to evaluate the role of this bursa-equivalent in the ontogeneic development of B lymphocytes.

Two other results obtained in our study need further discussion. First, we found in all organs investigated, except in the thymus, a small number of lymphocytes which reacted with both the ATC and the anti-Fab conjugate. Peak values were observed in the 15- and 15·5-week-old foetuses. As we postulated previously (Asma *et al.*, 1977), we think that those double-stained cells may represent a subpopulation of Ig-bearing T lymphocytes. It is not yet clear why the relative number of 'double' cells is highest around the 15th week of gestation. The expression of Ig on T lymphocytes may reflect a certain stage of maturation or activation (Moroz & Hahn, 1973; Marchalonis, Cons & von Boehmer, 1974) of those cells.

The second point concerns the relatively large number of 'null' cells present in liver and bone marrow during the entire investigated period of gestation and in the spleen up to the 16th week of gestation. Most certainly, they do not form a homogeneous population. Amongst them, precursors for B and T lymphocytes as well as cells bearing a receptor for complement and/or the Fc part of aggregated IgG may be found (Stutman & Good, 1971; Owen, Cooper & Raff, 1974; Gelfand, Asofsky & Paul, 1974).

We will extend our study on the ontogeny of membrane determinants on human foetal lymphoid cells, especially as found in the liver by further evaluation of the antibody specificities present in our antihuman T-cell conjugate.

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