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Anergy and not Clonal Ignorance Determines the Fate of B Cells that Recognize a Physiological Autoantigen[1]

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Autoantibodies to insulin arise spontaneously in the insulin autoimmune syndrome and in type I diabetes. In addition, administration of insulin to individuals without autoimmune disease routinely results in Abs that bind autologous hormone. These observations and findings in transgenic models of tolerance led to an inference that physiological levels of hormones and growth factors, such as insulin, are not sufficient to induce tolerance in B cells, a state termed clonal ignorance. In contrast, we have discovered that virtually all conventional B cells expressing a low affinity anti-insulin transgene interact with endogenous insulin and are effectively silenced for Ig production and for T cell-dependent immune responses. A fraction of transgenic B cells escapes silencing and functions autonomously to produce insulin Abs that may lower fasting blood sugars similar to an insulin autoimmune syndrome. These B cells have characteristics of a B1-like subset and are depleted by hypotonic peritoneal lysis. These findings question the concept of clonal ignorance and show that physiological concentrations of Ag may effectively silence conventional B cells even when the affinity for autoantigen is low. Self-reactivity may arise in the repertoire because of compartmental differences that govern the fate of B cells and not as a result of true clonal ignorance. ***The Journal of Immunology,*** **2001, 166: 3194–3200.**

Mice that express Ag-specific receptors as transgenes provide powerful tools for understanding immunological tolerance. Using this approach, several mechanisms are recognized to regulate the development and differentiation of B lymphocytes whose receptors (BCR)[3] bind self Ags. B cells reactive with cell surface molecules (e.g., MHC, membrane hen egg lysozyme (HEL)) or multivalent Ags (e.g., dsDNA) may be deleted from the repertoire unless they successfully edit their receptors (1–5). Soluble Ag, as exemplified by mice engineered to express both circulating HEL and anti-HEL BCR, does not eliminate self-reactive B cells (6). Rather, continuous Ags encounter results in a state of anergy or functional silencing that impairs competition for follicular niches and predisposes anergic B cells to programmed cell death (7, 8). Although highly effective in normal immune systems, these mechanisms of B cell tolerance often fail when transgenes are introduced into strains of mice with autoimmune disease. For example, transgenic B cells that express anti-globulin (rheumatoid factor) or anti-dsDNA do not differentiate in normal mice, but will produce autoantibodies when they develop in the genetic environment of lupus-prone strains (3, 9–11). However, the mechanisms of tolerance are not always protective in normal animals, as shown by the development of hemolytic anemia in mice that express a pathological anti-RBC Ab as a transgene (12, 13). In this model, conventional anti-RBC B cells were effectively eliminated from the spleen, but residual B1 type B cells in the peritoneal cavity produce autoantibody and cause disease (hemolytic anemia) in normal mice. Hypotonic lysis of peritoneal B cells or injection of RBC into the peritoneal cavity was sufficient to delete the autoreactive B cells and to prevent hemolytic anemia. The lack of Ag exposure and the unique features of the peritoneal environment are postulated to explain the failure of tolerance in this model.

In some circumstances, self Ag is found to have no measurable impact on the development and function of autoreactive B cells. Low levels of lysozyme (1 ng/ml) in high affinity anti-HEL transgenic mice and soluble H-2K^b in anti-H-2K^b transgenics failed to show any change in B cell development or differentiation (14, 15). These observations led to the conclusion that at low concentrations of Ag, the usual mechanisms that maintain B cell tolerance are ineffective, a state referred to as clonal ignorance (16). Immune responses to many self Ags present in low concentrations, such as growth factors, cytokines, or hormones, are often attributed to clonal ignorance. The high frequency of organ-specific autoimmune disorders associated with immune responses to such Ags suggests that clonal ignorance may provide a reservoir for expansion of autoreactivity in pathological states. Therefore, understanding the fate and function of clonally ignorant B cells is an important first step in discerning their contribution to autoimmune disease.

The immune response to insulin is an ideal tool for investigating clonally ignorant repertoires. The small molecule (5800 Da) is produced by the pancreatic β cells of all mammals, and it circulates postprandially in low concentrations as part of the physiological response to glucose. Abs that bind autologous hormone are routinely produced after administration of the hormone in the treatment of diabetes mellitus, and spontaneous Abs are detected following viral infections, hypersensitivity states, and in the prodrome of autoimmune endocrine disease (17–21). To directly investigate the nature of tolerance in the B cell repertoire for insulin, we introduced the V region genes from mAb125 into the germline of C57BL/6 mice as an IgMa transgene (125Tg). IgG mAb125 is derived from the primary response of a BALB/c mouse immunized with human insulin and uses a mutated V_H-IX gene

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[3] Abbreviations used in this paper: BCR, B cell receptor; HEL, hen egg lysozyme; 125Tg, mice that express V region genes from mAb125 as an IgMa transgene.

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(22, 23). mAb125 binds human insulin with an affinity of 3×10^{-8} L/M, and it binds autologous rodent insulin at 10^{-7} L/M (24). Based on the HEL/anti-HEL model system (10^{-9} L/M), the peak and basal levels of endogenous insulin (25) and the affinity of mAb125 are anticipated to generate B cells whose receptor affinities reside well within a range of clonal ignorance. In contrast to this expectation, most B cells from 125Tg mice are effectively silenced by endogenous insulin, as demonstrated by adoptive transfer and immunization with heterologous insulin and proinsulin. Although the majority of B cells in 125Tg mice are profoundly anergic to Ag challenge, some insulin Ab is autonomously produced in the absence of T cells or immunization as part of the natural autoantibody repertoire of 125Tg mice. Circulating insulin Abs in 125Tg mice are effectively depleted by hypotonic peritoneal lysis, suggesting that some B cells may have escaped tolerance by entering a B1-like compartment. Overall, the data indicate that for conventional B cells induction of tolerance may occur at intermittent low levels of physiological autoantigen, and continuous Ag production is not required to maintain the anergic state. These data do not support clonal ignorance as a central mechanism for insulin autoimmunity. We conclude that regulation within B cell compartments, such as the germinal centers from which mAb125 arose and the peritoneal cavity that supports constitutive insulin Ab production in 125Tg mice, is responsible for maintaining self-reactivity to insulin in the repertoire.

Materials and Methods

Mice and transgenic lines

Conventional C57BL/6 mice used in these studies were obtained from The Jackson Laboratory (Bar Harbor, ME). The V_H10-μ heavy chain and Vκ10-Cκ light chain constructs previously used to express anti-HEL transgenes in the pSVG-gpt vector were the gift of C. C. Goodnow (John Curtin School of Medical Research, Canberra City, Australia) (26). The V_H and Vκ regions derived from the HyHEL10 mAb were removed and replaced with the 5′ untranslated and L-VDJ or L-VJ DNA segments cloned from anti-insulin mAb125. The nucleotide sequences of the engineered 125V_H and 125Vκ chains were confirmed and the constructs tested for expression by in vitro transfection of B cells lines, as previously described (23). For microinjection, gene constructs were linearized with *Sal*I and purified chromatographically. To avoid the possible complications of anti-insulin Abs on gestation, V_H125-μ heavy chain and Vκ125-Cκ founder mice were initially generated individually from microinjection of B6 × DBA2 single cell oocytes (27). Founders were originally identified by Southern blot hybridization using cDNA probes on tail DNA digested with *Bgl*II and Pst for 125VH and with *Hin*dIII for 125Vκ genes. Based on hybridization signals relative to endogenous V genes, the number of transgene copies is estimated to be low (<3). Founder mice were backcrossed to C57BL/6 mice, and subsequent progeny were monitored for transgene transmission using PCR to identify V_H and Vκ transgenes or by flow cytometry to identify $IgMa^+$ B cells. Mice were intercrossed to obtain offspring that express both 125V_H and 125Vκ transgenes, 125Tg. 125Tg mice were subsequently found to mate and reproduce normally. All animals used in the current study were backcrossed >10 times to C57BL/6.

mAbs and flow cytometry

mAbs used in this study include: RS-3.1 (IgMa), AF6 (IgMb), 6B2 (B220), 57-3 (CD5), and M1/70 (Mac1) (BD PharMingen, San Diego, CA). B220 and isotype-specific reagents were biotin modified, and detection was with FITC avidin (Boehringer Mannheim, Indianapolis, IN) or PE streptavidin (Sigma, St. Louis, MO). FITC-labeled goat anti-mouse IgG was used for indirect detection of binding with some reagents (Cappel Laboratories, Cochranville, PA). Anti-insulin mAb123 was biotinylated on an insulin column to protect the binding site, as previously described (28). Pork insulin was biotinylated at its single lysine residue on the insulin B chain using normal human serum biotin (Pierce, Rockford, IL). Binding of insulin to the cell surface was detected using PE or FITC avidin. For flow cytometry, single-cell suspensions of spleens were prepared by lysis of RBC in NH_4Cl and were stained with the indicated Ab for 1 h at 4°C. For two-color analysis, cells were incubated sequentially with biotinylated reagents, followed by either PE or FITC conjugates. FACScan fluorescence data (Becton Dickinson, San Diego, CA) were displayed in two-dimensional dot plots.

Ab determinations

ELISA for detection of anti-insulin Abs were done using 96-well Immulon II plates (Dynatech Laboratories, Alexandria, VA) coated with biosynthetic human or pork insulin (Lilly, Indianapolis, IN) (1 μg/ml in borate-buffered saline, pH 8). Sera were diluted (1/100–1/1000) in PBS, pH 7.4, containing 0.5% Tween-20, and binding was measured at OD_{405} using an alkaline phosphatase-conjugated second Ab and para-nitrophenyl phosphate (Sigma) as substrate (17). For insulin-specific binding, results of binding to insulin-coated plates were reported as inhibitable OD_{405} U in the presence of 50 μg/ml of insulin. For detection of IgM allotypes in serum, microtiter wells were coated with goat anti-mouse sera (Cappel), and biotinylated isotype-specific mAbs were used to measure capture of IgMa and IgMb by OD_{405}. Control reagents for these studies include normal C57BL/6 and BALB/c sera, insulin-specific IgMa mAb301 (29), and polyclonal IgM anti-insulin obtained from B6 or BALB/c mice immunized with *Brucella* insulin (30).

Immunization and adoptive transfer

To provide maximum insulin-specific T cell help in H-2^b mice, animals were immunized i.p. with 0.1 cc of CFA containing 50 μg/ml of a mixture of beef insulin and pork proinsulin. Preliminary studies using trinitrophenyl-keyhole limpet hemocyanin-primed B cells and beef insulin-primed T cells were conducted to determine optimal kinetics for insulin-specific help. For adoptive transfer, B cells were purified from B6 or 125Tg mice by magnetic bead depletion of non-B cells (MACS; Miltenyi Biotec, Auburn, CA), and 2×10^7 B cells were transferred i.v. into irradiated (650 rad) recipients. Immune T cells were obtained from nontransgenic B6 mice by negative selection (MACS), and 10^7 T cells were used per recipient. The purity of these B and T cell populations as assessed by flow cytometry was >90%. After cell transfer, recipients were boosted with the same insulins in CFA. In some experiment cells, animals received a second injection of Ags in immunofluorescence assay. Insulin Ab in sera was measured by ELISA before and following immunization.

Glucose tolerance testing and pancreatic histology

To examine β cell function mice, a glucose tolerance test was performed after removing food for an overnight fast. Animals were administered i.p. glucose (1.8 g/kg) in PBS and blood sugars at 20- to 40-min intervals. Blood sugars were measured by the glucose oxidase method with an Elite Glucometer (Bayer, Elkhart, IN). For pancreatic histology, Formalin-fixed pancreata were embedded in paraffin, and 10–12 nonsequential sections were obtained from each gland. Slides were stained with hematoxylin and eosin and examined by light microscopy.

Results

Expression of anti-insulin transgenes

To investigate the role of clonal ignorance in Ab responses to a physiological autoantigen expressed at low levels, transgenic mice that express the V regions of anti-insulin mAb125 were produced, as described in *Materials and Methods*. The expression of transgenic Ig was first examined using B220 and IgM allotype-specific mAbs (Fig. 1). Mice that express only the heavy chain V region demonstrate high level of IgMa expression and effectively exclude >95% of endogenous IgMb on the surface of $B220^+$ spleen cells (*A* and *B*). Highly efficient allelic exclusion is also observed in mice when the V_H125 IgMa heavy chain is expressed with its Vκ partner from mAb125 (125Tg mice). Effective allelic exclusion in these mice is maintained for long periods (>40 wk). The finding that nearly normal numbers of B cells are present in 125Tg mice indicates that clonal deletion has not taken place. However, 125Tg mice (V_H + Vκ) routinely demonstrate a 10–20% reduction in mean fluorescent intensity of surface of IgMa compared with heavy chain-only transgenics (*D*). These findings suggest that acquisition of insulin binding through pairing of the IgMa heavy chain transgene with its Vκ125 partner may result in a modest down-regulation of surface BCR expression.

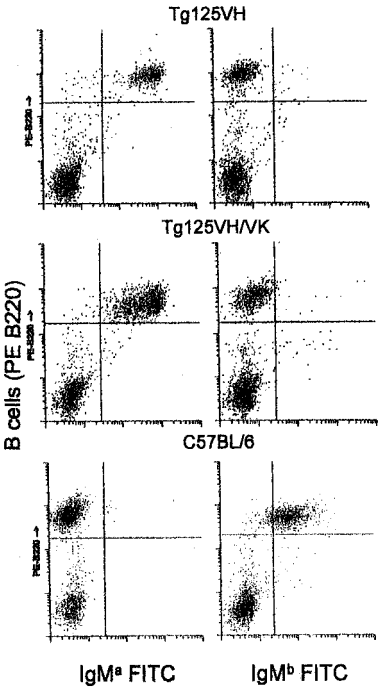

FIGURE 1. Anti-insulin transgenes exclude expression of endogenous Igs. Flow cytometry shows expression of transgene IgMa (*left panels*) or endogenous IgMb (*right panels*) in spleen B cells (B220, *left axis*). Data are shown for mice that express only the transgene (Tg) 125VH heavy chain (*top panels*), both anti-insulin V_H and Vκ Tg125 (*middle panels*), and normal B6 mice (*lower panels*). The reduced intensity of IgMa seen in V_H/Vκ transgenic B cells compared with V_H only transgenics is reproducible in multiple experiments.

Insulin binding by transgenic B cells

To examine Ag binding by the BCR in mice that express anti-insulin transgenes, FITC avidin and biotinylated insulin were used to examine interactions on the surface of 125Tg B cells. Using a range of insulin concentrations between 0.1 and 1000 ng/ml, spleen cells from normal mice have no detectable insulin-binding $B220^+$ B cells above that of background on avidin FITC alone (Fig. 2*A*). In addition, insulin-binding B cells are not detected in mice that express only the heavy chain transgene (identical with *A*, not shown). In contrast, essentially all B cells from mice that express H and L anti-insulin transgenes (125Tg mice) bind insulin (Fig. 2, *B–H*). The concentration of insulin that occupies ~50% of BCR (5 ng or 1 μM) is outside the physiological range. However, at nM concentrations of hormone, 5–15% of 125Tg B cells are observed to bind insulin (*B* and *C*). These concentrations are close to physiological levels of insulin in the postprandial period, and suggest that endogenous insulin may engage the Ag receptors on 125Tg mice.

A majority of BCR in 125Tg mice are occupied by endogenous insulin

To determine whether anti-insulin B cells encounter and bind endogenous insulin under physiological conditions, a biotinylated second anti-insulin mAb (mAb123) was used to detect insulin epitopes on the surface of B lymphocytes. mAb123 is structurally different from mAb125, and the binding sites of the two do not overlap (22, 24). Therefore, we used avidin FITC and biotinylated mAb123 to determine whether insulin epitopes are displayed on the surface of 125Tg and nontransgenic B cells. In these studies, mice received only normal laboratory chow, and manipulation of carbohydrate loads to increase endogenous insulin production was not necessary. As shown in Fig. 3, B cells from normal B6 mice (*A*) are not bound by mAb123. Furthermore, no binding of mAb123 is observed in B cells from mice expressing only the heavy chain transgene (identical with *A*, not shown). In contrast, a large majority of B cells from 125Tg mice are bound by mAb123 (Fig. 3*B*). All binding of mAb123 to 125Tg B cells is inhibited by excess soluble insulin (50 μg/ml). These findings indicate that most BCR in 125Tg mice are occupied by insulin as a consequence of exposure to hormone under physiological conditions. As discussed below, the function of insulin as a molten globule may contribute to its persistent association with 125Tg B cells, despite low affinity for rodent insulin (10^{-7} L/M) and limited Ag exposure.

Insulin Ab production in 125Tg mice

The data shown above suggest that anti-insulin B cells are not ignorant of endogenous insulin, but they encounter hormone and may be subject to functional silencing. Accordingly, relative binding in ELISA was used to measure circulating IgM allotypes, and competitive inhibition was used to detect insulin-specific Abs. The results show that in contrast to the effective allelic exclusion seen in B cells from 125Tg mice, endogenous IgMb accounts for greater than 98% of circulating IgM (Fig. 4*A*). The relative amount of IgMb is similar to that found in C57BL/6 mice (Fig. 4*B*). Although the preponderance of circulating IgMb suggests silencing of most

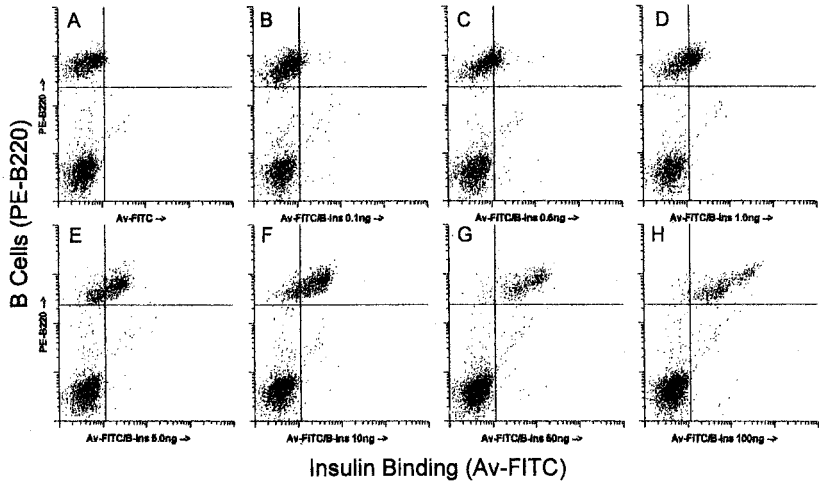

FIGURE 2. Most 125Tg B cells bind insulin. Biotinylated human insulin (B-ins) and avidin FITC (Av-FITC) were used to identify B cells (PE-B220, *left axis*) that bind insulin in flow cytometry. A binding profile is shown in *B–H*, with the amount of B-ins (ng/ml) used indicated below each panel. *A*, Background binding to Av-FITC alone, which is not different from B-ins binding in normal B6 mice or in 125VH-only transgenic mice.

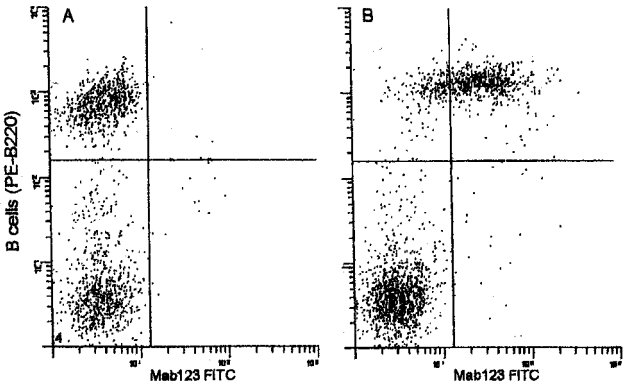

FIGURE 3. Endogenous insulin is bound by 125Tg B cells. Biotinylated anti-insulin mAb123 and avidin FITC (mAb123 FITC) were used in flow cytometry to identify B cells (PE-B220, *left axis*) that bind and display insulin on their surface. *A* (*left*), No binding in spleens of B6 mice; *B* (*right*), most 125Tg B cells are bound by mAb123, indicating the display insulin on their surface. The binding in *B* is totally inhibited by soluble insulin (50 μg/ml).

transgenic B cells, some insulin-specific Ab of the IgMa allotype is detected (Fig. 5*A*). Insulin-specific binding is never observed in nontransgenic B6 mice (Fig. 5*B*). To further test the function of transgenic B cells, 125Tg mice were immunized with combinations of beef insulin and pork proinsulin that are known to provide optimal T cell help for anti-insulin responses in H-2^b mice (31–33). The results show that in contrast to normal B6 mice (square), 125Tg mice (circles) fail to increase anti-insulin levels following immunization with a combination of beef insulin and pork proinsulin in adjuvant (Fig. 6*A*). Further evidence for the anergic state of 125Tg B cells is observed in adoptive transfer experiments using 125Tg B cells and insulin-immune T cells, followed by boosting with Ag (Fig. 6*B*). In these studies, transfer of 125Tg B cells alone results in the development of measurable levels of insulin Abs (closed circles). When immunized and boosted (arrows) in the presence of insulin-immune T cells (open circles), Ab levels do not exceed those reached in the absence of T cell help. The slight differences in Ab levels seen at early time points (day 5) are also observed in the absence of immunization and appear to reflect reconstitution differences and not Ag-specific response (unpublished observations). When naive B cells and insulin-primed T cells are transferred, a strong anti-insulin response develops after a single immunization (filled square) even though the precursor frequency of anti-insulin B cells in this population is $<10^{-5}$. Transfer of naive B cells alone (open square) does not yield measurable insulin-specific binding. Together, these findings indicate that large numbers of 125Tg B cells are functionally silenced by physiological concentrations of insulin. However, the presence of circulating insulin Abs of transgene origin (IgMa) indicates that some transgenic B cells may differentiate and produce insulin Abs as part of an endogenous repertoire of natural autoantibodies.

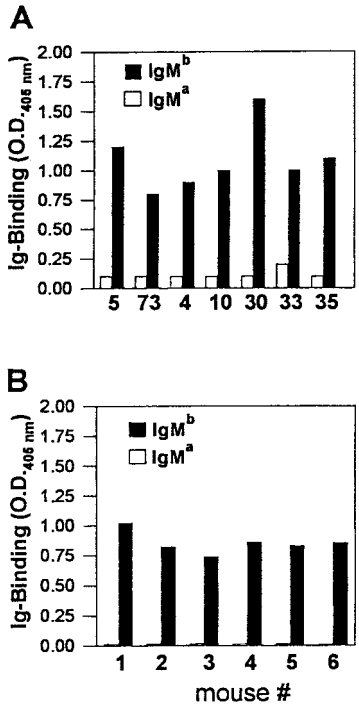

FIGURE 4. Circulating IgM in 125Tg mice is derived from endogenous B cells. Relative amounts of endogenous (IgMb, ■) and transgene (IgMa, □) Ig in serum (1/100) are compared in individual 125Tg mice (*A*, *top*) and in normal B6 mice (*B*, *bottom*). Data are reported as binding in ELISA (OD_{405}) with allotype-specific reagents, as described in *Materials and Methods*.

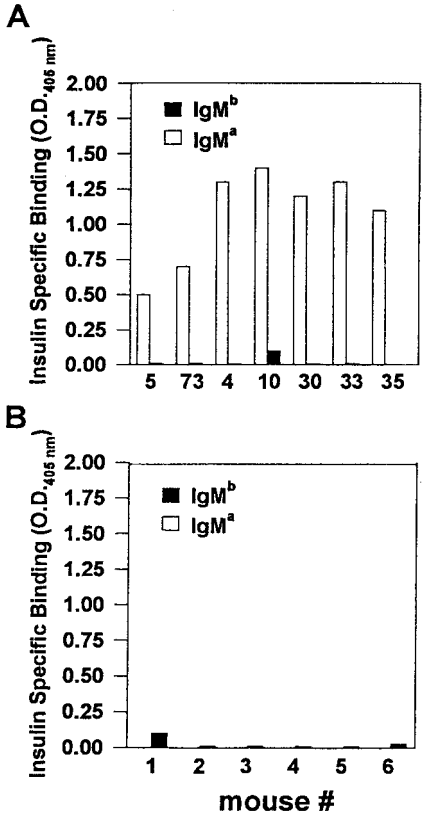

FIGURE 5. Detection of insulin Abs in 125Tg mice. Insulin-specific binding measured in ELISA (OD_{405}) is shown for individual 125Tg mice (*A*, *top*) and for normal B6 mice (*B*, *bottom*). Allotype-specific reagents were used to measure transgene (IgMa, □) and endogenous (IgMb, ■) Ig, as described in *Materials and Methods*.

B1-like characteristics of B cells producing insulin Abs in 125Tg

Autoantibodies present in the natural repertoire are often attributed to the B1 subset of B lymphocytes that can replicate autonomously in peritoneal and pleural spaces (34). The immunization-independent production of insulin Abs that we observe in anti-insulin

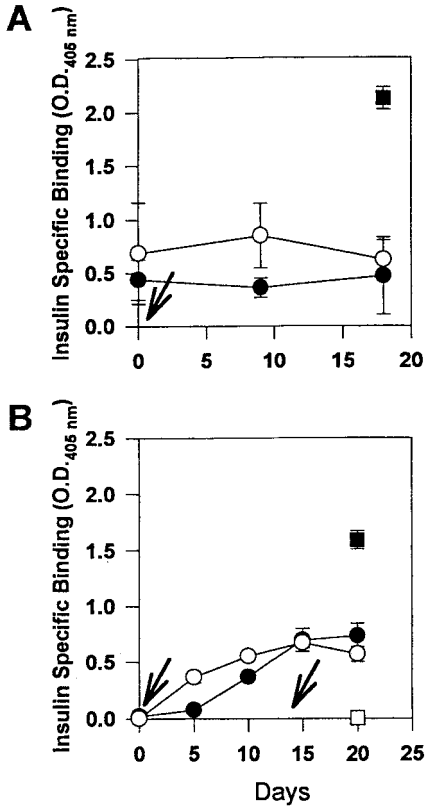

FIGURE 6. *A*, 125Tg mice do not respond to T cell-dependent immunization. Insulin-specific binding is measured in sera from 125Tg mice ($n = 5$) following immunization with heterologous insulins (●) and compared with CFA alone (○). The response of nontransgenic B6 mice ($n = 4$) on day 18 is shown (closed square). *B*, 125Tg B cells produce insulin Ab independent of T cell help in adoptive transfer. Irradiated recipients received 10^{-7} 125Tg B cells in the presence (○) or absence (●) of insulin-immune T cells from B6 mice. Animals were boosted with heterologous insulins in CFA (*left arrow*) and boosted in immunofluorescence assay (*right arrow*). The primary Ab response (without boosting) of mice receiving naive B6 B cells and immune T cells (■), and that following reconstitution with B cells only (□) is also shown.

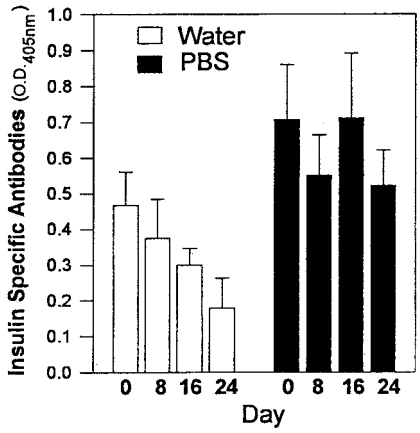

FIGURE 7. Hypotonic lysis depletes circulating insulin Ab in 125Tg B cells. Two groups of 125Tg mice ($n = 5$) received i.p. inject of sterile water (□) or PBS (■) weekly for 3 wk (*Materials and Methods*). The insulin-specific binding in serum (IgMa) was determined by competitive inhibition in ELISA (OD_{405}) the day following injection.

transgenics is characteristic of B1 type B cells. In a transgenic model of autoimmune hemolytic anemia, depletion of B1 B cells by distilled water lysis abolished autoantibody production and disease (12, 35). Therefore, to determine whether peritoneal B cells contribute to insulin autoantibody production in 125Tg mice, 125Tg mice were treated with i.p. injection of sterile water or PBS as a control (Fig. 7). Injection of the mice with PBS did not decrease the levels of IgMa anti-insulin, while hypotonic treatment anticipated to lyse peritoneal B cells resulted in a sharp and sustained decline in insulin binding. Examination of the peritoneal B cell population shows only small numbers (3%) of $CD5^+$ or $Mac1^+$ B cells in 125Tg mice compared with nontransgenic mice (30%). The majority of this small population is eliminated with the peritoneal injections (data not shown). Although 125Tg mice do not have an expanded population of B1 B cells, the findings are consistent with the interpretation that most circulating insulin Ab in 125Tg mice arise from a B1-like population, and suggest that B2 or conventional B cells are functionally silenced.

Insulin Abs in the natural repertoire of 125Tg mice may alter glucose homeostasis, but do not cause insulitis

Several autoimmune syndromes are associated with insulin Abs, and older studies suggest that passive transfer of insulin Abs may induce insulitis. To determine whether circulating insulin Abs have pathological effects on transgenic mice, we initially examined the pancreata of 125Tg mice for the presence of insulitis. None of a dozen mice ages 12–24 wk of age showed evidence of cellular infiltrates in the islets of Langerhans or in pancreatic tissue. In addition, random blood sugar determinations in larger number of animals have not shown hyperglycemia. To specifically examine β cell function, we measured fasting blood sugars and blood sugars 90 min following a glucose load (Fig. 8). The results suggest that these Abs may influence carbohydrate metabolism, as glucose levels after an overnight fast were significantly reduced in the 125Tg mice compared with control B6 mice. However, the β cell response to glucose challenge in 125Tg mice was not different from that of normal B6 mice. These data indicate that a large population of insulin-binding B cells and circulating insulin Abs in the repertoire of 125Tg mice do not alter β cell function. However, insulin Abs in 125Tg mice can interfere with the delivery of insulin to the periphery and result in lower blood sugars in fasting 125Tg mice. These findings are consistent with a phenotype of hypoglycemia and insulin autoimmunity recognized clinically as the insulin autoimmune syndrome (20).

Discussion

Abs that bind autologous insulin are found in several autoimmune disorders including the prodrome of type I diabetes mellitus and in hypoglycemic individuals with the insulin autoimmune syndrome (20, 21). In addition, IgG Abs that bind autologous insulin routinely accompany administration of the hormone to normal individuals or in mice that do not have autoimmune disease (17, 36, 37). These observations are interpreted to indicate that lack of tolerance reflects clonal ignorance in the B cell repertoire for insulin and that the mechanisms of clonal anergy or deletion are not active (16). In this state, B cells are said to be ignorant because the BCR encounters its cognate Ag too infrequently or with too low an affinity to induce tolerance. The present study questions the concept that B cells specific for low level self Ags develop and differentiate in an unregulated fashion. Contrary to the model of clonal ignorance, the bulk of B cells with modest affinity for endogenous insulin is effectively silenced by intermittent physiological levels of the hormone. The tolerant state induced by this physiological autoantigen is incomplete and may be coupled to the autonomous production of autoantibodies by a B1-like subset that

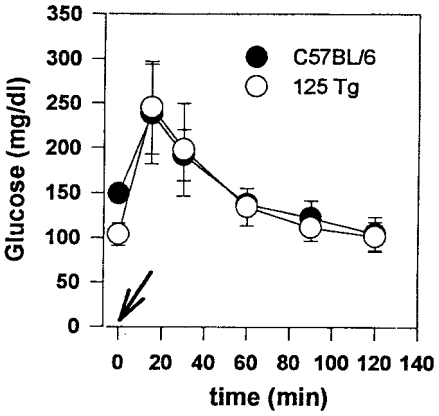

FIGURE 8. Fasting blood sugars are decreased in 125Tg mice. Glucose levels first were determined after an overnight fast of 125Tg mice (○, $n = 5$) and in nontransgenic B6 mice (●, $n = 4$). The animals then received an i.p. injection of glucose (1.8 mg/kg) in sterile PBS, and blood glucose was determined at the indicated intervals.

escapes silencing and contributes to the natural autoantibody repertoire. These circulating insulin Abs are able to induce metabolic changes consistent with an insulin autoimmune syndrome (38), but as a single factor they do not induce β cell destruction or insulitis. These findings indicate that self-reactivity to self insulin does not emerge unimpeded from the preimmune B cell repertoire. Rather, unique functions of microenvironments, such as the peritoneal cavity that may foster natural autoantibodies and immune follicles from which this anti-insulin specificity originated, maintain immune reactivity to insulin in the repertoire.

The transgene BCR used in this study (125Tg) expresses V_H and Vκ from mAb125 that binds human insulin with an affinity of 10^{-8} L/M and rodent insulin at 10^{-7} L/M. In the anti-HEL system, when the circulating concentration of the facultative autoantigen (HEL) was comparable with a physiological hormone (1 ng/ml), B cells were clonally ignorant and responded to immunization, even when the BCR affinity was high ($>10^{-9}$ L/M) (39). Thus, our anti-insulin transgene falls well below the affinity range expected for clonal ignorance. However, the bulk of 125Tg B cells are functionally silenced or anergic, as defined by their inability to differentiate and secrete Ab. The features of anergy include: preponderance of circulating IgMb derived from a small percentage (<5%) of residual endogenous B cells, failure to increase insulin Ab following immunization of 125Tg mice with heterologous insulin and proinsulin, and failure of insulin-primed T cells to induce responses in adoptive transfer with anti-insulin 125Tg B cells. In contrast to other models in which autoantibody transgenes are derived from animals with autoimmune disease, the anti-insulin specificity use by 125Tg mice is derived from a normal BALB/c mouse (23). Thus, an autoimmune specificity for insulin that is permitted in the immune repertoire of an adult mouse is effectively silenced when introduced into the germline. These observations question the concept of clonal ignorance and suggest that tolerance for insulin is effective in the preimmune repertoire at ranges of receptor affinity far below that in other transgenic models. This raises the issue of why the specificity of mAb125 was not silenced in the immune response from which it was generated. One potential mechanism may reside in the selection process that is part of germinal center reactions in T cell-dependent immune responses. Our previous studies on the origins of mAb125 suggest that it may arise from precursors that are not overtly insulin specific (23). Thus, B cell selection in follicles may be initiated with BCRs that somatically evolve to include insulin binding, and self-reactivity is permitted because of the unique properties (costimulation, cytokines, cell interactions, etc.) of the germinal center microenvironment. In the future, experiments using mice that express transgenes for the germline precursors of mAb125 may help resolve this issue.

The effective maintenance anergy in 125Tg mice correlates with the unexpectedly high level of BCR occupancy by endogenous insulin (Fig. 3). Sustained BCR occupancy occurs even though physiological exposure to insulin is not continuous and is limited to the postprandial period. The efficiency of tolerance induction to this natural Ag further contrasts other transgenic autoantigen systems (HEL, dsDNA, and RBC) in which Ag is present continuously. One possible mechanism is related to the structure of insulin as a molten globule that may change its conformation after receptor binding (40, 41). Accordingly, we hypothesize that the insulin molecule may reside in a more stable configuration once bound by the BCR. The induced fit between insulin and the Tg125 BCR may sustain a slow off rate and favor maintenance of tolerance especially when these interactions occur in the preimmune repertoire. Recent studies on the function of B cells expressing a rheumatoid factor transgene also indicate that features of BCR interaction with Ag other than affinity for ligand may determine the threshold for induction of tolerance (42).

Not all anti-insulin B cells are functionally silenced in 125Tg mice. Although greater than 98% of the circulating IgM detected in these transgenics is from the endogenous IgMb allotype, every transgenic mouse tested had measurable anti-insulin Abs of the IgMa allotype. Insulin-specific binding is not detected in normal B6 mice, in heavy chain-only transgenic mice, nor in the endogenous IgMb in transgenics. Although some anti-insulin IgMa is present, immunization does not increase these anti-insulin levels in 125Tg mice or in immunized recipients following adoptive transfers of 125Tg B cells with nontransgenic insulin-immune T cells. In adoptive transfer experiments, recipients of 125Tg B cells alone produce circulating insulin Ab independent of immune T cells. This self-regenerating capacity of B cells that produce insulin Ab suggests a relationship to the B1 B cell subset (34). Using the approach that was successful in anti-RBC transgenic mice (12, 35), we find that i.p. lysis reduces the level of insulin Ab in 125Tg mice. This finding is consistent with a B1-like origin for those anti-insulin B cells that escape silencing. The anti-insulin transgenes are not preferentially expanded in the B1 subset. Specifically, we do not observe increases in $CD5^+$ B cells in spleen compared with B6 mice. In the peritoneum, the numbers of $CD5^+$ and $Mac1^+$ B cells are low in our transgenic mice; however, both B1a and B1b B cells in the peritoneum are decreased after sterile water treatment (unpublished observations). These findings suggest that while the bulk of B2 B cells are anergic in 125Tg mice, a residual B1-like population may be responsible for the presence of circulating IgMa Abs detected in most transgenic mice. Recent data on anti-RBC transgenics in germ-free conditions indicate that environment signals may help maintain autoantibody expression in this compartment (13). Thus, physiological exposure to endogenous insulin in the context of an environment that favors B1-like differentiation pathways may permit some 125Tg B cells to contribute to the natural autoantibody repertoire. Anti-insulin B cells that have structural characteristics of a natural autoantibody are reported in human insulin-dependent diabetes mellitus, suggesting that similar B cells may contribute to an autoimmune process (43). Studies are in progress to determine whether differences in compartmental silencing of B cells in normal and in insulin-dependent diabetes mellitus-prone mice alter the fate and function of 125Tg B cells in autoimmune diabetes.

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