Are Small RNAs Associated with Crohn's Disease?

Reinhard Pechan*, Hans Kunert**, and Hans J. Gross

Institut für Biochemie, Bayerische Julius-Maximilians-Universität, Röntgenring 11, D-8700 Würzburg, Bundesrepublik Deutschland

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Twodimensional and bidirectional electrophoretic techniques previously developed for the specific detection of circular viroids and virusoids in plant material were used to analyze preparations of low molecular weight RNA from the granulomatous bowel tissue of patients with Crohn's disease and from corresponding tissue of healthy controls. A major and two minor RNA species of about 300 nucleotides length were detected in RNA samples from eight Crohn's disease patients and not in those from three healthy controls. It remains to be established whether these disease-associated RNAs with viroid-like electrophoretic properties play a causative role in Crohn's disease.

Introduction

Crohn's disease is a chronic inflammatory, painful human disease of unknown aetiology, which causes a slow destruction of the alimentary tract. Since its description in 1932 [1] it has been suspected to be an infection [2], probably with a long incubation time. It may be considered a civilization associated ailment, since it increased constantly during the last few decades, especially among young people in industrialized countries. The treatment, which includes dietary restrictions, medication and surgery, is often complicated by fluctuations of symptoms, *i.e.*, periods of severe suffering can be interrupted by intervals of presumable recovery. Moreover, the disease tends to recur after surgery or not to respond to medical treatment. This resistance against medication, together with its long incubation time of 9 to 27 months in laboratory animals [3] and the slow and chronic course of Crohn's disease may indicate an unconventional agent, as discussed for certain subacute spongiform encephalopathies of man and animals (reviewed in ref. [4] and [5]). The putative infectious agent present in the granulomatous ileum and colon and in mesenteric lymph nodes of patients with Crohn's disease passes through 0.2 μm filters [3] and thus appears not to be larger than a virus. The presence of antibodies against double-stranded RNA in patients and in their close personal contacts [6, 7] adds indirect evidence to the motion that Crohn's disease may be caused by a RNA virus-like or viroid-like pathogen, although such antibodies may suggest an autoimmune disease. All attempts to identify or to isolate the pathogen have been unsuccessful [8, 9]. Hence, this disease may be caused by an unconventional virus [4], a prion [5] or a viroid [10, 11]. This possibility has been investigated by Butcher *et al.* [8, 9], who found no differences when comparing human low-molecular-weight RNAs from appropriate controls and Crohn's disease derived blood, mesenteric lymph mode leucocytes and from Crohn's disease tissues using *in vitro* labeling and polyacrylamide electrophoresis. We show here that viroid-like RNAs are present in tissue samples from aereas of active Crohn's disease and not in healthy control tissue.

Materials and Methods

Tissue samples were frozen on dry ice immediately after surgery, and RNA was extracted according to Roe [12]. Twodimensional electrophoresis of 40 μg RNA (Fig. 1) was performed exactly as described by Schumacher *et al.* [13], except that 10% polyacrylamide gels (20 × 40 cm) instead of 5% gels were used for both dimensions. First dimension (20 cm gel length): non-denaturing electrophoresis at room temperature; second dimension (40 cm length): denaturing electrophoresis (8 M urea, 50 °C). Electrophoretic mobilities of the tracking dyes in the first dimension (in brackets: second dimension) were: Coomassie blue, 9.5 (22.5) cm; Xylene cyanol, 14 (30) cm; Bromphenol blue, 19 (37) cm. RNA was silver stained according to Sammons *et al.* [14].

Bidirectional electrophoresis [13] (Fig. 2): RNA samples (40 μg each) were separated on a 20 × 30 cm 10% non-denaturing polyacrylamide gel, allowing Xylene cyanol to move 15 cm. Then, a 1 cm broad gel strip was removed by two vertical cuts 8.5 and 9.5 cm from the bottom of the slots and polymerized into one end of a 20 × 20 cm denaturing 10% polyacrylamide gel (8 M urea). In the second electrophoresis (at 50 °C) Xylene cyanol is allowed to move to the opposite end of the gel.

Present addresses: * Institut für Pharmakologie und Toxikologie, D-8700 Würzburg, FRG, and ** St.-Josef-Krankenhaus, D-7800 Freiburg, FRG.

Reprint requests to H. J. Gross.

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Results and Discussion

In order to determine whether any viroid-like RNA is associated with Crohn's disease, we used screening techniques previously developed for the rapid, sensitive and unequivocal detection of viroids in plant material [13], which depends only on the thermodynamic properties of viroids, their electrophoretic behaviour and their sensitivity to silver staining, and which allows a clear distinction between circular RNA and normal cellular RNA or DNA. The combination of electrophoresis in nondenaturing and denaturing polyacrylamide gels was applied to produce twodimensional (Fig. 1) and bidirectional (Fig. 2) separations of RNA preparations from granulomatous intestinal tissue of Crohn's disease patients and from corresponding tissue of healthy controls. The analysis of RNA prepared from the ileum of healthy persons (Fig. 1A) indicates the absence of any viroid-like RNA. However, RNA from intestinal tissue with active Crohn's disease contains a major and two minor RNA species which show the electrophoretic behavior typical for circular RNA in that they are specifically retarded in the second dimension of the polyacrylamide gel and thus become located outside the diagonal of the bulk RNA (Fig. 1B). This result, *i.e.*, the presence of these three unique RNA species, was found in each RNA preparation from granulomatous bowel tissue of eight patients with Crohn's disease, and not in the healthy controls, *i.e.*, in RNA preparations from healthy ileum of a healthy patient and of two patients with colon cancer, respectively. Using circular Ω-RNA [15] derived from the 73 nucleotides long untranslated leader sequence of TMV and circular potato spindle tuber viroid RNA [16] as standards, we estimate a length of about 300 nucleotides or slightly less for the major Crohn's disease-associated RNA in case that it is also circular. We also applied

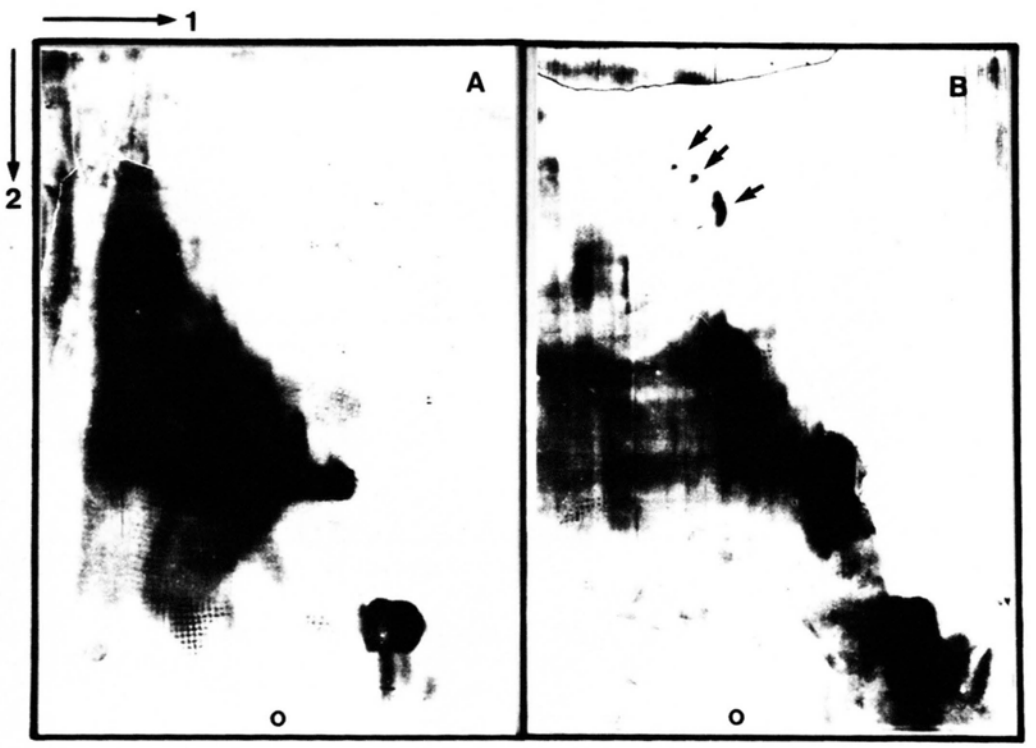

Fig. 1. Twodimensional electrophoretic analyses [13] of RNAs from intestinal tissues of a healthy control (A) and of a patient with Crohn's disease (B). Note that the Crohn's-specific RNAs (arrows) have the electrophoretic mobility of Coomassie blue in the first dimension. The amount of the major Crohn's disease-associated RNA in B is estimated to be in the range of about 1 to 5 ng. O, position of the Coomassie blue G250 dye marker.

Fig. 2. Bidirectional electrophoresis [13] of RNA from wheat germ (a), from intestinal tissue of a healthy control (b) and of two patients with Crohn's disease (c, d). Silver staining was as in Fig. 1. Arrows locate the RNA species which are specifically retarded in the second, denaturing electrophoresis.

the bidirectional procedure [13], which is a simplified version of the twodimensional electrophoresis. Fig. 2 shows such a gel with RNA preparations from two Crohn's patients and of a healthy control. Here again, a major and two minor RNA species appear in Crohn's RNA only (arrows), and were not detectable in the healthy controls. The same disease-associated RNAs were also detected in the RNA preparation of a tissue sample from an intestinal inflammation not classified as Crohn's disease. Interestingly, it has been argued that Crohn's disease and another inflammatory bowel disease, ulcerative colitis, may be caused by the same infectious agent [17].

As outlined in detail by Schumacher *et al.* [13], the two types of electrophoretic analyses used here identify small amounts of circular RNA molecules in the presence of excess linear RNA. At present we do not know of any type of RNA which behaves like the Crohn's RNAs without being circular, however, we do not want to exclude this possibility. Further work is required to establish whether these RNAs play a causative role for Crohn's disease, whether they are only a consequence of the chronic inflammation or whether they even derive from secondary infections.

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