Antineutrophil cytoplasmic antibodies (ANCA) are frequently associated with chronic inflammatory bowel diseases (IBD) and hepatobiliary disorders. However, their target antigens have not been identified yet. Recently, we observed an atypical perinuclear ANCA fluorescence (p-ANCA) together with an intranuclear staining using ANCA-positive sera from patients with IBD and hepatobiliary disorders. This observation suggests that the target antigens are localized within the nucleus of neutrophilic granulocytes. To further investigate this hypothesis, we examined sera from patients with ulcerative colitis, primary sclerosing cholangitis, autoimmune hepatitis or systemic vasculitis on ethanol or formaldehyde-fixed neutrophils using confocal laser scanning microscopy and immunoelectron microscopy. Counterstaining with propidium iodide, a DNA-specific dye, showed that ANCA-positive sera in IBD and hepatobiliary disorders react with intranuclear antigens at the nuclear periphery of the neutrophils. Double immunolabeling techniques revealed that nuclear lamina proteins, lamins A, C and B1, and lamin B receptor were colocalized with the antigen(s) recognized by atypical p-ANCA. No colocalization was observed with classical p-ANCA and antibodies against histones (H1-H4). Our study showed that atypical p-ANCA are antinuclear antibodies reactive with granulocyte-specific antigens present in the nuclear lamina. (Hepatology 1998;28:332-340.)

Abbreviations: ANCA, antineutrophil cytoplasmic antibody; IBD, inflammatory bowel diseases; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; p-ANCA, antineutrophil cytoplasmic antibody with perinuclear fluorescence; MPO, myeloperoxidase; PR3, proteinase 3; LBR, lamin B receptor; mPAN, microscopic polyarteritis; UC, ulcerative colitis; WG, Wegener's granulomatosis; ANA, antinuclear antibody; ASMA, anti–smooth muscle antibody; anti–LKM-1, anti–liver-kidney microsomal antibodies; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; LSM, confocal laser scanning microscopy; PBS, phosphate buffered saline; c-ANCA, antineutrophil cytoplasmic antibody with cytoplasmic fluorescence.

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PATIENTS AND METHODS

Patients. Serum samples were obtained from 80 ANCA-positive patients with ulcerative colitis (UC), PSC, AIH, and systemic vasculitis (Wegener’s granulomatosis [WG]), n = 10; microscopic polyarteritis [mPAN], n = 10) in whom ANCA serology was requested for routine diagnostic evaluation in the Department of General Internal Medicine, University of Bonn, Germany. Serum samples were stored at −20°C until analysis. All sera were investigated for the presence of autoantibodies by means of indirect immunofluorescence: antineutrophil cytoplasmic antibodies on ethanol-fixed and formaldehyde-fixed granulocytes (titers > 1:20), antinuclear antibodies (ANA) on Hep2 cells (titers > 1:80) or anti-smooth muscle antibodies (ASMA) on rat triple kidney, liver, and stomach sections (titers > 1:80). Anti-liver-kidney microsomal1 (anti-LKM-1) antibodies were determined by indirect immunofluorescence (titers > 1:10) as well as by enzyme linked immunosorbent assay (ELISA). To exclude false positive results for ANCA testing caused by the simultaneous presence of ANA, the ANCA titer had to be at least two-fold higher than the ANA titer. Therefore, the presence of ANA in our study population might not reflect the actual ANA prevalence in AIH type 1 or PSC.

The patients’ diagnoses were on the basis of generally accepted clinical, radiological, endoscopic, histological, biochemical and serological criteria.22-26 Characteristics of the patients are given in Table 1. Disease activities in IBD and hepatobiliary disorders were assessed by established scores using clinical and laboratory data such as the “Colitis Activity Index” for UC,22 the “MAYO Risk Score” for PSC23 and the “Scoring System for the Diagnosis of Autoimmune Hepatitis.”24 Laboratory data are given as mean (total). Eighty percent (16/20) of patients with PSC had active disease with elevated serum aspartate aminotransferase activities of 71.3 ± 2.1 mg/dL (normal, 0.1-1.1 mg/dL), alkaline phosphatase activities of 665 ± 19.8 ± 2.1 mg/dL (normal, 0.1-1.1 mg/dL), bilirubin concentration of 2.2 ± 1.1 mg/dL (normal, 0.1-1.1 mg/dL), alkaline phosphatase activities of 665 ± 6 mg/dL), globulin G concentrations of 26 ± 5 g/L (normal, 7-17 g/L). Sixteen of 20 patients (80%) with PSC showed histological features of pericholangitis, periporal hepatitis or periportal fibrosis and 4/20 (20%) had histological findings of cirrhosis. In patients with AIH, active disease was assessed in 16/20 patients (80%). Laboratory data revealed elevated serum aspartate aminotransferase activities of 155 ± 27 IU/L and immunoglobulin G concentrations of 19.8 ± 13 g/L. Histological findings assessed before therapy were consistent with chronic hepatitis in 14/20 (70%) patients, and in 6/20 (30%) patients the disease had progressed to cirrhosis. With respect to the disease-specific autoantibody profile,24 AIH type 1 was assessed in 14/20 patients (3/20 patients seropositive for ANA with titers > 1:80, 11/20 patients seronegative for ANA with titers > 1:80). Six of 20 patients were negative for all routinely investigated autoantibodies (ANA, ASMA, anti-LKM-1). However, tests to evaluate the presence of autoantibodies directed against soluble liver antigen, liver cytosolic antigen or the asialoglycoprotein receptor were not performed. Sera from patients with WG (n = 10) and mPAN (n = 10) with ANCA reactivity to proteinase 3 and MPO served as positive controls.

ELISA. All serum samples (diluted 1:100) were tested for reactivity to conventional cytoplasmic antigens such as cathepsin G, elastase, lactoferrin, lysozyme, MPO, proteinase 3 in commercially available ELISA (ELISA, Freiburg, Germany; EUROIMMUN, Gross Gruennau, Germany). ELISA testing was performed according to the manufacturer’s instructions.

Indirect Immunofluorescence Microscopy. ANCA were determined according to the guidelines of the first ANCA-workshop. Briefly, slides with ethanol-fixed or formaldehyde-fixed neutrophils (INOVA Diagnostics, San Diego, CA) were incubated with serum samples (diluted 1:20) in a humid chamber at 20°C for 20 minutes. After being washed twice, bound ANCA were detected with affinity-purified fluorescein isothiocyanate (FITC)-conjugated or Texas red-labeled antibodies (INOVA Diagnostics) at 20°C for 20 minutes followed by two further washing steps. After mounting with an antifading medium,28 slides were viewed with an epi-illumination fluorescence microscope Leitz SM-Lux with a 40× objective lens (Leica, Wetzlar, Germany) and photographed with Kodak Ektachrome 320 T color slide film (Rochester, NY). ANCA-positive sera (titers > 1:20) were titrated to their endpoint by serial dilution. The immunofluorescence patterns were read independently by two investigators who were not aware of the patient’s diagnosis.

Various nuclear labeling techniques were tested for better distinction of the neutrophilic cytoplasm from the nucleoplasm: propidium iodide, antibodies against nuclear lamina proteins or histones (H1-H4) which were detected with either tetramethylrhodamine isothiocyanate (TRITC)-conjugated or Texas red-labeled secondary antibodies.

**Propidium Iodide Staining.** Ethanol-fixed granulocyte slides were incubated with ANCA-positive sera as described above. Counterstaining with propidium iodide (diluted 1:20; 20 minutes, 20°C), a DNA-specific dye, was used to clearly mark the nuclear borders (SIGMA, St. Louis, MO).

**Antibodies to Nuclear Lamina Proteins and Histones (H1-H4).** Polyclonal antibodies against rabbit lamin A/C and B1,29 diluted 1:500, and polyclonal antibodies against rabbit lamin B receptor (LBR),30 diluted 1:200, were incubated (27°C for 30 minutes) on ethanol-fixed granulocytes followed by two washing steps. Bound antibodies were visualized by TRITC-conjugated affinity-purified goat anti-rabbit IgG (H+L) antibodies (diluted 1:200) (DIANOVA, Hamburg, Germany). Polyvalent affinity-purified antibodies against mouse histones (H1-H4) (Boehringer, Mannheim, Germany) were diluted 1:1000. Further incubation steps were performed as mentioned above for antibodies against nuclear lamina proteins. Texas red-labeled affinity-purified goat anti-mouse IgG (H+L) antibodies, diluted 1:200

### Table 1. Clinical Characteristics of the Study Population

<table>
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<th></th>
<th>UC</th>
<th>PSC</th>
<th>AIH</th>
<th>WG</th>
<th>mPAN</th>
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<td>20 (1:640)</td>
<td>20 (1:2560)</td>
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<td>11</td>
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*Immunosuppression: corticosteroids and/or azathioprine and/or cyclosporine.
ANCA Fluorescence Patterns in Relation to Nuclear DNA Staining. Three different patterns of ANCA fluorescence in relation to nuclear DNA staining by propidium iodide could be discerned in double-labeling experiments. In 11/80 sera (14%), a diffuse cytoplasmic ANCA fluorescence pattern without associated intranuclear staining was noted (pattern I; Fig. 1A). This staining pattern closely corresponded to the presence of conventional c-ANCA and was exclusively associated with systemic vasculitis (WG: n = 10, mPAN: n = 1). A peripheral rim-like fluorescence in the perinuclear cytoplasm, also negative for nuclear fluorescence, was detected in 10/80 sera (12%) (pattern II; Fig. 1B) and was identical to that of classical p-ANCA in systemic vasculitis (mPAN: n = 9). Additionally, classical p-ANCA could also be discerned in 1 patient with AIH. In 59/80 patients (74%), an inhomogeneous rim-like ANCA fluorescence in the nuclear periphery, partially overlapping with DNA staining by propidium iodide, was found (pattern III; Fig. 1C). Pattern III, corresponding to so-called atypical p-ANCA, was limited to patients with UC (20/20), PSC (20/20) and AIH (19/20; 95%), but was never observed in systemic vasculitis. Differences between the pattern I, pattern II and pattern III with respect to the underlying disease were highly significant (Fisher’s exact test, P < .001). No significant correlation was found between ANCA staining pattern and disease activity, extent, duration or immunosuppressive therapy (data not shown).

Relationship of p-ANCA Staining Patterns to Fixation of Neutrophils. The p-ANCA fluorescence pattern is known to result from an artificial redistribution of positively charged cytoplasmic antigens to the negatively charged nucleus during ethanol fixation. This redistribution is inhibited when neutrophils are fixed with cross-linking agents such as formaldehyde. Therefore, former p-ANCA with labeling of the perinuclear cytoplasm converts to a diffuse cytoplasmic staining after fixation with formaldehyde. This reactivity was regularly seen with all sera positive for pattern II, corresponding to classical p-ANCA (10/10). However, the very vast majority of the serum samples positive for pattern III p-ANCA, corresponding to atypical p-ANCA (57/59; 97%) showed a persisting fluorescence in the nuclear periphery after formaldehyde fixation, but no cytoplasmic staining.

Relationship of ANCA Target Antigens to the Nuclear Lamina and to Histones (H1-H4) Visualized by Confocal Laser Scanning Microscopy. To further characterize the subcellular localization of atypical p-ANCA antigens with respect to the nucleus, double staining experiments were performed with ANCA-positive sera and antibodies to nuclear lamins A, C, and B1 or to nuclear integral membrane proteins as LBR. p-ANCA were detected with FITC-conjugated secondary antibodies to give a green fluorescence signal, and antibodies to nuclear lamins or LBR were labeled with TRITC-conjugated secondary antibodies to give a red fluorescence. Areas of colocalization were optically superimposed with images of either propidium iodide, antibodies against lamins A/C, B1, LBR or histones (H1-H4) taken at 570 nm wavelength. With this technique, colocalization was identified by a yellow staining caused by mixed color of the green signal from FITC-labeled secondary antibodies to detect ANCA and the red signal of TRITC-labeled secondary antibodies to detect antibodies against nuclear lamina proteins, Texas red-labeled antibodies against histones (H1-H4) or propidium iodide-labeled DNA, respectively. Slides were photographed with Kodak Ektachrome 100 color slide film. All staining patterns were read independently by two investigators.

Immunoelectron Microscopy. Nine of the 80 well-characterized serum samples (atypical p-ANCA: n = 5, classical p-ANCA: n = 2) were further investigated by immunoelectron microscopy. For this purpose, neutrophilic granulocytes were isolated from peripheral blood of normal individuals by a one-step gradient centrifugation technique using PolymorphrepTM (NYCOMED, Oslo, Norway). The separation procedure was performed according to the manufacturer’s instructions. The cell pellet was washed in phosphate buffered saline (PBS), centrifuged (1,000 rpm, 3 minutes) and the cells were fixed with 4% formaldehyde/0.5% glutaraldehyde in PBS (30 minutes, 20°C). After centrifugation (15,000 rpm, 3 minutes), the cells were cryoprotected with 50% polyvinylpyrrolidone/2.3 mol/L sucrose in PBS overnight. The cell pellets were frozen in liquid nitrogen, and ultrathin sections were cut with a Leica EM FCS ultramicrotome (Leica). The cryosections were placed on grids and were stored in 3% bovine serum albumin in PBS. After being blocked with 0.05 mmol/L glycine (30 minutes, 20°C) and washed several times with PBS, the cryosections were incubated with the ANCA-positive human serum samples (diluted 1:100 to 1:300) or affinity-purified polyclonal rabbit lamin B1 antibodies (diluted 1:100 to 1:300). For double labeling experiments, the cryosections were incubated simultaneously with the aforementioned antibodies (20°C, 1 hour). After further washing steps (0.1% cold fish skin gelatin in PBS), bound primary antibodies were detected with affinity-purified immunogold (5 nm) labeled goat anti-rabbit IgG (H + L) secondary antibodies for lamin B1 antibodies or affinity-purified immunogold (10 nm) labeled goat anti-human IgG (H + L) secondary antibodies for ANCA detection (Amersham, Buckinghamshire, UK). Incubation (1 hour, 20°C) with the secondary antibody was again followed by further washes. Cryosections of fixed granulocytes were rehydrated with 0.4 aqueous uranyl acetate in 2% methylcellulose and finally examined by transmission electron microscopy with a Philips (Eindhoven, The Netherlands) CM120 Electron microscope (Philips, Netherlands) at 80 kV. Photographs were taken with an Agfa-Gevaert Scientia HJQ7B film (Agfa, Antwerpen, The Netherlands).

Statistical Methods. Statistical analysis was performed using the $\chi^2$ test and t test for matched pairs. P values of <.05 were considered to be significant.

RESULTS

ANCA Reactivity in Relation to Cytoplasmic Antigens. All serum samples were tested for reactivity to the putative cytoplasmic ANCA antigens such as elastase, cathepsin G, lactoferrin, lysozyme, MPO, and PR3 by ELISA. Reactivity to MPO and PR3 was restricted to ANCA-positive sera of patients with systemic vasculitis. However, this reactivity to MPO and PR3 was not found with sera positive for atypical p-ANCA in IBD, PSC and AIH. Furthermore, the sera did not yield any significant reactivity to the aforementioned cytoplasmic antigens of neutrophils (elastase: n = 2/80, cathepsin G: n = 1/80, lactoferrin: n = 4/80, and lysozyme: n = 0/80).
appeared yellow. Antibodies against nuclear lamins and LBR were used, because they display a characteristic peripheral rim-like staining pattern on mammalian cells, which resembles the “perinuclear” fluorescence seen with atypical p-ANCA.

All sera displaying ANCA staining pattern III in relation to propidium iodide showed colocalization of ANCA fluorescence with staining of antibodies against nuclear lamins and LBR as a fine rim-like peripheral nuclear fluorescence (Fig. 2A-2D). Foci of fluorescence in centrally located nuclear regions also revealed colocalization of p-ANCA fluorescence with staining of antibodies against nuclear lamins and LBR. In contrast, no colocalization of ANCA and antibodies against nuclear lamins and LBR could be shown with pattern I and pattern II reactive sera (Figure 2F). This phenomenon was independent of serial dilution.

Because antibodies against histones (H1-H4) induce an immunofluorescence pattern which partially resembles the staining pattern of atypical p-ANCA, we additionally investigated our ANCA-positive sera for putative colocalization with antibodies against histones (H1-H4). In double staining experiments, confocal laser scanning microscopy showed only partial colocalization. This colocalization was noted at several distinct spots which were likely to correspond to anchoring sites of the heterochromatin with the nuclear lamina (Fig. 2E). Therefore, the colocalization pattern of antibodies against histones (H1-H4) was in marked contrast to our findings with antibodies against nuclear lamins that were completely colocalized.

Relationship of ANCA Target Antigens to the Nuclear Lamina Visualized by Immuno Electron Microscopy. To further verify the hypothesis of nuclear antigen localization in atypical p-ANCA, cryosections of formaldehyde/glutaraldehyde-fixed neutrophils were reacted with immunogold-labeled ANCA-positive sera which were either positive for atypical p-ANCA, classical p-ANCA or c-ANCA. Bound gold particles were visualized by immunoelectron microscopy. The characteristic “perinuclear” fluorescence seen with atypical p-ANCA on formaldehyde-fixed neutrophils by LSM could clearly be assigned to the neutrophilic nucleus by immuno electron microscopy. In all sera positive for atypical p-ANCA, immunogold labeling was predominantly found in the nuclear periphery, closely associated with the heterochromatin (Fig. 3A). The euchromatin, however, did not show any relevant labeling. Most of the sera positive for atypical p-ANCA also displayed some cytoplasmic immunogold staining, which, however, was present in significantly lower amounts than the nuclear immunogold labeling. The ratio of cytoplasmic immunogold labeling differed significantly from that observed in the nucleoplasm (mean ratio 1:3.3, P < .001, t test for matched pairs). The distribution of nuclear versus cytoplasmic immunogold labeling was independent of serial dilution.

Furthermore, colocalization experiments were performed with immuno electron microscopy to assign the target antigens of atypical p-ANCA to nuclear substructures. Similar to LSM experiments, immunogold staining obtained with atypical p-ANCA was colocalized with immunogold labeling of lamin B1 antibodies and was predominantly found within the nuclear periphery (Fig. 3B). This immunogold labeling pattern could be demonstrated in all sera positive for atypical p-ANCA.

Fig. 1. Different ANCA-staining patterns on ethanol-fixed granulocytes relative to simultaneous propidium iodide counterstaining visualized by confocal laser scanning microscopy. ANCA were detected with FITC-conjugated secondary antibodies that produce a green signal at 525 nm wavelength. Propidium iodide gives a red signal at 570 nm wavelength. When the two signals are superimposed, colocalization appears yellow. (A) A diffuse cytoplasmic ANCA staining without associated intranuclear immunofluorescence in a patient with Wegener's granulomatosis (titer 1:640) was designated as pattern I, corresponding to c-ANCA with reactivity to proteinase 3. (B) A peripheral rim-like ANCA fluorescence without intranuclear fluorescence in a patient with microscopic polyarteritis (titer 1:1,280) was described as pattern II or “classical” p-ANCA with reactivity to myeloperoxidase. (C) A heterogeneous peripheral rim-like ANCA staining completely overlapping with propidium iodide marked by yellow nuclear fluorescence in a patient with primary sclerosing cholangitis (titer 1:2,560) was defined as pattern III or “atypical” p-ANCA with unknown target antigens. Size bars indicate 5 µm.
p-ANCA which have been investigated by immunoelectron microscopy.

In contrast, c-ANCA as well as classical p-ANCA displayed both a characteristic immunogold labeling of cytoplasmic structures (data not shown), because the formaldehyde fixation that is used for immunoelectron microscopy converts the artificial “perinuclear” distribution of cytoplasmic antigens in classical p-ANCA to a cytoplasmic pattern. No immunogold labeling was observed with sera of normal controls.

**DISCUSSION**

Our observation on the localization of atypical p-ANCA staining in the nuclear periphery as revealed by double labeling with propidium iodide supports the hypothesis that in IBD and hepatobiliary disorders target antigens of ANCA are localized in the neutrophil nucleus. In contrast, double staining with ANCA of sera from patients with systemic vasculitis and proquad iodide was restricted to the cytoplasm. Although antigens detected by classical p-ANCA and atypical p-ANCA were assumed to be localized in the perinuclear cytoplasm, in the case of classical p-ANCA this localization is known as an artifact caused by antigen relocation on ethanol-fixed granulocytes. Accordingly, a cytoplasmic staining has been reported to become detectable in classical p-ANCA staining when using cross-linking fixatives such as formaldehyde. In this study, we have shown that staining with atypical p-ANCA, however, remained predominantly localized in the nuclear periphery on formaldehyde-fixed granulocytes. More importantly, we showed the colocalization of nuclear lamina proteins A, B1, C and LBR with the target antigens of atypical p-ANCA.

In mammalian somatic cells, nuclear lamins polymerize to form the nuclear lamina that is a fibrillar meshwork on the inner aspect of the inner nuclear membrane. LBR is an integral protein of the inner nuclear membrane,58-60 or against LBR,56,60,61

Although nuclear lamins and their integral membrane proteins are expressed in all mammalian cells, reactivity of atypical p-ANCA in sera from patients with IBD and hepatobiliary disorders is confined to neutrophils. In this context, none of the sera positive for atypical p-ANCA stained Hep2 cells (data not shown). This indicates that the putative target antigens of atypical p-ANCA are either specific for granulocytes or present in other cell types below detectability.

The identification of the nuclear target antigens of atypical p-ANCA may have consequences for the clinical definition of IBD and hepatobiliary disorders. In this context, overlap syndromes between UC and PSC as well as between AIH and PSC were described.62,63 Such overlap syndromes could be demonstrated in 18 of 60 (30%) patients of our study population. With respect to the high prevalence of atypical p-ANCA in these overlap syndromes (18/18; 100%), this fact may point to a distinct subgroup of autoimmune diseases with a shared immunological alteration, even though the clinical and laboratory characteristics of these patients were not different from those without overlap syndromes. Future attempts to identify the responsible antigen(s) of atypical p-ANCA should be preferentially targeted at the nuclear lamina and heterochromatin-associated proteins adjacent to the nuclear lamina.

In conclusion, the terminology of “antineutrophil cytoplasmic antibodies” (ANCA) in IBD and hepatobiliary disorders appears to be a misnomer, and should be replaced by the term “antineutrophil nuclear antibodies” (ANNA).

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