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Characterization of C3a anaphylatoxin receptor on guinea-pig macrophages

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SUMMARY

We have characterized a C3a receptor on guinea-pig macrophages by ^{125}I–C3a binding and functional responses. Scatchard analysis applied to the ^{125}I–C3a binding to guinea-pig macrophages revealed the existence of two receptor classes; a high-affinity class with approximately $0{\cdot}63 \times 10^5$ binding sites/cell with a $K_d = 2{\cdot}7$ nM, and a relatively low-affinity class with approximately $1{\cdot}2 \times 10^5$ binding sites/cell with a $K_d = 51$ nM. The binding of C3a to macrophages was totally blocked when there was an excess of C3a. C3a triggered a transient intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in macrophages, which was accompanied by homologous desensitization. C3a was also capable of generating O_2^- from macrophages. The C3a-induced Ca^{2+} response and O_2^- generation were not detected in the pertussis toxin-treated macrophages, suggesting that G proteins are coupled with the C3a receptors of macrophages. Although the C3a-induced O_2^- generation was inhibited by staurosporine, it was more resistant to staurosporine than phorbol 12-myristate-13-acetate (PMA)-induced O_2^- generation, suggesting that a protein kinase distinct from protein kinase C may be associated with the C3a receptor.

INTRODUCTION

The activation of the complement system is accompanied by the production of various effector fragments that promote inflammatory responses. The polypeptides released from the third, fourth and fifth components of the complement, C3a, C4a and C5a, are collectively termed anaphylatoxins since they mimic anaphylatic responses, such as smooth muscle contraction and an increase in vascular permeability.[1] C5a, a basic glycopolypeptide of 74 amino acids,[2] is the most potent anaphylatoxin and has been shown to activate a variety of target cells to trigger liberation of effector molecules, such as histamine, lysosomal enzymes and active oxygen.[1] The structure of human and mouse C5a receptors has recently been determined.[3,4] C3a, a basic polypeptide of 77 amino acids,[5] is released in much larger quantities than C4a and C5a and subsequently should play an important role in inflammatory responses. However, C3a-induced cellular responses were observed with only restricted cells and species, such as human basophils,[6] rat mast cells[7] and guinea-pig platelets.[8,9] These cells are difficult to prepare in sufficient amounts for analysis of C3a-induced cellular responses. In addition, the number of C3a-binding sites on a guinea-pig platelet was reported to be as low as 200–500.[9] Information on the C3a receptor in the major inflammatory cells, such as polymorphonuclear leucocytes (PMN) and monocytes–macrophages is limited and controversial. Although there were reports on C3a-mediated liberation of lysosomal enzymes from PMN[10] and interleukin-1 (IL-1) from human monocytes,[11] C3a-binding analysis using a fluorescence-labelled C3a failed to prove the direct binding of the C3a to these cells.[12] Thus, the pathophysiological roles of C3a remain to be ascertained.

It is the purpose of the present study to investigate the biological effects of C3a on inflammatory cells. We found that guinea-pig macrophages express a large number of two types of C3a receptor, and that C3a induces cellular responses such as Ca^{2+} influx and O_2^- release in guinea-pig macrophages. These cellular responses were inhibited by pretreatment of macrophages with pertussis toxin, suggesting that G proteins may be coupled to the C3a receptor.

Abbreviations: BSA, bovine serum albumin; CVF, cobra venom factor; $[Ca^{2+}]_i$, intracellular Ca^{2+}; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PMN, polymorphonuclear leucocyte; SOD, superoxide dismutase.

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MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources: bovine serum albumin (BSA), ferricytochrome *c* and superoxide dismutase (SOD) from Sigma Chemicals Co. (St Louis, MO); phorbol 12-myristate 13-acetate (PMA) and pertussis toxin from Funakoshi Chemicals (Tokyo, Japan); Fura-2AM from Molecular Probes (Junction City, OR).

Purification of C3a and C5a

C3 and C5[13] and factors B[14] and D[15] were purified from human plasma by the reported methods, respectively, with minor

modifications. Cobra venom factor (CVF) was purified from lyophilized crude venom of *Naja naja kaouthia* (Sigma) by the method reported previously.[16] The alternative pathway C3/C5 convertase was assembled from CVF, B and D according to the method described previously.[17] Cleavages of C3 and C5 with C3/C5 convertase, CVFBb, were performed in 20 mM Tris buffer, pH 7·4, containing 35 mM $MgCl_2$, 150 mM NaCl, and 1 M ε-aminocaproic acid, with the C3 or C5, CVFBb ratio = 200:1 (w/w). C3a and C5a were separated from C3b and C5b by pass-through of a column of Q-Sepharose equilibrated with 20 mM Tris buffer, pH 7·4, containing 150 mM NaCl. C3a and C5a were recovered in the breakthrough fraction, while C3b and C5b remained bound to the column. The C3a and C5a fractions were dialysed against deionized water, freeze dried and finally purified by a reverse-phase high-performance liquid chromatography (HPLC) with a C18 column (YMC-Pack A-302, YMC, Kyoto, Japan), which had been equilibrated in 20% acetonitrile–0·05% trifluoroacetic acid. The column was developed at 1·0 ml/min with a linear gradient of 20–60% acetonitrile. C3a and C5a were eluted at about 33% and 37% acetonitrile concentration, respectively, lyophilized, and dissolved in deionized water. The amounts of C3a and C5a were determined by amino acid analysis after acid hydrolysis.

Radioiodination of C3a

This was performed by the chloramine-T method.[18] Approximately 30 μg of C3a was iodinated with 700 μCi of $Na^{125}I$. Specific activity of the ^{125}I–C3a was approximately 50 mCi/mg. Free ^{125}I was removed by gel filtration of the reaction mixture on a 1 × 25 cm column of Sephadex G-15 (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS) containing 1% BSA.

Isolation of macrophages

Peritoneal exudate cells were isolated from Hartley guinea-pigs, 4 days after i.p. injection of liquid paraffin. The cells were cultured in Eagle's minimum essential medium supplemented with 5% foetal calf serum (FCS) at 37°, using glass dishes.[19] After 2 hr, the adherent cells were collected and used as macrophages. PMN were isolated from peritoneal cavity of guinea-pigs injected with casein as described previously.[20]

Binding assay

All assays, except as noted, were carried out at 37°. ^{125}I–C3a saturation binding assays were performed as follows: serially diluted ^{125}I–C3a and macrophages (typically 1×10^6 cells) were mixed in polypropylene tubes to a final volume of 50 μl in PBS containing 0·2% BSA and 1 mM *o*-phenanthroline. After incubation for 15 min, separation of cell-bound and free tracers was achieved by centrifugation (3 min, 4°; 2000 ***g***) of the reaction mixture. Subsequently, cell pellets were washed three times by centrifugation as above and the cell-bound and free radioactivities were counted using an Autogamma spectrometer (Packard, Tokyo, Japan). All samples were performed in triplicate. To determine the competition with cold C3a, increasing concentrations of unlabelled C3a were mixed with a constant amount of ^{125}I–C3a prior to adding cells. Bound and free tracers were separated as in the saturation assay.

Measurement of $[Ca^{2+}]_i$ in macrophages

$[Ca^{2+}]_i$ in macrophages was determined with Fura-2 according to the modified procedure reported by Imamichi *et al.*[21] In brief, Fura-2-loaded macrophages, suspended in Krebs–Ringer PBS supplemented with 5·5 mM D-glucose, pH 7·4 (medium 1) were preincubated for 5 min at 37° and stimulated by the addition of C3a and C5a diluted with the above-mentioned buffer. The fluorescence of the cells was monitored at 497 nm; the exciting wavelength was 335 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence intensity of the cells (F) using the following equation:

$$[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$$

where K_d is an effective dissociation constant of the Ca^{2+} complex of Fura-2 (224 nM), and F_{max} and F_{min} are the fluorescence intensities obtained by the addition of 10 μl of 2% Triton X-100 or 10 μl of a solution containing 2% Triton X-100, 200 mM EGTA and 3·3 M Tris to the reaction mixture, respectively.

Measurement of the O_2^--generating activity of macrophages

The O_2^--generating activity of macrophages stimulated with C3a was determined by measuring the SOD-inhibitable reduction of ferricytochrome *c*.[22] For this purpose, a Hitachi double beam recording spectrophotometer U-3200 was used. Samples and reference cuvettes contained macrophages (1×10^6 cells) and 50 nmol of ferricytochrome *c* in 0·95 ml of medium 1. The reference cuvette also contained 50 μg of equine SOD. The reaction was initiated by the addition of C3a in 0·05 ml of medium 1. The difference spectrum was monitored at 550 nm, and the O_2^--generating activity of stimulated macrophages was estimated by measuring the initial velocity of the SOD-inhibitable reduction of ferricytochrome *c*; the amount of reduced cytochrome *c* was calculated by the use of an absorption coefficient of 21·1/mM/cm.[23]

RESULTS

^{125}I–C3a binding to guinea-pig macrophages

Figure 1a shows the kinetics and temperature dependence of ^{125}I–C3a binding to macrophages. Although no binding was detected at 4°, rapid C3a-binding occurred at 37°. The binding reached a plateau after 20 min and gradually decreased to 60% of the maximum binding after 60 min. Binding at 22° occurred slowly but progressively and reached a plateau at 60 min. Figure 1b shows the dose-dependent binding of ^{125}I–C3a to macrophages. A biphasic saturation curve was obtained with the first saturation at about 40 nM and the second saturation at about 100 nM of C3a. The binding of ^{125}I–C3a was almost completely inhibited by addition of cold C3a, suggesting that the binding was almost specific. Scatchard analyses of these data were carried out to calculate the dissociation constant (K_d) and the number of binding sites/cell (n). As shown in Fig. 1c, the resulting plot is non-linear but can be simulated well by the presence of two classes of receptor, one with a high affinity ($K_d = 2{\cdot}7$ nM, $n = 0{\cdot}63 \times 10^5$), and the other with a relatively low affinity ($K_d = 51$ nM, $n = 1{\cdot}2 \times 10^5$).

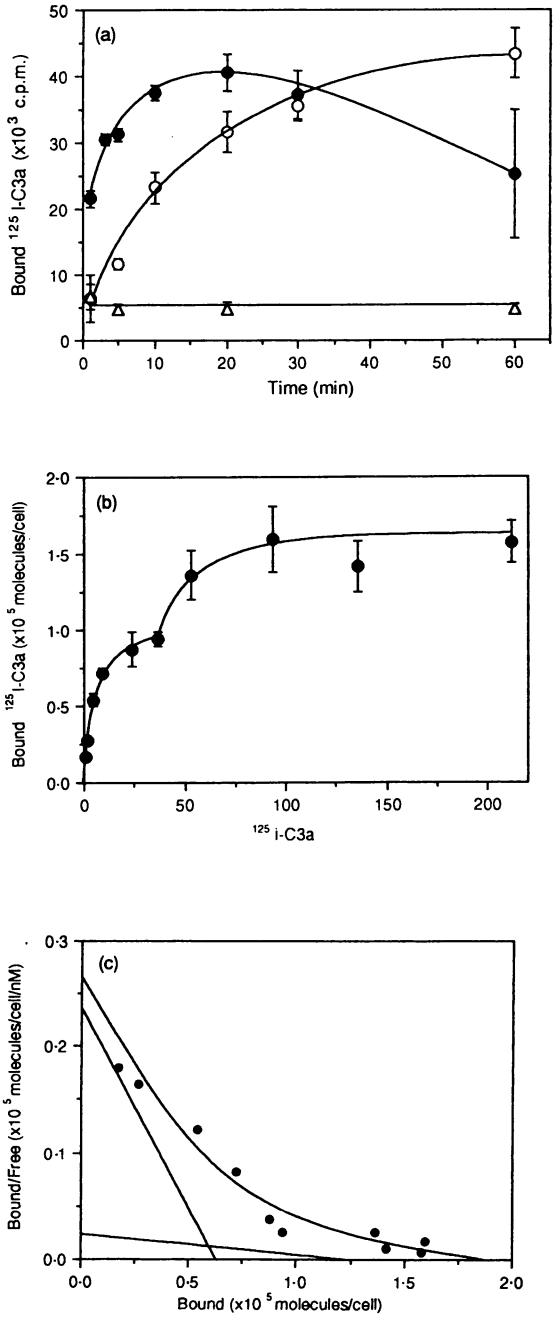

Figure 1. ^{125}I–C3a binding to guinea-pig macrophages. (a) Macrophages (1×10^6 cells) in PBS containing 0·2% BSA and 1 mM *o*-phenanthroline were incubated with 4×10^{-8} M ^{125}I–C3a. Incubations were performed at 4° (△), 22° (○) and 37° (●). After incubation at the indicated times, the cell-bound radioactivity was determined as described in Materials and Methods. Plotted are the means ± SEM of data from three cell preparations. (b) To estimate specific binding, macrophages (1×10^6 cells) were incubated with various concentrations of ^{125}I–C3a in the absence or presence of a 500-fold molar excess of unlabelled C3a. Specific binding was estimated by subtracting the non-specific binding (binding in the presence of unlabelled C3a) from the total binding. Plotted are the means ± SEM of data from three cell preparations. (c) The data of specific binding of ^{125}I–C3a to macrophages were processed by a Scatchard analysis. The resulting plot indicates two classes of C3a-receptor, a high-affinity class with $K_d = 2{\cdot}7$ nM and $n = 0{\cdot}63 \times 10^5$, and a relatively low-affinity class with $K_d = 51{\cdot}3$ nM and $n = 1{\cdot}2 \times 10^5$.

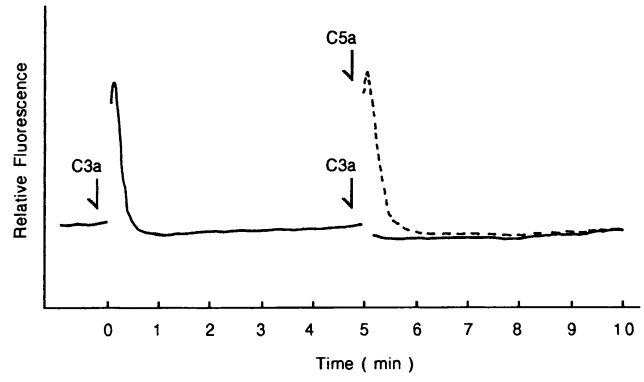

Figure 2. Increases in $[Ca^{2+}]_i$ in guinea-pig macrophages stimulated with C3a. Fura-2-loaded macrophages (5×10^6 cells) were initially stimulated with 300 nM C3a (——) and then with 300 nM C3a (——), or 100 nM C5a (– – –), after 5 min. The C3a and C5a were diluted in medium, which was also used for preparing the macrophage suspension.

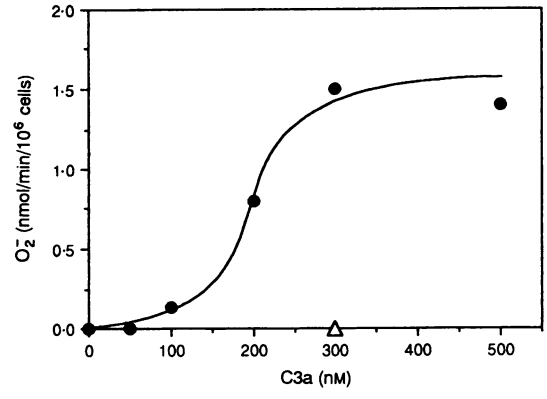

Figure 3. O_2^- generation from guinea-pig macrophages upon stimulation with C3a. Macrophages [1×10^6 cells (●)] were stimulated with varying concentrations of C3a and O_2^- generation was determined as described in Materials and Methods. O_2^- generation from PMN [1×10^6 cells (△)] was also determined with 300 nM C3a. The results presented here are from one of three similar experiments.

Cellular responses of guinea-pig macrophages to C3a

We first assessed the Ca^{2+} mobilization by C3a using Fura-2-loaded macrophages. C3a induced a rapid, but transient, increase in $[Ca^{2+}]_i$ (Fig. 2). The Ca^{2+} response was observed with 10 nM C3a and reached a plateau with 200 nM C3a (data not shown). Although the maxima of the $[Ca^{2+}]_i$ increase induced by C3a at a maximal dose (300 nM) varied from day to day, the average maximum for C3a was assumed to be approximately 695 ± 72 nM. After the increased $[Ca^{2+}]_i$ again reached baseline values, the same macrophages were stimulated again with the same amount of C3a. No Ca^{2+} response was observed, but the macrophages still showed Ca^{2+} response from stimulation with 100 nM C5a, which had been tested to cause maximum response. These results suggest that the desensitization observed with C3a was a homologous one and that the signal transduction pathway for C3a receptor is different from that of the C5a receptor.

Next, we assessed the generation of O_2^- from macrophages by C3a. O_2^- generation occurred at 50 nM C3a and reached a plateau at 300 nM C3a (Fig. 3). However, C3a failed to generate

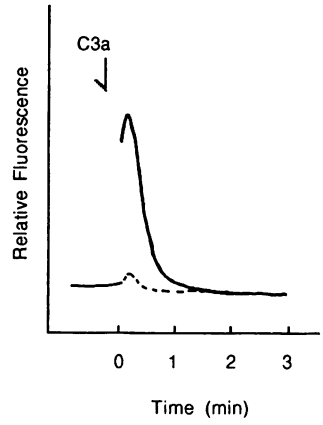

Figure 4. Effect of *Bordetella pertussis* toxin on $[Ca^{2+}]_i$ mobilization induced by C3a. Macrophages (2×10^7 cells/ml) were incubated with 100 ng/ml of the toxin for 2 hr at 37°. The toxin-treated (- - -) and untreated (——) cells (5×10^6 cells) were stimulated with 300 nM C3a in medium 1. The results presented here are from one of three similar experiments.

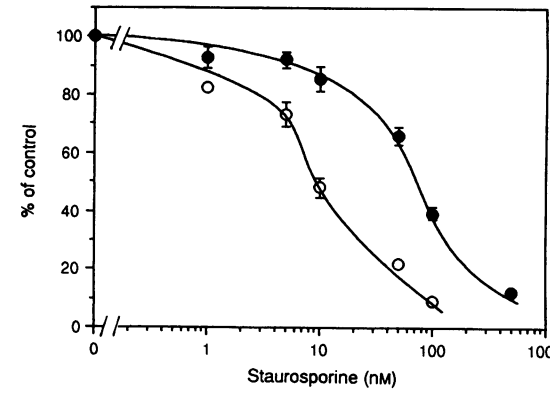

Figure 5. Inhibition of O_2^- generation with staurosporine. Macrophages (1×10^6 cells) were incubated with various concentrations of staurosporine for 5 min at 37°, and then stimulated with PMA [100 ng/ml (○)] and C3a [300 nM (●)]. The results are expressed as per cent of control values in the absence of staurosporine. Plotted are the means ± SEM of data from three different cell preparations.

O_2^- from PMN isolated from the peritoneal cavity of guinea-pigs. The maximum level of O_2^- released with 300 nM C3a was about 40% and 80% of those generated with 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and C5a, respectively (data not shown).

The chemotactic receptors, such as C5aR and FMLP-R have been shown to be coupled with a pertussis toxin-sensitive G protein.[3,24] The C3a-induced Ca^{2+} response was not detected with pertussis toxin-treated macrophages (Fig. 4). O_2^- generation by C3a was also inhibited by pretreatment of macrophages with pertussis toxin (data not shown). These results suggest that pertussis toxin-sensitive G proteins are coupled to the C3a receptor of macrophages.

We next examined the effect of staurosporine, a potent non-specific inhibitor of protein kinase C,[25] on the C3a-induced O_2^- generation. As a reference, the effect of staurosporine on O_2^- generation induced by PMA, a specific protein kinase C activator,[26] was determined. The dose–inhibition curves of PMA- and C3a-induced O_2^- generation are shown in Fig. 5. The PMA-induced O_2^- generation was more sensitive to staurosporine than that induced by C3a. The IC_{50} values, indicating concentrations of staurosporine causing 50% inhibition of the PMA-induced and C3a-induced O_2^- generations were approximately 8 nM and 80 nM, respectively. These data indicate that the signal pathway leading to O_2^- generation coupled with C3a may be different from that with PMA.

DISCUSSION

Biochemical information on the C3a receptor has been limited so far to that of guinea-pig platelets;[8,9] little information has been available on the role of the C3a receptor in phagocytes. In this investigation, we have attempted to characterize the C3a receptor of guinea-pig macrophages, because it is easy to prepare them in sufficient numbers for analysis of cellular responses. In addition, signal transduction pathways in guinea-pig macrophages leading to a Ca^{2+} response and O_2^- release have been well investigated in our laboratory.[21] Concerning the compatibility between human C3a and guinea-pig C3a receptor, it was shown that functional identity exists between human C3a and guinea-pig C3a in stimulating and binding properties to the C3a receptors of guinea-pig platelets.[9]

Equilibrium binding was established at 22° and time-dependent decrease of bound C3a was observed at 37°. This decrease is probably because of internalization of the C3a–C3a receptor complex as reported for the C5a receptor.[27] In the absence of *o*-phenanthroline, time-dependent binding of C3a was not observed, and the maximal C3a binding was only about 50% of that attained in the presence of *o*-phenanthroline (data not shown). These data suggest that C3a may be cleaved by metal-dependent peptidases on the cell membrane of macrophages.

Guinea-pig platelets have been reported to carry about 200 high-affinity C3a-binding sites/cell and 500 low-affinity sites/cell.[9] The saturation binding assay of C3a presented in this work revealed that guinea-pig macrophages carry high-affinity as well as low-affinity binding sites, and that their numbers are 200–300 times higher than those found on platelets.

After the completion of this work, a detailed description of the C3a receptors in human PMN and dibutyryl-cAMP-differentiated U937, a human cell line showing many characteristics of activated macrophages, was published.[28] Differentiated U937 are reported to express about 67,000 C3a-binding sites with a K_d of 4·5 nM. These data are comparable with the high-affinity C3a-binding sites of guinea-pig macrophages (63,000 sites/cell with a K_d of 2·7 nM). Thus, macrophages seem to express a high number of binding sites for C3a not only in guinea-pig but also in humans. Accordingly, the difference in the C3a-binding sites between guinea-pig platelets and macrophages seems to be due to the difference in cell types.

C3a was found to induce a Ca^{2+} response and O_2^- release in guinea-pig macrophages. One may argue that these cellular responses are because of trace contamination of the C3a preparation with C5a. However, this possibility is ruled out by the following observations:

(1) We prepared C3a from purified C3 using C3 convertase, CVFBb. C5 was not detected immunochemically in the purified C3 preparation. We also prepared C3a from C3, which had been treated with an affinity column fixed with anti-C5 antibody. C3a

prepared from this C3 preparation still induced similar cellular responses in macrophages.

(2) Reverse-phase HPLC is an effective method to obtain a clear-cut separation between C3a and C5a.

(3) Once treated with the C3a preparation, the macrophages did not respond to the C3a but continued to respond to C5a to induce Ca^{2+} mobilization.

(4) In contrast to C5a, the C3a preparation failed to induce O_2^- generation from human peripheral and guinea-pig peritoneal PMN.

The cloning of human and mouse C5a receptor cDNA has unequivocally shown that G proteins are coupled to the C5a receptors.[3,4] Structural and functional similarities between C3a and C5a suggested that G proteins could also be coupled to C3a receptors. Our observation that the cellular responses induced by C3a were almost completely inhibited by pretreatment with pertussis toxin support this assumption. Evidence that the macrophages which become tachyphylactic to C3a still respond to C5a suggests that C3a and C5a receptors are coupled to slightly different signal transduction pathways.

Studies of inhibition by staurosporine, a potent non-specific inhibitor of protein kinase C, suggested that the signal transduction pathway for C3a-induced O_2^- generation is different from that for PMA-induced O_2^- generation. Robinson *et al.* also observed that the FMLP-induced O_2^- generation is more resistant to staurosporine than that induced by PMA, and proposed that FMLP acts through a stimulatory pathway in PMN that contains a protein kinase distinct from protein kinase C.[29] The IC_{50} values of staurosporine to FMLP-induced and C3a-induced O_2^- generation were almost the same. This suggests that C3a acts through activation of another kinase associated with the FMLP-induced signal pathway.

Our data, together with the recent paper by Klos *et al.*,[28] strongly suggest that C3a is an important inflammatory mediator that modulates the cellular functions of macrophages. Although Klos *et al.*[28] described a C3a-dependent Ca^{2+} response with human PMN, little is known about the effector functions generated through activation of the C3a receptors of PMN. We found no evidence of C3a-dependent O_2^- generation from guinea-pig PMN isolated from the peritoneal cavity. Thus, the study of C3a-dependent cellular responses of PMN is essential to understand the pathophysiological roles of C3a.

ACKNOWLEDGMENTS

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