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Transient Absence of C5a-Specific Neutrophil Function in Inflammatory Disorders of the Skin*

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Chemotactic migration, production of superoxide anion (O_2^-), and the release of β-glucuronidase from azurophilic granules were determined in polymorphonuclear leukocytes (PMN) from 135 patients with infectious (e.g., pyoderma, acne conglobata, erysipelas) as well as noninfectious (psoriasis) skin diseases. Purified C5a and the formylated tripeptide FMLP were used as stimuli. In addition, longitudinal profiles of PMN activities were performed at daily intervals in several patients. There was a complete absence of PMN responses (chemotaxis, O_2^--production, and enzyme release) specifically induced by C5a in 25 patients suffering from various inflammatory diseases of the skin. In these patients PMN responsiveness for the tripeptide FMLP was either normal or increased. The C5a-dependent defect of PMN was transient and correlated with disease activity. When normal PMN were incubated with sera from C5a-defective patients, no inherent stimulatory or inhibitory activities compared to control sera were seen.

Pretreatment of normal PMN in vitro with various concentrations of C5a failed to *completely* deactivate PMN *without* affecting FMLP dependent functions.

These observations demonstrate the presence of a functional defect in circulating PMN during acute cutaneous inflammation. The in vitro experiments suggest transient blocking of C5a-dependent PMN functions by a cell-bound factor which seems not to be C5a or $C5a_{desarg}$.

Polymorphonuclear leukocytes (PMN) play an important role in inflammatory diseases associated with tissue damage. In vitro these cells demonstrate a number of functions, e.g., aggregation, adhesion, migration, production of oxygen radicals, phagocytosis, and enzyme release [1]. Neutrophil activities can be triggered by specific chemotaxins including the complement split product C5a and the synthetic tripeptide FMLP (formyl-methionyl-leucyl-phenylalanine). Both chemotaxins are multifunctional in stimulating chemotaxis, production of O_2^-, as well as release of enzymes, and both have been used extensively in the study of PMN functions.

In vivo regulation of PMN function is rather complex and triggering and inhibiting factors need to be considered. In addition, PMN functional responses to one cytotaxin are reported to vary in inflammatory conditions, e.g., in some diseases chemotactic migration is seen to be stimulated [2–7], whereas some authors have found these responses to be depressed [8–11].

In the present study we have compared C5a-induced neutrophil activities with those elicited by FMLP in patients suffering from PMN-related inflammatory skin diseases. As a result a transient, stimulus-specific defect was observed demonstrating complete absence of PMN responses to C5a. This functional defect of PMN is disease-nonspecific and becomes fully restored after resolution of disease.

MATERIALS AND METHODS

Patients

All patients were hospitalized for severe skin conditions. They accepted the proposed investigations by written consent. None of the patients was under therapy when the study was started and treatment was applied as indicated. The clinical diagnoses were confirmed by biopsy in psoriasis and pustular psoriasis as well as by routine laboratory tests in patients with erysipelas, acne conglobata, and pyoderma.

Isolation of PMN

Neutrophils were isolated as previously described [6] using a slight modification of the method of Henson [12].

Using this technique the final cell preparations contained more than 90% neutrophils with a viability of greater than 97% as assessed by trypan blue exclusion.

Chemotactic Factors

Purified C5a was prepared according to a combination of the methods described by Fernandez and Hugli [13] and Beebe et al [14]. Briefly, fresh human serum was activated with 20 mg/ml heat-inactivated baker's yeast for 30 min at 18°C. Then ε-aminocaproic acid (Sigma Chemical Co, Munich, F.R.G.) was added to give a 1 M solution and incubated for additional 1 h at 37°C.

Thereafter EDTA was added to give 10 mM. Ice-cold 1 M HCl was added resulting in a final pH of 3.7. The solution was kept cold, centrifuged, and the supernatant diafiltered against 0.1 M ammonium formate buffer, pH 5.0, using a YM 5 ultrafiltration membrane (Amicon Corp. Lexington, Massachusetts). Thereafter polyethylenglycol (M_r 8000, Sigma) was added giving a final concentration of 10%. The mixture was stirred, centrifuged, and the supernatant applied to a CM-cellulose column (2.6 × 10 cm) equilibrated in 0.1 M ammonium formate, pH 5.0.

PMN-O_2^--producing activity was eluted with a linear gradient of 0.5 M ammonium formate, pH 5.0. These fractions were collected, diafiltered against 0.1 M ammonium formate, lyophilized, and the residue applied to a Sephadex G-75 column (5.0 × 90 cm), equilibrated with 0.1 M ammonium formate buffer, pH 5.0.

Fractions inducing PMN-O_2^- release were collected (M_r 15,000), lyophilized and the residue chromatographed on a CM-cellulose CM 32 column (0.9 × 10 cm), equilibrated with 0.15 M ammonium formate buffer, pH 5.0. The O_2^--generating fractions were pooled, concentrated, diafiltered against 0.1 M ammonium formate (pH 7.0) and applied to a CM-Sephadex C-25 column (0.9 × 10 cm), equilibrated with 0.1 M ammonium formate, pH 7.0. C5a was eluted with the same buffer and showed a single line with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9%). For neutrophil function tests, highly purified as

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Abbreviations:
BSA: bovine serum albumin
C5a: complement split product C5a
$C5_{fr}$: low-molecular-weight fragment of complement factor C5
ECCA: endogenous component chemotactic assay
FMLP: formyl-methionyl-leucyl-phenylalanine
O_2^-: superoxide anion production
PBS: phosphate-buffered saline
PMN: polymorphonuclear leukocytes
ZAS: zyomosan-activated serum

well as partially purified C5a (pool of chemotactic activity after G-75 chromatography) was solubilized in 10^{-3} M HCl and stored below −70°C using a pool of small volumes.

Formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) was purchased from Sigma. It was dissolved in DMSO at 10^{-2} M, diluted with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1 glucose giving a 4×10^{-6} M solution, which was stored at −70°C in small portions.

Chemotactic and Random Migration

Chemotactic and random migration of PMN were assayed as described earlier [7] or by using a modification of the endogenous component chemotactic assay (ECCA) method described by Creamer et al [15], however using β-glucuronidase instead of lactic acid dehydrogenase as marker enzyme.

Both methods apply blind-well Boyden chambers (Bio Rad, Munich, F.R.G.).

Production of Superoxide Anions (O_2^-)

Superoxide anion production was measured using a slight modification of the method described by English et al [16]. In order to ascertain proper controls superoxide dismutase (bovine blood, Sigma) was added to one control tube in each experiment prior to the addition of cells to stop the reduction of cytochrome C by O_2^- [17]. Reduced cytochrome C was assayed in supernatants by measuring extinctions at 550 and 540 nm (Hitachi 100-80 spectrophotometer) vs blanks containing superoxide dismutase at the start of the reactions. O_2^- concentrations were calculated using an extinction coefficient of 15.5/mM·cm (ferrocytochrome c minus ferricytochrome c) [16]. When O_2^- production elicited by FMLP was measured, 5×10^5 cells per assay were used whereas for C5a-dependent O_2^- release 2×10^6 PMN, which were preincubated (10 min, 37°C) with cytochalasin B (5 μg/ml), were used.

Enzyme Release

The marker enzyme of PMN azurophilic granules, β-glucuronidase, was measured by a slight modification of the method described previously [7] using *p*-nitrophenyl-β-D-glucuronide instead of phenolphthalein-β-D-glucuronide as substrate. A 100% control was obtained by addition of Triton X-100 (final concentration: 0.1%) instead of the stimulus. Release of enzymes was expressed in percent of the Triton X-control (= 100%).

RESULTS

Abnormalities in C5a Responsiveness of Patients' PMN

Dose responses for C5a- (Fig 1) and FMLP-induced chemotaxis, O_2^- release, and enzyme release were studied in 136 patients with different diseases of the skin and 46 healthy persons. In all of these diseases inflammation was restricted to skin and had started within skin. Systemic symptoms such as fever, arthralgia, and high blood sedimentation rates were seen in patients suffering from erysipelas, acne conglobata, and psoriasis pustulosa.

In a total of 25 patients, isolated peripheral neutrophils were found to be unresponsive to C5a (Table I). Absence of functional responsiveness was seen when all three parameters—chemotaxis, O_2^- production, and enzyme release—were tested. Furthermore this phenomenon was noted with all C5a concentrations used and was still present at a 10- to 20-fold dose eliciting a half maximum chemotactic response (Table II).

Nonstimulated (random) migration was not inhibited, indicating that receptor-dependent stimulus recognition by PMN, but not cellular motility, was affected. Considering the diseases under investigation, the proportion of patients demonstrating absence of C5a-inducible responses was highest in erysipelas, pustular psoriasis, and in patients suffering from widespread pyoderma (Table I). On the other hand, we were unable to detect absence of dose responses in these patients when FMLP was used for activation of PMN function (Table II). Instead, most patients showed increased responsiveness of PMN against FMLP (data not shown). In 46 healthy control persons no evidence for functional PMN defects was found (Table I).

Longitudinal Profiles

In a patient with freshly developing erysipelas of the lower leg we examined PMN functions at near daily intervals (Fig 2). In this patient PMN responses to C5a remained normal 4 h after the patient became ill and showed rising body temperatures. However, on the next day C5a-dependent PMN functions were nearly absent and this included dose responses for the generation of O_2^-, chemotaxis, and release of β-glucuronidase. The responses to C5a were absent until day 6 (Fig 2). During this time PMN functions elicited by FMLP (chemotaxis, generation of O_2^-, and release of β-glucuronidase) remained unaltered (Fig 2). No correlation was seen between body temperature and the absence of PMN responses to C5a.

In other patients we observed this type of PMN nonresponsiveness lasting from 1 day to nearly 1 week (Table III). In all our patients periods of defective PMN function were followed by full restoration of C5a-induced reactions after variable lengths of time (one to several days). In patients showing systemic signs such as fever, arthralgias, and high blood sedimentation rates, the defect appeared to last longer as compared to patients without such symptoms.

Studies with "C5a Nonresponder" Sera

In order to further elucidate the nature of C5a nonresponsiveness, chemotactic activity of serum samples from affected patients was tested using PMN from healthy controls. As can be seen in Fig 3, there was no inherent chemotactic activity in sera from affected patients as compared to control sera or sera activated by zymosan. Furthermore, neutrophils from healthy persons when preincubated with fresh serum from C5a nonre-

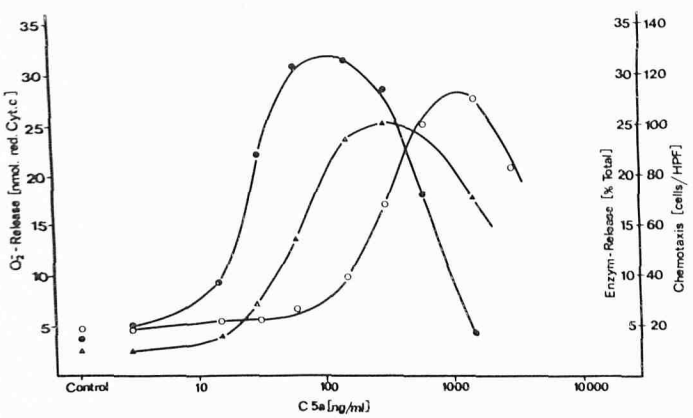

FIG 1. Dose responses for chemotactic migration (●), O_2^- production (▲), and β-glucuronidase release (○) induced by C5a (*abscissa*). PMN from healthy control donors were used.

TABLE I. *Number of patients with local inflammatory skin conditions showing absence of C5a-induced polymorphonuclear leukocyte (PMN) responses*

Diagnosis	No. of patients	C5a-nonresponders[a]
Erysipelas	12	8
Acne conglobata	9	4
Pyoderma	15	6
Psoriasis vulgaris	91	2
Psoriasis pustulosa	8	4
Urticaria vasculitis	1	1
Healthy controls	46	0
	182	25

[a] Meaning stimulus-specific absence of neutrophil responses to any dose of C5a (up to 800 ng/ml), e.g., C5a-induced chemotaxis of patients' PMN minus random migration of patients' cells ≦ 2 SD, C5a-induced release of O_2^- minus buffer control ≦ 2 SD, as well as C5a-induced release of β-glucuronidase minus buffer control ≦ 2 SD. C5a-receptor specific effect is ascertained by the fact that patients' PMN retain (a) cellular motility (= random migration) and (b) dose responses are seen with other stimuli (e.g., FMLP).

TABLE II. *Polymorphonuclear leukocyte (PMN) functions in a patient with erysipelas of the lower leg*

Stimulus	Concentration	Chemotaxis[a] (PMN × 10^4/h)		O_2^--production[b] (nmol red Cyt c)		Enzyme release[b] (% of total)	
		Patient	Control[c]	Patient	Control[c]	Patient	Control[c]
C5a (ng/ml)	800	—	—	1.8	24.5	9.0	22.2
	200	51 ± 3	44 ± 7	1.5	22.3	—	—
	100	50 ± 4	81 ± 6	1.7	17.6	—	—
	80	49 ± 3	74 ± 2	1.7	12.9	—	—
	20	47 ± 2	51 ± 4	1.7	3.4	—	—
	Buffer	45 ± 5	29 ± 5	1.3	1.3	8.6	2.8
FMLP (M)	10^{-6}	—	—	12.2	17.5	41.0	41.7
	10^{-7}	23 ± 3	25 ± 5	13.4	19.5	—	—
	10^{-8}	48 ± 4	42 ± 5	7.0	11.1	—	—
	10^{-9}	60 ± 1	67 ± 7	1.2	1.2	—	—
	10^{-10}	48 ± 2	37 ± 5	1.4	1.2	—	—

[a] Chemotactic migration as expressed in equivalents of migrated cells × 10^4/h using the ECCA-modification as described in *Materials and Methods*. Triplicate experiments were done.
[b] SD smaller than 5%.
[c] PMN from a healthy donor, which were tested simultaneously.

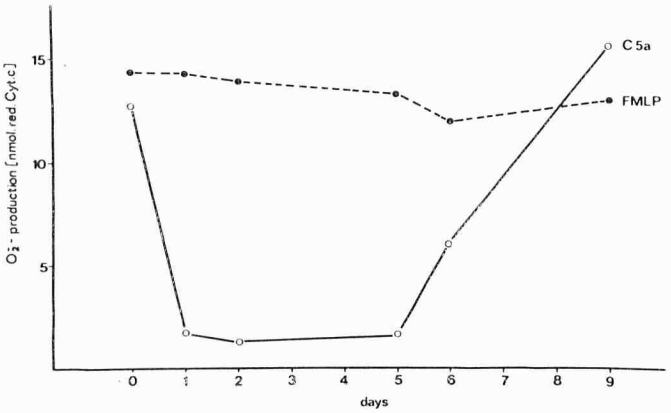

FIG 2. Follow-up of PMN functions in a patient with erysipelas. For C5a (○) and FMLP (●), each point represents maximum values of dose-response curves measuring production of O_2^-.

TABLE III. *Clinical diagnosis and duration of C5a-nonresponsiveness in affected patients*

Diagnosis	Age, Sex	PMN/μl[a]	Therapy[b]	C5a defect (days)[c]
Erysipelas	70, F	7,100	Erythromycin	4
Acne conglobata	15, M	6,800	Topical	2
Psoriasis pustulosa	53, M	6,300	Methotrexate	6
Psoriasis vulgaris	68, F	3,100	PUVA	1
Pyoderma	38, M	7,200	—	1
Urticaria vasculitis	42, F	2,900	—	7

[a] Neutrophil counts in peripheral blood at the first day of the C5a defect.
[b] Therapy has started after the patients were investigated.
[c] PMN functions were tested in nearly daily intervals.

sponders and thoroughly washed produced normal rates of O_2^- when stimulated with C5a (Fig 4). Also FMLP-induced responses remained unaltered and occasionally showed slight increase (data not shown). These results demonstrate, that sera from C5a nonresponders did not contain a cell-directed inhibitor of C5a-elicitable function.

Preincubation of PMN with C5a

In order to investigate the effects of preexposure of PMN with C5a, neutrophils from healthy controls were incubated with C5a. After washing, cells were again exposed to different concentrations of C5a or to FMLP and O_2^- production was investigated.

Fig 5 shows that, depending upon the concentration of C5a

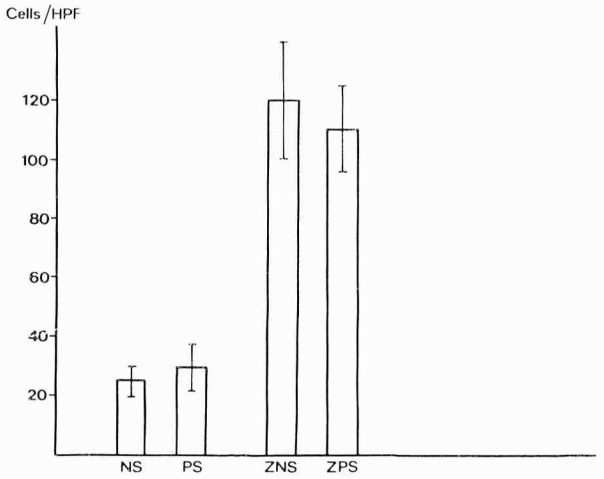

FIG 3. Chemotactic activity of patients (*PS*) and healthy control (*NS*) fresh serum as well as zymosan-activated serum of patient (*ZPS*) and healthy control (*ZNS*), each diluted 1:30 with PBS-GB. Average values from triplicate determinations in 4 serum samples are presented. PMN of healthy controls were used as indicator cells.

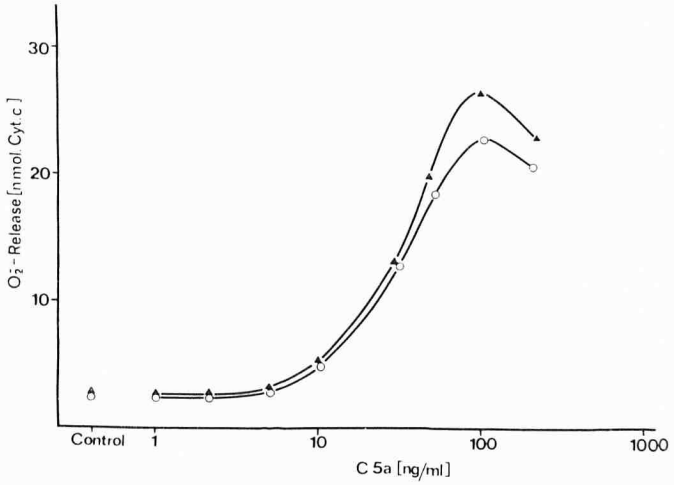

FIG 4. Incubation of normal PMN with nonresponder serum (○). PMN from healthy persons (10^7/ml) were incubated (30 min/37°C) with fresh serum from a nonresponder patient (final dilution: 1:2), washed twice, and, after counting, stimulated with various concentrations of C5a for production of O_2^-. Control experiments were performed by incubating PMN with 4% BSA (w/v) (▲). Six experiments of different patients were done. One typical experiment is shown.

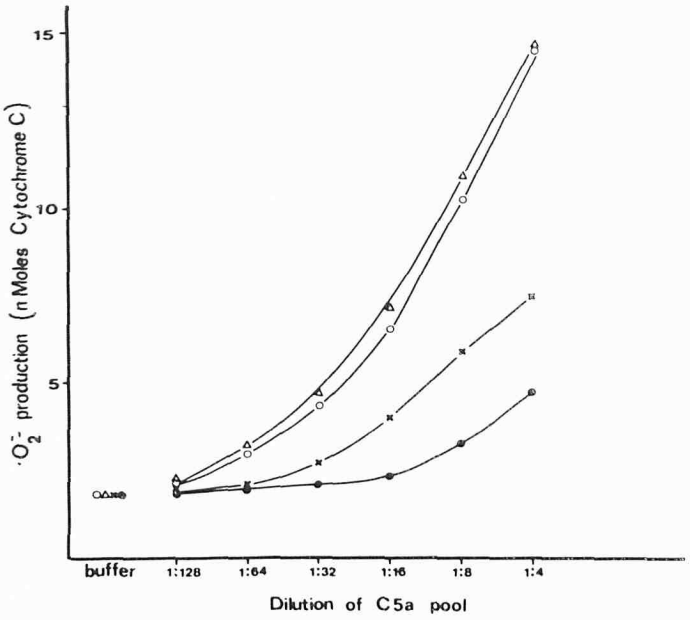

FIG 5. Deactivation of C5a-dependent function by preexposure with C5a. PMN from healthy control persons (5×10^6/ml) were preincubated with C5a (final conc: 1:2 (●), 1:4 (×), or 1:8 (○) dilution of a pool) or with buffer (△) in a constant volume (4 ml for 15 min at 37°C). After washing (2 ×) and counting of cells, PMN were stimulated with C5a and the amount of O_2^- was determined. The C5a pool contained partially purified C5a with a calculated concentration of 300 ng/ml. The curves describe one typical experiment.

during preincubation, PMN become *partially* deactivated against subsequent stimulation by C5a. Under identical conditions release of O_2^- induced by FMLP remained unaltered (data not shown).

In these experiments we were unable to *completely* deactivate normal PMN by preexposure to C5a in vitro at concentrations which were *without* effects on FMLP-dependent functions.

DISCUSSION

Our results show that in inflammatory skin diseases circulating PMN may become nonresponsive to C5a while cellular activities induced by FMLP remain unchanged. The absence of C5a-induced reactions persisted for variable lengths of time, e.g., 1–7 days, and thereafter no abnormality of PMN functional activities could be detected. In no case was there evidence that therapeutic agents were directly responsible for this defect.

Furthermore, this defect is seen in various inflammatory skin diseases showing either severe courses with extensive body involvement (acne conglobata, pyoderma) and/or vigorous onset with fever and acute illness (erysipelas, pustular psoriasis). This indicates that in correlation with disease activity, C5a-induced reactions in peripheral circulating PMN may become altered resulting in complete nonresponsiveness to C5a.

The reason for this defect could be a circulating factor, which specifically affects C5a-dependent neutrophil functions. In patients with systemic lupus erythematosus a specific serum inhibitor of C5 fragment-derived PMN activity has been found [18]. However circulating neutrophils from these patients were not affected, indicating that this factor is not responsible for the defect described here. In a patient with allergic drug reactions Bowers et al [19] found complement-induced reduction of C5 fragment-dependent granulocyte chemotaxis. Furthermore Skubitz and Craddock [20] observed that by contact with dialyser cellophane membranes plasma complement may become activated via the alternative pathway. In these studies $C5a_{desarg}$ was supposed to cause selective down regulation of PMN responses to the split product of the fifth complement component.

Whereas such findings may indicate receptor-specific deactivation of neutrophils by circulating chemotactic C5 fragments, it has been difficult to demonstrate directly the presence of C5a or $C5a_{desarg}$ in serum [21]. In fact in most studies (except studies of extracorporeal circulation) blood samples failed to show any $C5a_{desarg}$ activities [21]. The most likely explanation for the apparent failure to detect plasma C5 fragments is that plasma C5a or $C5a_{desarg}$ rapidly binds to PMN via membrane receptors and becomes internalized [22]. For this reason $C5a_{desarg}$ is hardly detectable in venous blood from patients who were likely to have experienced activation of complement. This may serve as an explanation for our failure to detect inherent chemotactic activity in C5a-nonresponder sera.

Interestingly we were unable to fully and preferentially deactivate PMN in vitro by treatment of PMN with C5a followed by washing (Fig 5). As shown by our experiments *complete* deactivation by in vitro incubation of PMN with C5a could not be obtained *without* affecting PMN responses to other chemotaxins, e.g., FMLP. Moreover a dose necessary to completely inhibit C5a-directed chemotaxis or O_2^- release in vitro would cause nonpreferential deactivation when applied in vivo, which is not seen in our patients.

These in vitro results are supported by the findings of Nelson et al [23]. Using a low dose of zymosan-activated serum (ZAS), these authors obtained only partial deactivation of neutrophil chemotaxis against this chemotaxin. However high doses of ZAS caused nonpreferential deactivation. Similarly Donabedian and Gallin [24] reported that neutrophils incubated with low concentrations of C5 fragment showed only partial inhibition of chemotactic activity against C5 fragments. When high doses (20 μg/ml) were used, again nonpreferential deactivation of subsequent chemotaxis against C5 fragments as well as FMLP was seen.

In view of these findings it appears unlikely that deactivation of PMN by C5 split products (down regulation) is the major cause for the absence of C5a responses seen here. This suggestion is supported by a recently published experimental study in rabbits which bears some similarities to our observations in humans. Rosenbaum et al [25] demonstrated that i.v. injection of bacterial lipopolysaccharide (endotoxin) in rabbits is followed by selective suppression of C5a-induced reactions in PMN while the cells retained their capacity to respond to FMLP. Similar to our observations no C5-derived chemotactic activity was seen in serum samples obtained at various time intervals. Plasma drawn from rabbits 24 h after a single i.v. injection of lipopolysaccharide could reduce the chemotactic response of normal PMN to C5-derived chemotaxins in a stimulus-specific manner. A factor different from $C5_{fr}$ was suggested to be responsible for this phenomenon [25].

Although we were unable to detect a causative factor in serum, this does not exclude the existence of very short-lived fluid phase agent(s). Ruddy et al [26] have shown that the alternative convertase C3bBb, which is formed from complement factors B and C3b by activation with D, causes deactivation of neutrophils to subsequent stimulation by $C5_{fr}$. This convertase is short-lived in plasma and rapidly binds to PMN where it is relatively stable. In conjunction with control proteins (e.g., factors I and H) this convertase may effectively modulate PMN responses against C5a and $C5a_{desarg}$ in vivo. Thus, by activation of the alternative pathway of complement, neutrophils passing the site may become nonresponsive for C5a.

In conclusion, our observations demonstrate that in severe cutaneous inflammatory disorders including psoriasis C5a receptor-dependent functions of neutrophils specific for C5a become impaired by a hitherto unknown principle. The nature of this effect apparently is related to a requirement for comple-

ment. Thus the recognition that C5a-dependent cell functions of PMN become modulated in acute local inflammation may represent a novel regulatory mechanism in inflammation. Work in our laboratory is in progress to verify this concept.

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