THE IN VITRO INDUCTION OF IMMUNOLOGICAL TOLERANCE IN THE B LYMPHOCYTE BY OLIGOVALENT THYMUS-DEPENDENT ANTIGENS*

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Many attempts have been made to study the induction of specific immunological unresponsiveness in vitro. In general, success has only been achieved with a special class of antigen, the polymeric, multivalent, thymus-independent antigen, examples of which are lipopolysaccharide (LPS)[1] from *Escherichia coli* (1) and polymerized flagellin (POL) (2), or dinitrophenyl (DNP) conjugates of POL (3). Attempts to induce unresponsiveness in dissociated immunocyte populations in vitro, using serum proteins or oligovalent haptenated serum proteins have been unsuccessful (3–5). However in instances where lymphoid cells have been treated in vitro and then transferred to irradiated hosts a degree of unresponsiveness has been induced with oligovalent antigens (6), though this form of unresponsiveness has been rather readily reversible (6).

In vitro tolerance induction by soluble oligovalent antigens is of considerable interest since such antigens have a greater resemblance to many self-antigens than do the thymus-independent antigens, that are mainly of microbial or artificial origin. Additionally, the mechanism is likely to differ in the case of thymus-dependent and thymus-independent antigens since the latter substances in fact nonspecifically abrogate B-cell tolerance induction by the former (7, 8). Questions particularly suited to an in vitro analysis of tolerance induction in splenic B cells concern the proposal of Diener and Feldmann (9) that a multivalent presentation of antigen is an obligatory requirement for tolerance induction in the B cell, and also the kinetics of tolerance induction in a defined pool of B cells that is not being altered by the B-cell neogenesis that occurs in the bone marrow (10). The results to be presented will demonstrate that B cells can be rendered specifically unresponsive by oligovalent thymus-dependent antigens in a wholly in vitro system, and will elucidate some of the characteristics of this process.

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; BSA, bovine serum albumin; DNP-CAP, DNP-ε-aminocaproic acid, DNP-LYS, DNP ε-lysine; FGG, fowl gamma globulin; HGG, human gamma globulin; LPS, lipopolysaccharide; POL, polymerized flagellin.

Materials and Methods

Mice. Noninbred congenitally athymic (nu/nu) mice of both sexes were used at 8–12 wk of age. In general they were the progeny of the mating of heterozygous (nu/+) females with (nu/nu) males.

Antigens. Fowl gamma globulin (FGG) was prepared as previously described (11) and stored at 4–8°C or frozen. Human gamma globulin (HGG) was obtained from the Commonwealth Serum Laboratories, Parkville, Victoria, Australia and bovine serum albumin (BSA) from the Armour Pharmaceutical Co., Kankakee, Ill. Dinitrophenylation of serum proteins was performed as described elsewhere (3) and average conjugation ratios were determined spectrophotometrically. DNP-ε-aminocaproic acid (DNP-CAP) and DNP ε-lysine (DNP-LYS) were both obtained from British Drug Houses, Ltd., Sydney, Australia. POL and DNP-POL were prepared as previously described (3, 7).

HGG was digested to $(Fab)_2$ fragments using pepsin, 2 mg/100 mg of HGG, for 24 h at 37°C in Na-acetate buffer, pH 3.9. The digest was purified and characterized by column chromatography, for which purpose a portion was labeled with ^{125}I using chloramine-T (12). The concentration of $(Fab)_2$ fragments was determined, and dinitrophenylation was carried out as for HGG and BSA.

Tissue Culture. Spleen cell suspensions were prepared and cultured using medium and fetal calf serum as previously described (3, 11). In brief, a modification of the Marbrook system was used, in which 15×10^6 viable nucleated cells were cultured in 1 ml of tissue culture medium containing 5% fetal calf serum in the inner chamber, the indicated amounts of antigen being added to this volume. Cultures were set up in quadruplicate and harvested after 3 days.

Preincubation of Cells. Spleen cells were preincubated in a 10% CO_2—air atmosphere for up to 24 h in 5-cm plastic dishes (Camalec, Adelaide, Australia) each containing 60×10^6 viable nucleated cells in 4 ml of tissue culture medium containing the appropriate antigen.

Washing of Cell Suspensions. After preincubation with antigen for the appropriate period, the cells were removed from the dishes, using vigorous pipetting to remove adherent cells, transferred to 10-ml plastic centrifuge tubes, and washed three times with ice-cold balanced salt solution.

Assay of Anti-DNP and Anti-FGG Antibody-Forming Cells (AFC). Sheep erythrocytes (SRBC) coated with either FGG (13) or dinitrophenylated rabbit-anti-SRBC-Fab' fragments (3) were used in a modified hemolytic plaque assay (14). Results are expressed as the arithmetic mean of the responses of a group of four cultures ± SEM.

Results

The In Vitro Induction of Tolerance to FGG and DNP-HGG. Spleen cells from congenitally athymic (nu/nu) mice were used as a source of B cells, uncontaminated by detectable T-cell function (15–19). The basic experimental protocol was to expose the suspension of spleen cells to a relatively high concentration of a thymus-dependent soluble serum protein such as FGG (13) for 24 h, to wash the cells thoroughly, and then to challenge in vitro. Challenge consisted of incubating the cell suspension for 3 days with 100 μg FGG plus 10 μg POL, this combination of FGG plus the thymus-independent antigen POL having previously been shown to give an optimal anti-FGG AFC response in cultures of spleen cells from nu/nu mice (20). As a specificity control the anti-DNP response to the thymus-independent antigen DNP-POL (18, 21) was also monitored in the same cultures. Table I shows a typical result. It can be seen that a 24 h exposure to 1 mg/ml of FGG induced a marked unresponsiveness to challenge with FGG. The response to DNP-POL on the other hand was unaffected, demonstrating the specificity of the effect. The effect was less marked when the cells were preincubated with a 10-fold lower concentration of FGG, and disappeared with the lowest concentration tested, 10 μg/ml.

The same effect could be demonstrated using a different antigen, DNP_5-HGG. The results of a group of cultures included in the experiment shown in Table I demonstrate the specific unresponsiveness induced by DNP_5-HGG. Fig. 1 shows

TABLE I
Tolerance Induction with Soluble Serum Proteins In Vitro

Preincubation with:	AFC/culture	
	Anti-FGG	Anti-DNP
FGG		
1 *mg/ml*	55 ± 13	832 ± 257
100 *μg/ml*	210 ± 115	579 ± 221
10 *μg/ml*	361 ± 129	817 ± 221
DNP_5-HGG		
1 *mg/ml*	340 ± 77	94 ± 44

nu/nu spleen cells were incubated (15 × 10^6/ml) with the indicated concentrations of either FGG or DNP-HGG for 24 h in plastic dishes. The spleen cells were then washed three times and cultured in flasks with POL (10 μg), FGG (100 μg), and DNP-POL (0.1 μg). After 3 further days of culturing the cultures were harvested and assayed. Results in this and succeeding tables are expressed as the arithmetic mean of the responses of quadruplicate cultures ± SEM.

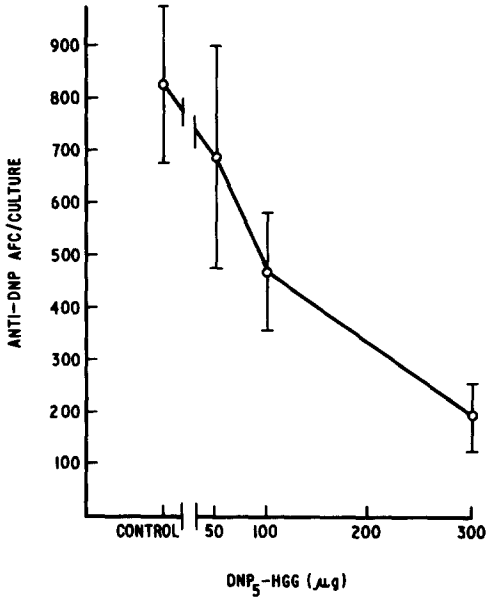

FIG. 1. The dose of DNP-HGG required to induce DNP-specific tolerance in vitro. nu/nu spleen cells were preincubated for 24 h with varying amounts of DNP_4-HGG. After washing the cells were cultured in quadruplicate for 3 days with 0.1 μg DNP-POL, 100 μg FGG, and 10 μg POL. Results are shown as mean anti-DNP AFC per group of four cultures, bars indicating SEM. Anti-FGG responses were equivalent in all groups.

the effect of decreasing the dose of DNP-HGG with which spleen cells were incubated. It can be seen that the effect became marginal with a dose of the same order as with FGG, that is 100 μg/ml.

The Kinetics of B-cell Tolerance Induction In Vitro. Previous work had

indicated that exposure of B cells to thymus-dependent antigens for short periods like 60 min did not lead to tolerance induction (11). To investigate the kinetics of tolerance induction in vitro in this system, spleen cells from nu/nu mice were set up in pairs of dishes, one of each pair containing 1 mg/ml FGG, the control dish medium alone. The mean anti-FGG responses obtained in three separate experiments were expressed as a percentage of the responses in the control cultures and the mean of these percentages are plotted against increasing time of incubation with FGG (1 mg/ml) in Fig. 2. There was no suppression at all after 4

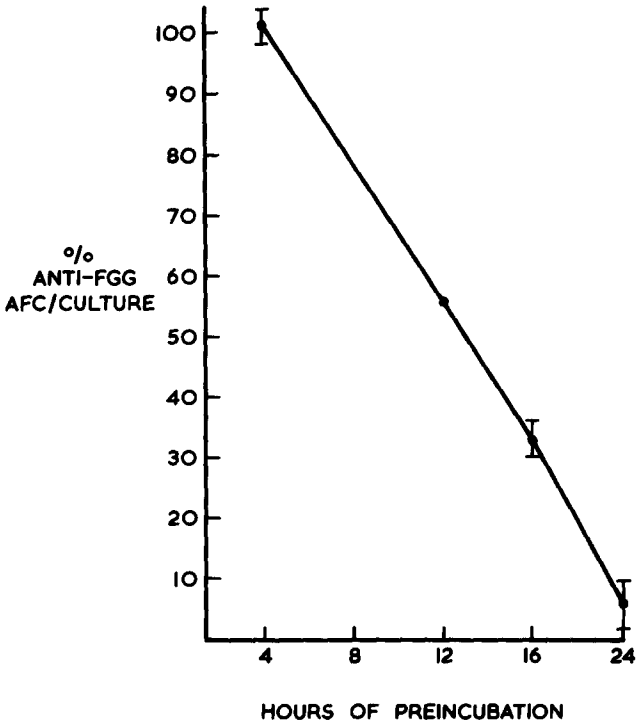

FIG. 2. The kinetics of the in vitro induction of tolerance by FGG. nu/nu spleen cells were incubated with or without 1 mg/ml FGG as in previous experiments, for varying time periods, after which the cells were washed and cultured with 0.1 μg DNP-POL, 10 μg POL, and 100 μg FGG. Cultures were set up in quadruplicate and harvested on day 4 of the experiment. The mean response of FGG-treated cells at each time point was expressed as a percentage of the respective control group and the results shown represent the means of the percentage responses of three individual experiments (except for the 12-h time point which is the result of a single experiment and thus four cultures). The anti-DNP responses were always equal in control and experimental groups.

h of pretreatment with FGG. It is apparent that 24 h were necessary for the development of a marked degree of unresponsiveness, although at 16 h the suppression was readily detectable, responses in experimental cultures being reproducibly only a third of those in control groups.

The Abrogation of Tolerance Induction by POL. It has been proposed on theoretical grounds (22) and subsequently established experimentally (23, 24), that endotoxin and other adjuvants can abrogate tolerance induction. Recently it

has been shown that this action of LPS and other thymus-independent antigens that are also active as adjuvants (unpublished observations), applies also to the abrogation of a pure B-cell tolerance induced in congenitally athymic mice by thymus-dependent antigens such as FGG (7). Therefore it was of interest to see whether a similar effect pertained in the present in vitro model. Table II shows that POL did indeed prevent tolerance induction in vitro both by FGG, and DNP-HGG.

The Lack of a Requirement for a Special Matrix of Antigen Determinants in Tolerance Induction. Previously it has been suggested that the configuration of antigenic presentation is critical for tolerance induction in the B lymphocyte, a high epitope density being recognized as a "tolerogenic" signal (3, 9). One possible way in which this mechanism could operate in the present system, is

TABLE II
Inhibition of In Vitro Tolerance Induction by POL

Pretreatment with:	AFC/culture
DNP_8-HGG	12 ± 12*
DNP_8-HGG + POL	192 ± 51*
—	279 ± 48*
FGG	12 ± 6‡
FGG + POL	229 ± 46‡
—	213 ± 29‡

Results of two separate experiments are shown. 60×10^6 nu/nu spleen cells were incubated for 24 h in 4-ml plastic dishes with either medium alone, DNP_8-HGG or FGG at 1 mg/ml, or either of the proteins at 1 mg/ml plus POL (10 μg/ml) as indicated. Spleen cells were then washed and each group was cultured in four flasks containing DNP-POL (0.1 μg), FGG (100 μg), and POL (10 μg). These cultures were harvested after 3 days.

* Mean anti-DNP AFC per culture.

‡ Mean anti-FGG AFC per culture.

that a matrix of determinants could be created by adsorption of the antigens onto cell surfaces, for example via for Fc receptor on B cells (25). One method of examining the proposed role of a matrix of critically spaced determinants in tolerance induction was to study a situation in which this spacing would be disturbed. Therefore spleen cells were incubated with 0.3 mg/ml DNP-HGG together with a threefold excess of aggregated HGG, the unconjugated HGG being designed to compete with DNP-HGG for sites within the hypothetical antigenic matrix, thus altering the spacing of DNP determinants. Table III shows that the addition of an excess of HGG had no effect on the degree of DNP-specific tolerance induced by 0.3 mg/ml of DNP-HGG.

The Lack of a Requirement for the Fc Piece in Tolerance Induction. Both HGG and FGG are immunoglobulins and one important theoretical problem that can be readily approached in vitro, where antigen dose and distribution can be matched without the considerations of differential clearance rates that pertain in

vivo, is that of the importance of the Fc piece of the immunoglobulin molecule. HGG was digested with pepsin, the major protein peak was obtained by fractionation with a G100 column, and this was concentrated and rerun to obtain a single peak. The protein in this peak was characterized by labeling a portion with ^{125}I and running this on a G-200 column with markers. The labeled material was found to elute between HGG and BSA markers, as expected for the mol wt of (Fab_2) fragments of 106,000 (26), Table IV shows that dinitrophenylated-(Fab_2) fragments [DNP-(Fab_2)] were efficient tolerogens, indicating that the Fc piece was not essential for tolerance induction in the present system.

The Ineffectiveness of DNP-LYS or DNP-CAP In Vitro Tolerogens. Watson et al. have reported that dinitrophenylated amino acids acted as in vitro immunogens, provided that LPS was added to the system (27). Since this observation paralleled that made in the system using FGG and POL (20), it was of interest to see whether DNP-amino acids could behave as tolerogens as did FGG in the experiments reported above.

The present study employed DNP coupled to one of two single amino acids,

TABLE III
The Lack of Effect of Aggregated HGG on The Induction of Tolerance by DNP-HGG

Cells incubated with:	AFC/culture	
	Anti-DNP	Anti-FGG
—	310 ± 86	213 ± 29
DNP_4-HGG (0.3 *mg*)	60 ± 51	229 ± 67
DNP_4-HGG (0.3 *mg*) + AGG-HGG (1 *mg*)	33 ± 40	205 ± 18

nu/nu spleen cells (60×10^6) were incubated for 24 h in plastic dishes in 4 ml of medium together with the indicated concentrations of soluble DNP_4-HGG and heat-aggregated HGG. After washing, each cell suspension was then cultured in quadruplicate with DNP-POL (0.1 μg) and FGG (100 μg) plus POL (10 μg). Cultures were harvested 3 days later.

TABLE IV
The Effectiveness of (Fab)$_2$ Fragments of HGG in Tolerance Induction In Vitro

Preincubation with:	Molarity of DNP	AFC/culture	
		Anti-FGG	Anti-DNP
—		2,020 ± 190	2,100 ± 102
DNP_4-HGG (0.3 *mg*)	1×10^{-5}	1,640 ± 370	355 ± 110
$DNP_{2.2}$-$(Fab)_2$ (1.4 *mg*)	3×10^{-5}	1,680 ± 230	195 ± 20
$DNP_{2.2}$-$(Fab)_2$ (0.5 *mg*)	1×10^{-5}	1,860 ± 360	470 ± 73

nu/nu spleen cells were incubated for 24 h as before with the indicated amounts per ml of DNP_4-HGG, $DNP_{2.2}$-$(Fab)_2$ or medium alone as shown. They were washed, challenged in culture, and harvested as above.

L-lysine or ϵ-aminocaproic acid, nu/nu spleen cells being incubated with either substance for 24 h, and the cells then washed and challenged with DNP-POL, FGG, and POL as before. These reagents were tested at concentrations from 10^{-7} to 2×10^{-4} M, at which level a nonspecific toxicity became manifest by a suppression of the anti-FGG response. The results of a representative experiment are presented in Table V. It is evident that DNP-CAP had only a marginal effect on the anti-DNP response at a 10^{-4} M concentration of DNP. This contrasts with marked suppression produced by 0.1 mg/ml DNP_5-HGG, the total DNP molarity in this situation being 30-fold less. A smaller molecule, DNP_3-BSA, was considerably less efficient than DNP_5-HGG at comparable DNP molarities, where the molarity of the BSA conjugate was twofold higher.

TABLE V
The In Vitro Induction of Tolerance by Various DNP-Conjugates

Preincubation with:	Molarity of DNP	Molarity of conjugate	AFC/culture	
			Anti-DNP	Anti-FGG
—	—	—	1,674 ± 126	741 ± 212
DNP_5-HGG (1 *mg/ml*)	3×10^{-5}	6×10^{-6}	123 ± 42	621 ± 113
DNP_5-HGG (0.1 *mg/ml*)	3×10^{-6}	6×10^{-7}	249 ± 71	574 ± 72
DNP_3-BSA (1 *mg/ml*)	4×10^{-5}	1.4×10^{-5}	652 ± 171	691 ± 186
DNP-CAP	2×10^{-4}	2×10^{-4}	460 ± 104	397 ± 38
	1×10^{-4}	1×10^{-4}	1,014 ± 128	813 ± 200

nu/nu spleen cells were incubated for 24 h in 4-ml plastic dishes with the indicated amounts of various DNP conjugates. After washing twice the cells were cultured in groups of four with FGG (100 μg), POL (10 μg), and DNP-POL (0.1 μg) for 3 days.

Discussion

An entirely in vitro induction of specific B-cell unresponsiveness by oligovalent thymus-dependent antigens has been demonstrated using a population of B cells from nu/nu mice. All evidence points to a lack of functional T cells in nu/nu mice (15–19), and recently evidence has also been presented against the presence of suppressor T-cell function in these animals (28). Thus B-cell tolerance induction occurs in this system, without any requirement for T cells. The features of this in vitro system quite closely parallel those of the induction of B-cell tolerance by thymus-dependent antigens in nu/nu mice in vivo (7). Thus the kinetics, 24 h being required for the development of maximal unresponsiveness upon in vitro challenge, are similar in each case, and the presence of POL during the pretreatment phase abrogates the induction of tolerance in both instances. Additionally, in neither case was it necessary to use deaggregated proteins, the preparations of FGG in particular often showing visual signs of aggregation, although being still effective.

The concept that immunological tolerance results from the direct access of antigen to the lymphocytes, due to a bypassing of accessory cells like the macrophage has much

experimental support (29–31). In the accompanying paper (32) I have reported the results of parallel experiments on B-cell tolerance induction in vivo in athymic and euthymic mice using nondeaggregated thymus-dependent antigens. Thus in the presence of functional T cells, the B-cell population tended to be immunized rather than tolerized, although this effect could be masked by T-cell dependent suppression (32). These experiments showed that B-cell tolerance was governed by the rules established for tolerance in mixed cell populations, namely that a small immunogenic influence due to aggregates of antigen could prevent tolerance induction (29–31), and furthermore pointed to the importance of T-cell activation in directing the overall response towards B-cell immunization. Tolerance induction in the present experiments, that used nu/nu spleen cells lacking functional T cells, may be explained by a direct interaction of antigen with the B cell in the absence of an immunogenic influence, normally dependent on both the presence of functional T cells (discussed by Katz et al. [6]), and the uptake of antigen by the reticuloendothelial system (29–31). It is likely that both T-cell and macrophage function are required for an efficient abrogation of B-cell tolerance in the absence of adjuvant-like B-cell stimulants such as LPS or POL. Elsewhere evidence has been provided that activated macrophages secrete a factor substituting for the action of substances like POL or LPS in allowing B-cell immunization in the absence of T cells (33). Since POL or LPS also block B-cell tolerance induction, both in vivo (7, 8) and in vitro (Table II), it is possible that it is this macrophage-produced factor that directly abrogates B-cell tolerance induction when a large dose of aggregated antigen is given to euthymic mice. The role of the activated T cell in the abrogation of B-cell tolerance induction may be to stimulate the macrophage to secrete this factor. Mitchell et al. (34) have suggested an alternative role of the activated T cell, postulating that the T cell prevents B-cell tolerance by reducing the amount of antigen on the B-cell surface.

The inability of Byers and Sercarz (4) to demonstrate an irreversible unresponsiveness after in vitro exposure to high doses of BSA for up to 3 days, may reflect the fact that they used lymph node fragments from primed animals, where firstly there were almost certainly many specific T cells present, and secondly the normal architecture of the lymphoid system was preserved, presumably allowing optimal interactions between T cells, and macrophages and B cells. The failure of Mitchison (5) to demonstrate tolerance in splenic lymphocytes after in vitro incubation for up to 24 h with BSA or human serum albumin is more difficult to understand, especially since Katz et al. (6) have more recently shown that in vitro exposure to DNP-ovalbumin could render DNP-reactive B cells at least temporarily unresponsive after adoptive transfer. It is noteworthy in considering Mitchison's negative results, that DNP-BSA was less efficient than DNP-HGG or DNP-$(Fab)_2$ in inducing tolerance in the present system (Tables IV and V).

It was shown that the dose of antigen used was critical (Table I and Fig. 1) the concentrations of the antigen required being of the order of 10^{-6} M. In the case of FGG this is 10 times higher than the concentration of antigen needed for a maximal in vitro response by nu/nu spleen cells in the presence of POL (20), although it should be noted that 1 mg/ml of FGG is perfectly immunogenic, provided it is given together with POL (20). With regard to the effect of antigen dosage on B-cell responsiveness, it is of interest that subtolerogenic doses of thymus-dependent antigens given alone to nu/nu mice in vivo induced B-cell memory.[2] On the other hand it has been recently shown that much smaller concentrations of antigen, e.g. 0.4 μg/ml of DNP_1-HGG induced specific

[2] Schrader. J. W. 1975. The role of T cells in IgG production: thymus-dependent antigens induce B-cell memory in the absence of T cells. *J. Immunol.* In press.

unresponsiveness in cultures of bone marrow cells, suggesting a difference in susceptibility between immature and mature B cells (35).

While there was a necessity for a certain dose of antigen in the present system, there was no evidence in favor of a requirement for a multivalent presentation of antigen for tolerance induction (3, 9). There is little likelihood that tolerance induction in the present system is due to the formation of antigen-antibody complexes as proposed by Diener and Feldmann (9). This is because antibody is unlikely to be secreted within the 24-h tolerance induction period, especially since nu/nu spleen cells do not respond with AFC formation to these antigens in the absence of additional factors like POL or supernates of cultures of activated macrophages (20, 33). The Fc fragment is unlikely to be involved in tolerance induction either directly or through the formation of matrices of the antigen since DNP-$(Fab)_2$, fragments and to a lesser extent, DNP-BSA were effective, and the Fc receptor on mouse cells is reported to have no affinity for FGG (36).

The kinetics of tolerance induction in the present system, where 16–24 h were required to induce unresponsiveness, contrasts with the 2–6 h required for in vitro tolerance induction using thymus-independent antigens (1–3, 37). The other pointer to what we have suggested is a fundamental difference in tolerance induction between thymus-dependent and multivalent thymus-independent antigens (7, 38) is the demonstration that in vitro (Table II) as in vivo (7, 8), thymus-independent antigens like LPS or POL act nonantigen specifically to prevent tolerance induction by thymus-dependent antigens. Whatever the mechanism of tolerance induction by thymus-independent antigens (9, 37), the present results suggest that there is a specific type of B-cell tolerance induced by oligovalent thymus-dependent antigens that depends on the direct access of antigen in a sufficient concentration over a sufficient period of time to the B cell. Additionally, there must be an absence of factors provided by thymus-independent antigens or activated macrophages, which will otherwise lead to immunity.

Summary

B-cell tolerance has been induced by oligovalent thymus-dependent antigens in an entirely in vitro system. Dissociated spleen cells from congenitally athymic (nu/nu) mice were preincubated for 24 h with 0.1—1 mg/ml of either fowl gamma globulin (FGG) or DNP-human gamma globulin (DNP-HGG). After washing, the cells were tested for the ability to mount in vitro, thymus-independent responses against FGG and DNP. A state of specific responsiveness to either FGG or DNP was thus demonstrated. Features of this wholly in vitro system that paralleled previous findings on the in vivo induction of B-cell tolerance in nu/nu mice were the kinetics, 24 h being required for tolerance induction in either case, the abrogation of tolerance induction by the presence of POL both in vivo and in vitro, and finally the observation that in neither case was there a requirement for the antigens to be deaggregated.

It was shown that DNP-$(Fab)_2$ fragments prepared from HGG induced DNP-specific tolerance indicating that the Fc piece was not required for tolerance induction in this in vitro system. DNP-bovine serum albumin was less effective than DNP-HGG or DNP-$(Fab)_2$. Preincubation with subtoxic concentrations of DNP-lysine or DNP-ϵ-capric acid had only a marginal effect on DNP responsiveness.

Since nu/nu mice, lacking in detectable T-cell function, were used as spleen cell donors, this work provides further evidence that B-cell tolerance to thymus-dependent antigens can be induced without the participation of T cells. It is suggested that B-cell tolerance to thymus-dependent antigens occurs when the antigen in a sufficient concentration and over a sufficient period of time has direct access to the B cell. This contact with antigen must be in the absence of an additional influence provided either by adjuvants like endotoxin or POL, or by activated macrophages, which may be stimulated by activated T cells; otherwise not tolerance but B-cell activation will occur.

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