EVIDENCE FOR THE CLONAL ABORTION THEORY OF B-LYMPHOCYTE TOLERANCE*

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There is now general agreement (1–3) that different models of experimentally induced immunological tolerance illustrate a variety of cellular mechanisms. Many models relating to antibody synthesis show that the tolerance-inducing antigen exposure has only affected thymus-derived (T) lymphocytes, leaving the compartment of bone marrow-derived progenitors of antibody-forming cells (B lymphocytes) normally reactive (reviewed in 4). This may sometimes reflect the activity of certain T lymphocytes (suppressor T cells), which possess the capacity to suppress the reactivity to antigen of a normal population of B cells (5, 6). In other examples, tolerance has been achieved at the B-cell level. Such models usually involve either the administration of relatively high doses of soluble, aggregate-free and poorly immunogenic antigens (e.g. 7–9), or the use of highly polymeric antigens with multiple antigenic determinants in supra-immunogenic concentrations (10, 11) or in molecular arrangements (12–14) peculiarly favorable for tolerogenesis. In some of these models, there is evidence that B cells have been rendered nonreactive because their receptors have been blockaded by antigen (reviewed in 1), permitting reversibility of the tolerance on removal of the antigen. In other cases, the tolerance appears to be due to some action of antigen on the B cell leading to its permanent inactivation or even elimination (15).

The above cellular mechanisms pertain to the injection of a tolerogen into a mature animal already equipped with competent T and B lymphocytes in its secondary lymphoid organs. The acquisition of self-tolerance to many autologous proteins may be different, as the protein towards which tolerance must be achieved may be present before lymphocytes develop. This consideration opens the possibility that self-tolerance is achieved through the existence of a phase of lymphocyte differentiation during which the cell is particularly sensitive to tolerogenesis on contact with antigen (16–18). To distinguish this concept from that of clonal deletion of competent lymphocytes, we have termed it clonal abortion (19). The clonal abortion theory predicts that lymphocytes, at a particular stage of their differentiation at which some receptors for antigen have already appeared, can be permanently switched off or eliminated if they encounter antigen in appropriate concentration. As for every other phenomenon in cellular immunology, there must be an avidity parameter to the postulate, with cells possessing receptors of low affinity for a particular "self" antigen requiring higher concentrations for clonal abortion.

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Recent studies from our laboratory (20–22) have pin-pointed a stage in B-lymphocyte differentiation that seemed particularly favorable for a test of the postulate. Adult mouse bone marrow was found to be a site in which large numbers of membrane immunoglobulin (Ig)-positive B lymphocytes were generated, with rapid renewal kinetics (20). Furthermore, radioautographic double-labeling studies in which animals received [^{3}H]thymidine in vivo and cells were exposed to [^{131}I]antiglobulin in vitro showed that mouse bone marrow small lymphocytes exited from the proliferative process (presumably involving large and medium lymphocytes) which generated them, lacking detectable Ig receptors. They acquired these receptors in progressively increasing amounts through a nonmitotic maturation period lasting approximately 2 days (21). When bone marrow was placed in tissue culture, i.e. when escape of mature, Ig-positive lymphocytes by emigration was rendered impossible, the proportion of Ig-positive cells rose (21), as did the performance of the cell population in an adoptive immune system where B-cell reactivity to a T-cell-independent antigen was measured (22). These studies revealed mouse bone marrow as being a major factory for B lymphocytes, and suggested that cells "caught" during the nonmitotic phase of progressive receptor display might be suitable targets for clonal abortion tolerogenesis.

In order to mimic in vivo realities as closely as possible, it was desirable to use monomeric, aggregate-free antigens. For most of the work, we have used deaggregated dinitrophenyl human gamma globulin (DNP-HGG)[1] at the low conjugation ratio of one hapten molecule per protein molecule. This paper presents evidence to show that hapten-specific tolerance in bone marrow cell cultures can be achieved with remarkably low antigen concentrations, the kinetics paralleling that of B-cell neogenesis, and the phenomenon differing crucially from observations made with spleen cell cultures.

Materials and Methods

Animals. CBA/H/Wehi mice aged 8–11 wk, bred and maintained under strict pathogen-free (SPF) conditions behind a sterile barrier, were used for most experiments. Congenitally athymic (nu/nu) mice in their fifth back-cross generation to BALB/c mice were also used.

Antigens. The DNP hapten was conjugated onto various proteins, including polymerized Salmonella flagellin (POL), human gamma globulin (HGG; Commonwealth Serum Laboratories, Melbourne), and, for preliminary experiments, bovine serum albumin (BSA), and fowl gamma globulin (FGG) using 2,4-dinitro-benzene-sulphonic acid (DNBS) (Eastman Kodak Co., Rochester, N.Y.) by Eisen's method (23). The DNP-POL possessed 4.2 mole of DNP per mole of monomeric flagellin (mol weight 40,000) in the POL. Three batches of DNP-HGG were used, bearing 0.9–1.1 mole of DNP per mole of HGG (DNP-HGG). HGG used as a hapten-unsubstituted protein carrier control in tissue culture studies was treated in a manner indentical to that used during dinitrophenylation of HGG, except that DNBS was not added. DNP_1-HGG and HGG were deaggregated by centrifugation at 145,000 *g* for 90 min. After some preliminary experimentation with freshly-deaggregated tolerogen, it was found that the tolerogenic properties were not altered if deaggregated antigens were stored in

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DNBS, 2,4-dinitro-benzene-sulphonic acid; FCS, fetal calf serum; FGG, fowl gamma globulin; HEM, Eagle's minimal essential medium buffered with Hepes; HGG, human gamma globulin; LPS, *Escherichia coli* lipopolysaccharide endotoxin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; PFC, plaque-forming cell; POL, polymerized flagellin from Salmonella strains SW1338 or SW871; SPF, specific pathogen-free.

diluted form at 4°C. The hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was prepared and conjugated to HGG or POL by standard methods (24, 25). The single batch of NIP-HGG used had a substitution rate of 1.5 mole NIP per mole HGG. The NIP-POL used was at 3.4 mole NIP per mole of monomeric flagellin in the POL. *Escherichia coli* lipopolysaccharide endotoxin (LPS) was obtained from Difco Laboratories, Detroit, Mich.

Preparation of Cell suspensions. The medium used for the preparation of cell suspensions was Eagle's minimal essential medium (MEM) (Catalogue F-15 Grand Island Biological Company, Grand Island, N.Y.) adjusted to mouse osmolarity and buffered with 20 mM Hepes (HEM) (Calbiochem, San Diego, Calif.). Bone marrow cell suspensions were prepared from femoral plugs, ejected with HEM using a syringe and a 21 gauge hypodermic needle, and the plugs were dissociated by repeated gentle aspiration through the needle. Spleen cell suspensions were prepared by gentle dissociation of the spleens with forceps into cold HEM, with 5% vol/vol fetal calf serum (FCS) using a stainless steel mesh sieve. Large cell clumps were removed from both cell suspensions by settling over 1 ml of FCS for 5 min, and the light debris was removed by washing the cells twice through FCS. Nucleated cell counts were performed in a hemocytometer and the viability determined by the eosin dye exclusion method.

Tissue Culture System. A Marbrook (26) system was used for cell culture. The culture medium used was MEM supplemented with 5% FCS and buffered with bicarbonate. 6×10^7 viable nucleated cells from either bone marrow or spleen were placed in an inner well (insert) in 4 ml of culture medium (containing the antigen, if any), and were separated from an outer reservoir of 50 ml of medium by a dialysis membrane. After tissue culture periods of 3 h to 5 days, the flask inserts were vortexed before cell harvest and the dialysis membranes were vigorously rinsed. The cells were washed once through FCS, resuspended in HEM, assessed for viability, and injected for measurement of adoptive immune performance.

Adoptive Immune Assay System. Cells were injected intravenously into lethally X-irradiated (850 rads) syngeneic SPF mice. A Phillips RT 250 X-ray machine was used (Philips-Electronic Instruments, Mount Vernon, N.Y.), operating at 250 KV, 15 ma, and a 0.2 mm Cu filter, (half value layer, HVL, = 0.8 mm Cu). Irradiation rate was 127 rads/min under conditions of maximum backscatter. Cells were injected within 3 h of irradiation, and, almost immediately after cell injection, the host mice received challenge antigen, namely an intraperitoneal injection of 5 μg DNP-POL, frequently mixed with 25 μg of NIP-POL. These challenge doses of the T-cell-independent antigens had previously been found to be the minimal doses giving a maximal plaque-forming cell (PFC) response by adoptively transferred cells. 6–10 (usually 9) days after cell transfer, host mice were killed and the spleen content of hapten-specific PFC determined. Anti-DNP and anti-NIP direct and enhanced PFC were determined by the Cunningham liquid monolayer plaque technique as previously described (22). As SPF mice were used, irradiation mortality in reconstituted mice was negligible. Nonreconstituted irradiated control mice died between 10 and 14 days, and were shown incapable of mounting any antihapten PFC response on antigenic challenge. Furthermore, background PFC numbers in irradiated, nonreconstituted mice were negligible. In a typical experiment, four–six cultures belonging to a treatment group were pooled and transferred to three–five irradiated mice. Most experiments were repeated two–three times.

Anti-θ Treatment of Mouse Bone Marrow Cells. This was as previously described (22), and conditions used were shown to be capable of completely abrogating "helper" or "suppressor" activity of authentic peripheral T-lymphocyte populations.

Results

Preliminary Experiments. In view of the complexities surrounding the adoptive immune response as a quantitative read-out system for the immune capacity of B lymphocytes (22, 27), preliminary experiments were performed to study the nature of the cell dose: PFC response relationship. Using transfers of normal, noncultured marrow, and with killing of host mice 9 days after transfer, the number of PFC rose linearly with cell dose from 2 to 6×10^7 viable cells transferred, after which an appreciably supra-linear increment occurred (see also 22). Similarly, the adoptive PFC response of marrow that had been cultured for 3

days showed an approximately linear relationship to cell dose from 5-40 $\times$ 10^6 viable cells transferred. Accordingly, 2 $\times$ 10^7 was chosen as the standard dose of viable cultured cells transferred. Preliminary work also showed the absence of a significant cross-reaction between the NIP and DNP haptens under the PFC-revealing conditions used. In a typical 3 day culture, the 6 $\times$ 10^7 input bone marrow cells yielded 3 $\times$ 10^7 recoverable nucleated cells of which 1.6 to 2 $\times$ 10^7 were viable.

Adoptively transferred bone marrow cells did not give rise to significant numbers of "enhanced" PFC at any day of killing between 6 and 10 days post-transfer. With spleen cell transfers, small numbers of enhanced plaques were occasionally found, but only when hapten-protein conjugates were present during the culture period. Accordingly, only the results for direct PFC are presented below.

The Effect of Tissue Culture on Adoptive Immune Performance. The effect of a period of tissue culture on the capacity of bone marrow or spleen cells to give an adoptive immune response, in the absence of any antigen (other than those present in FCS) added to the cultures, was studied. The results (Fig. 1) show that the performance of bone marrow cells rose considerably over the first 3 days of culture, whereas that of spleen cells fell off. Longer periods of culture signifi-

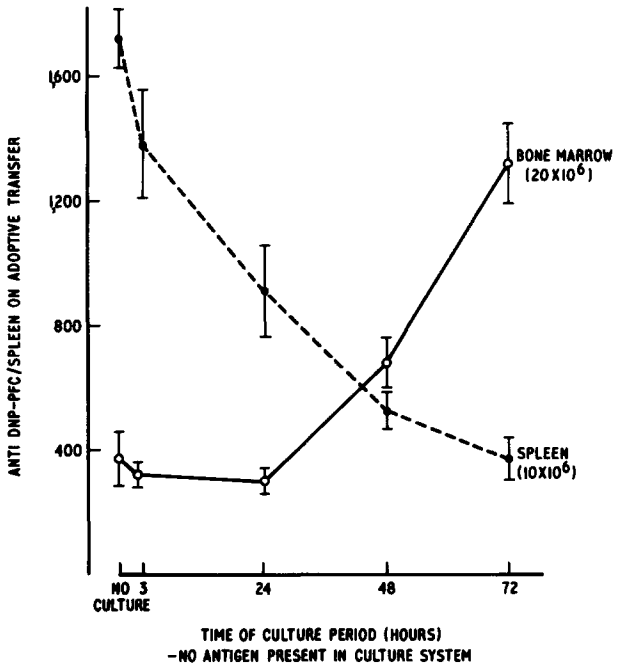

FIG. 1. The effect of tissue culture on adoptive immune performance. Adoptive hosts received 2 $\times$ 10^7 viable nucleated bone marrow cells (O—O) or 10^7 spleen cells (●---●) that had been cultured for periods of 3 h to 3 days, or that had not been cultured. They were immediately challenged with 5 μg of DNP-POL. 9 days later, the anti-DNP direct PFC content of host spleens was determined. Vertical bars here and elsewhere denote standard errors of the mean.

cantly decreased the potential of bone marrow cells. This confirmed previous experience (22).

The Effect of DNP_1-HGG on the Increased Adoptive Immune Performance of Cultured Bone Marrow Cells. Next, the effect of adding 4 μg/ml of DNP_1-HGG (2.5×10^{-8} M hapten) to bone marrow cultures was studied. The results (Fig. 2) show that this low concentration of hapten present in predominantly monomeric form completely abrogated the increment in adoptive immune capacity occurring on culture in the absence of antigen.

Influence of Day of Killing of Adoptive Host on DNP_1-HGG-induced Tolerance of Marrow Cells. To obviate the possibility that some subpopulation of B cells with abnormal maturation kinetics in the adoptive host might have been

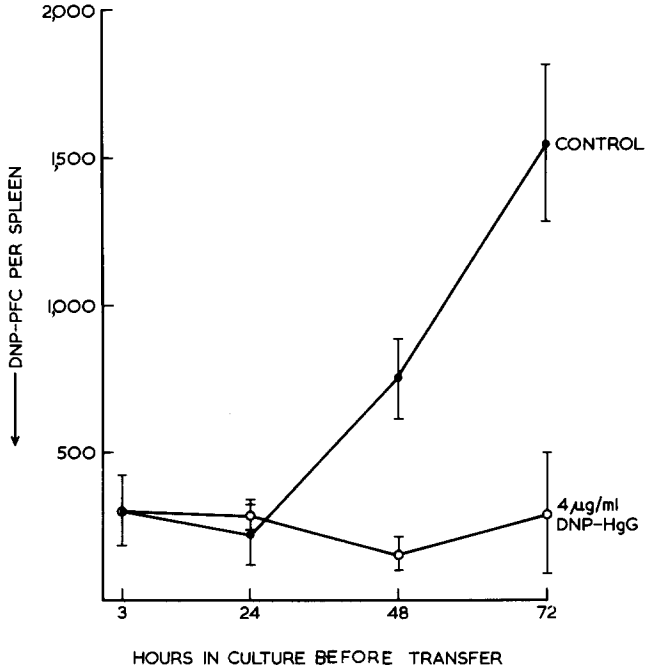

FIG. 2. The effect of DNP-HGG on the adoptive immune response of bone marrow cultures. Cells were incubated with or without 4 μg/ml of DNP_1-HGG for 3 h to 3 days before adoptive transfer.

escaping the postulated clonal abortion process, the time of killing of host animals was varied. Bone marrow cultures were held for 3 days with 4 μg/ml DNP_1-HGG, or HGG, or no added antigen. The results (Fig. 3) establish three points. First, control mice killed 9 days after cell transfer yielded the highest PFC numbers, and this day was chosen as the standard for subsequent experiments. Secondly, cultures containing HGG gave PFC numbers equivalent to those containing no added antigen. Thirdly, no matter which day of killing was considered, DNP_1-HGG present in culture substantially reduced the adoptive anti-DNP response.

Antigen Concentration Required for Tolerance Induction in Bone Marrow Cultures. Tissue cultures of mouse bone marrow or spleen cells were held with

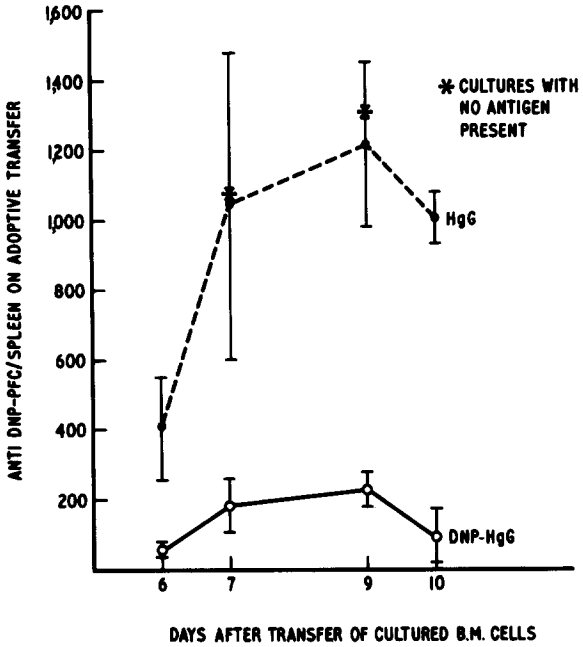

FIG. 3. The effect of varying the day of killing of adoptive hosts on the observed tolerogenesis of bone marrow cell cultures by DNP-HGG. All cultures were maintained for 3 days at 37°C with either no antigen (*) 4 μg/ml HGG (●---●), or 4 μg/ml DNP_1-HGG (O——O). Cells were then adoptively transferred, challenged with 5 μg DNP-POL, and hosts killed at intervals between 6 and 10 days after transfer.

various concentrations of DNP_1-HGG for 3 days of culture. The normalized results of a pool of experiments are presented in Fig. 4. It is shown that this antigen fails to cause tolerance in spleen cell cultures at any concentration up to 400 μg/ml (2.5×10^{-6} M). In other experiments, (not shown) spleen cells were held in culture for 1 day (a period during which better adoptive immune capacity is retained) with concentrations of DNP_1-HGG up to 400 μg/ml and again no tolerance was induced. Higher concentrations were somewhat toxic, as shown by reduction in irrelevant immune responses. In contrast, with bone marrow significant reductions in anti-DNP responsiveness was shown with concentrations as low as 0.4 μg DNP_1-HGG/ml (2.5×10^{-9} M), and marginal reduction may even have been present at 0.04 μg antigen/ml (2.5×10^{-10} M) though this did not reach statistical significance. These concentrations of DNP_1-HGG had no effect on the anti-NIP responsiveness of the cells.

Specificity of Tolerance Induction. To demonstrate the specificity of reduction in adoptive immune capacity, bone marrow cells were cultured for 3 days with either DNP_1-HGG or $NIP_{1.5}$-HGG. The adoptive hosts were challenged with both DNP-POL and NIP-POL. The results (Table I) show that tolerance is specific, the response being reduced only towards the hapten present in culture. This result has been obtained repeatedly. The normal anti-NIP response of DNP-HGG-treated cultures has been included as a specificity control in many experiments, the anti-NIP results not always being shown.

Antigen is Required During most of the Culture Period for Optimal

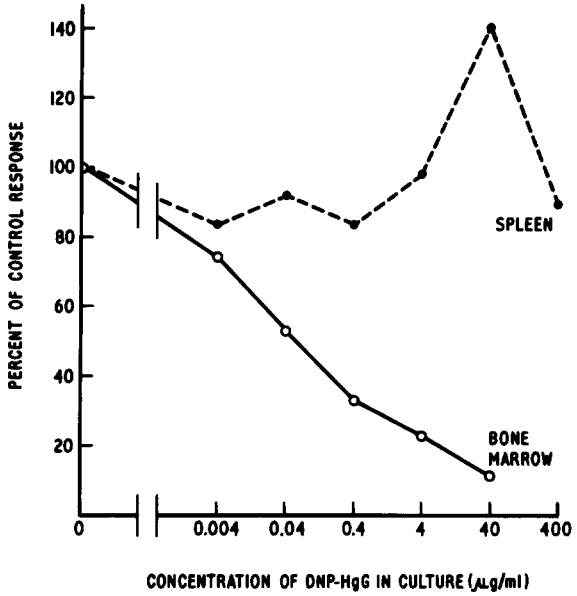

FIG. 4. Dose-response relationships in tolerogenesis of bone marrow cultures by DNP-HGG. Normalized, pooled results of several experiments. The control values for spleen (●---●) were lower in absolute PFC numbers than for bone marrow (O——O) (see Fig. 1).

TABLE I

Specificity of Tolerance Induced in Cultures of Bone Marrow Cells

Antigen present in culture system	DNP-PFC/spleen	NIP-PFC/spleen
	Mean ± SE	*Mean ± SE*
4 μg/ml DNP_1-HGG	315 ± 72	1,133 ± 152
4 μg/ml $NIP_{1.5}$-HGG	1,163 ± 282	248 ± 120

Adoptive transfer recipients were challenged with both DNP-POL and NIP-POL and spleens were assayed for both anti-DNP and anti-DNP PFC.

Tolerogenesis. On the working hypothesis, cultured cells would be maturing through the critical tolerance-sensitive phase asynchronously throughout the 3-day period. Accordingly, if antigen were added only late in the culture, some maturing small lymphocytes would have passed the critical stage in an antigen-free environment, and would have gained anti-DNP reactivity. Thus we reversed the experimental design shown in Fig. 2, maintaining a series of cultures for the full 3 days, but pulsing in antigen at variable times. The results (Fig. 5) show that 4 μg/ml of DNP_1-HGG had no significant effect on spleen cell cultures. With bone marrow cell cultures, it caused a partial reduction in response if present for the last 24 h; and it caused the usual 80% reduction if present for the full period of culture. With neither cultured nor noncultured bone marrow cells exposed to 4 μg/ml of DNP_1-HGG immediately before transfer, or for very brief culture periods, was a reduction in anti-DNP responses achieved.

The Tolerogenesis is not Dependent on Suppressor T cells. CBA mouse bone

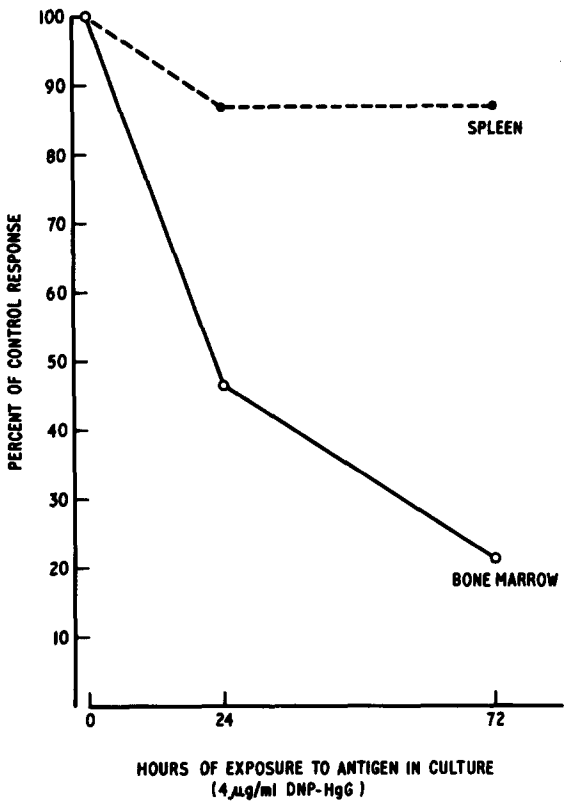

FIG. 5. The effect of addition of antigen at various times after initiation of culture on bone marrow cell tolerogenesis. Cultures of bone marrow to (O——O) or spleen (●----●) cells were maintained for 3 days. 4 μg/ml of DNP_1-HGG was added 24 h or 3 days before harvesting the cultures.

marrow only contains small numbers of θ-positive cells (20), but we wished to ensure that suppressor T cells were in no way involved in our system. Therefore, bone marrow cells were treated with anti-θ serum and complement before tissue culture in the presence of 4 μg/ml of DNP_1-HGG or HGG. The results (Table II) show that tolerogenesis was in no way affected by the anti-θ treatment.

To further document the irrelevance of T cells in this form of tolerance, cultures of bone marrow cells from partially inbred congenitally athymic mice were performed. The results yielded much lower PFC numbers than with CBA mice, but demonstrated the same order of tolerogenesis by 4 μg/ml of DNP_1-HGG.

The B-Cell Tolerance Induced in Bone Marrow Cultures is not "Infectious". To demonstrate whether tolerance might have been due to some active component, e.g. a suppressor B cell, an experiment was performed in which normal or tolerized bone marrow cell cultures were injected into lethally irradiated hosts either alone, or as a mixture. The results (Table III) show that the numbers of PFC appearing in adoptive recipients of cell mixtures did not differ significantly from the expected sums of PFC numbers in recipients of singly-treated cell cultures.

TABLE II

Tolerance Induction in Cultures of Anti-θ Treated Bone Marrow Cells

Antigen present in culture system	Anti-DNP-PFC/spleen	Anti-NIP-PFC/spleen
	Mean ± SE	*Mean ± SE*
4 μg/ml HGG	1,230 ± 95	1,103 ± 145
4 μg/ml DNP_1-HGG	245 ± 124	1,155 ± 110

TABLE III

Adoptive Immune Response of Mixtures of Tolerized and Nontolerized Bone Marrow Cultures

Reconstituting cell population	Anti-DNP-PFC/spleen	Anti-NIP-PFC/spleen
	Mean ± SE	*Mean ± SE*
20×10^6 HGG treated	589 ± 71	1,185 ± 120
20×10^6 DNP_1-HGG treated	250 ± 59	1,052 ± 108
20×10^6 HGG treated plus 20×10^6 DNP_1-HGG treated	613 ± 93	2,250 ± 160

Polyclonal B-cell Activators (28) do not Abolish Clonal Abortion. Part of the clonal abortion hypothesis is the concept that the lymphocyte matures from a stage during which it can only be tolerized on contact with antigen to a stage where it can be either immunized or tolerized depending on concentration, molecular form of antigen, and other factors such as co-mitogens. It has been shown (15, 29) that the tolerogenesis induced in mature B lymphocytes by monomeric antigens can be prevented by the addition of adjuvant substances with the capacity for polyclonal B-cell activation (28). Failure of such abrogation of tolerance in the present system would strengthen the probability that immature B cells were reacting in a unique way. Therefore, we investigated the effects of LPS added to bone marrow cultures undergoing tolerogenesis. Preliminary experiments were performed to show that the LPS batch used performed as would be expected in inducing appearance of PFC in antigen-free spleen cell cultures and in causing B-cell blastogenesis. When added to bone marrow cell cultures containing antigen (Table IV), 10 μg/ml of LPS did not diminish tolerogenesis. With 50 μg/ml LPS, the adoptive immune performance of tolerized cells increased but not to a statistically significant extent. Basically similar results were obtained when POL was used as a B-cell activator.

Discussion

The key findings of this study have been as follows: (*a*) Remarkably low molar concentrations, (c. 10^{-9} M) of an essentially monomeric antigen have induced specific immunological nonreactivity in B cells of bone marrow cell but not of spleen cell cultures. (*b*) The tolerogenesis follows a kinetics similar to that of

TABLE IV

Effect of LPS on Tolerance Induction of Bone Marrow Cells in Culture

Treatment in culture	Anti-DNP-PFC/spleen
	Mean ± SE
4 μg/ml HGG	780 ± 120
4 μg/ml DNP_1-HGG	167 ± 37
4 μg/ml DNP_1-HGG + 10 μg/ml LPS	132 ± 54
4 μg/ml DNP_1-HGG + 50 μg/ml LPS	270 ± 84

renewal of Ig-positive small lymphocytes in marrow. Full effects are achieved only if antigen is continuously present throughout culture. (*c*) The in vitro treatment always resulted in a residual immune competence in the cultured cells. In fact, the observed effect of antigen was really to prevent the emergence of new immune competence rather than to abrogate preexisting competence (Fig. 2). (*d*) The nonreactivity, tested through a T-cell-independent antigen challenge in a carefully calibrated adoptive immune model, could be induced as well in the absence of T lymphocytes as in their presence, and could not be transmitted to a population of untreated, cultured bone marrow cells. Thus, active suppression of one population of lymphocytes by another does not appear to be at work.

This series of results is entirely consistent with the predictions of the clonal abortion theory of B-cell tolerogenesis. The bone marrow lymphocyte population is seen as consisting of cells at diverse maturational stages. The large and medium lymphocytes in rapid mitotic cycle, as well as the nondividing small lymphocytes which are their immediate progeny, lack detectable Ig receptors (20, 21) and therefore cannot react with antigen. Over the subsequent 2 days or so, progressively increasing amounts of Ig receptors appear on the maturing small lymphocyte's surface (21), and we postulate that, for any given lymphocyte, all receptors are of identical antibody specificity. We suggest that any contact with an antigen capable of reacting with such receptors above a certain threshold of avidity of binding results in death or permanent functional suppression of that cell. This phase of the cell's maturation, which may last around 2 days, we refer to as the tolerance-sensitive, or obligatorily paralyzable phase. At its completion, the cell emerges as a fully mature, immunologically competent B lymphocyte with the density of surface Ig receptors characteristic of B cells in secondary lymphoid organs. At any given time, bone marrow samples will contain a small proportion of such mature B cells. Ex hypothesi, these show no special sensitivity to tolerogenesis and could account for the residuum of irreducible immune competence present in DNP_1-HGG-treated cultures. In culture, marrow cells appear to mature from Ig-negative to Ig-positive at approximately the same rate as in vivo (21), and their immune competence gradually builds up (22). However, in the presence of antigen, specifically reactive lymphocytes may be aborted as their receptors appear. Thus, while irrelevant immune competence increases normally, reactivity to the particular antigen present in culture does not increase.

If clonal abortion is a major physiological mechanism of tolerogenesis, this in no way negates the possibility that B-cell tolerance could also be achieved by other means. For example, recent studies from our own laboratory (29) have shown that in cultures of spleen cells from congenitally athymic mice, oligomeric antigens such as FGG or $DNP_{4.5}$-HGG

can cause tolerance, but in 1,000- to 10,000-fold higher threshold concentrations. Highly polymeric antigens can also functionally silence mature B cells (11–14). It may be that for many antigens present in extracellular fluids in concentrations above 10^{-9} M, clonal abortion at both T and B cell levels is the chief physiological guarantee against self-reactivity, and antigen-antibody complex-mediated tolerogenesis (12) may act as a useful "fail-safe" device. Tolerogenesis of mature B cells by antigens such as POL may represent an unphysiological counterpart of the latter phenomenon.

If clonal abortion of B cells indeed occurs in vivo, it is pertinent to ask why so many of the models of tolerance induced by the repeated injection of small amounts of antigen into intact animals have recently been shown to be exclusively at the T-cell level. We believe that, in some cases, such experimental protocols allow a degree of immunization to take place (especially amongst B cells of low avidity for the tolerogen) because of unavoidable presence of some aggregates or of contaminating endotoxinlike materials in proteins. The suppressor mechanisms documented in such experiments, while interesting and valid, may reveal more about feedback mechanisms in immune responses than about self-recognition.

The low concentrations of antigen shown capable of tolerogenesis of bone marrow cultures are striking when it is considered that our assay system measures the behavior of B cells whose progeny secrete relatively low-affinity IgM antibody. Our read-out system as presently constituted does not specifically highlight tolerogenesis of the subset of anti-DNP bone marrow B cells that might, with appropriate T-cell help and challenge, have matured into high affinity IgG-producing anti-DNP PFC. It is quite possible, in line with the speculations of Mitchell (30), that such cells would have been tolerized with even lower concentrations of antigen. As it is, we have documented B-cell tolerance with antigen concentrations usually regarded as more typical for T-cell tolerogenesis (7, 8). Experiments with comparable doses of tolerogens injected into living animals would have failed to induce B-cell tolerance because of the long-lived nature of many peripheral B cells. Any tolerogenesis of newly emerging B cells would have been obscured by the residual normal reactivity of the older B cells, not tolerized by that particular antigen dosage level. The present postulate explains why tolerance may sometimes be more readily induced in newborn animals, where the existing pool of mature lymphocytes is small; and why tolerance may sometimes be maintained (once established) by lower antigen doses than were needed to induce it.

We have not investigated the molecular mechanisms which may be involved in clonal abortion. The theory needs ad hoc explanations for the special sensitivity of immature cells to contact with oligomeric antigen; for example, if tolerogenesis were due to cyclic AMP synthesis, the immature B cell might display a much lower inhibitory threshold. It is worthwhile recording that the nature of the protein carrier in the induction of hapten-specific B-cell tolerance is significant. In vivo, DNP coupled to albumins is not as effective as coupled to globulins (9), and in the tolerization of spleen cell cultures with high concentrations of DNP-proteins (29), the same was true, although the B-cell Fc receptor was probably not involved as $F(ab)_2$ fragments were efficient carriers for hapten tolerogens. In the present bone marrow culture system, the role of the carrier has not been extensively investigated, but preliminary experiments indicate that FGG is as effective, but BSA much less effective, than HGG. A simple

methodology[2] for the preparation of functionally enriched populations of DNP- or NIP-reactive spleen cells has recently been developed in our laboratory. If this can be successfully applied to bone marrow lymphocytes, new approaches to the molecular biology of tolerance induction might become possible.

Summary

This paper deals with the behavior of adult mouse bone marrow cells placed in tissue culture with or without antigen, and subsequently assessed for immune competence after adoptive transfer into lethally X-irradiated, syngeneic hosts. Attention was focussed on B lymphocytes through using hapten human gamma globulin (HGG) preparations as putative tolerogens in tissue culture, the T-cell-independent antigens DNP-POL and NIP-POL as challenge injections in adoptive hosts, and numbers of hapten-specific PFC in host spleens for the quantitation of immune competence.

It was found that the capacity of bone marrow cells to mount an adoptive immune response rose by a factor of about fivefold over 3 days in tissue culture. This rise was completely abolished by the presence in the culture of hapten-HGG conjugates with about one mole of hapten per carrier molecule. The prevention of the emergence of immune competence amongst maturing B cells was termed clonal abortion tolerogenesis. Dose-response studies showed the lowest effective antigen concentration to be between 2.5×10^{-10} and 2.5×10^{-9} M, and a standard concentration of 2.5×10^{-8} M was chosen as producing near maximal effects.

The tolerance was antigen-specific and time-dependent, being maximal only when antigen was present continuously as the cultured cells were maturing. It did not depend on the presence of T lymphocytes in marrow, and was not of an "infectious" type. In contrast to tolerogenesis of mature B lymphocytes by high antigen concentrations, it could not be abolished by lipopolysaccharide. We speculate that clonal abortion may be a tolerance mechanism of great physiological significance for self-recognition, and discuss the results in the framework of other recent tolerance models, including those involving receptor blockade and suppressor T cells.

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