Commentary

Clonal Anergy of B Cells: A Flexible, Reversible, and Quantitative Concept

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At the conclusion of the 1986 Immunology Congress in Toronto, the late Georges Köhler presented a summary lecture which was, unfortunately, poorly attended. It dealt with the impact of the new genetics on immunology, and, among other wise things, he said that transgenic technology was set to revolutionize the way we studied immunologic tolerance (1). The greatest barrier to uncovering the details of what happened to anti-self cells in the repertoire was the heterogeneity of lymphocytes. Cells potentially reactive with a given self antigen were so rare that most attempts to study their fate relied on inferential rather than direct methods. Successful attempts to create mice transgenic for both heavy (H) and light (L) immunoglobulin (Ig) chains, or for the α and β chains of the recently discovered T cell receptor (TCR) in theory, at least, had the potential to create monoclonal mice, i.e., animals in which all or at least the greater part of the B or T cell population consisted only of cells bearing the transgenically imposed specificity. Anti-idiotypic reagents could identify such cells, or, even more easily, simple enumeration of B or T cell numbers under various experimental circumstances could yield information about the fate of anti-self cells.

How right Köhler was; but it took some time to prove it. Burnet first articulated the concept of clonal deletion as the key mechanism of tolerance, postulating that if an encounter between a self antigen and a cell reactive with it occurred in early life, while the immune system was immature, clonal deletion rather than clonal selection would be the end result (2). Lederberg refined this notion, placing the transition from paralyzability to inducibility at the level of each differentiating immunocyte, regardless of the age of the animal (3). When it first became possible to enumerate antigen-binding B cells by autoradiographic techniques, early results indeed favored deletion as a tolerance mechanism (4). For T cells, it was still necessary to rely on functional techniques to support repertoire purging as a tolerance mechanism as not even enumeration of antigen-specific precursors of effector T cells by in vitro cloning techniques could distinguish clonal deletion from some form of effective non-lethal silencing (5). However, the latter concept had articulate supporters on theoretical grounds (6). Careful work on antigen-specific B cells, involving both their enumeration and purification, showed that high doses of antigen could cause clonal deletion, but tolerance could be achieved with much lower doses, this non-deletional phenomenon being termed clonal anergy (7). Support for clonal anergy among T lymphocytes also gradually emerged (8, 9).

Enter transgenic mouse technology. As far as B cell tolerance was concerned, the first results were disappointing. Antigen-transgenic mice were either non-tolerant or variably tolerant (10, 11). However, antigen-transgenic animals were not the main game, as they left the investigator with the task of studying the minority of (unidentifiable) reactive lymphocytes. Things really took off when B (12, 13) or T (14) cell receptor transgenic mice were used. From the viewpoint of the present story (15), the critical model was an ingeniously devised double-transgenic strategy (12; reviewed in 16, 17). Mice were rendered transgenic for monoclonal B cell IgM and IgD receptors with high affinity for hen egg lysozyme (HEL) and such mice were mated with mice transgenic for HEL itself. Further features of the model were a capacity to distinguish transgenic from endogenous antibody via an allotype marker; the use of various founder lines constitutively producing various amounts of soluble HEL (s-HEL); a capacity to raise low HEL levels to high ones through zinc feeding in mice where the metallothionein promoter formed part of the HEL transgenic construct; and comparison of the effects of s-HEL with a membrane-anchored form of the same antigen (m-HEL). Some key findings of this model were as follows. s-HEL at very low concentrations caused T cell tolerance but with no discernible effects on the B cell compartment. Higher concentrations also resulted in T cell tolerance but in addition caused a non-deletional, clonal anergy type of B cell tolerance, readily observed as the great majority of B cells carried the transgenic Ig. This B cell tolerance was accompanied by a down-modulation of surface IgM and a marked shortening of the B cell's life-span (18). The transgenic B cells clustered chiefly in follicular areas of the spleen, especially the mantle zone of the follicles. Only when the strongly cross-linking signal of transgenic m-HEL was in place did a deletional type of tolerance, representing clonal elimination shortly after Ig expression (while the immature B cell was still in the bone marrow) become manifest. Still, the delayed deletion of the anergic cells at 3–4 d does raise the question of whether anergy in B cells is entirely discrete from "clonal abortion" (19).

The plot thickens as we move to studies that seek to track the migration of transgenic B cells after transfer into various kinds of host mice (20). Anti-HEL B cells trans-

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ferred into antigen-free hosts homed to lymphoid follicles, coming to occupy much the same location as within the donor animal. A similar trafficking pattern was noted when the transgenic B cells were transferred to doubly s-HEL–anti-HEL transgenic mice. However, when the anti-HEL (anti-neo-self) B cells find themselves in a minority among a diverse B cell repertoire, and still in the presence of the HEL antigen, something different happens. Transferred B cells still appear in the white pulp of the spleen, and soon move to the outer part of the T cell trafficking area, the so-called peri-arteriolar lymphocyte sheath or PALS, where they come to occupy an area just outside the follicle, at the follicle/PALS border. They do not penetrate into the follicle, but appear to die within a day or two.

To understand the interpretation that Cyster et al. (20) place on these findings, we must first present some further curious facts. Newly formed B cells leave the bone marrow in very large numbers every day and home predominantly to the spleen (21). At this stage of their lives, they are predominantly IgM^{high} IgD^{low} B cells, and these cells are rather short-lived, having largely disappeared by a week or so. If B cells from lymph nodes, from thoracic duct lymph, or from other parts of the recirculating lymphocyte pool are examined, these possess a different phenotype. They are $Ig\text{-}M^{low}$ IgD^{high}, recirculating, follicular mantle-seeking and they live for several weeks. These cells are not memory B cells, in that they display no or very few Ig V gene mutations, are not isotype switched, and do not carry memory if adoptively transferred from immunized mice. Yet they are different from virgin, just emerging B cells, in that they exhibit a different and more restricted Ig V gene repertoire (22). It is as though this population has either been positively selected by contact with antigen, but not in a way sufficient to induce isotype switch or Ig V gene hypermutation; and/or lacks cells (perhaps a very large population) that have been negatively selected through reactivity against self antigens. Against this background, Cyster et al. (20) postulate that the follicular micro-environment is necessary for B cells to undergo some final maturational steps. Only if they reach the follicles can B cells undergo these phenotypic changes. They explain their findings by suggesting that there is competition between B cells for access to the follicles. Anergic B cells manage to get to the follicles provided they are the only kind of B cell around, as in the doubly transgenic s-HEL–anti-HEL mice. Set up a situation (there are actually three different models in their paper) where the anergic cells, still "handicapped" by the presence of antigen, are in the minority among a normal, diverse B cell repertoire and the anergic cells fail to reach the follicles and soon die. This exclusion of cells with anti-self potential from follicles is seen as a potent weapon against autoimmunity as the cell does not have longevity conferred upon it and also does not reach the recirculating pool.

In the article that is the subject of the present Commentary, Fulcher and Basten (15) also examine the fate of self-reactive B cells after transfer in the same transgenic model, adding several elegant tricks to the experimental design. They track transferred cells by prelabeling them with the stable intracellular dye 5-carboxyfluoresceine diacetate-succinimidyl ester (CFSE). To give a calibration device comparing the fate of anti-HEL transgenic B cells and normal B cells, the former were treated with a concentration of CFSE four-fold higher than the latter. Thus, flow cytometry can easily distinguish the numbers of the two transferred populations in various organs under different experimental circumstances. To study the effects of antigen concentration, anti-HEL B cells were transferred into different founder lines, with zinc-induced antigen upregulation, or use of m-HEL transgenic mice, providing further variables. Transfer into non-transgenic mice given HEL by injection served as a check on s-HEL-transgenic results. To investigate the influence of effective cognate T cell help on trafficking patterns, survival, and fate of transferred normal or anergic cells, B and T cell cotransfer experiments were performed. CFSE-labeled-anti-HEL B cells were prepulsed with a peptide $MCC_{87\text{-}103}$ from moth cytochrome C. At the same time, $CD4^+$ T cells from a TCR-transgenic mouse, with specificity for $MCC_{87\text{-}103}$, preactivated in vivo one day previously, were also transferred. Appropriate F1 hybrid mice with a suitable class II MHC restriction element were used.

The following are the main results of the study. The shortened life-span of anti-HEL B cells on transfer into either the standard s-HEL transgenic or m-HEL transgenic hosts was confirmed. As neither cell division (which would have halved the CFSE labeling) nor migration to lymph nodes were noted, the cells probably died in situ in the spleen. Some enlargement of the anti-HEL cells by comparison with cotransferred normal B cells suggested a degree of activation before death. As noted by Cyster et al. (20), the anti-HEL B cells migrated to the PALS-follicle border. When anti-HEL B cells were transferred to s-HEL-transgenic strains showing lower HEL serum concentrations, below the threshold for induction of B cell anergy, the B cells did reach the follicle, and persisted there for at least 2 d. This did not occur if the hosts had the HEL levels raised by zinc feeding. As transfer to s-HEL–anti-HEL double transgenics lead to follicular localization, the question of the effective s-HEL concentration in the serum of such mice was raised. Two factors could lower these levels, namely some constitutive anti-HEL antibody formation, and the vast pool of B cells with anti-HEL receptors acting as an "antigen sink." In fact, both ELISA assays and serum capacity to downregulate the Ig receptors of anti-HEL B cells demonstrated that the doubly transgenic mice had significantly lower levels of available HEL than singly s-HEL transgenic mice. These findings showed antigen concentration to be an extremely important variable in the trafficking behavior and life-span of the anti-HEL B cells. When cells were transferred to normal mice, exogenously injected HEL could prevent entry into follicles provided the serum concentration reached above threshold levels.

The provision of T cell help had profound effects on the anergic B cells. Obviously in the doubly transgenic situation, B cells receive no T cell help because the T cell population is tolerant. When peptide-pulsed anergic anti-HEL B cells were transferred into s-HEL transgenic recipients to-

gether with activated, peptide-specific helper T cells, B cells migrated to two locations, namely follicles, where they generated immature germinal centers, and the red pulp, where proliferating foci of antibody-secreting cells were seen. By day 5, significant levels of HEL-specific IgM antibody had appeared.

The most significant difference of interpretation between this paper (15) and that of Cyster et al. (20) is the reason for the appearance of anti-HEL B cells within follicles upon transfer into doubly transgenic s-HEL–anti-HEL mice. Fulcher and Basten (15) claim there is no need to invoke competition for a follicular niche, as the effective concentration of HEL is lower in doubly as opposed to singly (s-HEL) transgenic mice, and this difference alone could alter migration patterns. Moreover, preincubation of anti-HEL B cells with HEL before transfer into doubly transgenic mice could negate follicular entry, again suggesting that B cell behavior is determined by exposure to antigen above a critical threshold concentration rather than by competition between B cells. It is of interest that a relatively small difference in the degree of receptor engagement in anti-HEL B cells, 47% following transfer into s-HEL transgenic mice, or 26% following transfer to doubly transgenic mice, could exert such a major difference in behavior (23).

It is in the outer PALS that B cells normally receive T cell help and it is perhaps not surprising that it is within this location that they die in the absence of T cell help if the partial receptor engagement, above a certain threshold, has given an early and only partial activation signal to them. Unencumbered by antigen they move to their preferred home in the follicles. Aided by T cell help, they embark on the dual proliferative and differentiative pathway of germinal center formation and creation of antibody-forming cell foci. The Fulcher and Basten paper (15) is silent on the question of what maturational or other process might occur in follicles, in the absence of obvious antigenic stimulation, to confer longevity, recirculating capacity and strong IgD positivity on B cells which reach the follicles. This part of the Cyster et al. (20) story thus appears to be unchallenged.

From both sets of studies, it seems that B cells with some degree of anti-self potential can reach the spleen and linger, at least for a while, in the PALS waiting for T cell help. Such anergic cells could represent a danger for autoantibody formation if some cross-reacting antigen, sharing a B cell epitope with the self antigen in question, but possessing a foreign T cell epitope, entered the body. The relevant T cell help would clearly break B cell anergy. Were that to occur, the chief danger would lie in germinal centers producing high affinity, hypermutated anti-self B cells. For this reason, it is fortunate that the germinal center appears to possess fail-safe mechanisms to prevent this occurring. Three independent and essentially simultaneous studies have shown that germinal center B cells are peculiarly sensitive to apoptosis should they encounter antigen unattached to follicular dendritic cells (24–26). Moreover, the more strongly receptor cross-linking this stimulus, the more extensive the apoptosis. This appears to be a powerful back-up to B cell deletion and anergy in ensuring that the secondary, hypermutated B cell repertoire is relatively uncontaminated by high affinity anti-self cells.

Given that the somatic minigene assembly process which creates the B cell repertoire is random, it is obvious that anti-self B cells will arise with highly variable affinities for the self antigen in question. Those most obviously dangerous to the body, namely exhibiting reactivity to some prominent self membrane-anchored protein, such as self MHC or self blood group antigen, will be deleted in the bone marrow and not allowed into the periphery. Those of lesser threat, e.g., directed against a soluble self antigen, requiring T cell help before activation and possible affinity maturation as well, enter the periphery but may display various degrees of anergy. The present work (15) prompts the reflection that anergy is a flexible, reversible, and quantitative concept. One could imagine one extreme, shading into central deletion, where the anergic cell is allowed to leave the marrow but dies very quickly. At the other extreme, shading into "ignorance" of the self antigen, the cell may suffer only a slightly shortened life-span. At either extreme, strong cognate T cell help can rescue the cell from anergy, but, as originally postulated by Linton and Klinman (27), the germinal center precursors to high affinity memory cells pass through a "second window" of tolerance susceptibility to protect the secondary repertoire.

As the person who originated the concept of clonal anergy, following the strong lead of Bretscher and Cohn (6), the present author compliments the two talented groups exploiting the HEL-anti-HEL transgenic model and is convinced that the moderate differences of interpretation and emphasis between them will soon yield to further explorations. He does wish to issue a challenge, however. Present transgenic constructs do not permit the study of isotype switching in transgenic B cells, nor has any evidence been presented that the immature germinal centers permitted with artificially provided T cell help actually support Ig V gene hypermutation among the still IgM and IgD expressing centroblasts. The effort to create transgenic B cells susceptible to isotype switching and affinity maturation must surely be worthwhile.

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