ONTOGENETIC DEVELOPMENT OF B-LYMPHOCYTE
FUNCTION AND TOLERANCE SUSCEPTIBILITY IN VIVO
AND IN AN IN VITRO FETAL ORGAN CULTURE SYSTEM*

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The study of the development of B cells in the various lymphoid tissues in vivo is
complicated by factors relating to cell migration. An in vitro organ culture system
provides an opportunity for analyzing the differentiation of B lymphocytes in a closed
system without the complication of cell migration. Moreover, it is possible to manip-
ulate the experimental conditions in vitro in an attempt to alter the development of
B cells and learn more about the process. One such manipulation is the addition of a
tolerogen (e.g., a hapten protein conjugate) to the culture medium to study its effect
on the development of hapten specific B cells.

It has previously been shown that some in vitro systems can support the growth
and differentiation of fetal lymphoid tissues. Melchers et al. (1) have demonstrated
that single-cell suspensions of fetal liver cells will develop mitogen-reactive B cells in
culture. Owen et al. (2, 3) have also presented evidence for maturation of B cells with
fetal liver or fetal spleen organ cultures. Explants of fetal tissue taken before the in
vivo appearance of B lymphocytes that bear surface immunoglobulin (s-Ig)1 generated
s-Ig-positive B cells in culture. In addition, as early as 11–12 d of gestation a putative
precursor of the s-Ig-positive cell, termed a pre-B cell, was identified by immunoflu-
orescence techniques and shown to contain cytoplasmic IgM but to lack functional s-
Ig receptors (1, 3–5). Other investigators have demonstrated the maturation of B cells
in fetal tissues by measuring the development of antigen-binding cells (6–8). Thus,
the majority of studies with fetal tissues, particularly in vitro studies, have followed B-
cell differentiation by the acquisition of mitogen reactivity, the development of
antigen binding cells, or the acquisition of s-Ig. Although these markers do indeed
reflect the maturation of B cells, they do not address the most important aspect of the
B cell, namely the production of antibody to a specific antigen.

In the past it has been very difficult to study the specific responses of fetal lymphoid
cells or even neonatal cells because of the small number of B cells present for a

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ogy, La Jolla, Calif.
1 Abbreviations used in this paper: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's
medium; DNP, 2,4-dinitrophenol; FITC, fluorescein isothiocyanate; HGG, human gamma globulin; KLH,
keyhole limpet hemocyanin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; OVA, ovalbumin; s-Ig,
surface immunoglobulin; SPF, specific pathogen-free.
particular antigen and the lack of functionally mature cooperating cells, e.g., macrophages and T cells (9). This constraint has recently been removed through B-cell cloning assays, in particular, the in vitro splenic focus assay developed by Klinman and Press (10, 11) in which the adult splenic environment is provided complete with carrier-primed T cells, thus facilitating the measurement of B-cell precursors in fetal and neonatal tissues. It is, therefore, of great interest to combine the technique of organ culture with this sensitive B-cell cloning assay to study the differentiation of B cells in relation to immune reactivity.

In this paper we compare the normal in vivo development of B-cell precursors specific for the hapten determinant 2,4-dinitrophenol (DNP) with the development of DNP precursors that occurs in the organ culture system. The results indicate that both fetal spleen and fetal liver contain lymphocyte progenitor cells that will differentiate into B cells as measured by the acquisition of clonable DNP precursors as well as by the acquisition of s-Ig-positive cells.

Evidence is presented for both a quantitative difference in the frequency of DNP precursors before and after culturing, and for a qualitative difference in that some of the B-cell precursors that mature in vitro acquire the ability to make antibody of the IgG isotypes. Finally, the effect of the presence of the DNP determinant during the process of B-cell maturation was studied, the result being near total elimination of potential anti-DNP clones.

Materials and Methods

Animals and Immunizations. The animals used in these studies were specific pathogen-free (SPF), inbred CBA/CaH/WEHI mice that were bred and maintained at The Walter and Eliza Hall Institute of Medical Research (Victoria, Australia). Spleens and livers were obtained from fetuses of dated gestational age ranging from 12 to 19 d. The age of gestation was determined by vaginal plugging; day 0 of gestation being the day that a vaginal plug was detected.

SPF female mice of 6–8 wk of age were injected with 0.1 mg i.p. of keyhole limpet hemocyanin (KLH) in complete Freund’s adjuvant. These mice were used in the splenic focus assay as recipients for transferred cells 6–8 wk later.

Hapten-Protein Conjugates. DNP was conjugated to KLH, bovine serum albumin (BSA), ovalbumin (OVA), or human gamma globulin (HGG) as previously described (12). DNP-KLH contained ~10 mol DNP/100,000 daltons of KLH. DNP-BSA, DNP-OVA, and DNP-HGG contained ~10 mol DNP/mol protein. The hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was conjugated to HGG according to standard procedures (13) and contained 10 mol NIP/mol protein.

Organ Culture Technique and Tolerance Induction. Spleens and livers were dissected from fetuses and placed in organ culture in a manner similar to that previously described for fetal thymus lobes (14). Briefly, intact fetal spleen or small pieces of fetal liver were placed on the surface of strips of sterile Millipore filter (Millipore Corp., Bedford, Mass.) that rested on surgical gelatin rafts (Gelfoam, The Upjohn Co., Kalamazoo, Mich.) immersed in Dulbecco’s modified Eagle’s medium (DMEM) that contained 15% fetal calf serum. The level of medium was adjusted such that the Millipore filters were at the gas/medium interface and the tissue was in the gas phase.

The fetal tissues were incubated for various times in 10% CO2 and 90% air, and the medium was changed every 3rd d. At the end of 3–9 d in culture the fetal tissue was dispersed into single-cell suspensions, and an aliquot was removed for counting and determining the percentage of s-Ig-positive cells by direct immunofluorescence with a fluorescein isothiocyanate (FITC)-conjugated, polyvalent, rabbit anti-mouse immunoglobulin (14). The cultured cells were then tested for the number of DNP-specific B-cell precursors in the in vitro splenic focus assay.

Tolerance induction was studied by incubating the organ cultures in DMEM that contained the tolerogen DNP-OVA or DNP-HGG at 5 × 10−7 M DNP, for the duration of culture. Similar results were obtained when either carrier was used.
Cell Transfers, Splenic Focus Assay, and Tolerance Induction. Mice that had been primed with KLH were given 1,300 rad of total body irradiation 4-6 h before cell transfer. Each irradiated, carrier-primed recipient received 2-20 × 10⁶ viable, nucleated cells from organ cultures or from uncultured fetal spleens or livers that were dispersed into single-cell suspensions. The recipients were sacrificed ~14-16 h later, and the number of DNP-specific precursors was determined in an in vitro splenic focus assay (10, 11).

The degree of tolerance susceptibility of cultured fetal B lymphocytes was determined by inducing tolerance in the organ cultures as described above. The susceptibility of uncultured B lymphocytes to tolerance induction, on the other hand, was determined by a modification of the splenic focus assay outlined by Metcalf and Klinman (15). In short, fragment cultures derived from recipient spleens were incubated for 24 h in high glucose DMEM plus 10% agamma horse serum with or without the presence of the tolerogen DNP-HGG or DNP-OVA at 5 × 10⁻⁷ M hapten. No significant difference was found in the degree of tolerance obtained with either DNP-HGG or DNP-OVA.

The tolerogen was washed out 24 h later, and DMEM that contained the antigen DNP-KLH (5 × 10⁻⁷ M DNP) was added. Culture supernates were subsequently replaced on day 4 and changed every 2-3 d thereafter. Supernates were collected at approximately day 12 and analyzed for the presence of anti-DNP by radioimmunoassay.

Radioimmunoassay and Isotype Analysis. Culture supernates (20-50 µl) collected on day 12 after stimulation were then assayed for anti-DNP antibody. The immunoglobulin was detected by a solid phase radioimmunoassay (16) through the use of a mixture of purified ¹²⁵I-labeled sheep anti-mouse Fab and purified ¹²⁵I-labeled rabbit anti-λ (17). Those supernates that were positive for anti-DNP were reanalyzed for the isotype(s) present. This was accomplished with the same radioimmunoassay except that the following purified ¹²⁵I-labeled, rabbit anti-mouse heavy-chain-specific antibodies were used: (a) anti-IgM, (b) anti-IgG1, (c) anti-IgA, (d) anti-IgG2a, (e) anti-IgG2b, and (f) anti-IgG3. The radioimmunoassay as well as the purification of class-specific reagents have been described in detail (17).

Results

Acquisition of s-Ig-Positive Cells in Fetal Lymphoid Tissues In Vivo and In Vitro. The normal maturation of B lymphocytes during fetal development was studied by analyzing the percentage of s-Ig-positive lymphocytes in liver and spleen suspensions obtained at various stages of gestation. The percentage of s-Ig-positive cells was determined by fluorescence microscopy with a FITC-conjugated, polyvalent, rabbit anti-mouse immunoglobulin (14), and ~500-1,000 cells were counted for each estimate. The results are presented in Fig. 1. Day 17 is the first day in which s-Ig-positive cells are reproducibly detected in both spleen and liver, and approximately the same percentage of s-Ig-positive cells are found in both tissues at this age. At day 18, however, there is a greater percentage of s-Ig-positive cells in the spleen compared with the liver; this difference is even greater at days 19 and 20. The greatest increase in the percentage of s-Ig-positive cells in both spleen and liver occurs between days 18 and 19 of gestation when there is a greater than fivefold increase in s-Ig-positive cells.

The development of fetal B lymphocytes was also studied in vitro with an improved organ culture technique (14). Spleens and livers were removed from fetuses at various ages of gestation and incubated in organ culture for varying periods of time. At the end of the culture period the tissues were dispersed into single-cell suspensions and an aliquot of cells was tested for the percentage of s-Ig-positive lymphocytes by fluorescence microscopy (Tables I and II).

The data in Table I demonstrate that organ cultures of spleen support the in vitro development of s-Ig-positive lymphocytes. Spleens taken at 17 and 18 d of gestation showed the greatest increase in s-Ig-positive cells after 3 d in culture (10- to 12-fold
increase). Cultures of fetal spleen explants taken at 15 d of gestation, on the other hand, produced variable results, and most cultures resulted in no maturation of s-Ig-positive cells.

The development of s-Ig-positive lymphocytes in these cultures is not a result of selective survival but rather to a net increase in the number of s-Ig-positive cells. For example, there are ~10^6 nucleated cells in a 17-d fetal spleen, and after 3–5 d in culture 4–6 × 10^5 cells per spleen are recovered, or about one-half the original cell number. As a maximum this could account for a two-fold increase in B cells by selective survival. However, because there is an ~10-fold increase in s-Ig-positive cells during the culture period there must be a net increase in the number of s-Ig-positive cells. It is not clear whether this increase is a result of differentiation of existing progenitor cells or if cell proliferation also occurs. However, in histological sections of cultured fetal tissues many mitotic figures can be identified (data not show).

Cultures of fetal liver explants also support the development of s-Ig-positive cells (Table II). Liver explants taken at 14 and 15 d of gestation (before the appearance of s-Ig-positive cells) contain 1.5–2% s-Ig-positive cells after 3–5 d in culture, and liver explants taken at 17 and 18 d of gestation show a five- to sixfold increase in s-Ig-positive cells after 3–7 d in culture.
**Table I**

Maturation of s-Ig-Positive Lymphocytes In Vitro – Fetal Spleen*

| Age of fetal spleen preculture | Number of experiments | Percentage of s-Ig-positive cells preculture | Duration of culture | Number of experiments | Percentage of s-Ig-positive cells postculture |
|-------------------------------|-----------------------|--------------------------------------------|--------------------|----------------------|--------------------------------------------|
|                               |                       |                                            |                    |                      | Control culture | DNP-treated culture |
| d                             |                       |                                            |                    |                      | 0.3 ± 0.3 | 0.8 ± 0.2 |
| 15                            | 6                     | 0 ± 0                                      | 5                  | 3                    | 3.0          | 3.8            |
| 17                            | 6                     | 0.6 ± 0.2                                 | 3                  | 5                    | 7.9 ± 0.7 | 5.3 ± 0.6 |
| 18                            | 4                     | 1.0 ± 0.3                                 | 7                  | 2                    | 3.0          | 3.8            |
| 19                            | 7                     | 5.3 ± 0.8                                 | 3                  | 3                    | 12.5 ± 4.8 | 9.4 ± 3.8 |

* Spleens were removed from CBA/H fetuses at the indicated ages of gestation and a small proportion of the spleens were dispersed into single-cell suspensions and analyzed for the percentage s-Ig-positive cells by direct immunofluorescence with FITC-anti-Ig. The remainder of the spleens were used intact as explants and incubated in organ culture in the presence or absence of DNP-HGG or DNP-OVA (5 × 10⁻⁷ M DNP) for the specified periods of time. After incubation, the explants were dispersed into single-cell suspensions, and an aliquot was tested for the percentage of s-Ig-positive cells. Between 500 and 1,000 cells were counted for each estimate. The data are expressed as the mean ± SE of the indicated number of experiments.

**Table II**

Maturation of s-Ig-Positive Lymphocytes In Vitro – Fetal Liver*

| Age of fetal liver preculture | Number of experiments | Percentage of s-Ig-positive cells preculture | Duration of culture | Number of experiments | Percentage of s-Ig-positive cells postculture |
|-------------------------------|-----------------------|--------------------------------------------|--------------------|----------------------|--------------------------------------------|
|                               |                       |                                            |                    |                      | Control culture | DNP-treated culture |
| d                             |                       |                                            |                    |                      | 1.7 ± 0.8 | 2.0 ± 1.0 |
| 12                            | 3                     | 0 ± 0                                      | ND                  |                      | 0.8          | 1.5            |
| 14                            | 7                     | 0 ± 0                                      | 3                  | 3                    | 1.4 ± 0.1 | 1.9 ± 0.5 |
| 15                            | 8                     | 0 ± 0                                      | 5                  | 5                    | 1.4 ± 0.4 | 2.2 ± 0.5 |
| 17                            | 6                     | 0.4 ± 0.2                                 | 3                  | 5                    | 1.8          | 2.0            |
| 18                            | 3                     | 0.3 ± 0.1                                 | 3                  | 2                    | 2.5          | 2.0            |
| 19                            | 5                     | 1.8 ± 0.4                                 | 5                  | 3                    | 2.4 ± 0.6 | 1.9 ± 0.3 |
|                               |                       |                                            | 7                  | 2                    | 1.0          | 1.7            |

* Fetal livers were analyzed in the same manner as described in Table I. Data are expressed as the mean ± SE of the indicated number of experiments.
† Not done.

Because it was planned to study the tolerance susceptibility of these developing B lymphocytes in organ culture to the DNP determinant, it was important to determine if the tolerogens DNP-HGG or DNP-OVA (5 × 10⁻⁷ M DNP) affected the development of s-Ig-positive cells. The data in Tables I and II demonstrate that the incubation of either fetal spleen or liver explants in the presence of the DNP protein conjugates does not alter the percentage of s-Ig-positive cells obtained.

Determining the Optimal Length of Incubation Time for Organ Cultures. It was necessary to determine if there was an optimal length of time for culturing the explant tissues so as to obtain maximum numbers of newly developed B lymphocytes. Consequently,
spleens and livers were removed from fetuses at 17 d of gestation and placed in organ cultures for varying periods of time. The results, presented in Fig. 2, indicate that there is an increase in the percentage of s-Ig-positive cells with time in culture with a peak occurring between 3 and 5 d. After ~5 d in culture, however, there is a decrease in the percentage of s-Ig-positive cells. Consequently, fetal organ cultures were incubated for 3-5 d unless specified otherwise.

Acquisition of B-Lymphocyte Function during Normal Fetal Development: Degree of Tolerance Susceptibility. So that the normal development of B-lymphocyte function could be studied, populations of spleen and liver cells of various ages of gestation were tested for the frequency of clonable DNP precursors by using a splenic focus assay (10, 11). The frequency of clonable DNP precursors in spleen increases as a function of gestational age (Fig. 3, open bars). Few, if any, DNP precursors are detectable in 15-d fetal spleens, but thereafter the frequency of DNP clones detectable steadily increases at 17, 18, and 19 d of gestation, respectively. The ontogenetic development of B-lymphocyte function in fetal liver was also studied (Fig. 4, open bars). Similar to fetal spleen there is an increase in the frequency of DNP precursors with increasing age of gestation. B-cell precursors specific for DNP were not detectable until day 14 of gestation, and by day 19 the frequency had increased by more than threefold.

The percentage of fetal B-cell precursors that were susceptible to tolerance induction was determined by a modification of the splenic focus assay (15) in which spleen
Fig. 3. Acquisition of clonable DNP-specific precursors during normal fetal development—fetal spleen. Spleens were removed at the gestational ages indicated, dispersed into single-cell suspensions, and transferred to lethally irradiated, carrier-primed recipients for analysis in the splenic focus assay. About 2-15 × 10⁶ cells were injected per recipient, 3-4 mice per experimental group. Fragment cultures were incubated in the presence or absence of DNP-HGG or DNP-OVA (5 × 10⁻⁷ M DNP) for 24 h followed by 3-d stimulation with DNP-KLH (5 × 10⁻⁷ M DNP). Supernates were collected at day 12, and anti-DNP-secreting clones were detected by radioimmunoassay with ¹²⁵I-labeled anti-Fab. The data are expressed as the mean frequency of DNP clones detected from 2-7 experiments, and error bars are shown where three or more experiments were done. [] frequency obtained with control fragment cultures; , frequency obtained with DNP-HGG- or DNP-OVA-treated fragment cultures; ], frequency obtained in tolerance specificity control where fragment cultures were treated for 24 h with NIP-HGG followed by 3-d stimulation with DNP-KLH.

Fig. 4. Acquisition of clonable DNP-specific precursors during normal fetal development—fetal liver. Fetal livers were analyzed in the same manner as described in Fig. 3. Fragments were incubated with the tolerogen DNP-HGG or DNP-OVA (5 × 10⁻⁷ M DNP) for the 1st 24 h of culture. The tolerogen was then washed out, the antigen (DNP-KLH, 5 × 10⁻⁷ M DNP) added, and the assay continued as usual to determine the resulting frequency of DNP clones. By use of this tolerance protocol, ~65-70% of
Acquisition of clonable DNP-specific precursors in vitro--fetal spleen. Spleens were removed at the gestational ages specified and incubated in organ culture in the presence or absence of DNP-HGG or DNP-OVA (5 × 10⁻⁷ M DNP) for 3–5 d. The explants were then dispersed into single-cell suspensions and transferred into lethally irradiated, carrier-primed recipients for analysis in the unmodified splenic focus assay. About 2–7 × 10⁶ cells were injected per recipient, 3–4 mice per experimental group. Fragment cultures were incubated in the presence of DNP-KLH (5 × 10⁻⁷ M DNP) for 3–4 d. Supernates were collected at day 12, and anti-DNP-secreting clones were detected by radioimmunoassay with ¹²⁵I-labeled anti-Fab. The data are expressed as the mean frequency of 2–7 experiments, and error bars are shown where three or more experiments were done. I-I, frequency obtained with control organ cultures; O, frequency obtained with DNP-HGG or DNP-OVA (5 × 10⁻⁷ M); ~, frequency obtained in tolerance specificity control where organ cultures were treated with NIP-HGG (5 × 10⁻⁷ M).

The potential DNP clones are eliminated when spleen cells are taken at 17 and 18 d of gestation, and >80% are eliminated at 19 d of gestation. When liver cells are tested in the modified splenic focus assay for tolerance susceptibility, ~50–60% of the potential DNP clones are eliminated at 14, 15, 17, and 18 d of gestation, and ~80% are eliminated at 19 d of gestation.

Acquisition of B-Lymphocyte Function during Fetal Development In Vitro.—Because some of the explants of fetal lymphoid tissue showed a >10-fold increase in s-Ig-positive cells, we were particularly interested in analyzing the development of B-lymphocyte function in these organ cultures. Fig. 5 (open bars) illustrates the frequency of DNP precursors obtained when fetal spleens taken at 15, 17, 18, and 19 d of gestation are incubated in organ culture for 3–5 d. At all ages of gestation tested, the frequency of DNP precursors detected is greater after 3–5 d in organ culture as compared with the age-matched uncultured controls (Fig. 3 vs. Fig. 5). However, spleens at 15 d of gestation that contain little or no B-cell activity before organ culture rarely show an increase in B-cell activity after 3–5 d in organ culture.

A similar analysis of various ages of fetal liver that were incubated in organ culture for 3–5 d is presented in Fig. 6. Although the data are not conclusive for all gestational ages tested, culturing fetal liver in vitro for 3–5 d results in an increase in clonable
DNP precursors compared with the age-matched uncultured controls (Fig. 4 vs. Fig. 6). As with the spleen cultures, the increase in B-cell activity is more apparent at the earlier ages of gestation tested.

**Effect of Tolerogen on the Acquisition of B-Cell Function during Fetal Development In Vitro.** To determine if the differentiation of DNP precursors occurring in vitro could be influenced by environmental factors, fetal spleen and liver explants were incubated in organ cultures in the presence or absence of tolerogen (DNP-HGG or DNP-OVA). It should be noted that the tolerance protocol used in the organ cultures is distinct from that used in the modified splenic focus assay that was used to study tolerance induction in uncultured fetal spleen and liver as described above. After incubating fetal tissues in organ culture in the presence of tolerogen for 3–5 d, the cultured tissues are then dispersed into single-cell suspensions and tested in the unmodified splenic focus assay for the frequency of DNP precursors.

The effect of the presence of the DNP determinant in the spleen organ cultures on the developing DNP-specific B-cell precursors is shown in Fig. 5. At all ages of gestation tested there is a reduction of ~80% in the frequency of DNP clones, although the day-15 result is not an accurate one because of the lack of a reproducible increase in the frequency of DNP precursors after culturing in vitro as mentioned above. Likewise, the incubation of fetal liver explants of various ages in the presence of DNP-HGG or DNP-OVA results in nearly total abrogation of potential DNP clones with usually an 80–90% reduction (Fig. 6).

The degree of tolerance obtained as a result of incubating spleen and liver organ cultures with the hapten for 3–5 d is consistently greater than the degree of tolerance obtained with uncultured fetal lymphoid cells as measured in the modified splenic focus assay in which the tolerogen is present for 24 h. Perhaps this difference is related to the length of time that the fetal lymphocytes are exposed to tolerogen.

**Analysis of the Isotypes Secreted by DNP Clones from Both Cultured and Uncultured Fetal B Cells.** DNP clones that were detected by radioimmunoassay with an ^125^I-anti-Fab
**DIFFERENTIATION OF FETAL B LYMPHOCYTES**

| Age of fetal spleen | Number of clones analyzed | Isotype distribution: Percentage of clones expressing |
|---------------------|---------------------------|--------------------------------------------------|
|                     |                           | IgM | IgM + IgG | IgA | IgM + IgG |
| Uncultured          |                           |     |           |     |           |
| 17                  | 10                        | 60.0| 30.0      | 10.0| 0         |
| 18                  | 34                        | 52.9| 26.4      | 14.7| 5.9       |
| 19                  | 18                        | 50.0| 11.1      | 11.1| 5.5  |
| Cultured¶           |                           |     |           |     |           |
| 17                  | 44                        | 50.0| 18.2      | 13.6| 11.3       |
| 18                  | 10                        | 60.0| 10.0      | 10.0| 0         |
| 19                  | 20                        | 60.0| 10.0      | 10.0| 0         |

*DNP-specific clones that resulted from the antigenic stimulation of both uncultured and cultured fetal spleen cells were detected in radioimmunoassay by 125I-labeled anti-Fab. The DNP-specific clones were then reanalyzed for isotype distribution with the following 125I-labeled purified anti-heavy-chain antibodies: anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, and anti-IgA.*

**Table III**

Isotypes Secreted by DNP Clones from Fetal Spleen*

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* Results expressed as the percentage of clones secreting the isotypes specified. Because the number of clones analyzed and the complexity of the data IgG1, IgG2a, IgG2b, and IgG3 were grouped as IgG.

† This grouping includes those clones positive for IgA + IgM + IgG.

‡ This grouping includes those clones positive for IgA + IgG.

§ Age of fetal spleen before culturing for 3–5 d.

were reanalyzed for the isotypes being secreted by repeating the radioimmunoassay with the following 125I-labeled anti-class-specific antibodies: (a) anti-IgM, (b) anti-IgG1, (c) anti-IgA, (d) anti-IgG2a, (e) anti-IgG2b, and (f) anti-IgG3. Because of the complexity of the data and the number of clones analyzed, however, the IgG classes were grouped together.

Table III presents the distribution of isotypes secreted by clones produced by both uncultured fetal spleen lymphocytes and those that had been incubated as explants in organ culture. The data suggest that uncultured spleen cells taken at 17 and 18 d of gestation rarely result in clones that secrete IgG classes (IgM plus IgG or IgG) even in the presence of adult T-cell help. By 19 d of gestation, on the other hand, the ability to produce IgG is more pronounced. If, however, the spleens at 17 and 18 d of gestation are incubated in organ cultures for 3–5 d before analysis in the splenic focus assay, the B cells appear to mature in vitro and acquire the ability to produce IgG.

The analogous experiments with fetal liver as the source of potential B cells are presented in Table IV. Consistent with the results obtained with fetal spleen, the proportion of uncultured liver cells that are able to produce IgG (IgM plus IgG or IgG) appears to increase with the age of gestation. Also consistent with the fetal spleen results is the finding that this maturation occurs in vitro because fetal liver explants incubated in organ culture before analysis for DNP precursors result in a greater proportion of clones that secrete the IgG classes.

Interestingly, both fetal spleen and fetal liver B-cell precursors, regardless of whether or not they are incubated first in organ culture, produce a substantial proportion of clones that secrete IgA (IgM or IgM plus IgA) or IgA. In fact, with the possible exception of uncultured 15-d fetal spleen, a temporal difference in the ability of fetal B cells to produce IgM vs. IgA could not be detected under the experimental conditions used, although the predominant isotype distribution in all cases was IgM.
**Table IV**

Isotypes Secreted by DNP Clones from Fetal Liver*

| Age of fetal liver | Number of clones analyzed | Isotype distribution: Percentage of clones expressing |  |
|--------------------|---------------------------|-------------------------------------------------------|---|
|                    |                           | IgM | IgM + IgA | IgA | IgM + IgG | IgG |
| d                  |                           | 14  | 15        | 17  | 18        | 19  |
| Uncultured         |                           | 14  | 56        | 28.6| 16.1      | 1.8 | 1.8 |
|                    |                           | 15  | 10        | 20.0| 10.0      | 0   | 0   |
|                    |                           | 17  | 35        | 45.7| 0         | 2.8 | 0   |
|                    |                           | 18  | 32        | 40.6| 3.1       | 0   | 0   |
|                    |                           | 19  | 24        | 62.5| 4.2       | 25.0| 4.2 |
| Cultured           |                           | 14  | 37        | 62.2| 21.6      | 8.1 | 5.4 | 2.7 |
|                    |                           | 15  | 15        | 53.3| 20.0      | 13.3| 6.7 | 6.7 |
|                    |                           | 17  | 19        | 73.7| 10.5      | 0   | 10.5| 5.3 |
|                    |                           | 18  | 10        | 60.0| 10.0      | 10.0| 10.0| 10.0|
|                    |                           | 19  | 20        | 60.0| 10.0      | 10.0| 10.0| 10.0|

* See Table III footnotes.

only. All of the DNP clones analyzed from 15-d uncultured fetal spleen secreted IgM only, but this result represents the analysis of only a few clones because of the small numbers of lymphoid cells present in 15-d fetal spleen and the low frequency of DNP-specific precursors.

**Discussion**

This study was undertaken to examine the maturation of B-cell function and tolerance susceptibility during fetal development at the level of individual B cells. Differentiation was assessed by determining the frequency of clonable DNP precursors with an in vitro splenic focus assay (10, 11) that had been previously shown to detect a range of B-cell subpopulations including immature tolerance-susceptible subpopulations (17-19). In addition, recent evidence indicates that the splenic focus assay can detect a subset of s-Ig-negative cells of the B-cell lineage, possibly pre-B cells, which is a further indication that this cloning assay is suitable for studies of B-cell development (20).

The antigen DNP was chosen for these studies because Klinman and Press (21) have previously demonstrated that there are at least three major neonatal anti-DNP specificities or clonotypes that are expressed in high numbers during neonatal development. This is in contrast to specificities directed against other haptens such as phosphorylcholine, which develop later in ontogeny (22). From these results Klinman and Press (21) predicted that the DNP clonotypes would be expressed as early as the 15th d of gestation in fetal spleen. Therefore, DNP appeared to be a suitable antigen for studies of B-cell function during fetal development.

B-cell differentiation can first be detected in fetal liver and fetal blood at the 11-12th d of gestation with the appearance of cells able to synthesize cytoplasmic IgM (1, 3, 4, 23). The acquisition of s-Ig-positive cells (3, 8) and antigen-binding cells (6-8) first occurs at 15-17 d of fetal life. In these studies of CBA/H mice it was shown that day 17 of gestation was the 1st d in which s-Ig-positive cells could be reproducibly detected by immunofluorescence in both fetal spleen and liver. However, by day 14
of gestation, before the appearance of s-Ig-positive cells, there was a small but
detectable frequency of DNP-specific precursors in fetal liver. This result lends
credence to the suggestion that the splenic focus assay may support the maturation
and subsequent cloning of a subset of pre-B cells (17, 20). After 15 or 16 d of gestation
there is a gradual increase in the percentage of s-Ig-positive cells in spleen and liver
with time of gestation, there being a marked increase occurring between 18 and 19 d.
An analysis of the frequency of DNP-specific precursors also shows an increase in B-
cell activity with time of gestation but there is no sudden increase of DNP precursors
between 18 and 19 d of gestation that would correlate with the marked increase in s-
Ig-positive cells. Perhaps this lack of correlation is a reflection of the contribution to
the DNP precursor frequency made by s-Ig-negative B-cell progenitors. The spleen of
the adoptive host appears to provide a suitable environment such that DNP progen-
itors mature and acquire receptors during the first few days of the assay and can
subsequently be stimulated with T-cell help (20). This finding is reminiscent of the
studies of Sherr et al. (24, 25) who found that fetal and neonatal B cells, if anything,
mature faster in adult irradiated hosts than in the intact donor. Maturation, in this
case, was assessed in terms of the ability to produce a heterogeneous response with
respect to antibody affinity.

Another factor that could contribute to the lack of correlation between s-Ig-positive
cells and precursor frequency around the time of birth relates to the mechanism by
which diversification of B-cell specificities may occur. Considerable evidence indicates
that there is a sequential appearance of various specificities or clonotypes (22, 26, 27),
and day 18 or 19 may mark the appearance of many later-developing, non-DNP
specificities.

In light of recent suggestions that the T-independent response develops first and
that the precursor for the T-dependent response is the more mature B cell that bears
both IgM and IgD receptors (28), it is interesting that a T-dependent response can be
obtained from 14-d fetal liver, 5-7 d before the in vivo appearance of s-IgD. This
finding tends to argue against the requirement for a more mature B cell as does the
study in which T-dependent responsive B cells were detected from a s-Ig-negative
subset even in the presence of anti-IgD (29).

The ability to study the differentiation of B cells in an organ culture, a closed
system, allows for the direct examination of B-cell progenitors that are present in the
fetal spleen or liver before organ culture. This removes the complication of the influx
of progenitors that may be at earlier stages of development as well as the efflux of
possibly more-mature cells.

Culturing spleen and liver of various gestational ages as explants for 3-5 d usually
results in substantial increases in the percentage of s-Ig-positive cells, a finding that
cannot be explained solely on the basis of selective survival. Because some of the
explants were taken very early in gestation, before the appearance of s-Ig-positive
cells, the results suggest that both fetal spleen and liver contain precursors that can
differentiate into B lymphocytes. This is consistent with the results and conclusions of
Owen et al. (2, 3) and Bruyns et al. (8).

By combining the techniques of organ culture and splenic focus assay the functional
capacity of these developing lymphocytes was ascertained by measuring the frequency
of DNP-specific B-cell precursors. It was determined that culturing explants of 14- to
19-d fetal liver or 15- to 19-d fetal spleen in vitro for 3-5 d resulted in an increase in
the frequency of clonable DNP precursors as compared with age-matched uncultured controls. However, the increases observed are greater at the earlier ages (i.e., 14- to 15-d fetal liver, 17-d fetal spleen), which suggests that the progenitors that are eventually capable of producing DNP clones are present at the early stages of gestation but require time to expand and mature before they can be detected functionally. In addition, with the exception of 15-d fetal spleen, it appears that the potential frequency of DNP clones after organ culture does not change with the gestational age of the tissue before organ culture. Thus, liver cultured at 14 d of gestation could give rise after 3–5 d to the same frequency of DNP clones as fetal liver cultured at 18 d of gestation for 3–5 d, again suggesting that the progenitors for the early-appearing DNP specificities are present as early as 14 d of gestation and that the expansion and maturation of these already-existing clonotypes can occur in vivo or in vitro resulting in an increased frequency of DNP clones.

An observed increase in B-cell activity after culturing 15-d fetal spleen, however, was variable. Perhaps at 15 d of gestation B-cell progenitors are just beginning to seed to the spleen and explants removed at this critical time may or may not contain progenitors that will give rise to DNP-specific precursors. Alternatively, 15-d fetal spleen simply may not have the requisite microenvironment for the maturation of progenitors of the B-cell lineage.

A further demonstration of the in vitro differentiation of B-cell progenitors during organ culture, in addition to the rise in the frequency of clonable DNP-specific B cells, was the increased proportion of clones that secreted IgG classes after organ culture. Thus, uncultured spleen and liver B-cell precursors gave rise to few, if any, DNP clones until 18 or 19 d of gestation. However, spleens or livers removed before 19 d of gestation and incubated in organ culture gave rise to a proportion of IgG-secreting clones greater than the age-matched uncultured controls. Because the same adult T-cell help is provided for both uncultured and cultured fetal cell populations, there must be an intrinsic difference in the B-cell precursor after in vitro maturation. Other investigators have provided evidence for the ontogenetic expression of isotypes both with regard to s-Ig (30) and to immunoglobulin secreted by stimulated cultures (31).

However, once this maturation with regard to isotype has taken place it would appear from the results herein as well as from the results obtained with adult B cells (J. M. Teale and H. Bathard. Manuscript in preparation.) that many of the B cells can give rise to clones that secrete several isotypes. This is inconsistent with the results of Lawton et al. (32), who concluded that B cells and their progeny are restricted with regard to the isotypes they or their progeny can express and that this restriction takes place early in the development of the B cell. On the other hand, there is considerable evidence that indicates that the isotypes secreted by antibody-forming cells are regulated extensively by T cells and/or T-cell factors (16, 33, 34). Therefore, the discrepancy may be partially explained by the effect of T cells because Lawton et al. (32) studied lipopolysaccharide-induced cultures.

One particularly interesting aspect of the class analysis is the ability of immature fetal B cells to produce the IgA isotype with adult T-cell help. This is reminiscent of previous studies in which a subset of s-Ig-negative cells from neonatal spleen contained clonable DNP precursors that gave rise to a considerable proportion of clones that secreted IgM plus IgA or IgA (20). The ability of 14-d fetal lymphoid cells to produce
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IgA-secreting clones is a further indication that lymphocytes capable of secreting IgA appear earlier in ontogeny than has previously been recognized and before the appearance of s-IgA (32). The ability to stimulate IgA production so early in ontogeny is probably related to the presence of adult T-cell help in the assay system. Indeed, the IgA response seems to be dependent upon the availability of T cells (33). Moreover, recent investigations have provided evidence for the T-cell regulation of IgA synthesis (36).

Notably, there appeared to be an ontogenetic relationship with regard to the degree of tolerance observed. Thus, uncultured fetal spleen and liver populations taken at earlier ages of gestation resulted in a lower degree of tolerance after a 24-h incubation with tolerogen than spleens or livers taken at 19 d of gestation or 1 d after birth (50–65% tolerance vs. 80–90% tolerance, respectively) as if the cells were in a pretolerant phase. This is in accord with a report by Szewczuk and Siskind (37), who found 14-d fetal liver to be particularly resistant to tolerance induction.

One possible explanation for the relative resistance to tolerance induction of B cells obtained at the earlier ages of gestation relates to the rate at which a population of fetal B cells acquires functional s-Ig receptors. Perhaps a 24-h incubation period is insufficient time to affect all of the potential DNP precursors from early fetal lymphoid tissues, and maturation and subsequent cloning can occur to a considerable extent after the removal of tolerogen. Consistent with this explanation is the finding that the degree of tolerance observed is increased to an 80–90% level when spleens or livers from the earlier ages of gestation are incubated in organ culture for 3–5 d in the presence of tolerogen. Presumably, most of the progenitors that can acquire s-Ig receptors in vitro have done so within the incubation period, and the presence of tolerogen during this maturation phase almost totally eliminates the potential anti-DNP clones. This is consistent with our previous results in which immature B cells in the absence of T cells are highly susceptible to tolerance induction (18, 20) and provides strong evidence in favor of the clonal abortion theory (38).

Although it is difficult to compare directly the normal development of fetal B cells with the development occurring in vitro because of the inability to account for cell migration in the former and cell death in the latter, it would appear that the in vitro development is substantial. Indeed, where comparisons can be made with the data available (e.g., 17-d fetal spleen cultured for 3 d vs. 1-d-old neonatal spleen), the DNP precursor frequencies are similar. Thus the organ culture system seems suitable for investigating B-cell differentiation, and it is planned to study this process further by examining individual clonotypes.

Summary

The maturation of B-lymphocyte function during fetal development was studied in vivo and in an in vitro organ culture system. The results indicated that the progenitors for 2,4-dinitrophenol (DNP)-specific B cells are present as early as 14 d of gestation in liver and possibly as early as 15 d in spleen. In addition, it was found that the organ culture system supports the development of B lymphocytes as measured by an increase in both the percentage of surface immunoglobulin-positive cells and the frequency of clonable DNP-specific B cells after culturing.

The majority of anti-DNP-secreting clones resulting from the antigenic stimulation of fetal B cells produced only the IgM isotype, and the ability to secrete the IgG
isotypes increased as a function of gestational age. Because fetal DNP precursors from spleens and livers that had been incubated in organ culture resulted in a greater proportion of clones secreting IgG compared with age-matched uncultured controls, it was concluded that the maturation with regard to the ability to secrete IgG can occur in vitro.

In studies relating to the ontogenetic development of tolerance susceptibility, it was found that up to one-half of the DNP-specific B-cell precursors from livers and spleens <18 or 19 d of gestation were resistant to tolerogen treatment for 24 h as if in a pretolerant phase. However, if tolerogen were present for 3–5 d during organ culture there was near total elimination of potential DNP clones. This finding suggested that the 24-h induction period was insufficient for affecting the DNP-specific precursors in livers and spleens from the earlier gestational ages, and that a proportion of precursors could subsequently form DNP clones in the splenic focus assay after the removal of tolerogen.

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