

Effects of skin-chamber fluids from human allergic reactions on neutrophil activation

Kaoru Sato, MD,* Burton Zweiman, MD, Anne R. Moskovitz, BS,
Carolyn von Allmen, BA, Andrew Lane, BA, and Paul C. Atkins, MD
Philadelphia, Pa.

We have found activated neutrophils (PMNs) in skin chambers overlying the site of ongoing human allergic reactions. To determine whether this activation is due to component(s) released early in these IgE-mediated reactions, we investigated the in vitro effects of skin chamber fluids (CF) on resting blood PMNs. These skin CF had been previously obtained, at different time periods, at pollen-antigen-challenged or buffer-control-challenged sites in sensitive human subjects. PMNs incubated with CF obtained during the first hour of antigen challenge (first-hour CF) generated significantly more superoxide (O_2^-) than spontaneous production ($p < 0.001$) and more than PMNs incubated with first-hour buffer-site CF ($p < 0.002$). A pattern similar to O_2^- generation was observed in lactoferrin secretion during the incubation of the three cell aliquots described above ($p < 0.001$). After these incubations, the subsequent responsiveness of the PMNs present in the cell buttons to opsonized zymosan, a PMN activator, was assessed. PMNs previously incubated with first-hour CF generated significantly more net O_2^- in response to opsonized zymosan than did PMNs previously incubated with first-hour buffer-site CF ($p < 0.001$) or buffered saline ($p < 0.001$). Again, a similar difference in the patterns of net lactoferrin secretion was observed ($p < 0.02$). These events were not associated with PMN damage. Analogous studies with CF previously obtained during the second- to first-hour of antigen or buffer challenge stimulated much less than first-hour CF. We conclude that one or more components released early in IgE-mediated skin reactions can activate PMNs. The nature of the activating component(s) is unknown, with no evidence of activation by histamine, antigen, or other components found to date in first-hour CF. (J ALLERGY CLIN IMMUNOL 1992;90:21-31.)

Key words: Allergic skin reaction, skin chambers, lactoferrin, superoxide, neutrophil activation

There is increasing interest in the possible role of inflammatory cell responses in human allergic reactions. Using a skin-chamber model, we have found greater numbers of PMNs exuding from antigen-challenged sites than from buffer-control-challenged sites in sensitive human subjects. These cells appeared activated, as evidenced by a polarized appearance, increased intracellular oxidative activity, and increased

Abbreviations used

Lf:	Lactoferrin
PMN:	Neutrophil
O_2^- :	Superoxide
SOD:	Superoxide dismutase
CF:	Chamber fluids
OZ:	Opsonized zymosan
LDH:	Lactic dehydrogenase
PAF:	Platelet-activating factor
LTB ₄ , LTC ₄ , LTD ₄ :	Leukotrienes B ₄ , C ₄ , and D ₄
PBS:	Phosphate-buffered saline
HBSS:	Hanks' balanced salt solution
PMA:	Phorbol myristate acetate
PNU:	Protein nitrogen control

From the Department of Medicine, Allergy, and Immunology Section, University of Pennsylvania School of Medicine, Philadelphia, Pa.

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Reprint requests: Burton Zweiman, MD, Johnson Pavilion, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6057.

*Present Address: Department of Otorhinolaryngology, Nagoya City University, Medical School, Nagoya, Japan 467.

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surface expression of the C3bi receptor.¹⁻⁴ Also, we have found increased release of specific granule components of the PMN (Lf and elastase) into overlying skin chambers during late-phase reactions.⁵ Further-

more, we found increased levels of PAF and LTB₄, LTC₄, and LTD₄ in these CF, starting after the first hour of antigen challenge in sensitive subjects.^{6,7} The nature of the stimulus for this local PMN activation was not known. Conceivably, one or more components released at the site could activate the PMNs attracted into the allergic-reaction site. The studies described here were designed to determine whether the CF overlying allergic-reaction sites and substances contained in such fluids can stimulate resting PMNs, either directly or by enhancing responses to a known PMN stimulant.

MATERIAL AND METHODS

Subjects

Five atopic subjects (aged 24 to 55 years), skin test reactive to 10 PNU/ml of ragweed- and/or grass-pollen extract (Greer Laboratories, Lenoir, N.C.), were studied after informed consent was obtained. All individuals had a history of allergic rhinitis during the fall and/or spring seasons. Four nonatopic subjects (aged 40 to 55 years) determined to be nonatopic on the basis of the absence of allergic symptoms and negative skin test responses to intradermal injection of protein nitrogen units per milliliter of pollen antigens were studied. No subject had received any medication for at least the previous 2 weeks and had never received allergy-injection therapy.

Skin-chamber challenge

Skin-chamber studies were performed according to the method described previously.⁶ In brief, two 1 cm diameter blisters were induced on each forearm by a combination of gentle heat and suction. The roofs were aseptically removed from the blister base at the dermal-epidermal junction. Collection chambers were taped into place over each denuded blister base. The chambers were filled with PBS to wash away any mediators generated by the blister induction. After 5 minutes, the buffer was removed and replaced with 0.3 ml of either PBS or 100 PNU/ml of pollen antigen with heparin (10 U/ml). After incubation for 1 hour, each CF was collected, the blister bases were washed thoroughly, and the collection chambers were filled with fresh solutions of pollen extract or buffer. After an additional 4 hours of incubation, the fluids were collected and immediately centrifuged (1000 rpm for 10 minutes at 4° C), and the supernatants were stored at -70° C.

In vitro basophil-challenge studies

Basophil-enriched blood leukocyte populations from five pollen-antigen-sensitive donors were obtained and incubated with serial tenfold concentrations of the relevant pollen antigens, using techniques that we previously reported from our laboratory.³⁰ After a 30-minute incubation, the supernatants were removed and stored immediately in aliquots at -70° C. Histamine levels were assayed in aliquots of each supernatant fluid, and the supernatant in the challenge of each subject's cells with the highest histamine level was

subsequently used for in vitro incubation with resting blood PMNs.

Reagents

HBSS was purchased from GIBCO, Grand Island, N.Y. Horse-heart ferricytochrome c, PMA, and SOD were purchased from Sigma Chemical Co., St. Louis, Mo. A stock solution of PMA was made in dimethyl sulfoxide and stored in aliquots at -70° C. OZ was prepared by boiling zymosan (10 mg/ml) in physiologic saline for 10 minutes, by washing twice in PBS, and then by incubating with autologous or pooled normal human serum for 30 minutes at 37° C. After centrifugation, the OZ was washed twice with PBS and resuspended (1 or 5 mg/ml) in HBSS containing 0.1% gelatin and Ca⁺⁺/Mg⁺⁺.⁸

PMN preparations

PMNs were obtained from heparinized venous blood of the volunteer pollen-sensitive subjects by modified density-gradient separation method with Hypaque-Ficoll columns.⁹ The cells were pelleted and washed twice in Ca⁺⁺/Mg⁺⁺-free Hanks' solution. Contaminating erythrocytes were removed by hypotonic lysis. The cells were washed twice with Ca⁺⁺/Mg⁺⁺-free HBSS and resuspended at 1 × 10⁷ cells per milliliter in HBSS containing 0.1% gelatin and Ca⁺⁺/Mg⁺⁺. These cell suspensions were >99% granulocytes, and viability was >97%, determined by trypan blue exclusion. PMNs from pollen-sensitive subjects were chosen so that the in vitro incubation of CF with these cells (described below) might stimulate the situation in which PMNs accumulating in the allergic-reaction site are exposed to factors released into the skin CF.

O₂⁻ production

O₂⁻ production was determined as the SOD-inhibited reduction of ferricytochrome c.¹⁰ Each tube contained, in a final volume of 1 ml, 2 × 10⁶ cells, 1.2 mg of cytochrome c in HBSS/gelatin/Ca⁺⁺/Mg⁺⁺ solution, and SOD (0.2 mg/ml). To each of replicate tubes was added 0.1 ml of one of the stimuli or of PBS (spontaneous secretion). After incubation for 30 minutes at 37° C in 5% CO₂ the tubes were centrifuged at 1000 rpm for 10 minutes at 4° C, and the supernatant was removed for spectrophotometric analysis. The O₂⁻ level was derived from the absorbance at 550 nm with an extinction coefficient of 2.1 × 10⁴/cm/mol/L.¹¹

Lf determination

Lf was determined by a slight modification of a published ELISA assay with the supernatant described above.¹² Polystyrene 96-well plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with rabbit anti-Lf antibodies in coating buffer overnight at 4° C, the fluid was removed, and 10% normal rabbit serum in PBS was added for 1 hour at room temperature as a blocking solution. The wells were drained and filled with either (1) test solution, appropriately diluted, (2) concentrations of purified exogenous Lf that were diluted in PBS containing 0.1% gelatin and 2.5%

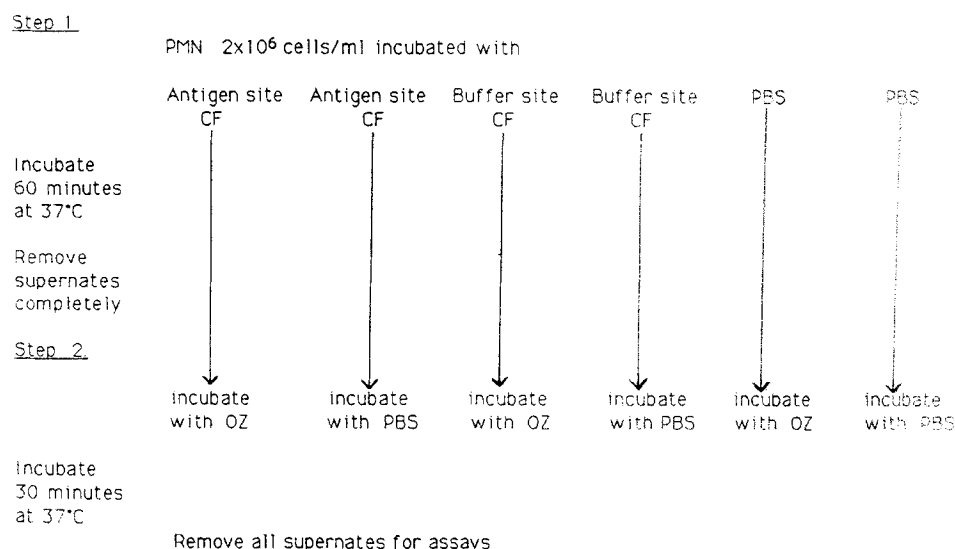


FIG. 1. In this experimental protocol for in vitro PMN stimulation, PMNs are incubated in pairs with antigen-site CF, or buffer-site CF, or PBS. After 60 minutes at 37° C, supernatants are removed. One cell button of each pair is resuspended in a suspension of OZ. After incubation at 37° C for 30 minutes, supernatants are removed.

ferricytochrome c to determine a standard concentration curve, or (3) PBS (negative control). After incubation for 90 minutes at 37° C, the fluids were removed from the wells and replaced with peroxidase-conjugated rabbit anti-Lf antibody, diluted 1:500. After further incubation for 90 minutes at 37° C, the fluids were discarded, the wells were washed three times with Tween/PBS, and a substrate solution (o-phenylenediamine hydrochloride in pH 5 citric acid buffer) was added. After incubation at room temperature for 20 minutes, absorbance was determined at 420 nm with an automated spectrophotometer (Dynatech Laboratories, Inc.). The concentration of Lf in test samples was determined by comparison with the standard curve. In previous quality control studies, we found that Lf detection was not affected by the presence of the cytochrome c gelatin/Ca⁺⁺/Mg⁺⁺ in the supernatant fluid.

LDH activity

Release of cytoplasmic LDH was used as a marker of cell damage. LDH activity was measured with a standard assay kit (Sigma Chemical Co.) and expressed in activity units per liter.⁵

Incubation of PMNs with skin CF

The effects of the skin CF obtained from antigen-challenged and buffer-control-challenged sites on PMN activation was investigated in a two-step protocol (Fig. 1).

Step 1. A direct stimulatory effect was assessed by incubating 2×10^6 resting blood PMNs in a 1 ml total reaction volume containing 50 μ l of either (1) different pools of first-hour CF obtained from antigen-challenged sites, (2) different pools of first-hour CF from buffer-challenged sites, (3) CF obtained after incubation during the second through

fifth hour at antigen-challenged sites, (4) CF obtained at the buffer-challenged sites after the second through fifth-hour incubation, (5) PBS (control diluent), (6) grass- or ragweed-antigen solutions, 100 PNU/ml, (7) histamine (Eli Lilly and Co., Indianapolis, Ind.), 70 ng/ml, (8) heparin (10 U/ml), and (9) normal human serum (diluted in PBS to a 4% vol/vol concentration). The concentrations of the components (5 through 9) listed above were chosen to approximate that found in first-hour CF at antigen-challenged sites. The rest of the 1 ml incubation-reaction solution consisted of the ferricytochrome/gelatin/Ca⁺⁺/Mg⁺⁺ solution, as described above. Each incubation was performed in quadruplicate, that is, two pairs were incubated with SOD and two pairs were without added SOD. Incubation was performed at 37° C for 60 minutes to approximate the in vivo conditions in which PMNs accumulating in skin-reaction sites would be exposed to components released into overlying CF. The supernatant fluids were removed completely, aliquots were analyzed within 1 hour for O₂⁻, and other aliquots were stored at -20° C for subsequent determination of Lf and LDH levels.

Step 2. The cells were then immediately resuspended in 1 ml of solution to investigate the possible enhancing effect of the prior incubation on the subsequent responses of the PMNs to OZ (Fig. 1). For example, one of the paired PMN aliquots previously incubated with antigen-site CF was now incubated with 100 μ l of OZ (final concentration, 0.1 mg/ml) in a total 1 ml volume of cytochrome/gelatin/Ca⁺⁺/Mg⁺⁺ solution; the other member of the pair was incubated in 100 μ l of PBS (instead of OZ) in cytochrome/gelatin/Ca⁺⁺/Mg⁺⁺. The latter incubation was performed to assess any residual stimulatory effects on the PMNs of the prior 60-minute incubation with CF. In a similar way, each of the paired PMN specimens previously

TABLE I. Comparison of spontaneous and agonist-stimulated O_2^- production and Lf secretion by PMNs

Stimulant	O_2^-	Lf	<i>p</i> Value*
PBS	$0.65 \pm 0.2^\dagger$	$227 \pm 24^\ddagger$	
OZ (0.5 mg/ml)	12.9 ± 0.7	2182 ± 175	<0.001
OZ (0.1 mg/ml)	5.6 ± 0.2	1309 ± 55	<0.001
PMA (0.01 μ g/ml)	19.5 ± 0.5	5200 ± 200	<0.001

PBS, Spontaneous O_2^- production and Lf secretion from PMNs incubated in PBS.

**p* Value compared with PBS incubations; N = 9 subjects.

† Mean $\pm$ SEM O_2^- generation in nanomolars per milliliter.

‡ Mean $\pm$ SEM Lf secretion in nanograms per milliliter.

incubated with buffer-site fluids, pollen antigens, histamine, etc. (see above), was incubated with OZ or PBS. After incubation of these paired specimens for an additional 30 minutes at 37°C in 5% CO₂/air, the tubes were centrifuged (1000 rpm for 10 minutes at 4°C), and the supernatants were removed and stored in aliquots for determination of O_2^- and Lf levels.

Statistical analysis

Since the findings were found to be normally distributed, the comparisons between PMN responses in test and control incubations in the group of subjects was analyzed by the Student's *t* test for unpaired values.

RESULTS

OZ- and PMN-induced O_2^- production and Lf secretion by PMNs

The O_2^- production of PMNs stimulated during 30 minutes by OZ was dose dependent. The (mean $\pm$ SEM) O_2^- production stimulated by OZ, 0.5 mg/ml, was 12.9 ± 0.7 nmol, significantly more than that stimulated by OZ, 0.1 mg/ml (5.6 ± 0.2 nmol; $p < 0.001$) (Table I). The latter was significantly more than spontaneous O_2^- production (0.65 ± 0.2 nmol; $p < 0.001$). Likewise, OZ-stimulated Lf secretion was also dose dependent; OZ, 0.5 mg/ml, induced significantly more secretion (2182 ± 175 ng/ml) than OZ, 0.1 mg/ml, stimulated secretion (1309 ± 55 ng/ml; $p < 0.001$), and, in turn, more than spontaneous Lf secretion (227 ± 24 ng/ml; $p < 0.001$). PMA (0.01 mg/ml) strongly stimulated O_2^- production (19.5 ± 0.5 nmol) and Lf secretion (5200 ± 200 ng/ml; $p < 0.001$).

The O_2^- generation and Lf secretion by individual OZ-stimulated PMN specimens were compared by linear regression analysis of the group of nine subjects. There was a statistically significant correlation between O_2^- production and Lf secretion by PMNs stimulated by 0.1 mg/ml of OZ ($r = 0.65$; $p < 0.05$).

There was a strong correlation between O_2^- production and Lf secretion stimulated by 0.5 mg/ml of OZ ($r = 0.89$; $p < 0.001$). However, no statistically significant correlation was observed between O_2^- production and Lf secretion in PMNs stimulated by PMA, 0.01 mg/ml ($r = 0.39$; $p < 0.26$).

O_2^- production and Lf secretion by the PMNs of atopic and nonatopic subjects

Unstimulated PMNs (2×10^6 cells) from atopic subjects produced 1.0 ± 0.3 nmol of O_2^- compared with that of 0.2 ± 0.1 nmol by the cells from nonatopic subjects ($p = 0.051$). There was no significant difference in O_2^- production by PMNs of atopic versus nonatopic subjects stimulated by OZ, 0.1 mg/ml (5.5 ± 0.3 nmol versus 5.8 ± 0.5 nmol; $p = 0.67$), or stimulated by OZ, 0.5 mg/ml (13.6 ± 0.9 nmol versus 12.1 ± 1.0 nmol; $p = 0.2$). Likewise, there were no significant differences observed between the two groups in O_2^- production stimulated by PMAs (0.01 mg/ml) (20.1 ± 0.7 nmol versus 20.2 ± 1.6 nmol; $p = 0.9$).

There was no significant difference in the spontaneous secretion of Lf by PMNs of atopic (252 ± 33 ng/ml) and nonatopic subjects (215 ± 49 ng/ml; $p = 0.53$). There was also no significant difference in the Lf secretion by PMNs of atopic versus nonatopic subjects stimulated by OZ, 0.5 mg/ml (2328 ± 270 ng/ml versus 2000 ± 217 ng/ml; $p = 0.39$), or by OZ, 0.1 mg (1328 ± 81 ng/ml versus 1285 ± 85 ng/ml; $p = 0.72$). Likewise, there were no significant differences observed between the two groups in Lf secretion stimulated by PMA (0.01 mg/ml) (5208 ± 475 ng/ml versus 5225 ± 270 ng/ml; $p = 0.97$). Thus, there was a trend for slightly more responses of PMNs from the atopic subjects, but not significantly so.

Effects of skin CF on PMN activation: Direct stimulatory effects of CF

First-hour CF. Aliquots of the resting blood PMNs (2×10^6 cells) incubated in the control medium alone for 30 minutes at 37°C spontaneously generated 1.5 ± 0.3 nmol of O_2^- . Aliquots of the same PMNs incubated with six different first-hour antigen-site CF, under the same conditions, produced significantly more O_2^- (3.5 ± 0.2 nmol; $p < 0.001$). The latter responses were also significantly more than the O_2^- generation induced by six first-hour buffer-site CF (2.3 ± 0.2 nmol; $p < 0.002$). The O_2^- response induced by buffer-site first-hour CF was significantly more than spontaneous O_2^- generation ($p < 0.05$) (Fig. 2).

Lf secretion induced in these PMNs by first-hour

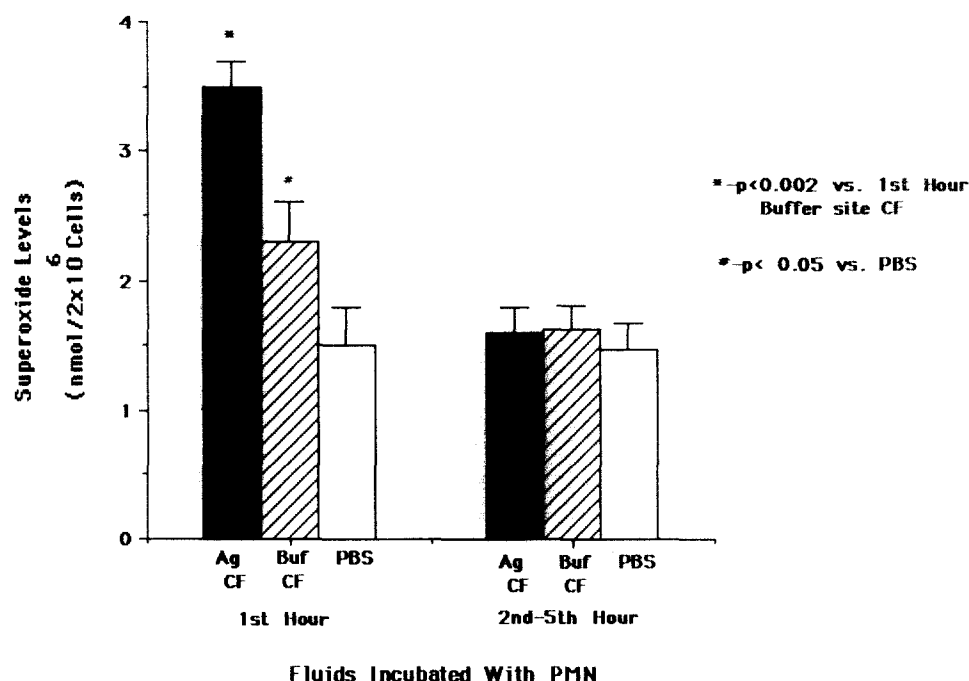


FIG. 2. O_2^- levels (mean $\pm$ SEM) in supernatants after incubation of PMNs with (1) antigen-site CF (Ag CF), (2) buffer-site CF (Buf CF), or (3) PBS. Columns (left panel), CF obtained after first hour of antigen or buffer challenge; columns (right panel), CF obtained after second- through fifth-hour of challenge.

antigen-site CF (567 ± 45 ng/ml) was also significantly more than spontaneous Lf secretion (227 ± 36 ng/ml; $p < 0.001$) and Lf secretion induced by first-hour buffer-site CF (372 ± 42 ng/ml; $p < 0.01$) (Fig. 3). The latter response was also significantly more than spontaneous Lf secretion ($p < 0.02$). These differences in Lf levels were not due to Lf present in the CF at the beginning of this study. In a separate analysis, we found that the mean Lf level in fluid from antigen-site first-hour CF was 453 ng/ml. Diluted 1:20 in the reaction volume described above, the Lf in these antigen-site first-hour CF itself would therefore contribute only an average 23 ng/ml.

Second- through fifth-hour CF. O_2^- production induced by PMNs (2×10^6 cells) incubated with four different second- through fifth-hour antigen-site CF was not significantly different from spontaneous O_2^- production (1.7 ± 0.2 nmol versus 0.2 nmol; p , not significant) and O_2^- production induced by four second- through fifth-hour buffer-site CF (1.6 ± 0.2 nmol; p , not significant) (Fig. 2).

In determination of Lf secretion induced by PMNs by second- through fifth-hour CF, we had to take into account the Lf already present in such CF. There was a large amount of Lf in the second- through fifth-hour antigen-site CF (18.8 ± 3.0 mg/ml), significantly

more than in the second- through fifth-hour buffer-site CF (5.5 ± 0.78 μ g/ml). Therefore, we determined the Lf levels present in each of the CF used in these studies. The amount of the Lf in individual fluids, diluted 1:20 (since 50 μ l of CF was contained in a total in vitro reaction volume of 1000 μ l), was subtracted from the respective supernatant Lf levels to obtain the secreted Lf levels depicted in Fig. 3. Secreted Lf induced by second- through fifth-hour antigen-site CF (220 ± 28 ng/ml) was not different from spontaneous Lf secretion (193 ± 27 ng/ml), and Lf secretion induced by second- through fifth-hour buffer-site CF (233 ± 32 ng/ml).

Effects of preincubation on subsequent responses to OZ

Preincubation with first-hour CF. After the incubation and supernatant removal described above, the paired PMN aliquots were then resuspended in the cytochrome/gelatin/ Ca^{++} / Mg^{++} medium with 100 ml of added OZ (in the suboptimal concentration of 0.1 mg/ml) or PBS and incubated for 30 minutes at 37° C (Fig. 1). PMNs (2×10^6 cells) previously incubated in medium containing first-hour antigen-site CF generated 7.1 ± 0.3 nmol of O_2^- . These responses were significantly more than responses of PMNs previously incubated with either (1) first-hour

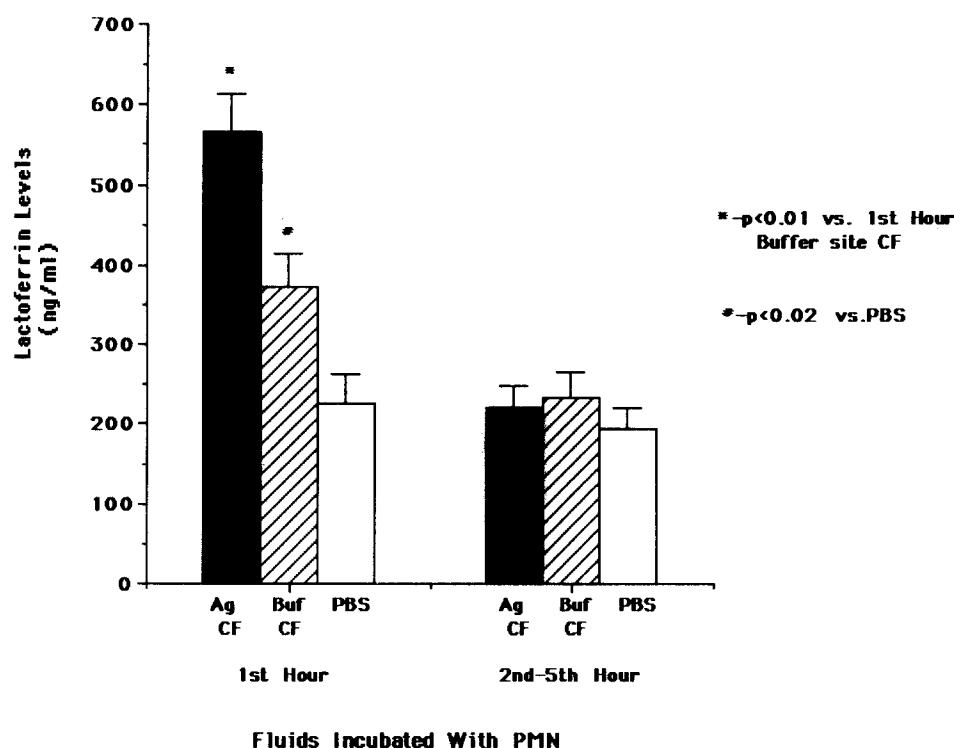


FIG. 3. Lf levels (mean $\pm$ SEM) in supernatants after incubation of PMNs with (1) antigen-site CF (Ag CF), (2) buffer-site CF (Buf CF), or (3) PBS. Columns (*left panel*), CF obtained after first hour of antigen or buffer challenge; columns (*right panel*), CF obtained after second- through fifth-hour of challenge.

buffer-site CF (6.0 ± 0.3 nmol; $p < 0.02$) or (2) with medium containing just PBS (5.4 ± 0.2 nmol; $p < 0.001$). The responses to OZ in the PMNs preincubated with first-hour buffer-site CF (6.0 ± 0.2 nmol) were not significantly more than in PMNs preincubated with PBS (5.4 ± 0.2 nmol).

A similar pattern was observed in comparison with Lf levels in these fluids. The Lf levels in the OZ-incubated PMNs previously incubated with antigen-site first-hour CF were 1613 ± 107 ng/ml, significantly more than responses to OZ of PMNs previously incubated with (1) first-hour buffer-site CF (1330 ± 567 ng/ml; $p < 0.05$) or with (2) PBS containing medium (1150 ± 54 ng/ml; $p < 0.01$). The OZ-induced Lf secretion was more in PMNs preincubated with first-hour CF (1330 ± 57) than in PMNs preincubated in PBS (1150 ± 54), but not significantly so.

The observed greater O_2^- generation during the incubation of OZ with PMNs previously exposed to antigen-site first-hour CF could reflect a persistent stimulation of O_2^- production because of the previous 60-minute incubation with the CF (even though the supernatants had been removed completely after the

first 60 minutes). Therefore, additional controls were performed in which PMN aliquots were incubated for 60 minutes at 37°C with either antigen-site first-hour CF, buffer-site first-hour CF, or PBS/medium. The supernatants were removed and cells were resuspended in PBS/medium instead of OZ solution for an additional 30-minute incubation at 37°C . The supernatants were then removed and assayed for O_2^- and Lf. We did find some generation of O_2^- and secretion of Lf in these fluids. Therefore, to determine the true effect of prior incubation on OZ-induced responses, we calculated "net" responses defined by the OZ-induced O_2^- or Lf response in the first tube of a pair (antigen-site CF incubation followed by OZ incubation) minus O_2^- or Lf levels in the second tube of the pair (antigen-site CF incubation followed by PBS incubation). A similar derivation of net responses was made for PMNs incubated first with buffer-site CF and for PMNs incubated first with PBS/medium.

Comparison of the net OZ-induced responses demonstrated a significantly higher O_2^- production by PMNs previously incubated with antigen-site first-hour CF (4.5 ± 0.2 nmol) than by those PMNs preincubated with either buffer-site first-hour CF

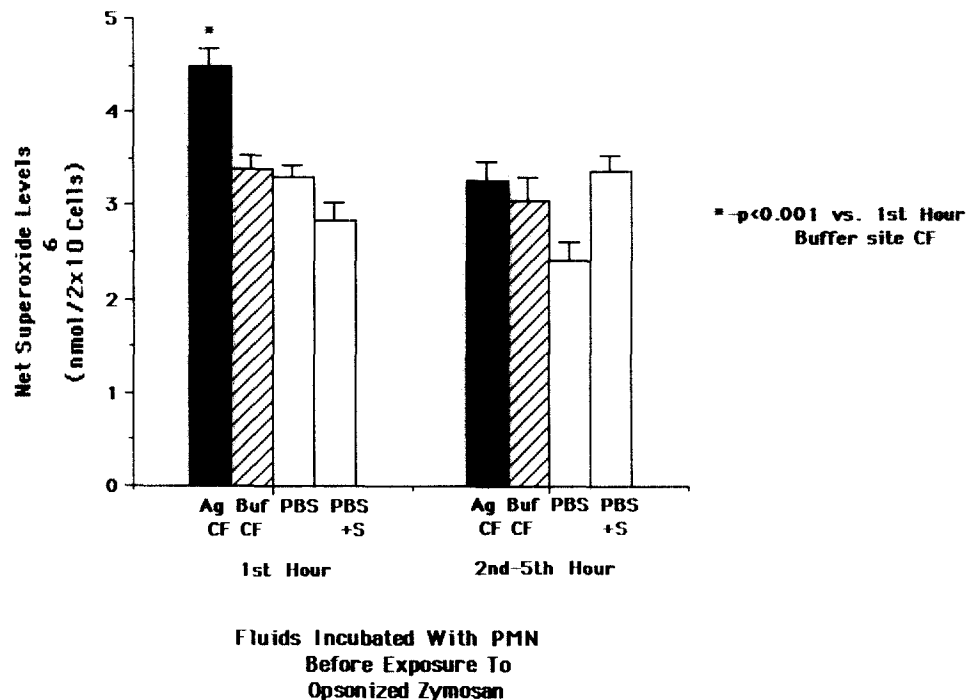


FIG. 4. Net O_2^- levels (mean $\pm$ SEM) in supernatants after incubation with OZ of PMNs previously incubated with (1) antigen-site CF (*Ag CF*), (2) buffer-site CF (*Buf CF*), (3) PBS, or (4) PBS plus 4% serum (*left panel*) or PBS plus 20% serum (*right panel*). Columns (*left panel*), CF obtained after first hour of antigen or buffer challenge; columns (*right panel*), CF obtained after second-through fifth-hour of challenge. (See text for definition of net O_2^- levels.)

(3.4 ± 0.1 nmol; $p < 0.001$) or with PBS/medium (3.3 ± 0.1 nmol; $p < 0.001$) (Fig. 4). Likewise, the net OZ-induced Lf secretory response was significantly higher in PMNs previously incubated with antigen-site first-hour CF (1353 ± 93 ng/ml) than in PMNs previously incubated with buffer-site first-hour CF (1067 ± 61 ng/ml; $p < 0.02$) or with PBS/medium (1043 ± 67 ng/ml; $p < 0.02$) (Fig. 5).

Preincubation with second- through fifth-hour CF. Comparison of the net OZ-induced responses did not reveal significantly more O_2^- production by PMNs (2×10^6 cells) previously incubated with second-through fifth-hour antigen-site CF (3.2 ± 0.3 nmol) than by PMNs preincubated with either second-through fifth-hour buffer-site CF (3.0 ± 0.3 nmol) or with PBS/medium (2.4 ± 0.2 nmol) (Fig. 4). The net OZ-induced Lf secretory response was significantly more in PMNs previously incubated with second- through fifth-hour antigen-site CFs (1256 ± 36 ng/ml) than in PMNs previously incubated with PBS/medium (930 ± 96 ng/ml; $p < 0.02$) (Fig. 5). However, there was no difference in the net Lf secretion in PMNs previously incubated with second-through fifth-hour antigen-site CF (1256 ± 36

ng/ml) compared with responses in PMNs preincubated with second- through fifth-hour buffer-site CF (1296 ± 63 ng/ml).

Effects of some agents present in CF

In an attempt to determine what component(s) of the first-hour CF led to the stimulatory effects described above, we assessed the effect on O_2^- production and Lf secretion by PMNs incubated with (1) the antigen (grass or ragweed) solutions (100 PNU/ml), (2) heparin (10 μ /ml), (3) histamine (100 ng/ml), and (4) serum (4%). None of these agents stimulated significantly more O_2^- generation or Lf secretion than occurred in PBS/medium. Likewise, preincubation of the PMNs with these components did not enhance net O_2^- or Lf responses during subsequent incubation with OZ, 0.1 mg/ml. PMNs were also incubated with PBS containing 20% human serum to approximate the serum levels sometimes found in second- through fifth-hour antigen-site CF. This solution did not directly stimulate O_2^- or Lf response but did enhance the OZ-induced secretion of Lf (1251 ± 58 ng/ml versus 930 ± 96 ng/ml; $p < 0.05$ for PBS preincubated PMNs). This enhancement was equiv-

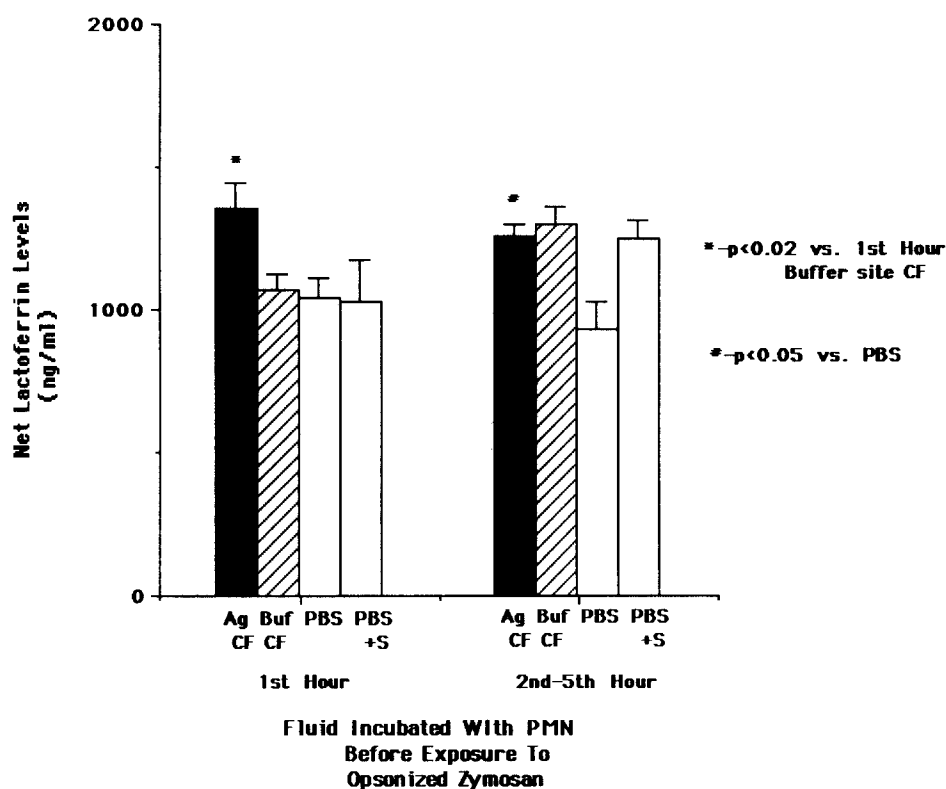


FIG. 5. Net Lf levels (mean $\pm$ SEM) in supernatants after incubation with OZ of PMNs previously incubated with (1) antigen-site CF (*Ag CF*), (2) buffer-site CF (*Buf CF*), (3) PBS, or (4) PBS plus 4% serum (*left panel*) or PBS plus 20% serum (*right panel*). Columns (*left panel*), CF obtained after first hour of antigen or buffer challenge; columns (*right panel*), CF obtained after second-through fifth-hour of challenge. (See text for definition of net Lf levels.)

alent to that observed in PMNs preincubated with second- through fifth-hour antigen-site CF.

Effects of basophil-activation supernatants

Because of the possibility that basophil activation contributes to continued histamine release that we have observed in antigen-challenged sites, we assessed the effects on blood PMNs of supernatants obtained after pollen-antigen challenge of basophil-enriched leukocyte populations from five sensitive cell donors. The histamine levels in these supernatants (3.6 ± 8) were comparable to levels observed in a number of our first-hour CF at antigen-challenged skin sites. When these fluids were used in vitro in the same vol/vol ratio as used for the CF in vitro studies described above, supernatants from antigen-challenged basophils induced no more O_2^- than did (1) supernatants of the same cell populations incubated with buffer or (2) PBS instead of supernatants (1.44 ± 0.5 versus 1.68 ± 0.6 versus ± 0.2 ; *p*, not significant).

LDH activity

Levels of LDH were not significantly different in the supernatants obtained from incubation of PMNs with the various CF compared with PMNs incubated with PBS/medium in the first step of the protocol (Fig. 6). Likewise, there were no higher levels of LDH in the supernatants incubated with OZ in the second step of the protocol. This finding suggests that the increased Lf levels did not reflect a nonspecific toxic release of intracellular PMN contents.

DISCUSSION

As noted previously, inflammatory cell responses may play major roles in the pathogenesis of late-phase reactions because of human IgE-mediated responses. Using a skin-chamber model, we have found a prominent accumulation of PMNs at the initiation of such reactions. These cells were activated, as witnessed by a polarized appearance and increased hydrogen peroxide.⁷⁻¹⁰ Furthermore, there was increased release of the PMN components, Lf and elastase, into the CF

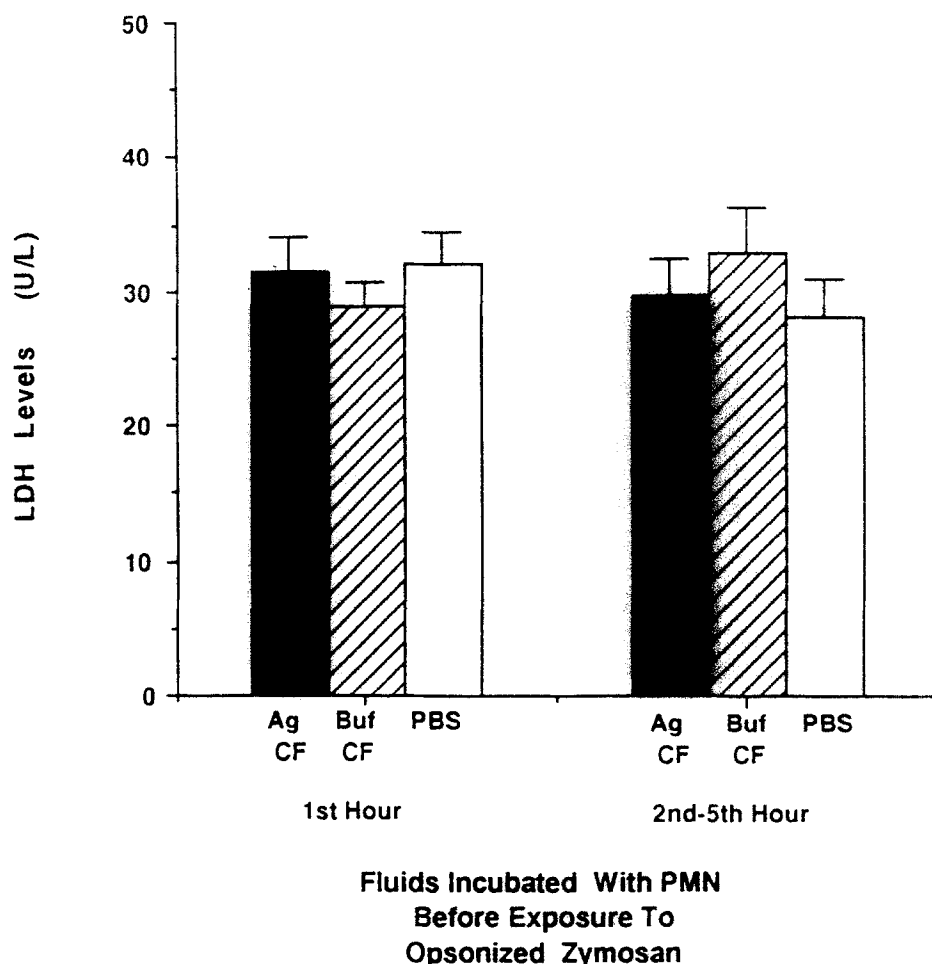


FIG. 6. LDH levels (mean $\pm$ SEM) in supernatants after incubation with OZ of PMNs previously incubated with (1) antigen-site CF (*Ag CF*), (2) buffer-site CF (*Buf CF*), or (3) PBS. Columns (*left panel*), CF obtained after first hour of antigen or buffer challenge; columns (*right panel*), CF obtained after second- through fifth-hour of challenge.

overlying the allergic-reaction sites.¹¹ There was no increased activity of blood PMNs obtained concomitantly. The nature of the stimulus for this local PMN activation was not known.

The findings reported in this study provide important information to help answer this question. Skin CF obtained after the first hour of antigen challenge consistently stimulated resting blood PMNs to generate O_2^- and secrete Lf. Furthermore, PMNs incubated with these antigen-site CF exhibited enhanced O_2^- and Lf responses during subsequent incubation with suboptimal concentrations of the well-known PMN stimulant, OZ. These direct and indirect effects of first-hour antigen-site CF were significantly more than effects induced by fluids obtained after first-hour at the buffer (control)-challenged sites. The latter responses were, in turn, significantly greater than re-

sponses observed in blood PMNs incubated with the diluent (PBS) solution alone. Our assay system may actually underestimate this stimulating effect, since the CF were diluted 1:20 in the in vitro incubation mixture used in this study.

Therefore, it could be postulated that PMNs accumulating in the skin site of an immediate IgE-mediated reaction are stimulated by agent(s) liberated in the early phase of this reaction. The nature of these components is still not defined. We incubated resting blood PMNs with substances we know to be present in the overlying CF after first hour of antigen challenge (pollen-antigen solutions, heparin, histamine, and serum proteins in concentrations similar to that found in the CF). No significant direct stimulatory effect or enhancement of OZ-stimulated PMN responses was observed. It is conceivable that a mixture of these

agents could be the responsible stimulus. However, in preliminary experiments, we found no stimulation of PMNs for a mixture of pollen antigen, heparin, histamine, serum protein (from donors sensitive to these pollens), and exogenous human skin tryptase, each in concentration similar to that present in the first-hour antigen-site CF.

In contrast to the first-hour fluids, the CF obtained after the second through fifth hour of antigen challenge did not stimulate resting blood PMNs nor consistently enhance OZ-induced O_2^- /Lf responses. This group of findings was rather surprising, since the second-through fifth-hour fluids were present when local PMN accumulation started and granule protein secretion had been noted by us.¹¹ It may be that products released earlier in the IgE-mediated response are responsible for local PMN activation.

We also found no stimulatory effect on blood PMNs of supernatants obtained after in vitro challenge by pollen antigens of basophil-enriched blood leukocyte populations from donors sensitive to those antigens despite the presence of prominent histamine released into such supernatants. This finding is of importance because of indirect evidence that basophils may be the source of histamine released into skin chambers after the first hour of continued antigen challenge of our subjects.

These findings can be considered in light of previous findings about other mediators released in these allergic-reaction sites. We have previously found increased levels of the LTB_4 , LTC_4 , and LTD_4 , and PAF in CF starting after the first hour of antigen challenge in sensitive subjects.^{1, 12} PAF can bind to PMNs with great affinity.^{13, 14} However, both PAF and LTB_4 are generally very weak stimuli of O_2^- generation in the absence of enhancing agents like cytochalasin B.¹⁵⁻¹⁷ We have confirmed these observations in our laboratory. PAF has been reported to enhance O_2^- production stimulated by suboptimal concentrations of PMA or the chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine.^{18, 19} We have found modest stimulation of Lf secretion by PAF or LTB_4 in the absence of cytochalasin B.²⁰ Zimmerli et al.²¹ described increased O_2^- and hydrogen peroxide production by PMNs exuding into chambers filled with undiluted autologous serum. However, in the present study, we have not found impressive in vitro stimulation of O_2^- or Lf responses by serum proteins in concentrations similar to that found previously by us in the CF overlying antigen-challenged sites.

Another relevant question is whether the PMN responses described above are due, in part, to an increased reactivity of PMNs from atopic subjects. One study described increased O_2^- generation by PMNs

from atopic subjects, both spontaneously and during incubation with calcium ionophore A23187 and suboptimal concentrations of *N*-formyl-methionyl-leucyl-phenylalanine.²² We found increased PAF-induced secretion of Lf by PMNs of atopic subjects not, apparently, caused by serum factors.²⁰ However, we and other investigators found no increased PAF-induced O_2^- generation or cytosolic Ca^{++} shifts in the PMNs of atopic subjects.²³ In the studies reported in this article, there was no greater O_2^- or Lf responses in PMNs from atopic subjects to OZ, a very consistent stimulus of PMN activation in our experience. The very limited amount of CF available precluded comparison of the effects of individual fluids on PMNs of atopic versus nonatopic subjects.

The consequences of PMN activation in allergic-reaction sites could be considerable. There was a strong correlation between O_2^- and Lf responses induced by these CF in individual PMN populations, suggesting a broad-based PMN activation. However, these events are not just due to release from damaged PMNs, since the increased Lf secretion was not accompanied by increased release of LDH, a marker of PMN damage. Reactive oxidants like O_2^- have been demonstrated to exert direct toxic effects on connective tissue.²⁴ Lf is a 78 kd iron-binding protein whose role in PMN responses is still uncertain. Some studies describe a down-modulatory effect of Lf,²⁵⁻²⁷ whereas other studies describe enhanced production of hydroxyl radical, a particularly potent oxidant.^{28, 29} This activity may be responsible for enhanced damage of vascular endothelium by reactive oxidants in the presence of Lf.

In conclusion, it appears likely that events occurring early in IgE-mediated responses attract and then activate PMNs accumulating in allergic reaction sites. The exact nature of the stimulatory components and the biologic effects of such PMN activation require further definition.

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