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Research Letter

# A Novel Antineutrophil Extracellular Trap Antibody Targeting Myosin Light Chain 6 in Microscopic Polyangiitis

*To the Editor:*

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by ANCA production in the serum. This disease entity includes microscopic polyangiitis (MPA). Previous studies have revealed that neutrophils excessively activated by ANCA and other serum factors are critically involved in AAV pathogenesis.[1] In particular, neutrophil extracellular traps (NETs) released from activated neutrophils play a pivotal role in tissue destruction in AAV.

NETs are extracellular web-like substances that consist of unraveled DNA coating with antimicrobial proteins.[2] Although NETs play roles in capturing and killing microbes invading the hosts, NETs degradation is necessary to avoid its pathogenicity.[3] The principal physiological degrader of NETs is DNase I in the serum.

Some patients with MPA possess antibodies to NETs (anti-NETs antibody [ANETA]),[4] and certain antibodies included in ANETA can affect NETs generation[5] and degradation.[6] Serum NETs degradation activity was lower in patients with MPA than in healthy controls.[4] The NETs degradation activity was significantly recovered using a protein G column in some ANETA-positive MPA sera after IgG depletion but not in ANETA-negative MPA sera. These findings suggest that some ANETA inhibit NETs degradation.[6] In a previous study by Hattanda et al,[6] such ANETA was present in 2 of the 19 enrolled patients with MPA, whereas ANETA was present in 10 of the 19 patients with MPA. To date, the following facts have been clarified: (1) the antigen recognized by the ANETA with NETs degradation inhibitory activity is an ANCA antigen because it is also present in the cytoplasm of neutrophils before NETs induction; and (2) the antigen is different from known ANCA antigens.[6]

The present study was conducted with the permission of the Ethical Committee of the Faculty of Health Sciences, Hokkaido University (permission no. 15-90 and 18-34) and in adherence with the Declaration of Helsinki. Written informed consent was obtained from all patients and healthy volunteers enrolled in this study.

ANETA with NETs degradation inhibitory activity could deteriorate NETs-related diseases, including MPA, and this study aimed to identify this antigen. First, Western blotting (WB) was carried out using neutrophil lysates as antigens and IgG eluted from sera using a protein G column as antibodies. Results showed that a 17-kDa band was detected by ANETA IgG with NETs degradation inhibitory activity eluted from the 2 MPA sera, but not ANETA IgG without NETs degradation inhibitory activity and IgG without ANETA (Figure 1A). A piece of gel corresponding to the 17-kDa band was cut from the sodium dodecyl sulfate (SDS)–polyacrylamide gel and subjected to nanoscale liquid chromatography coupled to tandem mass spectrometry. The antigen candidates are listed in Supplementary Table 1 (available with the online version of this article). Based on the order of priority and molecular weight, myosin light chain 6 (MYL6) was most likely the antigen recognized by the ANETA with NETs degradation inhibitory activity. WB using a commercially available anti-MYL6 polyclonal antibody as a primary antibody revealed that MYL6 was present in NETs and neutrophil lysates (Figure 1B). In addition, when recombinant MYL6 was electrophoresed through an SDS-polyacrylamide gel and subjected to WB, only ANETA IgG with NETs degradation inhibitory activity reacted with the corresponding 17-kDa band. Further, when the commercially available anti-MYL6 polyclonal antibody was allowed to react with phorbol myristate acetate (PMA)-induced NETs 30 minutes before digestion by DNase I, NETs degradation was significantly inhibited compared to when control rabbit IgG was applied instead (Figure 2). These findings were consistent with the observation of recovered NETs degradation activity in ANETA-positive MPA sera after IgG depletion.[6]

Myosin is a hexameric ATPase cellular motor protein composed of 2 heavy chains, 2 nonphosphorylatable alkali light chains, and 2 phosphorylatable regulatory light chains.[7] MYL6 is one of the nonphosphorylatable alkali light chains. Myosin mediates the morphological alteration and movement of cells by interacting with F-actin. F-actin is a cytoskeletal filamentous protein formed by the polymerization of spherical G-actin as a monomer. Because G-actin can bind to DNase I and absorb its enzymatic activity,[8] it is hypothesized that the anti-MYL6 antibody could interfere with the molecular association between myosin and F-actin. This might inhibit G-actin polymerization or promote F-actin degradation into G-actin. Consequently, the increased G-actin would absorb the NETs degradation activity of DNase I.

To our knowledge, serum anti-MYL6 antibody in autoimmune diseases has not yet been described in the literature. Anti-MYL6 antibody was detected in 2 of the 19 patients with MPA enrolled in the previous study.[6] Although it is speculated that the presence of anti-MYL6 antibody with NETs degradation inhibitory activity could aggravate NETs-related diseases, including MPA, the sample size was too small to lead to statistically significant conclusions. Therefore, a mass study using a Japanese nationwide cohort of patients with AAV (n > 100) is now in planning.[9] Currently, anti-MYL6 antibody can be detected only by WB. Because WB is not a high-throughput process, we recommend that an ELISA in which the recombinant MYL6 is immobilized be established. The correlation between the presence of anti-MYL6 antibody detected by ELISA and the clinical variables of patients with AAV is an important subject to be addressed in the next project.

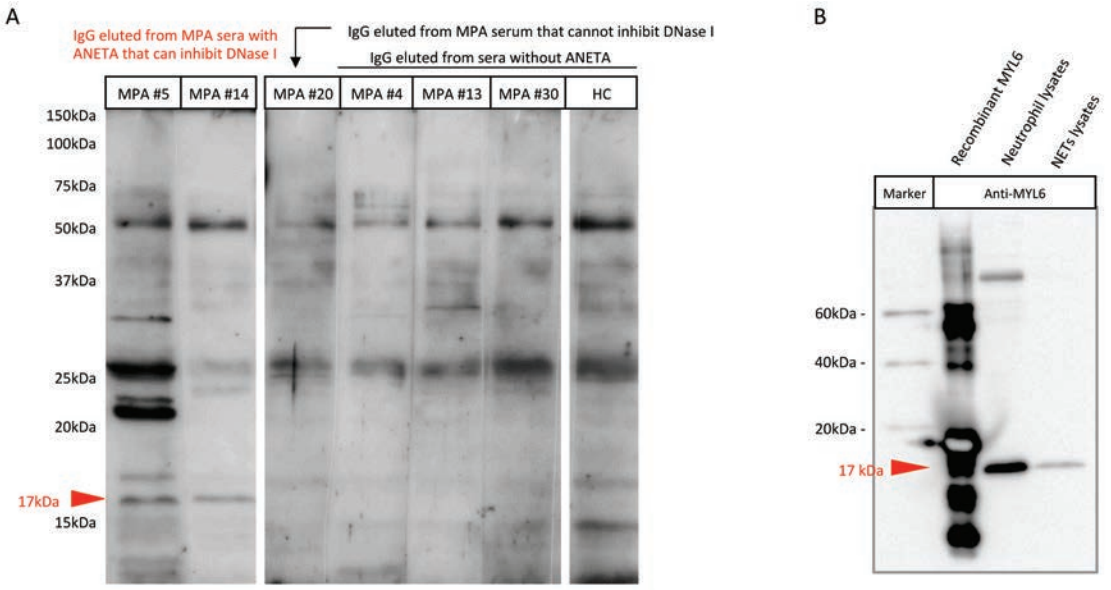


*Figure 1*. Western blot (WB). (A) Neutrophil lysates from healthy volunteers (5 μl/lane) heated under reducing conditions were applied to 12.5% SDS-PAGE. WB was carried out using IgG eluted from MPA sera containing ANETA with NETs degradation inhibitory activity (#5 and #14) as the primary antibody. For controls, IgG eluted from MPA sera containing ANETA without NETs degradation inhibitory activity (#20), MPA sera without ANETA (#4, #13, and #30), and HC sera were used. IgG was eluted from sera using Protein G HP SpinTrap (GE Healthcare) according to the manufacturer's instructions. (B) Recombinant MYL6 (Novus Biologicals; 0.5 μg/lane), neutrophil lysates (5 μl/lane), and NETs lysates (5 μl/lane) heated under reducing conditions were applied to 15% SDS-PAGE. WB was carried out using a commercially available anti-MYL6 polyclonal antibody (Abcepta) as the primary antibody. ANETA: anti-NETs antibody; HC: healthy control; MPA: microscopic polyangiitis; MYL6: myosin light chain 6; NET: neutrophil extracellular trap; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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The authors declare no conflicts of interest relevant to this article.

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## DATA AVAILABILITY

The data sheets used and/or analyzed in this study are available from the corresponding author on reasonable request.

## ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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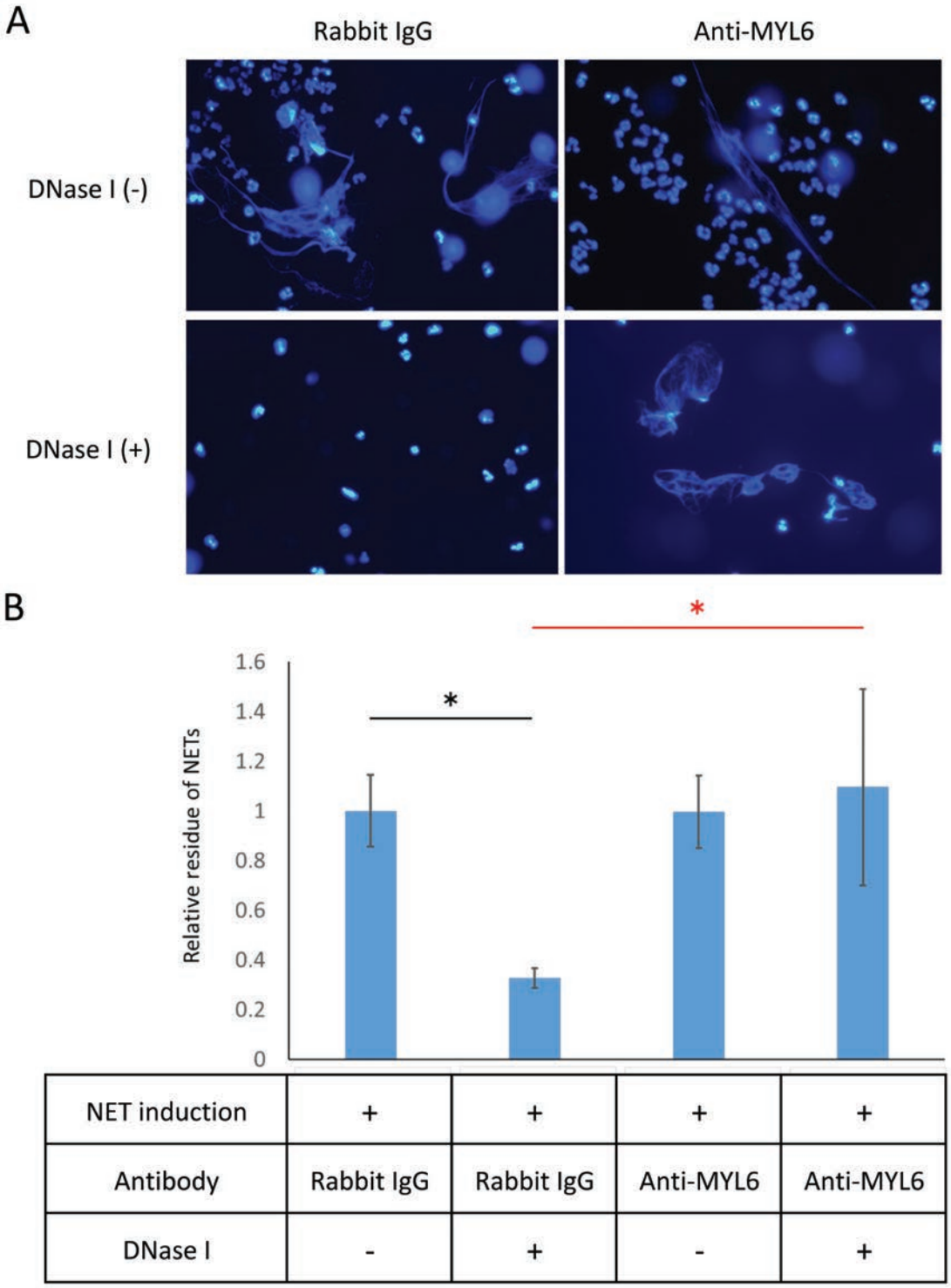


*Figure 2.* NETs degradation assay. (A) Peripheral blood neutrophils from healthy volunteers were seeded in the wells of 4-well chamber slides ($1 \times 10^6$/mL, 400 μL/well) and treated with 100 nM PMA for 3.5 h at 37 °C. The anti-MYL6 polyclonal antibody (Abcepta) or control rabbit IgG (Abcam) was applied to the wells (1 μg/mL), and the chamber slides were allowed to sit at 37 °C for 30 min. Thereafter, the medium was replaced by PBS with or without 1 U/mL DNase I, and the samples were incubated for 30 min at 37 °C. After rinsing with PBS, the samples were fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with PBS, the remaining samples were mounted with a 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting solution. Photomicrographs (magnification ×200) were taken randomly (6 fields per well of chamber slides). Representative photomicrographs are shown. (B) The DAPI+ area was measured using ImageJ version 1.50i (https://imagej.nih.gov/ij). The relative residue of NETs was calculated by setting the value of NETs without digestion under the presence of control rabbit IgG as 1. Experiments were repeated independently, and the reproducibility of results was confirmed. Data are shown as mean (SD). * $P < 0.05$ (1-way ANOVA, followed by Tukey-Kramer post hoc test). MYL6: myosin light chain 6; NET: neutrophil extracellular trap; PBS: phosphate-buffered saline.