Comparison of Inflammatory Events during Developing Immunoglobulin E-Mediated Late-Phase Reactions and Delayed-Hypersensitivity Reactions

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To compare cellular and mediator responses in early developing late-phase skin reactions (LPR) and delayed-hypersensitivity (DH) reactions in the same subjects, responses in skin chambers overlying sites of challenge with pollen antigen and Candida albicans antigens were compared in six humans with demonstrated prominent LPR and DH responses. Histamine levels in overlying chamber fluids at 1 h were much higher at LPR than at DH sites (P = 0.002). After the next 4 h, leukocyte exudation was higher at LPR than at DH sites (P = 0.005). Most leukocytes were activated neutrophils with greater frequency of superoxide-secreting cells and released lactoferrin at LPR than at DH sites (P = 0.01 and P = 0.02, respectively). The frequency of exuding eosinophils was higher, but not significantly so (P = 0.5), at LPR sites. Although significantly more eosinophils at LPR sites were activated (P = 0.02), the levels of released eosinophil cationic protein were not significantly higher at LPR sites (P = 0.09). The levels of interleukin-8 (IL-8), but not IL-6, were greater at LPR than at DH sites. During the first 5 h of challenge there was greater mast cell activation and subsequent exudation of activated neutrophils at sites of developing LPR than at DH sites, possibly related to greater local IL-8 levels. The frequency of activated eosinophils was also greater at LPR sites. These different initial inflammatory responses could play a role in determining expression of LPR or DH reactions.

Both late-phase immunoglobulin E-mediated reactions (LPR) and delayed-hypersensitivity (DH) responses to microbial antigens in the skin are characterized by erythematous, indurated reactions (1, 8, 22). Findings in sequential biopsy samples suggest that T lymphocytes play a pathogenic role in LPR as well as DH reactions (4, 5, 19). However, it is felt that different pathogenic mechanisms underlie LPR and DH responses. There may be different profiles of T-cell subpopulations in established LPR and DH (5, 15). Granulocyte accumulation is seen in biopsy samples obtained during the first hours after challenge in both LPR and DH (1, 19). However, there is a more prominent accumulation of eosinophils in LPR (4, 19).

We have focused our previous investigations on the inflammatory events occurring during the first 5 to 6 h after intradermal challenge with antigen leading to grossly well-expressed LPR by the sixth hour. Using a skin chamber model, we have noted the accumulation, by the sixth hour, of neutrophils and eosinophils, release of their granular proteins, and increased local levels of several proinflammatory mediators, including cytokines (12, 14, 20, 21, 24). It is felt by several investigators of DH reactivity that expression of this immune response is actually initiated in the first several hours after intradermal antigen challenge, even though the DH reaction may not be grossly apparent until at least 12 h later (reviewed in references 1 and 22). Because local accumulation of granulocytes is found in biopsy specimens obtained in the first several hours after intradermal antigen injection leading to both LPR and DH reactions, it is important to compare the profiles of inflammatory events occurring during these early hours after antigen challenge which may determine the subsequent gross expression of LPR or DH in the skin. We have used our skin chamber approach to make such comparisons for human subjects manifesting both types of responses.

MATERIALS AND METHODS

Subjects and skin testing. In screening studies, we identified six human volunteers, healthy except for seasonal allergic rhinitis, who manifested similarly sized erythematous, indurated reactions 24 h after intradermal injection of both grass or ragweed pollen extract (100 protein nitrogen units [PNU]/ml Greer Labs, Lenoir, N.C.) (induration diameter, 9 ± 2 mm) and Candida albicans extract (500 PNU/ml; Greer Labs) (induration diameter, 12 ± 3 mm). There were minimal reactions (≤1-mm diameters) at 24 h to intradermal injection with the buffered solution used to dilute these extracts. No subject was receiving medication at the time of the study.

Skin chamber studies. Skin chamber studies were performed outside the relevant pollenating season as previously described (12). In brief, four blisters were induced on the volar surfaces of the forearms by gentle heat and suction. The epidermal blister roofs were removed from the blister bases at the dermo-epidermal junction, and collection chambers were appended. After irrigation of each blister base with buffer diluent (phosphate-buffered saline [PBS] twice for 5 min time, 0.3 ml of grass or ragweed pollen antigens [Greer Labs] diluted to 100 PNU/ml in PBS containing heparin at 10 U/ml (solution B) was placed in the chambers at one site. Candida extract (500 PNU/ml) diluted in the same PBS-heparin solution was placed in chambers at two additional sites. As a control, the PBS diluent containing heparin at 10 U/ml (solution B) was placed in the chambers at the fourth site. All solutions had been shown to be endotoxin free by the Limulus test. After 1 h, the fluids were removed, and the fluids from duplicate chambers were pooled. They were then assayed for histamine levels with an enzyme-linked immunosorbent assay (ELISA) (Immunotech, Westbrook, Maine) sensitive to 0.1 ng/ml.

The chamber bases were then irrigated with PBS, and 0.5 ml of fresh PAG Candida solution, or solution B (all containing heparin at 10 U/ml) was placed in each of the chambers previously containing like solutions and allowed to remain for an additional 4 h. Fluids from the four challenge chambers were removed, and these fluids from duplicate sites of Candida challenge were pooled, and the total cell counts in the fluids at the PAG, Candida, and solution B challenge sites were assessed. These fluids were then centrifuged, and the supernatants were analyzed for levels of histamine, eosinophilic cationic protein (ECP) (measured with a radioimmunoassay [Pharmacia, Piscataway, N.J.] sensitive to 2 ng/ml), lactoferrin (measured with an ELISA sensitive to 2 μg/ml, as described by us [20]), interleukin-6 (IL-6) (measured with an ELISA [R&D, Minneapolis, Minn.] sensitive to 3.1 pg/ml), and IL-8 (measured with an ELISA [R&D] sensitive to 31
of cells exuding at the various challenge sites, the frequency of NBT
mined. Superoxide-secreting granulocytes are considered activated since resting
prior to induction of the skin blister on the day of the experiment. A predomi-
these cells were suspended in the NBT solution described above (10^6 cells/ml)
by density gradient centrifugation, as utilized previously by us (10). Aliquots of
at 37°C for 30 min in a CO2 incubator, the cells were cytocentrifuged, fixed in
Labs] and phorbol myristic acetate [10 ng/ml; P 8139; Sigma]). After incubation
either alone or with added agonists (tumor necrosis factor alpha [5 U/ml; R&D

| Sites                | Total leukocyte | % Eosinophils | % NBT^ cells | % EG2^ cells |
|----------------------|----------------|---------------|--------------|--------------|
| PAg challenge        | 6.1 ± 1.1^a    | 19 ± 9^b      | 60 ± 11^c,d  | 13 ± 3^e     |
| Candida challenge    | 0.5 ± 0.1      | 12 ± 5^b      | 25 ± 6^b     | 2 ± 1        |
| Solution B challenge | 0.8 ± 0.3      | 4 ± 2         | 9 ± 5        | 1 ± 1        |

^a P = 0.05 versus value for Candida challenge sites.
^b P = 0.01 versus value for solution B challenge sites.
^c P = 0.02 versus value for Candida challenge sites.
^d P = 0.01 versus value for Candida challenge sites.

pg/ml). The cell pellets were resuspended in PBS-heparin. Aliquots were either
counted for total cells (with a Coulter Counter [Coulter, Hialeah, Fla.], cyto-
centrifuged (apparatus from Shandon) for subsequent differential counting by
Wright's staining (Dif-Quik), or cytocentrifuged and studied by immunocyto-
chemical approaches to determine the percentage of cells binding the EG2
antibody (Pharmacia) as described by us (23).

Immediately after removal of the skin chambers and the fluid contained
therein, 10-mm-diameter sterile cover glasses were appended firmly to the blister
bases for 15 min to obtain the cell imprints. The cover glasses were then removed
and, while still moist, were immediately placed in 25-mm-diameter wells of a
tissue culture plate containing a solution of nitroblue tetrazolium (NBT)
(N 5514; Sigma, St. Louis, Mo.). After a 30-min incubation at 37°C in a CO2
incubator, the NBT solution was aspirated, and the cover glasses were kept for
1 min at room temperature to evaporate any residual fluid. Absolute methanol
was added for 5 min for fixation and then aspirated, and the cover glasses were
counterstained with 1% safranin to determine the total number of cells counted.
Stained cover glasses were mounted faceup on coded microscope slides, and a
fresh cover glass was appended to each with Permount. In each imprint the
percentage of safranin-stained cells which showed prominent deposition of the
formazan metabolite (>3 grains/cell) due to superoxide activity (17) was deter-
mained. Superoxide-secreting granulocytes are considered activated since resting
granulocytes rarely secrete superoxide prominently (17).

To help ascertain the significance of cells producing superoxide in populations
of cells exuding at the various challenge sites, the frequency of NBT^ cells in
these populations was compared with that in autologous blood leukocytes. Blood
specimens were collected into heparin-containing tubes from all subjects just
prior to induction of the skin blister on the day of the experiment. A predomi-
nantly granulocyte (>90%) subpopulation of the blood leukocytes was obtained
by density gradient centrifugation, as utilized previously by us (10). Aliquots of
these cells were suspended in the NBT solution described above (10^6 cells/ml)
either alone or with added agonists (tumor necrosis factor alpha [5 U/ml; R&D Labs] and phorbol myristic acetate [10 ng/ml; P 8139; Sigma]). After incubation
at 37°C for 30 min in a CO2 incubator, the cells were cytocentrifuged, fixed in
methanol, counterstained with safranin, and mounted in Permount, as described
above.

Statistical analyses. Cell counts and levels of cytokines and inflammatory
mediators in the skin chamber fluids at the PAg, Candida, and solution B sites in
the same individuals were compared by paired Student’s t test when the data
were normally distributed and by Wilcoxon’s rank sum test when nonparametric
statistics were required. Associations between different inflammatory responses
in the same chamber fluids were investigated by Spearman’s rank correlation
analysis.

RESULTS

Histamine levels after the first hour of challenge. The his-
tamine levels were much higher at the PAg sites (69 ± 10 ng/ ml) than at the Candida sites (2 ± 1 ng/ml). The level at the latter sites was not significantly different from that at the solution B (control) challenge sites (2 ± 2 ng/ml).

Inflammatory events during the second to fifth hours of challenge. The number of cells exuding from the PAg challenge sites into the overlying skin chamber fluid (6.1 × 10^5 cells) was significantly greater than the very low number of cells in the pooled chamber fluids from the two Candida challenge sites (0.6 × 10^5 cells/site) (P = 0.005) (Table 1). Indeed, we had set up a protocol to pool fluids from duplicate Candida challenge sites to get reliable cell counts in case the number of cells exuding from an individual Candida site was very low. If anything, the frequency of cells exuding at these sites was somewhat lower than that at solution B (control) challenge sites (Table 1).

The large majority of the exuding cells were neutrophils. The relative frequency of eosinophils in the cells at both the PAg and Candida sites was quite variable from subject to subject, and it was not correlated significantly with the percentage of eosinophils in the blood of individual subjects. Therefore, the mean frequency of eosinophils in fluids from the PAg sites (19% ± 9%) was higher than that in fluids from the Candida sites (12% ± 5%), but not significantly so (P = 0.5). The frequency of eosinophils in the solution B challenge sites was significantly lower than that at either the PAg or Candida challenge sites (4% ± 2%) (P ≤ 0.01). There were very few (<5%) lymphocytes in the chamber fluids obtained at the PAg or Candida challenge sites. We have consistently found few lymphocytes in chamber fluids obtained after 6 h of PAg chal-

Many of the neutrophils exuding in greater numbers at PAg than at Candida challenge sites were activated, so the levels of lactoferrin, released from the specific granules of neutrophils, were higher at PAg sites (13.2 ± 3.7 μg/ml) than at Candida challenge sites (3.8 ± 1.6 μg/ml) (P = 0.02) (Table 2). The levels at the latter sites were not significantly different from those at the solution B challenge sites (3.0 ± 2.0 μg/ml).

Also, the frequency of cells which were strongly NBT^, presumably due to secreted superoxide (17), was significantly higher at the PAg sites (60% ± 11%) than at the Candida challenge sites (25% ± 6%) (P = 0.01). The responses at both these sites were significantly greater than that in cells exuding at the solution B challenge sites (9% ± 5%) (P ≤ 0.01) (Table 1). The mean frequency of strong NBT^ cells in the exudate at the PAg challenge sites (60%) approached that (87% ± 8%) seen in autologous blood granulocytes which were obtained just prior to the skin chamber study and then stimulated in vitro by phorbol myristic acetate, a potent neutrophil activator. Very few of these autologous blood neutrophils were NBT^ when incubated without agonists (1% ± 1%).

Although the frequency of eosinophils in the exudate at the PAg challenge sites was only modestly greater than at the Candida sites, the majority of these eosinophils appeared to be activated, as indicated by the frequency of EG2^- cells at the PAg sites (12.5% ± 3%), which was significantly greater than the frequency of EG2^- cells at the Candida sites (2% ± 1%) (P = 0.02) (Table 1). However, the skin chamber levels of ECP, released from activated eosinophils, varied considerably among subjects. Therefore, the mean ECP level in fluid at PAg sites (71 ± 23 ng/ml) was higher than that at Candida sites (26 ± 12 ng/ml), but not significantly so (P = 0.09) (Table 2). The mean ECP level at the Candida sites was not much higher than that seen at the solution B (control) sites (18 ± 5 ng/ml).

Cytokine levels after the second to fifth hours of challenge. The mean IL-6 levels at the PAg, Candida, and solution B

| Sites                | Lactoferrin (μg/ml) | ECP level (ng/ml) | IL-6 level (pg/ml) | IL-8 level (pg/ml) |
|----------------------|---------------------|-------------------|--------------------|--------------------|
| PAg challenge        | 13.2 ± 3.7^a        | 71 ± 23           | 3.051 ± 632        | 5,871 ± 1,564^d    |
| Candida challenge    | 3.8 ± 1.6           | 26 ± 12           | 3.086 ± 1,178      | 1,787 ± 979        |
| Solution B challenge | 3.0 ± 2.0           | 18 ± 5            | 4.046 ± 1,435      | 1,242 ± 387        |

^a P = 0.02 versus value for Candida challenge sites.
^b P = 0.01 versus value for solution B challenge sites.
^c P = 0.04 versus value for Candida challenge sites.
challenge sites were quite similar (3.051 ± 0.632, 3.086 ± 1.178, and 4.046 ± 1.435 pg/ml, respectively), but with considerable variation in levels among the six subjects (Table 2). This lack of a significant difference between the IL-6 levels at the PAg and solution B challenge sites confirmed findings in other subjects recently reported by members of our group (24) but was different than the increased levels in PAg site chamber fluids reported previously by another group (7).

The skin chamber IL-8 levels after 2 to 5 h were significantly higher at sites of PAg challenge than at solution B challenge sites (5.871 ± 1.564 and 1.242 ± 0.387 pg/ml, respectively (\(P = 0.02\)) (Table 2). This finding confirmed earlier observations by members of our group (24). The IL-8 level at Candida challenge sites (1.578 ± 0.797 pg/ml) was significantly less than that at PAg challenge sites (\(P = 0.04\)) (Table 2). However, there was marked variation in IL-8 levels at Candida challenge sites among the study subjects, with the mean value not significantly higher than the levels seen at the control (solution B) challenge sites (\(P = 0.7\)). The levels of IL-8 correlated significantly with the levels of lactoferrin in the PAg site fluids of individual subjects (\(P < 0.05\)), confirming earlier observations by members of our group (24). However, there was no significant correlation between the IL-8 levels and neutrophil numbers in chamber fluids at individual sites.

DISCUSSION

The prominent LPR seen in the skin of some subjects following strong immediate immunoglobulin E-mediated reactions is often similar in gross appearance to the classic DH reactions to microbial antigens at 24 h. However, there are some differences between the patterns of gross inflammation in those two types of responses: (i) a prominent wheal-and-flare reaction at 20 to 30 min almost always precedes LPR but is very unusual before the onset of a true DH reaction (1); (ii) the LPR often starts as a continuation of the edematous reaction within 2 to 3 h of intradermal challenge, whereas gross inflammation is generally not apparent at DH reaction sites until at least 6 h, when LPR are generally already well developed (1, 23); and (iii) in our experience, gross LPR frequently begin to wane by 24 h and are present at 48 to 72 h in less than 50% of individuals with earlier LPR. In contrast, prominent DH reactions are grossly apparent for at least 48 h after challenge (1, 22).

The histologic patterns of LPR and DH seen in sequential biopsy samples also differ somewhat. Neutrophils frequently accumulate within several hours after challenge in both LPR and DH. Eosinophils are more prominent at LPR sites, while monocytes/macrophages are prominent at later periods in DH but not LPR. Mast cells are now thought to play important roles in the initiation of DH reactions as well as LPR (1, 8). Basophils are likely recruited into the sites of both DH and LPR (1, 3) and may be the source of the prolonged local histamine release seen in the latter (11).

Perhaps the most impressive histologic difference between LPR and DH responses involves T lymphocytes. Several groups have reported a significant increase in T-cell numbers in biopsy samples obtained from LPR sites 6 h after intradermal antigen challenge, persisting for up to 96 h (4, 5, 15). In situ hybridization studies suggested that these cells were of the TH-2 type, containing mRNA for IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (15). In contrast, T lymphocytes accumulated later (at 24 to 48 h) at sites of DH reactions, with predominantly mRNA for gamma interferon and IL-2 found by in situ hybridization (15). We have found a more variable pattern of T-lymphocyte accumulation at LPR sites at 6 h, with impressively greater numbers of T cells in antigen challenge sites than in buffer-treated control sites in some, but not all, subjects (23). In some individuals, there was a more prominent increase in the frequency of T cells at 24 h than at 6 h. We have found increased levels of mRNA for IL-5 by reverse transcriptase PCR at 6 and 24 h at LPR sites, but not at DH sites (9, 16). Therefore, further clarification of the mechanisms underlying LPR and DH responses was needed.

In the present study, we did find several significantly different responses occurring during the initiation of LPR and DH reactions. First, there was evidence of immediate mast cell activation leading to prominent histamine release at sites of developing LPR but not at sites of developing DH reactions. Such histamine release could be accompanied by secretion of other mast cell components that directly or indirectly lead to inflammatory LPR.

In the present study, we also found significantly greater neutrophil exudation into the skin chambers overlying sites of developing LPR than in those overlying sites of developing DH reactions during the first 5 h of challenge. Many of the neutrophils accumulating in greater numbers in fluids overlying sites of developing LPR were activated, secreting superoxide and releasing lactoferrin, a granular protein. The stimulus for this neutrophil response in LPR has not yet been defined. We have previously found agents released into skin chamber fluid early during PAg challenge, such as leukotriene B\(_4\), which will attract and/or activate neutrophils in vitro (10). There has been considerable interest in the possible roles of cytokines in skin inflammatory reactions (6). Lee et al. reported increased levels of IL-6 in the fluids of skin chambers overlying sites of developing LPR in a temporally biphagic pattern (7). In the present study, we found no difference in IL-6 levels at LPR and control sites. Members of our group have also found increased levels of several chemokines, including IL-8 and regulated-upon-activation, normal-T-cell-expressed and -secreted chemokine (RANTES), in chamber fluids overlying sites of developing LPR (24). Significantly greater IL-8 levels were found in chamber fluids at the sites of developing LPR than at the sites of developing DH reactions in the study reported here. IL-8 levels correlated with lactoferrin levels in the LPR site fluids, supporting the concept that IL-8 played a major role in the activation of neutrophils found at LPR sites. IL-8 is a potent neutrophil chemoattractant and activator and can be released by other cells at such reaction sites (13). There were very few lymphocytes present in the chamber fluid as a possible source of IL-8 or another neutrophil chemoattractant. However, it is conceivable that IL-8 could be released from lymphocytes in the underlying dermis or from keratinocytes in the surrounding epidermis.

Somewhat surprisingly, there was not a significantly greater exudation of eosinophils into the chambers at the LPR than at the DH sites, although the frequency of ECP\(^+\) (putatively activated) eosinophils was significantly higher in the LPR site exudates. However, the release of ECP, a cationic granule protein of eosinophils, was quite variable at both LPR and DH sites, resulting in a nonsignificant difference in mean ECP levels in these two response patterns. The ECP levels in the PAg site fluids, but not in the Candida site fluids, were significantly greater than at the solution B-treated (control) sites. A small percentage of the ECP found at both PAg and Candida sites could be derived from that present in the modest exudate containing plasma proteins released into the overlying skin chambers during the first 5 h of PAg and Candida challenge (2). However, previous immunofluorescence studies have shown marked deposition of eosinophil granule proteins in the dermis during the first 5 h of PAg challenge (21).
Members of our group have previously found elevated levels of RANTES in fluids from chambers overlying PAg challenge sites (24). RANTES is a potent attractant and activator of eosinophils (18). Unfortunately, there were insufficient amounts of chamber fluid available to compare RANTES levels at PAg and Candida challenge sites in the present study. mRNA for IL-5, a cytokine which can activate and prolong survival of eosinophils, has been found at LPR sites (15, 16). It has also been found that such IL-5 mRNA deposition correlates with the modest percentage of cells containing IL-5 protein (9). However, we have not found IL-5 protein released into the overlying chamber fluids at PAg challenge sites, possibly because of inadequately sensitive techniques.

Thus, it appears that LPR and DH reactions, which may appear grossly similar at 24 h after intradermal challenge, are characterized by different patterns of mast cell activation and granulocyte response during the first 5 h of challenge. The greater neutrophil responses at sites of developing LPR may be due to the higher local IL-8 levels at such sites. Further characterization of the causal inflammatory mediators may enhance our understanding of pathogenic mechanisms in these two types of immune responses.

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