RELATIVE SENSITIVITY OF FETAL AND NEWBORN MICE
TO INDUCTION OF HAPten-Specific
B CELL TOLERANCE*

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Recent work has emphasized the extreme sensitivity of immature B lymphocytes to the induction of immunological tolerance (1–3). In newborn mice, a marked lowering of splenic B cell responsiveness can be achieved without any demonstrable reduction in either the number of antigen-binding B lymphocytes or their antigen-binding avidity spectrum (4). Thus, concentrations of haptened-human gamma globulin (HGG) too low to saturate, to blockade, or to modulate the surface immunoglobulin (s-Ig) receptors and incapable of eliminating hapten-binding B cells were nevertheless sufficient to effect the transmission of a negative signal to the cells which rendered them incapable of responding to antigenic or mitogenic stimuli. Our study examines whether the sensitivity threshold for the induction of the anergic state in cells emerging from the pre-B (s-Ig negative) to B cell (s-Ig positive) status during fetal life before the development of immunocompetence was even lower than that of immature, but already s-Ig positive, B cells (1, 3) and examines the duration of anergy.

Materials and Methods

Mice. CBA/CaHWehi mice were used, either 2–3 d of age or as pregnant adults. (CBA × BALB/c)F1 hybrids, aged 4–6 wk, were used as thymus donors.

Antigens and Tolerance-induction Protocol. Fluorescein (FLU)-coupled HGG and polymerized flagellin (POL) were prepared as previously described (1, 5). Freshly deaggregated FLU-HGG was injected intraperitoneally into newborn mice (2) or intravenously into pregnant mice at 14.5 d of gestation (4).

Estimation of Serum Levels of FLU-HGG. FLU-HGG was lightly iodinated with 125I by the chloramine-T method, and serum levels estimated by gamma counting.

Preparation of Cell Suspensions and FLU-Gelatin Fractionation Procedures. These procedures were as previously described (1, 4–6).

Antibody Formation in Vitro. The frequency of clonable anti-FLU plaque-forming cell (PFC) precursors among unfractionated and FLU-gelatin-fractionated spleen cell populations was determined by in vitro limiting-dilution analysis as previously described (1, 6) with either the T-independent antigen FLU-POL (0.1 µg/ml) or Escherichia coli lipopolysaccharide (LPS) (batch 0111:B4; Difco Laboratories, Detroit, Mich.) (20 µg/ml) to stimulate antibody formation. The results are expressed as a percentage of the frequency value of the saline-injected (control) group.

Results

Serum Tolerogen Levels after a Single Injection. To enable comparison of the inductive phases of in utero (7) and neonatally induced tolerance, the serum levels achieved by

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a single injection of tolerogen were determined for each situation. Tolerogen doses were chosen so that approximately equivalent amounts were administered on a microgram/gram initial body weight basis. $^{125}$I-trace-labeled FLU-HGG was used, with pregnant mice (weighing ~25 g) receiving 1 mg intravenously at 14.5 d of gestation and newborn mice (weighing ~2 g) receiving 100 μg intraperitoneally 2-3 d after birth. When 1 mg of tolerogen was administered maternally, a fetal serum level of 34 μg/ml was achieved after 24 h and was maintained up to birth and for the next 3 d (8) after which it progressively declined (Fig. 1). Similar curves were obtained whether 1 mg, 10 μg, or 0.1 μg of trace-labeled FLU-HGG was injected. When 100 μg of tolerogen was administered to newborn mice, the 24-h serum level was 143 μg/ml, declining to 35 μg/ml by 7 d. After the initial equilibration period, the levels attained in both groups were similar, the biological half-life of the labeled protein was ~6–7 d.

**Degree of Tolerance Induced by Various Doses of Tolerogen.** 9 d after in utero exposure (3 d post-birth) and 7 d after neonatal exposure, the frequency of precursors of anti-FLU PFC clones among splenic cells of the treated mice was determined by limiting-dilution analysis in vitro using FLU-POL to stimulate PFC clone formation (Table I). For neonatally induced tolerance, it was calculated that a dose of 60 ng of FLU-HGG would have achieved a 50% reduction in the frequency of clonable anti-FLU PFC splenic precursors, which would have resulted in an imputed peak serum

![Fig. 1. Serum levels of tolerogen in mice at various periods after administration. Levels of $^{125}$I-FLU-HGG detected in fetal or offspring serum after in utero exposure to a single maternal dose of 1 mg injected intravenously into pregnant mice at 14.5 d of gestation (○) or in the serum of mice after neonatal exposure to 100 μg injected intraperitoneally 2-3 d after birth (●). Data presented for the in utero-exposed group represent the mean ± SE of 6-12 individual values obtained from two separate experiments. Neonatal data is derived from a single experiment, values represent the mean ± SE of values obtained with six to eight individual mice for each time point.](image-url)
Table I

Dose-Response Relationship of Induced B Cell Tolerance* as Assessed by the Reduction in the Frequency of Clonable Anti-Hapten Precursors

| Dose of FLU-HGG injected | Percentage of control anti-FLU PFC precursor frequency value | * In utero induction ‡ | P § | Neonatal induction ‡ | P § |
|--------------------------|------------------------------------------------------------|------------------------|-----|------------------------|-----|
|                          |                                                           |                        |     |                        |     |
| 0                        | 100                                                       | ND                     |     | 100                    | ND  |
| 1 mg                     | 6.42 ± 1.2                                                | 0.003                  |     | 20.3 ± 6.6             | 0.01|
| 100 µg                   | 11.5 ± 4.4                                                | 0.025                  |     | 21.4 ± 5.1             | 0.003|
| 1 µg                     | 21.5 ± 4.1                                                | 0.001                  |     | 26.1 ± 3.1             | 0.003|
| 100 ng                   | 45.4 ± 4.8                                                | 0.05                   |     | 32.3 ± 4.3             | 0.003|
| 10 ng                    | 74.1 ± 13                                                 | NS                     |     | 42.8 ± 5.5             | 0.005|
| 1 ng                     | 78.5                                                      | NS                     |     | 55.7 ± 8.1             | 0.025|
| 100 pg                   | ND                                                        |                        |     | 74.4 ± 7.8             | 0.05|

* Tolerance was induced either neonatally or in utero as described in Materials and Methods.
‡ Values given represent the mean ± SE of individually normalized percentages of saline-injected control frequency values obtained in four to nine experiments. Control frequency values were 39.3 ± 5.22 × 10⁻⁶ for neonatal-induction groups and 22.6 ± 4.0 × 10⁻⁶ for in utero-induction groups. ND, not done.
§ Values represent the significance of difference from control frequency value, obtained using the Student’s t test.

Concentration of tolerogen of 86 ng/ml (5.4 × 10⁻ⁱ⁰ M) after 24 h and a level of 20 ng/ml (1.3 × 10⁻¹⁰ M) at the time of killing. Doses ≥ 1 µg caused profound and increasing tolerance, 1 or 10 ng resulted in insignificant reductions. For in utero-induced tolerance, a maternal dose of 2.5 ng would have achieved a 50% reduction in the frequency of splenic anti-FLU PFC precursors in the offspring, which would have led to a fetal serum concentration of 80 pg/ml (5.4 × 10⁻¹⁰ M) during the period of exposure, a threshold of at least 240-fold lower than the minimum level in neonates. Maternal doses ≥ 10 ng resulted in substantial and significant tolerance.

**Failure to Obtain Absolute Tolerance In Utero.** In utero, 1 µg caused a 74% reduction in clonable anti-FLU B cells, and 1,000-fold more tolerogen did not significantly increase the degree of tolerance. The possibility that the residual activity was a result of s-Ig negative, pre-B cells acquiring immunocompetence and giving rise to anti-FLU PFC clones during the in vitro read-out period was investigated. The fluorescence-activated cell sorter (FACS) (BD FACS Systems, Mountain View, Calif.) was used to select the s-Ig negative population from spleen cells from 2- to 3-d-old donors. These cells were shown to turn 55% s-Ig positive within 24 h in vitro. In vitro cloning analysis using FLU-POL to stimulate antibody formation revealed the frequency of anti-FLU PFC precursors among the s-Ig negative spleen cells from both the untreated and 1 mg in utero tolerized to be 24% of that of control unfractionated spleen cells. This accounted for essentially all of the residual activity (Table I).

**Duration of Tolerance Induced by a Single Injection of Tolerogen.** Previous work had shown that hapten-binding B cells could readily be detected and isolated from the spleens of animals tolerized in the perinatal period (4). This allowed us to estimate the duration of anergy induced among both the unfractionated spleen cell populations and among hapten-specific B cells which were isolated by prefractionation on FLU-gelatin-coated dishes (4–6). Newborn mice received a single intraperitoneal injection of 100 µg of FLU-HGG, and at intervals thereafter the frequency of anti-FLU PFC precursors among unfractionated and FLU-gelatin prefraccionated spleen cells was...
determined. As expected, profound tolerance resulted (Table II) and this lasted for at least 10 wk. With a FLU-POL challenge, the rate of recovery of responsiveness in unfractionated spleen cells was slightly more rapid than that observed in the higher avidity (1) FLU-gelatin binding B cell population. Recovery of responsiveness among the FLU-gelatin-binding cells was slightly more rapid when a mitogenic (LPS) rather than antigenic (FLU-POL) triggering stimulus was used. From the studies on the serum levels of circulating tolerogen (Fig. 1), the estimated serum concentration of tolerogen after 10 wk would have been around 80 ng/ml (~5 × 10⁻¹⁰ M). Below this level tolerance broke down rapidly.

**Discussion**

The demonstration of high-avidity antigen-binding B cells, which are unable to be stimulated by either antigen or mitogen, within the spleens of mice rendered tolerant in early life (4) raises new perspectives on the mechanisms of induction of B cell tolerance. We must now ask not how a developing B cell can be eliminated as a consequence of premature contact with antigen, but, rather, how the B cell can register and store the negative signals that render it anergic as a result of that contact. Our study suggests that the B cell is most sensitive to tolerance induction at the time when its s-Ig receptors are first emerging. By exposure during fetal life before the appearance of any s-Ig positive cells in the fetal liver or elsewhere (9), concentrations of FLU-HGG as low as 5.4 × 10⁻¹³ M caused significant tolerance. Neonatal exposure,

| Time after FLU-HGG administration | Percentage of responsiveness of control spleen cells of Unfractionated + FLU-POL§ | FLU-gelatin binding cells + FLU-POL¶ + LPS** |
|----------------------------------|---------------------------------|---------------------------------|
| wk                              |                                |                                 |
| 1                               | 9.0 ± 2.0                       | 8.1 ± 2.1                       | 9.0 ± 4.8 |
| 2                               | 7.4                             | 10.3                            | 12.6     |
| 4                               | 17.0 ± 9.0                      | 8.5 ± 4.5                       | 14.3 ± 5.6|
| 6                               | 34.1 ± 4.3                      | 12.1 ± 2.8                      | 15.9 ± 2.4|
| 8                               | 34.9 ± 2.8                      | 21.3 ± 4.6                      | 38.6 ± 12 |
| 10                              | 38.6¶                           | 38.3                            | 70.0     |
| 12                              | 74.9§§                          | 66.1                            | 174      |
| 14                              | 117††                            | 89.1                            | 105      |

* Tolerance was induced neonatally by a single intraperitoneal injection of 100 µg of FLU-HGG.

§ As measured by enumeration of anti-FLU PFC precursors capable of responding to FLU-POL or LPS in vitro.

§§ Precursor frequency values for control groups rose from 40 × 10⁻⁶ at 1 wk (i.e., 10 d of age) to the adult levels of 150–200 × 10⁻⁶ at 4–6 wk.

¶ Antigen-binding cells isolated from spleens of tolerant mice by fractionation on FLU-gelatin dishes (4, 6).

† Control frequency values were 0.6–1% in young mice and 0.8–1.8% in the older mice.

** Control frequency values were 1–3.6% with this stronger stimulus, with no significant age variation.

†† Pooled results of two experiments.

§§ Results of a single experiment.
using 2- to 3-d-old animals, which already possess significant numbers of clonable anti-FLU B cells (1, 5), showed the threshold point for significant tolerance induction to be >240-fold higher. In other words, although immature B cells are highly susceptible to tolerance induction, their susceptibility is much less than that of cells caught in the pre-B to B cell transition phase. We have previously documented (1, 2) that adult mature B cells are 250- to 1,000-fold more resistant than immature B cells. The picture that emerges then, is not one of an abrupt all-or-none change in the behavior of the B cell at a particular stage of differentiation, but, rather, one of a progressive raising of the sensitivity threshold to tolerance induction as the B cell passes through various differentiation stages during ontogenic development.

The question of how long a B cell can store the negative signals it has accumulated is rendered complex because B cells cannot be maintained in tissue culture for long periods. Experiments on duration of tolerance were performed with a constantly falling level of antigen present in the circulation of living, tolerant animals. It appears that neonatally induced tolerance persisted so long as antigen levels remained above the threshold that could induce significant tolerance in newborn mice (~80 ng/ml or 5 × 10⁻¹⁰ M). However, our results cannot discriminate between a loss of the anergic state in given B cells, or a failure of induction of clonal anergy in newly developing B cells formed in adult bone marrow, or both.

The failure to induce tolerance in 20–25% of all the clonable cells in the spleens of 2- to 3-day old in utero-tolerized mice appears to be a result of their content of s-Ig negative, pre-B cells on the verge of gaining immunocompetence. These cells, with few if any s-Ig receptors thus cannot see the tolerogen in vivo, but are able to acquire s-Ig receptors rapidly in vitro, react with the T-independent immunogen FLU-POL and form anti-FLU PFC clones within the 3-d read-out procedure.

From these studies and previous papers in this series (1, 2), it is now clear that the degree of anergy induced in a given B cell as a result of exposure to hapten-HGG is a complex function of the maturity of the cell when it first encounters the antigen; the molar concentration of the antigen; the degree of its multivalency, which affects its capacity to bind to and cross-link receptors; the length of exposure to the tolerogen; and the presence or absence of concomitant stimulating signals. No attempt to explore the molecular biology of B lymphocyte signalling can proceed unless each and all of these variables are taken into account.

Summary

Mice were rendered tolerant to the hapten fluorescein (FLU) by a single injection of FLU-human gamma globulin (FLU₅HGG) 2-3 d after birth or via the maternal circulation at 14.5 d of fetal life. After 7-9 d, the degree of functional nonresponsiveness induced in vivo among splenic FLU-specific B cells of tolerized mice was assessed by limiting-dilution analysis in vitro, and the serum levels of trace-labeled tolerogen were determined. When tolerogen was introduced before the appearance of any B cells, and was thus present during the pre-B to B cell transition stage, a concentration of 5.4 × 10⁻¹⁸ M effectively silenced 50% of the clonable anti-FLU PFC precursors; but a similar reduction on newborns required a minimal tolerogen concentration of 1.3 × 10⁻¹⁰ M, >300-fold less than has previously been shown to equally affect adult B cells, but at least 240-fold more than in the in utero situation. Neonatally induced tolerance using a relatively high tolerogen dose lasted ~12 wk.
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