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V. Fineschi · R. Gambassi · M. Gherardi · E. Turillazzi

The diagnosis of amniotic fluid embolism: an immunohistochemical study for the quantification of pulmonary mast cell tryptase

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Abstract Recent clinical articles have suggested that amniotic fluid embolism (AFE) may be the result of anaphylactic reactions to fetal antigens and that the major part of this clinical syndrome is the result of mast cell degranulation and of the release of histamine, tryptase and other mediators. Tryptase, a neutral protease, is known to be the dominant protein component of the secretory granules of T and TC mast cells. In this paper we have examined the presence and the pulmonary distribution of mast cell tryptase utilizing specific immunohistochemical studies and morphometric evaluation in six cases of fatal amniotic fluid embolism compared to six subjects who died following anaphylactic shock and two control groups (five and six cases respectively) of traumatic death. The results demonstrate a numerical increase of pulmonary mast cells in the subjects who died of AFE (average cell number 54.095) with values corresponding to those encountered in cases of death due to anaphylactic shock (average cell number 51.378) compared with that of the traumatic control groups (average cell number 24.477 and 9.995 respectively). These results can shed light on additional criteria for the diagnosis of amniotic fluid embolism.

Key words Amniotic fluid embolism · Mast cell tryptase · Immunohistochemistry

Introduction

Amniotic fluid embolism (AFE) is an uncommon clinical occurrence with a reported case fatality rate of 61% [1] which has decreased over the past 10 years (86%) [2]. The thorough clinical observation that AFE has undergone in recent years has produced the conclusion that "there is no single clinical or laboratory finding which by itself can either diagnose or exclude AFE syndrome. The diagnosis must be made on the basis of clinical presentation and supportive laboratory studies"[3].

Recent clinical articles suggest that AFE may be the result of anaphylactic reactions to fetal antigens and that much of this clinical syndrome results from mast cell degranulation and the release of histamine, tryptase and other mediators [4].

The specificity of tryptase as a marker for human mast cells has been shown in previous studies by immunohistochemical techniques [5] but to date the effective participation of mast cells in deaths attributed to AFE has not yet been verified.

In this study we have investigated the presence and pulmonary distribution of mast cells utilizing specific immunohistochemical investigations for tryptase and morphometric evaluation in those cases of females who died during delivery or immediately after where the clinical and histopathological criteria led to a diagnosis of AFE.

Materials and methods

Study population

For this study 23 cases were selected from the autopsy records of the Department of Forensic Sciences, University of Siena, between 1985–1996. Among these, six cases of maternal death (Group A) meet the requirements on which a diagnosis of AFE can be made according to the 1988 national registry for amniotic fluid embolism established in the United States (Table 1) [1].

The AFE diagnosis in Group A was retrospectively supported by histopathological findings corresponding to the typical histomorphological pattern and defined, utilizing the necessary histological and immunohistochemical techniques, by the presence of amniotic fluid components (fetal squames, lanugo, vernix caseosa, fats, mucin) in the maternal pulmonary vessels [6].

In each case the main clinical symptoms were manifested after a completely uneventful course of pregnancy in a sudden and unexpected manner during labour (4 cases) or within 30 min postpartum (2 cases). Table 2 supplies the demographical data relative to the mother and fetus.

V. Fineschi (✉) · M. Gherardi · E. Turillazzi
Department of Forensic Sciences, University of Siena,
Policlinico Le Scotte, Viale Bracci, I-53100 Siena, Italy

R. Gambassi
Department of Educational Sciences, University of Siena,
Villa Godiola, Località San Fabiano, I-52100 Arezzo, Italy

Table 1 Amniotic fluid embolism group entry criteria

1. Acute hypotension or cardiac arrest
2. Acute hypoxia, defined as dyspnea, cyanosis, or respiratory arrest
3. Coagulopathy, defined as laboratory evidence of intravascular consumption or fibrinolysis or severe hemorrhage in the absence of other explanations
4. Onset during labor, cesarean section or dilatation and evacuation or within 30 minutes postpartum
5. Absence of any other significant confounding condition or potential explanation for the signs and symptoms observed

Table 2 Demographic data for patients with amniotic fluid embolism

Maternal age (y)	29.16 (range 22–39)
Gravidity	2.8 (range 1–6)
Parity	range 0–5
Maternal weight (kg)	mean 71 ± 10
Gestational age (weeks)	38.83 (range 36–40)
Birth weight (g)	3846.66 (range 2690–4650)

Table 3 Macroscopic and histological findings in AFE fatal cases

Organ	Weight (average)	Macroscopic findings	Histological findings
Lungs	680 g	Pulmonary edema and congestion, focal atelectasia. Subpleural blood extravasation (DIC)	Fetal squames and mucin in pulmonary vessels (demonstrated by special stains), acute emphysema, pulmonary edema. Subpleural haemorrhaging; microthrombotic formations in septal capillaries (DIC)
Uterus	Unremarkable	Surgical incisions (3 cases), lacerations of the lower uterine segment (one case). Submucosal blood extravasation (DIC)	Unremarkable
Other organs	Unremarkable	No pathologic findings. Extensive soft tissue blood accumulation around operative and intravascular catheter insertion sites (DIC)	Changes consistent with prolonged hypotension and anoxia. Microthrombotic formations in small cerebral and renal vessels (DIC)

Of the patients three died within an interval of 1–3 h from the onset of the clinical symptoms while the other three survived for a maximum period of 12 h. In two of the latter cases, the subjects developed a disseminated intravascular coagulation (DIC) documented both by clinical and laboratory tests and later by macroscopical and histological findings (Table 3).

We have examined histological samples from three control groups:

Group B: females (n = 6) where the cause of death was documented as anaphylactic shock

Group C: females (n = 5) where death occurred during pregnancy (gestational age varied from the 32nd to the 38th week) following traumatic events. These cases were selected on the bases of the medical history free of signs of hypersensitivity and on absence of medication with corticosteroids

Group D: females (n = 6) where the cause of death was clearly attributable to trauma

In all groups, the lapse of time between the pathogenic noxa or traumatic event and death varied from 1–16 h. The autopsy was performed within 36 h of death in all cases and a bilateral sample of pulmonary tissue was collected according to standard criteria (a sample of each lobe and a perihilar sample). All specimens were fixed in formalin and embedded in paraffin. In each case a routine H&E stain was employed. In those cases where death occurred during pregnancy we utilized more specific histochemical techniques aimed at selectively demonstrating the single amniotic fluid elements in the pulmonary vessels such as pinacyanole chloride, Alcian blue and Attwood's modified stains [6]. In each of the selected cases the mast cell population was estimated by immunohistochemical staining, utilizing the anti-tryptase antibody as a mast cell-specific marker [7].

Immunohistochemistry

The immunohistochemical technique was performed using the avidin-biotin-complex method (ABC-method) [8] on 5 μ thick paraffin sections. Enzyme pretreatment with proteinase K (0.01%; 37 °C) was necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies. The primary anti-tryptase antibody (DAKO) was applied in a 1:100 ratio and incubated overnight at 4 °C. The positive reaction was visualized by 3-diamino-9 ethyl-carbazole (AEC) (Sigma). The sections were counterstained with Mayer's hematoxylin and mounted in Aquatex (Merck) and examined under a light microscope.

Quantitative analysis

All specimens were examined by two investigators without any previous knowledge regarding the cause of death. In each histological section 10 observations in different fields per slide equivalent to 70 observations for each single case were performed. The positive mast cell count to the reaction of tryptase was made at a 6.3 × magnification, using a light microscope (Leitz Aristoplan) coupled to a high resolution color Sony 3CCD video camera. The video image was inputted to a computer programmed for quantitative morphometry (Quantimet 500 Plus, Leica, Cambridge). In each case a pulmonary area equivalent to 117.7 mm^2 was analyzed.

Statistical evaluation

The data were processed by both the descriptive statistics to obtain averages and standard deviations per subject and Group (A–D), together with more detailed methods of variance analysis (SPSS-WIN package utilized). Through opportune tests like the Bonferroni least-significant difference (modified LSD) and the Student-Newman-Kuels (SNK) methods it is possible to compare and test the distribution of the number of mast cells per field of view subdivided into single isolated groups. This enables the identification of the differences among the various groups, further confirmed by the application of non-parametric tests (U of Mann-Whitney and KS of Kolmogorov-Smirnov) regarding the distribution of the sums per sample and subject.

Results

With the staining procedure utilized in this study, mast cells could be easily identified and counted (Fig. 1). The results

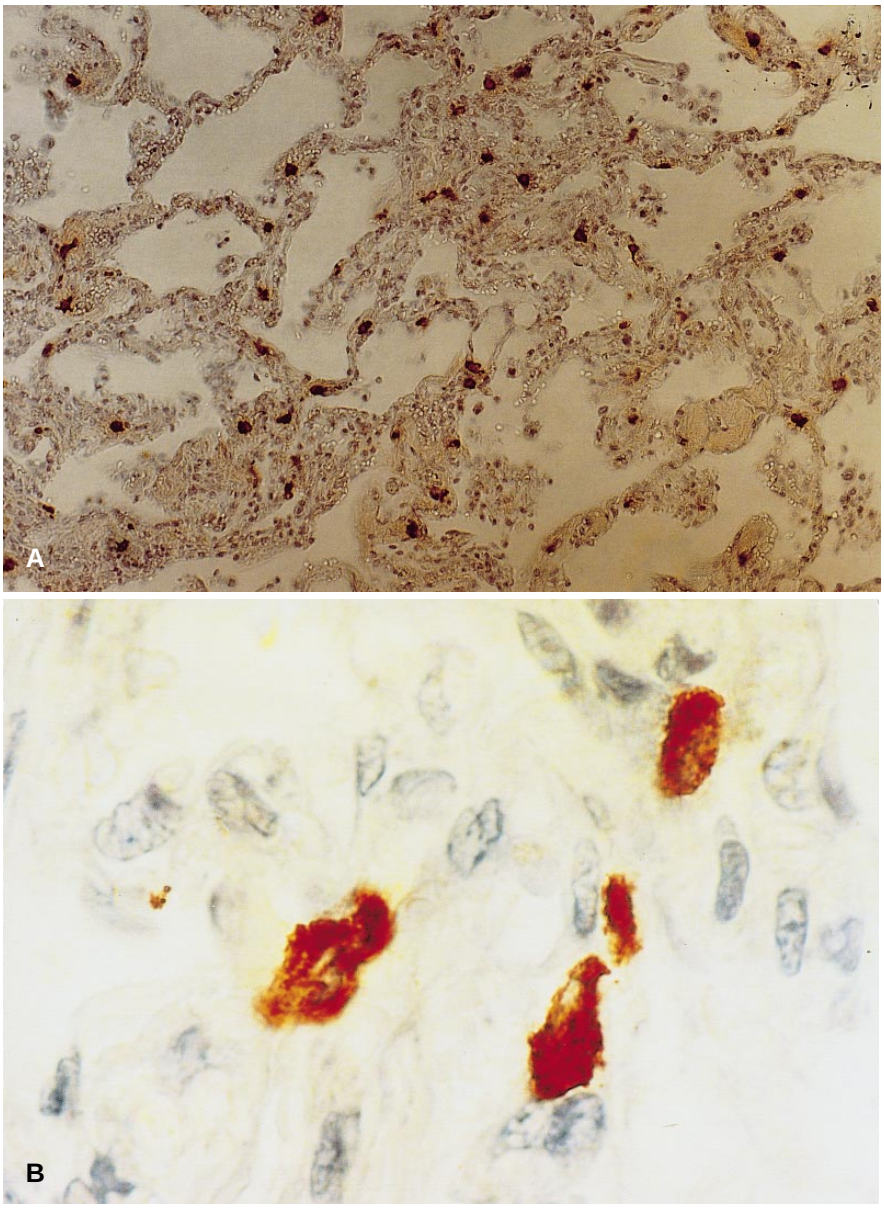

Fig. 1 **A** Pulmonary mast cells present in a 39-year-old female (Group A: AFE) Anti-tryptase, AEC, magnification × 190. **B** Interstitial mast cells present in a 39-year-old female (Group A: AFE) Anti-tryptase, AEC, magnification × 500

are given in the tables where the means and standard deviations per subject and group are highlighted. The elevated number of mast cells (an average of 54.095) present in the group where death was attributed to AFE is clearly evident, makes it possible to superimpose this group onto the group where death was clearly caused by anaphylactic manifestations (average mast cell number 51.378) (see Table 4).

The application of the One Way Analysis of Variance indicates statistically significant differences between groups treated globally (F-ratio = 439.7; $p < 0.0001$). Table 5 indicates a greater superimposition between the distribution of frequency of the first (AFE) and second group (anaphylaxis) compared to a marked difference of these two with groups C and D (deaths due to traumatic causes).

The application of the Bonferroni and SNK's multiple range tests with a 0.05 significance level confirm this newly emerged data. The significance levels reached through an analysis of the single samples and in the sum per subject are greater than 95% for combinations of groups indicated by an asterix (*) in Table 6 for the Bonferroni and for the SNK test.

The difference between control group D and the other groups proves to be statistically significant, while the distribution of groups A and B, homogeneous in their distributive dynamics, remains insignificant. The non-parametric tests confirm these results, quantifying with the level of probability P, and to a greater extent with the standardized mean difference of the Z ranges in the groups paired in order to compare the more of less marked difference among the distribution in the groups (see Table 7).

Table 4 Structural variables with the mast cells count, standard deviation and post mortem interval (P.M.I.) per subject and group

	Sex	Age	PMI	Mechanism	Average cell number	Standard deviation
Group A AFE						
Case 1	F	23	24 h	AFE	67.857	25.001
Case 2	F	26	16 h	AFE	51.357	19.185
Case 3	F	32	20 h	AFE	38.214	15.558
Case 4	F	39	30 h	AFE	50.286	16.830
Case 5	F	22	22 h	AFE	59.929	19.044
Case 6	F	33	15 h	AFE	56.929	21.030
					54.095	21.612
Group B Anaphylactic shock						
Case 1	F	66	30 h	Sulphamidic ingestion	71.643	29.300
Case 2	F	24	20 h	Wasp sting	40.643	20.798
Case 3	F	33	24 h	Wasp sting	35.152	19.344
Case 4	F	15	18 h	Peanut ingestion	25.043	14.136
Case 5	F	61	18 h	Sulphamidic ingestion	46.092	24.764
Case 6	F	69	15 h	Sulphamidic ingestion	80.286	24.817
					51.378	30.424
Group C Pregnancy						
Case 1	F	36	12 h	Shot	16.571	8.579
Case 2	F	42	22 h	Traffic accident	28.714	13.771
Case 3	F	18	34 h	Traffic accident	27.857	18.564
Case 4	F	43	18 h	Traffic accident	20.429	14.110
Case 5	F	33	20 h	Shot	34.600	13.581
					24.477	15.230
Group D Traumatic accidents						
Case 1	F	65	16 h	Traffic accident	6.800	5.497
Case 2	F	70	20 h	Traffic accident	13.086	5.439
Case 3	F	25	29 h	Traffic accident	10.657	6.195
Case 4	F	72	14 h	Traffic accident	13.186	8.545
Case 5	F	15	19 h	Traffic accident	7.700	5.974
Case 6	F	27	31 h	Traffic accident	8.543	6.121
					9.995	6.822

Table 5 Results of quantitative morphometry and statistics analysis

	Mean	Stand. dev.	Stand. error	Min.	Max.	95% Conf. Int. For Mean
Group A	54.095	21.612	1.054	15	150	52.024–56.168
Group B	51.378	30.424	1.554	5	145	48.322–54.435
Group C	24.477	15.230	0.865	5	85	22.775–26.179
Group D	9.995	6.822	0.333	0	40	9.340–10.649

Table 6 Statistical analysis of the mast cells count in the Bonferroni and S.N.K. tests

	Group D	Group C	Group B	Group A
Group D	–			
Group C	*	–		
Group B	*	*	–	
Group A	*	*		–

Table 7 Statistical analysis of the mast-cells count through non-parametric tests

	Mann-Whitney U		Kolmogorov-Smirnov KS	
	Z Corrected for ties	2-Tailed P	Z Corrected for ties	2-Tailed P
Group A–B	–0.8513	0.3946	0.788	0.563
Group A–C	–4.7818	0.0000	2.507	0.000
Group A–D	–5.9389	0.0000	3.464	0.000
Group B–C	–3.1214	0.0018	1.934	0.001
Group B–D	–4.5971	0.0000	2.831	0.000
Group C–D	–4.5619	0.0000	2.335	0.000

Discussion

The data that our study supplies indicate a pathogenetic mechanism of a systemic nature for AFE where the pulmonary mast cells are clearly involved. Numerical values of these cells have been identified much like those in subjects where death was definitely due to an anaphylactic reaction, while negative values are present in the control groups of pregnant women where death followed traumatic events and traffic accidents. However, the emerging data necessitates further laboratory proof and an immunohistochemical study on pulmonary mastcellular tryptase should accompany the routine stainings used in fatal cases of AFE.

Since the first descriptions of AFE, its post-mortem diagnosis had previously been based on the evidence of squamous cells or debris in the pulmonary artery vasculature [9]. An insufficiency of the routine staining methods has improved by the use of histochemical [6] and immunohistochemical [10–15] techniques for a correct forensic diagnosis [16].

One report postulates that an attempt should also be made to quantify the size of the embolus since not all amniotic fluid embolisms are fatal [17], even though no relationship has been demonstrated between the severity of the clinical features and the amount of particulate material in the lungs and even less so with the actual volume of embolized fluid [18]. Presently, the evidence of squamous cells in the maternal pulmonary artery circulation is no longer considered pathognomonic [2]and the introduction of amniotic fluid into the maternal circulation holds pathogenic characteristics of AFE only in certain situations tied to particular components of this fluid [3].

In a small percentage of women such exposure causes a pathological complex resulting, in many cases, in the death of the patient. The clinical variability of the onset of AFE can be compared with the antigenic variability of the amniotic fluid and with individual reactions similar to other anaphylactic or anaphylactoid reactions [1, 19].

An hypothesis has been put forward that AFE "may have an underlying immunologic source involving the presentation of pregnancy-associated antigen in the maternal circulation. Amniotic fluid leakage into maternal circulation per se seems an unlikely cause given the absence of a satisfactory animal model, the variable timing of symptoms after the presumed 'leak' and the highly variable presentation of patients. A more feasible attractive hypothesis could be an immunologically mediated response to the same type of pregnancy-associated antigen leaking into the maternal circulation" [19]. New laboratory tests are thus invoked, like the serum tryptase levels, which should supply scientific support to the presumed immunological mechanism of AFE [4]. Although the serum tryptase analysis is totally reliable when performed during a clinical course [20], a post-mortem comparison of tryptase values is discordant since the blood samples were collected at the autopsy many hours after death [21, 22].

In the current study pulmonary mast cells were identified and quantified by immunohistochemical techniques using antibody preparations against stored tryptase [23]. Tryptase, a neutral protease, is known to be the dominant protein component of the secretory granules of each of the two types of human mast cells identified as T mast cells, which contain tryptase rather than chymase and are found predominantly in the lung and TC mast cells which contain both neutral proteases and are found predominantly on the skin and intestinal submucosa [24]. Since tryptase is a mediator stored together with histamine and proteoglycan in the secretory granules of mast cells, the degranulation of mast cells is accompanied, either in immunological or non-immunological activation, by the release of this enzyme. Its localization in secretory granules is initially indicated by its release, together with histamine, from immunologically activated mast cells previously dispersed in human lung tissue [25].

The significance of the results obtained through quantitative morphometry prompts further studies adding, in all cases of death by AFE, the measurements of serum tryptase levels to the immunohistochemical study of tryptase.

A validation of these laboratory tests allows the forensic pathologist to obtain a more reliable diagnosis of AFE confirming that "the term amniotic fluid embolism appears to be a misnomer and should be discarded and the syndrome of acute peripartum hypoxia, hemodynamic collapse and coagulopathy should be hereafter described as (anaphylactic or) anaphylactoid pregnancy syndrome" [1].

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