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Rats Injected with Syngenic Rat Apoptotic Neutrophils Develop Antineutrophil Cytoplasmic Antibodies

YAËL C. PATRY,* DAVID C. TREWICK,† MARC GREGOIRE,§ MARIE A. P. AUDRAIN,* ANNE M. N. MOREAU,‡ JEAN-YVES MULLER,* KHALED MEFLAH,§ and VINCENT L. M. ESNAULT†

*Departments of *Immunology, †Nephrology and Clinical Immunology, and ‡Pathology, Hôtel Dieu, Nantes University Hospital; and §INSERM U419, Nantes, France.*

Abstract. Antineutrophil cytoplasmic antibodies (ANCA) are present in sera from patients with various forms of vasculitis-associated glomerulonephritis. Because autoantibodies may be directed against antigens presented by apoptotic cells, generation of ANCA using apoptotic neutrophils (PMN) in syngenic Brown Norway (BN) rats was attempted. These rats are T-helper type 2–prone animals, already used successfully in other ANCA-positive animal models. BN rats received repeated injections of buffer or of nonapoptotic or apoptotic PMN aged in cultures, in the footpad and once intravenously. Four of five rats that received injections of PMN aged for 48 h developed ANCA, which cross-reacted with human leukocyte elastase in three cases. None of the rats that received injections of freshly isolated neutrophils developed ANCA. One rat that received buffer injection and that exhibited chronic skin infection developed delayed ANCA. None of the rats showed signs of disease: no weight loss and no proteinuria. Then a subnephritogenic dose of antibody directed against rat glomerular basement membrane was injected. Rats then were killed, and different organs were frozen and studied. No significant lesions were found in kidneys or lungs. It is concluded that injections of apoptotic but not freshly isolated PMN can generate ANCA in BN rats. Additional studies are needed to elucidate the immunization mechanism and the ability of these autoantibodies to initiate vasculitis in these experimental animals.

Antineutrophil cytoplasmic antibodies (ANCA) are associated with systemic vasculitis, especially Wegener's granulomatosis and microscopic polyangiitis (1,2). These autoantibodies are directed against proteins in polymorphonuclear (PMN) granules and monocytes lysosomes (3). When detected by indirect immunofluorescence on ethanol-fixed PMN, there are two major patterns of ANCA staining: cytoplasmic (C-ANCA) and perinuclear (P-ANCA). The major C-ANCA and P-ANCA antigens are, respectively, proteinase-3 (PR3) and myeloperoxidase (MPO). Other minor ANCA antigens have been described, including lactoferrin, human leukocyte elastase (HLE), and cathepsin G.

ANCA may play an important role in the pathophysiology of their associated diseases; however, this remains controversial. Priming of PMN with tumor necrosis factor-α leads to primary granules' translocation to the cell surface, without release of their contents. ANCA can activate tumor necrosis factor-α–primed neutrophils, leading to the production of reactive oxygen species and cytokines, as well as the release of lysosomal enzymes (4). Furthermore, ANCA can promote the adhesion of PMN to monolayers of cultured endothelial cells and induce their lysis (5). In the absence of infectious events, aging neutrophils are constitutively programmed to undergo apoptosis, allowing removal of these cells without inducing inflammation (6). Apoptosis is an active form of cell death that is morphologically distinct from necrosis. During apoptosis, the nucleus of the affected cells condenses and becomes fragmented with vacuoles appearing in the cytoplasm. At the final stage of apoptosis, the cells themselves are fragmented into apoptotic bodies and phagocytosed by neighboring cells (6). Biochemically, it is accompanied by activation of endonucleases and fragmentation of DNA (7). Primary granule proteins are found on the surface of apoptotic PMN in the absence of priming (8). The mechanisms underlying surface expression of ANCA antigens on PMN after priming or apoptosis have not been elucidated.

Different animal models have been used in an attempt to elucidate the role of ANCA *in vivo*. Three animal models for MPO-ANCA–associated vasculitis have been described. In the first model, Brown Norway (BN) rats received an injection of mercuric chloride and developed gut vasculitis as well as MPO-ANCA (9). In the second model, BN rats that were immunized with MPO, perfused with a lysosomal extract and H_2O_2, developed proliferative glomerulonephritis and antibodies against human MPO (10). In the third model, BN rats were immunized with human MPO. After 2 wk, rats developed

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Correspondence to Prof. Vincent L. M. Esnault, Service de Néphrologie-Immunologie Clinique, Hotel Dieu, 30 Boulevard Jean Monnet, 44093 Nantes Cedex, France. Phone: +33-2-40-08-33-08; Fax: +33-2-40-08-33-12; E-mail: vesnault@nantes.inserm.fr

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autoantibodies to human and rat MPO but no signs of glomerulonephritis. Rats then received an injection of a subnephritogenic dose of rabbit anti-rat glomerular basement membrane (GBM) and subsequently exhibited proteinuria, hematuria, and severe glomerulonephritis (11).

The target antigens of lupus autoantibodies also are found on the surface of apoptotic blebs (12). Because injection of apoptotic thymocytes induced an autoimmune response in normal mice (13), we injected freshly isolated or apoptotic PMN into BN rats to see whether apoptotic PMN can generate ANCA.

Materials and Methods

Animals

Male BN rats were purchased from Iffa Credo (Orléans, France). They were housed conventionally and fed with standard rat chow and water *ad libitum*.

Isolation of Rat PMN

PMN were isolated from heparinized blood, obtained by intracardiac puncture. Five ml of blood was diluted 1:2 in Hank's balanced salt solution (HBSS), overlaid with 10 ml of ficoll, and centrifuged at $450 \times g$ for 30 min. Rat PMN obtained from the pellet were washed twice with HBSS, and red cells were lysed with ammonium chloride (0.83%) for 10 min at room temperature, followed by two washes with HBSS. PMN were resuspended in culture medium (RPMI 1640 with 10% autologous serum containing 1% glutamine) at 1.10^6/ml. The purity of the suspension was assessed by Wright-Giemsa staining of cytocentrifuged preparations. Viability was more than 91% as assessed by trypan blue exclusion. This technique provided 40% PMN, 55% lymphocytes, 5% eosinophiles, and 1 or 2% monocytes. Rats received injections either of freshly isolated PMN or of PMN that had been incubated for 24 or 48 h at 37°C in a humidified atmosphere containing 5% CO_2 to induce apoptosis.

Assessment of Rat PMN Apoptosis

At time 0 and at subsequent time intervals, cytocentrifuged preparations of rat PMN were fixed in methanol and stained with Wright-Giemsa. To assess the percentage of apoptotic PMN, we examined at least 500 PMN per slide. Apoptosis was defined as PMN containing one or more darkly stained pyknotic nuclei. Results are representative of four experiments. Lymphocytes were not included in the cell count and showed little apoptotic features during the time period of the experiment. Apoptosis also initially was assessed by flow cytometric analysis with staining for annexin V, propidium iodide, and a mouse monoclonal anti-rat neutrophil (HIS48; Pharmingen, San Diego, CA) that was partially lost during apoptosis. Therefore, flow cytometric analysis was abandoned because it proved to be less effective than morphologic studies for apoptosis assessment in this mixed cellular preparation.

Immunization Procedure

Four groups of five rats received injections of RPMI alone (group 1), freshly isolated PMN (group 2), apoptotic PMN aged for 24 h (group 3), or apoptotic PMN aged for 48 h (group 4). PMN (0.2×10^6) in 0.2 ml of RPMI was injected once weekly for 3 wk in the footpad. Rats received a booster intravenously once and then six times in the footpad, during a period of 9 mo. Only group 3 rats received five injections.

ANCA Indirect Immunofluorescence

Blood was drawn before and after each immunization procedure, and serum was harvested. ANCA were detected by indirect immunofluorescence on ethanol-fixed human PMN cytocentrifuged preparations. Positivity was confirmed on ethanol-fixed rat PMN and on formol acetone-fixed PMN. Rat sera were diluted 1:20, and FITC-conjugated goat anti-rat IgG (Immunotech 0827, Marseilles, France) was used at 1:50 to detect bound antibodies.

Exclusion of antinuclear antibodies as a source of false-positive ANCA indirect immunofluorescence was performed by testing all of the sera on HEp-2 (Biorad, Hercules, CA) cell lines. Antitissue activity was searched for on rat liver and stomach sections.

ANCA Enzyme-Linked Immunosorbent Assay

ANCA antigen specificity was searched for by enzyme-linked immunosorbent assay using both purified human antigens and rat MPO. Briefly, microtiter plates were coated overnight with human MPO (Calbiochem, La Jolla, CA), cathepsin G (Calbiochem), human leukocyte elastase (HLE; Calbiochem), lactoferrin (Sigma, St. Louis, MO), and lysozyme (Calbiochem) at 1 μg/ml in phosphate-buffered saline (PBS). PR3 and bactericidal/permeability-increasing protein were detected with a commercial kit according to the supplier's instructions (The Binding Site, Birmingham, UK). Nonspecific binding sites were blocked with PBS containing 2% bovine serum albumin. Rat sera were diluted 1:20 in PBS containing 0.1% Tween. After washing with PBS containing 0.1% Tween, bound antibodies were detected by alkaline phosphatase-labeled anti-rat IgG conjugate diluted 1:1000 (A-9654; Sigma, St. Louis, MO). P-nitrophenyl-phosphate disodium was used as substrate. A panel of nine negative rat sera was run on each plate. Positivity was defined as values greater than 2 SD over the panel of negative rat sera. The results are expressed as optical density (OD) values.

MPO-ANCA also were searched for with the use of purified rat MPO (kindly provided by R. Falk's group, University of North Carolina, Chapel Hill, NC). Briefly, rat MPO was coated overnight at 5 μg/ml in PBS. After blocking with PBS-bovine serum albumin 2%, a monoclonal mouse anti-rat MPO (provided by R. Falk's group) or rat sera were incubated diluted 1:20. Binding was detected with either alkaline phosphatase–conjugated anti-rat IgG (A8438; Sigma) diluted 1:30000 or alkaline phosphatase–conjugated anti-mouse IgG (BA 024 02222; Bioatlantic, Nantes, France) 1:1000, before the substrate was added. All incubations were 100 μl/well at 37°C for 1 h, with triple washes in PBS-Tween 0.1%.

Injection of Subnephritogenic Dose of Sheep Anti-Rat GBM

Nephrotoxic sheep anti-rat GBM was kindly provided by B. Fouqueray (Tenon Hospital, Paris, France) (14). IgG were purified from sera by protein G Sepharose affinity chromatography. Three naive BN rats received an injection of 50, 150, or 300 mg of sheep anti-rat GBM IgG and were killed on day 9. For direct immunofluorescence on rat kidney sections, a FITC-conjugated rabbit anti-sheep IgG (F 5137; Sigma) was added 1:50. Bright staining was observed in all rats with no signs of glomerulonephritis on day 9.

After 9 mo of observation, we injected a single intravenous dose of 300 mg of nephritogenic anti-GBM IgG in groups 1, 2, and 4 BN rats. All animals were killed 9 d later for histology.

Albuminuria

For estimation of proteinuria, urine was collected in metabolic cages during 24 h, before and after each immunization procedure, as

well as before and 4 and 9 d after injection of anti-GBM IgG. Microtiter plates were coated overnight with rat albumin at a protein concentration of 1 μg/ml to 0.0315 μg/ml, and rat urine was diluted 1:100 to 1:10^6. After saturation with PBS containing 0.5% Gelatin-0.1% Tween, a sheep anti-rat albumin (PC 341; The Binding Site) was added 1:2000 followed by a peroxidase-conjugated goat anti-sheep reagent (AP 360; The Binding Site) diluted 1:5000. Tetramethylbenzidine (SFRI, St. Jean D'Illac, France) was used as substrate. The results are expressed in milligrams per /milliliter using the calibration scale.

Histopathology

Nine d after injection of sheep anti-rat GBM IgG, different organs, including kidneys, gut, stomach, lungs, liver, skin, and pancreas, were collected for light microscopy and immunofluorescence studies. For light microscopy, renal tissue was fixed in formol diluted in water 1:10 and embedded in paraffin. Tissue sections of 5 μm were stained with periodic acid-Schiff, hematoxylin, and eosin and were examined by a pathologist who specialized in kidney disease (A.M.). For immunofluorescence studies, tissue samples were snap-frozen in isopentane and liquid nitrogen. Tissue sections of 5 μm were analyzed by direct immunofluorescence with a FITC-conjugated rabbit anti-sheep IgG reagent (Sigma).

Results

Rat PMN Apoptosis

At time 0 of culture, 4.75 $\pm$ 1.47% (mean $\pm$ SD) PMN showed features of apoptosis; at 24 h, 42.3 $\pm$ 4.27%; and at 48 h, 71.6 $\pm$ 3.33%.

ANCA Tests

Indirect immunofluorescence studies on human and rat ethanol-fixed PMN showed a strong P-ANCA pattern in three of five rats that received an injection of apoptotic PMN aged for 48 h, starting after the third injection, and P-ANCA titers increased in four of these five rats thereafter. In rats that received an injection of PMN aged for 24 h, one of five rats exhibited P-ANCA after three injections, but three of five developed P-ANCA late. Because this immunization procedure seemed less effective, injections were stopped after the fifth in this group. In rats that received injections of freshly isolated PMN, no ANCA were detectable at any time point. One aging BN rat that received an injection of RPMI exhibited P-ANCA late (Figures 1 and 2). Sera with P-ANCA activity on ethanol-fixed human and rat neutrophils exhibited C-ANCA staining on formol acetone-fixed human neutrophils.

Most P-ANCA–positive rat sera reacted with HLE in enzyme-linked immunosorbent assay. HLE-ANCA was present in three of the four P-ANCA–positive rats of group 4 (maximum OD values, 1.832, 0.768, and 0.457), two of the three P-ANCA–positive rats of group 3 (maximum OD values, 0.797 and 0.747), one of group 1 (positivity of the last two collected sera only; maximum OD value, 0.866), and none of group 2. The mean $\pm$ SD OD of the nine ANCA-negative rats was 0.222 $\pm$ 0.097, defining positivity over 0.416. No activity was found with the other antigens, including rat MPO.

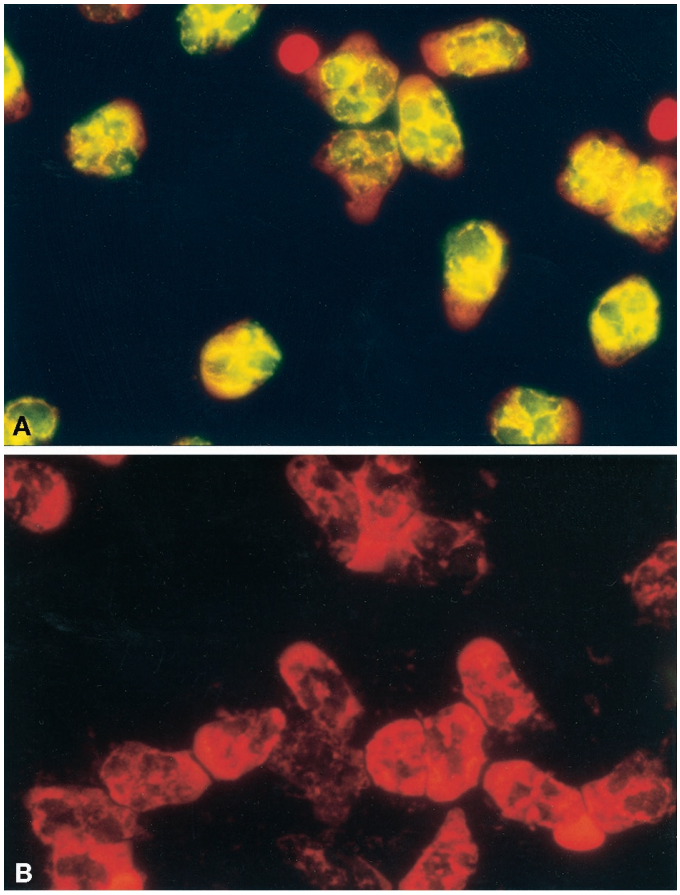

Figure 1. Immunostaining of neutrophils by indirect immunofluorescence microscopy. (A) Perinuclear antineutrophil cytoplasmic antibody (P-ANCA) staining on ethanol-fixed human polymorphonuclear granules (PMN) produced by a rat serum (diluted 1:20) from group 4. Yellow staining indicates binding of the FITC-conjugated reagent. (B) Negative staining on ethanol-fixed human PMN produced by a rat serum (diluted 1:20) from group 2. PMN with no ANCA binding are stained in red by Evans blue. Magnification, $\times$1000.

Immunofluorescence on HEp-2 Slides and Rat Liver and Stomach

No staining was found on HEp-2 cells, rat liver, or stomach sections.

Albuminuria and Histology

No obvious clinical changes were noted in the rats, except for chronic skin infection in the ANCA-positive RPMI injected rat. Albuminuria was not detected. Light microscopy showed no signs of tissue injury despite bright anti-GBM immunofluorescence with rabbit anti-sheep IgG in animals that received an injection of sheep anti-rat GBM IgG.

Discussion

There is growing evidence that apoptotic antigens are the natural targets for many autoantibodies (12,13,15,16). For example, many anti-DNA autoantibodies seem to be directed against nucleosomal DNA-histone complexes produced as a result of nucleosome cleavage during apoptosis. Nucleosomes

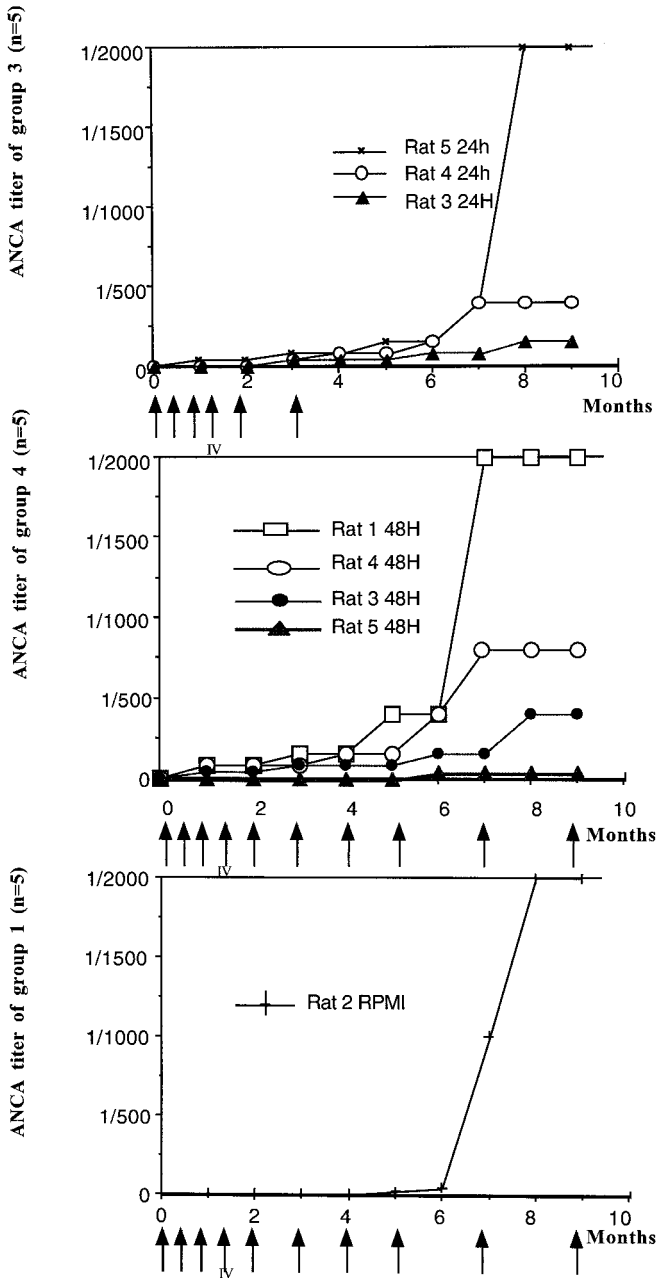

Figure 2. P-ANCA titers after injections (arrows) of RPMI (group 1), PMN aged for 24 h (group 3), and PMN aged for 48 h (group 4). Only the results of rats that exhibited ANCA are shown: four of five in group 4, three of five in group 3, and only one of 5 in group 1. No ANCA was found in rats that received injections of freshly isolated PMN (group 2).

are powerful immunogens for autoreactive T-cell clones derived from mice with systemic lupus erythematosus (15). Several prominent nuclear antigens targeted in systemic lupus erythematosus can be found at the surface of apoptotic keratinocytes as either apoptotic bodies or smaller so-called apoptotic blebs (12), and systemic injections of apoptotic thymocytes induce an antinuclear antibody response (13). ANCA antigens also are expressed on the surface of apoptotic PMN in the absence of priming. Translocation of primary granules occurs as a natural consequence of apoptosis without priming (8). Once expressed on the surface of apoptotic PMN, primary granule components may be recognized by the immune system. Surface expression of ANCA antigens seems most prominent during the later phases of apoptosis, thus explaining why late apoptotic PMN seemed more effective in generating ANCA in our model. Rats that received injections of apoptotic PMN developed ANCA in the long term. The intravenous boost, aimed at increasing immediate antibody response, showed little effect. Despite stopping injections in group 3 because of initial lower antibody response, ANCA titers still increased after 6 mo of observation. The mechanism of this slow immunization process remains unknown. However, we were able to break tolerance to neutrophils and documented the development of antibodies to at least one clinically relevant neutrophil granule antigen. We did so by the use of syngenic neutrophils without the use of adjuvant. No immunization was obtained using freshly isolated neutrophils; therefore, only apoptotic but not normal neutrophils can present the autoantigen effectively.

Indirect immunofluorescence on rat neutrophils confirmed ANCA activity. Most of the ANCA-positive sera reacted with HLE. The sequence of HLE largely has been conserved across species during evolution. HLE and PR3, the main antigen target in Wegener's granulomatosis not yet characterized in rats, are closely related neutral serine proteases. Therefore, rat ANCA reacting with HLE might be directed against a rat equivalent serine protease.

As previously reported, the development of anti-MPO antibodies after immunization with MPO is not sufficient to induce disease. Additional factors that induce a proinflammatory environment are needed for priming neutrophils and monocytes and for activating the endothelium. Heeringa *et al.* (11) showed that injection of a small amount of anti-GBM IgG, which was not enough to induce glomerulonephritis in the absence of anti-MPO antibodies, could trigger glomerular vasculitis in the presence of MPO-ANCA. Anti-GBM IgG injection in our model induced neither proteinuria nor histologic signs of glomerulonephritis or lung vasculitis, despite the presence of P-ANCA. However, the dose of nephritogenic IgG may be insufficient, because no inflammatory reaction was seen.

BN rats are T-helper type 2–prone animals, known to develop various autoantibodies (17). Surprising is that one of the control rats developed ANCA. However, it did so late and showed signs of severe skin infection after injury inflicted by its mates. This unusual infection may have led to the appearance of ANCA as observed in human subacute endocarditis (18). None of the rats that received injections of freshly isolated PMN developed ANCA.

We conclude that immunization with late apoptotic PMN may lead to ANCA activity. Additional studies are needed to elucidate the immunization mechanism and the ability of these autoantibodies to initiate vasculitis in these experimental animals.

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