Antineutrophil cytoplasmic autoantibodies penetrate into human polymorphonuclear leukocytes and modify their apoptosis

M. Deutsch¹, L. Guejes¹, N. Zurgil¹, O. Shovman², B. Gilburd², E. Afrimzon¹, Y. Shoenfeld

¹The Biophysical Interdisciplinary Jerome Schottenstein Center for the Research and the Technology of the Cellome, Department of Physics, Bar-Ilan University, Bar-Ilan; ²Department of Medicine B and the Research Unit of Autoimmune Diseases, Sheba Medical Center, Tel Hashomer, Israel

Mordechai Deutsch, PhD; Larissa Guejes, PhD; Naomi Zurgil, PhD; Ora Shovman, MD; Boris Gilburd, PhD; Elena Afrimzon, PhD, MD; and Yehuda Shoenfeld, MD. This research was supported by the Horowitz Foundation.

Please address correspondence to: Prof. Yehuda Shoenfeld, MD, Department of Medicine ‘B’ & the Research Unit of Autoimmune Diseases, Chaim Sheba Medical Center, (affiliated with Tel-Aviv University), Tel-Hashomer 52621, Israel.
E-mail: shoenfel@post.tau.ac.il or rtcellom@mail.biu.ac.il
Received on July 26, 2004; accepted in revised form on September 17, 2004.
© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2004.

Key words: Anti-neutrophil cytoplasmic autoantibodies (ANCA); polymorphonuclear leukocytes (PMN); apoptosis; anti-proteinase3 (PR3) IgG; normal human IgG.

ABSTRACT

Objective. The interaction of extracellular anti-neutrophil cytoplasmic autoantibodies (ANCA) with neutrophilic granules may play an important role in the pathogenesis of ANCA-related disorders. It has been confirmed that apoptosis is an essential trigger associated with translocation of the cytoplasmic granules to the cell surface, and with the expression of ANCA antigens. Since cell penetration by autoantibodies and apoptosis may be associated processes, we tested the hypothesis that penetration of ANCA-autoantibodies into polymorphonuclear leukocytes (PMNs) has an effect on apoptosis and thereby can influence surface antigen expression.

Methods. PMNs were isolated from the blood of healthy volunteers and incubated in the presence of anti-proteinase 3 (PR3) enriched IgG or normal human IgG. For each period of incubation (40 minutes or 12 hours) we evaluated: 1) PMN morphology by light microscopy (LM) and transmission electron microscopy (TEM) for general estimation of the apoptotic process, and 2) ANCA binding to the target antigen by immunogold electron microscopy (IgEM).

Results. Both normal and anti-PR3 IgG penetrate PMNs. The labeled PR3-ANCA were localized on PR3 granules, regardless of the granules’ location within the cell, and in the sites where the PMN destruction processes were most expressed. The destructive processes showed extensive apoptotic characteristics, in contrast to PMNs penetrated by normal IgG.

Conclusion. PR3 ANCA penetrate PMNs and, via the interaction between PR3-ANCA and PR3-containing granule components, initiate a modification of the apoptotic process.

Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCA) are considered to be a class of antibodies directed against proteins in both the cytoplasmic granules of neutrophils and the lysosomes of monocytes (1). ANCA are associated with small vessel vasculitis, especially Wegener’s granulomatosis and microscopic polyangiitis (2). Increasing evidence for the direct involvement of ANCA in the pathogenesis of this type of vascular inflammation has emerged from several lines of studies in vivo and in vitro (3-9). There have been substantial developments in the understanding of the pathogenetic role of ANCA and the mechanisms of ANCA-induced neutrophil activation (9). The most important and difficult issue connected with these investigations was to understand the interaction between extracellular ANCA and intracellular granule components. Several proposed mechanisms suggest that ANCA cause a release of toxic oxygen radicals and granule enzymes from normal human neutrophils with their subsequent activation (4). ANCA-induced neutrophil activation results from an engagement of neutrophil Fcγ receptors (5) or from crosslinking of ANCA-target antigens on the neutrophil surface with ANCA F(ab')2 fragments (6). The priming of neutrophils by cytokines, particularly by tumor necrosis factor-α (TNF-α), effects enhanced expression of the target antigens on their surfaces (4,7). Apoptosis of neutrophils also seems to lead to the surface expression of the ANCA target antigens (7, 8). Yang and associates found that neutrophils undergo priming and expression of ANCA antigens before any manifestation of apoptosis (7). Gilligan et al. have concluded that apoptosis of unprimed PMNs is associated with translocation of the cytoplasmic granules to the cell surface (8).
Alarcon-Segovia et al. described autoantibody penetration into living cells and opened a new way of explaining the different aspects of the interaction between autoantibodies and target cells (10-13). Several observations reinforce their views by showing that certain autoantibodies directed against intracellular antigens may modify the cell function with induction of immunodepletion (14-17). These experimental findings, coupled with evidence of autoantibody-induced apoptosis, led to the hypothesis that apoptosis may sometimes be linked to cell penetration by antibody (18). The purpose of our study was to define the influence of ANCA penetration on human PMN apoptosis using TEM and IgE.

Materials and methods
Human IgG preparations
The present study was conducted at the Sheba Medical Center, Tel Hashomer, Israel, in accordance with the principles of the Declaration of Helsinki. IgG fractions with anti-PR3 activity were purified from sera of active Wegener’s granulomatosis patients (n=3) with high level of anti-PR3 activity (100-150 ELISA units) by affinity chromatography on protein G column (Pharmacia) according to the manufacturer’s instructions. The normal human IgG fractions were purified from sera of healthy subjects (n=3) as described above. The purity of both IgG preparations was checked by polyacrylamide gel electrophoresis (PAGE). Restricted reactivity of patients IgG preparations to PR3 was confirmed by cross-ELISA assay with different (myeloperoxidase, elastase, lactoferrin, BPI) ANCA antigens (AESKULISA, Aeskus Diagnostic GMBH, Wendelsheim, Germany). All experiments were performed at least three times.

Isolation of human PMN
PMN were isolated from heparinized blood derived from healthy volunteers using the standard procedure of dextran (mol wt. 500,000, Sigma Chemical Co, St Louis MO) sedimentation and Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation as previously described (19). Contaminating erythrocytes were removed by hypotonic lysis with ice cold sterile H2O, and normal osmolality was restored by addition of 3.5% NaCl. PMNs were finally resuspended in complete culture medium (RPMI-1640 with 1% penicillin-streptomycin-nystatin solution). The suspension contained > 96% PMNs and the viability was > 99% as determined by trypan blue exclusion.

Cell culture
PMNs (1 x 10⁶ cells/ml) were incubated in RPMI 1640 culture medium in the presence of either 100 µg/ml of anti-PR3 enriched IgG or normal human IgG for up to 12 hours at 37º C in 5% CO2 in humidified atmosphere. After a careful investigation of samples at different periods of incubation, 40 min and 12 h cultures were selected as characteristic of short and long periods of incubation. At the different periods of incubation, the PMN cultures contained different proportions of normal, transitional and apoptotic forms. At both periods, the majority of cells had intact plasma membrane.

Light microscopy
PMN slides for light microscopy were prepared using a Shandon Cytospin cytancentrifuge. Following staining with May-Grunvald-Giernsa, a minimum of 1000 cells were analyzed due to the uneven distribution of normal and apoptotic neutrophils on the slides. The staining was used for the evaluation of the general morphological characteristics of the populations and for the determination of direction of the apoptotic process (20,21). Apoptotic cells at the light microscopic level were recognized by their rounded shape, even outlines, and clear, dense, round nuclei, or micronuclei (20).

Transmission electron microscopy
Portions of neutrophil sediments were fixed with 2.5% glutaraldehyde in Karnovsky’s buffer, washed in Serenson’s phosphate buffer (pH 7.4), and post-fixed for one hour with 1% osmium tetroxide in the same buffer. Samples were washed in phosphate and veronal acetate buffers and placed into a 1% solution of uranyl acetate. After dehydration and treatment with propylene oxide, the samples were positioned in a 1:1 Epon-propylene oxide mix and into Epon 812-Araldite epoxy-resin. Polymerization occurred overnight at 64º C. Ultrathin sections were prepared with an LKB-3 ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a JEOL1200 Ex EM at an operating voltage of 80 kV.

Immunogold staining
Immunogold labeling was performed on ultrathin sections of neutrophils derived from different populations incubated with PR3-ANCA or normal human IgG. After blocking with 1% BSA and 1% GSA in PBS (pH 8.2) PMNs were treated with 18 nm colloidal gold conjugated goat-anti-human IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) diluted 1:20.

Apopoptosis measurement
Apopoptosis of PMN was additionally assessed by image analysis using double staining with FITC-Annexin V and propidium iodide (PI). Briefly, one million cells were washed with PBS and resuspended in binding buffer (140 mM NaCl, 2.5 mM CaCl2, 1mM Hepes, and pH 7.4). FITC-Annexin V (R&D Systems, Minneapolis, MN) and PI (Sigma Chemical Co.) at final concentrations of 1 and 4 µg/ml, respectively, were added simultaneously and cells were incubated for 15 min in the dark at room temperature. About 300 cells were assessed in a sample. Cells were analyzed by Olympus motorized upright epi-fluorescence BX51 microscope, equipped with a Mercury light source. The emitted fluorescence was imaged using CoolSNAP HQ monochrome CCD camera, or DVC color camera. Digital image analysis from cellular fluorescence was performed by Image Pro plus software. Statistical analysis was performed by the ANOVA test.

Results
Fluorescence and light microscopy analysis of PMN apoptosis
The effect of PR3 ANCA on PMN apoptosis was initially evaluated by fluorescence and light microscopy.
Freshly isolated PMN contained 6 ± 1.5% Annexin V positive (early apoptotic) cells and 8 ± 1.5% of Annexin V and PI double positive (late apoptotic) cells. The percentage of Annexin V positive cells following 40 min incubation of freshly isolated PMNs in the presence of PR3 ANCA (Table 1) was significantly higher (p < 0.05) than the percentage of Annexin V positive cells following exposure to normal human IgG (46 ± 6.2 and 17 ± 4.5% apoptotic cells, respectively). Most PMNs which externalized phosphatidylserine were at the early stage of apoptosis, evidenced by their PI negative state. After 12 h incubation, high proportions of apoptotic cells were shown by Annexin V staining of PMNs incubated either with PR3 ANCA or normal human IgG (Table I, Fig. 1).

Light microscopy analysis revealed a similar pattern, but in this method, the distinction between PMNs after exposure to the different groups of antibodies was more pronounced after long incubation. PMNs exposed to PR3 ANCA for 12 hours showed a significantly higher proportion of apoptotic cells (Fig. 2C, Table I) than PMNs cultured with normal human antibodies (Fig. 2A, Table I). Moreover, out of the 57.4 ± 15% of PR3 ANCA exposed apoptotic cells, 38.08 ± 14.27% exhibited vacuoles of various sizes, whereas upon incubation with normal IgG no vacuoles were found – a phenomenon to be further examined by electron microscopy (Fig. 4).

Although the kinetics of the apoptotic process, as shown by Annexin V/PI staining and by light microscopy, were not the same, both techniques demonstrate a tendency toward the augmentation/alteration of PMN cell death upon exposure to PR3 ANCA.

Ultrastructure of PMNs incubated with PR3 ANCA or normal human IgG

Regardless of the IgG preparation used, following 40-minute incubation PMNs had normal neutrophil characteristics with moderately irregular contours; segments of nuclei were filled with both eu- and heterochromatin. The cytoplasm contained the elements of the cytoplasmic net, mitochondria, and a moderate quantity of granules (Fig. 3A). Incubation of PMNs with normal human IgG for 12 hours, yielded several predominant apoptotic appearances, mainly associated with the structural changes of the nucleus and cytoplasm. PMNs exhibited a coalescence of nuclear lobes into a single nucleus or formed 2-3 micronuclei filled with condensed chromatin. Alterations of the cytoplasm were characterized by a gradual increase in the density of the cytoplasmic matrix and a portion of the vacuolized organelles, as well as by an increased number of vesicles gradually moving to the cytoplasmic membrane (Fig. 3B).

Table I. The proportions of apoptotic cells within PMN cell populations exposed to normal human IgG or PR3 ANCA as determined by fluorescent and light microscopy. Freshly isolated PMN contained 6 ± 1.5% Annexin V positive (early apoptotic) cells and 8 ± 1.5% of Annexin V and PI double positive (late apoptotic) cells. The results are presented as mean ± SEM. About 300 cells were assessed by the fluorescence tests. At least 1000 cells were analyzed by light microscopy. Significance was determined by the ANOVA test. Results were considered to be significant at p < 0.05 (*).

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Fluorescence microscopy</th>
<th>Light microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Apoptotic cells</td>
<td>% Apoptotic cells</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>PR3 ANCA</td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>Annexin + PI</td>
<td>Annexin V</td>
</tr>
<tr>
<td>40 min</td>
<td>17 ± 4.5</td>
<td>10 ± 1.5</td>
</tr>
<tr>
<td>12 h</td>
<td>54 ± 6.2</td>
<td>9 ± 3.4</td>
</tr>
</tbody>
</table>

Fig. 1. Light (A) and fluorescence (B) micrographs of PS externalization and plasma membrane integrity in PMN population following 12h exposure to PR3 ANCA.

Fig. 2. Light microscopy shows that the PMN populations include: (A) mainly normal cells after 40 min incubation with normal human IgG; (B) mainly transitional (arrows) and apoptotic forms (double arrows) after 12h incubation with normal human IgG; (C) After 12 h incubation with PR3 ANCA, a considerable part of apoptotic cells is comprised of apoptotic forms with large autolytic vacuoles (arrows).
Apoptotic PMNs having altered granules with flocculent content (Fig. 4 A,B,C) were predominant after 12 hour exposure to anti-PR3 IgG. Notably, altered or destroyed granules were usually localized in the immediate proximity of the forming big autolytic vacuoles. At later stage, vacuoles and granule residues are translocated to the cell surface (Fig. 4C).

Immunogold electron microscopy of PMNs incubated with anti-PR3 enriched IgG or normal human IgG

Single gold labels on the cytoplasmic membrane and within distinct sites of the cytoplasm were found in PMNs that had undergone 40 min incubation whether with normal IgG or PR3 ANCA (data not shown). Similar individual gold label distribution was found in PMNs incubated for 12 h with normal human IgG (Fig. 5A). On the other hand, in PMNs exposed for 12 h to anti-PR3 IgG, the gold markers resided on altered granules, formed aggregates on decomposing granules, and also accumulated in large lytic vacuoles (Fig. 5B,C).

Discussion

Wegener’s granulomatosis is an autoimmune disease which is characterized by the presence of highly sensitive and specific ANCA (22). In recent years, considerable data have been accumulated regarding the direct involvement of ANCA in the pathogenesis of vasculitis. Special attention has been paid to the interaction of extracellular ANCA with intracellular primary granule components. Several experimental models established ANCA Ag expression by employing different priming events of apoptosis which might trigger translocation of primary granules to the surface of PMNs (3-8).

The aim of the present study was to show the antibody penetration into PMN and its relation to PMN apoptosis. In comparison to PMNs treated with normal human IgG, incubation of cells with anti-PR3 antibodies modified PMN apoptosis. The gold particles were associated with primary granules, formed aggregates on decomposing PR3-containing granules, and also accumulated in big lytic vacuoles. The first report that whole antibody molecules were actually competent to penetrate living cells was carried out by Alarcon–Segovia et al. in 1978 (10). Subsequent observations of intracellular events by the same group appeared to indicate that anti-ribonucleoprotein IgG entering T γ lymphocytes caused their deletion and the abrogation of suppressor function (12, 13). Since then, several additional studies have addressed a connection between these two processes: apoptosis and cell penetration by antibody (23-25). The same phenomenon of cell penetration specifically associated with anti-dsDNA antibody was demonstrated (23,24). One of the most interesting reports has been published by Adamus et al., which verified apoptotic death of retinal rod cells following entrance of IgG anti-recoverin antibodies (25). An exciting hypothesis, suggesting that apoptosis and cell penetration by autoantibodies may correspond to different stages of the
same linked process, was proposed by Williams and Peen (18). However, it has not yet been conclusively established which stage comes first. Recently, several investigators have provided experimental proofs indirectly supporting the validity of this hypothesis in ANCA-associated diseases. In a study of PMNs from patients with Wegener’s granulomatosis, Csernok et al. (26) have demonstrated both the presence of intra-cytoplasmic IgG antibodies and overexpression of the autoantigen, PR3, on the cell surfaces. This was in contrast to results seen in normal subjects. Gilligan’s group (8) have also noted that apoptosis of unprimed PMNs was associated with translocation of cytoplasmic granules to the cell surface. Moreover, it has been shown that autoantigens such as nucleosomal DNA, and small nuclear and cytoplasmic ribonucleoproteins targeted in SLE, can be found on the surface of apoptotic keratinocytes in the apoptotic blebs (27).

In the present study, we provide evidence of PMN penetration by anti-PR3 antibodies with a concomitant enhancement of the apoptotic process by: a) the appearance of immunogold particles associated with cytoplasmic granules, independent of the granules location within the cell; b) the difference between the dynamics of immunogold particle accumulation in PMN cultures incubated with PR3 ANCA and normal IgG; c) the clear correlation of immunogold accumulation with the intensity of the destructive process.

A variety of methodologies may support these findings. In our preliminary study (data not included in the present report), we used complete highly anti-PR3 active sera of Wegener’s granulomatosis patients, since isolated IgG fractions are known to sometimes form irreversible aggregates, and obtained similar results. However, in the reported study we used isolated purified IgG fractions and repeated the experiments at least three times to ensure that the specific ANCA penetrate the PMN with subsequent modification/enhancement of apoptosis. In the present study, in addition to the highly reliable electron microscopical analysis, we also assessed the early events of PMN apoptosis by image analysis using double staining with FITC-Annexin V and propidium iodide (PI), with an epifluorescent cytometric system. Although the dynamics of apoptosis, as indicated by light microscopy and Annexin V and PI staining, were not the same, there is a common tendency toward the enhancement of apoptosis following incubation with anti-PR3 IgG as compared to normal IgG. Our results show that ANCA penetration and apoptosis are interrelated processes, however, this does not exclude the possibility of subsequent or parallel antigen engagement by ANCA on the cell surface.

The obtained results may have relevance to the understanding of pathogenesis of ANCA-related disorders, particularly in view of the interference of these autoantibodies with intracellular granule components. Finally, our results may contribute to the understanding of the initiation of the autoimmune response in ANCA-associated vasculitis. Thus, our data are compatible with previously accumulated evidence that “apoptotic” autoantigens are the natural targets for different types of autoantibodies. We believe that the autoantibody penetration may be used in the future for specific delivery of conjugated drugs/toxins to the leukocytes.

References

6. KETTRITZ R, JENNETTE JC, FALK RJ: Crosslinking of ANCA-antigens stimulates...
ANCA penetration modifies PMN apoptosis / M. Deutsch et al.