The Human Fetal Omentum: A Site of B Cell Generation

By Nanette Solvason and John F. Kearney

From the Division of Developmental and Clinical Immunology, Department of Microbiology, and the Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

Summary

The fetal mouse omentum has been shown to be a source of precursors that exclusively reconstitutes $Ly1^+$ B cells and the closely related $Ly1^-$ sister population, but not conventional B cells or T cells. We have extended these studies to compare B cell development in the human fetal omentum, liver, and spleen, and to demonstrate that the pro/pre–B cell compartment ($CD24^+$, $sIgM^-$) is detected in the omentum and liver but not spleen as early as 8 wk of gestation. From 8 to 12 wk of gestation, the proportions of IgM^+ cells that were pre-B cells ($cIgM^+/sIgM^-$) in the omentum and liver were 53 ± 15% and 45 ± 13%, respectively, and IgM^+ cells were not detectable in the spleen. After 12 wk, the percentage of pre-B cells was unchanged in the fetal liver (41 ± 10%) but decreased significantly in the omentum (25 ± 14%); pre-B cells were now detected in the spleen but at much lower percentages (2 ± 3%) than either the omentum or liver. The nuclear enzyme, Tdt, was detected in approximately 25% of the $CD24^+$ cells in the omentum and liver during the 8–12-wk time period, however, Tdt^+ cells were not detected in the spleen. Approximately 40% of the mature B cells found in the omentum and spleen were $CD5^+$ compared with only 20% in the liver. These results demonstrate that the fetal omentum, like the fetal liver and bone marrow, is a primary site of B cell development.

The mouse fetal liver is an early site of hematopoiesis and a rich source of stem cells capable of reconstituting all hematopoietic lineages including $Ly1^+$ B cells (1, 2). At 16–17 d of gestation, hematopoiesis begins to shift from the liver to the bone marrow where it is maintained throughout adult life (3). However, adult bone marrow, in contrast to fetal liver, only poorly reconstitutes $Ly1^+$ B cells, which has led to the suggestion that Ly1 B cells were derived from distinct precursors that are present in the fetus but absent in the adult (2). More recently, it has been shown that mouse B cell precursors from fetal liver give rise in vitro and in vivo to $CD5^+$ B cells, whereas bone marrow precursors with the same phenotype give rise only to conventional B cells (4). In this connection we have recently demonstrated that the fetal mouse omentum contains precursors that give rise exclusively to $Ly1^+$ B cells and the closely related $Ly1^-$ sister population but not conventional B cells or T cells (5). These findings raise the possibility that very early during development $Ly1^+$ B cells are generated in a compartment distinct from that for conventional B cells.

$Ly1^+$ B cells differ from conventional B cells in their surface phenotype, tissue localization, and immunoglobulin repertoire (6–15). The most intriguing characteristic of $Ly1^+$ B cells in mice and $CD5^+$ (human Ly1 homologue) B cells in humans is the production of antibodies with affinity for self-antigens. Many antibody specificities, including rheumatoid factor activity, anti-DNA, and anti-T cell reactivity, appear to be enriched in this B cell subset (9–12). The best studied reactivity of these B cells in mice is the in vitro affinity for bromelain-treated mouse red blood cells (13). The overrepresentation of this specificity within $Ly1^+$ B cells is clearly the result of antigen selection, however, the mechanism for the segregation of this antibody specificity or the nature of its physiological counterpart in vivo is unclear (14, 15). Although there is an association between increased levels of $CD5^+$ B cells in certain autoimmune diseases such as rheumatoid arthritis in humans and in the autoimmune NZB mice (6, 16), $CD5^+$ B cells have not been shown to secrete pathogenic autoantibodies, and the exact role they play in the etiology of autoimmune disease is not known.

The human fetal liver is also an early site of hematopoiesis (17). Pre-B cells ($cIgM^+/sIgM^-$) are first detectable in human fetal life at ~8 wk of gestation, and it is not until ~12 wk that peripheralization of B cells occurs (18). A report from Bofill et al. (19) using immunohistochemical techniques described the appearance of CD5 B cells in the human fetal peritoneal and pleural cavity at 15 wk of gestation, however, these cells were absent from the liver before 16 wk and were rare in the bone marrow. This led the authors to comment that the "original derivation of these cells is a mystery."

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Since the mouse omentum has been shown to be a source of precursors for Ly1$^+$ B cells, we have compared B cell development in the human fetal omentum, liver, and spleen.

In this report, we demonstrate that B cell development occurs simultaneously in situ in the human fetal liver and omentum but not spleen, and that B cells from the omentum showed a bias towards CD5 expression.

Materials and Methods

Fetal Samples. Fetal liver, spleen, and omentum were obtained from elective abortion samples received from The Central Laboratory for Human Embryology, University of Washington (Seattle, WA), and gestational age was estimated by foot length.

Preparation of Single Cell Suspensions. The fetal liver, omentum, and spleen were dissected and placed in 1 ml of a 50% solution of Dispase II (Boehringer Mannheim Biochemicals, Indianapolis, IN) in complete RPMI with 5% FCS. The samples were placed in Eppendorf tubes and put on an orbital shaker (Fisher Scientific Co., Pittsburgh, PA), at speed 400 for 1 h at 37°C. The single cell suspensions were then centrifuged and washed two times in complete RPMI (Gibco Laboratories, Grand Island, NY) with 10% FCS and stained for flow cytometry as described below.

Antibodies. mAbs against CD5 and CD11b were purchased from Becton Dickinson & Co. (Mountain View, CA). The anti-CD24 (IgM, κ) mAb, BA-1 (20), was provided by Dr. Tucker Lebien (University of Minnesota), and the anti-CD24 (IgG1, κ) mAb, SN3, was provided by Dr. Ben Seon (Roswell Park Cancer Center). The anti-CD45RO antibody, HB11, has been described previously (21). The goat anti-mouse IgG1-PE, anti-mouse IgG-biotin, anti-human IgM-FITC, and anti-human IgM-RITC antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). The FITC and RITC anti-human IgM antibodies were extensively absorbed on mouse Ig coupled to Sepharose 4B before use. The anti-Tdt antibody was purchased from Supertechs (Bethesda, MD).

Immunofluorescence Staining. The protocol for staining pre-B cells and mature B cells has been described elsewhere (22). We also added 1 μl of 1 mg/ml Hoescht dye to 250 ml of the final PBS wash to facilitate counting of nucleated cells. To quantitate the percentage of cells with phenotypes described in Fig. 4, three aliquots of cells were stained with anti-CD24 (IgM, κ) followed by anti-mouse IgM-FITC. Cytocentrifuge preparations were made and fixed in methanol for 20 min. One slide was then stained with anti-human IgM-RITC to allow calculation of the percentage of the IgM$^+$/CD24$^+$ cells. The second slide was stained with anti-Tdt followed by anti-mouse IgG biotin and finally Streptavidin-Texas red (SATR)[1] for calculation of the percentage of Tdt$^+$/CD24$^+$ cells. To calculate the percentage of Tdt$^-$/IgM$^-$/CD24$^+$ cells, the last slide was stained with anti-Tdt, then anti-mouse IgG biotin followed by SATR, and finally the anti-human IgM-RITC.

For flow cytometric analysis, cells were stained with the antibodies indicated in the text using the following regimen. Cells were stained in a 96-well round-bottomed plate. 10 μl of the appropriate antibody was added for 10 min on ice and the cells were washed once with 200 μl of PBS/FCS followed by the next antibody as indicated. The samples were fixed in 1% paraformaldehyde (pH 7.4) and analyzed on a FACScan® (Becton Dickinson & Co.).

Immunohistochemical Analysis of Tissue. To detect IgM$^+$ cells in the fetal omentum, this tissue was embedded in paraffin, sectioned and processed for immunofluorescence staining as previously described (23). The sections were stained with goat anti-human IgM-FITC. Serial sections were also stained with hematoxylin and eosin (H&E).

Results

Histology of the Human Fetal Omentum. A photomicrograph of 10-wk-old human fetal omentum and spleen is shown in Fig. 1 *A*. During fetal life, the omentum is a thin transparent well vascularized membrane with clusters of erythrocytes associated with the vasculature (Fig. 1 *B*). Immunofluorescent staining using FITC-conjugated anti-human IgM showed that B lineage cells could be detected along the periphery of these erythroid areas (Fig. 1 *C*). B cells were also found in loose foci or as isolated cells throughout the mesentery without any association with the vasculature.

The Pro/Pre-B Compartment Is Present in Both the Fetal Omentum and Liver. CD24 is expressed on B lineage cells as early as the pro-B cell stage (24, 25) and was used in combination with anti-IgM to identify pro/pre-B cells (CD24$^+$, surface IgM$^-$ [sIgM$^-$]) and mature B cells (CD24$^+$, sIgM$^+$). This permitted us to determine the ratio of pro/pre-B cells to mature sIgM$^+$ B cells (pro/pre-B:B) during fetal development. Fig. 2 shows representative staining profiles of cell suspensions of omentum, liver, and spleen from 16 wk of gestation. Pro/pre-B cells are displayed in the upper left (UL) quadrant and mature B cells in the upper right (UR) quadrant. The ratio of pro/pre-B:B cells from 13–23 wk of gestation was 1.2:1 in both omentum (n = 8) and liver (n = 8); in contrast, the ratio of pro/pre-B:B cells in the fetal spleen (n = 5) during this time was 0.07:1. During the earlier 10–12-wk gestation period, this ratio was 4:1 in the omentum (n = 3) and liver (n = 3), while B lineage cells were not detected at this time in the spleen (n = 3). The pro/pre-B cell compartment was detected even earlier, at 8 wk of gestation in the omentum and liver, however, because of the low frequency of bright CD24$^+$ cells at this stage, we could not calculate the pro/pre-B:B cell ratio.

Pre-B Cell Development Is Similar in the Fetal Omentum and Liver at 8–12 wk of Gestation. The percentage of cytoplasmic IgM$^+$ (cIgM$^+$) cells that were pre-B cells (cIgM$^+$/sIgM$^-$) in the 8–12-wk period was determined by immunofluorescence microscopy (Fig. 3). In the fetal omentum and liver, the percentage of pre-B cells was 53 ± 15 and 45 ± 13, respectively, while B lineage cells were undetectable in the spleen during this time period. After the 8–12-wk period, the percentage of pre-B cells was unchanged in the fetal liver (41 ± 10) but was significantly lower in the omentum (25 ± 14).

These results may indicate that the development of B cells in the fetal omentum is transitory, however, we cannot rule out the possibility that the pre-B cells are simply being diluted by mature B cells seeding to the periphery during this time period. Analysis of fetal omentum from the last trimester is needed to address this question, however, fetal tissue from these late gestational ages are not available. Beginning at ~12 wk, pre-B cells were detected in the spleen (2 ± 3) but at much lower percentages than either the liver or omentum.

[1] *Abbreviations used in this paper:* c, cytoplasmic; s, surface; SATR, Streptavidin-Texas red.

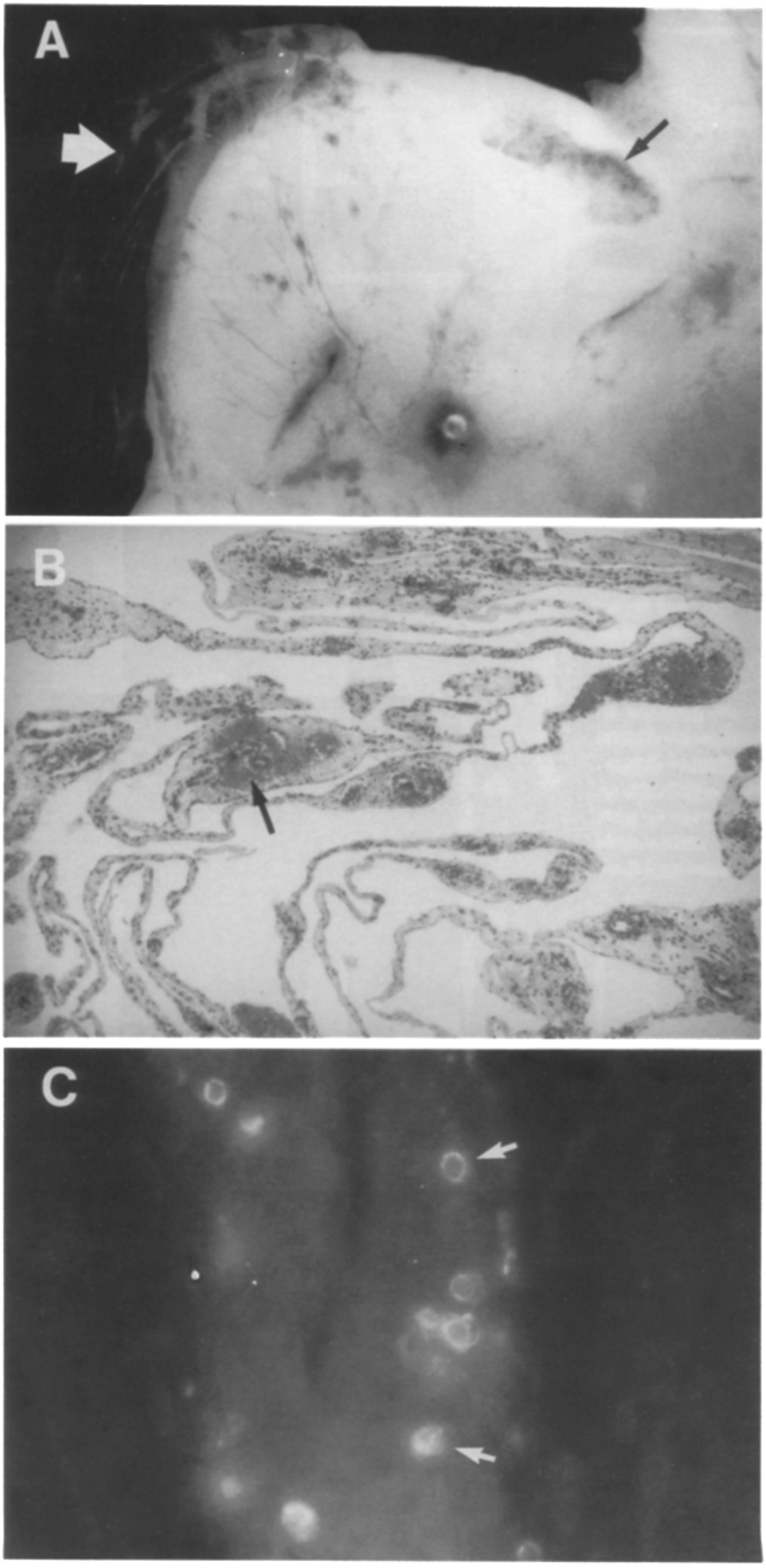

Figure 1. (*A*) Photomicrograph of 10-wk-old human fetal omentum, stomach, and spleen. The fetal omentum is a thin mesenteric membrane (*large arrow*) which hangs from the stomach into the peritoneal cavity. The spleen (*small arrow*) develops as a thickening of the mesenchyme of the omentum and is purely mesoderm in origin (×10). (*B*) Hematoxylin and eosin (H & E)-stained section of a folded 12-wk human fetal omentum, which demonstrates the membraneous nature of the fetal omentum and the presence of erythrocytes and lymphoid cells in the perivascular space (×100). (*C*) Immunofluorescence staining of sections of human fetal omentum. Sections were stained with FITC anti-IgM, and B cells can be seen in the perivascular spaces (×400).

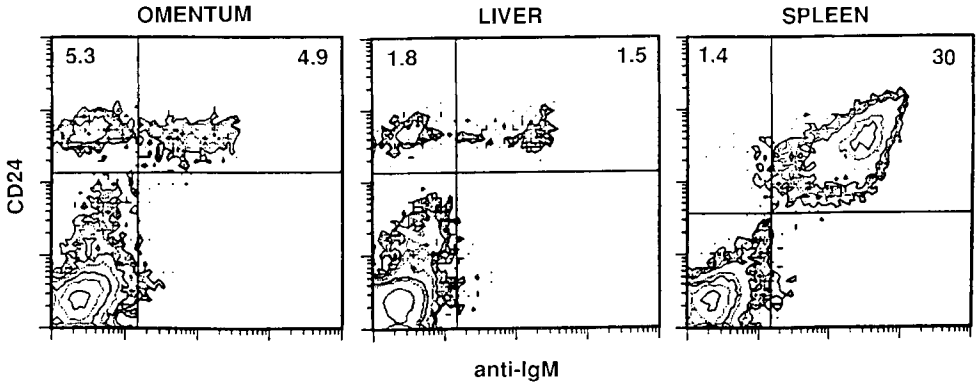

Figure 2. Pro/pre-B cell compartment is detected in 16-wk human fetal omentum, liver, and spleen. Cells were stained with anti-CD24 followed by anti-mouse IgG1 PE and finally anti-human IgM FITC. The pro/pre-B cell compartment is detected in the upper left quadrant ($CD24^+/sIgM^-$) and the mature $sIgM^+$ B cells are seen in the upper right quadrant. Relevant percentages are indicated.

The percentages of IgM^+ cells (both $cIgM^+$ and $sIgM^+$) per total nucleated cells in the omentum and liver were 0.74 ± 0.6 and 0.42 ± 0.5 during the 8–12-wk period and 2.1 ± 2.6 and 1.5 ± 1.3 during the 13–24-wk period, respectively. Thus, the omentum contains approximately twice as many IgM^+ cells per nucleated cells as the liver during both the early and late developmental periods.

Tdt^+ B Lineage Cells Were Detected at 8 wk of Gestation in Both the Fetal Omentum and Liver. Tdt has been associated with immunoglobulin gene rearrangement (26) and N region addition in vitro (27), and is believed to be expressed in B lineage cells at the pro-B cell stage immediately before the expression of cIgM (28). $CD24^+$ B lineage cells from fetal omentum and liver were analyzed for the presence or absence of Tdt and IgM. The results of this analysis are shown in Fig. 4. The phenotype of each group is indicated diagramatically in the order of the progression that is believed to occur during normal B cell development (28). It can be seen that the distribution of B lineage phenotypes within each group was remarkably similar between omentum and liver

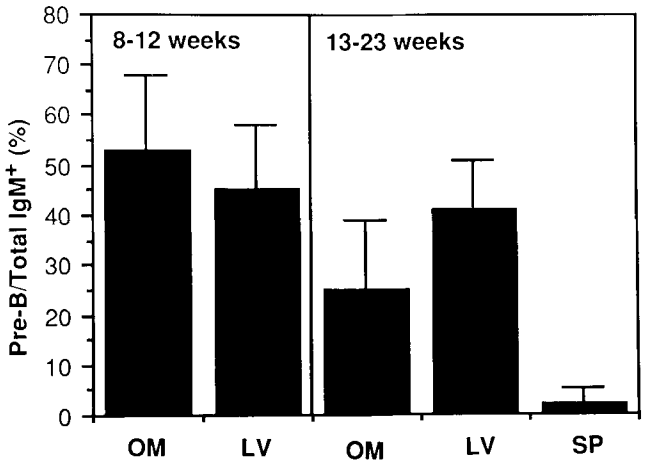

Figure 3. Percentage of pre-B cells total IgM^+ cells in omentum, liver, and spleen at 8–12-wk vs. 13–23 wk. Cells were stained first with FITC anti-human IgM, to detect surface IgM expression. Cytocentrifuge preparations were made and stained with RITC anti-human IgM to detect cytoplasmic IgM. Pre-B cells were identified as $cIgM^+/sIgM^-$, the data are presented as the percentage of pre-B cells/total IgM^+ cells, and SE bars are indicated. No IgM^+ cells were detected in the fetal spleen before 12 wk of gestational age.

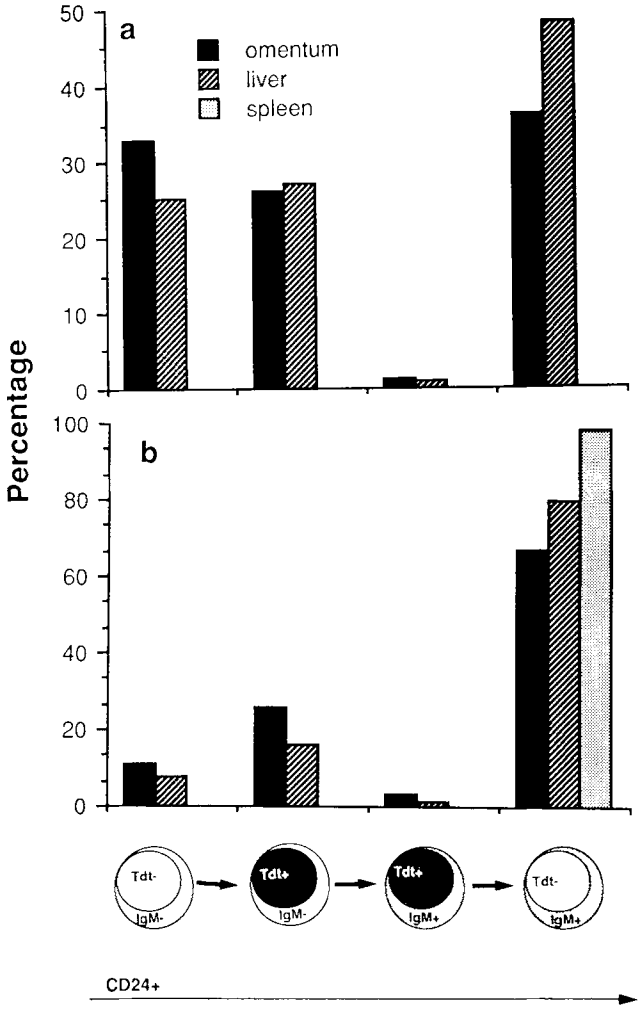

Figure 4. Percentage of $CD24^+$ B lineage cells expressing Tdt or IgM in omentum, liver, and spleen from 8–10 (*a*) and 18–20 (*b*) wk of gestation. Cell were stained first with anti-CD24, then anti-mouse IgM FITC. Cytopreparations were then stained with one of the following: anti-Tdt then biotin conjugated anti-IgG, followed by SATR, and finally anti-human IgM RITC to determine the percent of $Tdt^- Ig^-/CD24^+$; anti-Tdt, followed by biotin conjugated anti-IgG, and finally SATR to determine the percentage of $Tdt^+/CD24^+$; or anti-human IgM RITC to determine the proportion of $IgM^+/CD24^+$. One cytopreparation was stained with anti-Tdt, then biotin conjugated anti-IgG, followed by SATR, and finally anti-human IgM FITC to detect Tdt^+/IgM^+ cells. No $CD24^+$ cells were detected in the spleen during this time point. The phenotypes detected are indicated on the *x*-axis.

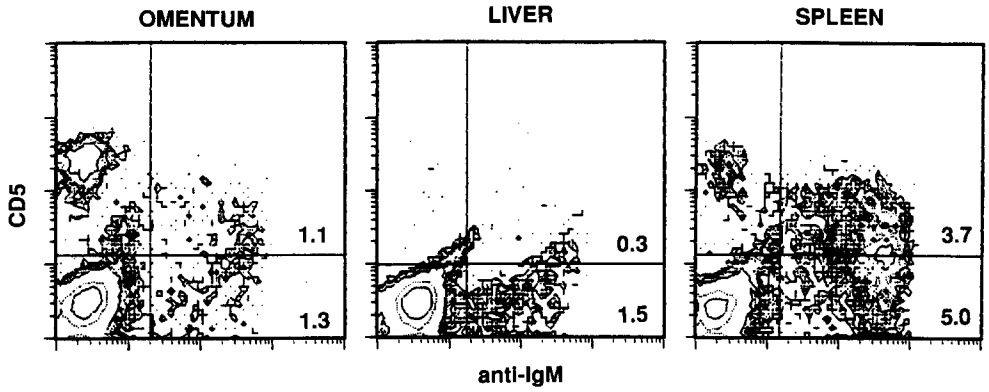

Figure 5. Presence of CD5 B cells in omentum, liver, and spleen at 14 wk. Cells were stained with CD5-PE followed by anti-human IgM FITC. Markers were set using an isotype-matched control (IgG2a-PE) for CD5 with anti-human IgM FITC (data not shown). Relevant percentages are indicated.

during both the early and late developmental time periods. One striking difference between the two age groups was the marked decrease in the large population of Tdt^-/IgM^- cells during the late time period; this decrease occurred concomitant with an increase in the percentage of $cIgM^+$ cells (both pre-B and mature B) during this time. $CD24^+$ cells were not detected in the spleen between 8 and 10 wk, however, $CD24^+$ cells were easily detected in the spleen at 18–20 wk. The phenotype of these cells was typically that of peripheral B cells, since ~98% of the B lineage cells in the spleen were $sIgM^+$, and Tdt was not detected.

Percentage of $CD5^+$ B Cells Is Higher in the Omentum than Liver. Mature B cells ($sIgM^+$) are detected in sufficient numbers to confidently calculate the percentage of $CD5^+$, IgM^+/total IgM^+ cells in the omentum and liver at ~14 wk of gestation. Cells from 14–17 wk gestation fetal tissues were stained with CD5-PE plus anti-IgM as indicated in Fig. 5. Markers were set for each tissue type based on staining with an irrelevant isotype-matched control and anti-IgM. The percentage of $CD5^+$ B cells/total B cells was equal in the omentum and spleen at 43 ± 11 (n = 6) and 41 ± 13 (n = 4), respectively. However, the percentage of $CD5^+$ B cells in the liver was substantially lower at 21 ± 3 (n = 5).

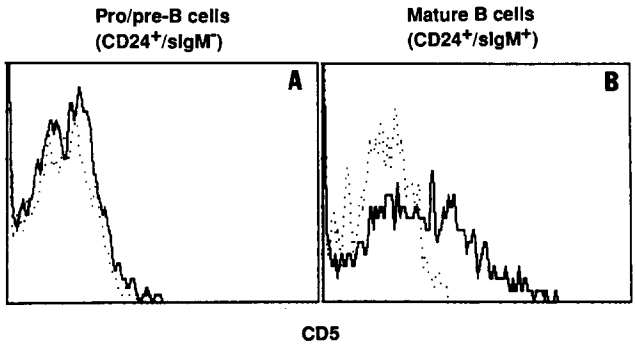

Figure 6. Analysis of pro/pre–B cells and mature B cells for expression of CD5. Three-color flow cytometric analysis was used to analyze the pro/pre–B cells and mature B cells separately. Cells were stained first with biotin-conjugated CD24, followed by SA-tandem; next, the cells were stained with anti-human IgM-FITC, and finally CD5-PE. Analysis gates were set to collect only the bright $CD24^+$ cells. The pro/pre–B cells ($CD24^+/sIgM$) are shown in *A*; the mature B cells ($CD24^+/sIgM^+$) are shown in *B*.

A variety of antibodies against other B cell surface markers were used in an attempt to determine if (1) the $CD5^-$ B cells in the omentum were the $CD5^-$ "sister" population and (2) omental B cells could be further distinguished from those in the fetal liver. Mac-1 is expressed on mouse $CD5^+$ B cells as well as the $CD5^-$ sister population but not on conventional B cells. However, <0.1% of the $sIgM^+$ B cells in either the omentum or liver expressed CD11b, the human analogue of Mac-1. In addition, there were no differences in the ratios of intensity of IgM and IgD expression by B cells in the omentum vs. liver. CD45RO, detected by mAb HB11, was expressed on the B cells from the omentum at a 20% higher level than B cells from the fetal liver (n = 3) (data not shown). Thus, using the available markers, we have been unable to determine if, in addition to the higher frequency of CD5 B cells present in the omentum, they are homogeneously distinct from those in the fetal liver.

As can be seen in both omentum and spleen, relatively large numbers of $CD5^+$ T cells were detected (UL quadrant) which were absent or rare in the fetal liver. The T cells found in the omentum and spleen expressed either α/β or γ/δ TCRs in a ratio that indicated that they were most likely mature T cells which at this time have migrated to the periphery.

Analysis of Pro/Pre–B Cells for the Presence of the CD5 Antigen. Three-color flow cytometry was used to determine when the CD5 antigen first appeared during development of B cells. Analysis gates were set to analyze exclusively B lineage cells using CD24 as a pan-B cell marker in conjunction with anti-IgM FITC and CD5-PE; the pro/pre–B cells ($sIgM^-$) (Fig. 6 *A*) and the mature B cells ($sIgM^+$) (Fig. 6 *B*) were analyzed separately for the expression of the CD5 antigen. The cells in the pro/pre-B cell compartment failed to express CD5, while it was easily detected on $sIgM^+$ B cells.

Discussion

The human perinatal and adult omentum is considered part of the lymphoid system because it contains loose unorganized lymphoid aggregates called "milk spots" (29). Despite its activity as a secondary lymphoid organ, there has been no analysis of the fetal omentum with respect to the generation of lym-

phocytes in situ in development or in the adult. In this study we show that the pro/pre–B cell compartment ($CD24^+/sIgM^-$), pre-B cells ($cIgM^+/sIgM^-$), and Tdt^+ B lineage cells can be detected as early as 8 wk of gestation in the human fetal omentum. These results demonstrate that B cell generation occurs in situ in the human fetal omentum concomitant with that occurring in the fetal liver.

Pro/pre–B cells were detected as early as 8 wk of gestation in the fetal omentum at a time when the pro/pre–B:mature B cell ratio is similar in the omentum and liver. In contrast, this compartment of the B cell lineage was not detected in the spleen until ~12 wk of gestation. In addition, the percentage of pre-B/IgM^+ cells in the fetal omentum and liver was approximately the same during the early 8–12-wk time period. However, during the 13–23-wk period, the percentage of pre-B cells/IgM^+ cells decreased significantly in the omentum, while remaining at the same level in the liver. These results are consistent with the idea that the development of B cells in the omentum is transitory, however, analysis of fetal omentum from later gestational ages is needed to determine the length of time that the fetal omentum maintains the ability to generate B lineage cells in situ. Additionally, during the 13–23-wk time period, pre-B cells became detectable in the spleen but at a much lower frequency than that seen in either the liver or omentum.

Tdt^+ cells have been detected in the fetal liver as early as 7 wk of gestation (30) and are believed to represent a stage of B cell development during which gene rearrangements occur (25–27). Here we report that ~25% of the $CD24^+$ cells in both the fetal omentum and liver from 8–12 wk of gestation express the nuclear antigen Tdt. Interestingly, almost 30% of the $CD24^+$ cells present in both tissues during the time period are negative for Tdt and IgM. This population was not described in a report from Nishimoto et al. (28) in analysis of $CD19^+$ B lineage cells from fetal bone marrow. This may reflect the presence of the CD24 antigen on B lineage cells at a more immature stage than CD19. Alternatively, this population may represent the earliest B lineage cells, and are present in large enough numbers to be detected only during very early gestation in fetal liver and omentum but not in later fetal bone marrow.

These results clearly demonstrate that every identifiable stage in B cell differentiation is detected in both the fetal omentum and liver as early as 8 wk of gestational age. This is in contrast to the fetal spleen, a peripheral secondary lymphoid organ, where little B cell generation appears to occur in humans and very few cells of immature phenotype are detected at any stage of development. The presence of B cell progenitors in liver and omentum at a time when they are undetectable in spleen also argues against the origin of these cell types in either organ as blood-borne contaminants.

Examination of mature B cells demonstrated a higher percentage of $CD5^+$ B cells in the omentum and spleen compared with the fetal liver. However, in the human fetal omentum and spleen, >50% of the B cells were $CD5^-$, similar to our results obtained by reconstitution of *scid* mice with fetal mouse omentum (5). In mice, the closely related $Ly1^-$ sister population is well characterized, and we were able to identify the $Ly1^-$ B cells as the sister population. Since, at the moment, there are no useful phenotypic markers to distinguish the $CD5^-$ sister population from conventional B cells in humans, we cannot determine whether the $CD5^-$ B cells present in the human omentum are equivalent to the $CD5^-$ sister population.

Although we have proposed that the mouse fetal omentum may provide a unique milieu that directs immature cells along the CD5 B cell lineage, there is evidence that CD5 B cell generation may be a general property of fetal B lineage differentiation (4). Our results, although suggestive that the omentum may constitute an enriched source of CD5 cells, obviously needs further comparative data with respect to differentiation antigen and V region usage by the B cell populations from liver and omentum. There is an alternative hypothesis related to CD5 B cell development proposed by Cong et al. (31), which suggests that crosslinking of sIgM on newly emerging B cells stimulates the expression of the Ly1 antigen. Although our results do not directly support this hypothesis, we have clearly shown that the CD5 antigen does not appear until or after the expression of sIgM. This is in contrast to CD5 expression by human and mouse T cells, which occurs early in thymocytes before TCR expression (32, 33). The contemporaneous expression of CD5 with sIgM during development of B cells suggests that the expression of both surface proteins may be linked.

Our earlier work in mice (5) and this report in humans show that the fetal omentum is a site where B cells are generated, thus raising the question as to the significance of the fetal omentum in B cell development in other species. B cell development as judged by the presence of pre-B cells also occurs in the fetal omentum in rabbits (our unpublished data). At this point we do not know if the B cells generated in the fetal rabbit omentum express the CD5 antigen. However, this observation, in addition to the work presented here and our previous work with fetal mouse omentum, demonstrates that this site is conserved through evolution in a variety of mammals as a site of B cell generation and should be considered along with the fetal liver, thymus, and bone marrow as a primary lymphoid organ during the development of the immune system.

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Address correspondence to Nanette Solvason, 263 Tumor Institute, University of Alabama at Birmingham, Birmingham, AL 35294.

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