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Delineation of producing ability of IgG and IgA subclasses by naive B cells in newborn infants and adult individuals

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SUMMARY

Neonatal B cells with the naive ($sIgD^+$) phenotype are able to generate IgG- and IgA-producing cells as well as IgM production in the presence of memory $CD4^+$ T cells expressing L-selectin (CD62L) in pokeweed mitogen-stimulated cultures. We used this system to examine comparatively the ability of naive B cells to produce IgG and IgA subclasses in newborn infants and adult individuals. Naive B cells were enriched from both donors on the basis of sIgD positivity, and memory ($CD45RO^+$) $CD4^+$ T cells with CD62L expression were isolated from adults. We here demonstrate some differences in profiles of IgG and IgA subclass production between neonatal and adult naive B cells. In neonatal B cells, IgG1 and IgG3 were predominantly produced, but IgG2 and IgG4 production was virtually absent. Similar to neonatal B cells, adult naive B cells produced mainly IgG1 and IgG3, although memory ($sIgD^-$) B cells from adults secreted all of the IgG subclasses. It should be noted that low but detectable levels of IgG2 and IgG4 were found in adults' naive B cell cultures. Although IgA produced by neonatal B cells was exclusively IgA1, IgA2-secreting cells were identifiable in adult naive B cells. The results suggest that further class switch of naive B cells to IgG2, IgG4 and IgA2 in addition to IgG1 and IgG3 may be controlled by their own age-dependent maturation process.

Keywords naive B cells IgG subclass IgA subclass L-selectin memory T cells

INTRODUCTION

Maturation of humoral immunity is preceded by a series of events that results in the efficient class switch of immunoglobulin from IgM to eight other classes and subclasses, namely IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE [1]. The class switch of immunoglobulin is associated with the recombination of immunoglobulin heavy chain genes and the somatic mutation of the variable regions. Recent findings suggest that at least part of the class switch process is regulated by direct signals provided by the activated T cells and cytokines produced by these T cells [2–4].

Neonates and infants are prone to suffer from severe and prolonged infections caused by organisms of relatively low virulence. Elicitation of the antigen-specific immune response is still poor during this period. Responses against organisms with polysaccharide antigens, such as *Haemophilus influenzae* and *Streptococcus pneumoniae* are especially delayed, partly because of the delayed maturation of IgG2 antibody response [5,6]. Deficient helper function of neonatal $CD4^+$ T cells is the major cause of immune dysfunction during this period [7–9]. Neonatal T cells are unable to induce effective immunoglobulin class switch in B cells, because they virtually lack the $CD45RO^+$ (memory) subpopulation of $CD4^+$ T cells [10–12]. In addition, neonatal B cells themselves are immature and produce only low levels of immunoglobulin even when the cells are stimulated by T-independent stimuli, such as *Staphylococcus aureus* Cowan I (SAC) and IL-2 [13]. Furthermore, immunoglobulin production is usually limited to IgM.

Recently, it was shown that $sIgD^+$ naive B cells can be induced to produce IgG and IgA when IL-10 and/or transforming growth factor-beta (TGF-β) are supplied together with anti-CD40 signal [14]. In addition, we have recently reported that neonatal B cells can produce IgG and IgA in pokeweed mitogen (PWM)-stimulated cultures, when these B cells are cultured together with $CD62L^+$ subpopulation of $CD4^+$ memory T cells [15]. The results indicated that neonatal B cells undergo efficient class switching in the presence of appropriate stimulation. In this study, we utilized this efficient culture system to elucidate the patterns of IgG and IgA subclass production by neonatal and adult naive B cells. The results indicate that a certain maturation mechanism exists for neonatal B cells to undergo class switching to IgG2, IgG4 and IgA2.

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MATERIALS AND METHODS

Reagents

FITC-conjugated anti-CD4 (Leu-3a) and PE-conjugated anti-CD20 (Leu-16) were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). PE-conjugated anti-CD45RA MoAb (2H4) and anti-CD62L (L-selectin) MoAb (TQ1) were from Coulter Immunology (Hialeah, FL). FITC-conjugated affinity-purified goat anti-human IgG, IgM, IgA and IgD and rhodamine-conjugated affinity-purified goat anti-human IgM were the products of Southern Biotechnology Associates, Inc. (Birmingham, AL).

Separation of lymphocytes

Heparinized whole blood was isolated from the placental end of the umbilical cord after uneventful delivery of full-term newborns. Adult peripheral blood was obtained from volunteers aged between 25 and 40 years. Mononuclear cells (MNC) were isolated by Ficoll–Hypaque gradient centrifugation. MNC were suspended in RPMI 1640 culture medium with 5% fetal calf serum (FCS) and incubated for 30 min at 37°C in plastic flasks. Plastic-non-adherent cells were collected and they were separated into E-rosetting (E^+) and non-rosetting (E^-) cells by rosette formation with 2-aminoethylisothiouronium bromide (AET; Sigma Chemical Co., St Louis, MO)-treated sheep erythrocytes, followed by Ficoll–Hypaque gradient centrifugation as described elsewhere [16]. Plastic-adherent monocytes were collected and suspended in RPMI 1640 culture medium with 10% FCS as described before [15]. $CD4^+$ $CD45RA^-$ memory T cells were negatively selected from E^+ cells by depleting $CD4^+$ $CD45RA^+$ naive T cells using a fluorescence-activated cell sorter. $CD62L^+$ fraction was further selected positively from these memory $CD4^+$ T cells. E^- cells were depleted of plastic-adherent cells and the non-adherent fraction was separated into $sIgD^+$ $CD20^+$ naive B cells and $sIgD^-CD20^+$ memory B cells. The sorting procedure for the $sIgD^+$ fraction was repeated twice to avoid contamination by the $sIgD^-$ fraction. Resultant populations were >99·7% pure, as determined by flow cytometric analysis.

Cell cultures

Culture medium consisted of RPMI 1640 medium containing 10% FCS, 20 mM HEPES, 2×10^{-5} M 2-mercaptoethanol (2-ME), 0·3 mg/ml L-glutamine, 200 U/ml penicillin G and 10 mg/ml gentamicin. Purified $sIgD^+$ B cells or $sIgD^-$ B cells were seeded at a density of 2×10^4/well together with autologous monocytes (6×10^3/well) and were cultured with or without added $CD4^+$ T cell fractions (4×10^4/well). PWM (Gibco, BRL Laboratories, Grand Island, NY) was added to the culture at 1 : 200 dilution. After 14 days of culture, supernatant fluid was collected and stored at −80°C until the time of assay. The frozen samples were thawed and supernatant immunoglobulin concentrations were determined by an ELISA.

Measurement of immunoglobulin production

Supernatant immunoglobulin concentrations were measured by an ELISA assay as described previously [17]. Distribution of IgG subclasses in the PWM-stimulated culture supernatants was determined by a sandwich ELISA assay kit (The Binding Site, Birmingham, UK). Lower detection limit of the assay was 5 ng/ml for IgG1 and IgG3, and 10 ng/ml for IgG2 and IgG4.

Immunohistochemical detection of immunoglobulin-producing cells

In some experiments, cultured cells were harvested 7 days after stimulation. B cells were enriched by depleting T cells by E-rosetting. Non-T cells were collected and cytospin preparations were obtained. Air-dried preparations were fixed in 95% ethanol with 5% acetic acid at −20°C for 30 min and extensively washed in PBS. Fixed cells were incubated with FITC-conjugated goat anti-human IgG and rhodamine-conjugated goat anti-human IgM for 30 min at room temperature. For determination of IgA-secreting cells, cells were first stained with mouse anti-human IgA2 for 30 min at room temperature and washed in PBS. They were further stained with rhodamine-conjugated goat anti-mouse IgG for 30 min at room temperature. After washing in PBS, FITC-conjugated goat anti-human IgA was added and incubated for a further 30 min at room temperature. Cytoplasmic immunoglobulin-positive cells were enumerated under immunofluorescent microscopy.

RESULTS

IgG subclass production by neonatal B cells

When neonatal B cells were cultured with $CD62L^+$ memory $CD4^+$ T cells from adult in the presence of PWM, low but significant levels of IgG were detectable in the supernatant. The concentration varied from 63 to 666 ng/ml (mean 288 ng/ml) (Table 1). Most of the IgG belonged to IgG1 and smaller, but significant proportion of the IgG was IgG3. Neither IgG2 nor IgG4 was detectable in most cultures. Mean levels of IgG1, IgG2, IgG3 and IgG4 were 152 ng/ml, < 10 ng/ml, 52 ng/ml and < 5 ng/ml, respectively.

IgG subclass production by adult naive and memory B cells

Distribution of the IgG subclasses produced by adult $sIgD^+$

Table 1. IgG subclasses produced by neonatal B cells combined with $CD62L^+$ memory $CD4^+$ T cells

	IgG produced (ng/ml)				
Experiment no.	Total	IgG1	IgG2	IgG3	IgG4
1	237	96	<10	<5	<5
2	63	33	<10	16	<5
3	111	76	<10	26	<5
4	630	305	15	146	5
5	98	39	<10	23	<5
6	178	66	<10	38	<5
7	666	390	<10	104	9
8	319	213	22	56	<5
Mean	288	152	<10	52	<5
(%)*	ND	(74·5)	(< 5.0)	(25·5)	(<2·5)

Neonatal $CD20^+$ B cells (2×10^4/well) were combined with autologous monocytes (6×10^3/well) and cultured with $CD62L^+$ memory $CD4^+$ T cells (4×10^4/well) from adults in the presence of optimum concentrations of pokeweed mitogen (PWM). Culture supernatants were collected on day 14 and immunoglobulin concentrations were measured by ELISA.

* Numbers in parentheses indicate the mean percentages of each IgG subclass within total IgG produced in the cultures.

Table 2. IgG subclasses produced by $sIgD^+$ and $sIgD^-$ adult B cells combined with $CD62L^+$ memory $CD4^+$ T cells

	IgG produced (ng/ml)				
Experiment no.	Total	IgG1	IgG2	IgG3	IgG4
$sIgD^+$					
1	628	370	<10	66	<5
2	474	360	<10	38	<5
3	1080	700	152	146	13
4	2280	1440	37	60	21
Mean	1116	718	47	78	9
(%)*		(84·3)	(5·5)	(9·2)	(1·0)
$sIgD^-$					
1	10 800	5000	4000	650	103
2	6880	3800	1500	425	165
3	9680	5700	4800	650	125
4	10 200	5900	1950	200	103
Mean	9390	5100	3063	481	124
(%)		(58·2)	(34·9)	(5·5)	(1·4)

Adult $CD20^+$ B cells were separated into $sIgD^+$ and $sIgD^-$ fractions. Each B cell population (2×10^4/well) was combined with autologous monocytes (6×10^3/well) and cultured with $CD62L^+$ memory $CD4^+$ T cells (4×10^4/well) in the presence of pokeweed mitogen (PWM). Supernatants were harvested on day 14 and immunoglobulin concentrations were measured by ELISA.

* Numbers in parentheses indicate the mean percentages of each IgG subclass within total IgG produced in the cultures.

Table 3. Frequency of immunoglobulin-producing cells in neonatal and adult B cell cultures combined with $CD62L^+$ memory T cells

	IgG-producing cells (%)			
Sample no.	IgG	IgM	IgA	IgA2
Neonate				
1	2·5	48·0	0·4	<0·1
2	0·8	18·2	0·5	<0·1
3	2·0	29·4	1·0	<0·1
Mean	1·8	31·9	0·6	<0·1
(%)*	(5·2)	(93·0)	(1·8)	(0)
Adult				
1	25·5	11·6	16·6	7·8
2	39·9	17·0	14·6	6·9
3	31·4	34·8	16·6	5·6
Mean	32·3	21·3	15·9	6·8
(%)	(46·5)	(30·6)	(22·9)	(9·8)

$CD20^+$ B cells from neonate and adult were cultured with $CD62L^+$ memory $CD4^+$ T cells for 7 days in the presence of pokeweed mitogen (PWM). T cells were depleted from the cultured cells, and cytocentrifuged cell preparations were stained for cytoplasmic IgG, IgM, IgA and IgA2.

* Numbers in parentheses indicate the mean percentages of each immunoglobulin isotype within immunoglobulin-producing cell populations.

naive B cells was similar to neonatal B cell cultures (Table 2). Of total IgG produced in the cultures (mean 1116 ng/ml), the vast majority belonged to IgG1 (mean 718 ng/ml). IgG3 was detectable at low levels (mean 78 ng/ml). IgG2 and IgG4 were detectable in some experiments, but at very low concentrations. In sharp contrast, total IgG produced by memory B cells was very high (mean 9390 ng/ml), and both IgG1 and IgG2 were produced abundantly (means 5100 ng/ml and 3063 ng/ml, respectively). IgG3 was produced at a much lower level, but concentrations ranged from 200 to 650 ng/ml and were significantly higher than levels produced by naive B cells. IgG4 production was also low, but levels were significant with mean concentration of 124 ng/ml.

IgA- and IgA2-producing cells induced in neonatal and adult B cell cultures

When $CD20^+$ B cells were cultured in the presence of adult $CD62L^+$ memory $CD4^+$ T cells, most of the neonatal B cells produced IgM in each experiment (Table 3). Only small fractions were producing IgG, and IgA-producing cells constituted < 1% of total cells. No IgA2-producing cell was detectable in these cultures. In contrast, IgG, IgA and IgM were produced similarly in adult B cell cultures. One third of IgA-producing cells were positive for IgA2. We next compared IgA and IgA2 production by adult naive and memory B cells in a similar culture system. As shown in Table 4, nearly half of the IgA-producing cells were $IgA2^+$ in memory B cell cultures, whereas only 12% of cells were producing IgA2 in naive B cell cultures.

DISCUSSION

As we have reported recently, neonatal or adult naive B cells can produce low, but significant levels of IgG and IgA when $CD62L^+$ memory $CD4^+$ T cells are added to PWM-stimulated cultures [15]. It has been also reported that neonatal B cells produce IgG and IgA upon stimulation with anti-CD40 and added cytokines [18]. These findings suggest that naive B cells are potentially capable of differentiating into IgG- and IgA-

Table 4. Frequency of IgA2-producing cells in cultures of $sIgD^+$ and $sIgD^-$ fractions of adult B cells

	IgA2-producing cells within IgA-producing cells (%)	
Experiment no.	$sIgD^-$	$sIgD^+$
1	33·7	5·5
2	48·5	19·7
3	44·5	12·1
Mean	42·2 ± 6·3	12·4 ± 5·8

$CD20^+$ B cells from adults were separated into $sIgD^+$ and $sIgD^-$ fractions and cultured with $CD62L^+$ memory $CD4^+$ T cells for 7 days in the presence of pokeweed mitogen (PWM). T cells were depleted from the cultured cells, and cytocentrifuged cell preparations were stained for cytoplasmic IgA and IgA2. Percentages of $IgA2^+$ cells among IgA-producing cells were calculated. At least 100 IgA-producing cells were calculated for each measurement.

producing plasma cells once required signals are provided appropriately.

We do not know, however, if this idea also holds true for IgG and IgA subclass production by neonatal B cells. Serum levels of IgG and IgA subclasses are known to be age-dependent [19,20]. IgG and IgA concentrations increase slowly with age. Among different IgG subclasses, IgG2 and IgG4 are produced much later than IgG1 and IgG3. IgA2 levels remain lower than IgA1 levels through different age groups, and cytoplasmic IgA2-positive cells are much fewer in frequency than IgA1-positive cells within the peripheral circulation [21–23]. The present study was undertaken to see if this particular pattern of serum immunoglobulin subclass levels is due to the functional immaturity of B cell itself, or to lack of appropriate stimuli, which can be overcome by certain *in vitro* culture conditions.

Low IgG and IgA concentrations during infancy are partly explained by the deficient helper functions of $CD4^+$ T cells *in vivo*. Namely, $CD4^+$ T cells from neonates and infants are predominantly $CD45RA^+$ naive T cells and produce few of the cytokines required for production of IgG and IgA [12,24]. Our culture system provided these signals by adding $CD62L^+$ memory $CD4^+$ T cells to PWM-stimulated cultures. They seem to produce significantly higher levels of IL-4 and IL-5 relative to IL-2, whereas the $CD62L^-$ subpopulation produces much higher levels of interferon-gamma (IFN-γ) (Kanegane *et al.*, submitted for publication). It is well known in the murine system that the addition of activated T cell membrane and appropriate combinations of these cytokines results in production of all IgG subclasses [2–4]. In particular, IgG2a and IgG2b production was efficiently induced by the addition of IL-4 and IL-5. It was expected from these studies that IgG2 and IgG4 production by naive B cells might be effectively induced in our culture system.

As shown in the results, IgG1 was the major subclass produced in neonatal B cell culture, and IgG3 was also produced at a significant level. However, IgG2 and IgG4 production was negligible, even when these cells were cultured with $CD62L^+$ memory $CD4^+$ T cells. Only few cells were IgA-positive within neonatal B cell cultures, and the IgA2-producing cell was undetectable among these cells. Importantly, adult naive B cells produced low but significant levels of IgG2 and IgG4 in some of the experiments. In addition, significant numbers of the IgA-producing cells were IgA2-positive in these cultures.

It could be argued that PWM stimulation resulted in skewed immunoglobulin responses, so that production of certain IgG subclasses was selectively induced, and the data presented here may not reflect the true nature of neonatal B lymphocyte functions. This is unlikely, because it has been repeatedly shown that the distribution of immunoglobulin classes and subclasses induced in PWM-stimulated cultures well reflects the levels of serum immunoglobulin concentrations [25–27]. In contrast, polysaccharide antigens, such as lipopolysaccharide (LPS), induced predominantly IgG2 and much less IgG1. Thus it is generally agreed that PWM provides a useful *in vitro* tool to evaluate the potential capacity of B cells to produce immunoglobulins.

It was recently shown that stimulation of $sIgD^+$ B cells with anti-CD40 and IL-10 resulted in the effective production of IgG1 and IgG3, but failed to induce IgG2 or IgG4 [28]. Neonatal B cells also produce significant levels of IgG1 and IgG3 when they are cultured with anti-CD3 activated T cells and cytokines [29]. Taken together, these findings suggest that neonatal B cells are inherently premature to produce IgG2 or IgG4. It is not clear why certain immunoglobulin subclasses are produced by naive B cells and other subclasses are not. One possible explanation can be provided by the fact that the genes for different immunoglobulin subclasses are aligned as duplicate tandem repeats in humans [30,31]. The first gene duplicate is composed of genes coding for IgG3, IgG1 and IgA1, and the second one is composed of those for IgG2, IgG4, IgE and IgA2. It is suggested that there exist distinct regulatory mechanisms for the class switches of the immunoglobulins from these different gene duplicates [32,33]. It is possible that additional stimulatory signals are required for the induction of the class switch to the immunoglobulins from the second gene duplicate. In this regard, it is intriguing that fetal B cells produced IgG4 and IgE in response to anti-CD40 and IL-4 [34]. Thus the addition of further ligands or cytokines might also elicit more IgG2 or IgA production by the neonatal naive B cell.

Another possibility is that there exist at least two distinct subpopulations within $sIgD^+$ B cells. Although it is generally agreed that $sIgD^+$ B cells are composed of naive B cells which produce little IgG or IgA upon stimulation [35,36], it is suggested that in the murine system adult $sIgD^+$ B cells are composed of heterogeneous populations of B cells, presumably of different maturation stages [37,38]. In this regard, it is noteworthy that significant numbers of IgA2-producing cells were detectable in the culture of adult naive B cells, whereas they were virtually undetectable in neonatal B cell cultures. In addition, much higher IgG2 and IgG4 concentrations were observed in adult naive B cell cultures than in neonatal B cell cultures.

It has been shown in the murine system that $sIgD^+$ cells from adults show preferential expression of certain V_H genes, indicating that they have already encountered environmental antigens and passed through a certain selection process *in vivo* [39,40]. It is also reported that $sIgD^+$ fraction from adult mouse contains antigen-specific memory B cells [41]. However, these antigen-specific $sIgD^+$ B cells are essentially of low affinity, and the affinity maturation seems to occur in parallel with the loss of IgD from the cell surface. These studies indicate that there exist, in addition to classical $sIgD^-$ memory B cells, a fraction of $sIgD^+$ B cells which have already gone through antigenic exposures and are ready to undergo class switch to each subclass of immunoglobulins, including IgG2, IgG4 and IgA2. Presumably, neonatal B cells are composed of naive B cells without an experience of antigenic exposure, whereas a large fraction of the adult $sIgD^+$ B cells are composed of antigen-primed B cells. Further study may delineate the different subpopulations of $sIgD^+$ B cells and shed light on the regulatory mechanisms of B cell class switching.

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