

## MECHANISMS OF CLONAL ABORTION TOLEROGENESIS

### I. Response of Immature Hapten-Specific B Lymphocytes\*

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Recent experiments from this laboratory have revealed a special sensitivity of immature, differentiating B lymphocyte populations to the induction of immunological tolerance. Thus, whether the cells were derived from newborn mouse spleen (1, 2); the differentiating pool from adult mouse bone marrow (2, 3); or the spleens of lethally irradiated mice being repopulated with fetal liver stem cells (4), they could be tolerized by minute concentrations of antigen, which left mature B cells either unaffected or immunized. These findings, which were interpreted as favoring the clonal abortion theory of immunological tolerance (3), have recently been independently confirmed and extended (5-9), (and some aspects disputed) using each of these three systems. Support for the basic concept has also come from the use of the universal tolerogen, anti- $\mu$  chain antibody (10-11).

Our attempts to study the cellular mechanisms underlying this tolerance-susceptible phase in the life history of the B cell have been handicapped by two related factors. First, all the cell populations that have been tolerized have been heterogeneous cell mixtures, including stem cells, pre-B cells, and Ig-positive lymphocytes of varying degrees of maturity; as well as B cells belonging to different functional subsets and displaying widely varying affinities for the tolerizing epitope. Second, in our hands, effective tolerance induction using such cell mixtures has required coincubation of cells with antigen for at least 36 h, in marked contrast to some models (5, 7), raising the possibility that the target cell for tolerance induction was a precursor and not itself a functional B cell.

For these reasons, we have developed methods for the enrichment of hapten-specific B lymphocytes applicable to both mature and immature cell populations (12, 13). We now report that such hapten-specific B cells, precultured at limit dilution, can be rendered tolerant within 8 h *in vitro* if derived from a newborn mouse spleen, while remaining normally reactive if derived from the adult.

### Materials and Methods

*Animals.* Inbred specific pathogen free mice were used in all experiments. CBA Ca/H Wehi mice, aged either 8- to 10-wk (adults), or 4-6 days (neonates), were used as spleen donors. (CBA  $\times$  BALB/c) $F_1$  hybrids, aged 4- to 6-wk, were used as thymus donors.

*Antigens.* The hapten fluorescein (FLU)<sup>1</sup> was coupled on to human gamma globulin (HGG)

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<sup>1</sup> *Abbreviations used in this paper:* B lymphocyte, bone marrow-derived lymphocyte; FACS, fluorescence-activated cell sorter; FLU, fluorescein; HGG, human gamma globulin; LPS, lipopolysaccharide; PFC, plaque forming cell; POL, polymerized Salmonella flagellin.

(Cohn Fraction II, Commonwealth Serum Laboratories, Parkville, Australia), and polymerized flagellin (POL) as previously described (13). The conjugate used as the tolerogen in all experiments except where otherwise specified, contained 3.6 mol of FLU per mol of HGG (FLU<sub>3.6</sub>HGG).

*Cell Suspensions.* Spleen and thymus cell suspensions were prepared as previously described (14). Thymus cells were treated with anti-Ia serum and complement to eliminate contaminating B cells before their use as filler cells in the microculture system.

*Cell Fractionation Methods.* FLU-gelatin fractionation, as previously described (12-14), was sometimes followed by a second cycle of enrichment of cells of high FLU-binding avidity using the fluorescence-activated cell sorter (FACS) (FACS II, Becton Dickinson Electronics Laboratories, Mountain View, Calif.), as previously described (13). The top 15-20% most fluorescent cells were sorted and after treatment with collagenase to remove adherent antigen, cells were placed into tissue culture.

*Tissue Culture Procedures.* The microculture system used for the limit dilution analysis of clonable anti-hapten precursors in both unfractionated and FLU-gelatin fractionated spleen cell populations has been described in detail elsewhere (13, 14). Briefly,  $5 \times 10^3$ - $2 \times 10^4$  unfractionated or 10-100 prefractionated spleen cells were cultured in the presence of anti-Ia treated thymus filler cells and 0.1  $\mu\text{g/ml}$  of FLU<sub>1</sub>-POL was used as the challenge antigen either immediately or after putative tolerogenesis. FLU-specific PFC determinations were performed 62-66 h after challenge.

*Basic Protocol for Tolerance Induction.* Unfractionated spleen cells or FLU-gelatin fractionated spleen cells were cultured at limit dilution in the presence of thymus filler cells ( $3 \times 10^6$  ml) in culture medium containing various concentrations of FLU-HGG.

After a period usually of 24 h, the supernatant medium of each well was carefully removed and replaced with 0.2 ml of fresh culture medium containing the immunogen FLU-POL (0.1  $\mu\text{g/ml}$ ), and further anti-Ia treated thymocytes, ( $7 \times 10^6$  cells/ml). Cultures were assayed after a further 62-66 h. In some experiments 20  $\mu\text{g/ml}$  *Escherichia coli* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) or 0.1  $\mu\text{g/ml}$  of 4-hydroxy-3-iodo-5 nitrophenyl-POL (NIP-POL) was used as a challenge.

*Assay for Anti-Hapten Plaque-Forming Cells (PFC).* Each individual culture well was assayed for directly hemolytic anti-FLU PFC as previously described (13, 14) using the Cunningham liquid monolayer method to reveal plaque formation. The frequency of clonable anti-FLU PFC precursors was then calculated by Poisson analysis (14).

## Results

*Tolerance Induction in Hapten-Specific Cells Prepared by FLU-Gelatin Fractionation.* Unfractionated newborn spleen cells showed only 30% reduction in clone frequency after 24 h with 5  $\mu\text{g/ml}$  of FLU-HGG (results not shown). Next, cells from newborn or adult spleen were fractionated with the FLU-gelatin technique. This achieves a mean enrichment of clonable anti-FLU-PFC precursors of 130-fold for adult spleen and 560-fold for newborn spleen (13). The FACS profile of the two enriched populations labeled with FLU<sub>4</sub> gelatin shows an identical degree of heterogeneity in antigen-binding avidity. Cells harvested by the technique are >95% Ig-positive in both cases. Using the same limit dilution prespreading technique, the cells were exposed for 24 h to various concentrations of FLU-HGG or to no added antigen prior to immunogenic challenge with FLU-POL. The frequency of anti-FLU PFC clones was determined 66 h after the addition of FLU-POL (Fig. 1). With adult FLU-enriched cells, no reduction in the frequency of anti-FLU PFC clones was detected at any concentration below 25  $\mu\text{g/ml}$ . Increasing concentrations of tolerogen caused increasing degrees of reduction in clone frequency among the newborn FLU-specific cells. A 50% effect was seen at a concentration of about 0.08  $\mu\text{g/ml}$  of antigen. With adult FLU-enriched cells (results of two experiments, data not shown) 50% reduction

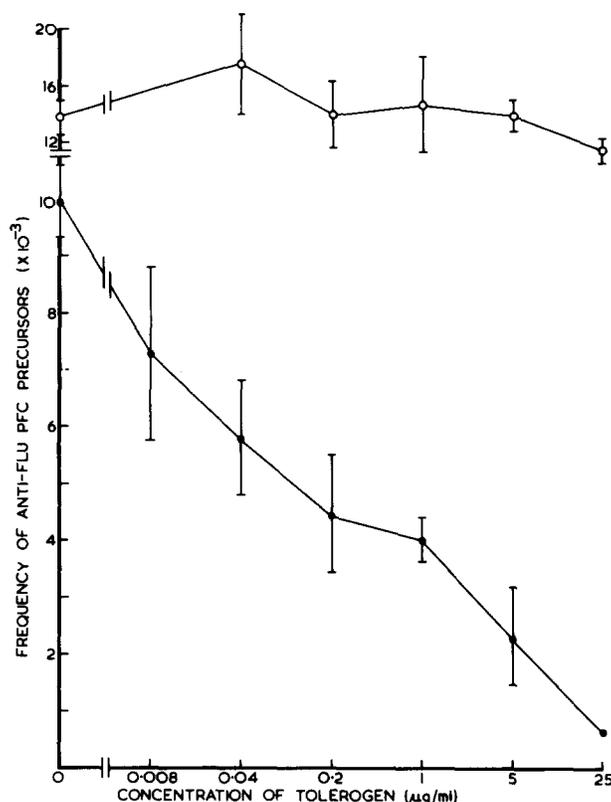


FIG. 1. Effect of 24 h exposure to increasing concentrations of FLU<sub>3.6</sub>-HGG before immunogenic challenge on the cloning frequency of anti-FLU PFC precursors in FLU-enriched cells from adult (○) and neonatal (●) spleen donors. Data shown for adults represents a pool of four to seven experiments and for neonates, a pool of four experiments. The average value obtained in two experiments is given where no error bars are shown.

required 80 μg/ml of antigen. Thus, despite their equivalent antigen-binding capacity, the immature hapten-enriched cells were 1,000-fold more sensitive to tolerogenesis than adult hapten-enriched cells.

*Effects of Challenge by Antigen or LPS after Tolerogenesis.* Studies by Cambier et al. (7) have claimed that immature T-dependent B cells are more sensitive to tolerance induction than their adult counterparts, but that both immature and mature T-independent cells are equally and extremely sensitive. As the polyclonal B-cell activator, LPS, appears to be capable of substituting for some T-cell functions, we challenged FLU-enriched B-cell populations (that had been tolerized with FLU-HGG) with either FLU-POL or LPS. The results (Table I) show that an equivalent degree of tolerance was obtained with either challenge system in the neonatal FLU-enriched cells without any reduction in responsiveness being shown in the adult population. Furthermore, the presence of LPS during the tolerogenesis phase abrogated the tolerogenic influence of FLU-HGG (results not shown), in agreement with Louis et al. (15).

*Tolerance Induction in Cells Sequentially Fractionated on FLU-Gelatin Dishes and FACS.* Previous work (13) has shown that when FLU-gelatin fractionated B cells are

TABLE I  
*Comparative Effects of Antigen and LPS as Challenge Stimuli for Tolerized, Hapten-Enriched B Cells*

Concentration of FLU <sub>3,6</sub> HGG during preculture, $\mu\text{g}/\text{ml}$	Frequency of anti-FLU PFC precursor ( $\times 10^{-3}$ )			
	Adult enriched cells challenged with:		Neonatal enriched cells challenged with:	
	FLU-POL	LPS	FLU-POL	LPS
0	25.0	23.9	11.0	15.6
5	22.4	29.5	3.73	4.29
1	22.4	29.5	4.74	8.17
0.2	19.4	19.2	5.11	6.06

FLU-enriched cells were precultured in the presence or absence of tolerogen as indicated for 24 h before immunogenic challenge with either 0.1  $\mu\text{g}/\text{ml}$  of FLU-POL or 20  $\mu\text{g}/\text{ml}$  of LPS. The frequency of anti-FLU PFC precursors was determined 3 days after immunogenic challenge. Results shown are from one representative experiment.

labeled with FLU-gelatin and sorted in the FACS such that the 10% of cells with the highest fluorescence intensity are collected, there is a further increment by a factor of 5 in the frequency of clonable anti-FLU PFC precursors. The next-lower 10% in fluorescence intensity show a slightly lower frequency. Using newborn spleen cells, there are some logistic difficulties in collecting enough cells for extensive experimentation, as 200 spleens yield only  $15 \times 10^8$  cells, of which  $2 \times 10^5$  can be recovered from FLU-gelatin fractionation. Therefore, in the sequential fractionation experiments to be described we used the 15–20% most intensely fluorescent cells. Again such cells were distributed at limit dilution and exposed to tolerogen for 24 h, challenged with FLU-POL as described, then assayed for anti-FLU PFC after 3 days of further culture. The results (Table II) indicate that the sequentially enriched, avidly FLU-binding cells from newborn mice were even more susceptible to tolerance induction than the more heterogeneous FLU-gelatin binding cells.

*Kinetics of Tolerance Induction in Newborn Hapten-Specific Cells.* The kinetics of tolerance induction were studied using FLU-gelatin fractionated cells. Neonatal FLU-enriched cells were exposed to tolerogen for varying time periods before challenge with FLU-POL. A no antigen control was included at each time point and the frequency of anti-FLU PFC precursors was determined 3 days after challenge. The pooled results of two experiments (Fig. 2) show that with 5  $\mu\text{g}/\text{ml}$  of FLU-HGG, no detectable tolerance was induced within 2 h, but by 8 h, 60% of the clonable hapten-specific cells had been tolerized. Progressively longer exposure periods caused tolerance in somewhat more cells, but a residuum of nontolerizable cells persisted even after 48 h tolerance induction. With 1  $\mu\text{g}/\text{ml}$  of tolerogen, the pattern was similar but a longer exposure period was required to induce a given degree of tolerance.

*Behavior of Hapten-Specific Cells from Mice of Various Ages.* Spleen cells from mice of various ages were fractionated on FLU-gelatin dishes. The FLU-enriched cell populations were subjected to 24 h preculture in the presence or absence of 5  $\mu\text{g}/\text{ml}$  of FLU<sub>3,6</sub>HGG, then challenged with FLU-POL as described followed by analysis for anti-FLU PFC clones 3 days later. The pooled results of two experiments showed that the susceptibility to tolerance induction decreased with increasing age of spleen

TABLE II  
*Tolerance Induction in FACS Sorted, FLU-Gelatin Prefractionated Neonatal Spleen Cells*

Concentration of FLU <sub>3,6</sub> HGG during 24 h preculture, $\mu\text{g/ml}$	Frequency of anti-FLU PFC precursors as percent of control response	
	FLU-GEL fractionated	Top 15-20% FACS sorted enriched cells
5	23 $\pm$ 8	ND
1	35 $\pm$ 4	22 $\pm$ 7*
0.2	41 $\pm$ 9	27 $\pm$ 9*
0.04	54 $\pm$ 12	31‡

\* Pool of three experiments.

‡ One experiment only.

donors. At 9 days of age, 50% of the FLU-enriched cells were able to be tolerized; at 16 days, only 25%; and by 30 days of age no tolerance could be shown.

*Effects of Highly Multivalent Hapten—HGG Conjugates in Tolerogenesis.* In all of our work on clonal abortion tolerogenesis to date, oligovalent hapten-protein conjugates have been used. In the work of Cambier et al. (7), which claims differences in the tolerizability of T-dependent and T-independent B cells, highly multivalent antigens were used. Accordingly, we tested the effects of increasing the hapten substitution rate on tolerance induction in B lymphocyte populations in our system. FLU-gelatin enriched cells were labeled at 0°C for 30 min with 5  $\mu\text{g/ml}$  of either FLU<sub>3,6</sub>HGG or FLU<sub>12</sub>HGG, and examined in FACS. The results (not shown) showed only a modest degree of enhanced fluorescence intensity among the cells exposed to the more highly substituted reagent. Next, the various B-cell populations were exposed to varying concentrations of FLU<sub>12</sub>HGG for 24 h in vitro before immunogenic challenge with FLU-POL and subsequent analysis for anti-FLU PFC precursors as described. It is evident (Fig. 3) that FLU<sub>12</sub>HGG is a much more effective tolerogen than the oligovalent material. For example, a concentration of 1  $\mu\text{g/ml}$  resulted in a 50% reduction in the frequency of anti-FLU PFC clones in unfractionated adult splenic B cells. A slightly lower concentration was required to produce an equivalent reduction in adult FLU-enriched splenic B cells. With this tolerogen, the neonatal B cells are considerably more sensitive. For example, 24 h exposure to 0.2  $\mu\text{g/ml}$  FLU<sub>12</sub>HGG barely affected the subsequent cloning frequency of adult FLU-enriched cells, whereas, with 0.2  $\mu\text{g/ml}$  the subsequent cloning frequency of neonatal FLU-enriched splenic B cells was reduced to <20% of the control value.

### Discussion

In its simplest form the clonal abortion theory of B lymphocyte tolerance states that B cells mature from a condition where they are tolerizable but not immunizable to one where they are immunizable but not tolerizable. That this absolute formulation is not correct has been clear for some time, for example, because of the tolerizability of adult spleen B cells by supraimmunogenic doses of multivalent antigens (16). Nevertheless, our previous work in this field (1-4) strongly suggested that there were circumstances under which the genesis of new, clonable B lymphocytes reactive to a particular antigen could be abrogated by exposure of the maturing cells to very low

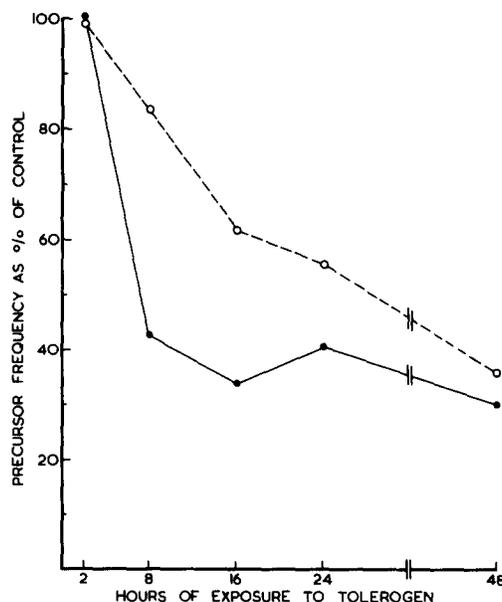


Fig. 2. Kinetics of tolerance induction in neonatal FLU-enriched cells exposed to either 5  $\mu\text{g}/\text{ml}$  (●) or 1  $\mu\text{g}/\text{ml}$  (○) of FLU<sub>3,6</sub>HGG before immunogenic challenge with FLU-POL. Precursor frequencies shown are normalized to the respective no tolerogen control frequency for each time point. Pooled results from two separate but identical experiments are shown.

concentrations of antigen. In those experiments, no evidence was obtained to suggest that the small number of clonable B cells in the heterogeneous, differentiating mixture of cells was being affected. The noteworthy difference between the previous studies and the present ones is that we have now placed emphasis on a much more homogeneous population of cells harvested from immature animals. We have eliminated from consideration all the cells incapable of binding antigen, i.e. all stem cells and pre-B cells, and furthermore have enriched for cells of high avidity for the FLU hapten. This has allowed the delineation of a functionally immature B cell, itself capable of tolerization by a nonimmunogenic hapten-protein conjugate at low concentrations that leave fully mature B cells unaffected. The existence of such a cell, simultaneously tolerizable and immunizable, had been strongly suggested by the work of Metcalf and Klinman (5, 8) but had not emerged through our prior work. In fact, our identification of this differentiation state removes the last major difference between their results and conclusions, and our own.

The clonal abortion theory must now be restated as in Fig. 4. We stand by the previous results and conclusions which showed that neogenesis of direct progenitors of antibody-forming cells can be aborted, presumably through an effect on those cells which first display a small amount of Ig receptors. The next stage of differentiation is an immature B cell. This differs from the pre-B cell in having sufficient Ig receptors to adhere to hapten-gelatin and to exhibit membrane immunofluorescence when stained with anti-Ig sera. This immature B cell is still capable of being tolerized by low concentrations of oligovalent hapten-protein conjugates that leave fully mature B cells of equivalent antigen-binding avidity unaffected (Fig. 1). However, this same

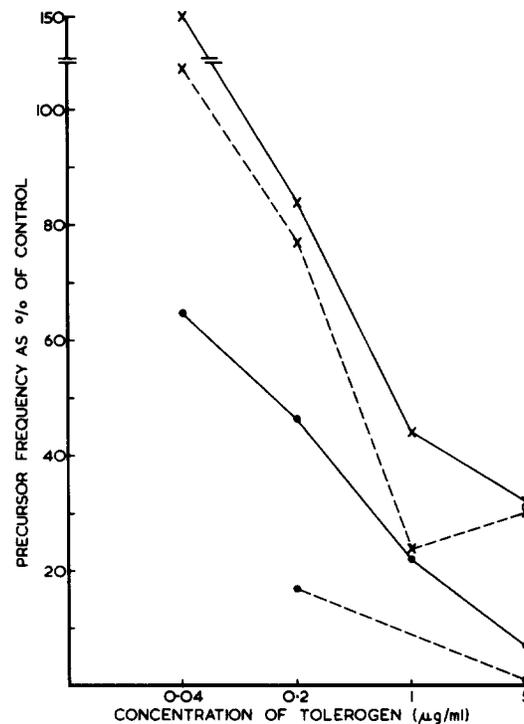


FIG. 3. Effect of 24 h exposure to FLU<sub>12</sub>HGG before immunogenic challenge with FLU-POL on the responses generated in unfractionated adult (X—X) and neonatal (●—●) spleen cells, and adult (X-----X) and neonatal (●-----●) FLU-gelatin enriched cells. Data shown represents normalized precursor frequency values from one experiment.

immature B cell is capable of being triggered by appropriate stimuli such as T-independent antigens or LPS. In contrast to a pre-B cell, which fails to mature into an Ig-positive clonable direct progenitor in the concomitant presence of tolerogen plus LPS (3), immature B cells as defined above have their tolerization threshold raised when tolerogen and polyclonal activator are exhibited simultaneously. It is clear that the difference in susceptibility to negative signals of the various differentiation stages is relative rather than absolute. This is well illustrated by the effects of FLU<sub>12</sub>HGG, an effective tolerogen of mature B cells (Fig. 3), or by the phenomenon of effector cell blockade (17, 18) where fully-differentiated antibody-secreting cells receive a negative signal from high concentrations of multivalent antigen, and lower their antibody secretion rate.

As regards the question of the relative tolerizability of T-dependent and T-independent B cells, we maintain, in contrast to Cambier et al. (7), that T-independent immature B cells are more susceptible than their adult counterparts, whether oligovalent or even more highly tolerogenic multivalent conjugates are used. In fact, preliminary investigations suggest that the tolerizability of B cells as measured by T-independent and T-dependent challenge systems closely resemble each other. We remain to be convinced that these two sorts of B cells really represent different lineages of differentiation rather than, for example, sequential maturation states. Even that

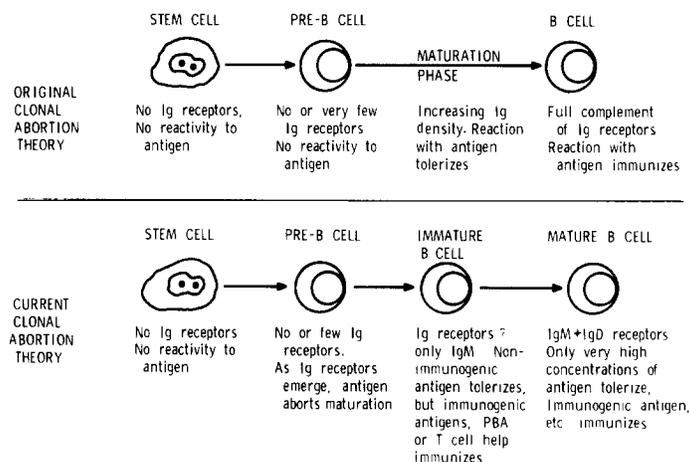


FIG. 4. Restatement of the clonal abortion theory.

possibility is rendered dubious by the efficient performance of fetal B cells in the T-dependent Metcalf and Klinman system (5).<sup>2</sup>

We consider that the present system, where B cells of relatively homogenous character and with high avidity for antigen, can be rendered tolerant within 8 h, represents a good opportunity to study the cellular mechanisms involved in tolerance induction of immature B cells. Our previous work was always handicapped by the long time in culture required for a significant effect. Not only did the *in vitro* maturation of cells make it difficult to determine which cell was actually being affected by the tolerogen, but also the death of cells in culture made it difficult to separate true effects from differential survival of cells. Within the 8-h period for the present tolerogenesis regime, nonspecific death in control cultures is much less, and studies of receptor dynamics and re-expression, or of effects on cell metabolism become much more feasible.

### Summary

B lymphocytes with receptors specific for the hapten fluorescein (FLU) were prepared from the spleens of mice of various ages. For most experiments, a one-step fractionation procedure based on the adherence of FLU-specific cells to FLU-gelatin was used. For some experiments, a subset of higher FLU-binding capacity was prepared from the FLU-gelatin binding population through the use of the fluorescence-activated cell sorter (FACS). FLU-specific B cells were placed into microculture with either FLU<sub>3,6</sub>-human gamma globulin (FLU<sub>3,6</sub>HGG) or FLU<sub>12</sub>HGG usually for 24 h at 37°C. The tolerogen was then removed and 0.1 μg/ml of a T-independent antigen, FLU-polymerized flagellin, was substituted. 3 days later, cells were harvested from the microcultures and assayed for FLU-specific plaque-forming cells to determine any reduction in clonable hapten-specific B cells which the tolerogenesis treatment might have induced.

The results showed that with FLU<sub>3,6</sub>HGG, hapten-specific newborn B cells could

<sup>2</sup> Teale et al. Manuscript in preparation.

be tolerized at 1,000-fold lower tolerogen concentrations than adult splenic B cells of equal antigen-binding capacity. The high-avidity subset was even more susceptible to tolerance induction. Tolerance could be induced within 8 but not within 2 h, and at lower tolerogen concentrations, longer periods of tolerogenesis were required for a given effect. Using a 24-h tolerogenesis phase, 50% reduction in clone frequency among newborn FLU-gelatin fractionated cells was achieved at 0.08  $\mu\text{g/ml}$  of FLU<sub>3,6</sub>HGG. Tolerance induction in immature B cells was inhibited by the concomitant presence of a polyclonal B-cell activator, *Escherichia coli* lipopolysaccharide (LPS) but tolerance once induced, was stable to challenge with LPS. Tolerogenesis was hapten specific. The proportion of tolerizable cells in spleens decreased with increasing age, reaching 50% at around 9 days.

FLU<sub>12</sub>HGG proved a more powerful tolerogen than FLU<sub>3,6</sub>HGG. It had an effect on adult cells, 50% reduction in clone frequency being noted at around 1  $\mu\text{g/ml}$ . However, and in contrast to results claimed for other T-independent systems, there still was a major difference between immature and mature B cells, the immature cells displaying much greater sensitivity to tolerogenesis.

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