B Cell Tolerance to a Minor, But Not to a Major, Antigenic Surface of the Self Antigen, Cytochrome *c*[1]

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To study B cell tolerance to the mitochondrial protein cytochrome *c* (CYT), the B cell response to pigeon CYT (PCC) was examined in mice transgenic for PCC. PCC was coupled to OVA to provide T cell help, since PCC-specific T cells in PCC-transgenic mice are deleted in the thymus. The frequency of secondary B cells responding to the minor antigenic surface around residue 44 on PCC was decreased about 10-fold in native PCC-transgenic mice compared with that in control mice or in transgenic mice expressing an altered form of PCC that lacked the heme and had a different amino acid sequence at the N-terminus. A similar decrease has been observed in the frequency of B cells in normal mice recognizing the site around residue 44 on mouse CYT compared with the frequency of B cells recognizing the corresponding site on foreign CYT. There were no major decreases but apparently were compensatory increases in the frequencies of B cells recognizing other sites on PCC in the native PCC-transgenic mice compared with those in other mice. These results indicate that B cells in mice are only partially tolerant to self CYT. A possible basis for this partial tolerance relating to the fate of CYT in cell death is discussed. This may be the first example of the use of a transgenic system to study B cell tolerance to a homologous self Ag. ***The Journal of Immunology,*** **1998, 161: 2841–2847.**

Our understanding of tolerance or negative selection of B and T lymphocytes in vivo has been dramatically enhanced by the use of transgenic mice expressing foreign Ag as self proteins (neo-self Ag) along with surface Ig or T cell transgenic receptors specific for those Ag (reviewed in Ref. 1; 2–4). Among the many concepts that have emerged from such studies is that negative selection is not absolute and depends on factors that affect the avidity of the interaction between Ag and their specific receptors on lymphocytes, including Ag concentration (5), whether the Ag is soluble or membrane-bound (6), and the affinity of the lymphocyte receptors for Ag (7). According to current models for tolerance induction in both B and T lymphocytes, high avidity interactions of Ag-specific receptors with self Ag lead to negative selection, while very low avidity interactions may be ignored (8–10). For T lymphocytes, low to intermediate avidity interactions actually lead to positive selection of maturing cells in the thymus (11, 12).

Although these models are useful in providing a framework for understanding immunologic tolerance, there are observations that are not readily explained by the current models. Functional self-reactive B cells are, in fact, often not rendered tolerant (13, 14). Their "escape" from tolerance induction is not necessarily due to their low affinity interaction with self Ag or to its sequestration. For example, B cells expressing a transgenic rheumatoid factor in an otherwise normal mouse are not rendered tolerant even though the Ag (IgG2a) is present in high concentration ($\geq$50 μg/ml) and the affinity for Ag (K_a) is approximately 5×10^5 M^{-1}, an affinity comparable to that of primary Ab (15, 16). IgG reactive to self Ag, including cell surface Ag, that have affinities on the order of 10^6 M^{-1} are present in naive mice and comprise as much as 20% of the total serum IgG (17). Furthermore, B cells reactive to some self Ag, e.g., thryoglobulin and bromelain-treated erythrocytes, are present in an activated state (18). A hypothesis that attempts to explain such a phenomenon is competitive tolerance, whereby autoreactive B cells that constitute a high fraction of the preimmune repertoire compete with each other for limiting amounts of self Ag (19). Accordingly, the B cells are not rendered tolerant and may even become activated, but they would not mature to produce high affinity Ab.

Another puzzling observation that may or not be explained by this hypothesis is partial B cell tolerance to an Ag. In a study of the Ab response to influenza hemagglutinin, which was expressed as a neo-self Ag (20), certain B cell clonotypes, including a population of IgG-secreting cells observed early in the primary response and another population expressing a particular pair of V genes, were found in lower frequencies in the transgenic mice. The basis for this partial tolerance is not clear. For T cells, partial tolerance, i.e., tolerance to some determinants and not to others on the same self Ag, may be explained by Ag-processing mechanisms. Those self peptides that are the dominant products of the Ag-processing machinery and bind most avidly to major histocompatibility molecules are the most tolerogenic, while those peptides representing more cryptic determinants may be ignored (21, 22). B cells do not require that the Ag be processed before engagement of their surface Ag, so a similar phenomenon probably does not occur for B cells. However, B cells can only respond to sites on an Ag that are accessible for recognition. If they encounter some self Ag in complex with other molecules or in a modified form, one or more epitopes on the native Ag might not elicit tolerance. This could explain the occurrence of B cells reactive with bromelain-treated erythrocytes, for example (18).

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Here we present the results of a study of B cell recognition of cytochrome *c* (CYT)[4] that indicate that B cell tolerance to this self Ag is only partial. While an explanation for this phenomenon remains to be found, the well-characterized antigenicity of CYT and its involvement in cell death processes may provide clues (see *Discussion*).

In previous studies of mouse B cell recognition of the self Ag mouse CYT in two different mouse strains (BALB/c and C57BL/6), the frequency of B cells responding to the surface around residue 44 on mouse CYT (coupled to OVA) was significantly less than the frequency of B cells responding to the corresponding site on the foreign Ag, horse CYT, coupled to the same carrier (23–26). The frequencies of B cells responding to epitopes on another surface of mouse CYT were not decreased. In fact, B cells reactive to this major antigenic surface on mouse CYT are present in peripheral lymphoid tissue of immunologically naive mice (26).

To determine whether the lower frequency of B cells responding to the site around residue 44 on mouse CYT is due to B cell tolerance, we examined the B cell responses to pigeon CYT (PCC) coupled to OVA in mice that were transgenic for either of two types of PCC (27), native endogenous PCC (e-PCC-transgenic mice) or membrane anchor-modified apo-PCC (m-PCC-transgenic mice), and compared the responses to those of control littermates. It has been demonstrated that in either of these transgenic mice, expression of PCC as a neo-self Ag causes deletion of PCC-specific T cells in the thymus (27). This suggests that mice are also T cell tolerant to mouse CYT, at least for any T cell determinants that are not cryptic (22). In this study PCC was coupled to OVA to render it immunogenic for B cells. Although the form of PCC in the m-PCC mice is probably not native, and B cell recognition of CYT is known to be conformationally dependent (28, 29), PCC as a membrane protein in m-PCC mice could bind PCC-specific B cells with sufficient avidity to induce tolerance. Indeed, very low affinity interactions of B cells with membrane-bound Ag have been shown to be tolerogenic (6, 30). Therefore, in this study we examined both m-PCC and e-PCC mice.

Quantitatively similar B cell responses to PCC were obtained in control and both types of PCC-transgenic mice. However, a 10-fold decrease was observed in the frequency of secondary B cells responding to the site around residue 44 in e-PCC-transgenic mice compared with that in control or m-PCC-transgenic mice. This indicates that B cells reactive to this site on endogenous native CYT are indeed rendered tolerant. Because of the low frequency of PCC-specific B cells, it was not possible to determine in this study whether the B cells were deleted or rendered anergic, or whether tolerance occurred in primary or memory B cells.

Materials and Methods

Transgenic mice

Two types of transgenic mice expressing either of two forms of PCC were derived by injection of C57BL/6 × SJL oocytes as previously described (27). In one type of mouse, referred to as m-PCC-transgenic, the nucleotide sequence corresponding to amino acid residues 1 to 19 of PCC was substituted by a sequence corresponding to the type II signal anchor of influenza virus neuraminidase to direct expression to the cell surface. This form of PCC also lacks the covalently bound heme of native PCC, which is only attached to the mitochondrial protein (31). Founder m-PCC-transgenic mice were backcrossed six or seven times onto the C57BL/6 background. In the other type of mouse, referred to as e-PCC, native PCC is expressed as a mitochondrial protein. Founder e-PCC-transgenic mice were backcrossed four or five times onto the C57BL/6 background. In a few experiments F_1 mice of a cross between the e-PCC/m-PCC and B10.A or between e-PCC and BALB/c were used. In transgenic mice the PCC transgenes were placed under the control of the MHC class I promoter and Ig enhancer, allowing expression of the transgenes in a variety of tissues. Offspring of C57BL/6 mice expressing the transgenes were identified by Southern analysis as previously described (27) or by using PCR. For PCR, oligonucleotide primers corresponding to nucleotides 105 to 133 (5′-TT GTTTGGCAGAAAGACTGGTCAAGCTGAAG-3′) and the reverse complement of nucleotides 285 to 312 (5′-CTTAGCGGTGGCTTGTT TCAAGTAAGCAAT-3′) of the synthetic PCC gene (32) were employed to amplify DNA from tail biopsies in 30 cycles using an annealing temperature of 62°C. These primers were selected from regions where PCC and mouse CYT differ (33) to prevent amplification of the mouse CYT gene. PCR products were electrophoresed in 0.7% agarose gels and visualized by ethidium bromide staining. A single band of 208 nucleotides was observed after amplification of DNA from PCC-transgenic mice.

Immunization

Transgenic mice and control littermates were injected once i.p. with 50 μg of PCC-OVA in PBS, pH 7.4, emulsified 1/1 in CFA. Immunogens were obtained from Sigma (St. Louis, MO). PCC was further purified by ion-exchange chromatography using carboxymethyl-Sephadex G-25. For immunization, PCC was covalently coupled to OVA using glutaraldehyde (Sigma) as previously described (25). For use as recipients in splenic fragment cultures, littermates of the PCC-OVA-primed mice were injected once i.p. with 100 μg of HY in PBS emulsified 1/1 in CFA and were used 4 to 10 wk later.

Ab titers

Blood was obtained by periorbital puncture on days 0, 11, 25, and, in some cases, 40 following immunization. Serum titers of PCC-specific Ab, defined as the dilution yielding an A_{492nm} reading equal to 1.0, were determined by ELISA.

Splenic fragment cultures

Individual PCC-specific secondary B cells were cultured in the splenic fragment system developed by Klinman (34, 35) and modified for analysis of CYT-specific B cells (25, 28). Briefly, B cells were prepared from splenocytes of mice immunized with PCC-OVA for 4 to 8 wk. RBC were lysed with 0.17 M ammonium chloride, and T cells were eliminated using anti-CD4 and anti-CD8 mAb and guinea pig complement (Life Technologies, Grand Island, NY). B lymphocytes ($1–3 \times 10^7$) were transferred by tail vein injection to HY-primed and irradiated (1300 rad) recipients. The next day, the recipients' spleens were removed, chopped into 1-mm^3 fragments, and cultured in DMEM containing 10% γ-globulin-free horse serum (Life Technologies), penicillin/streptomycin (Life Technologies), and 0.1 μM PCC-HY. PCC and HY were covalently coupled using glutaraldehyde as previously described (25). After 3 days the culture fluid was removed and replaced with medium without the immunogen. Culture fluids were collected on days 6, 9, and 12 and assayed for Ab production by ELISA. A splenic fragment culture was considered to be positive for Ab if the absorbance reading in ELISA was >3 SD above the background in assays of two consecutive collections of culture fluid. Generally, the absorbance readings in assays of 25 μl of culture fluid were between 1.0 and the maximum observable reading, 2.4. Based on the low frequency of cultures in which anti-PCC Ab was detected (<25%), the Ab can be considered essentially monoclonal.

ELISA

Ab were detected by indirect ELISA as previously described (25, 28). Briefly, PCC (0.5 μM in PBS) was adsorbed for 3 h to Nunc Immuno MaxiSorp 96-well microtiter plates (Life Technologies, Coon Rapids, MN). The plates were washed with PBS containing 0.1% Triton X-100, then 5% horse serum in PBS (25 μl) was added to the wells, followed by fluid collected from the splenic fragment cultures (25 μl). Ab binding to the microtiter plates was determined using horseradish peroxidase-conjugated anti-mouse IgG (whole molecule; Sigma).

mAb specificity

The specificities of PCC-specific mAb were determined by indirect ELISA employing several variants of CYT, including the natural variants, chicken

[4] Abbreviations used in this paper: CYT, cytochrome *c*; PCC, pigeon cytochrome *c*; e-PCC, transgenic mice expressing native pigeon cytochrome *c*; m-PCC, transgenic mice expressing a modified form of pigeon cytochrome *c* as a cell membrane-bound protein; HY, hemocyanin; E44P-PCC, pigeon cytochrome *c* with glutamic acid at position 44 replaced by proline; G60K-PCC, pigeon cytochrome *c* with glycine at position 60 replaced by lysine; A89D-PCC, pigeon cytochrome *c* with alanine at position 89 replaced by aspartic acid; Apaf-1, apoptotic protease-activating factor-1; SLE, systemic lupus erythematosus.

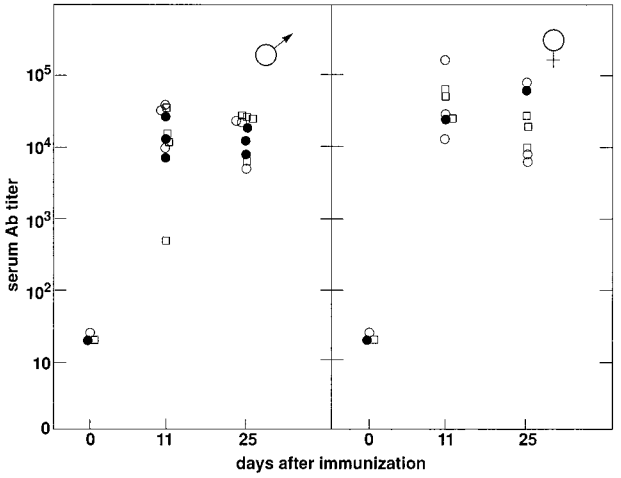

FIGURE 1. Titers of serum anti-PCC Ab during the immune response to PCC-OVA in control C57BL/6 mice (○), m-PCC-transgenic mice (●), and e-PCC-transgenic mice (□). Titers were determined as described in *Materials and Methods*. Before immunization (day 0), the titers were generally <15, the lowest serum dilution that was tested; for simplification, only one of the preimmune titers from each type of mouse is plotted.

and rabbit CYT (Sigma), and the single amino acid variants of PCC produced by site-directed mutagenesis, E44P, G60K, and A89D. Expression of the wild-type PCC gene in yeast allowed the isolation of native PCC as well as a modified form that is not acetylated at the amino terminus (32, 36). Similarly, both acetylated and nonacetylated forms of the PCC mutants were obtained. Only the acetylated forms of the mutants were used in this study. Due to peculiarities of post-translational modification of proteins in yeast, wild-type PCC expressed in yeast and the PCC mutants are trimethylated at lysine 72 (36, 37). The preparation and purification of the wild-type and PCC mutants have been described previously (38).

Results

Control and PCC-transgenic mice have indistinguishable anti-PCC serum Ab titers and similar frequencies of anti-PCC secondary B cells

Control, m-PCC-transgenic, and e-PCC-transgenic mice were immunized with PCC coupled to OVA, and sera were assayed at various times for the presence of Ab to CYT. The anti-PCC serum Ab titers were essentially the same in each group of mice regardless of whether they were male or female (Fig. 1). The titers reached a maximum by day 11 and were maintained at that level for at least several weeks. Among the sera tested 40 days after immunization, the titers had barely begun to decline (data not shown).

Between 4 and 8 wk after injecting the mice with PCC-OVA, the relative numbers of splenic PCC-specific secondary B cells responding in vitro in splenic fragment cultures were determined (Table I). Consistent with the indistinguishable serum Ab titers, there were no significant differences in the numbers of responding secondary B cells between control and either m-PCC- or e-PCC-transgenic mice regardless of whether they were male or female. Because it is known that approximately 5% of transferred B cells home to the spleen in the recipient mice (39), we estimate that the frequency of PCC-specific secondary B cells in C57BL/6 control and PCC-transgenic immunized mice is 1.2 to $1.3/10^5$ B cells.

The use of natural variants and site-directed mutants of PCC to map mAb binding sites in control and PCC-transgenic mice

In a previous study of PCC epitopes recognized by polyclonal Ab elicited in mice on the B10 background, most Ab were found to bind a discontinuous determinant on PCC that included residues 3, 103, and 104 (29). A smaller number of Ab recognized one or more determinants on the opposite surface of PCC around either or both residues 15 and 44. Studies of mAb binding to other CYT indicated epitopes in the region around residue 44 and residues 60 and 62 (23, 25, 40). To distinguish the epitopes of PCC in the present study we employed site-directed mutants of PCC with single amino acid substitutions at positions 44, 60, and 89; a modified form of PCC not acetylated at the amino terminus; and two natural variants, chicken and rabbit CYT, with amino acid differences from PCC at several positions, including 3, 100, 103, and 104 (Table II). We were able to distinguish among several epitopes using these variants.

Table I. Frequency of anti-PCC secondary B cells responding in splenic fragment cultures

Mouse Type[a]	No. of Mice	Number of Clones/10^7 B Cells Transferred
Control male	5	5.3 ± 1.8
Control female	3	6.8 ± 3.4
Control male + female[b]	8	5.9 ± 2.5
m-PCC male	3	6.4 ± 3.2
m-PCC female	1	6.0
m-PCC male + female[b]	4	6.3 ± 2.6
e-PCC male	4	5.2 ± 1.9
e-PCC female	5	7.0 ± 1.3
e-PCC male + female[b]	9	6.2 ± 1.8

[a] Data are from C57BL/6 mice. Similar frequencies of secondary B cells were observed with the few (C57BL/6 × B10.A)F_1 mice examined.
[b] Total from the two sexes

Most of the amino acid residue differences among these variants of PCC occur on the same surface of PCC, i.e., the opposite surface from the exposed heme crevice (Fig. 2*B*). The major exception is residue 44, which is situated on the lower right side from the heme (Fig. 2*A*). Use of the E44P-PCC variant allowed unambiguous identification of mAb binding this region. These mAb also bound rabbit CYT with lower affinity due to the substitution of valine for proline at position 44.

As previously shown with polyclonal Ab, most of the PCC-specific mAb were found to bind on the surface opposite from the exposed heme crevice (Table III, control column, and Fig. 2*B*). The variants of CYT we used allowed identification of several mAb specificities in this region that probably represent overlapping epitopes (25, 43). Many of the mAb bound chicken and/or rabbit CYT with reduced affinity relative to their binding to PCC. Since these mAb bound E44P-PCC, G60K-PCC, A89D-PCC, and native PCC similarly, it would appear that they bind in the region

Table II. *Comparison of amino acid sequences of CYT that were used to identify epitopes on PCC*[a]

	Amino Acid Residue Position								
CYT	3	15	44	60	89	92	100	103	104
PCC	I	S	E	G	A	A	Q	A	K
Mouse[b]	V	A	A	-	G	-	K	N	E
Chicken	-	-	-	-	S	V	D	S	-
Rabbit	V	A	V	-	D	-	K	N	E
E44P–PCC	-	-	P	-	-	-	-	-	-
G60K–PCC	-	-	-	K	-	-	-	-	-
A89D-PCC	-	-	-	-	D	-	-	-	-

[a] The CYT listed are identical except at the indicated positions (33) and at position 72, a lysine residue which is trimethylated in the yeast-produced variants of CYT (36).
[b] Provided for reference.

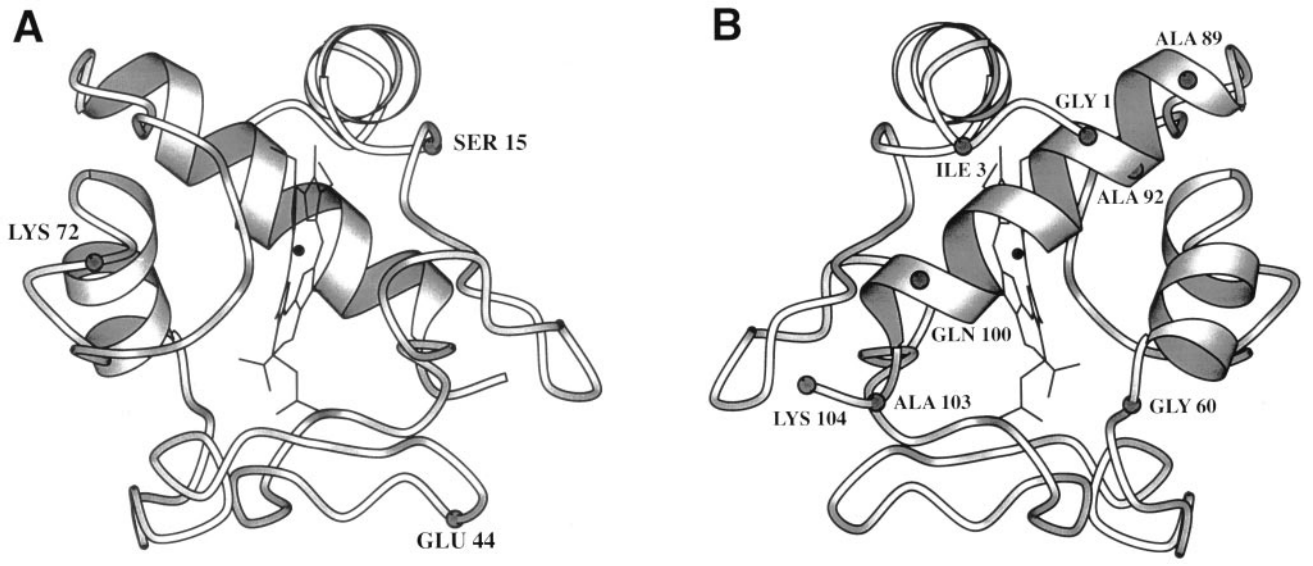

FIGURE 2. Computer-generated ribbon model of the PCC polypeptide chain based on the atomic coordinates of horse CYT (41). α-Carbons of amino acids that differ among the naturally occurring CYT employed in this study or that were altered by site-directed mutagenesis are indicated. In *A* the molecule is shown in the orientation in which the heme edge is exposed, and in *B* the molecule is rotated 180° around a vertical axis. The program MOLSCRIPT (42) was used to prepare this figure. Only the amino acid residue differences that were accessible on the surface being viewed are shown.

around residues 3, 100, 103, and 104 (see Table III, footnotes). Only a few of these mAb (1–2%) were affected in their binding to PCC by loss of the acetyl group at the amino terminus.

Another mAb binding site on PCC occurs in the vicinity of residue 60. mAb binding this site were affected by the substitution in G60K-PCC. Most of these mAb (80%) were also affected by one or more of residues 3, 100, 103, and 104 as well as residue 60, since these mAb bound rabbit, chicken, and G60K-PCC with reduced affinity relative to wild-type PCC and the other variants. Loss of the acetyl group at the amino terminus affected the binding of only a few of these mAb (11%).

A small number of mAb bound the A89D-PCC variant and rabbit CYT, which has aspartic acid at position 89, with reduced affinity indicating a minor epitope in the vicinity of residue 89. Some of these mAb (25%) were also affected by loss of the acetyl group at the amino terminus.

Most of the remaining mAb bound wild-type PCC and all of the variants tested similarly, indicating either that they bound yet another site on PCC or they bound one or more of the same sites that were identified but they did not distinguish between the amino acid residue substitutions between wild-type PCC and the variants.

Table III. *Frequency of secondary B cells in control and PCC-transgenic mice responding to different epitopes on PCC*

Epitope Defined by the Variation	Number (%)[a] of B Cells Responding		
	Control	m-PCC	e-PCC
E44P	18 (17)	12 (18)	2 (1.6)
G60K[b]	21 (19)	10 (15)	39 (31)
A89D	7 (6.4)	1 (1.5)	7 (5.5)
3/100/103/104[c]	21 (19)	17 (25)	43 (34)
Other[d]	42 (39)	27 (40)	36 (28)
Total	109	67	127

[a] Numbers in parenthesis represent percent of total PCC-specific B cells based on the assay of Ab produced in splenic fragment cultures such as discussed in Table I.

[b] Most of these mAb (80 ± 5% in each of the three groups of mice) were also affected in their binding by amino acid substitutions at one or more of positions 3, 100, 103, or 104.

[c] mAb which showed decreased binding to chicken and rabbit CYT but not to the site-directed mutants of PCC are included in this category. It is possible that these mAb are also affected by amino acid substitutions at positions 15 and/or 92. However, based on previous observations, regarding polyclonal Ab binding to PCC which indicated that residue 92 did not affect Ab binding and that residue 15 possibly affected the binding of only a small population of Ab, the two amino acid residues at positions 15 and 92 would not appear to be part of this or any epitope.

[d] Most of these mAb (90%) were not significantly affected by any of the mutations. A few of these mAb were only affected by loss of the acetyl group at the amino terminus.

Comparison of the frequencies of secondary B cells responding to the different sites on PCC in control and PCC-transgenic mice

The sites on PCC recognized by secondary B cells were determined from the specificities of their mAb as described above. The sites recognized and the numbers of B cells responding to each site were similar in control and m-PCC-transgenic mice (Table III). However, there were a few differences between these mice and the e-PCC-transgenic mice. While 17 to 18% of the B cells in control or m-PCC-transgenic mice responded to one or more epitopes in the region of residue 44, only 1.6% of the B cells in e-PCC-transgenic mice responded to this site. Among individual mice, in only 2 of 10 e-PCC-transgenic mice was a B cell observed that recognized this site (Table IV). In contrast, B cells responding to this site were observed in all 6 m-PCC-transgenic mice and in 8 of 11 control mice. (In two of the control mice in which B cells recognizing this site were not observed, seven B cells or fewer were obtained, perhaps explaining the apparent absence of B cells with that specificity in those mice.) The distinction between e-PCC-transgenic mice and control or m-PCC-transgenic mice is statistically significant by the Mann-Whitney test for population differences (44). In an additional experiment involving F_1 mice from a cross between C57BL/6 e-PCC- transgenic and BALB/c mice, 4 of 22 (18%) B cells responding to PCC (coupled to OVA) from a control littermate were specific for the region around residue 44, while only 1 of 45 (2.2%) B cells from an e-PCC-transgenic littermate had this specificity.

While the frequency of B cells responding to the site around amino acid residue 44 was reduced in e-PCC-transgenic mice, the frequencies of B cells responding to the sites around residue 60

Table IV. *Frequency of secondary B cells in individual mice responding to the epitope around residue 44*

	Male Mice								Female Mice					
Mouse Type	1	2	3	4	5	6	7	8	1	2	3	4	5	Average ± SD
Control	4/6	1/7	1/7	2/8	1/13	0/5[a]	3/14[a]	0/14[a]	3/10	0/7	3/18			0.18 ± 0.19
m-PCC	1/12	1/8	2/8	4/14[a]	3/16[a]				1/10					0.17 ± 0.083
e-PCC	0/5	0/12	0/9	0/14	1/15[a]				0/13	0/11	0/20	0/11	1/17	0.013 ± 0.027[b]

[a] (C57BL/6 × B10.A)F_1 mice.
[b] From the Mann-Whitney test for population differences, $p < 0.001$ in comparison with m-PCC transgenic mice and $p < 0.01$ in comparison with control mice.

and residues 3, 100, 103, and 104 were increased by as much as twofold in e-PCC-transgenic mice relative to those in control and m-PCC-transgenic mice.

Although most of the mice in the experiments summarized in Tables III and IV were C57BL/6, a few were F_1 progeny of C57BL/6 crossed to B10.A. Both strains were grouped together, since there appear to be no differences between these mice in their B cell responses to PCC. These mice are identical except in the MHC, and that difference would not be expected to affect the B cell repertoire for PCC, but could affect T cell recognition of the carrier molecules, OVA and HY. However, the Ab titers to the carriers were similar in C57BL/6 and F_1 mice (results not shown), suggesting that the T cell help was quantitatively similar among these mice.

Discussion

For many years it was believed that B cells were tolerant to self CYT and that the Ab response to foreign CYT was, therefore, directed to regions on an immunizing foreign CYT where the self and foreign proteins differ in amino acid sequence (45). This idea became untenable when it was found that Ab to self CYT are readily generated by immunizing animals with self CYT coupled to a carrier protein (23, 46). Indeed, B cells specific for mouse CYT and capable of producing unmutated Ab with affinities (K_a) on the order of 10^6 M^{-1} are present in peripheral lymphoid tissue of naive mice (26). These more recent observations have suggested that B cells are not rendered tolerant to self CYT, a concept consistent with the known sequestration of functional CYT between the inner and outer membranes of mitochondria (31). Recent evidence that CYT is translocated from the intermembrane space of mitochondria during apoptosis (47) and its appearance in small blebs at the surface of apoptotic cells[5] indicate that CYT may not be totally sequestered after all. These findings in conjunction with the lower frequency of B cells responding to the region around residue 44 on self vs foreign CYT (23–26) prompted the present investigation to determine whether there may be partial tolerance to CYT.

In the absence of a PCC-specific Ig transgenic mouse and due to the low frequency of PCC-specific B cells in normal mice, the conventional method for quantifying Ag-specific B cells by flow cytometry could not be applied in this study (8). Therefore, we used a functional approach in which secondary, PCC-specific B cells obtained from PCC-OVA-primed mice and then transferred in limiting dilution to HY-primed recipients were quantified in splenic fragment cultures on the basis of Ab produced in response to PCC-HY stimulation. The low frequency of primary PCC-specific B cells prevented their being assayed by this approach.

Although m-PCC-transgenic mice are tolerant to PCC by deleting PCC-specific T cells in the thymus (27), they are indistinguishable from control mice in their B cell response to PCC. Not only are the overall frequencies of responding secondary B cells and Ab titers similar between the control and m-PCC-transgenic mice, but the frequencies of secondary B cells responding to individual sites are essentially the same. Since m-PCC is expressed as a modified form of apo-PCC in the transgenic mice, it must have large conformational differences from native PCC. Formation of the amino terminal helix in native PCC is a nucleating event in the folding of the protein (48). This segment was replaced in the m-PCC mice by a membrane-spanning amino acid sequence that targeted the polypeptide for expression at the cell surface. Consequently, the m-PCC protein also lacks the heme of PCC that is crucial to proper folding of the polypeptide (49) and is only incorporated into CYT after it is transported into the mitochondrion (31). Since the epitopes on CYT, in general, are conformationally dependent (28, 29, 50), the B cells in m-PCC-transgenic mice that recognize native PCC would not have been expected to be rendered tolerant unless whatever affinity they may have for m-PCC was compensated for by the avidity resulting from the Ag being membrane bound. Apparently, this is not the case.

In contrast to m-PCC, e-PCC is identical in amino acid sequence to native PCC and, therefore, should be targeted to mitochondria, where it is modified by covalent attachment of the heme. Hence, e-PCC should be identical in conformation to PCC. Using one mAb specific for native pigeon CYT and another mAb reactive to both native pigeon and mouse CYT, we have determined by ELISA that the e-PCC mice do express native pigeon CYT but at lower levels than they express mouse CYT (<1%) (M. Daniels, S. Sonntag, and R. Jemmerson, unpublished observations). Although control and e-PCC mice were indistinguishable in the overall frequency of responding secondary B cells and in Ab titers, some distinctions were observed in the frequencies of secondary B cells responding to individual sites on PCC despite the low expression of PCC. Most notably, there was a significant decrease in the frequency of secondary B cells in e-PCC-transgenic mice responding to the site around residue 44. This effect is not due to differential processing of PCC T cell sites in e-PCC vs m-PCC mice. In these experiments T cell help was provided by the foreign protein OVA. Besides, PCC-specific T cells are deleted in both e-PCC and m-PCC mice.

The decreased frequency could be due to B cell clonal deletion or to the induction of an anergic phenotype. Which of these occurs could not be determined, since it is not presently possible to track the PCC-specific B cells that are present in low frequency. Thus, it is not known whether tolerance occurs in immature primary, mature primary, or secondary PCC-specific B cells. Tolerance can be induced in B cells at all three stages of maturation (51–55) and in precursors to memory B cells, if they do represent a separate B cell lineage (56, 57).

While the idea of tolerance to only a subset of sites on a protein Ag is unusual, there is precedence for such a phenomenon. In a

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study of the B cell response to influenza virus hemagglutinin expressed as a neo-self Ag, a substantial reduction was observed in the frequency of two populations of B cells, one secreting IgG Ab early in the primary response and the other expressing a particular V gene combination (20). The basis for the partial tolerance in the hemagglutinin transgenic system is not known.

It should be noted that PCC and mouse CYT are identical in amino acid sequence over a large portion of their surfaces (33), and their three-dimensional structures are likely to be extremely similar. Therefore, there could be other B cell epitopes on CYT to which the mice are tolerant that were not observed in the response to PCC-OVA due to tolerance to the corresponding sites on endogenous mouse CYT.

In the e-PCC-transgenic mice not only are the other sites around residue 60 and residues 3, 100, 103, and 104 nontolerogenic, but there are increases in the frequencies of B cells that respond to these sites. This could be due to immune regulatory mechanisms that compensate for the fewer B cells responding to the tolerogenic site(s).

The observed tolerance of B lymphocytes in e-PCC-transgenic mice to the epitope around residue 44 on PCC explains the low frequency of B cells in C57BL/6 and BALB/c mice that respond to the corresponding site on mouse CYT when mice are immunized with mouse CYT-OVA. In splenic fragment assays of C57BL/6 secondary B cells elicited against mouse CYT there were no B cells of 46 examined that recognized this site (26). However, as many as 18% of the B cells in C57BL/6 mice responding to the foreign Ag PCC recognized this site. In BALB/c mice, fewer than 10% of secondary B cells responding to mouse CYT-OVA recognized the site around residue 44 (23, 24). In contrast, approximately 40% of B cells from horse CYT-OVA primed BALB/c mice responded to this site on horse CYT (25), and nearly half the B cell hybridomas prepared against horse CYT were specific for this site (40, 58).

In a previous study, tolerance to the site around residue 44 was inferred from the observation that the frequency of bone marrow surface Ig^- B cells responding in splenic fragment cultures to the synthetic peptide containing residues 41 to 49 of mouse CYT (coupled to a carrier) was 30-fold greater than the frequency of splenic B cells that responded (52). However, the significance of this observation to the recognition of B cells specific for native CYT is not clear, since Ab elicited against peptides from the sequence around residue 44 do not bind native CYT (59).

For CYT to be a tolerogen of immature, primary B cells it would probably have to be exposed to developing B cells in the bone marrow, where massive apoptosis of B cells occurs. Recently, it was shown that native CYT does efflux from mitochondria in the early phase of apoptosis and appears to play a role in activating the apoptotic proteolytic cascade (47, 60, 61). Sufficient amounts of CYT could subsequently efflux from apoptotic cells or become exposed on the surface of dying cells for recognition by developing B cells. It is possible that steric hindrance by other molecules or the masking of certain sites by conformational changes in CYT itself could expose some sites more than others, leading to partial B cell tolerance. For example, CYT may be released from dying cells in association with some other molecule(s), e.g., apoptotic protease-activating factor-1 (Apaf-1) or a *bcl-2* family member. It has been shown that CYT (also known as Apaf-2) does bind Apaf-1 (62) as well as bcl-2 family members (63). Since CYT is a minor Ag of systemic lupus erythematosus (SLE) (64), it may also be modified during cell death, e.g., by phosphorylation, as are some other Ag of SLE (65). Even minor modifications could readily affect the antigenicity of a protein. Further investigation of the fate of CYT in cell death is planned to distinguish among these possibilities. The competitive tolerance hypothesis (19) could also be applicable in this case, since the B cells to the major antigenic surface of mouse CYT that are not rendered tolerant are present in relatively high frequency in naive mice.

The results of this study may have implications for understanding B cell recognition of CYT in humans with SLE. Ab to CYT have been observed in approximately 7% of patients examined for the presence of the Ab (66). From the cross-reactivity of these Ab with several CYT, it appears that many of them recognize a surface on human CYT corresponding to the nontolerogenic major antigenic surface we have identified on mouse CYT around residues 60 and 62. Since B cells to the major antigenic surface of CYT, but not to the minor antigenic surface, are present in normal mice and possibly in humans, B cells recognizing the major antigenic surface also escape tolerance induction in the bone marrow as do other autoimmune disease-associated, Ab-producing cells. They may become activated in peripheral tissues in response to some foreign Ag (67), but in certain individuals, e.g., SLE patients, the autoreactive B cells may be sustained in an activated state by an abnormality in peripheral regulatory mechanisms. Accordingly, it is not the presence of the autoreactive B cells in those patients that is unusual, i.e., the fact that they were not rendered tolerant, but, rather, that they became activated, perhaps in a dysregulated manner. This is consistent with the interpretation that the *lpr* mutation that makes mice susceptible to SLE-like disease does not adversely affect central tolerance induction, but may affect the regulation of peripheral autoreactive cells (68).

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