

# ONTOGENY OF MOUSE LYMPHOCYTE FUNCTION

## II. Development of the Ability to Produce Antibody is Modulated by T Lymphocytes

BY D. E. MOSIER AND B. M. JOHNSON

*(From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014)*

Spleen cells from neonatal mice stimulated *in vitro* with traditional antigens fail to respond by producing antibody. This failure of the immune response to occur is puzzling since spleen cells from mice 2 or 3 wk old possess normal adult numbers of  $\theta$ -bearing T lymphocytes (1, 2) and immunoglobulin-bearing B lymphocytes (1, 3).

Stimulation of neonatal spleen cells with a B-cell mitogen, bacterial lipopolysaccharide, partially restores their ability to form antibody (4), suggesting that neonatal B cells are capable (under some circumstances) of differentiating into antibody-forming cells. Neonatal T cells are functionally mature in terms of their ability to respond to mitogens or histocompatibility antigens by proliferation or generation of cytotoxic lymphocytes (5, 6, unpublished observations of R. Tigelaar and D. Mosier). Antigen-processing in neonatal spleen cells may be deficient (7), but evidence on this point is contradictory (8). The ease of tolerance induction in the newborn represents another difference in antigen reactivity compared to the adult. The cellular basis of the altered reactivity of neonatal lymphoid cells to antigen thus could be due to differences in maturity or functional activity at the level of the macrophage, T lymphocyte and/or B lymphocyte.

In view of the importance of understanding the contribution of lymphoid cell subpopulations to the unresponsiveness of the newborn animal, we have characterized the ability of different cell types from neonatal mice to participate in the antibody response of mouse spleen cells cultured *in vitro*.

A recently described (9) T-independent, adherent cell-independent antigen,  $\epsilon$ -dinitrophenyl-lysine-Ficoll (DNP-Ficoll), was used to evaluate the functional maturity of splenic B lymphocytes, while various cell fractionation procedures were applied to the analysis of the more complicated T-dependent, adherent cell-dependent response to SRBC or trinitrophenyl-substituted sheep red blood cells (TNP-SRBC).

The *in vitro* antibody response to DNP-Ficoll appears earlier in ontogeny than the response to SRBC or TNP-SRBC. T lymphocytes in neonatal spleen, however, were found to have a suppressive effect on antibody synthesis by B cells

of any age. Removal of neonatal splenic T cells by anti- $\theta$  and complement treatment or by nylon wool filtration increased the response to DNP-Ficoll and allowed the successful reconstitution of the SRBC or TNP-SRBC response by adult lymph node T cells. In the mouse spleen, therefore, B cells seem to mature by 1–2 wk of age, but the ability to produce antibody is limited by an excess of T “suppressor” cells. This imbalance of T-cell functions may explain the ease of tolerance induction and the difficulty in producing antibody in the neonatal animal.

## Materials and Methods

**Animals.** BALB/c or C57BL/6N mice were obtained from the NIH Animal Production Section. Term pregnant females were separated into single cages and litters maintained separately until 4 wk of age when the mice were weaned and separated according to sex. Animals of both sexes were used in all experiments.

**Cell Culture.** Spleen cell suspensions were prepared from pooled spleens of several mice of the same age and cultured according to a modification of the Mishell-Dutton technique (10) as previously described (9). Plaque-forming cells (PFC) were assayed after 3, 4, or 5 days of culture by a slide modification of the Jerne hemolytic plaque assay (11). Indirect (IgG) PFC were enumerated as previously described (9), using anti- $\mu$  serum to inhibit direct (IgM) PFC. TNP-coupled indicator erythrocytes were prepared according to the method of Rittenberg and Pratt (12). Assays were performed on triplicate cultures and the results are expressed as the geometric mean  $\pm$  standard error of the mean. Significance was assessed by Student's *t* test.

**Antigens.** Preparation of DNP-Ficoll has been described recently (9). DNP<sub>33</sub>Ficoll (32 mol DNP/400,000 average mol wt of Ficoll) was used for these experiments. TNP-SRBC were prepared by the method of Rittenberg and Pratt (12) to yield heavily-substituted erythrocytes.

**Cell Fractionation.**  $\theta$ -bearing spleen cells were deleted by treatment with AKR anti- $\theta$  C<sub>3</sub>H serum (prepared by Dr. C. A. Janeway, Jr.) and guinea pig complement. Deoxyribonuclease was added during complement-mediated lysis at 10  $\mu$ g/ml to prevent clumping of dead cells.

Spleen cells also were fractionated on a nylon wool column using the procedure of Julius (13) as modified by Handberger and Schwartz (14). The efficacy of this procedure was checked by staining recovered cells with fluorescein-conjugated anti- $\kappa$  serum. Column-passed cells averaged 3%  $\kappa$ -positive cells (T-cell-enriched) and the column-retained fraction averaged 82%  $\kappa$ -positive cells (B-cell enriched). The unfractionated cells had 35–45% labeled cells.

**Steroid Treatment.** Adult BALB/c mice were injected with 2.5 mg hydrocortisone acetate intraperitoneally. 48 h later, the thymuses were removed, mediastinal lymph nodes (which had been marked with colloidal carbon) carefully separated from the capsule, and cell suspensions prepared.

## Results

**Ontogeny of the In Vitro Antibody Response to “T-Dependent” and “T-Independent” Antigens.** The in vitro PFC response to DNP-Ficoll has been shown to be independent of T lymphocytes in adult (9) and neonatal (Mosier, unpublished experiments) mice. The response to SRBC or TNP-SRBC is quite dependent upon the presence of T lymphocytes (15). The ability of spleen cells from BALB/c or C57BL/6N mice varying in age from 1–8 wk to respond to either DNP<sub>32</sub>Ficoll or SRBC is shown in Fig. 1. Preliminary experiments had shown the culture conditions and time course of the two in vitro responses to be similar. Mouse spleen cells clearly are able to generate a substantial response to DNP-Ficoll earlier in ontogeny than they can respond to T-dependent SRBC. If

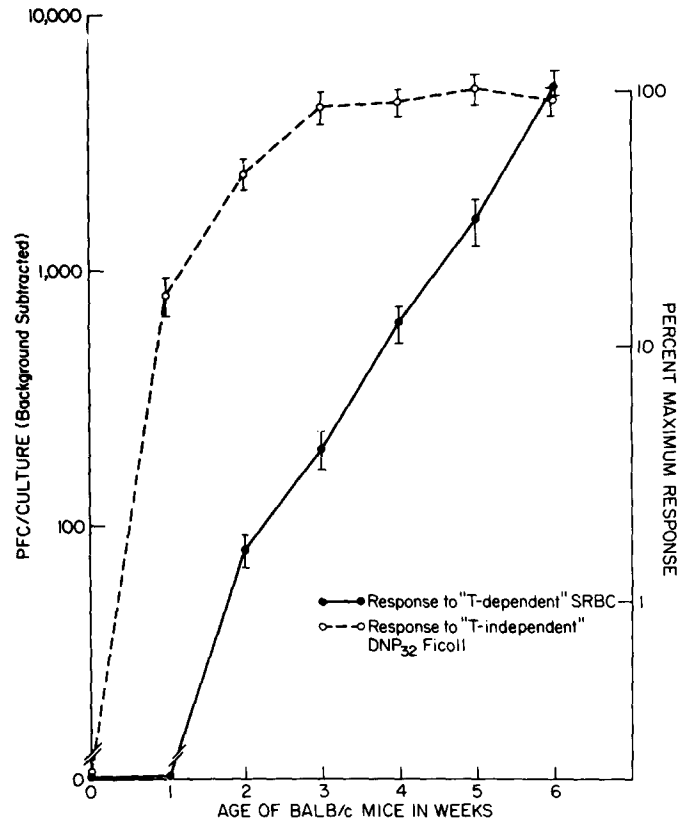


FIG. 1. Immune responses in vitro of BALB/c spleen cells from mice of varying ages stimulated with the thymus-dependent antigen SRBC or thymus-independent DNP-Ficoll. Results are expressed as mean  $\pm$  SE IgM plaque-forming cells of three replicate cultures in one representative experiment. Five replicate experiments gave similar results.

TNP-SRBC were used as antigen instead of SRBC and just the hapten-specific primary response measured, cells from young mice again failed to respond, but the age of onset of responsiveness was less clear because of the low magnitude and higher "background" of in vitro responses with this antigen.

*Which Cell Type is Functionally Immature in the Neonatal Spleen?* Our preliminary interpretation of these findings was that B cells mature earlier in mice than either T lymphocytes or adherent cells and thus the response to DNP-Ficoll, which appears to require only B cells, appears before the response to an antigen requiring all three cell types.

The cellular basis for the inability of neonatal BALB/c spleen cells to produce an in vitro response to SRBC was examined in more detail. That adherent cells from 2-wk old mice were functionally mature was shown by mixing them with adult nonadherent cells and showing a full reconstitution of the primary IgM response to SRBC in vitro. Table I shows the results of one of three replicate experiments. No functional defect is apparent in neonatal adherent cells. Our attention was therefore directed to the functional maturity of neonatal splenic T lymphocytes.

TABLE I  
*Neonatal Spleen Adherent Cells Support the In Vitro Immune  
 Response of Adult Nonadherent Cells*

Age of spleen donors		IgM PFC/culture vs. SRBC§
Adherent Cells*	Nonadherent cells‡	
8 wk	8 wk	1,880 ± 160
8 wk	2 wk	60 ± 15
—	8 wk	120 ± 40
—	2 wk	30 ± 10
2 wk	8 wk	1,830 ± 220
2 wk	2 wk	40 ± 15

\* Adherent spleen cells prepared by incubating normal spleen 1 h, decanting nonadherent cells, washing loosely adherent cells vigorously, and retaining only cells firmly adherent to plastic.

‡ Nonadherent spleen cells prepared by incubating normal spleen cells three times in plastic culture dishes, each time discarding adherent cells.

§  $2 \times 10^6$  SRBC was added to cultures after adherent and nonadherent cells combined.

|| Numbers are the mean PFC response of triplicate cultures in one representative experiment assayed against SRBC after 4 days of culture.

The failure of neonatal spleen cells to respond to SRBC could be due to an absence of T-cell function, immaturity of T-dependent subset of B cells, or an inappropriate environment for effective T-B-cell collaboration. In an attempt to evaluate these possibilities, an excess of adult T cells was added to 2-wk old spleen cells. 1 million lymph node cells or steroid-resistant thymocytes previously had been shown (16) to fully reconstitute the SRBC response of 10 million anti- $\theta$ -treated adult spleen cells. Accordingly, 1 million adult peripheral lymph node cells or steroid-resistant thymocytes were added to 10 million neonatal spleen cells, with the results shown in Table II. No reconstitution of the SRBC response was seen. If neonatal spleen cells and lymph node cells were mixed in equal numbers ( $5 \times 10^6 + 5 \times 10^6$ ), partial reconstitution of the SRBC response was seen (Exp. 2, Table II). It appears that neonatal spleen cells are refractory to reconstitution by adult T cells, suggesting either immaturity of B-cell cooperative function or an interfering neonatal T-cell influence.

In order to carefully evaluate the maturity of neonatal B cells potentially capable of responding to SRBC, it was required to first treat neonatal or adult spleen cells with anti- $\theta$  serum and complement to deplete intrinsic T-cell activity. Small numbers of adult peripheral lymph node cells were mixed with a fixed number of anti- $\theta$ -treated spleen cells and the response to SRBC, and DNP-Ficoll compared, as shown in Table III. Adult lymph node cells clearly are capable of reconstituting the SRBC response in the neonatal spleen, but only if preexisting T cells have been removed by anti- $\theta$  treatment (e.g., groups 2 and 4, Table III). Thus the response to SRBC is much increased when adult peripheral lymph node cells are added to anti- $\theta$ -treated spleen cells from 2-wk old mice. In addition, anti- $\theta$  treatment alone led to a small increase in the antibody response

TABLE II

*Failure of Adult T Cells to Reconstitute the SRBC Response of Neonatal Spleen Cells*

Source of cell populations:		4 day IgM PFC/culture*	
Spleen cells	Adult T cells	vs. SRBC	vs. DNP-Ficoll
Exp. 1 2-wk old BALB/c ( $10 \times 10^6$ )‡	—	20 ± 5	895 ± 40
“	$1 \times 10^6$ peripheral lymph node cells	10 ± 2.5	1,350 ± 95
“	$1 \times 10^6$ steroid-resistant thymocytes	20 ± 4	1,070 ± 80
8-wk old BALB/c ( $10 \times 10^6$ )	—	2,220 ± 180	2,385 ± 225
“	$1 \times 10^6$ peripheral lymph node cells	3,420 ± 260	3,475 ± 420
“	$1 \times 10^6$ steroid-resistant thymocytes	3,125 ± 195	3,250 ± 230
Exp. 2 2-wk old BALB/c§ ( $10 \times 10^6$ )	—	10 ± 5	950 ± 45
“	$1 \times 10^6$ peripheral lymph node cells	20 ± 5	1,030 ± 80
“	$5 \times 10^6$ peripheral lymph node cells	500 ± 25	1,295 ± 135
2-wk old BALB/c ( $5 \times 10^6$ )	$5 \times 10^6$ peripheral lymph node cells	395 ± 15	850 ± 50
None	$5 \times 10^6$ peripheral lymph node cells	20 ± 5	30 ± 10
8-wk old BALB/c ( $10 \times 10^6$ )	—	5,420 ± 360	3,620 ± 180
“	$1 \times 10^6$ peripheral lymph node cells	6,280 ± 545	4,000 ± 320
“	$5 \times 10^6$ peripheral lymph node cells	4,150 ± 220	3,465 ± 285

\* PFC were measured on the fourth day of culture against SRBC or TNP-substituted SRBC (for the response to DNP-Ficoll). Numbers are the geometric mean ± SE of triplicate cultures.

‡ Numbers of cells per culture. Changing the number of spleen cells in control cultures from  $10$  to  $11 \times 10^6$  per culture had no significant effect on the PFC response.

§ Adding  $5 \times 10^6$  peripheral lymph node cells to adult spleen cells resulted in modest reduction of the responses to both SRBC and DNP-Ficoll.

TABLE III

*Reconstitution of Neonatal Spleen Response to SRBC with Adult Cells Requires Prior Depletion of Neonatal T Cells*

Source of cell populations			Day 4 IgM PFC/culture*			
Group	Spleen ( $10 \times 10^6$ )	Lymph node‡ ( $1 \times 10^6$ )	Vs. SRBC		Vs. DNP-Ficoll	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
1	2-wk old normal	—	20	365	1,295	3,065
2	2-wk old normal	6-wk old	60	300	1,350	3,240
3	2-wk old anti- $\theta$ -treated§	—	0	0	1,500	4,945
4	2-wk old anti- $\theta$ -treated	6-wk old	625	1,100	830	4,200
5	6-wk old normal	—	2,200	2,205	2,385	6,045
6	6-wk old normal	6-wk old	3,420	2,425	3,475	6,805
7	6-wk old anti- $\theta$ -treated	—	315	175	1,845	6,215
8	6-wk old anti- $\theta$ -treated	6-wk old	2,115	1,595	2,730	6,985

\* PFC assayed on the fourth day of culture against SRBC or TNP-substituted SRBC. Numbers are mean PFC for three replicate cultures in the separate experiments.

‡ Peripheral lymph node cells from 6-wk old BALB/c mice.

§ Spleen cells were depleted of  $\theta$ -bearing cells by treatment with undiluted anti- $\theta$  C3H serum followed by 1:10 guinea pig complement. Approximately 40% of both 2-wk old and 6-wk old spleen cells were eliminated by such treatment.

against DNP-Ficoll in cultures of neonatal spleen, while little effect of anti- $\theta$  treatment was noted in adult spleen cells stimulated with DNP-Ficoll.

These observations gave rise to the concept that at least part of the inability of neonatal spleen cells to respond to SRBC *in vitro* might be due to excess of T lymphocytes which both interfered with helper function (*viz.*, the reconstitutive effect of adult lymph node cells for the SRBC response) and negatively influenced the response of B cells (to DNP-Ficoll).

*Are Neonatal T Cells Suppressive for Antibody Production in the Adult?* If neonatal unresponsiveness or hyporesponsiveness is actively mediated by suppressor cells as opposed to being a passive result of cellular immaturity, then mixing neonatal and adult spleen cells should result in a suppression of the responsiveness of the latter. The results of such an experiment are shown in Fig. 2

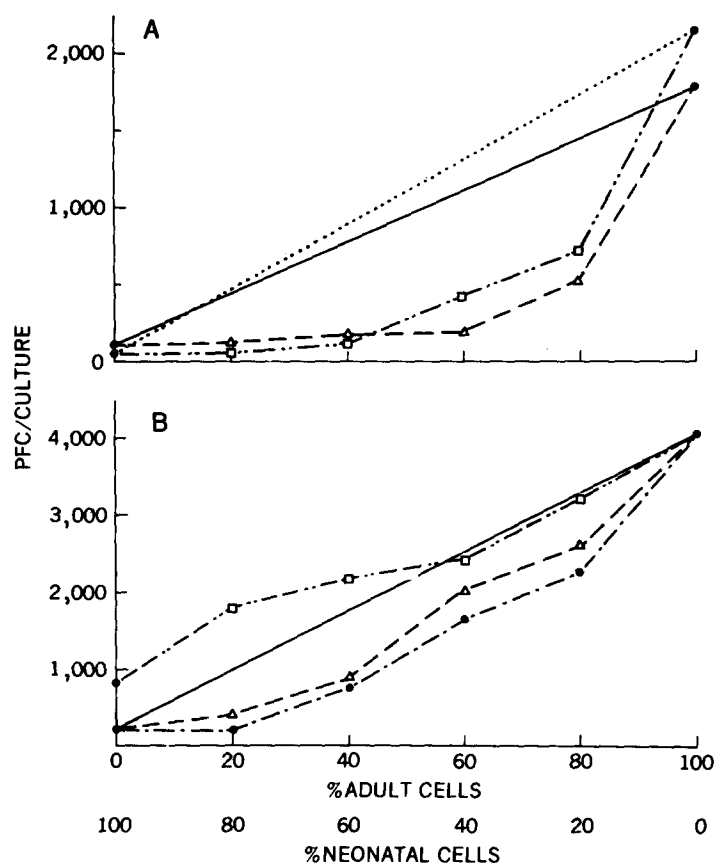


FIG. 2. Immune response of mixtures of neonatal and adult BALB/c spleen cells to SRBC (A) or DNP-Ficoll (A and B). The ratio of 2-wk old cells to adult cells was varied with the total number of cells in culture maintained constant at  $10 \times 10^6/\text{ml}$ . The expected lines represent the anticipated response if the two cell populations fail to interact either synergistically or antagonistically. (A) Expected response to SRBC (●---●); actual response to SRBC (□---□); expected response to DNP-Ficoll, (●---●); actual response to DNP-Ficoll (Δ---Δ). (B), expected response to DNP-Ficoll (●---●); mixture of normal neonatal spleen and adult spleen, (Δ---Δ); mixture of neonatal T cells and adult spleen, (●---●); mixture of neonatal B cells and adult spleen (□---□).

A. Spleen cells from 2-wk old and 8-wk old BALB/c mice were mixed in varying ratios with the total number of cells per culture kept constant at  $10 \times 10^6$  per ml. Cultures were stimulated with SRBC or DNP-Ficoll and PFC responses measured after 4 days. The expected result indicated represents the number of PFC expected if the two ages of spleen cells fail to interact either synergistically or antagonistically (e.g., if 2-wk old spleen cells produce 1,000 anti-DNP PFC/culture and 8-wk old cells 3,000 PFC/culture, then a 50:50 mix would be expected to give 2,000 PFC/culture). This calculation is straightforward in the case of the response to DNP-Ficoll, where the evidence suggests only one cell type is required, but involves assuming nonlimiting numbers of T cells and adherent cells in the case of the response to SRBC, so the main weight of the argument is based on the alteration of the response to DNP-Ficoll.

The data shows that small numbers of neonatal spleen cells significantly ( $P < 0.01$ ) depress the response of adult spleen cells to both DNP-Ficoll and SRBC. That this depression of the PFC response of adult spleen cells by neonatal cells was due to neonatal T lymphocytes was shown in two ways. Neonatal BALB/c spleen cells were depleted of T cells either by anti- $\theta$  and complement treatment or by passage over a nylon wool column (14). Cells passing through the nylon wool column are enriched for T cells (e.g. 4% Ig<sup>+</sup>), while cells mechanically eluted from the column are enriched for B cells (e.g. 76% Ig<sup>+</sup>). The results of mixing either T-cell enriched or T-cell depleted neonatal spleen cell populations with normal adult spleen cells in varying ratios are shown in Fig. 2 B. Increasing the number of neonatal T lymphocytes increased suppression, while decreasing their number abrogated suppression. The same results, i.e. reversal of suppression, were seen with anti- $\theta$  and complement treatment of neonatal spleen cells. Neonatal spleen thus seems to contain a T-lymphocyte population which somehow exerts a negative influence on the antigen-induced differentiation of B lymphocytes, be they from newborn or adult spleen. The suppressive population in neonatal spleen is not plastic-adherent since depleting adherent cells failed to reverse suppression, viz. neonatal nonadherent spleen cells mixed with normal adult spleen cells still reduced the anti-DNP PFC response.

*Neonatal Thymocytes also Suppress PFC Formation by Adult Spleen Cells.* Since the T lymphocytes which exert a suppressive influence on antibody formation in the neonatal mouse are presumed to be recently immigrated from the thymus, it was of interest to determine what effect small numbers of neonatal thymocytes added to a constant large number of adult spleen cells would have on antibody formation in vitro. The results shown in Fig. 3 show an age-dependent suppressive effect on the ability of BALB/c spleen cells to mount an anti-SRBC response, viz., the earlier the age of the mice from which thymocytes were obtained, the more profound was the suppression. In each case,  $1 \times 10^6$  thymocytes were added to  $10 \times 10^6$  adult spleen cells. For comparison,  $1 \times 10^6$  adult thymocytes caused an insignificant reduction in the PFC response compared to no thymocytes added ( $1,721 \pm 141$  PFC/culture vs.  $1,976 \pm 307$  PFC/culture). These findings confirm that T lymphocytes are the likely source for the suppressor cell found in neonatal spleen.

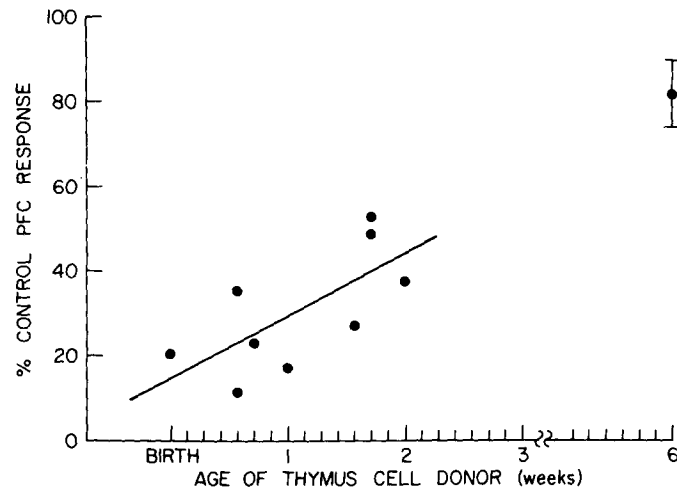


FIG. 3. Suppression of BALB/c 6-wk old spleen anti-SRBC PFC response by neonatal thymocytes. The response of adult BALB/c spleen cells ( $10 \times 10^6$ /ml) to SRBC in vitro as modified by the addition of  $1 \times 10^6$  BALB/c normal thymocytes of varying ages. The control of adding  $1 \times 10^6$  adult thymocytes resulted in a small but insignificant decrease in the IgM PFC response to SRBC. Each point represents the mean of three replicate cultures in one of three experiments.

## Discussion

These experiments show that at least two of the three cell populations involved in the in vitro immune response are functionally mature by about 2 wk of age in the BALB/c mouse. Thus macrophage-rich adherent cells 2-wk old mouse spleen were able to reconstitute fully the adherent cell-dependent response to SRBC when added to adult T and B cells, as has been previously reported by Fidler et al. (8). 2-wk old mouse spleen cells also were able to respond quite well to the T-independent adherent cell-independent antigen, DNP-Ficoll, suggesting that B cells are functionally mature by this age. This conclusion is in agreement with the recent finding of Spear and Edelman (4) that neonatal Swiss L strain mouse spleen cells could form antibody against SRBC if bacterial lipopolysaccharide was also added to the culture, suggesting functional maturity of B cells.

The functional activity of neonatal T lymphocytes seems, from these experiments, to be dominated by a suppressive influence exerted both on other T cells and on B cells. For example, adult T cells failed to cooperate with neonatal B cells to form antibody to SRBC unless neonatal T cells were first depleted. In addition, neonatal splenic T lymphocytes interfered with the ability of adult B cells to respond to DNP-Ficoll. In the presence of this dominant suppressive effect, the ability of neonatal T cells to perform a "helper" function is unknown.

On the basis of these findings in vitro, the failure of newborn animals to respond well to T-dependent antigens may be due not to an absence of antigen-reactive B cells but to an excess of suppressor T cells. The evidence that



neonatal T cells are responsible for the suppression observed in these experiments is fourfold: first, depletion of neonatal T cells by anti- $\theta$  serum and complement abrogated suppression; second, neonatal splenic cells enriched for T cells by passage over a nylon wool column showed enhanced suppression; third, neonatal spleen cells depleted of T cells had increased responses to DNP-Ficoll; and fourth, thymocytes from neonatal (but not adult) animals suppressed antibody formation *in vitro*.

The conclusion that neonatal B cells are nearing functional maturity by 2 wk of age is based upon two pieces of evidence. 1- or 2-wk old mouse spleen cells respond well to DNP-Ficoll, particularly if T cells have first been depleted, and they respond well to SRBC if neonatal T cells are replaced by adult lymph node cells. In the latter case, it is important to exclude the possibility that the  $10^6$  lymph node cells added to the culture of  $10^7$  neonatal spleen cells do not contribute the functional B cells. This possibility seems extremely remote since we have never observed even  $10^7$  lymph node cells to respond to SRBC under these culture conditions. Since lymphocytes bearing complement receptors are rare at 2 wk of age (e.g. 4% positive cells, reference 3), the finding that B cells can respond to T-dependent as well as T-independent antigens at this age suggest complement binding to B lymphocytes is not crucial in the response to T-dependent antigens. The recent evidence (17, 18) that C3 binding is involved in some T-dependent responses may be related more to C3 binding to macrophages than to direct C3 activation of lymphocytes.

Evidence is rapidly accumulating that T lymphocytes modulate the humoral immune response and possess the capacity both to augment and to suppress antibody formation (19, 20). Both thymus-dependent and thymus-independent responses (functionally defined by the amount of antibody formed in T-cell-deprived animals) seem to be subject to T-cell suppression (21). In neonatal mice, T-cell modulation of B-cell differentiation seems to err on the side of suppression. If T cells capable of augmenting antibody responses exist, their function is masked by the excess of suppressor cell activity. This imbalance suggests that augmenting or helper T lymphocytes may belong to a separate subpopulation of cells from suppressor cells, i.e., two distinct lines of differentiation for T cells may exist. Alternatively, one line of T cells may over express one function to the detriment of another during an intermediate stage of differentiation.

To the extent that suppressor T cells are involved in tolerance induction, the dominance of suppressor cells in the newborn may explain the relative ease of establishing tolerance. Whether this transient period of excess suppressor function is related to the initiation and maintenance of self-tolerance is unknown.

### Summary

The relative functional maturity of neonatal mouse spleen T- and B-cell populations was assessed by comparing the ability to respond to the thymic-independent antigen, DNP-Ficoll, or thymic-dependent SRBC by producing

antibody *in vitro*. Although mouse spleen cells responded to DNP-Ficoll at an earlier age than they responded to SRBC or TNP-SRBC, the reason for the lag in the T-dependent response was confounded by the finding of high numbers of suppressor T lymphocytes in the neonatal spleen. Thus, small numbers of neonatal spleen T cells or thymocytes significantly decreased the *in vitro* antibody response of adult spleen cells. Although B lymphocytes appear to be functionally mature soon after birth, their activity may be modulated by an excess of suppressor T cells; e.g., the reconstitution of helper cell function in the neonatal spleen required anti- $\theta$  treatment before addition of adult helper cells. Suppressing activity attributable to T cells seems to play a dominant role in determining the ability of the neonatal animal to react positively or negatively to antigenic stimulation.

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