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Formation of prostanoids during intravascular complement activation in the rabbit

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1 Plasma concentrations of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) were measured by radioimmunoassay in arterial blood before and after injections of the complement activator, cobra venom factor (CVF).

2 During the control period, the concentration of 6-oxo-$PGF_{1\alpha}$, which gives the sum of prostacyclin plus 6-oxo-$PGF_{1\alpha}$, and TXB_2 were, respectively, less than $20\,pg\,ml^{-1}$ and $70 \pm 15\,pg\,ml^{-1}$.

3 Intravenous injections of CVF induced dose-dependent, reversible elevations in the plasma levels of both prostanoids.

4 The time courses for the increases of 6-oxo-$PGF_{1\alpha}$ and TXB_2 paralleled the arterial hypotension and thrombocytopenia, suggesting the existence of a causal relationship between these parameters.

5 The results further support our hypothesis that complement-dependent formation of arachidonic acid metabolites contributes to some of the haemodynamic and haematological changes occurring during endotoxin shock.

Introduction

Intravenous administration of *E. coli* endotoxin to anaesthetized rabbits induces several haemodynamic and haematological changes e.g. pulmonary hypertension, arterial hypotension, thrombocytopenia and leukopenia (Bult & Herman, 1982). To some extent these events can be mimicked by intravenous injection of purified cobra venom factor (CVF) which is known to be a selective activator of the complement system (Ulevitch & Cochrane, 1977) and it is known that endotoxin activates complement *in vivo* (Gilbert & Braude, 1962) as well as *in vitro* (Galanos *et al.*, 1971). Recently, we demonstrated that activated serum complement, trypsin cleaved complement factor C5 and C3, and the anaphylatoxin C5a (and $C5a_{des\ Arg}$) stimulate the production of prostacyclin (PGI_2) by the endothelium of the rabbit isolated aorta (Rampart *et al.*, 1983a). The blood levels of this vasodilator metabolite of arachidonic acid rise markedly during endotoxin shock in rabbits, cats and pigs (Bult *et al.*, 1980; Coker *et al.*, 1983; Schrauwen *et al.*, 1983) and evidence has been presented showing that prostacyclin contributes to the decreased arterial blood pressure following the injection of endotoxin in rabbits (Bult *et al.*, 1980; Bult & Herman, 1982). Experiments with complement-depleted rabbits showed indirectly that activation of the complement system during endotoxin shock contributes to the generation of prostacyclin (PGI_2) and the concomitant arterial hypotension (Rampart *et al.*, 1982).

The aim of the present experiments was to investigate the effect of selective complement activation *in vivo* on the circulating arterial levels of prostacyclin and thromboxane A_2, two potent vasoactive prostanoids (Moncada & Vane, 1979), and the possible relationship with haemodynamic and haematological changes. Prostacyclin and thromboxane A_2 were assessed by radioimmunoassay of their non-enzymatic stable metabolites, i.e. 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2).

Methods

Preparation of animals and experimental protocol

Rabbits from a local breeding station (Dendermondse witte, of either sex, body weight 2.5 ± 0.2 kg) were anaesthetized with sodium pentobarbitone ($30\,mg\,kg^{-1}$) via a catheter in the marginal ear vein. Additional doses ($5-10\,mg\,kg^{-1}$) were administered when required. This catheter was also used for injection of saline or cobra venom factor. The animals were allowed to breathe spontaneously, through an en-

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dotracheal cannula to avoid suffocation. A catheter placed in the left carotid artery was used for continuous pressure measurement and blood sampling. Mean arterial blood pressure (MABP) was recorded with a SEM 4-88 (SE Labs. EMI) pressure transducer, connected to a Hewlett Packard 7758A recorder. All catheters were flushed with 0.154 M sterile pyrogen-free NaCl (saline), containing 8 units of heparin (LEO) ml^{-1}.

Eight rabbits were divided at random into 3 groups. Group 1 ($n = 4$) received saline at zero time ($1\,ml\,kg^{-1}$), group 2 ($n = 2$) received 250 u cobra venom factor (CVF, Cordis, U.S.A.), and group 3 ($n = 2$) 500 u CVF at zero time. The CVF was dissolved in saline.

Blood sampling and analysis

For determination of 6-oxo-$PGF_{1\alpha}$, TXB_2, haematocrits, and the number of circulating thrombocytes and leukocytes, samples (2.5 ml) of arterial blood were taken 15 min before and 5, 30, 60, 120 and 180 min after injection of saline (group 1) or CVF (groups 2 and 3). These samples (2.5 ml) were collected in plastic syringes containing EDTA (2.5 mg) and indomethacin (25 μg). For determination of complement titers, blood (2.5 ml) was collected in glass tubes at $t - 15$ min and $t + 120$ min and allowed to clot for 2 h at room temperature. After centrifugation (10 min, 4,000 *g*) serum was removed and stored at − 80°C. On each occasion, the volume of blood collected was replaced by an identical volume of saline containing heparin ($8\,u\,ml^{-1}$).

Platelets and white cells were counted with a Coulter Counter; white cells in whole blood (diluted 1/500 in isotonic counting buffer) after lysis of the erythrocytes with zapoglobin (Coulter Electronics, U.S.A.) and platelets in platelet-rich plasma (diluted 1/3000), prepared by centrifugation (10 min, 200 *g*). The results were corrected for changes in haematocrit, and expressed as the number of cells μl^{-1} blood. At − 15, 5 and 180 min blood smears were made for qualitative investigation of blood cells. The smears were stained with May-Grünwald-Giemsa stain.

Radioimmunoassay

Prostanoids were measured in plasma prepared by centrifugation (10 min, 4,000 *g*) of arterial blood. Prostacyclin was determined by radioimmunoassay of its non-enzymatic metabolite 6-oxo-$PGF_{1\alpha}$. The antiserum was raised in rabbits in our laboratory (Bult *et al.*, 1980). The cross-reactivity of the 6-oxo-$PGF_{1\alpha}$ antiserum with other prostaglandins (50% displacement of [^{3}H]-6-oxo-$PGF_{1\alpha}$) was 1% for $PGF_{1\alpha}$ and less than 0.1% for PGE_2, 15-oxo-PGE_2, TXB_2 and arachidonic acid (Beetens *et al.*, 1982). Therefore, plasma samples (200 μl) were used directly in the radioimmunoassay without further purification or extraction. The assay procedure has been described in detail elsewhere (Beetens *et al.*, 1982) and the results of the assay procedure have been corroborated by physicochemical methods (Claeys *et al.*, 1980; Rampart *et al.*, 1982).

Thromboxane A_2 was measured by radioimmunoassay of its non-enzymatic degradation product TXB_2. The TXB_2 antiserum (raised in rabbits) was a generous gift of Dr J.T. Flynn. Since the cross-reactivity with other prostaglandins was only 0.6% for PGD_2 and less than 0.01% for PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and arachidonic acid (Flynn, 1983), the plasma samples were assayed directly. Antiserum (0.1 ml) was mixed with 0.1 ml Tris buffer (Trisma base 50 mM, EDTA 1.1 mM, HCl 3N to pH 8.0) and 0.1 ml standard or sample. Subsequently, 0.2 ml of a 0.5% γ-globulin solution and tritiated TXB_2 (2,500 c.p.m. in 0.1 ml, New England Nuclear) were added. The final volume was always 0.7 ml and the final dilution of the antiserum was 10,500. After 16 h incubation at 4°C the bound fraction was precipitated by addition of 0.7 ml of an ice-cold 25% polyethyleneglycol 4000 solution. After centrifugation (60 min at 4°C) radioactivity was measured in the supernatant with a liquid scintillation counter. The assay was run in duplicate with 2 dilutions of the samples to be tested. Results were calculated after transformation to a logit plot and expressed as pg 6-oxo-$PGF_{1\alpha}$ or $TXB_2\,ml^{-1}$ plasma. The detection limit of both assays was $20\,pg\,ml^{-1}$.

Complement titers

The total haemolytic activity of the complement system was measured in serum (Rampart *et al.*, 1982), according to the method of Mayer (1961). The results were expressed as haemolytic units ml^{-1} undiluted serum (CH_{50}). The CH_{50} is the reciprocal of the serum dilution which contains the quantity of complement required for 50% lysis of sheep red blood cells (Biomérieux) coated with rabbit anti-sheep red cell serum (Biomérieux) in a haemolytic system. Serum concentrations of immunoreactive C3 were estimated by radial immunodiffusion (Mancini *et al.*, 1965) with agar plates containing anti-rabbit C3 antiserum (Nordic). The results were expressed as % of a standard serum (pooled rabbit serum).

Estimation of pulmonary oedema

At the end of the experiment, the rabbits were killed by an overdose of sodium pentobarbitone. The chest was opened and the right lung was carefully removed and weighed (wet weight). The dry weight was determined after heating the tissue for 48 h at 60°C. The water

content of the tissue (wet weight – dry weight) was expressed as gg^{-1} dry material. This ratio is independent of the total tissue weight, and increases when oedema formation occurs.

In vitro *experiments*

Commercial CVF preparations may still be contaminated with phospholipase A_2 (Waldman & Lachman, 1975), which could directly promote the formation of prostaglandins via liberation of arachidonic acid, thereby over estimating complement-mediated stimulation of prostanoid formation. Therefore we investigated the direct, complement-independent effect of CVF ($10\,u\,ml^{-1}$) on the prostacyclin production by a tissue with high PGI_2 forming capacity, i.e. the rabbit isolated peritoneum, according to the method described previously (Bult & Herman, 1983a).

Results

In the control period ($t = -15$ min to $t = 0$) there were no significant differences between the three groups of rabbits as regards complement titers, mean arterial blood pressure, number of circulating thrombocytes and leukocytes and plasma levels of TXB_2 and 6-oxo-$PGF_{1\alpha}$. The latter was below the limit of detection of the assay ($20\,pg\,ml^{-1}$) in all rabbits studied.

In the control group, intravenous injection of saline did not change any of these parameters in the subsequent 3 h (see Tables 1 and 2 and Figures 1 and 2). In the test groups, CVF (250 u, i.v.) activated the complement system, as indicated by the decreased complement titers measured 2 h later: 30 to 40% loss of functional value (CH_{50}) and 50 to 60% consumption of immunoreactive factor C3. The higher dose of CVF (500 u) did not further reduce immunoreactive serum C3, but the functional CH_{50} was almost completely consumed (Table 1), suggesting that a more profound activation had occurred.

Injection of CVF was followed by a biphasic drop in arterial blood pressure (Figure 1), thereby, closely resembling one of the characteristic features accompanying an injection of endotoxin in rabbits. The first phase of hypotension followed by a partial recovery took place within 5 min. This was followed by a modest, sustained hypotension, with a tendency to further decrease 60 to 120 min after administration of 500 u CVF. The 6-oxo-$PGF_{1\alpha}$ plasma levels reflected almost perfectly the changes in arterial blood pressure; after an initial sharp increase ($t + 5$ min), 6-oxo-$PGF_{1\alpha}$ plasma levels returned to undetectable levels (less than $20\,pg\,ml^{-1}$, $t + 30$ min). Levels of 6-oxo-$PGF_{1\alpha}$ were elevated again in plasma collected 60, 120 and 180 min after CVF. The arterial hypotension as well as the increase in plasma 6-oxo-$PGF_{1\alpha}$ were most pronounced with the highest dose of CVF (500 u).

Injection of CVF resulted in an immediate and dose-dependent thrombocytopenia (see Figure 2). The lowest platelet counts were observed 5 min after administration of the higher (500 u) dose of CVF; injection of 250 u resulted in a less pronounced but more prolonged thrombocytopenia (lowest values after 30 min). Thereafter the number of circulating free platelets gradually returned to control values which were reached 3 h after CVF injection.

As shown in Figure 2, the changes in TXB_2 concentration symmetrically paralleled the changes in the platelet count. CVF injection caused immediately a dramatic increase in TXB_2 plasma levels. The highest concentrations were measured in the samples collected 5 min after administration of the higher dose of CVF (500 u). Subsequently, TXB_2 plasma levels tapered off, reaching control values 2 h after injection of CVF.

The changes in circulating leukocytes observed after injection of CVF showed a pattern similar to the changes in platelet count (Table 2). In the control period (– 15 min), the differential leukocyte counts

Table 1 Activation of the complement system by intravenous injection of cobra venom factor (CVF)

CVF dose	*Haemolytic activity (CH_{50} units)*			*Immunoreactive C3* (%)		
(units/rabbit)	*– 15 min*	*120 min*	*ΔCH_{50}*	*– 15 min*	*120 min*	*$\Delta C3$*
0	165 ± 10	160 ± 10	3 ± 3	136 ± 10	135 ± 10	1 ± 1
250	184	130	54	123	50	73
	176	100	76	127	40	87
500	180	20	160	135	54	81
	160	20	140	127	50	77

For the control group ($1\,ml\,saline\,kg^{-1}$), the results show the mean ± s.e.mean ($n = 4$).
For the CVF-treated rabbits, the individual data are shown. Immunoreactive C3 was expressed as % pooled rabbit serum.

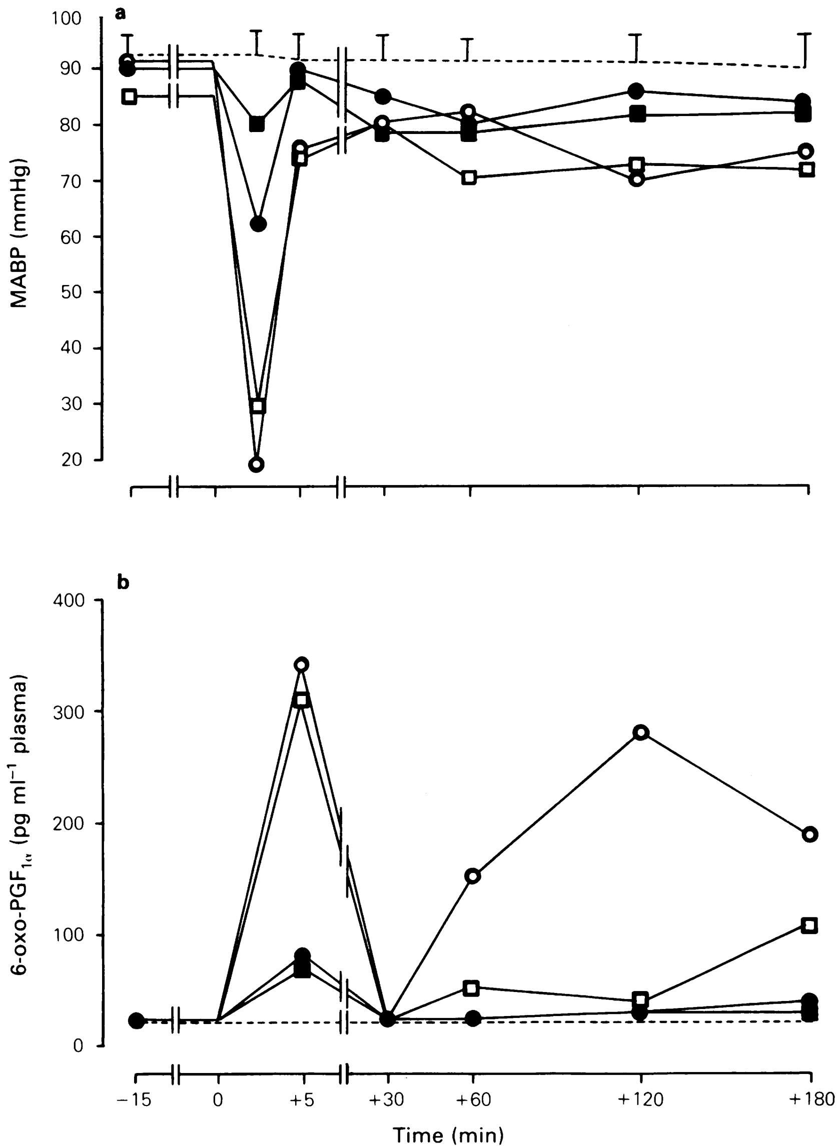

Figure 1 The effect of complement activation with cobra venom factor (CVF) on mean arterial blood pressure (a) and plasma levels of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) (b). For the control group (broken line) results are given as mean with vertical lines showing s.e.mean, of $n = 4$. For the CVF-treated groups (full lines) each symbol represents an individual rabbit (closed symbols (● and ■): animals receiving 250 u CVF; open symbols (○ and □): 500 u CVF).

were 58 ± 1, 30 ± 2 and 9 ± 2 (% ± s.e.mean, $n = 4$) for neutrophils, lymphocytes and monocytes, respectively. Inspection of blood smears obtained 5 min after CVF injection indicated that neutrophils (differential counts 33% and 8% after 250 units CVF, 1% and 3% after 500 units CVF) and monocytes (differential counts 10% and 8% after 250 units CVF, 1% and 4% after 500 units CVF) disappeared from the circulation, whereas a substantial number of lymphocytes (differential counts 56% and 83% after 250 units CVF,

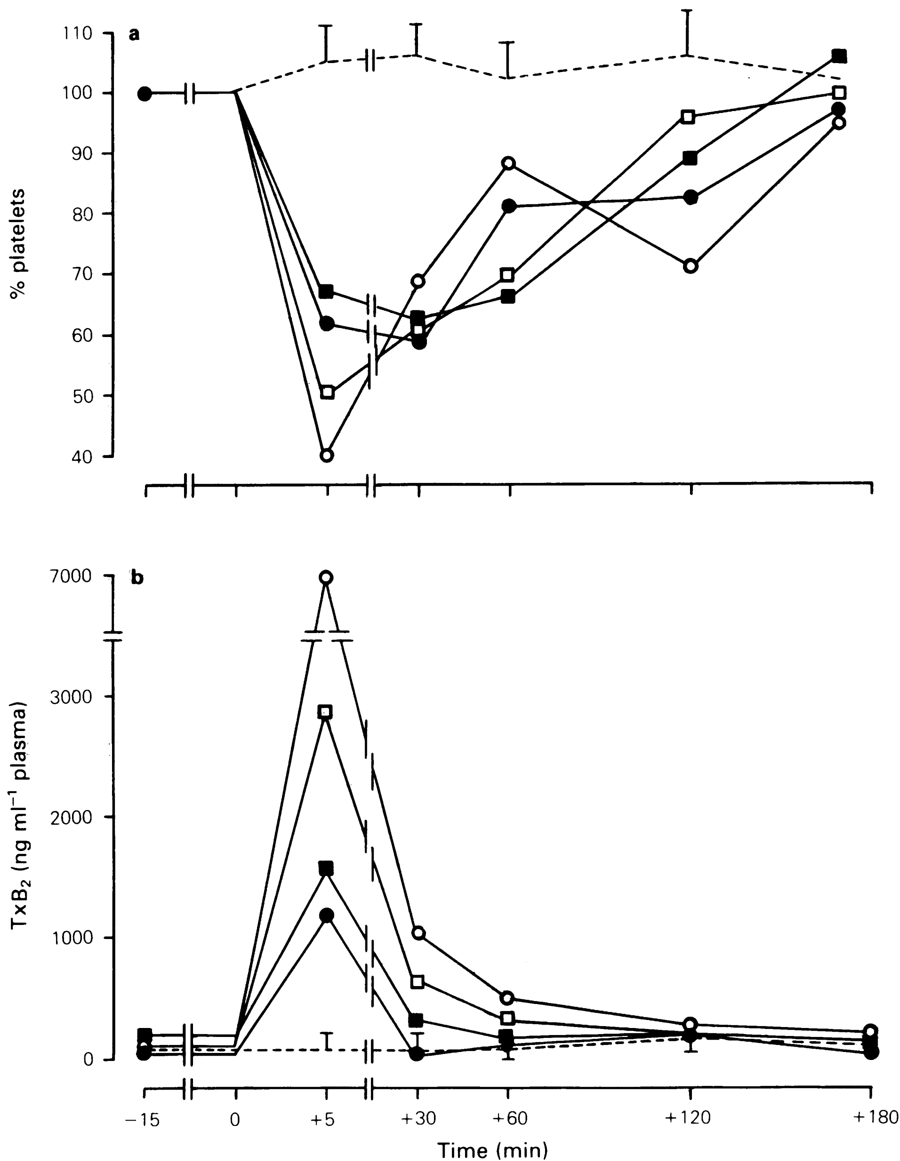

Figure 2 The effect of complement activation with cobra venom factor (CVF) on the number of circulating free thrombocytes (a) and plasma levels of thromboxane B_2 (TXB_2) (b). For reasons of clarity, platelet counts are shown here as percentage of the value during the control period (t − 15 min = 100%). For the control group (broken line) results are given as mean, with vertical lines showing s.e.mean of n = 4. For the CVF-treated animals (full lines) each symbol represents an individual rabbit (closed symbols (● and ■): animals receiving 250 u CVF; open symbols (○ and □), 500 u CVF).

and 97% and 92% after 500 units CVF) remained present. In addition, a few large clumps of platelets were observed in the blood smears obtained 5 min after CVF injection, indicating that agglutination and/or aggregation had occurred. Moreover, the morphology of a substantial proportion of the red blood cells had changed from the normal biconcave cells to acanthocytes, i.e. spheres with spiny projections. A number of acanthocytes was still present in the blood smears obtained 180 min after CVF injection. By that

Table 2 Effect of intravascular complement activation with cobra venom factor (CVF) on circulating leukocytes ($10^3\,\mu l^{-1}$ blood)

CVF dose (units/rabbit)	*Time* (min) *−15*	*5*	*30*	*60*	*120*	*180*
0	6.2 ± 0.6	6.2 ± 0.5	6.3 ± 0.5	6.5 ± 0.5	6.5 ± 0.4	6.6 ± 0.5
250	6.0	4.9	4.7	4.7	8.1	9.8
	7.5	1.0	3.9	4.2	4.4	7.8
500	3.6	0.9	2.5	1.7	1.3	3.4
	7.7	1.5	5.9	3.7	4.4	7.0

For the control group, results are the mean ± s.e.mean (n = 4). For the CVF-treated rabbits, the individual data are shown.

time, the leukocyte count had returned to baseline values (Table 2), and the differential leukocyte counts showed a tendency to normal proportions, but the percentage of lymphocytes remained elevated (40% and 78%, 180 min after 250 units CVF, and 71% and 97% after 500 units CVF).

Lungs from control rabbits contained 3.6 ± 0.1 g water g^{-1} dry weight. In the CVF-treated group no differences in oedema formation could be detected between high doses and low doses of CVF and, therefore, they were combined. The pulmonary water content of CVF-treated rabbits was 4.0 ± 0.1 g g^{-1} dry weight, and this was significantly higher than the control value ($P < 0.05$, Wilcoxon test).

Rabbit isolated peritoneal tissue, incubated in a mixture of 50 mM Tris buffer pH 7.5 with 10% saline, produced 23.5 ± 5.0 pmol 6-oxo-$PGF_{1\alpha}$ mg^{-1} tissue in 30 min. The presence of CVF (10 u ml^{-1}) did not alter peritoneal PGI_2 formation (25.0 ± 5.5 pmol 6-oxo-$PGF_{1\alpha}$ mg^{-1} tissue). Both values represent mean ± s.e.mean of 6 independent incubations.

Discussion

In the past few years evidence has been accumulating indicating that activation of the complement system may trigger the arachidonic acid cascade (for review see Bult & Herman, 1983b). This may then result in the formation of prostaglandins, a group of inflammatory mediators which have been claimed to contribute to the expression of the haemodynamic and haematological features of septic shock (Bult & Herman, 1982). The aim of the present study was to investigate the existence of a direct link between the activation of complement and the formation of vasoactive prostanoids *in vivo*.

An injection of CVF which could be shown to have activated complement also induced a dose-dependent biphasic arterial hypotension, reversible thrombocytopenia and leukopenia and the development of pulmonary oedema. These results are in good agreement with the observations of other investigators (Ulevitch & Cochrane, 1977; Jacob, 1980; O'Flaherty *et al.*, 1977). Radioimmunological estimation of 6-oxo-$PGF_{1\alpha}$ and TXB_2, the non-enzymatic metabolites of PGI_2 and TXA_2, respectively, revealed a marked and characteristic increase in plasma levels of these two potent vasoactive prostanoids during intravascular complement activation.

In vitro, a high concentration of CVF was devoid of PGI_2 stimulatory activity, indicating that the CVF preparation used was essentially free of phospholipase activity. Since prostacyclin was not detectable in the control period (less than 20 pg ml^{-1}), it cannot be considered as a circulating hormone, confirming previous reports (Bult *et al.*, 1980; Christ-Hazelhof & Nugteren, 1981; Haslam & McClenaghan, 1981). Therefore, the immediate rise of arterial prostacyclin plus 6-oxo-$PGF_{1\alpha}$ after CVF injection cannot be explained by reduced clearance of either compound, but must be due to stimulation of prostacyclin biosynthesis. The increase in prostacyclin plus 6-oxo-$PGF_{1\alpha}$ plasma levels occurred in parallel with complement activation, an observation also made in *in vitro* models (Rampart *et al.*, 1983a, b). It may be considered to be causally related to complement activation.

Plasma levels of 6-oxo-$PGF_{1\alpha}$ and TXB_2 peaked at the same time as arterial hypotension and thrombocytopenia. This suggests the existence of a causal relationship between PGI_2 formation and arterial hypotension on the one hand and between thrombocytopenia and TXB_2 production on the other hand.

The initial features of intravascular complement activation with CVF i.e. first phase of arterial hypotension, thrombocytopenia, leukopenia, and the first peak levels of 6-oxo-$PGF_{1\alpha}$ and TXB_2 closely resemble the early haemodynamic and haematological changes occurring during endotoxic shock (Bult *et al.*, 1985). Therefore, these experiments provide further evidence for the functional involvement of complement activa-

tion in the development of septic shock. Although CVF is a far more potent activator of the complement system than endotoxin, the haemodynamic and haematological changes occurring at later stages (1–3 h after CVF injection) were less pronounced. This is hardly surprising, since endotoxin activates not only complement but also the Hageman-kinin system, the clotting system, and by direct interaction affects, platelets, leukocytes or endothelial cells (Ulevitch & Cochrane, 1977; Ulevitch *et al.*, 1975; Semeraro, 1980).

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