FIBRIN SEALANT, APROTININ, AND IMMUNE RESPONSE IN CHILDREN UNDERGOING OPERATIONS FOR CONGENITAL HEART DISEASE

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Objective: Most commercially available fibrin sealants contain aprotinin in doses of 1500 kallikrein inactivator units per milliliter. They are used in many operative disciplines. An elevated risk of hypersensitivity reactions exists at reexposure to aprotinin. Our aim was to examine the immunogenic potency of aprotinin as a fibrin sealant content. **Methods:** We investigated 49 children with operatively treated congenital heart disease. All patients received aprotinin only topically as contained in fibrin sealant. Serum samples were drawn preoperatively, 1 week, 2 weeks, 6 weeks, and approximately 1 year after operation. They were analyzed for aprotinin-specific immunoglobulin G antibodies with a standard enzyme-linked immunosorbent assay and a fluorescence enzyme immunoassay for aprotinin-specific immunoglobulin E antibodies. **Results:** At 1 week, 2 weeks, 6 weeks, and 1 year, we found prevalences of 8% (2 of 26), 8% (2 of 24), 6% (3 of 49), and 0% for aprotinin-specific Immunoglobulin E, and for aprotinin-specific immunoglobulin G 8% (2 of 26), 17% (4 of 24), 39% (19 of 49), and 12% (5 of 41). The doses of aprotinin given did not differ significantly in antibody-negative and antibody-positive patients; no significant factors could predict the immune response. **Conclusions:** Our findings show the existence of a subgroup of patients who had aprotinin-specific antibodies develop after topical aprotinin application. Any use of aprotinin must be carefully documented. If aprotinin use is planned in patients who previously underwent a surgical procedure, preexposure to aprotinin in any form must be sought to avoid unexpected anaphylactic reactions. The necessity itself and alternatives for aprotinin as a stabilizing agent in fibrin sealants merit consideration. (J Thorac Cardiovasc Surg 1998;115:883-9)

Aprotinin is a polyvalent proteinase inhibitor from bovine organs. Since the 1980s, it has been used in high doses of several million kallikrein inactivator units (KIU) for its beneficial effect on perioperative blood loss and transfusion requirements in cardiac operations.^{1,2} It is also used in small concentrations of about 1500 KIU/ml in commercially available fibrin sealants because it significantly extends the

resorption of the fibrin clot in the physiologic environment.³ These biologic sealants are increasingly used in many surgical disciplines.^{4,5} In Europe and several Asian countries these products have been commercially available since the early 1980s. The U. S. Food and Drug Administration is presently considering approval of a commercially available fibrin sealant in the United States.

As a foreign protein composed of 58 amino acid residues with a molecular weight of 6512 daltons, aprotinin is capable of triggering hypersensitivity reactions, particularly after systemic reexposure.⁶ The frequency of severe allergic and pseudoallergic reactions after systemic reexposure is estimated to be between 0.1% and 5.8%.^{7,8} There are also reports of fatal adverse reactions.^{9,10}

Antigen-specific antibodies play a decisive role in adverse reactions of sensitized patients at reexposure to antigens: antibodies of the immunoglobulin (Ig) E class are responsible for "true" anaphylactic

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Table I. Distribution of patients according to age and lesions

	Age (mo)			Total
	0 ≤ 1	1 ≤ 12	>12	
Acyanotic <i>n</i> (% of total)	3 (6.1)	8 (16.3)	19 (38.8)	30 (61.2)
Cyanotic <i>n</i> (% of total)	4 (8.2)	7 (14.3)	8 (16.3)	19 (38.8)

reactions¹¹; antibodies of the IgG class may trigger anaphylactoid reactions through the involvement of the complement system.^{12, 13} Such anaphylactoid reactions cannot be clinically distinguished from true anaphylactic reactions.

The incidence of specific IgE antibodies within 8 weeks after a course of several infusions of aprotinin for acute pancreatitis was estimated to be about 32% (95% confidence interval: 19% to 48%) (14 of 44) of patients.¹⁴ The prevalence of aprotinin-specific IgG antibodies was estimated to be about 40% to 50% of adult patients exposed to one first systemic high dose of aprotinin during cardiac operation half a year earlier.^{15, 16}

Recent articles discussed the immunogenic effect of low aprotinin doses such as those contained in commercially available fibrin sealants.^{10, 17} There are two case reports of severe unexpected reactions accompanied by circulatory collapse in which the aprotinin component of fibrin sealant could be identified as the causative agent.^{18, 19}

The immunogenic effect of aprotinin contained in commercially available fibrin sealants has not yet been examined in a clinical study. No data are available for adults or children about the prevalence and time course of aprotinin-specific antibodies after a first and single exposure to commercially available fibrin sealant.

Patients and methods

Patients Forty-nine children (29 male, 20 female) undergoing cardiac operations between April 1995 and November 1995 and receiving fibrin sealant for the first time (median dose: 2.0 ml, 7090 KIU/m² body surface area) were the subjects of the study. This represents 38% of all children (49 of 130) operated in our division during that time frame. The median age of the patients was 15.2 months (50% range: 4 months to 3¹/₁₂ years). The corrective operations comprised the whole spectrum of congenital heart disease from direct atrial septal defect closure to complex reconstructions and definitive surgery in univentricular hearts.

Table I shows the distribution of the patients according to age and lesions.

Forty-three patients (88%) were operated on with the use of cardiopulmonary bypass, 17 of them with deep hypothermic circulatory arrest (17 of 43, 40%); there were six closed procedures, such as palliative shunts or coarctectomy.

Sixteen of the 49 children underwent cardiac reoperations. Most of the primary operations consisted of valvotomy for critical aortic stenosis or shunt procedures in cyanotic newborns with complex disease. Neither application of fibrin sealant nor infusion of aprotinin had been documented for these primary operations.

Methods All parents were informed about the aim of the study and consent was given in writing. The decision to use fibrin sealant was only made intraoperatively because of operative necessity. All patients received 0.7 to 12 ml of a commercially available fibrin sealant (Tissucol Duo S, Immuno, Vienna, Austria), consisting of two components: a plasma fraction (containing 70 to 110 mg fibrinogen, 2 to 9 mg plasma fibronectin, 10 to 50 U coagulation factor VIII, 0.02 to 0.08 mg plasminogen (all human), and 3000 KIU (i.e., 0.42 mg) bovine aprotinin per 1 ml and a thrombin fraction (containing 500 U human thrombin and 5.88 mg calcium chloride per 1 ml).

In all patients, aprotinin was applied only topically in the mediastinum, but not systemically. Children with previous contact with aprotinin, as ascertained from their history, documentation in the chart, and a preoperative serum sample positive for aprotinin-specific antibodies, were excluded from the study. To avoid allergic reactions or immunologic interference, we purposely did not perform skin testing.

A complete history of allergies was taken, including the patient's own and their family's history of atopic diathesis. The questionnaire also covered the patient's complete medical history emphasizing immunologically important factors such as susceptibility to infections (children having had two or more infections during the 3 months preceding the operation were considered as being susceptible to infections), the patient's history of long-term drug therapy, and vaccinations.

Serum samples were obtained preoperatively, 1 week, 2 weeks, 6 weeks, and approximately 1 year postoperatively. They were centrifuged for 15 minutes at 10° C at 1200 G, and stored at -20° C until the in vitro tests were performed.

One week after the operation we obtained the sera of 26 patients and after 2 weeks those of 24 patients. At a routine examination 6 weeks postoperatively we obtained the sera of all 49 patients. After 1 year, we obtained the sera of 41 children: two others had died and six were lost to follow-up or refused further blood sampling. Preoperative sera had been stored from all 49 patients. No child underwent reoperations during the observation time.

Analyses. Analyses included assays for the detection of total serum IgE and IgG levels and aprotinin-specific IgG and IgE antibodies with the techniques described below:

UniCAP System. Total serum IgE and aprotinin-specific IgE antibodies were quantified with the automatic Pharmacia UniCAP device (Pharmacia & Upjohn, Uppsala, Sweden) using a fluorescence enzyme immunoassay (FEIA). Quantitative expression of results was obtained by using standard reagents calibrated against World

Table II. RAST/FEIA classes

	RAST/FEIA classes						
	<i>0</i>	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>	<i>V</i>	<i>VI</i>
Aprotinin-specific IgE (kU _A /L)	<0.35	≥0.35	≥0.7	≥3.5	≥17.5	≥50	≥100
Antibody level	Absent or undetectable	Low	Moderate	High	Very high	Very high	Very high

Health Organization reference preparations for human IgE (75/502).

Total serum IgE levels greater than 120 kilo-Units per liter were considered elevated.

Concentrations of aprotinin-specific IgE are expressed in kilo-Units of allergen-specific IgE per liter (kU_A/L) and evaluated according to the conventional radioallergosorbent classes: values of 0.35 kU_A/L and greater represent a progressive increase in the relative concentration of allergen-specific antibodies (Table II). Values less than 0.35 kU_A/L represent absent or undetectable levels of allergen-specific antibodies.

Enzyme-linked immunosorbent assay Ninety-six-Well-Immuno-Plates (Maxisorp, Nunc GmbH, Wiesbaden, Germany) were coated with an antigen solution containing 5 µg aprotinin (36 KIU Trasylol, Bayer, Leverkusen, Germany) per milliliter Tris-buffered saline solution (Sigma Chemical Company, St. Louis, Mo.). The plates were incubated overnight at 4° C and washed five times with 300 µl TTBS (Tris-buffered saline solution plus 0.05% Tween 20; Merck-Schuchardt, Hohenbrunn, Germany) per well. This washing procedure was repeated after each subsequent step. The wells were blocked with 300 µl blocking reagent (Blocking reagent for enzyme-linked immunosorbent assay [ELISA], Boehringer Mannheim, Germany) per well and incubated for 60 minutes at room temperature. Then 100 µl samples or control serum diluted 1:100 in 0.9% NaCl were added per well. Serum with known concentrations of aprotinin-specific antibodies (IgG: 114 mg/L, RAST-FEIA), obtained from a patient who had had an anaphylactic reaction to aprotinin as described elsewhere,²⁰ served as a positive reference. Six dilutions from 1:25 to 1:1600 were used to establish the reference curve. Negative controls were obtained from a pool of 20 donors without prior contact with aprotinin. NaCl, 0.9%, was used as a blank. After 120 minutes of incubation at room temperature, 100 µl alkaline phosphatase-conjugated goat anti-human γ-chain specific IgG (Sigma) were added per well. The antibody-enzyme-conjugate was used in a dilution of 1:10,000 in 1% bovine serum albumin (Sigma) and TTBS. The plates were incubated again for 90 minutes at room temperature, followed by the addition of 100 µl *p*-nitrophenyl phosphate solution (Sigma Fast pNPP Tablets, soluble alkaline phosphatase substrate, Sigma) per well, and the absorbance was read at 405 nm using an ELISA-Reader (Dynatech Lab, Denkendorf, Germany). Because the semilogarithmic reference curve was linear for aprotinin-specific IgG concentrations of 10 to 400 mg/L and the mean plus three standard deviations of 20 negative controls was less than 10 mg/L, serum concentrations higher

than 10 mg/L of aprotinin-specific IgG were considered positive for aprotinin-specific IgG.

Western blotting. A solution of reduced aprotinin (Trasylol, heated for 5 minutes at 95° C with addition of 1 grain of dithiothreitol) was concentrated in a polyacrylamide gel by means of electrophoresis and subsequently transferred to a nitrocellulose membrane (Probind 45, Pharmacia, Uppsala, Sweden) with an electroblotting technique with the appropriate material (Mini-Protean II Multiscreen, Bio-Rad, Munich, Germany, and Multiphor II, Pharmacia-LKB, Bromma, Sweden). These membranes were then incubated with the test specimen and the control specimen in the appropriate test system (Mini-Protean II Multiscreen). Aprotinin-specific IgG antibodies contained in the 1:100 in TBS (Loewe Biochemica, Otterfing, Germany) diluted sera bind to the nitrocellulose, on which they are visualized by an enzymatic staining reaction (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Sigma Fast BCIP/NBT Tablets, Sigma) after incubation with a secondary enzyme-labeled anti-human IgG-antibody diluted 1:30,000 in TBS (goat anti-human IgG-AP, γ-chain specific, Sigma). The test bonds were compared with those of the control sera and divided by sight into the categories "positive," "questionably positive," and "negative."

Nephelometry. The evaluation of total serum IgG concentrations was performed in the clinic's routine laboratory with a Behring Nephelometer (Behringwerke AG, Marburg, Germany) according to the operating instructions and with the appropriate reagents purchased from Behring.

Statistical analysis. Values that are not normally distributed are shown as medians with 50% range; normally distributed values are portrayed as means with standard deviation. Percentages are presented with 95% confidence intervals for the probability based on the binomial distribution in brackets. The influence of antigen dose, age, body surface area, and preexisting allergies on the prevalence of antibody formation was tested with a multiple logistic regression analysis using the statistical package JMP.

Results

Aprotinin-specific antibodies. The number of patients positive for aprotinin-specific antibodies at the respective dates of blood-sampling (i.e., prevalence) is shown in Fig. 1.

The incidence of aprotinin-specific IgE antibodies within the observation time was 14.3% (5% to 24%),

Fig. 1. Prevalence of aprotinin-specific antibodies.

Table III. Course of aprotinin-specific IgE levels in the seven IgE-positive patients

Patients	Age (mo)	Fibrin sealant (ml)	Aprotinin dose (KIU/m ² BSA)	Aprotinin-specific IgE levels (kU _A /L, RAST-class)				
				Preoperatively	Postoperatively at			
					1 wk	2 wk	6 wk	1 yr
1	4	2	9375	<0.35	<0.35	<0.35	0.63 (I)	<0.35
2	8	4	17,647	<0.35	<0.35	<0.35	0.38 (I)	<0.35
3	44	3	6818	<0.35	<0.35	0.72 (II)	<0.35	<0.35
4	49	3	7031	<0.35	0.47 (I)	No serum	<0.35	<0.35
5	49	6	14,031	<0.35	6.08 (III)	No serum	<0.35	<0.35
6	146	2	6430	<0.35	No serum	No serum	2.6 (II)	<0.35
7	47	5	11,719	<0.35	No serum	0.41 (I)	<0.35	<0.35

KIU/m² BSA, Kallikrein inhibiting units per square meter body surface area; BSA, body surface area.

that is, 7 of 49 children. The course of aprotinin-specific IgE levels in the seven IgE-positive patients is shown in Table III. The children who were positive for aprotinin-specific IgG at 1 and 2 weeks remained positive through at least 6 weeks. Four of the five children who were positive at 1 year had already been positive at 6 weeks. Six children were positive for both IgG and IgE; this represents 32% (13% to 57%) (i.e., 6 of the 19 IgG-positive children).

Total antibody concentrations. Six children presented with elevated total serum IgE levels. Two of these presented with both aprotinin-specific IgE and IgG. Measurements of total serum IgG were all within the normal range for the respective age except for four children with too-low-for-age total

IgG concentrations. Only one of them presented with aprotinin-specific IgG.

Western blot versus ELISA. When we used the Western blot technique for the 49 sera sampled 6 weeks postoperatively, we detected 12 positive (24%), 13 questionably positive (27%), and 24 (49%) negative sera. The results of ELISA and Western blotting were comparable for very high concentrations and for negative sera. Low concentrations measured by ELISA mostly produced a "questionably positive" with the Western blot technique.

Clinical and pharmacologic influences. In Table IV we show antibody formation together with the factors aprotinin dosage, age, and body surface area.

Table IV. Clinical and biologic data

<i>n</i>	<i>Whole group 49</i>	<i>as-IgE positive 7</i>	<i>as-IgE negative 42</i>	<i>as-IgG positive 19</i>	<i>as-IgG negative 30</i>
Fibrin sealant					
Median (ml)	2	3	2	3	2
50% range	1-3	3-4.5	1-2	2-4.5	1-2
Aprotinin dosage					
Median (KIU/m ² BSA)	7090	9375	6410	8900	6440
50% range	4290-12,500	6930-12,900	3500-12,260	5340-13,850	4290-10,350
Significance			ns		ns
Age					
Median (mo)	15.2	46.6	14.0	44.0	8.0
50% range	4-49	26-49	4-44	10-62	3-30
Significance			ns		ns
Body surface area (BSA)					
Median (m ²)	0.41	0.64	0.4	0.58	0.35
50% range	0.32-0.64	0.49-0.65	0.31-0.62	0.39-0.68	0.27-0.53
Significance			ns		ns

as, Aprotinin-specific; ns, not significant.

Formation of aprotinin-specific antibodies was independent of sex, atopic diathesis, susceptibility to infections, and postoperative long-term medication.

Comments

In this prospective study we could clearly document that there is a systemic immune response to small topical doses of aprotinin. Our data show an initial rise of the frequency of antibody detection within at least 6 weeks, followed by a distinct decline after 1 year.

As has already been shown for high-dose systemic application, small topical aprotinin doses may also induce an aprotinin-specific IgE response. Yanagihara and Shida¹⁴ examined patients who received a course of several high-dose infusions of aprotinin for acute pancreatitis. They found aprotinin-specific IgE in 24% (11% to 39%) at 2 weeks (9 of 37) and 30% (12% to 54%) at 8 weeks (6 of 20).

The prevalence of aprotinin-specific IgG response after topical application is comparable to the prevalence reported after high-dose systemic aprotinin administration. Ruskowski and colleagues¹⁵ for a group of more than 250 patients with first time systemic high-dose aprotinin application reported a prevalence of aprotinin-specific IgG antibodies of 16.7% after 4 to 6 weeks and of 46.4% after 6 to 7 months. In a similar study Pfannschmidt and colleagues¹⁶ reported 3.6% aprotinin-specific IgG-positive after 3 to 4 weeks (2 of 56) and 46.6% after 6 months (26 of 56). In contrast to our findings, both studies revealed no aprotinin-specific IgE. This

might be due to procedural differences: they used immunoblotting and ELISA and we used FEIA. Another reason may be that they examined adult patients.

IgE antibodies sensitize mast cells and basophil granulocytes by binding to specific receptors on their surface. When an antigen cross-links two surface-bound IgEs, these cells release mediators that cause the clinical symptoms of anaphylaxis.¹¹ As for aprotinin, there are two documented cases of anaphylactic reactions with preformed aprotinin-specific IgE antibodies.^{20, 21}

The role of antigen-specific IgG for anaphylactic and anaphylactoid reactions is the subject of controversy. Its significance may depend on the distribution of its subclasses¹³ or the ratio of IgE/IgG.²² It has been shown that specific IgG subclasses may also trigger severe or even fatal reactions.^{13, 23} One explanation may be the so-called immune complex anaphylaxis in which anaphylactic mediators are released by activation of the complement system through the classical and/or the alternate pathway.¹² Another explanation may be the "homocytotropic activity" of the IgG₄ subclass (i.e., its capability of binding to mast cells and thereby acting as an anaphylactic antibody).^{22, 23}

In the literature from 1963 to 1997 we found 95 cases* of adult patients with anaphylactic or anaphy-

* Thirty-eight cases are presented in references 6, 8-10, 14, 17-21; the remainder cannot be cited for reasons of space because these 57 cases are reported in 33 other publications.

lactoid reactions to aprotinin. Preexposure to aprotinin was documented in 63 of these cases. From the clinical data given it was possible to infer the time interval between preexposure and reexposure in 40 cases: the large majority of 27 occurred within 1 week to 3 months, only 13 occurred within a long period of 4 months to 4 years after preexposure. The peak of specific IgE and IgG antibody detection in our patients is in accordance with the distribution of reports over time observed in the literature.

The presence of allergen-specific antibodies does not necessarily lead to anaphylactic reactions²⁴; they represent a risk factor for allergic reactions. In the literature we found reports of 27 patients with anaphylactic reactions to aprotinin whose sera had been examined for aprotinin-specific IgE, 17 of them were positive postreactively.^{9, 14, 15, 17, 18, 20, 21} Therefore especially in patients with a positive aprotinin-specific antibody screening test the indication for fibrin sealant containing aprotinin or systemic aprotinin should be reconsidered. If the indication is mandatory, we recommend precautionary measures such as pulse oximetry, monitoring of the arterial blood pressure, an intravenous line, and an antiallergic pretreatment at reexposure.

When we looked for predictive parameters for the aptitude to form aprotinin-specific antibodies, our data did not show any. This negative result can be attributed to the number of patients being too small for reliable epidemiologic calculations. Large clinical studies on anesthetic drugs revealed atopy and female gender as risk factors for IgE-mediated reactions.²⁵

The benefit of fibrin sealant for local hemostasis has been shown in extensive clinical studies.^{26, 27} Although aprotinin extends significantly the time of resorption of the fibrin clot from 3 to more than 28 days, it has not yet been investigated in a clinical study if the aprotinin component is indispensable for that indication. If a stable fibrin clot is necessary for more than a few days, possible alternatives such as ϵ -aminocaproic acid, which stabilizes the clot for 7 days,³ or an augmentation of the factor XIII component should be considered.

Contrary to Pfannschmidt and colleagues' findings¹⁶ that the Western blotting method was more sensitive for IgG detection than ELISA, we had clearer results using ELISA. This may be due to procedural differences.

In the absence of published data about the close time course of antibody formation, we chose four fixed intervals for blood sampling after a single dose

of aprotinin, considering the organizational practicality and the stress for the children and for their parents. Therefore the frequency of antibody formation in the period between 6 weeks and 1 year after exposure remains uncertain.

A limitation of our study is the small number of samples after 1 and 2 weeks because many children had been transferred early to other hospitals. After 6 weeks, all children were reexamined in the division of pediatric cardiology at our hospital. The parents' compliance after 1 year was excellent. All parents were informed in writing about the results and implications.

Conclusions

The existence of patients forming aprotinin-specific antibodies caused by a previous topical aprotinin application has been proven. There was an increase of aprotinin-specific antibody-positive patients during the first 6 weeks after application of fibrin sealant. Most anaphylactic and anaphylactoid reactions to aprotinin reported in the literature occurred in a comparable time lapse between preexposure and reexposure.

As a practical consequence, any use of aprotinin, even in fibrin sealant, must be carefully documented. If aprotinin use is planned in a patient who has undergone a previous operative procedure, especially during the past several months, previous exposure to aprotinin in any form must be sought.

For patients with recent aprotinin exposure, the indication for aprotinin readministration should be carefully reconsidered. If the expected benefit of fibrin sealant application in these patients outweighs the possible risk of anaphylaxis, we recommend stringent precautionary measures at reexposure.

The usefulness of aprotinin-specific antibody screening remains the subject of further research. The need for aprotinin in fibrin sealants and the use of possible alternatives as stabilizing agents merits consideration.

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