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Demonstration of Autoimmunity in the Tight Skin-2 Mouse: A Model for Scleroderma

Julie A. Gentiletti,* Laura J. McCloskey,† Carol M. Artlett,* Josephine Peters,‡ Sergio A. Jimenez,* and Paul J. Christner2*

The tight skin-2 (Tsk2+/+) mouse has been proposed as an animal model of systemic sclerosis (SSc) because this animal exhibits increased collagen synthesis and accumulation in the dermis. The Tsk2+/+ mouse also has been reported to have a mononuclear cell infiltrate in the dermis; however, to date no evidence of autoimmunity has been described in this animal model. We report here that Tsk2+/+ mice harbor numerous autoantibodies in their plasma including some, which are similar to those, present in SSc patients. Immunofluorescence with HEp-2 cells revealed the presence of anti-nuclear Abs (ANAs) in the plasma of 92% of the Tsk2+/+ mice. In contrast, <5% of cage-mated CAST/ei mice had a positive ANA and none of the C3H/HeJ age-matched controls were positive. Homogenous, speckled, rim, nucleolar, centromere as well as combinations of these patterns were observed. The proportion of Tsk2+/+ animals with a positive ANA increased slightly with age. ELISAs showed that 93% of the Tsk2+/+ animals were positive for anti-Scl70, 82% for anti-centromere, 5% for anti-RNP/Sm, and none were positive for anti-RNA-polymerase II Abs. Indirect immunofluorescence with Crithidia luciliae and ELISA for anti-dsDNA Abs showed that 76% of Tsk2+/+ mice were positive for this autoantibody. The high frequency of anti-Scl70 and anti-centromere autoantibodies indicates that Tsk2+/+ mice display some humoral immune alterations which are similar to those found in patients with SSc. However, the Tsk2+/+ mice also develop autoantibodies to dsDNA and a majority of the mice develop multiple autoantibody specificities (anti-Scl70, anti-CENP-B, and anti-dsDNA) indicating that the mouse may be a useful model to study autoimmunity in a wider spectrum of connective tissue diseases. The Journal of Immunology, 2005, 175: 2418–2426.

Systemic sclerosis (SSc) is an autoimmune disorder of unknown etiology and pathogenesis, characterized by the excess accumulation of collagen and other extracellular matrix proteins in skin and various internal organs, prominent alterations in the microvasculature, and cellular and humoral immunological abnormalities including the development of multiple autoimmune antibodies, some of which are disease-specific (1–3). The presence of anti-nuclear Abs (ANAs) is one of the most common manifestations of SSc, and >95% of SSc patients harbor ANAs (3, 4). There are numerous ANAs typically associated with SSc including anti-centromere, anti-topoisomerase I (also called ScI70), and anti-RNA-polymerase (3, 4). Anti-centromere Abs (ACAs) recognize three different centromere proteins: a 17 kDa (CENP-A), an 80 kDa (CENP-B), and a 140 kDa (CENP-C) (5). The frequency of ACAs in patients with SSc has been reported to be 20–30%, but it varies depending on the ethnicity of the patient (6). They are rarely found in healthy patients (7). However, they are found in ~80–96% of patients with the limited form of the disease (limited cutaneous SSc (lcSSc)) (4–7). Anti-Scl70 Abs, which were initially described as recognizing a 70-kDa nuclear protein, subsequently were found to react with a 105-kDa protein, which has been identified as DNA topoisomerase I (8, 9). Approximately 30–40% of patients with diffuse SSc harbor these autoantibodies (3, 4, 7). Anti-RNA-polymerase Abs have also been reported in SSc. They are directed against all three classes of RNA-polymerase, each multisubunit enzyme composed of two large distinct proteins with molecular masses greater than 100 kDa and several smaller proteins shared by either all three or two of these enzymes (10). In addition, anti-PM-Scl, anti-fibrillarin, anti-Th/To, anti-Ku, anti-phospholipid, anti-U1-ribonucleoprotein (RNP), and other autoantibodies are found in SSc (3, 4, 7, 11, 12).

Autoantibodies to dsDNA are highly specific for systemic lupus erythematosus (SLE), but not all the patients who harbor these Abs have active disease (13, 14). The anti-dsDNA autoantibodies have been strongly associated with immune complex disease, and are thought to contribute to renal inflammation and deterioration in patients with lupus nephritis (15–19). In addition to dsDNA, a number of specific epitopes within the nucleus have been shown to give rise to a positive test in SLE patients. These include small nuclear RNP s (RNP and Sm), SS-A/Ro, SS-B/La, ssDNA, the nuclear matrix, the nucleolar organizing region, the chromosomal coat protein, and histones (20, 21). The role of specific autoantibodies in the clinical progression of SSc remains controversial. The type of autoantibody found in the patient’s serum clearly correlates with the disease phenotype and is often used as a diagnostic tool to differentiate SSc from other connective tissue diseases (3, 4, 7, 22). For example, patients with diffuse SSc tend to have Abs against topoisomerase I, RNA-polymers, or U3 RNP s including fibrillarin (3, 4, 7, 22). Anti-topoisomerase I Abs are the most frequently observed autoantibodies in these patients and are associated with pulmonary fibrosis and...
cardiac involvement (3, 4, 7, 22–26). Anti-RNA-polymerase Abs are associated with severe skin involvement and renal crisis (3, 4, 7, 22, 27). Patients with lcSSc (calcinosis, Raynaud’s phenomenon, esophageal dysfunction, sclerodactyly, and telangiectasia (CREST) syndrome) harbor Abs against centromere proteins (28 – 31). However, the mechanism(s) by which these autoantibodies may be involved in the clinical manifestations (fibrotic and vascular) of this disease is not understood despite the recent studies suggesting that the titers of some of these autoantibodies may correlate with disease activity or clinical severity (32).

In the study of SSc, several animal models have been used. One of these is the tight skin-2 (Tsk2+/−/H11001) mouse, a model resulting from a mutagen-induced mutation localized on the proximal arm of mouse chromosome 1 (33). This mutation is inherited as an autosomal dominant trait and the homozygote (Tsk2+/−/H11001) is viable, degenerating in utero (33); Tsk2/+ displays histopathological and biochemical abnormalities similar to those present in the skin of patients with SSC, including increased collagen content and expression of type I collagen genes (34).

Histological examination of skin samples showed marked thickening of the dermis and excessive deposition of thick collagen fibers, which extended deeply into the subdermal adipose tissue and occasionally surrounded the fascicles of the panniculus carnosus (34). The Tsk2/+ mice display a mononuclear cell infiltrate with numerous mononuclear cells in the lower dermis and in the adipose tissue septa (34).

The more extensively studied Tsk1/+ mouse model for SSC differs from Tsk2/+ in several respects. The mutation for Tsk1 is on chromosome 2 and affects the fibrillin-1 gene (35). Tsk1/+ mice do not have an inflammatory mononuclear cell infiltrate in the dermis. However, Tsk1/+ mice develop ANAs including anti-Scl70 Abs, which were detected in supernatants from hybridomas established from Tsk1/+ splenocytes (36). The majority of the autoantibodies which arose in Tsk1/+ mice were shown to share a conserved heptapeptide sequence motif, YNEKFKG, in their H chains (37) and B cell clones producing autoantibodies in both the Tsk1/+ mouse and in SSC patients have been shown to share an interspecies cross-reactive Id (38).

It is clear that both Tsk1/+ and Tsk2/+ mice display some of the pathologic features of SSC, although neither model is a perfect mimic for the human disease. However, these mouse models can be useful in elucidating the alterations in the molecular pathway(s) that regulate a variety of physiologic processes leading to tissue fibrosis and in understanding the relationship between fibrosis and autoimmunity. The prudent interpretation of results obtained with the Tsk2/+ murine model for SSC will contribute to our knowledge.
of the mechanisms underlying the pathogenesis of this presently incurable disease.

In the present study, we examined the humoral immunologic features of the Tsk2/+ mouse in search of indicators of an autoimmune component in this mutant mouse. We report here for the first time the presence of numerous autoantibodies in the immune component in this mutant mouse. We report here for the mouse, a model for SSc.

### Materials and Methods

#### Mouse

Sixty Tsk2/+ mice (4–24 mo old) of both sexes were used to examine the presence of autoantibodies. The genetic background of the Tsk2/+ mice was (C3H/HeJ × CAST/ei)F1-Tsk2/+; MRL/lpr lupus prone (MRL) mice were used as positive controls and CAST/ei and C3H/HeJ mice as negative controls. The controls were age-matched with the Tsk2/+ mice.

#### Immunofluorescence assay for ANA

Slides precoated with HEP-2 cells (Bio-Rad) were used to evaluate the presence of ANA in dilutions of plasma obtained from retro-orbital bleeding of Tsk2/+ CAST/ei, and C3H/HeJ mice. A FITC-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnologies) at a 1/20 dilution was used as the secondary Ab. All mouse plasma samples were tested at a 1/20 dilution in PBS. Slides were examined on a Nikon Eclipse E600 fluorescence microscope. Plasma from MRL mice served as a positive staining control and plasma from C3H/HeJ and CAST/ei mice as negative controls. The controls were age-matched with the Tsk2/+ mice.

#### ELISAs for anti-Scl70, CENP-B, RNA-polymerase II, and dsDNA Abs

Microtiter plates (Costar/Corning) were coated with 100 μl of pure topoisomerase 1, CENP-B (Santa Cruz Biotechnologies), dsDNA or RNA-polymerase II (Sigma-Aldrich) at a concentration of 5 μg/ml in PBS and incubated overnight at 4°C. Plates were blocked with 1% BSA/PBS for 2 h (Sigma-Aldrich). Mouse plasma samples were diluted 1/20, 1/50, and 1/100 into 1% BSA/PBS; 100 μl was added to the microtiter plates and incubated for 1 h at room temperature. Plates were washed with PBS/ Tween 20 (Sigma-Aldrich) and 100 μl of goat-anti-mouse IgG HRP (Santa Cruz Biotechnologies) diluted 1/6000 into PBS was added to each well. Bound IgG was demonstrated by adding 100 μl/well of 3,3’5,5’ tetramethyl benzidine (Sigma-Aldrich) as substrate. The plates were read at 450 nm on a microplate reader (Labsystems Multiscan Plus; Fisher). To demonstrate the specificity of the Scl70 and dsDNA assays, inhibition studies with the appropriate Ag were conducted as above except that specific Ag, either Scl70 or dsDNA, was added to the plasma samples to a final concentration of 20 μg/ml and then incubated overnight at 4°C before addition to the microtiter plates. ELISA background determination was performed using pooled plasma samples from five C3H/HeJ mice and the OD readings from at least five microtiter wells were averaged.

#### ELISAs for RNP/Sm Abs

Plates precoated with the RNP/Sm Ag derived from calf thymus were obtained from TheraTest Laboratories. Mouse plasma samples at 1/20, 1/50, and 1/100 dilutions were added and incubated according to the manufacturer’s protocol. The bound IgG was demonstrated as described above with goat-anti-mouse IgG conjugated to HRP followed by the appropriate substrate.

#### Immunofluorescence assay for anti-dsDNA Abs

The Critecia luciiae kinetoplast substrate (Inova Diagnostics) was used to test for the presence of anti-dsDNA Abs in the 60 plasma samples from Tsk2/+ mice and all controls at a 1/20 dilution. FITC-conjugated goat-anti-mouse IgG was used as the secondary Ab at a 1/20 dilution.

#### Skin thickness measurements

Skin was removed from the interscapular region of the back, fixed embedded in paraffin, sectioned (4-μm thick), and stained with Masson’s Trichrome stain. The stained cross-sections of skin were viewed under the microscope, photographed, and printed such that the final magnification was ×200. The distance from the epidermis to the panniculus carnosus was measured directly from the photograph with a millimeter ruler and divided by 200 to obtain the actual skin thickness.

#### Statistical analysis

Values of p for a confidence level of 95% were obtained using the χ² method with the Yates correction (software program Instat, version 3.05; Graph Pad).

### Results

#### ANA patterns observed by indirect immunofluorescence (IIF)

All Tsk2/+ and control mouse plasma were tested first by IIF to determine whether they were positive for ANA and to determine the type of ANA pattern present. Fig. 1 shows the seven different patterns of ANA IIF with HEP-2 cells obtained with Tsk2/+ mouse plasma.

A total of 60 Tsk2/+, 60 CAST/ei, and 30 C3H/HeJ control plasma samples were tested. The results of the ANA assays are summarized in Table I. The results for the Tsk2/+ and CAST/ei mice are shown for three groups of mice: those mice from 4 to 6 mo of age, those mice from 7 to 12 mo of age and those mice from 13 to 24 mo of age. Overall 92% of Tsk2/+ mice tested positive in the ANA assay. In the youngest Tsk2/+ mice, 79% of the animals were positive and the percentage of animals with positive results increased with age, so that 100% of the Tsk2/+ mice older than 12 mo were positive for ANA. By comparison, the CAST/ei mice to which the Tsk2/+ mice were paired for breeding were all negative except for the plasma from one CAST/ei mouse ≤6 mo of age. The C3H/HeJ mice showed no positive ANAs at any age tested. These differences between Tsk2/+ mice and either CAST/ei or C3H/HeJ mice are statistically significant as shown in Table I.

#### Distribution of ANA patterns by sex in Tsk2/+ mouse plasma

The number of positive mouse plasma samples is plotted on the x-axis and the type of pattern observed is shown on the y-axis.

#### Distribution of ANA patterns by age in Tsk2/+ mouse plasma

The number of positive mouse plasma samples is plotted on the x-axis and the type of pattern observed is shown on the y-axis.
Patterns of ANA as a function of age and sex

As shown in Fig. 1, ANA reactions with Tsk2/+ plasma produced seven immunofluorescence patterns. The frequency of these patterns as a function of age is shown in Fig. 2. Although the number of plasma samples for the Tsk2/+ mice was small for each pattern, it appears that the number of mice positive with a homogeneous/rim and a homogeneous/speckled pattern increases after 1 year of age, but these changes were not statistically significant. Four mice of the 60 mice tested showed a centromere pattern and only 1 mouse of 60 showed the nucleolar pattern in the Tsk2/+ mouse plasma samples.

In addition to correlating age with the ANA patterns observed in Tsk2/+ plasma, the frequency of each immunofluorescence pattern was correlated to the sex of the Tsk2/+ mouse (Fig. 3). Overall the percentage of female mice which were positive (93%) was slightly higher than for males (86%). The majority of plasma samples from female Tsk2/+ mice had a homogenous/speckled or speckled pattern followed by a homogenous pattern. There were three plasma samples, which showed the centromere pattern, five that showed the rim pattern, and only one sample showed the nucleolar pattern. In plasma samples from Tsk2/+ males, the distribution of ANA patterns was similar but the difference between the more highly represented patterns (homogeneous/speckled and speckled) and the less represented patterns (rim and nucleolar) was minimal. Although these results suggest that the male and female Tsk2/+ mice may develop ANA patterns with different frequencies, the differences reported here are not statistically significant, because of the small number of animals in each group.

Identification of Ags recognized by autoantibodies in Tsk2/+ mice

Five ELISAs were chosen to identify the molecular targets recognized by the autoantibodies in Tsk2/+ mouse plasma samples. These ELISAs were performed using the following Ags: Scl70 (topoisomerase-I), centromere (CENP-B), dsDNA, RNA-polymerase II, and RNP/Sm. The decision to test for autoantibodies to these five Ags was made because it is known that specific immunofluorescence patterns correlate with the presence of a given autoantibody (7). The results of ELISAs performed to detect autoantibodies directed against Scl70 are shown in Fig. 4. From the 60 Tsk2/+ plasma samples, 56 (93%) were positive for Scl-70 at a 1/50 dilution (Fig. 4A). There was substantial variation in the titer observed for the different mice; the highest value was over 2.5 OD units and the lowest value was 0.42 OD units (only slightly above a background of 0.4 OD units). None of these mice showed signs of pulmonary fibrosis (data not shown). In contrast only 3 plasma samples...
samples from the 60 CAST/ei mice were above background and the highest titer observed in this group of animals was 0.45 OD units. The plasma samples from the 17 C3H/HeJ mice were all below background levels. The difference between the results obtained with Tsk2/+ and the controls was significant at \( p < 0.001 \) either when the average OD value for each group (0.867 for Tsk2, 0.273 for CAST and 0.198 for C3H) or the number of plasma samples higher than background were compared (Tsk2:CAST: C3H = 56:3:0). It can be seen that the titers are highest when plasma diluted at 1/20 was used and lowest when plasma diluted at 1/100 was used. In addition, the shape of the curves at all dilutions is similar, indicating the validity of these results (Fig. 4B).

To insure the specificity of these ELISA results, soluble Scl70 (20 \( \mu \text{g/ml} \)) or soluble dsDNA was incubated with the Tsk2/+ mouse plasma samples before their addition to the Scl70-coated ELISA plates. Preincubation of plasma samples with soluble Scl70 completely abolished the ELISA reaction whereas preincubation with dsDNA had no effect on the ELISA reaction (Fig. 4C). These experiments validated the specificity of the ELISA for the detection of Scl70 and showed that there was no cross-reactivity with dsDNA.

The results of the ELISA to detect autoantibodies directed against CENP-B are shown in Fig. 5. The number of positive Tsk2/+ plasma samples with an OD value above the background level of 0.18 OD units was 49 of the 60 tested (82%). In contrast, the number of positive CAST/ei plasma samples was 5 (8%) and all C3H/HeJ plasma samples were equal or less than background. However, it should be noted that whereas the CAST/ei mice all failed to develop a high titer of anti-Scl70 Abs, that is not the case for anti-centromere Abs. Three CAST/ei plasma samples had a titer as high as that observed in the 12 highest Tsk2/+ plasma samples. Nevertheless, the difference between Tsk2/+ plasma samples and CAST/ei plasma samples was significant at \( p < 0.001 \) when either the average OD value for each group (0.433 for Tsk2, 0.159 for CAST, and 0.157 for C3H) or the number of plasma samples higher than background were compared (Tsk2:CAST: C3H = 49:5:0). The significance of the few high titer CAST/ei plasma samples is not known. All C3H/HeJ plasma samples were below background. The ELISAs performed to detect autoantibodies to RNP/Sm showed that 5% of the plasma samples from Tsk2/+ and none of the plasma from the controls were positive; the ELISA results for RNA-polymerase II were negative in all samples (data not shown).

Abs to dsDNA were detected by immunofluorescence in 36 of 46 Tsk2/+ plasma samples assayed. The results are shown in Fig. 6. There were 29 females of 36 and 7 males of 10 positive for anti-dsDNA autoantibodies. Only two CAST/ei plasma samples were positive for anti-dsDNA autoantibodies (data not shown). The difference between the number of Tsk2/+ and CAST/ei plasma samples, which were positive for anti-dsDNA autoantibodies, was significant at the \( p < 0.001 \) level.

The results obtained by IIF showing the presence of autoantibodies to dsDNA were confirmed by ELISA. Twenty-one Tsk2/+ and CAST/ei representative samples were assayed. The results are shown in Fig. 7. Sixteen of the 21 Tsk2/+ plasma samples (76%) and 3 of the CAST/ei plasma samples (14%) were positive for autoantibodies to dsDNA. The difference between the two groups and between the average OD for each group was significant at the \( p < 0.001 \) level. It can be seen that the titers are highest when plasma diluted at 1/20 was used and lowest when plasma diluted at 1/100 was used. In addition, the shape of the curves at all dilutions is similar, indicating the validity of these results (Fig. 7B).

To insure the specificity of these ELISA results, soluble dsDNA (20 \( \mu \text{g/ml} \)) was incubated with the Tsk2/+ mouse plasma samples before their addition to the dsDNA-coated ELISA plates. Preincubation of plasma samples with soluble dsDNA completely abolished the ELISA reaction in all but one sample (Fig. 7C) whereas preincubation with normal C3H/HeJ control plasma had no effect on the ELISA reaction (Fig. 7D). These experiments validated the specificity of the ELISA for the detection of dsDNA and showed that control plasma had no effect on the assay.

**FIGURE 5.** ELISA to detect anti-centromere Abs in plasma. The absorbance in OD units at 450 nm is plotted on the y-axis and each plasma sample is plotted on the x-axis. Background absorbance not subtracted from the above values. A. Comparison of the amount of anti-centromere Ab in the plasma of Tsk2/+, CAST/ei, and C3H/HeJ mice. B. Comparison of the amount of anti-centromere Ab in Tsk2/+ plasma samples diluted 1/20, 1/50, and 1/100.
Correlation of autoantibody titer with skin thickness in Tsk2+/+ mice

The ELISA titers for anti-Scl70, anti-CENP-B, and anti-dsDNA for each Tsk2+/+ mouse sample (see Figs. 4A, 5A, 7A, and Table II) were ranked from highest to lowest. The 10 skin thickness measurements which corresponded to the highest ELISA titers were averaged and compared with the average of the 10 skin thickness values which corresponded to the lowest ELISA titers. The results are shown in Table III. There was a positive correlation between higher anti-CENP-B titers and greater skin thickness which was statistically significant. In contrast, no correlation was observed between the ELISA titers of either anti-Scl70 or anti-dsDNA and skin thickness.

![Correlation of autoantibody titer with skin thickness in Tsk2+/+ mice](image)
The numbers are absorbance units at 450 nM; ‘/’ the sample was not tested; ‘–’ the sample was negative for the given assay. Values ± variance are shown for anti-Scl70, anti-CENP-B, and anti-dsDNA.

**Discussion**

ANAs characterize the autoantibody response in SSc as well as SLE, mixed connective tissue disease, and other autoimmune diseases (39–41). ANAs are present in over 90% of patients with SSc. In these patients, autoantibodies are present, which target structures such as the kinetochore (centromere), topoisomerase I, or RNA-polymerase (40–42). It is believed that there is a correlation between the ANA profile and the type of connective tissue disease or the clinical subset of SSc, limited vs diffuse (3, 4, 7, 22).

It is expected that the identification of the Ags against which the autoantibodies are directed will lead to an understanding of the disease or the clinical subset of SSc, limited vs diffuse (3, 4, 7, 22).
The most striking difference between the mouse data and what has been reported in humans with SSc is the fact that the majority of Tsk2/+ mouse plasma samples were positive for multiple autoantibodies. Of the 60 mouse plasma samples shown (Table II), only a small number of the samples tested positive for only a single autoantibody. One sample was negative for all three autoantibodies and at least 38 samples showed a positive reaction for multiple autoantibodies. Two plasma samples were positive for Scl70 alone, five others for anti-dsDNA alone, and one for anti-CENP-B alone. The four plasma samples which showed a centromere pattern by ANA IIF were positive for anti-CENP-B as would be expected but were also positive for anti-Scl70 and anti-dsDNA. Such findings would be rare in a population of humans with SSc, in which the majority of patients would have developed autoantibodies only to Scl70 or CENP-B and not to multiple autoantigens (1, 3, 4). Although the finding that almost all of the Tsk2/+ mice develop autoantibodies to anti-Scl70 or anti-CENP-B is certainly similar to what has been reported for SSc, the fact that the mouse develops multiple autoantibodies to both of these Ags in 52 of the 60 Tsk2/+ plasma samples is a remarkable difference from what happens in humans (1, 3, 7, 22). In addition, it is of great interest that this mouse also has autoimmune features similar to those reported for SLE, namely autoantibodies to dsDNA (46) and these autoantibodies to dsDNA are present predominantly in the same mice, which develop anti-Scl70 and/or anti-CENP-B Abs. Although the significance of this unique display of autoantibodies in the Tsk2/+ mouse is not yet clear, it is probable that understanding the mechanism(s) responsible for the production of this autoimmune response in the Tsk2/+ mouse will lead to a better understanding of the role of autoantibodies in one or more autoimmune connective tissue diseases. The fact that the Tsk2/+ mouse has the phenotype of dermal fibrosis (34) that resembles the cutaneous alterations observed in SSc patients suggests that this mouse will be of interest in studying the interrelationship between the autoimmune response and fibrosis.

To this end, an important first question is whether there is a correlation between the appearance and/or titer of these autoantibodies and the fibrosis observed in the Tsk2/+ mouse. In Table III, the ELISA titers for anti-Scl70, anti-centromere (CENP-B), and anti-dsDNA have been divided into a high and low group and averaged. The corresponding average skin thickness for each group is shown and indicate that there was a statistically significant correlation linking increased skin thickness with higher titers of the ELISA titers for anti-Scl70, anti-centromere (CENP-B), and anti-dsDNA. The fact that the Tsk2/+ mouse develops autoantibodies to dsDNA and anti-centromere reactivity (42). Therefore, it seems likely that the discrepancy between the ANA results and the ELISA results are due to a more specific ELISA using purified mouse CENP-B and because we chose not to count nonclassical, coarse-speckled ANA patterns as positive for anti-centromere reactivity. In addition, human sera which are positive for anti-topoisomerase by ELISA have been reported to have a speckled pattern by ANA which can be accompanied by nucleolar staining (1, 3, 7, 22).

The Tsk2/+ mutation resides on the proximal arm of chromosome 1 within an interval of ~1 cM (47). Several candidate genes have been identified within this interval one of which might be responsible for the generation of both autoimmunity and fibrosis in other connective tissue diseases (22). The results presented here show for the first time that the Tsk2/+ mouse displays evidence of autoimmunity. Approximately 90% of the plasma samples from these mice had a positive ANA response (when the plasma was diluted 1/20), which included seven ANA immunofluorescence patterns. The majority of the mice had either a speckled, homogeneous, or homogeneous/speckled pattern. When the mouse plasma was tested at a 1/40 dilution (as human sera is tested) only 35% of the Tsk2/+ plasma samples were positive. We chose to report the results from the 1/20 dilution, because the assay using HEp-2 human cells is optimized for human sera, not mouse plasma, and the results we obtained at this dilution were in closer agreement with the ELISA results than when the mouse plasma was tested at 1/40. Further testing by ELISA demonstrated that almost all homogenous, homogeneous/rim, rim and centromere patterns were positive for both anti-dsDNA and anti-Scl70 autoantibodies (Table I). Almost all of the Tsk2/+ plasma samples with the speckled pattern were positive for Scl70 which correlates with what has been observed in SSc (7, 22, 26).

We also found CENP-B autoantibodies by ELISA in Tsk2/+ plasma, which correlates with the centromere pattern. Most studies show that a majority of SSc patients with this pattern have autoantibodies to centromere and exhibit clinical features of limited cutaneous SSC (6, 43). It should be noted that we detected a larger number of Tsk2/+ mouse plasma samples positive for anti-centromere Abs by an ELISA specific for mouse CENP-B than by ANA IIF using HEp-2 cells. With the ELISA, 82% of the Tsk2/+ plasma samples were positive whereas with HEp-2 cells only 5% were positive. Others have also reported increased sensitivity for anti-centromere Abs with an ELISA compared with the ANA IIF with HEp-2 cells (44, 45). This difference in our results was also influenced by the fact that we did not include 10 samples, which displayed the coarse-speckled ANA pattern as positive for centromere, although this pattern has been reported to indicate anti-centromere reactivity (42). Therefore, it seems likely that the discrepancy between the ANA results and the ELISA results are due to a more specific ELISA using purified mouse CENP-B and because we chose not to count nonclassical, coarse-speckled ANA patterns as positive for anti-centromere reactivity. In addition, human sera which are positive for anti-topoisomerase by ELISA have been reported to have a speckled pattern by ANA which can be accompanied by nucleolar staining (1, 3, 7, 22). In contrast, the topoisomerase positive Tsk2/+ mouse plasma samples did not display any nucleolar staining. The significance of these different staining patterns in Tsk2/+ mouse plasma and SSc patients' sera is not known.

Table III. Correlation between the titer of anti-Scl70, anti-CENP, and anti-dsDNA autoantibody skin thickness in Tsk2/+ mice

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Average Titer</th>
<th>Average Thickness in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA high</td>
<td>1.700 ± 0.734</td>
<td>0.083 ± 0.030</td>
</tr>
<tr>
<td>dsDNA low</td>
<td>0.385 ± 0.130</td>
<td>0.088 ± 0.032</td>
</tr>
<tr>
<td>Sc170 HIGH</td>
<td>1.507 ± 0.531</td>
<td>0.076 ± 0.034</td>
</tr>
<tr>
<td>Sc170 LOW</td>
<td>0.123 ± 0.024</td>
<td>0.084 ± 0.041</td>
</tr>
<tr>
<td>CENP HIGH</td>
<td>0.789 ± 0.186</td>
<td>0.104 ± 0.027</td>
</tr>
<tr>
<td>CENP LOW</td>
<td>0.186 ± 0.017</td>
<td>0.074 ± 0.030</td>
</tr>
</tbody>
</table>

**p** Value < 0.001 < 0.05

* The skin thickness values were divided into two groups. The 10 skin thickness measurements which corresponded to the highest ELISA titers for anti-dsDNA, anti-Scl70, and anti-CENP Abs were averaged and compared to the average of the 10 skin thickness values which corresponded to the lowest ELISA titers. A significant correlation between higher anti-CENP-B titer and thicker skin was demonstrated.
this mouse. One possible pathway recently proposed in the production of ANAs in SSc and other autoimmune diseases is molecular mimicry (48) in which persistent viral infection has been proposed as the causative event (49). Also Saito et al. (50) have suggested that B cells link the production of autoantibodies and fibrosis in the Tsk2+/+ mouse through a CD-19-dependent pathway. With further study of the Tsk2+/+ mouse, it will be possible to identify the causative gene and to elucidate the molecular pathway(s) leading to autoimmunity and fibrosis and to better understand how these two facets of SSc and other autoimmune connective tissue diseases are interrelated.

Disclosures
The authors have no financial conflict of interest.

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