Antitopoisomerase I Monoclonal Autoantibodies from Scleroderma Patients and Tight Skin Mouse Interact with Similar Epitopes

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Summary

We have generated for the first time monoclonal antibodies (mAbs) specific for topoisomerase I (topo I) from scleroderma patients, and tight skin mice which develop a scleroderma-like syndrome. The epitope specificity of these antibodies has been determined using a series of fusion proteins containing contiguous portions of topo I polypeptide. Western blot analysis demonstrated that both human and mouse mAbs bound strongly to fusion protein C encompassing the NH2-terminal portion of the enzyme, and weakly to fusion proteins F and G containing regions close to the COOH-terminal end of the molecule. This crossreactivity is related to a tripeptide sequence homology in F, G, and C fusion proteins. It is interesting that a pentapeptide sequence homologous to that in fusion protein C was identified in the UL70 protein of cytomegalovirus, suggesting that activation of autoreactive B cell clones by molecular mimicry is possible. Both human and mouse mAbs exhibiting the same antigen specificity, also share an interspecies cross-reactive idiotope. These data suggest that B cell clones producing antitopo autoantibodies present in human and mouse repertoire are conserved during phylogeny, and are activated during the development of scleroderma disease.

Progressive systemic sclerosis (PSS) is a connective tissue disease affecting skin (scleroderma) and internal organs such as lungs, heart, kidneys, and gastrointestinal tract. Autoantibodies to various nuclear proteins (1–4) and collagens (5) are found in 40–95% of patients with scleroderma. The major antinuclear antibodies found in patients with PSS are anti-Scl70 autoantibodies. Scl70 antigen represents a crude nuclear extract from thymus that is used for clinical diagnosis of scleroderma. Later studies show that topoisomerase I (topo I) is the major target autoantigen present in Scl70 preparation (2). These autoantibodies are not present in patients with other systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren’s syndrome, or rheumatoid arthritis. Recently, we have demonstrated the presence of anti-topo I antibodies in tight skin mice (TSK) (6). This mutant strain develops cutaneous hyperplasia with histopathological characteristics similar to those of human scleroderma (7), subsequent to hyperproduction of collagen. Furthermore, we have shown that the IgG fraction of scleroderma patients’ sera with antitopo I antibodies, inhibited the binding of TSK mAbs to topo I (6). These results strongly suggested that antitopo I autoantibodies present in the sera of scleroderma patients and TSK mice recognize similar or very closely related epitopes.

To define these epitopes, we have used antitopo I monoclonal autoantibodies obtained from scleroderma patients and TSK mice in competitive inhibition assays and Western blotting analysis using recombinant topo I fusion proteins containing defined portions of topo I. The results presented in this study demonstrate that human mAbs inhibit the binding of TSK mAbs to topo I, and that both human and mouse mAbs bind to the same peptide fragments of the enzyme expressed as fusion protein.

Abbreviations used in this paper: CMV, cytomegalovirus; GST, glutathione-S-transferase; PSS, progressive systemic sclerosis; SLE, systemic lupus erythematosus; Sm, Smith antigen; topo I, topoisomerase I; TSK, tight skin mouse; VH, variable region of heavy chain.
Materials and Methods

mAbs. The preparation and determination of the binding specificity of anti-topo I mAbs derived from TSK mice have been previously described (6). EBV-transformed B cell lines were prepared according to the method described by Davidson et al. (8). The cell lines producing antitopo I antibodies were subcloned by diluting one or three cells/well in RPMI medium supplemented with 10% FCS, and 20% of supernatant from murine macrophage cell line J774. The supernatants from the subclones were rescreened by RIA. GM17-1, GM3-16 are TSK antitopo I mAbs previously described (6). G17-13, a TSK mAb recognizing a cross-reactive Id on antitopo I antibodies was previously described (6).

Antigens. Highly purified calf thymus topoisomerase I of 70 kD used in this study was obtained from Bethesda Research Laboratories (Bethesda, MD). Sc170 Ag was obtained from Alpha Antigens, Inc. (Columbia, MO). Rat type I collagen was a kind gift from Dr. R. Holmdahl (Uppsala University, Uppsala, Sweden). Ag phage DNA was prepared in our laboratory and used as dsDNA. RNA polymerase was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Recombinant proteins consisting of various portions of topo I fused to glutathione-S-transferase (GST) have been described (Cram, D. S., N. Fisicaro, J. McVeilage, R. L. Coppel, and L. C. Harrison, manuscript submitted for publication). The peptide fragments of topo I, with corresponding amino acid residues contained in each fusion protein, are shown in Fig. 1.

Antigen-binding Studies. (a) Direct binding assay. Microtiter plates were coated with topo I (3 µg/ml) in carbonate buffer pH 9.6 at 4°C overnight. After washing, the plates were blocked with 1% BSA in PBS for 1 h, washed three times, and incubated with chromatographically purified TSK or human antibodies. After extensive washing, 50,000 cpm of 125I-labeled rat monoclonal anti-mouse κ antibody or rabbit F(ab′)2 antibody was added and incubated for another 2 h. After washing, the bound radioactivity was measured in a γ spectrometer. The purification of mAbs from the culture medium was carried out on Sepharose 4B, (Pharmacia Fine Chemicals, Piscataway, NJ) coupled with monoclonal rat anti-mouse κ antibody or rabbit F(ab′)2 antibody columns, as previously described (6).

Competitive Inhibition Assay. Pilot experiments were carried out to determine the concentration of mAbs giving 50% of maximal binding to topo I-coated plates. Antibodies at the concentrations corresponding to 50% binding were incubated for 30 min at room temperature with various amounts of topo I, type I collagen, RNA polymerase, or dsDNA, and then transferred to topo I-coated plates. The binding of antitopo I antibodies was measured as described above. Assay of inhibition of the binding of TSK mAb by human mAb was carried out by addition of various amounts of human mAbs and 50,000 cpm of 125I-labeled TSK mAb to microtiter plates coated with topo I.

Western Blotting. Purified topo I fusion proteins were run on 7.5% polyacrylamide gels in the presence of SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) at 100 V for 5 h at 4°C. The filters were blocked with 5% BSA and then incubated with TSK mouse or human mAbs (2 µg/ml) or 1:800 dilution of antitopo I positive serum from a scleroderma patient, in PBS containing 5% BSA and 0.1% Tween 20. After extensive washing with PBS containing 0.1% Tween 20, filters were incubated for 90 min at room temperature with 125I-rat anti-mouse κ mAb in the case of filters incubated with TSK mouse mAb or 125I-rabbit F(ab′)2 anti-human γ antibody in the case of filters incubated with human antibodies. Filters were washed, dried, and autoradiographed as previously described (6).

Results

Specificity of Monoclonal Antitopo I Autoantibodies. Among the panel of 14 TSK mouse antitopo I autoantibodies, two IgG2a antibodies were chosen for this study, since it was previously shown that their binding to topo I were inhibited by IgG from scleroderma patients positive for Sc170 antibody (6). The data presented in Table 1 show that TSK mouse mAbs GM17-1 and GM3-16, and human mAbs N3A, N36, and N188 obtained from scleroderma patients, exhibited significant binding to Sc170 and purified topo I, compared with control antibodies UPC10, a BALB/c myeloma protein of the same isotype, or BM5 mAb (IgG1) with unknown specificity, ob-

![Figure 1](image1.png)

Figure 1. Schematic diagram of topo I showing the regions enclosed by fusion proteins A and C-H.

![Figure 2](image2.png)

Figure 2. Dose-effect inhibition of binding of human mAb N188 to topo I by topo I, collagen I, dsDNA, and RNA polymerase.

![Figure 3](image3.png)

Figure 3. Dose-effect inhibition of binding of TSK mAbs GM3-16 (A) and GM17-1 (B) by human mAbs N3A, N36, N188, and BM5. 50,000 cpm of 125I-labeled TSK mAbs corresponding to 0.1 µg were incubated together with various amounts of cold human mAbs.

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Table 1. Binding Activity of TSK and Human mAbs to Scl 70 and Topo I

| Designation of mAb | Origin                  | Isotype | Binding to:   |
|-------------------|-------------------------|---------|--------------|
|                   |                         |         | BSA | Scl 70 | Topo I |
| GM17-1            | TSK mouse               | IgG2a   | 325 ± 11* | 5,809 ± 20 | 1,420 ± 65 |
| GM3-16            | TSK mouse               | IgG2a   | 556 ± 48  | 5,359 ± 325 | 3,998 ± 60 |
| UPC10*            | BALB/c mouse            | IgG2a   | 301 ± 0   | 509 ± 3     | 348 ± 37   |
| N3A               | Scleroderma patient     | IgG1    | 177 ± 34  | 2,123 ± 25  | 1,766 ± 45 |
| N36               | Scleroderma patient     | IgG1    | 54 ± 16   | 2,758 ± 50  | 1,951 ± 20 |
| N188              | Scleroderma patient     | IgG1    | 112 ± 4   | 5,881 ± 157 | 3,062 ± 17 |
| BM5*              | Multiple myeloma patient| IgG1    | 68 ± 6    | 179 ± 16    | 120 ± 3    |

* cpm average ± SD.

† Myeloma protein displaying β2-6 fructosan binding activity.

‡ Human mAb with unknown binding activity.

We have previously shown that the binding of TSK mouse antibodies to topo I was not inhibited by type II collagen, dsDNA, or RNA polymerase (6). Data depicted in Fig. 2 show that the binding of human mAb N188 to topo I was inhibited by more than 50% with 3 and 10 μg/ml of topo I, but not with type I collagen, dsDNA, or RNA polymerase. These data clearly demonstrate that both human and mouse antibodies are specific for topo I and Scl70.

In further studies, we investigated the ability of human antibodies to recognize topo I. We expressed recombinant topo I in E. coli and used it as an antigen to produce human mAbs. The binding activity of these mAbs to topo I was compared with that of the TSK mouse antibodies. We found that human mAbs were able to recognize topo I with high specificity, similar to the TSK mouse antibodies. These findings suggest that topo I is a promising target for the development of new therapeutic strategies for treating autoimmune diseases.

Figure 4. Western blot analysis.

(A) N188 human mAb; (B) scleroderma patient's serum; (C) TSK mAb GM3-16; (D) human myeloma IgG BM5. Lane 1, purified topo I; lane 2, fusion protein A; lane 3, fusion protein C; lane 4, fusion protein D; lane 5, fusion protein E; lane 6, fusion protein F; lane 7, fusion protein G; lane 8, fusion protein H; and lane 9, fusion partner protein GST. Each lane was loaded with 2 μg of protein estimated by the intensity of bands by Coomassie blue staining. Protein-transferred filters were incubated with 2 μg/ml of N188, GM3-16, and BM5 and 1:800 dilution of scleroderma patient's serum for 90 min at room temperature. The bound antibodies were revealed with rabbit F(ab')2 anti-human γ antibody, or rat monoclonal anti-mouse κ antibody labeled with 125I.
mAbs to inhibit the binding of $^{125}$I-labeled TSK mouse mAbs to topo I. The binding of mouse mAb GM3-16 was inhibited in a dose-related manner by all three human antitopo I mAbs with 50% inhibition at a concentration of 10 $\mu$g/ml (Fig. 3 A). In contrast, the binding of GM17-1 was poorly inhibited by human mAbs, with only 20% inhibition at a concentration of 10 $\mu$g/ml (Fig. 3 B). These results demonstrate that GM3-16 and the human mAbs probably recognize similar or closely related epitopes.

**Western Blot Analysis.** To define the epitopes recognized by the TSK mouse and human mAbs, seven GST-topo I fusion proteins (see Fig. 1) were used as antigens in immunoblotting experiments. The data depicted in Fig. 4 and summarized in Table 2 show that human mAb N188 binds to purified topo I (lane 1), binds strongly to fusion protein C (lane 3), and very weakly to fusion proteins F and G (lanes 6 and 7) in Fig. 4 A. Scleroderma patient's serum binds to purified topo I, and to fusion proteins A, C, F, G, and H (Fig. 4 B). Human myeloma IgG1 (BM5) did not bind to any of the fusion proteins (Fig. 4 D). TSK mAb GM3-16, like human mAb N188, binds to topo I, as well as to fusion proteins C, and weakly to F and G (Fig. 4 C). These results show that human and TSK mAbs that bind to purified topo I recognize a major epitope located on fusion protein C, and also interact weakly with epitopes present on fusion proteins F and G. By contrast, human polyclonal antibodies recognize additional epitopes found on fusion proteins A and H.

**Shared Id between TSK Mouse and Human mAbs.** In a previous study we have described the isolation of a TSK mouse mAb that recognizes a cross-reactive idiotype on TSK antitopo I antibodies (6). L350-7 is a prototype TSK mouse anti-topo I mAb expressing cross-reactive Id. A study of the expression of this Id in 20 sera positive for Scl70 antibody, and 16 sera negative for Scl70 antibody from scleroderma patients, and 11 sera from healthy subjects was undertaken. Results of this experiment showed that the reaction between Id and anti-Id antibody was inhibited by Ig from 16 of 20 Scl70 positive sera, but not by others (data not shown). Based on this information, in further experiments, we have studied the ability of human mAb N188 and TSK mouse mAb GM3-16 to inhibit the binding of G17-13 monoclonal anti-Id antibody to L350-7 mAb bearing cross-reactive Id. We have previously shown that L350-7 mAb bears cross-reactive Id of TSK antitopo I antibodies (6). The data depicted in Fig. 5 show both N188 and GM3-16 inhibited, to a similar extent, the binding of anti-Id antibody to L350-7 idiotype. These data indicate that human and mouse antibodies with the same fine specificity share an interspecies cross-reactive Id.

**Discussion**

Autoimmune diseases are commonly associated with antibodies against cytoplasmic or nuclear enzymes (e.g., antithyroid peroxidase autoantibodies in Hashimoto thyroiditis [10], antipyruvate dehydrogenase complex antibodies in primary biliary cirrhosis [11], antiglutamic acid decarboxylase antibodies in insulin-dependent diabetes [12], anti-H K ATPase antibodies in pernicious anemia [13], and antibodies to t-RNA synthetases in polymyositis [14]. There is a lack of information on the epitope specificities of autoantibodies against these enzyme autoantigens. Antibodies against topo I are found in scleroderma, but not in other systemic autoimmune diseases. D'Apra et al. (15) have shown that scleroderma sera contain antitopo I antibodies that bind to at least two sets of epitopes present in fusion proteins, one in amino acid residues 171-484, and the other in the 281 COOH-terminal residues. Piccini et al. (16), using a different panel of fusion proteins, found that a set of epitopes present between amino acid residues 405-485 are recognized by scleroderma sera. Our results presented here also show that polyclonal autoantibodies present in scleroderma sera react with several epitopes. It is not clear from these observations whether the antibodies that react with a dominant epitope also cross-react with other epitopes, or whether specific antibodies recognize each epitope.

**Table 2. Epitope Mapping of Human and Mouse Antitopoisomerase I Autoantibodies**

| Ab             | Topo I | GST | A  | C  | F  | G  | H  |
|----------------|--------|-----|----|----|----|----|----|
| Scleroderma serum | +      | -   | +  | +  | +  | ±  | +  |
| Human mAb      | +      | -   | -  | +  | ±  | ±  | ±  |
| TSK mouse mAb  | +      | -   | -  | -  | +  | ±  | ±  |

**Figure 5. Competitive inhibition of the binding of $^{125}$I-labeled G17-13 monoclonal anti-Id antibody to L350-7 antitopo I mAb by TSK mAb GM3-16 and human mAb N188 antitopo I antibody. L350-7 is a prototype of TSK antitopo I mAb expressing cross-reactive Id (see reference 6). The expression of cross-reactive Id was studied by competitive inhibition RIA, in which microtiter plates were coated with 10 $\mu$g L350-7 mAb, and then incubated with 50,000 cpm of $^{125}$I-GM17-13 anti-Id mAb, and various amounts of inhibitor antibodies, simultaneously. UPC10 is a BALB/c IgG2a with 92-6 fructosan binding activity, and BM5 is a human IgG1 myeloma protein.**
Antibodies against group A streptococci M protein are cross-reactive with cardiac myosin (19, 20). They also cross-react with the cytoplasm of neurons in rheumatic chorea (21). There are increasing examples of the presence of shared sequences between viral, bacterial, parasitic, and auto-antigens (22) that can be responsible for molecular mimicry.

Maul et al. (23) have reported that topo I and retroviral protein p30gag have some sequence similarity. The sequence of topo I contains six sequential residues shared with p30gag group specific protein of type C mammalian retroviruses. We compared the sequence of C fusion protein with those in the DNASIS database, and found that it shares five sequential residues 121–126 with UL70 protein of human cytomegalovirus. Studies are in progress to determine whether antitopo I autoantibodies from patients with scleroderma bind to the CMV protein containing these five homologous regions, or if the CMV peptide inhibits the binding of autoantibodies to topo I.

It is interesting to note that sequences of housekeeping enzymes in general, and enzymes involved in nucleic acid metabolism in particular, are quite conserved. Some of the autoantibodies against these enzymes even inhibit the catalytic activity. Thus, some antitopo I autoantibodies inhibit conversion of supercoiled DNA into a relaxed form (23), and anti-RNA polymerase antibodies inhibit the enzymatic activity (24). Because of the high degree of conservation of sequence homology between enzymes from different species, human and TSK mouse antitopo I autoantibodies bind to very similar or identical epitopes. Analysis of V gene repertoire and sequence determination of the mAbs will shed more light on the origin and generation of diversity of autoantibodies with similar fine specificities in different species.

Furthermore, our studies reveal that human and murine mAbs with the same immunochemical fine specificity, also share an interspecies cross-reactive Id. Similar observations have been made for anti-Sm antibodies in the MRL mouse and human SLE. Taket et al. (25), demonstrated that rabbit antiserum raised against a peptide corresponding to the CDR2 region of human anti-Sm antibody reacted with the corresponding peptide of mouse CDR2 region. They also showed that these anti-Id antibodies recognized mouse and human Ig H chains in Western blot analysis. Our studies suggest that V genes encoding autoantibodies specific for nuclear antigens are conserved during evolution, as is to be the case with anti-Sm (25) and anti-DNA antibodies (26).

Our results are important for three reasons. The first is the demonstration that B cell clones producing antitopo I antibodies with the same specificity are present in the human...
and mouse repertoire, and are activated during the course of disease, in scleroderma patients and TSK mice. Second, the presence of interspecies cross-reactive Id among antitopo I autoantibodies suggests a conservation of DNA segments encoding autoantibodies during evolution. Third, the use of fusion proteins could allow correlation between the presence of antibodies to specific epitopes, and the clinical features of autoimmune disease. Such studies may also provide insight into the pathogenesis of human scleroderma and TSK mouse disease.

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