Immunohistological localisation of staphylococcal toxic shock syndrome toxin (TSST-1) antigen in sudden infant death syndrome

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SUMMARY A polyclonal antiserum to toxic shock syndrome toxin (TSST-1) and a standard immunoperoxidase technique were used on formalin fixed tissues from 50 cases of sudden infant death syndrome (SIDS) to determine if the syndrome was associated with bacterial infection. There was strong specific staining in the renal tubular cells in nine (18%) cases. A similar pattern of staining was seen in three of a series of 50 kidneys selected for comparison from a wide range of necropsy cases. The staining was finely granular within the cytoplasm of proximal convoluted tubular cells and diffuse in tubular cell nuclei. In an attempt to validate the staining pattern the immunoperoxidase technique was also performed on formalin fixed kidneys from rats which had been given intravenous injections of crude bacterial products containing TSST-1. These showed coarse granular cytoplasmic staining in proximal convoluted tubules with some diffuse nuclear staining. This pattern was not seen in controls injected with saline. These results indicate that TSST-1 might have a pathogenic role in some cases of SIDS.

There are about 1500 annual cases of sudden infant death syndrome (SIDS) in Britain¹: it is the leading cause of infant mortality. Any hypothesis to explain the phenomenon must take into account the observed epidemiological characteristics²⁻⁴ which include the specific age distribution, with a peak at 2 to 3 months, the preponderance of winter deaths, and a high proportion of nocturnal deaths.

It has recently been suggested that bacterial toxins might be implicated in SIDS.⁵ The condition is associated with upper respiratory tract viral infections which lead to overgrowth of common toxigenic bacteria.⁶ When this coincides with a nadir in the level of protective antibody the combination might lead to sudden death with the characteristic age and temporal distribution of the syndrome.

As part of a programme of investigation of this hypothesis we attempted to localise specific bacterial toxins in stored tissues from cases of SIDS. This report concerns our work with a polyclonal antiserum to staphylococcal toxic shock syndrome toxin (TSST-1). This toxin was chosen because it occurs commonly in the community⁷ and can cause severe multisystem^{8,9} disease with profound hypotension and sudden death¹⁰ in adults.

Material and methods

Formalin fixed, paraffin wax embedded blocks of kidney, spleen, liver, thymus, heart and lung from 50 consecutive cases of SIDS were obtained. Detailed bacteriological data were available on 47 of these cases. This included results of bacterial culture of blood, cerebrospinal fluid, lung, spleen and nasopharyngeal secretions. All isolates of *Staphylococcus aureus* had been tested for production of TSST-1 at the Public Health Laboratory, Colindale.

A comparison series of formalin fixed kidneys was selected from a wide range of necropsy cases. These included three infants who had died from reasons related to prematurity, 12 cases of sudden traumatic death aged 3 to 47 years, and 35 cases of natural disease with an age range of 2 to 94 years. Bacteriological data were not available on these cases.

A standard peroxidase-antiperoxidase technique was used. Sections were trypsinised in 0.1% solution in 0.1% calcium chloride in TRIS buffer for 30 minutes. Polyclonal rabbit anti-TSST-1 (Toxin Technology, Wisconsin, USA) at a dilution of 1 in 30 was applied for one hour followed by swine anti-rabbit antibody at a dilution of 1 in 25 for 30 minutes. Finally, horseradish peroxidase complexed with rabbit anti-peroxidase was applied at a dilution of 1 in 50 for 30

minutes. The antigen and complexed antibody were visualised using 0.06 g of three amino-9-ethyl carbazole in 15 ml dimethylformamide.

All sections were stained in duplicate with one acting as a negative control in which specific antisera were replaced by rabbit immunoglobulin at a similar dilution. All positive results were repeated with primary antibody diluted 1 in 60, 1 in 120, 1 in 180. All positive staining was also blocked by preincubation with purified TSST-1 (Toxin Technology).

Crude mixtures of bacterial toxins were prepared by a cellophane-over-agar technique.¹¹ Plates were prepared by placing autoclaved cellophane discs on to brain-heart infusion agar in a standard 9 cm Petri dish. An overnight culture of bacteria on blood agar was suspended in 5 ml of sterile physiological saline and the suspension poured on to the prepared plates. After 24 hours' incubation at 37°C the growth was washed from the surface of the cellophane using 5 ml of Kreb's saline. The washings were centrifuged at 4500 rpm for 15 minutes and the supernate was sterilised by passing through an 0.2 micron Millex filter.

Male Wistar rats weighing 250 to 300 g were anaesthetised by intraperitoneal injection of nembutal (0.75 ml/100 g body weight of a solution of 0.5 ml nembutal in 5 ml of 1 in 10 ethyl alcohol in distilled water).

The freshly prepared bacterial products, which varied in volume from 2 to 6 ml, were given by slow

intravenous injection (three minutes) into the tail vein. Four rats received bacterial toxins from TSST-1 producing isolates of *S aureus*. Four rats were given intravenous injections of matched volumes of Kreb's saline. A further eight rats received bacterial products from nasopharyngeal bacterial isolates which were not known to produce TSST-1. These organisms included staphylococci, streptococci, and enterobacteria. The rats were observed for 45 minutes while under anaesthesia and then sacrificed by cervical dislocation. Dissection was carried out and heart, lungs, liver, kidneys and spleen were fixed in formalin. Sections were prepared from the tissues after paraffin wax embedding and immunoperoxidase staining was performed as for the cases of SIDS. The only difference was that the rat tissues did not require treatment with trypsin. A full range of controls, as in the cases of SIDS, was used.

Results

CASES OF SIDS

There was no consistent pattern of specific staining in the lung, myocardium, spleen, thymus and liver. There was, however, strong specific staining in renal tubular cells in nine (18%) cases. The staining was diffuse or finely granular in the cytoplasm of proximal convoluted tubular cells and within some tubular cells in the medulla (figs 1 and 2). A prominent feature was

Fig 1 Kidney from case of SIDS: finely granular staining of proximal convoluted tubule cytoplasm and strong diffuse nuclear staining. (PAP, anti-TSST-1, with partial bleach of the nuclear counterstain.)

Fig 2 Kidney from case of SIDS: granular cytoplasmic staining and diffuse nuclear staining in renal medullary tubules. The adjacent collecting tubules are negative. (PAP, anti-TSST-1, with partial bleach of the nuclear counterstain.)

strong diffuse staining of some renal tubular cell nuclei.

S aureus was isolated from the respiratory tract in 18 (38.3%) of the 47 cases in which bacteriological data were available. In three cases the isolates produced TSST-1, and in one of these cases there was strong positive renal staining. In fact, five of the nine positive cases had respiratory isolates of *S aureus* (55% against an expectation of 38.3%; not significantly different). There was no correlation between positive staining and age at death, month of death, sex, or the interval between death and necropsy.

COMPARISON CASES

Each of the three kidneys from premature infants showed diffuse positive staining within the tubules of the nephrogenic zone at a primary antibody dilution of 1 in 30. The staining was different in character from that of the cases of SIDS. It was faint, diffuse, affected all of the nephrogenic zone but not tubular nuclei.

Three of the remaining 47 cases showed positive tubular staining which was similar in character to that seen in positive cases of SIDS. In two of these, a case of chronic myeloid leukaemia and a cerebrovascular accident surviving several days, an infective element was clearly a possibility. The third case was a 42 year old woman dying of carbon monoxide poisoning in which there was no clinical or morphological evidence of infection. There was no consistent relation between

positive staining and the interval between death and necropsy.

RATS

Three of the four rats given TSST-1 died of respiratory arrest after seven, 13, and 19 minutes. Two of the rats given other bacterial toxins died after 14 and 21 minutes. All the other animals survived for 45 minutes after injection and were sacrificed by decapitation.

All the rats showed faint, diffuse positive staining of the cytoplasm of liver hepatocytes and renal distal convoluted cells. The four rats which had received TSST-1 by injection also showed coarse granular cytoplasmic staining in proximal convoluted tubules with some diffuse nuclear staining (fig 3). The nuclear staining was most pronounced in the rat which survived for 45 minutes. This pattern was not seen in the rats which received saline. One of the eight rats given other bacterial toxins, however, showed a similar pattern of cytoplasmic and nuclear staining in proximal convoluted tubules. This rat received products from an *S aureus* isolate which was TSST-1 negative. The rat had survived for 45 minutes under anaesthesia.

Discussion

In the first year of life infants encounter a large number of bacteria, many of which eventually colonise the

Fig 3 Rat kidney after intravenous TSST-1: coarse granular staining in the cytoplasm of proximal convoluted tubules with strong nuclear staining in renal distal convoluted cells. (PAP, anti-TSST-1, with partial bleach of the nuclear counterstain.)

body surfaces. These bacteria produce a wide range of toxins, some of which can be lethal. Thus the infant is at risk of fatal toxæmia in the interval between loss of protective maternal IgG and the acquisition of specific immunity. Any thing which disturbs the body flora, such as viral infections, can cause bacterial overgrowth and increase the risk of death. Several of the salient epidemiological features of SIDS, such as the age distribution and the winter peak in incidence can be explained in this way. This idea also fits with the observation that only minimal morphological changes are found at necropsy.

TSST-1 is one of a wide range of toxins produced by common commensal bacteria. It is a protein weighing 24000 daltons, produced by some strains of *S aureus* commonly found in the community.^{7,8} For example, in one study, anterior nares cultures from 11 of 22 healthy children grew *S aureus* and four of these were TSST-1 producers.⁷ The toxic shock syndrome is associated with this toxin in over 90% of cases. The syndrome is defined clinically as fever, rash, hypotension, desquamation of palms and soles and evidence of infection in three or more organ systems. The disease spectrum associated with the toxin, however, is probably broad. There are mild cases which do not fulfill stringent criteria^{7,9} and death may occur before they develop.¹⁰

In this study strong specific staining of renal tubular

cells in nine of 50 cases of SIDS was shown. In every case the sections were stained in duplicate, with one slide acting as a negative control. This slide received rabbit immunoglobulin rather than specific antiserum. Positive staining was only accepted as specific if there was a clear and consistent difference between test and control slides. Furthermore, all positive results were repeated at doubling dilutions and specific staining was blocked by preincubation of antisera with purified toxin. Thus we are confident that the staining is specific but we cannot conclude that it is necessarily TSST-1. The other possibility is that there is a cross reaction with a protein which has antigens in common with TSST-1. This could be another bacterial toxin or a host specific protein. In fact, cross reactions of both types are common. Several bacterial exotoxins are known to have amino acid sequences in common,¹² and several studies have shown that some antigens are shared between bacteria and human tissues.¹³

In an attempt to distinguish these possibilities we sought independent evidence from three sources. Firstly the findings were correlated with the results of nasopharyngeal bacterial culture. Three of the cases had TSST-1 producing *S aureus* isolates and one of these showed positive renal staining. There was no evidence of organisms producing TSST-1 in the other eight positive cases, although only nasopharyngeal *S aureus* isolates were tested. The possibility of TSST-1

Immunohistological localisation of TSST-1 antigen in SIDS

producing *S aureus* at other sites or of TSST-1 production by other organisms has not been excluded. This is of some importance because it has been proposed that TSST-1 production might be mediated by a temperate phage¹⁴ so that it need not be limited to strains of *S aureus*. Another factor to consider is that antigen loss is inevitable during routine fixation and processing, particularly in necropsy material. Thus the cases that stain might only be a subset of the true positive cases, and this could explain, at least in part, why only one of the three cases with TSST-1 producing isolates stained positively.

It is not possible to assemble an age matched control series of necropsy kidneys for SIDS as there are so few deaths in the first year of life in which one can be certain that infection has not played a part. Consequently we used 50 kidneys from a wide range of necropsy cases as a comparison group. Three of these cases showed a pattern of staining similar to that of the positive cases of SIDS. In two of the cases infection might have played a part, although in the third, infection was unlikely. Positive staining was also noted in the tubules of premature kidneys. Although this was different in pattern than the cases of SIDS in that it was faint, diffuse, and did not affect cellular nuclei, it does indicate a probable cross reaction between renal tubular proteins and TSST-1. There was no correlation between maturational age and positive staining in the cases of SIDS so it is unlikely that these positive results are simply due to the persistence of a fetal antigen.

In view of these equivocal results a further series of experiments was performed in which anaesthetised rats were injected with bacterial products containing TSST-1. Controls received bacterial products without TSST-1 or saline alone. All the rats showed positive staining in liver hepatocytes and renal distal convoluted cells which was presumably due to cross reaction. The rats injected with TSST-1 also showed coarse granular staining in proximal convoluted tubules with some diffuse nuclear staining. This pattern was similar to that seen in SIDS, although the granular staining was much coarser in the rats. The controls injected with saline did not show this latter pattern, although one of the rats injected with bacterial products from a non-TSST-1 *S aureus* isolate did. The latter result is presumed to have been due to an antigenically related bacterial product.

This pattern of localisation in proximal convoluted tubules is interesting and could have been predicted on theoretical grounds. This is because low molecular weight proteins are filtered in the glomeruli and then reabsorbed from the glomerular filtrate by proximal convoluted tubules. This process has been previously shown in rats¹⁵ and observed in man.¹⁶ Thus if bacterial exotoxins enter the circulation in excess of antibody,

which is envisaged in the toxin hypothesis of SIDS, clearance will be via the kidney and could be concentrated in proximal convoluted tubules. Consequently, further immunohistological studies of this site using antibodies to a range of bacterial toxins are indicated and hold considerable promise.

In view of the similar distribution of positive staining in the cases of SIDS and rats injected with TSST-1 at least some of the renal staining in SIDS is probably due to bacterial toxins. This particularly applies to the single case in which strong renal staining was associated with a heavy growth of TSST-1 producing *S aureus* in the nasopharynx. The presence of bacterial toxins in renal tubules does not necessarily imply that they have had a pathogenic role. But many bacterial toxins, including TSST-1, are potentially lethal and further studies of their role in SIDS are required.

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