Dissociation between the effects of zymosan on the systemic and pulmonary vessels of the rat

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1 Zymosan, an activator of the alternative complement pathway, (2 to 16 mg kg⁻¹) injected intravenously via the tail vein of anaesthetized rats, dose-dependently increased the vascular permeability of lung parenchyma, as measured by the accumulation of ¹²⁵I-labelled albumin in lungs.

2 Pretreatment of the animals with cyclo-oxygenase inhibitors, indomethacin or ketoprofen (3 mg kg⁻¹) or with the lipoxygenase and cyclo-oxygenase inhibitor, BW755C (40 mg kg⁻¹) abolished the vascular permeability changes induced by zymosan (16 mg kg⁻¹). Neither, the PAF antagonist, WEB 2086 (10 mg kg⁻¹) nor the antagonists of mast cell amines, mepyramine and methysergide (3 mg kg⁻¹) affected the plasma exudation in lungs. Zymosan did not induce any accumulation of labelled albumin in lungs of rats made leucopenic by rabbit anti-neutrophil serum.

3 Zymosan (16 mg kg⁻¹) increased the haematocrit. This increase was not modified by indomethacin but reduced by WEB 2086.

3 Intravenous injection of zymosan (3 and 8 mg kg⁻¹) in anaesthetized rats transiently increased right ventricular blood pressure and pulmonary arterial pressure, accelerated respiratory rate and decreased systemic blood pressure.

5 WEB 2086 largely reduced the systemic hypotension but did not affect the increase of pulmonary vascular resistance. Indomethacin inhibited the increase of blood pressure in the right ventricle and the modification of the respiratory rate. This drug did not inhibit but increased the systemic hypotension induced by zymosan.

6 Zymosan (16 mg kg⁻¹) reduced serum complement haemolytic activity by 46%.

7 These data suggest that the pulmonary vascular changes induced by intravascular complement activation with zymosan in rats are mediated by neutrophils and prostanoids while the systemic vascular effects depend mainly on PAF.

Keywords: Zymosan; leukocytes; PAF; prostanoids; rat systemic and pulmonary vessels

Introduction

Intravascular activation of complement induces thrombocytopenia, intrapulmonary sequestration of neutrophils, lung injury and systemic hypotension in several animal species (Hammerschmidt, 1983; Hugli *et al.*, 1987). In rats, intravenous injection of zymosan, an activator of the alternative complement pathway (Gewurz & Lint, 1977), induces neutropenia, thrombocytopenia, decrease of the mean systemic blood pressure, peripheral plasma extravasation with paw oedema (Damas, 1991). These vascular effects of zymosan in rats are largely reduced by a platelet activating factor (PAF) antagonist and abolished by pretreatment of the animals with a combination of drugs consisting of antagonists for PAF, mast cell amines and a lipoxygenase inhibitor (Damas, 1991). These results suggest that PAF is the main factor responsible for the systemic effects of zymosan but in addition, that mast cell amines and lipoxygenase products are also involved in the rat.

On the other hand, lung injury is observed after activation of complement with cobra venom factor in rats (Till *et al.*, 1982; Ward *et al.*, 1985). Therefore, we examined the pulmonary vascular effects of zymosan in rats. These effects were estimated by measuring the accumulation of ¹²⁵I-labelled albumin in pulmonary parenchyma and by recording haemodynamic changes.

Methods

Experiments were performed on Wistar rats of either sex. They were divided in two groups. The animals of the first group (body weight 230 g) were used for studies of plasma

exudation. Some of these animals were depleted of circulating blood leukocytes by the intraperitoneal injection of 1 ml serum obtained from rabbits immunized with rat neutrophils according to Till *et al.* (1982). These depleted rats were used for the experiments 12 h later. At this time, the blood neutrophil content was <500 neutrophils ml⁻¹ blood as determined by total and differential blood cell counts. Simultaneously, total cell count was reduced by 32 ± 3.2% (*n* = 6). Platelet count was not modified. The animals were treated intraperitoneally with saline or with a test drug. Thirty to sixty min later, anaesthesia was induced by intraperitoneal sodium pentobarbitone (35 mg kg⁻¹). ¹²⁵I-labelled bovine serum albumin (New England Nuclear) mixed with saline was injected intravenously into the tail vein either simultaneously or at different intervals after zymosan. Fifteen min later, a blood sample was withdrawn by cardiac puncture. The animals were exsanguinated with aortic trans-section and lungs perfused through the right ventricle with 10 ml of saline to remove blood remaining in the pulmonary vascular system. Then, the lungs were withdrawn and separated from the trachea, thymus and mediastinal tissues. The lungs were weighed. Blood samples were centrifuged at 1500 *g* for 10 min and plasma was separated. The radioactivity of plasma samples or lungs was counted with a gamma spectrometer (Packard Instruments Co.) and the results were calculated as µl of extravasated plasma into the lungs during the 15 min delay. The haematocrit of the blood samples was measured with capillary tubes centrifuged in a Haemofuge (Heraeus).

A second group of rats (body weight 340 g) was used to record cardiovascular and respiratory changes induced by zymosan. Some animals received no pretreatment (saline-treated rats), others were pretreated with WEB 2086 or indomethacin. They were anaesthetized with pentobarbitone (40 mg kg⁻¹, i.p.), placed in a supine position and tracheotomized. A catheter was inserted through a femoral vein into

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the inferior vena cava for drug administration. After heparinization (10 mg kg^{-1}), a second catheter was introduced into a femoral artery and connected to an electromanometric transducer (Elema) to record blood pressure. Similarly the pulmonary arterial pressure (PAP) or the right ventricular pressure (RVP) was obtained by the floating catheter technique. A Silastic tube (o.d. 0.9, i.d. 0.5 mm) with a lateral opening was inserted in the right jugular vein and pushed under manometric control into the pulmonary artery or remained in the right ventricle when the catheterization provoked important disturbances in heart rate. The position of the catheter was checked at the end of the experiments. A home-made Fleisch pneumotachograph (PTG) was inserted at the end of the tracheal cannula and connected to a differential pressure transducer (Validyne DP 45) with a CD 101 demodulator and an analog integrator (Pneumometer Elema) in order to obtain respiratory flow rate and tidal volume (Vt). The outputs of the manometers were fed into an ink-jet multi-channel recorder (Mingograph EEG) for continuous monitoring (5 mm s^{-1}) or calculations (50 mm s^{-1}). All data were means from 10 breaths, blood pressures were measured at the end-expiratory point, heart rate was the mean value of 20 beats. After a stabilization period of about 10 min, control values were recorded before and after (1, 3, 5, 10, 15 and 30 min) three successive injections of zymosan (3, 3 and 8 mg kg^{-1}) performed at 30 min interval.

Finally, serum complement activity was determined in some animals by a haemolytic assay. Washed sheep erythrocytes (Biomérieux, Belgium) were diluted to 20% in complement fixation buffer (Pasteur, Belgium) and sensitized by incubation with 1/100 sheep haemolysin (Biomérieux) for 10 min at 37°C . The sensitized erythrocytes were further diluted to 4% and incubated with an equal volume of diluted rat serum for 30 min at 37°C . The reaction was stopped by centrifugation and the degree of haemolysis was measured spectrophotometrically (541 nm).

The pharmacological agents used were as follows: mepyramine maleate and ketoprofen from Specia (Brussels, Belgium), BW755C (3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazoline) from Wellcome (Beckenham, England), methysergide bimalate from Sandoz (Brussels, Belgium) and indomethacin from Merck Sharp and Dohme (Brussels, Belgium). Zymosan was purchased from Sigma (St Louis, MI, U.S.A.), heparin from Leo (Therabel, Belgium). WEB 2086 (3-(4-2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)triazolo-(4,3-a)-diazepin-2-yl)-1-(4-morpholinyl)-1-propanone) was a kind gift of Boehringer Ingelheim KG (Germany). Indomethacin was dissolved in Tris HCl (0.15 M, pH 7.4). All the other drugs were dissolved in saline, with the aid of HCl 0.1 N for WEB 2086. Zymosan was boiled, washed and suspended in saline before use. Indomethacin, ketoprofen and BW755C were given intraperitoneally 30 to 60 min before zymosan.

Results are expressed as the mean $\pm$ s.e. mean. Student's paired *t* test was used to determine statistically significant differences in the same group. Comparison between groups was made by Student's *t* test for unpaired values and confirmed by analysis of variance. A *P* value less than 0.05 was regarded as significant.

Results

Vascular permeability

The effects of zymosan on the vascular permeability in lungs were examined by measuring the accumulation of ^{125}I -labelled albumin in the pulmonary parenchyma. Labelled albumin was injected 15 min after the intravenous administration of increasing doses (2 to 16 mg kg^{-1}) of zymosan and the animals were killed 15 min later. A group of rats received saline instead of zymosan and were used as controls. As shown in Figure 1, zymosan dose-dependently increased the extravasation of albumin. In other animals, labelled albumin

Figure 1 Exudation of ^{125}I -labelled albumin in lungs after intravenous injection of zymosan, as a function of dose (a) and as a function of time after 16 mg kg^{-1} (b). Exudation was expressed as μl of plasma per g of tissue (●) or as μl of plasma per lungs of one animal (■). Values measured in saline-treated rats are control values: 0 mg kg^{-1} and time 0. *n* = number of animals. Vertical bars indicate s.e.mean. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.005; **** *P* < 0.001.

was injected at various times after zymosan (16 mg kg^{-1}) and the rats were killed 15 min later. The increase of vascular permeability induced by zymosan was observed over the 1 h time period after its administration. Maximal effects were obtained during the first 15 min: at this time, the extravasation of albumin was increased by 301% (Figure 1).

The accumulation of ^{125}I -labelled albumin in lung parenchyma during the first 15 min after zymosan injection (16 mg kg^{-1}) was abolished by indomethacin (3 mg kg^{-1}) or ketoprofen (3 mg kg^{-1}), two cyclo-oxygenase inhibitors (Shen, 1979) and by BW755C (40 mg kg^{-1}), the dual inhibitor of cyclo-oxygenases and lipoxygenases (Higgs *et al.*, 1979).

However, WEB 2086 (10 mg kg^{-1}), a PAF antagonist (Cazals-Stenzels *et al.*, 1987), and the antagonists of mast cell amines, methysergide and mepyramine (3 mg kg^{-1}), did not modify the effects of zymosan in the lungs. The increase of permeability induced by zymosan was not observed in rats made leucopenic with a rabbit anti-neutrophil serum (Figure 2).

We estimated the effects of zymosan on the permeability of the systemic vessels by measurement of changes in the haematocrit. The haematocrit of blood samples withdrawn 15 min after saline injection was: $35.8 \pm 2.4\%$ ($n = 6$). This value was increased by zymosan (16 mg kg^{-1}): $54.8 \pm 0.8\%$ ($n = 6$, $P < 0.001$), indicating a large plasma extravasation. This increase was inhibited by WEB 2086 (10 mg kg^{-1}): $43.3 \pm 2.2\%$ ($n = 6$, $P < 0.001$) but was not modified by indomethacin (3 mg kg^{-1}): $57.0 \pm 1.5\%$ ($n = 6$).

Haemodynamic effects of zymosan

In the second series of experiments, cardiovascular and respiratory changes induced by zymosan were recorded. One min after the intravenous administration of zymosan (3 mg kg^{-1}), a rise of right ventricular systolic or of arterial pulmonary blood pressures was noted. Simultaneously, breathing rate was accelerated but with a reduced tidal volume so that no change of total ventilation was found. All these parameters returned to control levels before 3 min while systemic arterial blood pressure decreased to a minimum value (51% of control level) 3 to 5 min after the injection.

More pronounced changes of respiratory rate and of pulmonary arterial pressure were observed one min after 8 mg kg^{-1} of zymosan (Figure 3). Then, systemic blood pressure decreased to 33 and 37% of systolic and diastolic control levels respectively. Simultaneously, the right ventricular systolic pressure was transiently decreased to 60% of the control value. Right ventricular diastolic pressure did not change significantly after each dose of zymosan.

Figures 4 and 5 summarize the evolution of these responses after 3 and 8 mg kg^{-1} of zymosan and compare these effects with those obtained in rats pretreated with WEB 2086 or with indomethacin. WEB 2086 did not inhibit the increase in the right ventricular pressure nor the modifications of the breathing pattern induced by zymosan. But, WEB 2086 largely reduced the drop of the systemic blood pressure and suppressed the simultaneous decrease of the right ventricular systolic pressure. The rise of right ventricular systolic pressure and tachypnoea were abolished by indomethacin but this inhibitor increased the systemic hypotension and the fall of right ventricular pressure observed after the larger dose of zymosan. These later changes were accompanied by a significant relative bradycardia in indomethacin-treated rats.

Complement depletion

Previously, we observed that 4 and 10 mg kg^{-1} of zymosan induced a partial depletion of serum complement haemolytic activity (Damas, 1991). We measured this activity in some animals treated with 16 mg kg^{-1} in order to estimate the importance of the activation of the complement system. In these animals, complement haemolytic activity was reduced to $10.2 \pm 0.6 \text{ u CH}_{50} \text{ ml}^{-1}$ ($n = 8$; $P < 0.001$) or 54% of control values: $19.0 \pm 0.9 \text{ u CH}_{50} \text{ ml}^{-1}$ ($n = 6$).

Discussion

After intravenous injection of zymosan in rats, the first modification was a transient increase in pulmonary vascular resistances as measured by pulmonary arterial pressures and systolic right ventricular pressure. This was followed by a fall in systemic blood pressure and an increase in the permeability of pulmonary and systemic vessels. Simultaneously, a neutropenia could be measured (Damas, 1991). All these effects are the result of the activation of the complement alternative

Figure 2 Modification by different drugs of the exudation of ^{125}I -labelled albumin in lungs induced by zymosan (16 mg kg^{-1}). Exudation of albumin was expressed as μl of plasma per g of tissue in rats pretreated with saline (Z), indomethacin (I, 3 mg kg^{-1}), ketoprofen (K, 3 mg kg^{-1}), BW755C (B, 40 mg kg^{-1}), WEB 2086 (W, 10 mg kg^{-1}), methysergide and mepyramine (M, 3 mg kg^{-1}) or anti-neutrophil serum (S). Control rats (C) were not treated with zymosan but with saline. Vertical bars indicate s.e.mean $n =$ number of animals. * $P < 0.02$; ** $P < 0.005$; *** $P < 0.001$.

Figure 3 Variations expressed as percentage of control levels of tidal volume (V_t), respiratory rate (RR) and systolic (SPAP) and diastolic pulmonary arterial pressures (DPAP) recorded at the first min after intravenous injection of zymosan 3 mg kg^{-1} (open columns, $n = 4$) or 8 mg kg^{-1} (hatched columns, $n = 4$). Vertical bars indicate s.e.mean. * $P < 0.05$.

Figure 4 Changes in systemic blood pressure (BP), heart rate (HR), right ventricular pressure (RVP) and respiratory rate (RR) following i.v. injection of zymosan (Z, 3 mg kg^{-1}) in saline-treated rats (a, $n = 8$) or in rats pretreated with WEB 2086 (10 mg kg^{-1} , $n = 6$, b) or with indomethacin (3 mg kg^{-1} , $n = 5$, c). Values are mean with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, in comparison either with the initial values in saline-treated rats (a) or with the corresponding values in saline-treated rats (b and c).

Figure 5 Changes in systemic blood pressure (BP), heart rate (HR), right ventricular pressure (RVP) and respiratory rate (RR) following i.v. injection of zymosan (Z, 8 mg kg^{-1}) in saline-treated rats ($n = 8$, a) or in rats pretreated with WEB 2086 (10 mg kg^{-1} , $n = 6$, b) or with indomethacin (3 mg kg^{-1} , $n = 5$, c). Values are mean with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, in comparison either with the initial values in saline-treated rats (a) or with the corresponding values in saline-treated rats (b and c).

pathway as indicated by the reduction of serum complement haemolytic activity. Moreover, opsonization of zymosan in the vascular tree could be another stimulus for neutrophils which accumulated in the lungs (Till *et al.*, 1982; Kaslovsky *et al.*, 1990).

The vascular changes in lungs, increase in resistances and permeability, were inhibited by the cyclo-oxygenase inhibitors, indomethacin and ketoprofen. Similar results were obtained in sheep (Cooper *et al.*, 1980; McDonald *et al.*, 1983) and formation of prostanoids during intravascular activation of the complement system has been reported in sheep and rabbits (Cooper *et al.*, 1980; Perkowski *et al.*, 1983; Bult *et al.*, 1985). The prostanoids responsible for these changes in rat probably originate from leukocytes as leucopenia suppressed the accumulation of labelled albumin in lungs. Moreover, arachidonic acid is mainly converted to prostacyclin (PGI₂) by cyclo-oxygenase of rat pulmonary parenchyma (Pace-Asciak & Rangaraj, 1977). PGI₂ does not induce any increase in vascular resistance of rat pulmonary circulation (Damas & Troquet, 1978). However, the thromboxane mimic, U-44069 has vasoconstrictor effects in rat pulmonary circulation and, at high doses, induces pulmonary oedema (Damas *et al.*, 1979). Indeed, neutrophils stimulated by opsonized zymosan release thromboxane A₂ and other prostanoids *in vitro* (Weissmann *et al.*, 1979). All arachidonic acid metabolites which are generated by the cyclo-oxygenase pathway increase pulmonary vascular resistance, apart from PGI₂ (Gryglewski, 1987).

In a different model of lung injury, the accumulation of albumin in rat lung parenchyma induced by cobra venom factor, another complement activator, was inhibited by neutrophil depletion, catalase and hydroxyl radical scavengers (Till *et al.*, 1982; Ward *et al.*, 1985; Morganroth *et al.*, 1986). However, this enzyme and these drugs had no effect on the increase in pulmonary vascular resistance (Morganroth *et al.*, 1986) which, in our study, was suppressed by cyclo-oxygenase inhibitors. These results taken together suggest that complement activation stimulates neutrophils which release prostanoids and free radicals. The former agents would produce pulmonary hypertension while the latter would modify vascular permeability, but, acting together, both would trigger plasma exudation in lung parenchyma. Similar conclusions were drawn from study of the mediators responsible for lung injury induced by zymosan-activated plasma in sheep (Perkowski *et al.*, 1983).

On the other hand, systemic hypotension and plasma extravasation were not inhibited by indomethacin. These systemic effects were largely reduced by a PAF antagonist which

had no influence on the pulmonary changes induced by zymosan. Thus, in rats, the vascular reactivity of the pulmonary vessels to complement activation contrasted with the sensitivity of the systemic vessels. Recently, Sirois *et al.* (1990) reported that PAF increased vascular permeability in rat trachea and bronchi, as in other systemic vessels, but not in rat lungs. Our results agree with this observation.

Haemodynamic changes induced by zymosan in rats resulted from the release of several mediators, anaphylatoxins, PAF, prostanoids (this work), lipoxygenase products and mast cell amines (Damas, 1991). The systemic hypotension depends mainly on the release of PAF which induces systemic vasodilatation in rats (Feuerstein, 1989). However, after the high dose of zymosan, a simultaneous decrease of right ventricular systolic pressure is observed. This reduction would result either from a direct effect of PAF on cardiac muscles, as already described in some circumstances *in vitro* (Feuerstein, 1989), or from a decrease of cardiac filling due to haemoconcentration and transient accumulation of blood in vasodilated systemic vessels stimulated by PAF. Indeed, the reduction of right ventricular systolic pressure was inhibited by the PAF antagonist.

Indomethacin suppressed the initial rise of right ventricular systolic pressure but increased the fall in systemic blood pressure. Thus the increase in pulmonary vascular resistance, which was terminated before the beginning of systemic hypotension in untreated rats, was not important enough to provoke a reduction in cardiac output. The aggravation of systemic hypotension decreased coronary perfusion and could reinforce cardiac impairment inducing the relative bradycardia observed in indomethacin-treated rats. The influence of indomethacin on the systemic vessels would depend on the suppression of the inhibitory influence of prostaglandins on the release of mediators from mast cells (Raud, 1989) and leukocytes (Weissmann *et al.*, 1979).

In several mammalian species, intravascular activation of the complement system induces systemic hypotension which is inhibited by cyclo-oxygenase inhibitors (Hugli *et al.*, 1987). However, in rats, this hypotension is not reduced by these drugs but depends mainly on the release of PAF, although prostanoids trigger vascular changes in the lungs.

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