The loss of self-tolerance leading to autoimmune type 1 diabetes in the NOD mouse model involves at least 19 genetic loci. In addition to their genetic defects in self-tolerance, NOD mice resist peripheral transplantation tolerance induced by costimulation blockade using donor-specific transfusion and anti-CD154 antibody. Hypothesizing that these two abnormalities might be related, we investigated whether they could be uncoupled through a genetic approach. Diabetes-resistant NOD and C57BL/6 stocks congenic for various reciprocally introduced Idd loci were assessed for their ability to be tolerized. Surprisingly, in NOD congenic mice that are almost completely protected from diabetes, costimulation blockade failed to prolong skin allograft survival. In reciprocal C57BL/6 congenic mice with NOD-derived Idd loci, skin allograft survival was readily prolonged by costimulation blockade. These data indicate that single or multiple combinations of evaluated Idd loci that dramatically reduce diabetes frequency do not correct resistance to peripheral transplantation tolerance induced by costimulation blockade. We suggest that mechanisms controlling autoimmunity and transplantation tolerance in NOD mice are not completely overlapping and are potentially distinct, or that the genetic threshold for normalizing the transplantation tolerance defect is higher than that for preventing autoimmune diabetes. Diabetes 52:321–326, 2003

The nonobese diabetic (NOD) mouse is widely used to model human type 1 diabetes. Disease in NOD mice is characterized by T-cell–dependent autoimmune destruction of β-cells (2). NOD mice exhibit a number of immune defects that may contribute to their expression of autoimmunity. These include defective macrophage maturation and function (3), low levels of natural killer (NK) cell activity (4), defects in NKT-cells (5,6), deficiencies in their regulatory CD4+/CD25+ T-cell population (7), and the absence of C5a and hemolytic complement (8). Additionally, NOD mice are prone to development of other autoimmune syndromes, including autoimmune sialadenitis (9), autoimmune thyroiditis (10), experimental autoimmune encephalomyelitis (11), autoimmune peripheral polyneuropathy (12), and a systemic lupus erythematosus-like disease if exposed to killed mycobacterium (13).

Like humans, susceptibility to type 1 diabetes in the NOD mouse is a polygenetic trait, involving at least 19 different Idd loci on 11 chromosomes (14,15). When these NOD genetic susceptibility intervals, either alone or in combination, are replaced by congenic introgression with the corresponding interval from a diabetes-resistant strain, the individual contribution of these Idd loci to potential autoimmune phenotypes can be determined. In many cases, congenic introgression of one or a few Idd loci from resistant strains onto the susceptible NOD strain greatly reduces the incidence of insulitis and diabetes (16–20).

We have previously shown that treatment of normal mice with a single donor-specific transfusion (DST) plus a brief course of anti-CD154 (CD40L) monoclonal antibody (mAb) leads to permanent islet (21) and prolonged skin (22) allograft survival. However, transplantation in the NOD mouse presents a unique challenge. Although there are >125 protocols known to prevent diabetes in NOD mice (1), few tolerance-inducing interventions have been reported to be even partially effective in prolonging islet graft survival in diabetic NOD mice (23–26). For example, either anti-CD154 mAb monotherapy (27,28) or DST plus anti-CD154 mAb treatment (29), therapies that can induce permanent islet allograft survival in normal mice (30), fail to induce long-term islet graft survival in spontaneously diabetic NOD mice. Because our studies documented that DST and anti-CD154 mAb also failed to prolong the survival of skin allografts, a tissue that is not a target of the
autoimmune response, we have hypothesized that NOD mice have a generalized defect in costimulation blockade–based transplantation tolerance induction (29). This defect in tolerance induction was independent of the unique NOD H2B haplotype, but it did correlate with a defect in antigen-presenting cell maturation that has previously been described in NOD mice (3,29).

In the present study, we tested the hypothesis that the Idd loci that genetically control diabetes development in NOD mice would also control their resistance to transplantation tolerance. We tested this hypothesis using NOD and C57BL/6 mice with various Idd loci reciprocally introgressed onto each background. To avoid the confounding effects of simultaneously testing both tolerance induction and recurrent autoimmunity to islet-associated antigens, we used skin allografts for our experiments. Our data suggest that the genetic control of peripheral tolerance induction and autoimmunity differ, or are at least only partially overlapping, with the genes that control the induction of costimulation blockade–based peripheral transplantation tolerance.

TABLE 1
Idd congeneric intervals, cumulative diabetes frequency and insulitis, and candidate genes in each interval

<table>
<thead>
<tr>
<th>Congenic strain</th>
<th>Chromosomal location</th>
<th>Insulitis* (%)</th>
<th>Diabetes† (%)</th>
<th>Partial list of candidate genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD.B6 Idd3</td>
<td>Chr. 3</td>
<td>70</td>
<td>20</td>
<td>Idd2, Idd9, Fgf2</td>
</tr>
<tr>
<td>NOD.B10 Idd5 (Idd5.1 and Idd5.2)</td>
<td>Chr. 1</td>
<td>78</td>
<td>40</td>
<td>Idd5.1: Cd152, Cd28, Casp8, Flip; Idd5.2: Ccpx2, Il-4r</td>
</tr>
<tr>
<td>NOD.B6 Idd3 B10 Idd5</td>
<td>See Idd3/Idd5 above</td>
<td>10</td>
<td>1</td>
<td>(see Idd3 and Idd5 above)</td>
</tr>
<tr>
<td>NOD.B6 Idd10Idd18</td>
<td>Chr. 3</td>
<td>78</td>
<td>5</td>
<td>Csfml, Cdx53, Reraa, Rapla</td>
</tr>
<tr>
<td>NOD.B6 Idd5Idd10Idd18</td>
<td>Chr. 3</td>
<td>19</td>
<td>9</td>
<td>(see Idd3, Idd10, and Idd18 above)</td>
</tr>
<tr>
<td>NOD.B10 Idd9</td>
<td>Chr. 4</td>
<td>90</td>
<td>0</td>
<td>(see Idd5.1 and Idd5.2 above)</td>
</tr>
<tr>
<td>C57BL/6.NODctl</td>
<td>Chr. 1</td>
<td>0</td>
<td>0</td>
<td>(see Idd5.1 above)</td>
</tr>
<tr>
<td>C57BL/6.NODlec</td>
<td>Chr. 1</td>
<td>0</td>
<td>0</td>
<td>(see Idd3, Idd10, Idd18 above), Idd17</td>
</tr>
<tr>
<td>C57BL/6.NODc3</td>
<td>Chr. 3</td>
<td>0</td>
<td>0</td>
<td>nkrp1 (NKR-P1 complex)</td>
</tr>
<tr>
<td>C57BL/6.NODc6</td>
<td>Chr. 6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Specific genetic intervals, insulitis, and cumulative diabetes frequency, and a more complete listing of candidate genes contained within each interval can be found in (18,39–41,49,50). *Percent of females scoring positive for moderate (10 to 50% of islets positive for lymphocytic infiltrate) insulitis at 6–8 months of age; †cumulative diabetes frequency in females at 7 months of age. Chr., chromosome.

RESULTS

Costimulation blockade prolongs skin allograft survival in mice genetically deficient in C5a and hemolytic complement. The NOD mouse has numerous immune defects that may be involved in their resistance to the induction of transplantation tolerance. One defect that could impact this strain’s response to in vivo antibody therapy is the absence of hemolytic complement caused by their genetic deficiency in C5a (8). To test this, we attempted to tolerate B10.D2 congenic mice that genetically differ in the presence or absence of C5a (36). These congenic mice were treated with DST, anti-CD154 mAb, and C3H/HeJ skin allografts. As shown in Fig. 1, the
Costimulation blockade prolongs skin allograft survival in C57BL/6 congenic mice with NOD susceptibility loci. We next tested the hypothesis that selected NOD-derived Idd loci are responsible for the defect in peripheral transplantation tolerance induction by costimulation blockade. This was first assessed by determining whether the ability to prolong skin allograft survival was abrogated in C57BL/6 mice congenic for various NOD-derived Idd loci (22). None of the C57BL/6 congenic mice developed insulin or diabetes (Table 1). We tested four congenic strains. C57BL/6.NODc1c mice carry the NOD-derived Idd5.1 region, which includes genes encoding CD152 (CTLA4) and CD28, costimulatory molecules that are important for tolerance induction and immune activation (21). C57BL/6.NODc1t mice carry the NOD-derived Idd5.1 as well as the Idd5.2 region, which includes the CXCR2 and interleukin-8 receptor genes. C57BL/6.NODc3 mice carry the NOD-derived Idd3, Idd17, Idd10, and Idd18 loci, which include genes that control cell activation. C57BL/6.NODc6 mice carry the NOD Idd6 locus, which includes genes contained within the NK receptor protein-1a (NKR-P1) complex (Table 1).

The median skin allograft survival time of each of these C57BL/6 congenic strains ranged from 62 to >93 days and were statistically similar to that observed in C57BL/6 wild-type mice (MST = 81 to >94 days) (Table 2). Skin allograft survival in the C57BL/6 congenic mice was significantly greater than that observed in similarly treated NOD mice (MST = 20–24 days) (Tables 3 and 4).

Diabetes-resistant NOD congenic mice treated with DST and anti-CD154 mAb rapidly reject skin allografts. We next determined whether NOD stocks congenic for selected C57BL/6- or C57BL/10-derived Idd loci that mediate protection from insulitis and diabetes would also exhibit prolonged skin allograft survival after treatment with DST and anti-CD154 mAb. We tested three groups of NOD congenic mice with intervals that encompass the non–major histocompatibility complex (MHC) Idd loci with the greatest effect on diabetes expression (18, 37–39). The first of these were NOD congenic stocks carrying a C57BL/6-derived Idd3 or C57BL/10-derived Idd5 locus alone or in combination. These NOD single-Idd congenic mice each have a reduced frequency of diabetes, and when the Idd3 and Idd5 resistance loci are combined, the frequency of diabetes is reduced to 1%, and insulitis is absent in most mice (18) (Table 1). The median survival time of C3H/HeJ skin allografts in these NOD congenic mice treated with DST plus anti-CD154 mAb (24–31 days) was statistically significantly shorter than that of C57BL/6 mice (>115 days) (Table 3). Skin allograft survival in these NOD congenic mice was similar to that of NOD/Lt (MST = 20 days) or NOD/MrkTac (MST = 23 days) mice (Table 3).

We next tested NOD congenic mice that carried various combinations of C57BL/6-derived Idd3, Idd10, and Idd18...
resistance loci. The frequency of diabetes in these NOD congenic mice varies from 9 to 50% (Table 1). A significant reduction in the frequency of insulitis was also observed in NOD.B6 Idd3/10/18 triple congenic mice (40,41). Again, all of the NOD congenic strains with various combinations of these genetic intervals were resistant to tolerance induction and had median skin allograft survival times of 17–29 days (Table 4).

Finally, we tested an NOD stock congenic for the C57BL/10-derived Idd9 locus. This locus contains molecular variants of Cd30, Tnfr2, and Cd137 (39), and these mice have a frequency of diabetes of only 3% (Table 1). The MST of skin allografts in NOD.B10 Idd9 mice was 20 days, similar to that observed in NOD/Lt mice (MST = 24 days, Table 4).

**Discussion**

In the present study, we have investigated the relationship between genes that control autoimmune diabetes expression in NOD mice with those controlling their resistance to transplantation tolerance induced by costimulation blockade. We document that single or small combinations of the evaluated C57BL/6-derived Idd loci that dramatically alter diabetes expression are not able to correct the response of NOD mice to costimulation blockade and, conversely, that NOD-derived Idd loci do not shorten skin allograft survival in C57BL/6 mice. We further eliminate two other potential explanations: the genetic absence of hemolytic complement or an accelerated clearance rate of anti-CD154 mAb from the circulation.

Peripheral transplantation tolerance induction by DST and anti-CD154 mAb involves the deletion of alloreactive CD8+ T-cells (31,33). Because CD8+ T-cells in NOD mice appear to be resistant to tolerance induction (42), this may be one mechanism by which NOD mice are resistant to costimulation blockade–induced tolerance. However, we have recently determined that the majority of high-affinity alloreactive CD4+ T-cells are also deleted by treatment with DST and anti-CD154 mAb (D.L.G., unpublished observations). CD4+ T-cells express CD154 when activated (32). This suggests that one mechanism by which DST and anti-CD154 mAb induces tolerance could involve deletion of alloreactive CD4+ T-cells by antibody-mediated, complement-dependent lysis. However, the ability to prolong skin allograft survival in congenic B10.D2 mice that lacked C5a and hemolytic complement argues that this defect does not prevent tolerance induction in NOD mice. We have also documented that the circulating level of anti-CD154 mAb is inversely correlated with skin allograft survival in recipients treated with DST and anti-CD154 mAb (33). However, the clearance rate of anti-CD154 mAb from the circulation of NOD mice was similar to that of C57BL/6 mice. This data suggests that rapid clearance of anti-CD154 mAb, hence potentially lowering anti-CD154 mAb concentrations below effective tolerizing levels (~100 mcg/ml) (33), was not the basis for the resistance of NOD mice to tolerance induction.

We then tested the hypothesis that some of the genetic loci associated with development of autoimmune diabetes in NOD mice would also be important in their resistance to induction of peripheral transplantation tolerance. Prolonged allograft survival was not abrogated in C57BL/6 stocks congenic for any of the analyzed NOD-derived Idd susceptibility loci. We note, however, that no combination of Idd susceptibility loci introgressed into C57BL/6 mice to date has rendered them susceptible to the spontaneous development of insulitis or autoimmune diabetes.

Similarly, none of the analyzed C57BL/6- or C57BL/10-
derived Idd congenic intervals that confer various degrees of diabetes resistance to NOD mice restored their ability to be tolerized by DST and anti-CD154 mAb treatment. This observation was surprising because congenic introgression of even a few of the Idd resistance loci into NOD mice profoundly reduces the frequency of insulinitis and diabetes (17,18,38,39) (Table 1). Furthermore, many of the tested Idd congenic intervals are characterized by polymorphisms in genes important for costimulation and immune activation.

There is evidence that autoimmune diabetes in NOD mice is due primarily to defects in central tolerance (43). Bone marrow chimerism is known to prevent autoimmunity in NOD mice by this mechanism (44). In humans, bone marrow cells from diabetic donors have been documented to adoptively transfer disease to nondiabetic recipients, suggesting that central tolerance defects are also important in type 1 diabetes in humans (45). Additionally, there is data to suggest that manipulation of the peripheral immune system can affect self-tolerance and the expression of autoimmune diabetes in NOD mice (46–48). Mechanisms that control central and peripheral tolerance are different. Central tolerance is primarily mediated by intrathyMIC deletion of autoreactive T-cells during thymic development, whereas peripheral tolerance is mediated by multiple mechanisms, including deletion, anergy, and regulatory processes (21).

It is currently unknown whether improved central or peripheral tolerance is the mechanism by which the NOD congenic mice we studied in these experiments were rendered resistant to autoimmune diabetes. Our data suggest, however, that if the mechanism of protection from diabetes is due to restoration of the factors that permit peripheral regulation of autoimmunity, these mechanisms are not sufficient for the induction of peripheral allogeneic transplantation tolerance by costimulation blockade. Alternatively, it is possible that the results from the current study reflect the fact that more Idd resistance loci are required to genetically alter the phenotype of abnormal transplantation tolerance induction in NOD mice than are required to decrease the incidence of spontaneous autoimmune diabetes. Specifically, peripheral transplantation tolerance induction by costimulation blockade may be under the control of a complex combination of Idd loci not yet tested. Future experiments examining C57BL/6.H2b7 congenic mice bearing NOD diabetes-susceptibility loci will be required to identify potential interactions of the unique NOD H2b7 MHC with these genetic loci and their effect on tolerance induction. However, these data raise the possibility that the loss of self-tolerance leading to autoimmunity in NOD mice may be mediated by mechanisms that differ, in part, from their resistance to peripheral transplantation tolerance, a hypothesis we are currently testing.

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