Ig-specific T Cell Receptor-transgenic T Cells Are Not Deleted in the Thymus and Are Functional In Vivo

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Summary

The mechanisms that induce T cell tolerance to circulating self-proteins are still controversial, and both the deletion and selection of autoreactive T cells have been observed in the thymus of transgenic mouse models. To address the question of the induction of tolerance to circulating self-constituents, a T cell receptor-transgenic mouse specific for the serum protein immunoglobulin (Ig) γ and (IgG2a) was generated. The choice of an allotype-specific T cell also allowed the generation of transgenic control mice not expressing the self-antigen. It was found that the transgenic T cells were not deleted in the thymus, did not become tolerant in the periphery, and regulated the function of γ2ab-positive B cells as shown by the lack of IgG2a protein in the serum of the transgenic mice. In spite of this activity in vivo, the transgenic T cells did not proliferate in vitro in response to the allotype-specific peptide. Interestingly, antigen-specific T cell proliferation could be restored if the transgenic mice were previously challenged to induce IgG2a responses. After this challenge, IgG2a protein in the serum of the transgenic mice could be partially restored, although still remaining much lower than in control mice. In addition, there was a dramatic increase in serum IgE levels, suggesting that newly generated γ2ab-secreting B cells can be induced to switch to IgE in the presence of allotype-specific T cells. These results indicate that Ig-specific T cells may represent a late-acting form of T cell help for the regulation of the IgG2a-to-IgE class switch.

Tolerance is the property by which the immune system becomes unresponsive to self-constituents, and it can be achieved either by clonal deletion or functional silencing (anergy) (for reviews see references 1, 2). The tolerance-inducing capacity of three major groups of self-antigens—thymic presented, tissue sequestered, or circulating proteins—has been studied. In the thymus, negative selection of potentially autoreactive T cells has been extensively investigated. Indeed, T cells recognizing a self-protein epitope in association with MHC molecules are deleted during the T cell differentiation process (3–5). In the periphery, anergy or clonal deletion of T cells specific for tissue-sequestered self-antigens, not presented in the thymus and thus evading negative selection, has also been shown (6). On the other hand, several examples of association with MHC proteins that do not induce any thymic or peripheral tolerance have recently been described (7–9). In these studies, T cells specific for myelin basic protein or antigens expressed in pancreas β islets, induced autoimmunity in transgenic experimental allergic encephalomyelitis and diabetes-susceptible mice, respectively.

Regarding circulating self-proteins, the mechanisms that induce T cell tolerance are still controversial. It has been observed that transgenic mice expressing small amounts of hen egg lysozyme (10) or “physiological” amounts of human insulin (11) were tolerant to these antigens, but mice expressing a soluble transplantation antigen did not show any T cell tolerance (12). However, in all of these studies, T cell functional activity was investigated in vitro, and the fate of the antigen-specific T cells was not examined in vivo. Recently, thymic deletion of transgenic T cells specific for the fifth component of complement (C51 [a natural self-antigen regulated physiologically]) has been observed (13).

In a different transgenic mouse model, antidiotypic-specific T cells were deleted in the thymus only if the idiotype was expressed within the thymus as a transgene, or by the injection of nonphysiological amounts of Ig (14). When the endogenous idiotype was expressed at physiological levels, positive selection was observed in the thymus (15). Thus, the question of how the immune system becomes tolerant to circulating proteins, and in particular whether and how it becomes tolerant to its own Ig, is still open. Finding an answer to it could help us to understand
whether anti-Ig T cells are associated with autoimmune diseases, such as rheumatoid arthritis.

The possibility that circulating Ig-specific autoreactive T cells may be harmless for the organism should be considered. In line with the hypothesis that B cells cannot prime virgin T cells (16), it may be thought that most autointotype virgin T lymphocytes might develop tolerance to B cells presenting epitopes of their own Ig (17). However, it has also been shown that, if correctly activated by CD4+ helper T lymphocytes, B cells may acquire all of the costimulatory signals necessary to prime virgin T cells (18). Furthermore, it has been recently demonstrated that, if activated via the B cell receptor, B cells transduce all of the signals necessary for virgin T cell proliferation and lymphokine production (19).

To study the fate of T cells specific for Ig as circulating self-constituents, we have generated a TCR-transgenic mouse model specific for the serum protein IgG2b. There were two reasons for the choice of this model: first, an allotype-specific T cell would allow us to generate transgenic control mice that do not express the self-antigen; second, in the absence of tolerance induction, the fate of B cells after the interaction with allotype-specific T cells could be followed.

In the mouse model described in this study, the transgenic T cells are not deleted in the thymus, do not become tolerant in the periphery, and regulate the function of γ2b- or α-receptor B cells, as shown by the lack of IgG2b protein in the serum of the transgenic mice.

Interestingly, when the animals' immune system was challenged by immunization, the IgG2b protein in the serum remained much lower than in controls, and a dramatic increase in IgE was observed. This suggests that the γ2b-secretion B cells can be induced to switch to IgE in the presence of allotype-specific T cells.

Materials and Methods

Mice. BALB/c, DBA/2, and CD1 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and bred in our conventional animal facility. CB17 mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Construction of the α Transgene. The α and β chains of the TCR were both cloned from the B5 T cell clone. The α-rear ranged coding cDNA (1,300 bp) was amplified by anchored PCR as described (20), cloned in the XhoI-Sma1 restriction sites of pGEM7zf (Promega Corp., Madison, WI), and sequenced. The oligonucleotides used were 5'TACTCGACGTGACATCGATT TTCTTTTTTTTTTTTT3' and 5'TACTCGAGTCGACATCGA T3' (used in 5') and 5'CTGTTCACAAAGCTTTTCTCGGTCA ACGTG3' (used in 3'). The SalI-BamHI fragment containing the complete coding sequence for the α chain was subcloned in the pHSE3' transgenic vector (21).

Construction of the β Transgene. A 900-bp XhoI-Sma1 fragment containing rearranged Vβ14/Dα1.1/Jβ2.5/Cβ2 sequences was generated by reverse transcription (RT) PCR, cloned into pGEM7zf, and sequenced. The oligonucleotides used for the amplification were 5'GGTTCACGTGACATCGATTT TTTAGACACCTT- GAACTAT3' (used in 5') and 5'STTTRANSGENICTCTTT CGAAATTTTTTTCTGACC3' (used in 3').

amplification cycle was performed at the following conditions: 60 s at 94°C, 60 s at 60°C, 60 s at 72°C, for 30 cycles and 1 cycle at 72°C for 10 min to terminate the reaction. The SalI-BamHI fragment was inserted into the pHSE3'-transgenic vector. The two XhoI-XhoI fragments, from pHSE3'α and pHSE3'β, corresponding to the α and β chains, were used to produce transgenic mice.

Generation of Transgenic Mice. Transgenic mice were generated as described by Hogan et al. (22). DBA/2 (H-2ª) × BALB/c (H-2ª) mice were used as a source of oocytes, and the injected eggs were implanted in pseudopregnant CD1 females. Separate α and β chain-transgenic mice were established, and the two lines were then bred together to produce mice with the complete TCR. These mice were crossed to BALB/c mice for three generations (TGb– mice). TGb+ were generated by breeding TGb– male mice with CB17 females.

Flow Cytometry. Single-cell suspensions of 106 thymocytes or lymph node cells were pelleted and resuspended in 50 μl of PBS with 5% FCS and 0.05% sodium azide with the appropriate amounts of antibodies and incubated for 30 min on ice. Cells were then washed once with 1 ml of PBS. Second-reagent incubation was performed in 50 μl of streptavidin–PerCP (Becton Dickinson & Co., Mountain View, CA) conjugated at the concentration indicated by the manufacturer, for 15 min on ice in the dark. The first-step antibodies were CD4, FITC-coupled anti–mouse CD4 (20 μg/ml; PharMingen, San Diego, CA); CD8, PE-coupled anti–mouse Lyt-2 (20 μg/ml; PharMingen); Vβ14, biotinylated anti–mouse Vβ14 (20 μg/ml; PharMingen). Three-color analysis was performed on 2 × 106 viable cells with a FACScan® (Becton Dickinson & Co.); the stains were plotted on a quadradruple logarithmic scale.

T Cell Proliferation Assay. The cells were isolated from lymph nodes and cultured in 96-well plates for 4 d in IMDM (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin (all from Sigma Chemical Co.) and 5% heat-inactivated FCS (Boehringer Mannheim Corp., Indianapolis, IN), with serial dilution of synthetic peptide. The cells were incubated in triplicate wells, and 1 μCi of [3H]thymidine was added to the wells during the last 16 h of culture. The plates were then harvested and counts per minute were determined by means of liquid scintillation counting.

Measurement of Ig Isotypes and Cytokines. Serum Ig levels and cytokine amount were determined by solid-phase ELISA with the use of a sandwich assay. High-bind plates (Costar Corp., Cambridge, MA) were coated with different antiserum and anti-cytokine antibodies at 10 μg/ml in PBS and 50 μl/well. After incubation for 24 h at 4°C, the wells were washed five times with tap water (for isotype ELISA) or PBS, 0.05% Tween 20 (for cytokine measurement). The plates were blocked with 200 μl/well of PBS, 0.5% BSA for 1 h at 37°C, and then rinsed five times with tap water (for isotype ELISA) or PBS, 0.05% Tween 20 (for cytokine measurement). Mouse Ig or cytokine standards (50 μl/well) at various dilutions starting from 10 μg/ml in PBS, 0.5% BSA and serum samples or culture supernatants were loaded in the wells and incubated for 24 h at 4°C. After washing the wells with PBS–Tween or water, 50 μl of antisera or anti-cytokine biotin-coupled secondary antibodies were added for another 1-h incubation at 37°C. The wells were then washed six times and incubated 30 min with 100 μl of alkaline phosphatase–conjugated streptavidin (Boehringer Mannheim Corp.) at the manufacturer's recommended concentration. The developing substrate (0.1% p-nitrophenyl phosphate) was added for at least 30 min at room temperature, and the plates were read with a reader (Hewlett Packard Co., Palo Alto, CA). IFN-γ and IL-4 used as standard
curves were from Genzyme Corp. (Cambridge, MA). IgG1 serum level was measured by inhibition ELISA. After overnight coating with the N1G9 (IgG1) antibody and a 1-h blocking in PBS, 0.5% BSA, 0.05% Tween 20, the plates were incubated for 1 h at 37°C with 50 μl of a mixture of 50% sera and 50% biotin-conjugated goat anti-mouse IgG1 antibody (Southern Biotechnology Associates, Birmingham, AL) at a final concentration of 0.05 μg/ml. After PBS, 0.05% Tween 20 washing, the plates were treated, as previously described, with alkaline phosphatase-conjugated streptavidin and revealing substrate.

**Antibodies.** The isotype-specific antibodies were as follows: anti-mouse IgE, 95.3 and biotin-conjugated anti-mouse IgE (PharMingen); anti-mouse IgM, R33-42-12 and biotin-conjugated R 33-60; anti-mouse IgG1, biotin-conjugated anti-mouse IgG1 (Southern Biotechnology Associates); anti-mouse IgG2b, R 14-50 and biotin-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates); anti-mouse IgG3, 2E.6 and biotin-conjugated anti-IgG2a allotypes (all from PharMingen); anti-IgEβ, purified anti-total IgE and alkaline phosphatase-conjugated anti-IgEβ (all from PharMingen). All of the antibodies were kindly provided by K. Rajewsky (Cologne University, Cologne, Germany). Purified and biotin-conjugated anti-IFN-γ and anti-IL-4 antibodies were purchased from PharMingen, whereas the following antibodies used for the standard curves were provided by K. Rajewsky: IgG1, N1G9; IgE, 12.2, IgG2b, D3-13 F1; IgG2α, 42.1; IgG2α, S43.10; IgM, B1-8; IgG3, S24/63/63.

**Peptide-induced Thymic Deletion.** The peptide, not soluble in PBS, was resuspended in water at a concentration of 10 mg/ml. Mice were injected i.v. with 100 μl of peptide solution or 100 μl of water as a control.

**Propidium Iodide Analysis.** 10⁶ thymocytes were incubated 30 min on ice in 1 ml of 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100. Cells were then analyzed by FACSCalibur as described (23).

**Immunolocalization of the Apoptotic Thymocytes.** Fragments of thymuses were frozen in dry ice powder and stored at −80°C. 5-μm frozen sections were cut and mounted on gelatine-coated glass slides. The cryosections were dried at room temperature for at least 1 h and fixed in acetone for 5 min. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method was carried out using the Apoptag™ kit (Oncor Inc., Gaithersburg, MD). Briefly, the cryosections were incubated at 37°C with terminal transferase enzyme and digoxigenin-dUTP. The apoptotic cells were then detected by staining with 20 μg/ml of anti-digoxigenin-rhodamine antibody Fab fragments (Boehringer Mannheim Corp.).

**Immunization Procedure and Anti-CD8, Anti CD4 Treatment.** LT2 MC1 galE mutant of Salmonella typhimurium strain (24) was used in the experiments. The Tg- Tg- Tg+b- Tg+b+ mice were compared. The lower panel also shows the analysis of Vβ14 expression in the four thymic subpopulations: CD4+CD8− (CD4), CD4+CD8+ (DP), CD4−CD8+ (CD8), and CD4−CD8− (DN).

![Figure 1](image-url). Thymus T cell population in TCR transgenic mice. Thymocytes from 4-6-wk-old α/β Tg+b−, Tg+b+ transgenic mice were stained with antibodies specific for CD4 (horizontal axis) and CD8 (vertical axis, upper panel) and Vβ14 variable chain (lower panel). Nontransgenic and α/β-transgenic thymocytes were compared. The lower panel also shows the analysis of Vβ14 expression in the four thymic subpopulations: CD4+CD8− (CD4), CD4+CD8+ (DP), CD4−CD8+ (CD8), and CD4−CD8− (DN).
grown overnight in oxoid brain–heart (Sigma Chemical Co.) infusion (37 g/liter of distilled water) at 37°C. Bacteria were washed three times with 0.1 M sodium bicarbonate, pH 8.4, and injected intraperitoneally into the mice. The first immunization was performed with 2.5 × 10^6 cells in 500 μl and the second one with 10^6 LT2 M1C in 500 μl of sodium bicarbonate. The 3.168 anti-CD8 (25) and the GK1.5 anti-CD4 (26) mAbs were purified from supernatants by protein G affinity chromatography (Pharmacia Biotech Inc., Piscataway, NJ) according to recommended procedures. 500 μg of anti-CD8 and anti-CD4 antibodies were injected intraperitoneally into mice on days 0, 1, and 2.

Thymectomy and ELISPOT Assay. Thymectomy and ELISPOT assay were performed according to Sjokin et al. (27) and Czerkinsky et al. (28), respectively.

Results

Selection of the T Cell Clone for Constructing Transgenic Mice. The B5 clone was generated by Bartnes and Hansnestad (29) by the injection of a γ2aβ mAb into BALB/c mice. The T cell is a CD4+, Th1, I-Aα-restricted clone, and the epitope recognized by B5 TCR was mapped in the CH3 domain of IgG2αβ (residues 435–451) (30). We selected B5 because it has been shown to suppress the Igh-1b allotype in vivo (29); moreover, the choice of an allotype-specific T cell allowed us to generate transgenic control mice that do not express the self-antigen.

The rearranged α and β chains of the TCR were cloned by anchored PCR (20) or RT-PCR, respectively, sequenced, and then introduced into the pHE3′ transgenic vector (21). Transgenic mice were generated using the two XhoI–XhoI fragments corresponding to the functionally rearranged TCR genes containing Vα4DA33 (31) and Vβ14 (32) variable chains (data not shown). Separate α and β chain–transgenic mice were established by injecting (DBA/2 × BALB/c)F2 fertilized eggs, and the two lines were then bred together to produce mice expressing the complete TCR. Transgenic mice were generated on two different genetic backgrounds as described in Materials and
Figure 3. Lymph node T cell populations in TCR transgenic mice and specific proliferative response in vitro. (A) Lymphocytes from 4-6-wk-old Tg+b-, Tg+b+, and nontransgenic mice were stained with anti-CD4 (horizontal axis), anti-CD8 (vertical axis) antibodies (upper panel), and anti-Vβ14 variable chain antibody (lower panel). Analysis of Vβ14 expression in the two lymph node subpopulations, CD4+CD8- (CD4) and CD8+CD4- (CD8), is shown. (B) Peptide-specific proliferative response in Tg+b+ and Tg+b- mice was also tested: mesenteric lymph node (2 X 10⁶/well) from Tg+b+ (heavy squares), Tg+b- (light squares) and nontransgenic mice cells (solid diamonds), were cultured for 4 d at 37°C with the addition of synthetic peptide. Cultures were pulsed with 1 µCi of [3H]thymidine for the last 16 h and harvested. Incorporation was measured by means of scintillation counting.

Methods: IgH-1Vα (Tg+b- not expressing the self-antigen) and IgH-1Vβ (Tg+b+, expressing the self-antigen).

Thymic T Cell Populations in TCR-Transgenic Mice. Vβ14-expressing T cells could be followed by the 14.2 mAb (33); anti-Vβ14 staining showed that thymic Vβ transgene expression was comparable to the pattern of TCR expression in normal animals. Three different populations were observed in the thymus: high (CD4 or CD8 single-positive), intermediate (double-positive) and low (double-negative) Vβ14-expressing T cells (Fig. 1). No thymic deletion of the transgenic T cells was found as CD4, CD8, and Vβ14 triple staining showed a slight increase in the CD4+CD8- cells of both Tg+b- and Tg+b+ mice, compared with their nontransgenic littermates (Fig. 1).

Peptide-induced Apoptosis of Double-positive T Cells. To assess whether transgenic thymocytes were susceptible to deletion as a mechanism of tolerance induction, we compared the thymuses of the Tg+b+ mice for apoptosis after
injection with 1 mg/100 μl of the specific peptide (30) or water, and noninjected mice (the peptide, not soluble in saline, was dissolved in water). 10 h after injection, propidium iodide FACS analysis (23) showed <1% of apoptotic cells in both the noninjected and water-injected mice, whereas 10% of the total thymus cell population was apoptotic in those injected with peptide (data not shown). These data were confirmed by immunofluorescent staining of thymus cryosections (Fig. 2 C) using the TUNEL method (34). On sections, the preferential staining of the cortex is consistent with a deletion of double-positive T cells. An increase in the CD4 and CD8 double-negative subpopulation was detected in peptide-injected mice by means of CD4/CD8 immunofluorescence and FACS analysis (Fig. 2 E), indicating that, if deletion had occurred in those injected with peptide (data not shown). We therefore concluded that the transgenic T cells are not deleted in the thymus but released into the periphery.

Lymph Node T Cell Populations in TCR-transgenic Mice. Lymph node single CD4+ and CD8+ subpopulations were similar in the Tg+b- , Tg+b+, and control mice. The Vβ14 transgene was expressed in ~70–80% of CD4+CD8+ cells and in 50–70% of CD8+CD4- lymphocytes (Fig. 3 A). To determine whether the T cells in the transgenic mice were functional, we measured the capacity of lymph node-transgenic T cells to proliferate in vitro to response to the specific peptide. Lymph node Tg+b+ T cells proliferated, whereas Tg+b+ cells did not (Fig. 3 B). Transgenic T Cells Are Functional In Vivo. To test whether this nonresponsive status of Tg+b+ transgenic T cells observed in vitro was physiologically important, self-antigen serum levels were measured. Surprisingly, IgG2a+ serum levels (Fig. 4 A) were undetectable with ELISA (sensitivity 3 ng/ml), whereas IgG2a+ levels were comparable with those of nontransgenic mice (Fig. 4 B); furthermore, γ2a-secretory cells from lymph node and spleen were consistently undetectable as estimated by the ELISPOT assay, whereas 60 spot-forming cells/106 cells were measured in normal animals (data not shown). These results clearly indicate that transgenic T cells do not become tolerant in the periphery by clonal deletion or by induction of anergy but rather regulate the number of γ2a-producing cells.

As transgenic animals have a relevant CD8+CD4- population, a possible involvement of cytotoxic T cells in determining the observed phenotype was tested by injecting an anti-CD8-depleting mAb into mice (25): during the 7 d in which the CD8+ cells were not detectable in the peripheral blood, no increase in IgG2a+ serum levels was observed (Fig. 4 C), thus suggesting that CD8+ cells do not play any major role. In contrast, treatment with anti-CD4 mAb restored IgG2a+ control levels 2 d after injection (data not shown). Transgenic T Cells Are Functional in Thymectomized Tg+b+ Mice. Adult transgenic mice were thymectomized to investigate whether only newly generated transgenic lymphocytes were involved in determining the phenotype. If this were the case, then IgG2a+ control levels would have been restored once newly matured transgenic lymphocytes had been excluded. However, 5 mo after thymectomy, no increase in γ2a+ serum levels was observed (data not shown), suggesting that memory is preserved.

Altogether, these data indicate that transgenic T cells may be in an undefined functional state in vivo that is not measured in vitro by the antigen-specific proliferation assay. Transgenic T Cells Are Involved in an IgG2a+to-IgE Class Switch. On days 0 and 7, transgenic mice were immunized with S. typhimurium to challenge the immune system and increase IgG2a+ serum levels. 3 wk after immunization, a peak level of 10 μg/ml of γ2a+ protein was reached, which was almost 60 times lower than that reached in the control mice (Fig. 5, A and B). Interestingly, as the other isotypes and the IgG2a+ were unaffected (Fig. 6), IgE serum levels were five times higher than in the nontransgenic animals (Fig. 7 A). 4 wk after immunization, a decrease in

Figure 4. Serum levels of IgG2a+ in Tg+b+ mice. (A) IgG2a+ and (B) IgG2a+ serum levels in 4–6-wk-old mice were measured using solid phase ELISA. (C) Animals were treated on days 0, 1, and 2 with 500 μg of anti-CD8-depleting antibody. Serum levels of IgG2a+ in nontreated, PBS-treated, and antibody-treated mice were measured by ELISA.

Figure 5. IgG2a+ serum levels during an immune response. Transgenic mice were immunized on days 0 and 7 with S. typhimurium (arrows). (A) Nontransgenic and (B) transgenic mice IgG2a+ total serum levels, followed from 0–9 wk during immune response. IgG2a+ serum levels remained 60 times lower in transgenic compared with control mice.
IgM Serum levels

IgG3 Serum levels

IgG1 Serum levels

IgG2b Serum levels

IgG2a Serum levels

Figure 6. Total Ig serum levels during the immune response. Mice were immunized on days 0 and 7 with S. typhimurium. Isotype serum levels were followed for 9 wk during the immune response. No significant differences were observed in transgenic and control animals.

IgG2ab was associated with an increase in IgE levels. The increased IgE in the transgenic (Tg+ b+) (Igh-1b+) were all of the b allotype (Fig. 7 B), suggesting that they derive from IgG2a b-positive cells. A similar response was also observed with immunization of the transgenic mice with nonmicrobial protein antigens (data not shown).

The State of Transgenic T Cell Responsiveness Is Changed after Immunization. The immunization of the transgenic mice with S. typhimurium gave rise to another important finding aside from the increase in IgE serum levels. Lymph node cells from Salmonella-immunized transgenic mice could proliferate in vitro in response to the specific peptide (Fig. 8 A) and produce IFN-γ (Fig. 8 B). As late as 13 wk after immunization, the transgenic T cells still retained their different activation state, as shown by the capacity of the transgenic T cells to proliferate and produce lymphokines in vitro.

Discussion

As the mechanisms of tolerance induction to circulating self constituents are still not clearly defined, we generated a transgenic mouse model with a TCR specific for the serum protein IgG2ab. The original T cell clone from which the TCR-α and -β chains were derived was chosen because it...
immunized Tg+b+ mice. 5 wk after immunization, the lymphocytes (2 × 10^5/well) were cultured with the synthetic peptide for 4 d at 37°C. 1 μCi/well of [3H]thymidine was added for the last 16 h of incubation. The cultures were then harvested and their incorporation measured by means of scintillation counting. (B) Cytokine production by transgenic lymph node T cells. IFN-γ and IL-4 production was investigated by ELISA 3 d after in vitro culturing with the synthetic peptide.

**Figure 8.** Peptide-specific proliferative response and cytokine production in immunized mice. (A) Peptide-specific proliferative response in immunized Tg+b+ mice. 5 wk after immunization, the lymphocytes (2 × 10^5/well) of Tg+b+ mice (open squares) and nontransgenic mice (solid squares) were cultured with the synthetic peptide for 4 d at 37°C. 1 μCi/well of [3H]thymidine was added for the last 16 h of incubation. The cultures were then harvested and their incorporation measured by means of scintillation counting. (B) Cytokine production by transgenic lymph node T cells. IFN-γ and IL-4 production was investigated by ELISA 3 d after in vitro culturing with the synthetic peptide.

had been shown to suppress the IgH-1b allotype in vivo (29). Further, this allotype-specific T cell clone allowed us to generate transgenic control mice that did not express the self-antigen. Thus, transgenic mice were produced on two different genetic backgrounds: IgH-1^ab and IgH-1^a^b.

Thymic deletion has been described in TCR-transgenic mice specific for the blood-borne self-antigen C5 (13), and thymic or peripheral tolerance was observed with other serum proteins expressed as transgenes (10, 11). Surprisingly, in our transgenic model, peripheral T cell activity was observed rather than T cell tolerance, which is consistent with a previous finding from Bogen et al. (15) also showing positive selection for idiotype-specific transgenic T cells. However, in this model, given the small number of idiotype-expressing B cells, the influence of the T cells on the fate of the B cells could not be followed. The choice of an allotypic T cell allowed us to investigate the fate of the B cells after T cell interaction.

We have shown that anti-Ig-specific T cells are not deleted in the thymus and undergo positive selection on H-2^d haplotype. Although the small amount of circulating IgG2ab (below the level of detection) may have been insufficient to reach the thymus and be presented to transgenic T cells, there was no evidence of negative selection in the thymus of immunized mice when IgG2ab serum levels reached 10 μg/ml (data not shown). On the contrary, the skew toward CD4^+CD8^- cells (35) is greater in Tg+b+ mice expressing the self-antigen. One of the parameters that controls thymocyte selection is related to the number of TCRs engaged with peptide–MHC complexes, with negative selection occurring when this number is high (high avidity) and positive selection occurring when this number is low (low avidity) (36). As IgGs are not easily processed and presented (37), we can assume that the low avidity necessary for positive selection is attributed to the low density of peptide–MHC molecules on the surface of selecting cells. We cannot exclude that the transgenic T cells express different densities of the transgenic TCR because of expression in some cells of a second TCR using the transgenic β chain and a rearranged endogenous α chain (38). High density cells would have the highest avidity for the target and might have been deleted in the thymus, leaving only low avidity cells emigrating in the periphery. Alternatively, only a particular type of cell may be involved in Ig presentation and positive selection. If, as proposed in the literature (39), B cells are the principal Ig APC in the thymus, their number might be too low to induce negative selection.

Injection of specific peptide was found to lead to double-positive cell deletion, which may be explained by either an increase in peptide density in the thymus or the involvement of APC different from the selecting cells normally used.

Tg+b+ mice have very low or undetectable IgG2ab serum levels, and γ2a secreting cells are not detectable in either lymph nodes or spleen. This indicates that transgenic T cells do not become tolerant in the periphery by means of clonal deletion or the induction of anergy, but instead regulate the number of γ2a^+ cells. Because the b allotype of IgG2a appears to be easily suppressed (40, 41), it might be thought that the lack of tolerance of anti-b allotype-specific T cells could be a special case. Nevertheless, it has been shown that in mice transgenic for an Ig anti-IgG2a of the a allotype, the transgenic B cells do not become tolerant even at 50 μg/ml of IgG2a (42). Thus, this feature is not restricted to the b allotype and seems to be a common characteristic of anti-Ig-specific lymphocytes.

The activity of Ig-specific transgenic T cells could not be investigated in vitro by means of the proliferation function or lymphokine secretion because these cells did not respond to the specific peptide; surprisingly, the transgenic T cells did proliferate and produce IFN-γ after immunization with Salmonella. The presentation of self-Ig determinants has been investigated by Rudensky and Yurin (37) and Bikoff and Eckhardt (43); their results suggest that B cells are the major cell type presenting Ig because dendritic cells and macrophages are inefficient. We thus assume that, before immunization, it is the γ2a^b-positive cells that mainly present the CH3 domain of their Ig, and this determinant may not be available for other APC. After interaction with B cells, the T cells were not anergized in vivo, though they were not able to proliferate in vitro.

T cell activity was confirmed in vivo by the analysis of thymectomized mice: if the T cells had been deleted or anergized, γ2a^b serum levels would have increased given the absence of newly differentiated transgenic T cells, but this was not the case. The T cells remained functional for at
Figure 9. Fate of B cells during the immune response. Antigen-specific T cells interact with IgM⁺ B cells that induce their maturation and class switch. After this process, the IgM⁺ B cells become IgG2ab-expressing cells that can present epitopes of γ2a b and interact with allotype-specific transgenic T cells. A further class switch of the B cell is induced by this interaction. It cannot be excluded that this process induces B cell death. A possible involvement of non-B APC must be considered.

least 5 mo after thymectomy, thus indicating that T cell memory was preserved.

After immunization with Salmonella, the increase in IgG2a b serum levels made it available for other APC that could now present the IgG2ab epitope to the transgenic T cells. The signal given by non-B APC could drive the T cells to a different functional state, which could then be investigated in vitro by peptide-specific proliferation and lymphokine secretion. Once the T cells reached this state of maturation, they preserved it for at least 13 wk.

The fate of the B cells in immunized transgenic mice was also followed during the immune response; the γ2ab Igs reached a peak of 10 µg/ml 3 wk after immunization and started decreasing at 4 wk. At the same time, the level of IgE increased until it was five times higher than that in the nontransgenic animals. Interestingly, this increase is ascribable to IgE of the b allotype, indicating that IgGb-positive B cells might derive from IgG2ab-positive cells. To explain this observation, we propose that during the immune challenge, when IgM-expressing B cells become IgG2ab producers, epitopes of the γ2ab protein are presented by B cells to antiallotype transgenic T cells (Fig. 9). It can be hypothesized that the outcome of this interaction is the induction of a further switch to IgE; this is consistent with the genomic organization of the Ig locus, the e gene being downstream to the γ2a gene. However, at this point, we cannot distinguish isotype switching of activated B cells from differentiation (and secretion) of previously generated memory cells. In addition, since it has not been proven that memory B cells cannot switch to others isotypes, this might be the system to investigate this problem. Nevertheless, the possibility that B cells die after antiallotype transgenic T cell interaction cannot be formally excluded.

Given that transgenic T cells are not deleted in the thymus and are functional in the periphery, all of these findings indicate that Ig-specific T cells could exist in normal individuals and may represent a late-acting form of T cell help for the regulation of the IgG2a-to-IgE class switch.

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