Localization of Potential Binding Sites for the Edema Disease Verotoxin (VT2e) in Pigs

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ABSTRACT

The purpose of this study was to identify organs and cells to which the edema disease verotoxin (VT2e) could bind in pigs. Frozen 4–5 μm thick sections of organs usually affected in edema disease (colon, spinal cord, cerebellum and eyelid) and organs not usually affected (liver, ileum) from two 5- to 6-week-old weaned pigs were permeabilized with acetone, then exposed to VT2e. Unbound VT2e was removed by washing and bound VT2e was detected by immunohistochemistry. In the eyelid, double-label immunofluorescence was used to identify the cells to which VT2e bound. VT2e was shown to bind to all six organs that were examined. The toxin bound to arteries in all organs, to veins in all organs except the liver, and to enterocytes in the ileal crypts. Double labelling of eyelid with monoclonal antibodies specific for von Willebrand factor or α-smooth actin and VT2e showed that the toxin bound to endothelial and vascular smooth muscle cells. The binding of VT2e to endothelium is consistent with findings for other verotoxins but binding to vascular smooth muscle has not been reported for other verotoxins. It is concluded that i) factors other than the presence of receptors for VT2e influence the development of lesions in edema disease, and ii) smooth muscle necrosis, which is characteristic of the vascular lesions in edema disease, may be due to a direct action of toxin on smooth muscle cells.

RÉSUMÉ

Afin de déterminer les organes et cellules auxquels la vérotoxine (VT) 2e peut se lier chez le porc souffrant de la maladie de l'œdème, des sections congelées de 4 à 5 μm provenant d'organes affectés (côlon, moëlle épinière, cervelet, paupière) de même que d'organes non-affectés (foie, iléon) prélevés chez des porcelets sevrés âgés de 5 à 6 semaines ont été perméabilisés avec de l'acétone, puis exposés à la VT2e. La VT2e non-liée fut enlevée par lavage et la VT2e liée détectée par analyse immunohistochimique. Au niveau de la paupière, l'analyze par immunofluorescence à deux fluorochromes fut utilisée afin d'identifier les cellules auxquelles se liait la VT2e. Il a été trouvé que la VT2e se liait aux six organes examinés. La toxine se liait aux artères et aux veines de tous les organes sauf les veines du foie, et aux entérocytes des cryptes iléales. Le double marquage de la paupière avec un anticorps monoclonal spécifique au facteur de von Willebrand ou à l'actine lisse-alpha et à la VT2e a permis de démontrer que la toxine se liait aux cellules endothéliales et aux cellules des muscles lisses des vaisseaux. L'attachement de VT2e à l'endothélium est conséquent aux trouvailles faites pour les autres vérotoxines mais l'attachement aux cellules des muscles lisses des vaisseaux n'a pas été rapporté pour les autres vérotoxines. En conclusion, 1) des facteurs autres que la présence de récepteurs pour la VT2e influencent le développement des lésions lors de maladie de l'œdème, et 2) la nécrose des muscles lisses, qui est caractéristique des lésions vasculaires lors de maladie de l'œdème, pourrait être causée par une action directe de la toxine sur les cellules des muscles lisses.

(Traduit par docteur Serge Messier)

INTRODUCTION

Enteric infection with verotoxin (VT)-producing *Escherichia coli* (VTEC) is associated with edema disease (ED) in pigs (1,2), hemorrhagic colitis in calves (3) and humans (4), and the hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura in humans (4,5). Verotoxins (VTs, also called Shiga-like toxins) belong to 2 antigenic groups: VT1, which is identical to Shiga toxin produced by *Shigella dysenteriae*; and the VT2 group, which consists of VT2 and closely related antigenic variants (6). VT1 and VT2 are implicated in the diseases that occur in humans and calves. VT2e, a variant of VT2, is associated with ED of weaned pigs, which is characterized by neurological signs and edema formation in a wide variety of tissues (7), and has recently been detected in *E. coli* strains of O group 101, implicated in hemorrhagic colitis and HUS in humans (8).

The common feature of all VTEC-associated diseases is the presence of vascular lesions in target organs. The vascular lesions are considered to be due to VT, which can cause cell death

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This work was supported by a grant from the Natural Sciences and Engineering Research Council.

Received May 2, 1997.

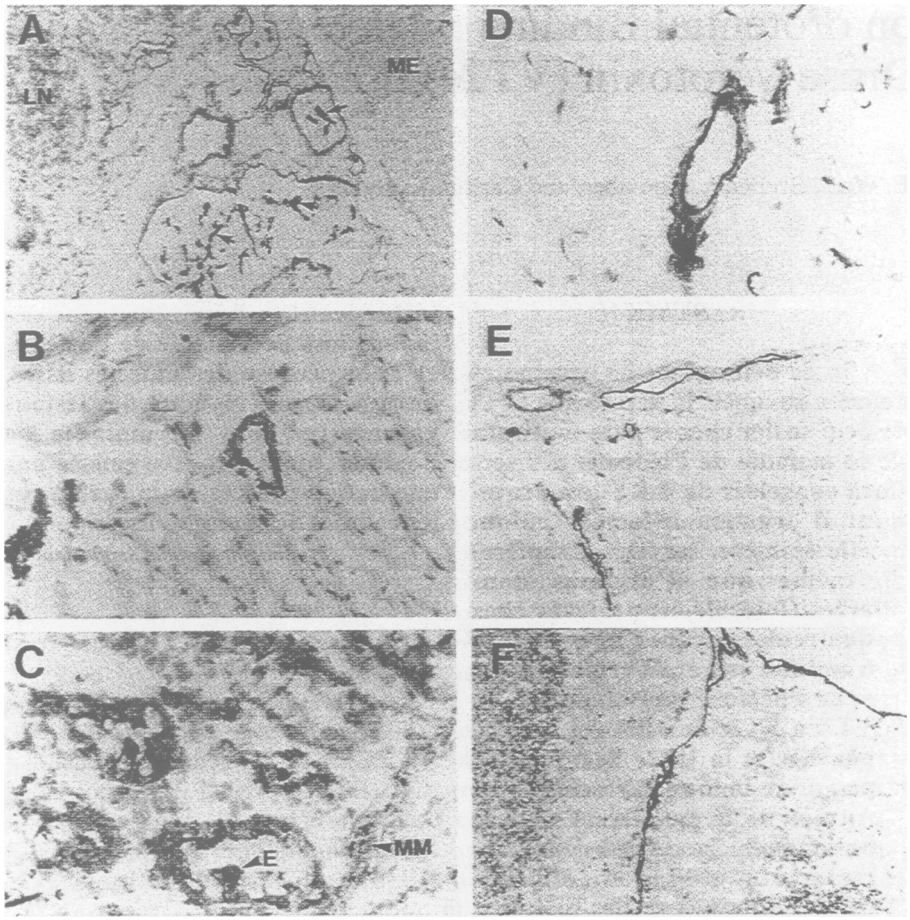

Figure 1. Immunohistochemical detection of VT2e binding to blood vessels in pig ileum (A,B,C), eyelid (D), spinal cord (E), and cerebellum (F). Frozen sections of tissue were overlaid with VT2e and bound toxin was visualized via immunohistochemical staining using rabbit anti-VT2e and aminoethylcarbazole substrate, which forms a red insoluble reaction product. Sections were counterstained with hematoxylin. In A, note the presence of toxin-binding red blood cells within the blood vessels (arrows). LN — lymphoid nodule of Peyer's patch, ME — muscularis externa, MM — muscularis mucosa, E — enterocyte. Magnification A,E,F 80 ×; B,C,D 275 ×.

by inhibiting protein synthesis in receptor-containing endothelial cells (9–11). VT can bind to other cell types, including lymphocytes (12) and pediatric glomerular mesangial cells in vitro (13). A role in disease has been suggested for binding to glomerular mesangial cells, in which there is dose-dependent inhibition of mitosis following adherence of VT1. The functional receptor for members of the VT1 and VT2 group of toxins is considered to be the glycosphingolipid globotriaosyl ceramide (Gb3) (14,15). However, VT2e (although capable of using Gb3 as a functional receptor) is thought to use globotetraosyl ceramide (Gb4) as its preferred functional receptor (16,17).

The vascular lesions in ED are characterized by endothelial cell swelling, vascular smooth muscle necrosis and perivascular edema (18,19). Certain organs, namely, brain, spinal cord, eyelids, the stomach, mesenteric lymph nodes, cecum and the colon are frequently damaged in animals with ED, whereas other organs such as liver, spleen and small intestine are less frequently affected (18). In verotoxemias involving VT1 or VT2, smooth muscle necrosis has not been reported as a feature of the vascular lesions. Thus, the development of smooth muscle necrosis in ED may be related to the cells to which toxin is targeted in pigs. This investigation, which involved detection of VT2e bound to swine tissues, was designed to test the hypothesis that endothelial cells and vascular smooth muscle cells are potential sites of direct action of VT2e in ED.

MATERIALS AND METHODS

PREPARATION OF PURIFIED VT2e

VT2e was purified from *E. coli* JM101(pGT110), which contains the VT2e genes in the expression vector pKK223-3. An overnight culture in brain heart infusion broth (BHIB, DIFCO, Detroit, Michigan, USA) containing 100 μg/mL ampicillin (Sigma, St. Louis, Missouri, USA) was seeded 1/400 into BHIB containing 100 μg/mL ampicillin. The culture was grown at 37°C with shaking at 200 revolutions per minute (rpm) to an optical density of 0.6 (λ = 600 nm) then induced with 1 mM IPTG (Xymotech, Montreal, Quebec). Approximately 12 h later the bacteria were collected by centrifugation, washed once with phosphate buffered saline (PBS, pH 7.2) and suspended in PBS to 1/10th the original volume. Polymyxin B sulfate was added to a concentration of 0.4 mg/mL and the suspension was shaken at 150 rpm for 2 h at 37°C; cells were then removed by centrifugation followed by filtration through a 0.45 μm cellulose acetate filter (Gelman Sciences, Montreal, Quebec). Solid ammonium sulfate (Fisher Scientific, Mississauga, Ontario) was added to 40% saturation and the solution was stirred at 4°C for 2 h. Precipitated protein was removed by centrifugation (24 000 × *g* for 30 min) then ammonium sulfate was added to make the supernatant 60% saturated. The stirring and centrifugation steps were repeated. The pellet was dissolved in water, centrifuged (24 000 × *g* for 10 min), filtered through a 0.2 μm syringe filter (Gelman), desalted through a Sephadex G50 column (Pharmacia, Baie d'Urfe, Quebec), and equilibrated with 10 mM Na_2HPO_4 buffer containing 100 mM NaCl (pH 6.0). The void volume was collected and applied, at a flow rate of 3 mL/min, to a 1 mL Econo-Pac S cation-exchange column (BioRad, Mississauga, Ontario) equilibrated with 10 mM Na_2HPO_4 buffer containing 100 mM NaCl (pH 6.0). After washing the column with 15 column volumes of equilibration buffer, bound toxin was eluted, at a flow rate of 3 mL/min, with 5 mL of 10 mM Na_2HPO_4 buffer containing 250 mM NaCl (pH 6.0).

Purified VT2e had a specific activity of 3.2×10^9 tissue culture cytotoxic doses per mg protein when assayed on Vero cells (20). SDS polyacrylamide gel electrophoresis of toxin preparations under reducing conditions showed 3 bands corresponding to the A, A_1, and B subunits of VT2e (20).

PREPARATION OF ANTI-VT2e ANTISERUM

Immune serum to VT2e was prepared by subcutaneous injection of rabbits with glutaraldehyde-inactivated purified VT2e adsorbed to aluminum hydroxide (Alhydrogel, Langford Inc., Guelph, Ontario) containing 0.01% saponin. Rabbits were injected at 2 sites with 25 μg VT2e per site 5 times at 3-week intervals. Preimmune and immune sera were collected, heat inactivated and stored at −20°C. The immune serum was specific for VT2e by Western immunoblot, radial immunodiffusion and immunoelectrophoresis. The anti-VT2e serum neutralized 4 CD_{50}'s VT2e with a titre of 1×10^6 in neutralization assays performed on Vero cells (21).

PREPARATION OF FROZEN SECTIONS

Frozen sections (4–5 mm thick) of ileum, colon, liver, spinal cord, cerebellum and eyelid from two 5- to 6-week-old weaned female Yorkshire-Landrace pigs (Arkell Swine Unit, Central Animal Facility, University of Guelph) were air-dried and permeabilized with absolute acetone at 4°C for 10 min. Acetone fixation was used because it does not remove glycosphingolipid from cells, but does expose the intracellular glycosphingolipid pool (22). Before acetone fixation some sections of eyelid were treated with chloroform/methanol (2:1, vol/vol) to remove glycosphingolipids from cells. Use of the pigs followed the guidelines established by the Canadian Council on Animal Care and the protocols were approved by the University of Guelph Animal Care Committee.

IMMUNOHISTOCHEMICAL PROCEDURE

Fixed sections were incubated with PBS containing 1% bovine serum albumin (Boehringer Mannheim, Laval, Quebec) (BSA-PBS) for 30 min and then overlaid with 1 μg VT2e per mL for 1 h. Following incubation for 1 h with anti-VT2e serum diluted 1/2000 (vol/vol), bound toxin was detected using an ImmunoStain-HP kit (aminoethylcarbazole substrate) (Zymed, San Francisco, California,

TABLE I. Summary of results of labelling pig organs with VT2e[a]

Organ	Vasculature labelled	Other sites labelled[b]
Ileum	Throughout the tissue but variable between and within vessels	Crypt enterocytes, areas of the muscularis mucosae, and cells in the lamina propria
Colon	Throughout the tissue, but variable within and between vessels	None
Liver	Arterial, but not venous vessels	None
Eyelid	Throughout the tissue	None
Spinal cord	Meningeal vessels and small arteries and capillaries	None
Cerebellum	Meningeal vessels and small arteries and capillaries	None

[a] Frozen sections of normal pig organs were air-dried, permeabilized with acetone at 4°C for 10 min, blocked with BSA, then overlaid with VT2e followed by rabbit anti-VT2e. Following washing, bound toxin-antibody complexes were detected with the reagents in an ImmunoStain HP kit
[b] The toxin also labelled red blood cells extensively when they were present in blood vessels

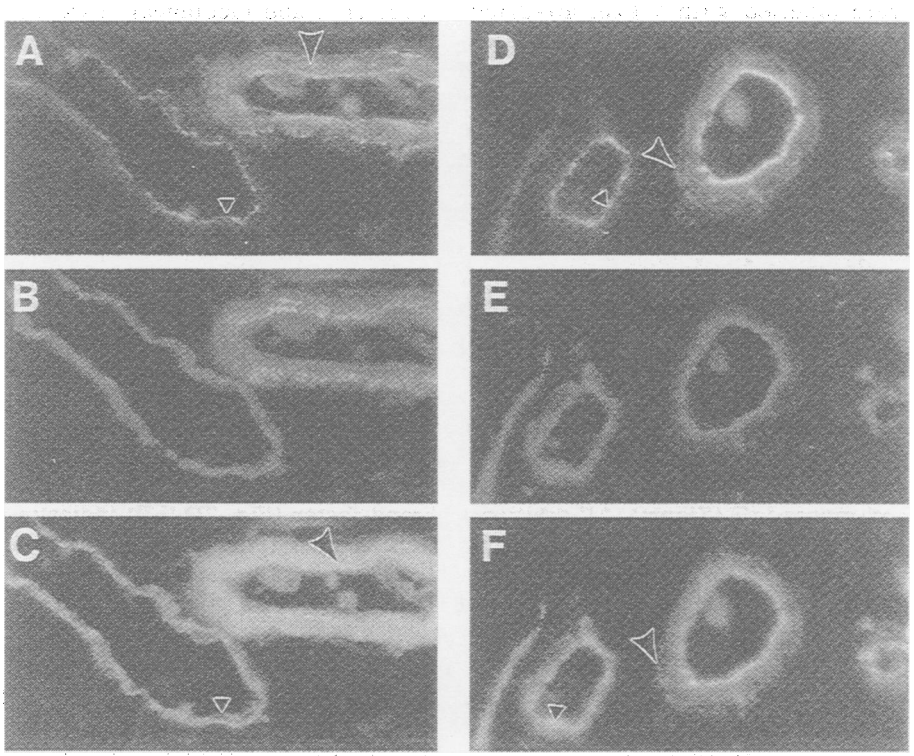

Figure 2. Immunofluorescent detection of VT2e binding to vascular smooth muscle and vascular endothelial cells in blood vessels in pig eyelid. Frozen sections of tissue were overlaid concurrently with VT2e and anti-α-smooth muscle actin (a marker for vascular smooth muscle cells) (A, B, C), or VT2e and anti-Von Willebrand Factor (a marker for vascular endothelial cells) (D, E, F). VT2e was visualized with rabbit anti-VT2e, FITC-conjugated secondary antibody, and MAb with a TRITC-conjugated secondary antibody. A — exposure for FITC showing VT2e binding to a vein (left) and artery (right); the toxin appears to label both endothelium (small arrow head) and smooth muscle (large arrow head). B — exposure for TRITC showing α-smooth muscle actin contained in vascular smooth muscle cells. C — sequential exposure for both FITC and TRITC showing co-localization of VT2e with α-smooth muscle actin (areas which appear yellow, large arrow head) and the outline of endothelial cells labelled with VT2e alone (small arrow head). D — exposure for FITC showing VT2e binding to a vein (left) and artery (right); the toxin appears to label both endothelium (small arrow head) and smooth muscle (large arrow head). E — exposure for TRITC showing von Willebrand Factor contained in vascular endothelial cells. F — sequential exposure for both FITC and TRITC showing co-localization of VT2e with von Willebrand Factor (areas which appear yellow, small arrow head) and the outline of vascular smooth muscle cells labelled with VT2e alone (large arrow head). Magnification 300 ×.

USA). Control sections were not incubated with VT2e, but were incubated with the reagents required to detect the toxin. Toxin and anti-serum were diluted in BSA-PBS. All incubations were at room temperature and the duration of incubation of slides with substrate was approximately 7 min. Slides were counterstained with hematoxylin.

DOUBLE-LABELLING TO IDENTIFY BINDING OF TOXIN TO ENDOTHELIAL AND VASCULAR SMOOTH MUSCLE CELLS

To determine the types of cells that were labelled with VT2e, air-dried sections of eyelid were overlaid concurrently with 1 μg VT2e per mL and either a mouse monoclonal antibody (MAb) specific for α-smooth muscle actin (to identify vascular smooth muscle) (Sigma) (1/300, vol/vol), or a MAb specific for von Willebrand factor (to identify vascular endothelium) (Boehringer Mannheim) (1/250, vol/vol). Bound toxin was visualized with rabbit anti-VT2e (1/1000, vol/vol), followed by fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit F(ab')2 (Boehringer Mannheim), and MAbs with rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma). All dilutions were in BSA-PBS, and incubations were for 1 h at room temperature. Control sections were not incubated with MAb and VT2e, but were incubated with secondary antibodies.

RESULTS

The results of labelling of eyelid, liver, ileum, colon, spinal cord and cerebellum with VT2e are summarized in Table I. In the ileum, VT2e labelled arteries and veins in the submucosa and between the lymphoid nodules of Peyer's patches (Fig. 1A), in the muscularis externa (Fig. 1B), and in the serosa (not shown). The toxin also labelled cells within the lamina propria of the villi, possibly endothelial cells (Fig. 1C), enterocytes at the lower portion of some villi, some areas of smooth muscle cells of the muscularis mucosa (Fig. 1C) and red blood cells (Fig. 1A). Pig red blood cells have been shown to bind VT2e in vivo (16). The toxin did not label enterocytes over the dome regions of lymphoid tissue, or lymphatic vessels. In the colon, VT2e labelled blood vessels in a manner similar to that observed in the ileum, but did not label enterocytes, or smooth muscle cells in the muscularis mucosa. In the colon, cells in the lamina propria of the villi were labelled with toxin, however the frequency of labelling of cells appeared to be less than that observed in the ileum. In the liver, VT2e labelled the arterial, but not venous blood vessels, nor bile ducts and sinusoids (not shown). VT2e labelled blood vessels in the eyelid (Fig. 1D), and meningeal vessels in the spinal cord (Fig. 1E) and cerebellum (Fig. 1F). In the grey matter of the spinal cord, and in the granular and molecular layer of the cerebellum, the toxin labelled small arterioles and capillaries (not shown). The labelling of vessels in the eyelid with VT2e was found to be sensitive to pretreatment of the sections with chloroform/methanol (not shown), which was consistent with the hypothesis that toxin bound to glycosphingolipid in blood vessels.

In blood vessels in the eyelid, VT2e co-localized with α-smooth muscle actin (Fig. 2A,B,C) and von Willebrand factor (Fig. 2D,E,F) indicating that toxin labelled both endothelial cells and smooth muscle cells.

DISCUSSION

There have been several studies to test the hypothesis that target organs in VT-mediated diseases are those with receptors for VT (16,23–26). Most of these studies have examined the concentration of receptor in whole organs rather than in vascular tissues in organs, but Richardson et al (23) examined binding of VT to blood vessels. What has emerged from these studies is that receptors must be present for an organ to be damaged in VT-mediated disease, but presence of receptors in an organ does not mean that the organ will be affected in disease. Thus, Richardson et al (23) and Zoja et al (24) showed that the small intestine and stomach of rabbits contained Gb3 and accumulated significant amounts of intravenously administered VT1 but did not show any VT1-induced lesions. A recent study in pigs examined the relationship between the sites of systemic distribution of intravenously inoculated $^{[125]}$I-labelled VT2e and the tissue content of Gb3 and/or Gb4 (16). The amount of labelled VT2e that was found in tissues after 2 h was not correlated with the tissue content of Gb3 and/or Gb4, but to the amount of blood flow to each tissue. After correcting for blood flow to tissues, the highest specific uptake of toxin occurred in the small intestine mesentery, eyelid, colon mesentery, liver and meninges. Similarly, in the present study, liver, which is not affected in ED and small intestine, which is infrequently damaged, both showed binding of VT2e to blood vessels. These findings indicate that there are factors other than presence of receptors which determine whether a tissue is damaged on exposure to VT.

Numerous studies have shown that cells may bind VT while being refractory to the effects of toxin and that extent of binding may not reflect extent of damage to the tissue (16,24, 27–29). Jacewicz et al (28) found that HeLa cell clones that differed approximately 10-fold in Shiga toxin binding capacity and Gb3 content differed more than 10^9-fold in susceptibility to the toxin. Furthermore, stage of growth of cells (29) and surface expression of receptor may influence susceptibility of cells. Samuel et al (30) have suggested that the density of receptors on the cell surface influences the number of receptor molecules engaged by a single VT molecule and that destabilization of the membrane and subsequent toxin internalization require a high density of receptors on the surface of cells.

The distribution of binding sites in vessels appeared to be patchy. Kurtz et al (18) also observed that only a few vessels in sections of affected tissues were damaged. In our study, we noted that most blood vessels in frozen sections bound toxin, suggesting that most vessels are potentially affected by toxin. It is possible that the variation in distribution and extent of vascular lesions observed in natural ED may be due to secondary signal-induced variation in surface expression of receptor by endothelium and smooth muscle cells. In this regard, it has been shown that Gb4 is associated with cytoskeletal intermediate

filaments in endothelial and smooth muscle cells, and that IFN-γ treatment of endothelial cells can cause a redistribution of the glycosphingolipid between intracellular and cell surface pools (22,31,32).

In natural and experimental ED endothelial and vascular smooth muscle cells show degenerative changes (2,18,19,33). The results presented here show that these types of cells are potential sites of systemic localization of VT2e in pigs and suggest that in natural ED damage to these cells may result from the direct effect of toxin.

VTs have been shown to affect a wide variety of cells which are hypothesized to be involved directly, or indirectly in the pathogenesis of disease associated with VTs. For example, VTs have been shown to be cytotoxic to endothelial cells (9–11) and certain lymphocytes (12). Tesh et al (34) have recently demonstrated that macrophages produce TNF-α in response to treatment with Shiga toxin. The cytokines TNF-α, Il-1β, and lipopolysaccharide have been shown to influence the susceptibility of cells to VTs by altering the cellular content of VT receptor (9,11,35–37). The present investigation has provided evidence which suggests that VT2e may be directly involved in the development of vascular lesions in ED by directly targeting both endothelial cells and vascular smooth muscle cells.

ACKNOWLEDGMENTS

We wish to acknowledge the generous contribution of *E. coli* JM101 (pGT110) by Drs. G. Tyrrell and J. Brunton.

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