Prostaglandin I₂ Plays a Key Role in Zymosan-Induced Mouse Pleurisy


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ABSTRACT

Zymosan, the cell wall of Saccharomyces cerevisiae, induces innate immune responses involving prostanooid production and complement activation. However, the roles of prostanooids in zymosan-induced inflammation and their interaction with the complement system remain to be determined. To clarify these issues, we examined zymosan-induced pleurisy in mice lacking receptors for prostaglandin (PG) E₂ (EP₁⁻/⁻ mice) or PGI₂ (IP⁻/⁻ mice). Zymosan-induced exudate formation was significantly reduced in IP⁻/⁻ mice compared with wild-type (WT) mice, whereas none of the EP₁⁻/⁻, EP₂⁻/⁻, EP₃⁻/⁻, and EP₄⁻/⁻ mice showed any significant difference from WT mice. Furthermore, indomethacin, an inhibitor of prostanooid biosynthesis, suppressed exudate formation in WT mice to almost the same level as that of IP⁻/⁻ mice. Accordingly, significant production of PG₁₂ in the pleural cavity, suggested to be cyclooxygenase-2-dependent, was observed after zymosan injection. Complement activation in the pleural cavity after zymosan injection was confirmed, and preinjection of cobra venom factor (CVF), to deplete blood complement C3, was significantly suppressed after zymosan-induced exudate formation in WT mice. Simultaneous treatment with indomethacin and CVF further suppressed exudate formation in WT mice compared with each treatment alone. Because, some degree of exudate formation was still observed, other factor(s) seem to be involved. However, platelet-activating factor, a promising candidate as one such factor, was not involved in zymosan-induced exudate formation. These results clearly indicate that the PG₁₂-IP system together with the complement system plays a key role in exudate formation in zymosan-induced pleurisy.

Zymosan, the cell wall of Saccharomyces cerevisiae, has been used extensively to investigate innate immune responses to fungal infections (Underhill, 2003). Zymosan is known as a potent activator of the complement system (Pillemer et al., 1954). In the lectin pathway, binding of mannose-binding lectin or ficolins to zymosan initiates complement activation in association with mannose-binding lectin-associated serine proteases (Fujita et al., 2004). Activation of the complement system liberates active components that enhance vascular permeability and induce inflammatory cell migration, contributing to the development of inflammation. In addition to complement activation, nonopsonized zymosan directly activates macrophages through glucan and mannann are responsible for both the alternative and lectin pathways of complement activation. In the alternative pathway, zymosan directly activates the complement system (Pillemer et al., 1954). In the lectin pathway, binding of mannose-binding lectin or ficolins to zymosan initiates complement activation in association with mannose-binding lectin-associated serine proteases (Fujita et al., 2004). Activation of the complement system liberates active components that enhance vascular permeability and induce inflammatory cell migration, contributing to the development of inflammation. In addition to complement activation, nonopsonized zymosan directly activates macrophages through...
several types of receptors, such as the type-3 complement receptor (Gordon, 2002) and the β-glucan receptor dectin-1 (Brown and Gordon, 2001). Activated macrophages then produce proinflammatory mediators, including eicosanoids, such as prostaglandins (PGs) and leukotrienes (LTs), platelet-activating factor (PAF), and inflammatory cytokines, further contributing to the development of inflammation.

Prostanoids, consisting of PGs and thromboxane, are the metabolites of arachidonic acid, and they exert a variety of actions throughout the body. Among these, the roles of prostanoids as inflammatory mediators have been extensively studied (Rocha e Silva, 1978), and it is well established that nonsteroidal anti-inflammatory drugs, such as aspirin and indomethacin, exert their anti-inflammatory effect by inhibiting cyclooxygenases (COXs), rate-limiting enzymes in prostanoid biosynthesis (Vane et al., 1998).

Among prostanoids, PGE\(_2\) and PGI\(_2\) have been implicated as the prostanoids most responsible for inflammation because they have been detected abundantly in inflammatory exudates and tissues (Harada et al., 1982), and because they show a range of proinflammatory activity when administered in vivo (Rocha e Silva, 1978). Prostanoids act on their specific receptors expressed in various organs and tissues (Narumiya et al., 1999). PGE\(_2\) acts on four types of receptors, EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\), and PGI\(_2\) acts on IP. Recent studies using mice lacking each of these receptors have confirmed some of the roles of PGE\(_2\) and PGI\(_2\) as inflammatory mediators, such as fever generation (Ushikubi et al., 1998) and inflammatory pain transmission (Murata et al., 1997), respectively. However, the role(s) of individual prostanoids is not precisely known in specific inflammatory conditions, including those accompanying innate immune responses.

An increase in vascular permeability is the hallmark for the presence of local inflammation, which then leads to edema formation or exudate accumulation. Zymosan-induced pleurisy and peritonitis have long been used as important models to examine the mechanisms of fungal-induced inflammation by evaluating exudate formation and leukocyte infiltration. Furthermore, a method to assess both the rate and degree of exudate formation has been developed in pleurisy models (Vinegar et al., 1973). In a recent study, zymosan-engaged dectin-1 has been reported to regulate the activity of type IVA cytosolic phospholipase A\(_2\), a key enzyme liberating arachidonic acid upon inflammation, and the COX-2 expression in mouse peritoneal macrophages (Suram et al., 2006). Furthermore, significant production of prostanoids in zymosan-induced peritonitis has been reported (Lundy et al., 1990; Rao et al., 1994), suggesting the participation of prostanoids in zymosan-evoked innate immune responses. However, in zymosan-induced rat pleurisy, we could not determine the participation of prostanoids, whereas an initial activation of the complement system followed by PAF formation was found to be critical for accumulation of pleural exudates (Imai et al., 1991). As a result, the role of prostanoids in zymosan-induced responses remains to be determined.

In the present study, we clarify the roles of prostanoids, as well as their interaction with the complement system, in zymosan-induced pleurisy using mice lacking EP\(_1\), EP\(_2\), EP\(_3\), EP\(_4\), or IP. We also demonstrate the important role of the PGL\(_2\)-IP system together with the complement system in zymosan-induced innate immune responses.

### Materials and Methods

**Mice.** The generation and maintenance of mice lacking EP\(_1\), EP\(_2\), EP\(_3\), EP\(_4\), or IP (EP\(_1^{-/-}\), EP\(_2^{-/-}\), EP\(_3^{-/-}\), EP\(_4^{-/-}\), and IP\(^{-/-}\) mice, respectively) has been reported previously (Murata et al., 1997; Segi et al., 1998; Ushikubi et al., 1998; Hizaki et al., 1999). All mice, including wild-type (WT) control mice, but with the exception of EP\(_1^{-/-}\), EP\(_2^{-/-}\), and IP\(^{-/-}\) mice, have the genetic background of C57BL/6 mice. EP\(_3^{-/-}\) and their control F2-WT mice have a mixed genetic background of 129/Ola and C57BL/6 (Segi et al., 1998). Mice lacking the PAF receptor (PAF\(^{-/-}\)) (Ishii et al., 1998) were kindly donated by Dr. T. Shimizu (Tokyo University, Tokyo, Japan). All experiments were approved by the Animal Research Committee on Animal Research, and they were carried out using 15- to 30-week-old male mice.

**Zymosan-Induced Pleurisy.** Zymosan (zymosan A from S. cerevisiae; Sigma-Aldrich, St. Louis, MO) was suspended in physiological saline at a concentration of 2%, and then it was autoclaved. Under ether anesthesia, pleurisy was induced by injecting 50 μl of 2% zymosan suspension into the mouse pleural cavity with a 25-gauge needle, whose tip had been blunted to avoid injury of the lung. Immediately after the mice had been killed at indicated times after the zymosan injection, pleural exudates were collected, and their volumes were measured. Next, the saline (0.5 ml) wash of the pleural cavity was collected, and it was combined with the collected exudates in a tube containing 50 μl of 3.8% sodium citrate solution. Leukocyte numbers in the pleural exudates were counted with a microcell counter (F820; Sysmex, Kobe, Japan), and leukocyte classification of a Giemsa-stained smear of the exudates was performed under a microscope (Imai et al., 1991). To assess the exudation rate, we injected pontamine sky blue (Tokyo Kasei, Tokyo, Japan) solution at a dose of 50 mg/kg into the tail vein 20 min before the designated time of sample collection. Concentrations of the dye in the collected pleural exudates and plasma were measured, and the rate of plasma exudation into the pleural cavity was calculated (Dozen et al., 1989).

To examine the involvement of prostanoids in zymosan-induced pleurisy, indomethacin (Sigma-Aldrich), which inhibits both COX-1 and COX-2, was prepared as a 1 mg/ml solution in 0.5% sodium carboxymethyl cellulose (Tokyo Kasei), and it was administered i.p. at a dose of 5 mg/kg 30 min before the zymosan injection. To examine the COX-2 contribution, SC58125 (Cayman Chemical, Ann Arbor, MI), a COX-2 inhibitor, was prepared as a 10 mg/ml solution in dimethyl sulfoxide, and it was administered i.p. at a dose of 10 mg/kg 30 min before the zymosan injection.

**Measurement of PGs.** The exudates collected at 1, 3, 5, and 24 h after the zymosan injection were frozen, and then they were stored at −80°C until use. The amounts of 6-keto-PGF\(_1α\), a stable metabolite of PGL\(_2\), and PGE\(_2\) in the exudates were measured using enzyme-linked immunosorbent assay kits (Cayman Chemical).

**Western Blot Analysis for C3-Related Peptides.** WT mice received injections intraperitoneally with 50 μl of 2% zymosan suspension, and pleural exudates and blood samples were collected at indicated times. Blood was collected in the presence of anticoagulants, and plasma was prepared by centrifugation of blood. The exudate sample at 0 h was prepared using a wash of the pleural cavity with 0.6 ml of saline containing anticoagulants, because the exudates could not be obtained at this time point. The pleural exudates and plasma were subjected to 10% SDS-PAGE under reducing conditions with 4 μg proteins/lane, followed by transfer onto an Immobilon-P membrane (Millipore Corporation, Billerica, MA). After a blocking procedure with nonfat dry milk (Carnation, Solon, OH), the membrane was incubated with a goat antiserum against mouse complement C3 (Cappel, ICN, Aurora, OH) for 1 h at room temperature, followed by incubation with a peroxidase-conjugated rabbit affinity-purified antibody to goat IgG (Cappel, ICN). After washing, C3-related peptides were detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).
Depletion of Blood C3. To deplete blood C3, cobra venom factor (CVF; partially purified compound from venom of Naja Kaouthia; Venom Supplies, Tanunda, SA, Australia) was prepared as a 0.1 mg/ml solution in physiological saline, and it was administered i.p. at a dose of 10 μg for 24 h before the zymosan injection according to standard methodology (Cochrane et al., 1970).

PAF-Induced Pleurisy. C16-PAF and methylcarbamyl (MC)-PAF (Cayman Chemical) were prepared as 0.1 mg/ml solutions in phosphate-buffered saline containing 1% ethanol and 0.1% bovine serum albumin, and they were stored at −30°C until use. At the time of use, C16-PAF or MC-PAF solution was diluted to a concentration of 4 μg/ml with phosphate-buffered saline containing 0.1% bovine serum albumin. PAF pleurisy was induced by injecting 25 μl of C16-PAF or MC-PAF solution into the pleural cavity. The volume of the pleural exudates was measured 30 min after an injection of C16-PAF or MC-PAF. TCV-309 (Takeda Pharmaceutical, Osaka, Japan), a PAF antagonist, was prepared as a 0.1 mg/ml solution in physiological saline, and it was administered i.p. at a dose of 1 mg/kg 30 min before the injection of C16-PAF or MC-PAF. When examining the involvement of PAF in zymosan-induced pleurisy, TCV-309 was injected i.p. at a dose of 1 mg/kg 30 min before the zymosan injection.

Statistical Analysis. Data were expressed as the mean ± S.E.M. Statistical analysis was carried out using Student’s t test or one-way analysis of variance followed by Dunnett’s t test. Differences with a value of p < 0.05 were considered statistically significant.

### Results

Time Courses of Zymosan-Induced Pleurisy in WT and IP^{−−} Mice. We first compared the time course of zymosan-induced pleurisy between WT and IP^{−−} mice by measuring the exudate volume, exudation rate, and total leukocyte number in the exudates. It was possible to measure pleural exudates in WT mice at 1 h, and levels increased rapidly peaking at 5 h, and then they decreased gradually until 24 h when they were barely detectable (Fig. 1A). In IP^{−−} mice (Fig. 1B), accumulation of pleural exudates was significantly less compared with those of WT mice; peak levels at 5 h were 524 ± 14 (n = 15) and 238 ± 24 (n = 10) μl in WT and IP^{−−} mice, respectively. Furthermore, pleural exudates in IP^{−−} mice were almost negligible at 16 h, when significant amounts of exudates (238 ± 48 μl; n = 6) were still detectable in WT mice. The exudation rate, which was assessed by the amounts of leaking dye during a 20-min interval, showed the highest value of above 100 μl/20 min at 3 h, levels declined quickly to around 40 μl/20 min at 8 h, and they stayed at a steady level of 20 to 40 μl/20 min up to 24 h in WT mice (Fig. 1C). In IP^{−−} mice (Fig. 1D), the exudation rates were significantly lower compared with those in WT mice.
mice throughout the time course up to 16 h; peak exudation rates at 3 h were 104 \pm 13 and 70 \pm 5 \mu l/20 min in WT and IP−/− mice, respectively. These results indicate that the PGL2-IP system increases vascular permeability and thus enhances exudate formation in zymosan-induced pleurisy.

Indomethacin treatment in WT mice attenuated exudate formation significantly throughout the time course (Fig. 1A); the peak exudate volume at 5 h was decreased from 524 \pm 14 (n = 15) to 209 \pm 9 \mu l (n = 9). Furthermore, indomethacin lowered exudation rates significantly throughout the time course up to 16 h in WT mice (Fig. 1C); it decreased the peak exudation rate at 3 h from 104 \pm 13 (n = 12) to 67 \pm 11 \mu l/20 min (n = 7), indicating again that prostanoids are involved in the facilitation of vascular permeability. It is noteworthy that indomethacin treatment showed no significant effect on the exudate volume (Fig. 1B) or the exudation rate (Fig. 1D) in IP−/− mice. Furthermore, exudate volume and the exudation rate in IP−/− mice were almost the same as those in indomethacin-treated WT mice, indicating that PGL2 is a major prostanoid involved in enhanced exudate formation in the present model of zymosan-induced pleurisy.

There was no significant difference in the number of pleural resident cells, consisting almost of mononuclear cells, between WT and IP−/− mice, 2.0 \pm 0.1 \times 10^6 (n = 7) and 1.9 \pm 0.3 \times 10^6 (n = 7), respectively. In WT mice, the leukocyte number in the exudates did not change at 1 h, it increased gradually up to 8 h, and it remained at a steady level thereafter (Fig. 1E), indicating that leukocyte recruitment starts later than exudate formation, although it occurs at a relatively early phase of zymosan-induced pleurisy. After 8 h, infiltrated cells were composed of 90% neutrophils and the remaining mononuclear cells, indicating that neutrophils are the main cell type recruited in zymosan-induced pleurisy.

In contrast to the significant differences found in the exudate volume and exudation rate, there was no significant difference in leukocyte number in the exudates for WT and IP−/− mice (Fig. 1, E and F), excluding the involvement of IP in leukocyte recruitment. Furthermore, indomethacin treatment had no significant effect on leukocyte number throughout the time course of both WT and IP−/− mice (Fig. 1, E and F), indicating that PGs, including PGL2, do not play a major role in leukocyte migration in zymosan-induced pleurisy. As a result, we focused our investigation on the regulation of exudate formation in zymosan-induced pleurisy in the following experiments.

Zymosan-Induced Pleurisy in EP−/− Mice. The findings in zymosan-induced pleurisy in IP−/− mice, as well as the effect of indomethacin on pleurisy, suggested that PGL2 is a major prostanoid component in zymosan-induced pleurisy. To further determine whether PGE2, another representative prostanoid mediating a variety of inflammatory responses, contributes to zymosan-induced pleurisy, pleurisy was induced in EP receptor knockout mice: EP1−/−, EP3−/−, EP2−/−, and EP−/−. The peak exudate volumes at 5 h were similar in all EP−/− groups, and these values were not significantly different from those of WT mice (Fig. 2A). Indomethacin treatment significantly suppressed exudate formation at 5 h in all EP−/− groups to levels similar to those in WT mice (Fig. 2A). Similar results were also obtained in the exudate volumes at an earlier time point of 1 h (data not shown). Accordingly, exudation rates at 1 h were similar in all the EP−/− groups, and these values did not differ significantly from those in WT mice (Fig. 2B). Indomethacin also significantly attenuated exudation rates at 1 h in all EP−/− groups to levels similar to those in WT mice (Fig. 2B). There was no significant difference in zymosan-induced exudate formation between WT and F2-WT mice (data not shown). These results clearly indicate that PGE2 does not play a role in the present model of zymosan-induced pleurisy.

Measurement of PGs in the Exudates. To clarify the reason for the differential involvement of PGL2 and PGE2 in zymosan-induced pleurisy, we measured the contents of 6-keto-PGF1α and PGE2 in the exudates at 1, 3, 5, and 24 h of zymosan-induced pleurisy. The contents of 6-keto-PGF1α had already increased at 1 h, they stayed at a peak level of 30 ng/mouse for at least up to 5 h, and they remained at a significant level even at 24 h (Fig. 3A). Estimated 6-keto-PGF1α concentrations in the exudates at 1 h were around 0.5 \mu M, a well working range of the PGL2-IP system. In contrast to the prominent production of PGL2, PGE2 production was almost negligible, and the level of PGE2 in the exudates was 1/20 to 1/30 of that of 6-keto-PGF1α at 3 and 5 h (Fig. 3B). This result suggests that preferential production of PGL2 in the pleural cavity supports PGL2 to be the main component in zymosan-induced pleurisy. Indomethacin treatment significantly reduced the levels of both 6-keto-PGF1α and PGE2 to barely detectable (Fig. 3, A and B), indicating almost complete COXs suppression by indomethacin.

Involvement of COX-2 in Enhanced Exudate Formation in Zymosan-Induced Pleurisy. To determine whether enhanced exudate formation in zymosan-induced pleurisy depends on PGL2 produced by COX-2, we examined the effect of SC58125 on exudate formation in WT mice (Fig. 2). There were no significant differences in exudate volume and the exudation rate of each type of EP−− and WT mice in zymosan-induced pleurisy. WT, EP1−/−, EP2−/−, EP3−/−, EP−/−, and EP−−−− mice received injections intrapleurally with 50 \mu l of 2% zymosan suspension. In some groups, indomethacin was injected i.p. at a dose of 5 mg/kg 30 min before the zymosan injection. A, exudate volume was measured at 5 h after the zymosan injection. B, exudation rate was estimated at 1 h after the zymosan injection. IM, indomethacin pretreatment. Data represent the mean \pm S.E.M. of the values obtained from four to 15 mice. *p < 0.05 compared with each indomethacin-nontreated control group.
SC58125 at a dose of 10 mg/kg, reported to be enough for suppressing COX-2 activity in mice (Rao et al., 1994), decreased peak exudate volume at 5 h significantly from 522 ± 25 to 150 ± 25 µl (Fig. 4A). Accordingly, the peak exudation rate at 3 h was suppressed significantly by SC58125 from 116 ± 36 to 36 ± 2 µl/20 min (Fig. 4B). These SC58125-suppressed levels of exudate volume and the exudation rate were almost the same as those in IP −/− mice (Fig. 1, B and D). Furthermore, the inhibitory effects of SC58125 on exudate formation were comparable with those of indomethacin (Fig. 1, A and C), suggesting that COX-2 is a responsible isof orm producing PGI2 in the present pleurisy model.

**Involvement of the Complement System in Zymosan-Induced Pleurisy.** To determine whether the complement system is activated in zymosan-induced pleurisy, pleural exudates and blood were collected, and they were examined for activation of the complement system using C3 activation as a marker. In the saline wash of the pleural cavity at 0 h, slight activation of the complement system represented by the presence of iC3b(a) and iC3b(b) segments was observed, showing spontaneous activation of C3 (Lachmann and Hughes-Jones, 1984). Although the bands in the lane at 0 h were apparently strong, absolute protein levels at this time point were much smaller, and they were 1/5 to 1/10 of those in the exudates at later time points, indicating that actual amounts of activated C3 was not so large at a basal condition. However, as early as 1 h, apparent activation of the complement system was observed in pleural exudates, as shown by an increase in iC3b(b) segment and the appearance of C3dg and C3c segments, degradation products of iC3b, which activation was observed up to 16 h (Fig. 5A). Because the production of iC3b(a) segment is dependent on the balance between the production and degradation of iC3b, amounts of iC3b(a) were apparently constant (Fig. 5A). In contrast, activation of the complement system was not observed in plasma up to 16 h, although a lesser extent of spontaneous activation of C3 was observed (Fig. 5B), indicating that the complement system was activated within the pleural cavity.

To evaluate the contribution of the complement system, we depleted blood complements using CVF and then examined exudate formation. CVF at 10 µg/mouse almost completely depleted blood C3 in both WT and IP −/− mice (Fig. 5C). In WT mice, complement depletion significantly suppressed the exudate volume at 3 h to a similar extent as that of indomethacin treatment (Fig. 6A). Furthermore, simultaneous treatment with CVF and indomethacin further decreased the exudate volume to more than that of each treatment alone (Fig. 6A). In IP −/− mice, CVF treatment alone significantly suppressed the exudate volume to a level comparable with that of WT mice treated simultaneously with CVF and indomethacin (Fig. 6B). As expected, additional treatment with indomethacin failed to influence the exudate volume in CVF-treated IP −/− mice (Fig. 6B). Similar results were obtained when examining the exudation rate at 1 h (data not shown). These results indicate that zymosan activated the PGI2-IP system through both complement-dependent and complement-independent pathways. In all EP −/− groups, CVF treatment significantly suppressed exudate volume to a similar extent as that of WT mice (Fig. 6C), showing once again the independent role of the complement system.

**No Involvement of PAF in Exudate Formation in Zymosan-Induced Pleurisy.** Although PGI2 and comple ment play essential roles in zymosan-induced pleurisy, simultaneous treatment with indomethacin and CVF did not suppress exudate formation completely, indicating that other factor(s) is involved in zymosan-induced pleurisy. Therefore, we finally examined whether PAF participates in exudate formation. In WT mice, C16-PAF and MC-PAF, a PAF analog resistant to metabolic degradation, induced exudate accumulation after being injected into the pleural cavity (Fig. 7A), indicating that PAF works as a potent inflammatory mediator that increases vascular permeability. PAF- and MC-PAF-induced formations of pleural exudates were suppressed significantly by pretreatment with a PAF receptor antagonist TCV-309 (Fig. 7A). Moreover, C16-PAF and MC-PAF did not induce exudate formation in PAFR −/− mice (Fig. 7A), indi-
cating that effects of PAF analogs were mediated exclusively by the PAF receptor. However, in zymosan-induced pleurisy, TCV-309 pretreatment did not show any suppressive effect on the exudate volumes at 3 and 5 h (Fig. 7B). Furthermore, there was no significant difference in the exudate volumes between WT and PAFR−/− mice at 3 and 5 h (Fig. 7B). These results clearly indicate that PAF plays an insignificant, if at all any, role exudate formation in the present zymosan-induced pleurisy model.
Discussion

Zymosan induces innate immune responses by activating the two major effectors, macrophages and the complement system. These two systems produce several kinds of proinflammatory mediators, among which prostanooids and activated complement components are thought to play a pivotal role in mediating zymosan-induced responses. However, it remains to be determined which types of prostanooids and their receptors participate in zymosan-induced responses, and whether these prostanooids interact with the complement system. In the present study, we clearly showed that the PGL_2-IP system plays an important role in concert with the complement system in zymosan-induced mouse pleurisy.

In the present study, selective involvement of IP, but not EPs, could be supported by a preferential production of PGL_2 compared with that of PGE_2 in the pleural cavity (Fig. 3). Low level of PGE_2 production was unexpected, because abundant PGE_2 production by zymosan-stimulated mouse peritoneal macrophages in vitro has been reported (Scott et al., 1980; Suram et al., 2006). However, resident peritoneal macrophages produced much greater amounts of prostanooids than elicited macrophages when stimulated by zymosan in vitro (Humes et al., 1980), indicating that production of prostanooids depends on the conditions of the macrophages prepared. Furthermore, infiltrated leukocytes affected the types of prostanooids produced by macrophages in zymosan-induced mouse peritonitis in vivo (Tripp et al., 1986), indicating that the types of prostanooids produced in response to zymosan in vivo would differ from those produced in vitro. In fact, selective production of PGL_2 has been reported in zymosan-induced mouse peritonitis in vivo (Lundy et al., 1990; Rao et al., 1994), indicating along with our results that the major prostanooid produced in zymosan-induced responses in vivo is PGL_2. In relation to this, it should be noted that the level of PGL_2 production in the present zymosan-induced pleurisy model was 10 times higher than that in the mouse pleurisy model induced by carrageenin (Yukhi et al., 2004), a potent activator of the kinin system.

In the present model, PGL_2 production depended mainly on COX-2, an inducible isofrom of COXs (Fig. 4). Accordingly, COX-2 has been reported to be significantly up-regulated with resultant prostanooid production in zymosan-stimulated peritoneal macrophages (Suram et al., 2006). It is interesting to note that a COX-2 inhibitor suppressed only a small part of PG production in lipopolysaccharide-pretreated, zymosan-stimulated peritoneal macrophages in vitro, whereas augmented PG production induced by an addition of exogenous arachidonic acid totally depended on COX-2, suggesting a more preferential usage of exogenous arachidonate by COX-2 (Rouzer and Marnett, 2005). These results indicate the possibility that COX-2 uses arachidonic acid, which is released abundantly into pleural exudates as reported in zymosan-induced peritonitis (Lundy et al., 1990), as a substrate to produce PGL_2 in the present pleurisy model.

In contrast to the important role of the PGL_2-IP system in the regulation of exudate formation, involvement of prostanooids, including PGL_2, in leukocyte recruitment into the pleural cavity is unlikely (Fig. 1, E and F). This result indicates that leukocyte recruitment occurs independently of an increase in vascular permeability and that factor(s) other than prostanooids works as a chemoattractant for the leukocytes, especially for neutrophils. As such a factor, LTB_4, the most potent chemoattractant known for neutrophils, was reported to be responsible for neutrophil recruitment in zymosan-induced mouse peritonitis (Byrum et al., 1999). Furthermore, a critical role for LTB_4 in neutrophil recruitment was reported in zymosan-induced mouse pleurisy (Takeshita et al., 2003), indicating an important role for an eicosanoid other than prostanooids in mediating zymosan-induced responses. However, another factor might be involved in neutrophil recruitment, because cytokine-induced neutrophil chemoattractant was found to increase in pleural exudates at an early phase of zymosan-induced rat pleurisy (Utsunomiya et al., 1998). In recent studies, the anti-inflammatory role of PGL_2 via the effects on dendritic and Th2 cells in a mouse asthma model has been reported (Idzko et al., 2007; Jaffar et al., 2007), indicating direct action of PGL_2 on immune-mediating cells. Although participation of these PGL_2 actions in zymosan-induced pleurisy is still unclear and remains to be determined, it might contribute to infiltration and activation of leukocytes in the present pleurisy model.

The complement system is another important effector system and mediates zymosan-induced innate immune responses (Morgan and Harris, 2003). The activated complement system generates anaphylatoxins, C5a and C5a, which play an important role in vascular permeability increase, as well as in leukocyte migration (Guo and Ward, 2005). In the present zymosan-induced pleurisy model, we confirmed the apparent activation of the complement system within the pleural cavity without an apparent activation of plasma C3 (Fig. 5). This indicates that the complement system was activated locally within the pleural cavity after blood complements had leaked into there, suggesting that an initial factor increasing vascular permeability would be required to...
induce a subsequent activation of the complement system. Accordingly, simultaneous treatment with CVF and indomethacin suppressed exudate formation more than each treatment alone in WT mice (Fig. 6), indicating that the PGL2-IP system and the complement system work together along with their independent actions in zymosan-induced exudate formation. This cooperation may result from the interrelation between the two systems so that initial production of PGL2 by zymosan-stimulated macrophages and the resultant increase in vascular permeability would be required for a subsequent activation of the complement system in the pleural cavity; and conversely, the activated complement components, such as C3a and C5a (Cooper et al., 1980), would further stimulate PGL2 production by macrophages. Although a cooperative action of PGL2 and the complement explained more than 70% of exudate formation, a small residual part was suggested to be mediated by other factor(s).

PAF was a promising candidate as such a mediator, because it participated in exudate formation in zymosan-induced rat pleurisy (Imai et al., 1991). However, in the present study, we could not detect any involvement of PAF in zymosan-induced exudate formation (Fig. 7), and insufficient production of PAF in response to zymosan was suggested. Although the precise mechanism of this species difference in rat and mouse is not clear and remains to be studied, there are some species differences in the expression pattern of phospholipase A2 that play an important role in PAF biosynthesis (Kudo et al., 1994). Furthermore, the zymosan-induced i.P. extravasation of plasma protein was significantly diminished in mice lacking LTC4 synthase (Kanaoka et al., 2001), suggesting the possibility that cysteinyl LTs are responsible for residual exudate formation remaining after simultaneous treatment with indomethacin and CVF in the present study.

Several investigators have reported the participation of prostanooids in fungal infection. Pathogenic fungi, such as Cryptococcus neoformans and Candida albicans, produced several types of prostanooids, and their growth was inhibited significantly by COX inhibitors (Noverr et al., 2001). In addition, survival of mice infected with C. albicans was significantly increased by treatment with LAAE-14, a novel anti-inflammatory drug that inhibits prostanoid synthesis (Lucas et al., 2004), suggesting the participation of prostanooids in the pathogenesis of fungal infection. Interestingly, it is known that fungal infection is prone to be chronic and dissemi-nating if the Th1-Th2 balance of cellular immunity is shifted toward Th2 preference (Romani and Kaufmann, 1998). Considering the proinflammatory effect of PGL2 presented in the present study and the suppressive actions of PGL2 on Th2 responses (Idzko et al., 2007; Jaffar et al., 2007), PGL2 might affect the course of fungal infection via these effects. However, the precise role of the PGL2-IP system in the pathogenesis of fungal infection remains to be determined. In conclusion, the present study clearly demonstrated the key role of the PGL2-IP system together with the complement system in zymosan-induced mouse pleurisy. This contributes to a better understanding of the mechanisms working in the zymosan-induced innate immune responses.

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References

Pillemer L, Blum L, Lepew IH, Ross OA, Todd EW, and Wardlaw AC (1954) The


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