

Determinants of the B-cell response against a transgenic autoantigen

(autoimmunity/pancreatic β cells/simian virus 40 large tumor antigen/major histocompatibility complex)

JACEK SKOWRONSKI*, CHRISTINE JOLICOEUR†, SUSAN ALPERT*‡, AND DOUGLAS HANAHAN†

*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; and †Department of Biochemistry and Biophysics, and Hormone Research Institute, University of California, San Francisco, CA 94143-0534

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ABSTRACT The failure to induce self-tolerance of simian virus 40 large tumor antigen (T antigen) expressed in the pancreatic β cells of transgenic mice results in an autoimmune response against this protein and the cells that synthesize it. In every transgenic mouse with delayed onset of T-antigen expression and consequent nontolerance, B cells, T cells, and macrophages are attracted to and infiltrate the pancreatic islets. In contrast, the incidence, onset, and intensity of the B-cell response to produce anti-T-antigen autoantibodies vary considerably with genetic background. Thus the initial attraction of lymphocytes to the cells synthesizing a non-self antigen can be separated from the activation of a B-cell response against it. Haplotypes of the major histocompatibility complex (MHC) differentially influence the character of the autoimmune response, with $H-2^d$ and $H-2^k$ conferring a high incidence of humoral autoimmunity. Additional non-MHC linked genes are also implicated in control of the B-cell response.

It is well established that the immune system develops a capacity to discriminate between foreign and self entities (1). The importance of tolerance toward self can be seen in a number of diseases of apparent autoimmune nature, in which tissues are selectively destroyed by the immune system (2). Common features to many autoimmune diseases are the development of circulating autoantibodies that are reactive to components of the tissue undergoing destruction (3) and the correlation of the autoimmune phenotype with specific haplotypes of the major histocompatibility complex (MHC) (4-6).

Recently, transgenic mice have been used in a number of experiments designed to test theories of self-tolerance or autoimmunity (7). We have developed one such transgenic model, which has allowed aspects of the relationship between self-tolerance and autoimmunity to be examined (8-10). The rat insulin gene regulatory region was used to selectively express the large tumor antigen (T antigen, or Tag) of simian virus 40 (SV40) in the insulin-producing β cells of the pancreatic islets in several lines of transgenic mice (RIP-Tag mice). Every transgenic individual eventually succumbs to pancreatic β -cell tumors. Besides this commonality, the lineages can be subdivided into two classes. In the first class, mice begin to express the hybrid gene during embryogenesis (developmental onset). In contrast, in several other lines, the insulin/T-antigen gene is first activated in a heterogeneous subset of the β cells beginning at 10-12 weeks after birth (delayed onset). The immune system responds in two different ways to the two distinct patterns of expression among these lineages (9). Mice with developmental onset of expression are nonresponsive (or self-tolerant) to this new self protein. In contrast, mice in the lines with delayed onset of expression are fully responsive upon immunization with

purified large T protein. This nontolerance to a new β -cell antigen is associated with the development of a spontaneous autoimmune response against T antigen and the cells that synthesize it, as evidenced by the appearance of circulating antibodies against the protein and cellular infiltration of the pancreatic islets. It is of note that the insulinomas that develop in these mice manage to escape this autoimmune response and, as a result, the mice become hyperinsulinemic and do not evidence frank diabetes. Here we examine the time course of the autoimmune response, as evidenced by the appearance of autoantibodies, a property that can be monitored noninvasively over time, and assess the possibility that genetic factors, including the MHC, contribute to the induction of autoimmunity.

MATERIALS AND METHODS

Mice. The inbred strains C57BL/6, C57BL/10, DBA/2, and C3HeB/Fe, the congenic strains B10.BR and B10.D2, and the hybrid C57BL/6 \times DBA/2 F_1 mice (B6D2F₁) were purchased from The Jackson Laboratory. The RIP1-Tag3 line of transgenic mice has been described (8).

Determination of H-2 Haplotype. Restriction fragment length polymorphisms for *Bst*EII and *Pvu* II at the A_β locus of the $H-2^b$ and $H-2^d$ haplotypes, visualized by hybridization to A_β^k cDNA probe, were used to determine $H-2$ composition of transgenic mice (11, 12).

Immunohistochemistry. T antigen was detected as described (9). Lymphocyte typing was performed on adjacent cryostat sections with rat monoclonal antibodies (mAbs) directed against the Lyt2 (clone 53.6.7), L3T4 (clone GK1.5), B220 (clone 6B2), and Mac1 (clone M170) surface determinants of the cytotoxic T cells, helper T cells, B cells, and macrophages, respectively, using the alkaline phosphatase system (ABC-AP kit, Vector Laboratories).

Quantitation of Anti-T-Antigen Autoantibodies. Blood samples were collected from the orbital plexus of transgenic or appropriate control mice. Solid-phase radioimmunoassays (RIA) were performed in Dynatech 96-well microtiter plates coated with purified SV40 T protein (13, 14). Plates were blocked with 1% bovine serum albumin and serial dilutions of test and control sera were absorbed onto coated plates. Bound antibody was allowed to react with ¹²⁵I-labeled rabbit anti-mouse IgG antibody (Amersham), and individual wells were cut out and assayed for radioactivity in a 1272 Clin-gamma counter (LKB). The titer is defined as the dilution of a test serum that results in 50% of the maximum signal obtained with mAb 416 (14), an anti-Tag mouse mAb used as a positive control. The titer of the positive control antibody

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Abbreviations: MHC, major histocompatibility complex; SV40, simian virus 40; T antigen or Tag, large tumor antigen; mAb, monoclonal antibody.

‡Present address: Beckman Center, Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305.

was stable (within 20%) within a period of >18 months when measured in this assay.

RESULTS

Genetic Control of the B-Cell Response in RIP1-Tag3 Mice. Transgenic mice of the RIP1-Tag3, RIP1-Tag4, and *RIR*-Tag1 lines, which are characterized by the delayed onset of T-antigen expression, develop antibody responses against this β -cell antigen with variable incidence (35–60%) (9). It is conceivable that the incomplete penetrance results from genetic differences. The founders of these transgenic lines were constructed in F₂ hybrids between C57BL/6 and DBA/2 inbred mice, and for the first few generations (six) the lines were propagated by brother/sister intercrosses and also by backcrosses to C57BL/6 and B6D2F₁ mice (8). Therefore, we first tested whether the C57BL/6 and DBA/2 genetic backgrounds could differentially affect the B-cell response in the RIP1-Tag3 line. Randomly selected RIP1-Tag3 males were mated with females of either the C57BL/6 or DBA/2 parental strains, and the development of serum antibodies reacting with SV40 T antigen in the transgenic progeny was monitored in samples collected periodically over a period of 30 weeks. Virtually all of the transgenic progeny derived from the DBA/2 cross developed high levels of serum anti-Tag antibodies by 20–25 weeks of age. In contrast, a smaller fraction (*ca.* 30%) of transgenic animals originating from the C57BL/6 cross developed humoral responses against Tag (Fig. 1).

Association of Autoimmunity with MHC Haplotype. Subsequent experiments sought to characterize the DBA/2 locus (or loci) that stimulates the autoantibody response in RIP1-Tag3 mice. One candidate is the MHC. The *H-2* haplotypes of a number of RIP1-Tag3 mice were determined, and brothers and sisters homozygous for the *H-2^b* allele were used to derive an *H-2^{b/b}* subline (RIP1-Tag3B), which has been maintained by continuous backcrossing to C57BL/6. RIP1-Tag3B males were mated to B6D2F₁ (*H-2^{b/d}*) hybrid females, and the activation of the B-cell response in their transgenic progeny was monitored periodically as before. Twenty of 27 mice (74%) developed autoantibodies by 30 weeks of age (Fig. 1). To test whether a specific MHC haplotype was being

co-segregated with the autoimmune phenotype, the MHC haplotypes of a subset of the transgenic progeny derived from this cross were determined. Interestingly, among the *H-2^{b/b}* offspring only 38% (5 of 13) had autoantibodies by 30 weeks of age. In contrast, a significantly higher incidence of autoantibodies was observed in mice carrying *H-2^b* and *H-2^d* haplotypes, where 75% (9 of 12 mice) evidenced serum antibodies reacting with T antigen at 30 weeks (Fig. 2).

The B-Cell Response Is Influenced by the MHC and a Second Locus. To further characterize the apparent association of the MHC haplotype with the incidence of B-cell autoimmunity, additional combinations of haplotypes were constructed by crossing RIP1-Tag3B mice with different histocompatibility congenic strains and with distinct inbred strains. RIP1-Tag3B males were crossed to C57BL/10 (*H-2^b*), to the congenic strains B10.D2 (*H-2^d*) and B10.BR (*H-2^k*), and also to DBA/2 (*H-2^d*) and C3HeB (*H-2^k*). The incidence of autoantibodies in the transgenic progeny from these outcrosses was assessed, and the data are summarized in Table 1. When the *H-2^{b/b}* transgenics derived from crosses to C57BL/6 or C57BL/10 are compared to the *H-2^{b/d}* or *H-2^{b/k}* offspring derived from crosses to C57BL/10 congenic strains, it can be seen that the *d* and the *k* haplotypes confer a higher incidence of autoantibodies ($\approx 16\%$ for *b/b* compared to $\approx 56\%$ for *b/d* and *b/k*). These results confirm the differential effects of MHC alleles on the activation of the B-cell response. However, the same haplotype combinations conferred a 100% incidence of autoimmunity when constructed by crossing RIP1-Tag3B mice to either DBA/2 or C3HeB. If one assumes identity of MHC haplotypes between B10.BR and C3HeB (which differ at the *Tla* and *Qa-1* loci), this observation implies that a second non-MHC locus contributes to the control of the B-cell response. This conclusion is consistent with the results obtained with the B6D2F₁ cross, where the *H-2^b* and *H-2^d* alleles are reassorting along with the other chromosomes from the C57BL and DBA/2 backgrounds. In this cross 67% of the *H-2^{b/d}* progeny evidenced autoantibodies at a comparable age (25 weeks), which is significantly lower than the 100% incidence in the DBA/2 and C3HeB crosses. Thus the incidence of B-cell activation to produce anti-T-antigen autoantibodies in these transgenic mice is influenced by at least

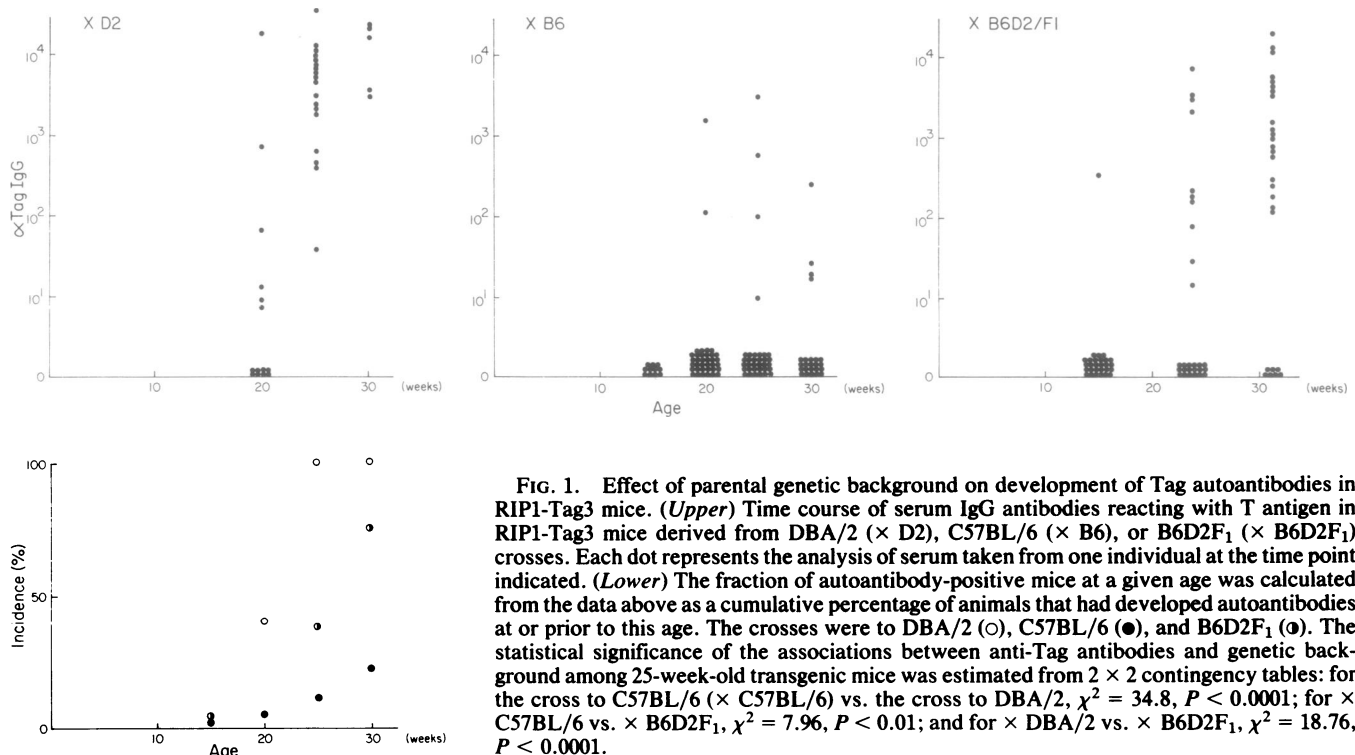


FIG. 1. Effect of parental genetic background on development of Tag autoantibodies in RIP1-Tag3 mice. (Upper) Time course of serum IgG antibodies reacting with T antigen in RIP1-Tag3 mice derived from DBA/2 (\times D2), C57BL/6 (\times B6), or B6D2F₁ (\times B6D2F₁) crosses. Each dot represents the analysis of serum taken from one individual at the time point indicated. (Lower) The fraction of autoantibody-positive mice at a given age was calculated from the data above as a cumulative percentage of animals that had developed autoantibodies at or prior to this age. The crosses were to DBA/2 (\circ), C57BL/6 (\bullet), and B6D2F₁ (\circ). The statistical significance of the associations between anti-Tag antibodies and genetic background among 25-week-old transgenic mice was estimated from 2×2 contingency tables: for the cross to C57BL/6 (\times C57BL/6) vs. the cross to DBA/2, $\chi^2 = 34.8$, $P < 0.0001$; for \times C57BL/6 vs. \times B6D2F₁, $\chi^2 = 7.96$, $P < 0.01$; and for \times DBA/2 vs. \times B6D2F₁, $\chi^2 = 18.76$, $P < 0.0001$.

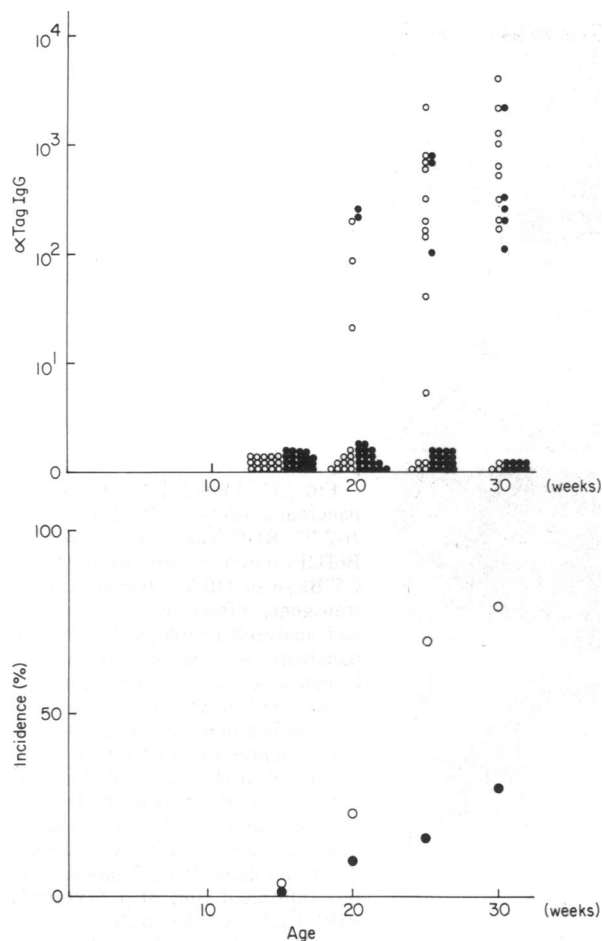


FIG. 2. Relation of humoral autoimmunity to segregating *H-2* haplotype. (Upper) RIP1-Tag3B males (of the N1 backcross to C57BL/6) were outcrossed to B6D2F₁ females (*H-2^{b/d}*) and their transgenic offspring were analyzed for T-antigen autoantibodies. The *H-2* haplotypes of the transgenic progeny were determined by analysis for a restriction fragment length polymorphism in the *A_β* locus. The time courses of autoantibodies arising in RIP1-Tag3 mice either homozygous for the *H-2^b* haplotype (●) or harboring *H-2^b* and *H-2^d* alleles (○) are shown here. (Lower) Incidence of humoral autoimmunity among RIP1-Tag3 mice carrying the *H-2^{b/b}* (●) or *H-2^{b/d}* (○) haplotype. The significance of the differences in incidences at 25 weeks of age is as follows: for the *H-2^{b/b}* progeny of × B6D2F₁ vs. the *H-2^{b/d}* progeny of × B6D2F₁, $\chi^2 = 8.08$, $P < 0.01$; for comparing *H-2^{b/b}* progeny of × B6D2F₁ with those of × DBA/2 (*H-2^{b/d}*), $\chi^2 = 24.8$, $P < 0.0001$; for the *H-2^{b/d}* progeny of × B6D2F₁ vs. progeny of × DBA/2 (*H-2^{b/d}*), $\chi^2 = 7.09$, $P < 0.012$; and for *H-2^{b/d}* progeny of × B6D2F₁ vs. progeny of × C57BL/6 (*H-2^{b/b}*), $\chi^2 = 14.25$, $P < 0.0005$.

two loci that are differential between C57BL and either DBA/2 or C3HeB. One is clearly linked to the MHC, and the other(s) appears to assort independently from it.

Characteristics of the Leukocyte Response to T Antigen in β Cells. The initial characterization of the autoimmune response in the RIP1-Tag3 mice revealed cellular infiltration within the islets of mice with circulating autoantibodies to T antigen (9). We have now characterized the cell types infiltrating the islets. Serial sections of transgenic and control pancreases were stained with hematoxylin/eosin or with mAbs that recognize the Lyt2 (CD8), L3T4 (CD4), B220, or Mac1 surface determinants on cytotoxic T cells, helper/inducer T cells, B cells, or macrophages, respectively. The analysis has revealed that leukocyte infiltration can be detected in the islets of anti-Tag autoantibody-positive and autoantibody-negative mice in each MHC background by 20

weeks of age, which is 2 months after T-antigen expression ensues in a subset of the β cells. Although islets in every mouse attracted leukocyte infiltration, only a fraction of the islets (10–40%) became infiltrated. Preliminary observations suggest that this nonuniform pattern of islet infiltration correlates with differences in the density of Tag-expressing β cells in different islets. The infiltrated islets were seen to have representation by B lymphocytes, CD8⁺ T cells, CD4⁺ T cells, and infrequent macrophages, in contrast to the islets of normal mice, where none of these leukocytes could be found in association with the islets. Examples of the analysis of infiltrated islets are shown in Fig. 3, which presents an autoantibody-negative mouse with an *H-2^{b/b}* MHC haplotype and an autoantibody-positive mouse with an *H-2^{b/d}* haplotype.

DISCUSSION

RIP1-Tag3 mice inherit a self antigen, SV40 T antigen, whose expression in the pancreatic β cells is inappropriately delayed into adulthood (8), thereby failing to induce immunological self-tolerance (9). The synthesis of this non-self antigen in the pancreatic β cells is clearly apparent to the immune system, as it provokes an autoimmune response against the transgenic antigen and the pancreatic β cells. Activation of the immune response against this autoantigen is modulated by the MHC and by additional genetic and/or epigenetic factors.

Lymphocyte Attraction Does Not Obligate B-Cell Activation. In all combinations of MHC haplotypes and genetic backgrounds tested thus far, T antigen elicits abnormal infiltration of the islets by the four major classes of leukocytes within 2 months of its appearance in adult β cells. However, the infiltration is not sufficient for induction of the B-cell autoimmune response against T antigen, given that every mouse evidences some degree of leukocyte infiltration, and yet by 20 weeks of age only 43% of the *H-2^{b/d}* mice developed autoantibodies, and a majority of the *H-2^{b/b}* mice never develop a detectable humoral response. The inactive status of the infiltrating B cells in autoantibody-negative mice is obvious. With regard to T cells and macrophages, their state of activation is presently unresolved. There are clear signs of β -cell destruction in occasional islets of mice at 20–25 weeks of age, which suggests focal activation of the infiltrating cells (e.g., Fig. 3). Taken together these observations indicate that homing of leukocytes to the site of a perceived abnormality can be separated from the activation of those immune cells to destroy the β cells that have attracted their attention.

MHC Association with B-Cell Activation. The B-lymphocyte response toward the transgenic β -cell protein ensues at high frequency when the delayed onset of expression of the transgene is combined with the *H-2^d* or *H-2^k* haplotypes of the major histocompatibility complex. In contrast, RIP1-Tag3 mice homozygous for the *H-2^b* haplotype develop a humoral response much less frequently. Furthermore, the intensity of the B-cell response is markedly weaker in the *H-2^{b/b}* mice, as seen in the serum autoantibody titers, which are characteristically 100-fold lower than those arising in transgenic mice with *H-2^{b/d}* or *H-2^{b/k}* haplotype. These results might suggest an involvement of the I-E region, since the *H-2^b* MHC encodes a defective *E_α* gene (15, 16) and, as a result, *H-2^{b/b}* mice do not express a functional I-E heterodimer on the cell surface (16). However, if I-E is involved, its absence does not completely abrogate presentation of Tag peptides by class II, since $\approx 16\%$ of *H-2^{b/b}* RIP1-Tag3 mice eventually develop anti-Tag autoantibodies (Table 1).

An Additional Locus Influences the B-Cell Autoimmune Response. Although a major component of the genetic control of the B-cell autoimmune response against T antigen is linked to the MHC, there were statistically significant differences in the incidence of autoantibodies among transgenic mice with

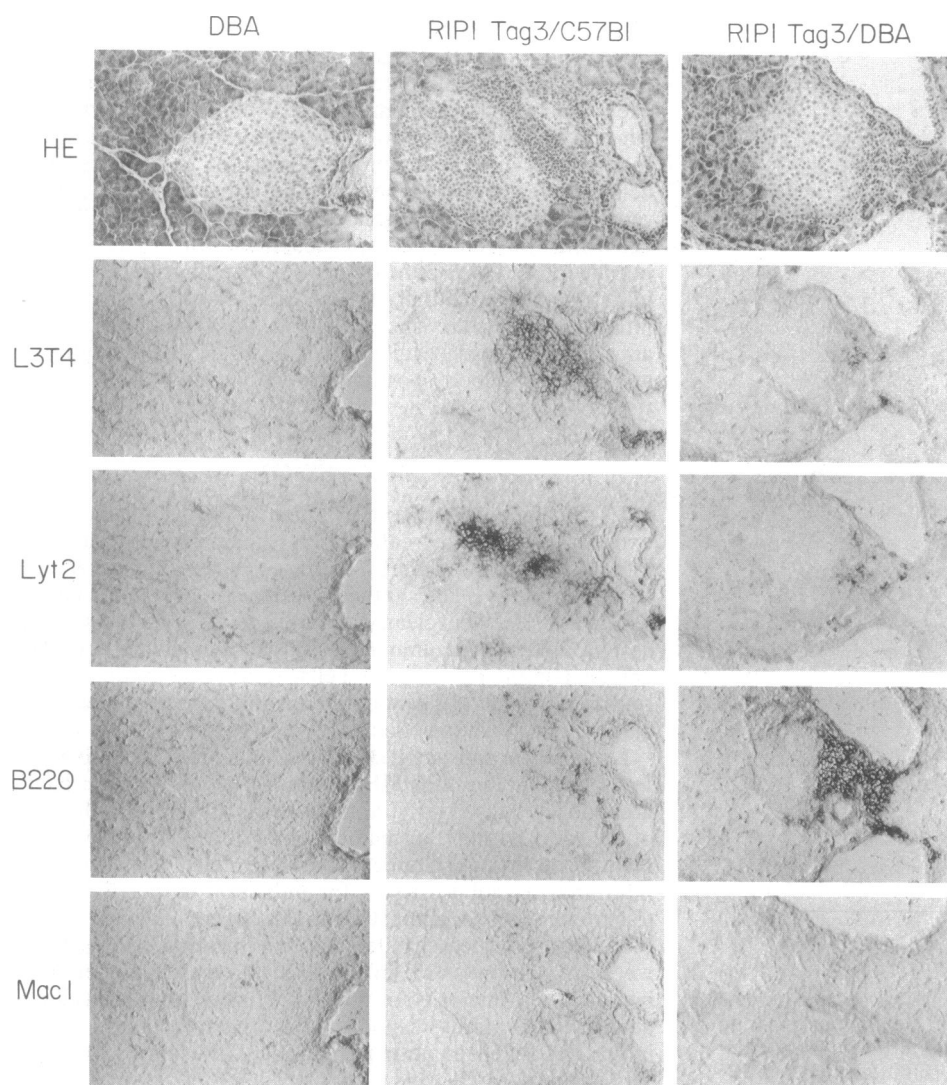


FIG. 3. Leukocyte infiltration of pancreatic islets in RIP1-Tag3 mice. $H-2^{b/b}$ RIP1-Tag3 males from the B6D2F₁ outcross were mated to either C57BL/6 or DBA/2 females, and the transgenic offspring were sacrificed and analyzed histologically. Adjacent pancreatic sections have been stained by hematoxylin/eosin (HE) or immunostained with mAbs specific for leukocyte surface determinants: Lyt2 (cytotoxic/suppressor T cells), L3T4 (helper T cells), B220 (B cells), and Mac1 (macrophages). In the three examples shown the islets are located next to a pancreatic duct. ($\times 80$.) (Left) Typical islet of a nontransgenic DBA/2 mouse. (Center) Infiltrated islet of a 5-month-old RIP1-Tag3 \times C57BL/6 ($H-2^{b/b}$) mouse that did not have a detectable titer of circulating anti-Tag autoantibodies. (Right) Infiltrated islet of a 5-month-old RIP1-Tag3 \times DBA/2 ($H-2^{b/d}$) mouse that had a high titer of circulating anti-Tag autoantibodies.

the same MHC haplotypes (either $H-2^{b/d}$ or $H-2^{b/k}$) but differing in strain background (Table 1). In addition, it is remarkable that only a small fraction ($\approx 16\%$) of the $H-2^{b/b}$ mice eventually develop a B-cell response against T antigen, despite the development of pancreatic β -cell tumors expressing this nontolerant antigen. These observations together

Table 1. The MHC and a second locus influence the autoantibody response in RIP1-Tag3 mice

Outcross	MHC		Time of sampling, weeks
	haplotype of progeny	Autoantibody incidence (%)	
C57BL/6	<i>b/b</i>	4/46 (9)	25
C57BL/10	<i>b/b</i>	8/35 (23)	16–28
B10.D2	<i>b/d</i>	7/13 (54)	23–32
B10.BR	<i>b/k</i>	4/7 (57)	25
DBA/2	<i>b/d</i>	27/27 (100)	25
C3HeB	<i>b/k</i>	9/9 (100)	25

RIP1-Tag3B males were mated to females of the indicated strains, and the incidence of autoantibodies was assessed in the transgenic progeny. A single male from the $H-2^{b/b}$ subline intercrosses was used for the B10.BR and the C3HeB outcrosses; the males used in the C57BL/10 and the B10.D2 outcrosses were N2 or N3 C57BL/6 backcrosses from the $H-2^{b/b}$ subline. The data for the C57BL/6 and the DBA/2 outcrosses are cumulative of the experiments presented in Fig. 1 and of a subsequent experiment in which a single male from the $H-2^{b/b}$ subline intercrosses was used. Haplotypes set in boldface type indicate the differential MHC haplotype among the genetic crosses.

support the proposition that an additional allelic locus (or loci) is involved in regulating activation of the B-cell autoimmune response. Although it is possible that these loci act by subtly affecting the level of T-antigen expression, immunohistochemical analysis of islets in 4- to 5-month-old transgenic mice from these different strain backgrounds indicates that each has similar levels and timing of transgene expression. Among other candidates loci are those that influence antigen processing (17) or antigen recognition. Precedents for this latter possibility come from studies implicating specific allotypes of the T-cell receptor α and β chains in human and animal autoimmunity (reviewed in ref. 6; ref. 18). Alternatively, biases in the T-cell repertoire produced by superantigens such as mls or other agents (19–21) could be influencing either positive or negative selection of the T cells involved in regulating the B-cell response to T antigen.

Multiple Requirements for Induction of Autoimmunity. Nontolerance toward T antigen is necessary for the attraction of leukocytes to the site of its synthesis, since none occurs in mice of the tolerant lineages RIP1-Tag2 and *RIR*-Tag2 (10). However, the leukocyte infiltration induced by this nontolerant antigen in RIP-Tag3 mice contrasts with other transgenic mouse models where the expression of non-self antigens has been targeted to peripheral tissues. Allogeneic MHC class I or class II molecules have been expressed in transgenic mice in various nonlymphoid tissues, including the pancreatic β cells of the islets of Langerhans (22–25), the exocrine pancreas (26–28), and also the liver and the kidney (27). Extrathymic expression of a non-self MHC molecule

resulted in the establishment of self-tolerance in some cases, but not in others, as assessed by different *in vitro* assays. However, regardless of their tolerance status, there is a consistent feature in these models: in all cases, the presence of a non-self MHC molecule did not trigger an autoimmune response against the tissue where it was expressed. Thus the model described here is clearly different, since the delayed expression of a non-MHC antigen, large T antigen, results in nontolerance and induces leukocyte infiltration of the islets of Langerhans in every RIP1-Tag3 mouse. In two other transgenic models, expression of interferon γ (IFN- γ) (29) or influenza virus hemagglutinin (HA) (30) in the pancreatic β cells also resulted in autoimmune responses that were concomitant with or preceded by abnormalities in the pancreatic β cells themselves. One interpretation of the dichotomy between the effects of MHC molecules, on the one hand, and T antigen/IFN- γ /HA, on the other, is that *both* nontolerance and other as yet undefined abnormal conditions are necessary for the induction of an autoimmune response. In this regard, it is notable that various abnormalities result from expressing the large T protein in β cells; these include increased β -cell senescence, extensive β -cell proliferation, and neovascularization (31, 32). Perhaps one or another of these abnormal conditions is necessary in addition to the immunogenic properties of a nontolerant self antigen to induce leukocyte infiltration of the islets and their subsequent functional activation to produce autoantibodies.

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