Analysis of the Factor(s) Involved in Pathogenesis of Zymosan-Induced Inflammation in Rats

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Abstract—The role of mast cell degranulation in increased vascular permeability in zymosan-air-pouch inflammation, an experimental model of inflammation induced by zymosan in rats, was investigated. The complement in the inflammatory pouch fluid was exhausted, and mast cells in the pouch wall subcutaneous tissues were degranulated. The histamine level in the pouch fluid was elevated immediately after application of zymosan in the preformed air-pouch and then quickly declined. Plasma exudation into the pouch fluid changed in close parallel with the change of histamine level. Application of compound 48/80 in the air-pouch also brought about liberation of histamine from mast cells, accompanied with elevation of vascular permeability similar to that observed in the zymosan-air-pouch inflammation. However, the amount of the plasma exudation in the zymosan-air-pouch inflammation was about twice as high as that induced by compound 48/80, though the quantity of histamine liberated in the two cases was almost equal. Rats depleted of histamine and serotonin were incapable of responding to compound 48/80, but zymosan still induced increased vascular permeability. A combination treatment with pyrilamine and methysergide did not abolish plasma exudation caused by zymosan, but brought about complete blockade of the vascular permeability response to compound 48/80. These results suggest that some mechanisms independent of degranulation of mast cells are responsible in part for the initial sudden elevation of vascular permeability in zymosan-induced inflammation.
Materials and Methods

Animals: Male Sprague-Dawley rats purchased from Charles River Japan, Inc., Kanagawa, Japan, specific pathogen free, 6 weeks old and weighing 150–210 g, were used.

Materials: Zymosan A, pyrilamine maleate and compound 48/80 were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. Sodium carboxymethyl cellulose (CMC, Cellogen F-3H, Dai-ichi Seiyaku Kogyo Co., Niigata, Japan) and methysergide hydrogen maleate (Sandoz Ltd., Basel, Switzerland) were generous gifts. Bovine serum albumin (BSA), fluoresceinisothiocyanate (FITC), histamine dihydrochloride and o-phthalaldehyde were purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. Sheep red blood cells (SRBC) sensitized with anti-SRBC antibody (EA, 1 x 10^9/ml) were purchased from Ishizu Seiyaku Co., Osaka, Japan.

Induction of zymosan-air-pouch inflammation: The zymosan-air-pouch inflammation was induced as described (1). In brief, rats were injected with 8.0 ml of air subcutaneously on the back under light ether anaesthesia to make an ellipsoid or oval air-pouch. Twenty-four hours later, 4.0 ml of 1.6% (w/v) zymosan suspension in 0.8% (w/v) CMC solution in saline, supplemented with antibiotics (0.1 mg penicillin G potassium and 0.1 mg dihydrostreptomycin sulfate per 1.0 ml), were injected into the preformed air-pouch to provoke an inflammatory response.

Induction of an inflammation of the air-pouch type with compound 48/80: The air-pouch was prepared on the back of rats as described above, and 24 hr later, 4.0 ml of 0.8% (w/v) CMC solution in saline containing a potent mast cell degranulating agent, compound 48/80, at a concentration of 1 μg/ml and supplemented with the antibiotics previously described were injected into the preformed air-pouch to induce an inflammatory response. According to the literature, compound 48/80 at the above concentration is harmless to mast cells (14).

Measurement of CH50: At suitable times after the injection of the zymosan suspension or the vehicle into the air-pouch, rats were killed by cutting the carotid artery under light ether anaesthesia, and the fluid in the pouch was collected in a plastic cup. The pouch fluid collected was diluted twice with ice-cold phosphate-buffered saline at pH 7.4 (PBS) and then centrifuged at 2,000 x g for 20 min at 4°C. The levels of complement in the pouch fluid supernatants were assayed according to the method of Mayer (15) with a modification (16) in which the volumes of samples and all the reagents were reduced to 1/5 of those in the original method, and the results were expressed in terms of CH50 in Mayer's unit.

Measurement of protein content: Protein content in the above supernatant of the pouch fluid was determined according to Lowry's method (17) using bovine serum albumin as a reference standard.

Measurement of histamine content: In order to remove protein from the above supernatant, 1.0 ml of it was mixed with 2.0 ml of 0.4 N HClO₄, and the mixture was centrifuged at 2,000 x g for 30 min at 4°C. Histamine content in the supernatant was assayed by the method of von Redlich and Glick (18) with a modification using each reagent and samples in 10 times the volume used in the original method.

Measurement of plasma exudation: Plasma exudation was measured with the aid of fluorescein-labeled bovine serum albumin (F-BSA) as a tracer (19) which was prepared in our laboratory. At a suitable time after injection into the preformed air-pouch of zymosan suspension, CMC solution containing compound 48/80 or the vehicle, the rats were injected intravenously with 20 mg of F-BSA dissolved in 0.2 ml saline. Thirty minutes later the rats were killed and the entire pouch fluid was collected, diluted twice and centrifuged to collect the supernatant and pellet separately. The supernatant was diluted four-fold with PBS and then fluorescence intensity was read at 521 nm under excitation at 490 nm. Total fluorescence intensity for the entire volume of the exudate was calculated and expressed in terms of percent of the amount of F-BSA injected and used as an index of the plasma exudation in the inflammatory locus.
Depletion of histamine and serotonin: Rats were depleted of their stores of histamine and serotonin by repeated injection of compound 48/80 according to the procedure described by Spector and Willoughby (20) and Feldberg and Talesnik (21). In brief, 0.1% (w/v) solution of compound 48/80 in sterile saline was administered intraperitoneally every morning and evening for eight doses, commencing with an evening dose. The dose of compound 48/80 was 0.6 mg/kg for the first six injections and 1.2 mg/kg for the last two doses. Those depleted rats were divided into 5 groups, and 5 hr after the last injection with compound 48/80, 5 groups of the animals were challenged in the preformed air-pouch with the zymosan suspension, vehicle CMC solution and the vehicle CMC solution containing compound 48/80, histamine and a combination of histamine with compound 48/80, respectively.

Histological examination: Pouch wall tissues were excised together with the dorsal skin, fixed immediately in phosphate-buffered (pH 7.0) 3.7% (w/v) formaldehyde solution for 48 hr, embedded in paraffin, sectioned into 2 μm thick slices and stained with toluidine blue for histological examination.

Drug treatment: A histamine-H1 antagonist, pyrilamine, and a serotonin antagonist, methysergide, were dissolved in a small volume of distilled water and then mixed with the zymosan suspension or CMC solution containing compound 48/80. Those mixtures were injected into the preformed air-pouch to clarify the roles of histamine and serotonin.

Statistical analysis: Statistical analysis of the data was done by means of the F-test, and P values smaller than 0.05 were considered as significant.

Results

Complement level and protein contents in the pouch fluid: A time course study on total protein contents and total CH50 titer for the entire pouch fluid collected from the inflammatory pouch was made and the results are shown in Fig. 1. The total CH50 titer of the pouch fluid from rats with zymosan-air-pouch inflammation was significantly lower than that from control rats injected with the vehicle CMC solution in the air-pouch. In contrast with the results for CH50, the total protein contents for the zymosan group were significantly higher than those for the control group.

Mast cell degranulation in the pouch wall tissues: Mast cells in subcutaneous tissues of the pouch wall in zymosan-air-pouch inflammation were degranulated in part and many free metachromatic granules were observed around mast cells in the histological specimens (Fig. 2a). In the control group, however, degranulation of mast cells in the pouch wall tissues was rarely observed in any of the specimens examined (Fig. 2b).

Plasma exudation and histamine content
in the pouch fluid: In the zymosan-air-pouch inflammation, the histamine level in the pouch fluid was abruptly raised immediately after injection with the zymosan suspension, and then it rapidly declined to approach the level of the control group injected with the vehicle CMC solution (Fig. 3b). In close parallel with the change of histamine level in the zymosan group, the vascular permeability of the tissues surrounding the pouch greatly increased in the first 30 min period, and then in the subsequent 30 min period, the vascular permeability dropped to half of the preceding level (Fig. 3a). In the control group injected with the vehicle CMC solution, the histamine level in the pouch fluid and plasma exudation were both maintained at very low levels throughout the experimental period of 6 hr.

Histamine release and plasma exudation by compound 48/80 and zymosan: Compound 48/80, a potent histamine liberator, dissolved in 0.8% CMC solution was applied in the preformed air-pouch to induce mast cell degranulation and to enhance plasma exudation. In this experiment, we attempted to investigate whether histamine liberated by compound 48/80 in an amount comparable to that in zymosan-air-pouch inflammation could induce increased plasma exudation to a level comparable to those observed in zymosan-air-pouch inflammation. Therefore, the dose-response relationship for the release of histamine and plasma exudation in the first 30 min periods after injection of various doses of compound 48/80 were investigated first. As summarized in Fig. 4, compound 48/80 at concentrations less than 0.1 μg/ml did not significantly increase plasma exudation, but 1 μg/ml of compound 48/80, which does
not injure mast cells at all (14), provoked release of histamine to the same extent as found in zymosan-air-pouch inflammation. In contrast, the amount of plasma exudation induced by 1 μg/ml of compound 48/80 was 50% less than that found in zymosan-air-pouch inflammation.

Plasma exudation in rats depleted of histamine and serotonin: Rats treated with repeated administration of compound 48/80 to deplete their tissue stores of histamine and serotonin demonstrated marked diminution in the amount of histamine liberated into the pouch fluid when stimulated with zymosan suspension and compound 48/80, respectively, as deduced clearly from comparing

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**Fig. 4.** Correlation of the histamine level in the pouch fluid with the plasma exudation into the pouch. Four ml of the zymosan suspension and 0.8% CMC solution containing various doses of compound 48/80 were injected into the preformed air-pouch. Thirty minutes later, rats were killed and histamine contents in the pouch fluid and plasma exudation into the pouch fluid were measured. Each column represents the mean±S.E.M. from 5 animals. Statistically significant differences against the corresponding control are shown by the following symbol: *P<0.001.

**Table 1.** Plasma exudation and histamine liberation in the air-pouch of rats whose mast cells have been depleted of histamine and serotonin

<table>
<thead>
<tr>
<th>Stimuli to the pouch</th>
<th>No. of rats</th>
<th>Exudate (ml)</th>
<th>Histamine content in the pouch at 30 min (ng/ml)</th>
<th>Plasma exudation (0–30 min) (% of F-BSA injected)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (control)</td>
<td>7</td>
<td>3.67±0.05</td>
<td>14±2</td>
<td>0.34±0.05</td>
<td>N.S.*</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>7</td>
<td>3.60±0.03</td>
<td>62±10</td>
<td>0.39±0.03</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Zymosan</td>
<td>7</td>
<td>3.66±0.10</td>
<td>63±15</td>
<td>1.08±0.31</td>
<td>N.S.</td>
</tr>
<tr>
<td>Histamine</td>
<td>6</td>
<td>3.42±0.12</td>
<td>–</td>
<td>1.16±0.13</td>
<td>N.S.</td>
</tr>
<tr>
<td>Histamine – Compound 48/80</td>
<td>7</td>
<td>3.49±0.03</td>
<td>–</td>
<td>1.51±0.17</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Rats were depleted of their stores of histamine and serotonin by repeated injection of compound 48/80 as described in the text. At 5 hr after the last injection of compound 48/80, 1.6% zymosan suspension in 0.8% CMC solution, compound 48/80 (1 μg/ml) dissolved in the vehicle CMC solution, histamine (30 μg/ml) or histamine (30 μg/ml) plus compound 48/80 (1 μg/ml) dissolved in the vehicle was injected into the preformed air-pouch. Then plasma exudation into the pouch fluid in the first thirty minute period and histamine level in the pouch fluid at the end of the thirty minute period were determined as described in the text. Figures represent the mean±S.E.M. Statistical significance: * compared with the vehicle group and † compared with the histamine group.
the data in Table 1 with those in Fig. 4. In spite of marked reduction in histamine liberation to such a level as observed in the vehicle CMC group, zymosan was still stimulating the induction of plasma exudation in histamine-depleted rats to the extent thrice as high as those in the control group (Table 1). In contrast with zymosan, application of compound 48/80 to the pouch caused no significant increase in the vascular permeability. A couple of experiments were additionally performed using histamine-depleted rats in an attempt to discover whether compound 48/80 reduces tissue reactivity to histamine. These experiments were designed in order to examine whether the difference shown in Fig. 4 with respect to plasma exudation between the two groups treated with compound 48/80 (1 μg/ml) and zymosan might have been due to a possible direct inhibitory effect of compound 48/80 on the vascular permeability response to histamine. The results summarized in Table 1 clearly excluded such a possibility.

Comparison of the effects of a combination treatment with pyrilamine plus methysergide on vascular permeability responses to zymosan and compound 48/80: In an attempt to determine to what extent histamine and serotonin from mast cells are responsible for the plasma exudation induced by zymosan and compound 48/80, experiments for combination treatment with a histamine-H1 antagonist and serotonin antagonists, pyrilamine and methysergide, were designed. The antagonists were administered locally in the air-pouch by dissolving them in the CMC solution containing zymosan and compound 48/80. The combination treatment with the two antagonists blocked vascular permeability responses almost completely in the case of compound 48/80, while it was only partially effective in blocking the vascular permeability response to zymosan (Fig. 5).

Discussion

We reported in the preceding paper that zymosan particles suspended in a solution of CMC, when injected into an air-pouch preformed on the back of rats, induced a typical inflammatory response characterized by accumulation of exudate fluid and polymorphonuclear leukocytes (PMNL) in the pouch (1). Moreover, zymosan caused the formation of an apparently aggregated layer of PMNL onto the inner surface of the pouch wall. Generation of proinflammatory fragments, C3a and C5a, of complement via activation by zymosan of the alternative pathway of the complement system was assumed to play an important role in the mechanism of induction of this inflammatory response, since the anticomplementary agent K-76COONa was effective in inhibiting leukocyte accumulation in the inflammatory site (1). The present experiments were undertaken to gain further insight into mechanisms of zymosan-induced inflammation.

Protein contents in the pouch fluid from
the zymosan-air-pouch inflammation was always higher than those of the control pouch fluid (Fig. 1a), reflecting the higher level of the vascular permeability throughout the experimental period (Fig. 3a). It is presumable, therefore, that the complement components exuded from the blood stream into the pouch fluid more actively in the zymosan group than in the vehicle CMC group. In marked contrast with the higher level of protein contents, however, the complement level as reflected by the CH50 titer in the pouch fluid from the zymosan-air-pouch inflammation was considerably lower throughout the experimental period than that of the control (Fig. 1b). It is likely, therefore, that the complement components exuded into the pouch fluid had been activated and consumed via the alternative pathway in the presence of zymosan particles generating biologically active fragments of the complement system such as C3a and C5a. The C3a and C5a anaphylatoxins thus generated are thought to stimulate mast cells to release their granular components containing histamine and serotonin. Liberation of histamine in the pouch fluid in the zymosan group has actually been confirmed, as shown in Fig. 3b. Degranulation of the mast cells in the zymosan group was also confirmed histologically (Fig. 2a). All of these experimental results appear to indicate that histamine and serotonin liberated from mast cells were responsible for the abrupt rise in vascular permeability observed in the initial phase of zymosan-induced inflammation.

In addition to the demonstration of a definite role for mast cell degranulation in the initial increase in vascular permeability in zymosan-air-pouch inflammation, the data summarized in Fig. 4 appear to indicate that some mechanisms other than mast cell degranulation are also responsible in part for the vigorous plasma exudation in the zymosan group, since plasma exudation of the zymosan group was significantly higher than those of the compound 48/80 groups, whereas the histamine level of the zymosan group was rather lower than that of the compound 48/80 (1 μg/ml) group. Elevation of vascular permeability induced by local application of compound 48/80 in the air-pouch was almost completely counteracted by a combination treatment with a histamine antagonist, pyrilamine, and a serotonin antagonist, methysergide (Fig. 5). In contrast, the combined treatment with pyrilamine and methysergide was much less effective in suppressing the vascular permeability response to zymosan (Fig. 5). These facts also support the above-mentioned concept that some factors independent of mast cell degranulation play certain important roles in the initial rise of the vascular permeability in zymosan-induced inflammation.

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