

# Autoimmune Response to Proteasome Activator 28 $\alpha$ in Patients with Connective Tissue Diseases

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**ABSTRACT. Objective.** To determine the autoimmune response against proteasome activator 28 $\alpha$  (PA28 $\alpha$ ) in patients with various connective tissue diseases, and to compare the immunoreactivity between anti-PA28 $\alpha$  and anti-Ki antibodies.

**Methods.** Serum samples were obtained from 219 patients with various connective tissue diseases. cDNA encoding full-length human PA28 $\alpha$  and Ki were produced by polymerase chain reaction. Antigens were expressed as glutathione S-transferase (GST) fusion proteins. The immunoreactivity of serum for PA28 $\alpha$  and Ki was studied by Western blotting. An inhibition test was performed by ELISA using purified Ki antigen.

**Results.** Anti-PA28 $\alpha$  antibody was detected in serum from 23% of patients with systemic lupus erythematosus (SLE) and 24% with Sjögren's syndrome (SS). These rates were significantly higher than those for the other rheumatic diseases. Since both PA28 $\alpha$  and Ki are elements of the PA28 complex and their amino acid sequences share 40.2% homology, immunoreactivity to PA28 $\alpha$  was studied further. Among 27 anti-Ki positive serum samples, 13 samples (48%) also reacted with PA28 $\alpha$ , suggesting a relationship between anti-PA28 $\alpha$  and anti-Ki antibodies. To investigate whether this finding was due to the presence of cross-reacting epitopes for PA28 $\alpha$  and Ki antigens, an inhibition test was performed by ELISA. The reactivity to purified Ki antigen was not inhibited by preincubation with recombinant PA28 $\alpha$ .

**Conclusion.** Detection of anti-PA28 $\alpha$  antibody was significantly higher in serum from patients with SLE and SS. The relationship between anti-PA28 $\alpha$  and Ki antibodies suggests the importance of an antigen-driven system in the induction of an autoimmune response to PA28 complex. (J Rheumatol 2004;31:252-9)

*Key Indexing Terms:*

AUTOANTIBODY    PROTEASOME ACTIVATOR 28 $\alpha$     PROTEASOME ACTIVATOR 28 $\gamma$   
ANTI-KI ANTIBODY    SYSTEMIC LUPUS ERYTHEMATOSUS    SJÖGREN'S SYNDROME

Proteasome is known as an ATP-dependent proteolytic enzyme involved in antigen presentation on class I major histocompatibility (MHC) molecules<sup>1-6</sup>, and autoantibodies to its element, Ki, which was later identified as proteasome activator 28 $\gamma$  (PA28 $\gamma$ ), were first reported by Tojo, *et al*<sup>7</sup> in 1981. Antibodies to Ki can be specifically detected in 6.7% to 21.4% of patients with systemic lupus erythematosus (SLE), and are related to clinical features such as persistent arthritis, pericarditis, sicca complex, and positivity for anti-Sm antibody<sup>7-9</sup>. Bernstein, *et al*<sup>10</sup> subsequently studied the clinical and biochemical characteristics of the sicca lupus autoantibody system, originally reported by Harmon, *et al*<sup>11</sup>, and suggested that the sicca lupus system was identical to

the Ki system<sup>10,11</sup>. We previously purified and characterized Ki, and found that Ki is a nonhistone nuclear protein (PI 4.3) that consists of a 32 kDa polypeptide that exists as a homopolymer (heptamer) in cell extracts based on analysis by high performance liquid chromatography<sup>8</sup>. To characterize Ki further, we isolated the bovine and human cDNA coding for Ki by using an anti-Ki serum as a probe, and found that the nucleotide sequence predicted the primary structure of a polypeptide with a molecular weight of 29.508 kDa<sup>12</sup>. The amino acid sequence was not completely identical with the reported sequence at that time, but molecular cloning of PA28 (11S regulator; REG) family proteins that are known to be proteasome activators has recently shown that Ki is identical to PA28 $\gamma$  (REG $\gamma$ )<sup>2,3,13,14</sup>. Subsequently, Tanahashi, *et al*<sup>15,15</sup> and Wilk, *et al*<sup>16</sup> showed that PA28 $\gamma$  forms a single homopolymer complex (hexamer or heptamer) and plays a role as a positive allosteric activator that binds to 20S proteasome the same as other members of the PA28 proteins<sup>2,17</sup>, PA28 $\alpha$  and PA28 $\beta$ , which share about 50% identity, with both proteins having about 40% homology to the Ki sequence reported in our study<sup>2,12</sup>.

Autoimmune reactions to proteasome were further studied by Feist, *et al*<sup>18-20</sup>, and it was shown that autoanti-

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bodies to the  $\alpha$  and  $\beta$  subunits of 20S proteasome that bind to PA28 can be detected in patients with SLE, primary Sjögren's syndrome (SS), and myositis. Although anti-Ki antibody is well characterized in patients with connective tissue diseases<sup>7-13</sup>, the autoimmune response to PA28 $\alpha$  and PA28 $\beta$  has not been reported. However, data on proteasome structure imply that such autoimmune responses could occur because there is a high level of shared amino acid identity with Ki, and because PA28 $\alpha$  and PA28 $\beta$  both bind to 20S proteasome, which is a target antigen recognized by autoantibodies in various connective tissue diseases<sup>1-4,18-20</sup>.

We investigated whether PA28 $\alpha$  is also a target antigen like Ki antigen. Accordingly, immunoscreening was performed using recombinant PA28 $\alpha$  antigen. In addition, we attempted to characterize the disease specificity of anti-PA28 $\alpha$  antibody and assessed the possible association between positivity for anti-PA28 $\alpha$  antibody and anti-Ki antibody.

## MATERIALS AND METHODS

**Human sera and rabbit polyclonal antibodies.** Serum samples from 219 patients with connective tissue diseases were used in this study, including 65 samples from patients with SLE, 25 from patients with rheumatoid arthritis (RA), 21 from patients with polymyositis/dermatomyositis (PM/DM), 21 from patients with systemic sclerosis (SSc), 20 from patients with mixed connective tissue disease (MCTD), 30 from patients with primary SS, and 37 from patients with secondary SS. All patients with SLE, RA, and SSc fulfilled the relevant American Rheumatism Association (ARA) criteria<sup>21-23</sup>. SS was diagnosed according to the criteria defined by the Research Committee on Autoimmune Diseases of the Ministry of Health and Welfare of Japan<sup>24</sup>, while the patients with PM/DM fulfilled the criteria proposed by Bohan, *et al*<sup>25</sup>. All patients with MCTD met the criteria proposed by the Special Research Committee of the Japanese Ministry of Health and Welfare<sup>26</sup>.

Rabbit polyclonal anti-PA28 $\alpha$  and anti-PA28 $\gamma$  (Ki) antibodies (Affinity Research Products, Exeter, UK) were used as the positive controls. Normal control serum samples were obtained from 20 healthy donors.

**Preparation of recombinant proteins.** PA28 $\alpha$  antigen was prepared as a glutathione S-transferase (GST) fusion protein. The cDNA encoding the full-length amino acid sequence of PA28 $\alpha$  was produced by polymerase chain reaction (PCR) as described<sup>1,2,17</sup>. Primers containing EcoRI sites at both the N- and C- terminals were designed according to the published sequence of the cDNA encoding human PA28 $\alpha$  (5'-, GAA TTC GAA TTC ATG ATG GCC ATG CTC AGG GTC CAG; 3'-, GAA TTC GAA TTC TCA TCA ATA GAT CAT TCC CTT TGT) and a human hepatoma cDNA library (Invitrogen, Carlsbad, CA, USA) was used as the template for PCR.

The PCR products were purified from agarose gel and digested by EcoRI, followed by insertion into an expression

vector (pGEX/EcoRI/BAP; Amersham Pharmacia Biotech, Buckinghamshire, UK). The plasmid clone was transfected into *Escherichia coli* DH-5 $\alpha$  and GST fusion protein was expressed after incubation for 4 h at 37°C in the presence of 1 mM isopropyl  $\beta$ -D-thio-galactopyranoside (IPTG). Recombinant GST and recombinant Ki were also prepared in the same manner<sup>12</sup>.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.** SDS-PAGE was performed to confirm the production of recombinant proteins, as described<sup>27</sup>. The slab gels consisted of 15% acrylamide and 0.1% SDS in Tris HCl (pH 8.8) with 15-mm stacking gels containing 5% acrylamide and 0.1% SDS in Tris HCl (pH 6.8). Samples were dissolved in 100  $\mu$ l of sample buffer [3% SDS, 5% 2-mercaptoethanol, 55 mM Tris HCl (pH 6.8), 10% glycerol, and bromphenol blue] and boiled for 5 min before electrophoresis. Then the gels were stained with Coomassie brilliant blue R250 to reveal protein bands.

For Western blots, proteins were subjected to electrophoresis on the polyacrylamide gel, and transferred to a nitrocellulose membrane at a constant voltage of 25 V overnight at 4°C in 12.5 mM Tris-HCl (pH 8.3), 96 mM glycine, and 20% methanol<sup>28</sup>. The membrane was blocked for 4 h at 4°C with phosphate buffered saline containing 0.1% Tween-20 and 5% powdered skim milk (PBS-T/milk). Then the membrane was cut into strips and incubated 90 min at room temperature with sera diluted to 1:100, or else with rabbit polyclonal anti-PA28 $\alpha$  or anti-Ki antibody diluted 1:2000 in PBS-T/milk. After incubation, the membranes were washed twice in PBS-T for 10 min each. After washing, strips were incubated 90 min at room temperature with alkaline phosphatase-labeled anti-human or anti-rabbit IgG (KPL, Gaithersburg, MD, USA) diluted 1:5000 with PBS-T/milk. After washing twice with PBS-T for 10 min each, the bound conjugate was detected by incubation with a substrate solution (BCIP/NBT Phosphate Substrate; KPL).

**ELISA using purified Ki antigen.** Purified Ki antigen was prepared from rabbit thymus extract (RTE) by anti-Ki affinity chromatography as described<sup>8</sup> and used as the antigen for ELISA. Fifty microliters of purified Ki antigen, diluted to 5.0  $\mu$ g/ml in 0.05 M bicarbonate buffer (pH 8.5), was added to the wells of an Immunoplate II (Nunc, Roskilde, Denmark), and incubation was done overnight at 4°C.

After the excess coating solutions were removed, plates were washed 3 times with PBS-T, and then were rinsed and blocked overnight at 4°C with 250  $\mu$ l of 3% bovine serum albumin in PBS-T. After washing 5 times with PBS-T, 100  $\mu$ l of serum diluted 1:1000 in PBS-T was applied and incubated 2 h at room temperature. Then 100  $\mu$ l of alkaline phosphatase-labeled goat anti-human IgG antibody (KPL), diluted to 1:5000 in PBS-T, was added and incubated 2 h at room temperature. After incubation, the plates were washed 6 times with PBS-T, 100  $\mu$ l of the enzyme substrate (1

mg/ml p-nitrophenylphosphate in diethanolamine buffer, pH 9.8) was added, and the optical density (OD) was measured at 405 nm.

**Inhibition test by ELISA.** An inhibition test was performed by ELISA with sera containing both anti-PA28 $\alpha$  and anti-Ki antibodies. Equal volumes of various concentrations of recombinant PA28 $\alpha$  antigen or recombinant Ki antigen (diluted from 12.74 to 0.11  $\mu$ g/ml) were added to sera diluted 1:1000 and preincubated for 2 h at room temperature. After incubation, samples were centrifuged and 100  $\mu$ l of each supernatant was used for ELISA as the first antibody.

The percentage inhibition was calculated as follows: % inhibition = [1 - (OD 405 nm of wells with blocking antigen/OD 405 nm of wells without blocking antigen)]  $\times$  100.

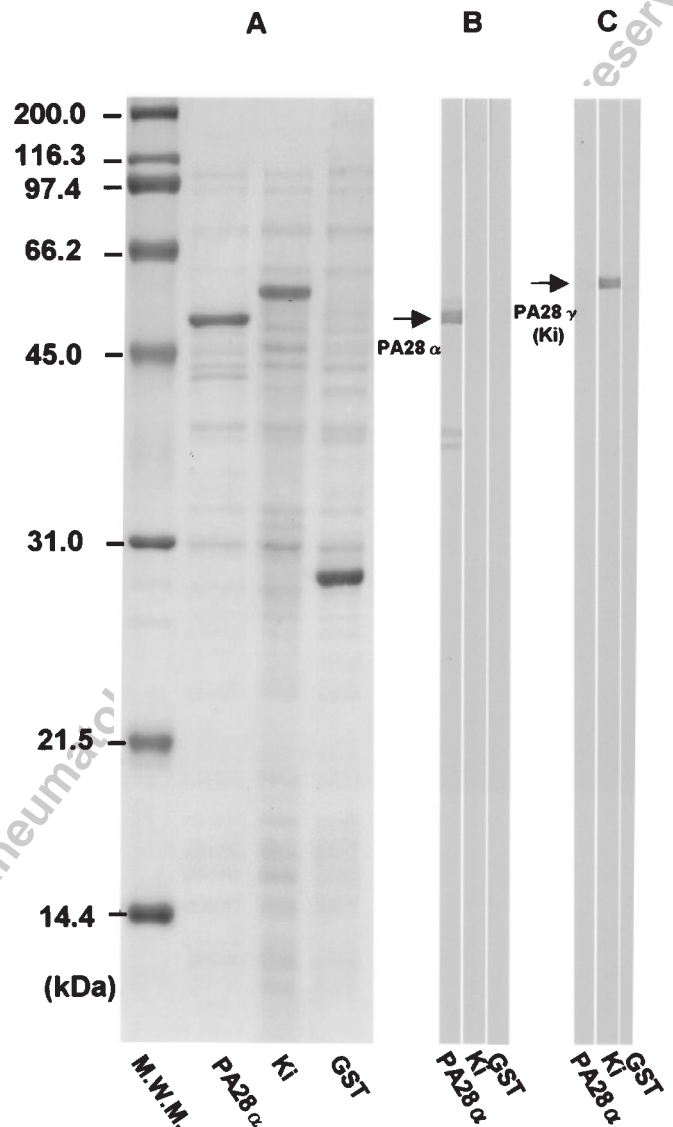
**Statistical analysis.** Statistical analysis was performed using the chi-square test with Yates' correction (2-tailed test).

## RESULTS

Expression of recombinant antigens and immunoreactivity to PA28 $\alpha$  on Western blot. SDS-PAGE analysis was performed to confirm the expression of recombinant antigens (Figure 1A). GST fusion proteins of PA28 $\alpha$  and Ki showed strongly stained bands at the molecular weights of 54 kDa and 58 kDa, respectively, which were identical to the predicted molecular masses based on the amino acid sequences. GST was also expressed as a control and showed a band at the molecular weight of 26 kDa. To determine whether these bands were recombinant PA28 $\alpha$  and Ki, Western blotting was performed using rabbit polyclonal anti-PA28 $\alpha$  (Figure 1B) and anti-Ki (Figure 1C) antibodies. The 54 kDa and 58 kDa antigens were specifically recognized by the respective rabbit polyclonal antibodies, as shown in Figure 1B and 1C.

Using recombinant proteins as described above, immunoreactivity of 219 patient sera and 20 control sera to PA28 $\alpha$  was studied by Western blot. Various patterns of immunoreactivity against recombinant PA28 $\alpha$  and Ki antigens were observed; representative results are shown in Figure 2. Serum 1 reacted with both PA28 $\alpha$  and Ki antigens, serum 2 only reacted with PA28 $\alpha$  antigen, and serum 3 only reacted with Ki antigen, while reactivity with GST was not observed for any serum sample. These observations suggested that immunoreactivity with PA28 $\alpha$  was specific. None of the control sera reacted with either PA28 $\alpha$  or GST. These results indicated that PA28 $\alpha$  was a novel target antigen recognized by sera from patients with autoimmune disease.

**Disease specificity of anti-PA28 $\alpha$  antibody.** To assess the clinical usefulness of anti-PA28 $\alpha$  antibody, the disease specificity of this antibody was studied (Table 1). Among serum specimens from 219 patients, 33 (15%) reacted with PA28 $\alpha$ , while none of the 20 control sera reacted with PA28 $\alpha$ . Anti-PA28 $\alpha$  antibody was detected in 15 out of 65 SLE sera (23%) and 16 of 67 SS sera (24%). Statistical



**Figure 1.** Detection of PA28 $\alpha$ , Ki, and GST by SDS-PAGE and reactivity to recombinant antigens in Western blots using rabbit anti-PA28 $\alpha$  and anti-PA28 $\gamma$  (Ki) antibodies. A. SDS-PAGE (15% gel) of recombinant PA28 $\alpha$ , recombinant Ki, and GST showed strong bands at molecular weights of 54, 58, and 26 kDa, respectively. These sizes corresponded with the predicted molecular masses based on the amino acid sequences of these antigens. B and C. Western blots using rabbit polyclonal anti-PA28 $\alpha$  (B) and anti-Ki (C) antibodies. Each rabbit polyclonal antibody specifically reacted with the corresponding proteins (arrows). MWM: molecular weight marker.

analysis revealed that the positive rate in SS was significantly higher ( $p < 0.05$ ) than in the other connective tissue diseases, except SLE, and the positive rate of anti-PA28 $\alpha$  antibody in SLE was significantly higher than in RA ( $p < 0.01$ ) and MCTD ( $p < 0.05$ ). Among the 67 SS patients, anti-PA28 $\alpha$  antibody was more frequently detected in patients with secondary SS (11 out of 37, 30%) than in patients with primary SS (5 out of 30, 17%), but this difference was not significant. The incidence of anti-PA28 $\alpha$  antibody was significantly higher in primary SS than in RA ( $p < 0.05$ ), and

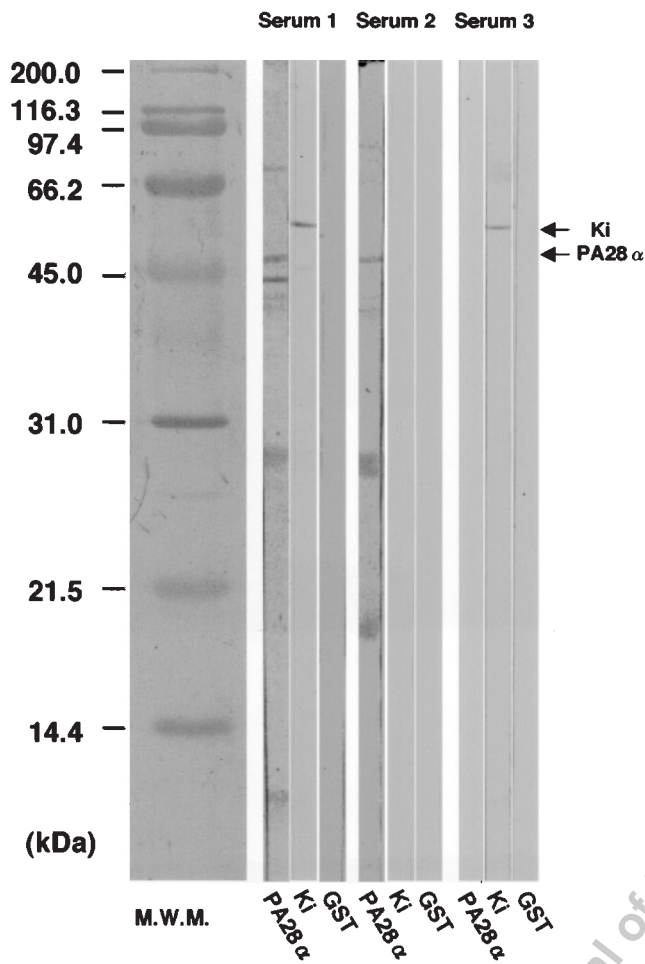


Figure 2. Reactivity of sera from patients with connective tissue diseases to PA28 $\alpha$  and Ki in Western blots. Various immunoreactive patterns against recombinant PA28 $\alpha$  and Ki antigens were observed and representative results are shown. Serum 1 reacted with both PA28 $\alpha$  and Ki antigens, serum 2 reacted only with PA28 $\alpha$  antigen, and serum 3 only reacted with Ki antigen. No reaction with GST was observed in any patient. MWM: molecular weight marker.

it was also significantly higher in secondary SS than in RA, MCTD ( $p < 0.01$ ), and PM/DM, SSc ( $p < 0.05$ ). These results suggested that the disease spectrum associated with anti-PA28 $\alpha$  antibody resembled that of anti-Ki antibody, as reported<sup>7-10</sup>.

In addition, we studied the existence of both antibodies in 65 SLE patients without SS and 15 SLE patients with SS, as well as in 30 patients with primary and 37 with secondary SS to assess the relationship between anti-PA28 $\alpha$  antibody and anti-Ki antibody. As shown in Table 2, anti-PA28 $\alpha$  antibody was predominantly found in sera from SLE patients with SS (33%) and secondary SS patients (30%). The positive rate of anti-PA28 $\alpha$  antibody in SLE patients with SS was higher than in those without SS, as is the case for anti-Ki antibody. In addition, it was notable that all of the anti-PA28 $\alpha$  positive sera from primary SS patients also contained anti-Ki antibody, although the number of patients

Table 1. Detection of anti-PA28 $\alpha$  antibody in various connective tissue disease.

Disease	Patients, n	Anti-PA28 $\alpha$ Antibody, %
Total	219	15
SLE	65	23*
SS	67	24**
Primary SS	30	17 <sup>†</sup>
Secondary SS	37	30 <sup>††</sup>
RA	25	0
PM/DM	21	5
SSc	21	5
MCTD	20	0
Normal	20	0

\* Significantly higher than RA ( $p < 0.01$ ) and MCTD ( $p < 0.05$ ). \*\* Significantly higher than the other connective tissue diseases, except SLE ( $p < 0.05$ ). <sup>†</sup> Significantly higher than RA ( $p < 0.05$ ). <sup>††</sup> Significantly higher than RA and MCTD ( $p < 0.01$ ) and PM/DM and SSc ( $p < 0.05$ ) (chi-square test with Yates' correction). SLE: systemic lupus erythematosus, SS: Sjögren's syndrome, RA: rheumatoid arthritis, PM/DM: polymyositis/dermatomyositis, SSc: systemic sclerosis, MCTD: mixed connective tissue disease.

with both anti-PA28 $\alpha$  and anti-Ki antibodies was limited (5% in secondary SS).

These data also suggested the similarity of disease distribution and positive rates between anti-PA28 $\alpha$  and anti-Ki antibodies in SLE and SS, so we studied the positivity rate for anti-Ki antibody in patients with and without anti-PA28 $\alpha$  antibody (Table 3A). Among 33 serum samples positive for anti-PA28 $\alpha$ , 13 sera (39%) were also positive for anti-Ki antibody. In contrast, only 14 sera negative for anti-PA28 $\alpha$  antibody were positive for anti-Ki antibody (8%), and the rate was significantly lower than in patients with anti-PA28 $\alpha$  antibody ( $p < 0.01$ ). When the positive rate for anti-PA28 $\alpha$  antibody was studied in patients with and without anti-Ki antibodies, anti-PA28 $\alpha$  antibody was detected at significantly higher levels in patients with anti-Ki (48%) than in patients without anti-Ki (10%) (Table 3B). These findings suggest there is significant association between anti-PA28 $\alpha$  antibody and anti-Ki antibody.

#### Inhibition of the reaction to purified Ki antigen detected by

Table 2. Anti-PA28 $\alpha$  and anti-Ki antibodies in patients with SLE and SS.

Disease	Patients, n	PA28 $\alpha$ Positive, %	Ki Positive, %	PA28 $\alpha$ and Ki Positive, %
SLE	80	25	21	10
With SS	15	33	27	13
Without SS	65	23	20	10
SS	54	26	22	10
Primary	30	17	20	17
Secondary	37	30	22	5

Statistical analysis was performed using the chi-square test with Yates' correction.

Table 3. Comparison of immunoreactivity between PA28 $\alpha$  and Ki in patients with connective tissue diseases.

A	Patients, n	Patients with anti-Ki Antibody, %	Patients without anti-Ki Antibody, %
Patients with anti-PA28 $\alpha$ antibody	33	39 }*	61
Patients without anti-PA28 $\alpha$ antibody	186	8 }*	92

B	Patients, n	Patients with anti-PA28 $\alpha$ Antibody, %	Patients without anti-PA28 $\alpha$ Antibody, %
Patients with anti-Ki antibody	27	48 }*	52
Patients without anti-Ki antibody	192	10 }*	90

\*  $p < 0.01$  (chi-square test with Yates' correction).

ELISA. Because the amino acid sequences of PA28 $\alpha$  and Ki antigen share 40.2% sequence similarity, the significant correlation between anti-Ki antibody and anti-PA28 $\alpha$  antibody positivity may have been due to the presence of cross-reactive epitopes in these molecules. To clarify this, we performed an inhibition test using recombinant PA28 $\alpha$  and Ki antigen as competitive inhibitors in an ELISA with purified Ki antigen.

Eight serum samples containing both anti-PA28 $\alpha$  and anti-Ki antibodies were preincubated with the inhibitors (at serial dilutions from 12.74 to 0.11  $\mu\text{g/ml}$ ) and the reactivity to purified Ki antigen was tested by ELISA. Representative results obtained using serum 1 and serum 2 are shown in Figure 3. The reactivity for purified Ki antigen was inhibited by preincubation with recombinant Ki antigen. On the other hand, preincubation with recombinant PA28 $\alpha$  did not inhibit the reactivity for purified Ki antigen, even at the highest concentration of PA28 $\alpha$ . These results suggested that the sera contained different antibodies specifically targeting PA28 $\alpha$  and Ki antigens.

## DISCUSSION

The frequency and the disease distribution of anti-PA28 $\alpha$  and anti-Ki antibodies was alike, and the positivity rate of anti-PA28 $\alpha$  antibody was significantly higher in serum with anti-Ki antibody than in serum without anti-Ki antibody. These data suggest that there may be association of the autoimmune response to anti-PA28 $\alpha$  and Ki antibodies in SLE and SS.

There are several possible mechanisms to explain an association of autoimmune response to PA28 $\alpha$  and Ki. First, the high amino acid sequence homology between PA28 $\alpha$  and Ki antigens suggests the existence of cross-reactive epitopes on both antigens<sup>29-31</sup>. To confirm this, we performed an inhibition test with serum containing both anti-PA28 $\alpha$  and anti-Ki antibodies, and found that reactivity to purified Ki antigen could not be inhibited by preincubation with recombinant PA28 $\alpha$ . These results suggested that there were 2 different antibodies specifically targeting PA28 $\alpha$  and Ki antigens, although there was an association

of autoimmune response to these antigens. This was an interesting finding because a similar observation was reported in patients with SS when the autoimmune response to  $\alpha$ - and  $\beta$ -fodrin was studied<sup>30,32</sup>. Autoantibody to  $\alpha$ -fodrin can be specifically detected in 29–95% of patients with SS<sup>30,32</sup>, and  $\alpha$ -fodrin is known to form a heterodimer with  $\beta$ -fodrin which shares a homologous 106 amino acid repeating motif<sup>31</sup>. This suggests that cross-reactivity of autoantibodies to  $\alpha$ - and  $\beta$ -fodrin may occur in SS, but Kuwana, *et al*<sup>31</sup> showed that anti- $\beta$ -fodrin antibodies detected in 70% of patients with SS were not cross-reactive with  $\alpha$ -fodrin. Similarly, the shared amino acid sequence may not explain the linked immune response among PA28 family proteins, but the possibility of cross-reaction inducing an autoimmune response to both PA28 $\alpha$  and Ki was not completely excluded, because epitopes recognized by the autoantibodies might alter to show specific reactivity to each autoantigen.

Another hypothetical mechanism for inducing anti-PA28 $\alpha$  and anti-Ki antibodies is a spread of epitopes involving "intermolecular-intrastructural help" that plays a key role in the immune response to multiprotein complexes<sup>33-39</sup>. Usually, the linked autoantibodies are strongly dependent on the structure of their target antigens. Many target antigens are part of large multimolecular complexes that consist of several different antigens, and it is well known that if an immune response against one part of the protein complex is elicited, other epitopes on the same complex also become antigenic proteins<sup>36,37,39</sup>. These examples are the linkage of autoimmune response between anti-SSA/Ro and anti-SSB/La antibodies, and between anti-Sm and anti-U1-RNP antibodies<sup>29,34,39</sup>. In addition, recent studies of the autoimmune response to proliferating cell nuclear antigen multiprotein complexes in patients with SLE have shown that the immune response can spread within the elements of the massive protein machinery such as a holoenzyme associated with DNA replication<sup>29,35,37,38</sup>. Therefore, it is possible that association of the autoimmune response among the elements of proteasomes can be explained in this manner.

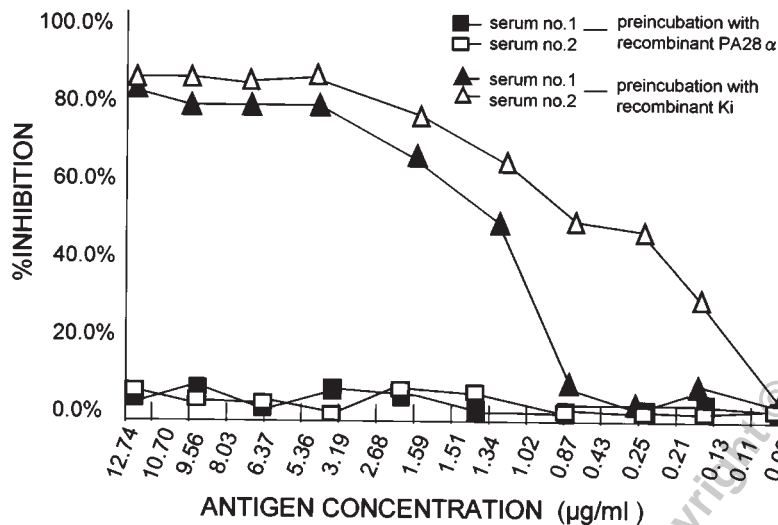


Figure 3. Inhibition test using recombinant PA28 $\alpha$  and Ki antigens as competitive inhibitors in an ELISA. Sera 1 and 2, containing both anti-PA28 $\alpha$  and anti-Ki antibodies, were preincubated with PA28 $\alpha$  or Ki antigen (serial dilutions from 12.74 to 0.11 mg/ml), and their reactivity to purified Ki antigen was tested. Reactivity was inhibited by preincubation with recombinant Ki antigen. Preincubation with recombinant PA28 $\alpha$  did not inhibit the reactivity to purified Ki antigen. These results suggested that the sera contained different antibodies specifically targeting PA28 $\alpha$  and Ki antigens.

The 20S proteasome is the catalytic core of proteasome, which has a barrel-like particle that forms as a stack of 4 rings made up of 2 outer  $\alpha$ -rings and  $\beta$ -rings, as shown in Figure 4<sup>3</sup>. Because the center of the  $\alpha$ -rings is almost closed to prevent the penetration of proteins into the inner surface of the catalytic center, the 20S proteasome needs to interact with activators such as PA28 and PA700 to open the proteasome channel, and there are at least 3 different specificities of the complexes, as shown in Figures 4A, 4B (B'), and 4C<sup>3,5</sup>. One is the 26S proteasome (Figure 4A), a dumbbell-shaped particle that consists of a 20S proteasome and 2 large V-shaped terminal PA700 modules attached to the 20S core particle in opposite orientations. The 26S proteasome can break down ubiquitinated proteins that are initially in their native state<sup>3</sup>. The second is the PA28 complex that consists of the 20S proteasome and another activator, PA28 (REG), which has an ovoid shape by binding of PA28 to both ends of the 20S proteasome<sup>3,5</sup> (Figures 4B and B'). In addition, it is known that there are at least 2 different PA28 complexes, one consisting of 20S proteasome and a ring-shaped particle, PA28 ( $\alpha_3 \beta_3$ ) or PA28 ( $\alpha_3 \beta_4, \alpha_4 \beta_3$ , or mixed) (Figure 4B), and the other consisting of 20S proteasome and PA28 $\gamma_6$  or PA28 $\gamma_7$  (Figure 4B'). Recent immunofluorescence studies using monoclonal antibodies suggest that the former is located in cytoplasm and the latter in the nucleus, and that these complexes are likely to be involved in different cellular functions of proteasome such as antigen presentation on class I MHC molecules, protein quality control, apoptosis, signal transduction, and cell cycle regulation<sup>1-5,14</sup>.

<sup>17</sup> The third type of proteasome is a so-called "hybrid proteasome" that consists of 20S proteasome, PA700, and PA28 (Figure 4C)<sup>3,5</sup>. Thus, PA28 $\alpha$  and PA28 $\gamma$  do not bind or interact directly, although they show an association of autoimmune response. However, recent studies by Feist, *et al*<sup>18-20</sup> explain the role of "intermolecular-intrastructural help" in inducing a linked set of autoimmune responses among PA28 family proteins. Feist, *et al*<sup>18-20</sup> showed that antibodies to the  $\alpha$  and  $\beta$  subunits of 20S proteasome can be detected in patients with SLE, primary SS, and myositis, and that the immune response to proteasome is "antigen-driven" because the elevation of the circulating antigen induced the increase of the titer of antiproteasome antibody, and the spread of the autoimmune response to the elements of 20S proteasome, in a longitudinal study<sup>18-20</sup>. These data suggest that the autoimmune response to the  $\alpha$  and  $\beta$  subunits of 20S proteasome may play a pivotal role in inducing the production of autoantibodies to PA28 $\alpha$  and PA28 $\gamma$  that bind to the 20S proteasome in patients with connective tissue diseases. Although it was possible that the cross-reactivity of the PA28 proteins could not be observed in an inhibition study by the alteration of epitopes due to the binding of recombinant PA28 $\alpha$  and GST fusion proteins, and the degeneration of antigens during the Western blot procedure resulted in a false-positive response showing the association between anti-PA28 $\alpha$  and Ki antibodies, further studies on the relationship of immunoreactivity with the elements of proteasome may shed light on mechanisms of autoimmunity in patients with connective tissue diseases.

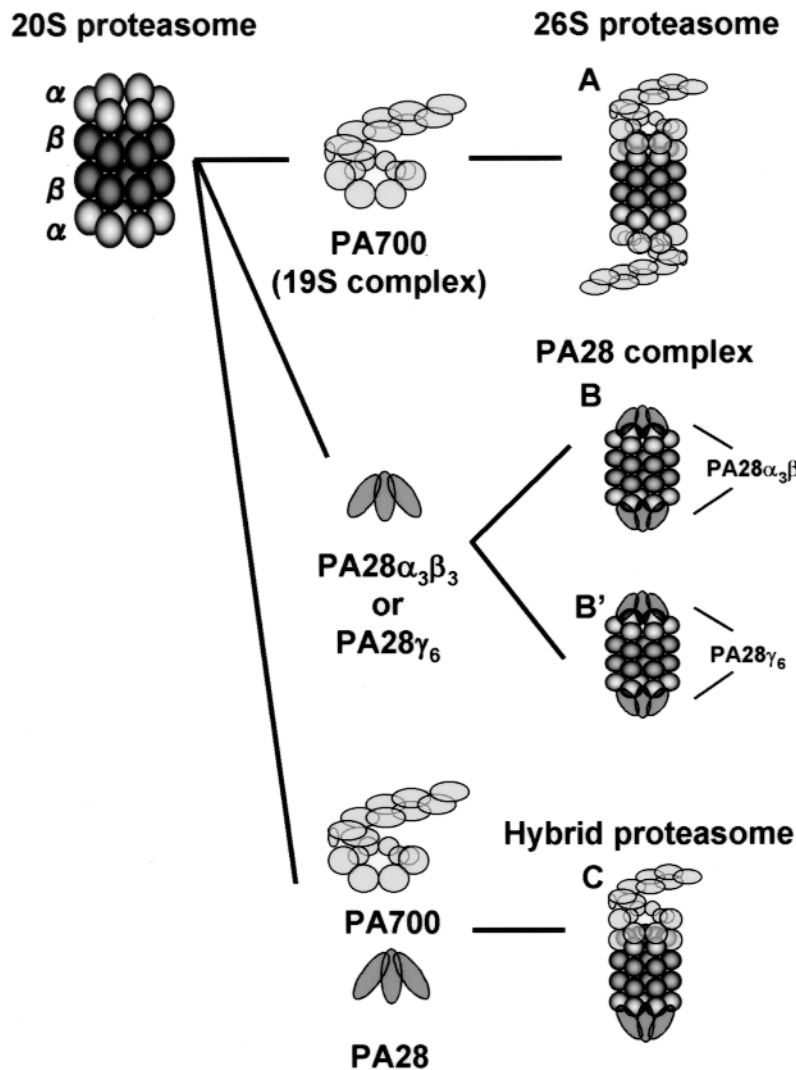


Figure 4. The 20S proteasome complexes; there are at least 3 types. A. The 26S proteasome. PA700, known as the 19S complex, binds to both ends of the 20S proteasome to form 26S proteasome. B and B' show PA28 complexes, which consist of 20S proteasome and another activator (PA28). There are at least 2 different PA28 complexes, one consisting of 20S proteasome and a ring-shaped particle, PA28 ( $\alpha_3\beta_3$ ) or PA28 ( $\alpha_3\beta_4\alpha_4\beta_3$ , or mixed) (B), whereas the other consists of 20S proteasome and PA28 $\gamma_6$  or PA28 $\gamma_7$  (B'). C. A hybrid proteasome made up of 20S proteasome, PA700, and PA28.

The other interesting observation in this study was the strong association of autoantibodies to proteasome in patients with SS, as revealed in a series of reports concerning anti-Ki and 20S proteasome antibodies<sup>7-13,18-20</sup>. In our patients with primary SS, all sera containing anti-PA28 $\alpha$  antibody reacted with Ki antigen. On the other hand, only 40% of anti-PA28 $\alpha$  sera reacted with Ki antigen in SLE samples. This characteristic pattern of reactivities in SS may be useful to diagnose patients with primary SS, and may help to illuminate the mechanisms of the autoimmune response to proteasome.

We need further investigations to confirm the clinical significance and pathogenic role of anti-PA28 $\alpha$  antibody in SS and SLE.

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