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SEA NETTLE AND MAN-O'WAR VENOMS: A CHEMICAL COMPARISON OF THEIR VENOMS AND STUDIES ON THE PATHOGENESIS OF THE STING*

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ABSTRACT

Sea nettle and Portuguese Man-O'War venoms have many common characteristics and several differences. The mouse lethal factors of both jellyfish have molecular weights of approximately 150,000 and isoelectric points of 4.0–4.5 and 6.3–6.5. At least nine lethal fractions can be isolated from each jellyfish by preparative gel electrophoresis. Only sea nettle venom could be easily purified by ammonium sulfate precipitation. The pain induced by the sea nettle sting may be produced by histamine, histamine releasers, serotonin, kinins, or prostaglandins, all of which are associated with the venom. At this time, no correlation can be made between the enzymatic content of these venoms and their toxicologic actions.

The nematocyst venoms of the Portuguese Man-O'War (*Physalia physalis*) (P) and the sea nettle (*Chrysaora quinquecirrha*) (C) contain toxic proteins and at least 6 and 7 injurious enzymes respectively [1–3]. These unpurified venoms have multiple toxic actions including: mouse lethality, dermonecrosis, neurotoxicity, hemolysis, cardiotoxicity, myotoxicity, mitochondrial lysis, and the ability to interfere with the transport of ions such as sodium and calcium [1, 2, 4–12]. Attempts to correlate these actions with the chemical components of the venoms have been unsuccessful because the chemical characteristics of the venoms render their purification by gel filtration difficult [1, 2]. For this reason, attempts to purify the venoms by ammonium sulfate precipitation, ultracentrifugation, isoelectric focusing, and gel electrophoresis were undertaken.

MATERIALS AND METHODS

Nematocyst suspensions from sea nettles and Portuguese Man-O'War jellyfish were prepared by density gradient centrifugation [1, 2]. The organelles were ruptured by extrusion pressure and the venoms (nematocyst venom—NV) were separated from the nematocyst capsules by centrifugation [1, 2]. The protein concentration and lethal activity of these venoms were determined according to previously described techniques [4].

The presence of the following enzymes in NV was quantitated by known methods: ATPase, AMPase, DNase, acid protease, alkaline protease, fibrinolysin, hyaluronidase, RNase, and nonspecific aminopeptidases [3]. All enzymatic determinations were performed on aliquots containing at least 0.5 mouse intravenous-LD_{50} doses.

Molecular weights were estimated by band velocity centrifugation in a Spinco Model E analytic ultracentrifuge (50,000 RPM, 20° C, AN E head). The venoms were suspended in a buffer containing 1 M NaCl, 10 mM EDTA, and 10 mM Tris, pH 7.2. Adsorption prints were taken during the run. These were later analyzed on a Joyce–Loebl microdensitometer. Preparative sucrose gradients (5–20% in the above buffer, 5.5 ml volume) were centrifuged in a Model L Spinco preparative ultracentrifuge equipped with a #SW39 rotor (39,000 RPM, 4° C, 26 hr). Fractions of 0.5 ml volume each were collected by puncturing the bottom of the plastic tube. The EDTA present in these fractions was neutralized with calcium before bioassay in the mouse system.

Electron microscopy was performed on negatively stained specimens by previously established procedures [13]. Specimens were photographed in a Siemens 101 electron microscope.

Some purification of the venoms was obtained by the addition of $(NH_4)_2\ SO_4$ to a 20% concentration. It was necessary, however, to remove the salt from both the resultant supernatant and sediment fractions by dialysis against neutral phosphate buffer before any toxicologic testing was done.

Isoelectric focusing and disc gel electrophoresis were carried out by previously described techniques [4, 5]. Preparative gel electrophoresis was conducted at 4° C using a jacketed column placed on a Buchler fractophorator apparatus. Overnight runs were made at 15 MA.

Oxidative phosphorylation of rat liver mitochondria preparations was measured polarographically using an oxygen electrode at 25°C as previously described [10].

Cutaneous pain was assayed on the volar forearm skin of 3 volunteers per experiment. One drop of the test or control material was placed on alcohol-cleaned skin which was pricked with a sterile needle in a manner similar to that used for vaccination. In order to evaluate the factors responsible for this cutaneous pain, the role of chemical mediators of inflammation was examined. Vascular permeability was measured by injecting the toxin (0.15 ml) or a control sample into rat, mouse, or guinea-pig skin. All specimens were diluted with 2–8 N saline to obtain isotonicity. Twenty min later Evans blue dye was injected intravenously (0.2 ml of 0.5% Evans blue dye or 5 ml/kg of 2% solution in mice and rats or guinea pigs, respectively). Twenty to 30 min after the dye injection the animal was sacrificed and the diameter of the blue spot on the under surface of the skin was measured. Histamine content was assayed chemically and by bioassay [14, 15]; the prostaglandin content of NV was measured by a radioimmunoassay technique [16]. SRS-A and kinin contents were measured by bioassay [17, 18].

Several experiments were performed in mice and rats to test the effect of various drugs upon the vasopermeability activity of C-NV. The test drug was administered intraperitoneally 10–20 min before subcutaneous injec-

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tion of the toxin or control solution. The drugs employed were obtained commercially and included: methysergide, diphenhydramine, hydrocortisone sodium succinate, and pyrilamine maleate. Serotonin, histamine, prostaglandin E_2, carboxypeptidase B (hog pancreas), and nitrogen mustard were also purchased commercially.

RESULTS

Centrifugation

Sea nettle nematocyst venom (C-NV) was initially examined in the analytic ultracentrifuge and found to contain proteins primarily in two density regions. A fraction of protein of average molecular weight 150,200 was detected but the bulk of the protein had an average molecular weight of approximately 19,000. Trace amounts of protein were present in two other molecular weight ranges (525–685 and 1925–3750). The venom was inoculated onto a 5–20% sucrose gradient and centrifuged in such a manner that a protein with a molecular weight of 19,000 would be in the center of the tube. Only those fractions at the bottom of the tubes possessed lethal activity. These data indicate that the protein with a molecular weight near 150,000 is lethal to mice.

Electron Microscopy

C-NV contained multiple ultramicroscopic fibrils after centrifugation at 50,000 $\times$ *g* for 1 hr. These fibrils were a few microns long and 0.1 mμ thick. All these structures could be precipitated with 20% $(NH_4)_2SO_4$. The resultant supernatant fraction after 20% $(NH_4)_2SO_4$ precipitation (NVS) contained only electron-dense round or oval particles (88 $\times$ 70 Å) representing protein structures free of particulate matter. This supernatant fraction (NVS) was repeatedly found to adsorb to Sephadex gels (pH 7–8.6) as did both C- and P-NV [1, 2].

Ammonium Sulfate Precipitation

The sediment after 20% $(NH_4)_2SO_4$ precipitation and the various fractions obtained by high-speed density gradient (5–20% sucrose) centrifugation of NVS from both jellyfish were analyzed for enzymatic activity, lethal potency, mitochondrial lysis, and the ability to produce human cutaneous pain (Tables I and II). The lethal, dermonecrotic and pain-producing activities were present in the denser portions of the supernatant fractions. Cutaneous pain was also detected in the lighter fractions of C-NVS. Mitochondrial damage and dermonecrosis were produced by the sediments.

Isoelectric Focusing and Preparative Electrophoresis

NVS from both jellyfish was subsequently purified by isoelectric focusing and preparative gel electrophoresis. Two peaks (isoelectric points 6.3 and 4.0) which contained lethal material were found after isoelectric focusing of C-NVS, thereby corroborating our earlier data [5]. Almost similar results were obtained with P-NVS which contained lethal activity in fractions having isoelectric points of 4.5 and 6.5. In both P and C-NVS, most of the lethal activity was present in the fraction having an isoelectric point above 6 [5]. Some lethal fractions obtained by isoelectric focusing of both C- and P-NVS also had atypical aminopeptidase and RNase activities (Table III).

Preparative gel electrophoresis separated at least nine lethal fractions in NVS from both jellyfish (Figs. 1, 2). Some of these lethal fractions contained atypical aminopeptidases. No other enzymes were detected and no exact correlation of lethal and enzymatic activity could be made.

Analytic disc gel electrophoresis was performed on various preparations. Four bands were visible in gels inoculated with C-NVS (0.5 mg protein) whereas three bands could be detected in gels inoculated with P-NVS (0.25 mg protein).

Single bands were visible in gels inoculated with lethal fractions obtained from *Chrysaora* or *Physalia* NVS purified by preparative gel electrophoresis (Fig. 3). Two bands were visible in a gel inoculated with 0.9 mg protein from the fraction of *Chrysaora* NVS purified by isoelectric focusing which had an isoelectric point of 4 (Fig. 3) and multiple bands were present in a disc gel prepared from the P-NVS fraction with an isoelectric point of 6.5 after isoelectric focusing.

The ammonium sulfate precipitation and preparative gel electrophoresis steps were adequate purification procedures for sea nettle venoms only (Table IV).

Cutaneous Reactions of the Toxins and Study of Chemical Mediators of Inflammation

The subcutaneous injection of 1 mouse IV LD_{50} was the threshold dose of C-NV required to consistently produce visible vasopermeability in mice or rats with Evan's blue dye. Nonlethal (heat inactivated, 56° C, 1 hr) C-NV was equipotent with fresh C-NV in producing vasopermeability. Hydrocortisone (10 mg IP to mice) prevented histamine (27.5 μg) from producing blue staining but was ineffective against C-NV. The antihistamines, diphenhydramine (0.25 mg IP to mice) and pyrilamine maleate (50 mg/kg IP to mice and rats), blocked the appearance of blue spots after both histamine and C-NV. Microgram quantities of histamine were detected in C-NV. In addition, each of 4 rats injected IP with C-NV (20 LD_{50} doses in 0.3 ml) and subsequently sacrificed released 2.5–3.0 mg histamine but no detectable SRS-A (technique sensitive to 1 unit) into their peritoneal cavity.

Serotonin was found to produce vasopermeability in mice if injected in skin at doses of 0.1 μg or larger. Methysergide (4 mg/kg), a serotonin antagonist, could prevent the vasopermeability effect of 1 LD_{50} C-NV as well as 0.5 and 10 μg serotonin in the mouse and rat respectively. The size of the vascular leakage resulting from local C-SU injection was not reduced in mice made leukopenic by nitrogen mustard (1.75 mg/kg IV, a dose which reduced the polymorphonuclear cell count by 80% in 5 days).

TABLE I

Chrysaora *nematocyst toxin*

Activity	Substrate	Supernatant fraction												
		1 (heavy)	2	3	4	5	6	7	8	9	10	11	12	Sediment
ATPase	ATP	1.15	1.15	0	0	0	0	0	0	0	0	0	0	0.0037
DNase[a]	DNA	0	0	0	920	0	410	720	350	120	0	0	0	20
Hyaluronidase[b]	Hyaluronic acid	1.70	1.85	1.20	0.98	1.02	1.37	0.67	0	0	0	0	0	0.287
RNase	RNA	0.002	0	0	0.005	0.001	0.005	0.004	0.003	0.002	0	0	0	0.03
Nonspecific aminoesterase	L leucyl-B napthylamide	0	.246	.645	1.43	0.62	0.17	0.05	0	0	0.03	0	0	0.52
	L alanyl-B napthylamide	0	0	0.43	0.46	0.26	0	0.09	0.04	0	0	0	0	0.15
Acid protease[c]	^{14}C hemoglobin	0	0	0	1+	2+	3+	4+	3+	2+	0	0	0	0
Alkaline protease[d]	Azocasein	0	0	0	2.23	2.65	3.14	2.69	0.69	0	0	0	0	0.40
Lethality		+	+	+	0	0	0	0	0	0	0	0	0	0
Pain		±	0	0	0	0	+	0	+	+	0	0	0	0
Mitochondral lysis		0	0	0	0	0	0	0	0	0	0	0	0	+
Dermonecrosis		+	+	0	0	0	0	0	0	0	0	0	0	+

Enzyme content and toxicity in the supernatant fractions and precipitate following ammonium sulfate treatment of *Chrysaora* nematocyst toxin.

All specimens contain 0.5 mouse intravenous LD_{50} doses. Ammonium sulfate was added to *Chrysaora* nematocyst venom to a concentration of 20%. The supernatant was centrifuged in a 5–20% sucrose gradient. These fractions plus the ammonium sulfate precipitate were analyzed.

[a] Activity expressed as Kunitz units/mg protein/min.

[b] Expressed as μg hyaluronic acid hydrolyzed/mg protein/min.

[c] In the absence of a recognized standard for acid protease only comparative values are given.

[d] Expressed as change of optical density/mg protein/min. Unless indicated all other values are expressed as μM/μg protein/min.

TABLE II

Physalia *nematocyst toxin*

Activity	Substrate	Supernatant fraction												
		1 (heavy)	2	3	4	5	6	7	8	9	10	11	12	Sediment
ATPase	ATP	0	0	0	0	0.048	0.047	0.055	0.015	0	0	0.040	0	0
AMPase	AMP	0.022	0.012	0.021	0	0	0	0	0	0	0	0	0.01	0.01
RNase	RNA	0	0	0	0	0	0	0	0	0	0.02	0	0	0.006
Nonspecific aminoesterases	L leucyl-B napthylamide	0.146	0.023	0	0	0	0.015	0.037	0	0	0	0.07	0	0.004
	L alanyl-B napthylamide	0.05	0.05	0.32	0.22	0.11	0.03	0.03	0	0	0	0	0.05	0.04
Lethality		+	+	+	0	0	0	0	0	0	0	0	0	0
Pain		0	0	+	0	0	0	0	0	0	0	0	0	0
Dermonecrosis		+	+	0	0	0	0	0	0	0	0	0	0	+

DNase, fibrinolysin, and mitochondrial lytic activity could not be found.

Enzyme content and toxicity in the supernatant fractions and the precipitate following ammonium sulfate treatment of *Physalia* nematocyst toxin.

All specimens tested contain 0.5 mouse intravenous LD_{50} doses. Ammonium sulfate was added to *Physalia* nematocyst venom to a concentration of 20%. The supernatant was centrifuged in a 5–20% sucrose gradient. These fractions were analyzed along with the ammonium sulfate precipitate.

For units, see legend to Table I.

TABLE III

Chrysaora

Fraction	pH	Lethality	RNase	Aminoesterases-hydrolysis of:	
				L alanyl-B-napthylamide	L leucyl-B-napthylamide
1	1.8	0	.33	0	0
2	2.6	0	0	0	0.0004
3	3.1	0	.67	0	0
4	4.5	+	.67	0	0.0006
5	5.5	0	.67	0.005	0
6	6.3	+	.50	0.0003	0
7	7.5	0	.45	0	0
8	8.8	0	.33	0	0.0013
9	10.0	0	0	0	0
10	11.3	0	0	0	0

Negative enzymes: DNase, hyaluronidase

Physalia

Fraction	pH	Lethality	RNase	ATPase	AMPase	Aminoesterases-hydrolysis of	
						L alanyl-B-napthylamide	L leucyl-B-napthylamide
1	1.9	0	0	.005	0	0	0
2	2.8	0	0	.015	.001	0	0
3	3.7	0	0	.017	.020	0	0
4	4.7	+	.33	.008	.029	.024	.0033
5	5.4	0	0	.006	.007	0	0
6	6.5	+	0	.077	.003	0	0
7	7.7	0	.095	.070	.003	0	0
8	8.2	0	0	.004	.0006	0	0
9	9.4	0	0	.002	.002	0	0
10	10.9	0	0	.001	.002	0	0
11	11.5	0	0	0	0	0	0

Negative enzymes: Fibrinolysin, DNase

A comparison of lethal and enzymatic activities in *Chrysaora* and *Physalia* NVS following isoelectric focusing. All specimens tested enzymologically contained at least 0.5 mouse intravenous LD_{50} doses. For units, see legend to Table I.

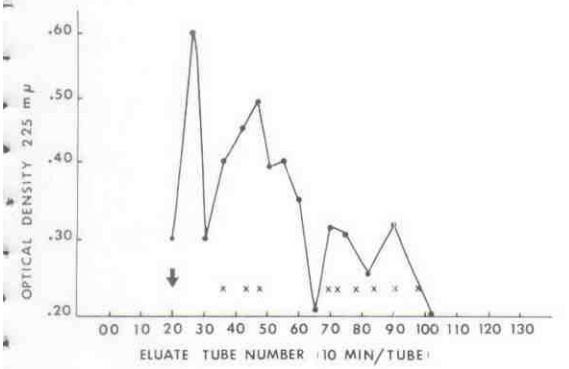

FIG. 1: Preparative gel electrophoresis of *Chrysaora* NVS. The arrow indicates the elution of bromophenyl blue (tracking dye). The × symbols identify lethal fractions. Electrophoresis of 200 mouse IV LD_{50}'s was at 15 MA 4° C. The optical density curve indicates relative protein concentration. (After treatment with 20% NH_4SO_4)

An aliquot of the same C-NV pool which contained histamine (67 LD_{50} and 14.4 mg protein/ml) also contained 4000 picograms of prostaglandin E and 370 picograms of prostaglandin A per ml. The etiologic role of prostaglandins is uncertain since the injection of prostaglandin E_2 (30–300 μg) into guinea-pig skin produced marked erythema but not urticaria and therefore did not clinically resemble the reaction produced by 1 LD_{50} C-NV.

Other aliquots of the identical C-NV pool were found to be equivalent to 500 mg bradykinin/ml in gut-contracting ability in the guinea-pig ileal loop preparation. However, carboxypeptidase B (42 units in 150 μg) in contact with 1.0 LD_{50} C-NV for 15 min at 37° C inactivated its vasopermeability and gut-contracting effects but did not alter its mouse lethality nor dermonecrotic potency, indicating that the kinin is not the etiologic agent for the latter two actions.

It appeared that the threshold for human pain

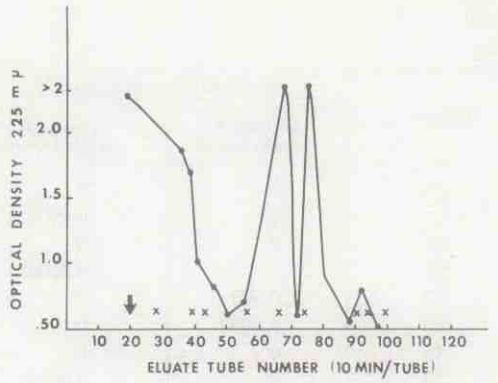

FIG. 2: Preparative gel electrophoresis of *Physalia* NVS. The arrow indicates the elution of bromophenyl blue (tracking dye). The × symbols identify lethal fractions. Electrophoresis of 200 mouse IV LD_{50}'s was at 15 MA, 4° C. The optical density curve indicates relative protein concentration. (After treatment with 20% NH_4SO_4)

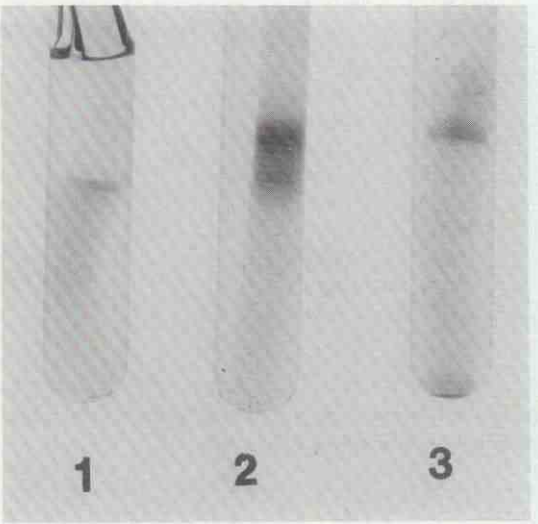

FIG. 3: Analytic disc gel electrophoresis. #1: The first lethal fraction of *Chrysaora* NVS after preparative gel electrophoresis. #2: The lethal fraction of *Chrysaora* NVS with an isoelectric point of 4 purified by isoelectric focusing. #3: The first lethal fraction in *Physalia* NVS after preparative gel electrophoresis.

by the method used was between 0.04–0.20 IV mouse LD_{50} when C- or P-NVS was employed. *Physalia* venom pain persisted for several minutes longer than that of nettle venom. If a similar dosage were inoculated intradermally into a human forearm, piloerection, local sweating, and sometimes erythema were seen. Dermonecrotic activity was present in both NV and NVS of both jellyfish as well as their sediments after 20% $(NH_4)_2SO_4$ precipitation and all lethal fractions obtained after isoelectric focusing. To date, neither vascular permeability nor human cutaneous pain have been detected in the more purified toxin preparations even though they could both be induced by C- or P-NVS having less mouse lethal activity.

TABLE IV

Effect of 20% $(NH_4)_2SO_4$ precipitation and preparative gel electrophoresis upon nettle and Man-O'War venoms

	Sea Nettle (CNV)		Man-O'War (PNV)	
	Fold increase in specific activity	% Loss of total toxicity	Fold increase in specific activity	% Loss of total toxicity
Before 20% $(NH_4)_2SO_4$	← baseline →			
After 20% $(NH_4)_2SO_4$	4.3 ×	0%	1.5 ×	50%
Preparative gel electrophoresis of 20% $(NH_4)_2SO_4$ supernatant	20 ×	30%	2 ×	30%

DISCUSSION

The lethal proteins in sea nettle nematocyst venom have molecular weights of approximately 150,000. These compounds are approximately 8-fold larger than the bulk of the protein present in NV. The fact that the lethal fractions of P-NV sedimented similarly to those of C-NV in a 5–20% sucrose gradient indicated that the lethal proteins of these two jellyfish have similar molecular weights.

The lethal toxins of the sea nettle and the Man-O'War resemble one another in several ways: (1) the NVS from both jellyfish contains two active lethal fractions with similar isoelectric points; (2) the majority of the lethal activity in NVS from each jellyfish has an isoelectric point over 6.0; (3) multiple active fractions from the NVS of each jellyfish can be isolated on preparative gel electrophoresis; and (4) the NVS of each animal as well as the NV adsorbs to Sephadex gels. In spite of these similarities, however, only C-NV was purified by 20% $(NH_4)_2SO_4$. The long fibrils which did not sediment at forces of 50,000 × *g* were found only in C-NV. These structures may be fragmented nematocyst threads and possibly contain lipids which account for their light density. The size of particles seen after ammonium sulfate precipitation is compatible with a structure having a molecular weight of 150,000.

It was previously shown that C- or P-NV contained more than one active agent [6, 11, 12]: one of which was lethal to mice, a relatively heat-labile dermonecrotic factor, and another which altered organelle function. Tables I–III illustrate that P- and C-NV contain multiple additional active fractions. No exact correlation between enzymatic activity and the factors producing mouse lethality, human cutaneous pain, dermonecrosis, and mitochondrial lysis is apparent.

Ammonium sulfate precipitation appears to have destroyed the *Physalia* DNase and fi-

brinolysin and partially inactivated its lethal factor. The DNase and hyaluronidase activities of *Chrysaora* were destroyed during isoelectric focusing. Human cutaneous pain was not detected in specimens from either jellyfish which had been subjected to isoelectric focusing or preparative gel electrophoresis. It is possible that some of these activities may be demonstrated later with more efficient purification techniques.

At this stage of our investigations it is not posible to determine whether the mouse lethal factors in C- or P-NV are one or multiple proteins acting singly or in combination. The observations that several active fractions can be eluted from Sephadex gels [11], isoelectric focusing columns, and preparative electrophoresis gels would support the hypothesis that multiple active proteins were present. However, similar results could be expected from venoms composed of one main active protein moiety which possessed side chains with different charges.

It seems probable that the lethal action in mice of C-NV is due to its action on the cardiac conducting system [9]. Further reports will demonstrate that the cardiotoxicity factor is present in only the lethal fractions after ammonium sulfate treatment.†

The animal skin experiments involving chemical mediators of inflammation demonstrate that histamine, serotonin, kinins, and prostaglandins are present in C venom. Crude nettle nematocyst preparations contained histamine (5 μg/gm dry tissue) [19] as did the C-NV. Moderate amounts of histamine were released after injection of nettle venom into the rat peritoneal cavity. Even though the cutaneous sting of the nettle was not characterized by pronounced erythema, significant amounts of prostaglandins were detected in nettle venoms. The presence of serotonin or a serotonin releaser in the C-NV was suggested by the observation that methysergide prevented the vasopermeability induced by the toxin. Finally, the observation that C-NV and C-NVS‡ contain kinins makes it probable that these substances could also be important in the pathogenesis of the jellyfish sting. It is not possible now to determine the relative importance of these agents in the production of human cutaneous pain.

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† Cranefield PF, Burnett JW, Calton GJ: Unpublished data

‡ Kaplan AP, Burnett JW, Calton GJ: Unpublished data

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