New Zealand Black (NZB) mice have long been exploited as a model of genetically controlled autoimmune diseases and immunoregulatory dysfunction (1–3). Of particular concern to us is the unusual development and function of their humoral immune system. There is reason to believe that spontaneous and polyclonal activation of B cells occurs independently of defects residing in thymus-derived (T) cells (2–4). For example, this occurs in cell transfer situations and in mutant athymic mice with the NZB background (5–6). In discerning which of the abnormal features of these animals are intrinsic and which represent changes that occur secondarily to defects in other systems, it is helpful to establish the chronology of events during ontogeny. Previous reports (7–9) indicated that NZB mice may become immunologically mature at an early age and that hypersecreting B lineage cells were detectable even from the time of birth. The present study, using a mitogen-dependent colony-forming cell assay, reveals that abnormalities affecting development of the humoral immune system occur much earlier in embryos of this interesting strain of mice.

**Materials and Methods**

**Animals.** NZB and CBA/H-T6T6 mice were bred in our own colonies. Pregnant A/J, BALB/cJ, CBA/J, C57BL/6J, and SWR/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Gestational ages of fetuses in this report are given as elapsed days since detection of vaginal plugs after overnight matings.

**Cell Suspensions.** Fetal liver cells were gently teased over metal sieves and large debris was removed by layering over fetal calf serum (FCS) (10). All manipulations of the cells were performed using RPMI 1640 medium containing 20 mM Hepes and 5% heat-inactivated FCS. Surface immunoglobulin (sIg)-positive cells were removed by incubating cells in goat anti-μ-coated plates in the cold (11). G-10-adherent cells were removed by passing cell suspensions through Sephadex G-10 columns (11). The average total nucleated cell recoveries in the column effluents were 40% for fetal liver suspensions. B cells, pre-B cells containing cytoplasmic μ heavy chains of IgM (cμ), and B lineage antigen (14.8)-positive cells were not preferentially decreased by adherent cell depletion.

**Cell Cultures.** Semisolid agar cultures containing 2-mercaptoethanol, FCS (lot #29101536; Flow Laboratories, Rockville, MD), and other additives were prepared as described elsewhere. Lipopolysaccharide (LPS) (25 μg/plate) was used to potentiate colony formation and give a linear dose response relationship. Colony numbers from four
replicates were averaged and plate to plate variability in a single experiment (standard error) was usually <10% and not stated. In some experiments, a liquid preculture system was used that permits maturation of B lineage precursors in vitro (10-11). Unseparated fetal liver cell suspensions were held for various times at a density of 10^6 cells/ml in RPMI 1640 medium with appropriate additives in 96-well microtiter plates. Then the cells were harvested, counted, examined for slg, and/or plated in semisolid agar cultures.

**Immunofluorescence.** Viable cells bearing slg were detected with purified, rhodamine- or fluorescein-labeled goat anti-mouse μ or κ antibodies. A macromolecular B lineage cell antigen was detected by monoclonal 14.8 antibody and purified, fluorescein-labeled mouse anti-rat Ig antibodies (11). This antigen is not expressed on all cμ⁺ pre-B cells at the earliest stages in normal embryos (A. Velardi and M. D. Cooper, manuscript submitted for publication and H. Jyonouchi, unpublished observations). However, from 16 d of gestation onwards, most cμ⁺ cells and slg⁺ cells were detectable by immunofluorescence with this antibody and were removable with 14.8 antibody-coated plates (12-14).

**Factors from Young NZB Serum.** Sera from 4-wk-old NZB mice contain factors that can enhance colony formation of slg⁻, 14.8⁺ normal B cell precursors and κ expression on the 70Z/3 pre-B cell line (14 and unpublished observations). Pooled young NZB serum was passed sequentially through Sephacryl S-200 and Sephadex S-100 superfine columns and fractions corresponding to apparent molecular weights of 18-48,000 were collected. This was then isoelectrofocused and fractions 1 (pI, 3.0–3.5), 23 (pI, 7.8–8.0), and 26 (pI, 8.4–8.5), which had significant activity, were used in this study. Upon neuraminidase treatment, fractions 23 and 26 converged into one peak in preparative isoelectric focusing. These factors were all stable at pH 2 but were heat-labile and inactivated by trypsin treatment. They had no enhancing activity on the cloning of mature B lymphocytes and had no detectable activity in granulocyte-macrophage colony-stimulating activity (CSA) or interleukin 2 assays (unpublished observations).

**Calculation and Expression of Results.** After each cell separation/manipulation step, numbers of nucleated cells were counted and values were adjusted to reflect incidences in the original cell suspension. For example, if 20% of the total cell sample was lost as a result of liquid preculture, all data obtained with this suspension would be multiplied by 0.8. Student’s t test was used to assess the significance of differences.

**Results and Discussion**

Cells that can respond to mitogens in semisolid agar cultures are normally first detectable at 16.5–17 d of gestation in liver of CBA/H strain embryos (10, 15-16 and Fig. 1). Our survey of embryos from a number of other “normal” strains of mice also revealed that at 16 d of gestation almost no colony-forming cells were detectable in the liver (Table I). However, these were present in NZB embryos 2–3 d earlier and in substantially higher numbers. Differences in overall embryo or liver sizes were not remarkable when NZB and CBA embryos of the same stages were compared. B cell precursors detected by monoclonal 14.8 antibody and cμ staining were found in low incidences in the normal strains as reported before (13) and such phenotypically defined B lineage cells were not significantly increased in NZB embryos (data not shown).

We previously found that precursors of B cells can give rise to functional B cells during liquid culture and their numbers and kinetics of appearance reflect the stage of embryonic development (10, 16). As illustrated in Fig. 2, colony-forming cells emerged earlier and in larger numbers within cultures of 15- and 16-d liver suspensions of NZB than CBA/H embryos. Functional (clonable) cells were present in cell suspensions of 15-d NZB fetal liver and these increased even more during liquid culture. Aliquots of cells were taken from the same cultures and examined by immunofluorescence (Fig. 3). Neither NZB nor CBA/H liver
Figure 1. Emergence of colony-forming cells in liver of NZB (○) and CBA/H (■) embryos. Each point represents the mean value ± SD of data obtained from three to four experiments.

Table 1
Analysis of Clonable Cells in Embryonic Liver Suspensions

| Mouse strain | Number of colonies after clonable cells cultured with: | Anti-μ* | Anti-CSA-1 |
|--------------|--------------------------------------------------------|--------|----------|
|               | -- Unseparated                                          | 1,050  | <2       |
|               | Adherent cell depleted                                 | 205*   | <2       |
|               | Serum factor added                                     | 650    | <2       |
| NZB           | Fraction 1                                             | <2     | 605      |
|               | Fraction 23                                            | <2     | 600      |
|               | Fraction 26                                            | <2     | 615      |
|               | A/J Unseparated                                        | <2     |          |
|               | BALB/cJ Unseparated                                    | <2     |          |
|               | CBA/H-T6T6                                             | <2     |          |
|               | CBA/J                                                  | <2     |          |
|               | C57BL/6J                                               | <2     |          |
|               | SWR/J                                                  | <2     |          |

* All data are expressed as numbers of colonies/10⁶ initial nucleated fetal liver cells of 16 d gestation. NZB data are from a single experiment. For other strains, three pregnant mice of each strain were sacrificed at 16 d of gestation.

* The final concentration of anti-μ antibodies was 10 μg/ml; anti-CSA-1 antiserum was added to a final dilution of 1:10⁴. The latter was sufficient to completely block macrophage colony formation in other cultures by CSA in L cell-conditioned medium.

* In four independent experiments, the average reduction of colony numbers in adherent cell-depleted suspensions was 78 ± 8.0%. Addition of fractions 1, 23, and 26 from NZB serum increased colony numbers 3.61 ± 0.85 times, 5.85 ± 1.17 times, and 4.01 ± 1.20 times, respectively, in adherent cell-depleted cell suspensions. They had no enhancing effects on unseparated fetal liver cell suspensions of NZB (unpublished observation).

* Partially purified NZB serum factors were prepared as described in Materials and Methods and added to the culture media as final dilutions of 1:10⁴.

suspensions contained significant numbers of slg* cells at 15–16 d of gestation but these emerged during liquid culture. Again they emerged earlier and in larger numbers in cultures of NZB embryonic liver.
FIGURE 2. Functional maturation of B lineage precursor cells during liquid culture of embryonic NZB (○) and CBA/H (●) liver cells. Cell suspensions were prepared from 15- or 16-d gestation embryos.

FIGURE 3. Acquisition of slg by B lineage precursor cells from NZB (○) and CBA/H (●) fetal liver during liquid preculture. Cell suspensions were prepared from 15- or 16-d gestation embryos.

Most of the cells in suspensions of lympho-hemopoietic tissues from adult mice that clone in semisolid agar are slg+ cells (16–17). However, recent studies with normal newborn and very young NZB mice revealed that under certain circumstances, slg− B precursors can form colonies (12, 14). There were very few slg+ cells in embryonic liver cell suspensions used in this study and depletion of slg+ cells with anti-μ-coated plates did not significantly diminish the numbers of colonies (Fig. 3 and data not shown). However, inclusion of anti-μ antibodies in the culture medium always prevented colony formation (12, 14 and Table I). Therefore, slg must be acquired by colony-forming B precursors in this situation before significant focal proliferation occurs.
Other studies indicate that maturation of B precursors in vitro is augmented by G-10-adherent regulatory cells in hemopoietic cell suspensions (12–14). An adherent cell type that is neither 14.8+ nor sIg+ seemed to be required for colony formation of sIg− B precursors in semisolid agar cultures of cells from normal neonates or very young NZB mice (12, 14). Table I also shows that G-10-adherent cell depletion greatly diminished numbers of colonies formed by NZB embryonic liver cells. Of particular interest, activity was at least partially replaced by humoral factors added to the culture medium (Table I). These were partially purified from serum of 4-wk-old NZB mice as described in Materials and Methods and have been briefly reported elsewhere (14). Further characterization of these factors in biological and biochemical terms are underway (manuscript in preparation). It is possible that G-10 column filtration removed some type of B lineage cells from the liver suspensions that lack markers such as 14.8 or Ig chain synthesis. But the fact that humoral factors at least partially replace the function of adherent cells makes this possibility less attractive. Anti-CSA-1 antibody, kindly given by Dr. E. R. Stanley, did not decrease colony numbers, which indicates that colonies of macrophage lineage cells were not mistakenly scored (Table I and reference 18). That only B lineage colonies were detected was also confirmed by this complete sensitivity to anti-μ antibodies.

These findings document that B lineage precursor cells arise at a very early stage of embryogenesis in NZB mice. We suspect that microenvironmental elements may be responsible for this. Such dysfunction could be important to the early emergence of hypersecreting cells and later production of autoantibodies (1–3, 8–9). Events that take place at 14–15 d of gestation are unlikely to be the direct result of defects in T lymphocytes or exposure to environmental antigens (19). Still to be excluded is the possibility that maternally derived autoantibodies or other humoral factors influence embryonic B lineage cell differentiation. Our preliminary experiments indicate that serum obtained from pregnant NZB or CBA/H mice (16 d gestation) had no enhancing or suppressive effects on B precursor cell maturation in vitro (unpublished observations). Further investigation of this model could be instructive in terms of mechanisms that normally control the programmed emergence of particular types of precursor cells, their expansion, and their maturation to functional cells.

Summary

Previous reports suggest that large numbers of immunoglobulin-secreting cells appear in tissues of NZB strain mice from the time of birth. In this study, we investigated the development of B lineage cells during embryonic life and found that they were present 2–3 d earlier and in higher numbers in NZB embryos than several other strains of mice. That is, liver cell suspensions from NZB embryos contained larger numbers of surface Ig (sIg)+ cells that could form B cell colonies in mitogen-dependent semisolid agar culture. Sephadex G-10-adherent cell depletion diminished numbers of colonies and this was partially restored by addition of humoral factors. The latter were partially purified from serum of very young NZB mice. These findings document that abnormal changes take place in B lineage cells and possibly also in cells that regulate their maturation in NZB strain mice at a very early stage of development.
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