EFFECT OF SODIUM NITROPRUSSIDE ON COMPLEMENT ACTIVATION INDUCED BY CARDIOPULMONARY BYPASS: A CLINICAL AND EXPERIMENTAL STUDY

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Complement activation and leukocyte stimulation were prospectively studied during and after cardiopulmonary bypass in 16 children receiving sodium nitroprusside—a nitrovasodilator releasing nitric oxide—for vasodilation during the cooling and rewarming periods of extracorporeal circulation. Results were compared with those in 29 patients who were not treated with sodium nitroprusside during the operation. Patients treated with sodium nitroprusside had significantly less C3 conversion during cardiopulmonary bypass as measured by the ratio C3d/C3 ($p < 0.05$) and significantly less C5a liberation immediately after cardiopulmonary bypass ($p < 0.005$) than patients not treated with sodium nitroprusside. C4 was not overtly consumed in our series. Leukocyte count during the rewarming period of cardiopulmonary bypass, but not leukocyte elastase release during cardiopulmonary bypass, was significantly reduced in patients treated with sodium nitroprusside ($p < 0.05$). In vitro experiments were conducted to analyze the effect of sodium nitroprusside on complement hemolytic activity initiated by the classic and the alternate pathways and on zymosan-induced C3 conversion by the activation of the alternate pathway. The in vitro experiments clearly demonstrate inhibition of complement hemolytic activity by sodium nitroprusside in the sera tested. The 50% inhibitory concentration of sodium nitroprusside on the available complement hemolytic activity was less through the alternate pathway than through the classic one (4.2 ± 0.8 mmol/L and 14.0 ± 2.88 mmol/L, respectively). The decrease of complement hemolytic activity measured was dose-dependent and was enhanced by the sodium nitroprusside preincubation of the sera tested. This effect was related to the duration of preincubation. Sodium nitroprusside photodegradation (enhancing nitric oxide release) increased the anticomplementary effect of the drug, reducing the 50% inhibitory concentration on complement hemolytic activity to 0.24 to 0.02 mmol/L for the alternate pathway and 2.74 to 0.3 mmol/L for the classic pathway. The zymosan-induced C3 conversion was inhibited by sodium nitroprusside. Nitroglycerin and isosorbide dinitrate (other nitric oxide donors) had in vitro effects on complement hemolytic activity similar to those of nonphotodegraded sodium nitroprusside at similar concentrations (1 mmol/L). Our results suggest that sodium nitroprusside, both in vitro and in vivo, has an inhibiting effect on complement activation initiated by both classic and alternate pathways and that this effect is mediated by

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This article is dedicated to the memory of Thibault Wolff.

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nitric oxide release from sodium nitroprusside. This is the first report on the anticomplementary effect of sodium nitroprusside by nitric oxide release. (J THORAC CARDIOVASC SURG 1996;111:882-92)

The complex generalized inflammatory reaction resulting from cardiopulmonary bypass (CPB) involves the liberation of various humoral and intracellular mediators that could be related to the development of postoperative organ dysfunction, the pathophysiologic basis of which includes the loss of endothelial integrity.^{1,2} Complement activation, by contact between blood and foreign surfaces and by its interactions with other biologic cascades,³ is thought to play a central role in this inflammatory reaction.^{1,4,5} Complement anaphylatoxins C3a and C5a are potent leukocyte chemotactic factors, inducing leukocyte stimulation with proteolytic enzyme and oxygen-derived free radical release and cytokine production,⁵⁻¹² which in turn may be involved in the development of postoperative organ injury.^{5,9,13,14} Several therapeutic interventions have been proposed to prevent or limit the magnitude of the inflammatory reaction occurring during CPB. These include nonspecific treatment with conflicting clinical and experimental results such as, among others, the preoperative administration of corticosteroids,¹⁵⁻¹⁷ of the protease inhibitor aprotinin,¹⁸⁻²⁰ or of methylxanthine pentoxifylline, which inhibits the inflammatory action of interleukin-1 and tumor necrosis factor- α .^{21,22} A more specific approach, the administration of human recombinant soluble complement receptor 1, which inhibits classic, alternate, and terminal pathway activation of the complement cascade, was recently investigated in an animal model.²³ Because complement activation may play a central role in the CPB-induced inflammatory reaction, the attenuation of complement activation makes this therapeutic approach rational.

We here report the effect of sodium nitroprusside (SNP), a long known vasodilator²⁴ and nitric oxide donor,²⁵ on complement activation and leukocyte mobilization occurring during CPB. Inasmuch as our clinical observations suggested an *in vivo* inhibitory effect of SNP on complement activation induced by CPB, we verified this hypothesis by *in vitro* experiments. We document for the first time the influence of this drug on both the alternate and the classic pathways of complement activation and discuss its mechanism of action.

Patients and methods

Clinical. Sixteen infants and children aged 6 months to 7 years (mean age 29 months) undergoing cardiac operations for congenital cardiac defects were included in this prospective study. Diagnoses and types of surgical procedures are shown in Table I. These 16 patients were operated on at the same time as a group of 29 patients aged 3 months to 17 years (mean age 40 months) studied in the same way and reported on previously.¹³ The CPB protocol in both patient groups was identical with one exception: for vasodilation during the cooling and the rewarming periods of extracorporeal circulation, the 16 patients studied here received SNP systematically instead of phentolamine or they received no vasodilator at all.

Anesthesia and antibiotic regimen. Conventional general anesthesia was performed in all patients. Cefotiam hydrochloride (25 mg/kg) and dexamethasone (3 mg/m²) given for prophylaxis of cerebral edema were administered before sternotomy.

CPB. The CPB protocol was identical for the 16 children examined. A roller pump, a disposable membrane oxygenator, and an arterial filter were used. Cooling and rewarming were done with a heat exchanger. The priming solution consisted of a crystalloid solution, mannitol (3 ml/kg), and fresh whole blood. The hematocrit value of the circulating volume averaged 25%. CPB was instituted after right atrial or bicaval and aortic cannulation with a perfusion index of 2.7 L/m². Heparinization was achieved with heparin sulfate (3 mg/kg). For vasodilation in the cooling period, all 16 children received a continuous infusion of SNP (0.6 to 2.9 μ g/kg per minute, average 1.7 μ g/kg per minute). Aortic crossclamping was done and cardioplegia was induced by a single intraaortic injection of cold (4° C) Bretschneider solution (30 ml/kg). In 12 children, the operation was performed under deep hypothermia (nasopharyngeal temperature averaging 16° C) and circulatory arrest. Low-flow CPB was conducted (perfusion index averaging 25% of the initial one) when circulatory arrest reached 60 minutes. The other four children were operated on with low-flow CPB (nasopharyngeal temperature averaging 26° C). In all children the lungs were reventilated when core temperature reached 30° C and a continuous infusion of SNP was reinstituted at the beginning of rewarming (0.6 to 2.9 μ g/kg per minute, average 1.6 μ g/kg per minute). Anticoagulation during CPB was monitored by determination of activated clotting time. Heparin was neutralized with protamine sulfate (6 mg/kg) injected into the aorta. Catecholamines were administered if necessary for weaning the patients from CPB.

Postoperative care. In the intensive care unit, sedation and analgesia were maintained with diazepam and morphine sulfate. The catecholamine and vasodilatory regimen was adapted to the particular hemodynamic situation.

Beyond standard determinations of blood gas and serum electrolyte values, laboratory tests including liver

Table I. Patient data

Patient no.	Age (mo)	Diagnosis	Surgical procedure	Complications	Outcome
1	6	Abnormal origin of LCA	LCA reimplantation	None	Discharged
2	12	VSDs, ASD	VSD + ASD closure	None	Discharged
3	2	LV tumor	Tumor extirpation	None	Discharged
4	12	Down, AVSD	Repair	None	Discharged
5	39	AoS, ASD	Repair	None	Discharged
6	54	TOF, ASD	Repair	None	Discharged
7	34	Down, VSD	VSD closure	PPS	Discharged
8	7	AVSD, MI	Repair	None	Discharged
9	11	Down, AVSD	Repair	None	Discharged
10	10	Down, AVSD	Repair	None	Discharged
11	16	D-TGA, ASD	Senning	None	Discharged
12	51	TOF, RVOT enlargement RV failure	Repair attempt		Died on operating table
13	11	TOF	Repair	None	Discharged
14	47	Single ventricle, TGA, CoA CoA repair + PAB	Fontan modified	MSOF	Died
15	66	DORV, L-TGA, hypoplastic LV, sub-PS	Fontan modified	MSOF	Died
16	83	TGA, VSD; Senning, VSD closure; sub-PS, VSD	VSD closure LVOT enlargement	AVB III PM	Discharged

LCA, Left coronary artery; VSD, ventricular septal defect; ASD, atrial septal defect; Down, Down syndrome; AVSD, atrioventricular septal defect; AoS, aortic stenosis; TOF, tetralogy of Fallot; MI, mitral insufficiency; TGA, transposition of the great arteries; RVOT, right ventricular outflow tract; RV, right ventricular; CoA, coarctation of the aorta; PAB, pulmonary artery banding; PS, pulmonary stenosis; PPS, postpericardiotomy syndrome; MSOF, multiple organ system failure; AVB, atrioventricular block; PM, pacemaker implantation.

Table II. Sample time schedule

1	Preoperative
2	After heparin administration
3	Ten minutes after onset of CPB
4	Lungs reventilated
5	Core temperature >30° C
6	After protamine administration
7	First postoperative day (9:00 AM)
8	Second postoperative day (9:00 AM)
9	Third postoperative day (9:00 AM)
10	Fifth postoperative day (9:00 AM)
11	Seventh postoperative day (9:00 AM)

enzyme and serum creatinine concentrations and coagulation times were routinely performed at least twice a day. Thiocyanate levels were monitored if indicated. Multiple system organ failure was diagnosed on the basis of our previous definition.¹³

Collection of samples. Venous blood was collected before and after the operation. During CPB blood was withdrawn from the arterial line of the circuit. For each sample time, 2 ml of blood taken in tubes containing ethylenediaminetetraacetic acid was necessary for analyzing complement factors and 0.3 ml for leukocyte count and differentiation. The sample time schedule is shown in Table II.

Determination of complement factors. The samples for complement analysis were immediately centrifuged for 3 minutes (3000 rpm) and the plasma was stored at -70° C until analysis.

C3 and C4 were determined by standard methods of turbidimetry²⁶ with the use of the immunoglobulin G fraction of a rabbit antihuman C3 or C4 antiserum with an

automated procedure on an RA 1000 turbidimeter (Technicon, Brussels, Belgium).

For C3d determination, the sample was incubated (volume/volume) with borate buffer containing 22% polyethylene glycol for 1 night at 4° C. After centrifugation to discard the C3 molecules, the supernatant was analyzed by turbidimetry as described for C3 and C4 with the following modifications: the sample diluent was supplemented with 20% distilled water to allow a reduction of polyethylene glycol to a maximum of 3.5% in the reaction mixture during immunoprecipitation.

C5a was determined by enzyme immunoassay (Enzygnost C5a, Behring, Hoechst, Brussels, Belgium). Normal range for healthy adults is 0.15 to 0.45 µg/L.

Elastase was measured with immunoactivation enzyme immunoassay (Merck, Darmstadt, Germany). Normal values for healthy adults are 22 ± 10 µg/L.

Leukocyte count and differentiation were determined by a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Inasmuch as several individual factors such as postoperative catecholamine administration possibly influence leukocyte count, circulating leukocytes were determined before CPB, during CPB, and after protamine administration, but not later. Leukocyte count was corrected for hemodilution according to the following formula: $L_c = L_s \times (H_t/H_{ts})$, where L_c = corrected leukocytes, L_s = sample leukocytes, H_t = control hematocrit, and H_{ts} = sample hematocrit.

The other values determined during CPB were not corrected for hemodilution, because we considered the measured concentrations to be biologically relevant.

In vitro experimentation

Experimental schedule. Sera taken from healthy blood donors from the "Centre Albert Hustin," Brussels, were

analyzed for complement hemolytic activity (CH_{50}) in the presence of increasing SNP concentrations (1 to 16 mmol/L; molecular weight of SNP = 298). CH_{50} also was measured after various periods of preliminary incubation (15, 30, 60, and 120 minutes) with a minimal active concentration of SNP (0.6 mmol/L). This was repeated after light degradation of SNP by ultraviolet exposure of the SNP solution for 20 minutes. The effect of SNP on zymosan-induced C3d generation and, finally, the effect of nitroglycerin and isosorbide dinitrate on CH_{50} were also studied.

CH_{50} . The alternate pathway was evaluated with a quantitative microassay, with rabbit erythrocytes used as target cells in the presence of ethyleneglycoltetraacetic acid- Mg^{++} -veronal gelatin dextrose buffer, in microtiter plates after six serial dilutions of serum samples. Hemolysis was measured with an MR 5000 microplate reader (Dynatech; van der Heyden, Brussels, Belgium) at 630 nm, according to our previously described methods.²⁷

The classic pathway was determined in the same manner as the alternate pathway with sheep erythrocytes sensitized with rabbit hemolysin in Ca^{++} - Mg^{++} -veronal gelatin dextrose buffer.

Chemicals. SNP (Nipride, Roche), nitroglycerin (Nysconitine, Bio-Therabel), and isosorbide dinitrate (Cédocard, Céocard, Byk Belga) solutions used were provided from the hospital pharmacy. Control solutions were prepared with ferrocyanide and potassium cyanide obtained from the laboratory (Merck, Belgolabo, Belgium) and the hospital pharmacy, respectively.

Statistical analysis. Results are expressed in the text and in the tables by the mean value $\pm$ standard deviation (SD) and in the figures by the mean $\pm$ standard error of the mean (SEM). For analysis of time-dependent variations of parameters within the same group, one-way analysis of variance for multiple comparisons with Scheffe's correction procedure was used. Comparisons of values between two groups in the clinical and experimental studies were done with the nonparametric Mann-Whitney U test or the paired Wilcoxon test. The paired t test was used for analysis of in vitro experiments on small series ($n = 5$). χ^2 Analysis was performed with Yates' correction for occasional low cell values. The data were computerized and analyzed with the SPSS software (SPSS Inc., Chicago, Ill.). All p values lower than 0.05 were considered significant.

Results

Clinical results. Clinical data of the 16 patients are shown in Table I. The results of the present study are described and briefly compared with the results of our previous work,¹³ which are presented as our own reference values.

Durations of CPB, aortic crossclamping, and circulatory arrest averaged 82 minutes (range 35 to 273 minutes), 63 minutes (range 29 to 124 minutes), and 48 minutes (range 6 to 59 minutes), respectively, and were identical to those of the previous group.

One child (patient 12) could not be weaned from extracorporeal circulation and died on the operating table because of severe right ventricular failure.

Multiple organ system failure developed in two children (patients 14 and 15) after modified Fontan procedures, and they died on the fifth and tenth postoperative days, respectively. Multiple organ system failure was less prevalent in the present study (2/16) than in the previous group (8/29), but this difference reached a significance level of only 0.1 ($1 - 2\alpha$; χ^2 with Yates' correction). Postpericardiotomy syndrome developed on the sixth postoperative day in one child. The other patients had no postoperative complications.

Hematocrit values. Hematocrit values fell from a preoperative value of $42\% \pm 11\%$ (mean $\pm$ SD) to $28\% \pm 5\%$ 10 minutes after induction of CPB. The hematocrit values remained stable during CPB and reached normal preoperative values from the immediate postoperative period on according to our previous observations.

Complement factors. All children had normal C3 values before the operation ($C3 = 121 \pm 25.5$ mg/dl) (mean $\pm$ SD). C3 concentrations fell to 56 ± 12.5 mg/dl after induction of CPB and remained stable up to the end of CPB, after protamine administration ($C3 = 67.8 \pm 12.7$ mg/dl). C3 values rose slowly from the first postoperative day on and reached normal values on the fifth postoperative day, in accordance with our previous results.

$C3d/C3$ was in the normal range before the operation in all children ($C3d/C3 = 0.75 \pm 0.36$) (mean $\pm$ SD). $C3d/C3$ rose from a value of 0.61 ± 0.34 10 minutes after institution of CPB (sample time 3) to 1.10 ± 0.45 after protamine administration (sample time 6). C3 conversion during CPB was significant ($p < 0.001$). $C3d/C3$ reached normal values from the first postoperative day on (sample time 7) ($C3d/C3 = 0.80 \pm 0.45$) and remained within the normal range during the first postoperative week. C3 conversion after protamine administration was significantly lower in the current patient group than in the previous one ($p = 0.02$). This was also true of the difference between $C3d/C3$ measured after protamine administration (sample time 6) and initiation of CPB (sample time 3) ($p = 0.02$). Fig. 1 compares the course of $C3d/C3$ in the current patient group with that in the previous one.

C4 was in the normal range before CPB in all children ($C4 = 19.8 \pm 8.35$ mg/dl) (mean $\pm$ SD). After institution of CPB (sample time 3), the C4 value fell to 9.53 ± 3.29 mg/dl. C4 remained stable during CPB up to the end of CPB, after protamine administration (sample time 6) ($C4 = 11.4 \pm 3.5$ mg/dl). C4 reached preoperative values on the third postoperative day and remained in this range (sam-

Fig. 1. Course of C3d/C3 before, during, and after CPB in patients treated with SNP (interrupted line) ($n = 16$ until sample time 5, $n = 15$ until sample time 10, and $n = 13$ at sample time 11), and in patients not treated with SNP (solid line) ($n = 29$ until sample time 7, $n = 27$ at sample time 8, $n = 26$ until sample time 10, and $n = 22$ at sample time 11). Results are expressed by the mean $\pm$ SEM. For sample time specification, see Table II. Asterisk indicates significant differences ($p < 0.05$) between the two groups (Mann-Whitney U test).

ple time 9) ($C4 = 15.1 \pm 7.3$ mg/dl). These results were identical to our previous results.

Compared with normal values in healthy adults ($C5a = 0.15$ to 0.45 $\mu\text{g/L}$), $C5a$ values were elevated in all children after protamine administration (sample time 6) ($C5a = 8.64 \pm 4.41$ $\mu\text{g/L}$) (mean $\pm$ SD). They were significantly lower, however, than values reported in our reference group ($C5a = 18 \pm 11.8$ $\mu\text{g/L}$) ($p < 0.005$).

Leukocytes and neutrophils. Leukocyte count fell from a preoperative value of $6.83 \pm 2.26 \times 10^9/\text{L}$ (mean $\pm$ SD) to a value of $3.3 \pm 0.9 \times 10^9/\text{L}$ 10 minutes after induction of CPB (sample time 3) ($p < 0.0001$). Leukocyte counts remained stable during CPB but increased at the end of the rewarming period (sample time 5) (leukocyte count = $3.7 \pm 3.3 \times 10^9/\text{L}$) ($p < 0.05$), increasing significantly after protamine administration (sample time 6) (leukocyte count = $11.3 \pm 5.9 \times 10^9/\text{L}$) ($p < 0.0001$). Leukocyte count after lung reventilation (sample time 4) and at the end of the rewarming period (sample time 5) was significantly lower than our reference values ($p < 0.02$ and $p < 0.05$, respectively).

Neutrophil counts fell from a preoperative value of $3.3 \pm 2.3 \times 10^9/\text{L}$ to $1.3 \pm 0.6 \times 10^9/\text{L}$ at sample

time 3 ($p < 0.0001$). Neutrophil counts remained stable during CPB but increased at the end of the rewarming period (sample time 5) (neutrophil count = $1.8 \pm 2.6 \times 10^9/\text{L}$) ($p < 0.05$), increasing significantly after protamine administration (sample time 6) (neutrophil count = $7.8 \pm 4.4 \times 10^9/\text{L}$) ($p < 0.0001$). Neutrophil count after lung reventilation (sample time 4) was significantly lower than our reference values ($p < 0.05$).

Leukocyte elastase. Leukocyte elastase rose in all children from 106 ± 59 $\mu\text{g/L}$ (mean $\pm$ SD) 10 minutes after institution of CPB (sample time 3) to 418 ± 244 $\mu\text{g/L}$ after protamine administration (sample time 6). These values were identical to our reference values.

The data of the current patient group are compared with our reference data in Table III.

In vitro experiments

Effect of SNP on CH_{50} (classic and alternate pathways) measured in the serum of seven healthy subjects (Fig. 2). In the presence of SNP, we observed in all sera a dose-dependent reduction of the complement hemolytic activity as measured by CH_{50} . The inhibitory effect of SNP on the alternate pathway occurred at a concentration that was three times less than the inhib-

itory concentration on the classic pathway (50% inhibitory concentration of SNP on CH_{50} , 4.2 ± 0.8 mmol/L (mean $\pm$ SD) and 14.0 ± 2.88 mmol/L for the alternate pathway and classic pathway, respectively).

Effect of photodegraded SNP on CH_{50} measured in the same sera. In comparison with the data shown before, the 50% inhibitory concentration of SNP on the available CH_{50} decreased to 0.2 ± 0.02 mmol/L (mean $\pm$ SD) for the alternate pathway ($p < 0.001$) and to 2.7 ± 0.3 mmol/L for the classic pathway ($p < 0.001$). Table IV summarizes the influence of non-degraded and photodegraded SNP on CH_{50} .

Effect of the preincubation time with photodegraded SNP (0.6 mmol/L) on CH_{50} (classic pathway) measured in the serum of six healthy subjects (Fig. 3). We observed in all sera a time-related decrease in CH_{50} according to the period of preincubation with a suboptimal concentration of SNP of 0.6 mmol/L.

Effect of SNP on zymosan-induced C3-conversion measured in the serum of five healthy subjects (Fig. 4). C3d averaged 4.3 ± 0.8 μ g/ml (mean $\pm$ SD) in the sera in which complement activation was blocked with ethylenediaminetetraacetic acid (10 mmol/L). In contrast, C3d increased to 22.8 ± 5.1 μ g/ml ($p < 0.001$) in control tubes in which sera were supplemented with magnesium-containing alternate pathway buffer, displaying an alternate pathway-induced C3 conversion caused by contact between serum and plastic surfaces. Addition of zymosan further increased C3d levels to 29.7 ± 5.0 μ g/ml ($p < 0.001$). The presence of SNP (8 mmol/L) for 1 hour at 37° C in sera incubated in plastic tubes significantly reduced C3d levels after zymosan addition, to a value of 17.0 ± 4.0 μ g/ml ($p < 0.0001$). This value also was significantly lower than the C3d value of the control sera ($p < 0.001$).

Effect of potassium ferrocyanide (2 mmol/L) and potassium cyanide (2 mmol/L) on CH_{50} measured in the serum of six healthy subjects. The SNP metabolites ferrocyanide and cyanide were not able to produce any inhibitory effect of SNP on CH_{50} (data not shown).

Comparative effect of nonphotodegraded SNP (1 mmol/L), nitroglycerin (1 mmol/L), and isosorbide dinitrate (1 mmol/L) on CH_{50} (alternate pathway) measured in the serum of six healthy subjects (Fig. 5). CH_{50} of the control sera was 74 ± 10 U/L (mean $\pm$ SD). In comparison, CH_{50} of the sera treated with different vasodilators was in all cases reduced and reached 50 ± 10 U/L for SNP ($p < 0.05$), 55 ± 11 U/L for nitroglycerin ($p < 0.05$), and 54 ± 11 U/L for isosorbide dinitrate ($p < 0.05$).

Table III. Comparative data analysis between patients treated with SNP and patients not treated with SNP

Variables	With SNP (n = 16)	Without SNP (n = 29)	p Value
Age (mo)	6-84, 29	3-213, 40	NS
(range, mean)			
CPB (min)	35-273, 82	31-152, 72	NS
(range, mean)			
ACC (min)	29-124, 63	7-105, 56	NS
(range, mean)			
CCA (min)	6-59, 46	6-60, 44	NS
(range, mean)	(n = 12)	(n = 18)	
MSOF (n)	2	8	0.1†
Deaths (n)	3	4	NS
C4 (mg/dL)			
T ₁	19.8 $\pm$ 8.3	21.6 $\pm$ 8.0	NS
T ₂	16.2 $\pm$ 6.8	18.2 $\pm$ 6.0	NS
T ₃	9.9 $\pm$ 3.3	9.5 $\pm$ 3.3	NS
T ₆	11.8 $\pm$ 4.3*	11.4 $\pm$ 3.5	NS
C3d/C3			
T ₁	0.75 $\pm$ 0.36	0.78 $\pm$ 0.33	NS
T ₂	0.65 $\pm$ 0.34	0.81 $\pm$ 0.46	NS
T ₃	0.61 $\pm$ 0.34	0.77 $\pm$ 0.37	NS
T ₆	1.11 $\pm$ 0.45*	1.52 $\pm$ 0.59	<0.05‡
C5a (μ g/L)			
T ₆	8.6 $\pm$ 4.4*	18.0 $\pm$ 11.8	<0.005‡
WBC ($\times 10^9$ /L)			
T ₁	6.8 $\pm$ 2.2	7.7 $\pm$ 2.5	NS
T ₂	7.2 $\pm$ 2.1	8.3 $\pm$ 2.1	NS
T ₃	3.3 $\pm$ 2.3	4.3 $\pm$ 5.7	NS
T ₄	2.8 $\pm$ 1.5	4.6 $\pm$ 2.3	<0.02‡
T ₅	3.7 $\pm$ 3.3	7.1 $\pm$ 6.1	<0.05‡
T ₆	11.3 $\pm$ 5.9*	14.5 $\pm$ 8.1	NS
PN ($\times 10^9$ /L)			
T ₁	3.3 $\pm$ 2.2	3.7 $\pm$ 1.5	NS
T ₂	3.4 $\pm$ 1.7	4.3 $\pm$ 0.67	NS
T ₃	1.3 $\pm$ 0.6	1.7 $\pm$ 0.9	NS
T ₄	1.0 $\pm$ 0.9	2.2 $\pm$ 1.5	<0.05‡
T ₅	1.8 $\pm$ 2.6	2.7 $\pm$ 1.8	NS
T ₆	7.8 $\pm$ 4.4*	8.7 $\pm$ 3.0	NS
Elastase (μ g/L)			
T ₃	106 $\pm$ 59	79 $\pm$ 37	NS
T ₆	418 $\pm$ 244*	355 $\pm$ 245	NS

SNP, sodium nitroprusside; CPB, duration of cardiopulmonary bypass; ACC, duration of aortic crossclamping; CCA, duration of cardiocirculatory arrest; MSOF, multiple system organ failure; WBC, white blood cells; PN, peripheral neutrophils; NS, not significant. For sample times 1, 2, 3, 4, 5, and 6, see Table II. Biologic results are expressed by the mean $\pm$ SD.

*n = 15.

† χ^2 analysis with Yates' correction.

‡Mann-Whitney U test.

Discussion

The clinical observations of this study suggest that SNP influences CPB-induced complement activation. These patients treated with SNP had significantly lower C3-conversion and C5a liberation than did patients belonging to our previous group, who were not treated with SNP.¹³ In the current study, as

Fig. 2. Effect of increasing concentrations of SNP on CH_{50} ($n = 7$). Results are expressed by the mean $\pm$ SEM. The 50% inhibitory concentration on CH_{50} for the classic (CP) and the alternate (AP) pathways are indicated by the projected lines.

Table IV. Effect of light-degradation on the 50% inhibitory concentration of SNP on the available CH_{50} , by the classic and the alternate pathways

	50% Inhibitory concentration on CH_{50} (mmol/L)		p Value
	Nonphotodegraded SNP	Photodegraded SNP	
Classic pathway	14.0 ± 2.88	2.7 ± 0.3	$<0.001^*$
Alternate pathway	4.2 ± 0.8	0.2 ± 0.02	$<0.001^*$

*Mann-Whitney U test.

in previous works,^{8, 11, 13} C4 levels were not significantly modified during CPB. The influence of SNP on the classic pathway of the complement cascade can therefore not be fully evaluated here. The leukocyte traffic during the rewarming period, but not after protamine administration, was affected by the treatment with SNP, inasmuch as the leukocyte count was significantly lower in the current patient group than in our reference group. Because leukocyte count during the rewarming period of CPB depends on the number of cells trapped in the lungs and also on the number of cells being demarginated at the same time owing to restoration of pulmonary flow,^{6, 28} the lower leukocyte count we measured in our patients treated with SNP could theoretically be explained in two opposite directions, namely higher intrapulmonary leukocyte trapping or lesser leukocyte mobilization. The net increase of leukocyte

mobilization after protamine administration leading to similar counts of circulating leukocytes and leukocyte elastase release in our patients treated with SNP and in those *not* treated with SNP suggests the participation of complement-independent factors of leukocyte stimulation, such as rewarming of the patient²⁸ and other chemotactic mediators liberated in response to CPB.²⁹

To verify the hypothesis of the complement-inhibiting effect of SNP and to investigate its mechanism of action, we conducted a series of in vitro experiments in our laboratory. These showed that SNP inhibits complement activation by both the classic and the alternate pathways, and that this inhibition is dose-dependent. Our experiments clearly show an inhibitory effect on the C3 to C9 steps of the complement cascade activated by the C3 convertase, independent of the path of initiation (classic or alternate), the effect of SNP on the alternate pathway occurring at a concentration that was three times less than the inhibitory concentration on the classic pathway. However, this difference must be interpreted with caution because it could be related to our experimental methods: activation of the alternate pathway measured with rabbit red cell hemolysis occurs with about ten times less efficiency than the classic pathway-induced hemolysis of sensitized sheep red cells, thus involving much less generation of C3 convertase. With similar sensitivity to inhibitory conditions, C3 convertase generation

Fig. 3. Effect of preincubation with photodegraded SNP (0.6 mmol/L) on CH_{50} by activation of the classic pathway (black circles). Results are expressed by the mean $\pm$ SEM. The open circle represents the control sera ($n = 6$).

or activity induced by the alternate pathway would be more easily inhibited than C3 convertase induced by the classic pathway. Because the zymosan-induced C3d generation is inhibited by SNP in buffer conditions allowing the alternate pathway to be activated exclusively, the C3 convertase of the alternate pathway must be one of the targets for this inhibitory effect. Specific site(s) of action of SNP on the complement cascade remain(s) to be elucidated in further studies.

Photodegradation of SNP and preincubation of the sera tested with SNP increased the inhibitory effect on the complement lytic activity in vitro, suggesting the role of a metabolite of SNP responsible for this effect. We excluded the SNP metabolites ferrocyanide and cyanide for this role. SNP containing a nitrogen in a low oxidative state releases nitric oxide by a nonenzymatic mechanism involving a simple one-electron reduction.³⁰ Light exposure of SNP, as well as a variety of reducing agents, induces nitric oxide release from SNP in this way.³¹ We suggest that the inhibitory effect of SNP on the complement lytic activity measured in vitro and on the C3 conversion and CSa liberation measured in vivo is mediated by nitric oxide. This contention is supported by the similar inhibitory effect on the complement hemolytic activity that we observed in vitro with nitroglycerin and isosorbide

dinitrate, which are other vasodilators releasing nitric oxide by mechanisms involving an enzymatic reaction.²⁵ To the best of our knowledge, this is the first report on the anticomplementary properties of nitric oxide demonstrated in vitro and in vivo, increasing the number of its previously described properties.³²⁻³⁴

In our clinical observations, complement activation during CPB and after protamine administration was reduced in patients treated with SNP but not totally abolished. It must be noted that the initial purpose of this clinical study was not to investigate the unexpected inhibitory effect of SNP on complement activation in patients undergoing cardiac operations. SNP was administered as a vasodilator, and its nonuniform dosage was adapted to each hemodynamic situation. The dose-dependency of the anticomplementary effect of SNP demonstrated in vitro could explain the attenuation but not the total inhibition of complement activation in our patients. In our in vitro experiments using hemolytic tests (CH_{50}) for the study of the inhibitory effect of SNP on complement activation, relatively large doses of SNP were necessary to show a positive effect. These concentrations (ranging from 0.2 to 1 mmol/L) correspond to about 60 to 300 mg/L, the 30- to 100-fold amount of SNP infused to a child receiving 2 μ g/kg per minute over 1 hour.

Fig. 4. C3d generation measured in plastic tubes containing ethylenediaminetetraacetic acid (EDTA) is significantly inhibited compared with C3d generation in plastic tubes where the alternate pathway is activated (*asterisk*) ($p < 0.001$). Zymosan-induced C3d conversion is significant compared with the control serum (*asterisk*) ($p < 0.001$). SNP significantly limits C3d generation caused by zymosan addition (*double asterisks*) ($p < 0.0001$), reducing C3d levels below the value measured in the control serum ($p < 0.001$) (paired t test). Results are expressed as mean $\pm$ SEM ($n = 5$).

Hemolytic tests leading necessarily to the presence of free hemoglobin inactivating nitric oxide³¹ introduce a negative bias in the study of the dose-response relationship of SNP as an anticomplementary substance. Efficient *in vivo* concentrations of SNP are probably far lower than those necessary to produce an equivalent effect *in vitro*, but at present the dose-response relationship of the anticomplementary effect of SNP would be difficult to establish. Indeed, liberation of nitric oxide from SNP *in vivo* is a dynamic and nonlinear process depending on the ubiquitous presence of several reducing (activating nitric oxide release) or oxidizing (inhibiting nitric oxide release) agents.³¹ Their concentrations vary from tissue to tissue and are necessarily different in the healthy subject and in the patient undergoing CPB; the latter is subjected to an inflammatory process associated with the extracellular liberation of substances able to locally inhibit or enhance the generation or the action of nitric oxide, as do, among others, free hemoglobin and the superoxide anion.^{31, 35} Furthermore, in our clinical model involving profound hypothermia, the temperature dependence of SNP reduction, which was clearly demonstrated *in vitro* in a range of temperatures

between 15° and 30° C,³¹ could certainly negatively influence nitric oxide release.

It was recently suggested in an animal model that SNP—by nitric oxide release—attenuates revascularization lung injury by inhibition of pulmonary neutrophil influx.³⁶ It is possible that attenuated C3 conversion and especially C5a liberation has been responsible for some of the SNP effects reported in that study. Moreover, endogenous nitric oxide has been demonstrated to inhibit leukocyte adhesion by a mechanism involving the regulation of the leukocyte adhesion molecules.³⁷ More recently, similar effects were shown with a novel nitric oxide donor.³⁸ Thus both exogenous and endogenous nitric oxide inhibit leukocyte adherence to the endothelium, and this effect is protective in experimental models involving ischemia and reperfusion and thus could be beneficial in the treatment of patients undergoing cardiac operations. Whether SNP had influenced perioperative leukocyte adhesion in our patients cannot be definitively assessed here, as discussed earlier.

The clinical relevance of attenuated complement activation by SNP could not be clearly demonstrated in the clinical part of our study because, although multiple system organ failure became less prevalent

Fig. 5. Comparative effect of nonphotodegraded SNP (1 mmol/L), nitroglycerin (NTG) (1 mmol/L), and isosorbide dinitrate (ISDN) (1 mmol/L) on CH₅₀ by the alternate pathway. Similar significant reduction of CH₅₀ is seen in all cases of sera treated with nitrovasodilators compared with the control sera ($p < 0.05$, Wilcoxon test) ($n = 6$). Results are expressed as mean $\pm$ SEM ($n = 6$).

in patients who received SNP than in the untreated patient group, the difference was not statistically significant. The indubitable demonstration of the clinical benefit of administering SNP during CPB needs a prospective randomized study on a much larger series, which we will not be able to conduct for ethical reasons. In our overall experience, multiple organ system failure became significantly less prevalent after SNP was introduced in our CPB protocol.³⁹ Thus we believe we cannot randomize to a non-SNP group. At present, SNP has numerous advantages over other intravenous nitric oxide donors available for clinical use.²⁵ Its pharmacologic and long known hemodynamic properties induce systemic and also pulmonary vasodilation.⁴⁰ SNP also was recently demonstrated to induce left ventricular relaxation and to improve left ventricular diastolic function in adults.⁴¹ Furthermore, in children undergoing cardiac operations it improved the postoperative cardiac index and did not cause related thiocyanate toxicity.^{42, 43} Last, SNP displays a low cost efficiency ratio.

In conclusion, we showed in vitro that SNP inhibits, most probably by nitric oxide release, complement system activation initiated through either the alternate or the classic pathway. In our clinical study, CPB-induced C3 conversion and C5a libera-

tion were less in patients receiving SNP for perioperative vasodilation than in patients who were not treated with SNP. The anticomplementary properties of SNP, combined with its hemodynamic effects, suggests that SNP is the vasodilator of choice for patients undergoing cardiac operations.

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