Laboratorio de Fotoquímica[1], Centro de Química, Instituto Venezolano de Investigaciones Científicas I.V.I.C., Caracas. BIOMED[2], Facultad de Ciencias de la Salud, Universidad de Carabobo-Núcleo Aragua, Venezuela

Clozapine prevents apoptosis and enhances receptor-dependent respiratory burst in human neutrophils

F. Vargas[1], C. Rivas[1], H. Perdomo[2], A. Rivas[2], L. E. Ojeda[2], M. Velásquez[2], H. Correia[2], A. Hernández[2], G. Fraile[2]

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Dr. Franklin Vargas, Laboratorio de Fotoquímica, Centro de Química, Instituto Venezolano de Investigaciones Científicas I.V.I.C., Apartado 21827, Caracas 1020-A, Venezuela
fvargas@ivic.ve

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The present study was undertaken to determine if the antipsychotic drug clozapine (CLZ) in the concentration range 2–50 μM can rescue polymorphonuclear cells (PMNs) from undergoing apoptosis. Our results indicate that 20 μM CLZ can rescue PMNs both from UVB-accelerated (28.0% vs. 45.9% for control without CLZ; $P < 0.05$) and from spontaneous (35.8% vs. 57.6%; $P < 0.05$) apoptosis whereas 50 μM CLZ could rescue PMNs from spontaneous (34.3% vs. 57.6%; $P < 0.05$) apoptosis only. Furthermore, since apoptosis has been reported to involve the impairment of PMN function, we evaluated the effects of CLZ on respiratory burst in UVB-irradiated and in unirradiated PMNs. When 20 or 50 μM CLZ-pretreated PMNs were aged in a culture during 4 h, the luminol-dependent chemiluminescence (CL) response was 3-fold ($P < 0.01$) and 2.5-fold ($P < 0.05$) increased, respectively, by subsequent exposure to serum opsonized zymosan (OZ). When 50 μM-pretreated PMNs were either UVB-irradiated or unirradiated, the CL response was 2.6-fold ($P < 0.05$) and 3.3-fold ($P < 0.05$) increased, respectively, after subsequent exposure to formyl-methionyl-leucyl-phenylalanine (fMLP). In contrast, the degree of enhancement was negligible upon subsequent exposure to ionomycin or phorbol myristate acetate (PMA). When incubation times were extended up to 22 h, the CL response induced by OZ in 20 μM CLZ-treated PMNs had a 4.9-fold increase ($P < 0.001$). This priming effect could be reverted when 20 μM CLZ-treated PMNs (aged 4 h in culture) were coincubated for 5 min with the protein tyrosine kinase inhibitor genistein as well as with the phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin. These findings suggest that CLZ primes respiratory burst and prevents PMN apoptosis as a consequence of tyrosine phosphorylation- and PI3-K activation-dependent signal transduction pathways.

1. Introduction

Clozapine (CLZ) is a dibenzodiazepine atypical antipsychotic agent that is more effective than standard neuroleptic drugs in the treatment of refractory schizophrenia. Unfortunately, its use has been limited by its propensity to cause lethal agranulocytosis, which occurs in approximately 1% of patients treated with this compound (Alvir 1994). The exact mechanism of this adverse reaction is not known although a good correlation exists between the incidence of agranulocytosis and drug activation by myeloperoxidase (MPO) (Fischer 1991) which is present in both neutrophils (PMNs) and bone marrow cells. Indeed, clozapine is oxidized *in vitro* by activated human PMNs to a free radical identified as a nitrenium ion (Liu 1995). This reaction was carried out by HOCl which is the major oxidant produced by MPO-H_2O_2-Cl^- system in these cells.

In a recent report it was shown that 30 μM CLZ can protect PC12 cells against serum withdrawal-induced cell death. These CLZ protective effects were observed after a 48 h-pretreatment with the drug which were related to its ability to modulate superoxide dismutase (SOD1) and p75 neurotrophil receptor (p75NTR) expression (Bai 2002). Moreover, in a more recent study it was demonstrated that either 25 or 50 μM CLZ, but not 100 μM CLZ, can protect PC12 cells against MPP^+-induced apoptosis and DNA damage (Qing 2003). However, preincubation with CLZ (0.1–3 μM) during 2 h in the presence of an activating system (horseradish peroxidase + H_2O_2) induced significant PMN apoptosis when these cells were incubated in drug-free medium for another 6 h as assessed by flow cytometry. On the contrary, CLZ (30–300 μM) in the absence of the activating system did not induce PMN apoptosis (Williams 2000). These seemingly contradictory findings prompted us to study the effects of prolonged incubation times of CLZ on PMN apoptosis.

2. Investigations and results

2.1. CLZ delays spontaneous or UVB-induced apoptosis in PMNs

To examine the effects of CLZ on PMNs apoptosis, two models were employed in this work: 1) spontaneous apop-

tosis at 4 h and at 22 h after the preparation of the *in vitro* culture and 2) UVB-accelerated apoptosis observed 4 h after irradiation. The latter model was employed to produce a rapid and synchronous progression of PMNs into the apoptotic pathway without inducing a significant amount of necrosis (Sweeney 1997). In the data presented in Fig. 1 A, apoptosis was quantified by the well established criterion of typical morphological changes apparent on examination of Wright-Giemsa cytospin preparations. Because it has been demonstrated that the increased percentage of necrotic PMNs with time represents secondary necrosis arising from apoptotic cells (Sweeney 1997), parallel assays of trypan blue exclusion were carried out as well. In our hands, the percentage of cells demonstrating necrotic features was between 4 and 17% and these values were not significantly different from controls (Fig. 1 B). Fig. 1 A reveals that preincubation with 20 μM CLZ generates a significant protective effect on both apoptosis evaluated after 22 h in culture and UVB-accelerated apoptosis evaluated 4 h after irradiation. However, significant protective effects with CLZ 50 μM were only observed in PMNs aged for 22 h.

2.2. CLZ inhibits DNA fragmentation

The protection of CLZ against apoptosis in PMNs, assessed morphologically, was confirmed by agarose gel electrophoresis (Fig. 2). DNA was extracted from PMNs 4 h after UVB irradiation and after 4 or 22 h of *in vitro* culture. At 4 h of *in vitro* culture, very little evidence of DNA fragmentation was observed (lanes 1 and 4). At 4 h after UVB irradiation, DNA extracted from control PMNs showed a pattern of DNA laddering very similar to that arising from DNA isolated after 22 h of *in vitro* culture (lanes 2 and 3). DNA extracted from CLZ-treated PMNs either 4 h after UVB irradiation or 22 h of *in vitro* culture showed inhibition of chromatin fragmentation in both models of apoptosis (lane 2 vs. lane 5; lane 3 vs. lane 6).

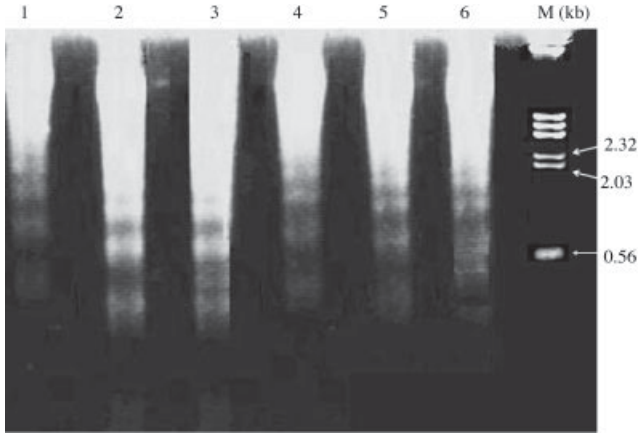

Fig. 2: Agarose gel electrophoresis of DNA extracted from PMNs 4 h after UVB irradiation and after 4 h or 22 h of *in vitro* culture. UVB-irradiated PMNs were preincubated with CLZ (20 μM) for 10 min before being UVB irradiated for 30 min at a dose of 3.0 mW/cm^2 as described in Material and Methods. Lane 1, control PMNs after 4 h *in vitro* culture; lane 2, control PMNs 4 h after UVB irradiation; lane 3, control PMNs 22 h after UVB irradiation; lane 4, CLZ-treated PMNs after 4 h *in vitro* culture; lane 5, PMNs pretreated with CLZ for 10 min before UVB irradiation; lane 6, CLZ-treated PMNs after 22 h *in vitro* culture; lane 7, DNA marker fragments are indicated on the right. This figure is representative of two other experiments that yielded the same results

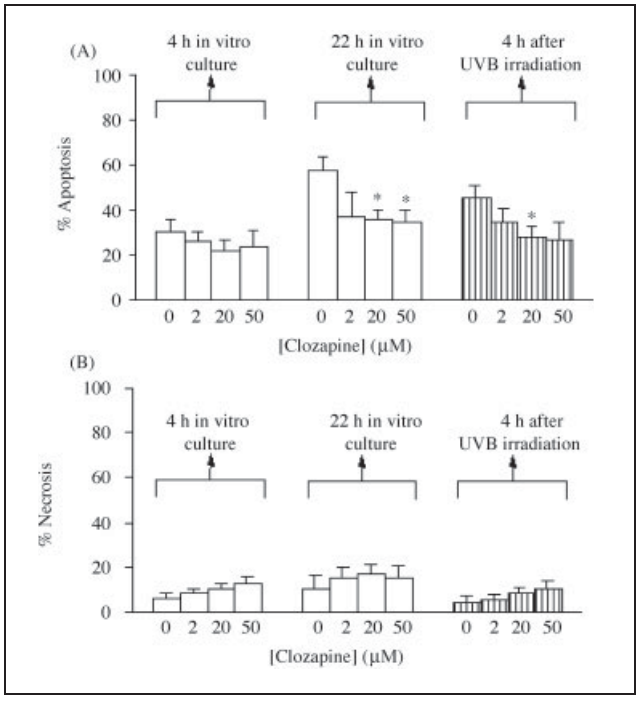

Fig. 1: A, dose-response of CLZ-induced apoptosis. PMNs (1×10^6/mL) were incubated with medium or with CLZ under indicated conditions. Apoptosis was determined by morphological observation. B, dose-response of CLZ-induced necrosis as assessed by trypan blue exclusion. The data are presented as the mean ± S.E.M. from four different experiments (carried out in duplicate). Statistical analysis was done by comparing incubations containing different concentrations of the drug with the diluent control: * $P < 0.05$, paired Student's test.

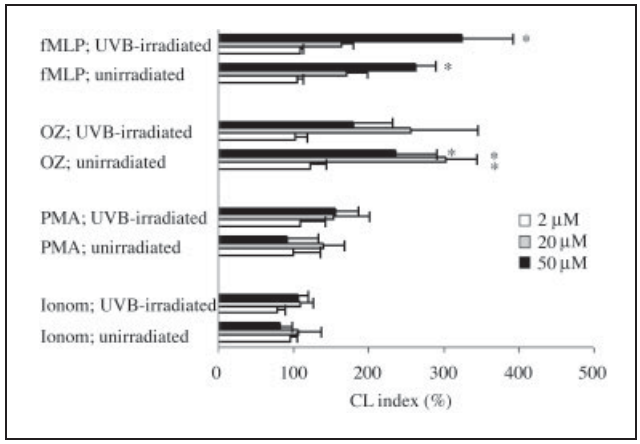

Fig. 3: Effect of different concentrations of CLZ on CL response of human PMNs (final concentration: 3.6×10^5/mL) irradiated for 30 min (or not irradiated) and then incubated for 4 h before activate with ionomycin (1 μM), PMA (0.22 μM), OZ (0.21 mg/mL) or fMLP (0.57 μM). CL Index = [(area under the curve of the CLZ-incubated cells)/area under the curve of the vehicle-treated cells)] × 100. Data are means from seven healthy donors ± S.E.M. For the control samples (vehicle-treated cells) from these donors, the means of the absolute values (mVsec) were: For ionomycin, 16405 ± 4237 and 6119 ± 455 for unirradiated or UVB-irradiated PMNs, respectively; for PMA, 138600 ± 10108 and 102009 ± 23980 for unirradiated or UVB-irradiated PMNs, respectively; for OZ, 329285 ± 24491 and 288875 ± 36093 for unirradiated or UVB-irradiated PMNs, respectively; for fMLP, 2875 ± 382 and 2691 ± 385 for unirradiated or UVB-irradiated PMNs, respectively. The areas under the curves were calculated over a time period of 30 min (PMA, OZ), 25 min (ionomycin) or 6 min (fMLP). * $P < 0.05$; ** $P < 0.01$

2.3. Effects of CLZ on respiratory burst in UVB-irradiated PMNs

Because neutrophil apoptosis has been reported to involve the impairment of neutrophil function (Whyte 1993) we tested whether the inhibition of apoptosis induced by CLZ contributes to preservation of CL response in UVB-irradiated and unirradiated PMNs. No reactive oxygen species release from resting PMNs could be detected under basal conditions during 40 min (not shown). On the other hand, when PMNs were activated by ionomycin or PMA, the CL response was not significantly affected in the presence of CLZ (Fig. 3). In contrast, when unirradiated PMNs were activated with OZ, the CL response was significantly greater in a medium containing 20 μM CLZ than in the control medium. In addition, Fig. 3 shows that when unirradiated or UVB-irradiated PMNs were activated with fMLP, the CL response was significantly potentiated only when cells were pretreated with 50 μM CLZ.

2.4. Time dependence on enhanced CL response in PMNs activated by OZ

Based on the most effective concentration of CLZ (20 μM, see Fig. 3) a time course study was done, and results are shown in Fig. 4. The extent of enhancement of CL response by OZ-activated PMNs incubated for 4–22 h with CLZ was significantly increased, with respect to the control without drug, according to the duration of incubation at 37 °C. In contrast, during the first 60 min there were no significant differences with respect to the control under the same conditions. However, CLZ-stimulated CL response at 4 h or at 22 h was more than that found with freshly isolated cells.

2.5. Effects of genistein and wortmannin on enhanced CL response by CLZ

PI3-K has been implicated in OZ-induced activation of neutrophil NADPH oxidase by virtue of its association with proteins containing intrinsic or associated tyrosine kinase activities (Chuang 2000). For this reason, we employed the tyrosine kinase inhibitor genistein as well as the PI3-K inhibitor wortmannin, to determine whether CLZ enhancement of CL response was dependent on this signaling pathway. Previously, we found that 5 min of pre-incubation with 10 μM genistein was sufficient to inhibit the CL response in PMNs activated with OZ by 71.0% ($P < 0.01$) whereas pre-incubation with 0.1 μM wortmannin under the same conditions inhibited the CL response by 78.9% ($P < 0.001$). On the other hand, because an approximately three-fold increases in CL response was consistently observed when 20 μM CLZ-treated PMNS (in absence of UVB irradiation) were activated with OZ after 4 h of in vitro culture (Fig. 3), the effects of selectivity of the CLZ-induced stimulation of CL were examined under these conditions. Fig. 5 shows that when CLZ-treated PMNs were incubated for 5 min with 10 μM genistein or 0.1 μM wortmannin before activating with OZ, the enhancements in CL responses were almost completely abolished.

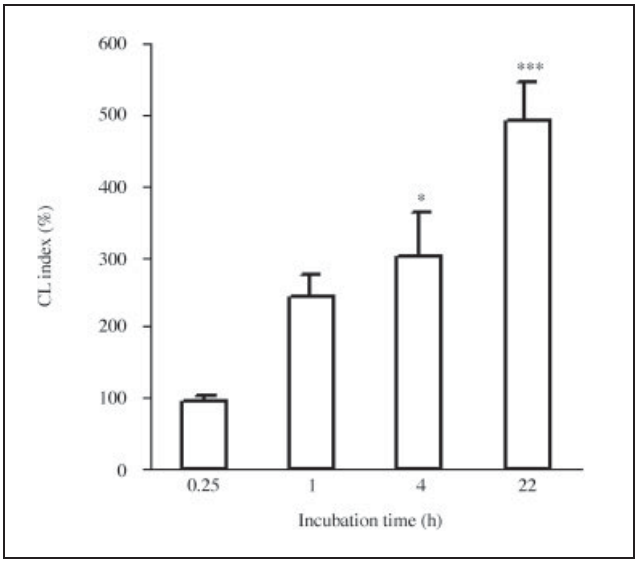

Fig. 4: Effects of incubation times on CL response in PMNs (3.6×10^5/mL) incubated with 20 μM CLZ at indicated times before activation with OZ (0.21 mg/mL). Values were estimated with respect to diluent control. For the control samples (without CLZ), the means of the absolute values (mVsec) were: 330150 ± 34990 at 15 min; 345500 ± 43125 at 1 h; 329285 ± 24491 at 4 h and 154412 ± 19330 at 22 h. * $P < 0.05$; *** $P < 0.001$

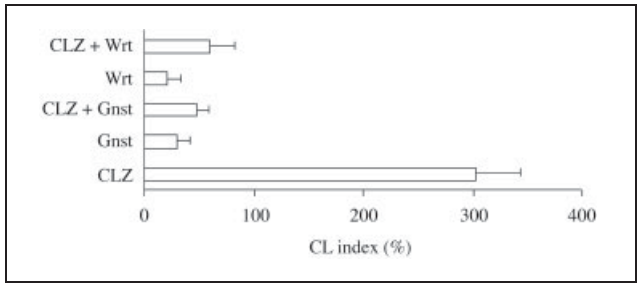

Fig. 5: Effects of genistein (Gnst) and wortmannin (Wrt) on enhanced CL response by CLZ. Human PMNs (3.6×10^5/mL) were exposed to 20 μM CLZ for 4 h before incubation with 10 μM genistein or 0.1 μM wortmannin. Then, CL response was evaluated after activation with OZ (0.21 mg/mL). Values were estimated with respect to diluent control. For the control, the mean of the absolute value (mVsec) was 329285 ± 24491

3. Discussion

In this report, we showed for the first time that supra-therapeutic concentrations of CLZ (20–50 μM) inhibit both spontaneous and UVB-accelerated apoptosis in PMNs. This effect of CLZ is in close agreement with recently published results using PC12 cells as substrates (Qing 2003) suggesting that this protective effect could be extended to other cell types. Indeed, because the morphological study did not reveal significant changes in necrosis (Fig. 1), it appears that CLZ can protect cells from apoptosis, but not from necrosis.

Furthermore, we also showed for the first time that the NADPH oxidase activity in human PMNs is significantly augmented by the addition of CLZ (20 or 50 μM), as measured by luminol-enhanced chemiluminescence following stimulation with OZ or fMLP, but not with PMA or ionomycin. This finding indicates that the effect of CLZ on the PMN-augmented response appears to be specific, since the effects were observed for those ligands which are bound to surface receptors.

Serum-opsonized zymosan (OZ) is a particle liable to undergo phagocytosis that binds to receptors existing on the neutrophil cell surface (Fcγ receptors and receptors for complement protein C3b). Although the signal transduction pathways by OZ leading to activation of NADPH oxidase remain obscure, there are evidences that cross-linking of the transmembrane FcγRIIA initiates a tyrosine kinase cascade-dependent upon the cytoplasmic tail of this receptor (Hazan-Halevy 2000). Furthermore, others have shown that OZ

is able to activate superoxide production in human PMNs by the PI-3-kinase dependent pathway (Baggiolini 1987). On the other hand, fMLP and PMA are two other potent activators of NADPH oxidase, and signaling by both stimuli involves tyrosine phosphorylation (Welch 1997). However, it has been shown that stimulation of human PMNs with fMLP activates the respiratory burst by activation of phosphatidylinositol 3-kinase (PI3-K), but when these cells are activated with PMA, superoxide production occurs without the corresponding activation of PI3-K (Vlahos 1995).

The above mentioned findings indicate that both fMLP and OZ induce reactive oxygen species (ROS) generation in PMNs by signaling pathways that involve increase in tyrosine phosphorylation and PI3-K activation. Interestingly, neutrophil priming agents such as a granulocytes-macrophage colony-stimulating factor (GM-CSF) delay neutrophil apoptosis both through PI3-K pathways and by an increase of protein tyrosine phosphorylation (Yousefi 1994). Therefore, because the enhancement of CL response by CLZ could be totally reverted by geninstein or wortmannin (Fig. 5), it is tempting to speculate that CLZ is a priming agent of human PMNs. Indeed, although inhibition of the CL response by CLZ required long incubation times (suggesting that this enhancement may be a consequence of inhibition of apoptosis only), CLZ-stimulated CL response at 4 h or at 22 h was more than that found with freshly isolated cells (Fig. 4).

On the other hand, in a detailed study about the consequences of apoptosis for PMN function, it was demonstrated that apoptotic PMNs (separated by elutriation of 24 h-aged cells) have a reduced ability to elicit a respiratory burst in response to activation with receptor-dependent stimuli such as fMLP and OZ (Whyte 1993). On the contrary, superoxide anion production in apoptotic PMNs activated with the receptor-independent stimulus, PMA, was preserved. These findings suggest that apoptotic PMNs exist in a state of functional isolation from the external milieu although preserving a degree of integrity of intracellular signal transduction pathways. Indeed, in our hands CLZ did not enhance the CL response in aged or UVB-irradiated PMNs activated with PMA (Fig. 3).

From our results and other published observations (Kitchen 1996) it would appear that CLZ is an agent that, (like GM-CSF or LPS) could be included into the category of largely irreversible primers. Strikingly, these types of primers are the only ones capable to modulate the rate of PMN apoptosis, which attests to the long duration of action in this type of agents (Lee 1993). Although our major findings were obtained at supratherapeutical CLZ concentrations, it is clear that the neutrophil response to CLZ is complex and further investigation is necessary to determine if the priming effect by CLZ can be extended to other PMN functions such as shape change, chemotaxis, degranulation and phagocytosis.

4. Experimental

4.1. Chemicals

Clozapine (CLZ), wortmannin, genistein, phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), ionomycin and luminol, were purchased from Sigma (St Louis, USA). All commercial reagents were used without further purification. Hank's balanced salt solution (HBSS) was prepared by dissolving the commercially available powdered medium (Sigma). Before use, 0.1% (w/v) gelatin was added (HBSS-gel).

4.2. Cells

After informed consent, venous blood was obtained from healthy volunteer donors. Neutrophils were isolated from EDTA-anticoagulated blood following a differential centrifugation method as previously described (Eggleton 1989). The cells were separated and suspended to 1×10^7/mL of HBSS.

4.3. Incubation conditions

PMNs (final concentration: 5×10^6/mL) were incubated with either CLZ (final concentrations: 2–50 μM) or an equivalent volume of DMSO (final concentration: 1% v/v) for the controls, in RPMI 1640 (pH 7.4) during different times at 37 °C and in the presence of 5% CO_2.

4.4. Neutrophil chemiluminescence

Samples for neutrophil chemiluminescence determinations were prepared by adding 50 μL of the clozapine-treated neutrophil suspensions in RPMI to HBSS supplemented with luminol (total volume: 700 μL. Final concentrations: 3.6×10^5 neutrophils/mL, 52 μM luminol). Chemiluminescence was measured in a Bio-Orbit 1251 luminometer using 4-mL polypropylene tubes. The tubes were equilibrated for 5 min at 37 °C after which 50 μL of opsonized zymosan (OZ; final concentration: 0.21 mg/mL), or 2.4 μL of PMA (final concentration: 0.22 μM), or 40 μL of fMLP (final concentration: 0.57 μM) were added. The light emission was continuously recorded and its intensity was determined by integrating the area under the chemiluminescence curve during 30 min (OZ, PMA) or at 6 min (fMLP). Percentage of chemiluminescence response at each CLZ concentration was expressed as the percent of the control value.

4.5. Opsonization of zymosan

Zymosan was boiled in isotonic NaCl solution for 1 h, washed three times by centrifugation, and resuspended (3 mg/ml) in HBSS. Opsonization was carried out by incubating 1 vol of the above suspension mixed with 1 vol of fresh human serum for 30 min at 37 °C and then washing the combined volumes three times with HBSS before dividing it in aliquots and keeping it at −80 °C until use (Kroes 1992).

4.6. UVB-accelerated PMN apoptosis

PMNs (5×10^6/mL), incubated in the presence and in the absence of CLZ, were illuminated from above using a 312-nm keeping a distance of 15 cm between the lamp surface and the solution, varying the time periods of exposure at 37 °C under continuous shaking. The UV a total irradiance at this distance was 3 mW/cm^2 as measured with a model of UVX Digital Radiometer after 1 h continued illumination. After the irradiation the cells were incubated at different times at 37 °C and in the presence of 5% CO_2. Features of the apoptosis were determined by using the methods described below.

4.7. DNA fragmentation

DNA fragmentation of PMNs was analyzed by using agarose gel electrophoresis. Neutrophils (2×10^6) were harvested and incubated in 100 μL of 10 mM Tris-HCl, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100 for 10 min at 4 °C, and then centrifuged at 20,000 × g for 20 min. The supernatant was treated with 2 μL of 20 mg/mL ribonuclease-A at 37 °C for 1 h. Then, 2 μL of 20 mg/mL proteinase-K were added and the incubation was continued for an additional 1 h. The mixture was kept at −20 °C overnight after adding 120 μL of isopropanol and 20 μL of 5 M NaCl. Then the mixture was centrifuged at 20,000 × g for 15 min, the supernatant was discarded and the remaining pellet was dissolved in 15 μL of 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25% bromophenol blue and 20% glycerol. Samples were loaded into each well of the 2% agarose gels, and electrophoresis was carried out at 100 V for 1 h. The DNA in gels was visualized under UV light after staining it with ethidium bromide (Hotta 2001).

4.8. Assessment of neutrophil apoptosis by morphological examination

For morphological assessment, 150 μL of pretreated neutrophils (5×10^6 cells/mL) were spun down (120 g, 6 min) onto glass slides using a cyto-centrifuge (Hermle, Z 323 K, Germany). The slides were air-dried and stained with Wright-Giemsa solution for light microscopic evaluation. The percentage of cells with characteristic apoptotic changes (nuclear condensation, vacuolation and blebbing) was assessed by counting at least 300 cells/slide with the use of a 40 × objective (Axiolab, Zeiss, Germany).

4.9. Statistical treatment of results

At least three independent experiments were performed except where indicated. The figures show either single representative results or means (± SD where appropriate).

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