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The second International Meeting on the Molecular Biology and Pathogenesis of the Clostridia was held at Seillac, France during the period 22-25 June 1997. The meeting was attended by leading researchers in the field from a variety of countries (list of attendees attached). The proceedings of the meeting were provided as a compilation of the abstracts of all oral and poster presentations at the meeting (attached). The meeting was considered a success and the third International Meeting will be held in Japan in Spring 2000.

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R. Tolcott 10/8/91

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CLOSTRIDIA 97

PATHOGENESIS

SECOND INTERNATIONAL MEETING ON THE MOLECULAR GENETICS AND PATHOGENESIS OF THE CLOSTRIDIA

Château Domaine de Seillac, 41150 Onzain (France)

June 22nd - June 25th 1997

Programme and Abstracts

SFM

FEMS

Congress Secretariat

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Letter from the Organizing Committee

Dear Colleagues,

On behalf of the Organizing Committee we have the pleasure to welcome you to the Second International Meeting on the Molecular Biology and Pathogenesis of the Clostridia, one of the leading FEMS meetings of 1997.

The location of the meeting, Chateau de Seillac in the Loire Valley is situated in one of most the picturesque regions of France, an area rich in history, culture and gastronomy. The facilities which include a swimming pool and tennis courts, opportunities for walks and bicycle riding should allow you to relax between sessions.

As you will see from the programme we have succeeded in attracting the leading scientists in the field to present their very latest work. The meeting should be exciting and stimulating.

We hope that the Symposium will provide an opportunity for you to meet old friends, make new acquaintances and enjoy the famous Loire Valley.

We look forward to welcoming you in Seillac,

With best wishes,

S. Cole
B. McClane

J.Rood
R. Titball

Organizing Committee

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SCIENTIFIC PROGRAMME

Sunday, June 22nd 1997

14:00 Meeting of the IAC

15:00 Registration

17:00 Reception

19:00 Opening

19:15 Keynote address : **Dennis Stevens** (VA Medical Centre, Boise, USA) : The pathogenesis of clostridial myonecrosis

20:30 Dinner

Monday, June 23rd 1997

Session 1: General Biology, Human and Veterinary Disease

Chair : Dennis Stevens

08:30 **E. Stackebrandt** (German Collection of Micro-organisms and Cell Cultures, Germany). The phylogenetic significance of pathogenicity in members of *Clostridium* and related species

08:50 X. Meng, K. Yamakawa, K. Zou, X. Wang, X. Kuang, C. Lu, C. Wang, T. Karasawa, **S. Nakamura** (Kanazawa Univ., Japon and Lanzhou Inst. of Biological Products, China): Isolation and characterisation of neutro-toxigenic *Clostridium butyricum* in Weisham lake area in China

09:10 **B.A. McClane**, J. Kokai-Kun, R. Collie (Univ. of Pittsburgh, USA) : Genotypic and phenotypic comparisons *of Clostridrium perfringens* isolates from foodborne enterotoxin-associated human gastrointestinal diseases

09:30 **G. Songer,** D. Bueschel (University of Arizona, USA) : Clostridial diseases of domestic livestock

09:50 E.M. Smidt, K.M. Hansen, **P.L. Frandsen**, K. Moller, A. Meyling (Danish Vet. Lab., Copenhagen, et Intervet Scandinavia, Skovlunde, Denmark) : Competitive ELISA for determination of antibodies to *Clostridium perfringens* beta-toxin in colostra and sera

10:10 **J. Sakurai**, M. Nagahama, S. Ochi, M. Mukai (Tokushima Bunri Univ., Japan) : *Clostridium perfringens* beta-toxin does not require a thiol group for lethal activity

10:30 Coffee break

Session 2: Genome organisation and Molecular genetics
Chair : Stewart Cole

11:00 **E. Cornillot**, P. Soucaille (Lab. de Biotechnologie-Bioprocédés, Toulouse, France) : Physical and genetic map of the genome of *Clostridium acetobutylicum*

11:20 **Per Granum** (Norwegian College of Veterinary Medicine, Norway) : The genetics of enterotoxin genes from *C. perfringens*

11:40 **S. T. Cole**, G. Daube, M.R. Popoff, B. Dupuy (Inst. Pasteur, Paris, France and Univ. of Liege, Belgium) : *Clostridium perfringens* urease genes are plasmid-borne

12:00 **D. Lyras**, P.K. Crellin, J.I. Rood (Monash University, Australia). Functional analysis of chloramphenicol resistance transposons, from *C.perfringens* and *C.difficile.*

12:20 **P.K. Crellin**, J.I. Rood (Monash Univ., Australia) : Evidence that Tn*4451* from *Clostridium perfringens* is a mobilizable transposon

12:40 **A. Roberts**, P. Mullany (Univ. of Aberdeen, UK) : Genetic organisation of the conjugative transposon Tn*5397*

13:00 Lunch

14:00 Plenary Research Discussions

17:00 Poster session A : Topics 1-4

Session 3: Membrane Active Toxins and Enzymes

Chair : Rick Titball

18:30 **M. Parker**, S.C. Feil, W.J. McKinstry, R.K. Tweten, J. Rossjohn (St Vincents Institute for Medical Research, Australia). The three dimensional structure of perfringolysin O

18:50 **L.A. Utt**, J. Rossjohn, M.W. Parker, R.K. Tweten (Univ. of Oklhoma, USA) : Utilizing the fluorescent properties of the dye NBD to map the functional domains of perfringolysin O

19:10 **J. Holley**, M. Flores-Diaz, A. Alape-Giron, R.W. Titball (CBD Porton Down, UK) : Function of the C-terminal domain of *C.perfringens* alpha-toxin

19:30 **I. Guillouard**, P.M. Alzari, B. Saint-Joanis, H. Sato, S.T. Cole (Institut Pasteur, Paris and National Inst. of Health, Tokyo, Japan) : The carboxy-terminal domain of the alpha-toxin from *Clostridium perfringens* contains a calcium-binding site required for membrane recognition

19:50 **V. Steinthorsdottir**, V. Fridriksdottir, E. Gunnarsson, H. Halldorson, O.S. Andresson (Inst. of Experim. Pathology, Iceland) : Structure and function of the beta-toxin of *C. perfringens*

20:10 **B.R. Sellman**, R.K. Tweten (Univ. of Oklohama, USA) : The propeptide from *Clostridium septicum* alpha toxin acts as an intramolecular chaperone

20:40 Dinner

Tuesday, June 24th 1997

Session 4: Neurotoxins

Chair : Nigel Minton

08:30 **R.C. Stevens**, F. Chen G. Kuziemko, A. Cohen, B. Lacy (University of California-Berkeley, USA) : The structure of botulinum neurotoxin in the complexed and uncomplexed forms.

08:50 **B. Lacy**, A. Cohen, R.C. Stevens (University of California-Berkeley, USA) : The three dimensional X-ray crystal structure of botulinum neurotoxin serotype

09:10 **T.C. Umland**, L.M. Vingert, S. Swaminathan, W.F. Furey, J.J. Schmidt, M. Sax (NIH, Bethesda, VA Medical Ctr, Pittsburgh, and USAMRIID, Frederick, USA) : The crystal structure of tetanus neurotoxin H_C fragment

09:30 **E. A. Johnson**, M. Bradshaw (Univ. of Wisconsin, USA) : Genetic characterization of neurotoxin complexes in *Clostridium botulinum* type A

09:50 **B.L. Singh**, Z. Zhang, B. Li, F.N. Fu, S.K. Sharma, X. Qian, C. Lafontaine, H.K. Sarkar (Univ. of Massachusetts Dartmouth and Baylor College of Medicine Houston, USA) : Molecular composition and topography of neurotoxin associated proteins (NAPS) of botulinum neurotoxin complex

10:10 **P. Washbourne**, R. Pellizzari, M. Wilson, C. Montecucco (Univ. di Padova, Italy) : Studies of the interaction of botulinum neurotoxins A and E with their common target SNAP 25

10:30 *Coffee break*

Session 5: Enterotoxins

Chair : Bruce McCLane

11:00 **K. Aktories** (University of Freiburg, Germany) : Molecular mechanisms of large clostridial toxins

11:20 **Ch. von Eichel-Streiber** (University of Mainz, Germany) : The pathogenicity locus of *Clostridium difficile* and its five genes *tcd A-E*

11:40 **J. Craggs**, A. Roberts, C. Choules, N.B.L. Powell, S.P. Borriello Univ. Hospital, Nottingham, UK) : Structure-function relationship of *Clostridium difficile* toxin A

12:00 **M. Popoff** (Institut Pasteur, France) : Clostridial ADP-ribosylating toxins : genetics and functional domains

12:20 **J. Kokai-Kun**, E. Wieckowski, B. McClane (Univ. of Pittsburgh, USA) : Topological analyses of *Clostridium perfringens* enterotoxin in membranes

12:40 **M. Matsuda**, J. Katahira, M. Inoue, Y. Horiguchi, N. Sugimoto (Osaka Univ., Japan) : Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin

13:00 Picnic lunch and plenary research discussions

16:30 Poster Session B: Topics 5-8

Session 6: Host - Pathogen Interactions
Chair : Jun Sakurai

18:00 **L.L. Simpson**, A.B. Maksymowych, N.J. Kiyatkin, N.M. Bakry (Thomas Jefferson University, USA) : Binding and transport of clostridial neurotoxins

18:20 **O. Dolly** (Imperial College, UK) : Novel effects and applications of botulinum neurotoxins

18:40 **D.M. Ellemor**, R.N. Baird, M.M. Awad, J.J. Emmins, R.L. Boyd, J.I. Rood (Monash Univ., Australia) : Immunohistological studies of acute clostridial myonecrosis in mice

19:00 **G. Donelli**, C. Fiorentini, P. Matarrese, L. Falzano, D.W. Payne, R. Titball (Istituto Sup. di Sanità, Rome, Italy and CBD Salisbury, UK) : Evidence for the involvement of actin cytoskeleton in the *in vitro* cell response *to Clostridium perfringens* epsilon toxin

19:20 **M. Flores-Diaz**, A. Alape-Giron, M. Moos, P. Pollesello, I. Florin, C. von Eichel-Streiber, M. Thelestam (Karolinska Inst., Stockholm, Sweden) : Hypersensitivity to *C. perfringens* PLC is linked to a low UPD-glucose level in Chinese hamster Don cells

19:40 **S. Johnson**, S. Sambol, J. Shim, C. Dileto, M. Merrigan, D. Gerding (VACHS, Northwestern Univ., Chicago, USA) : Unique susceptibility of clindamycin-treated hamsters to human epidemic-associated *Clostridium difficile* strains

20:30 Banquet

Wednesday , June 25th 1997

Session 7: Regulation of Virulence
Chair : Julian Rood

08:30 **T. Shimizu** (Tsukuba University, Japan) : Regulation of extracellular toxin production in *C.perfringens*

08:50 **J. Cheung**, J.I. Rood (Monash Univ., Australia) : Two-component regulation of toxin production in *Clostridium perfringens* : functional analysis of the VirR and VirS proteins

09:10 **S. Katayama**, C. Matsushita, O. Matsushita, J. Minami, A. Okabe (Kagawa Med. Univ., Japan) : Role of promoter upstream region in transcription of the *Clostridium perfringens* phospholipase C gene

09:30 **S Melville** (University of Tennessee, USA) : Analysis of promoters required for sporulation-dependent expression of enterotoxin

09:50 **B. Dupuy**, A.L. Sonenshein (Tufts Univ., Boston, USA) : Transcriptional regulation of *Clostridium difficile toxA* and *toxB* genes

10:10 **J.C. Marvaud**, M. Gibert, M.R. Popoff (Institut Pasteur, Paris, France) : *orf*21 is a positive regulator of botulinum neurotoxin and associated non toxic protein genes in *C. botulinum*

10:30 Coffee break

Session 8: Prophylaxis, Therapy and Diagnosis
Chair : Glenn Songer

11:00 **G. Dougan** (Imperial College, UK) : Modern approaches to tetanus vaccination

11:20 **P. S. Fishman**, D.A. Parks, R. Tully, A.J. Patwardhan (Univ. of Maryland, Baltimore, USA) : Protein delivery to neurons : tetanus toxin compared to fragment C

11:40 **L.A. Smith** (USAMRIID, USA) : Development of genetically engineered vaccines against botulinum neurotoxins

12:00 **O. Matsushita**, C.M. Jung, N. Nishi, S. Katayama, J. Minami, A. Okabe (Kagawa Univ., Japan) : Function of the N- and C-terminal domains of *Clostridium histolyticum* collagenase

12:20 **D.E. Mahony**, S. Lim-Morrison, G. Faulkner, P.S. Hoffman, N. Burford, L. Agocs (Dalhousie Univ., Halifax, Canada) : Antimicrobial activity of synthesized bismuth compounds on *Clostridium difficile*

12:40 **S.J. Ward**, G. Douce, G. Dougan, B.W. Wren (Royal School of Medicine & Dentistry, and Imperial College, London, UK) : Functional and immunological characterisation of fusion proteins containing defined numbers of C-terminal repeat sequences from *C. difficile toxin* A

13:00 Lunch and closing

POSTERS

Session 1

1. A. Rodriguez, C. Sahmkow : Incidence of clostridial infections compared with other anaerobic infections among patients hospitalized in a hospital of Caracas

2. M.R. Sarker, S. Billington, E. Wieckowski, G. Songer, B. McClane : *Clostridium perfringens* type E isolates associated with veterinary enteric infections carry an incomplete enterotoxin gene

3. D. Wrigley, S. Hanwella : Inhibition of Clostridium perfringens sporulation by Gram negative bacteria

4. F. Barloy, A. Delecluse, M. Lecadet : Genetic determinants involved in the mosquitocidal activity of *Clostridium bifermentans malaysia* CH18

Session 2

5. D. Lyras, S.B. Melville, J.I. Rood : Conjugative transfer of shuttle and suicide vectors from *Escherichia coli to Clostridium perfringens*

6. S. Brynestad, B. Synstad, P.E. Granum : Comparison of position and expression of the enterotoxin gene in different strains of *Clostridium perfringens*

7. T.O. Davis, I. Henderson, N.P. Minton : Gene transfer in non-proteolytic strains of *Clostridium botulinum*

8. C. Melville, P. Mullany : Demonstration of a copy of a plasmid closely related to pIP501 integrated into the *C. difficile* genome

9. N.G. Belogurova, E.P. Delver, E.E. Tupikova, S.D. Varfolomeyev, A.A. Belogurov : Characterization, sequence and replication of plasmid pNB2 from thermophilic bacterium *Clostridium thermosaccharolyticum*

10. M. Gibert, C. Jolivet-Renaud, M.R. Popoff : Beta2 toxin, a new toxin produced by *Clostridium perfringens*

Session 3

11. M. Jepson, J. Miller, J. Holley, A. Howells, B. Lingard, D. Moss, R. Titball : A comparison between *Clostridium perfringens* (NCT8237) alpha-toxin and *Clostridium bifermentans* (ATCC638) PLC

12. M. Nagahama, K. Michiue, J. Sakurai : Effect of 18 carbon-fatty acyl residues in phosphatidylcholine in liposomes on membrane-damaging action of *Clostridium perfringens* alpha-toxin

13. A.M. Howells, H.L. Bullifent, M. Jepson, J. Holley, J. Miller, R.W. Titball: Construction and characterisation of hybrid *C. perfringens*, *C. bifermentans* phospholipase C's

Session 4

14. R. Pellizzari, E.A. Johnson, C.C. Shone, C. Montecucco : Structural determinants in the interaction between tetanus toxin and botulinum neurotoxins type B, D, F, and G and their substrate, VAMP/synaptobrevin

15. K. A. Foster, M.J. Welch : Effects of botulinum neurotoxins on sensory afferent neurons

16. J. Herreros, E. Marti, B. Ruiz-Montassell, A. Casanova, H. Niemann, J. Blasi : Distribution of the clostridial neurotoxin receptors in rat brain

17. J. Minami, S. Katayama, O. Matsushita, A. Okabe : Activation of *Clostridium perfringens* epsilon-prototxin and an effect of epsilon-toxin on rat hippocampus

Session 5

18. A. Wagenknecht-Wiesner, M. Wiedmann, V. Braun, P. Leukel, C. von Eichel-Streiber : Reducing the catalytic domain of *Clostridium difficile* 's toxin B-10463 to a enzymatically active N-terminal 467 amino acid fragment

19. J. Kokai-Kun, B. McClane : Functional characterization of randomly-generated point mutants of the *Clostridium perfringens* enterotoxin

Session 6

20. B. McClane, T. Krakauer, B. Fleischer, J. Sipe, D. Stevens, B. Stiles : Evidence that *Clostridium perfringens* enterotoxin lacks superantigenic activity, but induces and IL-6 response

21. Y. Fujinaga, K. Inoue, S. Watanabe, K. Yokota, S. Koza, E. Nagamachi, K. Omuga : Structure and function of the nontoxic components of the *Clostridium botulinum* progenitor toxins

22. T. Karjalainen, M.C. Barc, H. Boureau, A. Collignon, M. Gomez-Trevino, A.J. Waligora, P. Bourlioux : Some aspects of intestinal colonisation by *C. difficile*

23. L. Petit, M.R. Popoff : Cytotoxicity of *Clostridium perfringens* epsilon toxin on MDCK cells

24. S. Johnson, S. Sambol, J. Shim, C. Dileto, M. Merrigan, D. Gerding : *Clostridium difficile* isolates from human epidemics are highly lethal to hamsters at low inoculum

Session 7

25. V. Braun, T. Hundsberger, P. Leukel, C. von Eichel-Streiber : Characterization and transcriptional analysis of the pathogenicity locus of *Clostridium difficile* 10463

26. M. Oe, S. Banu, K. Ohtani, H. Yaguchi, H. Hayashi, T. Shimizu : Identification of novel VirR/VirS-regulated genes in *C. perfringens*

Session 8

27. A. Bentancor, M. Rodriguez-Fermepin, L.D. Bentancor, R.A. De Torres : Improving the design of *Clostridium perfringens* components in conventional vaccines

28. F.A. Uzal, W.R. Kelly : Pathogenesis of *Clostridium perfringens* type D enterotoxaemia and enterocolitis in goats

29. F.A. Uzal, J.J. Plumb, L. Blackkall; W.R. Kelly : Polymerase chain reaction detection of different *Clostridium perfringens* in faeces of goats

30. T. Noren : PCR in monitoring *Clostridium difficile* in clinical treatment

31. V. Fridrikstottir, V. Steinthorsdottir, E. Gunnarsson, O.S. Andresson : Immunisation with recombinant *Clostridium perfringens* beta-toxin ; the ability of wild type and mutant strains to induce protective immunity

32. S.D. Singh : Molecular architecture and REA of a DNA fragment from *Clostridium septicum* genome and its implication in pathogen detection

ORAL COMMUNICATIONS

THE PATHOGENESIS OF CLOSTRIDIAL MYONECROSIS. D.L. Stevens, Infectious Disease Section, Veterans Affairs Medical Center, Boise, Idaho and The Department of Medicine, Univ. of Washington School of Medicine, Seattle, WA.

The classical features of gas gangrene caused by histiotoxic strains of the Clostridia are extensive local destruction of muscle (myonecrosis), shock, regional soft tissue destruction and ultimately, death. Crude toxins from Clostridium perfringens (Cp), purified and recombinant thiol-activated toxin (theta toxin) as well as phospholipase C (alpha toxin) induce myonecrosis in vivo. The importance of theta and alpha toxins was further proven by demonstrating that neutalizing monoclonal antibodies against each of these toxins provided partial protection against challenge with Cp. Myonecrosis caused by these toxins was further substantiated by in vivo studies using isogenic mutants lacking either alpha or theta toxin. In total, these studies support the notion that both toxins cause myonecrosis, and that alpha toxin is more potent than theta toxin.

Though shock is a common feature of gas gangrene, the mechanisms responsible for hemodynamic collapse have not previously been elucidated. Recently, we have demonstrated that crude toxins from Cp, recombinant alpha toxin and theta toxin have markedly different effects on cardiovascular function in rabbits. Alpha toxin induced a rapid decline in both mean arterial pressure (MAP) and cardiac index (CI). In contrast, theta toxin did not affect CI or MAP, but did induce a rapid drop in peripheral vascular resistance analogous to "warm shock" described following endotoxin infusion. Interestingly, crude toxin preparations produced greater effects than either toxin alone. Ex vivo studies using isolated atrial heart tissue demonstrated direct cardiodepressant effects in response to alpha toxin. The effects of these toxins upon human endothelial cell and mononuclear cell cultures were next studied. Briefly, theta toxin and alpha toxin both induced dose and time dependent production of the potent vasodilators, prostacyclin (PGI_2) and platelet activating factor (PAF) by endothelial cells. Alpha toxin but not theta toxin induced synthesis of tumor necrosis factor (TNF) by mononuclear cells. Thus, shock in gas gangrene is the culmination of direct cardiodepressant activity of alpha toxin and vasodilatory effects induced by both alpha toxin and theta toxin and mediated by PGI_2 and PAF. TNF may further amplify these effects by direct induction of these lipid autocoids

We have hypothesized that the regional tissue destruction in gas gangrene is the consequence of toxin effects upon vascular endothelium (EC). Briefly, we have demonstrated dose and time dependent upregulation of the EC adherence molecules, ICAM-1 and ELAM-1 by alpha toxin and theta toxin, respectively. Theta toxin also induced enhanced expression of the leukocyte adherence molecule CD11b/CD18. Thus, toxin-induced dysregulation of leukocyte/endothelial interaction could explain the accumulation of leukocytes within vessels adjacent to the site of infection and may play a key role in the fulminant regional destruction of tissue in gas gangrene.

THE PHYLOGENETIC SIGNIFICANCE OF PATHOGENICITY IN MEMBERS OF CLOSTRIDIUM AND RELATED SPECIES

Erko Stackebrandt, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany

The genus Clostridium, traditionally defined as containing Gram-positive, anaerobic rod-shaped and endospore-forming bacteria, constitutes a phylogenetically incoherent genus. Nevertheless, members of Clostridium can still be considered descendents of a common ancestor that emerged early in the evolution of Gram-positive bacteria. The formation of endospores appears to be a monophyletic trait that is expressed in a few genera of the Clostridium/Bacillus subphylum of Gram-positive bacteria, i.e., Clostridium, Bacillus, Desulfotomaculum and Heliobacterium (1), Sarcina (2), Sporomusa (3) and a few new genera that were recently described for certain members of the genera Clostridium and Bacillus. Loss of spore formation and changes in morphology led to the evolution of phenotypes that were clearly recognizable as being different from spore-forming species. The genus Clostridium contains 35 species which are considered pathogenic. About half of these pathogenic species are members of two phylogenetic clusters rDNA clusters, including those considered the major pathogenic. The species *C. botulinum* is defined by the botulinum neutotoxin (BoNT) complex. Comparative analysis of genes coding for 16S rDNA, BoNT and the nonhemagglutinin component indicate a different evolutionary fate for each of these molecules. 16S rDNA analyses and oligonucleotide probes developed against the variable 23S rDNA regions of *C. botulinum* were found to reliably identify (i) proteolytic strains with toxins of types A, B, and F as well as related species, (ii) non-proteolytic strains with toxins B and E, (iii) strains with toxin D, and *C. argentinense* (the former *C. botulinum*) with toxin G. These four lineages correlate with four physiological groups. Discordance in the phylogenetic trees inferred from 16S rDNA and BoNT groups indicate gene transfer of the latter gene between *C. botulinum* and non-botulinum species. This finding will be discussed in the light of a phylogeny-based classification approach.

ISOLATION AND CHARACTERISATION OF NEUROTOXIGENIC *CLOSTRIDIUM BUTYRICUM* IN WEISHAN LAKE AREA IN CHINA

X. Meng1, K. Yamakawa2, K. Zou1, X. Wang1, X. Kuang1, C. Lu1, C. Wang1, T. Karasawa2 and S. Nakamura2

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An organism identified as type *E botulinum* toxin-producing C. butyricumhad been obtained from the homemade food implicated in a food-borne type E botulism outbreak in Guanyun, China in 1994. Moreover, in the reexamination of our stock strains, we found that two strains isolated inthe botulism cases, in 1973 and 1983, in Weishan lake area locating in an eastern part of China show the property of *C. butyricum*. On the basis of these findings, we examined the lakeside soil of Weishan lake (660 km2) for the presence of neurotoxigenic *C. butyricum*. Type E toxicity in cultures of soil specimens was demonstrated in all 6 sampling regions and type E toxin-producing *C. butyricum* was isolated from soil specimens of 3 sampling regions. The PCR assay demonstrated a presence of the type E toxin gene in all the toxigenic isolates as well as the two stock strains. Biochemical properties of the isolates from the lakeside soil and the stock strains were identical each other except inulin fermentation property. Inulin fermentation of the isolates from the lakeside soil was positive whilst the stock strains negative. These findings suggest that neurotoxigenic *C. butyricum* with at least two different biochemical properties distributes in the soil of Weishan lake area with a considerable population and that the organism have its principal habitat in the soil as *C. botulinum* types A, B, E, F and G.

GENOTYPIC AND PHENOTYPIC COMPARIONS OF *CLOSTRIDIUM PERFRINGENS* ISOLATES FROM FOODBORNE VERSUS NONFOODBORNE ENTEROTOXIN-ASSOCIATED HUMAN GASTROINTESTINAL DISEASES.

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Clostridium perfringens enterotoxin (CPE) is considered the virulence factor responsible for the symptoms of *C. perfringens* type A food poisoning. Recently, CPE-producing *C. perfringens* have also been associated with several nonfoodborne human gastrointestinal (GI) illnesses that often involve more severe and longer-lasting symptoms than are typical of *C. perfringens* type A food poisoning. To improve upon our limited understanding of CPE-associated nonfoodborne human GI illnesses, the current study has genotypically compared *cpe*-positive isolates obtained from individuals suffering from both *C. perfringens* type A food poisoning and CPE-associated nonfoodborne human GI diseases. Results from restriction fragment length polymorphism and pulsed field gel electrophoresis studies reveal consistent genotypic differences between the food poisoning isolates and nonfoodborne human GI disease isolates examined in this study, with *cpe* apparently localizing to the chromosome of food poisoning isolates but to an episome in nonfoodborne human GI disease isolates. When the possible phenotypic consequences of these genotypic differences were explored, no consistent source- or genotype-related differences in *in vitro* CPE expression were observed. Further, all isolates examined, regardless of their source or genotype, were shown to carry a *cpe* open reading frame with an identical nucleotide sequence and to produce CPEs with equivalent cytotoxic activity. Finally, all *cpe*-positive isolates in this survey were shown to carry and express the gene encoding α toxin, but not the genes encoding other major lethal *C. perfringens* toxins, i.e., all CPE-associated nonfoodborne human GI disease isolates, as well as food poisoning isolates, examined in this study belong to *C. perfringens* type A. Considering these phenotypic findings, the basis for symptomology differences noted between the CPE-associated nonfoodborne human GI diseases and *C. perfringens* type A food poisoning remains unclear and will require further study.

CLOSTRIDIAL DISEASES OF DOMESTIC LIVESTOCK. JG Songer and Dawn Bueschel. Department of Veterinary Science, University of Arizona, Tucson, AZ 85721 USA

Clostridia persist as important pathogens of domestic animals, in spite of the extensive availability of immunoprophylactic products and putative knowledge of requirements for diagnosis. However, anecdotal reports of vaccine failure continue to accumulate, and the tenuous nature of at least some aspects of the diagnostic process is illustrated by the recent exhaustion, in the US, of supplies of reagents for diagnosis of certain clostridial diseases.

Results of work with polymerase chain reaction and gene probes for toxin genes of *C. perfringens* have provided insights into the etiology of clostridial enteric disease in the US and elsewhere. Of particular interest are the growing association between *C. perfringens* type A and disease of neonatal and weanling pigs and neonatal and adult cattle. In addition, organisms of genotype E, which have been thought to be virtually nonexistent in North America, represent less than 1% of the total number of isolates submitted to our laboratory, but more than 6% of isolates from hemorrhagic enteritis in calves. Genotyping data alone do not provide conclusive evidence of etiology or relative importance of various types, but results suggest that expansion of availability of immunoprophylactic products and improvement of diagnostic methods will be productive areas of work for the immediate future.

Competitive ELISA for determination of antibodies to *Clostridium perfringens* ß toxin in colostra and sera

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Clostridium perfringens (C.p.) ß-toxin produced by strains of C.p. C or B is known to play a major role in the pathogenesis of necrotic enteritis in humans ("pigbel") and in animals (piglets, lambs).

Determination of the activity of anti-ß-toxin antibodies in sera has been based on measurement of ability of neutralizing biological activities as dermonecrosis in guinea pigs or lethality in mice.

The present work describes the use of ß-toxin specific monoclonal antibodies in a competitive ELISA for determination of anti-ß-toxin antibodies in sera and colostra from all species of animals.

Samples for antibody analysis by anti-ß-toxin ELISA were prepared by mixing a fixed amount of a ß-toxin containing extract and a serial dilution of animal serum or colostrum. The mixtures of toxin and serum were transferred to the wells of anti-toxin monoclonal antibody coated microtiter plates. After incubation the amount of remaining not bound ß-toxin was determined by incubation with horse-radish peroxidase conjugated polyclonal antibody to ß-toxin and visualized colorimetrically.

The titer to ß-toxin in the anti-toxin ELISA was determined as international units (i.u.) of the most diluted sample which could decrease the absorbance by 50% of the non-inhibited ß-toxin. All calculations used "the first international standard for *Clostridium welchii (perfringens)* type B", established in 1954 as reference.

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CLOSTRIDIUM PERFRINGENS BETA-TOXIN DOES NOT REQUIRE A THIOL GROUP FOR LETHAL ACTIVITY

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Beta-toxin contains a single cysteine residue (C-265) in the protein. Treatment of the toxin with thiol modifying or oxidizing agents abolishes lethal activity, suggesting that the cysteine residue is important for lethal activity of the toxin. However, replacement of C-265 with alanine or serine by site-directed mutagenesis resulted in no loss of the activity. Treatment of C265A with N-ethylmaleimide, which inactivates lethal activity of wild-type toxin, led to no loss of the activity. Therefore, the cysteine residue was found not to be essential to the activity. The substitution of phenylalanine, histidine or tyrosine for C-265 significantly diminished lethal activity. In addition, treatment of C265H with ethoxyformic anhydride which is known to specifically modify histidyl residue resulted in significant decrease in the activity of the variant toxin, but that of wild-type toxin with the agent caused no loss of the activity. The results show that the substitution of amino acids containing large size of side chain for the cysteine residue or introduction of chemicals into the amino acid at position 265 affects the activity of the toxin. Moreover, double-gel immunodiffusion test showed no difference between wild-type toxin and these variant toxins. It is likely that the replacement of C-265 results in conformational change of the toxin. Accordingly the spatial position of the amino acid at position 265 plays an important role in the activity.

PHYSICAL AND GENETIC MAP OF THE GENOME OF *Clostridium acetobutylicum*.

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The genetic map of the type strain ATCC 824 of *C. acetobutylicum* was established by a physical approach. The strategy developed associates 2D PFGE with the use of the *Ceu*I restriction enzyme and indirect end labelling techniques. The two main structure-function features revealed in our study are:

- the great advantage of the use of *Ceu*I enzyme for rapid physical mapping and chromosome backbone description.
- the extra-chromosomal position of the solvent-forming genes[1].

The genome of *C. acetobutylicum* ATCC 824 is constituted by a 4.15 Mb chromosome and a 210 kb circular plasmid called pSOL1. The backbone structure of the chromosome is described by 11 ribosomic operons transcribed in a divergent direction from a putative origin of replication. It has been enlarged to conserved genes among low G-C%, sporulating Gram+ bacteria like *groESL*, *dnaKJ*, *recA*, *spo0A* or *sigEG*.

The genetic map constructed by hybridization with homologous and heterologous probes revealed the position of 40 genes of known functions. An important caracteristic of the genetic organization of strain ATCC 824 is the extra-chromosomal position of the genes coding for the enzymes of the acetone and butanol pathways. The plasmid pSOL1 also gives a molecular explanation of the degeneration process in the solventogenic clostridia whereby the cells lose the ability to produce solvent and to sporulate. By PFGE analysis, it is shown that two degenerated strains, DG1 and M5 have lost the 210 kb plasmid carrying the *sol* locus. This 210 kb extra-chromosomal element is specific of the *C. acetobutylicum* species.

[1] **Cornillot E. & P. Soucaille. (1996)** *Nature* **380**, p 489.

The genetics of enterotoxin genes from *C. perfringens*

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Clostridium perfringens type A is responsible for the classic mild type of food poisoning. Illness is caused by an enterotoxin (CPE) produced in the small intestine by sporulating bacteria with released of the toxin during mother cells lysis. The ability to produce enterotoxin is not associated with all *C. perfringens* type A strains, indeed only about 6% of isolated strains exhibit this capacity (Van Damme-Jongsten *et al.*, 1989). It also seems that the ability to produce enterotoxin can come and go within individual culture isolates. This can be explained by the fact that the enterotoxin gene (*cpe*) is on a transposon linked between two house-keeping genes (Brynestad *et al*, in press). This organisation seems to be the most common in the food poisoning strains. In non-food poisoning strains of *C. perfringens cpe* is usually on large plasmids, often linked to other virulence genes and to different IS elements than on the chromosome in type A food poisoning strains (Brynestad *et al*, in preparation & poster at this workshop). Expression of CPE and possible mechanisms of movement will be discussed.

Clostridium perfringens UREASE GENES ARE PLASMID-BORNE

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Although many bacteria are ureolytic, and in some cases urease acts as a virulence factor, this phenotype has not been analyzed in the anaerobic pathogen, *Clostridium perfringens*. In this study, ~2% of *C. perfringens* strains, representing the principal biotypes, were found to harbor the urease structural genes, *ureABC,* and these were localized on large plasmids that often encoded, in addition, the lethal ε or ι toxins, or the enterotoxin. This represents the first report of a plasmid-encoded urease in a gram positive bacterium. The *C. perfringens* enzyme was highly similar to the ureases of other bacteria and cross-reacted with antibodies raised against the urease purified from *Helicobacter pylori*. Urease production was inhibited by urea and induced under growth conditions where the availability of nitrogen sources was limiting. To date, this form of regulation has only been observed with chromosomal *ureABC* genes.

FUNCTIONAL ANALYSIS OF CHLORAMPHENICOL RESISTANCE TRANSPOSONS FROM *Clostridium perfringens* AND *Clostridium difficile.*

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The 6.3 kb transposon Tn*4451* confers chloramphenicol resistance in *C. perfringens*. Tn*4451* encodes a site-specific recombinase, TnpX, which excises the transposon as a circular molecule. TnpX is an unusual recombinase, having both integrase and resolvase/invertase motifs. Site-directed mutagenesis showed that only the resolvase/invertase domains are required for transposon excision. Nucleotide sequence analysis revealed that the transposon target site resembles the junction of the circular form of the transposon. A GA dinucleotide is found at this junction and on either side of the inserted transposon. Site-directed mutagenesis of these bases suggests that excision involves 2 bp nicks on either side of the dinucleotide with one copy remaining at the target site. These results support a resolvase/invertase model for the excision and insertion of Tn*4451*. PCR and cloning studies have shown that a similar transposon, designated Tn*4453*, is found in *C. difficile*. This element is excised in a similar manner to Tn*4451* but appears to transpose at a higher frequency. A cloned copy of the equivalent *tnpX* gene from Tn*4453* complements Tn*4451*Δ*tnpX* for excision, indicating functional similarity. Plasmids carrying Tn*4453* are also mobilised by RP4 in the same way that Tn*4451*-plasmids are mobilised. Mobilisation and transposition studies of a chromosomally integrated copy of Tn*4453* provide evidence that the circular form of the transposon is the transposition intermediate. Overall, these transposons seem to be complex elements which can be mobilised by conjugative plasmids and which are also capable of independent transposition. They may represent an intermediate between non-conjugative and conjugative transposons.

EVIDENCE THAT Tn*4451* FROM *Clostridium perfringens* IS A MOBILIZABLE TRANSPOSON.

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Tn*4451* is a 6.3kb chloramphenicol-resistance transposon from the conjugative *C. perfringens* plasmid pIP401. Tn*4451* has been sequenced and shown to contain six genes, one of which encodes a site-specific recombinase which excises the transposon as a circular molecule. A second gene, *tnpZ*, encodes a protein which has N-terminal sequence similarity with a group of plasmid-encoded mobilization and recombination proteins, the Mob/Pre family. Members of this family promote plasmid mobilization when appropriate transfer functions are provided by a coresident conjugative plasmid. Plasmids carrying *tnpZ* were found to be mobilized in *Escherichia coli* at a frequency of 5.15 transconjugants per donor cell in the presence of a chromosomally-integrated derivative of the promiscuous broad-host-range plasmid, RP4. During mobilization, Mob/Pre proteins interact with a palindromic sequence, designated RS_A, which is located upstream of the *mob/pre* gene. A putative RS_A site was identified upstream of *tnpZ* in Tn*4451*. TnpZ was shown to act *in trans* to mobilize plasmids carrying just the RS_A site, indicating that the protein interacts with this sequence during mobilization. This hypothesis was supported by the finding that a mutated RS_A site was mobilized at a dramatically reduced frequency. Tn*4451* therefore is a highly evolved non-conjugative element which is capable of being mobilized by conjugative plasmids.

GENETIC ORGANISATION OF THE CONJUGATIVE TRANSPOSON TN*5397*

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Tn*5397* is a conjugative transposon originally identified in *Clostridium difficile*. We have undertaken a physical analysis of this element and demonstrated that it is very closely related to Tn*916* in its central region, with some important sequences differences. The left end of the element is completely different to Tn*916*. This region of the transposon contains a site-specific recombinase that is closely related to *tnpX* from Tn4451 of *C. perfringens*. At the right end of Tn*5397* there are blocks of sequence that are closely related to Tn*916* but also sequence blocks that do not contain any homology to Tn*916*. Tn*5397* also contains a group II intron inserted into one of the sequence blocks that is similar to Tn*916* (Tn*916* does not contain an intron). The comparative maps of Tn*916* and Tn*5397* are presented.

THE THREE-DIMENSIONAL STRUCTURE OF PERFRINGOLYSIN O

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Perfringolysin O (PFO) is a virulence factor of *Clostridium perfringens*, a bacterium that causes gas gangrene, food poisoning, necrotizing enterocolitis of infants and pigel as well as a variety of diseases in animals. PFO belongs to a family of related toxins, the thiol-activated cytolysins, which are generated by a diverse range of gram-positive bacteria. Each toxin consists of a single polypeptide chain with molecular weights ranging from 50 kDa to 80 kDa and there is high pairwise sequence identity between them (40 to 70%) suggesting they all have similar three-dimensional structures. The toxins share a common mode of action. They interact with target cells via their receptor, cholesterol, followed by oligomerization and membrane insertion leading to membrane damage. The toxins form pores in membranes which have diameters exceeding 150 Å.

We have recently determined the three-dimensional atomic structure of PFO by X-ray crystallography and have modelled the membrane form of the toxin based on electron microscopy and biochemical data. The structures reveal a novel mechanism of membrane insertion and provide a molecular basis for understanding a plethora of published biochemical and mutagenesis data that are available for the thiol-activated cytolysin family.

UTILIZING THE FLUORESCENT PROPERTIES OF THE DYE NBD TO MAP THE FUNCTIONAL DOMAINS OF PERFRINGOLYSIN O. L.A. Utt, J. Rossjohn, A.E. Johnson, M.W. Parker and R.K. Tweten. The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190.

Perfringolysin O (PFO), a cytolysin produced and secreted by *Clostridium perfringens*, is a member of the thiol-activated toxin family. These toxins, which are produced by a variety of gram positive bacteria, are characterized by the presence of a single cysteine located within a conserved 11-residue peptide (ECTGLAWEWWR). The belief that this sulfhydryl group was absolutely necessary for cytolytic activity led to the designation "thiol-activated". It has subsequently been shown, however, in PFO and other toxins in this family that substitution of an alanine for the unique cysteine does not lead to any significant loss of activity. We have generated such a cysteine-less mutant of PFO, designated PFO^{ala}, for use in our studies. One of the enigmas surrounding PFO is the question of how a relatively hydrophilic molecule lacking any obvious transmembrane domains is able to aggregate into oligomers and insert into a membrane. In order to investigate the structural rearrangements which must occur in such a transition, we have generated a panel of PFO^{ala} mutants containing unique cysteines placed strategically along the primary sequence. This allows for the selective attachment of the environmentally sensitive fluorescent dye NBD to these key residues. The emission intensity, lifetime, and collisional quenching of the NBD-derivatized PFO mutants is then monitored, allowing determination of the environment in which each residue resides. Residues in a number of regions of the protein have been mutagenized to cysteine, including S206, L207, V209, D210, A215, I229, R450 and L462. Using this method, we have tentatively identified some of these residues in PFO to be in a region which is either an interfacial domain in the oligomer, or undergoes a conformational change during the oligomerization process.

FUNCTION OF THE C-TERMINAL DOMAIN OF C.PERFRINGENS ALPHA-TOXIN

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C perfringens alpha-toxin is a phospholipase C (PLC) which also has haemolytic and lethal activities. The lethality of alpha toxin is thought to be linked to its ability to hydrolyse membrane phospholipids, either directly, or indirectly, by activation of endogenous PLCs. Structurally, *C perfringens* alpha-toxin has two distinct domains, joined by a hinge region. the N-terminal domain (Cpa_{1-249}) has PLC activity, and the C-terminal domain ($Cpa_{247-370}$) is believed to confer the haemolytic and lethal properties on the toxin. The role of the C-terminal domain was investigated using several strategies.

The ability of the individual sub units (Cpa_{1-249} and $Cpa_{247-370}$) to directly hydrolyse sphingomyelin or phosphatidylcholine liposomes was measured. Cpa_{1-249} had minimal activity and $Cpa_{247-370}$ had no activity against both types of liposome. A mixture of the sub units increased the activity against sphingomyelin liposomes up to approximately 75% of that seen with alpha-toxin alone but showed no activity with phosphatidylcholine liposomes compared to alpha-toxin which demonstrated moderate activity.

The toxicity of Cpa_{1-249} and $Cpa_{247-370}$ to a mutant Chinese hamster ovary cell line was investigated. The individual sub units were non toxic, whereas when added together a clear cytotoxic effect was seen.

The effect of mutations in the C-terminal domain on the PLC (egg yolk assay) and haemolytic activities of alpha-toxin was investigated. None of the 6 mutants tested exhibited significantly altered activities. A mutation in the hinge region resulted in a slightly reduced egg yolk activity, but unchanged haemolytic activity. The hinge mutant gave the same activity as the sub units mixed together with both types of liposomes, indicating that a mutation in the hinge region reduces the activity compared to alpha-toxin.

These results suggest that both sub units of alpha-toxin are required for sphingomyelinase activity and cytotoxicity. The hinge region may also play an important role.

THE CARBOXY-TERMINAL DOMAIN OF THE α–TOXIN FROM *Clostridium perfringens* CONTAINS A CALCIUM-BINDING SITE REQUIRED FOR MEMBRANE RECOGNITION

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The lethal, cytolytic α–toxin (phospholipase C) of *Clostridium perfringens* consists of two distinct modules: the larger NH_2-terminal domain catalyses phospholipid (phosphatidylcholine and sphingomyelin) hydrolysis and its activity is potentiated by a smaller COOH-terminal domain. By means of site-directed mutagenesis, immunology, and biochemistry, functional evidence was obtained indicating that the COOH-terminal region of α-toxin mediates interactions with membrane phospholipids in a calcium-dependent manner. These results combined with sequence comparisons and molecular modeling suggest the COOH-terminal domain to belong to the growing family of C2 β-barrel domains, that are often involved in membrane interactions. Mutations to this domain may account for the natural lack of toxicity of the α–toxin homologue, phospholipase C of *Clostridium bifermentans*.

STRUCTURE AND FUNCTION OF THE BETA-TOXIN OF *C. PERFRINGENS*. V. Steinthorsdottir, V. Fridriksdottir, E. Gunnarsson, H. Halldórsson and Ó.S. Andrésson. Institute for Experimental Pathology, Reykjavik, Iceland.

The beta-toxin of *Clostridium perfringens* has some sequence homology with a group of pore forming toxins including alpha-toxin from *Staphylococcus aureus*. Alpha-toxin is a hemolysin and forms a heptameric pore in the cell membrane. We have established an expression system for the production of recombinant beta-toxin which facilitates functional analysis of the protein. Beta-toxin was expressed in *Bacillus subtilis* using its own regulatory sequences and secreted to the extra cellular medium. The recombinant toxin was lethal in mice but not hemolytic on rabbit or sheep erythrocytes. Work with other cell types indicates that beta-toxin is a cell specific pore-forming toxin.

We have used site directed mutagenesis to study the relationship of beta-toxin and the pore formers. Eleven single mutations of nine amino acids were made in the beta-toxin gene and cloned into the expression vector. Only seven mutant proteins were produced. Mutations of two amino acids, Y203 and R212, reduced the toxicity of beta-toxin in mice. Both amino acids have counterparts in the putative membrane binding region of alpha-toxin. Our results are consistent with previous data that beta-toxin is structurally and functionally related to the pore-forming alpha-toxin of *S. aureus*.

THE PROPEPTIDE FROM *CLOSTRIDIUM SEPTICUM* ALPHA TOXIN ACTS AS AN INTRAMOLECULAR CHAPERONE.

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The alpha toxin produced by *Clostridium septicum* is a lethal and cytolytic toxin. Alpha toxin is secreted from the cell as a protoxin which requires a proteolytic cleavage reaction for activation. This cleavage event removes a 5.1kD propeptide from the carboxy terminus of the toxin (~41kD). Alpha toxin is then capable to oligomerize into a supramolecular complex on the surface of the target cell membrane followed by membrane insertion and pore formation which results in the lysis of the affected cell. The role of the propeptide in the cytolytic mechanism of alpha toxin was investigated. Upon proteolytic activation the propeptide was found to remain associated with the toxin over a gel filtration column. When proteolytically activated alpha toxin was treated with excess purified propeptide the hemolytic activity of the toxin was decreased as the amount of propeptide was increased up to a 10-fold molar excess. The block in the hemolyitc activity was found to be at the point of oligomerization and not receptor binding or membrane insertion. The propeptide was also found to prevent the solution oligomerization/aggregation of the toxin while covalently attached. From these data a model is proposed in which the propeptide acts as intramolecular chaperone by preventing premature oligomerization/aggregation of alpha toxin prior to proteolytic activation.

THE STRUCTURE OF BOTULINUM NEUROTOXIN IN THE COMPLEXED AND UNCOMPLEXED FORMS

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The 900 kD complex form of botulinum neurotoxin serotype A has been determined using electron crystallography. The structure has a triangular shape with 6 distinct lobes. A series of biophysical experiments (electron microscopy, dynamic light scattering, gel electrophoresis, and circular dichroism) have been conducted aimed at understanding the relationship of the toxin in the complexed and uncomplexed forms. Various environmental conditions were examined ranging in pH from 1-10, and various protease cocktails. Although the toxin and the hemaglutinin portion of the complex are quickly degraded at acidic pH and protease cocktails, the complex form of the toxin is very stable at pH values below 1.0. Furthermore, very little effect is observed on the complex upon the addition of proteases. Based on the experimental results, a molecular mechanism for the toxicity of botulinum neurotoxin in the complexed and uncomplexed forms will be proposed.

Finally, these results are correlated to the three-dimensional structure of the holo 150 kD botulinum neurotoxin serotype A. The neurotoxin structure has been determined by x-ray diffraction at atomic resolution.

THE THREE DIMENSIONAL X-RAY CRYSTAL STRUCTURE OF BOTULINUM NEUROTOXIN SEROTYPE A

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Botulinum neurotoxin serotype A (BTA) is one of seven antigenically distinct proteins produced from strains of *Clostridium botulinum*. These serotypes (designated types A-G) are the causative agents of botulism, a potentially fatal condition of neuromuscular paralysis. Botulinum neurotoxin type A in its fully activated form exists as a di-chain protein consisting of a light chain (MW~50-kDa) and heavy chain (MW~100-kDa) linked by a disulfide bond. The protein can be further subdivided into three functional domains: a catalytic domain corresponding to the light chain, a translocation domain associated with the N-terminal half of the heavy chain, and a binding domain as the C-terminal half. To facilitate further functional studies on the mechanism of toxin binding, translocation and catalysis, we report here the three dimensional structure of the 150 kD BTA protein as determined by x-ray crystallography. This structure should serve as a key tool in elucidating the mechanism of toxin action for serotype A as well as the homologous BT serotypes and tetanus toxin.

THE CRYSTAL STRUCTURE OF TETANUS NEUROTOXIN H_C FRAGMENT.

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The crystal structure of tetanus neurotoxin H_c fragment has been determined at 2.7Å resolution and has been refined, with R=17.3%, and R_{free}=24.5%. The crystallized fragment is comprised of 452 amino acid residues, which are proportioned between two domains within the structure. The domain at the N-terminal end of H_c exhibits a topology reminiscent of that possessed by the legume lectins. The domain at the C-terminal end of H_c is folded in the β-trefoil motif. Details of the three-dimensional structure will be discussed, including possible receptor binding sites. In addition, structural implications for the H_c fragment of the various botulinum neurotoxin serotypes are suggested by this structure.

GENETIC CHARACTERIZATION OF NEUROTOXIN COMPLEXES IN *CLOSTRIDIUM BOTULINUM* TYPE A.

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Clostridium botulinum type A produces toxin complexes composed of neurotoxin, hemagglutinin, and nontoxic nonhemagglutinin. In this study, the structure and organization of the genes encoding the components of the toxin complexes was investigated in *C. botulinum* Hall A and 62A and in nontoxigenic mutants derived from the latter parental strain. Toxin mutants were generated by conjugative transfer of the transposon Tn*916* from *Enterococcus faecalis* to *C. botulinum*. Tn*916* stably integrated into random regions of the clostridial chromosome as shown by pulsed-field gel electrophoresis analyses and DNA hybridizations. Randomly selected tetracyline-resistant transconjugants of *C. botulinum* were examined for loss of toxin formation by colony immunoblots, and three nontoxigenic mutants were detected from about 7000 colonies screened. These mutants had a large deletion in the genomic DNA and all three genes of the toxin complex were deleted. The regions of *C. botulinum* chromosomal DNA adjacent to Tn*916* insertions were cloned using inverse PCR and their nucleotide sequences determined. Unexpectedly, both the right and left flanking regions cloned from two of the mutants were identical, and the right flanking region was identical in the third mutant. These results indicate that the toxin gene cluster lies within a region that can be lost from the genome by a deletion event. The deletion of such a large region (>12 kbp) suggests the genes of the toxin cluster is associated with a moveable genetic element. The mechanism of the deletion and potential for genetic transfer of the toxin gene cluster will be discussed.

MOLECULAR COMPOSITION AND TOPOGRAPHY OF NEUROTOXIN ASSOCIATED PROTEINS (NAPS) OF BOTULINUM NEUROTOXIN COMPLEX

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Botulinum neurotoxin (BoNT) is produced along with a group of neurotoxin associated proteins, or NAPs, which protect it from the low pH and proteases of the gastro-intestinal tract. From the type E M complex, we have isolated and characterized the neurotoxin binding protein (NBP) for its interaction with the type E neurotoxin. In addition, we have now identified a novel complex of type E BoNT that has five additional NAPs which makes BoNT/E complex similar to other types of BoNT complexes. Determination of nucleotide sequence upstream to the NBP gene of type E *Clostridium botulinum* has revealed open reading frames that correspond to the identified NAPs of BoNT/E. An open reading frame whose stop codon is only 18 bp apart from the start codon of the NBP gene has been cloned and sequenced.

We have also isolated, for the first time, one of the major components of type A NAPs in a pure form. The isolated protein is a 33 kDa single polypeptide that exhibits hemagglutinin activity. Polyclonal antibodies against hemagglutinin-33 (Hn-33) were able block the hemagglutinating activity of the type A neurotoxin complex completely, suggesting that Hn-33 is the only strong hemagglutinin present in the complex. Hn-33 showed complete resistance to the trypsin (under conditions that allowed digestion of the purified neurotoxin extensively), a biochemical feature which is consistent with the presumed role of Hn-33 in protecting the neurotoxin against the proteolysis in the gastro-intestinal tract. Supported by USDA (9437201-1167) and NIH (NS33740).

STUDIES OF THE INTERACTION OF BOTULINUM NEUROTOXINS A AND E WITH THEIR COMMON TARGET SNAP 25

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Botulinum neurotoxins type A and E (BoNT/A and /E) are metalloproteases with a unique specificity for an essential protein component of the neuroexocytotic machinery: SNAP 25 (synaptosomal-associated protein of 25 kDa). It has been suggested that at the basis of this specificity there is the recognition of a SNARE motif, consisting of nine residues and which is common to the other two SNARE proteins: VAMP (vesicle-associated membrane protein) and syntaxin. SNARE proteins are the only known substrates of clostridial neurotoxins. The SNARE motifs of VAMP are involved in its recognition by tetanus and botulinum types B, D, F and G neurotoxins. Here, we analyse the involvement of the four copies of the SNARE motif present in SNAP-25 in its interaction with BoNT/A and /E. Specifically SNARE motif deleted SNAP-25 mutants were expressed as GST fusion proteins and their kinetics of proteolysis were determined. Results indicate that BoNT/A and /E recognize SNAP-25 principally via the SNARE motif closest to the cleavage site. However, in its absence, the other more distant motifs may substitute to a certain extent, allowing a reduced rate of proteolysis to occur. In addition we report the susceptibilities of various isoforms of SNAP 25 to BoNT/A and E. The results suggest that SNAP 25 is a protein which can be made to adopt several different conformations.

MOLECULAR MECHANISMS OF LARGE CLOSTRIDIAL TOXINS

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The family of large clostridial cytotoxins ($M_r \geq 250$ kDa) consists of *C. difficile* toxins A (Tox A) and B (Tox B), *C. sordellii* lethal (LT) and haemorrhagic toxins (HT) and *C. novyi* α-toxin (α-toxin). The toxins monoglucosylate low molecular mass GTP-binding proteins. Tox A, Tox B, HT, and α-toxin modify Rho subfamily proteins Rho, Rac, and Cdc42, but not Ras subfamily GTPases. LT glucosylates Rac (Cdc42) but not Rho. In addition, LT modifies Ras subfamily proteins such as Ras, Rap, and Ral. Whereas UDP-GlcNAc serves as a cosubstrate for α-toxin, UDP-glucose is the cosubstrate for all other transferases. The toxins selectively glucosylate Thr-37/35 of the GTPases. The modification blocks stimulation of the GTPases by GTPase-activating proteins (GAPs) and inhibits the interactions with their effector proteins. To analyse the structure-functions relationship of *C. difficile* toxin B, three fragments of similar size of the holotoxins were constructed and expressed in *E. coli*. The N-terminal fragment possessed glucosyltransferase activity, whereas the middle and C-terminal parts were without activity. Further analysis of the active fragment of toxin B revealed that N-terminal 546 aa are essential for full enzyme activity. The structure-function-analysis of other large clostridial cytotoxins will be discussed.

THE PATHOGENICITY LOCUS OF *CLOSTRIDIUM DIFFICILE* AND ITS FIVE GENES tcdA-E

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Toxinogenic and non-toxinogenic *Clostridium difficile* isolates were compared and a genetic locus was found that was the integration site for 115 nucleotides in the case of non-toxinogenic strains or a segment of 19.6 kb when the strains were toxinogenic. Analysis of ten isolates revealed that the site was conserved and that integration of the segments was always oriented. Up- and downstream regions were sequenced and 7 open reading frames were defined. Some of them code for putative transport proteins. Since the 19.6 kb fragment is a prerequisite for pathogenicity of *C. difficile* isolates we designated it the pathogenicity locus (PaLoc). Most isolates harbored a complete PaLoc, while in some others it was shortened by up to 5.9 kb. The two toxin genes tcdA and tcdB are accompanied with three accessory genes tcdC-E. TcdD is an basic protein which contains helix-turn helix motifs and is homologous to DNA-binding proteins described in other clostridial species. TcdC is an acidic protein, with no homology to any known protein from the database (TcdE likewise). To resolve the function of these accessory genes we analysed transcription of the five genes. Two transcription units were defined (tcdC and tcdD,B,E,A), their transcription is conserted and related to cell growth. Transcription of tcdC is high when that of the others is low and vice versa. Genes tcdD,B,E,A are transcribed mono- and polycistronically, with the highest amount of transcript seen for tcdA. A transcriptional model is presented wherein tcdC has a negative and tcdD a positive influence on toxin expression.

Braun *et al.* (1996) Gene 181: 29-38
Hundsberger *et al.* (1997) Eur. J. Biochem. 244: 735-742

STRUCTURE - FUNCTION RELATIONSHIPS OF *CLOSTRIDIUM DIFFICILE* TOXIN A

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Clostridium difficile, the aetiological agent of pseudomembranous colitis in humans, produces two known toxins, A and B. Both toxins are potent cytotoxins, whilst toxin A is also a tissue damaging enterotoxin that probably causes most of the gastrointestinal symptoms associated with *C. difficile* induced colitis.

To study structure-function relationships of toxin A, seven overlapping DNA fragments representing the entire toxin A gene have been cloned 'in frame' with a maltose binding protein. One of these clones incorporates the entire repeat region of toxin A which is believed to be responsible for the binding of the toxin to its target cell. The protein products from these clones have been expressed in *E. coli* as maltose binding protein fusions. Each peptide has been purified from *E. coli* and cleaved from the maltose binding protein fusion product. The purified toxin A peptides have been analysed in a variety of biological assays including non - specific binding to monoclonal antibodies, haemagglutination of rabbit erythrocytes and cytotoxicity to tissue culture cells.

Recent observations have shown that toxin A glucosylates cytosolic Rho proteins which leads to actin filament breakdown and subsequently to a cellular cytopathic effect. Each of the purified toxin A peptides has been screened for the ability to glucosylate Rho A. One of these peptides, coded for by a 1.6 Kb fragment of the toxin A gene, was found to express glucosyltransferase activity. At the other extreme of the toxin, electron microscopy studies have revealed that the repeat region peptide has the ability to bind and be internalised into rabbit intestinal cells. This makes the repeat region peptide an ideal carrier molecule for transporting proteins into cells.

CLOSTRIDIAL ADP-RIBOSYLATING TOXINS: GENETICS AND FUNCTIONAL DOMAINS

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ADP-ribosylation is a common mechanism of cytotoxicity which is used by many bacterial toxins. Clostridial ADP-ribosylating toxins can be divided in two groups according to their substrate. One group (*C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin and *C. difficile* ADP-ribosyltranferase (CDT)) ADP-ribosylate actin, and the second group (*C. botulinum* and *C. limosum* C3 enzymes) ADP-ribosylate a small G-protein (Rho) involved in the regulation of actin polymerization. The toxins of both groups induce a disorganization of the actin cytoskeleton.

The actin ADP-ribosylating toxins are binary toxins and consist of two independent proteins one being the enzymatic component (about 50 kDa) ant the other being the binding component (about 80-90 kDa). The enzymatic and binding components of *C. perfringens* iota toxin, *C. spiroforme* toxin and CDT are immunologically and structurally related (80% identity at the amino acid level) and can be interchanged to form fully active toxins. In contrast, these toxins are not significantly related to *C. botulinum* C2 toxin. However, a common domain organization was found for the enzymatic component. The enzymatic and binding component genes are organized in operon and are localized on a large plasmid for *C. perfringens* iota toxin and on chromosome for *C. spiroforme* toxin, CDT and C2 toxins.

C3 enzyme consist of only one protein (about 25 kDa) and does not possess a binding component. This enzyme can not be internalized effciently into cells. A conserved ADP-ribosylation site to that of actin ADP-ribosylating toxins was found in C3 enzyme. In *C. botulinum* C and D, the C3 gene is localized on phage DNA which also harbor the botulinum neurotoxin genes. In some strains, the C3 gene has been localized on a transposon element.

FUNCTIONAL CHARACTERIZATION OF RANDOMLY-GENERATED POINT MUTANTS OF THE *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN.

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The *C. perfringens* enterotoxin (CPE) is a 319 amino acid, single polypeptide, membrane-active toxin. It has a unique multi-step mechanism of action that sequentially involves; specific binding of CPE to a 50kDa eukaryotic membrane protein to form a "small" complex, development of a post binding physical change in this small complex, association of this small complex with a 70kDa eukaryotic membrane protein to form "large" complex, and rapid development of membrane permeability alterations. To better understand this action, studies of the CPE structure/function relationship have been performed using deletion analysis. These studies demonstrated that, i) the N-terminal 44 amino acids of CPE are not required for CPE cytotoxicity, ii) amino acids between 45 and 53 are necessary for completion of the post-binding physical change step in CPE action, and iii) the extreme C-terminus (amino acids 290-319) of CPE contains an essential region for receptor-binding. In order to expand on this existing knowledge of CPE structure/function relationships, we sought to identify specific amino acid residues involved in the various steps in CPE action. To accomplish this, the cloned *cpe* gene was subjected to random mutagenesis in XL-1 Red® mutator *E. coli* (Stratagene). Using this technique, 88 clones that had altered cytotoxic activity were identified, from a total of 1000 clones screened. Six well-expressed mutants were further characterized for their ability to complete each of the four steps in CPE action, as mentioned above. These studies indicate that CPE variant 7B7 (Trp226ΔStop) is a noncytotoxic truncation variant blocked at the binding step, a result consistent with previous studies identifying the C-terminus of CPE as an essential binding region of the enterotoxin. Noncytotoxic variants 7H8 (Ser59ΔLeu) and 8C5 (Gly49ΔAsp) were shown to be able to undergo the post-binding physical change, but could not form large complex. Finally, 1A7 (Ser167ΔPro), 1E5 (Arg137ΔGly) and 3H6 (Arg116ΔSer) formed sharply reduced levels of large complex, consistent with the greatly reduced cytotoxicity exhibited by these variants. These results with 7H8, 8C5, 1A7, 3H6 and 1E5 support large complex formation being an essential step in CPE action, and also provide the first direct evidence that residues in the N-terminal half of CPE are involved in large complex formation.

MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF THE RECEPTOR FOR *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

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We attempted to identify a putative *Clostridium perfringens* enterotoxin (CPE) receptor (R)[CPE-R] by expression cloning. For this, we used flow cytometric analysis with a biotinylated recombinant C-terminal fragment peptide of CPE (amino acid residues 184-319 of CPE fused to 10 consecutive histidine residues) as a probe which binds specifically to CPE-sensitive cells but dose not induce cell lysis. Thus we could clone a cDNA encoding the CPE-R from an expression library of enterotoxin-sensitive Vero cells. The nucleotide sequence of CPE-R showed that the enterotoxin receptor consists of 209 amino acids with a calculated molecular weight of 22,029 dalton. This receptor is highly hydrophobic, contains four putative transmembrane segments, and has significant similarity to the rat androgen withdrawal apoptosis protein RVP1 and the mouse oligodendrocyte specific protein, the functions of which are unknown. The expression of CPE-R was detected in the enterotoxin-sensitive Vero, Hep3B, and Intestine 407 cell lines, but not in the enterotoxin-insensitive K562 and JY cell lines. The CPE-R gene product expressed in enterotoxin-resistant L929 cells bound to enterotoxin specifically and directly and with high affinity and rendered the cells sensitive to the toxin, indicating that the cloned receptor is functional. Results showed that enterotoxin could not assemble into a complex with a defined structure unless it interacted with the receptor. From these results, we propose that the enterotoxin receptor is required for both target cell recognition and pore-formation in the cell membrane.

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BINDING AND TRANSPORT OF CLOSTRIDIAL NEUROTOXINS

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A series of experiments were conducted using a human gut cell line, human neuromuscular junctions, mouse gastrointestinal preparations, and the mouse neuromuscular junction. Binding and transport experiments were done with unpurified preparations of botulinum toxin (i.e., neurotoxin, hemagglutinin, and non-neurotoxic, non-hemagglutinin components), homogeneous neurotoxin isolated from clostridial cultures, homogeneous recombinant neurotoxin, light chain obtained from clostridial cultures, and recombinant light chain. The data indicate that poisoning due to botulinum toxin involves two sequences of events and two principal cell types. The initial cell type, referred to as the transport cell, exists in the gut of vulnerable species. The sequence of events involves binding, translocation by a non-pH-dependent mechanism, and release of unmodified toxin. The second cell type, referred to as the target cell, is the peripheral cholinergic nerve ending of vulnerable species. The sequence of events here involves binding, penetration of the plasma membrane by receptor-mediated endocytosis, penetration of the endosome membrane by a pH-dependent mechanism with intracellular release of a presumably modified toxin, and expression of enzymatic activity. An appreciation of these events contributes to the understanding of several key issues, including: 1) the cellular basis for sensitivity or resistance to the various serotypes of botulinum toxin, 2) the techniques for generating an oral vaccine against botulinum toxin, and 3) the molecular basis for the signs and symptoms of botulism.

NOVEL EFFECTS AND APPLICATIONS OF BOTULINUM NEUROTOXINS

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The multi-functional activities of the several serotypes of botulinum neurotoxin (BoNT, A-G) are being exploited very effectively for clinical and research purposes. Success in treating human dystonias and dysphonias with type A toxin is due to: (a) its targeting to cholinergic nerve endings by binding avidly via its heavy chain (HC) to ecto-acceptors located exclusively on their presynaptic membrane; (b) endocytosis and translocation to the cytosol - steps requiring the HC to be disulphide-linked to the light chain (LC) and (c) irreversible blockade of acetylcholine release due to Zn^{2+}-dependent proteolytic cleavage of SNAP-25 by the LC. Eventual return of spasm to the treated muscles depends on toxin-induced nerve sprouting and the formation of functional synapses; this fascinating process has been demonstrated in vivo, for the first time, using image analysis of mouse sternomastoid muscle. Thus, BoNT is instrumental in attempts to decipher the molecular basis of synapse remodelling. Another potential therapeutic application has been created from the expression of an enzymically-inactive mutant of BoNT A. As the latter retains the targeting and trafficking abilities of the native toxin, it has been found to serve as an innocuous transporter for delivering a prototype drug inside motor nerve endings in biologically-active form. On the other hand, insights into the roles of SNAP-25 in exocytosis has been gleaned from the subtly different protease activities of BoNT A and E; their cleavage of SNAP-25 at distinct sites revealed that adjacent C-terminal domains contribute to pre- and post-priming stages of Ca^{2+}-elicited catecholamine secretion. Finally, the use of types B and C1 in studies on cultured adipocytes has provided evidence for the involvement of cellubrevin and syntaxin in insulin-stimulated trafficking of glucose transporter 4, while the contrasting lack of inhibition of insulin-induced glucose uptake by BoNT A led to detection in these cells of the homologue SNAP-23.

IMMUNOHISTOLOGICAL STUDIES OF ACUTE CLOSTRIDIAL MYONECROSIS IN MICE

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The pathology of clostridial myonecrosis (gas gangrene) commonly includes a paucity of leukocyte infiltration within areas of necrosis and the aggregation of leukocytes displaying altered morphology at the border of the necrotic tissues. This study aims to use immunohistological examination of gangrenous tissue to develop an understanding of the mechanisms involved in the inhibition of a normal inflammatory response to this disease state. *In vivo* analysis of the roles of extracellular bacterial toxins in the disease pathology is being performed using a range of α-toxin and θ-toxin mutant strains of *Clostridium perfringens*, the bacterium most commonly associated with human gas gangrene. Infections established using toxin negative mutants exhibited a markedly decreased severity of muscle necrosis compared with wild-type strain infections. Associated with this was a marked increase in leukocyte infiltration into the site of infection, particularly for the α-toxin negative mutant. Immunohistological staining of the wild-type infected tissues indicated a decrease in the staining levels of LFA-1, MHC-II, IFN-γ and IL-2 compared to levels observed in tissues injected with heat-killed *C. perfringens* cells. These effects could either be due solely to a destruction of infiltrating leukocytes, or may also involve a genuine down-regulation of the expression or secretion of these molecules, resulting in decreased leukocyte migration into the site of infection. Cellular staining levels for Mac-1 in wild-type infected tissues appeared comparable to, if not higher than, levels in tissues injected with heat-killed *C. perfringens*. Up-regulated expression of Mac-1 may also result in dysfunctional leukocyte migration. Further studies staining with a wider range of antibodies will determine whether these altered staining levels indicate genuine up- or down-regulations of adhesion molecule and cytokine expression. Immunohistological examination of tissues infected with the mutant strains should provide further information on the roles of α-toxin and θ-toxin in enabling *C. perfringens* to evade clearance by an inflammatory response.

EVIDENCE FOR THE INVOLVEMENT OF ACTIN CYTOSKELETON IN THE IN VITRO CELL RESPONSE TO CLOSTRIDIUM PERFRINGENS EPSILON TOXIN.

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Epsilon toxin, produced by Clostridium perfringens types B and D, is normally produced as a relatively inactive prototoxin which, after proteolitic hydrolysis, is converted to the fully active toxin. A basic property of this highly toxic protein is that it increases vascular permeability.

To elucidate the mechanism of action at subcellular level of the epsilon toxin we have evaluated in the present study its cytotoxic effect on different cell lines (HEp-2, HeLa, A431, Int 407, HT29, MDCK). Among the six different cell lines tested so far, only MDCK cells appeared to be supsceptible to epsilon toxin (1) being HEp2 cells only susceptible to very high doses of toxin (>100 µg./ml). Then, we focused our attention on the toxic effects on MDCK cells evidentiating, by scanning electron microscopy and fluorescence microscopy, a time-dependent effect on cell morphology and cytoskeleton organization. Upon 1 hour exposure to the toxin (4.5 µg/ml) the cells started to show a decrease in cell-cell and cell-substrate contacts, 3-6 hours of treatment causing a clear retraction of the cell body. The number of roundish cells increased by time and an high percentage of detached cells were observed after an overnight treatment. A parallel modification in the microfilament network was also detected. In fact, after 1 hour exposure to epsilon toxin the organization of stress fibers was already impaired, this damage becoming more evident prolonging the exposure time up to 12-18 hrs.

These preliminary results seem to indicate the actin cytoskeleton as a significant target of epsilon toxin and allows us to speculate that the actin-disrupting effect of epsilon toxin in MDCK cells might explain the increase of vascular permeability observed in vivo. Further studies are now in progress to address this question.

1. Payne et al., (1994). FEMS Microbiology Letters 116: 161-168.

HYPERSENSITIVITY TO *C.PERFRINGENS* PLC IS LINKED TO A LOW UDP-GLUCOSE LEVEL IN CHINESE HAMSTER DON CELLS

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A mutant cell line (Don Q) was previously isolated after mutagenization with ethylmethanesulphonate and a two-step selection procedure with the *Clostridium difficile* glucosyltransferase toxin B (TcdB). Don Q is 10^4 times more resistant to TcdB and 10^5 times more sensitive to *Clostridium perfringens* PLC than the wild type (Don wt). By growing Don Q cells in the presence of *C. perfringens* PLC, a spontaneous revertant (Don QR), was isolated, which has lost the hypersensitivity to this toxin. Don wt, Don Q and Don QR have the same sensitivity to *Bacillus cereus* PLC demonstrating that Don Q is not hypersensitive to all PLCs.

It was recently shown that Don Q has a UDP-glucose deficiency, which explains the resistance of this cell to TcdB. We have now found that the reason for the low UDP-glucose level in Don Q is a defect in its production due to a lowered activity of the UDP-glucose pyrophosphorylase enzyme (UDPG:PP). This enzymatic deficiency is caused by a single point mutation in the UDPG:PP gene. The $^{G}116^{D}$ mutation originally present in homozygous form in Don Q has reverted in only one of the alleles in Don QR.

Transfection of Don Q with a bovine UDPG:PP gene restores the enzymatic activity and UDP-glucose level. Furthermore transfectants are reverted with respect to their response to *C. perfringens* PLC.

Taken together our results demonstrate that there is a link between the hypersensitivity to *C. perfringens* and a low level of UDP-glucose. The importance of this finding in the context of *C. perfringens* induced gangrene is discussed.

UNIQUE SUSCEPTIBILITY OF CLINDAMYCIN-TREATED HAMSTERS TO HUMAN EPIDEMIC-ASSOCIATED *CLOSTRIDIUM DIFFICILE* STRAINS.

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Large hospital epidemics of diarrhea due to a unique *Clostridium difficile* strain identified by Restriction endonuclease analysis (REA) typing as J9 have been documented in widely diverse geographic regions within the U.S. (IDSA Annual Meeting 1995, Abstract #93). In each epidemic where antibiotic data were collected, clindamycin use was a significant risk factor for disease. This strain is highly resistant to clindamycin, erythromycin, and ciprofloxacin (MIC > 256 ug/mL for each), but susceptible to ampicillin (1.5 ug/mL) in vitro. Experiments with clindamycin-treated Syrian golden hamsters have demonstrated 100% mortality after oral feeding of this strain at low inoculum (100 CFUs or less). In order to determine the duration of susceptibility of hamsters following clindamycin treatment, groups of 3 hamsters were inoculated with 100 CFUs of a clindamycin-resistant epidemic strain at various time points following oral administration of a single dose of clindamycin (30 mg/kg). All treated hamsters were susceptible at days 5, 7 and 10 following clindamycin, but not at day 14 (3/3 survived) or at days 21 and 28 when challenged with a larger inoculum (2 x 10^4 CFUs). Prolonged host susceptibility following clindamycin therapy may help explain the variable timing of *C. difficile* diarrhea onset with respect to antibiotic exposure in hospitalized patients. In addition, clindamycin therapy may uniquely predispose hospitals to epidemics of *C. difficile* diarrhea due to particular strains.

REGULATION OF EXTRACELLULAR TOXIN PRODUCTION IN *C. PERFRINGENS*

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Production of extracellular toxins in *C. perfringens* is known to be regulated by a two-component regulatory system, VirR/VirS. The VirR/VirS system positively regulates the production of alpha-toxin (phospholipase C), theta-toxin (perfringolysin O), kappa-toxin (collagenase), and protease, which are thought to play an important role in the pathogenesis of *C. perfringens* type A.

We had isolated a *virR* mutant strain (TS133) using a homologous recombination mutagenesis from its parental strain 13 and analyzed the role of the VirR/VirS system on the production of extracellular toxins. The expression of the alpha-toxin (*plc*), theta-toxin (*pfoA*), and kappa-toxin (*colA*) genes was positively regulated by the VirR/VirS system at the transcriptional level, although the mode of the regulation somewhat differed among toxin genes. The transcription of the *pfoA* gene was totally dependent on the VirR/VirS system, while the expression of the plc and colA genes was shown to be partially VirR/VirS-dependent, because some constitutive expression of these genes was seen in TS133. Primer extension analysis revealed that the *pfoA* gene had two promoters one of which was totally VirR/VirS-dependent. The *colA* gene was shown to possess two kind of promoters, constitutive and VirR/VirS-dependent ones. However, the *plc* gene had only one promoter whose transcription was enhanced by the VirR/VirS system. Analysis of the flanking regions of these toxin gene promoters revealed that their promoter regions shared no similar sequence to which the putative phosphorylated VirR protein could bind. These data suggested that more complex regulatory networks might exist in the regulation of extracellular toxin production in *C. perfringens*.

TWO - COMPONENT REGULATION OF TOXIN PRODUCTION IN *Clostridium perfringens*: FUNCTIONAL ANALYSIS OF THE VirR AND VirS PROTEINS.

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The production of α-toxin, θ-toxin and κ-toxin in *C.perfringens* is regulated by a two-component signal transduction system consisting of the *virR* and *virS* genes. These genes are arranged in an operon, with *virR* encoding the response regulator and *virS* the sensor histidine kinase. VirS contains conserved motifs found in virtually all sensor histidine kinases. To ascertain whether these regions were essential for VirS function, site-directed mutagenesis was carried out on the His-255 residue, which is the postulated site of autophosphorylation, the DXGXG and GXGL motifs, and two Glu residues found in the putative transmembrane region. Plasmids containing the mutated *virS* genes did not complement the *virS* mutation, indicating that the altered residues were essential for VirS function. The cognate response regulator, VirR, regulates toxin production by activating transcription of its target genes. To localise the VirR binding sites, the *virR* gene was cloned into an expression vector and the resultant His-tagged VirR protein was overexpressed and partially purified by metal chelate chromatography under native conditions. His-tagged VirR was shown in gel mobility shift experiments to bind specifically to a region located upstream of *pfoA*, the θ-toxin structural gene, but not to the region located upstream of *pfoR*, a putative θ-toxin regulatory gene. These results imply that the activation of *pfoA* expression involves the direct interaction of VirR with the *pfoA* gene region and does not occur via the activation of *pfoR* transcription.

ROLE OF A PROMOTER UPSTREAM REGION IN TRANSCRIPTION OF THE *CLOSTRIDIUM PERFRINGENS* PHOSPHOLIPASE C GENE.

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The promoter upstream region stimulates the transcription of the *Clostridium perfringens* phospholipase C gene (*plc*). The stimulatory effect is more prominent at low temperature (25°C) than high temperature (45°C) probably through bent DNA structure formed by three polyA tracts existing between -66 to -40. The *in vitro* transcriptional activity of RNA polymerase purified from *C. perfringens* (Cp-RNAP) was determined using the *plc* gene with the three polyA tracts (3A) and that without them (0A). Cp-RNAP transcribed 3A 9-fold more than 0A at 25°C but only 1.4-fold at 45°C. Gel shift assay showed that 3A bound Cp-RNAP more efficiently (about 5.4-fold) than 0A at 25°C. Analysis by hydroxy radical footprinting revealed that Cp-RNAP was in contact with the region from -65 to +5 of 3A, extending to 10-bp upstream of the region attained with 0A. This may be responsible for an increase in the affinity of RNAP to the promoter region. RNAP purified from *Escherichia coli* (Ec-RNAP) transcribed 3A 2-fold less than 0A at 25°C, indicating that the stimulatory effect was specific to Cp-RNAP. Although Ec-RNAP was in contact with 3A similarly to Cp-RNAP and the binding of Ec-RNAP to 3A was significantly higher than that to 0A, its transcriptional activity against 3A was significantly lower than that against 0A. Thus, structure of the upstream region may be a device to form efficiently closed and open complexes with Cp-RNAP.

ANALYSIS OF PROMOTERS REQUIRED FOR SPORULATION-DEPENDENT EXPRESSION OF ENTEROTOXIN.

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Clostridium perfringens causes large scale outbreaks of food poisoning, due to the production of a potent enterotoxin by sporulating cells in the intestinal tract following ingestion of contaminated foods. We have shown that the enterotoxin gene, *cpe*, is controlled at the transcriptional level and is highly induced during sporulation. A *C. perfringens-E. coli* shuttle vector carrying a fusion of a ~500 bp *cpe* promoter region fragment to the reporter gene *gusA* from *E. coli* also was induced during sporulation. Primer extension experiments using RNA from sporulating cells identified four potential 5′ ends upstream of the *cpe* start codon (designated P1 to P4). Internal deletions in the cpe promoter region were made to isolate the promoters, except P1 and P2, which overlapped. P3 alone and P1 and P2 together showed significant levels of sporulation-dependent expression, but P4 alone showed almost none. A series of nested deletions of the cpe promoter were made. Deletions including P4 and P3 had little or no effect on the level of expression, while deletions to P1 and P2 drastically reduced the induction. Regions with homology to sigma E-dependent promoters from Bacillus upstream of the P3 and P2 5′ ends and another with homology to sigma K-dependent promoters upstream of the P1 5′ end were detected. Also, genes coding for *C. perfringens* homologs of spo0A, sigE, sigG, and sigK have been cloned and are currently being used to construct mutations in the chromosomal copies by homologous recombination to test their effects on sporulation and *cpe* regulation.

TRANSCRIPTIONAL REGULATION OF *CLOSTRIDIUM DIFFICILE* TOXA and TOXB GENES

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The exact 5' ends of *toxA* and *toxB* mRNAs were found by primer extension and RNase protection assays. These 5' ends were shown to correspond to promoter sites used in vivo by fusion of their putative promoters to the *Escherichia coli gusA* gene and measurement of β-glucuronidase activity in *C. perfringens*. A second mode of *tox* gene expression by readthrough from upstream gene(s) could also be detected, consistent with prior results of Hammond and Johnson. The symptoms of disease caused by *C. difficile* vary from mild diarrhea to lethal pseudomembranous colitis. This variation has been attributed to differing levels of toxin production. In strains that differ widely in toxin production and pathogenicity the content of *tox* mRNAs varied accordingly, suggesting that the rate of *tox* gene transcription is an important determinant of pathogenicity. Accumulation of tox mRNAs was strongly influenced by nutrient availability. Rapidly metabolized carbon sources (glucose, mannitol) strongly repressed *tox* gene transcription. Equivalent extents of repression were found in strains producing varying levels of toxin, indicating that high producers have more active promoters rather than less effective repressors. In the absence of repressing sugars, *tox* mRNAs accumulated rapidly as cells reached the end of exponential growth phase, suggesting that transcription responds to signals reflecting cell culture density, nutrient availability or growth rate. Wilkins and colleagues have identified TxeR, the product of the gene upstream of *toxB*, as a possible regulator of *tox* gene transcription. We have cloned the *txeR* gene in *E. coli* under the control of a T7 promoter, overexpressed TxeR and partially purified it. In vitro tests of specific binding of TxeR to the *tox* promoter regions and assays of transcriptional activation are in progress.

*orf*21 IS A POSITIVE REGULATOR OF BOTULINUM NEUROTOXIN AND ASSOCIATED NON TOXIC PROTEIN GENES IN *C. BOTULINUM*

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The botulinum neurotoxins (BoNT) are associated to non toxic proteins (ANTP) to form large complexes. The *bont* and *antp* genes are clustered in two operons. An additional gene (*orf*21) encoding a basic protein of 21-22 kDa, was found on the 5' part of the botulinum complex locus in *C. botulinum* C and D, and between the two operons in *C. botulinum* A and B. We constructed recombinant strains of *C. botulinum* A which overexpressed *orf*21 or produced antisense mRNA of *orf*21 gene. Using titration of the mouse lethal activity of the culture supernatants and Western blotting with specific antibodies against BoNT and ANTPs, we showed that *orf*21 regulates positively *bont* and *antp* genes in *C. botulinum* A. The results of the mRNA dot blot assays indicated that this regulation occured at the transcriptional level.

MODERN APPROACHES TO TETANUS VACCINATION

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Tetanus is still a common cause of morbidity and mortality in developing countries. Infections in the neonate are still a frequent, numbering in the hundreds of thousands. This is despite the fact that an effective vaccine is available. The existing vaccine is normally administered to children as part of the classical children's vaccine, along with diphtheria, Pertussis (cellular or acellular) and now Haemophilus influenzea type B. There is a move, prompted in part by WHO, to generate new forms of tetanus vaccines that can offer protection as a single dose and/or can be delivered mucosally, either as oral or nasal vaccines. Many of the new experimental tetanus vaccines exploit the use of novel delivery systems including livevectors, mucosal adjuvants or biodegradable particles. In this presentation I will attempt to describe some of the modern approaches to tetanus vaccine development, highlighting the underpinning technologies that can be applied in general to new vaccine developments.

PROTEIN DELIVERY TO NEURONS: TETANUS TOXIN COMPARED TO FRAGMENT C. P.S. Fishman, D.A. Parks, R. Tully, A.J. Patwardhan. Neurology Service, Baltimore Veterans Administration Medical Center., and Dept. of Neurology, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A.

The non-toxin 50 Kd C-terminus peptide of tetanus toxin (C-Fragment or CF) contains the ganglioside binding domain of tetanus toxin (TTX). CF retains much of the capacity of tetanus toxin for binding and transport by neurons. For this reason CF has been studied as a carrier for delivery of therapeutic proteins to neurons. However, other sites on TTX may also be involved in neuronal binding and internalization. We directly compared CF and TTX in the capacity to bind and be internalized by neurons by ELISA. Primary cultures of dissociated fetal cortical neurons were incubated with equimolar amounts of TTX or CF. Neuronal associated tetanus protein was 4-8 fold greater on a molar basis with tetanus toxin compared to CF (1 hr. incubation). This increase in neuronal tetanus protein was evident with incubation in concentrations of 0.1 μM to 2 μM of either TTX or CF. There were greater amounts of TTX delivered to the cultured cells at both 0°C (representing membrane bound tetanus protein) and 37°C (bound and internalized tetanus protein). Unlike CF, TTX showed significant continued accumulation of protein with increasing incubation durations. Neuronal associated TTX increased 2-3 fold over incubation times ranging from 1 to 8 hrs. Tetanus toxin appears to be clearly superior to the ganglioside binding fragment CF in the capacity for neuronal binding and internalization. Atoxic tetanus proteins containing additional molecular domains as well as CF may be more suitable vectors for linkage with therapeutic proteins and delivery to neurons. (Supported by NIA Grant 1PO1-AG12992-01).

DEVELOPMENT OF GENETICALLY ENGINEERED VACCINES FOR BOTULINUM NEUROTOXINS

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Synthetic genes encoding non-toxic, carboxyl-terminal regions (~50 kDa) of botulinum toxin (BoNT) serotypes A, B, and E (referred to as fragment C or Hc) were constructed and cloned into the methylotropic yeast, *Pichia pastoris*. Genes specifying BoNTA-, B-, and E(Hc) were expressed as both intracellular and secreted products. Recombinants, expressed intracellularly, yielded products with the expected molecular weight as judged by SDS-PAGE and Western immunoblot analysis, while secreted products were larger due to glycosylation. Gene products were used to vaccinate mice and were evaluated for their ability to elicit protective antibody titers *in vivo*. Mice given three intramuscular vaccinations with yeast supernatant containing glycosylated BoNTA(Hc) were protected against an intraperitoneal challenge of 10^6 50% mouse lethal doses (MLD_{50}) of serotype A neurotoxin. However, immunization with the glycosylated forms of BoNT B- and E(Hc) did not elicit protection. Vaccinating mice with cytoplasmically produced BoNTA(Hc) and BoNTB(Hc) protected animals from a challenge of 10^6 MLD_{50} of serotype A and B toxin, respectively. Because of the glycosylation problem encountered with secreted BoNT(Hc), our efforts are focused on the purification of products from intracellular expression. The BoNTA(Hc) and BoNTB(Hc) products were purified by using a series of conventional chromatography steps. The purification and characterization of other Hc serotypes is currently in progress.

FUNCTION OF THE N- AND C-TERMINAL DOMAINS OF *CLOSTRIDIUM HISTOLYTICUM* COLLAGENASE.

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The 116-kDa collagenase of *Clostridium histolyticum* consists of four segments, S1, S2a, S2b and S3. We have investigated the structure-function relationship of this enzyme using recombinant proteins. Full-length collagenase degraded both native type I collagen and a synthetic substrate, Pz-peptide, while an 88-kDa truncate containing only S1 and S2a (S1S2a) degraded only the latter. Unlike the full-length enzyme, S1S2a did not bind to insoluble type I collagen. To determine the molecular determinant or collagen-binding activity, various C-terminal regions were fused to the C-terminus of glutathione S-transferase (GST). S3 conferred collagen-binding activity on a fusion protein to an extent comparable to S2b plus S3. However, a GST-fusion protein with a region shorter than S3 exhibited reduced collagen-binding activity. S3 liberated from the fusion protein also showed collagen-binding activity. S1 exhibited the full Pz-peptidase activity but only 5% of the collagenolytic activity of the full length collagenase. These results indicate that S1 and S3 are the catalytic and binding domains, respectively, and that S2a and S2b form an interdomain structure. A zinc metalloprotease consensus motif, HEXXH, present in S1 was shown to form the catalytic center by an aa-replacement experiment. The third zinc-binding aa residue is currently under investigation. To exploit a new drug delivery system using the collagen binding domain, we constructed a fusion protein with mammalian epidermal growth factor (EGF). The fusion protein exhibited a prolonged effect of EGF upon injecting subcutaneously into mice.

ANTIMICROBIAL ACTIVITY OF SYNTHESIZED BISMUTH COMPOUNDS ON *CLOSTRIDIUM DIFFICILE*. D.E. Mahony[1], S. Lim-Morrison[1], G. Faulkner[1], P.S. Hoffman[1], N. Burford[2] and L. Agocs[2]. Departments of Microbiology and Immunology[1], and Chemistry[2], Dalhousie University, Halifax, Nova Scotia, Canada.

Clostridium difficile is a major nosocomial pathogen responsible for pseudomembranous colitis and many cases of antibiotic-associated diarrhea. Antimicrobial treatment of such disease has included metronidazole and vancomycin, but relapse is not uncommon. With an obvious need for more antimicrobial agents to treat *C. difficile* disease, we designed, synthesized and tested 14 new bismuth compounds with respect to their biological activity against *C. difficile*. Serial dilutions of these compounds were inoculated onto blood agar plates swabbed with *C. difficile* VPI 10463. The MIC was recorded as the highest dilution that inhibited growth and was expressed as μg/mL bismuth. Cytotoxicity of the compounds was tested against human foreskin cells (FSK cells).

Six compounds had good antibacterial activity against *C. difficile*. Of the 6 compounds, two had significantly higher activities. Based on the analyzed bismuth concentration in filtered compounds, the two compounds with highest activities had inhibitory activity at less than 1 μg/mL bismuth. These compounds showed lower cytotoxicity when tested against FSK cells than did the other compounds. Studies on compound no.1 showed that it bound to *C. difficile* and electron microscopy revealed bismuth aggregates on the surface of the bacteria. Thin sections showed aggregates inside the cells. Although we have shown that the bismuth compounds are bactericidal, we currently do not know how the bacteria are killed.

In conclusion, a number of new bismuth compounds have been synthesized which demonstrate anti-*C. difficile* activity. Testing more compounds for biological activity and determining how such compounds express antibacterial activity may lead to new therapeutic agents for *C. difficile*-related disease.

FUNCTIONAL AND IMMUNOLOGICAL CHARACTERISATION OF FUSION PROTEINS CONTAINING DEFINED NUMBERS OF C-TERMINAL REPEAT SEQUENCES FROM *C.DIFFICILE* TOXIN A

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Clostridium difficile produces two potent exotoxins, toxin A and B, which are the main virulence determinants for this major nosocomial pathogen. These mediate the tissue damage and inflammation seen at the colonic mucosa during infection. A striking feature of both toxins is the highly repetitive nature of the carboxyl-terminus, which in the case of toxin A has been shown to contain the receptor-binding domain of the toxin. Parenteral immunisation with either the C-terminal region of toxin A, or a conserved peptide from this region (IIB repeat) has been shown to generate toxin-neutralising antibodies. Our rationale is to generate a functional immune response at the point of action of the toxin (i.e.) gut mucosa, in order to circumvent the problems manifest in generating a systemic immune response against a mucosal pathogen.
Rationally attenuated Salmonella have been shown to be efficient at delivering heterologous antigens to the mucosal immune system. Using PCR, four overlapping fragments containing 8, 14, 20 and 36 toxin A C-terminal repeats were amplified, and subsequently expressed to high levels as fusions with the non-toxic fragment C of tetanus toxin within attenuated *S.typhimurium* (*htrA*). With the exception of the smallest fusion, the toxin A repeats retained their receptor-binding capability and promoted cold-haemagglutination of rabbit RBC. This receptor-binding capacity allowed fusions to be successfully purified using bovine thyroglobulin affinity chromatography. The immunological properties of these Salmonella-expressed fusions within mice will be presented in conjunction with preliminary function studies.

POSTERS

INCIDENCE OF CLOSTRIDIAL INFECTIONS COMPARED WITH OTHER ANAEROBIC INFECTIONS AMONG PATIENTS HOSPITALIZED IN A HOSPITAL OF CARACAS

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We realize a retrospective study to determine the incidence of Clostridial infections compared with other anaerobic infections among patients hospitalized in a hospital of Caracas. We determine the incidence of Clostridial infections, and we compared it with the incidence of other anaerobic infections. We used to determine the incidence of these infections, the results of the microbiologic cultures obtained from the clinical isolations of patients with a possible anaerobic infection, in the last two years, 1995 and 1996. We believe that, in our hospital, the anaerobic infections are predominantly due Clostridium sp., more than other anaerobic bacteria, or at least Clostridium sp. may be the most common anaerobic bacteria isolated from clinical samples in our experience.

CLOSTRIDIUM PERFRINGENS TYPE E ISOLATES ASSOCIATED WITH VETERINARY ENTERIC INFECTIONS CARRY AN INCOMPLETE ENTEROTOXIN GENE.

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Clostridium perfringens is a major cause of histotoxic and enteric infections in both humans and domestic animals. *C. perfringens* isolates can be classified into 5 types (A-E), based upon expression of four "major" toxins (α, β, ι and ε toxins). Type E isolates, which by definition are able to express α and ι toxins, are responsible for enterotoxemias in a number of species of domestic animals. In the current study, a multiplex PCR assay has been used to characterize several type E isolates associated with veterinary enteric disease. This analysis showed that all type E isolates examined carry sequences for the gene (*cpe*) encoding the *C. perfringens* enterotoxin protein (CPE), as well as for genes encoding α and ι toxins. However, while expression of ι and α toxins was demonstrated, no CPE expression was observed using either sporulating or vegetative cultures of these type E isolates. Preliminary Southern blot analyses suggested that the *cpe* sequences and ι toxin genes may be proximal in these type E isolates; this suggestion was confirmed by PCR amplification analysis, which showed that the *cpe* sequences in these type E isolates lie ~600 bp upstream, in the opposite orientation, from the iota toxin genes. Preliminary sequencing results suggest that these type E isolates are unable to express CPE for several reasons; i) there is no ribosome binding site and initiation codon upstream of the *cpe* sequences present in these isolates and ii) there are premature termination codons in the ORF of these incomplete *cpe* genes. Further analysis will be necessary to elucidate why many/most type E *C. perfringens* isolates associated with enteric veterinary disease are carrying incomplete *cpe* genes.

INHIBITION OF CLOSTRIDIUM PERFRINGENS SPORULATION BY GRAM NEGATIVE BACTERIA. D.M. Wrigley and S. Hanwella, Mankato State University, Mankato, MN 56002-0084, USA

C. perfringens gastroenteritis occurs when enterotoxin is released by C. perfringens during spore formation in the intestines. While enterotoxin and its activity are well defined, the conditions that lead to sporulation are not as well understood. The intestinal environment is a complex milieu of host secretions, a complex array of nutrients, and numerous transient and indigenous microorganisms. In this study, the effect of bacterial populations on sporulation was examined by co-culturing C. perfringens ATCC 12915 with the following gram negative bacteria: 2 strains of Escherichia coli; Enterobacter aerogenes; Salmonella typhimurium; and Bacteroides fragilis. One strain of E. coli, S. typhimurium and B. fragilis inhibited spore yield and enterotoxin production. Inhibition required a large initial concentration of each gram negative organism. The other gram negative bacteria tested did not inhibit spore yield, regardless of initial inoculum concentration. B. fragilis appears to modify the Duncan-Strong sporulation broth in that filter sterilized and heated Duncan-Strong medium in which B. fragilis had been cultured did not support C. perfringens sporulation. Since B. fragilis is saccharolytic, it is possible that inhibition was due to the ability of B. fragilis to degrade starch to glucose, a known inhibitor of sporulation. However, inhibition by E. coli requires a different mechanism which has not yet been determined. When C. perfringens was added to filter sterilized E. coli culture fluid, spore yield was not decreased. Glutaraldehyde fixed E. coli did not inhibit sporulation. A 1 1/2 hour delay in addition of E. coli to the sporulation culture still resulted in decreased spore yield. However, addition of E. coli 3 hours after the initiation of the C. perfringens sporulation culture did not affect spore yields.

GENETIC DETERMINANTS INVOLVED IN THE MOSQUITOCIDAL ACTIVITY OF*Clostridium bifermentans* subsp. *malaysia* CH18

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Clostridium bifermentans malaysia CH18 (*Cbm*) is the first strict anaerobic bacteria toxic for mosquito (*Anopheles* sp, *Aedes* sp et *Culex* sp) and blackfly larvae, vectors of tropical diseases. Comparative analysis realized with the non toxic strain ATCC 638 shows the presence of three major proteins (66 type, 18 and 16 kDa) in CH18 toxic extract.
Characterization of these proteins was realized through cloning of the corresponding genes and expression in the gram (+) *Bacillus thuringiensis* host. A plasmid, pCBM1, was isolated which contains an *Xba*I fragment of 7452 bp, carrying six ORFs: *orf1* and *orf2*, in opposite orientation, present homologies with genes encoding the transposase and resolvase of Tn3 family transposons, respectively. *orf3* and *orf4* are transcribed in the same orientation, separated by 91 bp and probably organized as an operon. These genes, named *cbm*71 et *cbm*72, respectively, encode proteins (66 and 67 kDa) 30 % identitical to *Bacillus thuringiensis* delta-endotoxins (proteins Cry). These identities are essentially located in the five conserved regions of the Cry family. Such homologies with entomopathogenic toxins may suggest the involvement of both proteins in the toxicity of CH18. Around 1 kb downstream from *orf4*, we can find *orf5* et *orf6*, also in the same orientation, called *cbm17-1* and *cbm17-2* and separated by 31 bp. These two orfs correspond to variants of the same gene, encoding P18 et P16. Analysis of amino acid sequences indicate that the 16 kDa protein is obtained after cleavage of the 17 first NH_2-terminal residues of the 18 kDa protein. These proteins are 30% similar to an hemolysin of *Aspergillus fumigatus*.
Structure of the cloned fragment and expression of the different genes will be presented.

CONJUGATIVE TRANSFER OF SHUTTLE AND SUICIDE VECTORS FROM *Escherichia coli* TO *Clostridium perfringens*.

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Although electroporation is a reliable and relatively efficient way of introducing shuttle plasmids into *C. perfringens*, only a small number of *C. perfringens* strains can be transformed using this procedure. In this study, two versatile *E. coli-C. perfringens* shuttle plasmids, pJIR1456 and pJIR1457, encoding chloramphenicol and erythromycin resistance, respectively, were constructed and characterised. These plasmids carry the origin of conjugative DNA transfer (*oriT*) from the broad host range Inc P plasmid RP4 and in mixed plate matings can be mobilised from an *E. coli* donor carrying RP4 into the *C. perfringens* recipient JIR325. The transfer frequencies observed were 1.1×10^{-3} and 2.2×10^{-3} transconjugants per donor for pJIR1456 and pJIR1457 respectively, which is similar to the frequency of intraspecies transfer of the conjugative *C. perfringens* plasmid pCW3. A suicide vector, pSM186, which carries the *oriT* region from RK2 has also been constructed. Following conjugal transfer of this plasmid from *E. coli* HB101, the plasmid was successfully integrated into the chromosome of the cpe^+ strain NCTC8798 by homologous recombination. This methodology represents a new means whereby plasmid DNA can be introduced into *C. perfringens* and, consequently, by which strains can be genetically manipulated. The development of these mobilisable shuttle and suicide vectors may allow *C. perfringens* strains which cannot be transformed by electroporation to be genetically manipulated.

COMPARISON OF POSITION AND EXPRESSION OF THE ENTEROTOXIN GENE IN DIFFERENT STRAINS OF *CLOSTRIDIUM PERFRINGENS.*

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The enterotoxin gene (*cpe*) in *Clostridium perfringens* can be chromosomally or plasmid bound. In human food poisoning strains the gene is located on the chromosome in a transposon integrated between two housekeeping genes. Other isolates have *cpe* on large plasmids. Three different IS elements, IS*1469*, IS*1470* and IS*1151*, have been found to be associated with *cpe*. The genetic configuration varies between type and strains *C. perfringens*. We have extended the known mapping information in various cpe+ strains. The production of enterotoxin in correlation to genetic location was examined, and there are apparent differences in the regulation of expression in some strains.

GENE TRANSFER IN NON-PROTEOLYTIC STRAINS OF *CLOSTRIDIUM BOTULINUM*

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Gene transfer systems have been developed to various degrees of sophistication for various *Clostridium* sp., including *Clostridium acetobutylicum* and *Clostridium perfringens*. There have been comparatively few reports of genetic exchange in *Clostridium botulinum*. Some progress has been made with certain group I, proteolytic strains, through the demonstration of electrotransformation of a limited number of plasmids and conjugative introduction of Tn*916*. There have, however, been no reports of gene transfer in group II, non-proteolytic *C.botulinum* strains.

We have investigated the ability of clostridial cloning vectors used in this laboratory to electrotransform various group II, *C.botulinum* type B strains. Although vectors based on a lactococcal plasmid replication origin were routinely found to result in low numbers of transformants, vectors which employed other clostridial replicons were apparently unable to transform. Analysis of extracts of one of the strains has demonstrated the presence of a restriction endonuclease activity. Its recognition sequence has now been determined and an appropriate methylase identified. The *in vitro* use of this methylase with plasmid DNA has been shown to significantly increase transformation frequencies, and in some cases allow plasmids to be transformed for the first time. The ability of a particular plasmid to transform appears to correlate with the frequency of the identified restriction enzyme recognition sequence.

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DEMONSTRATION OF A COPY OF A PLASMID CLOSELY RELATED TO PIP501 INTEGRATED INTO THE *C. DIFFICILE* GENOME

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A region of *C. difficile* 630 chromosomal DNA was cloned. Physical analysis of this region demonstrated that it contained a copy of a plasmid that was almost identical to pIP501. This plasmid was integrated into the *C. difficile* chromosome at the 23S RNA region. The integrated plasmid also contained a tet(L) gene. A map of the integrated element is presented and the repercussions of this finding for the evolution of antibiotic resistance and genetic manipulation of the clostridia is discussed.

CHARACTERIZATION, SEQUENCE AND REPLICATION OF PLASMID pNB2 FROM THERMOPHILIC BACTERIUM *CLOSTRIDIUM THERMOSACCHAROLYTICUM.*

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The 1882-bp nucleotide sequence of the cryptic plasmid pNB2 isolated from *Clostridium thermosaccharolyticum* was determined. pNB2 DNA has very low GC content (27%) and may serve as a model for studying the modes of maintenance and replication of AT-rich DNA under conditions of thermophilic growth. The plasmid sequence revealed three open reading frames (ORFs) which would encode polypeptides of 289, 68, and 59 amino acids, respectively, and these proteins were synthesized in E.coli extracts primed with the plasmid. We found that the product of ORF 289 may be initiated at the non-ATG start codon. TTG, and his similarities with the conserved motifs of Rep proteins encoded by rolling circle (RC) plasmids of the pC194/pUB110 family. Southern hibridization analysis of lysates of *C.thermosaccharolyticum* cells harboring pNB2 revealed single-stranded intermediates, suggesting that this plasmid is able to replicate in clostridial cells via the RC mechanism. The most significant similarities are found between pNB2 Rep proteins of three RC plasmids of the pC194 family (pTB913, pBC1 and pST1) isolated from thermophilic bacteria. Comparative analysis of these Rep proteins showed that despite the significant level of divergence, these Rep proteins share a high degree of similarity in the regions of five well-known conserved domains of RC Rep proteins and fall into two groups in accordance with the similarities found in their active sites.

BETA2 TOXIN, A NEW TOXIN PRODUCED BY *CLOSTRIDIUM PERFRINGENS*

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A new toxin (Beta2) and its gene have been characterized from a *Clostridium perfringens* strain isolated from necrotic enteritis in piglet. At the amino acid level, Beta2 toxin (27670 Da) presents no significant homology with the Beta toxin (called Beta1) (34861 kDa) from *C. perfringens* type B NCTC8533 (S. E. C. Hunter, J. E. Brown, P. C. F. Oyston, J. Sakurai, R. W. Titball, Infect. Immun. 1993, 61:3958-3965). Both toxins were lethal for mice and cytotoxic for cell line I407, and induced a rounding and a cell lysis without affecting the actin cytoskeleton. The genes of Beta1 and Beta2 toxins have been localized in large plasmids in *C. perfringens* but they were not closely linked. Beta2 toxin-producing *C. perfringens* strains were found associated to animal diseases such as necrotic enteritis in piglets and enterocolitis in horses.

A COMPARISON BETWEEN *CLOSTRIDIUM PERFRINGENS* (NCTC8237) ALPHA-TOXIN AND *CLOSTRIDIUM BIFERMENTANS* (ATCC638) PLC

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The phospholipase C's produced by *Clostridium perfringens* and *Clostridium bifermentans* are structurally related but exhibit different properties. *C. bifermentans* exhibits lower activity than *C. perfringens*. The work reported here was carried out in order to gain a better understanding of the molecular basis for the differences in the properties of these two proteins. The purification of recombinant-α-toxin isolated from the periplasmic space of *Escherichia coli* has previously been reported (1). Our initial studies showed that the cloned *C. bifermentans* PLC, expressed from the *C. bifermentans* promoter (2), was produced at a low level in *E. coli*. To achieve a higher level of expression , we used the *C. perfringens plc* promoter upstream of the *C. bifermentans plc* gene. The cloned *C. bifermentans plc* gene was nucleotide sequenced and several differences were found on comparison with the previously published sequence (2). The recombinant α-toxin and *C. bifermentans* PLC proteins were isolated from the periplasmic space of *E. coli*. Both proteins have been purified and their properties compared. The extracted proteins were purified using anion exchange chromatography (DEAE sephacel anion exchange column chromatography followed by FPLC Mono Q column chromatography). Native gel electrophoresis of the *C. bifermentans* PLC revealed four bands of increasing weight, suggesting multimerization. This multimerization was investigated using denaturing and non-denaturing techniques. Multimerization was not observed in *C. perfringens* NCTC8237 α-toxin.

1. Basak, *et al*, (1994), J. Mol. Biol., **244**:648-650.
2. Tso & Siebel, (1989), Infect. Immun., **57**:468-476.

Effect of 18 carbon-fatty acyl residues in phosphatidylcholine in liposomes on membrane-damaging action of *Clostridium perfringens* alpha-toxin

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The mechanism of membrane-damage induced by *Clostridium perfringens* alpha-toxin was studied using multilamellar liposomes prepared from phosphatidylcholines containing fatty acyl residues of 18 carbon atoms and cholesterol (molar ratio of 1 : 1). Alpha-toxin at the range over 0.1 and 60 ng/ml caused carboxyfluorescein leakage from liposomes composed of dioleoyl-L-α-phosphatidylcholine (DOPC) and dielaidoyl-L-α-phosphatidyl choline (DEPC), respectively, in a dose-dependent manner. Exposure of liposomes composed of β-oleoyl-γ-stearoyl-L-α-phosphatidylcholine (OSPC) or β-stearoyl-γ-oleoyl-L-α-phosphatidylcholine (SOPC) to alpha-toxin over 10 ng/ml resulted in the leakage. Furthermore, the toxin induced phosphorylcholine release from these liposomes and bound to these liposomes in the following order : DOPC>OSPC=SOPC>DEPC. However, these phosphatidylcholines solubilized by deoxycholate were just the same in the sensitivity to phospholipase C activity of the toxin. The toxin caused no effect on distearoyl-L-α-phosphatidylchoine (DSPC). These observations indicated that the action of alpha-toxin on membranes was dependent on membrane fluidity and double bond in fatty acyl residue.

CONSTRUCTION AND CHARACTERISATION OF HYBRID *C. PERFRINGENS, C. BIFERMENTANS* PHOSPHOLIPASE C'S

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Clostridium perfringens α-toxin is a phospholipase C which also possesses haemolytic activity and is toxic. In comparison the *Clostridium bifermentans* phospholipase C has a lower specific activity, is weakly haemolytic and is non toxic. There is 51% identity between the amino acid sequences of these enzymes (1). Evidence indicates that these phospholipases are composed of two domains (N and C domains). The roles of these domains were investigated by creating hybrid proteins. This was achieved using the PCR to generate a mutation in the DNA encoding the hinge region between the two domains. This approach allowed us to fuse the individual N and C domains in all possible combinations. The genes encoding the proteins were cloned into pBluescript downstream of the *C. perfringens* α-toxin gene promoter and electroporated into *E. coli*.

The authenticity of the constructs was confirmed by nucleotide sequencing. Expression of the proteins was demonstrated using western blotting. A preliminary study was carried out using crude periplasmic protein preparations of the hybrids and the results have provided new insights into the function of the two domains.

1. Tso & Siebel, 1989, Infect. Immun., **57**:468-476

STRUCTURAL DETERMINANTS IN THE INTERACTION BETWEEN TETANUS TOXIN AND BOTULINUM NEUROTOXINS TYPE B, D, F, AND G AND THEIR SUBSTRATE, VAMP/SYNAPTOBREVIN

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The isoform 2 of VAMP/synaptobrevin is an essential protein component of the synaptic vesicles directly involved in docking and fusion process by interaction with two other proteins of the presynaptic membrane, SNAP 25 and syntaxin. Tetanus toxin and botulinum neurotoxins type B, D, F and G are metalloproteases which block neurosecretion by a highly specific and efficient cleavage of VAMP/synaptobrevin. The high substrate specificity of such zinc-endopeptidases suggests an involvement of other regions of the target protein besides the cleavage site. VAMP contains two copies of a nine residue motif, termed V1 and V2, which is also present in SNAP25 and syntaxin, and has therefore been named SNARE motif.

We have obtained evidence that the acidic residues of V2 play a crucial role in the interaction of BoNT/B and BONT/G with VAMP, but have no effect on the proteolytic activity of tetanus neurotoxin, which hydrolyses the same peptide bond cleaved by BoNT/B. Conversely, the acidic residues of V1 are important for the activity of tetanus neurotoxin, and are not involved in the interaction with BoNT/B and G. The proteolysis of VAMP by BoNT/D is completely prevented by the replacement of Met46 with alanine, whereas that induced by BoNT/F is inhibited by replacement of the negatively charged residues of V1 and V2.

These results provide clear evidence for the involvement of the SNARE motif in the recognition of VAMP by the 5 VAMP specific clostridial neurotoxins. The different effect of the mutations of V1 and V2 on the proteolytic activity of the different neurotoxins indicate that different residues are present in the area of protein-protein contact between the metalloproteases and VAMP.

EFFECTS OF BOTULINUM NEUROTOXINS ON SENSORY AFFERENT NEURONS.

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A primary culture of embryonic rat dorsal root ganglia neurones is described that maintains an ability to secrete substance P in a calcium-dependent manner when stimulated by depolarisation with high potassium physiological buffer. The stimulated, calcium-dependent secretion was shown to be sensitive to botulinum neurotoxins, both in terms of substrate cleavage and inhibition of stimulated secretion. The relative sensitivities to botulinum neurotoxins A, B, C and F were assessed and found to be markedly different, with botulinum neurotoxin A displaying the greatest potency with an IC_{50} of 0.03nM, whilst BoNT/B was the least potent with an IC_{50} of >100nM. The temporal characteristics of the activity of the botulinum neurotoxins in the dorsal root ganglion neurones was also investigated. With BoNT/A effects were observed within one hour of toxin application and activity could still be observed in cells 15 days after toxin application, assessed as both inhibition of secretion and cleavage of substrate protein. Potentially these cells offer a highly sensitive system with which to study the botulinum neurotoxins.

DISTRIBUTION OF THE CLOSTRIDIAL NEUROTOXIN RECEPTORS IN RAT BRAIN

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Clostridial neurotoxins (tetanus and botulinum toxins) are potent blockers of neurotransmitter release. These toxins act specifically on the nervous system by interacting with still non-identified protein receptors together with gangliosides. Whereas many biochemical data is available on their binding properties to neuronal membranes *in vitro*, there is poor morphological evidence of their binding to mammalian central nervous system.

In the present study the binding of tetanus and botulinum neurotoxin type A to rat brain sections is reported. Both toxins bound to nerve terminals with a broad distribution in brain. Tetanus toxin additionally bound to nerve fibers. The staining patterns were clearly shown to be due to the interaction of the heavy chains, which contain the binding moiety, with the tissue.

In an attempt to investigate the nature of the acceptors present in the tissue, some sections were preincubated with periodic acid. This treatment resulted in the additional binding of botulinum neurotoxin type A to nerve fibers. Since the extended staining of nerve terminals was not modified by this pretreatment, it is suggested that protein receptors of clostridial neurotoxins are located at the nerve terminals, which may be common constituents of the synapses.

ACTIVATION OF *CLOSTRIDIUM PERFRINGENS* EPSILON-PROTOTOXIN AND AN EFFECT OF EPSILON-TOXIN ON RAT HIPPOCAMPUS.

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ε-Toxin is produced as prototoxin by *Clostridium perfringens* B and D strains and is activated by tryptic digestion. We examined activation of ε-prototoxin by λ-toxin, a metalloprotease produced by the same type B strain. Mouse lethality test showed that 50% lethal dose (LD_{50}) of the prototoxin with and without λ-toxin treatment were 110 and 70,000 ng/kg of body weight, respectively. The lethal activity of the prototoxin activated by λ-toxin was comparable to that by trypsin plus chymotrypsin and higher than that by trypsin alone: LD_{50} of the prototoxin treated with trypsin and trypsin plus chymotrypsin were 320 and 65 ng/kg of body weight, respectively. Determination of the N-terminal aa sequence of each activated prototoxin revealed that λ-toxin cleaved between the 10th and 11th aa residues from the N-terminus of the prototoxin, while trypsin and trypsin plus chymotrypsin did between 13th and 14th aa residues. The molecular weight of each activated prototoxin was also determined MALDI-TOF-MS. The C-terminus deduced from the molecular weight is located at the 23th or 30th aa residue from the C-terminus of the prototoxin, suggesting that removal of not only N- but also C-terminal peptides is responsible for the activation of the prototoxin. We also examined an effect of ε-toxin on rat brain by injecting trypsin-activated ε-toxin intravenously into mice at a dose of LD_{50}. Hippocampal neurons were shown to be damaged by HE stainining, immunostaining of microtuble associated protein 2 and zinc staining.

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REDUCING THE CATALYTIC DOMAIN *OF CLOSTRICIUM DIFFICILE'S* TOXIN B-10463 TO A ENZYMATICALLY ACTIVE N-TERMINAL 467 AMINO ACID FRAGMENT

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Abstract

In an attempt to directly approach the postulated toxic domain of *C. difficile's* TcdB-10463, eight subclones of different size and locations in the N-terminal third of the toxin were generated. Expression of these toxin fragments was checked in Western Blots and the enzymatic activity of the expressed proteins was analysed by glucosylating Ras related small GTP-binding proteins. Two polypeptides of 875aa (TcdBc1-3) and 557aa (TcdBc1-H) glucosylated their targets Rho, Rac and Cdc42 with unchanged activity and specificity. In comparison 516aa (TcdBc1-N) and 467aa (TcdBc1-A) protein fragments exhibited highly reduced activity, while Tcdc1 and TcdB2-3 (aa 1-243 and 244-890, respectively) were enzymatically inactive. Our results indicate that all structures involved in the catalysis are located at several different sites within the 557 aa fully active fragment. The shortest still enzymatically active protein covers aa 1-467 and obviously fullfills all minimal requirements for glucosylation. The data support the postulated three domain model of „large clostridal cytotoxins".

FUNCTIONAL CHARACTERIZATION OF RANDOMLY-GENERATED POINT MUTANTS OF THE *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN. J. Kokai-Kun and B. McClane, Univ. Pittsburgh Sch. Medicine, USA

The *C. perfringens* enterotoxin (CPE) is a 319 amino acid, single polypeptide, membrane-active toxin. It has a unique multi-step mechanism of action that sequentially involves; specific binding of CPE to a 50kDa eukaryotic membrane protein to form a "small" complex, development of a post binding physical change in this small complex, association of this small complex with a 70kDa eukaryotic membrane protein to form "large" complex, and rapid development of membrane permeability alterations. To better understand this action, studies of the CPE structure/function relationship have been performed using deletion analysis. These studies demonstrated that, i) the N-terminal 44 amino acids of CPE are not required for CPE cytotoxicity, ii) amino acids between 45 and 53 are necessary for completion of the post-binding physical change step in CPE action, and iii) the extreme C-terminus (amino acids 290-319) of CPE contains an essential region for receptor-binding. In order to expand on this existing knowledge of CPE structure/function relationships, we sought to identify specific amino acid residues involved in the various steps in CPE action. To accomplish this, the cloned *cpe* gene was subjected to random mutagenesis in XL-1 Red® mutator *E. coli* (Stratagene). Using this technique, 88 clones that had altered cytotoxic activity were identified, from a total of 1000 clones screened. Six well-expressed mutants were further characterized for their ability to complete each of the four steps in CPE action, as mentioned above. These studies indicate that CPE variant 7B7 (Trp226ΔStop) is a noncytotoxic truncation variant blocked at the binding step, a result consistent with previous studies identifying the C-terminus of CPE as an essential binding region of the enterotoxin. Noncytotoxic variants 7H8 (Ser59ΔLeu) and 8C5 (Gly49ΔAsp) were shown to be able to undergo the post-binding physical change, but could not form large complex. Finally, 1A7 (Ser167ΔPro), 1E5 (Arg137ΔGly) and 3H6 (Arg116ΔSer) formed sharply reduced levels of large complex, consistent with the greatly reduced cytotoxicity exhibited by these variants. These results with 7H8, 8C5, 1A7, 3H6 and 1E5 support large complex formation being an essential step in CPE action, and also provide the first direct evidence that residues in the N-terminal half of CPE are involved in large complex formation.

EVIDENCE THAT *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN LACKS SUPERANTIGENIC ACTIVITY, BUT INDUCES AN IL-6 RESPONSE.

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A previous report by Bowness et. al. (*J. Exp. Med.* 1992, 176:893-896) indicated that *Clostridium perfringens* enterotoxin (CPE) has superantigenic properties. The goal of the current study was to confirm this finding, since this putative CPE superantigenicity could conceivably play a role in CPE-mediated diseases. In light of the previous study's results, we were surprised to find that native CPE purified from *C. perfringens* was unable to stimulate *in vitro* proliferation of human lymphocytes from any of seven different donors, although a known superantigen, Toxic Shock Syndrome Toxin-1 (TSST-1) did have mitogenic effects on these same cell preparations. The failure of CPE to stimulate these human lymphocytes was not a result of CPE-induced cell death, since these lymphocytes continued to exclude trypan blue throughout the duration of these proliferation experiments. Identical results were obtained using recombinant CPE expressed in *E. coli.* Since these results are not those expected of a superantigen, we also evaluated whether CPE-treated lymphocytes produced IL-1, IL-2, IFNγ, or TNF, which are cytokines commonly associated with superantigenic stimulation. No increase in the concentration of any of these cytokines (or IL-4 or IL-10) was detected in the supernatants of CPE-treated lymphocyte cultures, although similar TSST-1 treatment of these cells did induce the changes in cytokine profiles expected of a superantigen. During this study we did observe stimulation of release of the proinflammatory cytokine IL-6 from CPE-treated lymphocytes. Collectively, these results bring into serious question whether CPE is a superantigen. Additonally, our observation that CPE induces a strong IL-6 response could be important if inflammation contributes to the gastrointestinal symptoms of CPE-associated diseases.

STRUCTURE AND FUNCTION OF THE NONTOXIC COMPONENTS OF *CLOSTRIDIUM BOTULINUM* PROGENITOR TOXINS.

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The neurotoxins produced by *Clostridium botulinum* are associated with the nontoxic components, and form three different-sized neurotoxin-nontoxic component complexes (designated as the progenitor toxins) in their cultures. Type A strain produces 19 S, 16 S, and 12 S progenitor toxins, types B, C, and D strains produce 16 S and 12 S progenitor toxins, and type E and F strains produce only 12 S progenitor toxin. The 12 S toxin consists of a neurotoxin (7 S) and a nontoxic component having no hemagglutinin (HA) activity designated as nontoxic-nonHA. The 19 S and 16 S toxins are formed by conjugation of the 12 S toxin with HA.

Food-borne botulism is caused by ingestion of foods containing the progenitor toxins. The nontoxic components are considered to be very important to the development of food poisoning because they protect the neurotoxin from the acidity and proteases in the digestive tract. However, the role of the nontoxic components on the oral toxicity has not been fully clarified. In this study, we report that the HA of the type C 16 S toxin is very important in binding and absorption steps in the small intestine of guinea pig. To study the structure/function relationship of the HA, all subcomponents of the HA have been expressed in *E. coli*. Works is in progress to identify the subunit needed for the intestinal binding of the HA component.

SOME ASPECTS OF INTESTINAL COLONISATION BY *C. difficile*

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Intestinal colonisation mechanisms by *Clostridium difficile* remain poorly elucidated. *C. difficile* must penetrate the intestinal mucus layer to attain its target cells to which it adheres. The aim of our studies is to elucidate different steps of the colonisation process by investigating mechanisms of adhesion to mucus and epithelial cells at the cellular and molecular level.

Three different techniques were used to study adhesive capacity of *C. difficile*: an adhesion assay to caecum of axenic mice *in vivo* and *ex vivo*; and *in vitro* adhesion assays to tissue culture cells. Furthermore, proteolytic activity of *C. difficile* was studied using different substrates: gelatine, collagen, Pz-peptide, casein, azocasein, and azocoll.

C. difficile can adhere to intestinal mucus and tissue culture cells. *In vivo*, the toxinogenic strains adhere better than non toxinogenic ones. Various environmental factors (pH, osmolarity, iron, oxydative shock) can modify adhesion to tissue culture cells. Adhesion is inhibited by glucose, galactose, di- and trisaccharides containing these sugars, and *N*-acetyl galactosamine, suggesting that the adhesin is a lectin.

Little inter-strain variability in the ability to produce proteases exists, regardless of toxinogenicity or serogroup. All strains studied are capable of producing two types of enzymes, a 35-kDa gelatinase and a 120-kDa collagenase. These hydrolytic enzymes could play a role in the degradation of the mucus core, thus permitting penetration of the mucus layer and access to underlying mucosa.

CYTOTOXICITY OF *CLOSTRIDIUM PERFRINGENS* EPSILON TOXIN ON MDCK CELLS.

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Epsilon toxin is produced by *Clostridium perfringens* types B and D and is responsible for a rapidly fatal enterotoxaemia in sheep and other animals. Epsilon toxin is lethal for mice, dermonecrotic and edematogen. A basic property of this toxin is that it increases vascular permeability and causes edema in lung, heart and kidney. In particular, epsilon toxin can across the brain barrier inducing cerebral edema and necrosis. *In vitro*, only the Madin Darby Canine Kidney cell line (MDCK) has been found to be sensitive to epsilon toxin.

We showed that epsilon toxin induces an increase of the MDCK cell volume and formation of blebs. Agents blocking endocytosis (benzyl alcohol and sucrose, or *C. perfringens* iota toxin which disrupts the actin cytoskeleton) did not prevent epsilon toxin cytotoxicity. Immunofluorescence studies provide additional evidence that epsilon toxin is localized on the cell surface during the intoxication process. These data strongly suggest that the internalization of the epsilon toxin into the cytosol is not required for the cytotoxicity and that the toxin remains associated to cell membrane.

Our findings with ^{125}I-labeled epsilon toxin showed that the toxin, and not the prototoxin, associates specifically with the membrane of sensitive MDCK cells and forms a large complex of about 155 kDa. The large membrane complex corresponds probably to the binding of several monomers of epsilon toxin with a protein receptor. The complex formation correlates with the cytotoxicity and could be involved in the alteration of membrane permeability by pore formation or by modification of a specific ion channel.

CLOSTRIDIUM DIFFICILE ISOLATES FROM HUMAN EPIDEMICS ARE HIGHLY LETHAL TO HAMSTERS AT LOW INOCULUM. S. Johnson, S. Sambol, J. Shim, C. Dileto, M. Merrigan, D. Gerding. VACHS, Lakeside Division & Northwestern U. Medical School, Chicago, IL, USA.

Clostridium difficile cecitis in the Syrian golden hamster is a well-studied model of *C. difficile* colitis in humans. Cecitis in hamsters is typically a fulminant illness with high associated mortality but a spectrum of virulence is often noted with different *C. difficile* strains. We have used this hamster model to study the virulence characteristics of 3 *C. difficile* strains responsible for major nosocomial epidemics in human patients locally and throughout the U.S.: Restriction endonuclease analysis (REA) types B1, J9, and K14. Initial dose ranging studies indicated that oral inoculation of 100 CFUs of B1 five days after administration of 30 mg/kg of clindamycin by gavage syringe was uniformly lethal. We observed 100% mortality 48 h after inoculation of 3 groups of 10 hamsters inoculated with 100 CFUs of each of the human epidemic strains. Death occurred within hours of developing symptoms which consisted of lethargy, and wet-tail (if witnessed at all). At necropsy in one hamster, the cecum was congested and hemorrhagic with a histologic picture of focal, but mild inflammation within the lamina propria. In addition, marked pulmonary hemorrhage was present with pulmonary vascular dilatation. These findings suggest a different mechanism of mortality in hamsters than cecal inflammation, per se. Disease is lethal in hamsters at low inoculum which may explain how these strains are easily spread in hospitals where exposure to low inoculum of *C. difficile* is likely.

CHARACTERIZATION AND TRANSCRIPTIONAL ANALYSIS OF THE PATHOGENICITY LOCUS OF *CLOSTRIDIUM DIFFICILE* 10463

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With a set of three paired primers used in polymerase chain reactions we succeeded in delineating the pathogenicity locus (PaLoc) of *C. difficile* coding for the toxin genes *tcdA* and *tcdB* and the three accessory genes *tcdC-E*. Sequencing of the appropriate stretch of DNA in *C. difficile* VPI10463 and four additional toxinogenic strains proved a high conservation of the borders of the PaLoc in all these strains. Our data define the locus as a distinct genetic element. Comparing the sequences of five toxinogenic and five non-toxinogenic strains the integration site of the PaLoc was defined. This showed that a stretch of 115 bp found in non-toxinogenic strains is replaced by the 19 kb locus in toxinogenic strains. To analyse the transcription of the PaLoc genes a RT-PCR-approach was established. Transcription analysis of the five genes *tcdA-E* showed that they were all transcribed. Analysing the growth dependence of transcription it was shown that the five genes are organized in two groups with an inverse transcription of *tcd*C versus *tcdA,B,D,E*. Primer extension analysis determining the transcription initiation sites of the genes *tcdA, B, D* and *E* and a RT-PCR approach searching for readthrough transcripts indicates that these genes are transcribed mono- and polycistronically in the late growth phases of a *C. difficile* culture. Based on our data we discuss a model for the growth-phase-related and coordinate transcription of the PaLoc genes.

IDENTIFICATION OF NOVEL VirR/VirS-REGULATED GENES IN *C. PERFRINGENS*

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In *C. perfringens*, a two-component regulatory system, VirR/VirS is known to regulate the production of alpha-toxin (phospholipase C), theta-toxin (perfringolysin O), kappa-toxin (collagenase), and protease. Since theVirR/VirS system globally regulates these virulence factors, it is possible that the VirR/VirS system regulates the expression of other unknown virulence factors or other unidentified regulatory genes. Here we tried to find other VirR/VirS-regulated genes using a differential display method in C. perfringens.

Total RNA prepared from strains 13 (wild type) and TS133 (*virR*-mutant of strain 13) was labeled with random primers, [α-32P]dCTP, and AMV reverse transcriptase and the cDNA probes were hybridized to a dot-blotted plasmid library prepared from strain 13. The densities of the DNA spots that hybridized with the strain 13-cDNA probe were compared with those hybridized with the TS133-cDNA probe. We selected the clones whose densities were apparently different between strain 13- and TS133-cDNA probes. After several screening, a total of five clones was selected as the VirR/VirS-regulated clones. Interestingly, there seemed to be two kinds of clones which were thought to be positively- or negatively-regulated by the VirR/VirS system. By Southern hybridization, two clones out of the five turned out to be the *pfoA* and *colA* genes which are known as the VirR/VirS regulated toxin genes. We analyzed other three clones further with plasmid DNA-cDNA hybridization, Northern hybridization, and nucleotide sequencing. These clones contained several genes which have not been reported and are expected to play some roles on the pathogenesis or signal transduction in *C. perfringens*.

IMPROVING THE DESIGN OF *CLOSTRIDIUM PERFRINGENS* COMPONENT IN CONVENTIONAL VACCINES.

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Recently, it was demonstrated that *Clostridium perfringens* type D was responsable of an outbreak of enterotoxaemia in goats, with a high rate of mortality.(Uzal, F.A. et al, Veterinary Record, 135 : 279-80, 1994).
There is a lack of reliable epidemiologycal data in our country and upon this report, it was necessary to review the status of the *C.perfringens* type D component, in current formulations for vaccine prevention in the area.
We have analyzed a group of vaccine that could be *C. perfringens* D strains (n=17) from our culture collection and one strain from a recent outbreak.
All strains were retyped by conventional microbiologycal methods, confirming Clostridium perfringens Type D in all cases.
The epsilon-toxin of *Clostridium perfringens* type D, is thought to be the major causative element of certain lethal enterotoxaemias encountered in sheeps and goats.
We also have explored in each strain the presence of epsilon-toxin gene (*etx*) using the appropiate primers, by polymerase chain reaction .
Only one of 18 strains showed a negative PCR signal for *etx*.
Strain SG analyzed by migration in agarose gels showed a DNA pattern compatible with the presence of cryptic high molecular weight plasmid..
SG have been chosen as the most proper to be incorporated in vaccine formulations. We have not been able, to establish if the *etx* gene is harbored in a chromosomal or plasmid position yet.

POLYMERASE CHAIN REACTION DETECTION OF DIFFERENT *CLOSTRIDIUM PERFRINGENS* TYPES IN FAECES OF GOATS.

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Traditionally the typing of *Clostridium perfringens* is performed by the mouse neutralisation test. This test is time consuming, expensive, and requires the use of living animals which, apart from ethical considerations, is prone to the lack of precision inherent in such biological tests. In addition, specific antisera are no longer commercially available. A polymerase chain reaction (PCR) was used to identify the genes encoding the major toxins of *Clostridium perfringens* in faeces of goats (alpha, beta, epsilon and iota). The samples were either processed directly by PCR or enriched by overnight culture in thyoglycollate broth prior to PCR. When used to identify *C. perfringens* (types A, B, C, D and E) in samples artificially spiked with these microorganisms, the PCR detected as few as 3.6 x 102 to 3.6 x 103 CFU/g of sample after enrichment. When faeces were processed directly by PCR, the different *C. perfringens* toxinotypes were detected only when present in concentrations of 1 x 107 CFU/g of sample or higher. Faeces were collected from 20 goats on a dairy farm and also from 9 colostrum-deprived kids that had been separated from their dams at birth. In the adult kids the genes encoding alpha and epsilon toxin were detected in 4 animals, while in other 5 goats only the gene encoding production of alpha toxin was detected. In the colostrum-deprived kids none of the genes encoding the major toxins of *C. perfringens* were detected. This technique showed high specificity and sensitivity to detect the different toxinotypes of *C. perfringens* in faeces of goats.

PATHOGENESIS OF *CLOSTRIDIUM PERFRINGENS* TYPE D ENTEROTOXAEMIA AND ENTEROCOLITIS IN GOATS

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Enterotoxaemia in goats is mainly characterised by enterocolitis, and it has been suggested that the poor efficiency of commercial vaccines in preventing this disease is due to the local action *of Clostridium perfringens* toxin/s at the intestinal level, where circulating antibodies might not exert their action. Five goats kids were vaccinated with an incomplete Freund's adjuvant *C. perfringens* type D epsilon toxoid vaccine on three occasions at 3 weeks intervals. Four similar additional kids were vaccinated with a commercial enterotoxaemia vaccine at the same times. Five other non-vaccinated kids were used as controls. All the animals were challenged with *C. perfringens* type D filtered culture supernatant intraduodenally one week after the last vaccination. At the time of challenge, the serum epsilon toxin antibodies in the oil-adjuvant vaccinated kids ranged between 2.45 and 230 IU/ml, while the kids that received the commercial vaccine had anti-epsilon toxin levels which varied between 0.22 and 1.52 IU/ml. The epsilon anti-toxin levels of the non vaccinated kids were below 0.03 IU/ml. No clinical or post-mortem alterations were observed in the kids that received the oil vaccine. Three of the four kids that received the commercial vaccine developed mild, pasty diarrhoea with a slight reddening of the colonic mucosa being the only post-mortem change observed. The non-vaccinated kids developed severe diarrhoae, respiratory distress and central nervous system signs, and were killed humanely between 6 and 24 hours after challenge. Post-mortem changes consisted on pseudomembranous colitis, lung oedema and perivascular oedema of the brain. Moderate to high serum levels of anti-epsilon antibody seem to protect goats against the systemic and also the intestinal effects of *C. perfringens* type D toxins.

PCR IN MONITORING CLOSTRIDIUM DIFFICILE IN CLINICAL TREATMENT

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Introduction

The role of Clostridium difficile as a patogen in AAD-antibiotic associated diarrohea is well established but patogenesis is not very well understood. The effectiveness of treatment with or without probiotics along with the great tendency for relapses demand a better scope of understanding during the clinical course.

Methods and Study group

Over a period during 1994-95 cases with suspicion of Clostridium difficile enteritis were enrolled and studied. Samples were collected pretreatment and then consecutive on day 2 and 3 followed by a post-treatment sample 4 weeks after the first one.Twenty samples from five patients were analyzed both with PCR on cultured colonies and directly on fecal sample.

Results and Discussion

Two of the clinical cases showed an interrresting pick-up of non-toxogenic Clostridium difficile in the posttreatment sample and did not relapse. Could this be a protective messure in the uncomplicated cases serving the healing process ?

There were also two patients negative in culture and tissueculturetest but with a typical clinical appearance and treated with metronidazole with success. Other etiology could be an explanation but in both cases the PCR directly on fecal sample showed faint activity in the toxin A gene alone respectively in the tox B, and this could as well stand for a true causal relationship indicating a increased sensitivity for the PCR test.

Further studies are in progress to expand these data and also typing the strains involved.

IMMUNISATION WITH RECOMBINANT *CLOSTRIDIUM PERFRINGENS* BETA-TOXIN; THE ABILITY OF WILD TYPE AND MUTANT STRAINS TO INDUCE PROTECTIVE IMMUNITY.

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The aim of this study was to improve the production of beta-toxin vaccines. A useful candidate would have greatly reduced toxicity but must at the same time induce protective immunity. In addition large scale vaccine production requires a candidate which is easy to produce and sample, without the need for complicated purification steps.

The beta-toxin gene of *C. perfringens* type C was cloned and the protein was produced as a 62 KD fusion protein (GST-beta) in *E. coli*. The fusion protein was non-toxic in mice. In addition wild type and mutant beta-toxin clones were expressed in *Bacillus subtilis* where the protein is secreted to the extra cellular medium. Mutant clones with two different substitutions of the same amino acid had considerably reduced biological activity compared to the wild-type protein expressed in *B. subtilis*.

Rabbits were immunised with the GST-beta fusion protein and the two mutant proteins. The antibody production was measured in an ELISA test and the protective immunity of the antiserum was tested in mice.

The GST-beta induced strong antibody response which neutralised the lethal action of beta-toxin of *C. perfringens* type C. The two mutant proteins secreted by *B. subtilis* both induced antibody response, but the protective immunity obtained appears to differ between the two strains.

MOLECULAR ARCHITECTURE & REA OF A DNA FRAGMENT FROM CLOSTRIDIUM SEPTICUM GENOME AND ITS IMPLICATION IN PATHOGEN DETECTION

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Clostridium septicum, a gram positive fastidious anaerobe, causes dreaded diseases in animals and man. Due to its nefarious association with certain human malignancies, this bacterium is also emerging as a potential pathogen of considerable medical significance. Animals may die within 1-2 days in severe infections with C. septicum and /or C. chauvoei / C. perfringens. Therefore, development of a more specific, reliable and rapid method for pathogen detection and disease diagnosis even in preclinical conditions is essentially needed for its application in better animal health and husbandry practices. In view of above, a recombinant plasmid Amp^r pSDS 12 (4.5 Kb) was selected at random from Hind III genomic library of C. septicum constructed in pUC18 after shotgun cloning and mapped by REA studies. A 1.8 Kb DNA fragment of C. septicum genome inserted out from pSDS12, was found to have three sites for Ssp I (at 0.55, 0.8, 1.6 Kb), one site each for Hinc II (at 1.8 Kb), EcoR I (at 1.4 Kb) and Xba I (0.7 Kb). Not I could not cleave this recombinant plasmid. Identical restriction genetic maps of pSDS 12 and insert (1.8 Kb) obtained from Et-Br stained gels and southern blots confirmed their unambiguous cleavage sites. Presence of a single unique site for Nsi I at 1.95 Kb of pSDS 12 is very interesting and significant finding useful for further cloning. DIG- labelled 1.8 Kb DNA insert gave positive result with C. septicum and no signal with Pasteurella multocida and Salmonella enteritidis DNA. These results may be useful for detection of C. septicum after further investigation.

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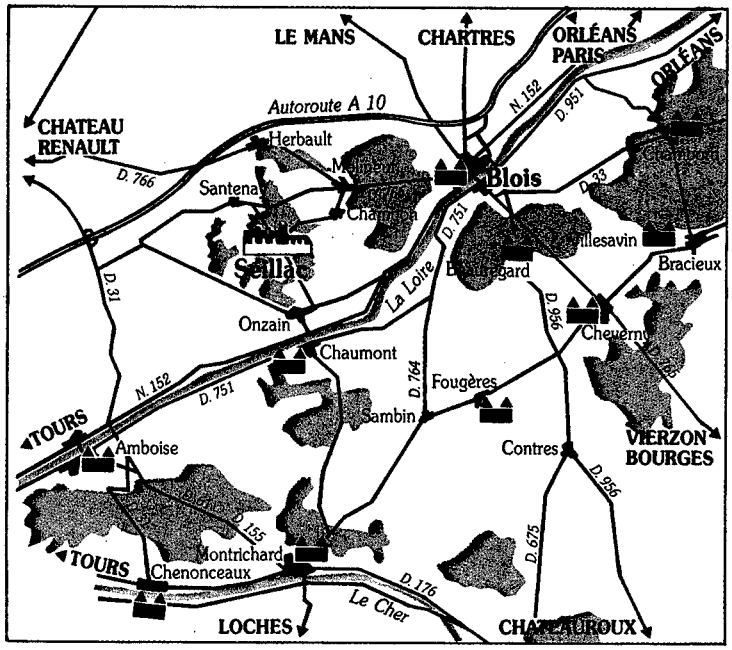

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