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Immunology

Ontogeny of lymphocyte subpopulations in human fetal liver

[surface immunoglobulins/mouse erythrocyte rosette-forming cells/complement receptors/IgG Fc receptors/thymus-processed (T) cells]

SUDHIR GUPTA, RAJENDRA PAHWA, RICHARD O'REILLY, ROBERT A. GOOD, AND FREDERICK P. SIEGAL

Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

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ABSTRACT **Lymphocytes were isolated from five fetal livers (13–17 weeks) and examined for different surface markers. Immunoglobulin M was found on 0.5–4.0% of lymphocytes. No membrane-bound surface IgD, IgA, or IgG was detected. Mouse erythrocyte rosette-forming lymphocytes ranged from 0.5 to 6.0%. Thymus-processed (T) lymphocytes, which were defined as those forming spontaneous rosettes with sheep erythrocytes, as well as lymphocytes with IgG Fc receptors, were present in a small proportion. No complement-receptor-bearing lymphocytes were found in the two cell populations of fetal liver studied. It is evident that during the ontogeny of bone-marrow-derived (B) lymphocytes IgM is the first surface immunoglobulin to appear. Receptors for the binding of mouse erythrocytes are present at the same time as surface IgM. The slight excess of mouse erythrocyte rosette-forming cells over cells having surface immunoglobulin M could mean that IgM appears later than do the receptors for the binding of mouse erythrocytes.**

Hematopoietic stem cells originate in the yolk sac from precursor hemangioblasts, and during fetal to postnatal life this pool of cells progressively moves from yolk sac to fetal liver and thence to the bone marrow (1). Fetal liver is the major hematopoietic organ during the greater part of embryogenesis in most mammals. During their journey from yolk sac to the adult bone marrow via fetal liver, lymphoid stem cells, under specific inductive influences, possibly derived from the local microenvironment, differentiate into several subpopulations of mature lymphocytes (2) and develop certain surface markers. Human T, or thymus processed, lymphocytes spontaneously form rosettes with sheep erythrocytes (3). B lymphocytes presumed to be derived from the bone marrow are characterized by the presence of intrinsic cell membrane immunoglobulin (4) and their spontaneous rosette-formation with mouse erythrocytes (5, 6). Recently a third population of lymphocytes has been described (7). These lymphocytes bear an avid receptor for the IgG Fc but lack surface immunoglobulin as well as characteristics of T lymphocytes.

The ontogeny of human lymphocyte surface receptors has not been investigated in great detail. Lawton *et al.* (8) demonstrated that during ontogeny IgM is the first surface immunoglobulin to appear on lymphocytes of fetal liver; however they did not examine membrane bound surface IgD. Rowe *et al.* (9) found more IgD-bearing lymphocytes than IgM in human cord blood lymphocytes and suggested that IgD may be the first immunoglobulin to appear on the surface of lymphocytes. In the present communication, by using a broad spectrum of surface markers on lymphocytes from fetal liver, we have demonstrated that IgM as a surface immunoglobulin develops prior to IgD.

Abbreviations: T lymphocytes, thymus-processed lymphocytes; B lymphocytes, bone-marrow-derived lymphocytes; MRFC, mouse erythrocyte rosette-forming cells; E-rosettes, T lymphocytes spontaneously forming rosettes with sheep erythrocytes; BSA/PBS, bovine serum albumin/phosphate-buffered saline; E, erythrocyte; A, antibody; C, complement; SRBC, sheep erythrocytes; R_n, rosette of a lymphocyte and n sheep erythrocytes.

MATERIALS AND METHODS

Human Fetal Liver Lymphocytes. Five intact nonliving human fetuses were obtained from normal pregnant adults following intravaginal-prostaglandin-induced abortions. The written informed consent of the aborted women for the use of the tissue was obtained in each case. Fetal age was determined by duration of gestation and by crown to rump length (10). Livers were removed and gently minced in Hanks' balanced salt solution and passed through wire gauze. Lymphocytes were isolated on a Ficoll–Hypaque gradient (11). Cells were washed three times in Hanks' balanced salt solution and finally resuspended in medium RPMI 1640 to adjust the concentration of cells to 5×10^6/ml. Cytocentrifuge preparations were also made from this cell suspension and stained with tetrachrome. These cell suspensions contained a small proportion of nucleated red cells and a few myeloid cells.

Membrane-Bound Surface Immunoglobulin. Surface immunoglobulins were determined by a technique previously described (12). A polyvalent rabbit antiserum to human immunoglobulin and antisera monospecific for γ, μ, δ, α, κ, and λ determinants conjugated with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate were used. Fluorescent antisera were prepared as described (12). They were appropriately absorbed with solid immunoabsorbants (CNBr-coupled agarose beads) and centrifuged to remove aggregated material immediately prior to use.

The specificity of the fluorescent antiglobulin reagents was defined by the following criteria: (*1*) In Ouchterlony analysis they were reactive only with myeloma proteins or Bence-Jones proteins of the appropriate class or type, and did not react with other human serum components. (*2*) On the cell surface, they exhibited class exclusion; in double fluorochrome experiments, the anti IgG, IgA, and IgM sera stained separate cells. The determinants for δ and μ appeared mostly on the same cells, but capped independently and gave discrete red and green spots. (3) Absorption with the appropriate antigens eliminated staining, although absorption with different antigens did not. Lymphocytes (5×10^5 per tube) were suspended in bovine serum albumin (20 mg/ml) in phosphate-buffered saline (BSA/PBS) containing 0.02% sodium azide. Staining was carried out at 4° for 30 min. The cells were washed three times in BSA/PBS and resuspended in a very small volume. Wet counts were made and 200 to 500 lymphocytes were counted with a Leitz Ortholux microscope equipped with vertical fluorescence illumination. Nucleated red cells and myeloid cells were excluded from counting, by morphological criteria by phase contrast optics.

Table 1. Surface markers of human lymphocytes on human fetal liver cells

Subject	Fetal age (weeks)	E-rosettes* (%)		C3 (%)	IgG Fc (aggregate) (%)	Ripley rosettes† (%)	Surface immunoglobulin determinant (%)							MRFC (%)
		(R_1–R_2)	(R_3)				PV‡	μ	δ	α	γ	κ	λ	
1.	13	0.0	2.0	—	1.5	1.5	2.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5
2.	14	1.0	0.5	0.0	3.4	11.0	4.9	2.5	0.0	0.0	0.0	0.9	1.9	4.0
3.	14	3.0	4.0	—	3.9	17.0	3.5	4.0	0.0	0.0	0.0	—	—	6.0
4.	15	0.0	1.5	0.0	2.0	8.0	4.5	1.5	0.0	0.0	0.0	1.5	0.5	4.0
5.	17	1.5	0.0	—	9.0	26.0	4.5	2.5	0.0	0.0	0.0	—	—	2.5

* T lymphocytes—see *text*.
† Most of these rosettes were with myeloid cells—see *text*.
‡ Polyvalent antiserum to human immunoglobulin.

Receptors for IgG Fc (Indirect Aggregate IgG Technique). Aggregated human IgG was prepared by a modification of the technique of Dickler and Kunkel (13). Lymphocytes (5×10^5 per tube) in BSA/PBS were incubated with 25 μl of unconjugated aggregated IgG at 4° for 30 min. Cells were washed three times in BSA/PBS and stained with fluorochrome-conjugated polyvalent antihuman immunoglobulin antiserum by incubating at 4° for another 30 min. These stained cells were washed and resuspended and wet mounts were prepared; 200 to 300 lymphocytes were counted. Nucleated red cells and myeloid cells were excluded from the counting.

High Affinity IgG Fc Receptors (Ripley Rosette Test). One hundred microliters of lymphocyte suspension (5×10^6/ml) were mixed with human erythrocytes (O Rh+) coated with 7S IgG of Ripley type (EAIgG) (7). The mixture was centrifuged at $50 \times g$ for 5 min followed by incubation at 4° for 30 min. A cell surrounded by three or more red cells was considered a positive rosette. Because of the large number of erythrocytes attached to a cell, it was difficult to identify the morphology of the central nucleated cell in suspensions and therefore cytocentrifuge preparations were made from rosette suspension and stained with tetrachrome to identify the cells forming rosettes with EA 7S IgG.

Complement (C3) Receptors (EAC Rosettes). C3 receptors were detected by the technique of Bianco *et al.* (14). One hundred microliters of suspension of sheep erythrocytes (SRBC) coated with 19S antibody against SRBC and fresh mouse serum (AKR) as a source of complement (final erythrocyte–antibody-complement complex termed EAC) were mixed with 100 μl of lymphocyte suspension (5×10^6/ml). The mixture was incubated at 37° for 30 min. Sheep erythrocytes coated with 19S antibody without complement (EA) were run simultaneously as a negative control. The mixture was vigorously resuspended and 200 lymphocytes were counted for rosette-forming cells.

Mouse Erythrocyte Rosette-Forming Cells (MRFC). MRFC were detected by the technique of Gupta *et al.* (*). Fifty microliters of lymphocyte suspension (5×10^6/ml) were mixed with 25 μl of fetal calf serum (heat-inactivated and absorbed with mouse erythrocytes) and 100 μl of 1% mouse erythrocytes. The mixture was then centrifuged at $200 \times g$ for 5 min followed by incubation at room temperature (24°) for 1 hr. The pellets were gently resuspended and 200 lymphocytes were counted. Cytocentrifuge preparations were made from rosette suspensions and stained with tetrachrome.

Sheep Erythrocyte Rosettes (E-Rosettes). T lymphocytes were enumerated by their spontaneous rosette-formation with sheep erythrocytes (SRBC). One hundred microliters of lymphocyte suspension were mixed with 25 μl of human AB serum (heat-inactivated and absorbed with SRBC) and 100 μl of 0.5% SRBC. The mixture was then incubated at 37° for 5 min and centrifuged for 5 min at $50 \times g$, then incubated at 4° for 18 hr. The pellets were gently resuspended and 200 lymphocytes were counted. Lymphocytes attached with 1-2($R_{1\text{-}2}$) and three or more (R_3) SRBC were both counted as positive rosettes. Cytocentrifuge preparations were made and stained with tetrachrome.

RESULTS

Results of the analysis of surface markers on lymphocytes from five fetal livers are presented in Table 1. Surface-immunoglobulin-bearing B lymphocytes as detected by a polyvalent antiserum to human immunoglobulin were 2.0–4.9%; IgM-bearing lymphocytes ranged from 0.5 to 4.0%. The number of lymphocytes with light chain immunoglobulin corresponded to that of IgM-positive cells. No cell with membrane-bound surface IgD, IgA, or IgG was detected. Mouse erythrocyte rosette-forming cells (a B-cell marker) were 0.5–6.0%. The proportion of lymphocytes with IgG Fc receptors as detected by the indirect aggregated human IgG method was comparable to that of surface-immunoglobulin-bearing cells. A considerably higher number of Ripley rosettes (which detect "high affinity" Fc receptors) was detected. It was not possible to exclude cells of the myeloid series from lymphocytes in wet preparations, because of large numbers of erythrocytes which almost covered the entire surface of the mononuclear cells. On stained centrifuge preparations, however, most of these rosette-forming cells were found to be of the myeloid series and only very few lymphocytes formed Ripley rosettes. Complement (C3)-receptor-bearing lymphocytes were not detected in either of the two liver cell preparations studied. The T lymphocyte surface marker was found in a small population of lymphocytes (1.5–7.0%).

DISCUSSION

The first recognizable step in the differentiation of B lymphocytes, in a special microenvironment such as the bursa of Fabricius in chickens and the fetal liver in mice, is the synthesis of immunoglobulin M which is incorporated into the plasma membrane (2, 15). Some daughter cells take further steps in differentiation by switching from IgM to IgG or IgA

* S. Gupta, R. A. Good, and F. P. Siegal, "Rosette-formation with mouse erythrocytes. II. A marker for human B and non-T lymphocytes," manuscript submitted.

synthesis (16). However, the early appearance during the ontogeny of cell surface IgD in humans confused the picture. Rowe *et al.* (9) demonstrated production of surface IgD by most of the IgM-bearing lymphocytes and also demonstrated a higher number of cells with IgD than with IgM in human cord blood. This led these investigators to postulate that IgD surface immunoglobulin may appear even earlier in ontogeny than IgM and therefore might be a primordial cell surface immunoglobulin. Vitetta *et al.* (16) demonstrated the presence of "IgD-like" immunoglobulin on the surface of murine lymphocytes. Their studies demonstrated that, during differentiation of B lymphocytes, IgD appears after IgM. They based their conclusion on the following observations: (*1*) Splenocytes from newborn BALB/c mice have only IgM on their surfaces for the first 10–14 days after birth. Subsequently, IgD appears and increases in amounts until the animal reaches about 3 months of age and accounts for 60–70% of surface immunoglobulin. (*2*) Bone marrow cells from adults bear only IgM. (*3*) The population of large lymphocytes in the spleen bears only IgM, whereas, the population of small lymphocytes has both IgM and IgD. Critical studies regarding the ontogenic relationship between IgM and IgD in the human heretofore have been lacking. In the present study, we have clearly demonstrated that, in human fetal liver, lymphocytes bearing IgM are present at 13–17 weeks of gestation when other surface immunoglobulins, i.e., IgD, IgA, and IgG, cannot be detected. Therefore, it is evident that in human B cell differentiation IgM is the first immunoglobulin antigen receptor to appear. The proposed model of B cell differentiation of Vitetta and Uhr (17) is in agreement with our observations. We have shown that, in human peripheral blood, mouse erythrocytes bind to IgM-bearing lymphocytes (*). In this study, the number of mouse erythrocyte rosette-forming lymphocytes was comparable to that of IgM-positive cells. The number of cells with IgM was 0.5–4.0%, whereas mouse erythrocyte rosette-forming cells were 0.5–6.0%. Lawton *et al.* (8) demonstrated the presence of IgM on human fetal liver cells as early as 9.5 weeks of gestation; however, the number of positive cells was not reported. Similarly, a small proportion of B lymphocytes (0–2.5%) was reported in human fetal liver (18). However, these authors did not mention the classes of immunoglobulin. A high number of IgM-bearing lymphocytes (14.1–30.1%) was reported in human fetal spleen (13.5–24 weeks) (8). Robert *et al.* (19) reported 0.5–2.4% antigen-binding cells in human fetal liver (8–24 weeks) using radiolabeled thyroglobulin as the antigen. Papamichail *et al.* (20) reported 6.0% lymphocytes with IgM in fetal blood (13–24 weeks). In the present study, the number of lymphocytes with IgG Fc receptors was found to be comparable to the number with surface immunoglobulin. We did not find any C3-receptor-bearing lymphocytes in either of the two fetal livers studied. Mukhopadhyay *et al.* (18) reported 5.0–10.5% C3-receptor-bearing cells in human fetal liver; however, it is unclear if these authors excluded the cells of erythrocyte and myeloid series from their determination, since both of these populations are known to have receptors for C3. It is probable that during the ontogeny of B lymphocytes C3 receptors appear later than do surface immunoglobulin and IgG Fc receptors, a relationship which would be consistent with the temporal relationship in differentiation established for mice (21). The small proportion of T lymphocytes found in the present study was also reported by other investigators (18).

In this communication, we have clearly shown that IgM as a cell surface immunoglobulin appears earlier in ontogeny than IgD. Further, binding sites for mouse erythrocytes are present at the same time as is IgM. The slight excess of MRFC over IgM could mean that IgM appears later than does the MRFC. These findings have an important counterpart in phylogenetic development, since an IgM-like molecule appeared earlier in phylogenetic development than did other immunoglobulins, including an IgD-like molecule (22, 23).

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