B-CELL TOLERANCE

II. Trinitrophenyl Human Gamma Globulin-Induced Tolerance in Adult and Neonatal Murine B Cells Responsive to Thymus-Dependent and Independent Forms of the Same Hapten*

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Neonatal splenic B cells which are responsive to thymus-dependent antigens (TD) are exquisitely susceptible to induction of tolerance (1, 2). This state of tolerance is not mediated by suppressor T cells and is not a result of suboptimal macrophage function (1 and footnote one). In adult mice, induction of B-cell tolerance is only achieved with doses of antigen 1,000-fold higher (1) than those required to produce the same degree of unresponsiveness in neonates.

In contrast to these results, studies with T-independent (TI) antigens indicate that neonatal and adult splenic B cells are equally susceptible to tolerance induction (3, 4). However, such studies have not ascertained whether the neonate is more resistant to tolerance induction or the adult is hypersusceptible, i.e., does the induction of tolerance in cells responsive to TI antigens resemble that of adult or neonatal cells responsive to TD antigens? The answer is pertinent to determining the relative maturity of the B cells which can be tolerated or respond to TI or TD antigens.

We report here the direct comparison of tolerogen sensitivity of adult and neonatal TD and TI responses by inducing tolerance in vitro with trinitrophenyl human gamma globulin (TNPγHgG) and assaying unresponsiveness with TD and TI forms of the TNP determinant.

Materials and Methods

Animals The BDF1 (C57Bl/6 female × DBA/2 male F1) mice, 8- to 10-wk of age (The Jackson Laboratory, Bar Harbor, Maine), or neonates 6–7 days of age (bred in our animal colony) were used in this study. Adult mice to be used as the helper T-cell source were carrier-primed by intravenous injection of low doses of sheep red blood cells (SRBC) (Colorado Serum Co., Denver, Colo.; 0.2 ml of 0.01% suspension) 7 days before sacrifice (5). Spleen cell suspensions from primed animals were irradiated (1,500 R; 132cesium source) before being cultured.

Antigens Human gamma globulin (HgG) (Cohn fraction II, Miles Pentex) was haptenated using 2,4,6-trinitrobenzene sulfonyl acid (TNBS) (Baker) at a substitution ratio of TNPγHgG (5, 6)

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as determined spectrophotometrically. Heat-killed *Brucella abortus* (NADL, Ames, Iowa) was haptenated with TNBS using the following procedure. 500 mg TNBS was added to 0.2 ml packed cells in 2.5 ml cacodylate buffer (0.2 M cacodylate-NaOH pH 10). The suspension was incubated at 26°C for 30 min before centrifugation (12,000 g, 10 min). The pellet was washed 5 times, once in cacodylate buffer, once in diluent glycylglycine (500 mg/300 ml), and 3 times in balanced salt solution (BSS). TNP SRBC were prepared as described previously (7).

**Assay.** Plaque-forming cells (PFC) to SRBC were determined by a microscope slide modification (8) of the hemolysis in gel technique (9). Anti-TNP PFC were determined using TNP-horse erythrocytes (HRBC) (10).

**Induction and Assessment of Unresponsiveness.** Antigen-specific unresponsiveness was induced as described previously (1, 11). Briefly, mouse spleen cells were incubated for 24 h at a density of 10⁶ cells/ml in the presence of graded concentrations of TNP₁₇HgG. Cells were washed 3 times and resuspended at a density of 10⁶ per ml in complete medium and plated in 35-mm Falcon dishes (Falcon Plastics, Oxnard, Calif). 0.5 ml per dish. 8 x 10⁶ viable, irradiated, low dose SRBC-primed spleen cells in 0.1 ml complete medium were added to each culture. Cultures were incubated with 0.1 ml of 0.1% TNP-SRBC or 0.1 ml of 0.01% TNP-*Brucella* in BSS. Antigen doses were chosen on the basis of their stimulation of optimal PFC response in control experiments. TNP-*Brucella* was determined to be TI on the basis of its ability to elicit normal PFC responses in spleen cell populations pretreated with anti-brain-associated Thy-1 + C' (12). During the immune response phase, cultures were incubated as described previously (13) to obtain primary PFC responses to TNP-SRBC. The number of direct PFC was determined 4 days after immunization.

**Results**

As shown in Fig. 1 A and as described previously (1), when mouse spleen cells are exposed to TNP₁₇HgG for 24 h before being immunized with TNP-SRBC in Mishell-Dutton cultures, antigen-specific suppression of the TNP response is observed in cell populations previously exposed to TNP₁₇HgG concentrations greater than 1 µg/ml. However, in parallel cultures immunized with TNP-*Brucella* (TI antigen), antigen-specific suppression of the TNP response was observed at much lower tolerogen doses with 50% suppression occurring in populations previously exposed to approximately 0.01 µg TNP₁₇HgG.

When the identical protocol is used with spleen cells from 6- to 7-day-old neonates, different results are observed (Fig. 1 B). In cultures immunized with either TNP-SRBC or TNP-*Brucella*, suppression is seen at tolerogen concentrations approximately equal to those causing suppression of adult cells responsive to TNP-*Brucella* and less than 0.1% of those that suppress adult cells responsive to TNP-SRBC.

**Discussion**

It has been more than two decades since Billingham et al. (14) demonstrated that tolerance is more readily induced in young animals than in adults. Yet, the explanation of their findings with regard to the role of T and B cells has not yet been clarified. The demonstration of heightened suppressor T-cell activity (15, 16) and minimal helper T-cell activity in neonates (15) has been interpreted as indicating that this imbalance of T-cell functions is solely responsible for the neonatal susceptibility to induction of tolerance. This explanation is excluded by recent observations (1, 2) that neonatal B cells are far more susceptible than adult B cells to induction of tolerance as determined by challenge with TD antigens. These findings suggest that clonal deletion of B cells occurs in early life and is a major mechanism for development of self-tolerance (17).
The evidence regarding age-dependent changes in susceptibility of B cells to tolerance induction as assayed by TI antigens contrasts with the above observations using TD immunogens. Using type 2 pneumococcal polysaccharide, Siskind et al. (3) established that newborn mice are no more susceptible than adults to tolerance induction. More recently, Howard and Hale (4) have reached the same conclusion using three other TI polysaccharide antigens.

In this report, we directly approached the question of the relative tolerance susceptibility of adult and neonatal splenic PFC precursors responsive to TD and TI antigens by using a nonimmunogenic hapten carrier complex (TNP-HgG) to induce tolerance in both precursor populations. The results indicate that whereas neonatal B cells responsive to TD antigens are hypersusceptible to tolerance induction as compared to adult B cells, B cells from both adults and neonates that are reactive to a TI form of the antigen display the same level of susceptibility. Moreover, this level of susceptibility is similar to that of neonatal responders to a TD antigen, i.e., the precursor cells are markedly susceptible to induction of tolerance.

The difference in susceptibility to tolerance induction between cells responsive to TD and TI forms of the antigen in adults can be explained by postulating that the two forms of antigen stimulate different B-cell populations. This concept is supported by the demonstration (18) that the precursors stimulated by
TI antigens to produce IgM PFC are on the average larger than IgM PFC
precursors stimulated by TD antigens. In addition, those cells responsive to TD
antigens apparently bear complement receptors, whereas those responsive to TI
antigens do not (19).

Ontogenetic studies indicate that the ability to respond to TI antigens precedes
responsiveness to TD antigens (20), but this interpretation is complicated by the
wide variation in time of onset of responsiveness within the TD and TI groups of
antigens. Nevertheless, responsiveness to TD antigens appears at approximately
the time that cells bearing IgD (21) and complement receptors (22, 23)
become readily detectable in spleens of neonatal mice. We have previously
demonstrated that the large blast-like cells in mouse spleens which are recently
derived from the bone marrow (24) bear only IgM (25). There is data to suggest
that these large, IgM-bearing cells which are minor constituents of adult spleens
and major constituents of neonatal spleens are predecessors of IgD-bearing cells
(21, 26-28).

The results of the present study and the other findings discussed above
suggest the model presented above. We suggest that large, immature cells
which bear IgM only are the major precursors for IgM PFC in neonatal spleens.
These precursors are responsive only to TI antigens and hypersusceptible to
tolerogen. As animals mature, a second population of precursors of IgM PFC
appear. These precursors are generally smaller, bear IgM, IgD, and C' recep-
tors, and respond only to TD antigens. These smaller cells which predominate
among adult splenic B cells are resistant to tolerance induction.

What cell accounts for the primary antibody response to TD of neonates? One

possibility is that a small number of B₂ are the responsive cells in neonatal spleens, that tolerance is induced only in B₁, and that the failure of B₁ to differentiate into B₂ as a result of tolerance induction results in a markedly decreased B₂ population and therefore a depressed primary response. Another major possibility is that there is a transitional cell between B₁ and B₂ which can respond to TD antigens and yet be readily tolerized. Further information about these hypotheses can be obtained by study of populations enriched for particular surface markers.

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