

Proteasome α -Type Subunit C9 Is a Primary Target of Autoantibodies in Sera of Patients with Myositis and Systemic Lupus Erythematosus

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Summary

Autoantibodies occur in low frequencies among patients with myositis characterizing only distinct subsets of this disease. Most of these known antibodies are directed to enzymatically active complexes. The 20S proteasome represents an essential cytoplasmatic protein complex for intracellular nonlysosomal protein degradation, and is involved in major histocompatibility complex class I restricted antigen processing. In this study we investigated whether the 20S proteasome complex is an antibody target in myositis and in other autoimmune diseases. 34 sera of poly/dermatomyositis patients were assayed for antiproteasomal antibodies using enzyme-linked immunosorbent assay, immunoblot, and two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE). Sera was from patients with systemic lupus erythematosus (SLE), mixed connective tissue disease, and rheumatoid arthritis; healthy volunteers served as controls. In 62% (21/34) of the cases sera from patients with myositis and in 58% (30/52) of the cases sera from patients with SLE reacted with the 20S proteasome. These frequencies exceeded those of sera from patients with mixed connective tissue disease, rheumatoid arthritis, and healthy controls. The α -type subunit C9 of the 20S proteasome was determined to be the predominant target of the autoimmune sera in myositis and SLE. Lacking other frequent autoantibodies in myositis, the antiproteasome antibodies are the most common humoral immune response so far detected in this disease entity.

Within eukaryotic cells the 20S proteasome (multicatalytic proteinase complex) represents the key enzyme complex responsible for intracellular nonlysosomal protein degradation, and is the proteolytic core of an even larger 26S protease complex which is involved in the ATP/ubiquitin-dependent protein degradation (1). Proteasomes are involved in the degradation or processing of transcription factors such as nuclear factor (NF) κ B, in cyclin degradation, cell cycle control, and apoptosis (1–4). Furthermore, the synthesis of the β -type subunits LMP2, LMP7, and MECL1, as well as the PA28 regulator, are inducible by IFN- γ , and the role of the 20S proteasome in the processing of antigens presented by MHC class I molecules is now well established. Analysis of the structure and subunit sequences revealed that the proteasome is highly conserved among species as far apart in evolution as bacteria and man (5–8). In eukaryotes the cylindrical structure of proteasomes is arranged in four staggered rings composed of 28 subunits whereby each ring contains seven different proteins. The α -type subunits are located in the outer rings and are the contact sites for regulatory complexes such as the 11S regulator PA28 and the 19S complexes. Four of

the α -type subunits carry putative nuclear location signals and several are substrates for protein kinases (9). The β -type subunits forming the two adjacent inner rings carry the proteolytically active sites. As deduced from the crystal structure of the thermoplasma proteasome and mutagenesis only three out of the seven mammalian β -type subunits within one ring form active sites with the NH₂-terminal threonine being the catalytically active residue (10, 11).

Autoantibodies play an important diagnostic role in many autoimmune diseases. Thus, anti-tRNA synthetase autoantibodies occurring in myositis patients characterize clinically different patient subsets (12). The cytoplasmatic aminoacyl-tRNA synthetases catalyze the binding of amino acids to their cognate tRNA, and five of them have been determined to be the antigens of specific autoantibody subtypes (13). Nevertheless, among myositis patients these specific antibodies are present at relatively low frequency (5–20%) (13).

The objective of the present study was to determine whether specific subunits of the abundant proteasome complex represent disease-specific autoantigens. Therefore, sera of different types of chronic inflammatory autoim-

mune diseases (polymyositis [PM]¹, dermatomyositis [DM], SLE, mixed connective tissue disease [MCTD]), rheumatoid arthritis (RA), and sera from healthy controls were tested in ELISA, immunoblot, and two-dimensional NEPHGE. The experiments show for the first time that sera from patients with PM and DM as well as SLE patients contain antiproteasome antibodies at high frequencies. Furthermore, we demonstrate that the proteasomal α -type subunit C9 represents a major immune target of reactive sera.

Materials and Methods

Patient Sera. Sera of the following individuals were investigated: 8 patients with DM and 26 patients with PM classified according to Bohan and Peter (14, 15), 52 patients with SLE fulfilling the 1982 revised ACR criteria (16), 10 patients with MCTD diagnosed according to Sharp et al. (17), 20 patients with RA (18), and 112 healthy controls. Materials were obtained at the university hospital of the Charité Berlin.

Purification of 20S Proteasome. 20S proteasomes were isolated from human erythrocytes following standard procedures, and analysis of proteasome subunits by two-dimensional electrophoresis (NEPHGE/SDS-PAGE) was carried out as described (19, 20).

Monoclonal Antiproteasome Antibodies. Mice were injected intraperitoneally with ~25 to 100 μ g proteasome protein in about 100–200 μ l PBS as a water in oil mixture in the same amount of CFA. Booster injections were given after a resting period of 2–3 mo. Antibody activity was determined in the serum of immunized mice in general 7 d after immunization. Spleen cells were taken 4 d after the last booster injection and fused with X63 Ag8.653 myeloma cells using polyethylene glycol. A small portion of the spleen cells was taken into culture for 7–10 d and antibody activity was measured in solid-phase binding assays after adsorbing the first reactant to polyvinyl chloride plates according to our general methods (21,22). The incubation sequences were as follows: proteasome, antibody-containing solution, biotin-labeled sheep anti-mouse Ig, streptavidin–galactosidase complex. The fluorescence of the substrate 4-methylumbelliferyl galactoside was measured at 355 nm.

Immunoblotting. For immunoblotting, purified proteins were separated by 15% SDS-PAGE and were transferred onto nitrocellulose membranes. The remaining binding sites were blocked for 2 h with PBS, pH 7.4, containing 5% skim milk. Subsequently, the patient's sera diluted 1:100 were added. The antibodies bound to the proteasome were detected by rabbit anti-human IgG antibodies coupled with horseradish peroxidase diluted 1:10,000 in PBS/1% Tween 20/1% skim milk. Antibodies bound were visualized using x-ray films exposed to enhanced chemiluminescence-treated blots (Boehringer Mannheim GmbH, Mannheim, Germany).

ELISA. Multititer plates were coated overnight with 1 μ g/ml of the purified proteasome in carbonate buffer, pH 9.6. Remaining binding sites were blocked with 0.5% skim milk and 0.05% Tween 20 in PBS, pH 7.4. Human sera were diluted 1:100 and applied to each well for 2 h at room temperature. Anti-human IgG peroxidase-labeled antibody (1 mg/liter) was used as the second reagent. Bound antibodies were detected enzymatically with

o-phenylenediamine as substrate using a microplate reader at 492 nm. Standard curves were established using the polyclonal proteasome-specific antibody MP-3 and mouse anti-rabbit IgG peroxidase as secondary reagent. MP-3 was diluted in linear steps starting with 1:400. OD units of the dilution step 1:12,800 were considered to represent a value of 10,000 arbitrary units. Therefore, it was possible to make a direct correlation between the antibody reactivity of patients sera of several assays. 112 sera from normal controls served to establish background reactivities. The mean value plus threefold SD were used as cutoff.

Monoclonal antiproteasome antibodies were used to support antiproteasome antibody specificity of patient sera. Multititer plates were coated with the antiproteasome antibody B65-BA5 diluted 1:1,000 in carbonate buffer, pH 9.6. Purified proteasome was applied at a concentration of 10 μ g/ml. Human sera were diluted 1:100 and applied to each well for 2 h at room temperature. Further, goat anti-human IgG (Fc specific) peroxidase-labeled antibody (affinity-isolated antibodies, no cross-reactivity with mouse or rat IgG; Sigma Chemical Co., St. Louis, MO) were added 1:10,000. The antibodies bound were detected using 2-acino-bis-ethylbenzthiazolin-6-sulfate diammonium as substrate and measured at 405 nm.

Statistical Analysis. Statistics were performed using the non-parametric U test (Mann and Whitney) and chi-square test with Yates correction to compare the qualitative and quantitative reactivities to the proteasome among the patient's groups investigated. *P* values of <0.05 were considered to be statistically significant.

Results and Discussion

Antiproteasome antibodies were detected by ELISA in 21 of 34 sera (62%) derived from PM (16/26) and DM (5/8) patients. Sera from patients with SLE reacted with proteasomes in 30 of 52 cases (58%) using ELISA. These frequencies of antiproteasome antibodies were significantly higher than those found in patients with RA ($P < 0.001$), MCTD ($P < 0.025$), or in healthy controls ($P < 0.001$) (Fig. 1). In

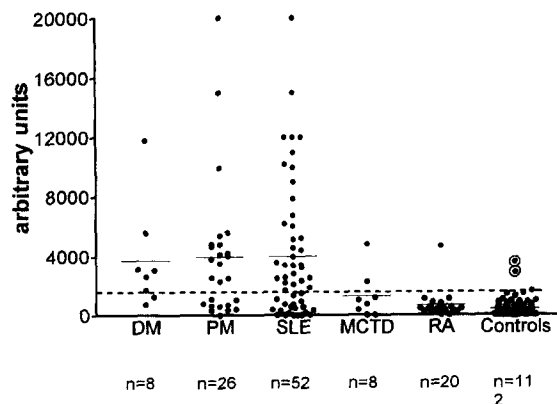


Figure 1. Autoimmune response against proteasome was detected in patients with PM, DM, MCTD, and SLE. ELISA reactivities and median values of patients and healthy controls sera to purified 20S proteasome. Note that the two antiproteasome-positive sera of healthy controls were not confirmed in further specific assays. The respective cut-off values are indicated (---). The strongest autoimmune response was detected in sera of patients with PM, DM, and SLE.

¹Abbreviations used in this paper: DM, dermatomyositis; MCTD, mixed connective tissue disease; NEPHGE, non-equilibrium pH gradient electrophoresis; PM, polymyositis; RA, rheumatoid arthritis.

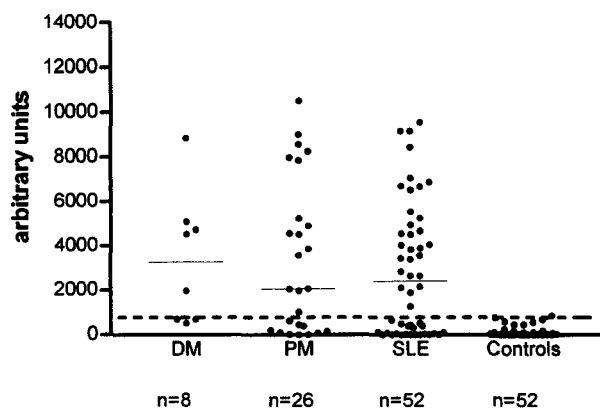


Figure 2. Antiproteasome antibodies were confirmed in a stringent sandwich ELISA. Multititer plates were coated with the monoclonal mouse anti-human proteasome antibody B65-BA5, which served as the proteasome-specific reagent. Reactivities and median values of the main patient groups are shown. The respective cutoff values are indicated (---). All sera of the healthy controls group were negative. The same sera of patients with PM, DM, and SLE revealed positive reaction in both ELISA assays.

fact, sera of patients with PM/DM and SLE revealed a much stronger reactivity with 20S proteasome protein than sera from patients with RA ($P < 0.0005$) and MCTD ($P < 0.025$). Sera of healthy controls gave a positive reaction in only 2 of 112 cases (1.78%).

To exclude the possibility that the above results were due to nonspecific binding of antibodies and to unequivocally establish the antiproteasome specificity of the autoantibodies of PM/DM and SLE patients, we performed sandwich ELISAs using the proteasome-specific mAb B65-BA5. In support of the data shown in Fig. 1, 21 of the 34 sera tested from both PM and DM patients and 30 of the 52 sera from SLE were also positive in the more stringent sandwich ELISA (Fig. 2). Furthermore, the two positive MCTD sera were confirmed in that test. Importantly, the same patient sera were positive in both ELISA systems. In

contrast, the two initially reactive sera from healthy controls as well as the one reactive RA serum were completely negative in the sandwich assay.

There was no evidence for a statistical correlation between the presence of antiproteasome antibodies and other routinely characterized antibody specificities. An exclusive presence of antiproteasome antibodies was only observed in sera from DM and PM patients. This contrasts with serum samples of SLE, MCTD, and RA patients, which predominantly reacted with other defined autoantigens such as anti-native DNA antibodies (ds-DNA), anti-small nuclear ribonucleoprotein Smith (Sm), anti-U1-ribonucleoprotein (U1-RNP), or contained rheumatoid factor. Interestingly, of the five sera which were positive for anti-histidyl tRNA synthetase (Jo-1) antibodies only one serum from the myositis group contained antiproteasome antibodies (Table 1).

To see whether the antiproteasome autoantibodies obtained from patients with PM/DM and SLE react with several or a specific type of proteasome subunits, the respective patient sera were probed on purified human erythrocyte 20S proteasome in immunoblot experiments. Of the 21 ELISA-positive myositis sera a total of 16 (PM 13/16; DM 3/5) were also positive in immunoblots. All five immunoblot negative sera were of low antiproteasome titer as determined by ELISA. 14 of the 16 antiproteasome autoantibodies of PM/DM patients tested exclusively identified a single proteasomal subunit of 28 kD and thus revealed a remarkable subunit specificity (Fig. 3). The remaining two sera, besides exhibiting specificity for the 28-kD protein, also revealed weak reactivity with a proteasome subunit of 23 kD. SLE patients recognized a single proteasomal subunit of 28 kD in 19 cases. Only three sera identified subunits of 28 and of 23 kD simultaneously. Both positive MCTD sera recognized subunits of 28 kD, whereas the negative sera revealed no reactivity. It is important to note here that all ELISA-negative sera, including the sera from patients with PM/DM and SLE, were also proteasome negative in immunoblots (data not shown).

Table 1. Distinct Autoantibody Profiles of the Different Disease Entities

		ANA		dsDNA					RF
		IF	IF	APA*	Jo-1*	Scl-70*	Sm*	U1-RNP*	ELISA
PM/DM	(n = 34)	26	0	21	5	2	0	0	5
SLE	(n = 52)	50	45	30	0	0	8	12	12
MCTD	(n = 8)	8	0	2	0	1	0	8	2
RA	(n = 20)	4	0	1	0	0	0	0	19

Antinuclear antibodies (ANA) detected by indirect immunofluorescence in human larynx epithelioma cancer cell line (Hep-2 cells). The dilution sera was started at 1:80. Anti-native DNA antibodies (dsDNA) detected in specific *Crithidia luciliae* immunofluorescence test; dilution of sera was started at 1:2. Antiproteasome antibodies (APA), antihistidyl tRNA synthetase (Jo-1), anti-DNA topoisomerase I (Scl-70), anti-small nuclear ribonucleoprotein Smith (Sm), and anti-U1-ribonucleoprotein (U1-RNP) specificities detected in ELISA and immunoblotting assays (dilution of the patients sera 1:100). Rheumatoid factors (RF) of the IgM type measured using ELISA. Statistical analysis indicate that APA is a candidate as a new useful diagnostic marker of autoimmune myositis. IF, immunofluorescence; IB, immunoblotting.

*Antigens identified by immunoblotting and ELISA.

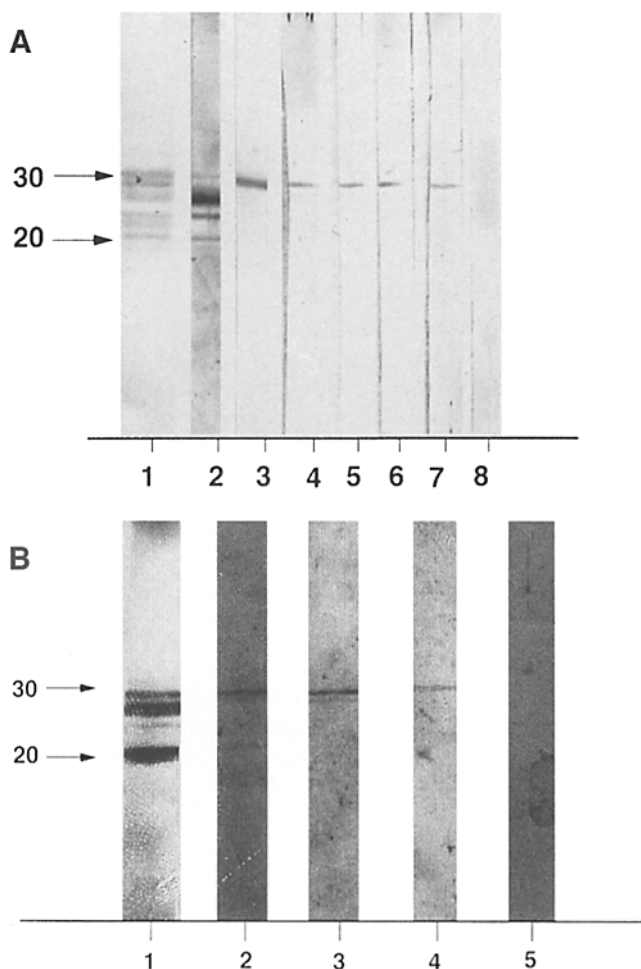


Figure 3. Patient sera detect a single proteasomal subunit in Western blot analysis. 20S proteasomes were isolated from human erythrocytes. Representative sera react with a 28-kD subunit of the proteasome. (A) Lane 1, 5 μ g of purified proteasome, Coomassie stained; lane 2, rabbit antiproteasome antibody MP-3 as a polyclonal Ab recognized several proteasomal subunits; lanes 3 and 4, sera of patients with PM; lane 5, serum of a patient with DM; lanes 6 and 7, sera of patients with SLE; lane 8, healthy control. All positive patients sera recognized a single subunit of 28 kD. (B) Lane 1, rabbit antiproteasome antibody MP3; lane 2, serum of a patient with PM; lanes 3 and 4, sera of patients with SLE; lane 5, healthy control.

The observation that the autoantibodies reacted predominantly with a single proteasome subunit after SDS-PAGE led us to determine the identity of the subunit after separation of proteasome subunits by two-dimensional electrophoresis (Fig. 4). The high titer antiproteasome sera of PM/DM and SLE patients used in these experiments identified a single protein which, as judged by its size, electrophoretic mobility, and amino acid sequence, represents the proteasome α -type subunit C9 (Fig. 4).

In this communication we have presented evidence that 62% of the sera from patients with PM/DM and 58% of SLE sera contain autoantibodies which react with the 20S proteasome representing one of the most abundant intracellular proteins. Furthermore, we have demonstrated that

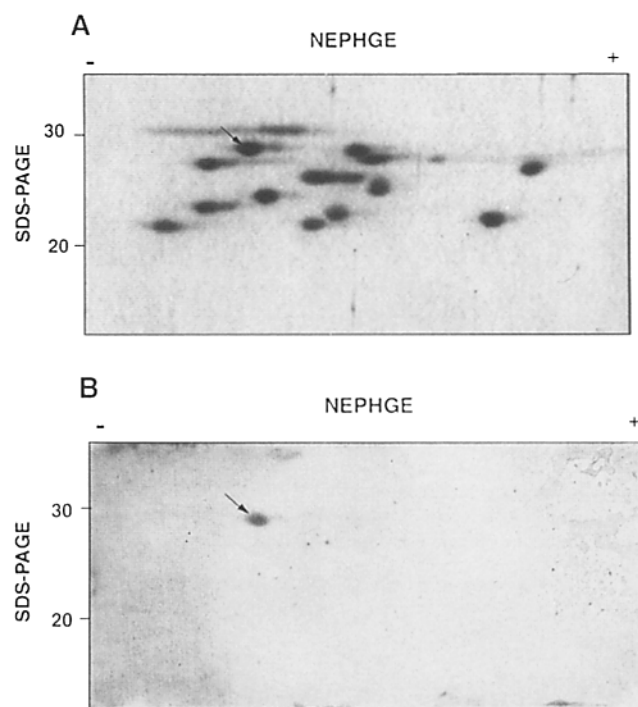


Figure 4. Subunit C9 is the major target of patients autoantibodies in two-dimensional electrophoresis of 20S proteasome. Analyses were performed with proteasome of human erythrocytes (first dimension, NEPHGE; second dimension, SDS-PAGE). Arrow indicates α -type subunit C9. (A) Coomassie staining of a two-dimensional gel shows the purified human proteasome. (B) Western blot analysis of two-dimensional electrophoresis detected by one representative serum of a patient with polymyositis. Rabbit anti-human IgG antibodies coupled with peroxidase served as the secondary reagents. Proteasomal α -type subunit C9 was recognized by the patient serum.

the proteasomal α -type subunit C9 represents the predominant target of the autoantibodies occurring in myositis patients. Skeletal muscle cells have been shown to represent APC by processing and presenting antigens to T cells via MHC class I and II pathways (23). In addition, skeletal muscle cells themselves can be targets of autoimmune attack in inflammatory muscle diseases such as polymyositis and dermatomyositis. Curiously, subunits of the proteolytic enzyme complex responsible for the processing of MHC class I antigens are themselves targets of autoantibodies. Previously reported myositis-specific autoantibodies recognize proteins of RNP complexes involved in RNA processing, protein translation, or protein translocation (24). The association of anti-tRNA synthetase antibodies with myositis, arthritis, and interstitial lung disease led to the description of an entity designated as antisynthetase syndrome or anti-Jo-1 syndrome and represents the most common disease of myositis subgroups (13).

Nevertheless, 80–85% of myositis patients do not possess autoantibodies to the previously reported antigens (24, 25). Interestingly, only 5 of 34 sera from PM/DM patients tested were anti-Jo-1 positive and of these only one also revealed reactivity with 20S proteasome. This demonstrates that there is an infrequent overlap in the induction of au-

toantibodies to Jo-1 and proteasome in myositis patients. This indicates a discrimination between both groups of patients and supports the role of antiproteasome C9 autoantibody as a serological marker of a myositis subset.

It is of special clinical interest that the presence of antiproteasome antibodies was observed in active inflammatory muscle diseases in the PM/DM patients group. We observed that the antiproteasome-positive cases of PM/DM revealed no manifestation of interstitial lung disease. Furthermore, we found that in these positive myositis patients a high frequency of other organ involvement due to vasculitis was seen. Remarkably, besides an active myositis the two antibody-positive MCTD patients revealed severe organ involvement. It is notable that all patients with SLE possessing antiproteasome antibodies also had severe organ manifestations like active nephritis and vasculitis of the skin. Since these patients did not show any signs of myositis, it may be concluded that the antiproteasome antibodies not only do not indicate muscle involvement in autoimmune diseases, but may also signify severe internal organ damage.

In a previous study Arribas et al. (26) reported an antiproteasome reactivity in 35% of SLE sera as judged by immunoblotting, but were not able to detect antiproteasome antibodies in sera of PM/DM patients. In that study it remained unclear, however, which of the proteasome subunits were recognized and whether they all were indeed subunits of the enzyme complex.

Here we demonstrate that antiproteasome antibodies

represent one parameter for humoral autoimmune response in myositis. In addition to clinical and serological signs, like elevated levels of creatinine kinase, antiproteasome antibodies are a candidate for a useful diagnostic marker. Initial data indicate a correlation with disease severity. Thus, clinical signs of an active myositis and severe organ involvement appear to be related to the presence of antiproteasome antibodies. However, the pathogenic importance of the antiproteasome antibodies and of the best-described autoantibodies in systemic rheumatic diseases remains unproven (27). One interesting aspect of ongoing studies in our laboratories is the modulation of the proteinase function by antiproteasome antibodies. Therefore, epitopes that represent functional domains of the C9 subunit could be antibody target sites. The C9 subunit possesses a nuclear location signal and is a component of the outer α -ring of the proteasome which interacts with a number of regulators of proteasome activity. One of these is the 11S activator PA28 which together with the 20S proteasome is directly involved in the MHC class I response of the cell. In recent reports it was shown that a large number of autoantibodies are also present within the cell (28). If this turns out also to be true for the C9 autoantibody, a direct influence on the proteolytic function of proteasomes appears possible. Nevertheless, more detailed studies will be necessary to determine the relevance of an immune response to proteasomes with the predominant recognition of the C9 subunit in patients with autoimmune diseases.

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