Thromboxane Synthesis by Sources Other Than Platelets in Association with Complement-Induced Pulmonary Leukostasis and Pulmonary Hypertension in Sheep

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SUMMARY. Infusion into sheep of plasma containing zymosan-activated complement produces leukopenia, pulmonary leukostasis, and pulmonary artery hypertension. We previously demonstrated a close relationship between the pulmonary vascular response and elevations of plasma thromboxane. We have investigated the source of thromboxane synthesis in this model. Plasma containing zymosan-activated complement added to whole blood did not stimulate thromboxane synthesis. This observation suggested that leukocytes do not synthesize thromboxane directly in response to complement. Sheep rendered severely thrombocytopenic by the administration of antiplatelet serum responded to complement infusion in the usual way. Pretreatment with aspirin (10 mg/kg) protected sheep against the pulmonary vascular response and completely blocked thromboxane synthesis. Transfusion of functional platelets did not restore these responses. Twenty-four hours after aspirin treatment, in vivo thromboxane synthesis was significantly greater than platelet thromboxane synthesis in vitro. Thromboxane is synthesized by a tissue which recovers cyclooxygenase enzyme activity at a rate that is more rapid than platelet turnover. Sheep lung synthesizes thromboxane actively in vitro. It is postulated that leukocytes exposed to activated complement components damage pulmonary vascular endothelial cells and stimulate synthesis of thromboxane A2 which causes pulmonary vasoconstriction. (Circ Res 52: 1-6, 1983)

THE leukopenia associated with hemodialysis is due to pulmonary sequestration of leukocytes aggregated in response to activation of complement by the alternative pathway (Craddock et al., 1977). Complement activation has been demonstrated in other clinical settings where plasma comes in contact with artificial membranes, as in leukapheresis units (Hammerschmidt et al., 1978; Nusbacher et al., 1978) and during cardiopulmonary bypass (Chenoweth et al., 1981).

Complement-mediated aggregation and pulmonary sequestration of leukocytes is accompanied by arterial hypoxemia and a marked rise in pulmonary vascular resistance. We have previously demonstrated in sheep that thromboxane synthesis is associated with the pulmonary hypertension which occurs in response to infusion of plasma containing zymosan-activated complement (Cooper et al., 1980) or in response to perfusion of an extracorporeal membrane oxygenator (Townsend et al., 1981). Aspirin (ASA), sulfipyrazone, or indomethacin blocked thromboxane synthesis and also protected against the pulmonary vascular response. These results and the close temporal relationship which existed between pulmonary artery pressure rise and elevation of plasma thromboxane B2 (TXB2) suggested that thromboxane A2 (TXA2) is the mediator of the hemodynamic change. TXA2 is the unstable and potent vasoconstrictor synthesized by platelets and other tissues from arachidonic acid (Hamberg et al., 1975). TXA2 is hydrolyzed to inactive, stable, TXB2. The present investigation was undertaken to attempt to identify the source of thromboxane synthesis associated with the pulmonary arterial response to infusion of activated complement.

Methods

Plasma Infusion in Sheep

Sheep of either sex, weighing 30-40 kg, were sedated with intramuscular ketamine (22 mg/kg). A carotid artery and jugular vein were cannulated using local lidocaine anesthesia to supplement the ketamine sedation. The carotid artery cannula was used for blood sampling and was attached to a Statham transducer for pressure monitoring. A no. 7 French Swan-Ganz catheter (modified by the manufacturer to have the proximal injection site 12.5 cm from the tip) was inserted via the jugular vein and advanced into the pulmonary artery, for measurement of pulmonary artery and pulmonary wedge pressures and for determination of cardiac output by the thermodilution method (cardiac out-
put model no. 9510 Edwards Laboratory). A separate central venous cannula was inserted for infusion of complement-activated plasma.

A period of 18 hours was allowed to elapse between cannulation and the performance of hemodynamic and biochemical studies. All studies were carried out in awake normoxic sheep, under conditions of stable baseline hemodynamics, blood counts, and blood gases. Indwelling catheters were continuously flushed with heparinized saline (1 U/ml) at a rate of 3 ml/hr. Platelet counts were measured on a Coulter Counter model 2F. In thrombocytopenic sheep, the count was checked by phase contrast microscopy. The white blood cell count was measured on a Coulter Counter model 5, and the differential white blood cell count was estimated from stained blood films. Blood gases and pH were measured on a Radiometer analyzer.

**Zymosan-Activated Plasma**

At least 40 ml of heparinized plasma (1 U/ml) were prepared from each sheep on the day of cannulation. The plasma was activated by incubation with 2.5 mg of zymosan (ICN Pharmaceuticals) per milliliter for 60 minutes at 37°C. The mixture was spun at 20,000 g for 10 minutes at 4°C, and the plasma was removed and centrifuged a second time to ensure complete removal of particles. The activated plasma was stored at −20°C for reinfusion on the following day via the central venous catheter at a rate of 5 ml/min for 5 minutes using a constant infusion pump (Harvard Apparatus).

**Preparation of Platelet Concentrates**

Four units of sheep blood were collected in citrate phosphate dextrose solution in double packs (Fenwall Laboratories). Anticoagulated blood was diluted 1:1 with sterile saline. Platelet rich plasma (PRP) was separated at 22°C within a few hours of blood collection by centrifugation at 4500 rpm for 15 minutes in an IEC, PR6000 centrifuge. Addition of 10% extra citrate phosphate dextrose solution was made at this time to reduce the pH to 6.5. Platelet clumping was prevented by this maneuver. The PRP was recentrifuged at 4500 g for 12 minutes at 22°C to pack the platelets. Packed platelets were resuspended in 300 ml of plasma and maintained overnight at 22°C with gentle agitation on a Medley platelet agitator. Platelet concentrates were infused into recipient animals 24 hours after preparation.

**Thrombocytopenic Sheep**

Antiserum against sheep platelets was raised in New Zealand white rabbits. Intramuscular injections containing 1 ml of sheep platelet suspension and 1 ml of Freund's adjuvant were made weekly for 10 weeks. The rabbit serum was then harvested, incubated at 56°C for 30 minutes to deactivate complement, and adsorbed against washed sheep white cells and red cells. The final serum was stored at −70°C. The antiplatelet serum produced complete lysis of platelets in samples of PRP to a titer of 1:100. The injection of antiplatelet serum was made using a volume of 1 ml intramuscularly and 1 ml subcutaneously. This produced within 18 hours severe thrombocytopenia (platelet count less than 10,000/μl) which persisted for an additional 24–36 hours. No alteration in hematocrit or white blood cell count resulted from the use of antiplatelet serum.

**ASA Preparation**

Acetylsalicylic acid solution was prepared freshly for each experiment. Ten grams of acetylsalicylic acid (Sigma Chemicals) were added slowly to 80 ml of water, the pH being maintained between 6.8 and 7.2 by the addition of 25% sodium bicarbonate. The solution was made up to 100 ml with water. If the preparation was not used within a few minutes, it was stored at −70°C and the pH rechecked prior to use.

**ASA Estimation**

Samples for estimation of ASA and salicylate in plasma were collected in vacutainers containing potassium oxalate and sodium fluoride (vacutainer #6470 MS 3204 PS). These were placed on ice and centrifuged at 18,000 rpm for 10 minutes at 0°C. The supernatant plasma was quickly frozen in a dry ice aceton mixture and stored at −70°C until subsequent analysis by high pressure liquid chromatography by the method of Ali et al. (1980). The method was sufficiently sensitive to detect ASA in a concentration of 0.05 μg/ml of plasma.

**Estimation of TXB₂**

Carotid arterial blood samples were placed in tubes containing 0.07 ml of 15% EDTA (vacutainer #5450 MS 3204 QS) and centrifuged at 18,000 rpm for 10 minutes at 0°C. The supernatant plasma was placed in a glass tube and stored at −20°C. Extraction, chromatography, and radioimmunooassay were carried out as previously described by Ali and McDonald, 1980.

**Thrombin-Induced Platelet Synthesis of Thromboxane Ex Vivo**

Whole blood (10 ml) was allowed to clot in glass test tubes at 37°C for 90 minutes. The serum was separated by centrifugation and stored at −20°C for subsequent TXB₂ assay. Thromboxane synthesis did not occur in heparinized blood samples standing under these conditions or in samples from severely thrombocytopenic sheep. Thromboxane synthesis under these conditions is assumed to be the result of thrombin stimulation of platelets. Synthesis is linear for 15 minutes, and then constant levels of TXB₂ are observed for at least 4 hours.

**In Vitro Synthesis of TXB₂ and 6-Keto-PGF₁α by Sheep Lung and Pulmonary Artery**

Fresh sheep lung and pulmonary artery were obtained from a local abattoir, washed, and chilled on ice immediately. The tissue was cut into approximately 1-g slices. Each slice was suspended in 2 ml of 0.05 M Tris-HCl buffer (pH 8.0). Prostaglandin synthesis was allowed to proceed in the presence or absence of added arachidonic acid (100 μM) for 15 minutes at 37°C. Incubation was terminated by addition of a mixture of ethanol and saline (2:1). The pH was adjusted to 3.0 with 1 N HCl and the lipids were extracted into chloroform. Chloroform extracts were taken to dryness under nitrogen, suspended in ethanol and stored at −40°C until used for radioimmunooassay of TXB₂ and 6-keto-PGF₁α.

**Results**

**Effect of Thrombocytopenia**

Figure 1 shows the effect of infusion of autologous plasma containing zymosan-activated complement into control sheep and sheep that had been rendered severely thrombocytopenic by antiplatelet serum. The leukopenia, hypoxia, rise in pulmonary artery pressure, and elevation of plasma TXB₂ were the same in...
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The effect of complement infusion in control and thrombocytopenic sheep. Plasma containing zymosan-activated complement was infused at a rate of 5 ml/min for 5 minutes into 11 control sheep (●—●) and 4 sheep rendered severely thrombocytopenic by treatment with antplatelet serum (○—○). The symbols represent the mean values ± 1 SE.

The two groups of animals. The possibility that antiplatelet serum resulted in platelet aggregation and sequestration within the pulmonary vasculature was excluded by electron microscopic studies of lung following administration of antplatelet serum. Platelets were very rare in preparations from thrombocytopenic sheep lungs compared with normal lung sections, and no aggregates were observed.

Effect of Platelet Transfusions in ASA-Treated Sheep

ASA and SA levels in plasma 2 hours after intravenous ASA injection in a dose of 10 mg/kg were, respectively, 38 ± 5.8 µg/ml and 54.8 ± 12.7 µg/ml. ASA was not detectable 2 and 5 hours after the injections and SA levels had fallen to 3.9 ± 2.6 and 1.2 ± 0.7 µg/ml at these times. The data in Figures 1 and 2 demonstrate that ASA treatment did not prevent the leukopenia associated with complement infusion, but blocked the changes in P<sub>02</sub>, pulmonary artery pressure, and the plasma thromboxane elevations. Thromboxane synthesis by platelets during blood clotting ex vivo was also strongly inhibited by ASA. Transfusion of platelet concentrates produced no significant change in hematocrit or polymorphonuclear leukocyte count but increased the platelet count by 36% (Table 1). The platelet transfusions restored thromboxane synthesis in vitro in blood samples from recipient animals to a degree which was appropriate to their effect on platelet count. However, the platelet transfusions did not restore the pulmonary vascular response or the elevations in plasma thromboxane in the ASA-treated animals (Fig. 2). Although there was no detectable ASA in the plasma at the time platelet transfusions were made, it is possible that the transfused platelets could be rendered nonfunctional by very low plasma levels of ASA which fell below detectable limits. This possibility was excluded by the observation that platelet thromboxane synthesis in vitro during blood clotting was efficiently restored by the platelet transfusions.

Recovery of Thromboxane Synthesis in Vivo and in Vitro after ASA

Maximal plasma elevations of TXB<sub>2</sub> in response to complement infusion were reduced from 8.9 ± 3.0 ng/ml in the control animals to 0.14 ± 0.12 ng/ml 5 hours after treatment with ASA. The synthesis by platelets during blood clotting exhibited a similar reduction from 10.6 ± 3 to 0.08 ± 0.1 ng/ml. In two sheep, experiments were carried out 24 hours after ASA treatment to determine whether the recovery of TXB<sub>2</sub> synthesis in vivo was the same as that exhibited by platelets in vitro. In vitro platelet synthesis of thromboxane during clotting was approximately 4% of the control value 24 hours after the ASA dose, whereas the mean peak plasma level after complement infusion in these animals had increased to approximately 18% of the control value.

In Vitro Synthesis of TXB<sub>2</sub> and 6-keto-PGF<sub>1α</sub> by Sheep Tissues

Sheep blood anticoagulated with citrate or heparin contained 0.20 ng TXB<sub>2</sub>/ml of plasma. This level did not increase on incubation at 37°C for 30 minutes with or without the addition of autologous plasma containing zymosan-activated complement. Negative
results were also obtained when heparinized blood was used.

The results of incubation of slices of lung and pulmonary artery are shown in Table 2. Lung synthesized much larger amounts of TXB₂ than of the prostacyclin hydrolysis product 6-keto-PGF₁α. Pulmonary artery also synthesized TXB₂ but in much smaller amounts relative to the synthesis of 6-keto-PGF₁α.

**Discussion**

The present investigation confirms our previous work (Cooper et al., 1980; Townsend et al., 1981) which indicates that the pulmonary vascular response to complement activation is mediated by thromboxane synthesis. Craddock et al. (1977) demonstrated that leukocytes are essential to the hypoxia caused by complement activation. Since leukocytes are known to synthesize thromboxane (Goldstein et al., 1977), it is possible that activated complement components stimulate leukocytes to synthesize the vasoconstrictor thromboxane. Presumably, this stimulation would in-
volve activation of phospholipase to make arachidonic acid substrate available to leukocyte cyclooxygenase. In the present investigation, zymosan-activated complement did not stimulate leukocyte thromboxane synthesis in whole blood in vitro. However, the possibility that leukocytes sequestered in the lungs are the source of thromboxane in vivo under conditions of stimulation by complement has not been entirely excluded.

Since platelets synthesize thromboxane actively (Hamberg et al., 1975) it is possible that platelets interact with pulmonary leukocyte microemboli to synthesize and release the mediator. Three lines of evidence in the present investigation indicate that platelets are not involved. Severe thrombocytopenia failed to reduce the thromboxane and pulmonary artery pressure responses. Transfusion of functional platelets failed to restore the responses in sheep rendered unresponsive by ASA treatment. It is possible that an insufficient number of platelets were infused or that the platelets were unresponsive in vivo. This possibility seems unlikely in view of the demonstrated restoration of thromboxane synthesis to about 50% of the control value in blood samples from recipient animals. A third observation which suggests that a tissue other than platelets is responsible for the in vivo thromboxane synthesis is the substantial recovery of the plasma thromboxane response observed 24 hours after a dose of ASA. At this interval after ASA treatment, platelet thromboxane synthesis in vitro remained almost totally inhibited. Vascular cyclooxygenase activity has been shown to recover more rapidly after ASA treatment than platelet cyclooxygenase activity (Preston et al., 1981). Platelet cyclooxygenase activity is irreversibly inhibited by ASA and recovers only as new functional platelets enter the circulation (Roth et al., 1975). Vascular cells may be capable of synthesis of new cyclooxygenase enzyme protein at a rate which is more rapid than the rate of platelet turnover.

Our conclusion that platelets are not the source of the thromboxane is consistent with recent findings of Wonders et al. (1981). They found that the pulmonary vascular response to the onset of venovenous membrane oxygenator perfusion in sheep was the same in normal sheep and in sheep rendered thrombocytopenic by the use of antiplatelet serum. In their experiments, however, the thrombocytopenia was not quite as profound as in our experiments. In addition, there was a concomitant leukopenia produced by the antiplatelet serum. Plasma thromboxane levels were lower in thrombocytopenic sheep than in control sheep in Wonders' report, but this may have related equally to the lower white blood cell count.

Guinea pig lung has been shown to synthesize thromboxane actively (Svensson et al., 1975), and the present investigation shows that sheep lung and pulmonary artery are also active in this respect. The synthesis by lung of thromboxane and of 6-keto-PGF_{1a}, the stable hydrolysis product of prostaglandin I_{2}, occurred in a proportion of approximately 20:1. This is similar to the proportion of TXB_{2} and 6-keto-PGF_{1a} previously observed in vivo following infusion of plasma containing activated complement (Cooper et al., 1980). Slices of the large branches of the pulmonary artery synthesize these materials in vitro in roughly reversed proportion. Therefore, the synthesis of thromboxane and 6-keto-PGF_{1a} probably occurs in cells of the pulmonary microcirculation or in cells in the pulmonary parenchyma. We postulate that aggregated leukocytes in the pulmonary microcirculation distort or damage endothelial cells in a manner which stimulates them to synthesize and release vasoconstrictor TXA_{2}. Although direct evidence of this sequence of events is not yet available, there is evidence for complement- and leukocyte-mediated vascular damage. Issekutz et al. (1980a, 1980b) have demonstrated that zymosan-activated complement causes microvascular damage by a process which involves polymorphonuclear leukocyte infiltration. They postulate that CSA_{ser} may cause leukocytes to release lysosomal enzymes or superoxide or OH radicals, which Sacks et al. (1978) have shown can damage endothelial cells. Endothelial cells have been shown by Ingerman-Wojenski et al. (1981) to synthesize thromboxane in addition to 6-keto-PGF_{1a}.

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