

Original article

The mechanisms of serum-treated zymosan (STZ)-induced oxidative metabolism by human eosinophils and the effects of IL-5 priming

Background: The aim of this work was to study the mechanisms of action of IL-5 on the subsequent stimulation of the oxidative metabolism of blood eosinophils by serum-treated zymosan (STZ), in terms of signal transduction characteristics, and by comparing the response of cells from healthy and allergic subjects during environmental exposure to birch pollen.

Methods: Eosinophils from healthy controls and allergic patients were purified to over 95% by Percoll gradients and the MACS system. Oxidative metabolism was measured by a lucigenin-enhanced chemiluminescence (CL) assay. Eosinophils were primed with IL-5 and subsequently stimulated with STZ. The signal transduction mechanisms of IL-5 priming were studied with the MEK inhibitor PD 98059, the PkC inhibitors staurosporine and Ro 318220, and the PI₃ kinase inhibitor wortmannin.

Results: IL-5 increased the maximum radical production ($P=0.0079$) and reduced the $t_{1/2}$ rise (0.000018) of the CL reactions. The $t_{1/2}$ rise was PkC dependent and MEK independent, while the maximum radical production was PkC, MEK, and PI₃ kinase dependent. During the pollen season, IL-5 reduced the total STZ-induced CL response in the patients' cells ($P=0.016$), but not in the control cells, whereas it primed the response to STZ of both cell populations in terms of the $t_{1/2}$ rise ($P=0.012$ and 0.00066, respectively).

Conclusions: STZ-induced oxidative metabolism consists of different stages. The initial stage ($t_{1/2}$ rises of the curves) is PkC dependent and MEK independent, while the end stage (maximum radical production) is PkC, MEK, and PI₃ kinase dependent. IL-5 shortened the initial stage, and increased the end stage. During allergen exposure, however, the end stage was reduced by IL-5. This could be due to increased amounts of hypodense eosinophils and/or some abnormality in cell responses.

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The eosinophil granulocyte is a cell involved in parasitic infections and inflammatory diseases, such as allergic rhinitis and allergic asthma (1–4). The eosinophil is attracted to and activated at the site of inflammation by various factors, such as the T-cell-derived cytokines IL-3, IL-5, and GM-CSF (5, 6).

The toxicity of the eosinophil can be either oxygen independent, through the secretion of different basic granule proteins, e.g., eosinophil cationic protein, eosinophil protein X, and eosinophil peroxidase, or oxygen dependent via oxidative metabolism and the thereby formed oxygen free radicals (2).

During the oxidative metabolism, the hexose monophosphate shunt activity increases and therefore also the amount of NADPH, which is used as the electron donor. Oxygen uptake is also increased, and various oxygen free radicals, such as $\cdot\text{O}_2$, H_2O_2 , $\text{OH}\cdot$, HOCL ,

and $^1\text{O}_2$, are formed (7, 8). The generation of oxygen metabolites is catalyzed by a membrane-bound enzyme complex, the NADPH oxidase (9).

The oxidative metabolism by the eosinophil can be induced by various agents, e.g., phorbol myristate acetate (PMA), platelet-activating factor (PAF), serum-treated zymosan (STZ), or immunoglobulins (10), but also by chemokines and cytokines, such as RANTES (11), TNF- α (12), eotaxin (13), and IL-5 (14).

In a previous study we found that the STZ-induced oxidative metabolism of eosinophils from asymptomatic allergics was reduced as compared to eosinophils from nonallergic subjects, before pollen exposure. This reduction was normalized during the season (15). This enhancement of the oxidative metabolism could be due to the *in vivo* priming of the cells by the presence of some cytokine, such as IL-5, during inflammation (5).

STZ is a preparation of the yeast cell wall. While the main opsonins are iC3b and IgG (16), STZ has been shown to bind mainly to the CR 3 receptor on eosinophils (17). The STZ-induced oxygen radical production could be enhanced by priming with either PAF (18) or the hemopoietic cytokines IL-3, IL-5, and GM-CSF (19).

Only IL-5 has been shown to be eosinophil selective among the hemopoietic cytokines (14, 20). IL-5 is a disulfide-linked dimer with a molecular mass of 45–60 kDa, produced mainly by activated Th₂ cells, but also by mast cells and eosinophils (21). The IL-5 receptor is a dimeric receptor without intrinsic tyrosine kinase activity. The β_c chain is a transmembrane protein with binding sites for signaling proteins, and is therefore considered to be the signal transducer of the receptor. The β_c chain is shared by all three hemopoietic cytokines. The α chain of the receptor complex is ligand specific and responsible for the high-affinity binding of the cytokine (22).

After binding to its receptor, IL-5 has been shown to activate different signal transduction mechanisms, such as the Ras–Raf–MEK–MAPK pathway (23), the JAK–STAT 1 signaling pathway (24), and phosphatidylinositol 3-kinase (PI₃K) (25), through the tyrosine phosphorylation of various intracellular proteins.

Another protein kinase present in granulocytes is protein kinase C, which is a serine/threonine kinase. The PkC family is a group of 12 different members, divided according to their dependence on or independence of Ca²⁺ and/or diacylglycerol (DAG) (26). In the eosinophil, 11 isotypes are expressed, of which the PkC- ζ has been reported to be activated after allergen challenge (27). PkC has been shown to be involved in the activation of the NADPH oxidase in eosinophils (28, 29).

Our preliminary results had shown that the oxidative metabolism by eosinophils from both allergic and non-allergic subjects, in response to STZ, consists of an initial stage, a progression, and a final stage. This is in line with the course of the respiratory burst of phagocytes (30, 31). The initial stage involves the onset of the process (lag phase), whereas the final stage has to do with the gradual switching-off of the burst. To study this further, we hypothesized that these phases might be differently affected by IL-5 and regulated by different signal transduction pathways, reflecting two different events, priming and activation.

To study the mechanisms of IL-5 action, we used four different protein kinase inhibitors, namely, staurosporine and Ro 318220, two PkC inhibitors (32, 33); wortmannin, a PI₃K inhibitor (34); and PD98059, a MEK inhibitor (35).

Material and methods

Reagents

Lucigenin (10, 10-dimethyl-9, 9-bicaridiniumdinitrate) (Sigma Chem-

ical Company, St Louis, MO, USA) was dissolved in DMSO (dimethylsulfoxide) and diluted to 0.5 g/l with TRIS (hydroxymethylaminomethane)-NaCl buffer (15 mM TRIS, 150 mM NaCl, pH 7.4). This stock solution was kept in the dark at 4°C for up to 1 month. Before use, lucigenin was diluted to 0.1 g/l with TRIS-NaCl.

STZ (ICN Biomedicals, Inc., Aurora, OH, USA) particles were suspended in TRIS-NaCl buffer to a concentration of 4 g/l. Opsonization was performed by addition of 20% (v:v) pooled normal serum and incubation for 30 min at 37°C. After centrifugation at 2000 g for 5 min, the supernatant was discarded, and the pellet was resuspended with TRIS-NaCl buffer to the original concentration. Opsonized zymosan particles were stored at 4°C for up to 4 days.

IL-5 (R & D Systems Europe, UK) was diluted in Gey's buffer (pH 7.4) before use to give a final concentration of 10.5 ng/ml.

Inhibitors (staurosporine, Ro 318220, wortmannin, and PD 98059) (Calbiochem, La Jolla, CA, USA) were dissolved in DMSO and kept in the dark at –18°C. Before use, the inhibitors were diluted in Gey's buffer, to the concentrations stated in the results and Tables 1–5.

The MACS-system and anti-CD16 beads were obtained from Miltenyi Biotec, Bergisch, Gladbach, Germany.

Percoll (Pharmacia, Uppsala, Sweden) was diluted with phosphate-buffered saline (PBS) to a 67% (v:v) solution before use.

Preparation of purified eosinophils from whole blood

Eosinophils were isolated from 60–80 ml of peripheral venous blood from each donor by the magnetic cell separation system (MACS), as described by Hansel et al. (36).

Venous heparinized blood was diluted 1:1 in PBS before being loaded on 67% Percoll (density 1.085 g/ml). After centrifugation at 1000 g for 30 min, mononuclear cells were removed, and the erythrocytes in the cell pellet were hypotonically lysed for 60 s. The PMNs obtained were incubated for 1 h at 4°C with anti-CD16 beads, and the suspension was placed in a MACS column for the final separation. Eosinophil purity was over 95% in all cases. Eosinophils were suspended in Gey's buffer to a final concentration of 1×10^6 /ml shortly before use.

Density separation of eosinophils

Normodense and hypodense eosinophils were isolated from peripheral venous blood from asymptomatic blood donors on a Percoll gradient, as previously described in detail (37).

Chemiluminescence (CL) assay

A volume of 100 μ l of purified eosinophil suspension was preincubated with IL-5 at 37°C, for 30 min. Thereafter, 100 μ l of the stimuli (STZ) and 100 μ l lucigenin were added, and the CL was recorded continuously with a luminometer (Berthold LB 9505 C, Bad Wildbad, Germany).

Fig. 1 illustrates the parameters measured throughout the present work. The peaks of the curves, expressed as counts per minute (CPM), were used as estimates of the maximum production of oxygen metabolites, which was usually equivalent to the total amount. The times to reach half the total amount of radicals ($t_{1/2}$ rise) are expressed in minutes, and their reduction is taken as an indication of IL-5 priming effects, with priming being defined as the process leading to an increased responsiveness of the cell to stimulation without activating the cell (2). Inhibitors were preincubated with eosinophil suspensions for 10 min, at 37°C, before incubation with IL-5 and further stimulation.

All experiments including patients were performed during the pollen season, while all experiments including inhibitors were done out of the pollen season. For the experiments with inhibitors, we used cells from both healthy and allergic donors. Since there was no difference in the results obtained, data are presented as belonging to one group only.

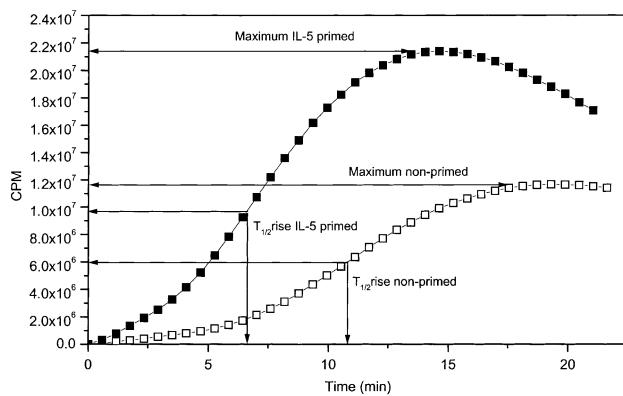


Figure 1. Kinetics of chemiluminescence responses illustrating measured parameters. Human blood eosinophils stimulated (open symbols) or IL-5 treated and subsequently stimulated (filled symbols) to produce oxygen free radicals. For details, see section under Material and methods.

Patient and control groups

Fifteen patients allergic to birch pollen were studied during the Swedish birch-pollen season. All of them presented symptoms of rhinoconjunctivitis, and some also of asthma. They were initially divided into two groups according to the presence or absence of asthma symptoms, but since no significant differences between the groups were shown, the results are presented as one group of patients only.

The control group consisted of eight healthy, nonallergic blood donors. All groups have been described in detail previously (15).

Statistics

The Wilcoxon signed rank test and the Mann-Whitney U-test were used, and *P* values <0.05 were considered significant. All calculations were performed on a personal computer by means of the statistical package Statistica (Statsoft, USA).

Results

Effect of IL-5 treatment on STZ-induced oxidative metabolism

IL-5 treatment changed the production of oxygen free radicals by blood eosinophils in two ways: it increased the peak of the CL curve, an estimate of the maximum radical production achieved, and it reduced the $t_{1/2}$ rise of the CL curve, which we consider to be an estimate of the priming state of the cell since it shows an earlier onset of the oxidative burst. Fig. 1 shows a representative kinetic CL curve illustrating the measured parameters, and Table 1 shows the results obtained.

Effect of protein kinase inhibitors on STZ-induced oxidative metabolism

Staurosporine (10^{-7} M) reduced substantially the total radical production by nonprimed ($P=0.028$) as well as primed ($P=0.028$) cells. No significant effect of IL-5 on this inhibition was observed (Table 2). The $t_{1/2}$ rise of both nonprimed ($P=0.028$) and primed ($P=0.043$) cells was reduced by staurosporine (Table 2); i.e., this inhibitor had a priming-like effect. IL-5 did not affect

Table 1. Effects of IL-5 treatment on STZ-induced oxidative metabolism of eosinophils out of season. Statistical differences are expressed according to Wilcoxon signed rank test. Results are presented as median (range), $n=24$

	Total radical production (CPM $\times 10^6$)	<i>P</i> value	$t_{1/2}$ rise (min)	<i>P</i> value
STZ	39.18 (10.61–77.10)	0.0079	8.40 (6.60–10.80)	0.000 018
STZ IL-5	52.28 (6.65–64.17)		5.40 (4.20–9.00)	

further the staurosporine-reduced $t_{1/2}$ rise. Similar results were obtained using Ro 318220 (10^{-6} M), another PKC inhibitor (Table 3).

Wortmannin (10^{-7} M) completely abolished radical production by nonprimed ($P=0.028$) as well as primed ($P=0.028$) cells (Table 4). Because of this strong inhibitory effect of wortmannin, $t_{1/2}$ rises could not be calculated.

PD 98059 (10^{-5} M) reduced the total radical production by both nonprimed ($P=0.028$) and primed ($P=0.028$) cells, but it did not affect the $t_{1/2}$ rises. IL-5 still reduced $t_{1/2}$ rises in the presence of PD 98059 ($P=0.028$) (Table 5).

Priming of the STZ-induced oxidative metabolism of eosinophils from control and allergic subjects during pollen exposure

It had been previously reported that a group of rhinitis patients showed a reduced total radical production before season as compared with the control group and the group of asthmatic patients, and that this reduction disappeared during the pollen season (15). Nevertheless, the $t_{1/2}$ rises of the CL responses showed no differences between the groups, either before or during the season (data not shown).

Table 2. Effects of staurosporine (10^{-7} M) on STZ-induced oxidative metabolism. Statistical differences are expressed according to Wilcoxon signed rank test. Results are presented as median (range), $n=6$

Stimuli	Inhibitor	Priming	Total radical production (CPM $\times 10^6$)	<i>P</i> value	$t_{1/2}$ rise (min)	<i>P</i> value
STZ			44.08 (11.66–64.71)	0.028	8.70 (6.60–10.80)	0.028
STZ	Staurosporine		12.73 (7.79–24.67)		4.80 (4.20–5.40)	
STZ		IL-5	57.05 (21.40–62.44)	0.028	5.70 (4.80–6.60)	0.043
STZ	Staurosporine	IL-5	23.73 (8.67–42.81)		4.80 (4.20–5.40)	
STZ	Staurosporine			NS		NS
STZ	Staurosporine	IL-5				

NS: nonsignificant.

Table 3. Effects of Ro 318220 (10^{-6} M) on STZ-induced oxidative metabolism. Results are presented as median (range), $n=3$

Stimuli	Inhibitor	Priming	Total radical production (CPM $\times 10^6$)	$t_{1/2}$ rise (min)
STZ			31.31 (16.73–44.27)	8.4 (7.8–9.0)
STZ	Ro 318220		14.49 (8.65–18.42)	7.2 (7.2–7.8)
STZ		IL-5	37.53 (15.21–59.38)	6.6 (6.0–6.6)
STZ	Ro 318220	IL-5	13.97 (10.53–33.36)	5.4 (4.8–5.4)

To determine whether the effect of priming on cells from allergics and healthy controls differed during allergen exposure, cells were treated with IL-5 prior to stimulation. Although preincubation with IL-5 did not significantly change the maximum oxygen radical production by control cells ($P=0.89$), there was considerable variability within the group (Fig. 2A). Instead, the response of patient cells was consistently reduced by IL-5 ($P=0.016$) (Fig. 2A). On the contrary, IL-5 treatment always primed eosinophils in terms of a reduction of the $t_{1/2}$ rise of the reaction both in control ($P=0.012$) and in patient cells ($P=0.00066$) (Fig. 2B).

STZ-induced oxidative metabolism of normodense and hypodense eosinophils

Allergic patients have been shown to have an increased amount of hypodense eosinophils in blood (38, 39). Here we show that hypodense eosinophils had a tendency toward a reduced total radical production, as compared with normodense eosinophils, while no differences in the $t_{1/2}$ rises between these cell populations were observed (Table 6).

Table 4. Effects of wortmannin (10^{-7} M) on STZ-induced oxidative metabolism. Statistical differences are expressed according to Wilcoxon signed rank test. Results are presented as median (range), $n=6$

Stimuli	Inhibitor	Priming	Total radical production (CPM $\times 10^6$)	P value	$t_{1/2}$ rise (min)	P value
STZ			39.01 (11.92–77.10)		8.70 (6.60–9.60)	
STZ	Wortmannin		0.07 (0.03–0.21)	0.028	n.a.	n.a.
STZ		IL-5	47.25 (6.65–63.95)		5.70 (4.20–9.00)	
STZ	Wortmannin	IL-5	0.21 (0.05–0.45)	0.028	n.a.	n.a.
STZ	Wortmannin				n.a.	
STZ	Wortmannin	IL-5		n.a.	n.a.	n.a.

n.a.: not applicable.

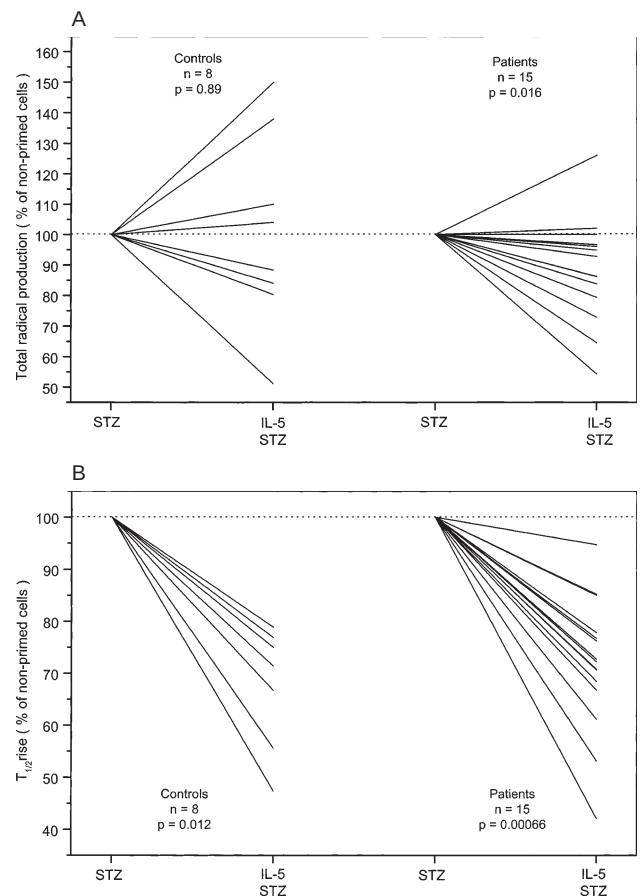


Figure 2. Effect of IL-5 treatment on STZ-induced lucigenin chemiluminescence of blood eosinophils from healthy controls and allergic patients during pollen season. A) Total radical production, measured as CPM and expressed as percentage of that of nonprimed cells, taken as 100%. Statistical differences within groups are expressed by Wilcoxon signed rank test. B) Priming effect of IL-5, measured as time to reach half total amount of radicals and expressed as percentage of $t_{1/2}$ rise of nonprimed cells, taken as 100%. Statistical differences within groups are expressed by Wilcoxon signed rank test. Results of some subjects were identical and therefore produced overlapping lines. This resulted in fewer lines than number of subjects actually investigated.

Table 5. Effects of PD 98059 (10^{-5} M) on STZ-induced oxidative metabolism. Statistical differences are expressed according to Wilcoxon signed rank test. Results are presented as median (range), $n=6$

Stimuli	Inhibitor	Priming	Total radical production (CPM $\times 10^6$)	P value	$t_{1/2}$ rise (min)	P value
STZ			40.41 (10.61–63.46)		8.40 (7.20–9.60)	
STZ	PD 98059		19.65 (6.16–40.35)	0.028	8.10 (7.20–9.60)	NS
STZ		IL-5	51.66 (36.15–64.17)		5.40 (4.20–6.60)	
STZ	PD 98059	IL-5	36.34 (11.29–51.14)	0.028	5.70 (4.20–6.60)	NS
STZ	PD 98059					
STZ	PD 98059	IL-5		NS		0.028

NS: nonsignificant.

Table 6. STZ-induced oxidative metabolism by hypodense and normodense eosinophils. Statistical differences are expressed according to Wilcoxon signed rank test. Results are presented as median (range), $n=7$

Density (g/ml)	Total radical production (CPM $\times 10^6$)	<i>P</i> value	$t_{1/2}$ rise (min)	<i>P</i> value
1.092	16.65 (8.52–32.16)	0.063	8.00 (6.4–8.2)	0.13
1.100	26.52 (7.08–44.37)		8.00 (6.0–8.4)	

Discussion

This study shows that different stages of the course of STZ-induced oxidative metabolism by blood eosinophils have different characteristics. The initial stage, measured as the $t_{1/2}$ rises of the CL curves, is an indication of the state of priming of the cell, and was PkC dependent and MEK independent. The end stage, measured as the peaks of the CL curves, is an estimate of the total radical production by the cells, and was PkC, PI_3 kinase, and MEK dependent. IL-5 priming affected differently these two stages. It shortened the initial stage, while increasing the end stage. During allergen exposure, however, the total radical production was reduced by IL-5, and this could be due to increased amounts of hypodense eosinophils and/or some inherent abnormality in the response of cells from allergics.

Priming has been defined as an increased reaction to a particular stimulus by an agent that, in itself, induces little or no response (40). Priming agents for eosinophils include PAF or the hemopoietic cytokines IL-3, IL-5, and GM-CSF. Several eosinophil functions are altered by cytokine priming, including migration (41, 42), degranulation (43, 44), the respiratory burst, and tyrosine phosphorylation (19).

In this study, we focused on the effect of IL-5 priming, since IL-5 has been shown to be eosinophil selective (14, 20). We found that treatment with IL-5 affected the STZ-induced CL in two ways. It increased the peak of the curves, which is an estimate of the total radical production by the cells, and it reduced the times to reach half the total amount of radicals ($t_{1/2}$ rise), which is an indication of priming. This led to the question of the mechanisms involved in the primed production of oxygen free radicals in eosinophils.

To study the mechanisms of STZ activation and IL-5 priming, we used four different protein kinase inhibitors: staurosporine and Ro 318220, two PkC inhibitors (32, 33); wortmannin, a PI_3K inhibitor (34); and PD 98059, a MEK inhibitor (35).

Staurosporine inhibited the STZ-induced total radical production by both nonprimed and primed eosinophils, indicating that it is PkC dependent, in keeping with previous findings (10). IL-5 did not counteract the staurosporine inhibition. On the other hand, staurosporine reduced the $t_{1/2}$ rises, i.e., a priming-like effect, in

both nonprimed and primed eosinophils, indicating that the initial stage of the oxygen radical production is positively regulated by PkC. Staurosporine has been previously reported to prime the STZ-induced radical production by eosinophils (45). These findings were confirmed with a more specific PkC inhibitor, Ro 318220.

Wortmannin totally abolished radical production by both nonprimed and primed eosinophils, thus showing a strong dependence on PI_3 kinase activity. This has been reported before for both nonprimed (10) and primed (25) eosinophils.

The MEK inhibitor PD 98059 reduced the total radical production by nonprimed as well as primed eosinophils, and IL-5 did not counteract this inhibition. PD 98059 had no effect on the $t_{1/2}$ rises of either nonprimed or primed eosinophils. On the other hand, IL-5 was able to prime cells in the presence of the MEK inhibitor. Thus, the effect of IL-5 was MEK independent, although IL-5 has been shown to result in the activation of MEK in eosinophils (23). These data indicate that the STZ-induced radical production shows a MEK-independent initial stage and a MEK-dependent end stage, and that the effect of IL-5 is MEK independent.

The STZ-induced total radical production by eosinophils from asymptomatic allergics has been previously shown to be reduced out of the pollen season, and to normalize during allergen exposure as compared to nonallergics (15). In analysis of the $t_{1/2}$ rises of the corresponding CL curves, no differences between the groups were seen either before or during the pollen season, indicating that different stages of the process of oxygen free radical production are affected differently in allergics and nonallergics.

Treatment of eosinophils isolated during the season with IL-5 showed also a dual effect, but somewhat different than out of season. The total radical production was reduced in patients' cells but either increased or reduced in nonallergics' cells. On the other hand, the $t_{1/2}$ rises were always reduced in eosinophils from both allergics and nonallergics. These results indicate that while IL-5 consistently anticipates the onset of the response in both allergics and nonallergics, total radical production does not follow a clear pattern of changes, particularly in control cells. We can only speculate that this is due to differences among cells prior to IL-5 priming, as in their state of activation or capacity to generate oxygen metabolites.

Eosinophil heterogeneity regarding cell density has been described, showing the existence of "normodense" and "hypodense" populations (46). We and others have previously shown that hypodense eosinophils have a reduced capacity for total radical production as compared with normodense cells when stimulated with PMA (37, 47), whereas no differences in $t_{1/2}$ rises among these cell populations were observed (unpub-

lished observations). The same pattern of response was obtained here when using STZ as a stimulant of eosinophils of different densities, indicating that the reduced radical generation capacity of hypodense eosinophils is due to something other than priming. A similarly reduced total radical production in response to STZ was observed in eosinophils from rhinitis patients during the pollen season (15).

The diminished capacity to produce oxygen metabolites during allergen exposure could be related to the presence of increased amounts of hypodense eosinophils, to an earlier action of the NADPH oxidase switch-off mechanisms, or to the signal transduction pathways activated after STZ binding to its eosinophil receptors. These factors could be acting alone or in combination, a problem that needs further clarification.

In conclusion, the STZ-induced oxidative metabolism by blood eosinophils consists of different stages. The initial stage seems to be PkC dependent and MEK independent, while the end stage seems to be PkC, PI₃

kinase, and MEK dependent. Apparently, PkC had a positive regulatory effect on the $t_{1/2}$ rises and a negative effect on the total radical production. IL-5 priming reduced the initial stage, while increasing the end stage. During pollen exposure, however, the end stage was reduced by IL-5, and this could be related to increased amounts of hypodense eosinophils and/or some inherent abnormality in the response of cells from allergics. Nevertheless, the interpretation of the clinical findings is complicated and requires further studies.

Acknowledgments

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