Failed Self-Tolerance and Autoimmunity in IgG Anti-DNA Transgenic Mice

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Transgenic mice were generated that express both the H and L chain genes derived from a hybridoma secreting an IgG2a mAb specific for ds- and ssDNA. This hybridoma is derived from a lupus mouse and can accelerate nephritis in young NZB × NZW F1 female mice and induce clinical nephritis in BALB/c mice. Some transgenic B cells did not exhibit allelic exclusion; they expressed both transgene-derived IgG and endogenous IgM intracellularly. Most of the B cells in transgenic mice expressed endogenous IgM, some of them expressed low levels of IgG on cell membranes. The transgenic mice, created in a strain not prone to SLE, expressed elevated serum IgG anti-DNA, and some developed clinical nephritis. The affinity of the spontaneously secreted IgG antibodies for dsDNA were similar in nephritic NZB IgG anti-DNA, and some developed clinical nephritis. The affinity of the spontaneously secreted IgG antibodies for dsDNA were similar in nephritic NZB × NZW F1, and transgenic mice. In contrast to the nontransgenic littermates, immunization of transgenic mice with murine DNA further enhanced serum levels of IgG anti-DNA in transgenic mice. Therefore, expression of transgene-encoded IgG anti-DNA mainly in the secreted form does not provide the signals necessary for allelic exclusion or self-tolerance. Expression of this Ig is sufficient to induce a mild form of autoimmune disease.

Studies of transgenic mice expressing rearranged Ig genes in a majority of their B cells have advanced our understanding of self-tolerance: B cells expressing transgenic surface IgM which binds self-Ag are deleted or anergic (1-3). In the autoimmune disease SLE, autoreactive B cells fail to be down-regulated. Among the autoantibodies produced, IgG reactive with dsDNA is characteristic of the disease, often fluctuates with disease activity, and may cause glomerulonephritis (4, 5). Only certain subsets of anti-dsDNA are pathogenic (6). Ig eluted from glomeruli of patients and mice with the disease are enriched for anti-DNA binding activity (7, 8), and that anti-DNA Ig has higher avidity for dsDNA than the anti-DNA in the sera of the same patients (9). We have derived a panel of IgG anti-dsDNA mAb from nephritic female BW mice. We characterized the pathogenicity of seven of these mAb based on their ability to accelerate nephritis in young BW3 mice and to induce it in BALB/c mice (6). Two mAb were classified as pathogens as they caused proteinuria and azotemia in both strains. Two others were classified as weak pathogens and three as nonpathogens. One of the pathogens, A6.1, deposited in glomeruli and caused histologic damage in renal tissue of young BW mice.

The defects in lupus individuals include the propensity to make pathogenic anti-DNA IgG and the inability to down-regulate these B cell clones. Transgenic technology enables us to introduce the "defect" of making pathogenic anti-DNA IgG into the genetic background of normal mice. We chose hybridoma A6.1 as the pathogenic mAb from which genomic DNA encoding the VH and VL were cloned to make transgene constructs encoding IgG2a anti-dsDNA. Our goals were to determine whether B cells expressing transgenic products would be deleted or anergized, and if not, whether the expression of a pathogenic autoantibody was sufficient to induce lupus nephritis in normal mice.

Materials and Methods

Constructs used to establish transgenic mice. Plasmid pY2aVH6 was constructed with a 5.5-kb BamHI-EcoRI fragment containing the productively rearranged A6.1 VH gene from bacteriophage HA6 (6) and a 5.0-kb EcoRI fragment (10, 11) containing the Cy2a gene from BALB/c plasmacytoma RPC5. This construct contained the 3.5-kb 5' region of the rearranged VH which should contain the endogenous promoter, and the 0.7-kb intronic XbaI-EcoRI endogenous enhancer region. These were cloned into pBluescript KS' (Stratagene). Plasmid pY6A6 had a 4.5-kb EcoRI (6) fragment from bacteriophage αA6 containing the L chain V-J region expressed by A6.1 and a 12.5-kb PstI-PvuII fragment from pB1-4 (12) containing the Ck gene and the flanking sequence (a kind gift from Dr. Ursula Storb) cloned into pBluescript. This construct contained the endogenous promoter and enhancer regions. Most of the vector sequence of these two plasmids was removed for microinjection: the H chain construct was digested with Nof and HindII and the L chain construct with XhoI. Fragments containing the Ig genes were gel-purified, mixed at equal molar concentration, and injected into C57BL/6 × DBA/2 F1, or C57BL/6 fertilized eggs. The transgenic mice were established as described elsewhere (13).

Southern and Northern blot analyses. Tail DNA was prepared (13), digested with PstI or XbaI and loaded into wells (15 μg/well) in 3% agarose gel.
0.8% agarose gel. After the completion of electrophoresis, DNA was blotted onto a nylon membrane (Stratagene, La Jolla, CA) according to protocols described by the manufacturer. Blots containing PstI-digested tail DNA gel fragments were hybridized with JH and V,6 probes synthesized from bacteriophage promoters (16, 17). DNA and probes were denatured at 85°C for 10 min, hybridized overnight at 50°C in 50% formamide, and treated with RNase. Protected DNA was denatured and electrophoresed in an acrylamide/7 M urea gel. The V,6 probe was labeled by using a randomprimer labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridized blots were hybridized with a 3ZP-labeled 2.8-kb PstI fragment containing VKA~, a cDNA encoding Cto (6) which hybridized to both C&, and C,b mRNA, a cDNA encoding Cp (14), and a cDNA containing the rat GAPDH gene (15). The results of Northern blots are representative of two experiments done using different mouse lines.

RsilC protection. Total splenic RNA (25 pg) isolated from hybridoma A6.1 cells, individual transgenic, or individual nontransgenic mice was combined with uniformly labeled &microglobulin and V&H6 probes synthesized from bacteriophage promoters (16, 17). RNA and probes were denatured at 85°C for 10 min, hybridized overnight at 50°C in 50% formamide, and treated with RNase. Protected RNA was denatured and electrophoresed in an acrylamide/7 M urea gel. The V,6 probe was labeled by using a random-primer labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridized blots were hybridized with a 3ZP-labeled 2.8-kb PstI fragment containing V,A6, a cDNA encoding Cto (6) which hybridized to both C&, and C,b mRNA, a cDNA encoding Cp (14), and a cDNA containing the rat GAPDH gene (15). The results of Northern blots are representative of two experiments done using different mouse lines.

RESULTS

Establishment of transgenic mice. To examine the effects of a pathogenic IgG antibody to DNA in normal mice transgenic for the appropriate genes, plasmids p2AvH6A and plg&H6A (Fig. 1) were constructed with VH and V,6 gene segments cloned from a genomic library of the B cell hybridoma A6.1 (6). This hybridoma which also binds to ssDNA but not cardiolipin, histone, or heparan sulfate (data not shown). After microinjection of the H and L chain gene fragments into (C57BL/6 x 129)F1 embryos, 6 pups out of 31 incorporated both chains of the A6.1 Ig genes, and one incorporated only the H chain gene, as determined by hybridization of Southern blots of tail DNA probed with either J, or J,, gene fragments (Fig. 2). The estimated copy number of both H and L chain transgenes incorporated into the host genome was estimated to be 20, 4, 5, 6, 1 and 10 in founders 1 to 6, respectively. Tail DNA from founder H showed the rearranged V,6, but not the V,6, band, thus the only ligH chain incorporated into the genome. The high m.w. bracketed transgenic bands were derived from flanking regions and were characteristic of each founder, and probably were integration site-dependent. Four founders (1–3 and H) were mated with BALB/c mice; the progeny of these crosses (F, mice) were used in all studies described below.

Tissue specificity of transgene transcription. Tissue-specific expression of the transgenes occurred (Fig. 3): total RNA from the spleen of transgenic mice hybridized strongly with probe V,6 (6) containing the V,6 gene

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segment expressed by A6.1, whereas RNA from lung, heart, and muscle did not. The nontransgenic littermates expressed V,6A in spleen cell RNA only. When the same blot was hybridized with a probe that detects both the γ2a and γ2b constant regions (6), both transgenic and nontransgenic spleen RNA showed that most hybridized γ2a/2b mRNA had the size characteristic of the message encoding the secreted form of the H chain (1.9 kb) although a small quantity had a size characteristic of the membrane form (3.9 kb) (19). Although hybridization of T kidney RNA with V,6A and γ2a/2b probes was unexpected: it may be due to the infiltration of lymphocytes into this tissue as part of the nephritic process, as observed in BW mice (20). Both transgenic and nontransgenic spleen RNA hybridized to a similar extent to a Ck (14) probe, suggesting that in transgenic mice significant numbers of B cells expressed endogenous μ message. As a measurement of quantity and integrity of mRNA from each tissue, the blot was hybridized with a probe encoding the “housekeeping” enzyme glyceraldehyde-3-phosphate dehydrogenase (15); the correct size 1.6-kb mRNA from all tissues showed hybridization. Densitometer tracing of the relative band intensity of V,6A transcripts normalized to the glyceraldehyde-3-phosphate dehydrogenase signal showed that in the transgenic mice spleen RNA had an increase of 1.7-fold of V,9 steady-state mRNA level compared to the nontransgenic spleen RNA. The blot was not hybridized with a V,6A-containing probe, because this V, gene segment belongs to the very large V,J558 family (21, 22). Therefore, in adult NT spleen it is expected that the expression level would be very high (23–25). Thus, a more specific RNase protection assay (Fig. 4) was performed by using 32P-labeled V,6A-D-JH4 antisense RNA hybridized to spleen RNA isolated from transgenic mice from lines 1, 3, and H and from two nontransgenic littermates. An antisense β2-microglobulin RNA probe was also added to the hybridization mixture; the intensity of the β2-microglobulin protected bands (a quadruplet centered at 230 nucleotides) was used to normalize the amount of RNA loaded in each sample. Total RNA isolated from hybridoma A6.1 cells showed that the transcription of the VH-D-JH4 region yielded a quadruplet of protected bands with the most intense band at 366 nucleotides (Fig. 4A). This was the size predicted from the sequence of the unspliced and the mature transcripts (Fig. 4B). The transgene H chain was actively transcribed in transgenic mice, as shown by the 29-fold higher intensity of the protected V,6A-D-JH4 bands normalized to the β2-microglobulin signal from transgenic line 1 mouse compared to a nontransgenic littermate. The normalized V,6A band intensity of lines 3 and H transgenic RNA were 1.1- and 7-fold more than the two nontransgenic samples. A small amount of the 366 nucleotide-protected fragments could be obtained from the nontransgenic mice, perhaps because of incomplete nuclease digestion of transcripts that were encoded by a similar J558 VH gene segment and JH4 and which differ at only a few junctional nucleotides. As a negative control, tRNA with no sequence homology to either probes showed no protected bands.

Transgenic B cells were not anergic: IgG anti-DNA was secreted and could be up-regulated by Ag stimulation. Protein products encoded by transgenes were secreted into the serum of transgenic mice, as indicated by F1 lines 1, 3, and H. Those lines had significantly higher serum IgG2a levels (which correlated positively with serum anti-DNA IgG levels) than the nontransgenic littermate (Table I). Transgenic line 1, which had the highest levels of transgene-derived H chain mRNA transcripts, nearly 30-fold increased over the background,
also had the highest levels of serum IgG2a antibody and anti-DNA antibody (Table I). Similarly, transgenic line 2 which had the lowest anti-DNA IgG, serum levels did not have significantly higher levels of serum IgG2a.

The serum levels of anti-DNA IgG in transgenic mice were lower than expected judging from the serum IgG2a levels. To determine whether perhaps some form of partial anergy is operative, line 1 transgenic and nontransgenic littermates were immunized with murine DNA and mBSA or mBSA only. Immunization with mammalian DNA/mBSA does not elicit significant levels of IgG anti-DNA in normal mice, but it enhances antinuclear antibodies in young BW mice (7, 26, 27). As shown in Figure 5, compared with nontransgenic littermates, DNA immunization significantly raised the serum levels of IgG anti-DNA in transgenic mice at wk 6, 8, and 10 (p < 0.05 by Student's t-test). Mice received mBSA only did not produce appreciable levels of anti-DNA IgG. On account of the spontaneous and Ag-induced secretion of IgG anti-DNA, transgenic mice appeared not to be anergic.

Transgene products failed to allelically exclude the expression of endogenous IgH chains. The H chain construct used to establish transgenic mice contained that encode both secretory and membrane forms of the Cγ2a message, but did not include the polyadenylation sites mapped 1.4- and 2.1-kb 3' of the second membrane exon (28). Therefore we expected that surface expression of the transgenes would be low. Surface expression of the γ-chain showed an increase in transgenic samples compared to their nontransgenic counterparts (Fig. 6). The mean fluorescence intensity was low, however, and cytophilic binding of the elevated transgenic serum IgG to spleen cells might be responsible for the observed increase of γ+ cells in transgenic spleen cells. FACS analysis indicated that however most of the transgenic splenic B cells expressed endogenous IgM on cell membranes; similar anti-μ staining profiles were obtained in transgenic and nontransgenic samples (41% vs 46%, Fig. 6). This suggests that allelic exclusion was not complete in transgenic mice perhaps on account of low surface expression. Consistent with this lack of allelic exclusion, serum IgM levels of all four transgenic lines were equivalent to those of nontransgenic littermates (Table I). To investigate this issue further, direct immunofluorescence was performed to determine if there were significant numbers of double positive (μ+γ+) B cells in transgenic spleen cells. After capping of surface Ig induced by the IgG fraction of a goat anti-mouse Ig antiserum, cells were permealized and stained. A significant fraction of transgenic but not nontransgenic spleen cells stained positively for both intracellular μ- and γH-chains (Fig. 7). The percentages of cytoplasmic μ+ cells only, γ+ only or both in Ig+ T spleen cells were 60, 22, and 18, respectively, compared to 88, 11, and 1 in NT Ig+ spleen cells (>250 Ig+ spleen cells were evaluated by using a fluorescent microscope). A high avidity anti-idiotypic antibody that clearly distinguishes transgene from endogenous Ig is not available. Nevertheless, the relative low frequency of μ+ γ+ cells in nontransgenic littermates makes it possible to use anti-γ antibodies to identify a population highly enriched for cells that express the IgH transgene. Expression of endogenous genes in some γ+ B cells is demonstrated by the simultaneous staining with anti-μ antibodies. Although it is possible that a small fraction of the μ+...
B cells are using transgene-encoded $\mu$A6 and an endogenous $\mu$ generated either by interchromosomal switch recombination or trans-splicing of mRNA as observed in other transgenic Ig models (29, 30), these mechanisms are not likely to account for the high frequency (18%) of transgenic spleen cells that express both cytoplasmic $\mu$- and $\gamma$-chains, or $\gamma$-chains alone (22%). In conclusion, most transgenic B cells probably failed to inhibit rearrangement and expression of endogenous Ig genes, and some of them probably produced both transgene-derived $\gamma$-2a- and endogenous gene-derived $\mu$H-chains in the same cells.
Figure 5. Time course of transgenic (T) and nontransgenic (NT) mice antibody response to DNA immunization. Sera from mice immunized with DNA/mBSA or with mBSA were assayed by ELISA at the dilution of 1/50. Heavy arrow depicts the time of the initial immunization, and light arrows indicate when boosters were given. Results are expressed as the mean ± SEM (n = 10 in each group).

Determination of serum IgG anti-DNA and clinical nephritis. Three of the four lines of transgenic mice studied, lines 1, 3, and H, exhibited significantly higher levels of serum IgG anti-dsDNA than their NT littermates at multiple time points during the first year of life (p < 0.05 or lower by Student’s t-test) (Fig. 8). Serum anti-DNA levels of the line 1, 3, and H transgenic mice fluctuated, but they never reached the high levels, a mean OD of 1.4 in a serum pool, typical of nephritic BW mice. Based on studies of line H, which incorporated the H but not the L chain from the A6.1 hybridoma, it is concluded that the A6.1 H chain can probably encode anti-DNA antibodies when combined with a variety of endogenous L chains. Line 2 transgenic mice expressed significantly higher serum anti-DNA levels than nontransgenic littermates only at the first two time points up to 20 wk of age and subsequently had low levels of both serum anti-DNA IgG and IgG2a which were not significantly different from nontransgenic mice. The mechanism(s) which suppressed sustained expression of transgenes in this line was not determined.

Comparison of affinity of serum IgG for DNA was made between pooled sera from line 1 transgenic mice and 30-34-wk-old (nephritic) BW mice. As shown in Figure 9, both samples showed similar competitive inhibition curves after preincubation with various amounts of DNA. Therefore, the serum IgG anti-DNA of transgenic mice and BW mice had similar affinities to DNA.

Some transgenic mice developed clinical nephritis, defined by proteinuria and azotemia. Compared with nontransgenic littermates, lines 3 (data not shown) and H
Figure 8. Serum IgG anti-DNA levels of 4 lines of transgenic (T) mice and nontransgenic (NT) littermates. Left panel, lines 1, 2, and NT mice. Right panel, lines 3 and H. Data are presented as the mean and SEM for each line. Numbers of T and NT littermates in each line analyzed were 10 (T) and 4 (NT) for line 1; 12 and 5 for line 2; 6 and 5 for line 3; and 10 and 2 for line H. The IgG anti-DNA levels from various lines was never significantly different from IgG anti-DNA in BALB/c mice. For simplicity of data presentation, all NT mice were grouped together (n = 16).

To standardize the anti-DNA titer over time, every plate of every assay contained a reference positive (pooled sera from nephritic BW mice which is diluted to 1/100 and set at 1.0 OD) and negative (pooled BALB/c sera at the same dilution is set at <0.025 OD) controls. The pooled BW sera (36 wk of age) contained anti-DNA-binding activity (OD = 1.4).

Lines connect mean values for all mice in each group; vertical bars indicate 1 SEM. Except for line 2, all values in T mice were significantly higher than values in NT mice, p < 0.05 or less by Student’s two-tailed t-test. The data of mice at 24 wk of age were also used in Table I.

DISCUSSION

The data presented here demonstrate that elevated levels of serum anti-DNA IgG antibodies and mild nephritis can be obtained in normal mice that have a repertoire skewed toward the production of IgG anti-DNA. This is consistent with our earlier work demonstrating that the A6.1 IgG2a is pathogenic (6). Although transcripts for the transgene-encoded V regions and serum IgG2a levels were highly elevated, the amount of serum anti-DNA IgG did not reach levels equivalent to diseased BW mice. This may be due to the formation of mixed molecules encoded in part by the transgenes and in part by endogenous Ig genes. Mixed molecules would be formed on account of the lack of allelic exclusion, which was clearly demonstrated by the coexpression of μ and γH chains by many of the B cells in the transgenic mice (Fig. 7). We believe that allelic exclusion and tolerance

Figure 9. Comparison of affinity of serum IgG for DNA between transgenic (T) and nephritic BW mice. Pooled serum samples were pretreated with DNase-1, incubated with dsDNA from calf thymus, and tested for binding by using dsDNA coated plates as described in Materials and Methods.

(Fig. 10A) showed significantly higher proteinuria. Elevated blood levels of urea nitrogen (azotemia) indicate renal dysfunction; lines 3 (Fig. 10B) and H (data not shown) showed significantly higher levels than nontransgenic littermates. Figure 8 shows IgG deposits in the basement membrane and mesangium of a glomerulus in a kidney from a transgenic mouse, and the absence of IgG in a nontransgenic littermate. The C component C3 also was found in glomeruli of transgenic but not nontransgenic mice (Fig. 11). These data suggest that deposition of transgene-encoded IgG2a anti-dsDNA could be responsible for nephritis in transgenic mice.
induction were not established in transgenic mice, on account of low surface expression of the transgenic Ig. The construct for the IgH chain did not include the most frequently used polyadenylation sites for membrane IgG2a H chain mRNA (28). Indeed, Northern blot analysis of transgenic spleen RNA showed that most of the Cγ2a/2b message had a molecular weight characteristic of the secreted form. Although a small amount of Cγ2a transcript with a size characteristic of the membrane form of Cγ2a was found, some of it could be derived from endogenous genes. Alternatively, some fully spliced, membrane form Cγ2a transcripts from the transgene could be present, in which case a cryptic polyadenylation site might be used. This latter possibility seems likely, because immunization with DNA could trigger an elevated antibody response to DNA in transgenic mice, suggesting that some transgenic B cells express transgenic Ig on their cell membrane as Ag receptors. Other possible explanations for the low level of serum anti-DNA IgG include masking by anti-idiotypic antibodies or induction of anergy in some B cells that express high levels of both the anti-DNA H and L chains. Although we cannot rule out these other possibilities, unlike the low surface expression, these possibilities do not predict the lack of allelic exclusion. Furthermore, the affinity for DNA of the IgG obtained from transgenic mice was equivalent to that of nephritic BW mice. Therefore it seems unlikely that most cells capable of producing high affinity anti-DNA IgG were subject to anergy.

In other transgenic mice systems, lack of expression of the membrane form of IgM or IgG2b results in the absence of allelic exclusion of expression of endogenous Ig genes (31–35). Our results (shown in Figs. 6 and 7) were consistent with findings of others; that some transgenic B cells failed to undergo allelic exclusion and expressed both transgene-derived IgG and endogenous gene-derived IgM. The results reported provide an interesting contrast with the recent report of IgM anti-ssDNA transgenic mice (3). Similar to other transgenic mice expressing the membrane form of IgM (29–33), rearrangement of endogenous Ig genes was effectively suppressed in those mice. Furthermore, although most B cells expressed the transgene-encoded anti-DNA IgM at high density on their surfaces, they were apparently anergic as judged by the low levels of both serum anti-DNA and total Ig. The difference in the effectiveness of tolerance induction in these two models may result from several factors. One possibility is that the transgenic IgM has greater avidity for ssDNA than our transgenic IgG2a. The avidity of the A6.1 Ig for either ss- or dsDNA is, however, fairly high. Most likely, the difference results from different densities of self-reactive transgenic Ig expressed on B cell surfaces in the two systems. The cross-linking of the high density surface IgM receptor by self-Ag-induced anergy (36). In our experiments, membrane-bound IgG was sparse and probably could not reach the threshold for tolerance induction. Thus abundant surface expression of Ig receptor may be required for tolerance and allelic exclusion. The data presented here suggest that B cells which can secrete IgG anti-DNA but have limited surface expression escape normal tolerizing mechanisms. In murine lupus excessive Th cell may block tolerance induction or may accelerate the differentiation of B cells into plasma cells, permitting the cells to escape the susceptible stages for tolerance induction (immature and emerging memory B cells) (37–43).

Although multiple lines of transgenic mice had high levels of circulating transgene-encoded IgG2a anti-DsDNA, in no case were the levels comparable to those observed in aged BW mice. After 30 wk of age BW mice had levels of serum anti-DNA IgG, proteinuria and azotemia greater than any of the transgenic mice; BW mice died of glomerulonephritis between 30 and 40 wk. Proteinuria and azotemia, which were less severe in transgenic mice, did not increase progressively over time and no transgenic mice developed fatal nephritis. The milder nephritis of transgenic mice correlates with their lower serum IgG anti-DNA levels compared to BW mice. Increased predominance of expression of transgene-derived IgG2a anti-DNA antibody can perhaps be achieved by including the known polyadenylation site for surface expression (28) or by crossing these transgenic mice with mice that lack functional genes for surface IgM expression (44). Alternatively, despite having a repertoire skewed toward anti-DNA Ig production, the normal mice may lack one or more additional factors present in BW mice that predispose toward disease. These could include stem cell defects, polyclonal activation of B cell population, up-regulation by Th cells, failure in immune suppression, defects in tolerance induction, or failure to effectively clear immune complexes (41, 45).

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