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The antigen receptor complex on cord B lymphocytes

P. J. MACARDLE‡, H. WEEDON*, M. FUSCO*, S. NOBBS†, J. RIDINGS*, L. FLEGO‡,
D. M. ROBERTON† & H. ZOLA* **Child Health Research Institute, Women's and Children's Hospital, North Adelaide, and †Department of Paediatrics, University of Adelaide, ‡Department of Clinical Immunology, Flinders Medical Centre, Adelaide, Australia*

SUMMARY

The neonatal immune system responds to a restricted range of antigens, producing largely IgM antibody of low affinity. Comparison of the components of the B-cell antigen receptor complex shows significantly elevated membrane levels of IgM in neonatal B cells, compared with adult cells. CD79, which acts as the signal transducer for membrane immunoglobulin, is elevated in parallel with IgM, while IgD is elevated to a lesser degree. CD19, CD21, CD22 and CD81, which are all involved in transmitting activation signals when immunoglobulin is engaged, are not elevated. CD32, which is involved in negative regulation of activation, is present at reduced levels on cord B cells. The elevation of B-cell membrane IgM persists during infancy. Neonatal B cells respond *in vitro* to interleukin-4 (IL-4) by further elevation of membrane IgM levels. The elevated level of membrane IgM may make neonatal B cells easier to trigger by low concentrations of antigen, but *in vitro* activation and immunoglobulin modulation experiments did not show significant differences between cord and adult B-cell responses to anti-IgM.

INTRODUCTION

The B-cell antigen receptor consists of membrane immunoglobulin (Ig) in association with a number of molecules that transduce signals to the cell. Membrane immunoglobulin is associated directly with Igα and Igβ (CD79α and CD79β), the products of the *mb-1* and *B29* genes.[1] A further series of molecules, CD19, CD21, CD22 and CD81, is associated with the Ig–CD79 complex.[2] B cells also express a receptor for IgG Fc (FcγRII, CD32), which functions as a negative regulator of B-cell activation.[3]

The interaction of antigen with surface immunoglobulin can result in three distinct outcomes. The B cell may internalize protein antigen, process it and present peptides to T cells; the B cell may be activated directly to proliferate and secrete antibody; or the interaction may result in B-cell anergy. The outcome depends on the nature of the antigen, on the state of differentiation of the B cell, and on the delivery of costimulatory signals. It is not clear whether the same set of receptor-associated molecules (CD79α and β, CD19, CD21, CD22, CD81, CD32) is involved equally in each of these reactions. Association with CD79 is essential for membrane expression of IgM, while IgD can exist in an alternative, glycolipid-anchored form.[4] CD19 appears to be necessary for triggering of B cells, particularly by antigen that binds the antigen receptor with low affinity.[2] CD19-deficient mice have a reduced capacity to respond to antigenic and other stimuli, while mice that overexpress CD19 show enhanced responses.[5] These effects are seen for T-dependent and for some T-independent responses, indicating that CD19 is involved in B-cell activation rather than T–B cognate interaction.[6] Activation requires receptor cross-linking, and the signalling pathway for activation depends on receptor aggregation.[7] In contrast, antigen internalization and presentation does not require receptor cross-linking,[8] making it highly probable that different signalling pathways are involved.

Infants have a limited ability to generate effective antibody responses. They make largely IgM antibodies, fail to respond to certain types of antigens, and have a limited capacity to develop a memory response when exposed to antigen.[9] Different aspects of the functional immaturity of the antibody response have been attributed to limitations in T-cell help and to intrinsic immaturity of the B cells.[10,11] Neonatal B cells differ from adult B cells in a number of characteristics. In particular, cord blood B cells express CD5,[12] which in adult B cells identifies a subset with a tendency to produce antibodies that are self-reactive, cross-reactive and generally of low affinity. This study explores the relationship between the phenotypic properties of neonatal B cells and their functional immaturity.

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Abbreviations: EBV, Epstein–Barr virus; FcγRII, receptor for IgG, type II (CD32); IL-4, interleukin-4; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NWSM, *Nocardia* water-soluble mitogen; PWM, pokeweed mitogen; TI-1, TI-2, thymus-independent antigens, types 1 and 2.

Correspondence: Dr H. Zola, Child Health Research Institute, Women's and Children's Hospital, North Adelaide 5006, Australia.

MATERIALS AND METHODS

Samples

Cord blood was obtained from healthy full-term babies, and adult blood from healthy laboratory volunteers. The mononuclear cell fraction was isolated by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). Red cell precursors were removed by lysis with isotonic ammonium chloride for 5 min at room temperature.[13] Staining and analysis of infant samples (and adult samples in parallel) was carried out on whole blood.[14] All human material was obtained with approval from the Ethics Committee of the Adelaide Women's and Children's Hospital.

Antibodies

The following monoclonal antibodies (mAb) were used: IgM, HB57;[15] IgD, HB70;[15] CD79b, SN8 (B037 in the 5th Human Leucocyte Differentiation Antigens Workshop);[16] CD19, FMC63;[17] CD21, HB5;[18] TAPA-1 (CD81): 5A6 (B068 in the 5th Human Leucocyte Differentiation Antigens Workshop);[19] CD22, 29.110;[20] CD32, 41H16;[21] CD98, 4F2;[22] CD40, G28/5;[23] and major histocompatibility complex (MHC) class II (FMC52; H. Zola, unpublished data). Mouse antibodies were detected using $F(ab')_2$ fragment of fluorescein-conjugated anti-mouse immunoglobulin (DDAF; Silenus Laboratories, Melbourne, Australia). In two-colour analyses, B cells were identified using CD19–CyChrome (Pharmingen, San Diego, CA). In three-colour experiments, B cells were identified using CD19–CyChrome and $CD5^+$ cells were identified using CD5–phycoerythrin (PE) (Becton Dickinson, San Jose, CA).

Immunofluorescence and flow cytometry

Marker expression was studied as described previously.[24] Flow cytometry was performed using a FACScan instrument (Becton Dickinson, San Jose, CA) with standard optics and settings, and results were analysed using Lysis II software (Becton Dickinson).

In vitro *functional assays*

Mononuclear cells, isolated as described above, were cultured at 1×10^6/ml, 3 ml per tube, in 10-ml round-bottomed culture tubes for 24 or 48 hr, with or without interleukin-4 (IL-4) (Genzyme, Boston, MA; used at 25 or 100 U/ml); polyclonal anti-IgM coupled to polyacrylamide beads [Bio-Rad Laboratories (Hercules, CA) cat. no. 170-5120; used at 10 μg/ml]; or both together, as described previously.[25] Cells were harvested and analysed by immunofluorescence. For capping and modulation studies, cells were incubated with reagents (see the Results) in the absence of azide. Samples were examined by fluorescence microscopy or flow cytometry, as detailed in the Results.

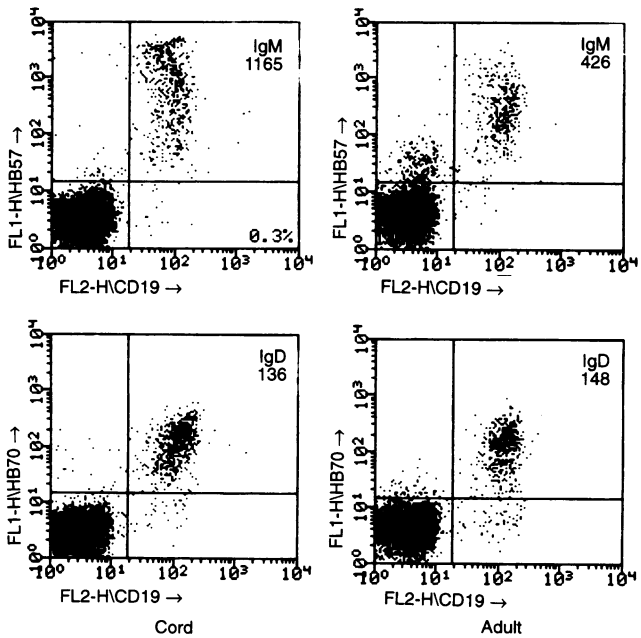

Figure 1. Staining of cord and adult B cells for IgM and IgD. The results are representative; Table 1 presents the statistical analysis for 31 samples.

RESULTS

Cord B cells express elevated levels of surface IgM

Cord cells expressed consistently higher levels of IgM than adult cells. Figure 1 shows one set of data, with IgD included for comparison. Thirty-one sets of data, each comparing one cord with one adult sample and using a single anti-human IgM antibody (HB57, see below), were analysed statistically, comparing the mean fluorescence intensity of IgM on cord B cells (MFI^{cord}) with the corresponding parameter on adult cells (MFI^{adult}). The mean ratio of MFI^{cord}/MFI^{adult} was 2·6, with a range of 0·95–7·2 ($P=0·0001$, derived using Student's paired *t*-test on paired cord and adult samples). For IgD the mean ratio MFI^{cord}/MFI^{adult} was 1·5 ($P=0·0005$). To ensure the result was not peculiar to a particular reagent, samples were examined with several reagents: HB57 (mouse monoclonal anti-IgM, detected with fluorescein-conjugated anti-mouse immunoglobulin), two fluorescein-conjugated polyclonal preparations from Silenus Laboratories [a $F(ab')_2$ fragment, MDF, and an affinity-purified preparation, MAF], and a polyclonal $F(ab')_2$ product from Zymed Laboratories (San Francisco, CA; cat. no. 62-7812). All the reagents showed higher staining intensities on cord compared with adult cells.

To ensure that the level of IgM seen on the cells did not reflect the time elapsed between birth and analysis of the sample, a fresh cord sample and a fresh sample of adult blood were tested immediately and again after 24 hr kept as whole blood at room temperature. There was no significant change in staining for IgM or IgD.

To estimate the number of antigenic sites per cell, cord and adult cells were stained with HB57 followed by conjugated anti-mouse immunoglobin. In the same experiment the same conjugate was used to stain 'Qifikit' beads (Biocytex, Marseille, France). The data, from two experiments using different donors, indicated that adult B cells expressed 12 000–48 000 molecules per cell, while cord B cells expressed 63 000–240 000 molecules per cell. IgD levels were 23 000–46 000 and 5500–11 000 molecules on cord and adult cells, respectively.

Cytoplasmic IgM content was examined by analysing cells with and without permeabilization, using Caltag Fix&Perm kit (cat. no. GAS003; Caltag, San Francisco, CA). While adult B cells contained relatively large amounts of IgM (MFI 1537 if stained after lysis, 566 if stained unlysed), in cord cells most of the IgM is on the membrane (MFI 2988 lysed, 2250

unlysed). A separate experiment with a different pair of donors showed a similar pattern.

The elevation of IgM levels on cord cells does not reflect a difference between CD5$^+$ and CD5$^-$ B cells

The majority of cord B cells express CD5, and are therefore regarded as belonging to the B1 sublineage. A possible explanation of the elevated IgM observed on cord cells is that this is a characteristic of CD5$^+$ cells. Comparison of the IgM levels of CD5$^+$ cord with CD5$^+$ adult B cells, however, showed that surface IgM levels are higher on cord CD5$^+$ cells (Fig. 2). Furthermore, IgM levels were much higher on cord CD5$^-$ cells compared with adult CD5$^-$ cells (Fig. 2). Adult CD5$^+$ and CD5$^-$ cells had equivalent levels of surface IgM (sIgM) (and IgD). Cord CD5$^-$ cells expressed higher levels of IgM than did cord CD5$^+$ cells (Fig. 2).

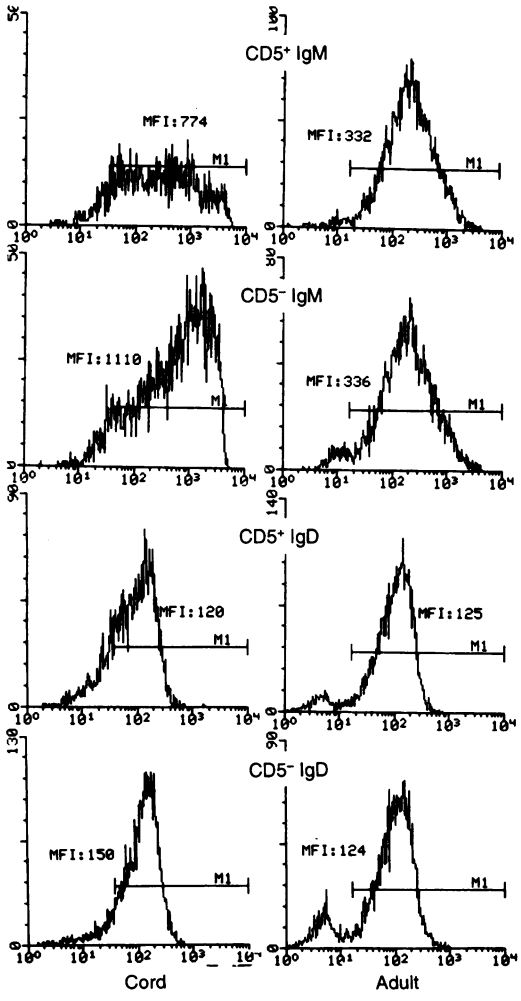

Figure 2. Expression of IgM and IgD by CD5$^+$ and CD5$^-$ B-cell subpopulations in cord and adult blood. Cells were stained with CD19–CyChrome, CD5–PE and HB57 (anti-IgM) or HB70 (anti-IgD) detected by fluorescein–anti-mouse immunoglobulin. Populations were gated using dual scatter parameters to select lymphocytes, and CD19 and CD5 to select CD5$^+$ or CD5$^-$ B-cell subsets.

Expression of the immunoglobulin-associated molecules of the B-cell receptor (BCR)

Cord and adult samples were analysed as paired samples. CD19, CD21, CD22 and CD81 showed cord/adult MFI ratios close to unity ($P>0{\cdot}1$) (Table 1). CD79β was, however, elevated to approximately the same degree as IgM. CD32 was lower on cord than adult cells.

The elevation of surface IgM levels persists in early childhood

IgM staining intensities were determined on 20 children ranging in age from 5 weeks to 8·5 years. There was a downward trend in IgM MFI values when plotted against age (Fig. 3). The Spearman rank correlation coefficient was $-0{\cdot}59$ with a P-value of 0·01. The downward trend was most pronounced in the first 2 years (Spearman rank correlation coefficient of $-0{\cdot}71$; $n=9$; $P=0{\cdot}035$).

Regulation of IgM and associated molecules by culture in IL-4

IL-4 stimulated upregulation of sIgM both in cord and in adult cells. CD23 and MHC class II were also upregulated by IL-4, in both cord and adult cells (Table 2). In each of seven

Table 1. Differences between cord and adult cells in expression of components of the antigen receptor

Marker	MFI^{cord}/MFI^{adult}	P-value	n
IgM	2·6	0·0001	31
IgD	1·5	0·0005	31
CD79β	1·9	0·04	10
CD19	0·9	>0·1	7
CD21	1·1	>0·1	7
CD81	1·2	>0·1	5
CD22	0·90	>0·1	10
CD32	0·76	0·01	17

MFI was measured for each indicated marker. Each assay consisted of one or more cord blood samples and an adult blood sample run in parallel; the ratio of MFI was calculated for each assay and the numbers in the MFI^{cord}/MFI^{adult} column represent mean values, for n samples, where n is in the last column of the table. The P-value was calculated by comparing cord and adult MFI values as paired samples.

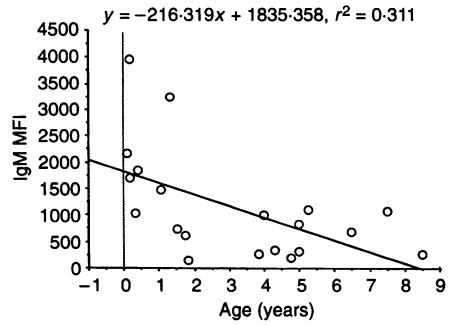

Figure 3. Plot of IgM expression (MFI) against age, for 20 infants aged from 1 month to 8·5 years of age. The tests were all carried out with the same batch of fluorescein–anti-IgM, and the flow cytometer was calibrated with fluorescent beads for each assay, to allow comparability of the data.

Table 2. Effect of culture in IL-4, anti-IgM and IL-4+anti-IgM on expression of surface IgM and other membrane markers

	Adult blood B cells			Cord blood B cells		
Marker	IL-4	Anti-IgM	IL-4+anti-IgM	IL-4	Anti-IgM	IL-4+anti-IgM
sIgM	2·04	0·92	1·93	2·35	0·55	1·17
sIgD	1·40	0·35	0·46	1·33	0·33	0·36
CD98	1·12	3·24	3·0	0·97	3·12	3·16
CD40	1·09	1·26	1·36	1·12	1·38	2·26
CD23	3·70	1·10	8·81	7·47	0·45	4·73
MHC II	1·64	2·79	2·23	1·07	1·78	1·55
CD19	0·99	ND	ND	1·25	ND	ND
CD21	0·94	ND	ND	1·11	ND	ND
CD79β	1·75	ND	ND	3·30	ND	ND

Antigen expression was analysed by two-colour immunofluorescence, with B lymphocytes selected on the basis of staining with CD19. The data represent the MFI for B-cell expression of the indicated marker, divided by the MFI for the same marker on B cells from control culture without added IL-4 or anti-IgM.

ND, not done.

experiments using different cord and adult samples, IgM was upregulated on cord, as well as adult cells, and the level of IgM expression remained higher on cord cells than on adult cells after IL-4 treatment.

Effect of ligating the antigen receptor with anti-IgM

Anti-IgM antibody activated B cells from both adult and cord blood (Table 2). IgM ligation characteristically upregulated CD98 and MHC class II expression, and these molecules were upregulated in cord as well as in adult cells. Quantitative differences were seen between three repeat experiments, but the changes were qualitatively similar.

Capping and modulation

Capping was examined by fluorescence microscopy, using anti-IgM and anti-IgD, and staining with a single layer (fluoresceinated anti-human IgM) or dual layer (mouse monoclonal anti-human IgM or IgD followed by anti-mouse immunoglobulin). Surface IgM was capped rapidly in both cord and adult cells. Cap formation in some cells was detectable after 5 min, particularly when using two layers of antibody. By 15 min capping was visible in a proportion of cells in all combinations. By 1 hr, the majority of cells were capped, although there were always some uncapped cells. IgD seemed to cap less extensively than IgM, particularly in cord cells, and cord cells seemed to cap their IgM more rapidly than adult cells. To obtain objective and quantitative information, antigen modulation was analysed by flow cytometry. Modulation was seen equally in cord and adult cells (Fig. 4). CD19 did not show any modulation in these experiments, and cells incubated at 4° showed little variation in MFI of either immunoglobulin or CD19.

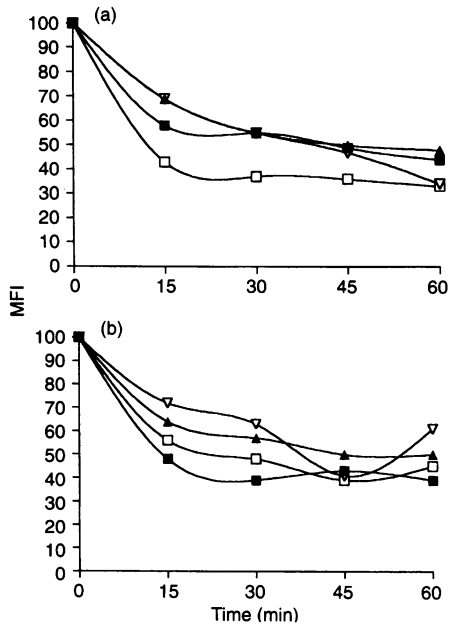

Figure 4. Modulation of B-cell surface expression of IgM (a) and IgD (b). B cells were identified with CD19–CyChrome while IgD and IgM were stained with the appropriate mAb detected with fluorescein–anti-mouse immunoglobulin. Two sets of paired samples were incubated at either 4° or 37° and the MFI of IgM or IgD on CD19$^+$ cells was recorded at 15-min intervals. Two independent adult (closed symbols) and two independent cord (open symbols) samples were examined; there were no consistent differences in the rate of modulation.

DISCUSSION

Antigen receptor functions by activating B cells and by internalizing antigen for subsequent processing and presentation to T cells. The differences between adult and neonatal cells in expression of membrane IgM and CD79, and the lack of difference for CD19, CD21, CD22 and CD81, suggests that the antigen receptor complex in cord cells is specialized to mediate functions that require CD79 but not the other receptor-associated components. CD79 is necessary for all IgM function, since IgM cannot be expressed on the surface without CD79. CD81, CD19, CD21 and CD22 are involved in B-cell activation through the antigen receptor,[26] but their role in antigen internalization for processing is not known.

Johnstone & Millard[27] measured the mean total IgM content (membrane and cytoplasmic) by radioimmunoassay; they found 351 ng/10^8 cord lymphocytes, compared with

200 ng/10^8 adult lymphocyte. The ratio of 1·75 is within the range of ratios we found for membrane expression. The data of Johnstone & Millard[27] can be used to calculate a value of 120 000 molecules of IgM per adult B cell and 210 000 molecules per cord B cell. The values are higher than estimated in our study using immunoglobulin-bearing beads and flow cytometry, but the two methods are different and probably associated with different systematic errors. In the present study, staining for IgM with or without membrane permeabilization suggests that cord cells have most of their IgM on the surface, while adult cells retain a higher proportion in the cytoplasm. Griffioen *et al.*[28] also noted a higher level of IgM on cord than on adult B cells, although their study concerned principally CD21, which they found to be present at reduced levels on cord cells. The reason for the difference between our results and those of Griffioen *et al.* with respect to CD21 is not clear.

Membrane IgM levels are sensitive to upregulation *in vitro* by IL-4.[29] Cord cells are responsive to IL-4.[30] A possible explanation for the elevated levels of IgM, therefore, is that cord cells are exposed to IL-4. However, culture of cord and adult cells in IL-4 raised surface IgM levels in both, and cord cells maintained a higher level than adult cells, indicating that the elevated IgM levels in cord do not simply reflect IL-4 preexposure.

Functional studies on human neonatal and infant B cells have produced a variety of findings. Andersson *et al.*[11] cultured neonatal and infant blood lymphocytes with Epstein–Barr virus (EBV), lipopolysaccharide (LPS) and pokeweed mitogen (PWM) as mitogens. Proliferative responses were similar to adult cells, while IgG secretion was always lower in cord cells. The functional deficits were interpreted as a combination of intrinsic B-cell immaturity and immaturity of T-cell help. It has recently been pointed out that neonatal T-cell function in turn depends on the antigen dose and the availability of costimulatory and non-costimulatory antigen-presenting cells.[31] Miyawaki *et al.*,[10] using *Nocardia* water-soluble mitogen (NWSM), found a very low production of IgM, IgG and IgA at birth. IgG and IgA production did not reach near-adult levels for several years. Durandy *et al.*[32] and Watson *et al.*[33] found that the proliferative response of neonatal B cells was comparable to adult cells, but maturation to antibody-secreting cells in response to several agents was much lower in newborn cells. Small *et al.*[34] found cord blood cells proliferated more strongly than adult cells in response to *Staphylococcus aureus* Cowan strain 1 (SAC) but less strongly in response to anti-IgM, and cord cells were capable of making IgM but not IgG *in vitro*. Splawski *et al.*[35] examined T-cell dependent B-cell stimulation *in vitro*. While activated T cells stimulated adult B cells to produce IgM, IgG and IgA, cord cells produced only IgM, in very low amounts. Mixing of neonatal and adult cells indicated that deficits existed in both B and T cells of the neonate. Yachie *et al.*[36] were able to elicit IgG and IgA responses from cord cells, cells in the presence of PWM and a $CD62L^+$ subset of CD4 cells. Levels of IgG production were lower than from adult cells in the same culture system. Pastorelli *et al.*[37] showed that cord blood cells cultured with IL-4 can switch to IgE production.

Van Tol *et al.*[38] performed antigen-specific culture experiments, examining the production of IgM plaques against a number of T-dependent antigens. Interestingly, cord blood cells showed maximum reactivity to antigen concentrations 100–1000-fold lower than the concentrations optimal for adult cells. The difference in optimal antigen concentration was attributed to differences in the handling of antigen by monocytes and the induction of suppressor T-cell activity. Nevertheless, it may be that the higher concentration of antigen receptor, demonstrated in the current study, contributes to the greater sensitivity of neonatal cells to triggering by these antigens.

Cord blood contains a high proportion of $CD5^+$ B cells, which are regarded as being largely T independent. The present study shows that membrane IgM is elevated both in neonatal $CD5^+$ and in neonatal $CD5^-$ B cells. While a relative inability to interact with T cells may explain much of the *in vitro* data summarized above, the response of neonatal cells to T-independent type 2 (TI-2) antigens, polymers, cross-link receptor, is also much smaller than that of adult cells.[39] The high concentration of antigen receptor on neonatal B cells may increase their sensitivity to cross-linking by polymeric antigens. Whether the result of cross-linking is triggering or anergy may depend on other factors, such as the ability to respond to cytokines. In our studies, the characteristic phenotypic changes seen when B cells react with anti-IgM (which acts by cross-linking the receptor) were seen in cord and adult cells. Capping and modulation, which also depend on cross-linking, also showed no clear difference between cord and adult cells. Thus receptor density is unlikely to account for the unresponsiveness of infant B cells to TI-2 antigens, which probably results from the lack of marginal zone B cells.[40]

TI-1 antigens, which are capable of activating B cells directly, include certain bacterial carbohydrates. Neonatal B cells can make antibody against TNP-*Brucella abortus*, a TI-1 antigen.[41] Blood group antibodies are made soon after birth (and sometimes before birth), apparently as a result of cross-reactive stimulation by bacterial carbohydrates.[42] Neonatal B cells appear to be specialized to respond to bacterial antigens of a class that includes the carbohydrates that stimulate cross-reactive blood-group antibodies.[43] The high concentration of IgM on the neonatal B-cell surface may enhance sensitivity to such antigens.

The published *in vitro* studies indicate that T-cell/B-cell collaboration can operate in the neonate, but does not give rise to the full set of functional consequences (mutation, selection, clonal expansion, class switch, differentiation and generation of memory cells). This conclusion is consistent with *in vivo* experience suggesting a relative inability to produce IgG and IgA and to develop memory in the perinatal period,[9] and the generally very low levels of IgE found in cord sera.[44] The elevated expression of antigen receptor, described in this study, may make neonatal B cells more sensitive to triggering by antigen, but the overall response is also dependent on additional factors.

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