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Dual pertussis toxin-sensitive pathway of zymosan-induced activation in guinea pig macrophages

An anti-CR3 antibody-inhibitable stimulation of phagocytosis and -resistant stimulation of O_2^- production and arachidonate release

Kaoru Hazeki[a,**], Koichi Tamoto[a,*], Michio Ui[b], Yoki Mori[a]

[a]*Department of Microbiology, Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Ishikari-Tobetsu 061–02, Japan*
[b]*Ui Laboratory, the Institute of Physical and Chemical Research, Wako-shi 351–01, Japan*

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Abstract

Complement receptor type 3 (CR3)-mediated cellular responses in guinea pig macrophages were investigated by using zymosan and serum-opsonized zymosan (SOZ) as the multivalent ligand for CR3. The ingestion of zymosan and SOZ was accompanied by O_2^- generation and arachidonate release. These responses were suppressed by prior exposure of macrophages to pertussis toxin (PT). Opsonization of zymosan gave rise to more than 6-fold activation of the ingestion, whereas the magnitude of either arachinonate release or O_2^- generation was unchanged. The Fab' fragment of anti-Z-1, a monoclonal antibody specific for the α chain of guinea pig CR3, inhibited the ingestion of zymosan by 60% without affecting zymosan-induced arachidonate release and O_2^- generation. These data suggested that there might be at least two functionally distinct binding sites for zymosan. O_2^- generation and arachidonate release might be regulated through one site and phagocytosis another. Both sites should be coupled to PT-sensitive GTP binding protein.

Key words: GTP-binding protein; CR3; Zymosan; Superoxide; Phagocytosis; Phospholipase A2

1. Introduction

CR3 is a well known leukocyte integrin and acts as the specific receptor for C3bi [1–3]. In addition, CR3 is involved in leukocyte adherence functions such as cell spreading on plastic surfaces, granulocyte aggregation, and chemotaxis [4,5]. CR3 further has lectin-like properties of binding to zymosan and bacteria that lack fixed C3bi [6,7].

In previous studies, we have prepared a monoclonal antibody, named anti-Z-1, which effectively inhibits phagocytosis of zymosan and SOZ by guinea pig macrophages [8]. The Z-1 antigen consists of an α-chain of 140 kDa non-covalently associated with a β-chain of 95 kDa. Recently, the Z-1 antigen has been identified as the guinea pig homologue of human CR3 [8,9]. The rationale for the identification is as follows: (i) the Z-1 antigen is predominantly expressed on macrophages and granulocytes and is involved in zymosan-phagocytosis, as is human CR3, (ii) anti-Z-1 binds to the α chain of human CR3, (iii) the β subunit of the Z-1 antigen is shared by two other surface antigens on guinea pig leukocytes that have their own unique α subunit (Nochi et al., in preparation), presumably the homologues of human LFA-1 and gp150, 95, respectively [10].

The expression of CR3 as well as the phagocytic capacity associated with CR3 is reported to be increased by C kinase [11,12]. We have previously found that C kinase-induced phosphorylation of CR3 (β) resulted in down-regulation of CR3-mediated phagocytosis [13]. Regulation of CR3-mediated responses by surroundings has been well characterized but the signalling pathways arising from stimulation of CR3 itself have not been clarified. To identify the CR3 signal transduction, the inhibitory effect of a monoclonal antibody to the receptor on zymosan-induced responses was examied. PT,

* Corresponding author. Fax: (81) (1332) 31669.

** *Present address:* Ui laboratory, The Institute of Physical and Chemical Research, Wako-shi 351–01, Japan.

Abbreviations: CR3, complement receptor type 3; C3bi, C3b treated with C3b inactivator; SOZ, serum-opsonized zymosan; PT, pertussis toxin; PMA, phorbol-12-myristate-13-acetate; fMLP, formyl-Met-Leu-Phe; BSA, bovine serum albumin; KRH, Krebs–Ringer HEPES; E, sheep red blood cell; EIgG, E coated with rabbit anti-sheep red blood cell; LFA-1, leukocyte function associated protein-1, C kinase; protein kinase C.

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which blocks agonist-induced responses by causing ADP-ribosylation of mediating GTP-binding proteins [14], was simultaneously examined. It was demonstrated that a PT-sensitive GTP-binding protein is involved in CR3-mediated responses in guinea pig macrophages. The anti-CR3 antibody distinguished the pathway involved in zymosan-induced phagocytosis from that for O_2^- generation.

2. Materials and methods

2.1. Materials

PT was purified from the 2-day culture supernatant of *Bordetella pertussis* [15]. Zymosan, PMA, fMLP, cytochalasin B, cytochrome *c*, and BSA (fatty acid free) were purchased from Sigma, [^{3}H]arachidonate, Na^{125}I, Na$_2{}^{51}$CrO$_4$, from New England Nuclear, and the GIT medium from Wako Chemical Co. (Tokyo).

2.2. Preparation of macrophages

Hartley guinea pigs weighing approximately 500 g were injected intraperitoneally with 20 ml of sterile thioglycollate broth 4 days before the collection of the exudate [8,13]. The cells in the exsudate were collected in the Krebs–Ringer HEPES (KRH) consisting of 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.1% BSA, 5 mM glucose, and 10 mM HEPES (pH 7.4). The cells were washed twice with KRH and finally suspended in the GIT medium at the concentration of 10^7 cells/ml. Cells were then seeded at the density of 2×10^5 cells/cm^2 in tissue culture wells and cultured at 37°C for 4 h in a humidified atmosphere containing 5% CO_2. Where indicated, the culture medium was supplemented with PT or [^{3}H]arachidonate (0.1 μCi/well). After this 4-h culture period, the non-adherent cells were removed by three times washing with KRH and monolayers of adherent cells were used as macrophages for measurements of O_2^- generation, arachidonate release and phagocytosis.

2.3. Preparation of monoclonal anti-guinea pig CR3

A mouse monoclonal anti-Z-1 antibody that recognizes the α subunit of guinea pig CR3 was purified from ascites fluid and its Fab′ fragment was prepared, as previously described [8].

2.4. Preparation of ^{125}I-labeled zymosan

Zymosan was labeled with Na^{125}I by the chloramine T method as previously described [13]. The labeled zymosan was washed five times with PBS, and dialyzed for 4 days with eight changes of 1,000 ml of PBS containing 10 mM KI. After dialysis, the zymosan was pelleted, washed, and stored in PBS at a concentration of 5 mg/ml at −20°C.

2.5. Preparation of serum-opsonized zymosan (SOZ) and EIgG

^{125}I-Labeled or unlabeled zymosan A prepared as above was incubated with freshly prepared guinea pig serum (10%) at 37°C for 30 min and washed three times with KRH immediately before use.

Sheep red blood cells (E) were labeled with ^{51}Cr and incubated with rabbit anti-E antibody as previously described [13,16].

2.6. O_2^- generation

O_2^- generation was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* [17]. The macrophage monolayers were preincubated in KRH at 37°C for 10 min and then fortified with PMA, fMLP, zymosan or SOZ together with cytochrome *c* (100 μM). Where indicated, the preincubation medium was fortified with monoclonal anti-CR3 antibody, anti-Z-1. After a 20-min incubation, the incubation medium was withdrawn to be determined for changes in absorbance of cytochrome *c* (550–540 nm).

2.7. Arachidonate release from [^{3}H]arachidonate-labeled cells

[^{3}H]Arachidonate-labeled macrophage monolayers were preincubated in KRH at 37°C for 10 min and then incubated with zymosan or SOZ for 20 min. Where indicated, the preincubation medium was supplemented with anti-Z-1. The radioactivity of ^{3}H released from the cells was measured as arachidonate release [18].

2.8. Phagocytosis of target cells

Macrophage monolayers in GIT medium were incubated with [^{125}I]zymosan, [^{125}I]SOZ, or ^{51}Cr-EIgG at 37°C in a CO_2-incubator for 60 min. For the assay of zymosan or SOZ phagocytosis, the cells were vigorously washed four times with PBS, which was withdrawn each time to remove free particles [13]. For the assay of EIgG phagocytosis, macrophage monolayers were washed twice with PBS, then exposed to 1/5 diluted hypotonic PBS for 30 s, and further washed twice with PBS to remove the EIgG bound at surface receptors [19]. These cells were finally solubilized with 2% Na_2CO_3/0.1 N NaOH and the cell-associated radioactivity was measured in a autogamma counter.

3. Results

3.1. Inhibition of fMLP- or zymosan-induced O_2^- generation by prior exposure of macrophages to PT

As reported earlier, PMA, fMLP or zymosan stimulated O_2^- generation in macrophages. fMLP- or zymosan-induced O_2^- generation was almost totally suppressed by the prior treatment of the cells with PT (Fig. 1). On the other hand, PMA-induced O_2^- generation was not affected by the PT treatment. The inhibitory effect of PT was dependent on the concentration of the toxin bin the culture medium. It has been well documented that the fMLP receptor is associated with a PT-sensitive GTP binding protein [18,20,21]. Since the degree of the inhibition of O_2^- generation by PT was indistinguishable between fMLP- and zymosan-treated cells at any PT concentration, zymosan receptor should be also coupled with the GTP-binding protein.

3.2. Inhibition of zymosan or SOZ ingestion by prior treatment of macrophages with PT

Zymosan has been demonstrated to bind to CR3 [6]. Opsonization of zymosan with fresh serum gives C3bi-coated zymosan (SOZ) which is a more efficient ligand of CR3. To determine CR3-dependent phagocytosis quantitatively, ^{125}I-labeled zymosan and [^{125}I]SOZ were

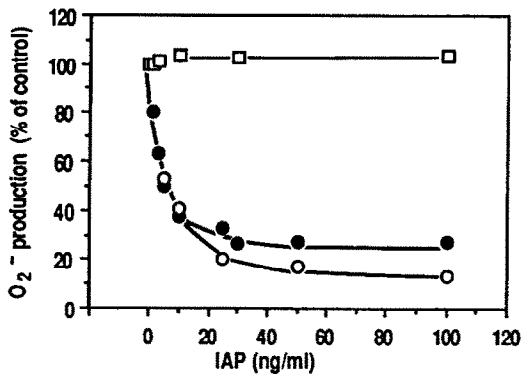

Fig. 1. Inhibitory effect of PT on fMLP- and zymosan-induced O_2^- production. Macrophages cultured with increasing concentration of PT were incubated with 1 mg/ml zymosan (●), 0.1 μM fMLP (○), or 0.1 μM PMA (□) for 20 min. O_2^- production was measured by the reduction of cytochrome *c*. O_2^- production in untreated cells were 36.6 nmol/5×10^5 cells for zymosan, 105.6 for fMLP, and 267.1 for PMA. Results were expressed as the percentage of these control value. Each point is the mean of duplicate observations in a typical experiment which was repeated more than three times essentially the same results.

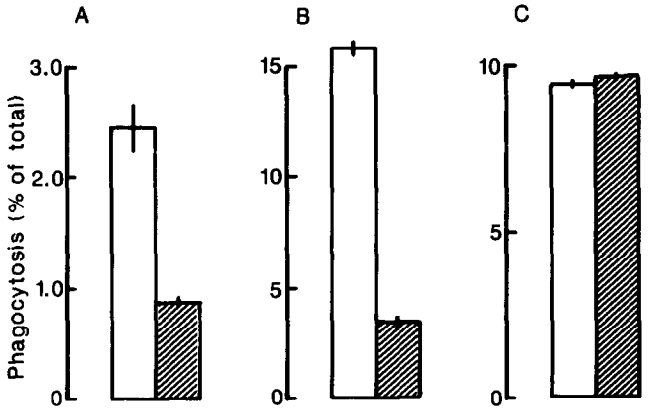

Fig. 2. Inhibitory effect of PT on phagocytosis of zymosan or SOZ by macrophages. Macrophages were first cultured with (shaded bar, 100 ng/ml) or without (open bar) PT for 4 h and then incubated with [^{125}I]zymosan (A), [^{125}I]SOZ (B), or ^{51}Cr-EIgG (C) for 60 min. Phagocytosis of these particles were expressed as percentage of total radioactivity added to the macrophages. Each bar shows the mean ± S.E.M. from triplicate observations in a typical experiment.

prepared and added to macrophage monolayers. Opsonization of zymosan gave rise to a more than 6-fold activation of the ingestion (Fig. 2). Both zymosan and SOZ phagocytoses were inhibited by the prior treatment of macrophages with PT. In contrast, the ingestion of antibody-sensitized sheep E, which binds to Fc receptor, was never inhibited by the PT treatment (Fig. 2), making it unlikely that PT susceptibility is a general event associated with particle ingestion. Together with the results shown in Fig. 1, CR3 should be coupled to a PT-substrate GTP-binding protein.

3.3. *Inhibition of zymosan- or SOZ-induced arachidonic acid release and O_2^- generation by prior exposure of macrophages to PT*

Both zymosan and SOZ induced arachidonic acid release as well as O_2^- generation (Fig. 3). These responses increased in a manner similarly dependent on the concentration of particles added. Both effects were markedly suppressed or mostly abolished by PT regardless of the concentrations of zymosan or SOZ. Apart from their differential effects on phagocytosis, both zymosan and SOZ caused essentially the same degree of increase in arachidonic acid release and O_2^- generation. The data would suggest that these particles were ingested via the C3bi binding site of CR3 whereas arachidonate release and O_2^- generation were activated through a different binding site of the receptor.

3.4. *CR3-dependent stimulation of phagocytosis and CR3-independent stimulation of arachidonate release and O_2^- generation by zymosan*

Macrophages were preincubated with Fab' of anti-Z-1 that is specific for the α subunit of guinea pig CR3 and then supplemented with zymosan. Ingestion of zymosan was inhibited markedly by the antibody fragment with the maximal degree of inhibition of 60% (Fig. 4). In contrast, zymosan-induced arachidonate release and O_2^- generation was hardly inhibited by the antibody. Together with the results shown in Fig. 3, zymosan particles were ingested through the C3bi-binding site of CR3, which was also an anti-Z-1-susceptible site on CR3. In contrast, zymosan-induced arachidonate release and O_2^- generation occurred through anti-Z-1-insusceptible site on CR3 or through other undefined receptor(s) for zymosan. At least two functionally distinguishable receptors were operative in the activation of macrophages by zymosan. Both were regulated through PT-sensitive G protein.

4. Discussion

4.1. *CR3-dependent and -independent responses of macrophages to zymosan stimulation*

CR3 plays a central role in host defence against microorganisms since, in many cases, bound C3b is converted

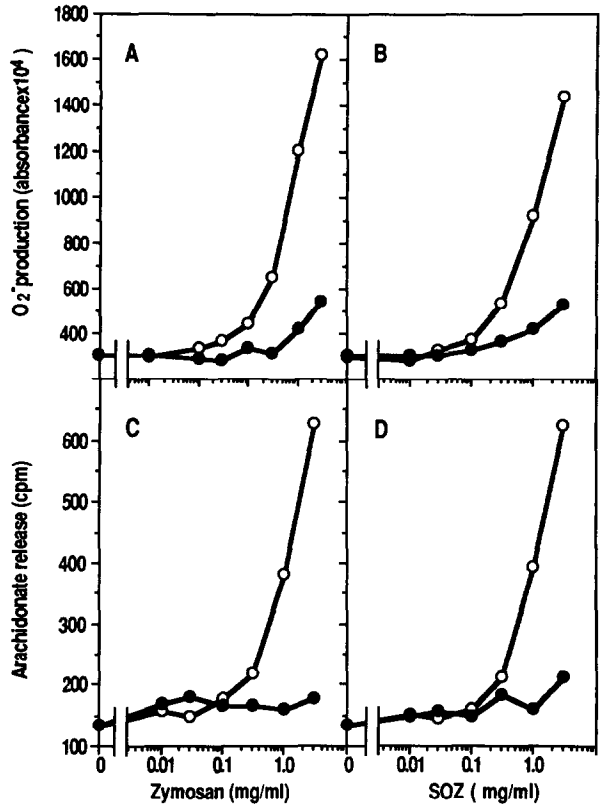

Fig. 3. Dose–response curves for zymosan and SOZ effects on O_2^- production and arachidonate release in PT-treated or non-treated macrophages. Macrophages were cultured with [^{3}H]arachidonate in the presence (●) or absence (○) of 100 ng/ml of PT for 4 h. The cells twice washed were incubated for 20 min with increasing concentration of zymosan (A,C) or SOZ (B,D). O_2^- production was measured by the reduction of cytochrome *c* (A,B). The radioactivity released into the medium is measured as arachidonate release (C,D) as described in section 2. Each point is the mean of duplicate observations in a typical experiment. Similar results were obtain in repeated experiments.

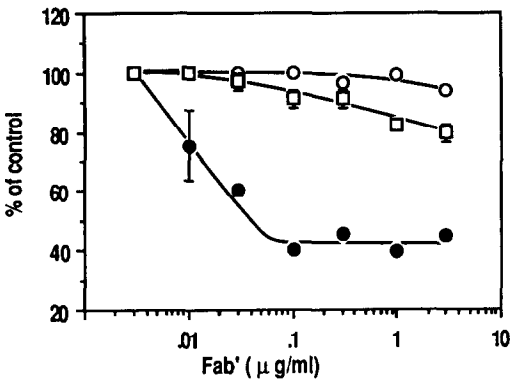

Fig. 4. Effect of anti-CR3 antibody on phagocytosis, arachidonate release, and O_2^- production in macrophages. Macrophages were cultured with (□) or without (○, ●) [^{3}H]arachidonate for 4 h. The cells washed twice were treated with increasing concentrations of Fab' of monoclonal anti-Z-1 for 15 min and then incubated with 1 mg/ml of zymosan for 20 min for arachidonate release (⊔), for O_2^- production (○) or for phagocytosis of [^{125}I]zymosan (1 mg/ml) for 60 min (●). Each point shows the mean value ± S.E.M. from three observations in a typical experiment. Error bars are within the size of symbols in some cases.

to C3bi on the surface of the foreign materials [22]. SOZ is also considered to be a ligand of CR3 because 84–87% of the bound C3 consists of C3bi [23]. Furthermore, unopsonized zymosan as well as microorganisms such as yeast and virulent pneumococci directly bind to CR3 [6,24,25]. In guinea pig, the regulatory system for the cleavage of the complement C3b is almost identical with that in humans [26,27]. Our data in this communication suggest that there might be at least two functionally distinct binding sites for zymosan in guinea pig macrophages. One would be a C3bi-binding site, which is occupied by anti-Z-1, and mediate phagocytosis only. Another putative binding site (referred to henceforth as Rz) should be involved in arachidonic acid release and O_2^- generation. Experimental supports for this assumption are as follows. (i) Opsonization of zymosan caused a 6-fold activation of the ingestion without increasing O_2^- production and arachidonate release. (ii) An antibody against guinea pig CR3 inhibited only zymosan phagocytosis without inhibition of accompanying O_2^- production or arachidonate release.

4.2. *Possible involvement of a GTP-binding protein in CR3-dependent and -independent process of zymosan stimulation*

It is now widely accepted that occupancy of fMLP receptors in leukocytes is coupled to activation of phospholipase C via a GTP-binding protein [18,20,21]. PT causes ADP-ribosylation of GTP-binding proteins and blocks fMLP-induced inositol phosphate formation. The dose and time required for PT treatment of macrophages to inhibit zymosan-induced O_2^- generation, arachidonic acid release, and phagocytosis were almost the same as those required to inhibit fMLP-induced responses. Hence, we could be convinced of the involvement of GTP-binding proteins in zymosan-induced activation of macrophages. The fact that PT inhibited not only O_2^- production but also phagocytosis might indicate the coupling of GTP-binding protein(s) with both C3bi-binding site and Rz. Recently, we found that spreading of macrophages to plastic dishes was dramatically inhibited by PT (in preparation) and that tyrosine phosphorylation of $p125^{FAK}$, a tyrosine kinase presumably associated with cell adhesion molecule, was dramatically decreased in PT-treated cells (unpublished data). These findings are suggestive of functional connection of a GTP binding protein with cell adhesion molecules like CR3.

It has been believed that phagocytosis is accompanied by other microbiocidal mechanisms such as superoxide production and vesicle degranulation. However, under the special conditions, superoxide production and degranulation were demonstrated to occur independently of phagocytosis [28,29]. Our data in this paper suggest that phagocytosis and the other responses do not always occur by one and the same mechanism, although these immuno-inflammatory responses might be functionally related to each other.

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