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Brief Communication

Enteral Nutrition and Microflora in Pediatric Crohn's Disease

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ABSTRACT. *Background:* Exclusive enteral nutrition (EN) is an established primary therapy for pediatric Crohn's disease (CD). The mechanism of action of such treatment is still conjectural. The aim of the present study was to investigate if EN-induced remission is associated with modification of the fecal microflora in CD. *Methods:* Stool samples were collected from 5 healthy children and adolescents over a period of 3 months, and from 9 children and adolescents with active CD. To induce disease remission, children with CD received a course of exclusive EN for 8 weeks with a polymeric formula (Modulen IBD, Nestlè). At the end of the course of exclusive EN, children returned to a free diet but continued to take 40% of the daily caloric intake as polymeric formula. Fecal microflora was analyzed by 16S ribosomal DNA polymerase chain reaction and temperature gradient gel electrophoresis (TGGE) with direct visual comparison of band profiles of PCR products. *Results:* In 8 of 9 children, the exclusive EN alone induced disease remission. In 1 child, it was necessary to add steroids to the exclusive EN course to achieve remission. In all children with CD, analysis of gel band distribution revealed profound modification of the fecal microflora after exclusive EN. Variations of band distribution corresponding to different bacterial species were observed also in children on partial EN and required time to achieve stability of the band profile. In contrast, control healthy children showed a host-specific and stable TGGE profile over time. *Conclusion:* These data suggest that a possible mechanism of action of EN in inducing disease remission in CD is the capacity of modification of gut microflora. Possible explanations of such capacity are both low residue and prebiotic properties of the polymeric liquid formula. (*Journal of Parenteral and Enteral Nutrition* **29:**S173–S178, 2005)

Exclusive enteral nutrition (EN) is an effective primary therapy for pediatric Crohn's disease (CD). Liquid diet, both elemental and more recently polymeric, can induce disease remission, promote linear growth and have steroid-sparing capacity in children and adolescents who are at risk of stunting.[1–4] The mechanism of action of such treatment is still conjectural. Proposed mechanisms have included the elimination of dietary antigen uptake, overall nutrition repletion, provision of important micronutrients to the diseased intestine, correction of abnormal intestinal permeability, and immunological downregulation.[5,6] There is considerable evidence that the intestinal microflora is of major importance in triggering and perpetuating chronic bowel inflammation.[7] Molecular analysis of the bacterial microflora based on the 16S ribosomal ribonucleic acid (rRNA) genes obviates the need of culture and can be used to characterize approximately 90% of the dominant fecal microflora.[8] The aim of the present study was to investigate if EN-induced remission was associated with modification of the intestinal microflora in CD. For this purpose, the biodiversity of the fecal microflora in patients treated with exclusive and partial polymeric EN was analyzed by temperature gradient gel electrophoresis (TGGE). This sensitive molecular technique has the advantage of creating a profile of complex microbial populations by separating mixed 16S rRNA polymerase chain reaction (PCR) amplification products. The pattern of separated bands illustrates the bacterial diversity in the sample.

METHODS

Nine children and adolescents (4 males and 5 females, age range 9–17 years) with active Crohn's disease were enrolled in the study. Patient disease activity was documented by the Pediatric Crohn's Activity Index (PCDAI). This index is a 0–100 point scale with remission defined as a score ≤15. It has been developed and validated for use in trials among children and adolescents.[9] Seven patients were at diagnosis and 2 on disease relapse. Six patients had ileocolonic disease; in 3 patients, the disease was localized in the small intestine. To induce disease remission, all patients received a course of exclusive EN for 8 weeks with a polymeric formula (Modulen IBD; Nestlè, Vevey, Switzerland). Eight out of 9 patients also received the immunosuppressive agent 6-mercaptopurine. At the end of the course of exclusive EN, all children returned to a free diet but continued a partial EN regimen (40% of the daily caloric intake as polymeric formula). Fresh fecal samples were obtained

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from each patient every 2–3 weeks since the beginning of EN, divided into aliquots and immediately stored at –70°C until analyzed. Fecal samples were also obtained from 5 healthy children and adolescents (age range 10–15 years) who provided 2 fecal samples each over a period of 3 months. None of the patients and healthy controls had taken antibiotics or undergone colon cleansing for at least 1 week before sample collection.

DNA was extracted directly from fecal samples and used as the target in PCR reactions in order to amplify the V6-V8 region of the bacterial 16SrRNA gene in PCR reaction using primers U968–GC and L 1401 after the procedure described by Zoetendal et al.[10] TGGE was performed with a DCode universal mutation detection system (BioRad). Six percent polyacrylamide gel was prepared and run with a 1× TAE buffer. The electrophoresis was conduced with a constant voltage of 120 V for 18 hours. A gradient of 37–46°C was applied parallel to the electrophoresis running direction. After the completion of electrophoresis, the gel was stained with Sybr-Green (Roche). The PCR products from fecal samples were electrophoresed alongside each other, allowing for direct visual comparison of band profiles between consecutive fecal samples from the same individual.

RESULTS

In all children, the polymeric formula was taken orally. In 8 of 9 children, the exclusive EN alone induced disease remission (PCDAI <15) within 2 weeks of exclusive diet. In 1 child with active ileocolonic CD complicated by erythema nodosum, after 2 weeks it was necessary to add steroid therapy to EN to induce disease remission (Fig. 1). All children were in disease remission after 4 weeks of exclusive EN. After the 8-week course of exclusive liquid diet, patients on partial EN were followed up for period ranging from 2 to 8 months. All patients maintained disease remission during the follow-up period.

At time 0 before starting the EN course, each patient showed his or her own TGGE band profile. Exclusive EN was characterized by drastic changes of the banding pattern in all patients. Example of TGGE profiles from 4 patients before and during exclusive EN are shown in Fig. 2. TGGE profiles varied greatly between subjects and required time to achieve stability of band profile in each subject during exclusive and partial EN. Partial EN with a free diet was associated with less drastic changes when compared with changes observed during exclusive EN. In Fig. 2,the TGGE profile of the patient with the longest follow-up is shown. After an 8-week course of exclusive EN and 12 weeks of partial EN, the banding pattern achieved stability.

Healthy children and adolescents showed a host-specific band profile that remained stable in all subjects during the 3-month period of sample collection. Fig. 3 shows a stable TGGE profile of an healthy child.

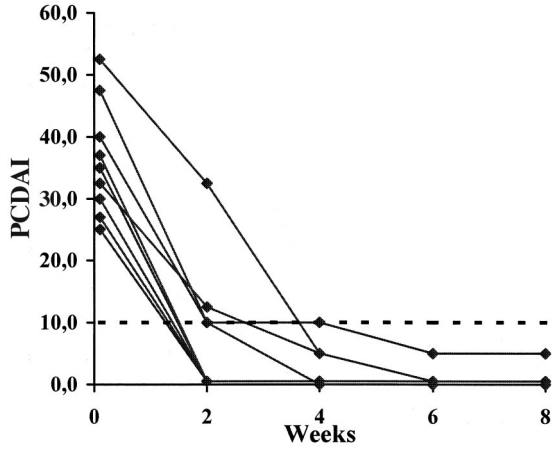

FIG. 1. Pediatric Crohn's Disease Activity Index (PCDAI) in 9 patients before and during exclusive enteral nutrition with the polymeric formula Modulen IBD. All patients except 1 achieved remission (PCDAI ≤15) in 2 weeks.

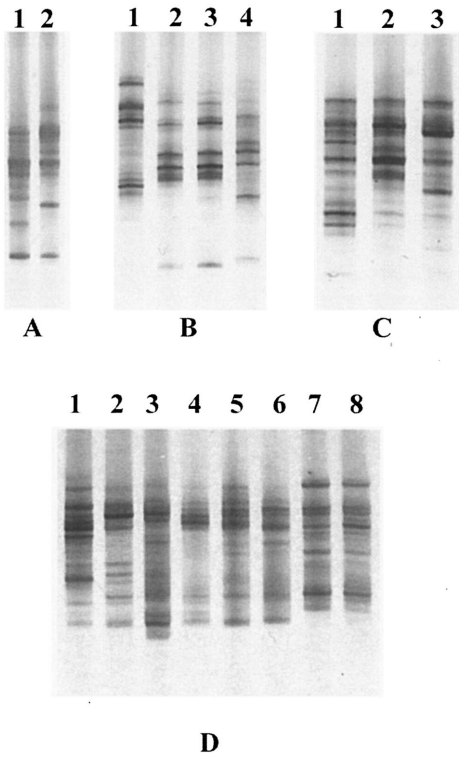

FIG. 2. Temperature gradient gel electrophoresis of 16S rRNA amplicons of fecal samples obtained from 4 patients. Patient A, B and C: lane 1 = time 0 before EN, lane 2 and 3 = 2 and 4 weeks of exclusive EN, respectively. Patient B: lane 4 = partial EN (40% daily caloric intake) since 4 weeks. Patient D: lane 1 = time 0, lane 2 and 3 = 4 and 8 weeks of exclusive EN, respectively; lanes 4, 5, 6, 7, and 8 = 2, 4, 8, 12, and 28 weeks of partial EN, respectively.

CONCLUSIONS

EN is considered an effective alternative to corticosteroids and is regarded as first-line therapy in many pediatric gastroenterology centers.[2] The mechanism of action of such treatment is still conjectural. It was initially hypothesized that the elimination of dietary antigen uptake was responsible for the efficacy of EN. Accordingly, elemental and semielemental formulas were used. Subsequent trials showed that polymeric formulas in which whole proteins are present are equally effective.[3,4] The overall nutrition repletion

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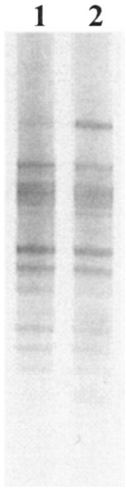

FIG. 3. Temperature gradient gel electrophoresis of 16S rRNA amplicons of fecal samples from an healthy child. Lane 1 = time 0; lane 2 = 3 months later.

induced by a course of exclusive EN could have a beneficial therapeutic effect in active CD; however, it has been demonstrated that significant improvement in inflammatory parameters precede any significant changes in nutrition parameters,[11] suggesting factors other than simple caloric intake are likely to be involved.[6] There is considerable evidence that the microflora of the distal ileum and colon contribute to the pathogenesis of CD.[7] Experimental animal models of inflammatory bowel disease have shown that colitis does not occur in a germ-free environment. In CD, inflammation is present in the part of the gut containing the highest bacterial concentrations, and diversion of the fecal stream prevents postoperative recurrence.[12] Modern molecular techniques based on nucleic acid sequence comparisons allow a culture-independent characterization of the complex ecosystem of the gut microflora. The TGGE analysis of 16S rDNA from human fecal samples from healthy individuals reveals stable and host-specific profiles.[10] Alteration of the dominant fecal bacterial groups has been demonstrated by the same technique in patients with colonic CD.[13] Using the same approach as previously demonstrated in adults,[10] we found that healthy children and adolescents have stable TGGE band profiles over a period of observation of 3 months. In contrast in children with CD, EN-induced remission was associated with profound modifications of the band profile corresponding to different bacterial species of the fecal microflora. Partial EN with a free diet was associated with less drastic variations of the band profile and required time to achieve stability in patients in disease remission.

Possible explanations of the EN capacity to induce variation of gut microflora are both low residue and prebiotic properties of the polymeric liquid formula. Such data further support the concept that intestinal microflora is indeed a key element in the pathogenesis of CD.

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Discussion

Dr Marteau: I also work on intestinal flora in IBD patients, but in adults. We didn't assess the efficacy of enteral nutrition but compared the flora in patients with active disease and in patients with remission. The flora is very different between active and inactive phases of the disease and the flora is unstable during activity. We used the TGGE technique and dot blot analysis, trying to describe more efficiently the differences, and also used molecular inventories to describe all the flora which is very difficult because you have >250 clones. We didn't have differences in biodiversity in the fecal flora between active disease and remission with TGGE. Are you sure that what you observe is due to enteral nutrition? What hap-

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pens when you stop enteral nutrition and there is no relapse, have you had the opportunity to follow these children?

Dr Lionetti: I must say that some of these children stopped the enteral nutrition and they are still in remission, and the profile is still stable, it doesn't change so much. We are waiting for a relapse, but I hope these children will stay in remission for a long time anyway. And we did also, I mean Dr Callegari did, all the experiments again, using DGGE and had exactly the same results. It's clear also, so maybe we will publish DGGE instead of TGGE. And as far as the other results, there is only 1 study, I think it is a French study, in which they found with the same technique a difference between active disease and remission but it was in the same patients going from active disease to remission that the flora was really different. Maybe that always happens, whatever you use, but I think that the way enteral nutrition works is by changing the flora. We know that antibiotics work for instance, but anyway I am sure that when you go from active disease to remission your flora changes drastically, and it never happens in normal individuals except if you have got terrible diarrhea.

Dr Saavedra: Can I ask a question with regard to the results in the trial that you are presenting? There are a number of bands that disappear at the end, which are present at the beginning. Were you able to identify the types that seem to be disappearing *vs* those that appear?

Dr Lionetti: I asked that, absolutely, I want to know which bands disappear, but I don't have the results yet; it is too early to have this talk at this time of the study.

Dr Saavedra: There are several people who have reported differences in terms of the type of flora that you have, whether you treat with antibiotics or whether you don't treat. Obviously we do use metronidazole as part of the therapy and we actually decrease biodiversity rather than increase biodiversity when we give a very broad-spectrum antibiotic. And the total mass of microorganisms of course drops drastically. So I think the association of events is very clear. I think that the pathophysiologic link is the harder part to demonstrate.

Dr Paerregaard: Your results are very exciting but the key question is the changes in the microflora, is it only a cophenomenon or does enteral nutrition lead to changes in bacterial flora that subsequently lead to whatever causes Crohn's disease to go into remission? Have you got a personal opinion about that?

Dr Lionetti: I don't have an answer, but I can tell you there are lots of experimental models. We all know these animals in which you have some genetic changes and they develop spontaneously something like IBD, and they don't get IBD if they live in a free germ environment. There are new studies now in which just manipulating specific types of flora in these animals, you can prevent the disease, so I think the luminal flora is really of importance and disease activity, it is not a reflection of what happens at the mucosal side, but there is a very important exchange between mucosal immune system and what happens in the lumen, I mean the luminal flora and also the adhesive bacteria.

Dr Paerregaard: I think it would be very interesting to perform a colonoscopy and obtain mucosal biopsies looking upon the bacterial flora that actually can be re-isolated from mucosal biopsies and the adherent flora.

Dr Lionetti: There is a lot of published data now about the mucosal flora, and that it is different in active Crohn's disease. We haven't done that yet.

Dr Saavedra: There are a couple of papers that have actually looked at this. Unfortunately the problem is that people look at specific strains, because it is just very hard to look at everything. Looking specifically at lactobacillus amounts in mucosa or mucosal adherent lactobacilli, and showing that there is a decrease in patients with active UC *vs* patients who have inactive disease may not be sufficient. Just to reiterate what you are saying, I think the kind of work that would be interesting would be related to what happens to the flora after you have stopped the enteral nutrition. Do they revert back to what they originally started with, like it has been shown with patients who get antibiotics? These patients basically go back to have the flora that they had before the antibiotic course.

Dr Lionetti: Our impression is that if the patients are still in remission the flora remains stable, I don't know what happens when there is a relapse; I think they would go back to the active disease flora.

Dr Schiffrin: What I think could also be interesting is the way you induce remission in these patients. These immunosuppressors seem to be such a strong option as we have heard and immunocompetence is so important for the composition of the microflora, this could give us some hint of the advantage to have a restitution of the ecology without immunosuppression. I think immunosuppression may really affect the composition of microflora in this group; it could be an additional argument for the use of enteral nutrition.

Dr Lionetti: We should have a control group without AZA or 6MP, but it is a little bit unethical to do that and I do prefer to add very straightforward the 6MP to prevent disease relapse. I found that I am probably the only person using 6MP in Europe; all use azathioprine, whereas in the United States 6MP is widely used.

Dr Saavedra: Yesterday we saw data showing the effects of Modulen within 3 days of administration. Would you expect changes in the gut microflora within 3 days?

Dr Lionetti: I don't know. It is easy to get the specimen because you give the vials to the mother to collect the stool samples, but not only after 3 days. We took these samples every 2 weeks so we didn't look at it so early. We are used to waiting for 2 weeks to see what is going on, if enteral nutrition induced remission or not, and after 2 weeks we decide to continue or not, so we

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took specimens every 2 weeks. Maybe in the future we can collect samples earlier.

Dr Huggett: I know there are people here working with probiotics. How long does it really take when you feed a diet to start to change the profile of the microflora?

Dr Saavedra: I can make a couple of comments on that. In some of the work done with oligosaccharides, for example, and looking at methods to modify intestinal flora, you do see changes relatively soon. It is hard to know and the quantification has not been adequate to do it over a period of less than 7 days, but if you are going to a see a bifidogenic effect, for example, you can see it within a period of 7 days. I don't know if anybody else has some other comment.

Dr Powell-Tuck: I just wanted to be a bit naïve if I may, forgive me, but if we were really interested predominantly in the effect in the flora surely shouldn't we just start with an intestinal lavage preparation, as for colonoscopy perhaps, before introducing our enteral feeds? I am not aware of any studies apart from some German studies which looked at this in the late 70s or early 80s which showed very dramatic drop in endotoxin levels after lavage, but it would surely produce the major effect you wanted, and then you can go in with your enteral feed or prebiotic or probiotic, whatever you wanted to do. Sorry, it is very naïve, but why don't we do that?

Dr Heuschkel: I think most of these children, before they start on enteral nutrition, they do have bowel prep to make the diagnosis, so essentially they do have a day or two of bowel cleansing? So they do start with a relatively empty colon.

Dr Lionetti: In children, we use polyethylene glycol (PEG) solutions before doing any colonoscopy, but I wait at least 1 week before sample collection just to have the situation normal without any gut lavage.

Dr MacDonald: Speaking as a referee, I think that there are lots of variables here and I think that the gut microbiologists will not be surprised to know that when you have got inflammation in the serum and neutrophils moving into the gut lumen, that changes the substrate and will change the flora, and if you stop this it is going to change the flora, and the difficulty is you are going to change transit time, you are going to change all sorts of things—the immune system, the effects on the normal flora. So you have got multivariant things happening in there and it is going to be very hard to tease out which ones of these especially are important, if you don't want to start segregating your patients into different treatment protocols. I guess the only way to see is if Modulen changes the normal flora in normal people. If you can show that it changes in normal people independent of all these confounding factors, you then have a very strong argument. This is actually part of the biologic basis for the efficacy, otherwise people are just going to say it is just secondary.

Dr Lionetti: I completely agree with you, and I already asked some people in my department if they want to do that and they said yes but they want to be paid, so I asked them to take 4 weeks of exclusive liquid diet.

Dr MacDonald: I don't think you need to do that because the flora changes in 2 weeks, the bands change in 2 weeks, so you could do a 2-week course of Modulen and give them £250.

Dr Saavedra: Obviously that is one of those experiments that can help. The problem is also that we would have to deal with the fact that if you change 40%–50% of the diet to a single other source of nutrition, you are going to change the substrate for bacteria. So literally, any diet that you increase to the point that it becomes the main source of substrate for whatever flora you have is going to change the flora, and we see that with lactulose, we see that with starch. In this particular case if you give a single substrate, single protein with a single carbohydrate composition, the flora is likely to change, so that alone again would still not answer that question that links changes in flora to disease.

Dr MacDonald: But it could explain why it doesn't really matter, what you give doesn't matter as long as it is soluble. If you give cows milk, it would work as well because it is all having the same effect, it is changing in different ways but it is the change that is important, not the way of the change.

Dr Roessle: In fact, Nestlé has already done the study you are asking for, except it has not been done with the Modulen IBD but with Nutren in healthy volunteers taking 2 weeks of exclusive nutrition with Nutren, which is a casein-whey based standard enteral nutrition formula, and the only change observed was a slight drop in the bifidogenic flora which could be reversed when you add prebiotics to the same formula. This study will be published or is under publication.

Dr Marteau: One of the points, which is very important when we have such data to discuss, is if we know what is reaching the colon when you ingest the product. What are the substrates which escape digestion in the small bowel? Do we observe a modification because we add something to the colon or because you remove something which is usually reaching the colon in the usual diet?

Dr Lionetti: I agree with you, it is also called prebiotic colonic food for bacteria, so this is a way of prebiotics working, most of the flora is in the colon.

Dr Saavedra: Just to add one point of complexity to the whole picture is the fact that we really don't know what the profile in stool really means for the rest of the gut. We use probiotics, for example, as a method of modification of flora that may lead to some kind of immunologic change that in turns leads to some clinical benefit. There is now I think increasing evidence that how much we change the flora in the colon by giving probiotics has very little relevance to how much we change clinically. Whether it comes to modifications of cytokines in peripheral blood, prevention in increased secretory IgA, actually most of the action

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seems to be happening in the small bowel rather than happening in the colon when you actually give probiotics.

Dr Lionetti: We know from clinical observations that the fecal stream is of importance for relapse in people who have surgery, when you have a diversion of the colon you don't have disease, when you again have the fecal stream; you again have disease, so it means that the fecal stream is of importance for disease relapse.

Dr Damiao: Have you thought about giving probiotics or prebiotics to these patients after the induction of intestinal microbiota alterations?

Dr Lionetti: I didn't add any probiotics to the children; I wanted to have only Modulen IBD, to see the change in the flora. Also amino sulfasalazine can change the flora probably, we don't know, but we intentionally didn't use other probiotics or prebiotics.

Dr Damiao: I meant after the treatment.

Dr Lionetti: The problem is that there are no data at all about the efficacy of prebiotics and there are some data on probiotics in pouchitis. There is no doubt it works, and concerning the relapse after surgery there is some data on the efficacy of probiotics in maintaining remission with probitotics, but although there are a lot of talks about probiotics and prebiotics, there is little published data.

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