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Piperlactam S suppresses macrophage migration by impeding F-actin polymerization and filopodia extension

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Abstract

This study was designed to evaluate the anti-inflammatory effect of piperlactam S on chemoattractant-induced migration, functions underlying leukocyte recruitment in vitro. Results showed that RAW264.7 macrophages migrate toward complement 5a, a powerful chemoattractant for macrophages. This phenomenon could be suppressed concentration dependently by piperlactam S (0.3–30 μM). Fluorescence staining demonstrated that piperlactam S and cytochalasin B both effectively reversed complement 5a-induced cell polarization, filopodia extension, as well as the increase in the cell content of F-actin. Functional inhibition by antibodies suggested that Mac-1 (CD11b) integrin plays a central role in complement 5a-induced migration. However, piperlactam S failed to modify Mac-1 expression. Furthermore, complement 5a triggered the activation of Cdc42, a Rho-family protein involved in the regulation of filopodia extension, with a time course that paralleled that of filopodia extension and which was inhibited by piperlactam S. In summary, piperlactam S exerts anti-inflammatory effects possibly by interfering with cell migration, impeding F-actin polymerization, filopodia formation, and/or Cdc42 activation. However, the detailed mechanism by which piperlactam S regulates the above processes needs further study.
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Keywords: Piperlactam S; Complement 5a; RAW264.7 macrophage; F-actin; Filopodia; Cdc42

1. Introduction

The inappropriate recruitment of macrophages to sites of inflammation or tissue injury is an essential process of inflammation. Crucial steps underlying this process include adhesion, cytoskeletal reorganization, cell polarization and spreading, and finally transmigration (Steeber and Tedder, 2001). A variety of products present at the site of inflammation can act as chemotactic agents, including bacterial products such as formylmethionyl peptides, complement products, platelet-activating factor, and leukotriene B4. Proinflammatory stimuli, such as complement 5a generated by tissue injury, could activate macrophages to up-regulate adhesive integrins and enhance the inflammatory response (Issekutz, 1995). Of the several families of leukocyte adhesion molecules, the monocyte/macrophage-specific integrins (β_2 integrins, CD11/CD18 molecules) are among the most important (Sànchez-Madrid and Corbi, 1992). The β_2 (CD18) integrins comprise three forms of heterodimeric glycoproteins, namely, lymphocyte function-associated (LFA)-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150, 95 (CD11c/ CD18), with Mac-1 being the predominant form in macrophages during inflammation (Arefieva and Krasnikova, 2001). Therefore, prevention of Mac-1-mediated adhesion and/or transmigration by macrophages is a potential target for drugs to control inflammation.

Leukocyte migration is mediated by the coordinated activation of the cytoskeleton and associated adhesive integrins (Laudanna et al., 1996). The major component of the cytoskeleton is actin which, in response to chemotactic stimuli, is rapidly and transiently converted from a monomeric, globular form, G-actin, to a needle-like filamentous form, F-actin (Gupta and Campenot, 1996). Although cell migration cannot be attributed to F-actin polymerization alone, the redistribution of F-actin fibers and the formation

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of pseudopodia are important events in cell locomotion (Watts, 1996). Furthermore, dynamic rearrangement of the actin cytoskeleton appears to be coordinated in space and time by intracellular signaling pathways involving small G protein activation. Recently, members of the Rho family of GTPases have been implicated in cytoskeletal alterations mediated by many agonists (Tapon and Hall, 1997). In particular, Rho induces the assembly of contractile actin-based microfilaments such as stress fibers, Rac regulates the formation of membrane ruffles and lamellipodia, and Cdc42 activation induces the formation of filopodia (Hall, 1998; Jones et al., 1998).

Piperlactam S is an alkaloid isolated from *Piper kadsura*, an anti-inflammatory Chinese herbal medicine used for the treatment of asthma and rheumatic arthritis (Han et al., 1990). A common feature of these conditions is chronic inflammation. In asthma patients, there is an accumulation of neutrophils, macrophages, and other immune cells in the air spaces. Furthermore, inappropriate recruitment of leukocytes from the blood to the site of infection, with subsequent phagocytosis of microorganisms, can result in a deleterious inflammatory response and tissue destruction in rheumatic arthritis. Thus, one of the therapeutic objectives in asthma and arthritic inflammation is to reduce the local inflammatory response by reducing inflammatory cell activation. Recently, piperlactam S was found to exert an immune regulatory effect on human T lymphocytes, by regulating cell proliferation and gene expression (Kuo et al., 2000). We hypothesized that piperlactam S may be one of the active components accounting for the anti-inflammatory activity of *P. kadsura*, and suggested that a putative beneficial effect of piperlactam S on asthma and arthritis might be mediated by interference with the inappropriate recruitment of macrophages. To accomplish the investigation, complement 5a-induced chemotaxis in the macrophage cell line RAW264.7 was used as an in vitro model. As remarked above, morphological change, F-actin reorganization and pseudopodia formation are important events in cell locomotion, and therefore, cell morphology and F-actin fluorescent stain were examined in particular.

2. Materials and methods

2.1. Cell culture condition

RAW264.7 macrophages (American Type Culture Collection, TIB 71, Rockville, MD) were cultured in 75-cm^2 plastic flasks (Corning Costar, Cambridge, MA, USA) with Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) supplemented with 10% heat-inactivated fetal calf serum, penicillin and streptomycin (Biological Industries, Israel) (Chiou et al., 2000). Peritoneal macrophages were collected from ICR mice anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Peritoneal macrophages were collected by washing the cavity with 4 ml sterile phosphate-buffered saline (PBS) containing heparin (50 U/ml). Peritoneal fluid was centrifuged and the cell pellet was washed twice with PBS. Cells were then plated onto 35-mm culture dishes ($4-5 \times 10^6$ cells per dish) containing DMEM. After 3 h at 37 °C, non-adherent cells were removed by washing with sterile PBS. Macrophages were removed from the culture dishes by vigorous pipetting and then centrifuged ($300 \times g$ for 10 min) and resuspended in DMEM at a concentration of 2×10^7/ml. Macrophage viability (95–98%) was determined by exclusion of Trypan blue.

2.2. Chemotactic migration

Cell migration in response to chemoattractant was assessed using a 24-well chemotaxis chamber with a membrane pore size of 5 μm (Transwell, Corning Costar). One-hundred microliters of cell suspension (2×10^7/ml) was added to each of the upper wells before and after treatment with piperlactam S, cytochalasin B, or colchicine for 15 min. For antibody functional blocking experiments, monoclonal antibodies to mouse CD18/β_2 (Pierce Endogen, MA, USA), CD11a (Pierce Endogen), and CD11b (Chemicon International, Temecula, CA) were first incubated with the cells for 60 min at room temperature. Complement 5a, a powerful chemoattractant for macrophages that has been shown to have an immune regulatory role and may stimulate mediators of both chronic and acute inflammation (Haynes et al., 2000), was added to the lower well of the chamber to assess chemoattractic activity. Then the entire chamber was incubated at 37 °C to initiate migration. Non-migrated cells were wiped off with a cotton swab and then the filter was fixed and stained with hematoxylin (Sigma) to define the cell nuclei. Chemotaxis was assessed by counting the number of migrated cells in five (at 400 × magnification) random microscopy fields per well. All experiments were performed in triplicate. Complement 5a-induced cell migration minus spontaneous migration in PBS served as control and was designated as 100% migration for each experiment.

2.3. Measurement of F-actin content by flow cytometry

Piperlactam S-pretreated macrophages were stimulated with or without complement 5a (1 μg/ml) for 15 min. Cells were then fixed at room temperature by addition of 4% paraformaldehyde supplemented with lysophosphatidylcholine (Sigma) for 10 min. They were stained for 30 min by adding 50 μl of fluorescein isothiocyanate (FITC)-phalloidin. After being washed twice in PBS, samples were evaluated by means of flow cytometry (FACSort; Becton Dickinson) for actin polymerization (Carulli et al., 1997). Data are expressed as mean channel fluorescence intensity for each sample, calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 6100/66 computer.

2.4. F-actin fluorescence stain to assay cytoskeleton distribution

FITC-labeled phalloidin (Molecular Probes, USA) was used to visualize changes in actin fiber organization in RAW264 macrophages (Cross and Woodroofe, 1999). Briefly, cells were seeded onto sterile coverslips in 24-well plates at a concentration of 5×10^4 cells/ml and pretreated with piperlactam S or cytochalasin B (Sigma) at 37 °C for 30 min. Cells were then stimulated with complement 5a for further 15 min. To stop activation by complement 5a, the solution was aspirated and cells were immediately fixed with 4% paraformaldehyde (Sigma) in PBS for 20 min. Cells were then permeabilized by incubation in 0.1% Triton X-100 (Sigma) for 15 min, washed twice in PBS containing 1% bovine serum albumin and incubated with FITC-conjugated phalloidin (200 ng/ml) at room temperature in a humidified atmosphere for 30 min. Cells were visualized under fluorescence microscopy (Olympus) and photographed.

2.5. Immunofluorescent staining for Mac-1 (CD11b)

Cells were seeded onto sterile coverslips in 24-well plates at a concentration of 5×10^4 cells/ml. The expression of Mac-1 (CD11b) on RAW264.7 macrophages stimulated with complement 5a (1 μg/ml, 30 min) before and after piperlactam S pretreatment was analyzed by adding rat anti-mouse CD11b (Chemicon). Isotype-matched control Ab, immunoglobulin G (IgG), was used to determine nonspecific binding. To stop activation by complement 5a, the solution was aspirated and cells were immediately fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.1% Triton X-100 for 15 min. After being washed twice in PBS containing 1% bovine serum albumin, cells were incubated with primary Ab for 60 min and then with FITC-goat anti rat IgG (Chemicon) for 60 min (Endemann et al., 1996). Cells were visualized under fluorescence microscopy (Olympus, Tokyo, Japan BX60) and photographed using Kodak 400 color film.

2.6. Measurement of Mac-1 up-regulation by flow cytometry

Expression of Mac-1 (CD11b) was analyzed as described in our previous study (Shen et al., 2002). Briefly, piperlactam S-pretreated macrophages were stimulated with complement 5a (1 μg/ml) for 30 min. The cells were then pelleted and resuspended in 1 ml ice-cold PBS containing 10% heat-inactivated fetal calf serum and 10 mM sodium azide. For staining of Mac-1, all subsequent steps were carried out in an ice bath. Cells were incubated in the dark for 60 min with FITC-conjugated anti-Mac-1 (anti-CD11b) antibody or a non-specific antibody (class IgG, Sigma) as a negative control. After two washes with PBS containing 5% fetal calf serum, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% of paraformaldehyde and analyzed on a flow cytometer (FACSort; Becton Dickinson) for Mac-1 expression. Data are expressed as the mean channel fluorescence intensity for each sample, as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 6100/66 computer.

2.7. Affinity precipitation using GST-PBD for Cdc42 activation assay

Cdc42 activation was analyzed according to the method described by Benard et al. (1999), using a commercial Cdc42 activation assay kit (UBI, Lake Placid, NY, USA). Cdc42 (~ 22 kDa) activates p21-activated kinases (PAK) through binding to the p21-binding domain (PBD), and the PAK protein exhibits a selective affinity for the GTP-bound form of Cdc42 (Benard et al., 1999). This was demonstrated using a GST fusion protein containing the p21-binding domain (PBD) of PAK that specifically binds the GTP-bound form of Cdc42. This protein was used to pull down activated Cdc42 from extracts of resting or complement 5a-stimulated RAW264.7 macrophages. The Cdc42 activation kit contains PAK bound to glutathione beads (GST/PBD), anti-mouse cdc42, 5 × Mg^{2+} lysis/wash buffer (5 × MLB) and recombinant GTPγS and GDP. Briefly, cells seeded in fibrinogen-coated flasks were stimulated with complement 5a (1 μg/ml) for 1, 5 and 10 min, respectively. The cells were lysed with 1 × MLB, and then 10 μg PAK/GST-PBD was added to 1-ml cell lysate and incubated at 4 °C for 60 min. Non-stimulated cell lysate was incubated with recombinant GTPγS and GDP and used as negative and positive control, respectively. The agarose beads were collected by pulsing (5 s, 14,000 × *g*) and draining off the supernatant. The agarose beads were resuspended in Laemmli sample buffer and boiled for 5 min. Supernatant was assayed by SDS-PAGE and immunoblotted with anti-Cdc42.

2.8. Identification of piperlactam S from P. kadsura

P. kadsura was collected at Pettou, Taiwan, and identified by Prof. Cheng-Jen Chou. A voucher specimen has been deposited in the herbarium of the Department of Botany of the National Taiwan University. High-performance liquid chromatography (HPLC) was used to extract pure active components from the *P. kadsura*. Piperlactam S was obtained from the third combined fraction as a yellow solid with m.p. of 242–244 °C. The chemical name of piperlactam S is 10-amino-3-hydroxy-4-methoxy-*N*-methoxyphenanthrene-1-carboxylic acid lactam ($C_{17}H_{13}NO_4$, MW: 295). The purity of piperlactam S was determined by HPLC with an ultraviolet (UV) detector (254 nm, Waters). Piperlactam S appeared as a single peak at 6.463 min retention time and its purity was 99.077% (Kuo et al., 2000). Before use, piperlactam S was first dissolved in dimethylsulfoxide at 10 mM and then serially diluted in PBS immediately prior to

experiments. Final concentration of solvent vehicle in the reaction mixture was less than 0.1% and did not show any significant effect on any assay system.

2.9. Statistical analysis

All values in the text and figures represent means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's *t*-test for multiple comparison. Concentration dependence was analyzed by simple linear regression analysis of response levels against concentrations of drug and by testing the slope of the regression line against 0 by Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of piperlactam S on complement 5a-induced chemotactic migration

Complement 5a (0.1–5 μg/ml) was able to induce significant migration of RAW264.7 cells in a concentration-dependent manner with a peak activity at 1 μg/ml (data not shown). Time course analysis from a preliminary study also showed an increase in cell migration with time: significant starting at 2 h and reaching a maximum between 4 and 6 h. Therefore, 1 μg/ml of complement 5a and a migration period of 4 h were selected for further experiments. As shown in Fig. 1A, non-stimulated control macrophages displayed spontaneous migration with a total cell number of 93 ± 14. The gradient generated by complement 5a induced an 11-fold increase in cell migration as compared with control. Piperlactam S (0.3–30 μM) alone did not influence spontaneous transmigration (data not shown), but concentration dependently inhibited complement 5a-stimulated chemotactic migration. The IC_{50} for piperlactam S to inhibit complement 5a-induced migration was 3.2 ± 0.3 μM. None of the concentrations of piperlactam S used reduced cell viability (>95%), as assayed by Trypan blue exclusion. Furthermore, we studied the effect of piperlactam S on C5a-induced migration of mouse macrophages isolated from peritoneal fluid. Result showed that piperlactam S also has the ability to inhibit C5a-evoked chemotactic migration in primary macrophage cultures with an IC_{50} of 1.9 ± 0.9 μM (four experiments performed in duplicate).

To assess the role of the actin cytoskeleton in cell migration after complement 5a stimulation, chemotactic migration was performed in the absence and presence of cytochalasin B (an inhibitor of actin polymerization) or colchicine (an inhibitor of microtubule polymerization). As shown in Fig. 1B, the complement 5a-induced response was suppressed concentration dependently by cytochalasin B (1 and 3 μM) to 72.3 ± 9.8% and 33.1 ± 14.7%, respectively, as compared with complement 5a control. In contrast, colchicine failed to interfere with complement 5a-induced migration, indicating that microtubules were not involved in the migratory response to complement 5a.

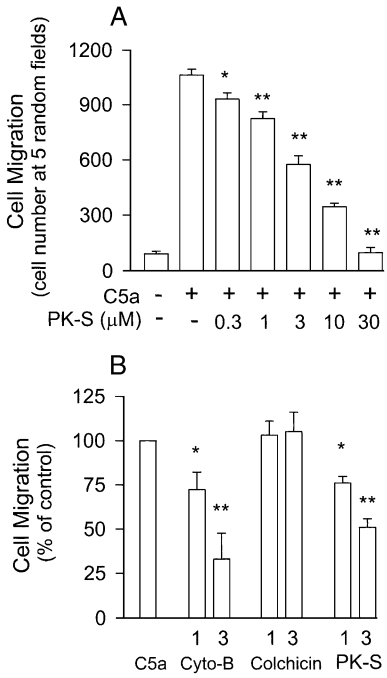

Fig. 1. Effects of (A) piperlactam S and (B) cytochalasin B or colchicine on complement 5a-induced chemotactic migration of RAW264.7 macrophages. Cells pre-incubated with drugs for 15 min were plated onto the upper wells of the chamber. Complement 5a (1 μg/ml) was then added to the lower wells for 4 h to induce cell migration. Results shown in (A) are the total cell number counted in five microscopy fields per well at 400 × magnification. Results shown in (B) are percentages of complement 5a control. Values are means ± S.E.M. of six experiments performed in triplicate. *$P < 0.05$ and **$P < 0.01$, as compared to samples receiving complement 5a alone (PK-S: piperlactam S; Cyto-B: cytochalasin B).

3.2. Effect of piperlactam S on complement 5a-stimulated F-actin content

The F-actin content of complement 5a-stimulated macrophages before and after piperlactam S treatment was measured with a flow cytometer. As shown in Fig. 2A, complement 5a caused a marked increase in F-actin fluorescence intensity, and an apparent shift-to-the-left of F-actin fluorescence intensity was observed in samples pretreated with piperlactam S. Piperlactam S concentration dependently inhibited complement 5a-stimulated F-actin content (Fig. 2B). A significant decrease in F-actin was also observed following exposure to cytochalasin B.

3.3. Effects of piperlactam S on cell spreading and filopodia extension in response to complement 5a

The ability of piperlactam S to affect complement 5a-induced F-actin content suggested the involvement of cytoskeletal reorganization and/or redistribution. Thus, the effect of piperlactam S on complement 5a-induced changes

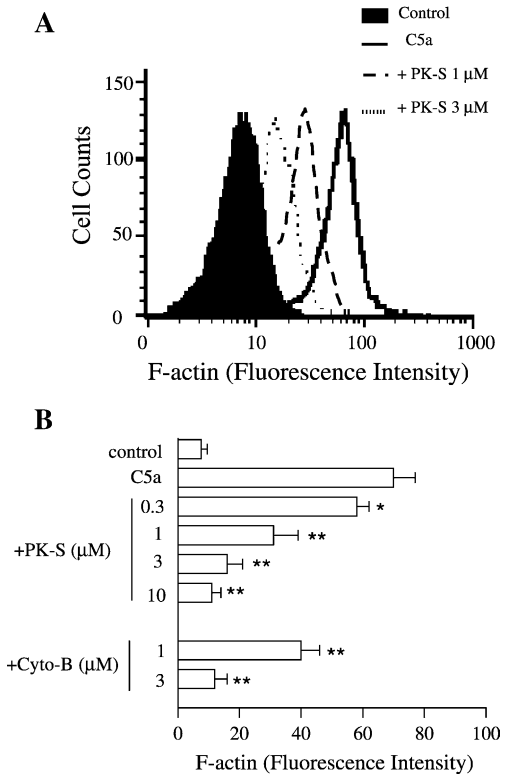

Fig. 2. Effect of piperlactam S on complement 5a (1 μg/ml)-induced F-actin content. (A) Flow cytometric analysis of intracellular F-actin content of RAW264.7 macrophages. Control cells received neither piperlactam S nor complement 5a treatment. Complement 5a-stimulated cells that were pretreated with piperlactam S are designated as '+PK-S'. (B) Statistical summary of F-actin fluorescence intensity of complement 5a-stimulated cells in the absence or presence of piperlactam S (0.3–10 μM) and cytochalasin B (1 and 3 μM). Values represent the mean of five experiments and horizontal lines show S.E.M. *$P<0.05$ and **$P<0.01$, as compared to samples receiving complement 5a alone (PK-S: piperlactam S; Cyto-B: cytochalasin B).

in F-actin redistribution was visualized by fluorescence microscopy. As shown in Fig. 3, non-activated cells (PBS group) were round and displayed a generalized distribution of F-actin fluorescence within the cell. After stimulation with complement 5a for 15 min, a net increase in phalloidin-associated fluorescence was detectable, with localization in the periphery of the cell. Complement 5a caused a shift in the distribution of F-actin toward the plasma membrane and induced filopodia formation; however, no stress fibers and lamellipodia were seen. The effects of complement 5a on F-actin redistribution and filopodium extension were fully reversed by the actin polymerization inhibitor, cytochalasin B. This suggests that complement 5a receptor-cytoskeletal communication initiated the events that led to altered cell morphology and spreading, due possibly in a large part to actin reorganization and realignment. Pretreatment of the complement 5a-activated cells with piperlactam S (3 and 10 μM) also inhibited the redistribution of the actin cytoskeleton and the filopodia extension in a concentration-dependent manner.

3.4. Effects of monoclonal antibodies to adhesion molecules on complement 5a-stimulated migration

This study was to determine the roles of LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) in the complement 5a-induced chemotactic migration of RAW264.7 macrophages. As shown in Fig. 4A, monoclonal antibody to CD18/β_2 (1, 10 μg/ml) concentration dependently blocked complement 5a-stimulated cell migration to 74.5 ± 5.6% and 12.3 ± 3.0% of the PBS control, respectively. Monoclonal Ab to CD11b (1, 10 μg/ml) also concentration dependently blocked complement 5a-evoked cell migration to 64.1 ± 4.6 and 10.4 ± 2.7%, respectively. In contrast, monoclonal antibody to CD11a and isotype-matched IgG did not impede the complement 5a-induced response. Cell migration blocked by anti-β_2 was as effective as treatment with monoclonal antibody to CD11b, suggesting that Mac-1 (CD11b/CD18) on RAW264.7 macrophages plays an important role in complement 5a-induced migration.

3.5. Effect of piperlactam S on the fluorescent-staining pattern of Mac-1 expression on complement 5a-stimulation macrophages

The distribution patterns of Mac-1 (CD11b) on permeable RAW264.7 cells before and after complement 5a stimulation were examined. Fig. 4B shows the nonspecific fluorescence binding to isotype-matched IgG and the specific fluorescence binding to Mac-1 in non-activated cells (PBS groups). The fluorescence patterns between the two

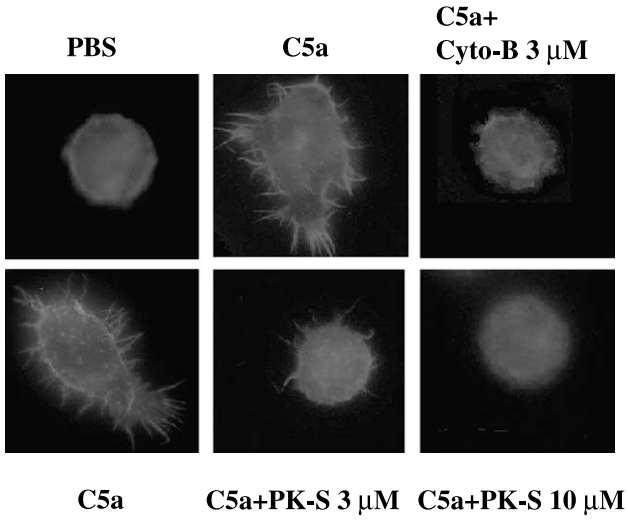

Fig. 3. Effects of piperlactam S and cytochalasin B on complement 5a-stimulated F-actin cytoskeleton reorganization. FITC-labeled phalloidin was used to visualize changes in F-actin fiber organization in RAW264.7 macrophages. Piperlactam S (3 and 10 μM) or cytochalasin B (3 μM) was added for 30 min and then cells were stimulated with complement 5a (1 μg/ml) for a further 15 min, as described in Materials and methods. Cells were subsequently fixed, permeabilized and finally visualized and photographed under fluorescence microscopy (PK-S: piperlactam S; Cyto-B: cytochalasin B).

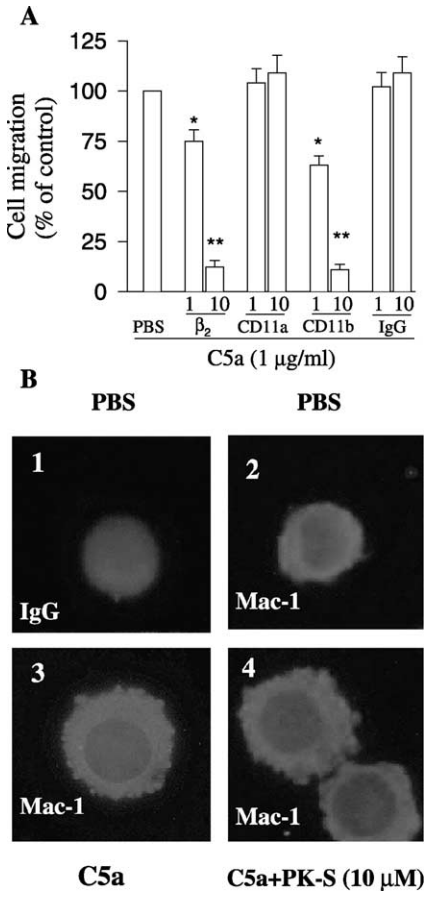

Fig. 4. (A) Effects of monoclonal antibodies to β_2, CD11a, CD11b, and isotype-matched IgG on RAW264.7 macrophage migration in response to complement 5a (1 μg/ml). Cells pre-incubated with monoclonal antibodies for 60 min were plated onto the upper wells of the chamber. Complement 5a was then added to the lower wells for 4 h to induce cell migration. Migration was assessed by counting migrated cells in five microscopy fields per well at 400 × magnification. Results are indicated as percentages of complement 5a control. Values are means and vertical lines S.E.M. of four experiments performed in triplicate. *$P<0.05$ and **$P<0.01$ denote degrees of statistical significance of differences as compared to samples receiving complement 5a alone. (B) Effect of piperlactam S on complement 5a (1 μg/ml)-induced Mac-1 (CD11b) up-regulation. Fluorescence microscopy of nonspecific immune fluorescence staining with an isotype-matched IgG antibody and processed for fluorescent immunocytochemistry using a monoclonal antibody to Mac-1 (CD11b) as described in Materials and methods (PK-S: piperlactam S).

were clearly different. In almost all of the PBS cells, anti-Mac-1 staining indicated that Mac-1 was present within the cell, giving a uniform staining that appeared as a diffused fluorescent ring along the plasma membrane at the peripheral edge of the cells. In contrast, no diffused fluorescent ring was observed in the IgG-stained PBS cells. A striking enhancement of Mac-1 labeling was observed in complement 5a-activated cells in which the granular distribution of Mac-1 gave the appearance of multiple small aggregates on their outer membrane surface, indicating an accelerated up-regulation of the surface expression of Mac-1 (Fig. 3). Although not tracked in the present study, a likely source for such accelerated up-regulation is translocation of Mac-1 from intracellular pools to the plasma membrane. The effect of 10 μM piperlactam S on complement 5a-stimulated Mac-1 up-regulation is illustrated in Fig. 4. Cells that were treated with 10 μM of piperlactam S showed no impairment of Mac-1 up-regulation: multiple small aggregates were visible on the cell surface.

Mac-1 quantification before and after piperlactam S treatment was also evaluated by flow cytometry (Fig. 5A). Complement 5a (1 μg/ml) caused a 2.5-fold increase in Mac-1 fluorescence ($P<0.05$). Piperlactam S, at the concentrations used, failed to affect the Mac-1 fluorescence intensity caused by complement 5a ($P>0.05$). A statistical summary is illustrated in Fig. 5B.

3.6. Effect of piperlactam S on complement 5a-stimulated Cdc42 activation

Because complement 5a induced filopodia formation, we hypothesized that complement 5a signaling involved a Cdc42-PAK pathway in complement 5a-induced chemotaxis in RAW264.7 macrophages. We reconfirmed that GST/PBD effectively interacts with the active GTPγS-bound form of Cdc42; however, there was little or no interaction with the inactive GDP-bound form (data not shown). Then, GST/

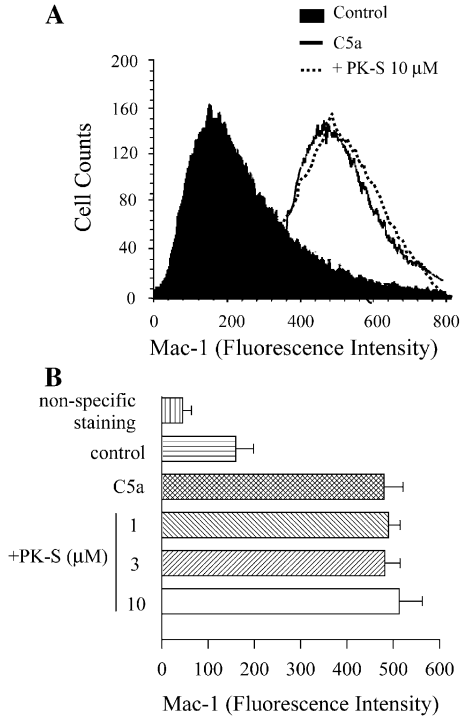

Fig. 5. (A) Flow cytometric analysis of total Mac-1 (CD11b) levels on the cell surface of RAW264.7 macrophages. Control cells received neither piperlactam S nor complement 5a treatment. Complement 5a-stimulated cells that were pretreated with piperlactam S are designated as '+PK-S'. (B) Statistical summary of complement 5a-upregulated Mac-1 expression in the absence or presence of piperlactam S (1–10 μM). Non-specific IgG was included to assess the specificity of anti-CD11b staining. Values represent the mean of five experiments and horizontal lines show S.E.M. (PK-S: piperlactam S).

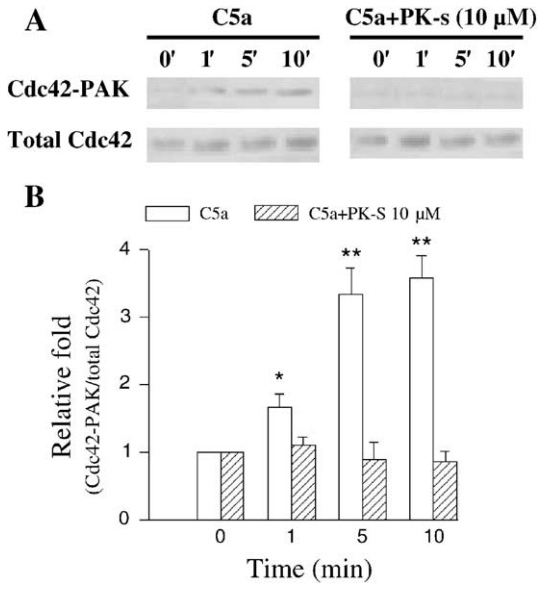

Fig. 6. Effect of piperlactam S on Cdc42 activation. (A) Time course of Cdc42 activation in RAW264.7 macrophages after complement 5a stimulation in the absence or presence of piperlactam S (10 μM), respectively. Cells were stimulated without (indicated as 0 min) or with 1 μg/ml complement 5a at 37 °C for 1, 5 and 10 min, respectively. The resulting cell lysate was used for the affinity precipitation assay in the presence of GST/PBD as described in Materials and methods. Proteins bound to GST/PBD or total proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted for Cdc42, followed by ECL detection. (B) Band intensities were quantified by densitometry and are given as relative fold of Cdc42-PAK/total Cdc42. This experiment was repeated four times with similar results (PK-S: piperlactam S).

PBD was used to pull down the Cdc42-GTP after complement 5a stimulation. As shown in Fig. 6A, stimulation of cells with complement 5a led to an increase in active Cdc42 in a time-dependent manner. Activation was clearly evident by 1 min and there was a marked increase by 5 min. A steady level was reached between 5 and 10 min after stimulation. Significantly, pretreatment of RAW264.7 macrophages with piperlactam S led to inhibition of Cdc42 activity. The relative amount of activated Cdc42 was compared with the total amount of Cdc42 in the cell lysate, normalized by calculating the ratio of activated Cdc42 to total Cdc42 (Fig. 6B). The normal expression of total Cdc42 in the cell lysate suggested that inhibition by piperlactam S was not due to nonspecific or cytotoxic effects.

4. Discussion

We report here that piperlactam S was able to inhibit complement 5a-stimulated chemotactic migration of RAW264.7 macrophages, functions underlying leukocyte recruitment in inflammation. This inhibition was caused by changes in the amount and structural distribution of F-actin in response to complement 5a. However, this effect of piperlactam S was not attributable to interference with the binding of complement 5a since the percentage of inhibition of migration was essentially the same at all complement 5a concentrations. Furthermore, piperlactam S also inhibited lipopolysaccharide-induced chemotactic migration and filopodia extension of RAW264.7 macrophages (unpublished data).

The actin cytoskeleton is believed to provide both the protrusive and contractile forces required for cell migration, via a combination of actin polymerization and depolymerization (Omann et al., 1995; Vicker, 2000). In the present study, complement 5a-induced chemotaxis was significantly suppressed by an actin polymerization inhibitor cytochalasin B (Baatout et al., 1998), but not by a microtubule polymerization inhibitor colchicine (Vandecandelaere et al., 1997). These findings suggest that actin filament, but not microtubules, was involved in the chemotactic response to complement 5a. Suppression of complement 5a-stimulated chemotactic migration by cytochalasine B was associated with an impaired polarity of macrophages. Results showed that the inhibition of cell polarity and chemotactic migration by piperlactam S were also closely correlated with a reduction in the relative amount of F-actin, as determined by flow cytometry. Therefore, the piperlactam S-induced suppression of polarity and chemotactic migration evoked by complement 5a may well be due to a decrease in F-actin as a result of inhibition of polymerization.

Although cell migration cannot be attributed to F-actin polymerization alone, the redistribution of F-actin fibers and the formation of pseudopodia are important events in cell locomotion. We found that the chemotactic response to complement 5a in RAW264.7 cells began with a change in cell morphology, notably cytoskeletal actin restructuring along with polarized formation of cytoplasmic projections resembling filopodia. That cytochalasin B prevented complement 5a-evoked chemotactic migration and filopodia extension provides the evidence that actin polymerization is involved in the redistribution of F-actin and in the formation of filopoda. Piperlactam S-treated cells were mainly round and did not appear to have undergone cytoskeletal actin rearrangement and filopodia formation.

Up-regulation of adhesion molecules is important to enable macrophages to adhere to and/or migrate in connective tissues in response to chemotactic factors generated in inflamed tissues (Lundgren-Akerlund et al., 1993). In the current study, inhibition by specific monoclonal antibodies helped define the functional involvement of Mac-1 integrin, while fluorescence staining confirmed its significant surface expression in the chemotactic response of RAW264.7 macrophages to complement 5a. Our results demonstrated that C5a induces chemotactic migration, F-actin polymerization, and filopodia extension of RAW264.7 macrophages and that this phenomenon can be attenuated concentration dependently by piperlactam S pretreatment. However, piperlactam S failed to modify Mac-1 up-regulation and and/or translocation indicating that the inhibitory effect of piperlactam S on chemotaxis is not due to an effect on Mac-1 expression. We suggest that piperlactam S may act

by inhibiting a cellular process that is downstream of Mac-1 signaling.

Studies of the regulation of cell migration, via a combination of actin polymerization and/or depolymerization within cells, has recently focussed on the Rho family of small GTPases (Allen et al., 1998). The roles of Rho-family GTPases, especially Rho, Rac and Cdc42, in regulating cytoskeletal organization have subsequently been investigated in a variety of other cell types, including leukocytes (Dharmawardhane and Bokoch, 1997). In Swiss 3T3 fibroblasts, Rho regulates the assembly of actin stress fibers, whereas Rac regulates membrane ruffling and the production of protrusive lamellipodia, and Cdc42 induces the formation of filopodia (Tapon and Hall, 1997; Jones et al., 1998; Allen et al., 1998). The precise contribution of each protein may well differ depending on the cell type, the chemotactic signal and the composition of the matrix upon which the cells are moving. In the present study, complement 5a induced filopodia formation, but no stress fibers and lamellipodia were seen. Also in chemotaxis assays, RAW264.7 cells without filopodia failed to detect a gradient of complement 5a and subsequently failed to polarize along the axis of the gradient. Studies have proved that Cdc42 activation is necessary for the formation of filopodia (Jones et al., 1998; Allen et al., 1998). Although a role for Cdc42 in human leukocyte function has been demonstrated (Schmitz et al., 2000), the ability of inflammatory mediators to stimulate the formation of Cdc42-GTP has not been well established. To date, few physiological activators of small GTPases have been reported. Therefore, we analyzed whether activation of Cdc42 by complement 5a occurred in RAW264.7 macrophages. By using a specific assay based on the GTPase-binding domain of PAK, we demonstrated for the first time, that the formation of filopodia in RAW264.7 macrophages in response to complement 5a is associated with Cdc42 activation. More interesting is the observation that the inhibition of filopodia extension by piperlactam S correlated with the breakdown of Cdc42 activation. The involvement of Cdc42 in actin cytoskeleton reorganization and chemotaxis induced by chemokines has been reported in monocytes (Weber et al., 1998). Whether Cdc42 functions to complement 5a-mediated cytoskeletal polarization and subsequent cell migration in RAW264.7 macrophages awaits further clarification.

In conclusion, we hypothesize that a change in the status of cellular F-actin and the suppression of filopodia extension might contribute to the inhibitory effect of piperlactam S on macrophage migration toward complement 5a. This effect of piperlactam S may be explained by down-regulation of Cdc42 activation to prevent filopodia formation. As an effective anti-migratory drug at pharmacological concentrations (0.3–10 μM), piperlactam S may be useful in inflammatory disorders by limiting the early phases of macrophage infiltration. The relative contributions of these activities to the potent anti-migratory effect of piperlactam S in vivo remain to be elucidated.

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