DIFFERENTIAL SUSCEPTIBILITY OF NEONATAL AND ADULT MURINE SPLEEN CELLS TO IN VITRO INDUCTION OF B-CELL TOLERANCE*

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It was recently hypothesized (1) that the two major immunoglobulin isotypes on the surface of B cells confer a different reactivity on the cell: IgM which appears early in ontogeny is a "tolerizing" receptor and IgD which appears later is a "triggering" receptor. As a first step in investigating this possibility, we have examined the relative susceptibility of neonatal B cells (which have virtually only IgM) and a population of adult B cells (which have both IgM and IgD) to tolerance induction.

Materials and Methods

Tolerance was induced in vitro using a variation of the technique described by Kettman (2) which is represented schematically in Fig. 1. Trinitrophenyl-human gamma globulin (TNP-HgG) was chosen as the tolerogen because of its well-characterized potential as an inducer of B-cell (and T-cell) tolerance in vitro (3). Spleen cells from adult (8- to 10-wk old) and neonatal (9- to 12-day old) (C57Bl/6 × DBA/2)F1 mice were harvested and treated with an antibrain ø-serum (4) and complement (C) to kill T cells. This treatment killed 46% of neonatal and 41% of adult spleen cells. The cells were washed and cultured at a density of 10^7 cells/ml in complete medium in the presence of varying concentrations of tolerogen (TNP17HgG). After an incubation period of 24 h, cells were washed three times with cold balanced salt solution (BSS) to remove the tolerogen and mixed with equal numbers of X-irradiated (1,500 R) spleen cells from adult mice which had been primed by intravenous injection of 200 µl of a 0.01% suspension of sheep erythrocytes (SRBC) 5-10 days before sacrifice (5). The addition of this cell population ensured that an excess of carrier-specific thymus-derived cells would be present during subsequent immunogen stimulation allowing measurement of functional bone marrow-derived anti-TNP and anti-SRBC precursor cells. The cell mixture was cultured at a cell density of 10^7 cells/ml in the presence of 100 µl of a 0.1% suspension of heavily substituted TNP-SRBC (6) per milliliter of culture. Cultures were incubated for 4 days (7) before being harvested and assayed (8, 9) for SRBC- and TNP-specific direct plaque-forming cells (PFC). In preliminary experiments, it was found that in the presence of excess helper cells, the anti-TNP and anti-SRBC PFC responses were linearly related to the number of B spleen cells cultured, irrespective of prior treatment of these cells with anti-ø and C'. Similarly, treatment of the helper cell population with anti Ly-2.2 and C' (which has been shown to eliminate suppressor T cells (10, 11)) did not affect the ability of these cells to cooperate with the B-cell population nor did it effect the ultimate responsiveness of the B cells. In other control experiments, addition of a large excess of underivatized HgG (50 times the amount of TNP17HgG) during the phase of tolerance induction did not effect the dose response of the B cells to tolerogen. This result argues against the involvement of Fc receptors in tolerance induction using TNP-HgG.

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Fig. 1. Protocol for the induction and assay of tolerance in murine splenic B cells. HRBC, horse erythrocytes.

Fig. 2. Effect of the dose of tolerogen on the induction of tolerance in neonatal and adult splenic B cells. The response to SRBC represents a control for the specificity of tolerance. PFC expressed per input B cell.

Results and Discussion

As shown in Fig. 2, adult TNP PFC were suppressed at tolerogen concentrations \( \geq 1 \mu g/ml \) with a suppression of 50% in PFC being reached at a dosage of \( \approx 10 \mu g/ml \). The SRBC response remained at constant levels regardless of the tolerogen dosage, indicating that the suppression is antigen specific. In the neonate, suppression was observed at tolerogen concentrations \( >0.001 \mu g/ml \) with 50% suppression being attained when tolerogen concentration reached \( \approx 0.01 \mu g/ml \). SRBC responses remained at constant levels except when TNP, HgG concentrations 1,000-fold greater than that giving detectable TNP suppression were used. At this point, the SRBC response began to decline. As the SRBC responses indicate, however, there is no inhibition of the response evident at the tolerogen levels which give optimal TNP-specific suppression.

Thus, there is a striking difference in the susceptibilities of neonatal and...
adult B cells to tolerance induction by TNP\textsubscript{11}HgG. 50% suppression required almost 10\textsuperscript{3} times more tolerogen in adult than neonatal cells. Previous to this report, heightened susceptibility of neonates to tolerance induction (12) was thought to result solely from an elevated suppressor cell activity in these animals (13). We have excluded suppressor T-cell effects in our system by treating cells to be used as the source of B cells with anti-\theta brain antibody and C before tolerance induction and cells to be used as the source of helper T cells with anti Ly-2.2 and C before mixing with B cells.

The mechanism(s) underlying the marked difference in tolerogen sensitivity of splenic B cells from 10- to 12-day-old and 8- to 10-wk-old BDF\textsubscript{1} mice is not known. The speculation that prompted this study, i.e., that a difference in the isotype of the receptor might determine susceptibility to tolerance is an attractive one for the following reasons. IgM first appears on murine lymphoid cells early during ontogeny and presumably mediates specific antigen binding by these cells, which occurs as early as 15 days of gestation (14). At this time the neonate appears to possess a repertoire of antigen specificities equal to that of the adult (14, 15); yet its splenic B cells are incapable of mounting an antigen-specific immune response of the magnitude predicted by the size of the antigen-binding cell population until at least 2-3 wk later (14). It is interesting that during the early period of relative antigen refractoriness the neonates' splenic B cells respond to lipopolysaccharide like their adult counterpart, i.e., they develop into antibody-forming cells (16). It is during the next phase of development when cells become responsive to antigen that the second cell surface isotype, the IgD-like molecule, reaches appreciable levels (17). The minimal PFC responses seen before 2 wk of age may reflect the size of the IgD\textsuperscript{+} cell population at the time of challenge. Cells bearing only IgM which come in contact with tolerogen may be prevented from acquiring IgD.

A finding that could be relevant to the observed variation in tolerogen susceptibility is that receptors on newborn murine splenocytes are not readily regenerated after "capping" by anti-Ig in contrast to splenocytes from adult mice (18, 19). Thus, the capacity to regenerate receptors could be a critical factor in determining B-cell susceptibility to tolerance. A restricted capacity to regenerate receptors is also suggested by the heightened susceptibility of neonates to anti-idiotypic antibody-mediated suppression (20). It would be informative to determine if receptor turnover is related to isotype or age of the animal or both.

Our results are equally consistent with other alterations in the B-cell population of the maturing mouse. For example, the C receptor which has been implicated in "triggering" of B lymphocytes also is acquired at about the time that IgD appears (21).

**Summary**

The relative susceptibility of neonatal and adult murine splenocytes to induction of B-cell tolerance was studied in vitro. Adult cells required approximately 1,000-fold more trinitrophenyl-human gamma globulin to be rendered tolerant than did cells from 9- to 12-day-old neonates. The potential effects of suppressor T cells were excluded by pretreating the cultured B cells with anti-Thy-1 and C and the helper T cells with anti-Ly-2.2 and C. The possible role of cell surface
immunoglobulin isotypes in contributing to this observed difference is discussed.

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