Onset of Lymphocyte Function in the Developing Human Fetus

CHARLES S. AUGUST, A. IZZET BERKEL, SHIRLEY DRISCOLL, AND EZIO MERLER[46]

Department of Pediatrics, Children's Hospital Medical Center and the Department of Pathology, Boston Hospital for Women (Lying-In Division), Harvard Medical School, Boston, Massachusetts, USA

Extract

Lymphocytes capable of responding to phytohemagglutinin (PHA) were present in human fetal thymus tissue by 12-week gestational age and in spleens 2–4 weeks later. In spleens, establishment of the PHA response was associated with the appearance of small lymphocytes in cuffs surrounding central arterioles.

Speculation

Onset of PHA responsiveness in lymphocytes obtained from the embryonic thymus precedes that of the spleen. It would be of particular interest to know when bone marrow cells and lymph node lymphocytes start to show this function.

Introduction

The onset of immunologic function in the developing human fetus has only recently been studied systematically [11, 12, 16, 32], and existing data mainly concern immunoglobulin synthesis. What is known about the onset of cellular immune function (delayed hypersensitivity) has been largely extrapolated from animal studies [15, 23, 32]. Accumulation of human quantitative data in this regard is essential not only for understanding the pathogenesis of many congenital infections, but also for rationalizing attempts to correct certain immunologic deficiency syndromes by the transplantation of fetal organs [15].

Studies of lymphocyte function in vitro [19], both clinically [2, 7, 9, 17, 20] and experimentally [22, 28], suggest that it is the so-called "thymus-derived" lymphocyte which proliferates in vitro when exposed to phytohemagglutinin (PHA). Such cells have been identified in the thymus [16] and peripheral blood [24] of the developing human fetus at 14- and 17-week gestational age (GA), respectively.

The present report describes studies undertaken in 18 human fetuses of 12- to 28-week GA. Cells from thymus and spleen were cultured and the responses to PHA were estimated quantitatively. In addition, portions of each organ were examined histologically. It was found that lymphocytes capable of responding to PHA were present in the thymus by 12-week GA and in the spleen 2–4 weeks later (14–16 weeks). In the spleen, onset of the PHA response was associated with the appearance of lymphocytes in cuffs surrounding central arterioles, the so-called "thymus-dependent" areas, as defined by Parrot, DeSousa, and East [25] and Goldschneider and McGregor [13].

Materials and Methods

Preparation of Cell Suspensions

Spleens and thymuses were obtained from noninfected fetuses which were the products of therapeutic abortions. Gestational age (from time of conception) was derived from crown-rump length measurements by referring to the data of Patten [26]. Employing sterile techniques, a small portion of each organ was placed

in Zenker's fixing solution and submitted for histologic examination. The remainder was placed in a Petri dish in medium 199 [34] and the cells were teased into the medium with a scalpel. The debris was allowed to settle for 5 min. The suspended cells were pipetted to achieve single cell suspension. Cells were sedimented by centrifugation at $200 \times g$ for 10 min and the cell pellet was resuspended in 50 times its volume of culture medium. Cells were counted in a Coulter particle counter ($100-\mu$ aperture) or with an hematocytometer and viability was estimated by the exclusion of eosin red dye [14]. Cell concentration was adjusted to $1-2 \times 10^6$ viable cells/ml. Viabilities ranged from 78-100% for thymus cells and from 21-64% for spleen cells.

Culture Technique

Medium 199 with Hanks' balanced salt solution was used throughout. For washing cells, 100 ml medium were supplemented with 2 ml antibiotic solution containing 10,000 units penicillin G, 10,000 μg streptomycin, 50 μg amphotericin B [35], and 1 ml heat-inactivated, AB, Rh-positive, human serum. For culturing cells, 18 ml serum were added, yielding a final serum concentration of 15%.

Whenever possible, cells were cultured in duplicate in 15-mm by 130-mm screw top tubes of either glass or plastic. Initially, all cultures in which sufficient cells were available were carried out in 5-ml volumes at cell concentrations of $1-2 \times 10^6$ viable cells/ml. In later experiments, volumes were reduced to 1 or 2 ml. The PHA [36] had been prepared by extracting red kidney beans (*Phaseolus vulgaris*) with 0.15 M NaCl. A sterile solution of this extract was added at a concentration of 0.05 ml PHA solution/ml culture. Occasionally, mixed lymphocyte cultures were carried out [3]. In these instances, equal numbers of cells from each donor were used with the total cell number per tube remaining at $1-2 \times 10^6$ viable cells/ml. When sufficient cells were available, ongoing DNA synthesis at the time of culture was estimated by incubating the cells with ^{3}H-thymidine for the first 16 hr of culture. In this way, it could be determined whether PHA had actually stimulated or merely prolonged ongoing DNA synthesis.

All cultures to which PHA had been added, as well as untreated controls, were allowed to incubate undisturbed for 72 hr in an atmosphere of moist, 5% CO_2-95% air at 37°. At that time, ^{3}H-thymidine [37] was added to each tube to achieve a final concentration of 1.0 μCi/ml. The cells were returned to the incubator for 16 hr (overnight) and harvested.

Quantitation of the Proliferative Response to PHA

Incorporation of ^{3}H-thymidine into DNA was estimated by an adaptation of the filter paper disk technique of Mans and Novelli for measuring protein synthesis [21]. At the time of harvest, cells were resuspended and 0.1-ml aliquots of the cultures were pipetted onto filter paper disks [38]. The disks were dried and transferred to cold 10% trichloroacetic acid (TCA) containing 10 mg unlabeled thymidine/100 ml TCA in a volume of at least 3 ml/disk. The disks were washed twice in 5% TCA for 15 min, twice in ethanol-acetone (1:1) at 37° for at least 30 min, and twice in acetone for 15 min. The disks were placed in vials and the radioactivity was estimated in a liquid scintillation spectrometer. Results were expressed as cpm/10^5 viable cells originally cultured. No corrections for the absorption of radioactivity to the disks were made. The average variation from the mean in cultures stimulated by PHA was 5.8% (range 0.4-11%). The average variation of unstimulated controls was 12.4% (range 0-38%). A "stimulation index" (SI) was calculated as the ratio of cpm/10^5 cells in the stimulated culture to that in the control.

Histologic Examination

All specimens were coded and examined without prior knowledge of the results of PHA stimulation. Thymuses were evaluated by two main criteria: (*1*) the demarcation into lymphoid cortex and medulla, and (*2*) the presence of Hassall's corpuscles. In the spleens, evidence was sought for the differentiation into red and white pulp by the presence of (*1*) arterioles, (*2*) small lymphocytes surrounding arterioles (cuffs) and (*3*) germinal centers. When histologic examination was complete, the results were correlated, both with gestational age and the results of PHA stimulation.

Results

Correlation of PHA Responses with Histologic Appearance

Thymus glands and spleens from 18 fetuses of 12- to 28-week GA were studied. The data from 16 technically satisfactory experiments using thymus cells and 15 using spleen cells are shown in Table I, together with a summary of the histologic appearance of each organ. Thymus cells responded to PHA by increased incorporation of ^{3}H-thymidine into DNA whenever they were tested, that is, from 12-week GA onward. Histologic examination revealed that all thymuses had

Table I. Correlation of PHA responsiveness, histologic appearance, and gestational age in 18 human fetal thymuses and spleens

Specimen	Crown-rump length, cm	Estimated gestational age, weeks	Thymus					Spleen					SI thymus/ SI spleen
			PHA	Control	SI[1]	Histology		PHA	Control	SI[1]	Histology		
			cpm/10⁵ cells			LC[2]	HC[3]	cpm/10⁵ cells			CA[4]	LC[5]	
1	7.5–8	12				+	0				0	0	
2	8.0	12				+	0				0	0	
3	8.0	12	1,180	93	13			78	73	1			13
4	8.0	12	24,274	873	28			881	321	3	0	0	9
5	8.2	12	2,641	377	7	+	0	452	574	0.8	0	0	9
6	8.5	12.5	16,399	727	23	+		1,568	527	3	+	±	8
7	10.0	13.5	4,203	126	33	+	+	617	239	3	+	0	11
8	10.7	14	7,817	213	37	+	+	2,832	1,019	3	±	0	12
9	11.0	14	5,337	57	94	+	0	9,154	904	11	±	0	9
10	11.0	14.2	4,388	202	22	+	+	1,820	1,067	2	±	0	11
11	11.5	14.5	1,889	159	12	+	±	14	5		±	0	
12	11.5	14.5	2,750	61	45	+	±	401	31	13	±	0-±	3
13	12.0	15	16,621	321	52	+	+	Contaminated			+	0	
14	12.5	15.5	1,885	18	106	+	±	611	57	11	+	±	10
15	14.0	16.5	11,688	762	15	+	+	12,416	1,402	9	+	±-+	2
16	15.3	17.5	2,872	46	63	+	+	4,048	91	45	+	±	1
17	15.5	17.5	2,045	88	23	+	+	5,780	600	10	+	+	2
18	36	28	3,442	182	21	+	0	7,042	360	20	+	+	1

[1] SI: Stimulation index; all numbers and ratios have been rounded off to the nearest whole number.
[2] LC: Lymphoid cortex.
[3] HC: Hassall's corpuscles.
[4] CA: Central arterioles.
[5] Lymph cuffs.

demarcated into a lymphoid cortex and medulla (Figs. 1 and 2). Hassall's corpuscles were found regularly beginning at 14.5-week GA. The absence of Hassall's corpuscles in the 28-week GA thymus was thought to be due to an artifact of section.

In contrast to the development in the thymus gland, the earliest spleen cells which responded to PHA with an SI greater than 3 were obtained from a fetus of 14-week GA. Indeed, the cells from only two of 11 spleens cultured prior to 16-week GA incorporated more than 4000 cpm/10⁵ cells. Histologic examination of the spleens revealed that the specimens obtained at 12-week GA were almost entirely red pulp with only scattered mononuclear cells (Fig. 3). Between 12- and 15-week GA, central arterioles began to appear which, at first, lacked the cuff of small lymphocytes which is characteristic of a mature organ (Fig. 4). In the next few weeks, more mononuclear cells appeared, at first scattered about the red pulp and then in cuffs only a few cells deep surrounding central arterioles. Touch preparations from these specimens showed the presence of many erythrocyte precursors. As early as 17-week GA, and particularly in the 28-week GA spleen, almost all central arterioles were surrounded by prominent cuffs of mononuclear cells easily identified as

small lymphocytes (Fig. 5). By that time, the proliferative response of spleen cells to PHA was well established. No germinal centers were found in any of the spleens examined.

The relation between the stimulation index (SI) calculated from the data in Table I and the gestational age is shown in Figure 6. Although the variation of the SI in cultures which responded was considerable, it was clear that PHA responsiveness of spleen cells lagged behind that of thymus cells by 2–4 weeks.

Intrinsic Rate of DNA Synthesis and PHA Stimulation

Cells derived from fetal thymuses were mixtures of medium and small lymphocytes, as well as of more primitive cells. It might be predicted and has been shown biochemically [6] that at least some of these cells actively synthesize DNA at the outset of culture. It was of interest to determine whether PHA had stimulated or simply prolonged ongoing DNA synthesis. In an experiment carried out for 27 hr, it was found that ³H-thymidine was incorporated into DNA linearly for 8 hr, after which time no new synthesis occurred. In the four thymuses and two spleens in which sufficient cells were available, DNA synthesis during the first 16 hr *in vitro* was compared with that observed after 72

Fig. 1. Thymus from a human fetus of 11-weeks GA. Note the demarcation into lymphoid cortex and medulla. No Hassall's corpuscles are present. Toluidene blue and eosin. × 80.
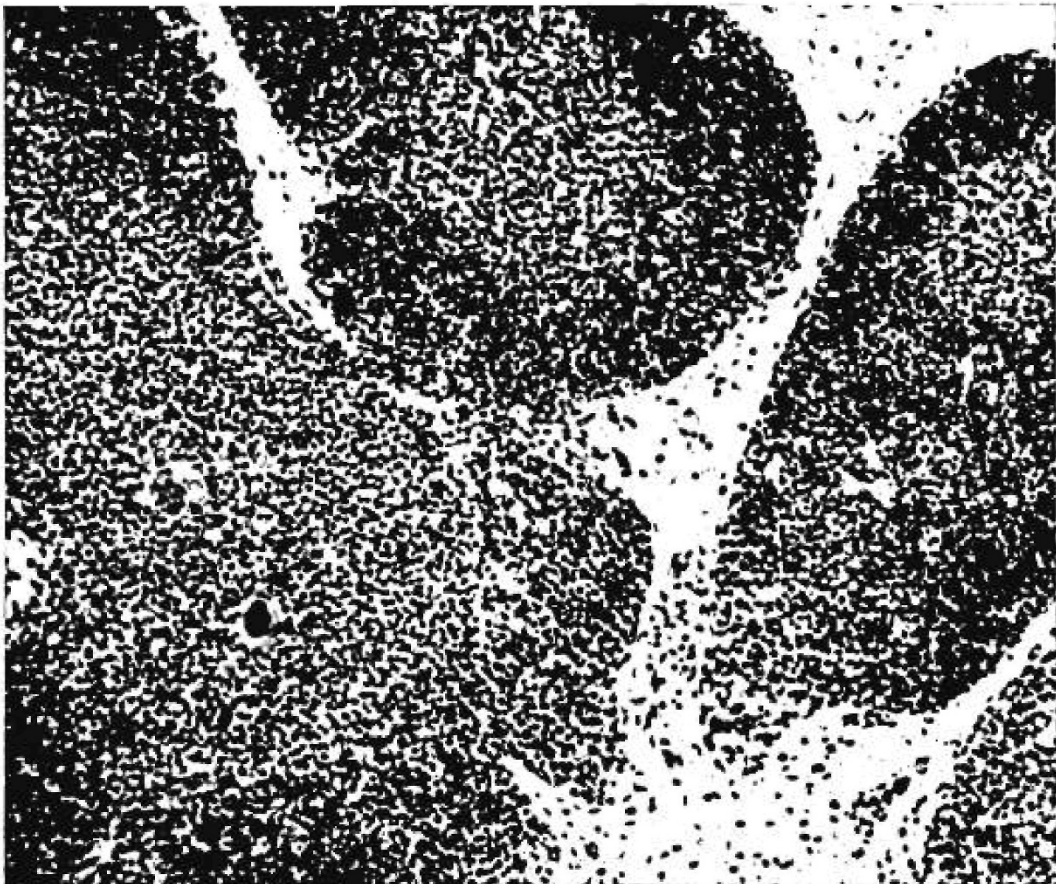

Fig. 2. Thymus from a human fetus of 14-weeks GA. Note the clear demarcation into lymphoid cortex and medulla. Hassall's corpuscles are present. Toluidine blue and eosin. × 32.
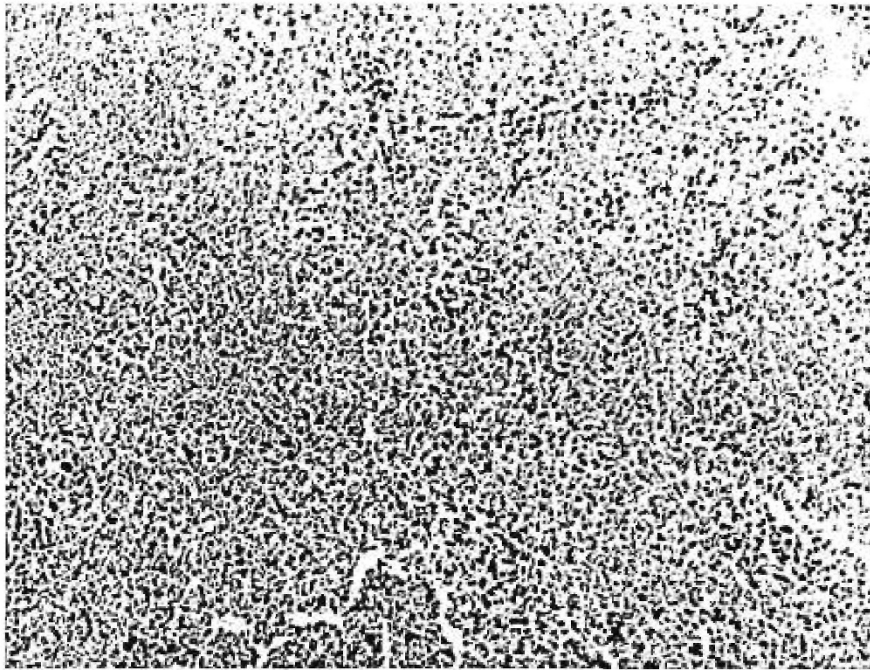
Fig. 3. Spleen from a human fetus of 11-weeks GA. Only scattered mononuclear cells are present. Toluidine blue and eosin. × 80.

hr in PHA-stimulated and control cultures. Table II indicates that in five of six cultures the magnitude of the PHA response during the 4th day exceeded the initial level of DNA synthesis by 200–1200%. Similarly, in five of six unstimulated controls, DNA synthesis declined from 3–21% of the initial value.

Responses of Fetal Lymphocytes to Allogeneic Cells

Mixed leukocyte cultures between fetal cells and adult peripheral blood leukocytes of the opposite sex or treated with mitomycin C were technically unsatisfactory. Specimens 13 and 15 became available simultaneously, and it was possible, therefore, to mix thymic cells from two fetuses of 15- and 16.5-week GA. Table III presents the results of this experiment, which clearly show that there had been vigorous proliferation in the mixture of allogeneic cells.

Discussion

This study of fetal thymus glands and spleens indicates that in man from at least the 12th gestational week onward the thymus has differentiated into functional lymphoid cortex and medulla. Starting at 12-week GA, fetal thymocytes were capable of responding to PHA by increasing DNA synthesis. In contrast, spleens of 12- to 13-week GA contained no recognizable lymphoid tissue and the cells teased from these organs responded

feebly to PHA. PHA responsiveness began in spleen cells at about 14-week GA and was fully established only after 16-week GA. These data correlated well with the appearance of scattered groups of lymphocytes in 14-week-old fetal spleen samples and with the appearance of larger numbers of lymphocytes found cuffing the central arterioles after 16-week GA. The morphologic data presented, indicating peripheral localization of lymphoid cells from thymus to spleen in the developing embryo, were identical with that described in a number of animal species [23], particularly rabbits [1] and opossums [4].

This study also includes some preliminary data which suggest that the ability of fetal thymus cells to proliferate in response to allogeneic stimuli is present around 15- to 16.5-week GA. This is consistent with the observations of Siegler and Metzgar [30], who found that at this time in fetal life the thymus is already endowed with histocompatibility (HL-A) antigens.

Our findings supplement earlier work concerning human maturation, the purpose of which was usually to obtain mitoses suitable for cytogenetic analyses. This work, summarized by Ling [19] and van Furth [10], suggested from morphologic studies that thymic lymphocytes at 14-week GA and spleen cells at 19- to 20-week GA responded to PHA. Recently, Papiernik
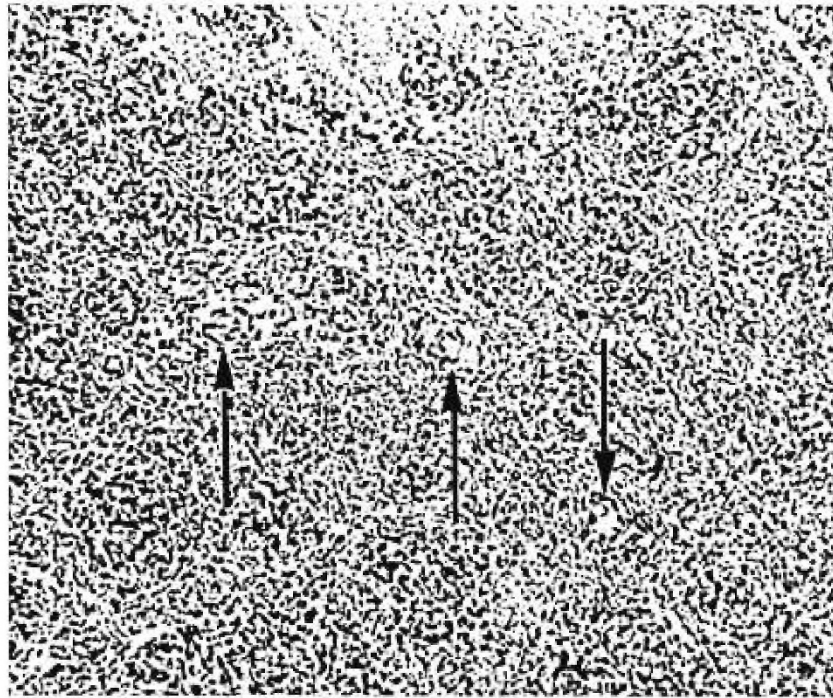
Fig. 4. Spleen from a human fetus of 14-week GA. Arterioles (*arrows*) are present but lack cuffs of small lymphocytes. Mononuclear cells are more numerous than at 11 weeks but still appear disorganized. Toluidine blue and eosin. × 80.
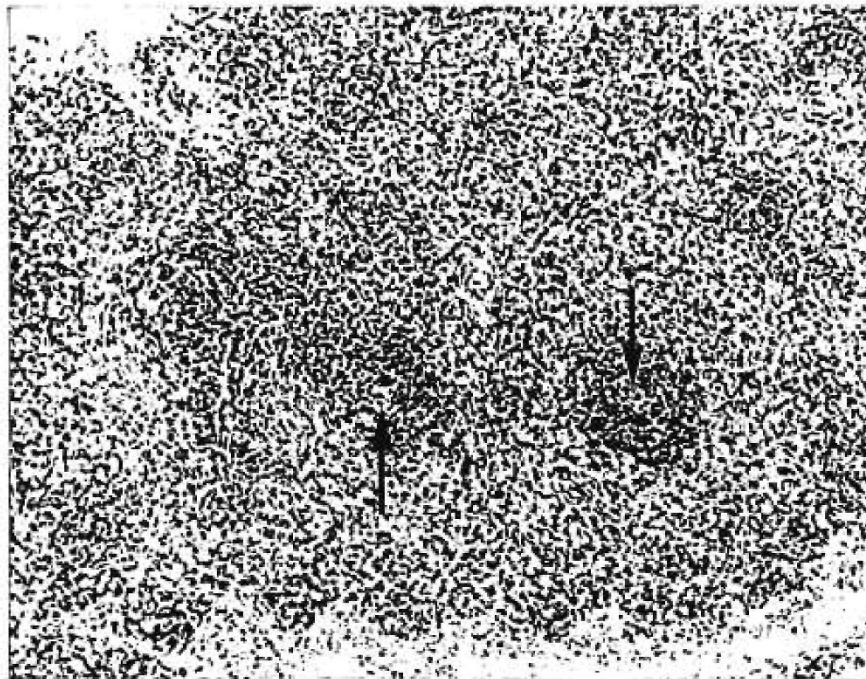

Fig. 5. Spleen from a human fetus of 28-week GA. Note the prominent cuffs of small lymphocytes surrounding central arterioles (*arrows*). No germinal centers are present. Toluidine blue and eosin. × 80.

[24] using autoradiography found that cells from thymus glands and peripheral blood from fetuses between 17- and 22-week GA proliferated in response to PHA. More recently, Kay, Doe, and Hockley [16] dated the onset of PHA responsiveness in human thymocytes at about 14-week GA. The quantitative methods employed in the present study and comparisons of the initial level of DNA synthesis with the values seen in stimulated and control cultures 3 days later appear to overcome the difficulty described by Kay [15] in quantitating "a system where only a few cells respond and where control cultures ... always contain some blast-like cells." Our data indicate that PHA stimulated DNA synthesis above and beyond the level which existed at the beginning of culture.

The immunologic significance of the capacity of lymphocytes to respond to PHA has recently been clarified. It has been shown in experimental animals that neonatal thymectomy, which impairs cellular immune function, also diminishes the ability of animal lymphocytes to respond in vitro to this mitogen [22, 28]. The lymphocytes of patients with congenital absence of the thymus are unable to proliferate normally in response to PHA [7, 9, 15, 20]. Correcting the immunologic deficiency in such patients by implanting fetal thymus tissue, however, has also restored the ability of lymphocytes to respond to PHA [2, 7]. Thus, it appears that normal lymphocyte response to PHA requires an intact population of peripheral lymphocytes which have been influenced by the thymus, and in vitro testing therefore appears to constitute a means of identifying this lymphocyte population.

It cannot be determined from the present study whether the PHA-responsive lymphocytes found in thymuses and spleens early in gestation are, in fact, immunologically competent. The results of the mixed leukocyte culture suggest that fetal thymocytes can proliferate in response to an allogeneic stimulus. Whether these cells can synthesize the mediators of cellular immune reactions [8] is unknown. Gitlin and Biascusi, however [12], have reported that cells obtained from fetal thymuses of 17- to 18-week GA were able to synthesize IgM and IgG. The results of our lymphocyte function studies correlate well with the studies of Silverstein and Kraner [31], who observed allograft rejections in fetal monkeys approximately one-third of the way through gestation. Our correlations of lymphocyte function with histologic appearance are reminiscent of the studies of Rowlands, LaVia, and Block [29], who found that antibody synthesis began in opossum embryos immediately after
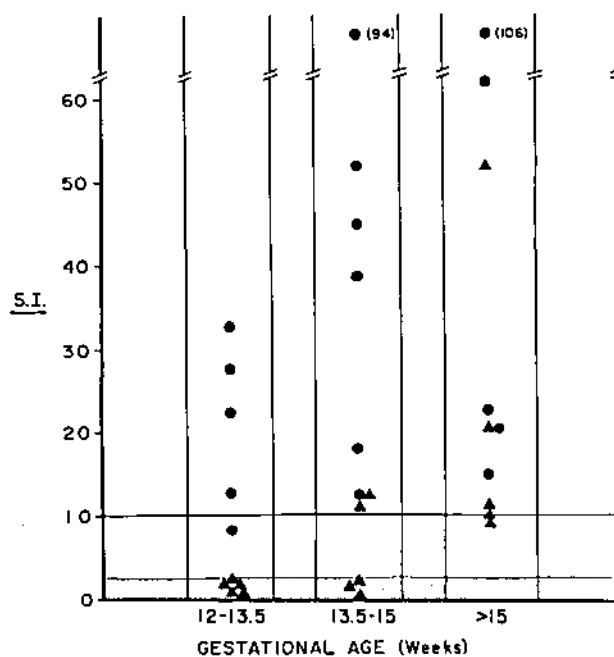

Fig. 6. Responses to PHA of human fetal thymocytes (●) and spleen cells (▲) of varying gestational ages. The stimulation index (SI) is calculated as in the text: SI of 0 to 3 (hatched area) represents little or no response; SI of 3 to 10 represents poor to fair response; SI greater than 10 represents the normal range for adult peripheral blood lymphocytes.

Table II. The DNA synthesis by fetal lymphoid cells on the 1st day of culture compared with PHA-stimulated DNA synthesis after 4 days

Specimen	Organ	Day 1 Unstim-ulated	Day 4 PHA	Day 4 Unstim-ulated	Ratio of day 4 to day 1 PHA	Ratio of day 4 to day 1 Unstim-ulated
		cpm/10⁵ cells				
12	Thymus	564	2750	61	4.9	0.10
14	Thymus	634	1885	18	3.0	0.03
14	Spleen	267	611	57	2.3	0.21
16	Thymus	665	2872	46	4.3	0.07
17	Thymus	2357	2045	88	0.9	0.04
17	Spleen	475	5780	600	12.2	1.30
Average					4.6	0.29

Table III. Mixed lymphocyte response of fetal thymuses

Specimen	Gestational age, weeks	Control	Mix
		cpm/10⁵ cells	
13	15	321	5925
15	16.5	762	

the appearance of lymphoid tissue in the thymus and a few lymph nodes.

The changing histologic appearance of the spleen suggests that the onset of thymus-derived lymphocyte

function in that organ occurred during the period encompassed by the present study. Studies in experimental animals [13, 25, 27, 33] have established that thymus-derived lymphocytes migrate out of the blood stream within the spleen and localize in sheaths surrounding central arterioles. These areas have been designated "thymus-dependent" [25]. The present data suggest that in the spleen the onset of PHA responsiveness accompanies the population of thymus-dependent regions during the 14th to the 16th week of gestation. It is noteworthy that no spleen showed germinal centers. These are thought to arise only after antigenic stimulation and to be related to immunoglobulin production rather than cellular immunity [5].

It is pertinent to note that LaVia *et al.* [18], using opossums as the animal model, found that antibody synthesis could not be detected when the thymus was the sole lymphoid organ. They concluded that some peripheral localization of lymphoid cells from the thymus was required for antibody synthesis. If this reasoning can be extrapolated to man, then on the basis of our data the onset of immunologic competence might be expected between the 12th and 16th week of gestation.

Summary

The onset of lymphocyte function in the developing human fetus was studied by culturing *in vitro* lymphoid cells obtained from spleens and thymus glands of 16 fetuses of 12- to 28-week gestational age (GA). The response of these cells to phytohemagglutinin (PHA) was determined by measuring the incorporation of ^{3}H-thymidine into deoxyribonucleic acid. In addition, each organ was examined histologically. It was found that PHA-responsive lymphocytes were present in the thymus at 12-week GA and in the spleen 2–4 weeks later (14- to 16-week GA). All thymuses examined had demarcated into lymphoid cortex and medulla. In the spleens, the onset of PHA response correlated with the appearance of small lymphocytes.

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34. Microbiological Associates, Bethesda, Md.

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37. New England Nuclear Corporation, Boston, Mass., specific activity, 2.0 Ci/mmole.

38. Whatman no. 3, 2.3-cm diameter.

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40. Requests for reprints should be addressed to: EZIO MERLER, Immunology, Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Mass. 02115 (USA).

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