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Induction of Autoimmunity in a Transgenic Model of B Cell Receptor Peripheral Tolerance: Changes in Coreceptors and B Cell Receptor-Induced Tyrosine-Phosphoproteins

Nili Feuerstein, Fangqi Chen, Michael Madaio, Michael Maldonado, and Robert A. Eisenberg

Abrogation of peripheral tolerance in transgenic mice that express a uniform B-cell receptor may create a powerful tool to examine the molecular mechanisms that underlie the autoimmune response in B cells. Here we report that processes that induce a systemic lupus erythematosus-like syndrome in normal mice, namely chronic graft vs host reaction, trigger systemic autoimmunity in a well-established transgenic mice model of B cell receptor peripheral tolerance. The induction of graft vs host reaction in mice that carry both a rearranged B cell Ag receptors specific for hen egg lysozyme and expressing chronically circulating hen egg lysozyme Ag resulted in induction of high and sustained levels of circulating anti-hen egg lysozyme autoantibodies and glomerulonephritis with proteinuria. This was associated with marked changes in expression of cell-surface proteins, such as CD23 and complement receptor 2. B cells from the graft vs host-induced mice could proliferate in vitro in response to self-Ag, and upon stimulation with anti-IgD demonstrated rapid phosphotyrosine phosphorylation of specific proteins, which could not be induced in the anergic double transgenic B cells. Conversely, loss of tolerance was not associated with a higher induction in the level of Syk kinase phosphorylation following stimulation with anti-IgD. Taken collectively, these data establish that 1) processes that induce a systemic lupus erythematosus-like syndrome in normal mice can abrogate peripheral tolerance in transgenic mice expressing self-tolerized B cells, and that 2) loss of tolerance in this model is associated with marked changes in surface expression of B cell coreceptors as well as with selective changes in IgD-induced signaling by discrete tyrosine-phosphoproteins, but not Syk kinase. The Journal of Immunology, 1999, 163: 5287–5297.

Lymphocytes that encounter self-Ag can undergo several distinct fates: they can be physically eliminated by deletion, undergo receptor editing, become functionally inactivated by anergy, or become activated leading to autoimmunity (1, 2). Understanding the mechanism of immunological tolerance to self-Ags and, conversely, the mechanisms involved in the activation of self-reactive cells and autoimmunity remains a fundamental problem in immunology.

Studies using the transgenic mice carrying rearranged B cell Ag receptor genes specific for hen egg lysozyme (HEL) and expressing chronically circulating HEL (i.e., Ig/soluble HEL (sHEL) double transgenic mice) have shed light on the molecular mechanisms of B cell tolerance to self-Ags (3–5). B cells from these double transgenic mice are exposed throughout development to HEL Ag. They mature and populate the peripheral lymphoid organs but they cannot differentiate into Ab-secreting cells in response to Ag stimulation in vivo and in vitro (Ref. 3; see review in Ref. 5). Ag binding to Ig/sHEL B cells fails to induce proliferation (6), B7.2 expression (7), or resistance to Fas-mediated apoptosis (8). In contrast, these cells can proliferate in response to LPS and retain responsiveness to stimulation via CD40 and IL-4 (6, 9), indicating that the cells are anergic only to stimulation via B cell receptor (BCR). The anergic state was characterized by down-regulation of IgM but not IgD (10) and by accumulation of the B cells in the splenic follicular mantle zone (11).

Studies compared the BCR-induced signaling pathways in tolerant B cells to those induced in B cells from mice transgenic only for the anti-HEL BCR (Ig single transgenic or “naïve” mice). These studies demonstrated that BCR ligation in tolerant B cells failed to activate signaling via the pathways involving Jun N-terminal kinase and the transcription factor NF-κB. Conversely, it activated the signaling pathways that involved the extracellular signal-regulated kinase and the transcription factor NF-AT (4). Furthermore, the BCR-induced activation of rapid phosphotyrosine phosphorylation of a number of proteins, as well as mobilization of intracellular Ca2+, were significantly diminished in tolerant B cells (6). These results implicate that differential biochemical processes characterize positive BCR-signaling by foreign Ag leading to immunogenicity, as opposed to negative BCR-signaling by self-Ag, which leads to tolerance and anergy (4).

The logical extension of these intriguing results would be the investigation of the molecular signaling pathways that distinguish self-Ag induction of tolerance from self-Ag induction of autoreactivity in B cells. The approach to this question in the Ig/sHEL double transgenic model requires a reliable method to break their B cell anergy. While most attempts to activate these anergic B cells
by provision of T cell help were unsuccessful (6, 12–14), other studies demonstrated that the anergic state could be at least partially reversed upon removal of the Ig/HEL anergic B cells from their environment to hosts expressing soluble or membrane bound HEL followed by provision of T cell help (9, 15). None of these reports demonstrate a breakdown of peripheral B cell anergy in the anergic environment of the transgenic mice themselves. In addition, the short-term nature of the protocols or the use of in vitro readouts alone make it difficult to extrapolate these results to the end-organ disease seen in models of chronic systemic autoimmunity. If mice that express a uniform anergic BCR could be made autoimmune, it would provide a powerful tool to examine molecular signaling mechanisms associated with the breakdown of tolerance in systemic autoimmunity.

We have previously shown that chronic graft vs host (GVH) reaction induced in normal mice by the transfer of Ia-incompatible spleen cells results in an autoimmune syndrome that closely resembles systemic lupus erythematosus (SLE) in the spectrum of autoantibodies and immunopathology (16, 17). It is thought that in this GVH model the allohelper T cells of the donor react against incompatible Ia structures of the host and generate abnormal help, which activates a subpopulation of B cells to become self-reactive (18–20). Although genetically determined SLE in mice and humans is probably not initiated by a GVH reaction, the serological and clinical similarities between the Ia-driven chronic GVH and the spontaneous disease are so striking that some of the fundamental mechanisms of loss of B cell tolerance must be comparable in the two models.

In this work, we examined whether the conditions that induce the development of SLE-like syndrome in normal mice can also induce systemic autoimmunity in Ig/HEL anergic mice. Here we report that induction of GVH in Ig/HEL double transgenic mice was associated with a marked increase in circulating autoantibody to HEL Ag as well as nephritis. This indicated not only loss of B cell tolerance in a homogeneous anergic population, but also the development of some aspects of autoimmune disease. B cells from the GVH-induced double transgenic mice demonstrated marked changes in expression of cell-surface proteins and could be induced to proliferate in response to stimulation with HEL in vitro. The loss of tolerance in the GVH-induced mice was also associated with the induction of phosphorylation of selective phosphotyrosine-containing proteins in response to BCR ligation. However, BCR-induced Syk kinase phosphorylation was not enhanced as compared with anergic cells.

Materials and Methods

Mice

MD3 × ML5 transgenic mice expressing HEL-specific IgM and IgD and soluble HEL (Ig/HEL double transgenic mice) on a C57BL/6J (B6) background were originally provided by Dr. C. Cambier (National University, Canberra, Australia) and were bred and typed in our mouse colony as previously described (21). B6 and coisogenic B6.C-H2ab/kKhEg (bm12) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) as pedigreed identified littermate pairs and were maintained in our mouse colony. 3A9 mice transgenic for a H chain of IgD (22)

Reagents

Affinity-purified polyclonal anti-Syk was kindly provided by Dr. John Cambier (National Jewish Medical Center, Denver, CO). Purified monoclonal anti-IgD Ab (H9/1) with specificity to the H chain of IgD (22) was kindly provided by Dr. Fred Finkelstein (University of Cincinnati Medical Center, Cincinnati, OH). Mouse monoclonal anti-phosphotyrosine was purchased from Upstate Biotechnology (Lake Placid, NY). HEL and protein A-Sepharose were obtained from Sigma (St. Louis, MO).

Experimental chronic GVH protocol

Chronic GVH was induced as previously described (20). Briefly, recipient mice between 2 and 4 mo of age were injected with 1 × 10^6 donor splenocytes (bm12 for induction of GVH or B6 for control), which were prepared by pressing donor spleens through a nylon-mesh cell strainer into HBSS. The resulting single-cell suspensions were washed, counted, and injected i.p.

ELISA

Blood samples were obtained from experimental mice at the time of the induction of GVH and at 2- to 4-wk intervals thereafter. Sera were stored at −20°C until further analysis. The expression of autoantibodies was assessed by ELISA, as previously described (19). Autoantigens were diluted in borate-buffered saline (BBS). HEL was used at 1 μg/ml; chromatin, purified from chicken erythrocyte nuclei, was used at 5 μg/ml; dsDNA and ssDNA were obtained from the National Institute of Arthritis (NIAID, Bethesda, MD). Experimental sera and standards were made by heating of calf thymus DNA at 97°C for 10 min and cooling on ice quickly, was used at 2.5 μg/ml. The autoantigens were added to polyclonal microtiter plates (Dynatech Laboratories, Alexandria, VA) and incubated 4 h at room temperature or overnight at 4°C. The plates were washed with BBS and blocked with BBS supplemented with Tween and BSA (BBS, 0.05% Tween-80, 0.5% BSA, and 0.1% NaN3) for 1 h at room temperature. Serum samples, diluted 1/500 (1:1000 for rheumatoid factor in BBT), were added in duplicate and incubated for 5 h at room temperature or overnight at 4°C. The plates were washed with BBS, and biotinylated rat anti-mouse Ig (k-chain specific) diluted 1/2000 in BBT was added and incubated 4 h at room temperature. The plates were washed and incubated for 1 h at with avidin-alkaline phosphatase (Zymed Laboratories, South San Francisco, CA). Plates were washed again, and paranitrophenyl phosphate substrate (Sigma), 1 mg/ml in 0.01 M diethanolamine, pH 9.8, was added. The plates were read at various time points with an automated ELISA reader (Dynatech Laboratories). Autoantibody results from individual ELISAs were standardized against the reference serum, and the result for each sample was defined as an equivalent dilution factor of standardized reference MRL/lpr sera, as previously defined by the formula: equivalent dilution factor = (dilution of standard reference sera, which gives equivalent OD of the test serum) × 10^6 (19).

Isolation of B lymphocytes

B cells were purified from murine spleens as previously described with some modifications to adjust for the transgenic mice. Briefly, T cells were depleted using mAb anti-Thy 1.2, clone MTTI (5B7), and 1.0 mM sodium orthovanadate. The insoluble material was pelleted, and the supernatant was defined as an equivalent dilution factor of standardized reference MRL/lpr sera, as previously defined by the formula: equivalent dilution factor = (dilution of standard reference sera, which gives equivalent OD of the test serum) × 10^6 (19).

Proliferation assay

Proliferation assays were performed as previously described (23, 24). In brief, 2 × 10^6 lymphocytes per well were cultured in 96-well flat-bottom microtiter plate in a total volume of 200 μl in RPMI 1640 that contained 1% FBS and 0.0036% 2-ME. Experimental samples performed in triplicate were incubated for 48 h and then pulsed with 1 μCi of [3H]thymidine. After 16 h, the cells were harvested using Harvester 96 Mach III M (EG & G Wallac, Gaithersburg, MD) and counted using liquid scintillation spectrometry.

Cell stimulation and Western blot analysis

Western Blot analysis was done as previously described (25). Murine lymphocytes were suspended in RPMI 1640 without FBS (5 × 10^6 per ml) and incubated for 10 min at 37°C before activation. HEL or anti-IgD was added, and the cells were further incubated for various periods of times (5–25 min) as indicated. At the end of the incubation, cells were pelleted by short centrifugation and lysed in 30 μl of RIPA buffer (0.15 M NaCl, 50 mM Tris pH 8.0, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM leupeptin, 100 mM NaF, and 1 mM sodium orthovanadate. The insoluble material was pelleted, and the supernatants were mixed with SDS sample buffer and boiled for 5 min. Lysates containing equal amounts of cells (3 × 10^6) were applied on 7.5% SDS polyacrylamide gel and analyzed by electrophoresis. Proteins were transferred onto nitrocellulose membranes and blotted with anti-phosphorylase Ab. In brief, following blocking overnight at 4°C with TBS supplemented with 20% FBS, the membrane was incubated with anti-phosphorylase Ab (0.35 μg/ml) for 1 h at room temperature. The immunoreactive Ab was detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) (25).
FIGURE 1. Circulating anti-HEL autoantibodies in Ig/sHEL double transgenic mice following induction of GVH. A, Ig/sHEL double transgenic mice or B6 mice (-10 mice per group) were injected with spleen cells from either control B6 donor mice (Ig/sHEL-C or experimental bm12 donor mice (Ig/sHEL-GVH). Blood samples were derived before the injection and at 8, 16, and 24 wk following the injection. Anti-HEL in the sera was tested by ELISA. Results represent means ± SD of one experiment. B, Ig/sHEL transgenic mice were injected with spleen cells from either control B6 donor mice (Ig/sHEL-C) or experimental bm12 donor mice (Ig/sHEL-GVH). Blood samples were derived before the injection and 2 wk following the injection. Anti-HEL in the sera was tested by ELISA. C, Ig/sHEL-H2-k mice were injected with splenocytes from different donors: “bm12,” (bm12 × B6-H-2k)F1 (I-A incompatible); from “B6,” (B6 × B6-H-2k)F1 (I-A compatible); or from “TCR(B6-H-2k) × TCR(B6 × B6-H-2k)F1, (TCR transgenic) mice. Anti-HEL Abs in sera determined by ELISA. Results represent means ± SD of one experiment.

Immunoprecipitation

Immunoprecipitation assays were performed as previously described (26). Ten million B cells were incubated for 5 min at 37°C with anti-Ig in 0.5 ml of RPMI 1640 without FBS. The cells were pelleted by centrifugation and lysed in 300 μl of RIPA buffer supplemented with 1 mM PMSF, 1 mM leupeptin, 100 mM NaF, and 1 mM sodium orthovanadate. The insoluble fractions were pelleted, and the supernatants were incubated with 12 μg/ml of affinity-purified rabbit polyclonal anti-Syk (a kind gift of Dr. John Cambier) and 35 μl of protein A-Sepharose for 90 min at 4°C. At the end of the incubation, the Sepharose beads were pelleted, washed three times with RIPA buffer, suspended in SDS sample buffer, and boiled for 5 min. The immunoprecipitates were analyzed on 7.5% acrylamide gel SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and blotted with anti-phosphotyrosine Ab, as described above.

Immunofluorescence staining

Two-color flow cytometric staining of spleen cells was performed as previously described (27). The Abs used for staining were purchased from PharMingen (San Diego, CA); FITC-anti-B220, FITC-anti-CD21, PE-anti-CD23, PE-anti-CD24, and SAPE-anti-I-Ak. In all experiments 10,000 live events were acquired, and positive staining with specific Abs was analyzed using FACSscan flow cytometry and CellQuest software (Becton Dickinson, Mountain View, CA).

Kidney histopathology

The presence and severity of nephritis was determined on hematoxylin-eosin-stained sections, as previously described (28, 29). The severity of nephritis was based on a semiquantitative scale (0–4+: absent, mild, moderate, severe). The evaluation was based on parameters previously described and performed independently by one observer (Maidaio), who was blinded to the origin of the kidneys (28, 29). Briefly, light microscopic evaluation of two to four sections of kidneys from different levels in each animal was visualized to determine the level of inflammation and fibrosis in the glomerular, vascular, and interstitial compartments. A minimum of 50 glomeruli, 50 interstitial fields, and 25 medium to large vessels were evaluated for disease in each animal.

Proteinuria

Proteinuria was determined in urine samples that were derived before the GVH injection and at different time points following the injection using Uristix reagent strips (Miles Laboratories, Elkhart, IN).

Results

Systemic autoimmunity in Ig/sHEL transgenic mice is induced by GVH

Circulating anti-HEL autoantibodies in Ig/sHEL double transgenic mice. Studies were initiated to examine whether chronic GVH reaction could reverse B cell tolerance in double transgenic mice that express profound B cell anergy. Recipient Ig/sHEL double transgenic mice received a single i.p. injection of donor splenocytes from either B6 mice (control, syngeneic) or from bm12 mice (experimental, I-A incompatible). Blood samples were obtained at intervals following injection of splenocytes and analyzed by ELISA for the presence of anti-HEL autoantibodies. A marked induction in systemic anti-HEL autoantibodies was observed in Ig/sHEL double transgenic mice injected with bm12 splenocytes, but not in mice injected with compatible B6 splenocytes (Fig. 1A). The high levels of systemic anti-HEL autoantibodies in the Ig/sHEL double transgenic mice could be detected at 2 wk after the induction of GVH (Fig. 1B), and they remained elevated at 24 wk (Fig. 1A). In contrast to the transgenic Ig/sHEL mice, B6 mice injected with bm12 splenocytes did not develop anti-HEL Abs (Fig. 1A), but they demonstrated high levels of antinuclear autoantibodies (Fig. 2).

Control groups of Ig-HEL single transgenic mice and sHEL single transgenic mice were also included in the experiment shown in Fig. 1A (data not shown). In contrast to the anergic mice, single transgenic Ig-HEL (“naïve”) mice already have detectable levels of anti-HEL before GVH (OD: 0.274 ± 0.04). Injection of bm12 splenocytes into the Ig-HEL single transgenic mice resulted in a 3.6-fold increase of the OD level of anti-HEL. The moderate elevation in the level of anti-HEL in these mice parallels the polyclonal activation previously documented in the early phases of the chronic GVH (16, 17). By contrast anti-HEL Abs were not found in sHEL transgenic mice before or after injection of bm12 splenocytes (data not shown).

The striking breakdown of tolerance by chronic GVH driven by
allorecognition raised the question of whether similar breakdown of tolerance would result from different conditions of T cell help. Thus, in additional experiments, we examined whether adoptive transfer of splenocytes from transgenic mice carrying rearranged αβ TCR specific for HEL (TCR hel) would reverse tolerance in Ig/sHEL anergic mice. Because the TCR hel transgenic cells recognize a HEL-derived peptide in the context of I-Ek, (B6 × B6.H2k)F1 Ig/sHEL double transgenic mice were used as recipients in these experiments. Recipients were injected with donor splenocytes from (bm12 × B6.H2k)F1 (I-A incompatible); from (B6 × B6.H2k)F1 (I-A compatible); or from TCR hel-(B6 × B6.H2k)F1 (TCR transgenic, I-A compatible) mice. Blood samples were assayed for the presence of anti-HEL autoantibodies. Transfer of I-A incompatible splenocytes (bm12 × B6.H2k)F1 induced loss of tolerance as described above (Fig. 1C). However, transfer of splenocytes from TCR hel mice, failed to induce the production of anti-HEL Abs in recipient anergic mice (Fig. 1C). These negative results suggest that a difference may exist in the T cell help delivered through allorecognition of Ia vs recognition of Ia plus Ag, and that this difference may determine whether or not self-tolerance can be broken.

Evidence for nephritis in the absence of the characteristic SLE autoantibodies. The serum samples in the experiments shown in Fig. 1A, which demonstrate an increase in serum anti-HEL autoantibodies following GVH induction, were further analyzed for the presence of several autoantibodies that characterize SLE in humans and are found in systemic autoimmunity induced by chronic GVH in normal mice (16, 17). The transfer of bm12 spleen cells induced anti-chromatin, anti-ssDNA, and anti-dsDNA in nontransgenic mice, but not in Ig/sHEL double transgenic mice (Fig. 2, A–C). Importantly, in spite of lack of increase in these nuclear autoantibodies, the induction of GVH in Ig/sHEL mice was associated with a significant development of nephritis as demonstrated with histological analysis of the kidneys (Fig. 3) and further supported by evidence of proteinuria (Fig. 2D).

Distinct changes in expression of cell-surface proteins are associated with breakdown of tolerance

Further studies examined whether breakdown of tolerance in Ig/sHEL mice was associated with changes in expression of cell-surface proteins. Flow cytometry analysis (Fig. 4A) compared the expression of CD23 (FcεRII), CD24 (heat stable Ag) and I-Aα on...
following stimulation with LPS (Fig. 5). Our results confirmed that tolerant B cells demonstrate normal proliferation following the presence of submitogenic concentrations of LPS. We concluded that tolerant B cells can respond in response to stimulation with self-Ag. As shown in Fig. 5A, stimulation of B cells from GVH-induced mice with HEL resulted in ~12- to 18-fold induction in [3H]thymidine incorporation, which was similar to the increase (10.7-fold) found in B cells from “naïve” Ig HEL single transgenic mice. Fig. 5C demonstrates a dose-response effect of HEL Ag on proliferation of B cells from GVH-induced mice. Notably, 100 ng/ml of HEL Ag induced a ~10-fold increase in proliferation of B cells from GVH-induced Ig sHEL mice, while increasing amounts of HEL up to 6000 ng/ml did not induce proliferation in the B cells from the tolerant Ig sHEL double transgenic mice. It should be noted that two GVH-induced mice of eight tested in in vitro proliferation assays at different experiments did not show any proliferation response despite changes in expression of cell-surface proteins. B cells from the other two GVH-induced mice showed a proliferation response that was lower than in naïve Ig HEL controls, but significantly higher than in non-GVH Ig sHEL mice. The reason for lack of in vitro proliferation response in certain GVH-induced mice is currently under investigation.

FIGURE 3. Evidence for kidney disease in Ig sHEL anergic mice induced with GVH. The kidneys from mice in the experiment shown in Fig. 1 were analyzed 24 wk after the induction of GVH for kidney disease as described in Materials and Methods. Results show mean scores for histopathology ± SEM. Statistical significance of the difference between control and GVH in either normal mice or Ig sHEL mice was evaluated using Wilcoxon/Mann-Whitney U test. *p < 0.03; **p < 0.01 (n = 5).

B cells from “naïve” mice (Ig HEL single transgenic), “tolerant” (Ig sHEL) and “GVH” (Ig sHEL mice induced with GVH).

Intriguingly, tolerant B cells demonstrated high level of MHC class II as compared with “naïve” B cells, but breakdown of tolerance was associated with even further increase in MHC class II expression. Tolerant B cells were reproducibly characterized with very low level of expression of CD23 as compared with “naïve” cells (Fig. 4A). Upon GVH induction, CD23 expression increased to a level higher than that on “naïve” cell. Conversely, the expression of CD24 demonstrated a marked decrease upon breakdown of tolerance to a level that is lower than in “naïve” cells. These data demonstrate profound differences in the expression of cell-surface proteins on B cells from GVH-induced Ig sHEL mice, as compared with self-tolerized, or to “naïve” B cells.

Because profound decrease in B cell expression of complement receptor 2 (CR2) (CD21) was reported in human patients with SLE (30–32), we further examined whether loss of tolerance by GVH is also associated with a decrease in the expression of CR2. Indeed, we found a marked decrease in expression CD21 on B cells following breakdown of tolerance by GVH in Ig sHEL transgenic mice as well as in normal mice (B6) that underwent a chronic GVH (Fig. 4B). Comparison of GVH-induced changes in transgenic vs normal B6 mice showed that, although the anergic B cells were phenotypically very different from the normal B cells, they underwent similar changes in expression of MHC class II, CD23, CD21, and CD24 (Fig. 4B). This suggests that certain biochemical processes that underlie the breakdown of tolerance in the anergic mice resemble those that trigger SLE in normal mice.

Proliferation in response to self-Ag in vitro distinguishes self-reactive from self-tolerized Ig sHEL B cells

B cell tolerance in Ig sHEL mice is characterized by the inability to proliferate in response to stimulation with self-Ag, despite proliferation responses to LPS (1). In further studies, we investigated whether induction of GVH changed the capability of B cells to proliferate in response to self-Ag in vitro. Proliferation assays were performed 4–6 wk postinduction of GVH and followed the protocol as previously described by Goodnow (6). In this protocol, it has been established that naïve cells, but not tolerant cells, can proliferate in response to the HEL Ag when the cells are primed in the presence of submitogenic concentrations of LPS. We confirmed that tolerant B cells demonstrate normal proliferation following stimulation with LPS (Fig. 5B), but could not be induced to proliferate in response to stimulation with increasing amounts of HEL (Fig. 5C). In contrast, B cells from GVH-induced mice acquired the ability to proliferate in response to stimulation with self-Ag. As shown in Fig. 5A, stimulation of B cells from GVH-induced mice with HEL resulted in ~12–18-fold induction in [3H]thymidine incorporation, which was similar to the increase (10.7-fold) found in B cells from “naïve” Ig HEL single transgenic mice. Fig. 5C demonstrates a dose-response effect of HEL Ag on proliferation of B cells from GVH-induced mice. Notably, 100 ng/ml of HEL Ag induced a ~10-fold increase in proliferation of B cells from GVH-induced Ig sHEL mice, while increasing amounts of HEL up to 6000 ng/ml did not induce proliferation in the B cells from the tolerant Ig sHEL double transgenic mice. It should be noted that two GVH-induced mice of eight tested in in vitro proliferation assays at different experiments did not show any proliferation response despite changes in expression of cell-surface proteins. B cells from the other two GVH-induced mice showed a proliferation response that was lower than in naïve Ig HEL cells, but significantly higher than in non-GVH Ig sHEL mice. The reason for lack of in vitro proliferation response in certain GVH-induced mice is currently under investigation.

Loss of tolerance in GVH Ig sHEL double transgenic mice is associated with induction of specific phosphotyrosine proteins upon BCR activation

A question of major importance is: what are the biochemical processes associated with BCR response to self-Ag leading to autoimmunity as compared with negative signaling leading to anergy? Our results demonstrated that transgenic B cells from anergic mice induced by GVH became self-reactive in vivo and in vitro. Therefore, it appears that these cells may be useful in studying activation pathways associated with positive signaling by self-Ags. To address this question, we compared phosphotyrosine protein phosphorylation following BCR activation in B cells from Ig HEL single transgenic ("naïve"), Ig sHEL double transgenic ("tolerant"), and GVH-induced Ig sHEL mice ("self-reactive"). We found that BCR ligation in B cells from GVH-induced mice was associated with induction in the phosphorylation of discrete proteins, which could not be induced in tolerant cells under the same experimental conditions (Fig. 6, A and B). Particularly, phosphorytosine-containing proteins at molecular masses of ~78 kDa and ~60 kDa were reproducibly induced under the resolution conditions of these experiments. The preferential induction of these two proteins in "self-reactive" B cells was prominently demonstrated in Fig. 6C.

While the identity of these two proteins is not yet known, we excluded Lyn kinase based on its lower electrophoretic mobility and high level of constitutive phosphorylation that was not induced following BCR ligation in either tolerant or GVH-induced mice. This was found in immunoprecipitation studies with anti-Lyn Ab (data not shown). We also excluded Syk kinase as shown in further studies.

Syk kinase phosphorylation following BCR activation does not distinguish anergy from self-reactivity in Ig sHEL B lymphocytes

Syk kinase plays an essential role in mediating the early propagation of signaling following BCR activation of naïve B cells (33, 34). Thus, it was important to determine the role of Syk kinase in breakdown of tolerance. To address this question, initial studies were performed to compare quantitatively the level of BCR-induced Syk phosphorylation in "tolerant" vs "naïve" B cells. Following BCR activation of "naïve" and "tolerant" cells, portions of the cell lysates were analyzed in Western blot with anti-phosphotyrosine Ab to view a wide range of phosphorylated proteins on B cells from GVH-induced Ig sHEL mice, while increasing amounts of HEL up to 6000 ng/ml did not induce proliferation in the B cells from the tolerant Ig sHEL double transgenic mice. It should be noted that two GVH-induced mice of eight tested in in vitro proliferation assays at different experiments did not show any proliferation response despite changes in expression of cell-surface proteins. B cells from the other two GVH-induced mice showed a proliferation response that was lower than in naïve Ig HEL cells, but significantly higher than in non-GVH Ig sHEL mice. The reason for lack of in vitro proliferation response in certain GVH-induced mice is currently under investigation.

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proteins (Fig. 7A). The rest of the lysates were used in immunoprecipitation assays with anti-Syk Ab (Fig. 7B).

Interestingly, unstimulated tolerant cells expressed high levels of phosphorylation of several proteins as compared with naive cells (particularly ~55 kDa and 135–145 kDa; Fig. 7A). Immunoprecipitation of Syk kinase from the same cell lysates demonstrated equal amounts of Syk kinase in naive and tolerant cells. BCR ligation in tolerant cells was associated with significant but diminished induction of Syk kinase phosphorylation (Fig. 7B). Densitometry analysis demonstrated that the amounts of phosphorylated Syk kinase in “tolerant” cells following BCR ligation was diminished by ~67% as compared with the amount in “naive” cells.

In further studies, we examined whether induction of GVH restored a higher level of Syk kinase phosphorylation upon BCR ligation in Ig/sHEL B cells. To investigate this possibility, B cells from tolerant mice or from GVH-induced mice were stimulated with anti-IgD, and Syk kinase was immunoprecipitated from lysates containing equal amounts of cells. Fig. 8 demonstrates that BCR ligation in self-reactive B cells was not associated with any increase in the amounts of phosphorylated Syk kinase as compared with tolerant cells. This evidence was reproducible in several different experiments. Interestingly, when the immunoprecipitates were washed less stringently (Expt. 3 in Fig. 8), significant differences were observed in the proteins that coprecipitated with Syk in “self-reactive” as compared with “self-tolerized” cells, indicating differences in self-reactive cells that warrant further investigation.

**Discussion**

In this work, we demonstrate that MHC class II-directed allogeneic T cell help is sufficient to induce marked peripheral breakdown of tolerance in mice that genetically express profound BCR tolerance. Induction of chronic GVH in Ig/sHEL double transgenic mice was associated with the emergence of circulating autoantibody to HEL Ag and with manifestations of nephritis indicating development of autoimmune disease. B cells from the GVH-induced double transgenic mice demonstrated profound changes in the expression of cell-surface proteins, most prominently an increase in B cell expression of CD23 and a decrease in expression of CR2. The B cells from the GVH-induced transgenic mice could be induced to proliferate in response to in vitro stimulation with HEL self-Ag. Upon BCR ligation, these autoreactive cells demonstrated rapid phosphotyrosine phosphorylation of specific proteins, which could not be induced in anergic cells. Intriguingly, while tolerant B cells expressed diminished Syk kinase phosphorylation following BCR ligation compared to naive B cells, the induction of self-reactivity by GVH was not associated with any change in the level of BCR-induced Syk kinase phosphorylation. This demonstrates that breakdown of B cell tolerance does not require a change in the level of Syk kinase phosphorylation, but is associated with selective activation of signaling pathways leading to phosphorylation of other discrete tyrosine-containing phosphoproteins.

Taken collectively, these results establish that processes that induce a SLE-like syndrome in normal mice can lead to breakdown of peripheral tolerance in mice expressing profound B cell anergy.
We suggest that this model will be a powerful tool in dissecting signaling pathways associated with breakdown of tolerance in B cells and therefore should provide important insights into the immunological mechanisms of SLE.

From profound anergy to systemic autoimmunity

The mechanism of failure of tolerance is perhaps the key issue for understanding the pathogenesis of SLE. In chronic GVH in normal mice, a SLE-like syndrome is induced by transfer of allogeneic T cells (16, 17). It is thought that the allohelper T cells of the donor react against incompatible Ia structures of the host and generate aberrant T cell help, which activates a subpopulation of self-reactive B cells (18–20). In the current work, the same allo-Ia recognition led to breakdown of B cell anergy in Ig/sHEL double transgenic mice. This suggests that one possible mechanism of SLE involves the conversion of a subpopulation of mature silenced self-tolerized B cells into self-reactive B cells. Indeed, the ability to activate these mature anergic B lymphocytes by T cell help was previously reported using adoptive transfer of Ig/sHEL anergic B cells to other hosts (9, 15). Anergy in Ig/sHEL B cells can also be reversed in vitro by costimulation with CD40 ligand, IL-4, and IL-5 (9). Perhaps host T cell activation during GVH results in expression of B cell stimulatory ligands as well as secretion of lymphokines, which would contribute to the breakdown of B cell self-tolerance.
Finally, the development of proteinuria and nephritis in the absence of the wide spectrum of the nuclear autoantibodies that characterizes SLE is intriguing. This evidence is consistent with previous suggestions of an important role of the B cells themselves in the pathophysiology of the glomeruli and the blood vessels in SLE (35). In this respect, the autoimmune transgenic mouse model that is presented in this work may serve as a useful tool to investigate other mechanisms that are involved in SLE and particularly the role of self-reactive B cells in the development of glomerulonephritis associated with SLE.

Changes in expression of complement receptor and CD23 characterize breakdown of B cell tolerance

Our data show that the entire population of B cells demonstrated changes in expression of cell-surface proteins following induction of chronic GVH. The striking similarity and the reproducibility of these changes in the transgenic mice and in normal mice induced by GVH may suggest that chronic GVH activates a specific “program” of gene expression in B cells that makes them prone to become autoimmune. The ability to define the primary changes in protein expression, which drive B cell autoreactivity in this model, will be a fundamental goal in future studies.

The data demonstrate that the expression of MHC class II in anergic cells is considerably higher as compared with “naive” cells, indicating that maintenance of anergy requires a certain level of activation. Breakdown of tolerance by GVH was associated with a further increase in MHC class II expression. By contrast, low level of CD23 expression characterized the anergic cells as compared with naive cells. Breakdown of tolerance was associated with a marked increase in CD23 expression to a level that is considerably higher than in naive cells. CD23 is not expressed on immature B cells, on Ly1+ (B1), or on marginal zone B cells (36). Because these double transgenic anergic B cells are mature and were shown to be eliminated from the spleen marginal zone (11), it is possible that the low level of CD23 represents an intrinsic characteristic associated with B cell anergy. Indeed, low levels of CD23 expression were reported in anergic B cells from double transgenic mice expressing IgHEL and membrane bound HEL (6, 13). Increase in CD23 expression characterizes not only the transition from immature to mature B cells, but also is associated with T cell-dependent activation of B cells by stimulation with IL-4, IFN-γ, and CD40 ligation (38, 39). Thus, the marked increase in CD23 expression associated with breakdown of tolerance by GVH may be due to T cell-dependent activation of B cells.

A potential importance of CD23 in tolerance was recently suggested with evidence indicating that anti-CD23 mAb induced Ag-specific B-cell tolerance in vivo (40). In addition, co-cross-linking of CD23 and soluble Ig was found to inhibit B cell proliferation and induce apoptosis (41), indicating a regulatory role of CD23 in BCR-mediated immune responses. Curiously, our own work with lpr mice has shown a decrease in CD23 expression on B cells associated with spontaneous autoimmunity (42). CD23 undergoes proteolysis, giving rise to soluble CD23 molecules that act as ligands (see Refs. 38 and 43). Increased level of soluble CD23 (sCD23) has been demonstrated in the serum of patients with SLE and rheumatoid arthritis (44, 45). Ranges of cytokine activities presented in this work may serve as a useful tool to investigate other mechanisms that are involved in SLE and particularly the role of self-reactive B cells in the development of glomerulonephritis associated with SLE.

In this work, we demonstrate for the first time that mice that are functionally anergic could be induced to become functionally autoimmune by MHC class II-directed allogeneic T cell help. It is not clear why our results differ from previous adoptive transfer studies, which reported that tolerant IgHEL single transgenic mice ("naive") and from IgHEL double transgenic mice ("tolerant") were stimulated with 5 μg/ml of anti-IgD for 5 min at 37°C. A, Portions of the cell lysates (1 x 10⁶ cells) were analyzed on SDS-PAGE and blotted with anti-phosphotyrosine Ab. B. The rest of the cell lysates (equal amounts of cells) were exposed to immunoprecipitation with anti-Syk Ab. The immunoprecipitates were analyzed by Western Blot using anti-phosphotyrosine Ab. The membrane was then stripped and rebotted with anti-Syk Ab.

**FIGURE 7.** Comparison of Syk phosphorylation following BCR ligation in “naive” and “tolerant” B cells. Splenic lymphocytes (10 x 10⁶ cells/ml) from Ig-HEL single transgenic mice ("naive") and from IgHEL double transgenic mice ("tolerant") were stimulated with 5 μg/ml of anti-IgD for 5 min at 37°C. A, Portions of the cell lysates (1 x 10⁶ cells) were analyzed on SDS-PAGE and blotted with anti-phosphotyrosine Ab. B. The rest of the cell lysates (equal amounts of cells) were exposed to immunoprecipitation with anti-Syk Ab. The immunoprecipitates were analyzed by Western Blot using anti-phosphotyrosine Ab. The membrane was then stripped and rebotted with anti-Syk Ab.

Interestingly, we show that the induction of autoimmunity in the anergic mice only occurred when the donor cells recognized host MHC class II as foreign. In an experiment in which the donor T cells bore a TCR transgene directed at HEL, no autoreactivity could be induced by transfer to double transgenic recipients (IgHEL). These negative results imply that a difference may exist in the help delivered through allore cognition of Ia vs recognition of Ia plus Ag, and that this difference may determine whether or not self-tolerance can be broken.

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another potential physiological significance to the increase in CD23 expression on B cells that became autoreactive in the GVH model.

In this study, we further report that a marked decrease in CR2 (CD21) on B cells is associated with breakdown of tolerance in anergic mice and with induction of an SLE-like syndrome in GVH-induced normal mice. A previous striking finding is that patients with SLE reproducibly demonstrated a significant decrease in the expression of CR1 and CR2 on B cells and that this decrease was correlated with disease activity (30–32). Decreases in CR1 and CR2 expression were most recently reported during development of autoimmunity in MRL/lpr mice (49). While complement receptors were implicated in regulation of B cell activation and in normal and pathological conditions (50, 51), there was no evidence as to whether the activation of complement system plays a protective or deleterious role in autoimmunity. Recent studies suggested an answer to this dilemma by providing evidence to indicate a critical role for complement in maintenance of self-tolerance. These studies demonstrated that combining of mice that are genetically deficient of CR1/2 or C4 with lupus prone strain of lpr mice resulted in an exacerbation of the disease due to an increase in autoantibody (52). Furthermore, Ig/sHEL double transgenic cells that are deficient in CR2 or transferred to mice deficient in complement protein C4 failed to anergize in response to soluble self-Ag (52). Thus, the marked decrease in expression of CR2 in anergic B cells following GVH induction may play a role in driving breakdown of tolerance.

Induction in phosphorylation of discrete phosphotyrosine proteins, but not in Syk kinase, distinguishes self-reactive from self-tolerized B cells

The striking shift from peripheral anergy to systemic autoimmunity in transgenic mice with a uniform population of B cells highlights the potential of this model to dissect the molecular mechanisms associated with breakdown of tolerance. Previous studies have shown that self-Ag engagement in Ig/sHEL double transgenic B cells activates selective signaling pathways while others are blocked (4). This differential pathway activation reveals a remarkable plasticity in signaling by BCR and suggests that the same receptor can signal positively to promote immunity or negatively to enforce self-tolerance. Studies in the present work demonstrate that loss of B cell tolerance in Ig/sHEL double transgenic mice is associated with selective restoring of the ability to phosphorylate discrete phosphotyrosine proteins following BCR ligation. This finding parallels a previous report that human patients with SLE display abnormal Ag receptor-mediated early tyrosine phosphorylation when compared with normal control (53). Thus, identification of these proteins may unravel important signaling pathways that are involved in breakdown of tolerance and induction of self-reactivity.

![Figure 8](http://www.jimmunol.org/)
Attempts to identify these proteins revealed that none of these proteins is Syk kinase. Immunoprecipitation studies confirmed that Syk kinase phosphorylation does not distinguish self-tolerized from self-reactive B cells in the GVH model of autoimmunity. Thus, we show that the breakdown of tolerance was not associated with an elevation in the amounts of phosphorylated Syk kinase following BCR ligation in self-reactive cells as compared with anergic cells. In B cells, the pathway leading to the activation of Syk is initiated by the phosphorylation of tyrosine residues within a conserved motif, the immunoreceptor tyrosine-based activation motif, located on the cytoplasmic domains of the receptor components Ig-α and Ig-β (reviewed in Refs. 34 and 54). When phosphorylated, this domain acts as a docking site for the recruitment of Syk to the receptor that results in an increase in Syk phosphorylation and activation. The recruitment of Syk to the receptor is mediated via two Src homology 2 domains and is necessary for Syk phosphorylation (33). The receptor-mediated activation of Syk in B cells is invariably associated with an increase in its state of phosphorylation. The primary candidates for the kinase responsible for Syk phosphorylation are Syk itself and one or more members of the Src family. Syk kinase activation leads to additional downstream signaling events such as mitogen-activated protein kinase activation, Shc phosphorylation, phosphoinositide signaling pathway, and cooperation with Rac1 to activate Jun N-terminal kinase, which is required for transcriptional activation (55, 56).

The importance of Syk to receptor-mediated signaling in B cells is underscored by the signaling defects that adversely affect B cell differentiation in mice that lack Syk (57, 58).

In view of the crucial role for Syk kinase in B cell activation, the absence of any change in the amounts of phosphorylated Syk kinase in self-reactive cells as compared with tolerant cells is surprising. In contrast, the striking increase in the phosphorylation of other, as yet unidentified, phosphoproteins in BCR-activated self-reactive B cells from GVH-induced double transgenic mice indicates that breakdown of tolerance is associated with selective activation of signaling pathways that bypass the sequence of events leading to the induction of Syk kinase phosphorylation. Thus, an increase in Syk kinase phosphorylation above the level observed in anergic cells is not required for the induction of self-reactivity in B cells driven by MHC class II-directed allogeneic T cells. This finding implies another dimension to the plasticity of the BCR signaling, i.e., engagement of the same receptor may trigger different signaling pathways leading to induction of immunity, tolerance, or autoreactivity. Further studies to dissect the mechanisms and the molecular basis for induction of self-reactivity by GVH in this transgenic mouse model will promote our understanding of breakdown of tolerance and SLE autoimmunity.

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