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Cytokine production of neutrophils is limited to interleukin-8

J. ALTSTAEDT, H. KIRCHNER & L. RINK *Institute of Immunology and Transfusion Medicine, University of Lübeck School of Medicine, Lübeck, Germany*

SUMMARY

Since granulocytes are one of the first cell types at sites of inflammation, investigation of their capacity to produce cytokines has concentrated on interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α (TNF-α). However, the results are subject to controversy. Incapability to produce cytokines as well as a broad panel of cytokines induced by isolation procedures are reported. The purpose of this study was to investigate the capacity of non-prestimulated neutrophils to produce the above-mentioned cytokines in response to stimulation with lipopolysaccharide or zymosan. In reverse transcription–polymerase chain reactions, we found IL-8 mRNA directly after isolation in unstimulated cells, whereas mRNA for IL-1β, IL-6 and TNF-α only appeared after stimulation. By means of flow cytometry we ruled out the possibility of prestimulation of the neutrophils during isolation, proving that IL-8 mRNA is produced constitutively by neutrophils. In enzyme-linked immunosorbent assays we found that, compared with controls, only IL-8 was released at significantly higher levels after 24 hr of stimulation, giving a further indication that neutrophils have an immunoregulatory influence driven by IL-8. We can confirm neither a constitutive nor a post-stimulatory release of IL-1β, IL-6, or TNF-α by neutrophils, as had been reported by others. These observations may be due to prestimulation, handling and culturing of the granulocytes, or to monocyte contamination. Collectively, our results show that granulocytes have a preformed capacity to produce the cytokine IL-8 and that the production of proinflammatory cytokines by neutrophils is limited to IL-8.

INTRODUCTION

Granulocytes (polymorphonuclear cells; PMN) are the major population of leucocytes (40–75%). Together with macrophages they represent the primary effector cells in acute infection.[1] Their known responses to microbial invasion are migration from the blood stream to the site of infection and phagocytosis of the micro-organisms. Granulocytes leave the bone marrow as terminally differentiated cells and persist in peripheral blood for only 48–72 hr. Furthermore, they possess rather few ribosomes and endoplasmic reticulum compared to mononuclear cells, and for these reasons they have long been regarded as incapable of producing proteins. However, during the last 20 years some research on this subject has been done, and it seems that this view should be reassessed.[1]

As granulocytes play an important role in inflammation, most studies so far have concentrated on the inflammatory cytokines interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α (TNF-α),[1,2] but nevertheless the results are still quite controversial. For IL-1β, mRNA has been found in stimulated PMN[3,4] as well as cytokine production in their supernatants.[4,5] Messenger RNA for IL-6 has been obtained after stimulation of PMN[6] and even constitutively,[7] but there are also reports that it could not be detected at all.[8] Secretion of IL-6 has been measured after some of the used stimuli.[6,9] Furthermore, TNF-α mRNA has been demonstrated in stimulated PMN,[10] but the measured cytokine secretion[10,11] could not be confirmed by other authors.[9] Most interesting are the results concerning IL-8, a potent stimulator of PMN. The mRNA of IL-8 has been detected under control conditions[9,13,14] and even constitutively directly after isolation,[9,14] and secretion of IL-8 has also been reported to occur after stimulation[8,12–14] as well as constitutively.[9] However, some authors presume that the phenomenon of constitutive IL-8-mRNA and cytokine release is probably due to prestimulation of the PMN during isolation,[9,12] but the state of activation of PMN in correlation to their cytokine production has not yet been investigated.

One problem in working with granulocytes is their isolation even though several comparable isolation procedures exist. Furthermore, some authors refer the measured cytokine production only to neutrophils, others to all granulocytes as the examined cell group, i.e. neutrophils, eosinophils and basophils.

The focus of our study was to investigate the capacity of non-prestimulated neutrophils to produce the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in response to bacterial and fungal stimuli.

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Abbreviations: HES, hydroxy ethyl starch; IL, interleukin; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leucocyte; RT-PCR, reverse transcription polymerase chain reaction; TNF, tumour necrosis factor; ZY, zymosan.

Correspondence: Dr L. Rink, Institute of Immunology and Transfusion Medicine, Ratzeburger Allee 160, D-23538 Lübeck, Germany.

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MATERIALS AND METHODS

Cell isolation

Blood from buffy coats from healthy donors was mixed with hydroxy ethyl starch (HES, Plasmasteril, Fresenius AG, Bad Homburg, Germany) and allowed to sediment for 30 min at room temperature until a clear separation between the leucocyte-rich supernatant and the red-cell fraction was visible. The supernatant was collected, washed twice with phosphate-buffered saline (PBS; Gibco, Berlin, Germany), and exposed to hypotonic lysis of the remaining erythrocytes. The cell pellets were suspended in 10 ml sterile aqua bidest and mixed once, and immediately afterwards isotonic conditions were restored by addition of the same volume of 2× concentrated PBS prepared from 10× PBS (Gibco). A three-layer gradient was prepared from Percoll (Pharmacia, Freiburg, Germany) adjusted to the densities 1·082 g/ml (66%), 1·092 g/ml (75%) and 1·106 g/ml (85%). Gradient densities were determined by density beads (Pharmacia). The cell suspension was carefully layered on top of the gradient and centrifuged for 20 min at 600 *g* and 20°. The interface A above the 75%-layer was collected, washed once and layered again on 66% Percoll to extract the remaining PBMC, and the interface B above the 85%-layer was collected, washed once and layered again on 75% Percoll to separate the remaining eosinophils. After centrifugation for 20 min at 600 *g* and 20° the pellet from A and the interface from B were collected, washed twice and adjusted to the appropriate cell concentrations in RPMI-1640 (Biochrom KG, Berlin, Germany) with 10% low-endotoxin fetal calf serum (Gibco, myoclone quality), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all Biochrom KG).

Purity

The mean purity achieved by this isolation technique was 98% ± 0·9 neutrophils, determined by May–Gruenwald–Giemsa-staining (Merck, Darmstadt, Germany) of cytospins and 99·8% by flow cytometry analysis (Coulter XL, Coulter Electronics, Krefeld, Germany). The contaminating cells were found to be mainly eosinophils (approximately 1%).

Flow cytometry analysis

Preparations were made directly after isolation and after each stimulation interval to determine the purity and degree of activation of the neutrophils. The cells were pelleted, resuspended in PBS with 1% bovine serum albumin (BSA, Fluka Chemie AG, Buchs, Switzerland) and incubated with CD66b–FITC (fluorescein isothiocyanate) (formerly CD67, clone 80H.3, Immunotech, Hamburg, Germany) and CD62L–PE (phycoerythrin; alternative names: LECAM-1, L-selectin; Pharmingen, Hamburg, Germany) for 15 min. As negative control we used IgG1-FITC/IgG1-PE Mouse (Immunotech). The cells were washed again with 1% PBS/BSA, and fixation was done automatically in a Multi-Q-Prep (Coulter electronics).

Stimulation of the cells

Stimulation of the neutrophils was performed in 10 ml Petri dishes with two different stimuli: lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (1 μg/ml; Sigma, Deisenhofen, Germany), and zymosan (ZY; 0·16 g/ml; Serva, Heidelberg, Germany), a mixture of magnesium-containing glycoproteins from cell walls of *Saccharomyces cereviseae* yeast, as representative for fungal infection. Time intervals were 0, 1, 3 and 24 hr.

Reverse transcription–polymerase chain reaction (RT–PCR)

After each time-interval the RNA was prepared as follows: total cellular RNA was isolated by a procedure modified from that described by Chomczynski & Sacchi.[15] Briefly, 3×10^7 cells were lysed in 5 ml of a 4 M guanidium thiocyanate solution, containing 25 mM sodium citrate, 0·5% sarcosyl and 0·1 M 2-mercaptoethanol (GTC-solution) and squeezed through the 25G needle of a syringe three times. Lysed cells were frozen and stored at −20°. Samples were thawed simultaneously and mixed with 0·5 ml 2 M sodium acetate (pH 4), 5 ml water-saturated phenol and 1 ml chloroform/isoamylalcohol (49 : 1), mixed thoroughly and incubated on ice for 15 min. Phenol extraction was performed by 20 min of centrifugation at 10 000 *g*, after which the water phase was mixed with 5 ml 2-propanol and stored at −20° for at least 1 hr. RNA was then pelleted and resuspended in 300 μl GTC-solution, mixed with the same volume of 2-propanol and stored again at −20° for a minimum of 1 hr. RNA was pelleted again, washed twice with 80% RNAase-free ethanol, dried in a vacuum and dissolved in RNAase-free water. One microgramme of isolated total RNA was used for reverse transcription by using a reverse transcription kit from Promega (Serva, Heidelberg, Germany). PCR was performed in a Perkin-Elmer thermal cycler Cetus 480 (Vaterstetten, Germany). We used a cycle program of 40 seconds denaturation (95°), 60 seconds annealing (60°), and 3 min amplification (72°) for 35 cycles with a final 5 min amplification. As a control, all cDNA samples exept those for IL-6 were amplified by using β-actin primer. IL-6 was done without β-actin because the two primers inhibit each other (Fig. 2a). The primers used were the same as those previously described.[16]

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were harvested after appropriate times, stored at −80° until measuring and only thawed once for cytokine detection. To measure IL-1β, IL-6, IL-8 and TNF-α in the supernatants, we used ELISA kits obtained from Biermann (Bad Nauheim, Germany). All ELISA were quantified using an ELISA-reader (Anthos, Labotec, Salzburg, Austria).

Statistical analysis

Only preparations with a CD62L expression of ≥ 95% to exclude prestimulation of the neutrophils and a CD66b expression of ≥ 99·5% to exclude monocyte contamination were included into the study.

Kolmogorow–Smirnov Goodness of Fit Test was used to evaluate normal distribution of the measured cytokine amounts. In the case of normal distribution of spot checks, significances of differences were analysed by Student's *t*-test of the program Sigma Plot from JandelScientific. Otherwise, the two-tailed Wilcoxon Matched-Pairs Signed-Ranks Test was carried out using the program SPSS for Windows.

RESULTS

Purity and state of activation of neutrophils

To define exactly our isolated cell population we checked the neutrophils for purity and, in contrast to others, also for preactivation by using flow cytometry. CD62L is a surface molecule that disappears during activation of the cell and therefore may be used as an indicator for cell activation.[17] To determine the purity of our preparation we chose the pan-granulocyte-marker CD66b.[18] As

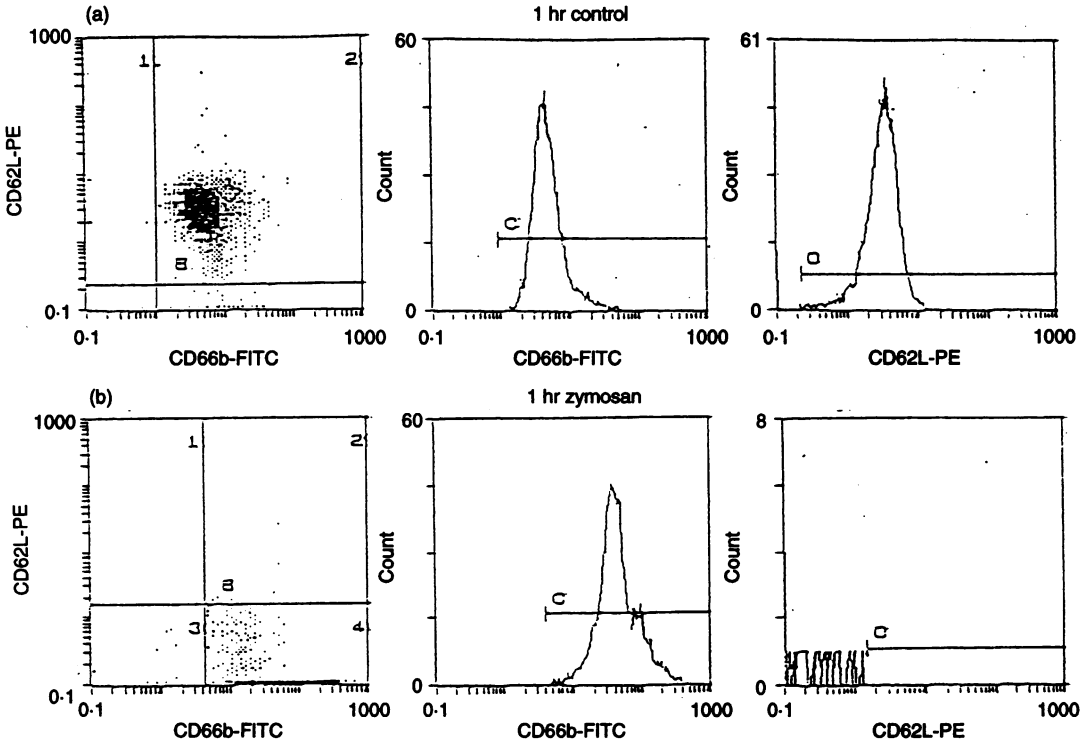

Figure 1. Purity and state of activation of neutrophils: 1×10^6 neutrophils were incubated with monoclonal antibodies CD66b and CD62L for 15 min and flow cytometry analysis was performed. Representative experiments ($n = 6$) are shown. (a) Flow cytometry analysis after 1 hr of incubation under control conditions. (b) Flow cytometry analysis after 1 hr of stimulation with zymosan (0·16 g/ml). Mean values of flow cytometry analysis are given in Table 1.

shown in Table 1, directly after isolation the cells were highly positive for both CD66b and CD62L: the mean level of CD66b was 99·8%, the one of CD62L 97·3%. Under control conditions, CD62L remained high (98·6% after 1 hr; Fig. 1a) whereas as early as 1 hr after stimulation with LPS only 5·8% of the PMN were still CD62L-positive (Table 1), and after 1 hr of incubation with ZY, CD62L had nearly disappeared (0·2%; Fig. 1b, Table 1). Therefore, we can exclude the possibility of prestimulation of the neutrophils during our isolation procedure.

Cytokine production by neutrophils

To determine the production of IL-1β, IL-6, IL-8 and TNF-α by neutrophils after stimulation with LPS or zymosan, we isolated the mRNA and collected the supernatants of the neutrophils after each time-interval and stimulant to perform RT-PCR and ELISA.

The mRNA for IL-1β appeared after 1 hr or 3 hr of incubation in stimulated cells (Fig. 2a) as well as in unstimulated controls (Table 2), which had not been described yet. Directly after isolation we could not detect any IL-1β-signal in RT-PCR (Fig. 2b). Messenger RNA for IL-6 and TNF-α was detectable after stimulation of the cells for 1 or 3 hr (Fig. 2a), but we did not find a signal for IL-6 in unstimulated controls (Table 2) as had been described elsewhere.[7]

For IL-8, we detected mRNA in cells stimulated with LPS or ZY for 1 or 3 hr (Fig. 2a) as well as in unstimulated controls (Table 2) and, most interestingly, in our experiments IL-8 was the only cytokine-mRNA detectable directly after isolation of the neutrophils without any incubation or stimulation (Fig. 2b).

Although the mRNA for all four investigated cytokines was measurable in our experiments, the only protein secreted at a significantly elevated level after 24 hr of stimulation compared to controls was IL-8 (Fig. 3). In contrast to the results of others who also described the production of IL-1β,[4,5] IL-6[6] and TNF-α[10,11] by

Table 1. Purity and state of activation of neutrophils

	CD66b (%)	CD62L (%)
Mean values of flow cytometry ($n = 6$)		
0 hr	99·8 ± 0·07	97·3 ± 1·69
24 hr control	84·4 ± 27·9	36·13 ± 25·2
24 hr LPS	99·0 ± 0·68	0·78 ± 0·15
24 hr ZY	97·0 ± 1·4	0·47 ± 0·4
CD62L down-regulation: one representative kinetic		
0 hr	99·7	99·0
Control		
1 hr	99·9	98·6
3 hr	99·8	97·8
24 hr	99·4	51·4
LPS		
1 hr	99·7	5·8
3 hr	99·5	2·7
24 hr	99·2	0·7
ZY		
1 hr	99·6	0·2
3 hr	96·1	0·7
24 hr	95·8	0·5

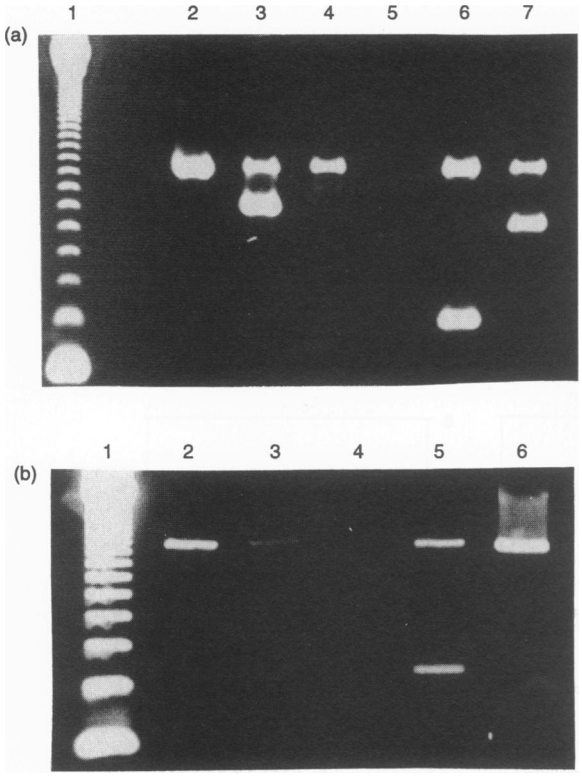

Figure 2. Messenger RNA production of neutrophils: RT-PCR of neutrophils (3×10^6/ml) was performed with β-actin as control. One representative experiment (out of three) is shown. (a) After stimulation with LPS (1 μg/ml) for 3 hr: lane 1, 123 base pair (bp) ladder; lane 2, β-actin; lane 3, IL-1β + β-actin; lane 4, IL-6 + β-actin; lane 5, IL-6; lane 6, IL-8 + β-actin; lane 7, TNF-α + β-actin. (b) Directly after isolation without incubation: lane 1, 123 bp ladder; lane 2, β-actin; lane 3, IL-1β + β-actin; lane 4, IL-6; lane 5, IL-8 + β-actin; lane 6, TNF-α + β-actin. Complete data of RT-PCR are given in Table 2.

PMN, we could not detect any significant levels of either of these cytokines in the supernatants of stimulated neutrophils. In our experiments, average levels of IL-1β, IL-6 and TNF-α were 4·8 pg/ml, 4·7 pg/ml and 5·4 pg/ml, respectively (Fig. 3). Under control conditions we did not measure a significant secretion of IL-8 as had

Table 2. Production of cytokine-mRNA by neutrophils

	IL-1β			IL-6			IL-8			TNF-α		
Experiment	1	2	3	1	2	3	1	2	3	1	2	3
0 hr	−	−	−	−	−	−	+	+	+	−	−	−
Control												
1 hr	+	+	+	−	−	−	+	+	+	−	−	−
3 hr	+	+	+	−	−	−	+	+	+	−	−	−
LPS												
1 hr	+	+	+	+	+	+	+	+	+	+	+	+
3 hr	+	+	+	+	+	+	+	+	+	+	+	+
ZY												
1 hr	+	+	+	+	+	+	+	+	+	+	+	+
3 hr	+	+	+	+	+	+	+	+	+	+	+	+

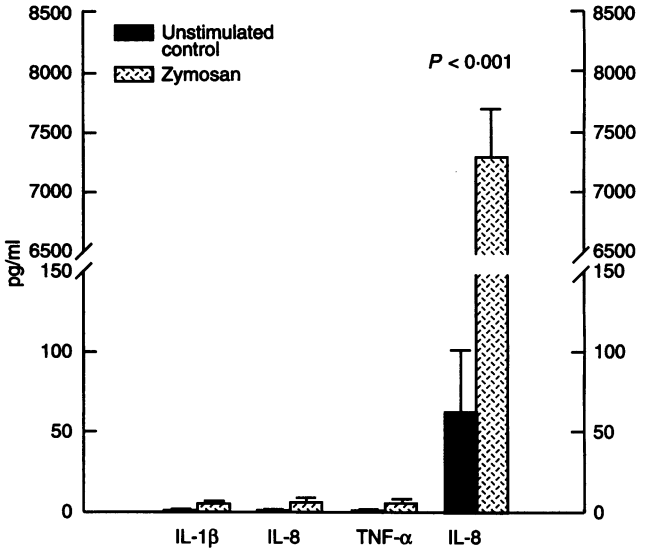

Figure 3. Production of cytokines: neutrophils (3×10^6/ml) were stimulated with zymosan (0·16 g/ml) for 24 hr (shaded bars), controls remained unstimulated (solid bars). Cytokines in the supernatants were measured by ELISA. Mean values ± SEM of $n = 6$ experiments are shown.

been described elsewhere.[9,14] Average control levels in our experiments were 52 pg/ml (Fig. 3). In contrast to the findings with PBMC, the IL-8 levels secreted by neutrophils were higher after stimulation with ZY (7279 pg/ml) than with LPS (5040 pg/ml; Fig. 4), implying that the IL-8 was definitely produced by neutrophils.

DISCUSSION

PMN are the most prominent cells in early inflammation. Together with macrophages they are the first to appear at sites of infection.

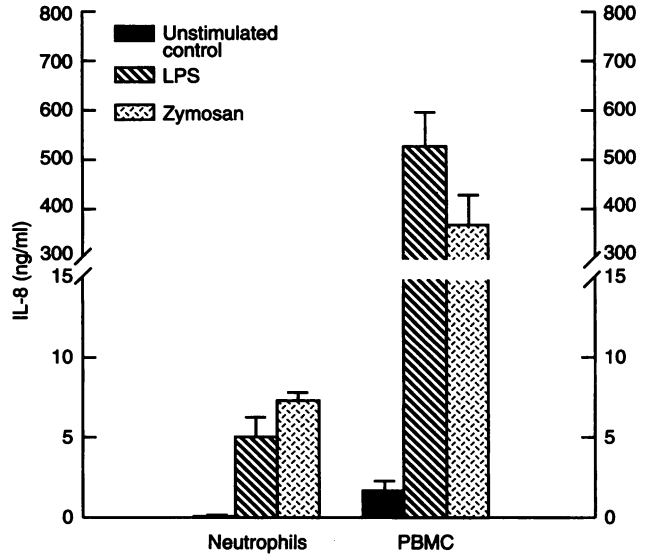

Figure 4. Production of IL-8: neutrophils (3×10^6/ml) and PBMC (3×10^6/ml) were stimulated with LPS (1 μg/ml; hatched bars) or ZY (0·16 g/ml; shaded bars) for 24 hr, control cultures remained unstimulated (solid bars). IL-8 in the supernatants was measured by ELISA. Mean values ± SEM of $n = 6$ experiments are shown.

Presuming that PMN could be able to partly regulate the further recruitment of other cells of the immune system by releasing cytokines, some research has been done on this subject during the last years. As PMN are the primary effector cells in inflammation, most studies have investigated the production of the proinflammatory cytokines IL-1β, IL-6 and TNF-α and the chemokine IL-8,[1,2] which is strongly chemotactic for PMN.[21] Despite the similarity of the described experiments, the results reported by the different authors are varying and even contradictory.[6,8–11]

The present study was performed to define the capability of PMN to produce and release IL-1β, IL-6, IL-8 and TNF-α in response to LPS and zymosan. In RT-PCR we obtained signals for IL-8 directly after isolation, in unstimulated controls and after stimulation, for IL-1β after incubation under control conditions and after stimulation, and for IL-6 and TNF-α only after stimulation of the cells with LPS or ZY. By flow cytometry we demonstrated that the existence of mRNA for IL-8 directly after isolation was not due to prestimulation of the neutrophils by the isolation procedure as had been presumed by others.[9,12] Prestimulation of PMN during isolation may be driven by the use of ammonium chloride for erythrocyte lysis[19] or dextran sulphate for sedimentation.[20] In our experiments, 97·3% of the cells were positive for CD62L after isolation, indicating that our isolation procedure with HES-sedimentation, Percoll density gradients and hypotonic lysis with aqua dest. does not have any stimulatory effects on neutrophils. Therefore the production of IL-8-mRNA by neutrophils is constitutive, whereas mRNA for IL-1β, IL-6 and TNF-α only appears after stimulation.

These differences in mRNA-production correspond well to the measured cytokine secretion by neutrophils. The only cytokine measurable at significantly higher levels after stimulation than under control conditions was IL-8. For the release of IL-8, neutrophils only have to start translation of the already existing mRNA, whereas for the secretion of IL-1β, IL-6 and TNF-α they first have to transcribe the genes. The fact that even after 24 hr of stimulation there are no significant levels of IL-1β, IL-6 and TNF-α measurable in the supernatants is probably due to the limited lifetime of PMN. We presume that the release of IL-8 beginning immediately after stimulation takes all sources of the cell leaving no more capacity to produce one of the other cytokines by the time their transcription is finished. Moreover, the small amounts of IL-1β, IL-6 and TNF-α measured in the supernatants of stimulated neutrophils showed the same stimulus-dependence as was found in supernatants of equally stimulated PBMC. The levels were higher after incubation with LPS than with ZY (data not shown). Therefore it is not clear if the small amounts of these cytokines measured in the supernatants, and maybe also the signals in RT–PCR, originated from neutrophils or from the few contaminating PBMC, whose number was below the discrimination level of flow cytometry. In contrast to this, the IL-8-production in the supernatants of the neutrophils was higher after stimulation with ZY than with LPS, giving additional proof that neutrophils were definitely the source of the measured IL-8.

Our data suggest that, of those cytokines investigated in our study, IL-8 is the only cytokine neutrophils are definitely able to release at significant levels, and that PMN-directed immune regulation is driven by IL-8. On the one hand, IL-8 elicits changes in the internal calcium ion concentrations and the respiratory burst in monocytes and a rapid and long-lasting increase in inositol phosphates in lymphocytes (reviewed by Baggiolini *et al.*[21]). On the other hand, IL-8 has important autocrine effects: it is a potent chemoattractant for PMN themselves, and it is the only cytokine capable of activating all different functions of the PMN, i.e. maturation and recruitment from the bone marrow, the mechanism of rolling and diapedesis from the vessels to the sites of infection, respiratory burst, exocytosis, phagocytosis and intracellular digestion of incorporated particles (reviewed by Baggiolini *et al.*[21]). Thus, by just releasing this one cytokine, neutrophils can have important influences on the recruitment and activation of other cells of the immune system at inflammatory lesions.

In conclusion, we cannot confirm that neutrophils stimulated with ZY or LPS do secrete IL-1β, IL-6 and TNF-α, as had been reported by others.[5,6,9–11] According to our findings, they only produce IL-8. The constitutive mRNA pool for IL-8 accelerates the appearance of the mature protein after stimulation of the neutrophils, and the amount of the secreted IL-8 is high enough to enable the neutrophils to have regulatory effects on the immune response upon inflammation.

The capacity of neutrophils to produce other cytokines which has been described in single instances for IL-3, IL-12, transforming growth factor-β and for granulocyte, macrophage and granulocyte–macrophage colony-stimulating factors[1,2] as well as the cytokine production of eosinophils and basophils has to be investigated under controlled conditions for purity and prestimulation as shown in this study.

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