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Modification of endotoxin-induced haemodynamic and haematological changes in the rabbit by methylprednisolone, $F(ab')_2$ fragments and rosmarinic acid

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1 The effects of methylprednisolone, $F(ab')_2$ fragments of human gamma globulins and rosmarinic acid, an inhibitor of complement activation, were tested on endotoxin-induced haemodynamic and haematological changes in the rabbit. Their effects were compared with complement depletion by cobra venom factor (CVF) pretreatment.

2 The results provide further evidence for the role of complement activation and the concomitant triggering of the arachidonic acid cascade in the early phase of shock. The formation of vasoactive prostanoids (prostacyclin and thromboxane A_2), the arterial hypotension and the thrombocytopenia were largely dependent on the presence of the intact complement system.

3 $F(ab')_2$ fragments (150 mg kg^{-1}, i.v.) diminished the second fall in blood pressure to some extent but failed to alter any of the other endotoxin-induced changes.

4 Methylprednisolone (40 mg kg^{-1}, i.v.) given 10 min before endotoxin significantly reduced the activation of complement, the second rise of prostacyclin and the secondary hypotension, but was without effect on the early thromboxane peak of the haematological features of endotoxin shock.

5 Rosmarinic acid (20 mg kg^{-1}, i.v.) may be of potential interest for treatment of septic shock, since the drug suppressed the endotoxin-induced activation of complement, the formation of prostacyclin, both hypotensive phases, the thrombocytopenia and the concomitant release of thromboxane A_2.

6 The role of leukocytes and their arachidonic acid metabolites in plasma exudation deserves further investigation, because leukopenia and pulmonary oedema were not complement-dependent and were not affected by any of the treatments.

7 Our results indicate that drugs, interfering with complement activation and/or prostaglandin biosynthesis, may be beneficial in endotoxin shock, provided that they are administered at an early stage.

Introduction

Since the pathological effects of intravenous administration of endotoxins resemble the clinical symptoms of septic shock, experimental endotoxin shock is often employed as a model for shock induced by gram-negative micro-organisms (Guenter *et al.*, 1969; Webb *et al.*, 1981; Bult & Herman, 1982). The endotoxin-induced haemodynamic changes are to a considerable extent caused by vasoactive substances released as a result of activation of Hageman factor-dependent pathways (Morrison & Cochrane, 1974; Webb *et al.*, 1981) and the complement cascade (Gilbert & Braude, 1962; Fearon *et al.*, 1975; Whaley *et al.*, 1979).

The potent vasodilator prostacyclin (PGI_2), the main metabolite of arachidonic acid in vascular tissue, has been reported to contribute to the endotoxin-induced arterial hypotension in several species, i.e. rabbits (Bult *et al.*, 1980), pigs (Schrauwen *et al.*, 1983), cats (Coker *et al.*, 1980), baboons (Harris *et al.*, 1980) and rats (Wise *et al.*, 1980). In rabbits, both arterial hypotension and the enhanced blood levels of prostacyclin and/or its non-enzymatic metabolite 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) were largely reduced when animals were depleted of complement before the injection of endotoxin, suggesting a mediatory role for complement activation in the formation of prostacyclin and the concomitant drop in

arterial blood pressure (Rampart *et al.*, 1982). This assumption was further supported by the observation that activated human and rabbit serum complement, trypsinized complement factor C3 and C5, and purified porcine anaphylatoxin C5a (and C5a des Arg) potently stimulated endothelial and mesothelial PGI_2 production *in vitro* (Rampart *et al*, 1983a, b). *In vivo* too, selective activation of the complement system is associated with formation of PGI_2, and to a number of other changes (arterial hypotension, thrombocytopenia, thromboxane formation and leuko-penia) resembling early endotoxin shock (Bult *et al.*, 1985).

For these reasons, the main objective of our experiments was to investigate the effect of three types of pharmacological agents on the activation of the complement system and the formation of vasoactive prostaglandins during endotoxic shock in rabbits. To this end, we tested both methylprednisolone, a corticosteroid used in the treatment of human septic shock, which under some circumstances may interfer with complement activation (O'Flaherty *et al.*, 1977), and $F(ab')_2$-fragments of human IgG, which have been employed against severe gram-positive infections in rabbits (Ronneberger & Zwisler, 1979) and which protect mice against experimental septicaemia (Klesel & Limbert, 1981). We also tested whether rosmarinic acid, a recently described inhibitor of *in vitro* complement activation (Hadding, Etschenberg, Graf, Leyck, Winkelmann, Parnham, personal communication) was active *in vivo*. Due to the fact that corticosteroids are reported to interfere with the complement system only when they are present at the time of activation (O'Flaherty *et al.*, 1977) all drugs were administered 10 min before endotoxin. A number of complement-depleted rabbits were used as positive controls, since selective anti-complement drugs were not available and the acute hypotensive reaction pattern may vary between different batches of rabbits.

Methods

Experimental animals and procedures

Experimental animals were prepared using previously described procedures (Bult *et al.*, 1985). In the first experiment 43 rabbits of a local breeding station (Dendermondse Witte; 2.6 ± 0.2 kg) were divided at random into 6 groups: group (1) ($n = 7$), received saline (1 ml mg^{-1}) at $t = -10$ min and at $t = 0$ min (control group); group (2) ($n = 8$) received saline (1 ml kg^{-1}) instead of drugs at $t = -10$ min, and endotoxin (lipopolysaccharide *E.coli* 0111: B_4, 0.5 mg kg^{-1}) at zero time (LPS group); group (3) ($n = 8$) received methylprednisolone (40 mg kg^{-1}) at $t = -10$ min, and endotoxin (0.5 mg kg^{-1}) at $t = 0$ min; group (4) ($n = 8$) identical to group 3, but $F(ab')_2$ fragments (150 mg kg^{-1}) were administered at $t = -10$ min; group (5) ($n = 8$) identical to group 3, but treated with rosmarinic acid (20 mg kg^{-1}) at $t = -10$ min; group (6) ($n = 4$) rabbits were depleted of complement by treatment with cobra venom factor (CVF, 200 u kg^{-1}), divided over 2 i.v. injections, 72 and 60 h before the administration of endotoxin (0.5 mg kg^{-1}, $t = 0$ min).

In the second experiment 12 rabbits were divided at random into two groups: group A ($n = 6$) received saline (1 ml kg^{-1}) at $t = -60$ min and endotoxin (0.5 mg kg^{-1}) at $t = 0$ min; group B ($n = 6$) received methylprednisolone (40 mg kg^{-1}) at $t = -60$ min and endotoxin (0.5 mg kg^{-1}) at $t = 0$ min. All drugs to be tested were dissolved in sterile, pyrogen-free saline (dose per kg = concentration per ml) and injected via the marginal ear vein, after the solutions had been warmed up to body temperature (± 38°C).

Mean arterial blood pressure (MABP), complement titers (CH_{50} and C3), the numbers of circulating thrombocytes and leukocytes, the plasma levels of 6-oxo-$PGF_{1\alpha}$ and TXB_2, and the formation of lung oedema were determined using previously described methods (Bult *et al.*, 1985). At each sampling time blood smears were prepared for microscopic inspection after staining with May-Grünwald-Giemsa.

Materials

Rosmarinic acid (2-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propinyl]-oxy]-3-(3,4 dihydroxyphenyl)-propionic acid) was a gift from Nattermann & Cie (Cologne, FRG) and dissolved in saline by addition of 0.1 N NaOH to pH 7.4. Methylprednisolone (Solu-Medrol, $125 \text{ mg } 2\text{ml}^{-1}$) was a gift from Upjohn (Puurs, Belgium). $F(ab')_2$-fragments obtained by pepsin treatment of human plasma gamma globulins (Gamma Venin, 500 mg) were a gift from Behringwerke-Hoechst (Brussels, Belgium). Cobra venom factor was obtained from Cordis (Miami, Fl., USA).

Statistical analysis of data

All results are presented as mean $\pm$ s.e.mean. In order to evaluate drug effects the data from group 2 to 5 and the data from group A and B were subjected to one way analysis of variance (ANOVA) for each sampling time. The data from group 1 and group 6 were separately subjected to ANOVA to investigate time-effect relationships. Duncan's New Multiple Range test was used to compare individual means when the ANOVA indicated an overall effect (Diem & Lentner, 1970). The logarithms of the plasma concentration of 6-oxo-$PGF_{1\alpha}$ and TXB_2 were used for statistical analysis; plasma levels below the detection limit of the radioimmunoassay (20 pg ml^{-1}) were considered to be 20 pg ml^{-1}. The consumption of CH_{50} and C3 within

Table 1 Activation of the complement system during endotoxin shock in rabbits

Group treatment	*(1) Saline + saline*	*(2) Saline + endotoxin*	*(3) Methylpredn-isolone + endotoxin*	*(4) F(ab')$_2$fragments + endotoxin*	*(5) Rosmarinic acid + endotoxin*	*(6) Complement-depleted + endotoxin*
n	7	8	8	8	8	4
	(A) Decrease in total haemolytic activity: CH_{50}					
− 15 min	160 ± 8	179 ± 13	167 ± 13	190 ± 18	175 ± 13	<4
120 min	153 ± 7	122 ± 9*	145 ± 10*	146 ± 14*	156 ± 12	<4
ΔCH_{50}	6 ± 3	58 ± 8	22 ± 5†	48 ± 8	18 ± 6†	—
	(B) Consumption of immunoreactive C3					
− 15 min	129 ± 11	132 ± 6	129 ± 4	141 ± 7	125 ± 6	<2
120 min	128 ± 10	101 ± 6*	119 ± 3*	109 ± 5*	117 ± 5	<2
ΔC3	1 ± 1	30 ± 2	10 ± 3†	32 ± 7	8 ± 4†	—

CH_{50}, haemolytic units per ml undiluted serum; C3, % of a standard reference serum. − 15 min: complement titers in the control period; 120 min: complement titers 2 h after injection of saline or endotoxin. ΔCH_{50}, ΔC3: complement consumption over the 2 h period.
* $P < 0.05$, paired Student's t test, + 120 min versus control period.
† $P < 0.05$, Duncan-test, group 3 and group 5 versus group 2: ANOVA ΔCH_{50}, F(d.f. 3, 28) = 6.96, $P < 0.01$; ANOVA ΔC3, F(3, 28) = 5.82, $P < 0.01$.

each group was tested with the paired Student's t test. A 5% level of significance was chosen.

Results

Complement titers

The haemolytic activity (expressed as CH_{50}, i.e. the serum dilution with the quantity of complement required for 50% lysis of sheep red blood cells in a standardized haemolytic assay) and the level of immunoreactive C3 in serum samples from arterial blood in all six groups of rabbits of the first experiment are shown in Table 1. In the control sample (t = − 15 min) complement titers (CH_{50} and C3) in group 1 to 5 were not different from each other. Injection of saline and blood sampling (group 1) did not lead to statistically significant reduction of complement titers. Endotoxin (group 2) clearly induced activation of complement, as indicated by the significantly (20–30%) decreased complement titers, 2 h after its injection.

$F(ab')_2$ fragments did not prevent endotoxin-induced activation of the complement system: the decrease in total haemolytic activity (ΔCH_{50}) and the consumption of C3 (ΔC3) were as high as in rabbits receiving only endotoxin (group 2). Pretreatment of rabbits with methylprednisolone (group 3) or rosmarinic acid (group 5), significantly (− 70%) reduced the consumption of complement (both ΔCH_{50} and ΔC3) after administration of endotoxin.

The complement titers in animals previously depleted of complement (group 6) were below the limit of detection of the assay system used, and as a result, administration of endotoxin did not result in a measurable reduction in complement titers.

Plasma levels of 6-oxo-$PGF_{1\alpha}$ and changes in mean arterial blood pressure (MABP)

The results from groups 1, 2, 3, 5 and 6 are summarized in Figure 1. Injection of methylprednisolone, $F(ab')_2$ fragments or rosmarinic acid at t = − 10 min did not affect MABP in the control period (from t = − 15 to t = 0) During this period plasma levels of prostacyclin plus 6-oxo-$PGF_{1\alpha}$ were below the limit of detection of the radioimmunoassay (< 20 pg ml^{-1}) in all animals studied. In animals treated only with saline (group 1), MABP remained at the control level during the entire experiment (only decreasing to 3 mmHg after 3 h). None of the samples (up to 3 h) taken from these animals (group 1) contained detectable amounts of prostacyclin plus 6-oxo-$PGF_{1\alpha}$.

Endotoxin (group 2) induced a characteristic biphasic arterial hypotension. The first phase occurred during the first 3 min, and a partial recovery took place within 5–10 min. This was followed by the second phase in which blood pressure dropped more slowly (60–180 min). Injection of endotoxin clearly

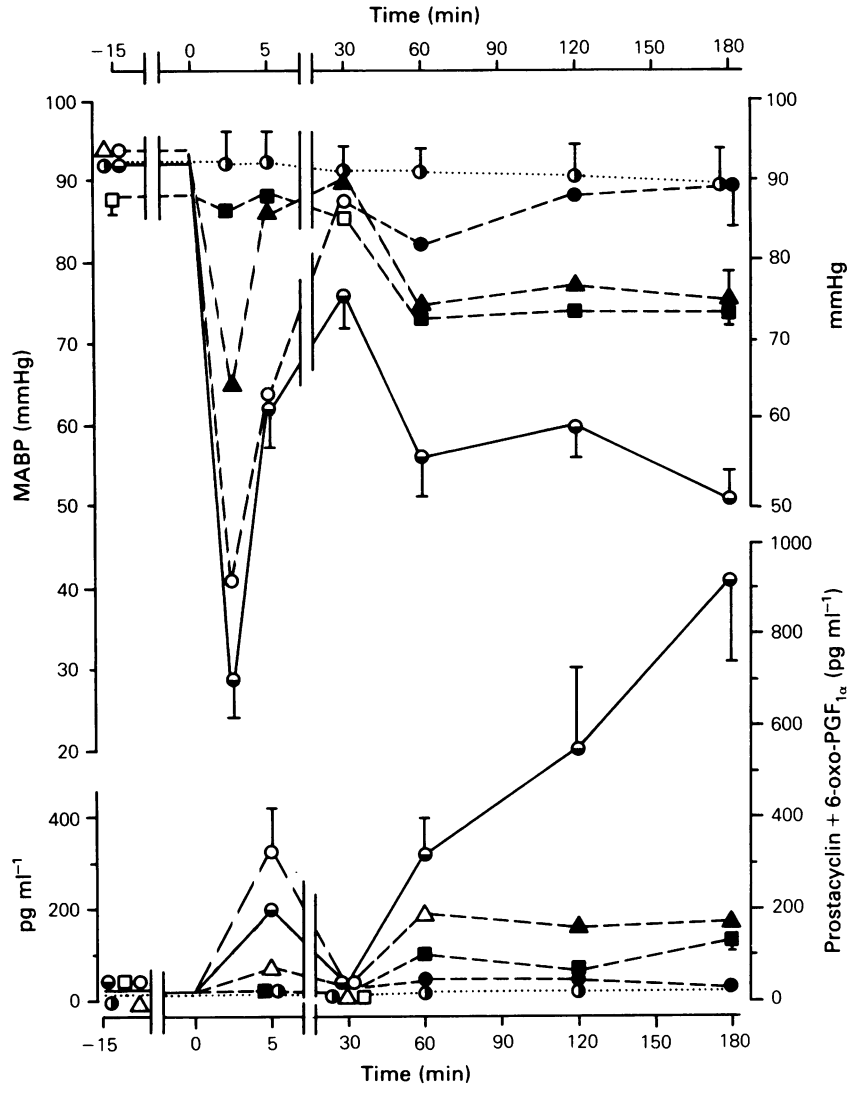

Figure 1 The effect of methylprednisolone (40 mg kg^{-1}, --○--), rosmarinic acid (20 mg kg^{-1}, --△--) and cobra venom factor pretreatment (200 units kg^{-1}, --□--) on endotoxin-induced arterial hypotension (upper panel) and plasma level of prostacyclin plus 6-oxo-PGF$_{1\alpha}$ (lower panel). Controls (....◑....; group 1, $n = 7$) received saline only, endotoxin animals (—◒—, group 2, $n = 8$) received saline at − 10 min and endotoxin at zero time. Methylprednisolone (group 3, $n = 8$), and rosmarinic acid (group 5, $n = 8$) were given 10 min before endotoxin. The cobra venom factor pretreatment (group 6, $n = 4$) started 72 h before endotoxin. The limit of detection of 6-oxo-PGF$_{1\alpha}$ in plasma was 20 pg ml^{-1}. Closed symbols are significantly different from group 2. For reasons of clarity s.e.mean of groups 3, 5 and 6 are shown only at 180 min.

resulted in the presence of PGI_2 in arterial blood. After a first peak, measured at + 5 min, plasma levels returned to baseline (30 min). This was followed by a gradual increase of the 6-oxo-PGF$_{1\alpha}$ plasma levels between 60 and 180 min after injection of endotoxin. Since all samples were collected on indomethacin (10 μg ml^{-1}), the amounts of 6-oxo-PGF$_{1\alpha}$ measured reflect *in vivo* circulating PGI_2 + 6-oxo-PGF$_{1\alpha}$.

Pretreatment with methylprednisolone for 10 min (group 3) did not affect the early fall in blood pressure (3–5 min) nor the initial peak in 6-oxo-PGF$_{1\alpha}$ plasma levels after 5 min. However, 1, 2 and 3 h after injection

of endotoxin, methylprednisolone strongly reduced 6-oxo-$PGF_{1\alpha}$ plasma levels to near the detection limit. The corresponding MABP (60–180 min) remained almost at control values in these animals (Figure 1).

The $F(ab')_2$ fragments (group 4) caused a small reduction of the second phase of arterial hypotension. Blood pressure was significantly higher at 60, 120 and 180 min (MABP respectively 69 ± 3, 75 ± 5 and 75 ± 5 mmHg) as compared with the rabbits receiving only endotoxin (group 2, MABP 56 ± 5, 60 ± 5 and 52 ± 5 mmHg). The modified human gamma globulins did not influence the first hypotension and failed to reduce the early and late increases in plasma 6-oxo-$PGF_{1\alpha}$ (results not shown).

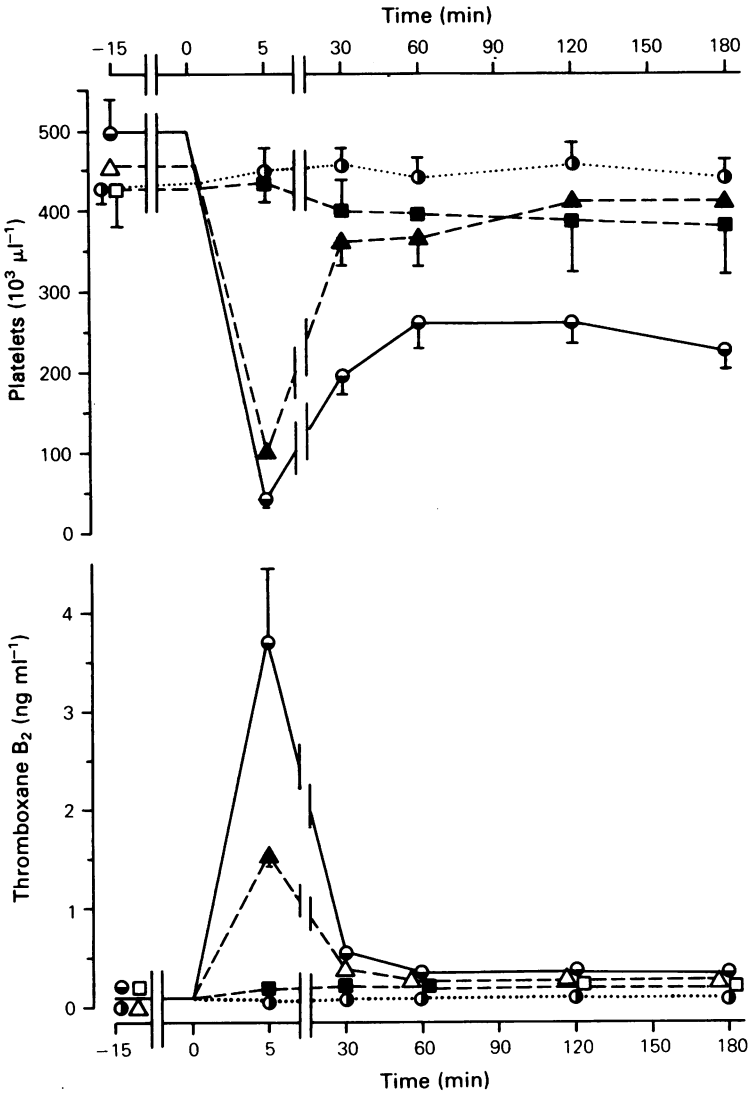

Figure 2 The effect of rosmarinic acid (20 $\mu g\,kg^{-1}$, --Δ--) and cobra venom factor pretreatment (200 units kg^{-1}, --□--) on endotoxin-induced thrombocytopenia (upper panel) and plasma level of thromboxane B_2 (lower panel). Controls (...◑..., group 1, $n = 7$) received saline only; endotoxin animals (——◒——, group 2, $n = 8$) received saline at − 10 min and endotoxin at zero time. Rosmarinic acid (group 5, $n = 8$) was given 10 min before endotoxin, whereas the cobra venom factor treatment (group 6, $n = 4$) ended 60 h before endotoxin. Closed symbols are significantly different from group 2. S.e.means not shown when they fall within the limits of the symbols used.

Rosmarinic acid (group 5) reduced both episodes of arterial hypotension to a large extent, and MABP was at all times significantly higher than in animals receiving only endotoxin (group 2). Although plasma 6-oxo-$PGF_{1\alpha}$ was elevated over control values (group 1), the increase in the second phase was significantly reduced by pretreatment with rosmarinic acid (Figure 1).

In complement-depleted rabbits (group 6), the initial drop in MABP and the concomitant rise in 6-oxo-$PGF_{1\alpha}$ plasma levels were completely absent. Between 1 and 3 h, the decrease in MABP and the elevation of arterial 6-oxo-$PGF_{1\alpha}$ still occurred, but were markedly diminished by complement depletion (Figure 1).

Platelet counts

At the beginning of the experiments ($t = -15$ min), platelet counts in the different groups were not significantly different from each other (ANOVA, F(d.f. 5,37) = 0.21, $P > 0.05$) being on average 463.000 μl^{-1} blood. Administration of saline and blood sampling (group 1) did not change the number of circulating platelets (see Figure 2).

Injection of endotoxin (group 2) resulted in an immediate drop of the platelet count, and after 5 min only 10% of the initial number was present. Afterwards platelet counts recovered partially, but they never exceeded 40 to 50% of the value recorded in the control period (Figure 2). Pretreatment for 10 min with methylprednisolone (group 3) or $F(ab')_2$ fragments (group 4) failed to alter the endotoxin-induced thrombocytopenia at any time (results not shown). Platelet counts from animals pretreated with rosmarinic acid (group 5) were always substantially higher than the corresponding counts in animals receiving only endotoxin (group 2), as shown in Figure 2.

In complement-depleted rabbits, the administration of endotoxin did not lead to statistically significant changes in platelet count, although this number tended to decrease in the samples taken after 2 and 3 h (Figure 2).

Leukocyte counts

Before administration of drugs or endotoxin, leukocyte counts were similar in groups 1 to 5, the mean count being 6300 μl^{-1}. Injection of saline did not affect the number of circulating leukocytes (ANOVA, F(d.f. 5,30) = 0.1, $P > 0.05$). Endotoxin (group 2) induced an immediate drop in the number of circulating white cells per μl blood (from 6320 ± 200, 15 min before endotoxin to 930 ± 130 after 5 min), i.e. a decrease of about 85% within 5 min. Leukocyte counts were somewhat elevated after 30 min (1970 ± 380 leukocytes per μl) but remained very low (20–30% of control period) for the rest of the experiment (i.e. after 3 h, 1430 ± 200 leukocytes per μl). Neither methylprednisolone (group 3) nor $F(ab')_2$ fragments (group 4) affected the leukopenia, the decrease being respectively 83% and 82%, 5 min after endotoxin. Rosmarinic acid (group 5) diminished the decrease in leukocyte count to some extent; the difference was, however, only statistically significant 5 min after endotoxin injection, when the leukocyte count was 1660 ± 200, the decrease being 74% as compared to 85% in group 2.

Pretreatment with CVF (group 6), resulted in leukocytosis. In the control period (i.e., 3 days after CVF injection) the number of white cells in these rabbits (11150 ± 1080 leukocytes per μl, $n = 4$) was about twice the normal value. In complement depleted rabbits endotoxin induced a very similar, severe leukopenia (e.g. 1770 ± 170, 1450 ± 320, and 1520 ± 180 leukocytes per μl, respectively, 5, 30 and 180 min after endotoxin) which means that in these animals about twice as many leukocytes were sequestered.

The immediate and profound leukopenia was caused by a disappearance of granulocytes, whereas some lymphocytes were still present in the samples obtained after 5, 30, 60, 120 and 180 min (microscopic inspection of blood smears). After 60, 120 and 180 min aggregates of granulocytes were sometimes observed.

Plasma levels of thromboxane B_2

Levels of immunoreactive TXB_2 ranged between 20 and 190 pg ml^{-1} in all animals (group 1 to 6) in the control sample ($t = -15$ min). Injection of saline (group 1) did not alter these basal TXB_2 levels (Figure 2). Endotoxin administration (group 2) resulted in a transient arterial TXB_2 peak, from the control level of 100 ± 20 pg ml^{-1}. The highest concentrations (2–9 ng ml^{-1}) were measured after 5 min (Figure 2). Subsequently, TXB_2 levels tapered off, but were still significantly above the control value after 3 h.

Pretreatment with methylprednisolone (group 3) or $F(ab')_2$ fragments (group 4) for 10 min did not interfere with the endotoxin-induced burst in TXB_2 levels (results not shown). In rosmarinic acid pretreated rabbits (group 5), the control TXB_2 level was 75 ± 20 pg ml^{-1}, and the plasma concentration measured 5 min after endotoxin was significantly lower as compared to saline-pretreated rabbits (Figure 2). Thereafter (30–180 min), the rosmarinic acid-treated rabbits displayed lower TXB_2 levels, but the differences were statistically not significant.

Depletion of complement before the endotoxin injection abolished the immediate increase in plasma TXB_2 levels (Figure 2). In these animals, TXB_2 concentrations were significantly elevated above the con-

trol period ($t = -15$ min) at 30–180 min, but remained under the levels in group 2 at 30 and 60 min after endotoxin (Student's t test).

Estimation of pulmonary oedema

Lungs from control animals (group 1, receiving only saline) contained 3.70 ± 0.09 g water per g dry weight. Administration of endotoxin (group 2) caused a small increase in this ratio to 3.99 ± 0.08 ($P < 0.05$, Student's t test). None of the treatments tested (methylprednisolone, $F(ab')_2$ fragments, rosmarinic acid, complement depletion), reduced the pulmonary fluid accumulation after endotoxin injection.

Effect of prolonged pretreatment with methylprednisolone

In a second experiment saline or methylprednisolone was given 60 min before endotoxin. In the saline pretreated rabbits (group A) endotoxin induced similar changes to those in the first experiment (group 2). The prolonged pretreatment with methylprednisolone failed to interfere with endotoxin-induced consumption of haemolytic activity (CH_{50}), but it reduced primary and secondary hypotension (Table 2). Furthermore, both the first and the second increase in arterial 6-oxo-$PGF_{1\alpha}$ levels were diminished, but not abolished, and the acute TXB_2 peak in response to endotoxin was significantly inhibited (Table 2). The thrombocytopenia, the leukopenia, and the pulmonary fluid accumulation were not affected by methylprednisolone.

Discussion

Complement-dependent and -independent haemodynamic and haematological features in endotoxin-treated rabbits

Intravenous injection of *E.coli* endotoxin in rabbits induced: (1) activation of 20 to 30% of the complement system, (2) a biphasic arterial hypotension coinciding with increased plasma levels of prostacyclin plus 6-oxo-$PGF_{1\alpha}$, (3) an immediate, partially reversible thrombocytopaenia, (4) a short-lasting peak in plasma TXB_2, (5) a severe and long-lasting leukopaenia, and (6) fluid accumulation in the lungs. In the present experiments the rabbits displayed an immediate drop in arterial blood pressure (between 2 and 5 min) in response to *E.coli* 0111:B4 lipopolysaccharide (cf. Bult *et al.*, 1980), which was absent in our first study on the involvement of complement in rabbit endotoxin shock (Rampart *et al.*, 1982), in which rabbits from the same breeding station and the same lot of endotoxin were used. This unexplained, seasonal variation between different batches of rabbits made the inclusion of a positive control for the study of drug effects on the initial blood pressure changes indispensable. To this end, CVF pretreated animals were used to distinguish between complement-dependent and complement-independent effects of endotoxin. Three days after CVF injection, complement titers (i.e. haemolytic activity and immunoreactive C3) were below the limit of detection of the assay systems used, indicating that alternative and terminal pathways were eliminated. Apart from a doubling of the number of

Table 2 Effect of prolonged pretreatment with methylprednisolone (60 min) on changes in CH_{50} consumption, mean arterial blood pressure (MABP) and plasma levels of prostacyclin plus 6-oxo-$PGF_{1\alpha}$ and thromoboxane B_2 in response to endotoxin (0.5 mg kg^{-1}, given at 0 min)

Group pretreatment		(A) *Saline* (− 60 min)	(B) *Methylprednisolone* (− 60 min)
Parameter	*Time* (min)		
ΔCH_{50} (units ml^{-1})	− 15 to 120	48 ± 11	59 ± 11
MABP (mmHg)	0	94 ± 3	97 ± 4
	2	52 ± 7	78 ± 6*
	180	68 ± 6	88 ± 5*
6-oxo-$PGF_{1\alpha}$ (pg ml^{-1})	− 15	<20	<20
	5	208 ± 66	64 ± 17*
	180	842 ± 192	276 ± 69*
TXB_2 (ng ml^{-1})	− 15	0.15 ± 0.04	0.15 ± 0.06
	5	5.48 ± 1.57	1.82 ± 0.34*

Mean ± s.e.mean of 6 rabbits; * ANOVA $F(1,10) > 4.96$, $P < 0.05$

circulating leukocytes (see below), the other parameters (i.e. MABP, plasma levels of prostacyclin and thromboxane B_2, haematocrit and platelet count) were not altered in the decomplemented rabbits.

In the control period or after saline injection, prostacyclin was not detectable (less than 20 pg ml^{-1} plasma) in arterial blood. This confirms that in rabbits (Bult *et al.*, 1980; Rampart *et al.*, 1982) as well as in humans (Christ-Hazelhof & Nugteren, 1981; Blair *et al.*, 1982) prostacyclin is not a circulating hormone under normal conditions, but appears in the circulation as a consequence of pathological alterations such as septicaemia in humans (Christ Hazelhof & Nugteren, 1981; Rie *et al.*, 1983) and experimental endotoxin shock in animals (Bult *et al.*, 1980; Harris *et al.*, 1980; Rampart *et al.*, 1982; Webb *et al.*, 1981; Schrauwen *et al.*, 1983; Coker *et al.*, 1983).

Previous work indicates that complement-activation in response to endotoxin or cobra venom factor is accompanied by increased biosynthesis of prostacyclin and that purified C5a and C5a des Arg can stimulate endothelial and mesothelial prostacyclin biosynthesis in the absence of neutrophils (Rampart *et al.*, 1982; 1983a; Bult *et al.*, 1985). The symmetrical time course of arterial hypotension and plasma 6-oxo-$PGF_{1\alpha}$ concentrations, which are associated with complement activation, support the existence of causal relationships between these three factors during the early phase of experimental endotoxin shock, as postulated previously (Bult *et al.*, 1980; Rampart *et al.*, 1982). The experiments with complement-depleted rabbits clearly demonstrated that the development of the first hypotensive phase (2–5 min) and the early increase in plasma levels of prostacyclin plus 6-oxo-$PGF_{1\alpha}$ are complement-dependent phenomena. The second phase of arterial hypotension (60–180 min) and the concomitant increase of prostacyclin plus 6-oxo-$PGF_{1\alpha}$ were reduced by complement depletion, thereby confirming previous results (Rampart *et al.*, 1982), although the extent of inhibition after 180 min may vary. Complement depletion in dogs also abrogated the initial fall (2–5 min) in blood pressure after endotoxin-injection, whereas the inhibition of the secondary hypotension (60–180 min) was variable (From *et al.*, 1970; Garner *et al.*, 1974).

In contrast to prostacyclin, small amounts of thromboxane B_2 were detected in the arterial blood samples obtained during the control period or after injection of saline. This may be explained by activation of platelets or other cells during blood sampling before indomethacin in the collecting syringe could block thromboxane synthesis. These basal levels of TXB_2 were, however, negligible in comparison with the concentration (2–9 ng ml^{-1}) present 5 min after endotoxin injection. The short-lasting but dramatic increase in TXB_2, also observed in similar shock models (Webb *et al.*, 1981; Coker *et al.*, 1983; Revenäs & Smedegård, 1981; Feuerstein & Ramwell, 1981, McDonald *et al.*, 1983) and the acute thrombocytopenia (cf. Ulevitch & Cochrane, 1977; Rampart *et al.*, 1982) were completely absent in complement-depleted rabbits. Thus, endotoxin-induced complement activation is necessary for and may have mediated the burst in thromboxane A_2 biosynthesis, but the present experiments did not identify the cell types responsible for its synthesis. The thrombocytopenia is thought to be due to platelet sequestration in pulmonary and other vascular beds (Myrvold & Lewis, 1977). It should be mentioned that thromboxane formation is rather the result than the cause of the C3-dependent platelet sequestration, since blockade of thromboxane formation by pretreatment with indomethacin has little effect on the endotoxin-induced thrombocytopenia (Bult *et al.*, 1980; Semeraro, 1980). Moreover, endotoxin-induced activation of rabbit blood platelets *in vitro* is also accompanied by release of thromboxane A_2, but blockade of thromboxane formation does not reduce aggregation or the release reaction (Bult *et al.*, 1983), which confirms that thromboxane biosynthesis is of little importance for endotoxin-induced platelet activation. However, the release of thromboxane A_2 by platelet emboli in the pulmonary circulation could be relevant to the development of acute pulmonary hypertension (Coker *et al.*, 1983), which may contribute to the transient, acute systemic hypotension by reducing left ventricular filling.

Leukocytes, the pulmonary vasculature as well as interstitial lung tissue could be other sources for TXB_2 (Salzman *et al.*, 1980; Feuerstein & Ramwell, 1981; McDonald *et al.*, 1983). Our results with complement-depleted animals, in which TXB_2 formation was almost completely prevented, may explain why other investigators failed to see an effect of endotoxin on prostaglandin formation by lung tissue *in vitro* (Feuerstein & Ramwell, 1981).

The immediate and profound leukopenia in response to endotoxin was caused by a disappearance of granulocytes, whereas lymphocytes remained present in the circulation during the entire experiment, which is in accordance with more detailed studies by Koníčková *et al.* (1980). Pretreatment with CVF resulted in a doubling of the number of circulating leukocytes, which may be explained by formation of large quantities of C3e, an acidic fragment of C3b, which triggers the release of new leukocytes from the bone marrow (Rother, 1972; McCall *et al.*, 1974; Ghebrehiwet & Müller-Eberhard, 1978). In spite of the leukocytosis, endotoxin administration lowered the number of circulating leukocytes in the CVF pretreated animals to the same extent as in normal rabbits. The experiments with the decomplemented animals further indicate that the immediate leukopenia is not directly related to the acute (2–5 min), transient systemic

hypotension and the concomitant prostacyclin formation. The leukopaenia could have been caused either by aggregation of granulocytes and embolization of these aggregates in the lungs (Craddock, 1982) or by margination of granulocytes in pulmonary vessels (Semeraro, 1980). In other experimental models of inflammation, polymorphonuclear leukocytes (PMNLs) have been associated with increased vascular permeability and interstitial fluid accumulation (Williams & Jose, 1981; Till *et al.*, 1982; Jacob, 1980). It is well-documented that complement activation may trigger aggregation and sequestration of PMNLs and concomitant plasma extravasation (Hammerschmidt *et al.*, 1979; Jacob, 1980; Semeraro, 1980; Till *et al.*, 1982; Bult *et al.*, 1985). However, the experiments with complement-depleted rabbits demonstrated unequivocally that endotoxin-induced leukopenia and pulmonary oedema were not dependent on complement activation. The presence of endotoxin 'binding sites' on granulocytes (Brunning *et al.*, 1964; Springer & Adye, 1975) could possibly explain the complement-independent leukopenia (Semeraro, 1980; Ulevitch & Cochrane, 1977).

Modulation of endotoxin shock by pharmacological agents

The effects of methylprednisolone, $F(ab')_2$ fragments of human gamma globulins and rosmarinic acid on endotoxin-induced complement activation were investigated. The experiments with complement-depleted rabbits demonstrated that the first phase (2–5 min) of arterial hypotension, the thrombocytopenia, and the early increase in plasma levels of 6-oxo-$PGF_{1\alpha}$ and TXB_2, are complement-dependent phenomena in the present model. The second phase of arterial hypotension (60–180 min) and the concomitant increase of plasma 6-oxo-$PGF_{1\alpha}$ were only partly reduced or postponed by complement depletion, whereas leukopenia and accumulation of fluid in the lungs appeared to be independent of complement activation.

The use of massive doses of glucocorticoids (e.g., methylprednisolone, 30 to 40 mg kg^{-1}) in combination with antibiotics in the treatment of human septic shock remains controversial, but could possibly lead to a favourable outcome of the septic shock, provided that methylprednisolone is given at the very early stages of the developing shock syndrome (Hardaway, 1980; Sheagren, 1981). For this reason, the treatment with methylprednisolone (40 mg kg^{-1}) was started just before the injection of endotoxin and it apparently reduced complement activation, an effect also reported by O'Flaherty *et al.* (1977) and Imai *et al.* (1982). The inhibition is probably not due to a direct effect of methylprednisolone on the enzymes of the complement cascade, since 1 mg ml^{-1} (i.e. approximately the estimated plasma concentration) failed to reduce complement activation *in vitro* (results not shown). Methylprednisolone failed to influence endotoxin-induced thrombocytopenia, leukopenia and fluid accumulation in the lungs. The lack of effect of methylprednisolone on endotoxin-induced leukopenia is a further indication that it is a complement-independent phenomenon, since it has been reported that high doses (30 mg kg^{-1}) and concentrations (1 mg ml^{-1}) of methylprednisolone inhibit complement-induced leukopenia in rabbits (O'Flaherty *et al.*, 1977) and neutrophil aggregation *in vitro* (Hammerschmidt *et al.*, 1979). Our results and those of others indicate that the effects of high concentrations of methylprednisolone on complement activation (and on complement-induced leukocyte aggregation) cannot be explained by an intracellular process involving receptor mediated transport, DNA transcription and RNA translation of proteins like macrocortin or lipomodulin (Blackwell & Flower, 1983), because the inhibition is observed within 10 min, is absent if methylprednisolone is given at a more remote time (− 60 min, see Table 2, cf. O'Flaherty *et al*, 1977) and is not shared by dexamethasone (O'Flaherty *et al.*, 1977, Hammerschmidt *et al.*, 1979).

The formation of one or more peptides like macrocortin which suppress the release of arachidonic acid and thus prostaglandin formation (Blackwell & Flower, 1983) could further contribute to the almost complete suppression of prostacyclin production and the concomitant hypotension in the second phase of shock (60–180 min). Since this induction process needs 20 min or more, it is not surprising that methylprednisolone, administered 10 min before endotoxin, did not reduce the primary increase in prostacyclin biosynthesis. Likewise, the immediate burst of thromboxane formation was not reduced by methylprednisolone. In accordance with this assumption, the formation of both prostanoids in the first minutes after endotoxin administration was indeed reduced to some extent when methylprednisolone was given at an earlier time (− 60 min). The observation that the inhibition of the secondary increase in prostacyclin production and the secondary hypotension were less pronounced when methylprednisolone was given one hour before endotoxin could possibly be due to its failure to diminish complement activation under this condition.

Immunoglobulin substitution therapy with $F(ab')_2$ fragments from human IgG reduced the secondary hypotension but did not affect endotoxin-induced complement activation, or any of the other changes. These negative results are in accordance with the observation that $F(ab')_2$-fragments were without effect on acutely developing gram-positive or -negative septicaemia in mice, whereas IgG substitution therapy diminished lethality in protracted forms

of septicaemia in mice (Klesel & Limbert, 1981) and rabbits (Ronneberger & Zwisler, 1979).

Rosemarinic acid (20 mg kg^{-1}) inhibited the activation of complement after endotoxin injection by about 70% (cf. Table 1), confirming recent *in vitro* results (Hadding *et al.*, personal communication). The complement-dependent features of endotoxin, i.e. the stimulation of prostacyclin and thromboxane biosynthesis, both hypotensive phases and the primary thrombocytopenia, were largely reduced after treatment with rosmarinic acid. The platelet effect is interesting since drugs which increase the number of free, circulating platelets during endotoxin shock have not yet been reported. Even infusion of prostacyclin, the most potent anti-aggregating agent known, failed to influence thrombocytopenia during endotoxin shock in pigs (Webb *et al.*, 1981), dogs (Krausz *et al.*, 1981) and rabbits (Bult & Herman, unpublished results). The complement-independent effects of endotoxin (leukopaenia, formation of lung oedema) were hardly influenced by rosmarinic acid.

These results indicate that pharmacological interference with complement activation may offer new possibilities for the modulation of septic shock and possibly for the treatment of inflammatory diseases in general.

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