Patients with diabetes are at significant risk of developing corneal lesions, such as superficial punctuate keratitis, recurrent corneal erosions, persistent epithelial defects, and microbial keratitis. These lesions are all due to lacrimal gland dysfunction [1]. Even proliferative diabetic retinopathy appears to have a more severe course in patients with diabetes and declined tear film function [2]. Whether or not lacrimal gland dysfunction is metabolic or autoimmune in origin is still controversial [3].

Proliferative and non-proliferative retinopathies are metabolic complications of diabetes. These metabolic complications are caused by microangiopathy, the severity of which in turn determines the development of retinopathy as well as nephropathy and neuropathy. However, a causative relationship between metabolic complications of diabetes and non-retinal ocular disease (i.e., lacrimal dysfunction) is not well established. On the contrary, evidence exists for the association between “tear film” dysfunction (characteristic of diabetes-associated dry eye disease) and autoimmune diabetes [4]. Studies have recently established that the prevalence of symptoms, signs, and definitive diagnosis of dry eye syndrome (DES) are higher, and basal tear secretion and tear film stability are lower, in children with autoimmune (type 1) diabetes mellitus (T1D), years before metabolic complications are even expected [4].

Animal models of autoimmune diabetes have provided the strongest evidence for a causative relationship between autoimmunity and diabetes-associated DES. Non-obese diabetic (NOD) mice, the classic model of human T1D, usually develop lacrimal gland dysfunction. Autoimmune lymphocytic infiltration of lacrimal glands accompanies lymphocytic infiltration of pancreatic islets in NOD mice [5]. A possible explanation for this association of pancreatic islets and lacrimal gland lymphocytic attacks has been based on the existence of cross-reactive antigens in both glands. Recently, a special group of CD4+ helper T cells was observed to be enriched in the inflamed pancreas and salivary glands of NOD mice as well as in the circulation of Sjögren’s syndrome patients [6].
We have developed a novel model of TID using double-transgenic mice carrying HLA-DQ8 diabetes-susceptibility haplotype instead of mouse MHC-class II and expressing the human beta cell autoantigen Glutamic Acid Decarboxylase (GAD65) in pancreatic beta cells. Double-transgenic mice immunized with DNA encoding human GAD65 develop insulitis, glucose intolerance, and diabetes, while controls are insulitis-free and glucose-tolerant [7,8]. We report here the development of DES (classically associated with diabetes in NOD mice and proposed to be associated with TID in humans) after diabetes induction in our humanized transgenic model.

**METHODS**

*Mice:* HLA-DQA1*0301/DQB1*0302 (DQ8; murine MHC-class II molecule-deficient) and RIP7-hGAD65 line 1 transgenics in the BTBR background were used for all experiments [7,8]. All mice used in this study were >N6 crosses. All animal protocols were approved by the University of Wisconsin and the Veterans Affair animal research committees.

*Adenoviral constructs for immunization:* Adenoviral constructs were made using the Gateway system (Invitrogen, Carlsbad, CA) as previously described [8]. All viruses used in this study were from the same preparation and were stored in aliquots at -80 °C. hGAD65 expression from pAD-CMVhGAD65 was confirmed by western blot. A pAD-CMV empty vector was used for control immunizations. Immunizations were performed two times at two-week intervals. Mice were intraperitoneally injected with 100 µl of PBS containing 10^10 particles of pAD-CMVhGAD65.

*Histology, immunohistochemistry, and immunofluorescence:* Pancreatic and lacrimal/Harderian gland sections were stained with hematoxylin/eosin for histological identification and localization of lymphocytic infiltration. Sections were then stained for markers of T cells (CD3) and B cell (CD20) lineage as well as macrophages (CD68 and MAC-1) using immunohistochemical techniques [8,9]. CD4 and CD8 markers were used to characterize helper versus cytotoxic T cell responses (CD4/CD8 ratio), mostly using immunofluorescence techniques and confocal microscopy, but also using histomorphometric microscopy [8,9]. The primary antibodies used were anti-CD3, CD4, and CD8 (1:100; BD PharMingen San Jose, CA).

IL-17 detection (anti-IL-17; 1:200; Santa Cruz Inc., Santa Cruz, CA), which identifies another subset of helper T cells known as Th-17 cells, was also performed. Finally, the presence of Foxp3 (anti-Foxp3; 1:200; Abcam, Cambridge, UK), a marker for T-regulatory cells, was investigated [8,9].

Lymphocytic infiltration was quantified by analyzing 15 microscopic fields. In the pancreas, it was expressed as percent of pancreatic islets infiltrated by lymphocytes. In lacrimal/Harderian glands, it was scored as severe when more than 50% of glandular tissue was affected by lymphocytic infiltration, and as mild when less than 50% was affected.

**Antibody measurement:** Anti-GAD65 antibodies from mice serum samples were quantified with an ELISA assay according to manufacturer instructions (Kronus, Boise, ID). The ELISA results were validated with a parallel immunoprecipitation assay previously standardized in our laboratory [8]. Briefly, the immunoprecipitation method has been substituted entirely by the ELISA method. Control mouse serum samples showed less than 5 U/ml of anti-GAD65 antibody concentration. At least two independent experiments in duplicated wells were performed, and average values for each sample were obtained.

**Measurement of aqueous tear production:** Tear production was measured with cotton threads (Zone-quick; Oasis, Glendora, CA). The threads were held with jeweler’s forceps and applied to the ocular surface in the lateral canthus for 60 s. Wetting of the thread was measured in millimeters, using the scale on the cotton thread under a light microscope [10].

**Statistical analysis:** Two-tailed probability of chi-square distribution was used to compare results. P values were considered significant when <0.05.

**RESULTS**

We recently developed a new humanized animal model of autoimmune diabetes [7,8]. We conducted the current studies using ten transgenic and control mice. We followed an immunization protocol already shown to induce autoimmune diabetes in these mice [8]. Our animals developed eye problems as they developed glucose intolerance and diabetes. The glycemic level used to establish the diagnosis of diabetes was glucose ≥250 mg/dl in two consecutive days (as previously reported [8]). We observed a correlation between the severity of the pancreatic infiltration and severity of ocular disease (Table 1).

**Dry eye syndrome:** Our animals developed dry eye signs several weeks after immunization for diabetes induction. From mild corneal erosions to severe keratitis, our animals manifested obvious clinical signs of ocular disease (Figure 1 is representative of early tear film dysfunction). DQ8/ hGAD65 transgenic mice do not spontaneously develop diabetes or DES. However, upon immunization with a vector carrying hGAD65 coding DNA (induction), most animals develop insulitis, glucose intolerance, and diabetes [7,8]. Ten
to twenty-four weeks post-immunization, animals were noted to develop clinical signs of tear film dysfunction (Figure 1). On further examination of histological specimens of the retro- and peri-bulbar areas, lymphocytic infiltration of glandular structures was revealed (Figure 2, early infiltrates). Indeed, severe histopathological lesions were observed in lacrimal and Harderian glands within weeks following development of glucose intolerance. Lymphocytic infiltration ranged from focal lesions to complete acinar destruction of the glands involved.

**Aqueous tear production defect:** We performed two modified Schrimer’s tests using cotton threads [10] two weeks apart to assess for tear production defects ten weeks after triggering immunization with hGAD65. Most experimental
animals demonstrated lower tear production when compared to controls (Figure 3). A statistically significant decrease in aqueous tear production for four animals (mean, 0.57±0.7 mm) in the experimental group, as compared with control animals (mean, 3.30±0.05 mm), was observed (Figure 3). Additionally, most experimental animals showed non-significant worsening of aqueous tear production within the two-week measurements. Notably, the mice with statistically significant defects in aqueous tear production also had the most severe lymphocytic infiltrates in lacrimal/Harderian glands (Table 1).
We observed a syndrome immediately followed by the development of Sjögren’s syndrome in 1973, when they published a case of Sjögren’s syndrome. Rudobielska et al. first suggested this association in 1973, when they published a case of Sjögren’s syndrome. However, correlation was found with the antibody titers and either lacrimal/Harderian or pancreatic islets lymphocytic infiltration/destruction.

Immune characterization of lacrimal gland lesions: Lymphocytic infiltrates were characterized by immunohistochemistry and immunofluorescence. CD3-positive cells (T cells Figure 4B) were the dominant population in the infiltrates (Figure 4A). CD4+ T cells predominated over CD8+ in areas of heavy infiltrates (Figure 4C). CD8+ T cells were much less abundant and mainly present in inter-acinar spaces (Figure 4D).

We also studied the presence of IL17 and Foxp3 markers in an eight-year-old girl [1]. Another case report followed in 1975, in which diabetes shortly preceded Sjögren’s syndrome. In a larger 1990 study of 102 unselected T1D patients, 55% were affected by Sjögren’s syndrome. Of those patients, 25% tested positive for antinuclear antibodies (HEp-2) and 32% were positive for anti-Ro antibodies, reinforcing the suspicion of an overall autoimmune rather than metabolic cause of DES. Cases have been reported in which a common triggering event for both diabetes and DES was postulated. Others have shown the concomitant occurrence of several autoimmune problems, highlighting the possibility that the association of autoimmune diabetes and DES was linked to a common autoimmune target. The first large clinical study designed to compare the symptoms, signs, and results of objective tests for DES in T1D patients and controls was undertaken recently [4]. A total of 104 children with T1D and 104 age- and sex-matched controls were studied. A total of 15.4% of diabetic children complained of dry eye symptoms, versus 1.9% of controls (p=0.029). Dry eye signs were clinically detected in 7.7% of diabetic children, versus 0.96% of controls (p=0.034). Tear break-up time and Schrimer test of tear production were significantly lower (p=0.018, p=0.024, respectively) in the T1D group than in the control group. These results clearly demonstrated a statistically significant association of DES with T1D that preceded the metabolic complications of diabetes.

Lacrimal gland dysfunction has also been associated with autoimmune diabetes in animal models. Asamoto et al. observed in 1984 that lymphocytic infiltration of lacrimal glands accompanied lymphocytic infiltration of pancreatic islets in the NOD mouse model of autoimmune diabetes. Furthermore, the NOD mouse has been proposed as a valid animal model for diabetes-associated DES or diabetes-associated Sjögren’s syndrome in the setting of autoimmune diabetes. Unlike other mouse strains with reported infiltration of the lacrimal glands, such as MLR/lpr, MLR/+, and NZBxNZW F1 mice, gland dysfunction observed in NOD mice is concomitant with progressive cellular infiltration and tissue destruction of these organs. A possible explanation for the coexistence of pancreatic islets and lacrimal gland lymphocytic attacks has been based on the presence of candidate cross-reactive antigens in both pancreatic islets and lacrimal glands. Unfortunately, the nature of the primary target antigen of the immune attack to the lacrimal glands in NOD mice is not known.

We have developed a diabetes mouse model, in which a known human autoantigen is presented to effector cells in the context of human diabetes-susceptibility genes. After a triggering event, our transgenics develop insulitis and
diabetes. A proportion of our animals also develop clinical DES in close association with their diabetes phenotype. Although the target antigen for the lacrimal gland attack is not known, GAD65 antigen is the target of the pancreatic islet attack. We first noticed dry eye signs when glucose tolerance tests confirmed incipient glucose homeostasis abnormalities.

A modified Schrimer’s test confirmed the lack of proper tear secretion (tear film dysfunction). In the tested experimental group, most animals showed impaired tear secretion when compared to control mice, which was statistically significant for some. Mild corneal lesions evolved into severe keratitis in some animals. Upon development of diabetes, histological
examination of the retro- and peri-bulbar specimens revealed lymphocytic infiltration of glandular structures that ranged from focal lesions to complete acinar destruction. As expected, the worse the tear secretion dysfunction, the more severe the lymphocytic infiltration in lacrimal/Harderian glands. The lymphocytic infiltration was predominantly of the T-cell type. CD4+ T helper cells seemed to be overrepresented compared to CD8+, and their distribution appeared somewhat different (focal for CD4+; sparse for CD8+). The proportion of cytotoxic (CD8+) T cells was not consistent with the tissue destruction in some cases. On further analysis of the T-helper (Th) cell type, we noticed subtle differences depending on the severity of the orbital gland infiltration. While lacrimal glands in severely affected animals showed the dominant presence of Th-17 cells (IL17 positive), mildly affected animals showed the presence of clusters of T regulatory cells (Foxp3 positive). Th-17 cells are thought to be good markers for autoimmune components of immune responses [23]. On the other hand, the presence of Foxp3 (a marker for T regulatory cells [24]) is believed to identify regulatory responses that when dominant, control autoimmunity. These differences may explain why we noticed more severe clinical features with more severe histology in animals lacking T regulatory cells.

We also noticed that more severe lacrimal gland dysfunction phenotypes accompanied more severe diabetes. Severity of lymphocytic infiltration of orbital glands correlated with severity of infiltration of pancreatic islets. This finding is in keeping with similar findings in the NOD model. In the NOD model, the severity of lymphocytic infiltration is directly correlated with the diabetes and DES phenotype [17]. This correlation points toward a common target between the orbital glands and pancreatic islets. In the NOD model, however, the target antigen is uncertain. In our model, GAD65 is the target antigen for the pancreatic islet attack. The immune response in our model is triggered by a peripheral immunization with the target antigen, which in turn homes to the pancreatic islets where the antigen is expressed. The transgenic expression of GAD65 outside the pancreatic beta cell has been studied, and non-measurable amounts of GAD65 have been detected anywhere else [25]. However, GAD expression around acinar cells in salivary glands has been demonstrated, and suggests that the GABAergic system plays a role in autonomic regulation of these and other glands [26]. Other cross-reactive antigens may also be implicated. Obvious and less obvious cross-reactive candidate antigens that have been investigated include islet cell autoantigens (ICA) 69, prominin-1 (CD133), and insulin. ICA69 is a self-antigen expressed in brain, pancreas, salivary, and lacrimal glands. An inactivated genomic ICA69 locus in the NOD strain generated NOD-congenic mice that were deficient in ICA69; in addition, it prevented lacrimal gland disease. Immunotherapy with a
high-affinity mimicry peptide targeting ICA69-specific T-cells reduced established Sjögren's syndrome in wild-type NOD mice [20]. Prominin-1 (CD133) is expressed by various stem and progenitor cells originating from diverse sources. In addition to stem cells, its mouse ortholog is expressed in a broad range of adult epithelial cells. Two independent laboratories have demonstrated expression in secretory serous cells, mucous cells, and intercalated ducts of lacrimal glands, as well as all duct-lining cells of the pancreas [21,27]. Insulin has been shown to be secreted in the tear film; its mRNA is expressed in the lacrimal gland and its receptor in tissues of the ocular surface [28,29]. Furthermore, insulin production in the lacrimal glands, showed: (i) the presence of insulin and C-peptide, (ii) glucose- and carbachol-induced insulin secretion ex-vivo and (iii) biochemical and histological characteristics of diabetic lacrimal glands that would support this possibility, have been demonstrated [30]. Indeed, after inducing diabetes with a streptozotocin injection, diabetic male Wistar rats had rising insulin levels in the tear film after glucose stimulation. Ex vivo static secretion assays demonstrated that higher glucose and carbachol significantly increased mean insulin levels from lacrimal glands. Insulin and C-peptide were expressed in diabetic rats’ lacrimal glands, as determined by RIA. All of these candidate cross-reactive antigens still need to be further investigated to find the cause of the association between diabetes and DES.

Our results demonstrate development of DES in association with antigen-specific insulitis and diabetes following immunization with clinically relevant human autoantigen, concomitantly expressed in mouse beta cells. As in the NOD mouse model and as in human T1D, our animals developed diabetes-associated DES. This specific finding stresses the relevance of our model for studying the human disease. We believe our model will facilitate studies to prevent human diabetes and other associated syndromes, such as DES.

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REFERENCES

1. Cousen P, Cckett P, Bennett H, Swa K, Dhillon B. Tear production and corneal sensitivity in diabetes. J Diabetes Complications 2007; 21:371-3. [PMID: 17967709].
2. Yu L, Chen X, Qin G, Xie H, Lv P. Tear film function in type 2 diabetic patients with retinopathy. Ophthalmologica 2008; 222:284-91. [PMID: 18560249].
3. Jaume JC. Endocrine autoimmunity. In: Gardner DG, Shoback DM, editors. Greenspan’s Basic and Clinical Endocrinology. McGraw-Hill Media; 2011. p. 27–46.
4. Akinci A, Cetinkaya E, Aycan Z. Dry eye syndrome in diabetic children. Eur J Ophthalmol 2007; 17:873-8. [PMID: 18050110].
5. Humphreys-Beher MG, Hu Y, Nakagawa Y, Wang PL, Purushotham KR. Utilization of the non-obese diabetic (NOD) mouse as an animal model for the study of secondary Sjögren’s syndrome. Adv Exp Med Biol 1994; 350:631-6. [PMID: 8030546].
6. McGuire HM, Vogelzang A, Ma CS, Hughes WE, Silveira PA, Tangey SG, Christ D, Fulcher D, Falcone M, King C. A subset of interleukin-21+ chemokine receptor CCR9+ T helper cells target accessory organs of the digestive system in autoimmunity. Immunology 2011; 34:602-15. [PMID: 21511186].
7. Elagin RB, Balijepalli S, Diacovo MJ, Baekkeskov S, Jaume JC. Homing of GAD65 specific autoimmunity and development of insulitis requires expression of both DQ8 and human GAD65 in transgenic mice. J Autoimmun 2009; 33:50-7. [PMID: 19289270].
8. Elagin RB, Jaume JC. Glucose intolerance and diabetes following antigen-specific insulin in diabetes-susceptible “humanized” transgenic mice. Biochem Biophys Res Commun 2010; 395:99-103. [PMID: 20350527].
9. Mirocha S, Elagin RB, Salamat S, Jaume JC. T regulatory cells distinguish two types of primary hypophysitis. Clin Exp Immunol 2009; 155:403-11. [PMID: 19077086].
10. Dursun D, Wang M, Monroy D, Li DQ, Lokeshwar BL, Stern ME, Pflugfelder SC. A mouse model of keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 2002; 43:632-8. [PMID: 11867577].
11. Rudobielska M, Lewicka J, Krasowska I, Zaleski B. Case of Sjögren’s syndrome associated with diabetes in an 8-year-old girl. Wiad Lek 1973; 26:857-60. [PMID: 4706382].
12. Wesolowska H, Ludwiczak H, Ferdynus J. Sjögren’s syndrome with progressive systemic sclerosis and primary diabetes in a 12-year-old girl. Reumatologia 1975; 13:215-9. [PMID: 1179065].
13. Binder A, Maddison PI, Skinner P, Kurtz A, Isenberg DA. Sjögren’s syndrome: association with type-1 diabetes mellitus. Br J Rheumatol 1989; 28:158-68. [PMID: 2590806].
14. Takeuchi K, Hori Y, Hayakawa T, Hashimoto H, Atsumi T, Hirose K, Sakai K, Nishikawa T, Miwa A, Takashiro Y, Suzuki T. Sjögren’s syndrome associated with insulin-dependent diabetes mellitus. Ryumachi 1996; 36:769-74. [PMID: 8969557].
15. Shimomura H, Nakase Y, Furuta H, Nishi M, Nakao T, Hanabusa T, Sasaki H, Okamoto K, Furukawa F, Nanjo K. A rare case of autoimmune polyglandular syndrome type 3. Diabetes Res Clin Pract 2003; 61:103-8. [PMID: 12951278].
16. Kocaman O, Aygun C, Guruz Y, Cefle A, Konduk T, Senturk O, Celebi A, Hulagu S. Burst of autoimmunity with the
emergence of primary Sjögren syndrome, cholestatic autoimmune hepatitis and latent autoimmune diabetes of adults (LADA). South Med J 2006; 99:1014-5. [PMID: 17004542].

17. Leiter EH, Serreze DV. Autoimmune diabetes in the nonobese diabetic mouse: suppression of immune defects by bone marrow transplantation and implications for therapy. Clin Immunol Immunopathol 1991; 59:323-34. [PMID: 2029789].

18. Pozzilli P, Signore A, Williams AJ, Beales PE. NOD mouse colonies around the world-recent facts and figures. Immunol Today 1993; 14:193-6. [PMID: 8517916].

19. Jabs DA, Prendergast RA. Murine models of Sjögren’s syndrome. Adv Exp Med Biol 1994; 350:623-30. [PMID: 8030545].

20. Winer S, Astsaturev I, Cheung R, Tsui H, Song A, Gaedigk R, Winer D, Sampson A, McKerlie C, Bookman A, Dosch HM. Primary Sjögren’s syndrome and deficiency of ICA69. Lancet 2002; 360:1063-9. [PMID: 12383988].

21. Karbanová J, Missol-Kolka E, Fonseca AV, Lorra C, Janich P, Hollerová H, Jászai J, Ehrmann J, Kolár Z, Liebers C, Arl S, Subrtová D, Freund D, Mokry J, Huttner WB, Corbeil D. The stem cell marker CD133 (Prominin-1) is expressed in various human glandular epithelia. J Histochem Cytochem 2008; 56:977-93. [PMID: 18645205].

22. Atkinson MA, Leiter EH. The NOD mouse model of type 1 diabetes: as good as it gets? Nat Med 1999; 5:601-4. [PMID: 10371488].

23. Bettelli E, Oukka M, Kuchroo VK. Th-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007; 8:345-50. [PMID: 17375096].

24. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008; 9:239-44. [PMID: 18285775].

25. Baekkeskov S, Kanaani J, Jaume JC, Kash S. Does GAD have a unique role in triggering IDDM? J Autoimmun 2000; 15:279-86. [PMID: 11040068].

26. Kosuge Y, Kawaguchi M, Sawaki K, Okubo M, Shinomiya T, Sakai T. Immunohistochemical study on GABAergic system in salivary glands. Eur J Pharmacol 2009; 610:18-22. [PMID: 19292982].

27. Lardon J, Corneil D, Huttner WB, Ling Z, Bouwens L. Stem cell marker prominin-1/AC133 is expressed in duct cells of the adult human pancreas. Pancreas 2008; 36:e1-6. [PMID: 18192867].

28. Rocha EM, Cunha DA, Carneiro EM, Boschero AC, Saad MJ, Velloso LA. Insulin, insulin receptor and insulin-like growth factor-I receptor on the human ocular surface. Adv Exp Med Biol 2002; 506:607-10. [PMID: 12613967].

29. Myers MG Jr, White MF. Insulin signal transduction and the IRS proteins. Annu Rev Pharmacol Toxicol 1996; 36:615-58. [PMID: 8725404].

30. Cunha DA, de Alves MC, Stoppiglia LF, Jorge AG, Módulo CM, Carneiro EM, Boschero AC, Saad MJ, Velloso LA, Rocha EM. Extra-pancreatic insulin production in RA lachrymal gland after streptozotocin-induced islet beta-cells destruction. Biochim Biophys Acta 2007; 1770:1128-35. [PMID: 17561349].