Enterotoxigenic Intestinal Bacteria in Tropical Sprue

IV. Effect of Linoleic Acid on Growth Interrelationships of Lactobacillus acidophilus and Klebsiella pneumoniae

MICHAEL J. MICKELSON and FREDERICK A. KLIPSTEIN

Tropical Malabsorption Unit of the Universities of Rochester and Puerto Rico, University of Rochester Medical Center, Rochester, New York 14642

Received for publication 15 May 1975

The factors responsible for colonization of the small intestine by enterotoxigenic coliform bacteria in Puerto Ricans with tropical sprue are unknown, but epidemiological observations have suggested that they may be related to an increased dietary intake of long-chain unsaturated fatty acids, particularly linoleic acid, which is known to exert an inhibitory effect on the growth of gram-positive organisms that normally comprise the flora of the small intestine. We have examined, by using a glucose-limited continuous-culture system, what effect this fatty acid exerts on the growth relationships of enteric gram-positive and coliform bacteria. In this system, colonization by an invading strain of Klebsiella pneumoniae was prevented by the presence of an established culture of Lactobacillus acidophilus, principally by virtue of a lowered pH of the medium that was incompatible with Klebsiella growth. However, when the population density of L. acidophilus was reduced by the presence of a sufficient concentration of linoleic acid, the invading K. pneumoniae successfully colonized the system and, once established, suppressed the growth of L. acidophilus. These observations indicate that, under the conditions of our chemostat, gram-positive enteric bacteria suppress coliform growth and that this effect is reversible by the presence of linoleic acid. It remains to be established, however, what pertinence these in vitro observations have to conditions within the intestinal tract of persons living in the tropics.

Investigations conducted in India, London, and the West Indies indicate that the proximal small intestine of persons with tropical sprue is usually colonized by coliform bacteria (2, 13, 22, 35). Among Puerto Ricans with sprue, these bacteria are most often Klebsiella pneumoniae and, less commonly, Enterobacter cloacae and Escherichia coli (22). Most strains of these bacteria that have been examined elaborate an enterotoxin that produces, in experimental animal models, a number of structural and functional abnormalities that are similar to those present in persons with tropical sprue (23, 24), and it has been suggested that the intraluminal production of this toxin may be important in the pathogenesis of the intestinal lesion in tropical sprue (22–24).

Persons living in the tropics clearly are frequently exposed to coliform bacteria, and diarrhea due to transient colonization of the upper intestine by toxigenic forms is common among persons living in or visiting these areas (12, 14). The factors responsible for persistent colonization of the small intestine by such bacteria in subjects who develop sprue remain to be identified, however. Several, separate epidemiological observations have raised the question as to whether a relationship may exist between the occurrence of sprue (1, 8, 9, 39), as well as the presence of coliform overgrowth (21), and the dietary intake of long-chain, unsaturated fatty acids. These fatty acids, particularly linoleic acid, are known to have an inhibitory effect on the growth of gram-positive bacteria (10, 19, 25), and it has been suggested (21) that coliform overgrowth in persons with sprue might occur as a sequel to depression by these dietary lipids of gram-positive bacteria, which represent the principal flora populating the small intestine under normal circumstances (29). Interrelationships between certain bacterial species are recognized as one of the mechanisms responsible for maintaining a balanced gastrointestinal ecosystem that is relatively resistant to colonization by alien bacterial species (3, 16), but there is scant information regarding the effect of gram-positive bacteria on the growth of coliforms and no information whatsoever as to whether such a relationship could be modified

1 Present address: Bigelow Laboratory for Ocean Science, West Boothbay Harbor, Me. 04575.
by the presence of long-chain, unsaturated fatty acids.

Because of the formidable technical problems that would be associated with examining these bacterial interrelationships in human subjects, we have conducted a preliminary inquiry into this by using a continuous-culture system in which we have tested whether the presence of *Lactobacillus acidophilus*, the most commonly encountered organism in the small intestine of asymptomatic Puerto Ricans, affects the growth of *K. pneumoniae*, the predominant organism isolated from the jejunum of most Puerto Ricans with tropical sprue, and whether the interrelationship between these two species can be altered by the presence of linoleic acid.

**MATERIALS AND METHODS**

*Bacteria.* *L. acidophilus* (VPI 6106-1) and *K. pneumoniae* serotype 5 (VPI 927) were isolated from a Puerto Rican, in the fasting state, with normal intestinal function (case PR 6) and from a patient with overt, untreated tropical sprue (case TS 7), respectively. Clinical aspects and details of the laboratory investigations in these two persons have been described previously (22).

**Medium.** Lyophilized organisms were recovered and maintained in micro inoculum broth (BBL). The organisms were grown in a chemically defined medium (4) that was modified to contain 0.1 g of Tween 80 per liter and 1 g of glucose per liter for continuous-culture studies. Organic chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The linoleic acid used was 99% pure; when added to the culture medium, it was emulsified to a maximum particle size of 10 μm by Vortexing under nitrogen. In the concentration used, the addition of this fatty acid did not affect the pH of the medium.

**Culture conditions.** For batch cultures, anaerobe tubes (Bellco Glass, Inc., Vineland, N.J.) containing 10 ml of medium were inoculated with 2 x 10^7 cells (washed once with 0.95% saline), purged with 95% nitrogen-5% CO₂ before stoppering, and then incubated at 37°C. For continuous cultures, the medium was pumped with a model 1201 peristaltic infusion pump (Harvard Apparatus Co., Millis, Mass.) at 12 ml/h, and 95% N₂-5% CO₂ was delivered at 10 ml/min to 30-ml magnetically stirred chemostats placed in a 37°C water bath. All chemostats were operated anaerobically at a redox potential (Eh) of -150 to -250 mV, as measured with platinum and calomel electrodes on an Accumet model 230 pH meter. Chemostats were inoculated with 2 x 10^7 exponential-phase, washed cells. Chemostats in which one species was growing were operated for a minimum of 12 h, during which time the hourly turbidometric readings and pH were constant, before they were inoculated with cells of the other species.

To identify some of the factors limiting growth, 10-ml portions of the effluents of chemostats growing single species of either bacteria were supplemented, after removal from the continuous-culture system, with either 10 mg of glucose or 1 mg of Tween 80, after which the portions were incubated as batch cultures. Only the addition of glucose enhanced the growth of either species of bacteria, indicating that the energy source (glucose) was growth limiting.

**Analytical assay.** Growth of duplicate batch cultures was followed by turbidimetry at 600 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Chemostat effluents were serially diluted and plated on LBS agar (Baltimore Biological Laboratory, Cockeysville, Md.) as recommended by González et al. (11) for the enumeration of lactobacilli. The dilutions were also spread (0.1 ml) onto Trypticase soy agar (BBL) plates, and *K. pneumoniae* was enumerated after incubation at 37°C for 8 to 12 h, at which time no growth of *L. acidophilus* was apparent. A minimum of 100 colonies was counted for each data point. The pH of chemostat effluents was measured at 25°C with a Radiometer combination electrode and pH meter.

The theoretical rate of clearance ("washout rate") for a nondividing organism or inert substance from the chemostat system was calculated by the method of Jannasch (18).

**RESULTS**

**Batch cultures.** Initially, batch cultures were used as a guide for setting up the chemostat experiments by establishing the appropriate conditions in which *K. pneumoniae* growth is inhibited in the presence of *L. acidophilus* and the necessary dosage of linoleic acid required to reduce growth of the lactobacilli. These studies indicated that *K. pneumoniae* will not grow, even after a period of 96 h, when the pH of the medium is acidified to 5.6 prior to inoculation by the addition of 100 μmol of either hydrochloric or lactic acid to 10 ml of medium, whereas maximum growth occurs within 20 h when the initial pH is 6.8. The amount of glucose in the medium required for *L. acidophilus* to produce enough lactic acid to reduce the pH of the medium from 6.8 to 5.6 was found to be 1 g/liter.

It is well recognized that small amounts of certain fatty acids are required for the growth of lactobacilli (25, 28) and that the optimal growth of many strains of this bacterium is achieved by the presence of Tween 80 (polyoxyethylene sorbitol monooleinate), which supplies these acids in a nontoxic form. Such proved to be the case for the strain of *L. acidophilus* used in the present study. At the same time, Tween 80 is recognized to detoxify the inhibitory effect of larger doses of linoleic acid on lactobacillus growth (38). Therefore, a series of experiments was conducted to determine the effect on *L. acidophilus* growth of varying doses of linoleic acid in the presence of different doses of Tween.
80. The results indicated that 0.1 g of Tween 80 per liter was sufficient to produce maximum growth and that the minimum tested amount of linoleic acid necessary to inhibit growth at this concentration of Tween 80 was 100 μg/ml. This concentration of linoleic acid had no effect on the growth of K. pneumoniae. Oleic acid at the same concentration and linoleic acid at 10 μg/ml were not found to have an inhibitory effect on L. acidophilus growth.

Single-species chemostats. When inoculated separately into sterile chemostats, L. acidophilus and K. pneumoniae established population densities of $4 \times 10^7$ to $8 \times 10^7$ and $2 \times 10^8$ to $4 \times 10^9$/ml, respectively (Fig. 1A). After the establishment of a steady state within these limits, the pH of the effluent from the L. acidophilus chemostat was 5.6 to 5.7 and that from the K. pneumoniae chemostat was 6.1 to 6.3. Sterile, uninoculated chemostats had a pH of 6.6. The Eh of $-150$ to $-250$ mV was not measurably changed by bacterial growth.

Two-species chemostats. The population density of K. pneumoniae decreased rapidly when this organism was inoculated into a chemostat containing L. acidophilus in the steady state (Fig. 1B). The rate of decrease in the population density of K. pneumoniae growth approximated that of the clearance ("washout") of an inert substance from the system, suggesting that K. pneumoniae growth had largely ceased.

The above procedure was repeated in a new chemostat, except that in this reactor the concentration of linoleic acid in the medium was changed from 0 to 0.1 mg/ml at 14 h after the introduction of K. pneumoniae. The pattern of bacterial growth of the two species was unchanged from that shown in Fig. 1B, except that the concentration of L. acidophilus in the effluent decreased slightly to $2 \times 10^7$ cells per ml after a 48-h exposure to this concentration of linoleic acid, but the pH of the medium remained at 5.6 to 5.7, and K. pneumoniae growth continued to be markedly suppressed.

K. pneumoniae was again introduced into another chemostat containing L. acidophilus in the steady state, but this time the concentration of linoleic acid introduced into the medium 14 h after the invasion of K. pneumoniae was increased to 1 mg/ml (Fig. 2). This resulted in a prompt decrease in the population density of L. acidophilus, which 40 h after the introduction of linoleic acid was estimated at 3 viable organisms per ml. The decreased population of L. acidophilus was associated with a rise in the pH to 6.1 within 10 h after the introduction of linoleic acid into the medium. Subsequent to this change in pH, there was a marked increase in the population density of K. pneumoniae.
The introduction of *L. acidophilus* into a linoleic acid-free chemostat system in which *K. pneumoniae* had been established resulted in a decrease in the population density of *L. acidophilus* (Fig. 3). This decrease proceeded at a rate that was slower than the projected rate of clearance of an inert substance from the system, suggesting that the rate of *L. acidophilus* growth was being restricted as a result of competition with *K. pneumoniae* for the available supply of glucose (37).

DISCUSSION

A large body of experimental evidence (both in vitro and in vivo) indicates that the indigenous flora, mostly of the distal bowel, serve as one of the stabilizing influences on gut ecology by interfering directly or indirectly with the establishment of pathogens by means of a variety of mechanisms, including competition for essential nutrients, elaboration of antibiotic substances or toxic metabolites, or alteration of the physical environment. These observations have recently been summarized (3, 5, 16). Under normal circumstances, the small bowel of humans is populated with microaerophilic lactobacilli and enterococci (3, 5, 29). Scant attention has been paid to date as to whether these organisms have any effect on the growth of other bacterial species. Investigations concerning this have been limited to, in continuous-culture systems, the demonstration that enterococci reduce the growth of *Vibrio cholerae* (31) and that streptococci have an inhibitory effect on the growth rate of, and production of enterotoxin by, *Staphylococcus aureus* (15). Investigations regarding the effect of gram-positive organisms on the growth of *coli* species have been confined to the demonstration, by using stationary-plate cultures, of an inhibitory effect of *L. acidophilus* on the growth of *E. coli* (32, 36). In the present study, when the relationship between *L. acidophilus* and *K. pneumoniae* was examined under defined conditions with a glucose-limited continuous-culture system, we found that colonization by invading *K. pneumoniae* is inhibited in the presence of an established colony of *L. acidophilus*. Our observations indicate that this inhibitory relationship is related to a lowered pH of the medium that is incompatible with *Klebsiella* growth although other unrecognized mechanisms may be of relevance.

Our interest in the possible effect of linoleic acid on bacterial interrelationships stems from two clinical observations. First, the scattered geographic distribution in tropical climates of tropical sprue, as well as the occurrence of this disorder among expatriates visiting the tropics whose dietary intake has been analyzed, appears to coincide with a dietary lipid intake that consists principally of long-chain unsaturated fatty acids (1, 8, 9, 39). Second, the peak seasonal occurrence of the onset of both overt and subclinical tropical malabsorption among Puerto Ricans appears to occur both at a time when colonization of the small intestine by *coli* forms takes place among members of this population and at the time of year during which the dietary intake of pork among residents of the island is markedly increased (21).

The fatty acid composition of the small-intestinal fluids is principally a reflection of the lipid composition of the diet (40), and pork not only contains a high proportion of long-chain, unsaturated fatty acids but it is also unique among both vegetable and animal fats in that these acids are located in the α position of the dietary triglyceride (27) such that pancreatic hydrolysis within the small intestine liberates an unusually large quantity of oleic and linoleic acid (20). Long-chain fatty acids have been shown in stationary batch culture experiments to have an inhibitory effect on gram-positive bacteria (10, 19, 25), but not on gram-negative bacteria, due to the protective action of their lipopolysaccharide layers (33). Increased unsaturation up to the point of a double bond enhances the effect

**Fig. 3. Growth of L. acidophilus** (●) after inoculation into a linoleic acid-free medium containing *K. pneumoniae* (□, before invasion; ■, after invasion) in a steady state. Broken line indicates the projected rate of clearance of an inert substance from the medium.
of the fatty acids; linoleic acid (C₁₈:₂) has the most potent antibacterial activity (19).

Chemical constituents of the diet are thought to exert, under certain circumstances, an influence on the nature and distribution of the bacterial flora in the intestinal tract (6, 26, 30, 34), and it seemed a possible consideration that the coincident occurrence of intestinal abnormalities, colonization of the small intestine by coliform bacteria, and increased pork intake might be related to an inhibitory effect of linoleic acid on the normally resident gram-positive flora of the small intestine, thus establishing an environment permissive for coliform overgrowth. Because of the technical problems that would be present in testing this relationship in human subjects, we conducted a preliminary evaluation by using a continuous-culture system, which is considered to be the best laboratory approximation of in vivo conditions (17). The results of our studies indicate that the addition of a sufficient quantity of linoleic acid permits the successful establishment of invading K. pneumoniae in a continuous-culture system populated by an established colony of L. acidophilus and that, once established, the K. pneumoniae suppressed the growth of L. acidophilus.

Although these observations confirm the fact that linoleic acid can modify interrelationships between gram-positive and coliform bacteria, it remains uncertain as to what relationship, if any, these in vitro observations bear to conditions within the intestinal tract of humans. For example, although the pH found necessary to inhibit Klebsiella growth is within the range present in postprandial intestinal aspirates from humans (7), it is unknown whether the presence of the relatively low concentrations of gram-positive bacteria normally resident there has any influence on the pH of luminal contents. Nevertheless, we believe that the observations made in the present, preliminary in vitro investigations indicate that further evaluation of the effect of linoleic acid on the intestinal flora is now warranted in human subjects.

ACKNOWLEDGMENTS

This work was supported by a grant from the Williams-Waterman Fund of the Research Corporation, New York, N.Y., and Public Health Service training grant 5-TO-1-AM-05177 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

LITERATURE CITED


