Wegener's Granulomatosis Autoantigen Is a Novel Neutrophil Serine Proteinase

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Circulating IgG autoantibodies that produce cytoplasmic immunofluorescence staining of ethanol-fixed normal neutrophils have recently been found in a large percentage of patients with active Wegener's granulomatosis. Such autoantibodies are rarely found in other diseases and are therefore virtually diagnostic of Wegener's granulomatosis. The nature of the neutrophil antigen defined by these autoantibodies is controversial and the roles of the antigen and/or autoantibodies in the pathogenesis of Wegener's granulomatosis are unknown. We studied serum samples that produce the cytoplasmic pattern of staining from 10 patients with a diagnosis of Wegener's granulomatosis. By Western blot analysis, all 10 sera reacted with a 29-Kd neutrophil protein (p29). We generated a mouse monoclonal antibody directed against this antigen. The monoclonal antibody produced the same immunofluorescence staining pattern as the serum autoantibodies and was used to affinity-purify p29. The purified antigen had a novel N-terminal sequence homologous to members of the serine proteinase family and bound to radiolabeled diisopropyl fluorophosphate (DFP). We conclude that the neutrophil antigen responsible for the cytoplasmic staining pattern produced by autoantibodies in patients with active Wegener's granulomatosis is a distinctive serine proteinase.

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Fig 1. Western blot of an 8% to 12% polyacrylamide gel showing the 29-Kd neutrophil antigen (arrow) recognized by sera from 10 patients (Lanes 2 through 11) with Wegener's granulomatosis. Lane 1 is an India Ink stain of neutrophil acid extract (see Materials and Methods). Lane 12 shows background reactivity of normal human serum. The latter does not recognize the p29 antigen but reacts weakly with a faint band at 27 Kd. Arrowheads represent molecular weight markers β-galactosidase, 116 Kd; phosphorylase-b, 92.5 Kd; bovine albumin, 66 Kd; ovalbumin, 45 Kd, and carbonic anhydrase, 31 Kd (obtained from Biorad).

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WEGENER'S GRANULOMATOSIS is a disease of unknown cause and pathogenesis. Its major pathologic features are necrotizing granulomatous lesions, which most often affect the upper and lower airways, vasculitis, necrotizing glomerulonephritis, and pulmonary capillaritis. Wegener's granulomatosis is a devastating illness, and without immunosuppressive therapy it can lead to rapid loss of renal function or massive pulmonary hemorrhage. Death occurs in over 80% of patients within 1 year. Since treatment with cyclophosphamide and steroids is generally effective in preventing progression, reliable and rapid diagnosis is critical. Definitive diagnosis has depended on demonstration of characteristic histologic findings. However, it has recently been shown that detection of circulating autoantibodies against neutrophils and monocytes is useful in the diagnosis of Wegener's granulomatosis and related conditions.

The autoantibodies have usually been detected by indirect immunofluorescence assay, employing ethanol-fixed normal neutrophils as substrates. Two staining patterns have been described: (1) cytoplasmic, and (2) nuclear or perinuclear. The cytoplasmic pattern is detected in the majority of patients with active Wegener's granulomatosis and is rarely found in other diseases. In contrast, the nuclear or perinuclear staining pattern is seen in only a very small percentage of patients diagnosed as having Wegener's granulomatosis, and is also observed in some patients with idiopathic necrotizing and crescentic glomerulonephritis, and in periarteritis with glomerulonephritis (so-called microscopic periarteritis). The antigen associated with the nuclear staining pattern has been shown to be myeloperoxidase (MPO), a primary granule component. The nuclear staining pattern results from artifactual redistribution due to ethanol fixation. The nature of the antigen(s) associated with the cytoplasmic staining pattern seen in patients with Wegener's granulomatosis have...
been controversial. In one report, the antigen was considered to be alkaline phosphatase; however, this finding has not been confirmed. Others have found that the autoantigen is a soluble protein of 27 to 29 Kd localized to the primary or secondary granule fractions.

In the present report, we found that sera producing the cytoplasmic immunofluorescence staining pattern from patients with Wegener's granulomatosis contain autoantibodies directed against a neutrophil 29-Kd protein, in confirmation of other reports. These autoantibodies gave a cytoplasmic staining pattern by indirect immunofluorescence. Using a mouse monoclonal antibody that we generated against the 29-Kd protein, we purified and characterized the antigen. Our findings indicate that the 29-Kd protein recognized by Wegener's granulomatosis autoantibodies is a novel serine proteinase.

MATERIALS AND METHODS

Patients' sera. Serum samples were obtained from 10 patients diagnosed as having Wegener's granulomatosis. Clinically, all patients had upper or lower airway disease (nasal erosion, sinusitis, hemoptysis) with or without rapidly progressive renal failure. Pathologically, three patients had characteristic necrotizing granulomatous lesions in nasal biopsies. The remaining seven patients had pathologic evidence of nasal vasculitis or pulmonary capillaritis with or without necrotizing and crescentic glomerulonephritis with scanty or absent immunoglobulin deposits. Sera were also obtained from normal volunteers. All sera were frozen at -20°C until used.

Indirect immunofluorescence analysis. Antineutrophil cytoplasmic antibodies were detected by indirect immunofluorescence using cytocentrifuged and ethanol-fixed neutrophils from normal individuals. Neutrophils were isolated by centrifugation on Ficoll-Hypaque gradients, followed by hypotonic lysis as described. Cytocentrifuge preparations were made using a Shandon Southern cytocentrifuge (Cheshire, England). Each preparation was fixed for 5 minutes in 100% ethanol, dried, and incubated for 1 hour at room temperature (RT) with serum (1:16 dilution). After two washes, cells were stained with fluoresceinated goat anti-human Ig (60 minutes, RT), washed, and examined using a fluorescence microscope.

Western blot analysis of Wegener's granulomatosis autoantibodies. A sodium acetate extract from normal human neutrophils was prepared as described. Briefly, 1 x 10⁶ cells were treated with diisopropylfluorophosphate (DFP) (5 mmol/L, 10 minutes on ice), washed, and sonicated for 5 minutes in 0.2 mol/L sodium acetate buffer, pH 4.2 at 0°C. After centrifugation at 20,000 g for 20 minutes at 4°C, the supernatant was adjusted to pH 7.4, or dialyzed against phosphate buffered saline (PBS), pH 7.4. Concentrations...
tration of protein in the sample was determined by the method of Lowry. The acid extract (25 μg/lane) was separated by electrophoresis on sodium dodecyl sulfurepolyacrylamide gels (SDS-PAGE). Proteins from unstained gels were transferred electrophoretically onto nitrocellulose membranes. The membranes were cut into strips and stained with patients sera (1:10 dilution) followed by biotinylated secondary antibody and avidin biotin peroxidase complex. The bound antibodies were detected by the chromagen 3- amino-9-ethylcarbazole as a substrate for peroxidase.

Preparation of monoclonal antibody (MoAb). To generate MoAbs, 6-week-old female Balb/c mice were immunized each with 10 μg of neutrophil acid extract (in complete Freund's adjuvant) intradermally in the lower leg. After three boosts over a 2-week period, popliteal lymph nodes were isolated, and lymphocytes used for fusion with the NS1 mouse plasma cell line as described. After 10 to 14 days of growth in hypoxanthine-aminopterin-thymidine (HAT)-selected culture medium, culture supernatants from hybridomas were evaluated for antineutrophil activity by Western blot analysis as described above. MoAbs that stained a 29-Kd band (p29) that comigrated with the band identified by Wegener's granulomatosis autoantibodies were selected. Hybridomas with the desired activity were subcloned twice and one MoAb, I E8, was successfully isolated.

p29 affinity purification. The I E8 MoAb was used to affinity purify p29 using the method of Schneider et al. I E8 (of the IgG1 subclass) was bound to sepharose-protein A beads by coupling with dimethylpimmelimidate. A 10-mL column of settled sepharose beads was extensively washed, then incubated with 30 mg of neutrophil acid extract (prepared as detailed above) for 3 hours at RT. The column was washed with five bed-volumes of PBS followed by five bed-volumes of PBS containing 500 mM NaCl. After re-equilibration in PBS, the column was eluted with 0.2 mol/L citric acid, pH 2.75, in 1-mL fractions and neutrality was immediately established using Tris base. Eluted protein was detected spectrophotometrically at an optical density of 280 (OD280). The desired fractions were pooled and incubated with protein A sepharose (to remove trace amounts of contaminating MoAb). Pooled fractions were concentrated and dialyzed against distilled water using collodion bags (Schleicher & Schuell, Keene, NH). Seven hundred micrograms of protein were recovered in the eluate.

Isoelectric focusing. This was done in 0.75-mm thick gels using a vertical gel apparatus (Hoeffer Scientific, San Francisco, CA) and a pH range 3.5 to 11. Gels were run at 2.5 mA constant current for 16 hours at 4°C, fixed, stained with Coomassie Blue R-250, and destained.

N-terminal sequence determination. One hundred micrograms of purified p29 were exhaustively dialyzed against distilled water, concentrated to 200 μL and 20 μL subjected to SDS-PAGE, dry-blotted onto Immobilon-P (Millipore, Boston, MA) and stained with India Ink. The major p29 band was excised with a razor blade and subjected to 20 cycles of Edman degradation using the Applied Biosystems (Foster City, CA) Model 470 A sequenator. Phenylthiohydantoin (PTH) amino acid derivatives were resolved by high performance liquid chromatography (HPLC) using an IBM Cyano column and Permechip ETH precolumn, with gradient elution (solvent A: 70 mmol/L sodium acetate, pH 5.5, 5% vol/vol tetrahydorufuran; solvent B: acetonitrile, gradient 11% to 48% over 20 minutes, at a flow rate of 1 mL/min). A search for protein homology was performed using National Biomedical Research Foundation and SWISS protein data banks.

3H-DFP binding assay. MoAb I E8 was used in a sandwich radiomimunoassay to detect binding of 3H-DFP to p29. A sodium sulfate cut of I E8 ascites was diluted to 10 μg/mL in PBS and 35 μL/well were incubated for 1 hour at 37°C in a 96-well polystyrene microtiter plate. Unoccupied binding sites were blocked with 1% nonfat dried milk. An acetate extract of human neutrophils, prepared without DFP, was diluted to 100 μg/mL and incubated with [3H-DFP (3.3 nmol/L at 3 μCi/μmol/L, NEN, Boston, MA) for 30 minutes at RT. The extract was then added (35 μL/well) to I E8 precoated wells, or wells precoated with an irrelevant MoAb, or with an anti-MPO MoAb (developed in our laboratory) as controls. After a 4-hour incubation period at RT, wells were washed in PBS, dried, cut, and immersed in β-fluor and counted in a β counter.

RESULTS

Wegener’s granulomatosis sera identify a 29-Kd neutrophil cytoplasmic antigen. Sera from 10 patients with Wegener’s granulomatosis were screened for the presence of autoantibodies reactive with normal neutrophil lysates by Western blots. All patients’ sera were obtained within 1 month of tissue biopsy. Sera from all 10 patients contained autoantibodies against a 29-Kd antigen (p29) (Fig 1) and produced a cytoplasmic staining pattern in ethanol-fixed...
Fig 4. N-terminal sequence of p29 and its homology to the N-termini of other serine proteases such as neutrophil elastase, cathepsin G, complement factor D, trypsin, chymotrypsin, plasmin, β-chain, coagulation factors, and thrombin.

Identical residues are indicated by (:) and conserved residues by (×). X represents an unassigned residue.

**Discussion**

The major finding in this report is that antineutrophil cytoplasm autoantibodies in patients with Wegener's granulomatosis are directed against a distinctive serine proteinase.
of 29 Kd. Our conclusion that the antigen is a serine proteinase is based on the homology of its N-terminal sequence with other serine proteinases and its DFP-binding ability. p29 is distinct from the neutrophil DFP-inhibitable neutral proteases elastase and cathepsin G. However, it is possible that the p29 antigen is identical to a third major neutral serine proteinase of neutrophils, proteinase 3.22-25 The two proteinases are major neutrophil constituents, have similar molecular masses and isoelectric points, and bind DFP. However, no information is yet available on the protein sequence of proteinase 3, its reactivity with Wegener’s granulomatosis autoantibodies, or on the substrate specificity of p29 to permit a more accurate comparison between the two proteinases.

The relationship between the p29 serine proteinase and its autoantibodies to the pathogenesis of Wegener’s granulomatosis is unclear at present. It has been proposed that the autoantibodies have a pathogenic role, because their presence and titers usually correlate with disease activity and because of in vitro evidence that they may activate neutrophils, whose products, including proteinases, are known to be capable of inducing tissue injury, as in the lung or glomerulus.26,28 It is conceivable that the autoantibodies promote release of the p29 proteinase or potentiate its tissue-damaging potential, perhaps either by stabilizing its enzymatic activity or through an antibody-mediated effect. Identification of the serine proteinase nature of p29 should now permit testing of these hypotheses.

The usual method of detection of anti-neutrophil cytoplasmic antibodies is indirect immunofluorescence. This method is also useful in the distinction between antibodies against p29 and MPO, since the former produce a cytoplasmic pattern in ethanol-fixed neutrophils and the latter generally produce a nuclear-staining pattern. However, interpretation of immunofluorescence findings requires considerable experience and may vary from one laboratory to another. Isolation of Wegener’s granulomatosis autoantigen should permit the development of more specific and quantifiable assays, based either on the use of MoAbs (Fig 5) or of the purified antigen.

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