

ANTI-NEUTROPHIL CYTOPLASM ANTIBODIES IN
WEGENER'S GRANULOMATOSIS RECOGNIZE
AN ELASTINOLYTIC ENZYME

BY JENS LÜDEMANN, BERT UTECHT, AND WOLFGANG L. GROSS

From the I. Medizinische Universitätsklinik, 2300 Kiel, Federal Republic of Germany

Recently, autoantibodies directed against a cytoplasmic antigen of human neutrophil granulocytes (anti-neutrophil cytoplasm antibodies [ACPA; also known as ANCA]) have been described to be closely associated with Wegener's granulomatosis, a granulomatous disorder associated with systemic necrotizing vasculitis (1-3). Furthermore, ACPA serum concentration appears to be correlated with disease activity (1, 4-7). Together with the known involvement of granulocytes in granuloma formation, a hallmark of Wegener's granulomatosis, this correlation supports the hypothesis that ACPA play a pathogenetic role (8). Therefore, identification of the target antigen of ACPA is of crucial importance for further investigations into the pathogenesis of this disease. Here we report that ACPA are directed against an elastinolytic neutral serine proteinase, which is most probably identical with the recently described proteinase 3 (9-11), the third neutral serine proteinase of human neutrophils.

Materials and Methods

Purification of the Target Antigen of ACPA. The target antigen of ACPA was released from human neutrophils during degranulation induced by PMA (12) and was further purified by affinity chromatography using a column with bound IgG from an ACPA-positive serum as described previously (13).

SDS-PAGE and Immunoblotting. The affinity-purified antigen was separated in 20% acrylamide gels under reducing or nonreducing conditions using a PhastSystem (Pharmacia/LKB Biotechnology, Uppsala, Sweden). After electrophoretic transfer to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) by semi-dry blotting (14), the membrane was cut into strips and blocked in PBS containing 2% casein (PBS/casein). Serum pools from ACPA-positive sera or from healthy donors (13) were added in dilutions of 1/100 in PBS/casein and incubated for 2 h, followed by four washings with PBS containing 0.05% Tween 20 (PBS/Tween). After incubation for 2 h with gold-linked anti-human IgG (Janssen Life Sciences Products, Olen, Belgium) diluted 1/100 in PBS/casein, the membrane strips were washed four times with PBS/Tween and two times with distilled water. To enhance the gold signal, a silver enhancement mixture (Janssen Life Sciences Products) was added for 10 min, followed by washing with distilled water and airdrying.

In some experiments human neutrophil elastase and cathepsin G (Calbiochem-Behring Corp., San Diego, CA) were separated in parallel under nonreducing conditions. Immunodetection was done as described above with sheep anti-elastase and sheep anti-cathepsin G an-

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Address correspondence to Jens Lüdemann, I. Medizinische Universitätsklinik, Schittenhelmstr. 12,
2300 Kiel 1, Federal Republic of Germany.

tisera (Serotec, Oxon, England) followed by rabbit anti-sheep IgG (Sigma Chemical Co., St. Louis, MO) and gold-linked goat anti-rabbit IgG (Janssen Life Sciences Products).

Silver staining of gels was done with a staining kit (Sigma Chemical Co.) according to the manufacturer's instructions. Molecular weights were estimated by means of a low molecular weight calibration kit (Pharmacia/LKB Biotechnology) separated under reducing conditions.

Partial Amino Acid Sequence Analysis. To obtain sequence data the antigen preparation was separated in SDS-PAGE under nonreducing conditions and transferred electrophoretically to a polyvinylidene difluoride membrane as described above. The membrane was stained with Coomassie Blue and the band containing the antigen was cut out and subjected to sequence analysis in a gas-phase sequencer (model 470; Applied Biosystems, Inc., Foster City, CA) as described elsewhere (15). The MIPSX database (Martinsrieder Institut für Proteinsequenzen, Martinsried, FRG), which contains all available protein and nucleic acid databases, was used for sequence comparison.

Cleavage of Substrates. Cleavage of α -naphthyl acetate and synthetic peptides by the antigen and the influence of enzyme inhibitors on esterase activity were analyzed as described in detail elsewhere (11). Assays for cleavage of synthetic peptides included the positive controls elastase, cathepsin G, and trypsin. To investigate elastinolytic activity, the antigen was separated in SDS-PAGE under nonreducing conditions and the electrophoresis gel was layered on top of an agarose plate containing finely pulverized (smaller than 37 μ m) bovine neck ligament elastin labeled with fluorescein (Elastin Products Company, Inc., Pacific, MO), prepared according to the manufacturer's instructions. After an incubation period of 16 h at 37°C the gel was removed and the plate was photographed with indirect illumination against a black background.

Results and Discussion

The affinity-purified antigen was applied to SDS-PAGE under reducing or non-reducing conditions followed by immunoblotting. Under reducing conditions SDS-PAGE revealed two main protein bands and a minor one with molecular masses between 26 and 28 kD (Fig. 1, lanes 1 and 2). Using sera containing ACPA in immunoblotting, no reaction could be observed with any of the reduced proteins (Fig. 1, lane 3). Separation of the antigen under nonreducing conditions revealed a relatively diffuse band in protein staining and immunoblotting (Fig. 1, lanes 4 and 5), with the main density in the molecular weight range of 38 kD. A serum pool of 10 healthy donors showed no reaction with the antigen separated under nonreducing conditions (Fig. 1, lane 6). The observation that the immunoreactivity of the antigen was totally abolished when disulfide bonds were cleaved by reduction with 2-ME indicates that the autoantibodies are directed against a conformational epitope of the protein.

The amino acid sequence of the NH₂ terminus of the antigen was obtained in two independent determinations. The first sequence analysis revealed 9 NH₂-terminal amino acids and the second analysis with antigen prepared from different donors extended the sequence information to the 17 NH₂-terminal amino acids Ile-Val-Gly-Gly-His-Glu-Ala-Gln-Pro-His-Ile-Arg-Pro-Ile-Tyr-Met-Ala. This sequence was not identical with any of the sequences listed in the January 1989 release of the MIPSX database. However, some considerable homologies with known serine proteinases were detected (Fig. 2), especially the highly conserved NH₂-terminal sequence Ile/Val-Ile/Val-Gly-Gly in connection with Pro in position 13. Interestingly, the two well-characterized neutral serine proteinases of human neutrophils, elastase (16) and cathepsin G (17), and the recently described proteinase 3 (10) share

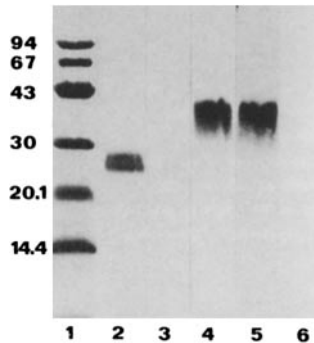


FIGURE 1. SDS-PAGE and immunoblotting of the target antigen of ACPA. Silver-stained molecular weight markers (lane 1) and target antigen of ACPA separated under reducing (lane 2) and non-reducing (lane 4) conditions. Immunoblot of the antigen separated under reducing (lane 3) or nonreducing (lanes 5 and 6) conditions probed with a serum pool containing ACPA (lanes 3 and 5) or a serum pool from healthy donors (lane 6). Relative molecular masses of the marker proteins are shown on the left in kilodaltons.

considerable NH_2 -terminal sequence homology with the antigen (Fig. 2). Evidence that the antigen is a serine esterase has been reported recently by Goldschmeding et al. (18) who were able to immunoprecipitate a 29-kD protein labeled with tritiated diisopropyl fluorophosphate (inhibitor of serine proteinases) using ACPA-positive sera.

These results encouraged us to investigate the enzymatic activity of the antigen. Initially we found that the antigen is able to cleave α -naphthyl acetate, a substrate that is hydrolyzed by many proteinases. The pH optimum for esterase activity with α -naphthyl acetate was between 6.75 and 7.25, indicating that it is a neutral proteinase. Assays using this substrate at pH 7.0 demonstrated that the specific activity of the antigen was $\sim 60\%$ that of elastase and $\sim 130\%$ that of cathepsin G. The specific substrates for human leukocyte elastase (Suc-Ala-Ala-Ala-pNA, Suc-Ala-Ala-Val-pNA, and MeO-Suc-Ala-Ala-Pro-Val-pNA), for cathepsin G (Suc-Ala-Ala-Pro-Phe-pNA), and for trypsin (Tosyl-Arg-Methyl-ester) were not hydrolyzed by the antigen. To investigate if the antigen is active against elastin, it was separated in SDS-PAGE under nonreducing conditions and the electrophoresis gel was layered on top of an agarose gel containing insoluble elastin particles. Under these conditions the antigen had considerable elastinolytic activity (Fig. 3, lane 3). The positions of elastase and cathepsin G in the electrophoresis gel were clearly distinct from that of the antigen, as demonstrated by immunological detection of these enzymes with specific antisera (Fig. 3, lanes 4 and 5), which did not react with the antigen (not shown). The conclusion drawn from the sequence data that the target antigen of ACPA is a serine protease was further substantiated by using inhibitors with relative enzyme class

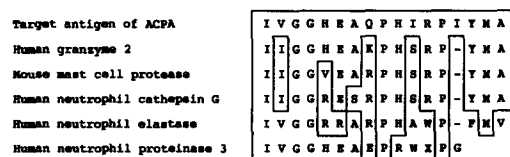


FIGURE 2. NH_2 -terminal sequence of the target antigen of ACPA compared with the human lymphocyte protease granzyme 2 (22), mouse mucosal mast cell protease (23), human neutrophil cathepsin G (17), human neutrophil elastase (16), and human neutrophil proteinase 3 (10). Of proteinase 3 only 14 NH_2 -terminal amino acids have been published in a Ph.D Thesis (10). The one letter amino acid code is used (X = not identified). The gap (-) in position 14 is introduced to optimize identity.

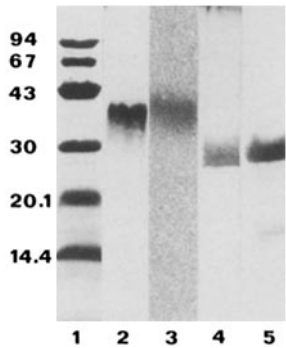


FIGURE 3. Degradation of elastin by the target antigen of ACPA (lane 3) and immunoblot of the antigen (lane 2), human neutrophil elastase (lane 4), and human neutrophil cathepsin G (lane 5), all separated in SDS-PAGE under nonreducing conditions. The dark band in lane 3 shows loss of light scattering under indirect illumination due to degradation of the elastin particles. Relative molecular masses of the marker proteins separated under reducing conditions (lane 1) are shown on the left in kilodaltons.

specificity. The α -naphthyl acetate esterase activity was not inhibited by 10 μ M pepstatin (inhibitor of carbonyl proteinases), 1 mM *N*-ethylmaleimide (inhibitor of thiol proteinases), or 5 mM EDTA (inhibitor of metalloproteinases) but by 1 mM PMSF (inhibitor of serine proteinases). In addition we were able to inhibit esterase activity of the antigen with 5 μ M soybean trypsin inhibitor, another inhibitor of serine proteinases.

Since the molecular weight of the three isoforms of the antigen, its substrate specificity, and its inhibitor profile reported in this study are identical with those reported recently for proteinase 3 (10, 11), we conclude that ACPA are most probably directed against proteinase 3. This was further substantiated by purifying the antigen with the same chromatographic techniques described by Kao et al. (11) for isolation of proteinase 3. The differences in the NH_2 -terminal amino acid sequence (Fig. 2) could be due either to microheterogeneity or inaccuracies in one of the sequence analyses. Very recently the NH_2 -terminal amino acid sequence of a 29-kD antimicrobial protein from human neutrophils, which is almost identical to that of the antigen, has been reported (19). It is very likely that this protein is also identical with proteinase 3, but that must be proven by functional studies.

Proteinase 3 possesses elastolytic activity and therefore may play a role in the destruction of tissue. It has been shown that proteinase 3 is, along with elastase, only the second enzyme purified from human phagocytes that causes experimental emphysema (10, 11). Elastase has been reported to be partially responsible for tissue injury in vasculitis (20) and to function as a major proteinase in granulomatous tissue remodeling (21). Proteinase 3 also digests elastin and may therefore induce pathological alterations similar to those induced by elastase. In preliminary experiments we found that binding of ACPA to the antigen did not inhibit its enzyme activity, but possibly the autoantibodies prevent the enzyme from being inactivated by its natural inhibitors. A thus uninhibited proteinase digesting elastic fibers in vessel walls could cause a necrotizing vasculitis and may also be involved in granuloma formation, which are the two major histopathological findings in Wegener's granulomatosis. Further investigations into the biological functions of proteinase 3 and its physiological inhibitors might give more insight into the pathomechanisms not only of vasculitis and pulmonary emphysema but also of other inflammatory disorders.

Summary

The target antigen of anti-neutrophil cytoplasm antibodies (ACPA; also known as ANCA) was isolated by affinity chromatography from supernatants of human neutrophils, stimulated with phorbol ester to induce degranulation. Sequence analysis of the antigen revealed 17 NH₂-terminal amino acids (IVGGHEAQPHIR-PIYMA), which have considerable sequence homology with known serine proteinases. Investigation of the enzymatic activity showed that the antigen is a neutral serine proteinase that is able to cleave elastin. Since the molecular weight of the antigen, its substrate specificity, and its inhibitor profile reported in this study are identical with those reported recently for proteinase 3, we conclude that ACPA are most probably directed against proteinase 3.

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