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Original Article

Immunohistochemical Studies on the Development of Human Foetal Spleen

Rajeev Mukhiya[1], Rahul Singh[2], Rakesh Kumar Mukhia[3], Jay Prakash Thakur[4], Anjali Sabnis[5]

[1,2] Associate Professor, Department of Anatomy, Hind Institute of Medical Sciences, Sitapur.
[3] Associate Professor, Department of Microbiology, Hind Institute of Medical Sciences, Sitapur.
[4] Assistant Professor, Department of Anatomy, World College of Medical Sciences Research and Hospital, Jhajjar.
[5] Professor and Head, Department of Anatomy, MGM Medical College, Navi Mumbai, India.

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Corresponding Author:

Rajeev Mukhiya
Associate Professor, Department of Anatomy, Hind Institute of Medical Sciences, Sitapur.

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ABSTRACT

Background: Spleen is one of the organs of interest for researchers since a long time. Though, detailed morphometric of spleen is there in the literature but immunohistochemical studies on spleen at different stages in the foetal period is less available. Hence, the present study aims to find out the immunohistochemical development of human spleen in different gestational weeks.
Materials and Methods: The study was carried out on 40 human foetuses of known gestational age in the Department of Anatomy, MGM Medical College. After dissection the spleens were removed and fixed in 10 % buffered formalin solution for 10 days. Tissue blocks were cut and then embedded in paraffin after dehydration in a graded ethanol series. Serial sections were prepared for haematoxylin and eosin for histology and immunohistochemical examination.
Results: In the spleen of the 16th gestational week, T and B lymphocytes were scattered in the splenic parenchyma. At 22nd gestational week, T and B lymphocytes became denser. After 24th gestational week white pulp was specialized into T-cell and B-cell region. CD3 positive T-lymphocytes around the arteriole formed periarteriolar lymphatic sheath. CD20 positive B-lymphocytes around the arteriole to form lymphoid follicle. At later stages immunoreactive CD3 positive cells were reduced as compared to the CD20 lymphocytes in the spleen.
Conclusion: The study concludes that the density of immunoreactive CD3 T-lymphocytes was less as compared to the CD20 B-lymphocytes in the spleen throughout the gestation. The T and B lymphocytes increased in the later gestational stages.

Keywords: *Gestational weeks, Foetal spleen, CD3 T-lymphocytes, CD20 B-lymphocytes.*

INTRODUCTION

The human spleen consists of an extracellular matrix composed of the reticular fibers and proteins. These fibers and proteins develop early in the foetal life. The characteristic architecture of human spleen changes dramatically during the foetal life. Since much of the architecture of the spleen is not fully formed in the embryonic period, significant development of the red and white pulp occurs during the second and third trimesters of fetal life with additional changes occurring Postnatally [1, 2].

During the development of human foetuses, the site of lymphocyte proliferation and differentiation moves from the yolk sac blood island, via the thymus, liver, spleen, and intestinal wall to the bone marrow. T cells or T lymphocytes are a type of lymphocyte that plays a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells, by the presence of a T-cell receptor (TCRs) on the cell surface. CD3 is a membrane complex associated with the T-cell receptor [3].

B cells or B lymphocytes are a type of lymphocyte that plays a role in the immunity by secreting antibodies. Additionally, B cells present antigen (they are also classified as professional antigen-presenting cells (APCs)) and secrete cytokines. They can be distinguished from other lymphocytes, such as T cells and natural killer cells, by the presence of a B-cell receptor (BCRs) on the cell surface. CD20 is a membrane complex associated with the B-cell receptor [4].

The white pulp of the spleen is essentially the lymphoid component of the organ, which in the adult spleen contains mature T- and B-lymphocyte compartments. The B-cell areas include the germinal center, mantle zone, and marginal zone and the T-cell area is the less well-organized parafollicular zone, but T-cells are also a component of the periarteriolar clusters [5]. In the fetal spleen, these lymphoid compartments are just beginning to develop and do not achieve the histological architecture that is recognizable in the mature, adult spleen, even by the end of full-term gestation [6].

Unfortunately, there are only few immunohistological studies of the human foetal spleen in literature, and while they provide some insight into prenatal splenic development, more comprehensive analyses are needed to fully understand the changes that occur in this complex organ during fetal life. Though, detailed morphological and morphometric features about the adult spleen is available the immunohistochemical studies on the human foetal spleen have still a large scope for researchers.

MATERIALS AND METHODS

Study Design

The present study was carried out on 40 normal human foetuses, aged between 10^{th} to 38^{th} gestational weeks in the Department of Anatomy, MGM Medical College, Navi Mumbai from March 2014 to January 2017. The normal foetuses were obtained from the Department of Obstetrics and Gynaecology, MGM Medical College, Navi Mumbai. After ethical review and permission from the concerned authorities of the Institute, the foetuses were collected in 10 % formalin for carrying the study.

Inclusion and Exclusion Criteria

The foetuses included the spontaneous abortion and still born foetuses. Cases with any anomaly or pathology were not included in the study.

Determination of the age of foetuses

The age of foetuses was calculated from the obstetrical history, crown rump length (CRL) and crown heel length (CHL).

Dissection of foetuses

The dissection of the anterior abdominal wall of foetuses was done to expose the spleen.

Staining procedures

Spleens were fixed in 10 % buffered formalin solution for 10 days. Tissue blocks were cut (4 μm in thickness) from the fixed materials and then embedded in paraffin after dehydration in a graded ethanol series. Serial sections were prepared for hematoxylin and eosin for histology and immunohistochemical examination.

Immunohistochemistry

1. Sections cutting: 3-4 micrometer thick sections of the slides were taken on saline or poly-L-lysine coated slide.
2. It was then transferred to three changes of xylene for 30 minutes.
3. Later rehydrate with decreasing grades of alcohol absolute, 95 %, 70 %, 50 %.
4. Finally sections were washed under tap water for 20-30 minutes.
5. Antigen Retrieval- The demonstration of many antigens can be significantly improved by the pre-treatment with the antigen retrieval reagents that break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. This was done by adding citrate buffer pH 6 and antigen retrieval reagents. Heat mediated antigen retrieval was done in the microwave oven at 800 watt for 10 minute, following 420 watt for 10 minute and 360 watt for 5 minute.
6. Immunostaining:
    a) Peroxidase block with 3% hydrogen peroxide in methanol for 5 minute.
    b) Incubate sections for 10 minute for power block. It is done to mask the other antigens which are not required.
    c) Primary Antibody Incubation: Incubated with primary antibody depending upon manufacturer's instruction either for 30 minute at room temperature or overnight incubation (we incubated for 30 minute at room temperature).
    d) Washed in Tris buffer solution pH 7.4 - 10 minute.
    e) Superenhancer: Incubate with super enhancer for 10 minute.
    f) Wash in Tris buffer solution pH 7.4 - 10 minute.
    g) Incubate with polymer horse reddish peroxide (poly HRP) for 30 minute.
    h) Wash in Tris buffer solution pH 7.4 - 10 minute.

i) Substrate: incubated with substrate DAB and check for the colour change, brown colour appears within 5-10 min, depending upon intensity of antigen-antibody reaction.
j) Again wash in Tris buffer solution pH 7.4 - 10 minute.

7. Transfer to tap water for 10-20 minutes.
8. Put into increasing grades of alcohol 50 %, 70 %, 95 % and absolute alcohol.
9. Transfer to three changes of xylen.
10. Mount with DPX.

Structures analyzed and semiquantitative scale used to evaluate the staining reactions

All the sections were studied under the compound light research microscope and microphotography was done with USB camera. All the digital microphotographs was stored and analyzed.

The amount of staining reaction by the immunohistochemical procedure were observed and semiquantitated as

+ (mild) when there was only few positively stained cells,
++ (moderate) when there was moderate numbers of positively stained cells,
+++ (strong) when there was large numbers of positively stained cells,
++++ (high strong) when there was very large numbers of positively stained cells,
Neglible staining reaction was marked as absent.

RESULTS

Lymphocyte subpopulations were examined in the fetal spleen of various gestational ages. We used CD3 as a marker of T-lymphocytes subpopulations and CD20 as a marker of B-lymphocytes subpopulations to find the immunoreactivity of T and B lymphocytes in the fetal spleen. In the spleen of the 16th gestational week foetuses, T and B lymphocytes were scattered in the splenic parenchyma. No accumulation of T and B lymphocytes was found around the arterioles (Fig. 1). At 20th g.w. B-lymphocytes condensations began to form around the central arterioles with scattered T-lymphocytes. In the spleen of 22nd gestational week, the population of T and B lymphocytes became denser as compared to earlier groups. The B-lymphocyte aggregates were larger and better formed than earlier groups. Also, slightly more scattered T-lymphocytes are present (Fig. 2). At 24th gestational week, the B-lymphocytes aggregates were well formed and were associated with scattered T-lymphocytes, which also begin to form clusters. After the 24th gestational week (Fig. 3), the reticular framework of the white pulp was specialized into two distinct compartments (T-cell region and B-cell region). CD3 positive T-lymphocytes were distributed in the reticular framework around the arteriole and formed the periarteriolar lymphatic sheath (PALS). CD20 positive B-lymphocytes aggregated around an eccentrically placed arteriole to form the lymphoid follicle (Fig. 4). At later stages immunoreactive CD3 positive cells were reduced as compared to the immunoreactive CD20 lymphocytes in the spleen. The staining reaction of the CD3 (T-lymphocytes) and CD20 (B-lymphocytes) at different gestational age is shown in following Table 1 (Fig. 5).

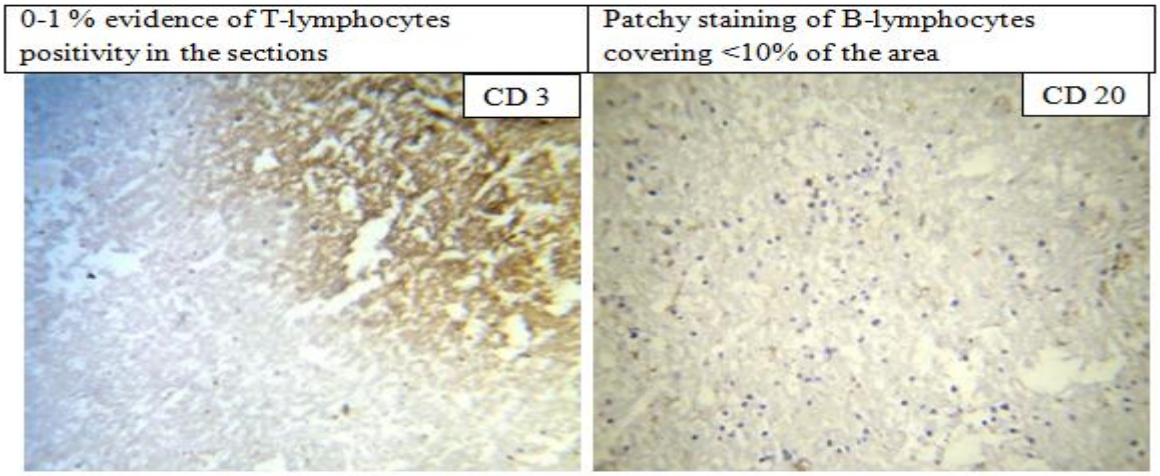

Figure 1: Immunohistochemistry of the foetal spleen at 16th g.w. for CD3 + T-lymphocytes and CD20 + B-lymphocytes. Note that the B-lymphocytes are beginning to form small aggregates. Also scattered T-lymphocytes are present.

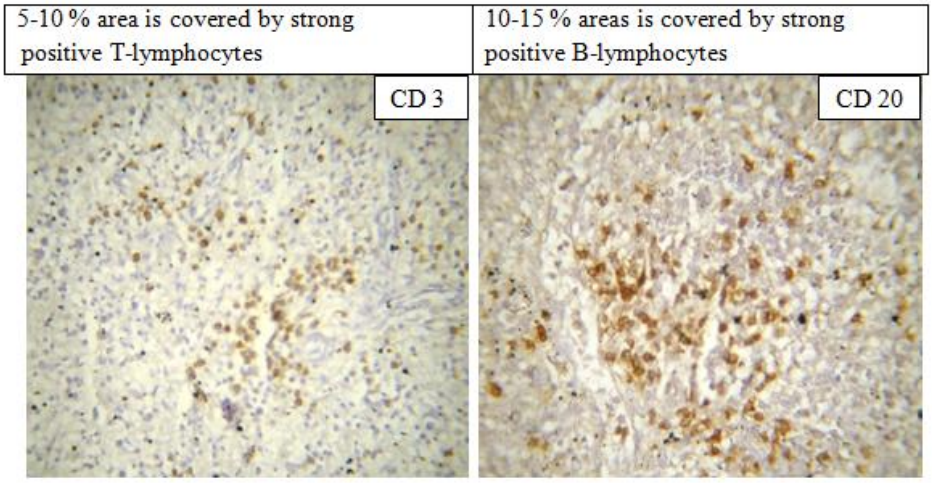

Figure 2: Immunohistochemistry of the foetal spleen at 22nd g.w. for CD3 + T-lymphocytes and CD20 + B-lymphocytes. Note that the B-lymphocyte aggregates are larger and well formed. Also, slightly more scattered T-lymphocytes are present.

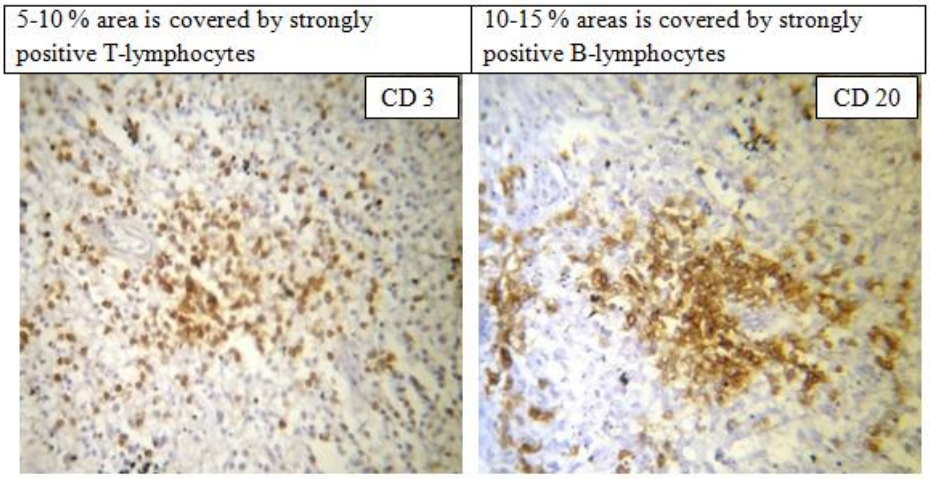

Figure 3: Immunohistochemistry of the foetal spleen at 28th g.w. for CD3 + T-lymphocytes and CD20 + B-lymphocytes. Note that the B-lymphocyte aggregates are well formed and are associated with scattered T-lymphocytes, which also begin to form small clusters.

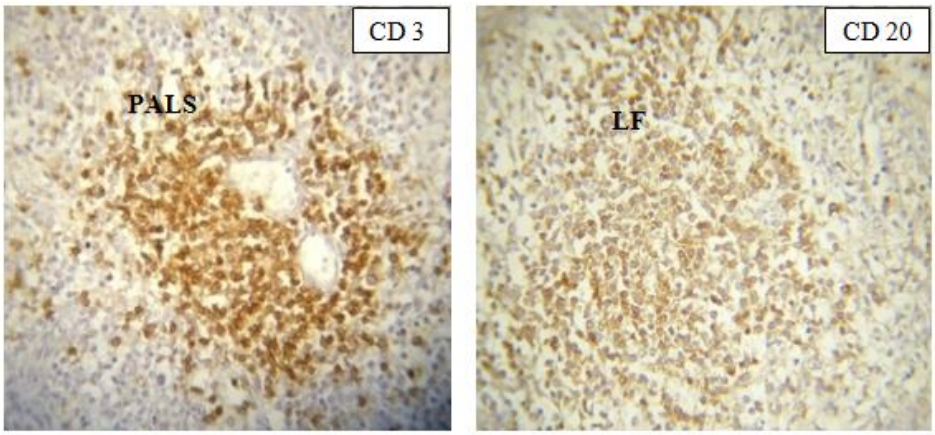

Figure 4: Immunohistochemistry of the foetal spleen at 32nd g.w. for CD3 + T-lymphocytes and CD20 + B-lymphocytes. Note that B-lymphocyte aggregates now are seen adjacent to clusters of periarteriolar T-lymphocytes.

Table 1: Density of CD3 (T-lymphocytes) and CD20 (B-lymphocytes) at different gestational age.

Gestational ages	CD3 T-lymphocytes		CD20 B-lymphocytes	
	Density	Staining Reaction	Density	Staining Reaction
14	-	-	-	-
16	0-1 %	+	5-10 %	++
18	5-10 %	++	5-10 %	++
20	5-10 %	++	5-10 %	++
22	5-10 %	++	10-15 %	+++
24	5-10 %	++	10-15 %	+++
26	5- 10 %	++	10-15 %	+++
28	5-10 %	++	10-15 %	+++
30	10-15 %	+++	20-25 %	++++
32	10-15 %	+++	20-25 %	++++
34	10-15 %	+++	20-25 %	++++
36	5-10 %	++	15-20 %	++++
38	5-10 %	++	15-20 %	++++

++++: More than 15 %, (High Strong positivity) when there was very large numbers of positively stained cells,
+++: 10-15 % (Strong positivity) when there was large numbers of positively stained cells,
++: 5-10 % (Moderate positivity) when there was moderate numbers of positively stained cells,
+: < 5 % Mild positivity when there was only few positively stained cells.
- Not seen

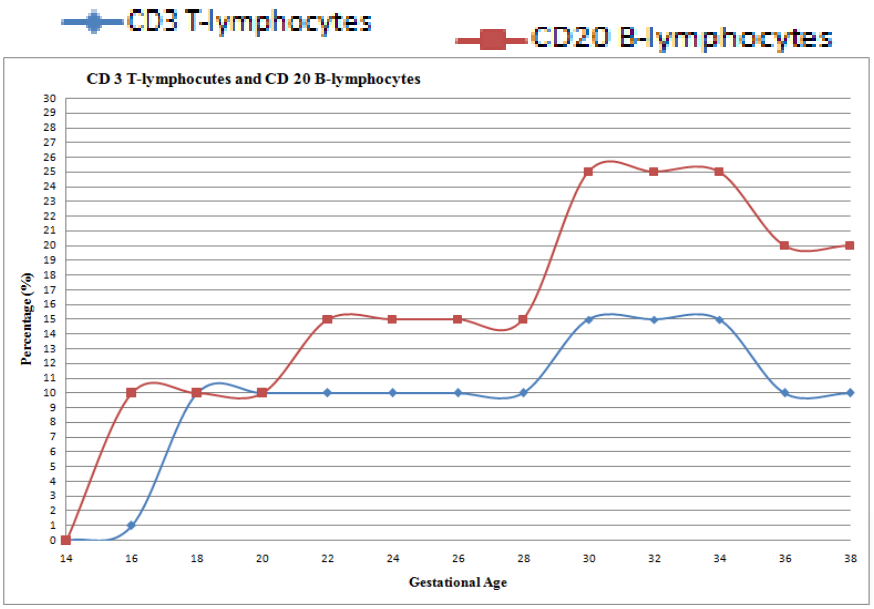

Figure 5: Line graph showing density of CD3 T-lymphocytes and CD20 B-lymphocytes against gestational age.
The density of immunoreactive CD3 T-lymphocytes is less as compared to the immunoreactive CD20 T-lymphocytes in the foetal spleen.

DISCUSSION

Numerous previous studies dealing with the development of the human spleen in foetal period mainly contain qualitative descriptions of this processes and report changes in the weight of the gland.

Lymphocytes in the spleen include T and B lymphocytes. CD3 and CD20 lymphocytes are important differentiation antigens on T- and B-cell membranes. Immunohistochemical staining of CD3 and CD20 lymphocytes is ideal for analyzing the distribution and count of T and B lymphocytes in tissue [7].

In the present study, an initial scattered invasion of lymphoid cells throughout the splenic reticulum and an accumulation of lymphocytes around the central arteries (PALS) can be recognized during the 18th and 20th g.w. Some of them show morphological criteria of T-precursor cells as described in the fetal thymus, lymph node and tonsil (V. Gaudecker and Muller-Hermelink 1980) [8]. Immunohistochemical labelling experiments (V. Gaudecker et al. 1985) [9] reveal that many lymphoid cells in the forming PALS have the same surface antigen as T-cells in postnatal lymphoid tissue

(Ledbetter et al. 1981; v. Gaudecker et al. 1984) [10,11]. This indicates that the periarterial lymphoid sheath (PALS), in this early developmental stage, can be regarded as the T-cell region.

In the present study during the 20th week of gestation, lymphoid cells clearly have colonized the PALS. Between the 20th and 24th GW, the white pulp extends continuously. During this time a remarkable growth of the lymphatic tissue can be followed, while the red pulp seems to cease its extension. In the 34th week of gestation, the white pulp occupies about two fifth of the organ volume, which is in flagrant contradiction to the proportion in the postnatal spleen, where the red pulp makes up more than 80 % of the volume, Ono (1930) and Tischendorf (1969) [12].

The assemblage of primary follicles i.e., the development of the B-cell region, begins later in the human spleen than in all other lymphatic organs. The time interval between the formation of the T-cell region, and the differentiation of the B-cell region, is also larger in the spleen than in lymph nodes and tonsils, taking about 5 weeks and from 2 to 3 weeks, respectively (Markgraf et al. 1982; V. Gaudecker and Muller-Hermelink 1982) [13,14]. In our study around the 23rd g.w., the assemblage of B-cell regions first begins at the periphery of PALS. These small primary follicles contain almost homogeneous type of lymphatic cells, eccentrically located in relation to the PALS, represent the precursors of B-cells.

During postnatal life, the manner in which lymphocytes recirculate from the blood to the stroma of lymphoid organs is different in the spleen than in lymph nodes or tonsils. In the latter, lymphoid organs lymphocyte diapedesis takes place in high endothelial venules. It is supposed that in the spleen recirculating lymphocytes leave the circulation via arteries terminating in the marginal zone of the PALS. These penicillar arteries often bifurcate and form a funnel shaped orifice (Weiss 1977) [15]. T-cells home into the PALS, the T-cell region. B-cells migrate through the PALS and home into the follicles, the B-cell region (Pabst 1981) [16].

The Table-2 shows a comparative study of CD3 and CD20 markers at 16th g.w. with Hwang et al.[17] They are almost similar with our study.

Table 2: Comparison of CD3 and CD20 markers with other study.

Researchers	CD 3 Marker Staining Reaction	CD 20 Marker Staining Reaction
Hwang et al. (2014)	±	+
Present study (2016)	+	++

Hwang et al (2014) semiquantitated the staining reaction as
±: when there were 1-24 positive cells.
+: when there were 25-50 positive cells.
Present study (2016) semiquantitated the staining reaction as
++: 5-10 % when there was moderate numbers of positively stained cells,
+ : < 5 % when there was only mild/few positively stained cells.

Limitation: The limitation of the present study is the less numbers of foetuses in different groups ranging from 10th to 38th weeks of gestation.

CONCLUSION

From our study we conclude that the spleen of foetuses stained positively for CD3 T-lymphocytes and CD20 B-lymphocytes by 16th g.w. By the 24th g.w the white pulp showed two distinct components T-cell and B-cell regions. The density of immunoreactive CD3 T-lymphocytes was less as compared to the immunoreactive CD20 B-lymphocytes in the spleen throughout the gestation. The T and B lymphocytes, the immunological component increased in the later gestational stages. The density of T lymphocytes remained lesser than B lymphocytes throughout gestation. Nevertheless, a working knowledge of the normal splenic appearance at certain gestational ages can be helpful in recognizing abnormal development of the spleen that has been retarded or accelerated by a pathologic process.

Declaration:
Conflicts of interests: The authors declare no conflicts of interest.
Author contribution: All authors have contributed in the manuscript.
Author funding: Nill.

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