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The ontogeny and functional characteristics of human B-1 (CD5+B) cells

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We demonstrate that, on average, >90% of B lymphocytes in fetal spleen express CD5 at gestational ages of 17–23 weeks. Similarly, CD5+ B cells (B-1 cells) are the major B cell subset in umbilical cord blood. These findings depend on the optimization of fluorochrome conjugated anti-CD5 reagents for multiparameter fluorescent-activated cell sorter (FACS) analysis. From infancy through childhood the percentage of B-1 cells gradually diminishes in both spleen and peripheral blood. Stable adult levels, 25–35% of the total B cell population, are reached in late adolescence. The decrease in the percentage of B-1 cells in spleen is accompanied by an increase in conventional (CD5-) B cells, keeping the percentage of total B cells per mononuclear cells relatively constant. In contrast, in peripheral blood, the concentration of both B-1 cells and total B cells decreases, while T cells increase. At the functional level, we show that polyreactive IgM autoantibodies are produced by FACS-sorted CD5^{high} B cells, but not by CD5- B cells from adolescent spleen. In contrast, fetal splenic CD5^{high} and CD5- B cells appear functionally uniform, both producing IgM autoantibodies that are typical of B-1 cells. The apparent level of CD5- B cells in fetal spleen, on average 10% of total B cells, may still result from limitations of our reagent. The prominence of B-1 cells in fetal spleen and cord blood, the gradual reduction of B-1 cells with increasing age, and its characteristic repertoire, all suggest a role for this cell type in immunologically immature hosts.

Introduction

Murine Ly-1 (CD5+) B cells, which are now called B-1 cells (see nomenclature note in Methods), are physically and functionally distinct from conventional (CD5-) B cells. These two B cells differ in size, surface phenotype, anatomical location, and the repertoire of antibodies they produce. Murine B-1 cells predominate in neonatal spleen and adult peritoneum, but represent only a few percent of total B cells in adult spleen (1). They secrete IgM autoantibodies that react with DNA, bromelain treated mouse erythrocytes (phosphatidylcholine) (2), thymocytes (3), and with micro-organismal coat antigens such as $\alpha 1-3$ dextran (4), phosphatidylcholine, and undefined determinants on *Escherichia coli* (5).

The human homolog of the murine B-1 cells, sometimes referred to as the Leu-1 B cell subset, or simply as CD5+ B cells, shares a similar repertoire with its murine counterpart. Like murine B-1 cells, adult human peripheral blood B-1 cells produce IgM autoantibodies, including rheumatoid factor, antibodies to single-stranded (ss) DNA, and to bacterial antigens such as lipopolysaccharides (LPS) (6–9). These autoantibodies, many of which are polyreactive, are readily detectable in healthy normal individuals and in patients with autoimmune disorders (10,11) The precise physiologic role of these antibodies is still not clear, but because of their reactivity to phosphatidylcholine, LPS, and other epitopes present on bacterial cell walls, it has been hypothesized that these so called 'natural antibodies' could provide the first line of defense against infections (6,7).

B-1 cells constitute roughtly 1–7% of peripheral mononuclear cells in normal human adults (9,12). Although there is heterogeneity among unrelated individuals, repeated analysis of blood samples from the same healthy adults demonstrates that the proportions of B-1 cells are relatively constant for at least a few months (12). Monozygotic twins and triplets have nearly identical proportions of B-1 cells, suggesting that this stable phenotype is genetically controlled (12). Although rare in adult bone marrow

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(13), these cells have been reported to constitute 10 – 25% of the B cell population in adult secondary lymphoid organs such as spleen, tonsils (14), and lymph nodes (15).

Studies by several investigators indicate that a large proportion of B cells early in ontogeny are $CD5^+$. B-1 cells in fetal lymphoid tissues have been reported as early as 15 weeks of gestation in spleen, lymph nodes, and peritoneal and pleural cavities (16). The reported fraction of fetal splenic B cells which are $CD5^+$ varies, but is generally near 50% (13,16,17). Immunohistology of second trimester lymph nodes suggests 40 – 60% of IgM^+ cells are $CD5^+$ (18). However, in cord blood samples obtained at full term, most B cells express CD5 (9). Here we reconcile some of the differences in reported B-1 levels and demonstrate the importance of optimal staining reagents.

B lymphocytes are often regarded as a homogeneous population, except for the specific idiotype expressed by each cell. The expression of CD5 on a subset of B lymphocytes and their characteristic antibody repertoire contradicts the established paradigm and suggests an important functional niche for B-1 lymphocytes. Mouse studies have established that B-1 cells represent a distinct lineage from conventional B cells and have a distinct repertoire (1,19). To further address the development and function of human B-1 cells, we undertook a detailed analysis of the distribution and repertoire of this cell type from fetus to adulthood. We demonstrate differences in both the ontogeny and antibody repertoire between B-1 and conventional B cells. Hybridomas generated from fetal spleen produce IgM autoantibodies, many of which are polyreactive. Although the expression of CD5 is very broad, the two extreme populations produce similar IgM autoantibodies, suggesting that all B cells in fetal spleen are functionally B-1 cells. In contrast, in a spleen from a normal 14 year old, IgM autoantibodies are detected only from B-1 cells and not from conventional B cells. We suggest that the predominance of B-1 cells in the human fetus, and the gradual decrease in the frequency of this subset with age, represents an ontological shift from reliance solely on cells that produce a low affinity, broad specificity antibody response, towards functional utilization of cells capable of mounting a high affinity, fine specificity, memory antibody response. The data is consistent with the model that the highly evolved conventional B cell response is layered on a more primitive immune system that predominates early in ontogeny (20).

Methods

Nomenclature

The participants at the New York Academy of Sciences meeting on CD5 B cells adopted workshop designations for mature B cells. B-1 cells encompass what have previously been known as CD5 B cells (Ly-1 B cells in mice and Leu-1 B cells in humans). These cells arise early in ontogeny in both mouse and man. Conventional B cells arise later and constitute the majority of B cells in adult lymphoid organs (21).

Cells

Human fetal spleens were obtained from medically approved pregnancy terminations, performed by dilation and extraction or suction curettage. Fetal age, as determined by measurements of foot length (22), ranged from 17 to 23 weeks of gestation. Human adult spleens from different age groups were obtained from staging laparotomies for Hodgkin's lymphoma (only spleens that showed no involvement were used), or from patients who had splenectomies for conditions that were not immunologically related (e.g. trauma). Heparinized adult peripheral blood was obtained from normal donors of 20 – 50 years of age. Citrated peripheral blood from individuals under 15 years of age was obtained when blood was drawn for other medical conditions. Heparin and citrate harvested peripheral blood specimens gave comparable percentages of T and B lymphocytes. Heparinized umbilical cord blood was obtained from normal deliveries at term (>37 weeks). The above specimens were all obtained with the approval of the Committee for the Protection of Human Subjects at Stanford University.

Individual organs were gently teased apart in Hanks balanced salt solution (HBSS) with 1% FCS and 0.2% DNase, and passed through sterile nylon membranes to obtain single cell suspensions. Peripheral blood and the single cell suspensions obtained from individual organs were separated on a Ficoll-hypaque gradient (Histopaque-1077, Sigma, St Louis, MO), the mononuclear cell population washed 3 times in HBSS with 1% FCS, and resuspended in staining medium (RPMI with 3% FCS, 1 mM EDTA, and 0.01 M HEPES) at 2.5×10^7 cells/ml. All specimens except adult splenic cells were analyzed within 24 h. Adult splenic mononuclear cells were frozen in 10% DMSO and 90% FCS. The cells were thawed and maintained overnight in 10% FCS in Iscove's-modified Dulbecco's medium (IMDM, Sigma, St Louis, MO) before analysis. All thawed specimens used in this study had >80% viability.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-Leu-16 (CD20), phycoerythrin (PE)-conjugated anti-Leu-16 (CD20), biotin-conjugated anti-CR_2 (CD21), biotin-conjugated anti-Leu-20 (CD23), and PE-conjugated anti-IL-2R (CD25) were obtained from Becton-Dickinson Immunocytometry Systems (San Jose, CA). 4F2 was a kind gift from Dr Anish Sen-Majumdar, Stanford University. Mouse anti-human IgD (∂TA4-1) (23), mouse anti-human IgM (DA4-4) (24), mouse anti-human Leu-12 (CD19) (25), and mouse anti-human Leu-1 (17F12) (26) were purified from ascities fluid by ion-exchange chromatography on DEAE – Sephacel or QAE – Sephadex (27). Antibody conjugation to FITC (fluorescein-5-isothiocyanate, F-1906, Molecular Probes, Eugene, OR), biotin (*N*-hydroxysuccinimidobiotin, Pierce, Rockford, IL), and preparation of texas red (TR, Molecular Probes) – avidin have been described previously (27). Allophycocyanin (APC) was purified from *Spirulina platensis* and PE from red algae and crosslinked with succinimidyl 4-*N*-maleimidolmethyl cyclohexane-1-carboxylate (SMCC, Pierce) to the hinge region sulfhydryls of dithiothreitol reduced antibodies (method modified from R. Hardy, personal communication). Six different conjugates of mouse anti-human Leu-1 (CD5) were used in the study including one PE- and one FITC-labelled reagent obtained from Becton-Dickinson.

Flow cytometry and cell sorting

Multi-parameter flow cytometric analysis has been described in detail (28). In brief, 5×10^5 cells were suspended with predetermined saturating concentrations of each of the conjugated antibodies in a final volume of 125 μl and incubated on ice for

15 min. After washing, the cells were incubated with TR – avidin for another 15 min on ice. The cells were washed and resuspended in 200 μl of staining medium and analyzed or sorted on a highly modified dual-laser FACS II (Becton-Dickinson, Mountain View, CA), interfaced with a VAX 6300 computer (Digital Equipment, Maynard, MA) running FACS/desk software (29). In order to ensure reproducibility, the sorter was calibrated for each experiment with standard polystyrene microspheres (Pandex) with well defined scatter and fluorescence in all channels.

Epstein – Barr virus transformation

EBV stock was prepared from cultures of EBV-transformed marmoset cells, strain B95-8, as described elsewhere (30). The virus stock was added at 1/10 final dilution to the cell suspensions containing 2×10^6 cells/ml (30). The virus was allowed to adsorb to the cells for 2 h at 37°C. Cells were then pelleted at low speed centrifugation and resuspended in IMDM with 15% FCS (heat inactivated), penicillin (100 IU/ml), and streptomycin (50 μg/ml). EBV-infected cells were immediately cultured at different cell concentrations (from 50 to 1000 cells/well) in U-bottom plates (Falcon, Oxnard, CA), and the supernatant analyzed for presence of Ig and reactivity to the panel of autoantigens after 3 weeks in culture.

Generation of human hybridomas

EBV-transformed cells or fresh B lymphocytes mixed with half the number of logarithmically growing SHM-D33 cells (31) were washed twice with PBS (calcium – magnesium free). The washed cells were resuspended in 1 ml of 44% polyethylene glycol 1450 (PEG, American Type Culture Collection) at 37°C with gentle agitation for 45 s. The fusion mixture was diluted with warm serum-free IMDM, first 2 ml for 2 min, then with 8 ml for 5 min and centrifuged (2000 *g*, 5 min). The fused cells were resuspended in IMDM with 15% FCS and dispensed into flat-bottom 96-well plates (Costar, Cambridge, MA) at $1-2 \times 10^4$ lymphocytes and 10^5 human thymocytes as feeders per well. After 24 h at 37°C, 5% CO_2, an equal volume of selection media [3×10^{-4} M hypoxanthine, 5×10^{-7} M aminopterin, 1.6×10^{-6} M thymidine, 5×10^{-7} M ouabain, penicillin (100 IU/ml), and streptomycin (50 μg/ml) in IMDM with 15% FCS] was added and thereafter fed with selection media every 4 days. Hybridoma cells form fusion wells testing positive in the screening assays for Ig and autoantibody production were transferred to larger wells and subcloned at 3 cells/well with 10^5 human thymocytes as feeders.

Ig detection

The amount of IgG and IgM produced in the culture supernatants of hybridomas and EBV-transformed cells was determined by a isotype-specific ELISA. Briefly, 96-well polystyrene plates were coated with 100 μl of 20 μg/ml of affinity purified goat anti-human IgG or IgM antibodies (CalTag, South San Francisco, CA) in PBS overnight at 4°C. Wells were blocked by 10% FCS in HBSS for 30 min at room temperature. After washing with 0.1% Tween 20 in PBS, 100 μl of culture supernatants diluted 1:2 in PBS was added to plates. After 90 min incubation at 37°C the plates were washed and 100 μl of affinity purified goat anti-human IgG or IgM antibodies conjugated with horse radish peroxidase were added to respective plates and incubated for 90 min at 37°C. After washing the plates, 100 μl of substrate ABTS [1 mg/ml, 2,2′-azino-di(3-ethylbenzthiazolone) sulphonic acid with 0.03% hydrogen peroxide in citrate buffer (0.1 M, pH 4)] was added. The color development was measured after 15 min at OD_{414}.

Assay for autoantibodies

ssDNA was prepared by heat denaturation of calf thymus DNA. For the preparation double-stranded (ds) calf thymus DNA was dissolved in 0.06 M NaCl, 0.1 M acetate, 1 mM $ZnCl_2$, and 100 U/mg S1 nuclease, incubated at 37°C for 45 min, and the reaction stopped with EDTA (10 mM). The dsDNA was reprecipitated with ethanol and stored in saline citrate buffer (0.15 M NaCl, 0.02 M sodium citrate, pH 7.5). Polystyrene plates were first coated with polylysine (50 μg/ml), then coated with antigen (dsDNA, ssDNA, cardiolipin, chondroitin sulfate) at

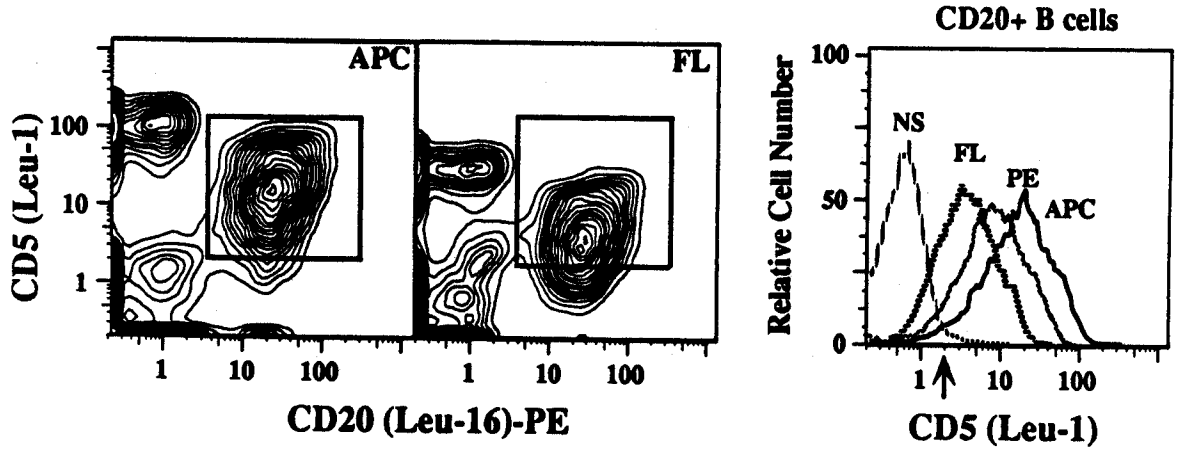

Fig. 1. Better resolution of CD5 expression on B cells with an optimal APC – anti-CD5 reagent. Data are from multiple stainings of the same 20 week fetal spleen. FACS contour plots are shown after staining with PE – anti-CD20 and either APC – anti-CD5 or FITCFL – anti-CD5. Histograms of CD5 expression are shown after gating for B cells by CD20. The arrow defines the cutoff for $CD5^+$ B cells. The measured value of B cells which are $CD5^+$: 90% with APC – anti-CD5, 80% with PE – anti-CD5, and 62% with FITC – anti-CD5. Gates are used such that <2% of the B cells are positive with CD20 alone in the FITC channel (NS) or in other channels. Isotype matched controls labeled with APC or FITC give the same background levels (<2%). All plots presented here have 5% probability contours.

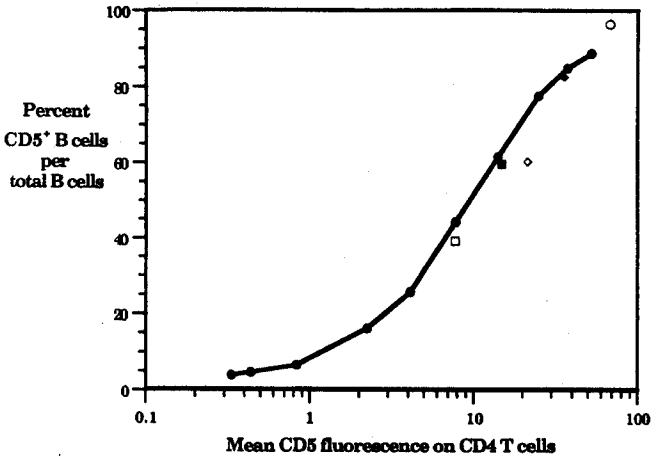

Fig. 2. The calculated level of CD5⁺ B cells depends on the anti-CD5 reagent. Data are from analysis of a single 21 week fetal spleen. Anti-CD19 (APC or FITC) is used to identify all B cells; anti-CD4 (FITC or PE) is used to identify a subset of T cells with the highest and most uniform CD5 expression (CD8 and γδ T cells are duller for CD5 and the ratio of CD4 to CD8 T cells varies among individuals). The anti-CD5 reagents are ○, ● APC, the ● reagent is used for the age related studies and in Fig. 1; ◇, ◆ PE, ◆ is also used in Fig. 1; □, ■ FITC, ■ is also used in Fig. 1. Points for the solid curve were generated by titrating unlabeled anti-CD5 in the presence of a constant, optimal amount of APC – anti-CD5.

2.0 μg/ml, and finally with polyglutamate (50 μg/ml). Transferrin, myelin basic protein, insulin, BSA and thyroglobulin were coated at 20 μg/ml. All chemicals were obtained from Sigma. *E. coli* J5 Rc LPS (List Biological, Campbell, CA) was dissolved in PBS, pH 8, at 10 μg/ml on Covalink ELISA plates (Nunc, Naperville, IL). Lipid A (List Biological) was diluted in 95% ethanol to 10 μg/ml and air dried overnight. The above coatings, except Lipid A, were performed in PBS at 37°C for 2 h and then transferred at 4°C overnight. Testing for specific antibody in culture supernatant of hybridomas and EBV-transformed cells was performed by ELISA as described above.

Results

Expression of CD5 on fetal splenic B cells

Evaluation of the number of B-1 cells is highly dependent on the anti-CD5 reagent. Contour plots reveal striking differences in the perceived level of B cells (CD20⁺) which are CD5⁺ when two different reagents are used (Fig. 1). Our best APC – anti-CD5 reagent, which is also used for all the age related data described below, indicates that at least 90% of the B cells are CD5⁺ in this fetal spleen. In comparison, the fluorescein reagent (one of ours or the one obtained from Becton-Dickinson) tested on the same fetal spleen sample yields only 62% CD5⁺ B cells. The entire B cell population in fetal spleen shifts as an ensemble to higher levels of CD5 with increasingly bright anti-CD5 reagents, suggesting one CD5⁺ cell population. In contrast, the majority of B cells in adult spleen or adult peripheral blood do not shift above the autofluorescence level with the same increasingly bright anti-CD5 reagents.

The dependence of the calculated percent of CD5⁺ B cells on reagent brightness is further emphasized in Fig. 2. CD4 T cells, which are bright and uniform for CD5 expression, provide an excellent internal measure for the anti-CD5 reagent brightness. A well-defined curve is generated by titrating unlabelled anti-CD5 in the presence of a standard, optimal amount of APC – anti-CD5. APC-isotype and unstained controls are quite clean in the APC channel, with <2% of the CD20⁺ cells being CD5⁺. Other anti-CD5 reagents, including those labelled with PE and FITC also fall on this curve. In our hands, APC – anti-CD5 reagents are generally better than PE reagents, which in turn are better than FITC-conjugated reagents. Imperfect stains deviate from the curve, e.g. the data point designated with an open circle in Fig. 2 was from a slightly sticky APC – anti-CD5 reagent, which also bound non-specifically to non-T and non-B cells. Similar deviations are seen if the reagent is used in great excess or if aggregates are not removed from the reagent prior to staining.

Table 1. Most B lymphocytes in human fetal spleen are B-1 cells

Gestational age (weeks)	Percent B lymphocytes of total mononuclear cells		Percent CD5⁺ B cells of total B cells
	CD5⁺ B cells	CD5⁻ B cells	
18	30	5	86
18	44	0	100
19	53	2	96
19	39	10	80
20	42	3	93
20	61	4	94
20	52	6	90
21	49	9	85
21	41	5	89
21	34	3	92
22	62	1	98

Previous investigators reported that 40 – 60% of the B cells in fetal spleen are CD5⁺ by fluorescent-activated cell sorter (FACS) analysis (17) or immunohistology (13). Our data indicate these estimates are low, probably because of duller reagents. Using the bright APC – anti-CD5 reagent described above, we show that on an average 91 ± 6% of the B cells in fetal spleen (18 – 22 weeks) express CD5. The values for all 11 individual spleens analyzed are above the highest value reported previously and range from 80 to 100% (Table 1).

Direct comparison of our FACS results with previous immunohistology is difficult; however, FACS analysis is clearly more sensitive and quantitative. Using immunohistological staining of frozen tissue sections, Bofill *et al.* (16) report a dramatic increase in B-1 cells from <2% at 16 weeks, 3 – 6% at 22 weeks, to 60% at 24 weeks of gestation. They correlate the appearance of B-1 cells to the formation of follicles in fetal spleen. In contrast, Freedman *et al.* (13) report 40% of the fetal B cells in single cell suspension as CD5⁺ by dual label immunohistology at <24 weeks of gestation. More work is needed to clarify the discrepancy in the tissue section results and to localize the splenic B-1 cells.

Age distribution of human B-1 cells in spleen

Analysis of spleens from fetuses, children, and adults shows that there is a gradual decrease in the percentage of B-1 cells, both per total mononuclear cells and per total B cells, with increasing age. As demonstrated above, the majority of B cells in fetal spleen

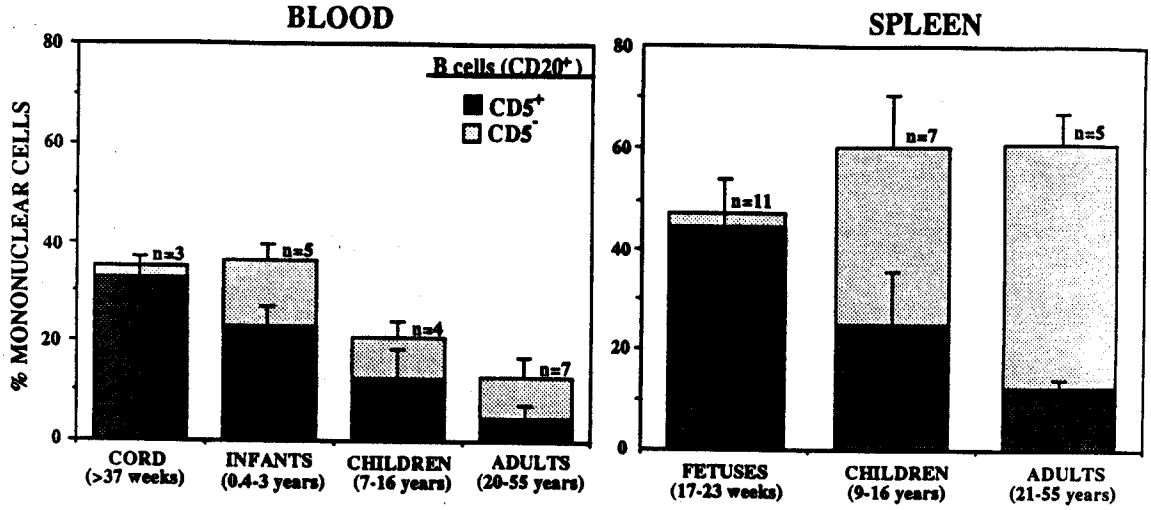

Fig. 3. The percentage of B-1 cells decreases with age in peripheral blood and spleen. Cells were stained with PE – anti-CD20 and APC – anti-CD5, and the mean percentage ± SD of B-1 and conventional B cells in each of the age groups is presented in the bar diagram. Years and weeks in parenthesis indicate the lower and upper age limit of samples analyzed in each group. Student's *t*-test analysis of B-1 cells between groups in both peripheral blood and spleen is statistically significant. (*P* values for peripheral blood: 0.01 for cord/infant, 0.035 for cord/child, 0.001 for cord/adult, 0.001 for infant/child, 0.001 for infant/adult, and 0.005 for child/adult; *P* values for spleen: 0.005 for fetus/child, 0.001 for fetus/adult, and 0.04 for child/adult.)

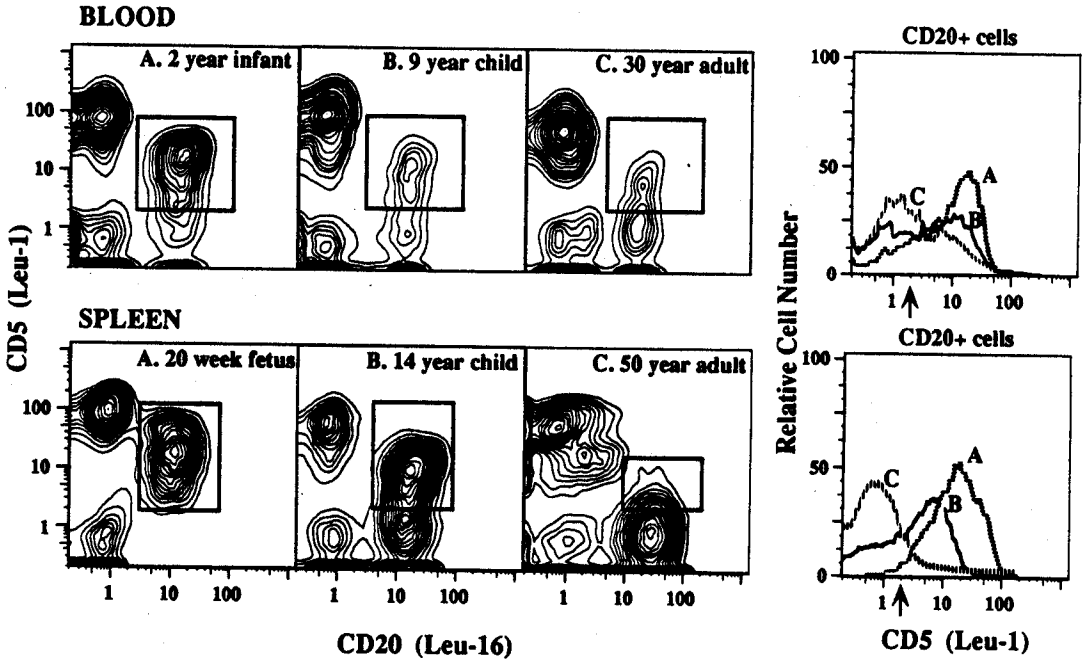

Fig. 4. Immunofluorescence profiles of representative samples from each age group in peripheral blood and spleen demonstrating the gradual decrease in B-1 cells. The box shows the gates used to measure $CD5^+$ B cells. Expression of CD5 after gating for $CD20^+$ cells is shown in the last panel for each sample analyzed. The arrow defines the cutoff for $CD5^+$ B cells.

express CD5. In childhood, ~50% of the total B cells are $CD5^+$. In fact, the probability contour plots resolve relatively distinct $CD5^+$ and $CD5^-$ B cell populations at this stage (Fig. 4). The appearance of the two resolvable populations is in marked contrast to the dominance of $CD5^+$ B cells in fetal spleen and $CD5^-$ B cells in adult spleen. In adults, B-1 cells constitute ~20% of total B cells and 10 – 15% of total mononuclear cells (Figs 3 and 4). The anti-CD5 staining on the reported spleen and peripheral blood samples from individuals of all ages is relatively consistent as judged by the CD5 levels on T cells. Statistical analysis using Student's *t*-test demonstrates that B-1 cells between age groups are significantly different (Fig 3). The percentage of total B cells (B-1 + conventional B cells) within the mononuclear population does not differ significantly within

Table 2. Subsets of lymphocytes in peripheral blood of children and adults

Age (years)	% B cells[a] ($CD20^+$)	% B-1 cells[a] ($CD5^+$)	% T cells[a]	Mononuclear cell count/μl	B-1 cells/μl	B-2 cells/μl	T cells/μl
<16	30 (15–42)	18 (8–26)	51 (34–57)	3200 ± 1100	580 ± 80	400 ± 100	1400 ± 100
>20	13 (8–21)	5 (3–9)	68 (56–83)	1200 ± 600	60 ± 40	120 ± 40	950 ± 70

[a]Percent of total mononuclear cells (mean with range in parenthesis).

groups ($P > 0.05$), due to the simultaneous rise in conventional B cells.

Age distribution of human B-1 cells in peripheral blood

Analysis of umbilical cord blood obtained at time of delivery (>37 weeks) and peripheral blood obtained from infancy through adulthood shows that there is a gradual reduction in the percentage of B-1 cells per total mononuclear cells with increasing age (Figs 3 and 4). In cord blood, 90% of the B cells express CD5 in agreement with previous results (9). During infancy, 75–80% of the B cells are $CD5^+$. From early childhood to adolescence, the percentage of B-1 cells gradually decreases until it stabilizes in late adolescence at 25–35% of the total B cell population (Figs 3 and 4). Thus, in the adult peripheral blood, as reported earlier (9,12), B-1 cells range from 2 to 6% of the total mononuclear cells and from 25 to 35% of the total B cells. The decrease in B-1 cells with increasing age is also evident when calculated as number of cells per milliliter of blood. The number of mononuclear cells and B-1 cells recovered per milliliter of peripheral blood from children and adolescents (<16 years) is significantly higher than from adults (Table 2) (32–34).

Statistical analysis using Student's *t*-test demonstrates that the percentage of peripheral blood B-1 cells are significantly different between each of the age groups (Fig. 3). Unlike spleen, the percentage of total B cells in peripheral blood is also significantly different between age groups, except for cord blood versus infants and children versus adults. Conventional B cells, in contrast, appear after birth and their levels do not differ significantly in peripheral blood among infants, children, and adults ($P > 0.05$). Thus, the progressive decrease in the percentage of total B cells in peripheral blood with increasing age is due to the reduction of the B-1 cell subset (Fig. 3). The percentage, but not the concentration, of T cells in peripheral blood increases simultaneously with the drop in the percentage of B-1 cells (Table 2).

Expression of other surface antigens on human B-1 cells

The majority of unstimulated $CD20^+$ B cells in human fetal spleen and adult peripheral blood express other B cell associated antigens such as CD21 and IgM, but do not express certain activation antigens like CD23 (Fcε II R) (35,36), CD25 (IL-2R) (37), and 4F2 (38) (Figs 5 and 6). B-1 cells also express high levels of IgD, which has been reported to be down regulated on activated B lymphocytes (39). The size of B-1 and conventional B cells from spleen and blood, as measured by forward and obtuse scatter with FACS analysis, are similar and typical of small, resting lymphocytes. Consistently, unstimulated B-1 and conventional B cells from normal individuals secrete similar low levels of Ig (9,40,41). When stimulated with LPS or *Staphylococcus aureus* Cowan I, a considerable portion of B-1 and conventional

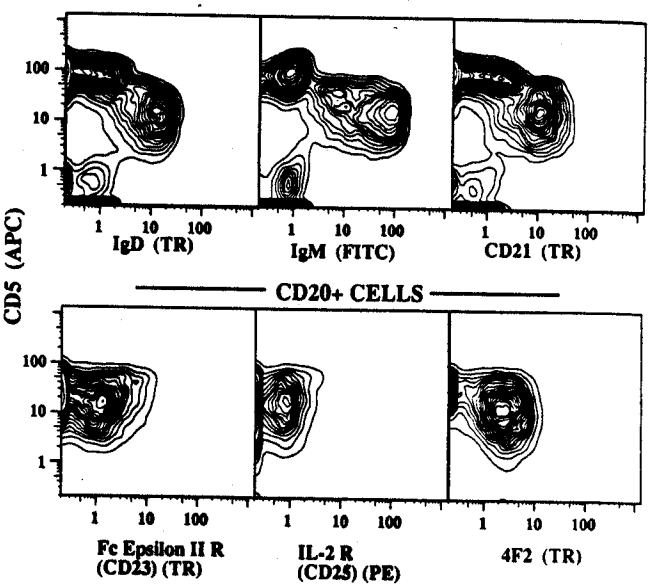

Fig. 5. B-1 cells in fetal spleen express other common B cell antigens but do not express activation antigens. Immunofluorescence profiles of mononuclear cells of a 20 week human fetal spleen stained with anti-CD5 and either anti-IgD, anti-IgM, or anti-CD1 is shown in the top panel. Expression of the three activation antigens with respect to CD5 is shown in the bottom panel after gating for $CD20^+$ cells.

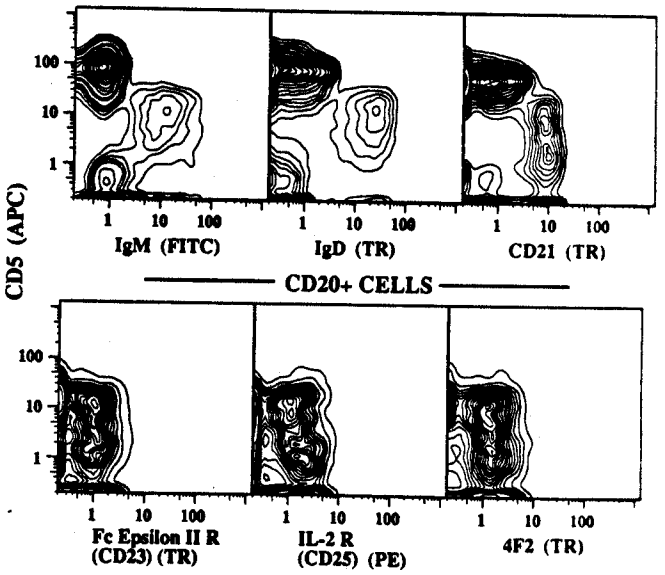

Fig. 6. B-1 cells in adult peripheral blood express other common B cell antigens, but do not express activation antigens. Immunofluorescence profile of mononuclear cells from peripheral blood of a 30 year old stained as in Fig 5.

Table 3. Autoantibody specificities of selected hybridomas generated from human fetal spleen

MAb[a]		Anionic polymer antigens[b]				Protein antigens[b]			
Fusion	Clone	ssDNA	dsDNA	Cardiolipin	Chondroitin sulfate	Thyroglobulin	Transferrin	Myelin basic protein	Insulin
A	1	+ + + +†	±	+ + +	+	+ +	+ +	+ +	+ + +
	2	+ + + +	±	+ + +	+	+ +	+ +	+ +	+ + +
	3	+ + + +	±	+ + +	+	+ +	+ +	+ +	+ + +
	4	+ + + +	±	+ + +	±	+ +	+ +	+ +	+ + +
B	1	+ + + +	−	+ + +	±	−	−	−	+
	2	+ + + +	−	+ + +	−	−	−	−	+
	3	+ + +	−	+	+	−	−	+	NT
C	1	+ + +	−	+	+	−	−	+	NT
	2	+ + + +	−	+	+	−	−	+	NT
D	1	+ + + +	+ +	+	±	−	−	+	+ +
	2	+ + + +	−	+ +	−	−	−	−	−
	3	+ +	−	±	−	−	−	−	−
	4	±	−	+ + +	−	+	±	−	+
	5	±	−	−	−	−	−	±	−
	6	−	−	−	+ +	−	−	−	−

[a]Culture supernatants from clones; 50 ng. All culture supernatants negative for reactivity to BSA, and positive for Rc LPS and Lipid A (+ to + + +)
[b]ELISA Score (based on OD reading at 414 nm): − = 0.000 – 0.159; ± = 0.160 – 0.299; + = 0.300 – 0.499; + + = 0.500 – 0.699; + + + = 0.700 – 0.999; + + + + = ≥ 1.000; NT = not tested.

Table 4. Production of IgM autoantibodies by human adult and fetal B cell subsets

FACS-sorted splenic B cells (EBV transformed)[a]	Autoantigen reactivity[b]		
	ssDNA	Chondroitin sulfate	Thyroglobulin
21 week fetus			
$CD5^{high}$	97	29	8
$CD5^-$	100	32	NT
14 year adolescent			
$CD5^{high}$	100	NT	19
$CD5^-$	0	NT	2

[a]Sorted fetal $CD5^-$ B cells were expanded *in vitro* after EBV transformation.
[b]Percentage of wells containing IgM that reacted with the indicated antigen; 1000 cells plated per well; all wells contained IgM; all wells with spleen from the 14 year old also contained IgG.

B cells from both human fetal spleen and adult peripheral blood increase in size and show a 10- to 15-fold increase in the expression of CD25, 4F2 (data not shown), and CD69 (Leu-23) (41). The expression of IgD, the absence of the three activation antigens, and the small size of the majority of unstimulated B-1 and conventional B cells isolated from fetal spleen and adult peripheral blood, indicates that human B-1 cells do not simply represent a stage in B cell activation.

Human fetal splenic B cells produce IgM antibodies that react with multiple autoantigens

B lymphocytes from fetal spleen between gestational ages of 17 and 23 weeks were EBV-transformed or fused directly to generate hybridomas, and the supernatants tested for Ig isotype and autoantibody production as described in Methods. Only IgM was detected. IgG production was undetectable in the supernatants of both EBV-transformed cells (n = 2) and hybridomas (n = 4) generated from fresh, independent fetal spleen cells. One-third of the hybridomas generated from four independent fetal spleen specimens secreted IgM antibodies that reacted with two or more autoantigens tested. Specificities of 65% of the IgM producing hybridomas are unknown. Specificities of representative hybridomas generated from four spleens that reacted to the panel of autoantigens tested are described in Table 3. All of the antibodies from the hybridomas in Table 3 also reacted to bacterial Rc LPS and Lipid A, but not with BSA. The reactivity pattern of these monoclonals demonstrates that B-1 cell antibodies are polyspecific and are similar to those described by others as 'natural autoantibodies' (10).

Repertoire differences in B-1 and conventional B cells from spleen of a 14 year old

Analysis of the antibodies produced by B-1 and conventional B cells shows that only the B-1 cell subset yield detectable IgM autoantibodies. $CD20^+CD5^{high}$ and $CD20^+CD5^-$ cells were sorted into two separate fractions from a spleen of a 14 year old

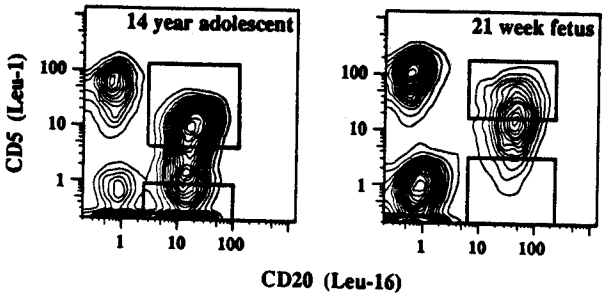

Fig. 7. Sorted B cells: 21 week fetal and 14 year old adolescent splenic cells were stained with FL – anti-CD20 and APC – anti-CD5. $CD20^+CD5^{high}$ and $CD20^+CD5^-$ B cells from the two spleens were sorted using gates shown. Re-analysis of sorted cells for $CD5^{high}$ fraction from fetal spleen showed 98% purity. Sorted $CD5^-$ B cells from fetal spleen were not re-analysed due to the low number of cells in the fractions. $CD5^{high}$ and $CD5^-$ B cells from the spleen of the 14 year old were 92 and 95% pure respectively.

(Fig. 7). Spleen from an adolescent was chosen because the proportion of B-1 and conventional B cells are approximately equal at this stage. The two extreme populations of B cells with respect to CD5 expression were sorted to minimize contamination from the overlapping populations in the middle. Re-analysis of the sorted cells demonstrated 95% purity for the $CD20^+CD5^-$ sort and 92% for the $CD20^+CD5^{high}$ sort. Most of the contaminating cells in the later sort were T cells, which do not bias the subsequent EBV-transformation and antibody production. Unlike fetal spleen, which produced only IgM, both the B cell subsets of the 14 year spleen secreted IgM and IgG; however, only the IgM fraction from the $CD5^+$ B cell subset reacted to ssDNA and thyroglobulin, representative antigens of the autoantigen panel (Table 4). Casali and Notkins (8) have reported a similar segregation of reactivity between the normal adult peripheral blood B-1 and conventional B cells, and have further shown that no selection exists in the EBV transformation of different B cell subsets (42).

Fetal splenic $CD5^{high}$ and $CD5^-$ B cells produce polyreactive autoantibodies

Although 80 – 100% of fetal splenic B cells express CD5, the density of expression varies at least 100-fold. To test whether the antibody repertoire varies with CD5 expression, as observed in adult spleen, FACS-sorted $CD20^+CD5^{high}$ and $CD20^+CD5^-$ fetal spleen cells where EBV-transformed, cultured, and screened for autoantibody production. Both $CD5^{high}$ and the apparently $CD5^-$ B cells produced IgM antibodies that reacted with ssDNA and chondroitin sulfate, representative antigens of the autoantigen panel (Table 4). Thus, unlike adult spleen, where $CD5^{high}$ B-1 cells are functionally distinct from $CD5^-$ (conventional) B cells, fetal splenic B cells appear functionally uniform. The measured level of '$CD5^-$' B cells in fetal spleen (0 – 20%) may still result from the limitation of the current reagent. In fact we suggest that all B cells in fetal spleen are functionally B-1 cells.

Discussion

We have presented a detailed analysis of the age related distribution of human B-1 cells from fetus to adult. The ontogeny of human B-1 cells is very similar to murine B-1 (Ly-1 B) cells. In both species, the earliest detectable mature B lymphocytes in fetal secondary lymphoid organs express CD5 and, in both the species, the percentage of B cells that express CD5 progressively diminishes with age.

Reconstitution experiments with congenic mice have established that murine B-1 cells constitute a distinct lineage from conventional B cells (19). Unlike conventional B cells, B-1 cells are poorly constituted by transfers from adult bone marrow. Instead, they are reconstituted by transfers of fetal liver, fetal omentum, or by self-replenishing Ig^+ B-1 cells from adult peritoneum or spleen (43 – 47). More recent studies reveal a feedback mechanism through which mature B-1 cells prevent further B-1 cell development from Ig^- precursors (48). This feedback mechanism cannot be triggered prior to weaning or prior to the onset of puberty, implying that an hormonal signal may be required. We hypothesize that a similar feedback mechanism may stabilize the levels of B-1 cells in humans around puberty.

The higher concentration of B cells in peripheral blood of children compared with adults has been reported earlier (32 – 34). We demonstrate it as the decrease in B-1 cells that leads to the gradual reduction of both the number of mononuclear cells and B lymphocytes in peripheral blood with age. Stable adult levels are reached in adolescence. Simultaneous with this decrease in B cells, there is a gradual increase in the percentage of T cells with respect to mononuclear cells in peripheral blood. Although a similar decrease in the levels of B-1 cells is seen in spleen, the percentage of B cells with respect to mononuclear cells does not change because of a gradual increase in conventional B cells. We thus demonstrate a differential replacement of cell types in human spleen and peripheral blood with increasing age.

Human B-1 cells from fetal and adolescent spleen produce IgM autoantibodies, many of which react with multiple antigens. Similar results have previously been shown with adult peripheral blood cells (8). The segregation of polyspecific autoreactivity to the IgM fraction of human B-1 cells implies that the two subsets of B cells may serve different, albeit overlapping, functions. The majority of B-1 cells display the phenotype of mature resting cells, rather than activated cells. This, together with the characteristic repertoire of B-1 cells, suggests that, like murine B-1 cells, human B-1 and conventional B cells may have evolved as two distinct subsets. Accumulating evidence shows that conventional B cells produce high affinity, fine specificity antibodies, which have the characteristics of an acquired immune response. In contrast, B-1 cells produce low affinity, broad specificity antibodies that participate in 'natural immunity'.

The predominance of B-1 cells in human fetal spleen suggests a role for this cell type in early development, possibly in maintaining tolerance towards maternal anti-fetal antibodies (e.g. for anti-blood group or anti-HLA) that enter the fetus or in shaping the developing immune repertoire. In fact, there is some evidence that natural autoantibodies may maintain the dynamics of the immune response through a network of idiotypic and anti-idiotypic interactions which modulate the T and B cell receptor specificities and avoid pathological autoimmunity (49,50). Timed administration of selected polyreactive anti-idiotypic autoantibodies to neonatal mice has dramatically increased or decreased the expression of idiotope-bearing antibodies in adult mice (51,52).

Several laboratories have shown that the majority of antibodies produced by B-1 cells are encoded by germline genes without somatic hypermutation (7,53). Phylogenetically, such non-

mutated germline genes and the low affinity broad specificity antibodies first appear in sharks and torpedo fish (54). These primitive immune systems are devoid of the typical conventional B cells that undergo affinity maturation to yield high affinity, fine specificity antibodies. The V_H elements found in these early vertebrates, and in birds and rabbits are all V_H3-like (55 – 57). Biased use of V_H3-like segments is also seen in fetal and neonatal mice (58,59). Thus the preferential usage of the V_H3 locus by fetal B-1 cells that we (manuscript in preparation) and others demonstrate appears to be a conserved component of the primitive antibody repertoire (60 – 62).

Besides recognizing certain self antigens, B-1 cell antibodies also react with common bacterial cell wall components. This reactivity, in conjunction with their strategic distribution in mice, led to the hypothesis that these antibodies could provide the first line of defense against infections (6,7). The antibody data with the age related decrease suggests that B-1 cells produce a library of specificities evolved and preserved through evolution for immediate protection in immunocompromised or naive hosts before the antigen-specific conventional B cell system matures.

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Abbreviations

APC	allophycocyanin
B-1 cells	$CD5^+$ B cells
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
EBV	Epstein – Barr virus
FACS	fluorescent-activated cell sorter
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
IMDM	Iscove's-modified Dulbecco's medium
LPS	lipopolysaccharide
PE	phycoerythrin
SSC	sodium chloride – sodium citrate buffer
TR	texas red

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