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Immunology

Clonal anergy: Persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen

(fluorescence-activated cell sorter/antibody formation/fluorescein-conjugated human gamma globulin)

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ABSTRACT **The purpose of these experiments was to determine the degree of reduction in the number of antigen-binding B lymphocytes in the spleens of mice that had been rendered tolerant in the perinatal period. Newborn or pregnant mice were injected with fluorescein (Flu) coupled onto human gamma globulin, and the spleen cells of the neonatally injected mice, or of the offspring of the pregnant mice, were analyzed 1–6 weeks later. Tolerogen doses were chosen so as to achieve either a two-thirds reduction (low dose) in the number of anti-Flu B cells capable of yielding anti-hapten plaque-forming cell clones after *in vitro* stimulation, or as representing a supra-optimal tolerogenic stimulus (high dose). Antigen-binding B cells were studied by a two-cycle procedure, namely an initial cycle of binding to Flu-gelatin thin layers, followed by analysis of the binding cells in the fluorescence-activated cell sorter (FACS) after suitable staining with Flu-protein conjugates. With the high dose of tolerogen, a modest diminution in Flu-binding cell numbers down to 56–71% of control values could be induced. When these residual Flu-specific B cells were analyzed in the FACS to quantitate their spectrum of Flu-binding avidities, profiles identical to those of controls were obtained. The reduction proved transient in nature, binding cell numbers having returned to 80% of normal by 2 weeks and to normal by 6 weeks. Nevertheless, the Flu-specific B cells were incapable of responding to antigen or mitogen by antibody formation. With the low dose of tolerogen, despite the desired degree of functional silencing of Flu-specific B cells, the numbers and avidity spectra of antigen-binding cells were entirely normal in both the neonatally injected and *in utero*-injected groups. The results indicate that tolerance induced amongst immature B lymphocytes is not due to a physical elimination of the relevant B cell clones or to a modulation or blockade of their surface Ig receptors. Rather, it is due to the recognition and storage of negative signals amongst cells that continue to display a normal complement of receptors. We therefore propose that the term "clonal anergy" is a more accurate description than either "clonal deletion" or "clonal abortion."**

It is now well established that immature B lymphocytes are extremely sensitive to tolerogenic signals both *in vitro* and *in vivo* (1–13). The question remains, however, whether induction of tolerance is accompanied by an actual elimination of the immature lymphocyte or rather by a modification of its function. One modification that would effectively prevent a B lymphocyte from responding to immunogenic antigen would be a disappearance of the immunoglobulin (Ig) receptors from the plasma membrane, and immature B cells can in fact have their Ig receptors readily and irreversibly removed through anti-Igs activating the receptor patching–capping–endocytosis cycle (14, 15). If a tolerogen acted in either of these two ways, the result would be a decreased number of B cells with receptors for the epitope in question. The literature on antigen-binding cell numbers in animals rendered tolerant in early life is confused. Some authors have observed substantial reductions (16–18), whereas others have reported only modest (19) or no (20) diminution. Some of the above studies were performed before the importance of suppressor T cells in tolerance was appreciated, and may not have represented B cell tolerance. Furthermore, the radioautographic enumeration of rare antigen-binding cells is tedious, rendering accurate quantitation difficult. The availability of the fluorescence-activated cell sorter (FACS) prompted a new look at the question.

MATERIALS AND METHODS

Mice. Inbred CBA Ca/H Wehi mice were used in all experiments. Mice used as newborns were 2–3 days old. Pregnant CBA mice, gestating syngeneic fetuses, were used 14.5 days after impregnation was deemed to have occurred through the finding of a vaginal plug. (CBA × BALB/c) F_1 hybrids, 4–6 weeks old, were used as thymus donors for tissue cultures.

Antigens. The hapten fluorescein (Flu) was coupled onto human gamma globulin (HGG) (Commonwealth Serum Laboratories, Parkville, Australia), gelatin, and polymerized flagellin (POL) as described (21). The freshly deaggregated (4) Flu-HGG conjugates used as tolerogens in these studies had a substitution ratio of 5 mol of Flu per mol of HGG (Flu_5-HGG) unless otherwise specified. For analyses of antigen-binding cells, Flu_5-gelatin and Flu_1-POL were used as antigens. Antibody formation *in vitro* was induced by either the "T-independent" antigen Flu_1-POL at 0.1 μg/ml or the polyclonal B cell activator *Escherichia coli* lipopolysaccharide (LPS) (batch 0111:B4, Difco) at 20 μg/ml.

Isolation of Flu-Gelatin-Binding Cells. The methods used for the preparation of spleen cell suspensions and the subsequent fractionation procedure for the isolation and recovery of Flu-gelatin-binding B cells, greatly enriched for specific anti-hapten reactivity, have been described (21–24). Briefly, spleen cells were subjected to erythrocyte and damaged cell removal procedures, suspended in balanced salt solution containing 0.1% gelatin, rocked for 15 min at 4°C in petri dishes coated with a thin layer of Flu-gelatin, and immediately washed extensively with balanced salt solution (0°C) to remove all unbound cells. Each dish was then placed on a grid under a dissecting stereo microscope, the number of bound cells was counted, and the cells were recovered by melting the gelatin at 37°C. Finally, the cells were treated with collagenase (100 μg/ml, 15 min at 37°C). The number of gelatin-binding cells was also determined for each experimental and control group, by fractionation on processed but nonhaptenated gelatin. This number (about 30% of the Flu-gelatin-binding number) was then subtracted from the Flu-gelatin-binding number of the identical group to arrive at a net figure for Flu-binding cells.

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Abbreviations: Flu, fluorescein; FACS, fluorescence-activated cell sorter; HGG, human gamma globulin; LPS, *Escherichia coli* lipopolysaccharide; PFC, plaque-forming cell; POL, polymerized flagellin; s-Ig, surface immunoglobulin.

FACS Analysis of Flu-Gelatin-Binding Cells. The FACS (FACS II, Becton Dickinson Electronics Laboratory, Mountain View, CA) was used to analyze the glancing angle light-scattering behavior and fluorescence intensities of the Flu-gelatin-binding cells, detailed techniques being published elsewhere (1–3, 21, 25, 26). Briefly, Flu-gelatin-binding cells were exposed to the carrier POL (500 μg/ml, 15 min at 4°C) labeled with Flu-POL (5 μg/ml, 15 min at 4°C), washed by centrifugation through fetal calf serum, resuspended in balanced salt solution/fetal calf serum and subjected to FACS analysis. The fluorescence intensity distributions for all viable cells falling within the lymphocyte peak measured by using low angle light-scattering characteristics (3, 27) were reprocessed and are shown as the percent cells above a specified fluorescence intensity channel.

***In Vitro* Assay for Antibody Formation.** The microculture system used for the assessment of the functional capacity of the Flu-gelatin-binding cell populations has been described in detail (23, 24). Briefly, Flu-gelatin-binding cells were cultured for 3 days at limiting dilution (50–100 cells per culture) in the presence of anti-Ia-antigen-treated thymus filler cells (24) in medium containing either Flu-POL at 0.1 μg/ml or LPS at 20 μg/ml, after which each culture was assayed for anti-Flu plaque-forming cells (PFCs) and the frequency of anti-Flu PFC precursors was determined by Poisson analysis (23).

RESULTS

Numbers of Flu-Gelatin-Binding Cells in Mice Rendered Tolerant as Newborns. Previous work (4) had shown that 1 μg of Flu_4-HGG injected into newborn mice produced a significant reduction in the number of clonable splenic anti-Flu B cells within 1 week, and that 10 μg produced a maximal effect of over 90% reduction. Accordingly, newborn mice were given either 1 μg as representing a dose in a sensitive portion of the dose–response curve, or 100 μg as representing a supra-optimal tolerogen dose. Spleens were analyzed for their content of Flu-binding cells (Table 1). With the low tolerogen dose, there was no reduction and with the high dose there was only a modest reduction to 56% of control numbers by 1 week. When a more highly tolerogenic (1), heavily haptenated conjugate was used instead of the oligovalent Flu_5-HGG, no greater reduction in antigen-binding cell numbers was obtained. With Flu_5-HGG at 100 μg, the reduction proved to be transient, antigen-binding cell numbers having returned to 80% of the control value by 2 weeks and to 93% by 6 weeks. In contrast, the B cell tolerance as measured by reduction in clonable anti-Flu B cells persisted considerably longer (unpublished data).

Numbers of Flu-Gelatin-Binding Cells in Mice Rendered Tolerant *In Utero*. Previous workers (11, 13) have shown the free passage of analogous tolerogens across the placenta. To study the effect of tolerogen introduced prior to the first appearance of B lymphocytes in the mouse at 16.5 days of fetal life (28), we injected Flu_5-HGG intravenously into pregnant mice at 14.5 days gestation. Low and high doses were chosen to approximate those used for newborns on a weight-adjusted basis. The results (Table 2) show that both doses induced only a modest reduction in Flu-binding cells.

FACS Analysis of Antigen-Binding Cells from Tolerant Mice. Amongst the population of Flu-gelatin-binding cells, there is heterogeneity in both amount of surface Ig and avidity of receptors for Flu. Cohorts of cells capable of binding the most Flu-POL also exhibit the highest *in vitro* cloning efficiency (21). A possibility exists that the nonresponsiveness might simply be due to the removal of a subpopulation of B cells with high avidity for Flu. Accordingly, cells harvested from Flu-gelatin dishes were labeled with Flu-POL and analyzed in the FACS. The results (Fig. 1) showed that for both the neonatally injected and *in utero* injected groups, and with both the low and the high tolerogen doses, the spectrum of Flu-binding intensities of the various groups did not differ significantly from their respective controls.

Given that the curves in Fig. 1 reflect the combined results of surface immunoglobulin (s-Ig) density and anti-Flu avidity, a possibility (however remote) existed that the tolerant groups of Flu-gelatin-binding cells might have exhibited a lower median avidity for Flu but a higher median Ig density, the two effects cancelling each other out. Accordingly, we analyzed the s-Ig density spectrum of Flu-gelatin-binding cells from tolerant mice through FACS analysis after labeling with rhodamine-conjugated anti-s-Ig. The results (not shown) demonstrated that both tolerant and control populations consisted of 97% B cells, and that the s-Ig density spectrum of the two sets of cells was identical. The results therefore indicate not only that the putatively tolerant mice possess antigen-binding B cells but also that these cells exhibit as high avidity for the Flu hapten as do controls.

Confirmation of Nonresponsiveness in Flu-Gelatin-Binding B Cells. Previous work had shown the sensitivity to tolerance induction of immature B cells both *in vitro* (1–3) and *in vivo* (4), whether the cells were challenged with T-inde-

Table 1. Numbers of Flu-binding cells from spleens of mice rendered tolerant to Flu-HGG in the newborn period

Tolerogen injected	Dose, μg	Interval between tolerogen and sacrifice, weeks	No. Flu-binding cells/10^6 spleen cells*	% of control value	P†
0	—	1	119 ± 6.1	100	—
Flu_5-HGG	1	1	149 ± 13.6	125	NS
Flu_5-HGG	100	1	66.8 ± 5.9	56.1	<0.001
Flu_{25}-HGG	100	1	72.2 ± 9.9	60.6	<0.005
0	—	2	146 ± 12.1	100	—
Flu_5-HGG	100	2	117 ± 10	80.1	$0.1 > P > 0.05$
0	—	4	147 ± 7.2	100	—
Flu_5-HGG	100	4	118 ± 6.1	80.3	<0.005
0	—	6	161 ± 18.5	100	—
Flu_5-HGG	100	6	150 ± 19.3	93.2	NS

* As determined by specific adherence to thin layers of Flu-gelatin. Values are mean ± SEM here and in the following tables.

† In this and the following tables, P was determined by Student's t test. NS, not significant.

Table 2. Numbers of Flu-binding cells from spleens of mice rendered tolerant to Flu_5-HGG *in utero*

Dose of tolerogen,* μg	Age of mice at sacrifice,† days	No. Flu-binding cells/10^6 spleen cells	% of control value	P
0	3‡	132 ± 11.7	100	—
10	3‡	68.6 ± 19.7	52	NS
1000	3‡	94.0 ± 17.2	71.2	NS
0	7	108 ± 7.6	100	—
10	7	116 ± 8.1	107	NS
1000	7	73.4 ± 7.9	68	<0.005

* Injected intravenously into pregnant mice at 14.5 days of gestation.

† Because CBA mice are born at 19.5 days gestation, this meant an interval of 8 or 12 days between injection and analysis.

‡ Because the spleens of 3-day-old mice were small, only four dishes were prepared for each dosage in two experiments and therefore the number of samples was too low for the differences to reach statistical significance.

pendent antigen, polyclonal B cell activators, agar mitogens (2), or T-dependent antigen in the presence of helper T lymphocytes (29–31). It remained to document formally that the particular antigen-binding B lymphocytes from the tolerant animals used in the present study were indeed deficient in their capacity to form anti-Flu clones. Accordingly, Flu-gelatin-binding cells from the various groups of putatively tolerant mice were placed into microculture at limiting dilution in the presence of either Flu-POL or LPS to generate anti-Flu antibody-forming cell clones. The results (Table 3) show that the low dose of tolerogen induced substantial partial tolerance, in mice treated both neonatally and *in utero*, as judged by an 81–63% reduction in the number of clonable precursors. As previously observed (21), the PFC clone frequency was higher with LPS as the triggering stimulus. With the high tolerogen dose, more profound tolerance was observed.

DISCUSSION

When the present program of research into B cell tolerance was initiated (5), we coined the term "clonal abortion" to capture the idea that a tolerogen acted not to delete an immature B cell with receptors preadapted for that antigen, as Burnet (32) had predicted, but rather to prevent an s-Ig-negative pre-B cell from maturing into an immunocompetent B cell. To date, none of our extensive exploration of the clonal abortion hypothesis (1–5, 29–31) had concerned itself directly with the question of whether the early encounter with tolerogen actually led to death of the tolerogen-binding B cell, to a failure of receptor appearance with maintenance of a normal life span, or to some other mechanism of its functional silencing. The former alternatives would necessarily imply a reduction in the number of B cells capable of binding the relevant epitope. The accurate quantitation of antigen-binding B cells is fraught with technical difficulties (20). The analytical capacity of the FACS in principle allows more accurate and convenient detection of rare ligand-binding cells. However, our previous studies (21) using the FACS to provide populations of spleen cells enriched for Flu-specific precursor B cells failed to give the degree of enrichment anticipated from a knowledge of the Flu-binding characteristics of cells obtained by other hapten-affinity fractionation procedures. This problem was overcome through the use of the hapten-gelatin fractionation technique (22) as a prelude to FACS analysis.

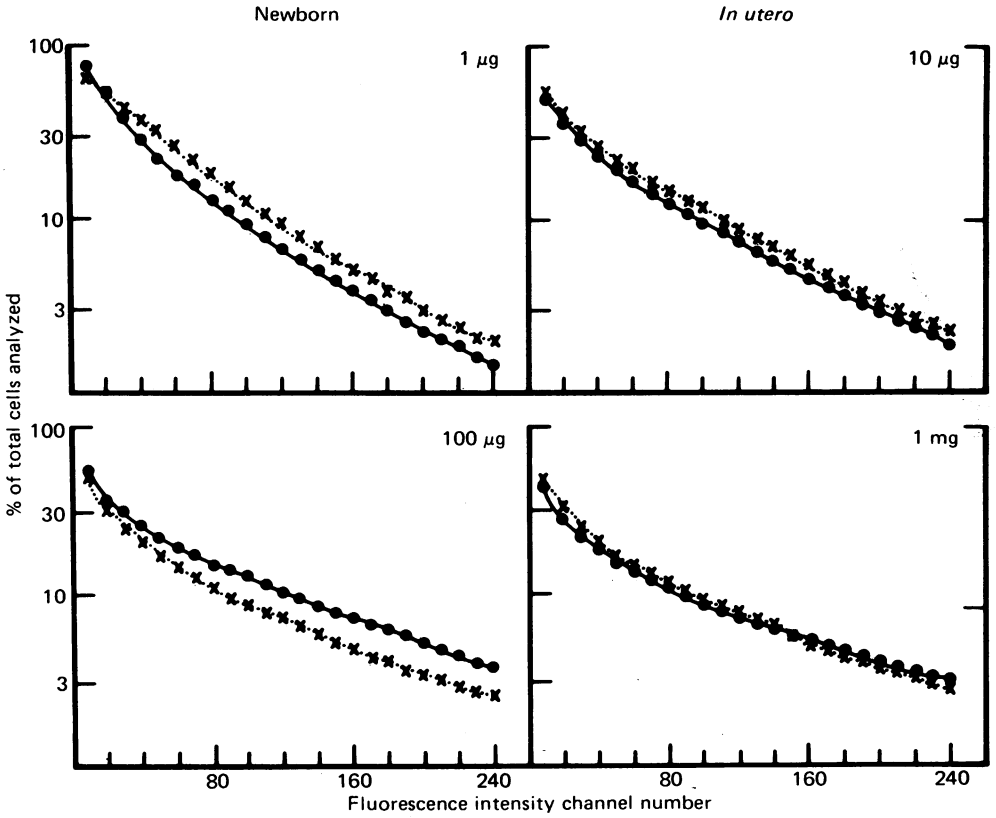

FIG. 1. FACS-generated fluorescence intensity profiles of Flu-gelatin-binding cells from control mice (●) or those rendered tolerant while newborn or *in utero* by injection with Flu-HGG (X). Pooled, normalized results from two experiments are given. Tolerogen doses are indicated.

Table 3. Capacity of Flu-binding cells from tolerant mice to form anti-Flu PFC clones*

Dose of Flu_5-HGG, μg	Timing of tolerance induction	Flu-POL challenge % of control response†	P	LPS challenge % of control response†	P
1	Newborn	37.4 ± 6.5	<0.01	26.8 ± 11.7	<0.05
100	Newborn	6.84 ± 1.92	<0.001	7.78 ± 3.7	<0.001
10	*In utero*	34.0‡	—	18.5	—
1000	*In utero*	13.8 ± 1.61	<0.001	9.45 ± 3.92	<0.001

* Cells harvested from Flu-gelatin dishes were placed in microculture at limiting dilution with thymus "filler" cells, stimulated with Flu-POL or LPS, and harvested 3 days later and assayed for the presence of Flu-specific PFC clones, the number of which was determined by using Poisson statistics. There was no difference in PFC clone size between control and tolerant groups (data not shown).

† Flu-gelatin-binding cells are greatly enriched for anti-Flu PFC precursor B cells. Controls gave the following clone frequencies per 10^4 cells: Flu-POL-stimulated, 95 for newborn and 102 for *in utero* groups; LPS-stimulated, 206 for newborn and 190 for *in utero* groups.

‡ Results of two experiments only.

Flu-gelatin-binding cells from the spleens of either control or tolerant mice were 97% s-Ig-positive. Those mice rendered tolerant through amounts of Flu-HGG greater than those required to induce maximal B cell tolerance in this system resulted in only a slight reduction in adhering cells. This could have reflected either a modulation of s-Ig receptors or both modulation and blockade (33) amongst a minority of the B cells. Even under these rather extreme circumstances, readily detectable numbers of Flu-binding cells persisted. FACS analysis of these cells after rebinding of the epitope linked to POL showed an unaltered anti-Flu avidity profile. These studies therefore proved that physical elimination, receptor modulation, or receptor blockade through saturation, while possibly contributing to B cell tolerance, cannot represent the chief mechanisms. Of even greater interest is the observation that low concentrations of tolerogen achieve substantial partial reduction of B cell responsiveness while causing no alteration at all in either the antigen-binding B cell numbers or their anti-Flu avidity profiles. The only conclusion that can be drawn is that such immature B cells have received and stored some negative signal from their encounter for tolerogen, without having manifested the complete receptor modulation that could have been induced with much higher concentrations of tolerogen.

To a degree, this conclusion could have been predicted through previous work. The threshold concentrations of Flu-HGG required for induction of B lymphocyte tolerance were three orders of magnitude lower than those required to achieve good staining of B cells for immunofluorescence studies, using the identical ligand (34). It seems likely that those few receptors that do bind the tolerogen at low concentration are rapidly aggregated, pinocytosed, and shed. However, they must be rapidly replaced, because with the low tolerogen doses there are normal numbers of antigen-binding cells with a normal complement of receptors present at all times. It is noteworthy that the receptor-bearing tolerant B cells cannot be triggered by LPS. Obviously the negative signals they have received prior to immunogenic stimulation cancel not only potentially positive signals registered via the Ig receptors but also those from other sources—e.g., mitogens or T lymphocyte help (6, 29–31).

The rules governing negative signaling might be different depending on whether the B cell's s-Ig receptor coat was present at the time of introduction of the antigen or was just about to emerge. From that viewpoint, it was of interest that the results of the experiments in which mice were exposed to tolerogen *in utero* differed little from those involving neonatal exposure. Evidently, cells caught in the transition phase from pre-B cell to immature B cell can receive tolerogenic signals without detriment to the emergence of their s-Ig receptor coat. This being the case, the persistent presence of an autologous antigen analogous to the Flu_5-HGG model tolerogen might functionally abort the emergence of immune reactivity whilst nevertheless allowing the development of s-Ig-positive B lymphocytes with anti-self receptors.

The behavior of antigen-binding cells from mice rendered tolerant through low doses of antigen agrees well with our previous *in vitro* studies in which anti-μ chain antibody was used as a "universal" tolerogen (2). This work showed that immature, but not mature, B cells could receive and store negative signals after anti-μ chain antibody concentrations far too low to cause complete Ig receptor modulation. If careful attention was given to ligand concentration and duration of action, nonclonable B cells with an essentially normal Ig receptor complement resulted. On the other hand, if relatively much higher antiglobulin concentrations were used, the irreversible receptor modulation previously noted by others (14, 15) was obtained.

The present studies have essentially reconciled the previous conflicting results about numbers of antigen-binding B cells in tolerant animals (16–20, 35). Unnecessarily high doses of a multivalent tolerogen can lead to reductions in relevant B cell numbers, though not of as great a degree as the functional inhibition achieved. Tolerogen doses closer to the threshold of effectiveness result in no change in numbers of antigen-binding cells.

Given that antigen-binding B cells incapable of reacting to antigen or mitogen can exist in an animal in a functionless state, even when the relevant antigen had been introduced into the tissues before any B cells had emerged, it now appears that "clonal abortion" is not a strictly descriptive metaphor for the chain of events. The state wherein a B cell with a full complement of receptors exists but has been rendered incapable of reacting to stimuli that normally cause clonal expansion and the differentiation of antibody-secreting progeny is better described by the term "clonal anergy." The molecular mechanisms underlying clonal anergy can presently only be guessed at, but the fact that the present studies support previous ones (2) using anti-μ chain antibody is reassuring. The latter reagent, reactive with every virgin B lymphocyte, presents many potential advantages as a model universal tolerogen.

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