Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool

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Abstract Proteinase 3, which is known as an intracellular serine protease of neutrophils, was detected at the surface of a subpopulation of freshly isolated PMN. The proportion of PR3-positive and -negative PMN, observed by flow cytometry with anti-PR3 mAbs or ANCA autoantibodies, varies among individuals but is extremely stable for each individual over prolonged time periods. After PMN degranulation by FMLP with cyt. B, membrane PR3 expression increases but the proportion of low and high PR3-expressing cells remains stable. The existence of a subset of PMN which spontaneously expresses PR3 and varies among individuals, may be relevant to the pathogenesis of anti-PR3 ANCA autoantibody-related vasculitis.

Key words: Proteinase 3; Polymorphonuclear neutrophil; PMN serine protease; ANCA; Vasculitis

1. Introduction

Proteinase 3 (PR3) is a serine protease present in azurophil granules of polymorphonuclear neutrophils (PMN) and originally described as an enzyme which degrades elastin and causes pulmonary emphysema after intratracheal instillation in hamsters [1,2]. It was subsequently identified to myeloblastin, an enzyme involved in the proliferation and the control of differentiation of HL-60 cell lines [3]. Most importantly, PR3 is the main antigenic target of antineutrophil cytoplasmic autoantibodies (ANCA) observed in Wegener's granulomatosis (WG) [4,5]. It belongs to the family of neutrophil serine proteases with bactericidal activity, also called 'serprocidins', which includes neutrophil elastase, cathepsin G, PR3 and the enzymatically inactive azurocidin [6,7].

These enzymes are supposed to function mainly intracellularly after the fusion of azurophil granules with phagocytic vacuoles and to be only released into the extracellular space by 'leakage' during extensive phagocytosis [1,8,9].

On the other hand, the close correlation observed in WG between systemic vasculitis and anti-PR3 ANCAs [10–12] suggests that anti-PR3 autoantibodies either trigger or amplify neutrophil activation, resulting in cell aggregation and release of inflammatory mediators responsible for endothelium damage. This hypothesis implies an extracellular localization of PR3 accessible to anti-PR3 ANCAs.

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Abbreviations: PR3, proteinase 3; PMN, polymorphonuclear neutrophils; ANCA, antineutrophil cytoplasmic autoantibodies; WG, Wegener's granulomatosis; HBSS, Hanks' balanced salt solution; cyt. B, cytochalasin B; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

While analysing the conditions of in vitro neutrophil activation resulting in PR3 exocytosis, we observed that a proportion of unactivated, freshly isolated PMN expressed PR3 on their surface as shown by flow cytometry. Most surprisingly, this proportion of PR3-positive cells was found to vary between 0 and 90% among the blood donor population while being extremely stable for one given individual. We here analyse this phenomenon which reveals a phenotypic heterogeneity of neutrophils and could be relevant to the pathogenic role of anti-PR3 ANCAs.

2. Materials and methods

2.1. PMN isolation and in vitro activation

PMN were isolated from EDTA-anticoagulated blood from healthy donors by depletion of the platelet-enriched plasma, centrifugation on Polymorphprep (Nycomed, Oslo, Norway) and lysis of contaminating erythrocytes. Cells were washed in Hanks' balanced salt solution (HBSS) without Ca^{2+}/Mg^{2+} (Gibco, Paisley, UK) and resuspended, either in ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS/BSA/azide) for immediate incubation with antibodies for flow cytometry analysis or in warm HBSS with Ca^{2+}/Mg^{2+} (Gibco) for cell activation. For in vitro cell activation, PMN 10^6/ml in HBSS with Ca^{2+}/Mg^{2+} were incubated at 37°C in BSA-coated tubes, in the presence of protease inhibitor soyabean trypsin inhibitor and oxidant scavenger methionin, 5 min with 10 μg/ml cytochalasin B (cyt. B; Sigma, St Louis, MO) followed by 1 h with 1 μM FMLP (Sigma). PMN were then centrifuged and resuspended in PBS/BSA/azide for flow cytometry analysis.

Two healthy volunteers were injected s.c. with 1 mg glucagon. Blood samples were taken before and 2 h after the injection and PMN isolated as described above.

PMN were isolated from the blood and synovial fluid of rheumatoid arthritis patients as described [13] by centrifugation on Ficoll Hypaque (Pharmacia) preceeded, for blood cells, by erythrocyte sedimentation on plasmagel (Roger Bellon Laboratory, Neuilly-sur-Seine, France).

2.2. Antibodies

Murine mAb anti-PR3 CLB 12.8 was from CLB (Amsterdam, The Netherlands) while WGM.2 was a gift from E. Czernok (Bad Bramstedt, Germany); anti-elastase AHN-10 was from Pharmingen (San Diego, CA), anti-elastase NP-57 from Dako (Glostrup, Denmark); anti-CD63 CLB-gran/12, control mouse immunoglobulin IgG1 and FITC-conjugated $F(ab')_2$ fragment of goat antihuman IgG were from Immunotech (Marseille, France); FITC-conjugated $F(ab')_2$ antimouse IgG were from Caltag Laboratories (San Francisco, CA) while FITC-F(ab')2 antihuman $F(ab')_2$ was from Jackson (West Grove, PA); Anti-FcγRII (IV.3) and anti-FcγRIII (3G.8) mAbs were from Medarex (West Lebanon, NH). Anti-GPIIbIIIa mAb PI873 was a gift from C. Kaplan (CNTS, Paris). Normal goat IgGs were from Sigma. They were heat-aggregated by incubating them at a concentration of 10 mg/ml for 30 min at 60°C.

IgGs from a patient with WG with anti-PR3 ANCA and from a normal control were isolated by chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden). $F(ab')_2$ fragments were obtained after digestion with 2% w/w pepsin (Worthington, Freehold, NJ) at pH 3 for 4 h at 37°C and further absorption of uncleaved IgGs and Fc fragments on protein A-sepharose. Purity of $F(ab')_2$ fragments was assessed by PAGE analysis with PHAST system (Pharmacia). When

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mentioned, antibodies were labelled with NHS-LCbiotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.3. Immunofluorescence flow cytometry

10^6 PMN in PBS containing 1% BSA and 0.1% sodium azide were first incubated for 30 min at 4°C with 1 mg/ml heat-aggregated goat IgGs to block Fcγ receptors. Cells were then treated with dilutions of monoclonal antibodies followed by FITC-conjugated $F(ab')_2$ fragments of goat antimouse IgG. Cells were fixed with 1% formaldehyde and analysed for fluorescence on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with light scatter gate set.

For whole-blood analysis, anti-PR3 antibodies were added to EDTA-anticoagulated blood for 15 min at room temperature. After a single wash in PBS/BSA/azide, the FITC-antimouse IgG antibody was added for 15 min at room temperature, then red cells were lysed in Becton Dickinson FACS lysis solution according to the manufacturer's instructions and fixed with 1% formaldehyde.

2.4. Statistical analysis

The distribution of the proportion of PR3-positive PMN in the normal population was analysed using the Statistica Software package (Tulsa, OK).

3. Results

3.1. Heterogeneous PR3 membrane expression on resting PMN

Flow cytometry analysis of membrane-bound PR3 on in vitro activated neutrophils revealed that a proportion of PMN

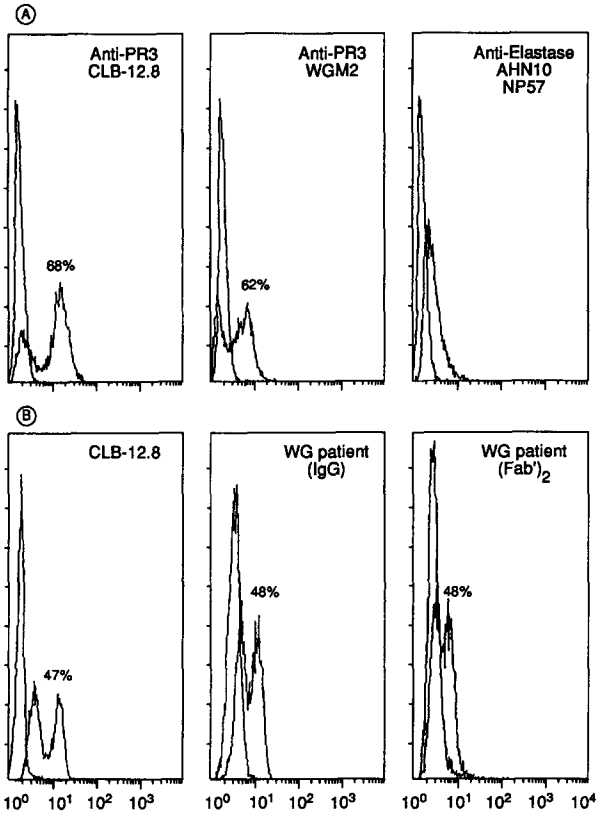

Fig. 1. Heterogeneous expression of PR3 on freshly isolated neutrophils. PMN from individual (A) were analysed by flow cytometry with clone CLB-12.8 or clone WGM2 anti-PR3 mAbs or with a mixture of two anti-elastase mAbs. Individual (B) was tested either with the CLB 12.8 mAb or with whole IgGs from a WG patient with anti-PR3 ANCA or with the $F(ab')_2$ fragments of this WG patient IgGs. The percentage of PR3-positive cells is mentioned. The thin peak at the left of each diagram is the overlayed control histogram obtained with mouse IgG1, normal human IgGs or $F(ab')_2$ according to the antibody tested.

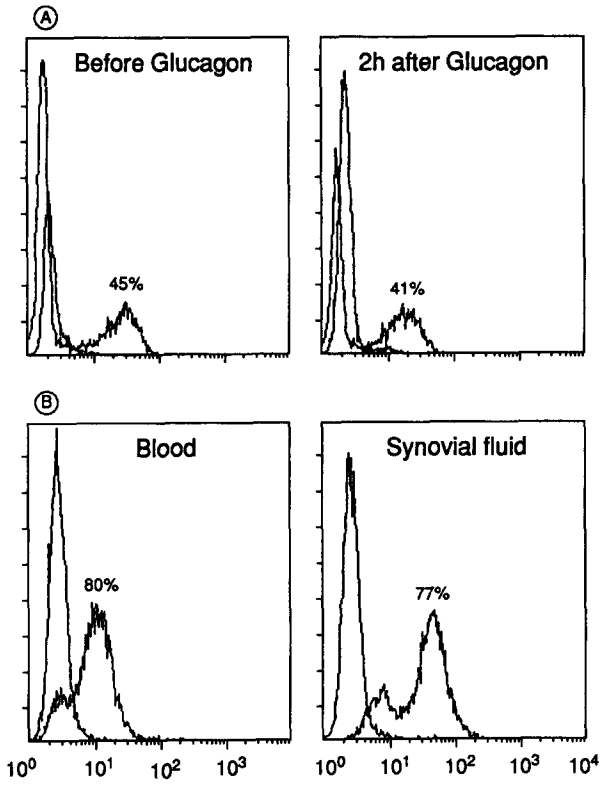

Fig. 2. Expression of PR3 on PMN newly recruited from the marrow or having migrated to an inflammation site. PMN were analysed by flow cytometry with CLB 12.8 anti-PR3 mAb. The thin peak at the left shows the control histogram obtained with mouse IgG1. PMN from individual (A) were tested before and 2 h after glucagon injection. Individual (B) was a patient with rheumatoid arthritis for whom blood and synovial fluid PMN were compared.

was labelled with anti-PR3 antibodies in the absence of in vitro activation. A bimodal distribution was constantly observed when freshly isolated PMN were labelled with two different anti-PR3 mAbs, CLB 12.8 and WGM.2 clones, or with IgGs from two WG patients with anti-PR3-ANCAs (only one is shown) (Fig. 1). A proportion of cells was not or were weakly labelled while the others clearly expressed PR3. PMN tested in parallel with a mixture of two anti-elastase mAbs (Fig. 1) or with a polyclonal anti-elastase antibody (data not shown) were not or were weakly and homogeneously labelled. Futhermore, PMN analysed similarly with mAbs directed against various neutrophil markers, such as integrins CD11a, CD11b/CD18 and CD29, L-selectin, complement receptor CR1, FcγRII and III, CD31, CD43 and CD44, resulted in our hands in a single homogeneously labelled cell population (data not shown).

The proportion of PR3-positive PMN varied from one individual to another but the same percentage was obtained, for one given individual, with the two anti-PR3 mAbs and the IgGs from the two WG patients (~65% for individual A, 47% for individual B on Fig. 1).

Similar results were obtained whether or not Fcγ receptors were saturated with aggregated IgGs before anti-PR3 labelling, except that a better resolution of the two cell peaks was obtained after Fcγ receptor blocking. The same percentage of PR3-positive PMN was obtained with IgGs from a WG patient and with the $F(ab')_2$ fragments of these patient IgG revealed

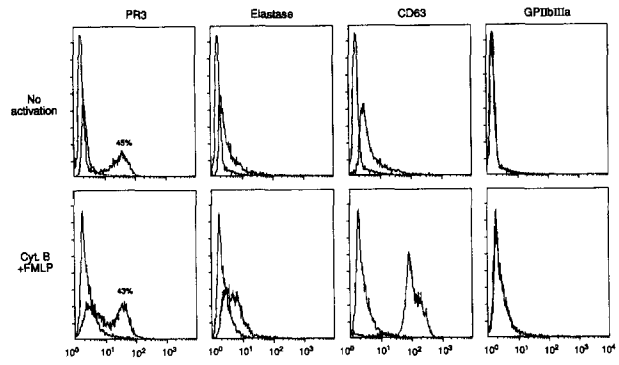

Fig. 3. Expression of azurophil granule markers on the plasma membrane of activated PMN. PMN were incubated for 1 h at 37°C, as described in Section 2, with HBSS or with 10 μg/ml cyt. B and 1 μM FMLP. Unactivated and activated cells were analysed by flow cytometry with CLB 12.8 anti-PR3, AHN10 anti-elastase, CLB-gran/12 anti-CD63 and anti-GPIIbIIIa PI873 mAbs. The peak on the left represents the overlayed control histogram obtained with mouse IgG1.

with FITC-F(ab')$_2$ antihuman F(ab')$_2$ as secondary antibody (Fig. 1B).

When isolated PMN were incubated with autologous plasma, PR3 was no more detected at the cell surface. This explains the negative results obtained in whole-blood analysis (data not shown).

3.2. *Proportion of PR3-expressing PMN is stable for one given individual*

When the percentage of PR3-positive cells of 7 different individuals was measured on two occasions distant of 12 ± 9 weeks, the results of the two evaluations were strikingly similar (correlation $r = 0.998$). Two individuals tested 5× over a period of 5 months resulted in 29.64 ± 2.68 and 41.26 ± 2.12 PR3-positive cells, respectively (data not shown).

For one given individual, the observed heterogeneity in the blood neutrophil pool does not seem to be related to the amount of neutrophils newly released from the marrow or, on the contrary, to the amount of senescent PMN. The same proportion of PR3-positive cells was observed in individual A before and after artificial massive recruitment of marrow neutrophils by in vivo injection of glucagon (Fig. 2A). This was observed in two different individuals (only one is shown) for whom the number of blood PMN was increased 4× by the glucagon injection. Conversely, on Fig. 2B is shown the comparison of PMN at the inflammation site, i.e. in the synovial fluid of a patient with rheumatoid arthritis, with blood PMN from the same patient. The amount of membrane PR3 was increased on all cells from the synovial fluid but the percentage of PMN with high levels of membrane-bound PR3 was similar to that of PR3-expressing cells in the PMN-circulating pool.

3.3. *Two PMN populations with different expression of membrane PR3 remain distinct after in vitro cell activation*

In vitro cell activation by FMLP with cyt. B resulted in an increased amount of membrane PR3 on all PMN with two clearly distinct populations of high and low PR3-expressing cells in the same proportion than PR3-negative and -positive PMN before activation (Fig. 3). The mean fluorescence intensity of the two cell peaks was 2 and 29 units, respectively, before activation, 5 and 43 after activation. Membrane-bound elastase was also detected after cell activation but resulted in a single labelled peak. Stimulation by cyt. B and FMLP resulted in a marked de novo expression of CD63 on the cell surface, showing that indeed azurophil granules have fused with the plasma membrane [14]. The negative results obtained with anti-GPIIbIIIa confirmed that the positive CD63 labelling was not due to activated platelets which strongly express CD63 and may bind neutrophils [15].

3.4. *Distribution of PR3-positive subpopulation in normal donors*

The proportion of freshly isolated PMN expressing membrane PR3 among 42 blood donors varied within the range of 0–95% (mean ± SD = 49.64 ± 20.39). When the distribution was depicted as a frequency histogram (Fig. 4), the pattern seemed to be normal-unimodal and statistical analysis was consistent with a normal distribution ($\chi^2 = 0.7$, df = 3, P = NS).

4. Discussion

The present data show that normal circulating neutrophils express PR3 on their membrane with an unusual PR3 expression phenotype which defines two distinct populations, one unlabelled while the other clearly expressing PR3. A remarkable feature of this PR3 expression is the high variability of the proportion of PR3-expressing cells among individuals, contrasting with the striking stability of this proportion, over prolonged periods of time, in one given individual both on resting and on activated PMN. An heterogeneous expression of membrane PR3 after neutrophil activation had previously been mentioned by Czernok et al. [16] but variations among individuals were not studied by these authors.

This peculiar type of distribution seems to be restricted to PR3 since neutrophils analysed by flow cytometry with a wide variety of antineutrophil marker mAbs resulted in our hands in a single homogeneously labelled cell population. The existence of two discrete populations of PR3-positive cells was not due to an artefactual interaction of anti-PR3 antibody with the neutrophil membrane since the same bimodal distribution and the same proportion of PR3-positive cells were observed with two different anti-PR3 mAbs or with IgGs from two WG patients with anti-PR3 ANCAs. In addition, the binding of anti-PR3 antibodies did not involve the Fc portion of antibodies

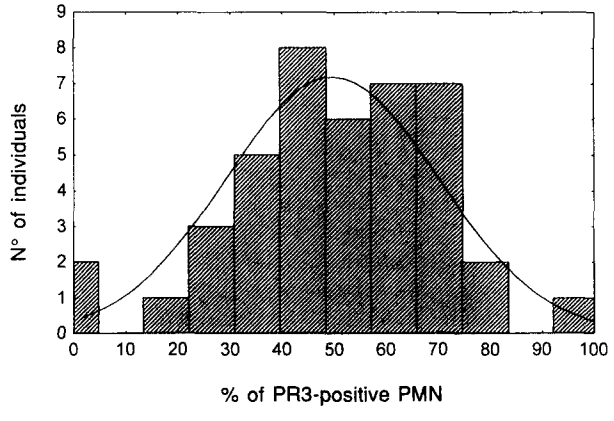

Fig. 4. Distribution of the proportion of PR3-positive PMN among 42 blood donors.

since: (1) PMN labelling with anti-PR3 mAbs was not modified by Fcγ receptor blocking; and (2) the same proportion of PR3-positive cells was measured with WG patient IgG and with their $F(ab')_2$ fragments. Altogether, these results strongly suggest that the bimodal binding of anti-PR3 antibodies to neutrophils is due to a true heterogeneity in PR3 membrane expression.

Neutrophil isolation procedures result in limited cell activation, which modifies the expression of various markers [17] and could potentially induce artefactual PR3 expression on freshly isolated PMN. This is, however, very unlikely for two reasons. (1) When cells were fully activated in vitro by cyt. B and FMLP, which induces the release of azurophil granule content, the level of membrane PR3 was found to increase proportionally on all PMN and two populations of low and high PR3-expressing cells remained clearly distinct, in the same proportion as in resting cells. If freshly isolated cells which express PR3 were simply a proportion of cells activated during the purification procedures, one would expect that extensive degranulation would result in an homogeneous population with maximal PR3 expression. (2) PMN from different individuals isolated on the same day and in the same way resulted in different proportions of PR3-positive cells while this proportion appeared as an extremely stable characteristic of one given individual tested over a period of up to 4 months. This stability strongly argues against the hypothesis that variations in the cell isolation procedures would explain the differences observed among individuals. We do not exclude, however, that purification steps allow to reveal the constitutive PMN heterogeneity described here, since we were not able to detect membrane PR3 on PMN in whole blood, presumably because of interferences of plasma factor(s) with anti-PR3 antibody binding.

Antigenic and functional heterogeneities of mature neutrophils have been previously described [18]. Various techniques of cell fractionation by density centrifugation or flow electrophoresis revealed physicochemical and functional heterogeneity in the circulating neutrophil pool [19–21]. Some of these variations concerned azurophil or specific granule components defining subpopulations of PMN with differences in myeloperoxidase or lactoferrin activity [22,23]. None of these studies, however, pointed out to individual and possibly genetic differences in the proportion of PMN subsets. Specific monoclonal antibodies (31D8, PMN 6, AML.2.23) also allowed to distinguish PMN subpopulations with maturational and/or functional differences [24–26]. The percentage of cells bearing these antigens, which to our knowledge have not been characterized so far, vary among individuals but the stability of this distribution for a given individual was not mentioned by these authors.

By analogy with reported heterogeneities in the PMN pool related to cell maturation differences or to the presence of apoptotic cells [26,27], the expression of PR3 might be related to the 'age' of neutrophils. This seems unlikely since the proportion of PR3-expressing cells was the same in PMN newly recruited from the bone marrrow by in vivo glucagon treatment as in the normal circulating cells of the same subject before treatment. Furthermore, the subpopulation of PMN expressing high levels of PR3 among cells recovered from the synovial fluid of a rheumatoid arthritis patient represented the same percentage of the total PMN pool having reached the inflammatory site than the PR3-positive subset in the blood from the same patient.

Both the origin of membrane PR3 and the interaction between PR3 and the neutrophil membrane remain to be elucidated. Membrane PR3 is unlikely to result from azurophil granule mobilization since CD63, which is a sensitive marker of azurophil granule exocytosis [15], was absent or weakly expressed on freshly isolated PMN independently of the importance of the PR3-positive population. This contrasted with the massive membrane expression of CD63 induced by degranulating stimuli, such as cyt. B and FMLP, which resulted in a modest increase of membrane PR3 level. Can PR3 be inserted in the membrane or is it secreted then bound to a receptor? A membrane receptor for elastase, another azurophil granule enzyme closely related to PR3, has indeed be reported on PMN [28]. Furthermore, the highly basic PR3 molecule could bind to negatively charged components of the cell glycocalyx and charge heterogeneities have been described in the neutrophil pool [21].

The molecular basis of the heterogeneity among mature neutrophils is not known from this study but the stability, for one given subject, of the proportion of this PMN subset suggests that it may be under genetic control. Family studies are now required to ascertain this point. If such a genetic control exists, it could be relevant to the occurrence of WG vasculitis which in most cases is associated with anti-PR3 ANCA autoantibodies. The existence of a PMN subpopulation naturally expressing PR3 and its variability among individuals may be involved in the pathogenesis of ANCA-related diseases. Indeed, the exposure of PR3 on the cell surface could favour the autoimmunization and/or allow the binding of anti-PR3 autoantibodies to neutrophils which may amplify the PMN-induced vascular inflammation. The analysis of PR3 expression on PMN from WG patients will be required to see if a high proportion of PR3-expressing cells predisposes to the disease.

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