Clonal anergy: The universally anergic B lymphocyte

(immunological tolerance/monoclonal anti- μ /B cell differentiation/fluorescence-activated cell sorter)

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ABSTRACT The clonal anergy theory of induction of immunological tolerance states that differentiating B lymphocytes that encounter multivalent antigen at the pre-B to B cell transition stage can receive and store a negative signal, which renders them anergic to later triggering stimuli. The theory was tested by using an anti- μ chain monoclonal antibody, E4, as a model tolerogen. The fluorescence-activated cell sorter was used to select B cell-free cell populations from adult murine bone marrow or newborn spleen, and later, to analyze B cell neogenesis *in vitro*. The presence of E4 at $\geq 1 \mu\text{g/ml}$ was required to impede the development of normal numbers of B cells with full receptor status. The subsequent capacity of these B cells to respond *in vitro* to mitogens was assessed in a filler-cell free microculture system that allows single B cells to proliferate and differentiate. Concentrations of E4 far below those required to affect B cell neogenesis had profound inhibitory effects on the subsequent functional capacity of the B cells. In fact, $10^{-3} \mu\text{g/ml}$ of E4 markedly impaired both proliferation and antibody formation, and $10^{-1} \mu\text{g/ml}$, which had no effect on Ig receptor development, abrogated functional capacity. Thus B cells formed in the presence of E4 at $10^{-1} \mu\text{g/ml}$, though possessing the receptor status typical of B cells, were functionally entirely anergic. Exposure to E4 appeared to accelerate the spontaneous death rate of newly formed B cells *in vitro*. Whether the anergic cell would also have a shortened life span *in vivo* is not known.

It is now well established that immature cells of the B lymphocyte lineage can be rendered immunologically tolerant by certain multivalent antigens both *in vitro* and *in vivo* (1–8). Recently, we provided evidence that the stage at which the cell displayed its greatest sensitivity to negative signaling was that of first emergence of the surface membrane immunoglobulin (mIg) receptors, that is, during the pre-B to B cell transition (7). It is possible to introduce tolerogens into the tissues of the developing fetus by transplacental transfer (6) and thus ensure interaction between lymphoid cells and tolerogen at the first appearance of specific antigen binding receptors. When fluoresceinated human gamma globulin (FLU-HGG) was injected into mice at 14.5 days of gestation, effective B cell tolerance was induced with doses far lower than those required to reduce the number of FLU-specific antigen-binding B cells in the offspring (8). In other words, B lymphocytes expressing a normal range of FLU-binding avidities appeared to emerge from the pre-B cell pool in normal numbers, but were incapable of giving rise to anti-FLU antibody-forming cell (AFC) clones when challenged with a B cell mitogen or a T lymphocyte-independent antigen. This suggested that interaction between the newly emerged anti-FLU receptors and antigen had neither impeded the subsequent development of a standard complement of mIg nor directly led to the death of the anti-FLU B cell. Rather, the cell, though still alive and capable of binding antigen, appeared

to have received some signal rendering it incapable of responding to appropriate triggering stimuli. We termed this phenomenon clonal anergy.

This conclusion was dependent on a technology that enumerated and characterized FLU-binding B cells, by counting cells adhering specifically to thin layers of haptened gelatin, and then analyzing the cells' capacity to bind fluoresceinated proteins by using the fluorescence-activated cell sorter (FACS) (8, 9). We wished to strengthen the conclusion by using an experimental design that avoids the complexities inherent in work with rare antigen-binding cells. Accordingly, we explored the properties of antibodies to the μ chain of murine Ig as a "universal" B cell tolerogen. It has long been known that the injection of anti- μ antibody in very early life can inhibit the emergence of B lymphocytes and produce an agammaglobulinemic animal (10, 11). In contrast, we wished to determine whether concentrations of anti- μ antibody too low to impede the development of a B cell population with normal mIg receptors could nevertheless functionally impair the cells, creating a population of universally anergic B cells. We developed an *in vitro* strategy because transplacental transfer of anti- μ chain IgG would be inhibited by the large amount of IgM in the maternal circulation, leading to immune complex formation; and intra-fetal injections are accompanied by uncertainties relating to leakage into the amniotic fluid.

In this paper, we document the effects on maturing B cells of very low concentrations of a monoclonal anti- μ chain antibody, E4, the product of a rat-murine hybridoma and the preparation and properties of which we have described (12). Adult bone marrow or newborn spleen cells were fractionated in the FACS to obtain mIg⁺ cells, which were cultured in the presence or absence of E4 for a period of 1 or 2 days, allowing many cells to mature into functional B lymphocytes in control cultures (13). The cells were then analyzed in the FACS to determine their mIg density spectrum. The capacity of these cells to divide or differentiate into AFC was also assessed by using a filler cell-free system devised by Wetzel and Kettman (14–16), which in the presence of the two mitogens *Escherichia coli* lipopolysaccharide (LPS) and dextran sulfate (DXS) allows a substantial proportion of single B cells to proliferate to form a cluster of B cell blasts. The studies showed that concentrations less than 1/1000th of those needed to inhibit mIg appearance could prevent the cells from dividing or forming antibody, consistent with the clonal anergy theory.

MATERIALS AND METHODS

Mice and Cell Suspensions. Mice from the inbred strains CBA/CaHWehi and BALB/c AnBradleyWehi were used. All

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Abbreviations: AFC, antibody-forming cell; DXS, dextran sulfate; FACS, fluorescence-activated cell sorter; FLU, fluorescein; LPS, *Escherichia coli* lipopolysaccharide; mIg, surface membrane immunoglobulin; PA-SRBC, staphylococcal protein A coupled to sheep erythrocytes.

bone marrow donors were 8- to 10-week old CBA. Newborn spleen donors were 1–2 days old. Cell suspensions were prepared with erythrocyte and damaged cell removal steps as described (13, 17).

Antisera. Polyvalent anti-mouse Ig antiserum raised in a sheep was purified on a mouse IgM (μ, κ)-Sepharose column and then fluoresceinated as described (18). This preparation, which had high anti- κ activity, was used in all FACS selection and analysis procedures.

Monoclonal Antibodies. The monoclonal anti- μ chain antibody used, E4, was prepared by fusion of spleen cells from a rat immunized with FLU-IgM-1, a murine IgM (μ, κ) hybridoma antibody specific for FLU (19), and the mouse myeloma NS-1 as described (12). Anti- μ antibody was purified by adsorption to staphylococcal protein A-Sepharose 4B. As a control, B7, a monoclonal anti-idiotypic antibody against murine FLU-IgM1, was used.

FACS Selection of mIg⁻ Cells. The FACS (FACS II, Becton Dickinson Electronics Laboratory, Mountain View, CA) was used to analyze the forward light-scatter behavior and fluorescence intensities of both cell populations as described (9, 13). The small cell peak was selected by forward light scatter (usually channels 80–130), the fluorescence profile was then analyzed, the threshold for positivity was set at fluorescence channel 10, and all cells below were collected as described (13). Essentially the same parameters were used for selection of mIg⁻ bone marrow and newborn spleen cells.

In Vitro Culture Procedures. FACS-selected mIg⁻ cells were cultured for 1 or 2 days in the presence or absence of E4 antibody at 50,000–80,000 cells in 10 μ l of RPMI 1640 medium (GIBCO), supplemented with 10% (vol/vol) fetal calf serum (Flow Laboratories, Australia) and 50 μ M 2-mercaptoethanol in 60-well Terasaki trays (Lux Scientific). After the maturation period, cells were harvested, counted, and recultured at approximately 100–800 viable cells in 10 μ l of medium containing LPS at 100 μ g/ml, DXS at 10 μ g/ml, and E4 as in the primary “bulk” culture. All cultures were held at 37°C in a humidified atmosphere of 10% CO₂ in air.

FACS Analysis of mIg Status. After the “*in vitro*” maturation step, cells were washed, labeled with fluoresceinated anti-Ig antisera, and analyzed in the FACS as described (13). Propidium iodide was added to allow the exclusion of nonviable cells in the analysis procedure (20).

Antibody Formation in Vitro. Antibody formation was assessed by using the protein A-sheep erythrocyte (PA-SRBC) “reverse” plaque assay (21). A polyvalent rabbit anti-mouse Ig serum was used as the developing antiserum to enable the detection of all Ig-secreting cells. Cultures were assayed 4–5 days after mitogenic stimulation by transfer to 96-well flat-bottomed microculture plates and 50 μ l of plaque-revealing mix (PA-SRBC, complement, and developing antisera) was added to the wells as described (22, 23). Plaques were counted under a dissecting microscope after 4–6 hr at 37°C. In some experiments aliquots of pooled cultures were assayed. In limiting diluting analysis experiments, the frequency of AFC precursors was calculated by Poisson analysis as described (17, 23).

Assessment of Clonal Proliferation. Cultures were examined at the indicated intervals, using an inverted phase-contrast microscope at $\times 100$ magnification as described (16, 23). The frequency of cluster-forming cells was determined by Poisson analysis as above.

RESULTS

Concentrations of E4 Required to Reduce mIg Expression Amongst Maturing B Cells. The FACS was used as described (13) to prepare B lymphocyte-free cell populations greatly en-

riched for pre-B cells capable of turning into B cells after 1 or 2 days of culture. Because the mIg on immature B cells can be readily and irreversibly removed by anti- μ antibodies (24), relatively low concentrations of E4 could be expected to prevent a normal set of mIg receptors emerging from the pre-B cells, essentially by continuous removal of mIg molecules on their placement into the membrane. Accordingly, FACS-selected mIg⁻ pre-B cells from either adult bone marrow or newborn spleen were exposed for 1 day *in vitro* to medium alone, to graded concentrations of E4, or (as a control) to B7, an irrelevant anti-idiotypic monoclonal antibody, at 10 μ g/ml. Fig. 1 gives the results of a single experiment each on adult bone marrow and newborn spleen. In medium alone, or with B7, 30–40% of the cells developed above-background numbers of mIg receptors. Exposure to E4 at 10 μ g/ml was markedly inhibitory, and few cells of high mIg density appeared. With 1 μ g/ml, much less inhibition was noted, and 0.1 μ g/ml failed to affect mIg emergence, as did still lower concentrations (data not shown). Essentially identical results were obtained in repeated experiments, and the pooled results are shown in Fig. 2 as percent inhibition of mIg expression. With a purified conventional anti- $(\mu + \lambda)$ antibody from a hyperimmunized rabbit or a 2-day culture period, the patterns were not substantially different (data not shown). Note that Fig. 1 shows a high proportion of cells with a relatively low mIg density, suggesting the presence of many cells capable of further maturation.

Inhibition of Antibody Formation Amongst B Cells Exposed to E4 During the pre-B to B Transition. FACS-selected mIg⁻ cells were exposed to graded concentrations of E4 during the

FIG. 1. FACS analysis of selected mIg⁻ adult CBA bone marrow or newborn CBA spleen cells matured *in vitro* for 1 day prior to assessment of total mIg density. Dotted presentation, no inhibitor present; continuous lines, indicated inhibitor present. (A) Irrelevant monoclonal antibody, B7, at 10 μ g/ml; (B) E4 at 10 μ g/ml; (C) E4 at 1 μ g/ml; (D) E4 at 0.1 μ g/ml. (Left) Adult bone marrow; (Right) newborn spleen.

FIG. 2. Inhibition of antibody formation (----) and B lymphocyte appearance (—) by the presence of graded concentrations of E4 during the mIg⁻ to mIg⁺ transition. ○, Adult CBA bone marrow; ×, newborn CBA spleen. Control values: adult bone marrow, 33% mIg⁺, 27 ± 5 PA-SRBC plaque-forming cells per 100 cells; newborn spleen, 40% mIg⁺, 38 ± 11 PA-SRBC plaque-forming cells per 100 cells.

pre-B to B (mIg⁻ to mIg⁺) transition period of 1 or 2 days. Cells were recultured at 70–800 cells in 10 μl of medium with LPS at 100 μg/ml and DXS at 10 μg/ml. The presence of E4 was maintained throughout to prevent any pre-B cells that might acquire mIg and functional capacity somewhat later from biasing the results (7). Cultures were assayed after 4–5 days for the presence of Ig-secreting cells as detected by the PA-SRBC plaque method so that any B cell stimulated into antibody production by the polyclonal activators LPS plus DXS would be detected, regardless of specificity. Fig. 2 compares inhibition of antibody-forming capacity with inhibition of mIg appearance as a function of E4 concentration. Whereas 10 μg/ml of E4 achieved a substantial reduction in mIg expression, 1 μg/ml caused only about a 40% reduction, but abrogated AFC capacity. The data for E4 at 10⁻¹ μg/ml are particularly interesting, because B cell numbers are entirely normal but the AFC capacity is abolished. Similarly, 10⁻² μg/ml of E4 virtually abrogated AFC capacity whilst allowing a normal B cell number to emerge. The mIg⁻ cells from bone marrow were somewhat more sensitive to functional incapacitation by E4 than were those from newborn spleen (about 50% reduction with 10⁻⁴ and 10⁻³ μg/ml, respectively), requiring 10,000-fold rather than 1,000-fold higher concentrations to achieve a 50% reduction in mIg receptor status than in AFC capacity. In contrast, experiments with unfractionated adult spleen cells showed that 10 μg/ml abrogated function and about 10⁻¹ μg/ml achieved a 50% reduction. Unfractionated newborn spleen or adult marrow cells were intermediate in sensitivity, reflecting mainly the activity of the cells already mIg⁺ at the moment of killing, showing 50% functional impairment with E4 at about 10⁻² μg/ml.

Reduction in Numbers of B Cells Capable of Giving AFC Clones. Because the LPS plus DXS system allows clonal growth of isolated B cells (14–16, 23), we also examined the AFC capacity of the *in vitro* matured cells at a clonal level by culturing the cells at limit dilution at challenge. The actual input cell number was determined by counting viable cells 18 hr after resspreading. As shown in Table 1, the AFC clone frequency is reasonably high, considering the immaturity of the cells, of which only a third actually were B cells. When analyzed at the single-cell level, the recently matured B cells were extremely

Table 1. Capacity of single B lymphocytes to develop into AFC clones after polyclonal activation: Inhibition by a monoclonal anti-μ chain antibody, E4

E4, μg/ml	% of <i>in vitro</i> matured cells capable of generating AFC clones with LPS plus DXS*		
	mIg ⁻ bone marrow, 1 day preculture†	mIg ⁻ bone marrow, 2 days preculture‡	mIg ⁻ newborn spleen, 1 day preculture†
0	3.4	5.4	11.8
10 ⁻²	0.06	0.36	0.32
10 ⁻³	0.84	1.9	2.6
10 ⁻⁴	Not tested	2.6	4.6

* FACS-selected mIg⁻ cell populations were precultured for 1 or 2 days with or without E4 as indicated, then recultured at limit dilution with LPS plus DXS with or without E4 and assayed 4–5 days later for AFC clones. Frequencies were determined according to the Poisson equation, using the number of viable cells 18 hr after reculture as the input cell number.

† Mean of two experiments.

‡ One experiment only.

sensitive to the induction of clonal anergy, 10⁻² μg/ml of E4 virtually abrogating the AFC response to LPS plus DXS and even 10⁻⁴ μg/ml appearing to have some effect. The mean number of hemolytic plaques per AFC clone was around 10, and because it was common to find small clumps of cells at the center of plaques, we believe the true number of AFC was higher, perhaps of the order of 20 (17). There was no systematic difference in clone size between E4-treated and control groups.

Frequency and Characteristics of Proliferating Clones After LPS plus DXS Stimulation of *in Vitro* Matured pre-B Cells. The filler cell-free Kettman system (14–16, 23) allows the detailed daily examination of cultures, so that blastogenesis and formation of clusters can be directly detected. Table 2 presents relevant data on frequencies of generated B cells capable of proliferation and shows that the proliferative capacity is just as readily inhibited as antibody formation. In fact, these results closely resemble those of Table 1.

Table 3, which represents a single large experiment typical of up to four experiments performed, describes the kinetics of proliferation of mIg⁻ adult bone marrow cells. In the absence of E4 or mitogens, these cells gradually die in culture, only 16% surviving to 4 days after resspreading (i.e., 5 days total *in vitro*), with no cluster formation being noted. This basic process was not greatly affected by E4 at 10⁻³ μg/ml, although at day 3, these cells were perhaps slightly smaller and fewer than control cells. With E4 at 10⁻² μg/ml, there was a definite acceleration of cell death, with virtually no viable cells left at day 4. When

Table 2. Capacity of single B lymphocytes to give rise to proliferating cell clusters following polyclonal activation: Inhibition by E4

E4, μg/ml	% of <i>in vitro</i> matured cells capable of generating proliferating clones with LPS plus DXS*		
	mIg ⁻ bone marrow, 1 day preculture	mIg ⁻ bone marrow, 2 days preculture†	mIg ⁻ newborn spleen, 1 day preculture‡
0	4.0 ± 0.1§	8.0	14.0
10 ⁻²	0.01 ± 0.01§	0.18	0.32
10 ⁻³	1.1 ± 0.08§	1.5	3.6
10 ⁻⁴	2.0†	3.1	8.9

* As assessed morphologically 4–5 days after challenge.

† One experiment only.

‡ Mean of two experiments.

§ Mean of three experiments ± SEM.

Table 3. Kinetics of proliferation amongst mIg⁻ bone marrow cells maturing in culture in the presence of E4 and then challenged*

Time after challenge, days	Nature of challenge	Normalized [†] cell number and morphological comment		
		No anti- μ antibody	E4 at 10^{-3} μ g/ml	E4 at 10^{-2} μ g/ml
1	None	100 small	100 small	100 small
	LPS + DXS	100 mainly small, some medium, a few large blasts	100 small	100 small
2	None	62 small	51 small	33 small
	LPS + DXS	Numerous transformed cells, doublets, clusters of 3–4 cells	Some transformed cells, fewer doublets or clusters than control	24 small
3	None	31 slight increase in median size	20 small	7 small
	LPS + DXS	Many large clusters	Clusters smaller than controls	4 predominantly small, very rare blast or small cluster
4	None	16 as day 3	13 small	0.2 small
	LPS + DXS	Clusters still larger than on day 3	Definitely less extensive proliferation than controls, clusters smaller	0, no viable cells

* Cells were cultured in bulk for 1 day with or without E4, then spread at approximately 100 cells per well and challenged with LPS plus DXS. Accurate cell counts were performed at 1 day after spreading, at which time no effect of E4 on cell numbers had been detected.

[†] The actual cell number for a particular group on a particular day as a percentage of the number present 1 day after respreading. Where no number appears, cluster formation prevented accurate counting.

control cells (no E4) were stimulated with LPS plus DXS, there was a lag period of at least 1 day before significant blastogenesis was noted. By day 2, cell division was clearly evident, and this proceeded rapidly over the next few days. Control cultures generated between 300 and 380 cells at day 5 from the input 100 at day 1. This blastogenesis and division was detectably inhibited by E4 at 10^{-3} μ g/ml, and was virtually abrogated at 10^{-2} μ g/ml. With E4 at 10^{-2} μ g/ml cell death was slightly more rapid in the presence of mitogens. The kinetics of the LPS plus DXS-induced proliferative response of mIg⁻ newborn spleen cells were similar, the major difference being that significant blastogenesis and cell division was evident in control (no E4) cultures within 1 day (rather than 1–2) of stimulation. The presence of E4 at 10^{-2} μ g/ml accelerated cell death. Control cultures yielded 390–603 cells from 100 input cells at day 4. Similar inhibition was achieved in the presence of E4 (Table 2).

DISCUSSION

This paper seeks to address the question of what happens to a B lymphocyte when it encounters molecules capable of cross-linking the mIg receptors as they first appear on the plasma membrane. A monoclonal rat IgG antibody (resulting from a rat-mouse cell fusion) with specificity for murine μ chains, termed E4, was used as a model ligand, because of its capacity to react with all emerging B lymphocytes, regardless of specificity. To ensure that all cells studied first encountered the ligand during the pre-B to B cell transition, small mIg⁻ cells were selected from the heterogeneous mixture of differentiating cells present in adult bone marrow or newborn spleen by FACS sorting prior to culture. Both cell sources used contain numerous cells capable of developing into B cells without division during a relatively short culture period (13, 25, 26). When anti- μ antibody was present during this transition, various phenomena were observed, depending on the concentration of the ligand. A moderate concentration, 10 μ g/ml, inhibited the appearance of the full complement of mIg receptors, consistent with previous observations on the special susceptibility of immature B cells to mIg receptor modulation (24). This effect could not be achieved with anti- μ monoclonal antibody at 10^{-1} μ g/ml (Fig.

1), and it is the effects of low concentrations of E4 on functional capacity that are of special interest.

B lymphocyte function can now be probed in a different way by the use of the mitogens LPS and DXS acting together (14–16, 23). In the absence of filler cells or added interleukins, a high proportion of single B cells can respond by blast transformation and division, which can be observed microscopically. The Kettman group (14–16) has reported that, with adult splenic B cells, only a small proportion of LPS plus DXS-stimulated clones form detectable antibody, unless a few macrophages are added to the cultures. In a recent paper (23), we showed that when adult splenic hapten-affinity fractionated B cells were stimulated with LPS plus DXS, 13.6% formed a visible clone and, of these, a quarter were capable of anti-hapten antibody production. When, instead of the input cell number, the cell number observed 18 hr after initiation of culture was used to calculate cloning efficiency, 32% of the surviving B cells proliferated. The present studies demonstrate that newly formed B cells, emerging in culture from mIg⁻ precursors, can also respond to LPS plus DXS with a reasonably high cloning efficiency. When mIg⁻ B cells from adult bone marrow were precultured in bulk for 1–2 days in the absence of E4 to allow B cells to emerge, 4–8% of all cells surviving this initial preculture period formed clusters (Table 2), and, because only 32% of these surviving cells were B cells, the actual cloning efficiency on a per B cell basis was considerably higher. With newborn spleen mIg⁻ cells matured *in vitro*, the cloning efficiency was 14% of all cells and 35% of B cells surviving the bulk preculture. Interestingly, with these sources of new B cells, the majority of LPS plus DXS-stimulated clones formed antibody. We do not yet know whether this represents a true difference between immature versus mature B cells or a difference in the assay methods.

When the anti- μ monoclonal antibody E4 was present during the maturation of the mIg⁻ cells, their capacity to proliferate and to differentiate into AFC could be profoundly inhibited. Most importantly, this negative signaling was achieved with anti- μ concentrations that had no effect on mIg emergence. Thus, B cells were produced that had a normal mIg receptor coat but that could not be stimulated to divide or differentiate.

This finding materially strengthens the clonal anergy theory. It then becomes pertinent to ask what might be the long-term fate of such cells. This is a difficult question to approach by tissue culture techniques, because unstimulated B cells die rather rapidly in culture. Some information relevant to the issue is presented in Table 3, which shows that the spontaneous death of unstimulated, newly emerging B cells is accelerated by anti- μ at 10^{-2} $\mu\text{g/ml}$, though this concentration had not reduced the number of B cells formed in the first instance. This death rate may have been increased still further by mitogenic stimulation. The functional life span of virgin B lymphocytes *in vivo* appears to be short (27, 28), the B cell pool being constantly replenished from the bone marrow on the one hand, and enlarged by antigenic stimulation and clonal expansion of selected elements of the repertoire on the other. Whether exposure to low concentrations of an mIg receptor-linking ligand early in its differentiation shortens the life span of the B cell *in vivo* is not known. To the extent that it were to do so, the differences between the notions of clonal anergy (8) and clonal abortion (1) would diminish. This question, as well as the question of whether the negative signal would decay if the ligand were removed from the cell or its environment, deserves further study.

It is now clear that the mIg receptors play a decisive role in signal transduction in the B lymphocyte. In the case of a mature B cell, cross-linking of the receptors can initiate the cascade of events that leads to proliferation and antibody production, but only under some circumstances, such as are provided by the conjoint use of a T-independent antigen and appropriate interleukins (24). Frequently, crosslinking in the absence of circumstances favoring lymphocyte activation leads to the opposite effect, namely the delivery of a negative signal. The differentiation state of the cell profoundly influences its sensitivity to this negative signal. Further, it is now clear that the cell's capacity to receive and store the signal is not connected to its capacity to generate and maintain its mIg receptor status. Obviously clonal anergy can be induced through only a minute proportion of the receptors being aggregated. The immature B cell, first seeing the ligand when the mIg receptors are already plentiful, is less susceptible to negative signaling, but still much more so than the typical mature adult splenic B cell (1). This cell can still readily be rendered nonreactive, as indeed can an actual antibody-secreting cell in the phenomenon we have termed effector cell blockade (29, 19), though this, of course, requires higher ligand concentrations still. It is of interest to compare the threshold concentration of our monoclonal reagent, E4, required for induction of clonal anergy, around 10^{-4} $\mu\text{g/ml}$ (Fig. 2), with that required for effector cell blockade, namely 30 $\mu\text{g/ml}$ (unpublished data) a difference of about 5.5 $\log_{10}$. The gene control mechanisms that transmit the signal to the nucleus remain entirely obscure, but the sensitivity differences observed must influence any strategy aimed at their eventual elucidation.

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