SHORT ARTICLES

CATECHOLAMINE AND CYCLIC NUCLEOTIDE RESPONSE OF SHEEP TO THE INJECTION OF *CLOSTRIDIUM* *WELCHII* TYPE D EPSILON TOXIN

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THE HYPERGLYCAEMIC response of sheep to *Clostridium welchii* (*C. perfringens*) type D epsilon toxin (Bullen and Scarisbrick, 1957) is well known. Demonstration of glucosuria is therefore a useful aid to the post-mortem diagnosis of enterotoxaemia. Gardner (1973c) showed that the hyperglycaemia was due to a mobilisation of glucose from liver glycogen reserves. Recently Buxton (1978) showed that plasma cyclic adenosine 3',5'-monophosphate (cAMP) levels were significantly raised after intravenous injection of epsilon toxin. He felt that epsilon toxin could be causing a direct activation of adenyl cyclase in a manner similar to that of cholera toxin and other enterotoxins. We, however, feel that there is little similarity between the action of epsilon toxin and that of cholera toxin. Cholera toxin attaches to gut mucosa cells and activates adenyl cyclase which results in an increased production of cAMP. This is followed by an increased secretion of salt and water into the lumen of the intestine resulting in severe diarrhoea and dehydration (Finkelstein, 1976). The primary effect of epsilon toxin in the intestine appears to be quite different. It affects the permeability of the mucosa in such a way that large molecules, including epsilon toxin itself, are absorbed into the bloodstream (Bullen and Batty, 1956, 1957). Secretory diarrhoea has not been described. In the case of cholera toxin increased absorption of large molecules has not been described.

Absorbed epsilon toxin causes rapid and dramatic changes. Griner and Carlson (1961) showed that the blood-brain barrier became disrupted and brain oedema developed. This observation has subsequently been confirmed (Gardner, 1973b; Morgan, Kelly and Buxton, 1975). It was also shown that these changes took place with astonishing rapidity. Large doses of toxin caused death of mice within 2-3 min. and in this time 1.5% of the labelled albumin had already entered the brain (Worthington and Mülders, 1975). Epsilon toxin seems to have a strong affinity for brain tissue but not for other tissues (Worthington, Mülders and Van Rensburg, 1973b). Studies of the structural and ultrastructural alterations caused by epsilon toxin led Gardner (1973b) to conclude that "The primary morphological lesion produced in mice and lambs during *Clostridium welchii* type D epsilon toxin intoxication is severe vascular endothelial damage". He further stated that severe oedema frequently developed in brain, heart and lungs. Gardner (1973a) also gave an excellent account of the biochemical and haematological alterations that occurred; the most dramatic were an increase of blood glucose and lactate, an acidosis and a marked haemoconcentration.

It therefore seems that the primary lesion produced by epsilon toxin is a severe and rapidly developing brain oedema. This could be expected to cause acute pain and a severe state of anxiety which could result in an increased release of catecholamines. Because catecholamines are potent activators of adenyl cyclase, an increase of cAMP and release of glucose from glycogen stores would follow. The purpose of this study was to test this hypothesis. Accordingly, lambs were given injections of epsilon toxin and changes in the levels of adrenaline, noradrenaline, dopamine, cAMP and cyclic guanosine 3',5'-monophosphate (cGMP) in their blood were measured.

Received 7 Mar. 1979; revised version accepted 3 May 1979.

MATERIALS AND METHODS

Experimental animals. Five crossbred lambs weighing 10–20 kg were used. Epsilon proto-toxin was purified by ion-exchange chromatography and activated as described previously (Worthington, Mülders and Van Rensburg, 1973a). The final solution for injecting into the sheep contained activated epsilon toxin (0.1 mg/ml) in 0.1M phosphate buffer (pH 8.0) containing trypsin 0.25% w/v. All lambs were bled three times at 30 min. intervals 1–5 days before the epsilon toxin was injected. Measurements on these samples provided the normal levels of the various substances under investigation and the changes attributable to the effect of repeated bleeding. Two lambs were each given 0.1 mg epsilon toxin intravenously; a third received 0.5 mg via the same route; the remaining two lambs received 0.2 mg of toxin subcutaneously.

Sampling technique and storage of samples. Blood samples were collected from the jugular vein before injection of toxin and at 30-min. intervals thereafter or immediately before death. The following samples were collected in evacuated tubes: 3 ml of blood in tubes containing 0.06 ml of EDTA 7.5% w/v (Venoject Tubes, Terumo Corporation), for cAMP determinations and haematology; 3 ml of blood in tubes containing 6 mg of potassium oxalate and 7.5 mg of sodium fluoride (Venoject Tubes, Terumo Corporation), for blood glucose determinations; 5 ml of blood in tubes containing EDTA and glutathione for catecholamine determination (Vacutainer, Becton Dickinson). Immediately after collection the tubes were placed on ice. Sufficient whole blood was removed for the haematological determinations. All samples were then centrifuged in a refrigerated centrifuge. Portions of plasma for catecholamine and cyclic nucleotide determinations were stored at -20°C . Plasma for glucose determinations was held for not more than 2 h at 4°C before glucose analyses were performed.

Assay methods. Assays for adrenaline, noradrenaline and dopamine were performed according to a modification of the radioenzymatic method of Passon and Peuler (1973) (Upjohn Diagnostics, Kalamazoo, USA). cAMP was assayed by a protein-binding radioassay (Radiochemical Centre Amersham). cGMP levels were determined by a radioimmunoassay method (Radiochemical Centre Amersham). Glucose was determined by an enzymatic (glucose oxidase, peroxidase) method (Boehringer Mannheim). Haemoglobin determinations were made in a Coulter Electronics haemoglobinometer and red-blood-cell counts (RBC) with a Coulter Counter. Haematocrits were determined by the microhaematocrit method.

RESULTS

Bleeding of five normal sheep at 30-min. intervals resulted in little change of plasma glucose, cAMP and cGMP levels. Haematocrit, haemoglobin concentration and RBC were significantly reduced. The results are summarised in table I.

The three sheep that were given intravenous injection of epsilon toxin died within 5 min. and the catecholamine and cAMP levels increased dramatically during this time (table II). Blood glucose concentration fell in the two sheep that received 0.1 mg toxin and was raised in the sheep receiving 0.5 mg toxin (table II). In the latter sheep, which also showed the greatest adrenaline and noradrenaline response, a distinct haemoconcentration occurred which probably indicated rapidly developing oedematous changes. The haematological pictures did not change appreciably in the two sheep given 0.1 mg of epsilon toxin (table II).

The two sheep that were given toxin by subcutaneous injection died 55 min. and 90 min. later. Sheep no. 5 developed distinct signs of discomfort within 10 min. of the injection and discomfort became apparent in sheep no. 6 after about 40 min. Both animals became restless and anxious, frequently lying down and getting up; they became incoordinated, then weak and unable to rise and finally collapsed completely. Respiratory rates increased markedly. No clinical examination of the animals was made because this might have affected catecholamine levels. Changes in catecholamine and cAMP levels appeared to coincide with the onset of symptoms. The increases in catecholamine and cAMP levels were remarkable; sheep no. 5 showed a 30-fold increase in cAMP and a 200-fold increase in adrenaline levels (table III). Blood glucose concentrations were greatly increased. Haematological changes indicated a moderate haemoconcentration.

TABLE I
Mean values of some biochemical and haematological measurements in five normal sheep bled three times at 30-min. intervals

Time of bleeding (min.)	cAMP		cGMP		Glucose		Haematocrit		Haemoglobin		RBC	
	nmol/litre (% change)	SD*	nmol/litre (% change)	SD	mmol/litre (% change)	SD	cell/blood ratio (% change)	SD	g/litre (% change)	SD	10 ⁶ /µlitre (% change)	SD
0	44	10.7	15.0	3.1	3.3	0.62	0.31	0.05	112	20	11.8	2.7
30	38	8.7	14.0	5.1	3.6	0.72	0.29	0.05†	105	18†	11.0	2.1
60	41	10.3	16.0	3.2	3.4	0.83	0.28	0.05†	102	17†	10.6	2.0†
	(-6.8)		(+6.7)		(+3.0)		(-9.7)		(-7.1)		(-8.9)	

*SD = Standard deviation.

† † test between 0 min. value and 30 or 60 min. value; $p < 0.05$.

TABLE II
Biochemical and haematological changes in three sheep before and after intravenous injection of *Clostridium welchii* epsilon toxin

Sheep no.	Dose of toxin (mg)	Dopamine		Noradrenaline		Adrenaline		cAMP		cGMP		Glucose		Haematocrit		Haemoglobin		RBC	
		(ng/litre) at min.		(ng/litre) at min.		(ng/litre) at min.		(nmol/litre) at min.		(nmol/litre) at min.		(mmol/litre) at min.		(cell/blood ratio) at min.		(g/litre) at min.		(10 ⁶ /µlitre) at min.	
1	0.5	120	250	528	12653	67	10849	46	118	15	17	3.8	5.8	0.33	0.38	116	137	11.0	13.0
2	0.1	0	297	617	5692	119	3066	58	172	16	17	2.7	1.8	0.30	0.31	108	109	10.2	10.2
3	0.1	4	53	222	2999	48	222	34	111	12	30	4.1	2.5	0.34	0.33	128	124	14.0	13.5

TABLE III
Biochemical and haematological changes in two sheep after subcutaneous injection of 0.2 mg of C. welchii epsilon toxin.

Sheep no.	Time of bleeding (min.)	Dopamine (ng/litre)	Noradrenaline (ng/litre)	Adrenaline (ng/litre)	cAMP (nmol/litre)	cGMP (nmol/litre)	Glucose (mmol/litre)	Haematocrit (cell/blood ratio)	Haemoglobin (g/litre)	RBC ($10^6/\mu\text{litre}$)
5	0	10	1427	72	35	16	3.4	0.28	91	9.7
	30	586	4503	4887	360	21	9.6	0.24	88	9.5
	50	1413	11876	15355	1120	28	11.7	0.30	96	10.5
6	0	11	354	64	40	9	3.4	0.22	80	8.2
	30	0	364	47	52	17	5.9	0.22	80	8.3
	60	262	2998	1741	108	22	6.8	0.25	92	8.9
	90	498	8948	4852	520	25	7.0	0.26	89	9.1

DISCUSSION

Because of the acute nature of the disease a detailed investigation of field cases of enterotoxaemia is seldom possible. Injection of epsilon toxin appears to reproduce all the known symptoms and pathological changes encountered in the natural disease. We therefore believe that the results obtained in our experiments should closely parallel those seen in natural cases, in which similarly activated toxin is absorbed from the small intestine.

The most remarkable findings in this study were the dramatic increases that occurred in cAMP and catecholamine levels. The rise in plasma cAMP in response to epsilon toxin confirms the findings of Buxton (1978) in mice. Buxton reported increases of only 47% whereas we measured increases of up to 30-fold. This may be because our animals were allowed to die and were bled in the terminal stages of the disease. Buxton's mice were killed at a fixed time after injection, possibly before severe symptoms were established. It was clear from our results that there was a correlation between the onset of symptoms and changes in the various plasma constituents tested. AMP levels increased sharply and progressively until the time of death. The finding that massive increases in catecholamine levels also occurred suggests that cAMP increases were due to catecholamine activation of adenylyl cyclase. Exhaustion of liver glycogen as seen by Gardner (1973c) and hyperglycaemia are thus readily explained.

cGMP levels were also increased in one lamb in which the toxin was injected intravenously and in both lambs given epsilon toxin subcutaneously. It has been suggested that adrenaline stimulation of β -adrenergic receptors could give rise to activation of adenylyl cyclase while stimulation of α -adrenergic receptors causes activation of guanylyl cyclase (Goldberg, O'Dea and Haddox, 1973). The rises in cGMP can thus also be explained in terms of a response to catecholamine stimulation. In this experiment the number of animals used was small, and the comparatively small increases in cGMP levels will need to be confirmed in further experiments. The remarkable increases in catecholamine and cAMP levels leave no doubt about their significance and render statistical analysis superfluous. Catecholamine assays were not performed on control plasma samples because they showed no rise in glucose or cAMP levels.

Adrenaline and noradrenaline levels were greatly increased. This suggests a generalised activation of the sympathetic nervous system resulting in a stimulation of the adrenal medulla. A rapidly developing brain oedema might provide such a stimulus. To avoid disturbing the animals more than absolutely necessary clinical examinations were not performed. We have, however, observed that intravenous injection of 2 mg of adrenaline into sheep causes the heart rate to increase to over 250 beats per min. It is thus conceivable that the levels of catecholamines encountered in our experiments even cause cardiac flutter or fibrillation and ultimately contribute to the death of the animal. Further investigations of the effect on heart function are indicated.

Small but definite changes in haematological values indicate that there is a significant leakage of plasma from the blood vessels. Although the numbers of sheep used were too small to warrant statistical analysis, our findings do confirm those of Gardner (1973a). In Gardner's experiments the changes were much more dramatic with haematocrit and haemoglobin concentration increasing by about 50%, whereas in our experiments increases were in the region of 20% during the last 30–60 min. before death (table III). At necropsy our animals showed only slight congestion and oedema of the lungs whereas Gardner considered lung oedema to be a prominent finding. We are unable to explain this difference; it could be due to differences in the toxins or in the breeds of sheep used.

SUMMARY

Injection of *Clostridium welchii* (*C. perfringens*) type D epsilon toxin into sheep caused large increases in catecholamine and cyclic adenosine 3',5'-monophosphate levels and moderate increases in cyclic guanosine 3',5'-monophosphate levels. Haemoconcentration also occurred. It is suggested that a rapidly developing brain oedema is the stimulus for a release of catecholamines which in turn activates adenylyl cyclase. The resulting rise in cAMP causes glycogenolysis and hyperglycaemia.

We wish to thank the University of Pretoria Research Fund for financial assistance, the Weil Organisation for cAMP and cGMP kits and Professor J. W. Nel of the Faculty of Agriculture, University of Pretoria for the lambs. We would also like to thank Dr K. J. Kühne for helpful discussions.

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