Biliary Secretory IgA Levels in Rats with Protein–Calorie Malnutrition

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Malnutrition is a recognized cause of failure of host defense mechanisms. In the past 5 years, it has been demonstrated that the gut, long known to have significant morphologic changes with protein calorie malnutrition (PCM), is an immune organ affected by malnutrition. To assess the role of biliary immunoglobulin A (S-IgA), part of the barrier to bacterial invasion from the gastrointestinal tract, the following study was performed. Seventy-eight Fisher female inbred rats weighing 110–130 g were randomly separated into two groups. The control rats were fed standard rat chow. The experimental rats were fed a 2% agar protein depletion (PCM) diet (USP XV). The biliary tract of the rat was cannulated with Silastic tubing and bile flow and rat secretory immunoglobulin A (S-IgA) was sampled at intervals. S-IgA was measured by the Elisa method. Total bile protein was measured by micro-Kjeldahl. Bile was collected from the rats on day 0, 7, 14, 21, 29, 36, 42, and 49. During the study, the weight of rats fed the PCM diet decreased from 127.4 ± 14.5 g at day 0 to 83 ± 2.6 g on day 37. Control rats gained weight from 124.4 ± 14.5 g at day 0 to 153.6 ± 3.8 g at day 37. Total biliary protein at day 0 was 2.52 ± .05 mg/ml and at day 36 was 2.51 ± 11 mg/ml for PCM rats and 2.57 ± 10 mg/ml for control rats. Normal rats and control rats both had an initial increase of S-IgA from 2.74 ± 73 mg/ml on day 0 to 5.75 ± 1.75 mg/ml on day 37. Both PCM and control rats demonstrated an increase in S-IgA levels despite significant loss of weight in the experimental group. Similarly, total biliary protein was not decreased in either group. The results suggest that gut immune system is preserved despite significant protein calorie malnutrition.

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INFECTIONS ARE STILL THE MAJOR CAUSE of morbidity and mortality in our society.[1] Most of these infections occur after initial exposure of foreign proteins, bacteria, or viruses to mucous membranes. In response to the continual threat of infection, the body has developed an efficient host immune defense system concentrated in the mucous membranes. In the intestinal tract, because of the continous exposure to microbes and food antigens, it has been estimated that there are about 7.5 (10^{10}) anti-body-producing cells lining the mucosa of the gastrointestinal tract. Heremans calculated that this represents approximately 50 g of immunoglobulin-producing cells in the gut, which is comparable to the mass of the immunoglobulin-producing cells in the spleen.[1]

Although immunoglobulin A (IgA) had been observed as a precipitate in immunoelectrophoresis of human serum,[1] its purification allowed its role to be defined. IgA was also found to be the predominate immunoglobulin in human milk and human saliva as well as in many other secretions.[2] Tomasi demonstrated that secretory IgA was a dimer of IgA molecules with an additional polypeptide chain, the secretory component (SC).[3] It was later discovered that S-IgA also contained another polypeptide structure called the J-chain. The J-chain adds to the compact structure of S-IgA, and thus S-IgA antibodies are more resistant to enzymatic action than are serum IgA antibodies.

IgA with the J-chain is produced by specialized lymphoid cells (B-cells). It is then linked to SC on the surface of specialized epithelial cells (M-cells).[1] According to Brandtzaeg, the binding of Sc to the IgA dimer requires the presence of the J-chain.[4] After the binding of the IgA dimer to the SC polypeptide, the complex is transported through the epithelial cell and is then released at the apical surface of the cell. Thus, S-IgA is found in secretions like milk, saliva, tears, bile, as well as in fluids of the nasal, bronchial, intestinal, and genitourinary surfaces. The levels of IgA are indirectly regulated by the amount of exposure of foreign antigen.

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According to Walker and Isselbacher, diminished levels of S-IgA may be related to mucosal atrophy.[5] Patients with S-IgA deficiency have an increased incidence of circulating antibodies to food and bacterial antigens, which suggests an increased permeability of the mucosal barrier. Patients with protein–calorie malnutrition may have an increased incidence of intestinal infections, circulating antibodies to food and bacteria, and higher incidence of autoimmune disease.

Malnutrition affects the humoral immune system in many ways. The B lymphocyte subpopulations and immunoglobulin synthesis of serum IgG, IgM, and IgA are usually normal or increased; however, secretory IgA (S-IgA) levels in the respiratory and gastrointestinal fluids are generally decreased as are secretory IgA antibody responses.[6]

Secretory IgA binds and neutralizes viruses and bacterial enterotoxins, S-IgA agglutinates bacteria and prevents the attachment of bacteria to epithelial cells. However, the bacteriolytic action of S-IgA antibodies *via* lysozyme and the complement system has been verified by some investigations and not by others. Successful immunization for poliomyelitis involves the complex gut immune system. The polio virus (Sabin) oral immunization has led to a predominately IgA response in intestinal secretions, whereas parenteral immunization (Salk) did not cause elaboration of intestinal antibody.[7] The local immune response is dependent on route of administration (oral or parenteral) as well as the amount of antigens. Secretory IgA is the predominant antibody in bile. Polymeric IgA is transported into bile after the addition of the SC portion by the hepatic cells[1] and is a major source of intestinal S-IgA.

From previous work, it has been demonstrated that malnourished patients exhibit an increased susceptibility to infection, and thus, one might expect a decrease in the amount of S-IgA.[8] Protein–calorie malnutrition has a profound effect on the structure of the gastrointestinal (GI) tract. Moreover, atrophy of mucosal epothelium, Peyer's patches, and gut-associated lymphatic tissue (GALT) could diminish the cell numbers and function for maintaining GI immune function, specifically S-IgA.

Moreover, earlier studies have shown that route of nutrition (parenteral or enteral) influences gut morphology. Gut atrophy occurs when food is absent from the GI tract and is associated with increased susceptibility to infection.[9,10] Acknowledging that S-IgA is only one component of the complex GI immune system, these studies were done to determine if S-IgA was affected by malnutrition in rats. Because S-IgA is prominent in bile, the model used evaluates S-IgA in bile.

Materials and Methods

Seventy-eight female inbred Fisher rats weighing 110–130 g were randomly separated into two groups of 38 and 40. The control group included 38 rats, the test (PCM) group included 40 rats. The control rats were fed a standard rat chow diet and water *ad libitum*. The PCM rats were fed a 2% agar protein depletion diet (USP XV) and water *ad libitum*. This diet was selected as the test rats will lose a predictable amount of weight. Moreover, few rats will die because of malnutrition.

During the experimental period, five rats from each group were studied every 7 days. The rats were weighed and anesthetized intraperitoneally with sodium pentathol, 0.01 ml/100 g body weight (BW), and intramuscularly with ketamine, 0.01 ml/100 g BW. A celiotomy was performed and the common bile duct was cannulated with Silastic tubing (O.D. 0.025″, I.D. 0.012″). Bile was collected over a 30-minute period and the rats were killed.

The rat bile specimens were then measured and the bile flow rate was calculated. It is important to measure bile flow so that obstruction of the bile flow by cannulation can be avoided. The samples were stored in plastic vials at −20 C until the total protein and S-IgA assays were performed (Fig. 1).

Rat S-IgA used for standards in the assay was purified from rat bile courtesy of the Rosalind Russell Laboratory at the University of California–San Francisco. Standards were prepared by diluting rat S-IgA with 0.01 M PBS— 0.1% Tween 20 pH 7.4 at concentrations of 12.5–200 mg/ml on the day the assay was performed.

Immulon II 96-well microtitre plates (Dynatech Labs, Alexandria, VA) were coated with goat anti-rat S-IgA (1 μg/ml in 0.01 M PBS pH 7.4, 200 μl/well). The goat antisera was purchased from Miles Laboratories (Ames Division, Elkhart, IN) and purified. The purification of the antisera involved dialysis with 0.02 Tris-HCL. 0.028 M NaCl buffer pH 7.2, purification of the IgG fraction *via* affinity chromatography with DEAE Affigel blue (Bio Rad Labs, Rockville, NY), concentration with Lyphogel (Gelman Sciences, Inc., Ann Arbor, MI), dialysis with 0.01 M phosphate buffered saline (PBS) pH 7.4, and stored at 4 C in a borosilicate glass vial. The optical density at 280 mm of the antisera was used to calculate the protein concentration. Plates containing the goat anti-rat S-IgA were placed in a 37 C humid incubator for 1–2 hours, then stored at −20 C. Before being used, the plates were thawed and washed three times with PBS-Tween 20, allowing 2–3 minutes equilibrium time per wash, then drained and blotted. Bile samples were thawed and diluted 1:20,000 with PBS-Tween 20. Two hundred microliters per well of blank solution (PBS-Tween) was pipetted to the first two columns of a coated microtitre plate. To avoid the "edge effect," the edge rows were not used, and 200 μl/well of various concentrations of standard was pipetted into two rows of the plate. Adjacent wells were filled with 200 μl/well of PBS-Tween 20 and the remaining wells received diluted

TABLE 1. *Biliary Flow, IgA Values—Normal* Versus *Control*

Day	Control (N = 5) Flow (ml/hr)	Control (N = 5) Total Protein (mg/ml)	Control (N = 5) S-IgA (mg/ml)	Protein Calorie Malnutrition (N = 5) Flow (ml/hr)	Protein Calorie Malnutrition (N = 5) Total Protein (mg/ml)	Protein Calorie Malnutrition (N = 5) S-IgA (mg/ml)
0	1.42 ± 0.59	2.52 ± 0.05	2.74 ± 0.73			
7	1.54 ± 0.58	2.61 ± 0.10	1.68 ± 0.77	1.44 ± 0.49	2.40 ± 0.07	1.95 ± 0.54
14	1.62 ± 0.16	2.55 ± 0.05	2.46 ± 1.34	1.56 ± 0.70	2.47 ± 0.10	3.14 ± 0.91
21	1.91 ± 1.29	2.44 ± 0.04	4.13 ± 0.40	1.27 ± 0.40	2.32 ± 0.09	3.90 ± 2.40
29	2.19 ± 0.88	2.52 ± 0.08	3.68 ± 1.24	1.88 ± 0.52	2.30 ± 0.08	4.21 ± 1.27
36*	3.06 ± 2.16	2.57 ± 0.10	5.44 ± 0.36	1.86 ± 0.23	2.51 ± 0.11	5.75 ± 1.75
42	2.36 ± 0.96	2.57 ± 0.05	1.50 ± 0.43	2.83 ± 1.04	2.50 ± 0.72	2.19 ± 0.79
49	2.59	2.53 ± 0.09	2.81	3.35 ± 0.96	2.46 ± 0.06	3.28 ± 1.38

* The malnutrition phase of the experiment was terminated at day 36. On day 37, five PCM rats were refed and samples during the refeeding made at day 42 and 49. Six PCM rats died between days 32 and 36.

bile, four replicas per sample. The plate was placed in a humid 37 C incubator for 1 hour, and then washed three times with PBS-Tween 20. Conjugated horseradish peroxidase-IgG (IgG specific for rat S-IgA) (Jackson Immunoresearch Labs, Inc., West Grove, PA) was diluted 1:5000 with PBS-Tween 20, then added to the plate, 200 μl/well. The plate was incubated 1 hour at 37 C and washed three times with PBS-Tween 20. During this interim, a horseradish peroxidase substrate solution was prepared using 0.04% o-phenylenediamine dihydrochloride and 0.04% of 30% H_2O_2 in a 0.1M citric acid 0.2 M Na_2HPO_4 buffer. After the plate was washed, 200 μl/well of substrate solution was added and the rection was allowed to proceed 15 minutes ± 2 seconds at room temperature. The reaction was stopped by the addition of 50 μl/well of 2.5 N H_2SO_4 and the optical density of each well was determined with a Multiskan microtitre plate reader blended with air using the 492 mm filter (Flow Laboratories, Inc., McLean, VA). Sample values were obtained by interpolation from linear regression of the standard curves. Total bile protein was measured by a colorimetric micro-Kjeldahl method (SIGMA bulletin 690).

Results

On day 0, 10 rats were operated on and their bile ducts were cannulated. Bile was collected for 30 minutes. The average flow of bile was 1.42 ± 0.59 ml/hr. The average total protein content of the bile collected was 2.52 ± 0.05 mg/ml, whereas the average concentration of S-IgA was 2.74 ± 0.73 mg/ml (Table 1).

The 2% agar diet used in many previous experiments produced a predictable weight loss with minimal malnutrition-related mortality.[8-10] The female Fisher rats in the control group weighed 124.4 ± 14.5 g at the beginning of the experiment. These rats fed a "normal" diet of rat chow gained weight, 29.1 ± 9.3 g. Rats fed a 2% agar protein depletion diet (USP XV) loss weight (44.4 g) to 83.0 ± 2.6 g during the experiments. The weight changes at the extreme of the experiment (36 days) are statistically significant ($p < 0.001$). Immune function such as survival from *Escherichia coli*-Hb peritonitis usually differs from control after 14 days of the 2% agar protein depletion diet.[7,9,10] The experiment represents an extreme nutritional challenge to the immune system.

On day 7, another two groups of five rats, one control and one PCM, were studied. The bile ducts were cannulated and bile was collected for 30 minutes. The control rats had an average flow rate of 1.54 ± 0.58 ml/hr, an average total protein content of 2.61 ± 0.10 mg/ml, and an S-IgA concentration of 1.68 ± 0.77 mg/ml. In the PCM rats, the average bile flow rate was 1.44 ± 0.49 ml/hr, an average total protein value was 2.4 ± 0.07 mg/ml, and an average S-IgA concentration was 1.95 ± 0.54 mg/ml.

On day 14, another five rats from each group were operated on and bile was collected. The control rats had an average flow rate of 1.62 ± 0.16 ml/hr, an average total protein value of 2.55 ± 0.05 mg/ml, and an S-IgA concentration of 2.46 ± 1.34 mg/ml. The PCM rats had an average flow rate of 1.56 ± 0.70 ml/hr, an average total protein value of 2.47 ± 0.10 mg/ml, and an average S-IgA concentration of 3.14 ± 0.91 mg/ml.

On day 21, another five rats from each group were operated on and bile was collected. The control rats had an average flow rate of 1.91 ± 0.29 ml/hr, an average total protein value of 2.44 ± 0.04 mg/ml, and an S-IgA concentration of 4.13 ± 0.40 mg/ml. The PCM rats had an average flow rate of 1.27 ± 0.40 ml/hr, an average total protein value of 2.32 ± 0.09 mg/ml, and an average S-IgA concentration of 3.90 ± 2.40 mg/ml.

On day 29, another five rats from each group were

TABLE 2. *Data Analysis: Day 36*

	PCM	Control	p value
Wt (g)	−44.4 ± 12	19 ± 10.7	<0.001
Total protein (bile) mg/ml	2.51 ± 11	2.57 ± 10	NS
S-IgA (mg/ml)	+3.01 ± 1.02	1.69 ± 63	NS

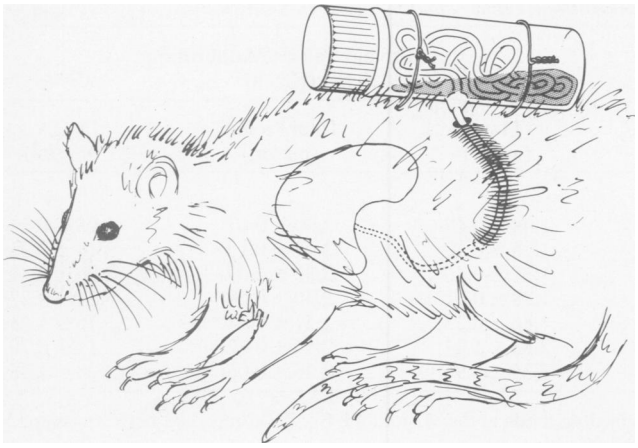

FIG. 1. Rat biliary sampling preparation.

operated on and bile was collected. The control rats had an average flow rate of 2.19 ± 0.88 ml/hr, and average total protein value of 2.52 ± 0.08 mg/ml, and an S-IgA concentration of 3.68 ± 1.24 mg/ml. The PCM rats had an average flow rate of 1.88 ± 0.52 ml/hr, an average total protein value of 2.30 ± 0.08 mg/ml, and an average S-IgA concentration of 4.21 ± 1.27 mg/ml.

On day 36, another five rats from each group were operated on and bile was collected. The control rats had an average flow rate of 3.06 ± 2.16 ml/hr, an average total protein value of 2.57 ± 0.10 mg/ml, and an S-IgA concentration of 5.44 ± 0.36 mg/ml. The PCM rats had an average flow rate of 1.86 ± 0.23 ml/hr, an average total protein value of 2.51 ± 0.11 mg/ml, and an average S-IgA concentration of 5.75 ± 1.75 mg/ml.

On day 37, five PCM rats were refed with standard rat chow and on day 42 bile was again collected. The five control rats had an average flow rate of 2.36 ± 0.96 ml/hr, an average total protein value of 2.57 ± 0.05 mg/ml, and an S-IgA concentration of 1.50 ± 0.43 mg/ml. The PCM rats had an average flow rate of 2.83 ± 1.04 ml/hr, an average total protein value of 2.50 ± 0.72 mg/ml, and an average S-IgA concentration of 2.19 ± 0.79 mg/ml.

On day 49, the experiment was terminated and samples of bile were collected from the rest of the rats. The control rats had an average flow rate of 2.59 ml/hr, an average total protein value of 2.53 ± 0.09 mg/ml, and an S-IgA concentration of 2.81 ± mg/ml. The PCM rats had an average flow rate of 3.25 ± 0.96 ml/hr, an average total protein value of 2.46 ± 0.06 mg/ml, and an average S-IgA concentration of 3.28 ± 1.38 mg/ml.

Statistical analysis, using 95% confidence interval, was established using a pooled t-test and the levels of IgA and total protein for the PCM and control rats were found to be statistically the same ($p < 0.05$). These results suggest that protein–calorie malnutrition minimally affects the levels of biliary IgA and protein, which did not significantly ($p < 0.05$) change during a 36-day period of a protein depletion diet (Table 2).

Discussion

One of the major defense mechanisms against bacterial and viral invasion through mucosal surfaces is from secretory immunoglobulins, *i.e.*, S-IgA and S-IgM, and secondarily, IgE, IgG, and IgD. Immunoglobulin A (IgA) is in secretions of the gastrointestinal and respiratory tracts as well as in milk and saliva.

The origin of IgA in the gastrointestinal tract appears to be from lymphoblastic cells of the gut-associated lymphoid tissue (GALT) which differentiates to precursor IgA producing plasma cells.[11,12] The plasma cells located in the lamina propria of the intestine are stimulated by intestinal antigens that "leak" or are transported by specialized membranous epithelial cells (M-cells) to produce S-IgA (dimeric form). In the presence of IgA deficiency, S-IgM will be increased as compensatory measure.

Secretory IgA differs from serum IgA in that IgA is bonded tightly to the J-chain and is secreted into the crypts of the mucosa. It is also linked to the secretory component (SC), which protects the molecule from proteolytic enzymatic action in the intestine.[13,14]

In the bile, approximately 60% of the IgA is in the S-IgA form. Serum IgA is transported into the hematocytes and is transformed into the polymeric form.[15,16] It is then secreted into the bile. In bile duct obstruction, S-IgA is increased in the serum. On relief of the obstruction, the level of S-IgA in the serum returns to normal levels.

The action of S-IgA is protective and binds with intestinal antigens. It neutralizes bacterial and viral enterotoxins along with agglutinization of the organisms. It also functions to prevent the attachment of bacteria to the surface epithelium. This function is important in infections such as Streptococcal pharyngitis or cholera. S-IgA does not seem to have any clearcut effect on the complement system, although bacteriolytic action *via* the complement system has been described.[17] Human serum antibody responses are variable and are probably due to the nature of the antigen, the amount involved, the age of the patient, and the route of infection.

Protein–calorie malnutrition (PCM) is associated with many immunologic defects. PCM causes an increased gastrointestinal permeability to antigenic factors. In severely malnourished children, a selective decrease in secretory IgA levels in intestinal secretions occurs. PCM is associated with an increased incidence of intestinal infection, circulating antibodies to food and bacterial antigens, and evidence of autoimmune disease. Paradoxically, some studies of children who have

kwashiorkor reveal T-cell lymphokin levels—migration inhibition factor (MIF) and leukocyte migration inhibition factor (LMI), which are similar to normal children.[18] However, studies by Sirisinha et al. found S-IgA levels decreased in children who have PCM.[19]

Our study was undertaken to determine if the S-IgA levels of bile in rats were lowered by malnutrition. The weight of the Fisher rats in PCM and control rats was predictable. The weight of PCM rats decreased from an average weight of 127.4 ± 14.5 g at day 0 to 83 ± 2.6 g on day 37, whereas the control rats' weight increased from 124.4 ± 14.5 g on day 0 to 153.6 ± 3.8 g on day 37. The control rats, who were fed a normal diet, increased in weight and appeared healthy. The levels of S-IgA in PCM rats increased from 2.74 ± 0.73 mg/ml on day 0 to 5.75 ± 1.75 mg/ml on day 37, as did the control rats' S-IgA levels increase from 2.74 ± 0.73 mg/ml to 5.44 ± 0.36 mg/ml. Both the PCM and control rats' S-IgA levels similarly increased despite the PCM rats losing weight and the control rats gaining weight. These results suggest that the immune system of the gut may be of great importance and appears to be maintained, whereas other bodily systems deteriorate in the presence of malnutrition.

The total biliary protein level on day 0 was 2.52 ± 0.05 mg/ml and on day 36 was 2.51 ± 0.11 mg/ml for PCM rats and 2.57 ± 0.10 for the control rats. The total biliary protein levels remained constant in both the PCM and control rats. Even in the absence of sufficient protein, PCM rats maintained a constant biliary protein level. No differences occurred between the control rats and the PCM rats in terms of total protein and S-IgA concentrations in their bile.

Prior experiments of *E. coli*-Hb adjuvant peritonitis and other immune studies show that rats of this breed, age, and sex become highly susceptible to infection at 14 days. During 14–21 days of a 2% agar diet, weight loss is approximately 25% of starting weight. One to two weeks of the diet causes substantial loss of body protein and fat. The model results in pronounced loss of weight and protein in the small intestine as well.

It is surprising that IgA, the "antiseptic paint" of the gastrointestinal tract is maintained in severe malnutrition. It is especially surprising that IgA is well preserved in PCM because of the well-known atrophy of the small intestinal mucosa associated with malnutrition. Because the intestinal mucosa comes into greatest contact with foreign antigens, bacteria, and viruses, constant levels of S-IgA aid in maintenance of protection against ingested bacteria, viruses, and other antigens. It appears the biliary S-IgA is a protein of high biologic priority.

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Discussion

DR. GEORGE L. JORDAN, JR. (Houston, Texas): We are indebted to Drs. Sheldon and Lim for the information contained in this paper. Immunology has been a subject of interest to surgeons over a good many years now, but actually only a few surgeons are conducting serious experimental studies. The subject of secretory immunoglobulins is almost nonexistent in the surgical literature. In fact, I had a computer search of the recent literature and found only one other recent article, and that was by Dr. Sheldon and his group. That particular article showed that secretory IgA in the bile decreased in animals fed intravenously and that oral food intake was necessary in maintain-