ANTIBIOTIC-INDUCED COLITIS IMPLICATION OF A TOxin NEUTRALISED BY CLOSTRIDIUM SORDELLII ANTITOXIN

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Summary
A toxin(s) has been demonstrated in the stools of two patients with antibiotic-associated colitis. This toxin(s) was heat-labile, was rapidly lethal for hamsters, increased vascular permeability in rabbit skin, and was cytotoxic for cells in tissue-culture. It was neutralised by Clostridium sordellii antitoxin but not by antitoxins prepared against other clostridia; Escherichia coli, and Vibrio cholerae toxins. These characteristics were identical to those of a toxin implicated in the aetiology of antibiotic-induced colitis in the hamster. One patient improved rapidly after treatment with oral vancomycin, and at the same time the toxin disappeared from the stool.

Introduction
Pseudomembranous colitis has been recognised as a complication of antimicrobial therapy since 1952.1 In recent years, clindamycin, lincomycin, and ampicillin have been most frequently associated with this disease.2,3 While the pathogenesis has remained unclear, Larson et al. have implicated a heat-labile toxin found in the faeces of patients with pseudomembranous colitis.4 We have demonstrated a similar toxin and its neutralisation by polyvalent clostridial antitoxin in hamsters with clindamycin-induced ileo-cæcitis.5 This study shows the presence of toxin(s) in faeces of two patients with antibiotic-associated colitis and the neutralisation of the toxin's activities by in-vitro incubation with Clostridium sordellii antitoxin. Resolution of the illness and disappearance of the toxin followed oral vancomycin in one patient.

Patients and Methods
Case I.—A 13-year-old girl was transferred from another hospital on Feb. 5, 1977, with fulminant HBsAg-negative hepatitis. Stage-iv encephalopathy developed despite treatment with corticosteroids, oral neomycin, fluids, and nutritional support. Two exchange transfusions were carried out within 72 hours of admission and within 30 hours she became conscious and had no abnormal neurological signs. 3 weeks later, an 8-day course of ampicillin was given (total dose 70 g) for a urinary-tract infection. Intermittent abdominal pain, vomiting, and fever developed during treatment. On March 23, 1977, the urinary-tract infection recurred, and she was treated again with ampicillin (10 g/day) and gentamicin (240 mg/day) for 14 days. Urinary symptoms and pyuria cleared up, but her temperature rose to 39-4°C, the abdominal pain increased, and diarrhoea developed. Sigmoidoscopy with biopsy on April 13, 1977, revealed pseudomembranous colitis. Treatment with oral vancomycin (500 mg every six hours) was started, and within 24 hours her diarrhoea ceased. Within 2 days she wasafebrile and without abdominal pain. Vancomycin was continued for 10 days without complications. Stools were obtained for study shortly before and again on the 2nd and 4th days of therapy with vancomycin.

Preparation of stool filtrates.—Fecal samples from the two patients were incubated at room temperature (21°C) for 30 minutes with gas-gangrene polyvalent antitoxin (P.C.A.) (Lederle, Pearl River, N.J., control no. 339-244); the individual U.S. standard clostridial antitoxins contained in P.C.A. (Cl. perfringens, Cl. septicum, Cl. histolyticum, Cl. adenia-tiens, and Cl. sordellii) (Dr E. Seligmann, F.D.A., Bureau of Biologics, Rockville, Md.); Cl. perfringens, Cl. novyi A and B, and Cl. septicum antitoxins (Burroughs Wellcome Co., Research Triangle Park, N.C.); human immune serum globulin (H.I.S.G.) (Merck, Sharp and Dohme, West Point, Pa., control no. 1170); or mycoplasma-free horse serum (Difco, Detroit, Mi.).

Preparation from case I was also tested for Esch. coli enterotoxin-like activity, neutralisation of his filtrate was attempted in Y-1 adrenal cells with heat-labile Esch. coli enterotoxin antiserum and Swiss Serum Vaccine Institute cholera antiserum (N.I.H., Bethesda, Md.) by the method of Sack.7

Physicochemical treatment of filtrates.—Filtrates were heated at 56°C for 30 minutes, incubated with trypsin (100 µg/ml) or pronase (100 µg/ml) (Sigma Chemical Company, St. Louis, Mo.) at 37°C for 24 hours, or had their pH adjusted with 1 mol/l HCl, or 1 mol/l NaOH.

Control solutions.—These consisted of solutions of P.C.A., H.I.S.G., Cl. sordellii antitoxin, other clostridial antitoxins, pronase, trypsin, and saline.

Toxicity studies in hamsters.—Male golden (Syrian) hamsters (Charles River Breeding Laboratories, Newfield, N.J.) weighing 60-90 g were injected intraperitoneally with 10-200 µl of the filtrates or control solutions. Hamsters were weighed daily, if they became moribund they were killed, and submitted to necropsy, otherwise necropsies were carried out within 24 h of death. Tissues were sent for histological examination.

Assay of vascular permeability factor in rabbit skin.—New Zealand white rabbits (Langshaw, Augusta, Mi.)
were depilated and injected intradermally with 100 µl of filtrate or control solutions. Evans blue dye (2%) was injected intravenously 24 hours later; diameters of areas of oedema, of haemorrhage, and of increased permeability (seen as "bluing" due to dye leakage) were measured after 2 hours according to the method of Evans et al. Each solution was tested in at least two rabbits and the mean diameter of the lesions was recorded. Solutions giving a mean diameter of bluing of at least 5 mm were considered positive for permeability factor.

**Assay of cytotoxicity in tissue cultures.**—Confluent monolayers of WI-38 human diploid fibroblasts and hela cells (HEM Research Inc., Rockville, Md.), were inoculated with 100 µl of filtrate preparations or control solutions and examined microscopically at 24 and 48 hours. Reactions were graded visually as: negative = no change in cells; weakly positive = 50–75% cells rounded; positive = 75–100% cells rounded and monolayer disrupted.

Stool filtrate from case I was tested for Esch. coli heat-labile enterotoxin-like activity in Y-1 adrenal cells by the method of Sack and Sack.

**Results**

**Toxicity for hamsters.**—All hamsters injected intraperitoneally with 100 µl of filtrate from case I died within 24 hours (table I). Weight loss, diarrhoea, haematuria, and death within 4 to 10 days occurred in 40% of animals receiving 10 µl of filtrate from case I. Necropsy on hamsters receiving 10 or 100 µl of filtrate revealed haemorrhages in the caecum and omental and mesenteric fat, ileal and caecal distension, and pleural effusions; blood clots were found in the bladders of those receiving 10 µl of filtrate. Microscopic examination confirmed purulent and hemorrhagic inflammation of the caecum and serosa.

When 100 µl of filtrate from case I was incubated with 100 µl of P.C.A. or H.I.S.G. before injection, the rapidly lethal response was no longer demonstrable (table I). Instead, a toxic syndrome similar to that induced by 10 µl of untreated filtrate was noted. These observations suggested partial neutralisation of filtrate toxicity. Hamsters injected with 100 µl of P.C.A. or H.I.S.G. remained well.

Filtrate prepared from the stool of case II (before vancomycin therapy) was also lethal for hamsters. Intraperitoneal injection of six animals with 200 µl of filtrate from case II killed half the hamsters within 48 hours. Because only a limited quantity of filtrates from both cases was available, further toxicity tests in hamsters could not be carried out.

Stool specimens obtained from case II 2 and 4 days after starting vancomycin were not toxic for hamsters (each specimen was tested in ten hamsters). Groups of ten hamsters injected with control filtrates or solutions of P.C.A. or H.I.S.G. in saline remained well.

**Vascular permeability factor assay in rabbit skin.**—When rabbits were injected intradermally with stool filtrate from either patient, localised induration and oedema developed within 4 hours and reached their maximum size by 18–24 hours. Vascular permeability factor was produced by filtrates from both patients (table II). Volumes of filtrate (10 µl of filtrate from case I and 100 µl of filtrate from case II) that induced areas of oedema

<table>
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<tr>
<th>TABLE I—EFFECT OF ANTISERA ON MORTALITY OF HAMSTERS GIVEN CASE-I FILTRATE* INTRAPERITONEALLY</th>
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<tbody>
<tr>
<td><strong>Solution incubated† with case-I filtrate (amount)</strong></td>
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<tr>
<td>Normal saline (100 µl)</td>
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<tr>
<td>Polyclonal clostridia antitoxin (100 µl)</td>
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<td>Human immune serum globulin (100 µl)</td>
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*Filtrate derived from the faces of case I. | †Filtrate incubated with saline or antiserum at 21°C for 30 min before intraperitoneal injection.

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<tr>
<th>TABLE II—NEUTRALISATION OF FILTRATE TOXICITY BY ANTISERA AS ASSAYED IN RABBIT SKIN AND TISSUE CULTURES</th>
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<tr>
<td><strong>Filtrate source (10 µl per test)</strong></td>
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<tr>
<td><strong>Antiserum</strong></td>
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</tr>
<tr>
<td>Assay</td>
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<tr>
<td>Rabbit skin*</td>
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<td>WI-38† and hela cells</td>
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with average diameters of at least 15 mm also produced intradermal haemorrhage and areas of increased permeability with mean diameters of at least 8 mm. These effects were neutralised (zero mm bluing) by incubation of the filtrates with P.C.A., H.I.S.G., and Cl. sordellii antitoxin. Intradermal reactions produced by the filtrates were not altered by incubation with normal horse serum or antisera produced against toxins of Cl. perfringens, Cl. septicum, Cl. cedematiens, and Cl. histolyticus.

Vascular permeability factor(s) present in these filtrates was destroyed by heat, alkalinisation to pH 10 and acidification to pH 6. Trypsinisation did not affect the permeability factor but incubation with pronase decreased the extent of oedema produced by both filtrates and the capillary permeability produced by 10 µl of filtrate from case 1 (5 mm bluing).

Filtrates prepared from the stool of case II while she was receiving vancomycin therapy did not contain permeability factor. Control filtrates, clostridial antitoxins, and solutions of trypsin, pronase and saline were also inactive.

Tissue culture assay.—The toxicity of filtrates from the two patients for WI-38 human diploid fibroblasts and hela cells corresponded to activity noted in the rabbit skin assay (tables II and III). Cl. sordellii antitoxin was the only clostridial antitoxin that neutralised the toxicity produced by the two patients' filtrates. In this assay, filtrate from case II was more toxic; as little as 1 µl of filtrate from case I (5 mm bluing).

Filtrates prepared from the stool of case II while she was receiving vancomycin therapy did not contain permeability factor. Control filtrates, clostridial antitoxins, and solutions of trypsin, pronase and saline were also inactive.

Discussion
This investigation confirms and extends the findings of Larson et al. that a heat-labile toxin(s) is present in the feces of patients with pseudomembranous colitis induced by clindamycin and ampicillin.4 Our results suggest that the toxin(s) is derived from Cl. sordellii. We found that this factor(s) was rapidly lethal for hamsters, produced oedema, haemorrhage and increased vascular permeability in rabbit skin, and was cytotoxic for WI-38 and hela cells. Toxic properties were neutralised by in-vitro incubation of the stool filtrates with Cl. sordellii antitoxin but not with other clostridial, Esch. coli or V. cholerae antitoxins. The lethal effect of our toxin on hamsters, its effect on vascular permeability in rabbit skin, its heat lability, and the pH at which it is effective, all resemble the properties of Cl. sordellii toxins which cause either oedema or haemorrhage.10 Differences in reactivity noted with the three tests we used might be due to different amounts of one or more Cl. sordellii toxins in the two filtrates. In addition, Cl. sordellii toxin obtained from the F.D.A., when tested in hamsters, rabbit skin, and WI-38 and hela cells produced effects identical to those of filtrates from cases I and II (unpublished observations).

Cl. sordellii, although pathogenic for man, has not previously been associated with gastrointestinal disease. Cohn, however, has demonstrated Cl. sordellii toxin production in dogs with bowel obstruction,11 a recognised cause of colitis.3 His observation and our findings may point to a common pathogenic mechanism responsible for the varied illnesses associated with pseudomembranous colitis in man and animals.

We have demonstrated that clindamycin-induced colitis can be prevented in hamsters by oral vancomycin.12 Furthermore, stool filtrate from hamsters protected by vancomycin is not toxic by our assays (unpublished observation). Case II was treated with vancomycin after the onset of colitis and improved within 24 hours. At the same time toxin disappeared from stool filtrates. Vancomycin has been reported to be effective in staphylococcal enterocolitis and pseudomembranous colitis.13 A recent review has questioned an aetiological role for Staphylococcus aureus in pseudomembranous colitis;2 we did not isolate staphylococci from stools of cases I and II, nor from hamsters with colitis. We do not believe staphylococci are pathogenically important. Moreover, the response to vancomycin therapy reported previously, in hamsters, and now in one of our patients, warrants a controlled clinical trial of this drug for the treatment of antibiotic-induced colitis.

We hope this brief report will stimulate microbiological and toxicological studies of all cases of pseudomem-

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**TABLE III—PHYSICOCHEMICAL PROPERTIES OF STOOL FILTRATES AS ASSAYED IN RABBIT SKIN AND TISSUE CULTURES**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Filtrate source (10 µl per test)</th>
<th>Heated at 56°C for 30 mins</th>
<th>Physicochemical treatment of filtrates</th>
<th>Effect of pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case I.</td>
<td>Pos.</td>
<td>Proteolytic enzymes</td>
<td>pH 4</td>
</tr>
<tr>
<td></td>
<td>Case II</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Neg.</td>
</tr>
<tr>
<td>Rabbit skin*</td>
<td></td>
<td></td>
<td>Pos.</td>
<td>Neg.</td>
</tr>
<tr>
<td>WI-38† and hela cells</td>
<td>Case I.</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Pos.</td>
</tr>
<tr>
<td></td>
<td>Case II</td>
<td>Pos.</td>
<td>Weak pos.</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

*Pos.=≥5 mm average diameter of bluing; Neg.=0 mm diameter of bluing.
†Pos. = 100% rounded and monolayer disrupted; Weakly Pos.=50–75% rounded; Neg. = normal monolayer.
CLOFIBRATE INCREASES LIPROTEIN-LIPASE ACTIVITY IN ADIPOSE TISSUE OF HYPERTRIGLYCERIDÆMIC PATIENTS

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Summary Clearance of plasma-triglycerides and activity of lipoprotein lipase in adipose tissue were studied in six hypertriglyceridæemic patients before and after a week of clofibrate therapy (2 g/day). Plasma-triglycerides decreased significantly from 6.85±1.1 to 2.66±0.29 mmol/l and triglyceride clearance increased significantly from 1.3±0.2 to 2.4±0.4%/min. There were concomitant significant increases in heparin-releasable lipoprotein lipase (95±16 to 181±34 nmol free fatty acids/10⁶ cells/h) and in extractable lipase (88±14 to 179±28 nmol free fatty acids/10⁶ cells/h). It is concluded that an important effect of clofibrate may be to increase the levels of adipose-tissue lipoprotein lipase and thereby improve the clearance of plasma-triglycerides.

Introduction

CLOFIBRATE is extensively used in the treatment of hypertriglyceridæmias. However, its mechanism of action is not clearly understood. The possibility that clofibrate may improve the clearance of very-low-density lipoprotein (V.L.D.L.) was suggested by the demonstration of increased levels of post-heparin plasma-lipo-protein-lipase activity after treatment with this drug.¹⁻³ Plasma-lipoprotein-lipase catalyses the rate-determining step for the removal of triglycerides from the blood.

An increase in levels of lipoprotein lipase in adipose tissue during clofibrate therapy has previously been observed in the rat,⁴ but not in man. We have studied the effect of clofibrate therapy on lipoprotein-lipase activity in adipose tissue, and plasma-triglyceride clearance in six hypertriglyceridæmic patients.

Patients and Methods

Six male patients (mean age 51±2 years, mean weight 76.2±3.5 kg) with hypertriglyceridæmia were studied while the men were fasting. Secondary causes of hypertriglyceridæmia such as excess alcohol intake and renal disease were excluded. Three patients were slightly glucose intolerant. All were classified as having W.H.O. type iv lipoproteinemia by electrophoresis. Informed consent was obtained before hospital admission. None of the patients was receiving drug therapy, and they were all on unrestricted diets before and during the period in hospital. The mean weight of the group decreased to 73.6±3.1 kg (p<0.01) during the week of the study.

On the day after admission, after an overnight fast, adipose tissue samples were taken by subcutaneous needle biopsy from the anterior abdominal wall after 2% lignocaine local anaesthesia. After a further overnight fast, blood was taken to determine fasting plasma-triglyceride. Patients remained in hospital and received clofibrate 1 g twice daily for 7 days, and the studies were repeated.

Lipoprotein lipase released by heparin in vitro was determined by incubating samples of adipose tissue (100 mg) in Earle’s bicarbonate buffer pH 7.4 containing 2.5%, crystalline bovine serum albumin with and without heparin (2 U/ml) at 37°C for 4 min at maximum power by means of a MSE 100 W ultrasonic disintegrator with titanium probe, in a solution of 6 ml of 0.16 mol/l "tris"/hydrochloric-acid buffer pH 8.1 containing 20 mg of crystalline bovine serum albumin. 50 µl of this substrate was used in each assay with 100 µl of normal human serum taken while fasting, to activate the enzyme. Incubations were at 37°C for 1 h, and then 200 µl was extracted in a Belfrage Vaughan partition system. 1 ml of the upper phase was counted in a Packard liquid scintillation counter model 2420.

Extractable lipase was determined by treating an homogenate of adipose tissue (100 mg) with acetone/ether (2/1 v/v) and sodium bicarbonate buffer pH 8.6, and the homogenate assayed for lipoprotein lipase as above. Adipose-cell size and triglyceride content were determined,³ enabling results of enzyme activity to be expressed as a unit free fatty acid (F.F.A.)/10⁶ cells/h. Results are expressed as means±S.E.M., and significance of differences calculated by Student’s t test.

Results

Mean fasting plasma triglyceride decreased from 6.85±1.1 to 2.66±0.29 mmol/l (p<0.01) and the in vivo measurement of triglyceride clearance (k₂) increased in all patients (see accompanying table) on clofibrate therapy. This was associated with an almost twofold increase in the activity of adipose-tissue heparin-releasable and extractable lipoprotein lipase (table). The heparin-releasable lipase increased in all six

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