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This information is current as of December 5, 2009

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J. Immunol. 1998;161;3315-3324

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Neonatal Murine B Lymphocytes Respond to Polysaccharide Antigens in the Presence of IL-1 and IL-6¹

Ralph L. Chelvarajan, Nikole L. Gilbert,² and Subbarao Bondada³

Unlike adults, neonates are unable to respond to polysaccharide Ags, making them especially vulnerable to pathogenic encapsulated bacteria. Since the Ab response to polysaccharides in adult mice requires certain cytokines, it was hypothesized that neonatal murine B cells may be competent to respond to such Ags, but may fail to do so due to a deficiency of cytokines. Neonatal splenocyte cultures, which were otherwise unresponsive to trinitrophenyl (TNP)-Ficoll, a haptened polysaccharide Ag, mounted an adult-like Ab response when supplemented with IL-1. However, IL-1 failed to induce such a response to TNP-Ficoll when purified B cells were used instead. Although IL-6 alone did not induce a response in whole spleen cells or purified B cells from neonates, it synergized with IL-1 in inducing purified neonatal B cells to respond to TNP-Ficoll. The avidity of the cytokine-induced neonatal anti-TNP Abs was comparable to that of Abs made by adult splenocyte cultures. One effect of IL-1 may be at the level of clonal expansion, since it induced neonatal B cells to proliferate in response to anti-IgM, which was further enhanced by IL-6. The spontaneous secretion of IL-1 by neonatal splenocytes was below the detection limit, while adult splenocytes secreted 30.8 ± 5.2 U/ml, which is of the same order of magnitude as what was required to stimulate neonatal B cells to respond to TNP-Ficoll. Thus, the neonatal unresponsiveness to polysaccharide Ags could be due to the inability of a non-B cell population resident in the neonatal spleen to secrete sufficient quantities of IL-1. *The Journal of Immunology*, 1998, 161: 3315–3324.

Neonates from a number of species, including humans and mice, cannot mount a humoral immune response against encapsulated bacterial pathogens such as *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Hemophilus influenzae* (1–4). This is due to the inability of neonates to respond to capsular polysaccharides despite responding well to protein Ags. As such, after the loss of maternally transferred Abs to the capsular polysaccharides, neonates become susceptible to infections caused by these pathogens (5, 6). Similar to normal healthy neonates, B cells from adult CBA/N mice with an X-linked recessive immunodeficiency (Xid)⁴ due to a mutation in the protein tyrosine kinase, Btk, also fail to respond to polysaccharide Ags. This led to the classification of polysaccharides as thymus-independent type 2 (TI-2) Ags (7). The unresponsiveness of neonates and Xid mice to polysaccharide Ags has been well studied and has previously been shown to be due to intrinsic B cell defects (2, 3, 8, 9).

TI-2 Ags stimulate B cells without the requirement for MHC class II-restricted presentation to T cells and subsequent cognate T cell-B cell interactions (2, 10, 11). Nevertheless, the responses of B cells to this class of Ags is dependent on cytokines secreted by T cells and accessory cells (2, 12–16). When

TI-2 Ags are added to cultures of adult splenic cells, the Ag-specific B cells readily respond by secreting Abs to the immunizing Ag. However, when purified adult B cells are used instead of total splenocytes, the B cells respond to TI-2 Ags only when cultures are provided with certain cytokines. In agreement with the multiple cellular sources of cytokines, purified splenic B cells from adult mice overcome their unresponsiveness in vitro to TI-2 Ags when stimulated with cytokines such as IL-1, -2, -4, -5, and -6 and IFN- γ (13–16). These cytokines are required for a number of steps in the B cell response to Ags, including clonal expansion, differentiation into Ab-secreting cells, the level of Ab secretion, and isotype switching (2, 3, 17–22).

Recent studies have emphasized the predominance of the Th2 type of cytokine profile in neonatal immune responses. Thus, both in the neonatal transplantation tolerance model and in the immune responses to Cas-Br-M murine leukemia virus, Th2 cytokines are primarily produced in neonates (23). Despite this, neonates are deficient in TI-2 Ag responses, which have been shown to be enhanced by Th2 cytokines such as IL-4, -5, and -6 (13–15, 24–26), suggesting that another cell type or cytokine critical for activation of such Th2 cells or TI-2 Ag-specific B cells is deficient in neonates. Since numerous previous studies have shown TI-2 Ag responses to be accessory cell dependent (26–29), it is conceivable that neonates have a deficiency in such a cell type or in cytokines produced by these cells. In this context, similar to neonatal mice, peripheral lymph node cells from normal healthy adult mice do not respond either to the haptened TI-2 Ag, TNP-Ficoll, or to the pneumococcal polysaccharide-containing Pnu-Imune vaccine (26, 29). Moreover, aged mice and humans are hyporesponsive to TI-2 Ags, increasing their susceptibility to infections with capsulated bacteria. Extensive analyses of these models in our laboratory showed that the unresponsiveness of both young adult murine lymph node cells and aged mouse spleen cells to TI-2 Ags is due to a defect in an accessory cell population, which can be compensated by the addition of young mouse splenic accessory cells or IL-1, an accessory cell-derived cytokine (22, 25, 26, 29). In vivo, this defect is overcome by the injection of accessory cell-activating

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Received for publication March 5, 1998. Accepted for publication June 1, 1998.

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¹ This work was supported by Grants AG05731 and AI21490 (to S.B.) from the National Institutes of Health, and by a Howard Hughes Undergraduate Research Fellowship (to N.L.C.).

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⁴ Abbreviations used in this paper: Xid, X-linked immunodeficiency; TI-2, thymus-independent type 2; TNP, trinitrophenyl; HSA, heat-stable Ag; AFC, antibody-forming cell; PFC, plaque-forming cell.

agents, such as monophosphoryl lipid A (30). Even Xid B cells can be stimulated to respond to TNP-Ficoll when cultures are provided with synovial fluid from arthritis patients (31), which is an enriched source of a number of cytokines, including the accessory cell-derived IL-1 and IL-6 (32).

Despite the immune-compromised status of neonates with regard to capsular bacterial infections, neonatal B cells are not completely unresponsive to polysaccharides. Mosier (33) established that neonatal B cells do indeed proliferate *in vivo* in response to TNP-Ficoll and could subsequently be induced to secrete TNP-specific Abs upon stimulation *in vitro* with LPS. Furthermore, certain polysaccharide Ags upon conjugation to a protein carrier, become immunogenic in neonates, a strategy that has been used very effectively in designing vaccines against certain diseases caused by encapsulated bacteria, such as *H. influenzae* (5, 34, 35). Both these studies imply that TI-2 Ag-specific B cells are present in neonates but are unable to respond to these Ags. Since adult B cells require cytokines to respond to polysaccharide Ags, the lack of responsiveness in neonates to these Ags could be due to a cytokine deficit.

In this study we have measured the Ab-secreting cell responses to TNP-Ficoll, a prototype polysaccharide TI-2 Ag, after supplementing neonatal splenocyte cultures with a panel of accessory and T cell-derived cytokines shown previously to modulate B cell function. Our study demonstrates that neonatal B cells, when provided with the right mixture of cytokines, such as the accessory cell-derived IL-1 and IL-6, respond to polysaccharide Ags *in vitro* by secreting Abs of appropriate avidity against the immunizing Ag. We also show that although adult splenocyte cultures respond to TNP-Ficoll without the addition of exogenous cytokines, once purified, the adult B cell Ab response to TNP-Ficoll is modulated by cytokines in a remarkably similar fashion to that of purified neonatal B cells.

Materials and Methods

Mice

Pathogen-free, 2- to 3-mo-old BALB/c mice were purchased from the National Institute of Aging (Bethesda, MD), while 6-wk-old BALB/c C57BL/6 and mice were purchased from the National Cancer Institute (Frederick, MD). All mice were housed in microisolator cages in our American Association for Laboratory Animal Accreditation and Certification-approved rodent facility. Neonatal BALB/c (10–12 days old, unless otherwise stated) were bred in our rodent facility.

Cytokines and Ags

TNP-Ficoll was prepared from the aminoethyl carbamylmethyl derivative of Ficoll as described previously (36) and was a gift from John Inman, National Institutes of Health (Bethesda, MD). The F(ab')₂ goat anti-IgM heavy chain was purchased from Organon Technika (Aurora, OH). To obtain D10 T cell supernatant, 10 × 10⁶ D10.G4.1 cells (C. A. Janeway, Yale University, New Haven, CT) were cultured with irradiated splenocytes (2000 rad) from one C57BL/6 mouse in 10 ml of RPMI 1640 nutrient medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT) at 37°C in 5% CO₂. On the fourth day, D10 supernatant was collected and stored at -20°C until needed. For the IL-1 bioassay, the activated D10 cells were depleted of dead cells and then cultured in RPMI in the presence of supernatant (10%, v/v) derived from Con A-stimulated rat splenocytes. The cells were ready for use 7 days after antigenic stimulation. Human rIL-1β, which was functionally interchangeable with murine IL-1 (37), was obtained through the Biologic Response Modifiers Program of the National Cancer Institute (Bethesda, MD). Mouse rIL-4 and -6 were purchased from Genzyme (Cambridge, MA). All three cytokines were certified to be free of endotoxin, and the tissue culture components were routinely tested for endotoxin using the *Limulus* amoebocyte lysate kit (BioWhittaker, Walkersville, MD). IL-10 was a gift from Dr. Jay Fine (Schering-Plough Research Institute, Kenilworth, NJ).

Isolation of splenocytes and B cells

Lymphocytes were isolated from spleens of unimmunized mice and were processed according to protocols described previously (26, 29, 30). Briefly,

adhering fat and connective tissue were removed from spleens, and lymphocytes were dispersed by pressing the spleens against the bottom of a tissue culture dish containing HBSS (Life Technologies) using the flat surface of a syringe plunger. Cells were left undisturbed for 1 min to let the splenic debris settle and then were collected and washed by centrifuging for 5 min at 400 × g. RBCs were depleted with Gey's solution according to a previously described protocol (38).

B cells were enriched by panning with goat anti-IgM immobilized on tissue culture-treated dishes using a modification of the protocol reported by Mage (39). Briefly, plastic adherent cells were removed by incubating 120 × 10⁶ splenocytes in 13 ml of Iscove's modified Dulbecco's medium supplemented with 5% newborn bovine serum on a 150 × 25-mm polystyrene tissue culture dish (Corning Glass Works, Corning, NY) for 1.5 h at 37°C. The nonadherent cells were then transferred to a dish that had previously been precoated with a mixture of 10 μg/ml anti-IgM and 90 μg/ml goat IgG (Sigma, St. Louis, MO) and were incubated at 4°C. After 70 min, the unbound cells were removed, and the dish was gently rinsed with HBSS. The bound cells were dislodged by vigorous vortexing of the dish, and the cells were recovered after repeated flushing with HBSS. The purity of the cells was determined by staining aliquots with anti-IgM-FITC (Southern Biotechnology Associates, Birmingham, AL), anti-B220-phycoerythrin (clone RA3 6B2; Sigma), anti-Thy1.2-FITC, and anti-Mac-1-phycoerythrin (PharMingen, San Diego, CA). The cells were also occasionally stained with biotinylated Abs to CD5 (Becton Dickinson, San Jose, CA) and heat-stable Ag (HSA; CD24) (PharMingen). The cells were then fixed with 1% paraformaldehyde (Sigma) and analyzed on a FACS-Calibur flow cytometer (Becton Dickinson).

Cell culture

Lymphocytes were cultured in IF-12 tissue culture nutrient medium, that had previously been shown to support *in vitro* responses from B cells (29, 40). It consisted of a 1/1 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 nutrient mixture (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), insulin (Life Technologies), L-glutamine, 50 μM 2-ME (Sigma), transferrin (Sigma), progesterone (Pfaltz & Bauer), and gentamicin (Fujisawa, Deerfield, IL). For *in vitro* immunization, cultures contained 2.0 × 10⁶ total splenocytes or 0.8 × 10⁶ purified B cells/0.5 ml of IF-12 in 48-well tissue culture plates (Costar, Cambridge, MA). For proliferation assays, cultures contained 2.0 × 10⁵ cells/0.2 ml in 96-well tissue culture plates (Costar). Cultures were set up in triplicate and placed in a 5% CO₂ humidified incubator (Forma Scientific, Marietta, OH) maintained at 37°C.

The radioactive based assay to determine cell proliferation was performed as described previously (41, 42). Briefly, cultures were pulsed with 1 μCi of [³H]thymidine (New England Nuclear, Boston, MA) 2 days or, in the case of bioassays, 3 days after initiation of cultures, and 4 h later the cells were harvested onto filter mats (Skatron, Sterling, VA) using a cell harvester (Packard, Meriden, CT). The levels of tritium trapped in the mats was measured with a Matrix 96 beta particle counter (Packard).

Hemolytic plaque-forming cell (PFC) assay

The number of IgM anti-TNP-secreting cells was determined on the fourth day of culture using a glass slide version of the technique of localized hemolysis in a gel (43). Briefly, a 1-ml packed cell volume of SRBC (Colorado Serum, Denver, CO) was coupled with 2,4,6-trinitrobenzene sulfonic acid (Eastman Kodak, Rochester, NY) following published protocols (44). The cultured splenocytes were washed with HBSS and then mixed with 50 μl of 13.5% (v/v) TNP-coupled SRBC, 200 μl of 2× basal Eagle's medium (Life Technologies), and 200 μl of 1.6% agarose (FMC Bioproducts, Rockland, ME) and poured onto a glass microscope slide (Goldseal, Portsmouth, NH). The slides were incubated for 1 h at 37°C, and the plaques were developed during an additional 1-h incubation at 37°C with guinea pig complement (Pel-Freez Biologics, Rogers, AR). The plaques were viewed under a low power microscope. Ag-specific Ab-forming cells (AFC) were calculated by taking the number of plaques for any given treatment and subtracting the number of plaques obtained in the corresponding cultures incubated without Ag. Results are provided as the arithmetic mean ± SE, and the statistical significance of different treatments was evaluated by Student's *t* test (two tailed, unequal variance).

A comparative Ab avidity assay was employed to determine the avidity of Abs produced in neonates and adult culture total splenocyte cultures. Briefly, the PFC assay was performed in the presence of increasing concentrations of the monovalent TNP-lysine (Research Organics, Cleveland, OH) or the multivalent TNP-Ficoll, which resulted in reduced plaque formation. The results were plotted as the concentration of free hapten on the abscissa vs the percentage of maximum response on the ordinate axis. A

measure of the relative avidity of Abs secreted due to the different treatments was obtained by comparing the amounts of free hapten needed to obtain 50% inhibition, which was termed average avidity (45).

Apoptosis assay

The level of apoptosis was determined using the protocol of Reid et al. (46). Briefly, cells were stained with the DNA-binding viable dye, Hoechst 33342 (Molecular Probes, Eugene, OR), for 30 min at 37°C. The resulting level of fluorescence was an indicator of the amount of DNA per cell, thus determining the position of each cell in the cell cycle at the time of harvest (47). The cells were washed and stained with the naturally fluorescent lipophilic merocyanine 540 (Molecular Probes) for 20 min at room temperature. This dye selectively stained disordered or loosely packed membrane bilayers, and its level of fluorescence upon binding was a measure of the degree of packing of membrane lipids (48), which was an early characteristic of apoptosis (49). The cells were finally stained with anti-B220-FITC (Sigma), and cells positive for B220 and Hoechst were analyzed using a FACStar (Becton Dickinson) as described previously (50).

Determination of cytokine levels

Splenocytes or B cells were cultured for 1 or 2 days, and then the supernatants from replicate cultures were pooled and immediately frozen at -20°C. The bioassays were performed according to recently published methods (51, 52). Briefly, a twofold serial dilution of a culture supernatant or cytokine standard was conducted in a 96-well tissue culture plate in triplicate. For the IL-1 assay, each dilution was incubated with 4×10^4 activated D10 cells (see *Cytokines and Ags*) and 1.25 $\mu\text{g}/\text{ml}$ Con A. For the IL-6-dependent assay, each dilution was incubated with 2×10^3 B9 cells, and proliferation was determined 3 days later. The amount of cytokine in the supernatant was calculated by comparing the dilution of the culture supernatant required to achieve 50% of the maximum proliferation with that required for the cytokine standard (53). IL-1 was also estimated by ELISA at the Cytokine Core Laboratory (Baltimore, MD). The minimum detection limits were 1 U/ml (IL-6 bioassay), 3 U/ml (IL-1 bioassay), and 3 pg/ml (ELISA).

Results

IL-1 stimulated neonatal splenocytes to respond to TNP-Ficoll

The inability of neonates to respond to polysaccharide Ags was best exemplified when splenocytes from both adult (2- to 3-month-old) and neonatal (10- to 12-day-old) mice were cultured with TNP-Ficoll, a haptenated prototype TI-2 Ag, for 4 days. Using the PFC assay, a good anti-TNP response of 208 ± 64 AFC/culture was detected in adult cultures, which was about 20-fold higher than the neonatal response of 11 ± 9 AFC/culture in eight experiments ($p < 0.001$), one of which is represented in Figure 1A.

The fact that purified adult splenic B cells were dependent on cytokines to respond to TI-2 Ags (54) suggested that the unresponsiveness in neonates might be overcome in vitro by supplementing neonatal cultures with an appropriate mixture of cytokines. Initially, we supplemented neonatal splenocyte cultures with supernatant harvested from freshly activated D10 cells, a Th2 cell line that had previously been used successfully by Peçanha et al. (55) to elicit a TI-2 Ag response from purified adult splenic B cells. In the presence of D10 supernatant, neonatal splenocytes now responded to TNP-Ficoll at a level comparable to that of adult splenocytes (data not shown), implying that if neonatal B cells were provided with the right mixture of cytokines, they would overcome their intrinsic defect to mount an Ab response to TI-2 Ags.

To determine which cytokine or cytokines present in D10 supernatant could be responsible for stimulating neonatal B cells to respond to TNP-Ficoll, we substituted defined cytokines in place of D10 supernatant. Since D10 supernatant was collected immediately after stimulating the D10 cells by coculturing them with irradiated C57BL/6 splenocytes, this supernatant served as a good source of both Th2 cytokines secreted by the D10 cells (56) as well as accessory cell-derived cytokines from the irradiated C57BL/6 splenocytes. Thus, we initially screened a number of Th2 and ac-

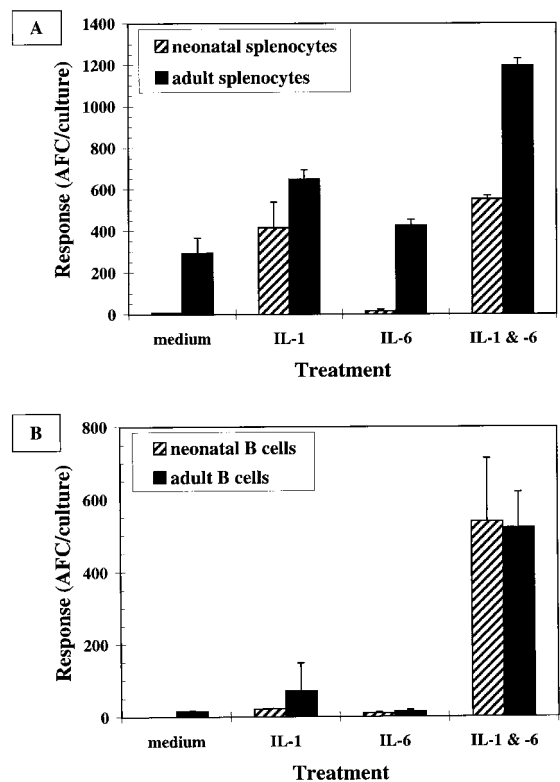


FIGURE 1. IL-1 enabled neonatal splenocytes to respond to TNP-Ficoll, but purified B cells also needed IL-6. Unseparated splenocytes (A) and purified B cells (B) from both age groups were stimulated with TNP-Ficoll in the presence of optimal concentrations of IL-1 and IL-6 (400 U/ml). The immune response was detected by a PFC assay on the fourth day of culture. The bars represent the mean of triplicate cultures \pm SE. These data are representative of three separate experiments showing similar results.

cessory cell-derived cytokines and finally some Th1 cytokines that were known to modulate B cell function, both alone and in combination. Individually, granulocyte-macrophage CSF and IL-2, -3, -4, and -10 did not stimulate neonatal spleen cells to respond to TNP-Ficoll (data not shown), whereas IL-1 alone consistently induced a robust response that was >50-fold higher than that of neonatal splenocyte cultures in the absence of IL-1 (hatched bars in Fig. 1A). In this experiment, IL-6 did not induce any response from neonatal splenocytes, but in one of three experiments, it had a modest effect (data not shown). IL-5, like IL-1, was also able to consistently overcome the neonatal unresponsiveness, but this is the subject of another manuscript.

Purified B cells only responded with a mixture of IL-1 and IL-6

Since the in vitro immunization with TNP-Ficoll was performed using total splenocytes, IL-1 might have overcome the unresponsiveness of neonatal B cells to TNP-Ficoll either by directly stimulating neonatal B cells, by activating other splenic cells to secrete cytokines that, in turn, activated the appropriate B cells, or both. To elucidate whether B cells were indeed directly responding to the exogenous IL-1, purified neonatal B cells would have to be cultured instead of total splenocytes.

First we characterized the lymphocyte population in neonates and adults by flow cytometry. As expected, 3-day-old mice had about 18% B cells, which increased with age (Table I). They were further characterized by HSA expression (57), which showed that the number of immature B cells ($\text{IgM}^+\text{HSA}^{\text{high}}$) was greater in neonates than in adults. The frequency of the HSA^{high} population

Table I. Cellular composition of murine splenocytes^a

Population	Adult	10 Day Old	3 Day Old
	% of B Cells		
CD5 ⁺ B cells	4 ± 1	7 ± 1	5 ± 1
Immature B cells	22 ± 3	53 ± 13	52 ± 12
Mature B cells	78 ± 3	47 ± 13	48 ± 1
	% of Splenocytes		
B cells	63 ± 1	34 ± 2	18 ± 3
T cells	31 ± 3	9 ± 0	3 ± 1
Mac-1 ⁺ cells	6 ± 0	18 ± 0	18 ± 0

^a RBC-depleted splenocyte preparations were subjected to flow cytometry. The numbers of B cells, macrophages, and T cell were determined by staining with IgM, Mac-1, and Thy1, respectively. B cells were further characterized for CD5 expression and immature (*i.e.*, IgM⁺HSA^{high}) and mature (*i.e.*, IgM⁺HSA^{low}) phenotypes.

was about the same in 3- and 10-day-old neonates. In general, the 10- to 12-day-old neonates had fewer B and T cells, but more Mac-1⁺ cells (Table I) and pre-B cells (15% of spleen cells as opposed to 2% in adults), with about 25% of the spleen cells not in any of these categories. Thus, any procedure employing negative selection would yield neonatal B cells contaminated with the uncharacterized IgM⁻ population, implying that IgM⁺ B cells would have to be enriched by positive selection. Consequently, we purified B cells by positively selecting IgM⁺ cells by panning with the F(ab')₂ fragments of goat anti-IgM immobilized on tissue culture-treated dishes, yielding B cells of 85 to 95% purity. Positive selection using anti-IgM has been previously employed to purify neonatal B cells without affecting B cell function (58–60).

Panned B cells from neonates and adults were then cultured with IL-1 and TNP-Ficoll. IL-1, which was very effective in stimulating neonatal splenocyte cultures to respond to TNP-Ficoll, did not stimulate purified B cells (Fig. 1B). However, this was not unique to neonates, since purified adult B cells also did not respond to TNP-Ficoll when cultured with just the Ag alone or with Ag plus IL-1. One possibility for the absence of an Ab response by purified B cells was that IL-1 alone was not sufficient, but that one or more cytokines secreted by the non-B cell population of the neonatal spleen were required to complement IL-1. To test this hypothesis, various combinations of Th2 and accessory cell-derived cytokines were added to cultures of purified B cells, TNP-Ficoll, and IL-1. Of the various cytokines examined, IL-6, which had no effect on purified B cells from both age groups, when added together with IL-1 enabled both neonatal and adult B cells to respond very effectively to TNP-Ficoll at a level comparable to that induced by IL-1 alone in total splenocyte cultures (Fig. 1). Interestingly, the response of purified neonatal B cells was as high as that of purified adult B cells and was about twice the response seen in adult splenocyte cultures in the absence of exogenous cytokines (Fig. 1, A and B). To confirm the hypothesis that the additional requirement for rIL-6 by purified neonatal B cells compared with that of the unseparated neonatal splenocytes was due to the elimination of an IL-6-producing cell but was not due to their exposure to anti-IgM during the panning procedure, we performed an add-back experiment. Thus, panned neonatal B cells cocultured with adherent cells from neonates needed only IL-1 to respond to TNP-Ficoll (0 ± 0, 1 ± 1, 8 ± 11, and 180 ± 36 AFCs/culture for panned neonatal B cells with medium, with adherent cells alone, with IL-1 alone, and with adherent cells plus IL-1, respectively), which is the same as the unpurified neonatal spleen cells.

Since IL-1 and -6 together stimulated a response to TNP-Ficoll from panned neonatal and adult B cells, this suggested that these two cytokines were directly acting on B cells to overcome their

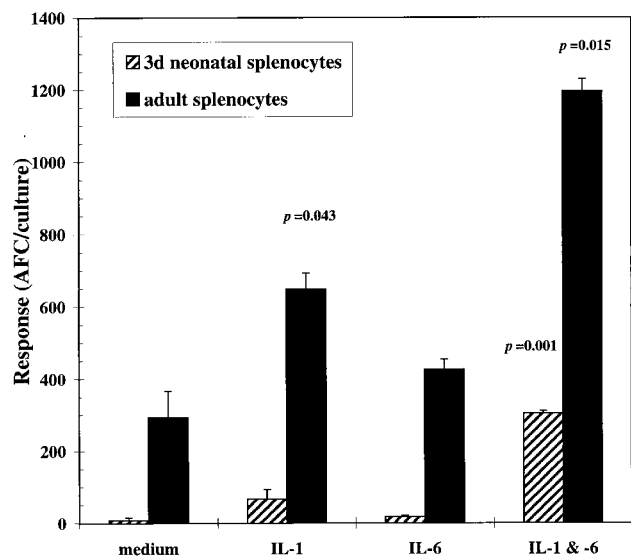


FIGURE 2. IL-1 and IL-6 were effective even in splenocyte cultures from neonates as young as 3 days. Total splenocytes from 3-day-old neonatal and adult mice were cultured with optimal concentrations of IL-1 and IL-6 (400 U/ml). The bars represent the mean of duplicate cultures ± SD. These data are representative of three separate experiments showing similar results.

unresponsiveness. To show conclusively that B cells were indeed the target of both cytokines, this experiment was repeated twice with 98% pure IgM⁺ adult B cells obtained by flow cytometry. In both instances, IL-1 and IL-6 together induced B cells to produce an Ab response against TNP-Ficoll (431 ± 124 AFC/culture with cytokines and 10 ± 3 AFC/culture without cytokines), implying that these cytokines were indeed acting directly on B cells, enabling them to overcome their unresponsiveness.

IL-1 and -6 together were effective even in 3-day-old neonates

Having established that IL-1 and -6 together could drive neonatal B cells to produce a good Ab response to TNP-Ficoll, we were curious to see how early in the ontogeny this combination of cytokines would work. Total splenocytes from 3-day-old murine neonates responded to TNP-Ficoll only when cultures were provided with Ag plus IL-1 (Fig. 2). The response was small and not significantly above background. Interestingly, the adult response increased over twofold due to IL-1 treatment ($p < 0.05$). However, if 3-day-old splenocytes were provided with both IL-1 and -6, an excellent Ab response to TNP-Ficoll was detected on the fourth day of culture ($p < 0.001$), and IL-6 also improved the adult IL-1-stimulated response ($p < 0.02$). The response of 3-day-old splenocytes was less than that of adults or even that of 10- to 12-day-old neonates, presumably because 3-day-old mice contain fewer B cells (Table I).

Similar thresholds for IL-1 and IL-6

Since the concentrations of IL-1 and IL-6 used up to this point were determined from titrations performed with only neonatal splenocyte cultures, such concentrations could possibly mask a lower threshold for one or both cytokines in purified adult B cell cultures. To determine whether there was a reduced threshold in adult B cells, panned B cells from both age groups were cultured with increasing concentrations of either IL-1 or IL-6. Since purified B cells would only respond in the presence of both cytokines, the second cytokine was maintained at its optimal concentration. A

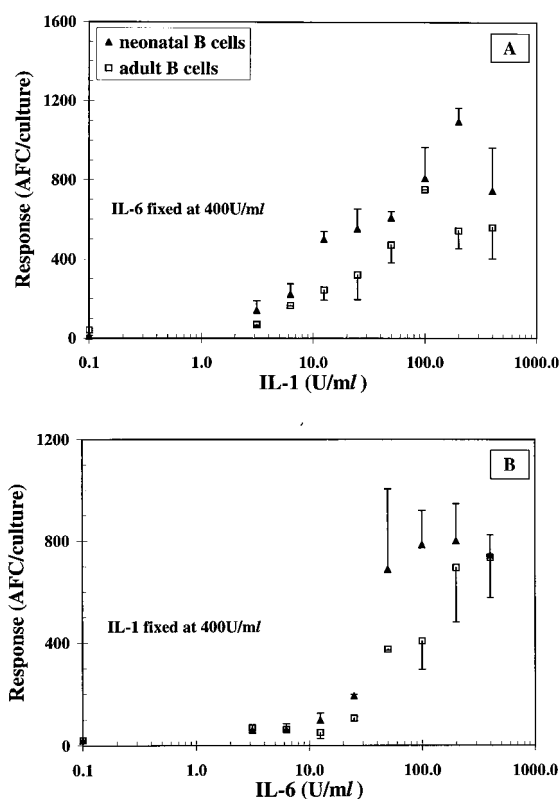


FIGURE 3. Neonatal and adult B cells responded similarly to titrations of IL-1 and IL-6. Purified B cells were cultured with TNP-Ficoll and varying concentrations of one cytokine while the other was maintained at its optimal dose. *A*, A titration of IL-1 while IL-6 was held constant at 400 U/ml. *B*, A titration of IL-6 while IL-1 was held constant at 400 U/ml. Data points represent the mean of triplicate cultures plus (neonatal) or minus (adult) the SE. Similar results were obtained in three separate experiments.

reduced threshold for either cytokine in adult B cells was not observed (Fig. 3), and surprisingly, for any given concentration of cytokine, the neonatal B cells tended to produce a higher anti-TNP response.

Neonatal and adult TNP Abs have comparable avidities

The avidity of Abs secreted against some Ags, including TNP-Ficoll, was reduced in aged mice (61, 62). To determine whether the cytokine-induced TNP Abs made by neonates were immunologically compromised, the avidities of the IL-1- and IL-6-induced TNP Abs secreted by neonatal and adult splenocyte cultures were compared. We could not use the Abs secreted by purified adult B cells as the benchmark for avidity, because these cultures also needed to be stimulated with the same pair of cytokines as the purified neonatal B cells. Hence, we used Abs secreted by adult total splenocyte cultures as the standard, to which we also added IL-1 and IL-6, to determine whether these cytokines modified the avidities of the secreted Abs. The relative avidities of the anti-TNP Abs produced by the PFC assay were determined by inhibiting plaque formation in the presence of varying concentrations of free hapten. Free hapten competes with the hapten coupled to the target SRBC for the plaque-producing Abs, thus reducing the incidence of plaque formation (63). The profiles of the inhibition curves of the Abs secreted by both adult and neonatal splenocyte cultures were very similar, suggesting that the avidities to the TNP determinant were comparable (Fig. 4) and indicating that this cytokine treatment enabled neonatal B cells to secrete immunologically effective Abs against a TI-2 Ag.

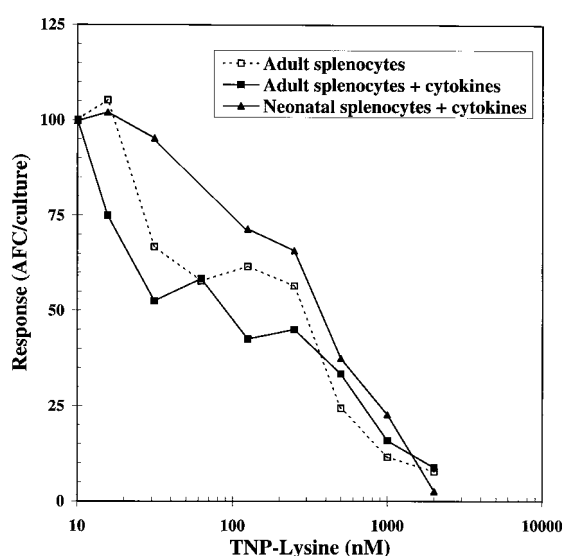


FIGURE 4. Neonatal and adult Abs to TNP have comparable avidities. Neonatal and adult splenocytes were immunized in vitro with TNP-Ficoll in the presence or the absence of a mixture of IL-1 (400 U/ml) and IL-6 (400 U/ml). During the PFC assay, TNP-lysine was added at increasing concentrations. These data are representative of two separate experiments showing similar results.

IL-1 and IL-6 required by the second day

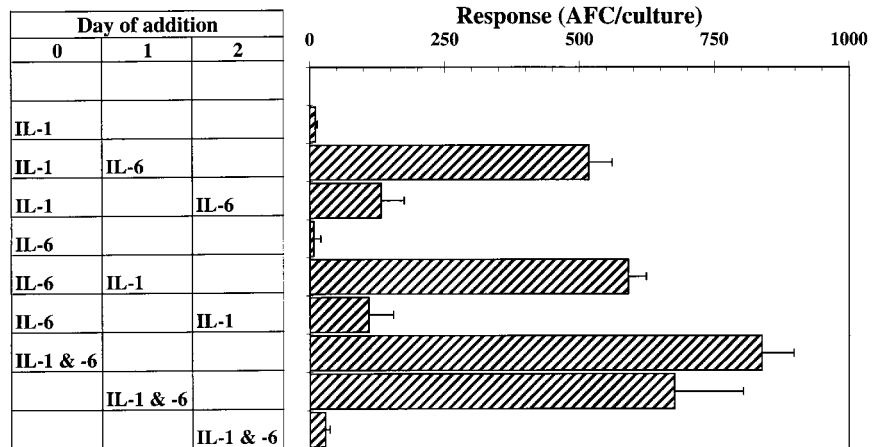
Since purified murine B cells needed both IL-1 and IL-6 to respond to TNP-Ficoll, this raised the interesting possibility that one cytokine may be required for the second to exert its function, such as up-regulation of the second cytokine's receptor. To address this issue, a number of B cell cultures containing TNP-Ficoll were established on day 0, and IL-1 and/or IL-6 were added either immediately or after 1 or 2 days. The best anti-TNP response was observed when both cytokines were added at the initiation of the 4-day culture (Fig. 5). If the addition of both cytokines was delayed by 1 day, there was a small, but insignificant, reduction in the PFC response, but a further 1-day delay completely abrogated the response. If either cytokine was added at the start of the culture, and the second cytokine was added a day later, the response was comparable to that to adding both cytokines on the first day of culture. However, when the addition of the second cytokine was delayed to day 2, the response was significantly reduced, but still higher than that when adding both cytokines on day 2. In summary, the best Ab response to TNP-Ficoll occurred if both cytokines were added within 1 day after initiation of culture, and the order of addition of cytokines was immaterial.

IL-1 and IL-6 induce anti-IgM-dependent proliferation in neonates

Since IL-1 was also known to amplify the proliferation of mature adult B cells in response to anti-IgM (37, 64), we were interested to determine whether IL-1 and IL-6 were acting by overcoming the inability of neonatal B cells to proliferate in response to TNP-Ficoll. Since TNP-Ficoll-induced B cell proliferation cannot be measured reliably due to the small number of TNP-specific B cell clones, we used anti-IgM instead, which had previously been established to be a polyclonal TI-2 Ag (65, 66). Immature B cells, including neonatal splenic B cells, unlike mature B cells, do not proliferate in response to anti-IgM unless cytokines such as IL-4 are provided (59).

Although IL-1 on its own did not induce B cell proliferation, when added together with anti-IgM, neonatal B cells responded at

FIGURE 5. IL-1 and IL-6 were required by the first day for maximal effect. Multiple cultures of purified B cells were set up on day 0, and TNP-Ficoll was added immediately. On days 0, 1, and 2, 400 U/ml IL-1 and IL-6 were added to different sets of cultures, and the PFC assay was performed on day 4. Although this figure only depicts an experiment performed with neonatal B cells, in three previous experiments cultures of purified adult B cells were also included, and purified B cells from both ages behaved very similarly. The bars represent the mean of triplicate cultures \pm SE.



a rate comparable to that of adult B cells stimulated with anti-IgM alone (Fig. 6). While IL-6 alone did not stimulate neonatal B cells to proliferate in response to anti-IgM, it did augment adult B cell proliferation. When both cytokines were added together, this resulted in an additive effect in adult B cell proliferation and a good synergy in neonatal B cell proliferation in response to anti-IgM, bringing the neonatal B cell response close to that of adult B cells. The combination of IL-1 and IL-6 was more effective than IL-4 alone or IL-4 with IL-1 or IL-6 (Fig. 6). Interestingly, there was no cooperative effect between IL-6 and IL-4, a cytokine whose effects on both neonatal and adult B cell proliferation were very similar to those of IL-1, nor did IL-4 influence the IL-1-induced or IL-1- plus

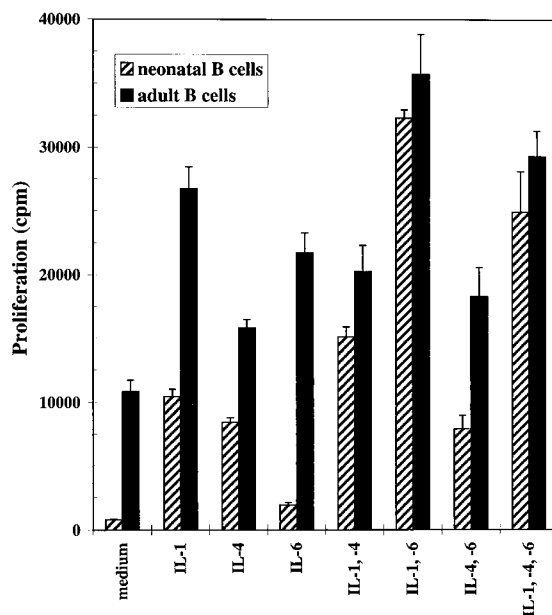


FIGURE 6. Neonatal B cells proliferate in response to anti-IgM when cultured with cytokines. Purified B cells ($250 \times 10^3/200 \mu\text{l}$) were cultured for 48 h with or without F(ab')_2 goat anti- μ ($50 \mu\text{g/ml}$) in the presence of 400 U/ml of IL-1 and IL-6 or 50 U/ml of IL-4. Cultures were pulsed with $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine 4 h before harvesting. Proliferation due to the cytokine in the absence of anti-IgM was considered the background and was subtracted from proliferation in the presence of anti-IgM and the corresponding cytokine. Depending on the treatment, the proliferation in the absence of anti-IgM was <1500 cpm (one or no cytokines added), 600 to 2200 cpm (two cytokines added together), or 600 to 4300 cpm (all three cytokines added together). The bars represent the mean of triplicate cultures \pm SE. Results are representative of three experiments.

IL-6-induced proliferation in response to anti-IgM, suggesting a special relationship between IL-1 and IL-6. Preliminary results from whole splenocyte cultures from adult Xid mice, which did not respond to anti-IgM (40, 65), indicated that just as with neonatal BALB/c B cells, IL-1 induced proliferation of Xid B cells in response to anti-IgM, and IL-6 enhanced the effect of IL-1 (R. L. Chelvarajan, H.-J. Wu, and S. Bondada, manuscript in preparation).

IL-1 improves viability of B cells in vitro

Neonatal lymphocytes have been reported to have a higher rate of spontaneous apoptosis in vitro (67). Friedlander et al. (68) demonstrated that if IL-1 was provided exogenously to HeLa cells in culture before an apoptotic stimulus, it had the ability to stave off apoptosis. It was thus possible that IL-1 improved the survival of neonatal B cells in culture, thus increasing the numbers of B cells that could respond to anti-IgM or TNP-Ficoll. To test this hypothesis, splenocytes were cultured in the presence or the absence of IL-1, and an apoptosis assay was performed immediately after harvesting the cells. After about 2 days in culture, the proportion of B220^+ cells undergoing spontaneous apoptosis in the unstimulated neonatal cultures was slightly higher than that in adult splenocyte cultures (52 ± 5 vs $39 \pm 10\%$; data not shown). If cultures were provided with IL-1 at the initiation of culture, this reduced the level of apoptosis in both neonatal and adult B220^+ cells to $39 \pm 4\%$ ($p = 0.057$) and $29 \pm 7\%$ ($p = 0.335$), respectively (data not shown). Addition of the IL-1 and IL-6 together did not improve the level of survival afforded by either cytokine on its own (data not shown). Thus, the reduction of spontaneous apoptosis might play some role in the ability of IL-1 to overcome neonatal B cell unresponsiveness in vitro.

Neonatal splenocyte cultures do secrete IL-6, but not IL-1

Since neonatal splenocyte cultures responded to TNP-Ficoll when supplied with IL-1, while purified B cells needed IL-1 and IL-6, could a deficiency of IL-1 and/or IL-6 account for the unresponsiveness of neonatal B cells to polysaccharide Ags? Both neonatal and adult splenic cells spontaneously secreted IL-6 (Fig. 7A). The level of IL-6 in neonatal cultures was about 4 times higher than that in adult cultures. Since macrophages were known to readily take up TNP-Ficoll among other carbohydrates (69, 70), cultures were also incubated with TNP-Ficoll to determine whether this polysaccharide would activate macrophages, leading to a boost in IL-6 production. However, this treatment did not increase the level of IL-6 secreted (Fig. 7A). Even though B cells have been shown previously to secrete IL-6 (71), in our hands, supernatants from

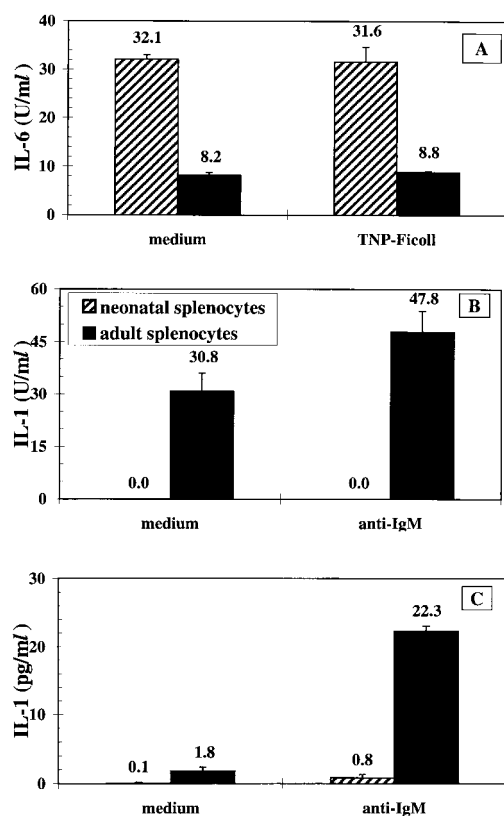


FIGURE 7. Neonatal splenocytes secrete IL-6 but not IL-1. Supernatants were collected after 2 days from splenocyte cultures stimulated with anti-IgM (50 μ g/ml) or TNP-Ficoll (16 ng/ml). Samples were analyzed using bioassays for IL-6 (B-9 cell growth response; A) and IL-1 (D10 cell growth response; B). IL-1 was also determined by ELISA (C). Note that the concentrations of IL-1 are depicted in units per milliliter in B and in picograms per milliliter in C. Results are representative of three experiments for A and B and two experiments for C.

cultures of purified B cells (with or without TNP-Ficoll) from either age group contained undetectable levels of IL-6 (data not shown).

The level of IL-1 present in neonatal and adult splenocyte 1-day cultures was too low to be estimated accurately (data not shown). However, by the second day of culture, a significant level of spontaneous IL-1 secretion was detected in adult splenocyte cultures, while the concentration in neonatal cultures was below the detection limit (Fig. 7B). It had previously been shown that anti-IgM stimulated IL-1 secretion by mature adult B cells (72). Adult, but not neonatal, splenic cells produced slightly higher levels of IL-1 upon treatment with anti-IgM. To verify these findings, IL-1 was also measured by ELISA (Fig. 7C). The results from the ELISA confirmed the trend seen previously that adult spleen cell cultures spontaneously secreted more IL-1 than neonatal spleen cells and this level could be increased by culturing cells with anti-IgM (Fig. 7C). It is important to note that 0.1 pg/ml IL-1 in neonates is at the level of sensitivity of the assay. Since the standard used to calibrate the bioassay (Fig. 7B) was human rIL-1 and not murine native IL-1, we could not convert the IL-1 results from its functional concentration (i.e., units per milliliter) to its protein concentration (i.e., picograms per milliliter). Nevertheless, the trends portrayed by both techniques were similar, in that adult splenocytes secrete more IL-1 than neonates, and this could be up-regulated by anti-IgM only in adults. The fold increase in IL-1 secretion due to anti-IgM stimulation was much higher in Figure 7C than in Figure

7B. This discrepancy might be due to a variation between the two assays or the different supernatants used for the two assays. Despite this, in the presence of anti-IgM, adult splenocytes secrete considerably more IL-1 than neonatal splenocytes.

Discussion

The unresponsiveness of neonates to TI-2 Ags has been well characterized using both polyclonal stimuli, such as anti-Ig, and specific Ags, such as phosphocholine, pneumococcal polysaccharides, and TNP-Ficoll. Specific defects intrinsic to neonatal B cells in terms of expression of surface Ig, CD21 and CD23; increased susceptibility to tolerance; and inability to activate specific signaling cascades via the Ig receptor have been identified (73). Despite these deficiencies, the immune competence of neonatal B cells is shown by their ability to respond to T-dependent forms of TI-2 Ags (e.g., conjugated to proteins) as well as to direct stimulation with CD40L and a mixture of cytokines (5, 34, 35, 60). However, no previous studies have reconstituted neonatal responses to TI-2 Ags in the absence of T cell-derived signals provided by Ag-primed T cells, CD40L, or T cell-derived cytokines. In this report we demonstrate for the first time that neonatal spleen cells can be induced to mount an adult-like response to TNP-Ficoll and anti-IgM when supplemented with a single cytokine, IL-1. Upon purification, the neonatal B cells require both IL-1 and IL-6 to mount a good immune response. Furthermore, the purified adult B cells behave like neonatal B cells in requiring both IL-1 and IL-6. The quantity and the quality of the Ab response in the presence of the cytokines are similar in the neonate and the adult, suggesting that any intrinsic difference between neonatal and adult B cells can be overcome by this cytokine mixture. The primary reason for the neonatal unresponsiveness appears to lie in their reduced production of IL-1 compared to that by adult spleen cells.

The additional requirement for IL-6 by purified B cells from either age group is in agreement with previous observations of synergy between these two cytokines in inducing the adult B cell response to dextran sulfate (74). Our ability to detect IL-6 production in cultures of whole spleen cells but not of purified B cells explains the need for addition of IL-6 for purified B cells to respond to TNP-Ficoll. Our observation that neonatal splenocytes secrete more IL-6 than those from adults contrasts with the report by Schibler et al. (75) that human neonatal monocytes are defective in IL-6 secretion. The authors considered neonatal monocytes to be defective, not because they failed to make any IL-6 at all but because they made about half as much as adults. The authors, in fact, showed that upon stimulation, human neonatal monocytes are quite competent in up-regulating the secretion of IL-6. Although many transformed B cells have been shown to produce IL-6 (76) and IL-6 mRNA is detected in anti-Ig-stimulated B cells (77), its production by TNP-Ficoll-activated B cells must be insufficient to induce a PFC response. The non-B cell populations in the neonatal spleen are at least as efficient as those in the adult (if not better) in producing sufficient IL-6 in whole spleen cultures, such that additional supplementation is unnecessary.

Unlike IL-6, IL-1 production is deficient in neonatal splenocytes. Previous studies with B cells from young mouse adult lymph nodes and aged mouse spleens suggest that the source of IL-1 is a non-B cell, most likely an accessory cell, such as macrophages and/or dendritic cells (26, 29, 78). These B cells also fail to respond to the TI-2 stimuli unless supplemented with splenic accessory cells or IL-1 (26, 29). The level of IL-1 is low despite the presence of a significantly larger macrophage population (Mac-1⁺ and F4/80⁺) in neonatal spleens vs adult spleens (Table I and data

not shown), which suggests that these accessory cells may be deficient in IL-1 secretion, and such a deficiency has been reported in accessory cells of aged mice (79). Dendritic cells can also produce IL-1, but have been shown to be functionally immature in neonates (78, 80), which could contribute to the defective IL-1 production in the neonate. In support of this possibility, we were unable to detect IL-1 in neonatal splenocyte cultures stimulated with TNP-Ficoll (data not shown) or anti-IgM, which can activate most B cells. Before this report, only one other publication reported IL-1 production by normal B cells (72), although that study was performed with human B cells. However, the levels of IL-1 produced in an Ag-specific response do not appear sufficient to drive the differentiation of purified B cells from adults.

Although the causes of the lack of a neonatal response to TI-2 Ags have not been characterized in detail, one early study showed that neonatal injection of TNP-Ficoll induces clonal expansion of TNP-specific B cells, which could be subsequently induced to differentiate into Ab-secreting cells with mitogens such as LPS (33). Similarly, we have shown that lymph node B cell unresponsiveness may be due to a defect in differentiation, since *in vivo* priming with TNP-Ficoll expands TNP-specific B cell precursors without Ig secretion (22). The ability of accessory cell-derived cytokines to induce neonatal B cell responses to TNP-Ficoll is consistent with these observations. It also explains the finding that another macrophage activator, monophosphoryl lipid A, can overcome neonatal unresponsiveness to type III pneumococcal polysaccharide (81).

In contrast to these studies that point to a defect in the differentiation step of B cells, experimental models that use anti-Ig have found neonatal B cells to be defective at the clonal expansion step (82, 83). Although neonatal B cells enter into early G₁ phase, they fail to progress into late G₁ and S phases upon B cell receptor cross-linking. In these systems, neonatal B cells are defective in B cell receptor-mediated signal transduction compared with adult B cells. Thus, neonatal B cells are defective in the production of inositol 1,4,5-triphosphate; expression of the protein tyrosine kinases, Fgr and Fyn; induction of the immediate early gene *c-myc*; as well as induction of cdk2, cdk4, and cyclin E proteins (84–86).

IL-1 has been implicated in the initial activation of B cells (87) and, later, in promoting Ag-induced clonal expansion (64, 88). This is in agreement with our finding that IL-1 was required early, during the first 1 to 2 days of culture. What is surprising is that IL-6, a cytokine that acts directly on preactivated B cells (89) to induce the final maturation of B cells into Ab-producing cells (90, 91), is required during the same time frame as the early acting IL-1. Maybe IL-1 hastens the activation and clonal expansion of the TNP-Ficoll-stimulated B cells, such that the differentiation occurs 1 day earlier. If these clonally expanded cells are not stimulated with IL-6 immediately, they probably become unresponsive and undergo apoptosis. Alternatively, IL-6 could be another B cell growth factor. There is ample evidence that IL-6 supports the growth of transformed murine and human B cells (92, 93). Moreover, Vink et al. (74) noticed that IL-6 enhances the IL-1 plus anti-IgM-induced proliferation in adult murine splenic B cells, while Mizutani et al. (94) showed that IL-6 enhanced the IL-1 plus Con A-induced proliferation of the D10.G4 T cell line. Since neither these studies nor ours noticed significant proliferation induced by IL-6 in the absence of IL-1, it appears that IL-6 only amplifies the growth response delivered by IL-1. Further evidence, albeit circumstantial, for the early requirement for IL-6 is provided by Freeman et al., who reported that, upon anti-IgM stimulation, adult splenic B cells rapidly expressed the IL-6 gene, with the peak of expression occurring about 4 h after stimulation (77).

Neonatal splenocytes have a greater tendency to undergo spontaneous apoptosis *in vitro* (data not shown) (67), and this might

play a major role in inhibiting the response to TI-2 Ags. In both cultured primary cortical neurons (95) and in a murine fibrosarcoma cell line (96), pretreatment with IL-1 significantly improved cell viability upon stimulation with an apoptotic signal. The expression of *egr-1* is also found to be deficient in immature B cells from the bone marrow (97). Induction of the *egr-1* gene may be a key event, since our recent studies with an immature B cell lymphoma cell line showed that *egr-1* expression is causally related to the apoptotic vs the growth response induced by anti-IgM (98). In agreement with these models we are also unable to demonstrate the proliferation of neonatal B cells upon treatment with anti-IgM Abs unless they are supplemented with IL-1. Thus, the accessory cell-derived cytokines can completely overcome the proliferation defect in neonatal B cells. It is conceivable that IL-1 promotes neonatal B cell resistance to apoptosis by facilitating *egr-1* expression.

In our study, IL-1 reduces the proportion of B220⁺ cells undergoing apoptosis, but this reduction is rather small compared with the level of Ab response to TNP-Ficoll encountered in the presence of IL-1. Therefore, the antiapoptotic activity of IL-1 might play a minor role in overcoming neonatal unresponsiveness to TI-2 Ags. The major role may be in overcoming the block in the proliferation step, as outlined above, although our IL-4 data show that this alone is not sufficient.

The Th2 cytokine, IL-4, also induces anti-IgM-stimulated neonatal B cells to proliferate to a level comparable to that induced by IL-1, but, unlike IL-1, IL-4 does not induce neonatal splenocytes to differentiate into anti-TNP-secreting cells. When IL-4 was added in combination with IL-1 (neonatal splenocytes) or with IL-1 and IL-6 (purified neonatal B cells), it frequently inhibits the differentiation response to TNP-Ficoll (data not shown). In fact, Alderson et al. (99), using a single cell study, showed that although IL-4 induces TI-2 Ag-dependent proliferation of single B cells, it is a poor agent for differentiation. Interestingly, they too noticed that IL-4 inhibited IL-1-induced Ag-dependent differentiation of B cells into Ig-secreting cells induced by IL-1. In our hands, the level of inhibition caused by IL-4 varies from experiment to experiment, and this phenomenon is being further investigated.

Recently, Snapper et al. (60) were able to induce adult-like levels of IgM secretion upon stimulation of purified neonatal murine B cells with anti-IgD-dextran, a prototype TI-2 Ag, in the presence of IL-4, IL-5, and CD40L (i.e., CD154). The ability of neonatal B cells to respond to anti-IgD-dextran in the presence of a plethora of T cell-derived stimuli is not surprising, since it is known that neonatal B cells can respond to protein-polysaccharide conjugates that can activate T cell help (34, 35). Our system is unique in that unseparated spleen cells need only IL-1 to respond to TNP-Ficoll, while just one other cytokine, IL-6, is required to activate purified B cells, and no T cell signal, such as CD40L, is required. Since many agents can augment IL-1 or IL-6 production from a variety of cell types in the body (100), it may be much easier to devise a means to induce neonatal responses to polysaccharide Ags with nontoxic IL-1- and/or IL-6-elevating adjuvants. This has a very important clinical significance, since it had been difficult to extend the success of *H. influenzae*-diphtheria toxin conjugate to the pneumococcal polysaccharide system due to the large number of serotypes of pneumococcal bacteria that vary in their capsular polysaccharide structures (2).

In summary, we have shown that immature neonatal B cells, long considered to be intrinsically incapable of mounting an immune response to TI-2 Ags, differentiated into Ig-secreting cells at a level comparable to that of adult B cells, and secreted TNP Abs of appropriate avidity when stimulated with TNP-Ficoll in the presence of IL-1 and IL-6. The defect in neonates might be due to

the inability of accessory cells in the neonatal spleen to secrete sufficient quantities of IL-1.

Acknowledgments

We thank E. C. Snow, G. Bikah, C. Venkataraman, A. Simmons, and G. Baumann for critical review of manuscript, and D. A. Robertson for excellent technical assistance.

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