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A Standard Immunoglobulin Preparation Produced from Bovine Colostra Shows Antibody Reactivity and Neutralization Activity against *Shiga*-like Toxins and EHEC-Hemolysin of *Escherichia coli* O157:H7

Summary: Enterohemorrhagic *Escherichia coli* (EHEC) causes a variety of clinical conditions, the most important being hemorrhagic colitis and hemolytic uremic syndrome. A curative therapy of EHEC diseases is not yet feasible. This study investigates the antibody reactivity of Lactobin®, a standardized immunoglobulin (Ig) preparation, obtained from the colostra of non-immunized cows. Three different batches of Lactobin® exhibited equally high titers of specific antibodies against *Shiga*-like toxins (SLTs, verocytotoxins) and EHEC hemolysin (EHEC-Hly) produced by *E. coli* O157. In addition, Lactobin® blocked the cytotoxic effect of SLT-I and SLT-II on Vero cell monolayers and inhibited the cytolytic effects of EHEC-Hly on human erythrocytes. Since Lactobin® contains high levels of antibodies and neutralizing activity against important virulence factors of EHEC O157, this drug has potential use in the treatment of diarrhea and the prevention of EHEC-associated hemolytic uremic syndrome.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are now recognized as important pathogens of the human gastrointestinal tract. Approximately 10% of children with EHEC-associated diarrhea develop extraintestinal sequelae of which hemolytic uremic syndrome is the most frequent [1]. Severe complications such as long-term renal or cerebral damage occur in up to 30% of patients with hemolytic uremic syndrome [2]. *Shiga*-like toxins (SLTs), comprising an enzymatic A-subunit and multiple B-subunits, are thought to be associated with both the development of hemorrhagic colitis and the occurrence of systemic complications [3].

However, some of the clinical symptoms cannot be explained by the action of SLT alone [1]. It is of interest in this regard that, in addition to SLT, most EHEC strains express the plasmid-borne EHEC-hemolysin (EHEC-Hly) [4]. EHEC-Hly belongs to the pore-forming branch of RTX (Repeats in ToXin) exoproteins, and is cytolytic for erythrocytes and cultured cells of human and animal origin [5].

Cows are a natural reservoir for EHEC, and the prevalence of these organisms in apparently healthy cattle can be high [6–8]. The transfer of SLT-specific Ig via colostrum to the newborn calf is thought to be an important mechanism in the early acquired passive immunity against EHEC infections [9]. Furthermore, it is reported that in the human, bovine colostrum preparations are safe and effective agents for the prevention and treatment of diarrheal disease caused by rotavirus [10], enterotoxigenic *E. coli* [11] and the protozoan parasite *Cryptosporidium parvum* [12]. Since bovine colostrum has potential use in the treatment of EHEC infections, this study describes the antibody reactivity and neutralizing activities of bovine colostrum against the potentially pathogenic factors associated with the disease.

Materials and Methods

Expression and purification of recombinant SLT subunits: Both SLT-IIB and SLT-IB were expressed as fusion proteins with the glutathion-S-transferase (GST) under the control of an IPTG-inducible promoter [13]. Strain H1469/pFG4 was used for expression of a subgenic SLT-IIB fusion protein [13]. For expression of SLT-IB we used *E. coli* H1469/pAM2 harboring (by means of frame insertion) a subgenic fragment of the SLT-IB gene, fused to the 3′ end of the GST gene of plasmid pGEX-2T. Expression and purification of both recombinant proteins are essentially the same as described previously [13].

Preparation of Lactobin®: Colostrum was obtained within the first 10 h after calving from at least 100 non-immunized cows maintained under conditions subject to regular veterinary control. The preparation of the colostral Ig fraction was carried out using the customary techniques of the dairy industry [14]. After the removal of fat, the casein was precipitated with HCl, and the whey spray-dried. This technique yielded between 2 and 5 kg of dry powder from 100 kg of colostrum. The immunoglobulin content of Lactobin® was measured by radial immunodiffusion, using specific antisera (Nordic, Hamburg) as previously described [14]. The freeze-dried preparations were stored at 4°C.

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The final product contained approximately 51 mg IgG (predominantly IgG1). 8.6 mg IgM and 1.0 mg IgA per 100 mg [14]. Stock solutions of Lactobin® were prepared in sterile, distilled water and adjusted to a final concentration of 0.1 g per ml. For immunoblot analyses and neutralization assays, the stock solution was diluted further, as described below.

Preparation of hemolytic culture supernatants: Hemolytic culture supernatants were prepared from overnight cultures of laboratory *E. coli* strain HB101, transformed with recombinant plasmid pEO40. Plasmid pEO40 carries a 11.9 kb BamHI fragment derived from plasmid pO157 of EHEC O157:H7 strain EDL933 which harbors the complete EHEC-hemolysin operon [4]. EHEC-hemolysin can be prepared extracellularly from culture supernatants of late log cultures of *E. coli* HB101/pEO40. Briefly, overnight cultures were diluted in 30 ml of fresh, prewarmed Luria-broth to an initial optical density (600 nm) of 0.1 and grown at 37°C with 180 rpm on a rotary shaker to a final optical density of 1.0. Cultures were cooled without delay on ice and centrifuged for 10 min with 3,800 × g at 4°C. The supernatants were transferred to a fresh tube, supplemented with 20 µg per ml gentamicin and used within 3 h of preparation.

Neutralization of EHEC-Hly: Neutralization of the cytolytic activity of EHEC-Hly by Lactobin® was determined using inhibition of erythrocyte lysis where the degree of lysis was expressed as percent lysis according to *Bauer* and *Welch* [5]. Briefly, human erythrocyte concentrates were first defibrinated by carefully mixing with an equal volume of Alsevers solution (2.05% [w/v] dextrose, 0.8% [w/v] sodium citrate, 0.055% [w/v] citric acid, 0.42% [w/v] sodium chloride, pH 6.1) and incubating this mixture at 4°C for 3 days. After centrifugation for 5 min with 1,700 × g at room temperature, the upper phase was discarded, the erythrocytes were carefully washed three times with an equal volume of phosphate buffered saline (PBS) and finally a 2% solution was prepared in PBS. Hemolysis assays contained 100 µl erythrocyte suspension, 10 mM $CaCl_2$ and 20 µl culture supernatant of *E. coli* HB101/pEO40 in a total volume of 200 µl. After incubation at 37°C for 1 h and centrifugation for 5 min at 1,500 × g, the release of hemoglobin was measured spectrophotometrically at 540 nm. "Total hemolysis" was determined by mixing erythrocytes with an equal volume of distilled water, and "background lysis" by adding growth media and/or Lactobin® instead of culture supernatants. In the neutralization assay, toxin-containing culture supernatants were preincubated with different Lactobin® dilutions for 30 min prior to mixing with erythrocytes. The neutralization titer of Lactobin® was defined as the highest dilution inhibiting 50% erythrocyte lysis.

Cytotoxicity assay and neutralization test: Culture filtrates containing SLT-I and SLT-II were prepared from laboratory *E. coli* strains C600(H19J) and C600(933W), respectively, and tested for cytotoxic activity on Vero cells as previously described [15]. The titer of the cytotoxic activity was defined as the reciprocal of the highest dilution of the culture filtrates causing 50% cell death (CD_{50}) per well after two days of incubation at 37°C. The CD_{50} was estimated by microscopic examination of the Vero cells and confirmed by staining residual Vero cells with crystal violet as described by *Gentry* and *Dalrymple* [16]. The results were expressed as percent Vero cell-survival compared to survival of control cells treated with non-toxin. The neutralization titer of Lactobin® was defined as the reciprocal of the highest dilution producing 50% inhibition in cytotoxicity. The neutralization tests were carried out as described [15]. Briefly, a SLT-I or SLT-II stock solution was incubated with 100 µl of Lactobin® solution (diluted geometrically from 1:200 to 1:6,400 with cell culture medium) at 37°C for 1 h, 100 µl of which were then added to the Vero cells and incubated for 2 days. The following controls were added on each plate: Vero cells, Vero cells incubated with anti-SLT-I and anti-SLT-II antisera, and Vero cells incubated with Lactobin® to ensure that the latter is not toxic for the cells.

Immunoblotting: Immunoblot analyses with the purified SLT fusion proteins and the EHEC-hemolysin were essentially performed as described by *Gunzer* et al. [13] and *Schmidt* et al. [4], respectively. After blotting the antigens on nitrocellulose, filters were blocked and allowed to react overnight with colostral immunoglobulin diluted geometrically from 1:200 to 1:25,600 in PBS-Tween. All immunoblot analyses were performed in duplicate with three Lactobin® batches (no. 5511090, no. 5512090 and no. 5511021). In control experiments, Lactobin'® solutions were subjected to immunoblot analysis with purified diphtheria toxin (Calbiochem, La Jolla) as antigen.

Results

Antibody Reactivity of Lactobin® against SLT-I, SLT-II and EHEC-Hemolysin

Antibodies against SLT-I and SLT-II were measured in three different batches of Lactobin® by immunoblot analyses with subgenic fragments of SLT-IB and SLT-IIB expressed as fusion proteins with GST. Figure 1 shows an immunoblot analysis with SLT-IIB using serial dilutions of a 0.1 g per ml Lactobin® stock solution in the range of 1:200 (lane 1) to 1:25,600 (lane 8). Reactivity against both SLT-IIB (Figure 1, lane 6) and SLT-IB (both proteins appear at 33.5 kDa) could be detected up to a dilution of 1:6,400. When EHEC-Hly was used as an antigen in the

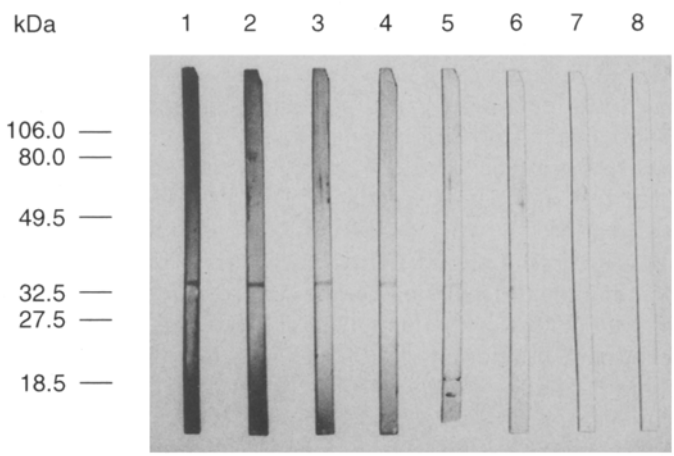

Figure 1: Immunoblot reactivity of Lactobin® with the SLT-IIB fusion protein (33.5 kDa). The Lactobin® stock solution (0.1 g per ml) was diluted geometrically from 1:200 to 1:25,600, and each of these dilutions was analyzed. Eight immunoblot strips incubated with the different Lactobin® dilutions (from 1:200 in lane 1 to 1:25,600 in lane 8) are shown. The molecular weight marker is indicated at the left.

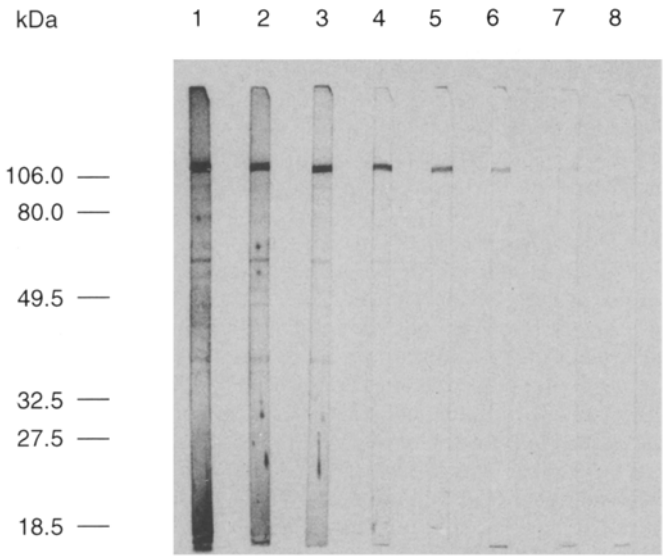

Figure 2: Reactivity of Lactobin® with EHEC-Hly (107 kDa) demonstrated by immunoblot analysis. Lactobin® was diluted geometrically from 1:200 (lane 1) to 1:25,600 (lane 8). The molecular weight marker is indicated at the left.

immunoblot analysis with Lactobin®, one specific band of 107 kDa was shown to react with Lactobin® and could be detected up to a dilution of 1:12,800 (Figure 2, lane 7). Titers against the SLTs and EHEC-Hly within three different batches were identical. Lactobin® did not react with the purified GST carrier protein, indicating that the response was directed against the SLTs but not against the GST protein. Moreover, we observed no immunoblot reactivity against the diphtheria toxin which was included as negative control.

Neutralization of Toxic Activities of SLT-I, SLT-II and EHEC-Hly by Lactobin®

The SLT neutralizing activity of three batches of Lactobin® was determined in a standard neutralization assay. The capacity of various dilutions of Lactobin® to neutralize the effects of SLT-I and SLT-II are shown in Figures 3A and 3B, respectively. Dilutions of 1:800 neutralized SLT-I, whereas SLT-II was neutralized by dilutions up to 1:1,600. Hemolytic activity of EHEC-Hly was inhibited depending on the dose of Lactobin®. Inhibition of 50% of the hemolytic activity was achieved by Lactobin® dilutions of 1:100. Titers within the three Lactobin® batches were constant in two different experiments. Appropriate controls showed that growth media and Lactobin® itself had no significant influence on the proliferation of Vero cells or the background lysis of erythrocytes.

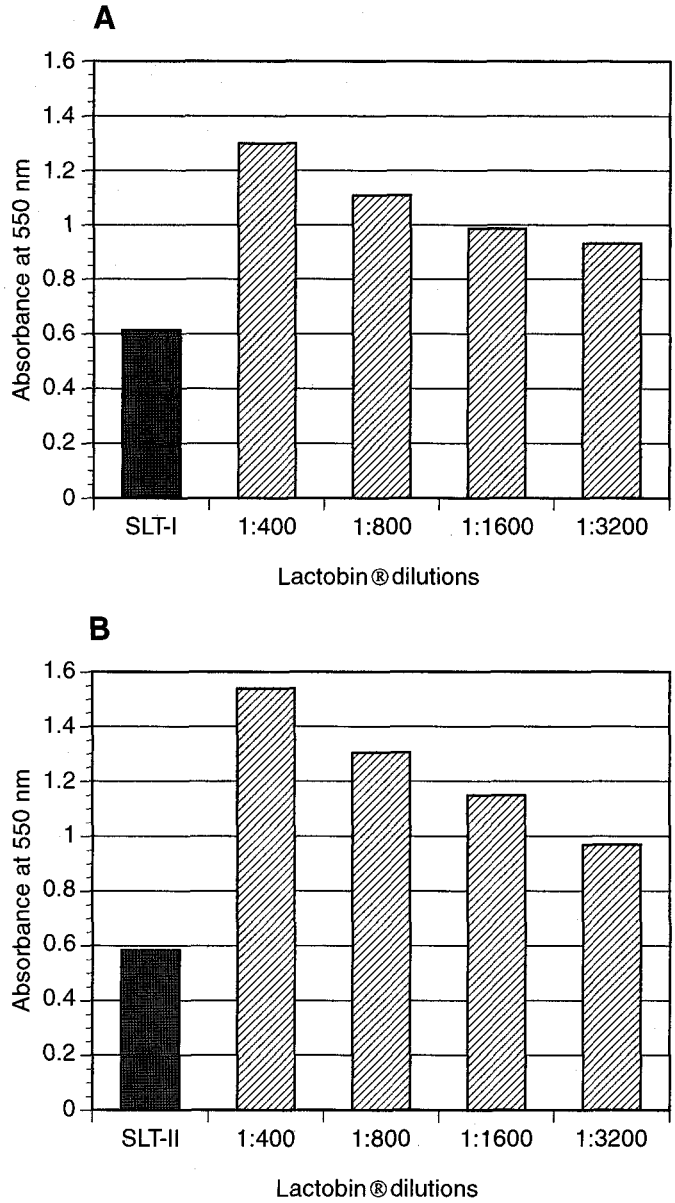

Figure 3: Dose-dependent neutralization of SLT-I (3A) and SLT-II (3B) by Lactobin®. Neutralization of a SLT-I solution containing 100 CD_{50} or a SLT-II stock solution containing approximately 320 CD_{50} by four different Lactobin® dilutions (1:400, 1:800, 1:1,600 and 1:3,200) are shown. The neutralization assay was performed according to *Gentry* and *Dalrymple* [16]. The toxic activity of *Shiga*-like toxins causes detachment of disturbed cells from the Vero cell monolayer. After removal of the detached cells, residual Vero cells were stained with crystal violet. Decreased cell detachment due to the neutralizing activity of Lactobin® causes increase in the absorbance at 550 nm. Data are means of three experiments.

Discussion

These investigations with Lactobin® using immunoblot techniques showed the presence of high IgG reactivity against potentially pathogenic factors associated with EHEC infections. Lactobin® also neutralized the toxins produced by EHEC. These findings have therapeutic implications because the oral administration of Lactobin®

may modify the severity of diarrheal illness in humans and prevent hemolytic uremic syndrome.

Hemolytic uremic syndrome usually develops 1 week after the onset of EHEC-associated diarrhea. After the onset of diarrhea, one of the most critical questions is whether the infection will lead to life-threatening complications of hemolytic uremic syndrome. Unfortunately, there is no therapeutic regimen currently available for the treatment of EHEC-associated diarrhea or to prevent the extraintestinal sequelae. Antibiotic therapy is not recommended for several reasons. First of all, antibiotic treatment early on in the infection may kill or disrupt intracolonic EHEC, resulting in release and possibly systemic absorption of SLT [1]. Moreover, subinhibitory concentrations of some antibiotics have been reported to increase SLT production *in vitro* [17]. Secondly, a prospective controlled study in children with EHEC enteritis demonstrated no significant effect of trimethoprim-sulfamethoxazole on the duration of symptoms and fecal excretion of the pathogen, or on the risk of hemolytic uremic syndrome progression [18]. From these results, the investigators concluded that antibiotic therapy was not indicated in the treatment of EHEC-associated diarrhea. Thirdly, in retrospective analyses, patients who received antibiotics were found to have a greater risk of developing hemolytic uremic syndrome compared to those who received no antimicrobial agents [19–21]. In two studies, the mortality of patients with hemolytic uremic syndrome receiving antibiotic therapy was 50% [20] and 88% [19]. In patients with EHEC infections, treatment with antimotility agents is also a risk factor for the development of hemolytic uremic syndrome [22] and is therefore contraindicated.

Ideally, in order to prevent progression to more severe disease, intervention should be initiated at the early diarrheal stage. Synthetic trisaccharide sequences homologous to the SLT receptor, attached to chromosorb (Synsorb-Pk) have been developed in the hope that these agents will prevent hemolytic uremic syndrome in EHEC-infected patients [23]. The investigators expect that this agent, if given early enough after the appearance of diarrhea, would absorb free fecal SLT and prevent subsequent injury at extraintestinal sites. This synthetic oligosaccharide was well tolerated by healthy adult volunteers [24]. However, the results of a study that is being carried out on children with bloody diarrhea treated with this SLT receptor analogue have not yet been reported.

Another approach under investigation to prevent extraintestinal manifestations of EHEC disease is the antibody neutralization of SLT. There is evidence from animal models that antibodies against SLTs are protective [25, 26]. *MacLeod* et al. [25] demonstrated that pigs could be protected against the lethal effects of SLT-IIe by intravenous administration of immunoglobulins from a pig immunized with inactive purified SLT-IIe toxoid. However, passive immunization must be initiated before microvascular damage has occurred, i.e. in the early diarrheal stage. The reason for this may be that SLT can escape antibody detection by binding to its functional cell receptor and undergoing rapid internalization [27].

Unfortunately, specific SLT-II serum antibodies are absent in most humans. In a previous study we demonstrated that only 15 of 472 (3.2%) serum samples from healthy adults reacted with recombinant SLT-IIB [28]. In line with these findings, *Bitzan* et al. [29] reported that commercial therapeutic Ig preparations, although effective in neutralizing SLT-I, were ineffective in neutralizing SLT-II. However, most EHEC isolates from patients with hemolytic uremic syndrome in Europe and North America produce members of the SLT-II family, occasionally in combination with SLT-I [30–32]. The reasons for the low prevalence of neutralizing antibodies in the population are open to speculation. It is possible that significant levels of specific antibodies have not yet developed in man because EHEC is a new pathogenic entity, introduced to the population during the last decade. On the other hand, we observed that only one of seven sera from patients convalescing after an EHEC infection showed an immune response against SLT-II [13]. This indicates that the toxin is produced in quantities insufficient to generate antibody responses and/or that it causes immune suppression and blockade of seroconversion [33].

The presence of antibodies that react with and neutralize SLT-I and SLT-II has been demonstrated in the colostra from cows [9]. In a study of 225 randomly collected samples, *Pirro* et al. [9] observed SLT-I neutralizing activity in 69.3% of colostrum samples. However, only 14.7% of the colostrum samples neutralizing SLT-I also neutralized SLT-II [9]. In the present study, the Ig concentrate Lactobin® exhibited not only high antibody titers against SLT-I but also high activity against SLT-II, the toxin produced by the majority of EHEC strains. Since Lactobin® also contains high levels of antibodies against EHEC-Hly, this drug may have future applications in modifying the severity of diarrhea and preventing hemolytic uremic syndrome in EHEC-infected patients.

Acknowledgement

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Zusammenfassung: Eine Standardimmunpräparation von bovinem Kolostrum weist Antigenreaktion und neutralisierende Aktivität gegenüber *Shiga*-like-Toxine und EHEC-Hämolysin von *Escherichia coli* O157:H7 aus. Enterohämorrhagische *Escherichia coli* (EHEC) verursachen eine Reihe klinischer Zustandsbilder. Von besonderer Bedeutung sind die hämorrhagische Enteritis und das hämolytisch-urämische Syndrom (HUS). Eine Kausaltherapie der durch EHEC verursachten Infektionskrankheiten ist bisher nicht verfügbar. In der vorliegenden Untersuchung wird über die Antikörperreaktivität von Lactobin®, einem standardisiert hergestellten Immunglobulin (Ig)-Präparat aus Kolostren nicht immunisierter Kühe berichtet. Drei unterschiedliche Produktionschargen zeigten gleichmäßig hohe Titer spezifischer Antikörper gegen *Shiga*-like-Toxine (SLTs, Verotoxine) und EHEC-Hämolysin (EHEC-Hly) aus *E. coli* O157. Lactobin® blockierte den zytotoxischen Effekt von SLT-I und SLT-II auf Verozellen (Monolayers) und hemmte die zytolytischen Effekte von EHEC-Hly auf menschliche Erythrozyten. Da Lactobin® hohe Antikörpertiter und neutralisierende Eigenschaften gegen wichtige Virulenzfaktoren von EHEC O157 enthält, besitzt dieses Arzneimittel möglicherweise ein pharmakodynamisches Potential für die Behandlung von EHEC-bedingten Durchfallerkrankungen bzw. für die Prophylaxe eines assoziierten HUS.

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Book Review

P. Murray
The Widening Circle
A Lyme Disease Pioneer Tells Her Story
321 pages
St. Martin's Press, New York, NY 1996
Price: $ 23.95

Polly Murray tells the story of a disease from the viewpoint of the patient. She has lived in the Lyme area for many years and may have suffered from Lyme disease for 20 years. She, her husband and their four children led an outdoor life, all of them eventually experiencing varied, unusual and in part severe ailments, many of which were probably related to the disease.

Part 1 of the book is entitled "Focusing on Lyme Disease" and encompasses 13 chapters; part 2, "The Scope Broadens," consists of six chapers. The book is mainly in chronological order and six chapters contain a diary covering important events in the life of the members of the family in general as well as those applying specifically to Lyme disease.

The book would be of interest not only to persons who have been affected by Lyme disease but to anyone who spends time outdoors in the many parts of the world where ticks are endemic. It is a saga, and a well-written one, that exemplifies how a patient's determined investigation can eventually help to bring on medical recognition of a disease. Therefore the book can also be recommended to physicians, especially those involved with Lyme disease, and they might want to mention it to their patients.

As a young woman, Murray, whose main interest was art history and painting, worked summers for the WHO in the Tuberculosis Research Office and thus developed an early interest in medicine. In her long and frustrating quest for a definite diagnosis (some doctors told her, "It's all in your head.") and cure she started, in the early 70s, to read medical texts on diseases with symptoms similar to her own. They included fever, sores, nausea, stiffness, joint pain, blurry eyesight, weight loss (several times she had to be hospitalized) and she eventually described them in a nine-page medical history.

Murray began to hear of similar symptoms among her friends and their children, which, together with her medical history, she presented at an appointment with a physician in 1974 at the Yale University School of Medicine. This was the turning point at last for the Murrays and triggered an investigation at Yale. The initial study involved 51 residents in or near Lyme, Connecticut, with "Lyme arthritis." A media blitz (the title of one of the chapters of this book) ensued and climaxed with an article in "Science" in 1982, "Lyme Disease – A Tick-Borne Spirochetosis?" by *W. Burgdorfer* et al., whom the author met the same year. From the beginning, she participated in conferences and symposia, despite recurring manifestations of the disease, and in 1988 she attended the First Annual Yale Conference on Lyme Disease.

As pointed out in the afterword by Dr. *B. A. Murray,* the author shaped medical history.

The book includes a foreword by a Lyme disease specialist, Dr. *K. B. Liegner.* The index covers ten pages and is carefully done. It contains references from scientific journals and from publications for lay readers.

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