

The anaphylaxis hypothesis of sudden infant death syndrome (SIDS): mast cell degranulation in cot death revealed by elevated concentrations of tryptase in serum

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Summary

A series of cases of sudden unexpected post-neonatal deaths from two centres in the UK have been investigated for evidence of mast cell activation using the biochemical markers tryptase and $9\alpha,11\beta$ -PGF₂. Tryptase was selected as a possible marker because it is a component of mast cell secretory granules and, unlike histamine, it is not released from basophils. The prostaglandin $9\alpha,11\beta$ -PGF₂ is an initial and pharmacologically active metabolite of PGD₂, the major mast cell-derived cyclooxygenase product. This prostaglandin was chosen to serve as a marker of newly generated mediator release. In the study, unexplained infant deaths were associated with a higher concentration of tryptase in serum compared with cases of unexpected, but subsequently explained death. However, $9\alpha,11\beta$ -PGF₂ was found to be an unsuitable post mortem marker in this situation. These results provide direct evidence that mast cell degranulation, possibly as a result of anaphylaxis, may be occurring around the time of death in some cases of cot death.

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Introduction

The mechanistic basis of the sudden infant death syndrome (SIDS) has remained elusive despite intensive efforts encompassing epidemiological survey and laboratory based investigations.

One explanation to account for a proportion of sudden unexplained postperinatal deaths is the so-called 'anaphylaxis hypothesis'. The basic premise of this hypothesis is that infants become sensitized to protein components of cows' milk whilst feeding and that during sleep the fatal reaction occurs by regurgitation of recently ingested cows' milk into the airways [1–3].

Evidence for this hypothesis is largely circumstantial, although there are compelling features which command attention. Direct support for the operation of an anaphylactic reaction in cot death has been severely hampered by the inability to obtain adequate evidence of mast cell activation, either by assay of specific chemical markers or by assessment of cell ultrastructure [1]. However, some evidence for the anaphylaxis hypothesis comes from studies in an animal model in which guinea-pigs fed cow's milk in their drinking water can, under appropriate conditions, be shown to undergo a fatal anaphylactic reaction that bears certain similarities to sudden unexplained death in infants [4–6].

Infantile hypersensitivity to cows' milk is a well-recognized phenomenon, although it may only be transient in nature [8,9]. Manifestations of hypersensitivity range from anaphylactic shock and bronchospasm to skin

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rashes. In a study of *ex vivo* basophil responses to diluted cows' milk, McLaughlan and Coombs demonstrated that among a sample of 54 infants aged 4–26 weeks, three responded by releasing 36–63% of their total basophil histamine, indicating the presence of a marked hypersensitivity state in these cases [10]. Although no study has linked the degree of basophil response to clinical signs, these observations raise important questions concerning a possible link between hypersensitivity and the clinical manifestations of SIDS.

Certain similarities have been noted in the pulmonary pathology of anaphylactic shock, chronic immediate hypersensitivity diseases and sudden infant death (e.g. oedema, inflammatory cell infiltration and disruption of the architecture of the airway epithelium). These pathological features are not unique markers of anaphylactic shock, but their presence is certainly of interest in the context of other supporting evidence. The presence of cows' milk antigens in the lungs of a high percentage of SIDS cases [3] is of note, but it is not possible to distinguish whether this is a cause or consequence of death.

As fatal pulmonary anaphylaxis has an obligatory requirement for mast cell activation and degranulation, it is not surprising that the presence and appearance of these cells have received some attention [10]. However, the results of these studies have been equivocal because the techniques employed did not offer the requisite degree of ultrastructural resolution to provide a definitive comment on their state of degranulation.

An alternative source of evidence for mast cell activation is the measurement of chemical mediators derived from mast cells. Although attractive, this approach has, until very recently, suffered from major limitations imposed by the sensitivity of analytical techniques and the mast cell specificity of certain biochemical markers [11]. For example, the anaphylactic mediator histamine is an inappropriate marker in post mortem samples because of its potential artefactual release from basophils in blood. Prostaglandin (PG) D_2 , a mast cell derived mediator and powerful airway smooth muscle spasmogen, offers potential as a relatively specific marker of mast cell activation [12,13]. Unfortunately, it is unsuitable for analysis as it occurs in small quantities and it undergoes facile isomerization and dehydration in biological fluids that contain serum albumin [14]. However, assay of more stable enzymatic metabolites of this prostaglandin may be possible and might overcome some of these difficulties [11].

Tryptase, a 134 kDa tetrameric neutral proteinase, is one of the major protein constituents of mast cell, but not basophil secretory granules [15–18] and is released upon their IgE-dependent activation. We reasoned that

measurement of this granule-derived mediator might enable us to test biochemically for the possible involvement of a type I anaphylactic hypersensitivity reaction in unexplained infant deaths. Monoclonal antibodies to human tryptase were therefore employed to identify and enumerate mast cells in lung sections and to quantify tryptase in serum from postperinatal deaths.

Methods

Collection of samples

Sample of serum from babies who died suddenly and unexpectedly aged between 1 week and 2 years were collected by teams in Sheffield and Avon as part of ongoing studies into possible causes of SIDS. Investigations were performed on consecutive cases where sufficient sample material was available. The serum was obtained from blood samples aspirated from the right side of the heart, although in some cases samples were taken from the left side of the heart or (rarely) the superior sagittal sinus. The time after discovery of death at which the samples were collected was noted in each case. Each case was subject to detailed post mortem examination and categorized into explained or unexplained deaths using the criteria adopted in the study regions. The criteria for inclusion encompassed the definition for sudden unexpected death proposed at an international conference and incorporated the age range surveyed by the Department of Health multicentre study of postperinatal deaths [19,20]. Unexplained deaths were those in which there was insufficient evidence to demonstrate an adequate cause of death. Biological fluids were coded and sent for biochemical investigation. Aliquots of serum were separated for the two assay techniques. In some cases samples had to be rejected from certain assays due to inadequate volume or the presence of haemolysis or other factors that were known to interfere with assay performance. Division of samples and all assays were performed without the knowledge of the post mortem findings. Sufficient quantities of sample were not available for all assays, and this accounts for the differences in the sample sizes for the prostaglandin and tryptase measurements.

Lung tissue from a selection of the Avon centre cases was subjected to immunohistochemical analysis for the identification of mast cells and eosinophils.

Analytical methods

Tryptase was quantified in duplicate aliquots of serum using a radioimmunoassay with tryptase specific monoclonal antibody AA5 [21] coupled to cyanogen bromide

activated agarose (Pharmacia, Uppsala, Sweden). Samples or tryptase standards over the range 2–50 ng/ml were incubated with AA5-agarose (2.5 mg in 100 μ l) for 16 h at room temperature with constant shaking. After washing with pH 7.6 Tris buffer, 25/ μ l of ^{125} I-labelled tryptase monoclonal antibody (Pharmacia) was added for 16 h at room temperature. At the end of the incubation samples were washed with 0.9% saline and radioactivity quantified by gamma counting. The standard curve was usable for quantification over the range 2–50 ng/ml and the intra- and interassay coefficients of variation were 6.2% and 13.1% respectively, determined at a concentration of 3.1 ng/ml.

9 α ,11 β -prostaglandin F₂ was used as an index of PGD₂ production. This compound is formed by the stereoselective, 11-ketoreductase dependent metabolism of PGD₂ and is the initial metabolite formed from this prostaglandin under the majority of circumstances [22–24]. Like PGD₂ it is a powerful contractile agonist on airway smooth muscle and may therefore contribute to anaphylactic bronchoconstriction [25]. Samples of serum were diluted with water and applied under gentle negative pressure to immunoaffinity matrix columns for the selective capture of the target analyte. Extraction matrices were constructed by carbamate linkage of a rabbit polyclonal anti-9 α ,11 β -PGF₂ IgG fraction onto an agarose support. A total of 44 mg of purified rabbit IgG was obtained from animals that had a high titre of specific antibodies after immunization with a keyhole limpet haemocyanin-9 α ,11 β -PGF₂ conjugate. The IgG was allowed to react with a washed slurry of Reacti-Gel (original volume 20 ml, Pierce, Rockford, Illinois, USA) in 100 ml of borate/phosphate buffer, pH 8.5. Initial reaction was allowed to proceed, with gentle stirring, for 2 h at room temperature, followed by overnight incubation at 4°C. Unreacted sites on the gel were blocked by treatment with pH 8.0 2 M Tris-HCl for 1 h. The derivatized gel was then packed into glass columns to a bed volume of 3 ml and these were covered with a porosity three sintered glass filter. Column performance and specificity was determined prior to the study using solutions containing radioactive prostanooids and by extraction of unlabelled standards. When the columns were loaded with individual serum samples the captured analyte was then washed with water and then released by elution into double distilled acetone. Acetone was dried by evaporation under nitrogen at room temperature and the extract resuspended in radioimmunoassay (RIA) buffer prior to quantification of 9 α ,11 β -PGF₂ using a second rabbit polyclonal antiserum of high specificity. Recovery of analyte through the sample work of procedure was 63 $\pm$ 7% and un-

extracted standards were processed with a coefficient of variation of 8% (intra) and 14% (inter) respectively. Tritiated 9 α ,11 β -PGF₂ for use in the RIA was prepared biosynthetically from [^{3} H₇-n]-PGD₂ using guinea-pig liver cytosol as a source of NADPH-dependent 11-ketoreductase. The 11 α -isomer (approximately 5–10% of the product yield) was removed by reversed phase high performance liquid chromatography using techniques derived from those detailed elsewhere [22].

Immunohistochemistry

Monoclonal antibody AA1 directed against tryptase was used to enumerate mast cells in sections that had been trypsinized to reveal the epitope recognized by the antibody [21]. Visualization was achieved using the avidin-biotin horseradish peroxidase technique according to methods described in detail elsewhere [17,26]. Activated eosinophils were quantified in plastic-embedded semithin sections using the monoclonal antibody EG2 that recognizes the secreted form of eosinophil cationic protein in activated cells [26]. For both immunohistochemical markers, cell counts were made in peribronchial and sub-pleural regions and expressed per mm² of lung tissue.

Statistics

Data are presented as the median values with percentiles or mean $\pm$ standard error of the mean (SEM). Differences were evaluated by a two-tailed Mann-Whitney two sample unpaired test. Correlation analysis was by the Spearman rank procedure.

Results

Tryptase measurements

Tryptase was detectable above the assay threshold in 94 of 106 serum samples analysed. The results are shown in Fig. 1. There was a statistically significant difference between the tryptase concentrations present in the two unexplained death groups when compared with the Sheffield explained group. Of the 56 SIDS cases from Sheffield (median tryptase concentration 5.9 ng/ml, interquartile range 3–8.3), five specimens (9%) had values at or below the limit of assay sensitivity, compared with 1 from 26 (4%) in the Avon group (median concentration 6.3 ng/ml, interquartile range 3.7–8.8). In contrast, tryptase concentrations were not resolvable above the limit of detection in six out of the 24 (25%) deaths in the Sheffield explained group (median 3.9 ng/ml, interquartile range 2–6.5).

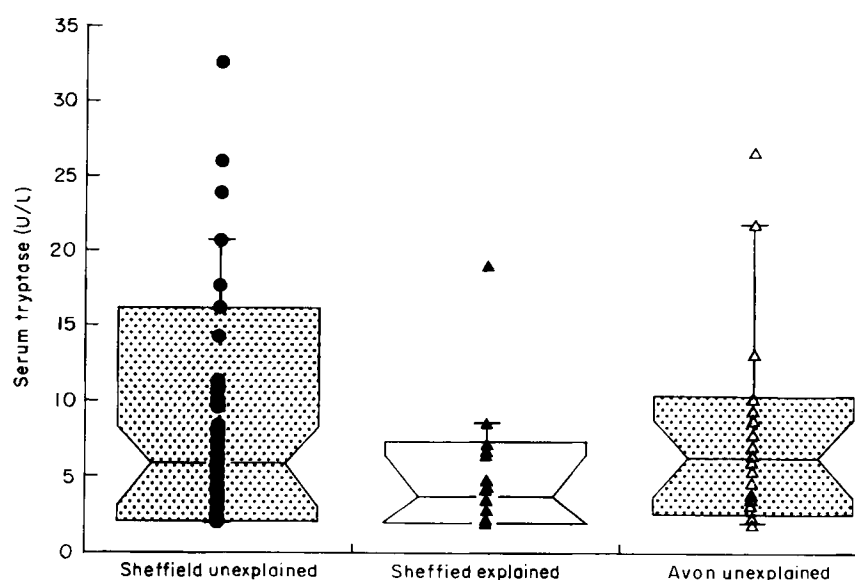


Fig. 1. Box-whisker plot showing the concentrations of immunoreactive tryptase in serum from victims of sudden explained and unexplained infant death in the two study centres. Horizontal lines within the box indicate median values, notches indicate the interquartile range, box ends the 10th and 90th percentiles and the whiskers the 5th and 95th percentiles. Fifty-six and 24 cases were in the Sheffield unexplained and explained groups, 26 cases were in the Avon group. The median values of the Sheffield and Avon unexplained groups are significantly different than the explained group ($P = 0.03$, $P = 0.02$ respectively). One tryptase unit/litre = 1 ng/ml.

With the exception of two explained deaths (which have not been included in the Sheffield control group), the assay data for the samples from the Avon group all relate to unexplained deaths. The serum concentrations of tryptase in the samples from explained deaths were 3.8 ng/ml (a case of non-accidental death) and 4.1 ng/ml (asplenia) and based on a one sample Wilcoxon signed rank comparison, the median of the Avon unexplained deaths was significantly greater ($P < 0.002$) than both these values. The cases provided by the Avon centre had been sub-divided on an established three point post mortem classification system based on histological, microbiological and biochemical criteria (I: no abnormal findings; II: associated contributory factors; III: death fully explained by these findings). There was no significant difference between the tryptase concentrations in the category I and category II cases with median values of 6.5 (range 2–26.7, $n = 18$) ng/ml and 4 (range 2–17.8, $n = 7$) ng/ml respectively.

There was no significant correlation between the amount of tryptase present in serum and the time after discovery of death at which sampling was made (Spearman rank correlation probability values, $P = 0.47$, $P = 0.65$ and $P = 0.63$ for the Sheffield unexplained, Sheffield explained and Avon unexplained cases respectively). Similarly, there was no apparent relationship between serum tryptase and age of the infants in any of the study groups, despite there being significant differences between the Sheffield explained death group (median age 32 weeks) and the Sheffield unexplained (median age 15 weeks, $P = 0.03$) and Avon unexplained (median age 12 weeks, $P = 0.004$) groups.

Prostaglandin measurements

The serum concentrations of $9\alpha,11\beta$ -PGF₂ are illustrated in Table 1. This substance was detectable above

Table 1. Concentration of $9\alpha,11\beta$ -PGF₂ (pg/ml) in serum in cases of explained and unexplained sudden infant death

	<i>n</i>	Median	Interquartile range	Minimum	Maximum
Sheffield explained*	23	51.3	14.9–108.9	ND	437.55
Sheffield unexplained†	60	59.9	17.6–108.8	ND	565.4
Avon unexplained	30	146.4	66.3–240	9.8	1063.6

ND. = None detectable

* $P = 0.003$ with respect to median of Avon group.

† $P < 0.001$ with respect to median of Avon group.

the assay threshold in the majority of the 118 samples investigated, although there was a considerable heterogeneity in the measured values. There were no significant differences between the median values of the Sheffield explained and unexplained groups. However, there was a significant ($P < 0.0001$) difference between the median of the two unexplained groups. The data for the Avon group (mean 225 ± 44 pg/ml) are more similar to the concentration of $9\alpha,11\beta$ -PGF₂ found in fresh adult serum (216 ± 18 pg/ml, $n = 16$), suggesting decomposition of the compound in the Sheffield sample groups during the longer time interval between discovery of death and sampling in this group.

Discussion

In this study we report the use of the neutral proteinase tryptase as a marker of mast cell activation in cases of sudden postperinatal death. Tryptase is not usually detectable in plasma or serum in healthy individuals. Its presence in sera from most explained and unexplained deaths in this study indicates that some degree of mast cell degranulation was associated with death in the majority of all these cases. However, the important feature of these results is that, when compared with the explained deaths group, the concentrations of tryptase were significantly elevated in those cases where no adequate cause of death could be rigorously established. These findings thus provide evidence of unusually high degrees of mast cell activation in some cases of sudden infant death, but we are nevertheless unable to exclude the possibility that this is secondary to an unknown primary fatal event that occurs in this syndrome.

In the Avon cases where information on feeding was available, there was no obvious relationship between the post mortem concentration of tryptase in serum and whether the baby had been fully breast fed or had been bottle fed; one of the highest concentrations of tryptase was found in a baby said to have been entirely breast fed before being weaned onto solids. We are unable to comment on the serological status of this infant for responses to milk antigens acquired by maternal transfer or after the commencement of solids because such data were not obtained at the time in this case. Moreover, although the anaphylaxis hypothesis as originally proposed relates to hypersensitivity to cows' milk, it is entirely possible that other allergens or infections might be capable of initiating mast cell degranulation as either a primary or secondary cause of death in SIDS. Cases in the Avon series had been examined in detail for the presence of viral or bacterial pathogens in the upper

respiratory tract. Of the 26 cases studied, only three tested positive for viral and nine positive for bacterial infections and the highest tryptase concentration was recorded in a case which was negative for both.

One of the difficulties that has prevented progress in understanding a possible anaphylactic basis to cases of cot death has been the inability to test directly for evidence of mast cell activation using a marker that is pathognomic of fatal anaphylaxis. Most chemical mediators released by mast cells do not have a unique mast cell origin or they are subject to artefactual generation or decomposition under the conditions that are encountered in specimens available for investigation of postperinatal deaths [11]. Tryptase and prostaglandin D₂ are mast cell-derived mediators that are released by IgE-dependent mechanisms and which are released in greater amounts by these cells than most other potential sources. Basophils contain only trace quantities of tryptase and do not have the capacity to synthesize PGD₂ [18,11]. Although PGD₂ has been measured during IgE-dependent reactions *in vivo* [27] it has not to our knowledge been employed as a marker in studies of post mortem samples. Furthermore, because it undergoes facile isomerization and dehydration in the presence of serum albumin [14] there are sound chemical reasons to believe that it would not be a suitable analyte under such conditions [11]. We reasoned that it might nevertheless be possible to use a PGD₂ metabolite, $9\alpha,11\beta$ -PGF₂, as an index of PGD₂ turnover in these cases. Despite this prostaglandin metabolite being detectable there are grounds to suggest that it is less satisfactory than the performed marker tryptase. In particular, the finding in biological fluids of multiple isomeric forms of substances with the $9\alpha,11\beta$ -PGF ring structure that arise by enzymatic and non-enzymatic pathways [28] suggests difficulties in interpreting their route of formation and contribution to measurements of immunoreactive $9\alpha,11\beta$ -PGF₂ made using polyclonal antisera. Consequently, the absence of a clear correlation between the tryptase and prostaglandin measurements is unsurprising. It is also noteworthy that serum concentrations of $9\alpha,11\beta$ -PGF₂ in all of the groups studied are substantially higher than the amounts found in normal human plasma (which are undetectable with this assay system, [Robinson & Shell, unpublished]) implying that there are significant, but unknown, coagulation-dependent sources of materials that are immunoreactive in this highly specific assay. Clearly, this possibility severely restricts the application of this prostaglandin as a specific marker of mast cell activation under these conditions.

One concern of any retrospective analysis of biochemical events preceding death is the possibility that

measured changes may be due to post mortem artefact. Whilst this is a possibility for the prostaglandin measurements (compare the data for the Sheffield and Avon groups) this seems less compelling for tryptase. The reason for our view is the similarity of the tryptase concentrations for the unexplained deaths in both the Sheffield and Avon groups despite the large discrepancy in the time after discovery of death at which sampling took place (median 27 h, range 4–145 h in Sheffield and median 3.4 h, range 2–8.5 h in the Avon group). Furthermore, the explained deaths from Sheffield were sampled a median 30 h (range 4–120 h) after discovery of death, a range not significantly different from the corresponding unexplained group. There was no correlation between the measured concentration of serum tryptase and the time after discovery of death at which samples were obtained in any of the three groups. Previous studies by Schwartz *et al.* [29] also have a bearing on the relative stability of endogenous tryptase in serum. These workers found that approximately 50% of immunoreactive tryptase was still assayable after incubation of serum at 22°C and 37°C for 4 days [29].

The difference between serum tryptase concentrations in the explained and unexplained deaths was detected despite the likelihood that the majority of cases of unexplained death are unrelated to anaphylactic responses and mast cells. Moreover, the data from the control group of explained deaths demonstrate that this degree of mast cell activation and tryptase release are not secondary to all forms of sudden infant death, although it is clear that some degree of mast cell degranulation must have occurred in the majority of the deaths to account for the presence of tryptase in these sera. Causes of death in the explained group were heterogeneous and several cases of traumatic death were present. The highest serum tryptase concentration recorded in this group (19 ng/ml) was a case of trauma, and it is possible that this is an effective stimulus for mast cell degranulation.

The significance of tryptase as reported here now extends the evidence of its suitability as a biochemical index of mast cell degranulation [29,30]. A comparison of our data with 19 cases of anaphylactic reactions to insect stings, food and therapeutic agents [30] suggest that our SIDS cases have lower post mortem serum tryptase concentrations than result from systematic anaphylaxis in juveniles and adults, although the range of measured values in the previous study is very large (< 2.5–150 000 ng/ml) [30]. Whilst the reason for this difference is unknown, it is noteworthy that even according to the definition of normal post mortem range used by Yunginger *et al.* [30] (i.e. < 10 ng/ml) some of our cases exhibit abnormally high concen-

trations of serum tryptase. Whether such values are associated with anaphylactic reactions that are life-threatening primary causes of death must for the moment remain speculative.

Evidence for the operation of the anaphylaxis hypothesis implies functioning of immunological mechanisms in the major shock organ. Previous studies have documented in sudden infant deaths the presence of increased concentrations of IgG in bronchoalveolar lavage fluid [31]. Cows' milk antigens have also been demonstrated in the lung in some cases of cot death [3], although we are not aware of any studies where the source of this has been unambiguously demonstrated, or where this was related to the presence of antigen-specific IgE. In the present study we confirmed by immunocytochemical staining for tryptase that in 15 cases from the Avon unexplained deaths AA1⁺ mast cells are concentrated in the airways at a site where their activation could lead to profound bronchoconstriction (data not shown). In the same lungs there were also significant numbers of EG2⁺ eosinophils whose granules contained the secreted form of cationic protein, indicative of their activation (data not shown). The recruitment and activation of eosinophils are now well-documented features arising from immediate hypersensitivity reactions and imply the presence of other activated cell types, most notably T lymphocytes and mast cells, in providing the required chemical mediator and cytokine signals that result in eosinophil recruitment, priming and activation. It is noteworthy that a very recent study has highlighted the preferential recruitment of eosinophils into the lungs in cases of sudden unexplained infant death when compared with a control group of a comparable age [32]. In the light of recent advances in understanding the relationship between T cell cytokine phenotype and the acquisition of allergic responses it seems likely that a careful application of molecular biological techniques will yield further insights capable of establishing the contribution of the anaphylaxis hypothesis to the enigma of cot death.

In summary, we have demonstrated that elevated concentrations of serum tryptase are associated with sudden unexplained infant death. This observation is consistent with anaphylaxis as a mechanism in some cases of SIDS, but further work will be necessary to establish whether mast cell degranulation in these cases might occur secondary to terminal respiratory failure or some other event common to most cases of SIDS. On the basis of our data we suggest that measurement of serum tryptase will now provide the basis for further studies in which the precise relationship to hypersensitivity to cows' milk and possibly other allergens can be determined.

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