LOW ZONE DESENSITIZATION: A STIMULUS-SPECIFIC
CONTROL MECHANISM OF CELL RESPONSE

Investigations on Anaphylatoxin-induced Platelet Secretion*

By STEFAN MEUER, BERND ZANKER, ULRICH HADDING, AND DIETER BITTER-SUERMANN

From the Institute for Medical Microbiology, University of Mainz, D-6500 Mainz, West Germany

A variety of cells known to be involved in acute inflammatory reactions (mast cells and basophils, neutrophils, platelets, and macrophages) respond to the anaphylatoxic polypeptides C4a, C5a, or C5a with secretion of biogenic amines and secretion of various enzymes (1-11). These biological activities of anaphylatoxic complement fragments were studied mainly in vitro by use of purified or even synthetic substances (12-17).

Calculations from experiments (18) performed in a recently developed guinea pig platelet system led to the conclusion that cleavage of <1% of the serum C3 or C5, leading to generation of C3a and C5a, could induce a marked release of platelet granule constituents. Sufficient amounts of anaphylatoxins may physiologically occur by the attack of various enzymes on C3 or C5 in the circulation or at a local site. Such high levels of activity require efficient control mechanisms.

Investigations (19-21) with serum provided evidence for control mechanisms represented by humoral factors such as carboxypeptidases or proteases, which rapidly destroy the biologic activity of anaphylatoxins once they are generated from the native complement components. However, anaphylatoxin activity can be detected in vivo in several species (22, 23) in spite of fluid-phase regulatory mechanisms. Besides these humoral factors, a regulatory phenomenon on the target cell level has been observed (3, 24, 25): after incubation of cells with an anaphylatoxic stimulus, they do not respond a second time to the same or a related stimulus. Desensitization turned out to be stimulus specific. In addition, desensitization could be achieved with low amounts of stimulating substances that were lower than those required to lead to a detectable release reaction (18).

The experiments described in this study were performed to provide information on how cell reactivity to anaphylatoxic stimuli is influenced by the phenomenon of specific desensitization.

We were especially interested in investigating how platelets respond to an anaphylatoxic stimulus generated in their presence, which seems more likely to happen under in vivo conditions than from exposure to preformed anaphylatoxic molecules.

For these investigations, the platelet system was chosen because platelets can be

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obtained in high purity, they represent very sensitive targets, and they offer the possibility of exact quantitation of biological anaphylatoxin activity (11, 18).

Materials and Methods

Chemicals. Imipramine, aqueous solution (2 mg/ml), was obtained from Ciba Geigy, Basel, Switzerland. Aquasol and [3H]serotonin (NET 398, 1 mCi/ml) were obtained from NEN-Chemicals, Dreieich Germany.

Guinea Pig C5, Guinea Pig C5a, and Guinea Pig C3a. These were prepared according to the procedure described by Meuer et al. (18, 26). Specific activity of the C5-preparation was $2.0 \times 10^3$ site forming units/mg.

Synthetic Hexapeptide C3a 72–77. This was synthesized according to a previously described method (17) by Dr. Andreatta (Research Department Pharmaceuticals Division, Ciba Geigy, Basel, Switzerland).

Cobra Venom Factor-C3 Convertase (VF-Bb). This was generated by incubating purified cobra venom factor (VF) (27) with factors B (28) and D (29) of guinea pig complement in the presence of Mg$^{2+}$ and was purified as described (30). A 1:10 dilution of the particular preparation used led to a loss of 90% of hemolytic activity of guinea pig C5 (20 µg/ml) within 60 min at 37°C.

Hemolytic C5 Assay. This was performed as described (31).

Preparation of [3H]Serotonin-labeled Platelets and the [3H]Serotonin Release Assay. These were performed as described (18). Calculations were performed as follows:

$$\text{percent specific release} = \frac{cpm\ (test\ sample) - cpm\ (buffer\ control)}{cpm\ (total\ label) - cpm\ (buffer\ control)} \times 100.$$ 

Results

Purified guinea pig C5 (final concentration 20 µg/ml) was incubated with minute amounts of the purified alternative pathway enzyme VF-Bb for 5, 15, and 25 min at 37°C in the absence of target cells. After these various incubation periods, [3H]serotonin-labeled platelets were added to the samples and incubated for an additional 5 min at 37°C. Depending on the time of the first incubation, increasing amounts of anaphylatoxin activity could be detected in the incubation mixture, measured as release of [3H]serotonin (Fig. 1).

In a hemolytic test that was run parallel, no C5 turnover was observed, indicating a high sensitivity of the guinea pig platelet assay for detecting C5 cleavage.

However, when amounts of C5 and VF-Bb equal to those used in the first experiment were incubated in the presence of [3H]serotonin-labeled platelets for 5, 15, and 25 min at 37°C, no release of the biogenic amine could be detected during the whole incubation period. The supernatants of these samples induced a strong release reaction, similar to that described above, when added to fresh 3H-labeled platelets, indicating that high amounts of anaphylatoxin had been generated and maintained their activity. Controls with C5 in the absence of VF-Bb or with VF-Bb alone were negative.

To determine whether low amounts of freshly generated C5a might have desensitized the platelets to higher C5a concentrations appearing subsequently in the incubation mixture, the characteristics of the desensitization phenomenon were also investigated with purified C5a.

Platelets were incubated with various amounts of C5a for 5 min at 37°C, followed by determination of [3H]serotonin release. Fig. 2 A demonstrates the described dose dependency (17).
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Fig. 1. Influence of time on platelet reactivity against C5a generated in their absence or presence. Δ, C5 (20 μg/ml) + VF-Bb (1:80) were incubated for various times at 37°C. Then [3H]-labeled platelets were added and incubated for additional 5 min at 37°C, followed by determination of serotonin release. ○, C5 (20 μg/ml) plus VF-Bb (1:80) were incubated for various times at 37°C in the presence of [3H]-labeled platelets, followed by determination of serotonin release.

Fig. 2. Platelet reactivity against varying doses of purified C5a given as first and second stimulus. (A) ○, platelets were incubated with various doses of guinea pig C5a (molarities shown on the abscissa) for 5 min 37°C. Then PBS was added to each sample and incubated for additional 5 min at 37°C, followed by determination of serotonin release. (B) ○, platelets were incubated with various doses of guinea pig C5a (molarities shown on the abscissa) for 5 min at 37°C. Then guinea pig C5a (1.5 × 10^{-9} mol/liter) was added and incubated for additional 5 min at 37°C, followed by determination of [3H]serotonin release. Fig. 2 B shows the additional release induced by the second stimulus (additional release is the total release minus release of first stimulus, see Fig. 2 A). The dotted line shows the release induced by the second stimulus alone (C5a 1.5 × 10^{-9} mol/liter).

To examine the desensitizing potency of C5a, platelets were incubated with the same amounts of C5a for 5 min at 37°C, as above. Then a high dose of C5a (1.5 × 10^{-9} mol/liter), which alone would induce ~50% specific release (see dotted line Fig. 2 B), was added to all the test samples. After incubation for an additional 5 min at 37°C, the total [3H]serotonin release was measured. Fig. 2 B demonstrates that
preincubation with C5a negatively influenced platelet reactivity against a second C5a stimulus. In this figure, only the additional release mediated by the second stimulus is plotted (see legend to Fig. 2). Whatever concentration of C5a was used above 1.5 × 10^{-10} mol/liter in the first step, there was no additional release upon addition of a high dose of C5a (1.5 × 10^{-9} mol/liter). This means the response to the second stimulus was completely inhibited. Below the level of 1.5 × 10^{-10} mol/liter, desensitization of the platelets to the second C5a stimulus could still be observed, even at concentrations of C5a, which did not lead to a detectable release alone. Desensitization, however, was no longer complete.

In a control experiment, platelets were washed after the first incubation period and then exposed to a second C5a stimulus. The results were the same as described above. Therefore, for practical reasons, a washing procedure was usually omitted. The stimulus specificity of desensitization was controlled by an unaltered response of the platelets to guinea pig C3a.

In a next series of experiments, we tried to discover conditions under which platelets could respond to an anaphylatoxic C5a stimulus generated in their presence. Constant amounts of C5 (20 μg/ml) were incubated with various doses of the enzyme VF-Bb (1:1500, 1:500, 1:100, 1:9) together with [3H]serotonin-labeled platelets for 5 min at 37°C, and then the release was measured (Table I, first column). Additionally, the response of guinea pig platelets after this first incubation step to purified C5a (3 × 10^{-9} mol/liter) as a second stimulus was determined in the various samples (Table I, 3rd column). Table I, 2nd column, shows the amount of C5a generated from C5 in the absence of platelets. The amount of C5a generated from C5 by VF-Bb was the same whether or not platelets were present in the incubation mixture.

The responsiveness of the platelets to C5a depended on the speed and the amount of C5a-generation from C5, which was a function of the concentration of VF-Bb applied. The more enzyme VF-Bb was present, the more C5a was generated, and thus the degree of platelet response to a second C5a stimulus decreased as a result of desensitization induced during the first incubation step. C5 cleavage in the presence of the target cells led to platelet secretion only if a high concentration of the enzyme VF-Bb was used—conditions under which high amounts of C5a were rapidly generated (Table I, 1st column). Under these experimental conditions, 10.4% of C5 had been cleaved after 5 min, as calculated by loss of hemolytic C5-activity.

If this interpretation is correct, it should be possible to desensitize platelets to high concentrations of an anaphylatoxic stimulus by previously adding stepwise increasing amounts of a related stimulus. Thereby, a state of stimulus-specific unresponsiveness could be induced without any preceding secretory event.

In the next experiments, we investigated platelet reactivity to C3a after pretreatment with a synthetic C3a-related hexapeptide comprising the amino acids 72-77 of human C3a (His-Leu-Gly-Leu-Ala-Arg). This substance has been described (17) as inducing platelet secretion and desensitization qualitatively like the natural C3a molecule.

Platelets were treated with increasing concentrations of the hexapeptide. These various doses were added stepwise in 2-min intervals to the samples. At each step, platelet reactivity to an optimum dose of C3a (1.1 × 10^{-7} mol/liter) as second stimulus was determined.
Table I

Platelet Activation and Desensitization When C5a Was Generated in Their Presence

| Specific release | Platelets present during C5a generation | Platelets added after C5a generation | Platelets present during C5a generation, then addition of C5a as second stimulus (3.0 × 10⁻⁸ mol/liter) |
|------------------|----------------------------------------|--------------------------------------|--------------------------------------------------------------------------------|
|                  | %                                      |                                      |                                                                                 |
| Guinea pig C5*   | 0                                      | 0                                    | 58.6                                                                           |
| 0.9% NaCl        |                                        |                                      |                                                                                 |
| VF-Bb (1:9)‡     | 0                                      | 0                                    | 56.5                                                                           |
| Guinea pig C5    |                                        | 0                                    | 60.2                                                                           |
| VF-Bb (1:1,500)  |                                        |                                      |                                                                                 |
| Guinea pig C5    |                                        | 0                                    | 21.8                                                                           |
| VF-Bb (1:500)    |                                        |                                      |                                                                                 |
| Guinea pig C5    |                                        | 0                                    | 18.6                                                                           |
| VF-Bb (1:100)    |                                        |                                      |                                                                                 |
| Guinea pig C5    | 46.2                                   | 53.2                                 | 45.7§                                                                          |
| VF-Bb (1:9)†     |                                        |                                      |                                                                                 |

The samples of C5 and VF-Bb were incubated for 5 min at 37°C. When platelets were present during this incubation period, release was measured after this time (first column). When platelets were absent during the first incubation period, platelets were added after 5 min at 37°C and incubated for an additional 5 min at 37°C (second column). If desensitization to C5a should be measured, C5a (3.0 × 10⁻⁸ mol/liter) was added after the first incubation period (platelets present) and incubated for an additional 5 min at 37°C (third column).

* Final concentration of C5 was always 20 µg/ml.
‡ Final dilutions of VF-Bb.
§ This amount of [³H]serotonin had been released during the first incubation period (compare first column).

The initial concentration of the hexapeptide in the sample was 6.4 × 10⁻⁸ mol/liter and was increased to 3.1 × 10⁻⁷ mol/liter in the second step. It was further increased to 1.3 × 10⁻⁶ mol/liter, 5.6 × 10⁻⁶ mol/liter, and 2 × 10⁻⁵ mol/liter. Finally a concentration of 7.9 × 10⁻⁵ mol/liter was reached.

Fig. 3 demonstrates that when the concentration of hexapeptide was increased stepwise, no release of [³H]serotonin occurred. As expected (17), the same concentrations of the hexapeptide, if added in a single dose, led to secretion.

Analogously to Fig. 2 B, where C5a was added in an optimum dose after pretreatment, platelet response to C3a as additional stimulus was also measured (not shown in Fig. 3). The degree of desensitization of the platelets pretreated with low doses of hexapeptide (below 3.1 × 10⁻⁷ mol/liter) was identical whether the synthetic substance was applied in a single dose or accumulated stepwise. With increasing concentra-
FIG. 3. Influence of repeated vs. single doses of a synthetic hexapeptide on platelet reactivity. ○, dose-response curve of $[^{3}H]$serotonin release from guinea pig platelets by the synthetic hexapeptide C3a 72-77. The various concentrations were added as single doses to the platelets. ●, $[^{3}H]$serotonin release from guinea pig platelets when the various doses of C3a 72-77 were accumulated stepwise in 2-min intervals in the presence of the target cells.

FIG. 4. Kinetics and dose dependence of desensitization by hexapeptide at 37°C. The abscissa indicates the time at which the second high dose of hexapeptide (3.38 $\times$ 10$^{-6}$ mol/liter) was given to platelets that were pretreated (time 0) with different low concentrations of hexapeptide (first stimulus). After addition of the high dose, all samples were incubated for a further 5 min at 37°C. ○, $[^{3}H]$serotonin release by four different, low doses of hexapeptide alone (first stimulus). ●, $[^{3}H]$serotonin release by the high dose of hexapeptide alone (second stimulus). ●, $[^{3}H]$serotonin release by the first plus second stimuli.

FIG. 5. Influence of repeated vs. single doses of a synthetic hexapeptide on platelet reactivity. ○, dose-response curve of $[^{3}H]$serotonin release from guinea pig platelets by the synthetic hexapeptide C3a 72-77. The various concentrations were added as single doses to the platelets. ●, $[^{3}H]$serotonin release from guinea pig platelets when the various doses of C3a 72-77 were accumulated stepwise in 2-min intervals in the presence of the target cells.

Next we looked for the kinetics of desensitization and for approaches to dissociate the process of deactivation from the events leading to secretion. Figs. 4 and 5 yield further information on the interdependence of both processes. In Fig. 4, the first low dose of hexapeptide (1.35 $\times$ 10$^{-7}$ mol/liter up to 5.4 $\times$ 10$^{-7}$ mol/liter) does not mediate a release reaction, whereas a concentration of 1.35 $\times$ 10$^{-6}$ mol/liter allows a mild release. The release mediated by this and by the second, high dose alone (3.38 $\times$ 10$^{-5}$ mol/liter) is complete within 5 s at 37°C (data not shown in Fig. 4). Looking
at desensitization against the high dose, the first three subthreshold doses of hexapeptide cause an increasing desensitization, with a reproducible short lag phase up to 15 s. The fourth dose ($1.35 \times 10^{-6}$) that per se has $[^3]$H serotonin-releasing activity causes a complete and instantaneous desensitization concomitantly with the rapid release reaction. This small time-dependent increase in desensitization at $37^\circ$C was the first hint that activation and deactivation may be separable. Therefore a comparable experiment was done at $22^\circ$C with a first dose of $1.35 \times 10^{-6}$ ml/liter (Fig. 5). Whereas the parallel values obtained at $37^\circ$C equal the data given in Fig. 4 (lowest section), and in general are slightly higher than at $22^\circ$C, the time dependency of desensitization at room temperature is obvious. The velocity of the $[^3]$H serotonin

**Table II**

**Kinetics of Desensitization to Hexapeptide in the Absence or Presence of Ca$^{++}$**

|                      | Without Ca$^{++}$ | With EDTA (10$^{-2}$ M) | With Ca$^{++}$ |
|----------------------|-------------------|-------------------------|----------------|
| 1st dose*            | 0                 | 0                       | 8, 6           |
| 2nd dose‡ with Ca$^{++}$ | 54, 2             | 63, 8                   | 48             |
| 1st dose + 2nd dose after§ | 47, 8             | 46, 7                   | 16, 3          |
| 10 s                 | 31, 6             | 24, 3                   | 9, 1           |
| 1 min                | 1, 1              | 3, 8                    | 5, 7           |
| 3 min                | 0                 | 0                       | 8, 2           |
| 5 min                |                   |                         |                |

* Release measured 5 min after addition.
‡ Platelets preincubated for 5 min under the conditions indicated were mixed with the 2nd dose, and release was measured 5 min later.
§ Release measured 5 min after addition of 2nd dose.
¶ This amount of $[^3]$H serotonin was already released by the 1st, low dose of hexapeptide.
release at 22°C by the high dose alone is as rapid as at 37°C and complete within 5 s.

A recovery from deactivation in a similar test system as above (first stimulus 5.4 x 10^-7 ml/liter), but with a time gap to the application of the second, high stimulus up to 60 min, was not measurable. But from data obtained in a platelet aggregation system (with washed platelets as well as PRP) under comparable experimental conditions, we could demonstrate a recovery from deactivation by C3a after 2-3 h (unpublished results).

Because activation to secretion by the anaphylatoxic peptides is absolutely Ca++ dependent, we tested the Ca++ dependency of the deactivation process at 37°C. As shown in Table II, hexapeptide induces a deactivation not only in the presence of Ca++, but also in the absence of Ca++ and in the presence of EDTA (0.01 mol/liter, to capture free Ca++ released by the platelets). Under the latter conditions, no release reaction can be triggered by any dose. Although the deactivation is markedly retarded in comparison to a Ca++ sufficient system (third column and Fig. 4), it finally reaches the maximum level.

Discussion

Considerations concerning the regulation of anaphylatoxin activity in vivo generally focused on the cleavage of C3a or C5a by fluid-phase enzymes such as serum-carboxypeptidase or other proteases. Inactivation of anaphylatoxins by these enzymes, however, occurs within minutes (20), whereas platelet activation by C3a and C5a is a process of a few seconds (18). Thus, the ability of anaphylatoxins to stimulate target cells in spite of these regulatory processes in vivo is probably maintained by the fact that interaction of anaphylatoxins with platelets, for instance, occurs more rapidly than cleavage of these stimuli by their fluid-phase control enzymes. This concept is supported by an experiment in which platelets and C3a had been incubated in the presence of excess carboxypeptidase B (8). Under these competitive experimental conditions, C3a exerted unaltered biological activity. Furthermore, environmental conditions at local inflammatory sites (low pH) may protect anaphylatoxins from being inactivated rapidly. The function of fluid-phase control, therefore, should be considered as preventing a longer persistence and the systemic accumulation of active anaphylatoxins in vivo and acting as a second-line regulatory mechanism. If this assumption is correct, there should be a cell-associated first-line regulatory mechanism to prevent continuous cell activation.

We conclude from our experiments that regulation of the stimulus-cell interaction by the mechanism of specific desensitization, which is located at the target cell level, might be responsible for maintaining an essential balance: secretion from platelets induced by anaphylatoxins occurs only in pathologic but not physiologic situations.

The results presented in this study suggest that the mechanism of desensitization prevents platelets from responding to any low anaphylatoxic stimulus generated in their presence. Consequently, platelet reactivity towards preformed anaphylatoxins is then reduced.

It is conceivable that platelets in vivo are constantly desensitized to a certain extent as a result of C3a and C5a that are generated during the physiologic turnover of complement. Therefore, a stimulus, to be capable of inducing a secretory event, has to fulfill prerequisites of a "second stimulus," according to Fig. 2. This means that
platelets could be activated by preformed anaphylatoxic molecules, provided these stimuli occur in concentrations that are ~50-100-fold higher than those present in the platelets' physiologic environment.

It is more likely, however, that in vivo an anaphylatoxic stimulus is generated in the vicinity of the target cell so that the concentration of the stimulus increases continuously. Kinetic studies reported here (Fig. 1, Table I) clearly demonstrate that secretion can occur only if the gradient of anaphylatoxin concentration is steep enough to exceed a threshold value within a critical, very short period of time.

If the gradient is too gentle, this necessary concentration will not be built up within the required interval, and only desensitization ensues. In this situation, a target cell will no longer be able to subsequently respond to very high concentrations of anaphylatoxins with secretion of granule constituents. The accumulated concentrations of anaphylatoxins, therefore, cannot be correlated to their biologic efficacy in vivo.

This concept is illustrated in Fig. 6. Induction of desensitization and of secretion can be clearly separated by varying the steepness of concentration gradient of an anaphylatoxic stimulus in the platelets' environment. The process of activation to secretion thus depends on both concentration and time, whereas the degree of

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![Image of a diagram illustrating the concept of stimulus-specific low-zone desensitization as a cell-bound regulatory mechanism for control of the biological effects of AT-peptides.](image-url)
desensitization is only a function of anaphylatoxin concentration in the vicinity of the target cell. Therefore, it is possible to induce C3a-specific unresponsiveness of the platelets without a preceding secretory event by adding the hexapeptide C3a 72-77 stepwise to the samples (Fig. 3). The state of platelet desensitization is reversible within ~2-3 h (unpublished results).

The experiments performed to dissect the process of desensitization from activation to secretion yielded positive results by only manipulating the physiological conditions of ≤37-22°C and by deprivation of Ca++ (Fig. 4 and Fig. 5, Table II). The Ca++ independence seems to be a general feature of desensitization (33, 35), indicating that for binding of the ligand to its receptor and for the signal to switch off the secretory response there is no need of extracellular Ca++. The use of enzyme inhibitors to block distinct enzymatic steps within the chain of signal processing as well as electron microscopic studies on the fate ligand-receptor complexes will give further information on the interdependence of desensitization and activation.

A principally similar phenomenon of specific desensitization has been reported for platelets measured as release reactions (18, 32) or as platelet aggregation (33) and for neutrophils in their response to anaphylatoxic stimuli (3, 24, 34). Moreover, a state of stimulus-specific unresponsiveness has been found in a great number of inflammatory cells (for review see ref. 32). Some of the most interesting studies regarding the regulatory function of the phenomenon of low zone desensitization have been performed with rabbit and human neutrophils and human eosinophils in their response to stimuli such as C5a, the peptide Formyl-Methionyl-Leucyl-Phenylalanine (3, 35, 36), the high molecular weight neutrophils chemotactic factor (HMW-NCF), (37) and the acidic tetrapeptides of eosinophil chemotactic factor (38, 39). Similar to our results, these authors demonstrated that desensitization is a time and concentration-dependent process. Moreover, desensitization could be induced using concentrations of stimuli lower than those required for optimum activation. In particular, the reported examples of the tetrapeptides of eosinophil chemotactic factor and of HMW-NCF underline that the striking dose-response relationship between deactivating and activating concentrations of stimuli, as described in the present paper, are not unique.

In this context, one might envisage that slowly increasing concentrations of C3a-related substances applied in vivo could induce a selective state of unresponsiveness for C3a without exerting secretory activity themselves. This may allow studies of in vivo effects of the C3-derived anaphylatoxic polypeptide. Synthetic C3a analogs may represent suitable substances for in vivo desensitization because they are C3a specific and they are not immunogenic. Moreover, the dose range between secretory and inhibitory effect is considerably greater for the synthetic substances than for C3a itself (17).

The concept of in vivo desensitization by synthetic analogs could be extended to C5a as well, which has recently been reported (23, 40, 41) to exert severe systemic effects in the course of complement activation in vivo. Therefore, the present investigations seem to have a broader relevance for understanding how secretory and chemotactic target cells control inflammatory stimuli.

Summary

The biologic activity of the anaphylatoxic peptides C5a and C3a is regulated efficiently at the target-cell level by the phenomenon of desensitization. Desensitiza-
tion of platelets is stimulus specific and can be induced by low concentrations of anaphylatoxins without any preceding secretory event. In contrast to activation to secretion, desensitization is Ca++ independent but much more time consuming, especially at lower temperatures where both processes differ markedly in reaction velocity. This low zone desensitization insures that secretion from platelets only occurs when high amounts of anaphylatoxins are rapidly generated in the vicinity of the target-cell. Consequently, stimulus-specific unresponsiveness of the target cells can be induced by slowly increasing the concentration of the respective stimuli in their vicinity. Cellular control seems to act as a first-line mechanism of regulation, whereas the role of fluid-phase control is considered as preventing longer persistence and systemic accumulation of active anaphylatoxins.

Note added in proof: After this manuscript had been submitted, a paper by K. M. Skubitz and P. R. Craddock was published (J. Clin. Invest. 67:1383, 1981) that supports our concept of low-zone deactivation as a stimulus-specific cellular control mechanism and demonstrates its validity under in vivo conditions with C5a<sub>desens</sub> and human granulocytes.

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