SUPPLEMENT ARTICLE

Gastrointestinal Microflora Studies in Late-Onset Autism

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Some cases of late-onset (regressive) autism may involve abnormal flora because oral vancomycin, which is poorly absorbed, may lead to significant improvement in these children. Fecal flora of children with regressive autism was compared with that of control children, and clostridial counts were higher. The number of clostridial species found in the stools of children with autism was greater than in the stools of control children. Children with autism had 9 species of *Clostridium* not found in controls, whereas controls yielded only 3 species not found in children with autism. In all, there were 25 different clostridial species found. In gastric and duodenal specimens, the most striking finding was total absence of non–spore-forming anaerobes and microaerophilic bacteria from control children and significant numbers of such bacteria from children with autism. These studies demonstrate significant alterations in the upper and lower intestinal flora of children with late-onset autism and may provide insights into the nature of this disorder.

Autism is characterized by delays in understanding and use of language, unusual response to sensory stimuli, insistence on routines and resistance to change, and difficulties with typical social interactions. Some children are aggressive, and some have even been described as animalistic. It is a devastating disease for the children and their families. The disease usually manifests itself in early infancy [1], but in at least one-third of patients, the onset is delayed until age 18–24 months [2]. Autism occurs in 1 of every 500 births [3], but some recent studies have indicated an increasing incidence. Some 10% of cases have a genetic background, but no underlying etiology can be determined in the majority of patients [4]. Therapy of autism has centered around intensive speech and language therapy, intensive psychological intervention, and behavior modification. This treatment helps, but it is labor-intensive and expensive. Anecdotal reports have indicated that patients on a gluten- and casein-free diet may show improvement. It has been hypothesized that antimicrobial use might disrupt the indigenous intestinal flora and allow colonization by ⩾1 organisms that produce a neurotoxin [5], because a number of parents date the onset of the regressive form of the disease to antimicrobial administration that was followed by chronic diarrhea and gradual evolution of autistic symptoms. On the basis of this hypothesis, an open-label trial of oral van-

Financial support: Intrabiotics Pharmaceuticals, Inc.; personal funds (S.M.F.); VA Merit Review funds; Charles Pfizer, Inc.; Bristol-Myers Squibb Co.; several parents of children with autism; and Cure Autism Now.

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Clinical Infectious Diseases 2002; 35(Suppl 1):S6–16

1058-4838/2002/3505S1-0004$15.00

comycin was done [6] that led to improvement in a number of parameters in 8 of 10 children studied. The purpose of the present study was to do microbiological studies of the intestinal contents of autistic and control children in hopes of detecting ⩾1 organisms unique to the flora of the children with autism.

PATIENTS, MATERIALS, AND METHODS

Study patients. Stool specimens were obtained at Rush Children's Hospital, Chicago, under the jurisdiction of its Institutional Review Board (IRB) and with written informed consent by a parent or guardian. Specimens of gastric juice and duodenal/jejunal fluid were collected at the Children's Hospital Medical Center, Cincinnati, by upper gastrointestinal endoscopy (via the mouth) under the jurisdiction of its IRB and with written informed consent of a parent or guardian. The patients with autism had late-onset disease, and all had gastrointestinal symptoms, primarily diarrhea and/or constipation. Many of these patients were on a gluten-free, casein-free diet; we are not aware of any studies that have indicated whether such a diet influences the makeup of the bowel flora. All patients had received no antibacterial agents for at least 1 month before the study. A number of the patients were on oral nystatin or fluconazole.

Fecal flora studies. Stool specimens were shipped to the VA Medical Center, West Los Angeles, by overnight air express. The entire stool specimen was collected in a grocery store–type Ziploc bag; all the air was squeezed out manually and closure effected. Specimens were then frozen at −70°C until they could be shipped, packed in dry ice. All microbiological manipulations were done in an anaerobic chamber, and all media were prereduced. The entire stool specimen was homogenized by use of a sterile stainless steel blender with 1–3 volumes of peptone (0.05%) added as diluent, if needed. An aliquot of the specimen of ~1 g weight was used, and serial 10-fold dilutions were made in prereduced, anaerobically sterilized (PRAS) dilution blanks (Anaerobe Systems). Another aliquot was weighed before and after thorough drying in a vacuum oven to permit the calculation of counts on a dry-weight basis. Initially, culturing was done on 27 different media or incubation setups; this proved to be so formidable that we subsequently elected to culture primarily for clostridia. Various dilutions were plated (100 μL/plate) onto brucella blood agar (BAP; Anaerobe Systems), CDC ANA blood agar (BBL Microbiology Systems), egg yolk agar (EYA; Anaerobe Systems), and brain-heart infusion blood agar (SBA; Becton Dickinson) with trimethoprim (Sigma Chemical; final concentration, 4 mg/L) and sulfamethoxazole (Sigma; final concentration, 1 mg/L). Additional sets of dilution tubes, 1 heated at 70°C and the other at 80°C for 10 min, were also inoculated onto sets of the above media. Plates were incubated in the anaerobic chamber at 37°C for 120 h. Single colonies were selected, described, and identified according to standard methods [7, 8]. Analysis of metabolic end products, analysis of cellular fatty acids, PCR of the 16S–23S spacer region, and 16S rRNA sequencing were performed on all gram-positive anaerobic rods and selected other organisms.

Gastric and small-bowel specimens. These specimens were collected in 6-mL crimped-top anaerobic vials with silicone-coated polytetrafluoroethylene septa, maintained at room temperature, and shipped to the VA Medical Center, West Los Angeles, by overnight air express. All work was performed in anaerobic chambers with prereduced media. Specimens were serially diluted in PRAS dilution blanks and plated to BAP, CDC, EYA, SBA, and chocolate agar with 10 mg/L pyridoxal (CAP; Becton Dickinson). Dilution blanks were then heated at 70°C for 10 min and plated onto another set of the above plates. CAP plates were incubated in a CO_2-enriched atmosphere for 48 h. The other plates were incubated anaerobically at 37°C for 120 h. Isolates were selected and identified as described above, except that aerobic isolates were identified by other standard methods [9]. The pHs of gastric specimens were determined with a pH meter probe unless the volume was too small, in which case litmus paper was used.

16S rRNA gene sequencing. Genomic DNA was extracted and purified from cells in the midlogarithmic growth phase with a QIAamp DNA Mini kit (Qiagen). The PCR products of the 16S rRNA gene fragments were generated using universal primers to the 16S rRNA gene. The almost complete 16S rRNA gene was amplified between positions 8 and 1485 (*Escherichia coli* numbering) with 2 pairs of primers (8UA, 907B and 774A, 1485B). The amplification was performed for 35 cycles at 95°C for 30 s for denaturation and at 50°C for 2 min for extension. The PCR product was excised from a 1% agarose gel after electrophoresis and purified, using a QIAquick Gel Extraction kit (Qiagen). It was then sequenced directly with an ABI 377 sequencer (Applied Biosystems). Sequences were compared with sequences in the Ribosomal Database Project (RDP) 16S rDNA database (release 7.0), using the SIMILARITY_RANK and CHECK_CHIMERA software, and with GenBank sequences, using BLAST software, and the percentage similarity to other known sequences was determined.

Statistical analysis. The difference between the mean counts of the clostridia and ruminococci, taken together, for the stools of the children with autism and the control children's stools was analyzed by Student's *t* test, using the logs of the numbers and assuming both equal and unequal variance.

Institutional review. The protocols and informed consent forms were approved by the IRBs on human experimentation and the research committees of all institutions involved, and all research was done in accordance with the ethical standards of these committees and with the Helsinki Declaration of 1975, as revised in 1983.

RESULTS

Stool data. The clostridia and ruminococci (the latter organisms often survive the procedures designed to select out clostridia from mixtures) recovered from the fecal samples of 13 children with autism and of 8 control children are listed in table 1. In all, we encountered 25 different species of *Clostridium* and 6 of *Ruminococcus*. There were 23 species of *Clostridium* and 5 of *Ruminococcus* found in the autistic group and 15 clostridial species and 5 ruminococcal species in the control group. The number of these strains per specimen ranged from 2 to 10 in the children with autism and from 3 to 12 in the controls; the average number of strains per specimen was 6 in both groups. The peak counts of these organisms ranged from 10^2 to 10^9 in the children with autism and from 10^4 to 10^8 in the controls; the geometric mean count was 1 log higher in the stools of children with autism (2.1×10^6 vs. 1.6×10^5). The statistical analysis of the counts showed that they were significantly different ($P = .0393$ under the assumption of equal variance and $P = .0289$ under the assumption of unequal variance). A value of ⩾98% similarity to the closest RDP/GenBank organism indicates identification to the species level or to a genomically closely related species, and this figure applied to 21 (67.8%) of 31 species isolated. A value of 99% similarity is essentially diagnostic of identification to the species level; this applied to 17 (54.8%) of 31 isolates. Organisms that displayed >2% sequence divergence with described species were considered novel species. Figure 1 is a phylogenetic tree that shows the relationships of the clostridia and ruminococci isolated from both the autistic and the control children.

Gastric and small-bowel data. Data on 7 children with autism and 4 controls are presented in table 2 and table 3, respectively. The gastric pH was elevated in 2 of 4 children with autism who had this measurement (these children had not been receiving H_2 blockers or proton pump inhibitors). Two children with autism (patients 1 and 2) had only duodenal/jejunal fluid studied, one (patient 1) of whom also had jejunal biopsy material studied. These were the first specimens we received, and they were not collected or transported in optimum fashion. One of the control children did not have gastric juice sampled. Two patients with autism had no organisms recovered from either the gastric or the duodenal specimens, and a third had only 3 species of aerobes recovered. All 3 of these subjects and all control subjects had normal gastric pH. One of these 3 patients had never had either diarrhea or constipation, and a second had had a number of courses of antimicrobial agents for recurrent sinusitis, although he had not had any antimicrobials during the month prior to his endoscopy. The most striking finding was that non–spore-forming anaerobic and microaerophilic bacteria were totally absent from the control children's specimens. In contrast, 4 of 5 children with autism whose specimens yielded any growth at all had such organisms present.

Table 1. *Clostridium* and *Ruminococcus* species isolated from stool specimens of children with autism and control children.

Identified or nearest known species	Similarity,[a] %	Highest counts (cfu/g) in stool specimens	
		From autistic children	From control children
Clostridium spp.			
C. aminobutyricum	92.7[b]	2.0×10^5	—
C. bifermentans	95.0	2.0×10^9	—
C. butyricum	100.0	1.6×10^3	3.2×10^4
C. clostridioforme	95.0	4.0×10^5	—
C. cocleatum[c]	93.5	3.0×10^4	—
C. difficile[d]	100.0	8.0×10^2	—
C. disporicum 1	98.3	—	1.8×10^4
C. disporicum 2	98.1	3.0×10^6	4.2×10^6
C. glycolicum	97.5	4.0×10^5	9.0×10^5
C. innocuum	98.7	2.0×10^9	3.0×10^4
C. lactifermentum	99.7	8.0×10^5	3.0×10^5
C. nexile	96.0	3.0×10^7	—
C. orbiscindens 1	99.8	8.0×10^7	3.0×10^6
C. orbiscindens 2	97.9	4.0×10^4	9.0×10^2
C. orbiscindens 3	97.0	4.6×10^4	—
C. paraputrificum	99.9	2.1×10^6	3.0×10^6
C. perfringens	99.9	1.8×10^4	2.1×10^5
C. ramosum	100.0	6.0×10^7	—
C. roseum	99.6	3.2×10^3	—
C. scindens	99.2	9.0×10^7	—
C. sordellii	98.6	4.8×10^4	1.2×10^4
C. spiroforme	99.5	4.0×10^4	1.8×10^6
C. subterminale	99.4	1.0×10^8	3.0×10^3
C. symbiosum	99.0	4.0×10^9	6.0×10^3
C. tertium	99.9	—	2.7×10^6
Ruminococcus spp.			
R. albus	94.9	—	1.3×10^6
R. flavefaciens	92.0	1.2×10^7	1.2×10^3
R. gnavus	99.8	4.0×10^9	1.7×10^4
R. lactaris	99.3	6.0×10^5	9.0×10^5
"R. luti"	100.0	3.0×10^9	1.2×10^8
R. torques[c,d]	99.1	4.0×10^5	—

[a] Similarity to the closest Ribosomal Database Project/GenBank organism, as determined by use of nearly full-length 16S rDNA sequences.
[b] Isolates with <98% sequence similarity with described species can be regarded as novel species.
[c] Not shown in figure 1: the sequences are not available.
[d] Isolated from a child with autism after mebendazole treatment.

Two of those 4 children with autism had 7–9 different species of non–spore-forming anaerobes or microaerophiles in each of their gastric and duodenal fluids.

In terms of clostridia, both the gastric and small-bowel specimens from children with autism were more likely to have clostridia and were more likely to have a higher number of

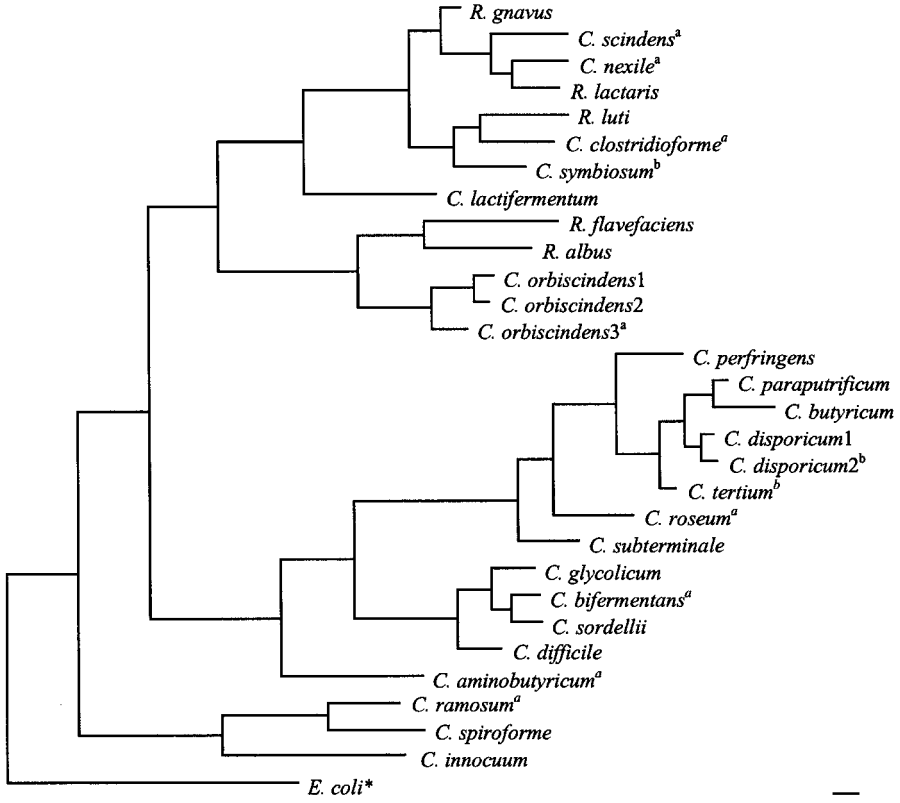

Figure 1. Dendrogram showing the interrelationships within the *Clostridium* and *Ruminococcus* species isolated from autistic and control children's stool specimens. The bar denotes 1% sequence divergence. The asterisk (*) denotes the outgroup standard. [a]*Clostridium* species isolated only from the stool specimens of children with autism. *Clostridium cocleatum* is not shown in this figure because the sequence data are not available. [b]*Clostridium* species isolated only from the stool specimens of control children.

species of clostridia than was true for control specimens of these types. Geometric mean counts, however, were somewhat higher in the control specimens. All the clostridial species isolated from the gastric and/or small-bowel specimens were also recovered from fecal samples (different patients) except for *Clostridium acetobutylicum/beijerinckii, Clostridium ghonii/Eubacterium tenue,* and *Clostridium cochlearium. Candida albicans* was found in 1 gastric fluid and 2 duodenal fluid samples from control patients; non-*albicans Candida* was found in the gastric fluid of a child with autism.

DISCUSSION

Reasons to consider that microorganisms may be involved in late-onset autism include the following: (1) onset of the disease often follows antimicrobial therapy, (2) gastrointestinal symptoms are common at onset and often persist, (3) other antimicrobials (e.g., oral vancomycin) may lead to a clear-cut response and relapse may occur when the vancomycin is discontinued, and (4) some patients have responded to several courses of vancomycin and relapsed each time it was discontinued. The issue can be raised as to whether the effectiveness of vancomycin might be related to some unknown property of the drug aside from its antimicrobial activity (e.g., an effect on the CNS). Because vancomycin is only minimally absorbed when given orally, it is much more likely that the effect is mediated through its activity on intestinal bacteria. The relapse after discontinuation of therapy may be related to the persistence of spores from a spore-forming organism such as *Clostridium* during therapy and then germination of the spores after the drug is stopped [10]. Although waxing and waning of autistic symptomatology is well known [11], the repeated responses and relapses in the same patient treated on several occasions and the degree of improvement on vancomycin therapy argue against a coincidental improvement. However, a double-blind, placebo-controlled trial should be performed with an agent effective in patients with autism in an open-label trial. The reasons that we suspected clostridia initially were (1) anecdotally, the antimicrobial that most commonly predisposes to late-onset autism is trimethoprim/sulfamethoxazole, a drug that is notably poorly active against clostridia, (2) the patients had responded to oral vancomycin and, again anecdotally, to oral metronidazole, which suggests the possibility that a gram-positive anaerobic organism is involved (although the very high

Table 2. Microbiological data from examination of gastric and small-bowel specimens obtained from children with autism.

Specimen pH level and organism isolated	Microbiological data, by patient number and specimen number (type)											
	Patient 1, 1324 (J/D)	Patient 2, 1327 (D)	Patient 3		Patient 4		Patient 5		Patient 6		Patient 7	
			1333 (G)	1334 (D)	1337 (G)	1338 (D)	1339 (G)	1340 (D)	1346 (G)	1347 (D)	1350 (G)	1351 (D)
Specimen pH level	no pH	no pH	6.3	6.8	1.25	4.5–5	4	5.7	1.8	7.2	1.25	2.6
Clostridium spp.												
C. acetobutylicum/beijerinckii			2.0×10^2	20			1.0×10^4					
C. bifermentans		1.0×10^3										
C. cochlearium	10^a											
C. ghonii/Eubacterium tenue				4.5×10^6								
C. glycolicum			10									
C. orbiscindens	10^a											
C. perfringens	10											
C. ramosum		1.0×10^4										
C. subterminale			1.0×10^4									
Nonclostridial anaerobes												
Bacteroides ovatus			1.0×10^5	5.0×10^5								
Prevotella sp.								1.1×10^2				
Selenomonas sp.			1.0×10^4									
Actinomyces D01							2.1×10^4					
Actinomyces odontolyticus				1.0×10^5			2.0×10^4					
Actinomyces sp.		1.0×10^3		1.0×10^5								
Probable *Eubacterium* sp.	2.4×10^3											
Propionibacterium acnes			1.0×10^5	1.0×10^6								
Anaerobic GPR							1.1×10^4					
Anaerobic rod								40				
Veillonella sp.			2.0×10^3				1.37×10^5	1.0×10^3				
Anaerobic GPC							9.7×10^4					
Anaerobic coccus							7.0×10^3					
Anaerobic GNCB								30				
Microaerophilic isolates												
Campylobacter concisus			5.0×10^2									
Campylobacter gracilis			2.0×10^3									

Lactobacillus sp.			1.0×10^3	3.5×10^6		9.0×10^3	50	
Sporolactobacillus racemicus			3.0×10^5	1.0×10^5				
Streptococcus intermedius				3.0×10^5				
Large gram-positive cocci						1.0×10^3		
Aerobes								
Enterococcus faecium						4.9×10^3	10	
Enterococcus sp.	20[a]		2.0×10^6	6.0×10^6				
α-Hemolytic *Streptococcus*	30,[a] 2000					3.0×10^3	3.0×10^1	
Streptococcus parasanguinus				1.0×10^6				
Streptococcus salivarius				7.0×10^5				
Streptococcus sp.	10,[a] 1400					7.0×10^2	10	
Aerobic GPC		1.7×10^5						
Micrococcus sp.						2.0×10^2		
Staphylococccus aureus				8.0×10^4				3.6×10^5
Coagulase-negative *Staphylococcus*	2.0×10^2			1.0×10^5		1.0×10^5	10	1.0×10^3
Neisseria perflava			1.0×10^4					
Neisseria sp.			1.43×10^4					
Haemophilus sp.								1.45×10^6
Pasteurella sp.			1.0×10^4					
Bacillus sp.				1.0×10^6				
Cellulomonas sp.				2.0×10^3				
Corynebacterium singulare								
Coryneform sp.				2.0×10^6		1.0×10^4		
Oerskovia sp.				2.5×10^7				
Aerobic gram-positive rod	2.0×10^3							
Yeast								
Candida albicans								
Non-*albicans Candida*			2.0×10^5					

NOTE. D, duodenal fluid; G, gastric juice; GNCB, gram-negative coccobacillus; GPC, gram-positive coccus; GPR, gram-positive rod; J/D, jejunal/duodenal fluid.

[a] Jejunal biopsy.

Table 3. Microbiological data from gastric and small-bowel specimens obtained from control children.

Specimen pH level and organism isolated	Microbiological data, by patient number and specimen number (type)						
	Subject 8		Subject 9		Subject 10	Subject 11	
	01-1341 (G)	01-1342 (D)	1343 (G)	1344 (D)	1345 (D)	1348 (G)	1349 (D)
Specimen pH level	1	6.4	1.5	6.6	6.8	1.5	6.3
Clostridium spp.							
C. acetobutylicum/beijerinckii							2.0×10^2
C. bifermentans				4.0×10^2			
C. cochlearium							
C. ghonii/Eubacterium tenue							
C. glycolicum							
C. orbiscindens							
C. perfringens							
C. ramosum							
C. subterminale			1.0×10^3	2.0×10^4			
Nonclostridial anaerobes							
Bacteroides ovatus							
Prevotella sp.							
Selenomonas sp.							
Actinomyces D01							
Actinomyces odontolyticus							
Actinomyces sp.							
Probable *Eubacterium* sp.							
Propionibacterium acnes							
Anaerobic GPR							
Anaerobic rod							
Veillonella sp.							
Anaerobic GPC							
Anaerobic coccus							
Anaerobic GNCB							
Microaerophilic isolates							
Campylobacter concisus							
Campylobacter gracilis							
Lactobacillus sp.							
Sporolactobacillus racemicus							
Streptococcus intermedius							
Large gram-positive cocci, slightly microaerophilic							
Aerobes							
Enterococcus faecium							
Enterococcus sp.							
α-Hemolytic *Streptococcus*		10			1.96×10^6		
Streptococcus parasanguinus							
Streptococcus salivarius							
Streptococcus sp.					1.0×10^3		
Aerobic GPC							
Micrococcus sp.							
Staphylococccus aureus							1.7×10^4
Coagulase-negative *Staphylococcus*		7.0×10^4	3.0×10^2	6.0×10^4	4.0×10^6		
Neisseria perflava							
Neisseria sp.							
Haemophilus sp.					1.5×10^6		
Pasteurella sp.							
Bacillus sp.				6.0×10^4			
Cellulomonas sp.							
Corynebacterium singulare							7.8×10^3
Coryneform sp.							
Oerskovia sp.							
Aerobic gram-positive rod							
Yeast							
Candida albicans			20	3.0×10^7			2.14×10^7
Non-*albicans Candida*							

NOTE. D, duodenal fluid; G, gastric juice; GNCB, gram-negative coccobacillus; GPC, gram-positive coccus; GPR, gram-positive rod; J/D, jejunal/duodenal fluid.

levels of vancomycin achieved in the bowel when it is given orally are sufficient to eliminate the *Bacteroides fragilis* group and most other gram-negative anaerobic bacteria), (3) unusually high tetanus antitoxin titers have been noted in several patients with late-onset autism [12], and (4) clostridia are the principal bacteria that produce both an enterotoxin and a neurotoxin and are generally very active metabolically (e.g., may produce potentially toxic metabolites such as phenols, p-cresol, or certain indole derivatives). Other antimicrobial agents may also lead to overgrowth of clostridia; for example, *Clostridium perfringens* and *Clostridium innocuum* were found in the salivary flora of patients treated with clindamycin [13]. For the above reasons, then, early during the course of our studies we focused primarily on clostridia in stool specimens that we studied. In our initial article [6], however, we noted a relative scarcity of peptostreptococci in stools of children with autism compared with control children. This suggested the possibility that peptostreptococci might be protective. We have elected not to follow up on this until we develop a good selective medium for these anaerobic cocci.

The studies reported herein should be interpreted in light of the following considerations: (1) there may be $\geqslant$1 key organisms other than clostridia and ruminococci, (2) uncultivatable clostridia or other organisms may be present, (3) the key organism(s) may be present in small numbers (considering that autism is a low-grade, chronic process) and cannot be detected without the use of a selective medium or the availability of some special marker, (4) the organisms involved in autism may be mucosa-associated and therefore not cultivatable by the methods we used, (5) the key organisms may be farther down in the small bowel than we are able to sample, (6) the organisms of concern may be cell wall–deficient and therefore unable to grow on conventional media (although the response to vancomycin, a cell wall–active drug, indicates otherwise), and (7) different types of autism and/or different degrees of severity of the process may be different in terms of whether there is an abnormal flora either in the colon (reflected in the feces) or in the small bowel, and perhaps these factors may even be associated with different specific flora and/or sites or degrees of abnormal colonization.

The stool flora studies showed higher counts of *Clostridium* and *Ruminococcus* spp. in the stools of the children with autism than in those of the control children. There were also a number of species of these genera present only in the stools of children with autism. Additional studies are needed to further analyze the differences in the flora recovered or differences in toxin or toxic metabolite production.

We were interested in the possibility of there being an abnormal microbial flora in the small bowel in autism and, of course, recognized that if that should prove to be the case, our task would be much easier than analyzing stool flora because the flora of the small bowel, even under abnormal circumstances, would likely be considerably less diverse and profuse than would be true of the large bowel and feces. To the extent that toxins were produced by an abnormal flora and gained access to the CNS via the vagus nerve (as hypothesized by Bolte [5]), the small bowel would be a logical site for the abnormal flora because the vagus nerve innervates primarily the small bowel. Also, Wakefield et al. [14, 15] have described pathology in the terminal ileum and proximal colon in autism. Some studies [16] have reported increased small-bowel permeability in autism, and, finally, some children with autism have watery diarrhea, which is characteristic of small-bowel disease.

Older studies from the literature have shown a relatively sparse flora, with relatively few anaerobes and no clostridia in the proximal jejunum [17–20], and even less flora in the duodenum [17, 18] in healthy adult and pediatric subjects. In most cases, the specimens for these studies were obtained by oral intubation. Our laboratory's previous studies of material obtained from the proximal jejunum [21] yielded similar results; these specimens were obtained by aspiration with needle and syringe at the time of surgery. All patients in the latter study were adults. Studies of the gastric flora [17, 18, 22] revealed many sterile specimens in 1 study [17] and counts that were usually in the range of up to 10^4–10^6, when positive. A positive correlation between elevated pH and higher counts in gastric specimens was noted in all these studies, as it was in our patients. Studies of the bacterial content of saliva from 132 subjects were done in one of the above investigations [17]; a wide variety of aerobes and anaerobes were found, with counts as high as 10^7/mL. No clostridia were recovered.

Our current study of upper gastrointestinal specimens has revealed definite abnormality. Nevertheless, there were some children with autism whose specimens yielded no growth or only a few aerobes. Therefore, the possibilities listed above with regard to other types of studies that might be indicated (see above) deserve exploration in this setting as well. Clostridia, as well as non–spore-forming anaerobes, were recovered from 4 upper gastrointestinal tract specimens of children with autism. After the first 2 patients yielded clostridia from duodenal/jejunal contents, we obtained gastric specimens as well, when possible, with the thought that if we found clostridia in the duodenum but not the stomach, this would likely rule out an oral source of these organisms. Clostridia have been reported in the oral cavity on occasion, in conjunction with gingivitis or periodontal disease [23] and in relation to antimicrobial therapy [13]. We were surprised to find elevated gastric pH and bacterial overgrowth in the stomach in 2 of the patients subsequently studied. In 1 of these cases, cholecystokinin had been administered during the endoscopy, and there was likely some reflux of duodenal contents into the stomach; however, the flora at these 2 sites were not at all identical. Cholecystokinin

was not used with any of the other endoscopies. We were also surprised to find clostridia in 2 of 4 control children, despite normal gastric pH; however, none of the 4 controls had non–spore-forming anaerobes or microaerophiles present. Thus, a normal gastric pH may provide a reliable negative screening test for significant bacterial overgrowth. Non–spore-forming anaerobes and microaerophilic bacteria were found in 4 of 5 children with autism whose specimens yielded any growth at all but not in any of the control children. The relatively greater frequency of recovery of yeasts from the control children may reflect the fact that children with autism are not uncommonly given antifungal agents, with the feeling that yeast may contribute to the clinical picture of autism.

The reasons for abnormal colonization of the stomach and upper small bowel in some patients with autism remain to be determined. One factor, already documented in our studies, is hypochlorhydria, but it may be that other factors are responsible in addition to or instead of that factor in some patients. The 2 most reasonable possibilities are impaired gastrointestinal motility and IgA deficiency. Either a genetic predisposition (or defect) or an acquired defect might be involved in any of the above scenarios. There is an excellent recent review of the immune abnormalities noted in patients with autism, including the association of major histocompatibility complex genes with autism [24].

Finally, the question as to how the bacteria in the gut effect the damage that results in the picture of autism is important to consider (see table 4). As noted, one possibility is the production of a toxin or toxins. One organism might produce both an enterotoxin and a neurotoxin, or different organisms might produce these or other toxins separately. One or more of the species of *Clostridium* or of *Ruminococcus* that were found in the fecal flora of the children with autism but not the controls could be a toxin producer. Additional studies of stool samples may narrow the possibilities (i.e., there may be fewer organisms of these types found only in the children with autism). The same points made with regard to fecal flora may also pertain to the clostridia found in the gastric or small-bowel flora of children with autism but not in the control children.

A second possibility, mentioned in our previous study [6], is that autoantibodies or some other bacterial-antibody interaction might lead to autism. Autoantibodies to neuron-axon filament protein [25], glial fibrillary acidic protein [25], and myelin basic protein [26] have been found in patients with autism and might contribute to the pathology and clinical picture of the disease. Such autoantibodies related to *Proteus mirabilis* have been postulated to be involved in rheumatoid arthritis [27], and autoantibodies to *Campylobacter jejuni* are felt to be important in associated Guillain-Barré syndrome [28].

Finally, it may be that the pathogenesis of autism related to abnormal microbiology in the gut relates to microbial production of toxic metabolites. An elegant article by Elsden et al. in 1976 [29] discussed the end products of metabolism of the aromatic amino acids phenylalanine, tyrosine, and tryptophane by clostridia. Twenty-one species of clostridia plus 2 toxin types of *Clostridium botulinum* were studied, and the amounts of phenylacetic, phenylpropionic, phenyllactic, hydroxyphenylacetic, hydroxyphenylpropionic, indole acetic, and indole propionic acids as well as the amount of phenol, p-cresol, and indole produced were given. Another excellent article, by Smith and Macfarlane in 1997 [30], reported similar studies that investigated the effects of pH and carbohydrate availability and compared the effects of free amino acids and peptides as substrates on aromatic amino acid metabolism and the production of toxic metabolites. The latter investigators also showed interconversion of some of these products. They worked with batch cultures of colonic anaerobes rather than with pure cultures. It is interesting to note that urinary myelin basic protein-like material is increased in progressive multiple sclerosis, and p-cresol sulfate is an immunologic mimic of myelin basic protein [31]. Elevated whole blood levels of serotonin (5-hydroxytryptamine), the precursor of which is tryptophan, have been noted in 30%–40% of children with autism, although there are no clear-cut studies to indicate that such elevation plays a role in the disease [32]. Serotonin is not formed by bacterial action, but the extensive activities of bacteria related to other indole derivatives is interesting, particularly in the light of serotonin-function effects on social interaction, mood, obsessive-compulsive activity, motor stereotypies (spinning activity, etc.), and little reaction to painful stimuli [33, 34].

A curious phenomenon reported by parents of some children with autism is a mothball-like odor to the stools of their children. A study by Moore et al. [35] suggested that this odor is not uncommon in healthy adult subjects and is due to the presence in such stools of indole, skatole (3-methyl-indole), or both. These compounds are formed in the human gut from

Table 4. Possible pathways for bacteria-related autism.

Immune-mediated
Toxin
Toxic metabolic product(s)
p-cresol and phenols
Casomorphin, gliadomorphin, and endorphins
Products required for sulfation (e.g., glycosaminoglycans and sulfotransferases) or interfering with sulfation (sulfatase and sulfate-reductase)
Indole derivatives, including serotonin and indolylacryloyl glycine
Naphthalene derivatives
Naphtha derivatives
Mercury (especially methyl mercury) or other metallic ions
Interferons and cytokines

tryptophan by microbial action. Skatole and indole are rapidly absorbed in the jejunum, ileum, and colon of humans [36]. It should be noted, however, that other compounds (e.g., naphthalene and naphtha and their derivatives) also give the odor of mothballs. After the infusion of tryptophan or casein into the colon of normal subjects, indican and indole-3-acetic acid are excreted in large amounts in the urine [36]. Indolylacryloylglycine excretion in urine has been noted in several situations, including autism and other behavioral disorders [37].

Sulfate metabolism is very important in the normal physiology of the body. Specifically, large macromolecules called proteoglycans bear chains of carbohydrate called glycosaminoglycans (GAGs) that are modified and patterned posttranslationally by sulfate. The body makes sulfate from the amino acid cysteine and indirectly (via cysteine) from methionine. The regulation of sulfate may play a significant role in autism. There is depressed phenolsulfotransferase activity in autism, and patients with autism excrete significant amounts of sulfate in the urine [38], despite abnormally low plasma sulfate levels [39]. Colonic bacteria may produce sulfide, which is a neurotoxin [40]. Quite a few bacteria are known to desulfate mucins, including *B. fragilis, Bacteroides thetaiotaomicron, Prevotella, Bifidobacterium, Helicobacter pylori, Clostridium, Ruminococcus torques,* and various oral streptococci [41]. Twenty-three isolates of human fecal bacteria were studied, and some or all produced sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase, all of which are implicated in mucin degradation in the human colon [42]. A *Bacteroides* strain from the human intestinal tract produced polysaccharide lyases (including heparin lyase and chondroitin sulfate lyase) that could degrade GAGs [43]. The effect of gastrointestinal surgery and bacterial overgrowth on the urinary excretion of sulfur amino acids and their degradation products has been studied [44].

Caseinomorphin, gliadomorphin, and various endorphins may play a role in autism. The benefits of a casein-free and gluten-free diet may derive from the elimination of some of these compounds.

There has been much speculation about the role of mercury in autism, particularly in relation to the use of mercury-containing compounds in vaccines administered to children. In vitro studies have shown that the bowel microflora can transform mercury, sometimes to more toxic compounds such as methylmercury (by methylation of mercuric salts), and, in other cases, to less toxic compounds (by demethylation of methylmercury), but such metabolism seems to be of significance to the host only in the case of demethylation. Elimination of the bowel flora by antimicrobial administration in the rat leads to increased mercury content in tissues and a greater proportion of the total mercury as methylmercury [45]. Thus, an intact bowel flora is protective. On the other hand, methylation of mercury has been demonstrated in rats with an experimental jejunal blind loop [46]. Studies with individual components of the human intestinal microflora have indicated that facultative bacteria were more often able to methylate mercury than were lactobacilli, *Bacteroides,* and *Bifidobacterium* [47]. More information is needed on the relative importance of methylation and demethylation by specific elements of the bowel flora and, therefore, the impact of abnormal flora in autism on the toxicity that might be encountered.

The present study provides up-to-date information on the gastric, small-bowel, and fecal flora of young children, using current techniques for identification of the various taxa. In all, 28 species of *Clostridium* were encountered. The geometric mean count of clostridia in stools of children with autism was 1 log greater than that in control children ($P = .0393$). Nine clostridial species were found only in children with autism compared with only 3 in control children. Nonclostridial anaerobes and microaerophilic bacteria were common in upper gastrointestinal specimens of children with autism but were absent from controls. Two children with autism had hypochlorhydria with attendant bacterial overgrowth in gastric and small-bowel specimens. These significant alterations in bowel flora may provide insight into the nature of some types of autism.

Acknowledgments

We are indebted to many colleagues, parents of children with autism, and others who have provided assistance of various types to us. In particular, we are grateful to the parents of children with autism for their constant encouragement and their great help in educating us about autism and providing numerous outstanding references in the scientific literature, as well as their own thoughts about this disease. A number of them have also had fund-raisers that have helped support our laboratory. Included among those who should be acknowledged are Dr. Marvin Ament, Doug Benson, Rita J. Bigelow, Holly Bortfeld, Dr. Debbie Darnley, Maura Fisk, Mary and Harry Higinbotham, Bram Hornstein, Calida Li, Dr. John (Zeb) Little, Cindy and Jeff Mays, Dr. Woody McGinnis, Susan Owens, Stuart Samuels, Judy Sidell, Shahera St. John, Ann Vu, and Penny and Syd Yallen.

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