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A Large Subset of Neutrophils Expressing Membrane Proteinase 3 Is a Risk Factor for Vasculitis and Rheumatoid Arthritis

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Abstract. It has been shown previously that proteinase 3 (PR3), a neutrophil intracellular protease that is the main antigen of antineutrophil cytoplasm (ANCA) autoantibodies, is present on the plasma membrane of a subset of freshly isolated neutrophils. This study shows that the size of this subset of membrane PR3-positive ($mPR3^+$) neutrophils is a stable feature of a given individual, most likely genetically controlled. It ranges from 0 to 100% of neutrophils and allows us to define a new polymorphism in the healthy population, with three discrete phenotypes corresponding respectively to less than 20% $mPR3^+$ neutrophils ($mPR3^{low}$) or to a mean percentage of 47% ($mPR3^{intermediate}$) and 71.5% ($mPR3^{high}$) $mPR3^+$ neutrophils. The frequency of the $mPR3^{high}$ phenotype was significantly increased in patients with ANCA-associated vasculitis (85% *versus* 55% in healthy subjects). The percentage of $mPR3^+$ neutrophils was not affected by disease activity, relapses, or therapy, and did not reflect *in vivo* cell activation. In addition, $mPR3^+$ phenotypes were normally distributed in cystic fibrosis patients, indicating that infection and/or inflammation *per se* do not lead to a high percentage of $mPR3^+$ neutrophils. The frequency of the $mPR3^{high}$ phenotype was not related to anti-PR3 autoimmunization, since it was increased in vasculitic patients regardless of the ANCA specificity (anti-PR3, anti-myeloperoxidase, or unknown). Interestingly, the frequency of the $mPR3^{high}$ phenotype was also increased in patients with rheumatoid arthritis. It was normal in type I-diabetes, a T cell-dependent autoimmune disease. It is proposed here that a high proportion of membrane PR3-positive neutrophils could favor the occurrence or the progression of chronic inflammatory diseases.

Proteinase 3 (PR3) is known as an intracellular serine protease, localized in azurophilic granules of polymorphonuclear neutrophils, and as the main target of antineutrophil cytoplasm antibodies (ANCA), observed in Wegener's granulomatosis (1–4). The conditions that allow PR3 to become accessible to the immune system and later to ANCA antibodies remain an intriguing question.

We initially observed that normal unactivated neutrophils express on their membrane this so-far considered intracellular enzyme and pointed out the peculiar features of this membrane expression (5): (*1*) Membrane PR3 expression is heterogeneous in the circulating neutrophil pool and defines two distinct cell subsets. No PR3 can be detected on the surface of neutrophils of the first subset ($mPR3^-$ neutrophils), whereas in the other subset ($mPR3^+$ neutrophils) substantial amounts of membrane PR3 are present; (*2*) The proportion of neutrophils presenting PR3 on their membrane varies among individuals but is highly stable over prolonged periods of time in a given individual, suggesting that it might be genetically controlled.

The aim of the present study was to evaluate whether the presence of PR3 on the membrane of a neutrophil subset could be relevant to the appearance and/or to the pathogenesis of ANCA autoantibodies. Indeed, the exposure of PR3 on the cell surface could favor the autoimmunization and/or allow the binding of anti-PR3 autoantibodies to neutrophils, which may amplify neutrophil-induced vascular inflammation.

We show here that three phenotypes can be defined in the healthy population, corresponding to low ($mPR3^{low}$), intermediate ($mPR3^{inter}$), and high ($mPR3^{high}$) percentage of $mPR3^+$ neutrophils. The occurrence of the $mPR3^{high}$ phenotype is increased in patients with ANCA-related vasculitis, when compared to the healthy population. However, this high proportion

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of $mPR3^+$ neutrophils is not restricted to the vasculitic patients with anti-PR3 ANCA, but is also observed in patients with anti-myeloperoxidase (MPO) ANCA and with nonvasculitic patients with rheumatoid arthritis. This suggests that, rather than a role strictly limited to ANCA pathophysiology, a large subset of $mPR3^+$ neutrophils may favor the occurrence or the development of chronic inflammation.

Materials and Methods

Healthy Control Subjects

Normal healthy adult blood donors from the blood transfusion center ($n = 126$) (mean age 39.9 ± 16.7).

Patients with Inflammatory and/or Autoimmune Disorders

ANCA-related vasculitis ($n = 37$, mean age 62.3 ± 19.8): Wegener's granulomatosis (WG) ($n = 16$); microscopic polyangitis (MPA) ($n = 21$). The diagnoses were established according to the Chapel Hill criteria (6). ANCA were determined by PR3 and MPO-specific enzyme-linked immunosorbent assays (ELISA) as described (7). At the time of mPR3 phenotype determination, clinical activity was present in eight patients and absent in 29 patients. Seventeen patients were without therapy. Eleven patients received maintenance regiment of therapy (prednisone <15 mg/kg per d) either alone ($n = 4$) or with azathioprine ($n = 5$) or with cyclophosphamide ($n = 2$). Nine patients received induction regimen of therapy (prednisone 1 mg/kg per d and monthly intravenous cyclophosphamide).

Rheumatoid arthritis (RA) ($n = 34$, mean age 58.8 ± 14.8): Patients fulfilling the 1987 criteria of the American College of Rheumatology for RA diagnostic (8) were analyzed. Disease activity was present in 21 patients and absent in 13 patients. Patients received either no treatment ($n = 7$), methotrexate alone (7.5 to 15 mg/wk, $n = 2$), corticosteroids alone (prednisone ≤12.5 mg/d, $n = 11$; prednisone >12.5 mg/d, $n = 2$), or both methotrexate and corticosteroids (prednisone ≤12.5 mg/d, $n = 6$; prednisone >12.5, $n = 4$). In addition, two patients received corticosteroids associated with either azathioprine ($n = 1$) or cyclophosphamide ($n = 1$).

Spondylarthropathy ($n = 8$, mean age 39 ± 15): Patients fulfilling the criteria of the European Spondylarthropathy Study Group (9) were studied.

Cystic fibrosis (CF) ($n = 24$, mean age 12.2 ± 5.7): CF was diagnosed according to standard criteria including a sweat chloride test and CF transmembrane conductance regulator genotyping (10). All of the CF children were hospitalized for severe pulmonary infections; 16 had chronic *Pseudomonas aeruginosa* and seven had *Staphylococcus aureus* infection. Pulmonary function was evaluated by measuring the forced expiratory volume per 1 s (FEV1, mean 55.1 ± 4.5%, $n = 24$).

Type I diabetes ($n = 28$, mean age 28.3 ± 10.5): Patients presenting with type I insulin-dependent diabetes were diagnosed according to the World Health Organization criteria.

Disease Control Group

Renal patients presenting with nonvasculitic conditions ($n = 29$, mean age 59.4 ± 16.1): renal stone disease ($n = 8$); minimal change nephropathy ($n = 6$); autosomal dominant polycystic disease ($n = 4$); type 2 diabetic nephropathy ($n = 4$); renal arteriosclerosis ($n = 3$); renal dysplasia ($n = 1$); amyloidosis ($n = 1$); Von Hippel Lindau disease ($n = 1$); primary hyperaldosteronism ($n = 1$).

Rheumatologic patients with noninflammatory diseases ($n = 24$, mean age 60.6 ± 14.3): lumbar sciatic pain ($n = 16$); knee osteoarthritis ($n = 8$).

Informed consent was obtained from patients or from the parents for children under 15 yr old. This study was approved by the Ethics Committee of Necker-Enfants Malades Hospital.

Antibodies

Antibodies used for flow cytometry were: murine monoclonal antibody (mAb) anti-PR3 CLB 12.8 (CLB, Amsterdam, Netherlands), which was used to measure PR3 surface labeling and to define the $mPR3^+$ subset. Identical results were obtained with other anti-PR3 mAb such as sera from two Wegener's patients and with WGM0.2 (5) (a gift from E. Czernok, Bad Bramstedt, Germany ([11])) or with anti-PR3 monoclonal antibodies 4A3, 4A5, and 6A6 (12) (a gift from J. Wieslander, Wieslab, Lund, Sweden) (data not shown); murine mAb clone DREG56 anti-CD62L (L-selectin), clone Bear-1 anti-CD11b, control mouse Ig IgG1, and FITC-conjugated $F(ab')_2$ fragment of goat antihuman IgG were from Immunotech (Marseille, France); FITC-conjugated $F(ab')_2$ anti-mouse IgG were from Caltag Laboratories (San Francisco, CA). Normal goat IgG were from Sigma (Sigma-Aldrich, St. Louis, MO). They were heat-aggregated by incubating them at a concentration of 10 mg/ml for 30 min at 60°C.

Neutrophil Isolation and in Vitro Activation

Neutrophils were isolated from ethylenediaminetetra-acetic acid-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+} (Life Technologies, Paisley, Scotland) and resuspended, either in ice-cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BSA/azide) for immediate incubation with antibodies for flow cytometry analysis, or at 37°C in HBSS with Ca^{2+}/Mg^{2+} (Life Technologies) for cell activation. For *in vitro* cell activation, neutrophils (10^6/ml) in HBSS with Ca^{2+}/Mg^{2+} were incubated at 37°C in BSA-coated tubes for 5 min with 10 μg/ml cytochalasin B (Sigma) followed by 10 min with 1 μM *N*-formyl-methionin-leucin-phenyalanin (fMLP) (Sigma). Neutrophils were then centrifuged and resuspended in PBS/BSA/azide for flow cytometry analysis (5).

Immunofluorescence Flow Cytometry

A total of 10^6 neutrophils in PBS containing 1% BSA and 0.1% sodium azide was first incubated for 30 min at 4°C with 1 mg/ml heat-aggregated goat IgG to block Fcγ receptors. Cells were then treated with dilutions of monoclonal antibodies followed by FITC-conjugated F(ab′)2 fragments of goat anti-mouse IgG. Cells were fixed with 1% formaldehyde and analyzed for fluorescence on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with light scatter gate set.

For whole blood experiments, platelet-rich plasma from ethylenediaminetetra-acetic acid-anticoagulated blood was removed, and blood cells were washed in PBS/BSA/azide. Anti-PR3 antibodies were incubated for 15 min at room temperature followed by the addition of the FITC-anti mouse IgG antibody (15 min at room temperature). Red

cells were then lysed in Becton Dickinson FACS lysis solution according to the manufacturer's instructions and fixed with 1% formaldehyde. Measurements were made after gating on neutrophils, which were distinct from lymphocytes and monocytes in the whole blood samples by their characteristic forward/sideways scatter.

Determination of membrane PR3-negative and membrane PR3-positive neutrophils was done by integration of the surface of the respective $mPR3^-$ and $mPR3^+$ peaks. The intra-assay variability of the phenotype determination was virtually negligible. Indeed, we determined the percentage of $mPR3^+$ neutrophils in eight individuals (31 determinations, three to six determinations per individual on the same sample tested on the same day). The ANOVA showed that the variance was almost entirely (99.4%) due to the interindividual variability (intra-assay, SS = 49, df = 23, MS = 2.1; interindividual, SS = 8039, df = 7, MS = 1147). The contribution of the intra-assay variability to the total variance was only 0.6%.

Statistical Analyses

Data are expressed as mean ± SD. The distribution of the proportion of $mPR3^+$ neutrophils in the healthy population was analyzed by using an expectation-maximization algorithm adjusted for two Gaussian distributions. Healthy subjects and patients were compared by the Wilcoxon rank test (significant at 0.05 for $Z \geq 1.645$). Correlations with age were assessed in each group using Pearman's correlation coefficient (r) obtained by regression analysis.

Results

mPR3 Phenotype in the Healthy Donor Population

As mentioned previously (5), the percentage of neutrophils expressing PR3 on their membrane ($mPR3^+$) is highly variable among individuals, ranging from 0 to 100% of total neutrophils. A given individual conserves a constant $mPR3^+$ subset over prolonged periods of time. Indeed, when the percentage of $mPR3^+$ neutrophils in 21 different individuals was quantified on two occasions 12 ± 9 mo apart, the results of the two evaluations were identical (Figure 1A). Two individuals that were tested, respectively, 7 and 9 times over a period of 20 mo resulted in 30.9 ± 2.2 and 42.9 ± 4.4 percent of $mPR3^+$ neutrophils (data not shown). The distribution observed in 126 healthy blood donors is shown in Figure 1B. Eleven individuals had an $mPR3^+$ subset ≤20% of the total neutrophil circulating pool, while the remaining 115 healthy donors fitted highly significantly with a two-Gaussian distribution, with respective means of 47.3 and 71.5% of $mPR3^+$. This allows us to define three phenotypes corresponding, respectively, to 0 to 20% of $mPR3^+$ neutrophils (low phenotype, $mPR3^{low}$), to 21 to 58% of $mPR3^+$ neutrophils (intermediate phenotype, $mPR3^{inter}$), and to 59 to 100% of $mPR3^+$ neutrophils (high phenotype, $mPR3^{high}$). These three phenotypes were present in 9, 36, and 55%, respectively, of the 126 healthy subjects. In the healthy population, regression analysis showed no relationship between $mPR3^+$ phenotype and age ranging from 18 to 62 yr ($r = 0.159$; $P = 0.171$). No influence of gender could be found in the healthy population (Wilcoxon test: $Z = -0.29$; $P = 0.767$).

The stability of the mPR3 phenotype prompted us to study mPR3 expression in two independent families. As shown in Figure 2, the low phenotype, which represents only 9% in the healthy population, was overexpressed in family L (5 of 14), whereas no high phenotype was observed. By contrast, in family N, no low phenotype was observed, whereas the high phenotype was largely represented. The distributions of the three phenotypes in those two families are compatible with our hypothesis of a Mendelian inheritance regulated by two codominant alleles, with one exception, *i.e.*, subject 14 from family N. Indeed, the mPR3 phenotype of the daughter was

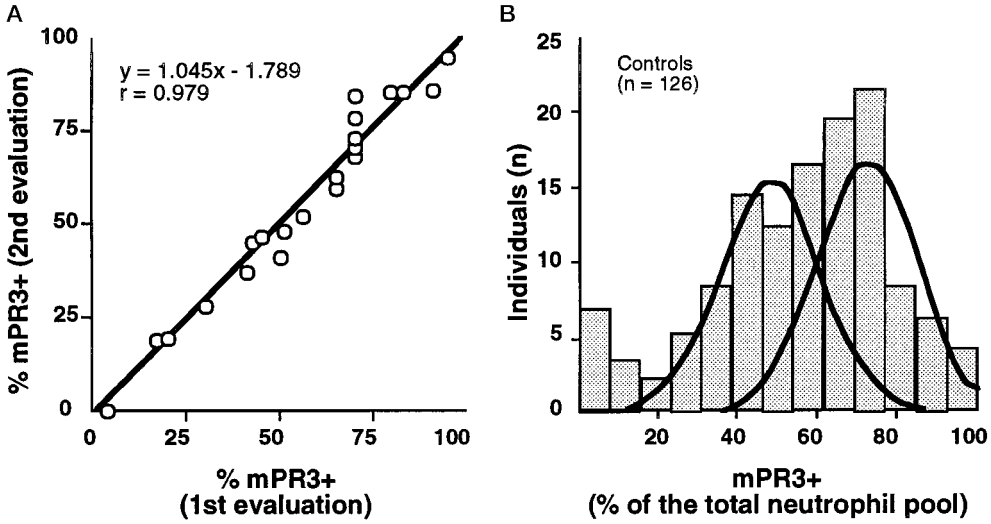

Figure 1. Stability of the membrane proteinase 3-positive ($mPR3^+$) subset in a given individual and distribution of the $mPR3^+$ subset in the healthy population. (A) The $mPR3^+$ subset, which is the percentage of neutrophils expressing membrane PR3 of each subject ($n = 21$), was quantified by flow cytometry using clone CLB12.8 anti-PR3 monoclonal antibody (mAb), on two occasions 12 ± 9 mo apart. The result of the first determination is plotted on the x-axis and of the second on the y-axis. Regression analysis shows a close correlation between the first and the second evaluation of the $mPR3^+$ subset. (B) Histogram of the $mPR3^+$ subset in 126 healthy donors.

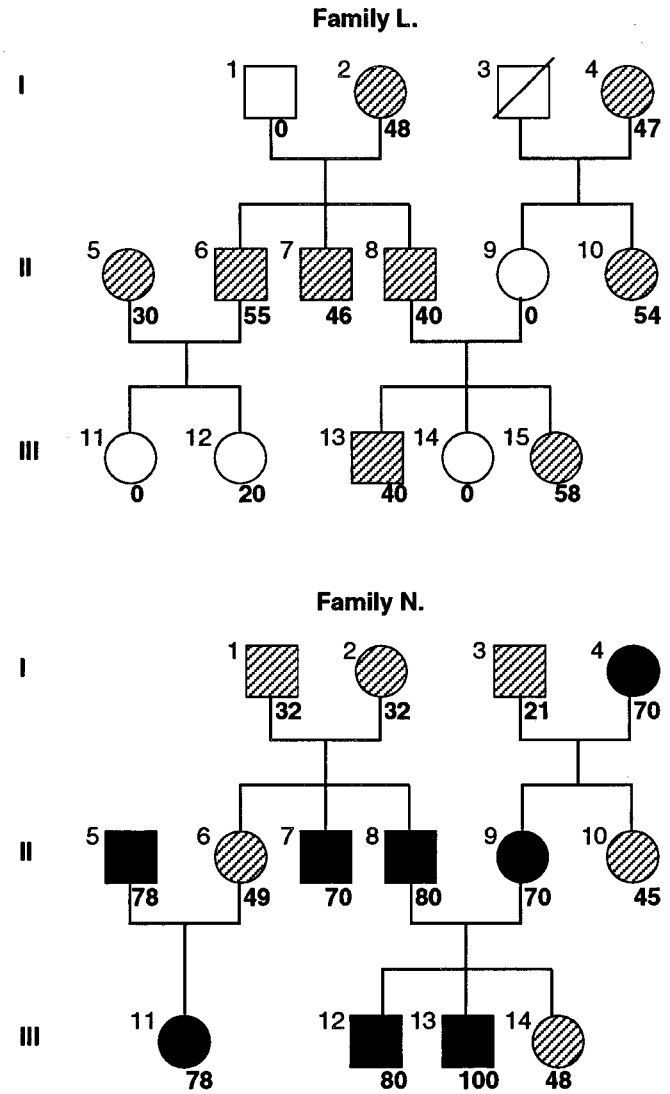

Figure 2. Determination of the mPR3 phenotype in two representative families. $mPR3^+$ phenotypes were defined according to the statistical analysis and the distribution depicted in Figure 1: $mPR3^{low}$ phenotype (0 to 20%; open symbols), $mPR3^{inter}$ (21 to 58%; hatched symbols), and $mPR3^{high}$ (59 to 100%; filled symbols). Individuals are identified by generation (roman numerals at right margins of each pedigree) and individually (arabic numerals at top left of each individual). The mPR3 phenotype is indicated at the bottom right of each individual.

intermediate (48%), whereas both of her parents belonged to the high phenotype group (70 and 80%, respectively). This apparent discordance may be due to the overlap of the Gaussians defining intermediate and high phenotypes.

We then further characterized the relationship between the $mPR3^+$ subset and cellular activation in neutrophils from healthy donors.

We first investigated the influence of neutrophil isolation procedure. PR3 was undetectable by surface labeling of whole blood cells (data not shown). However, removal of the plasma followed by a single wash of blood cells and 1 h incubation at 37°C was sufficient to visualize PR3 membrane expression (Figure 3A). The proportion of neutrophils labeled for surface PR3 was identical to that of purified neutrophils isolated from the same blood sample (Figure 3B). Interestingly, PR3 membrane expression was detected on freshly isolated neutrophils maintained at 4°C. Addition of autologous plasma to isolated neutrophils led to the disappearance of surface PR3 labeling. This was dependent on the plasma dilution added to purified neutrophils and could also be obtained by addition of purified α1-anti-trypsin at physiologic concentrations (data not shown). These experiments suggest the presence of a serum factor(s) hindering the detection of surface PR3 on whole blood cells. In addition, they show that PR3 surface labeling in the positive subset of resting neutrophils appears under very mild conditions, including physical stress induced by centrifugation or by incubation at 37°C of plasma-deprived blood cells.

The percentage of $mPR3^+$ neutrophils of 10 individuals was tested after *in vitro* cell activation by 10^{-6}M fMLP in the presence of cytochalasin B, conditions that are known to lead to maximum exocytosis of azurophil granules (13). In these activation conditions, the two $mPR3^+$ and $mPR3^-$ subsets of neutrophils remained totally distinct. This is clearly illustrated in Figure 4, which shows PR3 membrane expression of neutrophils of each of the three phenotype ($mPR3^{low}$, $mPR3^{inter}$, and $mPR3^{high}$) before (top panel) and after (bottom panel) *in vitro* activation with fMLP and cytochalasin B. The effect of this *in vitro* activation is an enhanced fluorescence intensity (shown on the abscissa), *i.e.*, an increased number of PR3 molecules on the cell surface in the $mPR3^+$ neutrophil subset. By contrast, mPR3 remained barely detectable on the $mPR3^-$ subset. The respective increase of mPR3 fluorescence on the two subsets is further detailed in Figure 5A. The major point of these results is that the proportion of $mPR3^+$ neutrophils over the total neutrophil population remained unchanged in all 10 healthy individuals tested, after *in vitro* activation with cytochalasin B and fMLP (Figure 5B, open diamonds). This allows us to conclude that the mPR3 phenotype is clearly independent of neutrophil activation.

Distribution of the mPR3 Phenotype in Patients Presenting with Vasculitis

Compared to the phenotypic distribution observed in the healthy population, the distribution in patients presenting with primary vasculitis (WG and MPA) was significantly skewed toward the $mPR3^{high}$ phenotype (normalized Wilcoxon rank test, $Z = 2.810$) (Figure 6). The same abnormal distribution was observed in the subgroups of patients with WG and with MPA (data not shown). In addition, we found no significant differences in the phenotypic distribution in vasculitic patients according to the ANCA status (Table 1) (normalized Wilcoxon rank test, PR3 positive *versus* PR3 negative, $Z = -0.71$; MPO positive *versus* MPO negative, $Z = 0.80$; PR3 and MPO ELISA negative *versus* PR3 or MPO ELISA positive, $Z = 0.45$). Comparison of the group of the 29 nephrologic disease control subjects with the population of 126 healthy donors showed no significant difference ($Z = -0.554$). The phenotypes in patients as well as in disease control subjects were not related to gender and age (data not shown).

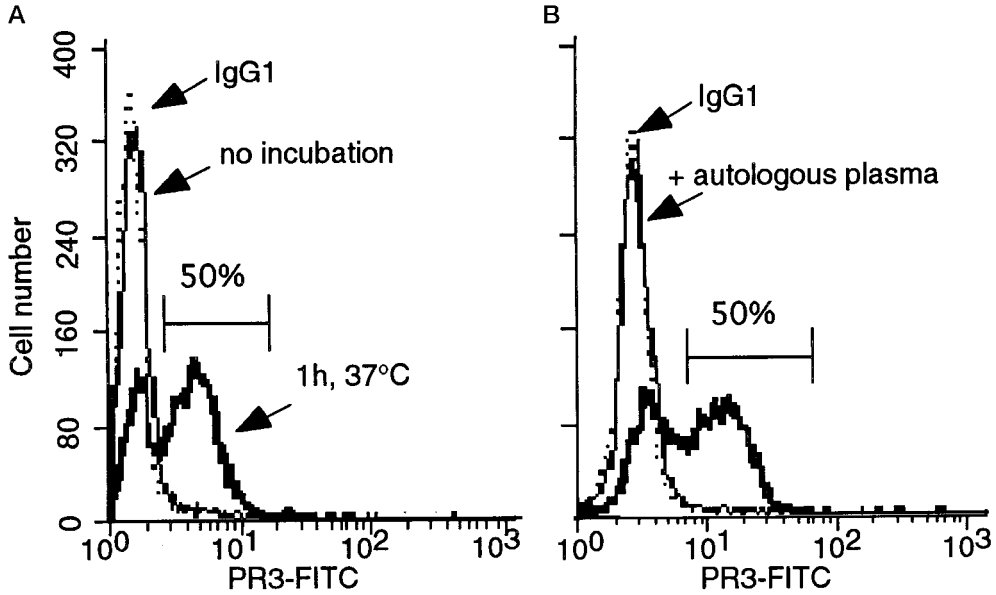

Figure 3. Membrane expression of PR3 within whole blood and in isolated neutrophils from the same individual. (A) mPR3 expression is not detectable in washed whole blood cells (plain line) compared with control IgG1 (dotted line). After incubation at 37°C for 1 h, PR3 membrane expression appears in 50% of neutrophils (bold line). (B) mPR3 expression in isolated neutrophils from the same individual shows the same $mPR3^+$ subset, which constitutes 50% of the whole neutrophil population (bold line). Addition of autologous plasma to isolated neutrophils suppresses the detection of mPR3 (plain line) to those background levels seen with control IgG1 (dotted line).

It was then critical to assess the stability of the mPR3 phenotype in patients with vasculitis, to see whether the high proportion of $mPR3^+$ could be related to *in vivo* neutrophil activation and to the disease activity.

The $mPR3^{high}$ phenotype was not related to *in vivo* cell activation evaluated by the membrane expression of L-selectin (CD62L) or CD11b (14,15). Indeed, flow cytometry results, expressed as mean fluorescence units (MFI), obtained with anti-CD62L mAb were 13.5 ± 4.1 *versus* 14.1 ± 3.5 MFI, or with anti-CD11b mAb were 24 ± 15 *versus* 26 ± 20 for vasculitic patients ($n = 19$) and control subjects ($n = 31$), respectively, thus showing no difference in the *in vivo* neutrophil activation state in vasculitis patients *versus* control subjects.

As in healthy control subjects, the percentage of $mPR3^+$ neutrophils in five vasculitic patients was found to be highly stable, over prolonged periods of time, in a given patient (data not shown). The mPR3 phenotype was not influenced by relapses and therapy, as illustrated by Figure 7, showing a representative longitudinal study of $mPR3^+$ in a vasculitic patient presenting with necrotic and crescentic glomerulonephritis, multineuritis, and alveolar hemorrhage. The percentage of $mPR3^+$ neutrophils remained strikingly stable after prednisone and cyclophosphamide treatment, while ANCA titer decreased to baseline levels, and in spite of the increase in neutrophil counts, which was probably induced by corticosteroids. We performed 32 iterative determinations in 11 patients with vasculitis (two to six determinations per patient). Again as in healthy subjects, the variance was almost entirely (95.2%) due to the interindividual variability. The contribution of the intraindividual variability to the total variance was only 4.8%, indicating that the variability in a given patient was very low, even when determinations were performed during relapse episode (in four of 11 patients) or remission and was not influenced by induction or maintenance regimen of immunosuppressive therapy (normalized Wilcoxon rank test between untreated and treated patients, $Z = -1.02$). Additionally, we studied in two healthy subjects, the effect of 25 mg of oral prednisone sufficient to induce a maximal increase of the neutrophil count (16). The $mPR3^+$ subset remained identical to the baseline level (data not shown).

Taken together, our results indicate that the proportion of $mPR3^+$ in vasculitic patients is independent of the disease activity and of neutrophil activation.

Distribution of the mPR3 Phenotype in Patients Presenting with other Inflammatory and/or Autoimmune Disorders

The high frequency of $mPR3^{high}$ phenotype observed in vasculitis, regardless of the ANCA antigen specificity, prompted us to assess whether the proportion of $mPR3^+$ neutrophils might also be elevated in other autoimmune or inflammatory diseases. As shown in Figure 8, the distribution of $mPR3^+$ neutrophils was normal in a T cell-dependent autoimmune disorder such as type I diabetes.

On the other hand, a strikingly high frequency of the $mPR3^{high}$ phenotype was observed in a group of 34 patients with rheumatoid arthritis (normalized Wilcoxon rank test, $Z = 6.56$) compared with healthy donors (Figure 8). Furthermore, eight patients with spondylarthropathy were all of the $mPR3^{high}$ phenotype (data not shown). Comparison of the

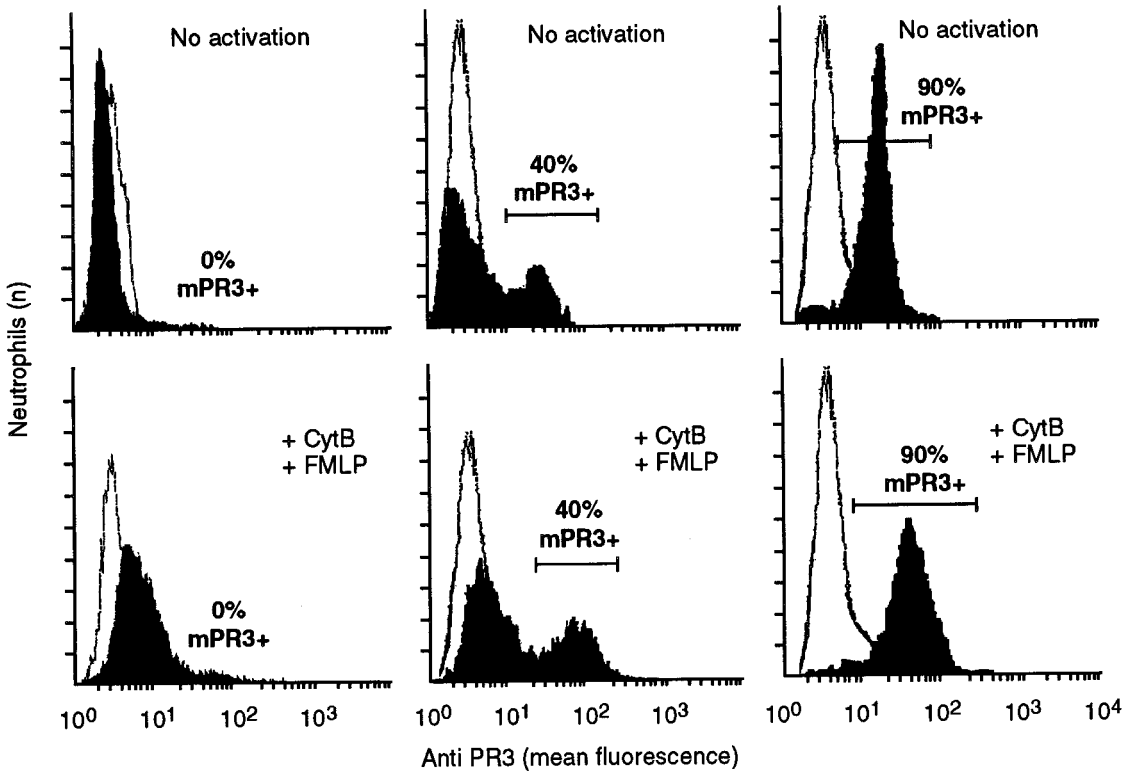

Figure 4. Membrane expression of PR3 either freshly isolated or *in vitro* activated by cytochalasin B and *N*-formyl-methionin-leucin-phenyalanin (fMLP). The top panels show flow cytometry histograms of unactivated isolated neutrophils from three different donors having either 0% (left), 40% (middle), or 90% (right) $mPR3^+$ neutrophils, labeled with anti-PR3 mAb and FITC-conjugated secondary antibodies. The bottom panels show the effect of *in vitro* activation on the same neutrophils, which were activated for 10 min at 37°C with 1 μM fMLP in the presence of 10 μg/ml cytochalasin B in order to induce azurophilic degranulation.

group of 24 control rheumatologic patients with the population of 126 healthy donors showed no significant difference ($Z = 1.35$).

No difference was observed between rheumatoid patients with active or inactive disease (normalized Wilcoxon rank test, $Z = -0.801$). Furthermore, as for vasculitic patients, iterative determinations in two patients with rheumatoid arthritis showed that the level of $mPR3^+$ was not affected by relapses or by therapy (data not shown). It also was not related to cell activation. Indeed, we had previously shown that blood neutrophils of patients with rheumatoid arthritis do not show signs of *in vivo* activation (17). By contrast, synovial fluid neutrophils from rheumatoid patients are strongly activated *in vivo*, as shown by their membrane expression of CD63, a marker of azurophilic granules. In two patients with rheumatoid arthritis, having, respectively, 45 and 90% of $mPR3^+$ neutrophils, we found that the percentage of $mPR3^+$ in neutrophils from the synovial fluid was identical to that observed for circulating resting neutrophils (data not shown).

Finally, in vasculitic or rheumatoid arthritis patients, as in healthy control subjects, *in vitro* neutrophil activation with fMLP and cytochalasin B did not modify the $mPR3^+$ ratio. This is shown in Figure 5B, where closed symbols represent patient neutrophils: five with rheumatoid arthritis (closed squares) and one with vasculitis (closed circle), chosen equally between intermediate ($n = 3$) or high ($n = 3$) phenotypes. These data further confirm that the size of the $mPR3^+$ subset is independent of neutrophil activation.

We then tested $mPR3^+$ neutrophils in a chronic idiopathic inflammatory disease, namely, cystic fibrosis, to determine whether pyogenic infections and chronic pulmonary inflammation might induce an increased subset of $mPR3^+$ neutrophils (18). As shown in Figure 8, 24 cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa* or *Staphylococcus aureus* showed a normal distribution of $mPR3^+$ subset. The proportion of $mPR3^+$ was unrelated to the severity of the pulmonary disease—since no correlation was found between the forced expiratory volume FEV1 and the proportion of $mPR3^+$ neutrophils—and as for control subjects was not related to age (data not shown).

Discussion

The analysis of PR3 membrane expression on circulating resting neutrophils reveals the existence of two distinct subsets of neutrophils, one expressing ($mPR3^+$) and the other not expressing ($mPR3^-$) membrane PR3. The proportion of these two subsets is a stable feature of a given subject, not related to age or gender. We show here that the distribution of the $mPR3^+$ neutrophil subset among healthy individuals corre-

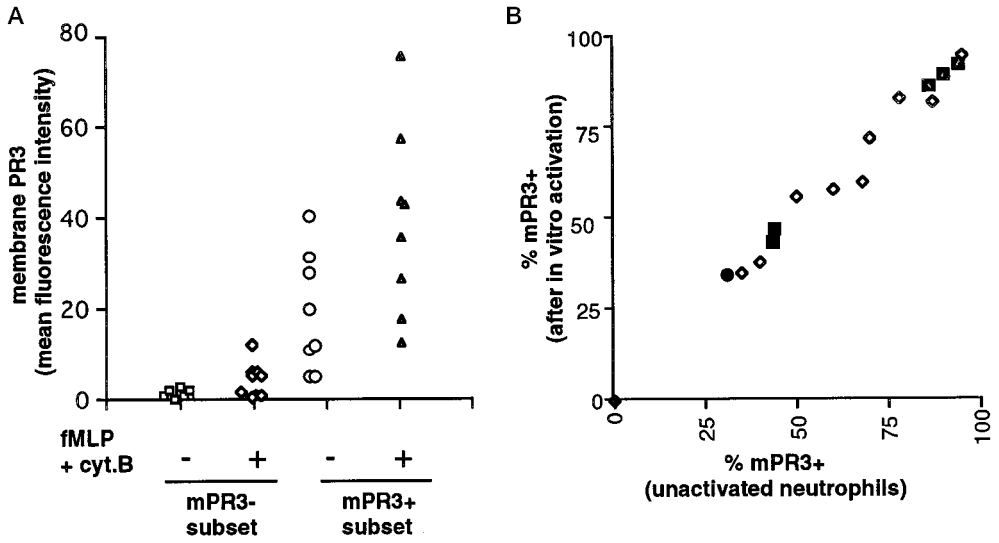

Figure 5. Influence of *in vitro* neutrophil activation on membrane PR3 expression. Neutrophils from 10 healthy control subjects (open symbols) and six vasculitic or rheumatoid patients (filled symbols) were analyzed by flow cytometry of neutrophils labeled with anti-PR3 mAb and FITC-conjugated secondary antibodies before and after activation 10 min at 37°C with 1 μM fMLP in the presence of 10 μg/ml cytochalasin B. (A) The mean fluorescence intensity of $mPR3^-$ and $mPR3^+$ subsets was measured in the presence or in the absence of activation in 10 different healthy donors as described in Figure 4. (B) The $mPR3^+$ subset measured in unactivated (x-axis) and fMLP-activated conditions (y-axis) were closely correlated. Filled circles and filled squares represent vasculitic patients and rheumatoid patients, respectively.

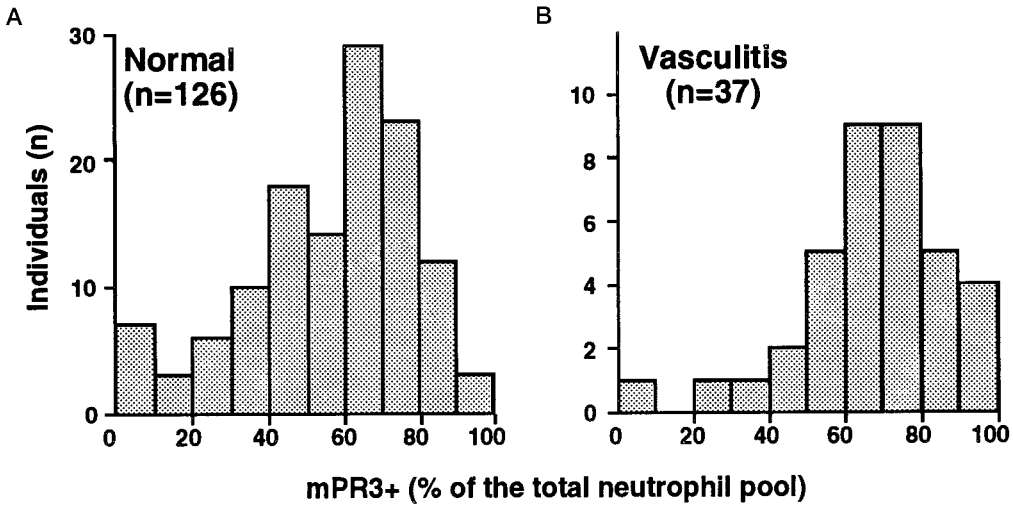

Figure 6. $mPR3^+$ phenotype in vasculitic patients. Distribution histogram of the $mPR3^+$ phenotype in patients with vasculitis, compared with the healthy population.

sponds to three discrete phenotypes, $mPR3^{low}$, $mPR3^{inter}$, and $mPR3^{high}$, respectively present in 9, 36, and 55% of the healthy individuals. Indeed, after sorting out individuals having less than 20% $mPR3^+$ neutrophils (corresponding to 9% of the healthy population), the histogram of the remaining healthy individuals fitted with a two-Gaussian distribution with respective mean percentages of 47 and 71% $mPR3^+$ neutrophils. A mode of inheritance compatible with the present data would be a regulation by two codominant alleles, with $mPR3^{low}$ or $mPR3^{high}$ individuals homozygous for the low or high expression alleles, and $mPR3^{inter}$ individuals heterozygous. With such a hypothesis, the gene frequency estimated from the numbers in these three phenotypes are 0.72 for the allele for high PR3 expression and 0.28 for the allele for low PR3 expression. These frequencies satisfy the Hardy–Weinberg law for a population at genetic equilibrium. Our study of two representative families is compatible with such a mode of inheritance.

Since PR3 is the main antigen of ANCA autoantibodies associated with Wegener's granulomatosis, we analyzed the distribution of the mPR3 phenotypes in vasculitic patients. Our data show that the frequency of the $mPR3^{high}$ phenotype is strikingly higher in patients with ANCA-related vasculitis than in the healthy control population. The proportion of $mPR3^+$ neutrophils in a given patient is remarkably stable during

Table 1. Phenotypic distribution in vasculitic patients according to ANCA status[a]

Subjects	Percentage of Subjects (%) of the Three mPR3 Phenotypes[b]		
	$mPR3^{low}$	$mPR3^{inter}$	$mPR3^{high}$
Healthy population ($n = 126$)	9%	36%	57%
Vasculitis (WG + MPA), ($n = 37$)	5.5%	11.1%	83.3%
PR3 ANCA ($n = 12$)	0%	27.2%	72.7%
MPO ANCA ($n = 16$)	6.25%	18.75%	68.8%
ANCA ELISA negative ($n = 9$)	0%	22.2%	77.8%

[a] ANCA, antineutrophil cytoplasmic antibody; mPR3, membrane proteinase 3; WG, Wegener's granulomatosis; MPA, microscopic polyangitis; MPO, myeloperoxidase; ELISA, enzyme-linked immunosorbent assay.

[b] The three phenotypes corresponded, respectively, to 0 to 20% of $mPR3^+$ (low phenotype: $mPR3^{low}$), to 21 to 58% of $mPR3^+$ (intermediate phenotype: $mPR3^{inter}$), and 59 to 100% of $mPR3^+$ (high phenotype: $mPR3^{high}$).

follow-up and is not influenced by relapses or by therapy, in spite of variations of ANCA titers. The presence of PR3 on the plasma membrane does not reflect an activation state of neutrophils, which present normal levels of CD62L or of CD11b (14). Furthermore, the $mPR3^+$/total neutrophil ratio is not modified after *in vitro* activation of patients' neutrophils. In addition, a high $mPR3^+$ subset is not a consequence of inflammation and infection since normal ratios of $mPR3^+$ neutrophils were observed in cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa* or *Staphylococcus aureus* (18). Thus, the increased frequency of the $mPR3^{high}$ phenotype in vasculitic patients cannot be explained by neutrophil alterations resulting from chronic inflammation. As in the healthy population, the $mPR3^{high}$ phenotype is very likely genetically determined.

It is worth noting that the normal distribution of $mPR3^+$ phenotypes observed in the young population of cystic fibrosis patients (mean age 12.2 ± 5.7), as in the older groups of disease control patients (mean age 59.4 ± 16 and 60.6 ± 14.3 for renal and rheumatoid patients), further demonstrates that mPR3 expression is not related to age.

Our first hypothesis was that the presence of PR3 on the neutrophil surface could be relevant to the pathogenesis of ANCA-related vasculitis and, specifically, to the preferential autoimmunization against PR3 over similar neutrophil intracellular enzymes such as elastase or cathepsin G. However, the fact that the $mPR3^{high}$ phenotype is abnormally frequent in vasculitis regardless of the ANCA antigen specificity (PR3, MPO, ANCA negative) does not support the idea that a large subset of neutrophils presenting PR3 on their membrane would specifically favor the occurrence of anti-PR3 autoantibodies. Another possibility was that a high proportion of membrane PR3-positive neutrophils might be a more general risk factor for autoimmune responses. The normal distribution of mPR3 phenotypes observed in type I diabetes would argue against this hypothesis.

Anti-PR3 ANCA antibodies have been shown to directly activate neutrophils, leading to degranulation and superoxide production (19,20). Our data are compatible with a possible role of PR3 at the plasma membrane of unactivated neutrophils in ANCA-mediated vascular inflammation, particularly in Wegener's granulomatosis, usually associated with anti-PR3 autoantibodies. Moreover, we show that PR3 is present on the membrane of freshly isolated neutrophils from the $mPR3^+$ subset, while it is generally admitted that a step of cell priming by tumor necrosis factor-α is required for PR3 to be translocated to the cell surface and to become accessible to ANCA autoantibodies (21). On the other hand, the high frequency of $mPR3^{high}$ phenotype in patients with anti-MPO vasculitis as well as in rheumatoid arthritis and spondylarthritis suggests that a high proportion of membrane PR3-positive neutrophils might be more generally relevant to the progression of the inflammatory lesions, particularly when neutrophils are involved.

Neutrophil activation is known to result in the translocation of PR3 from intracellular granules to the plasma membrane. The presence of a serum factor(s) that hinders membrane PR3 detection does not allow us to state that PR3 is present on the membrane of *in vivo* circulating neutrophils belonging to the $mPR3^+$ subset. It may appear on the membrane as a consequence of the mild cell activation occurring during the cell isolation procedure or the *in vitro* incubation of washed blood cells. Indeed, we have recently demonstrated the existence of a highly mobilizable intracellular pool of PR3, distinct from azurophilic granules (22). Such mild neutrophil activation may occur *in vivo* at the initial phase of inflammatory cell recruitment. Furthermore, we show here that after neutrophil *in vitro* intense activation by fMLP and cytochalasin B, or after *in vivo* neutrophil migration and activation—such as in rheumatoid arthritis synovium—the $mPR3^-$ and $mPR3^+$ subsets remain clearly distinct, with the $mPR3^+$ subset of neutrophils being the only one to express substantial amounts of membrane PR3 upon cell activation. In a subject with a high proportion of $mPR3^+$ circulating neutrophils, most neutrophils recruited at the site of inflammation will thus express high levels of membrane PR3. Significant levels of membrane PR3 have indeed been reported on freshly isolated neutrophils from patients with ANCA vasculitis and with other intravascular inflammation such as sepsis (11).

The potential role of mPR3 in the pathogenesis of chronic inflammation is still unclear. Although we do not have evidence that membrane PR3 is enzymatically active, closely related neutrophil enzymes such as elastase and cathepsin G have been shown to retain their activity and to be resistant to plasma protease inhibitors, when bound to the neutrophil surface (23,24). PR3 is able to degrade extracellular matrix proteins (25) and to activate cytokines such as tumor necrosis factor-α or interleukin-8 (26,27). As such, it could be involved in tissue lesions and in sustained inflammatory reactions.

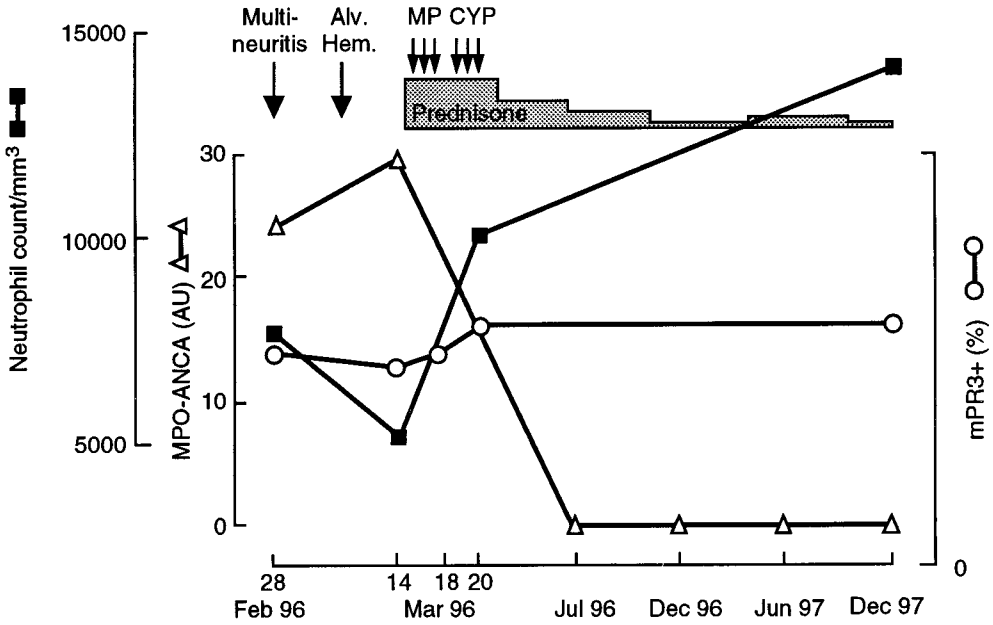

Figure 7. Follow-up study of a patient presenting with vasculitis. Neutrophil count (filled squares), antineutrophil cytoplasmic autoantibody titers (open triangles), and mPR3$^+$ neutrophil subsets (open circles) were sequentially measured over a 10-mo period in the same vasculitic patient. This patient had necrotic and crescentic glomerulonephritis, multineuritis, and alveolar hemorrhage. Alv.Hem, alveolar hemorrhage; MP, methylprednisolone; CYP cyclophosphamide.

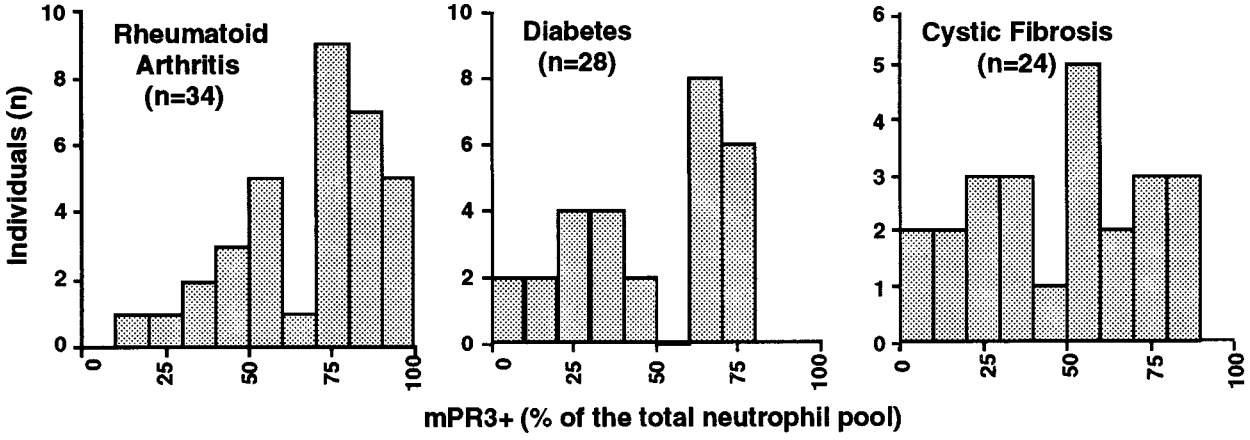

Figure 8. Analysis of mPR3$^+$ phenotype in autoimmune or inflammatory diseases. Histograms of the mPR3$^+$ phenotype (see Figure 1) in patients with rheumatoid arthritis, with type I diabetes, or with cystic fibrosis.

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