Immunology 1983 **49** 69

IgE and IgD antibodies to cow milk and soy protein in duodenal fluid: effects of pancreozymin and secretin

S. FREIER,* E. LEBENTHAL, MIRIAM FREIER, P. C. SHAH, B. H. PARK & P. C. LEE
Department of Pediatrics, State University of New York at Buffalo, Children's Hospital of Buffalo, Buffalo, New York, U.S.A.

Accepted for publication 20 October 1982

Summary. Duodenal fluid IgE was reported to be increased in food allergy and in inflammatory conditions of the bowel. We studied the presence and specificity of IgE and IgD antibodies against α-casein, β-lactoglobulin A, α-lactalbumin, bovine serum albumin and soy bean agglutinin using an enzyme-linked immunoassay (ELISA). Thirteen children with various intestinal diseases and thirteen normal adult volunteers were examined. In resting duodenal fluids, 8/13 of the children had IgE and 5/13 had IgD, while only 1/13 of the adults showed detectable IgE and IgD. After pancreozymin, 4/6 of the children and 4/8 of the adults showed detectable IgE and IgD in their duodenal fluids. After secretin, the duodenal fluids from 1/8 of the children and 2/8 of the adults had detectable IgE, while 6/13 children and 1/10 of the adults had IgD. The results indicate an increase in duodenal contents of IgE and IgD antibodies specific to cow's milk and soy protein after pancreozymin. Since this mediator is normally released during digestion, it is suggested that IgE and IgD antibodies specific for food proteins, may be involved in the physiological processing of foods in the intestine. In infants and children with gastrointestinal disease, the incidence of IgE and IgD antibodies specific for milk and soy proteins is higher in basal and pancreozymin-stimulated duodenal fluid when compared with control adults.

INTRODUCTION

Although only IgA and IgM are considered to be the secretory immunoglobulins, plasma cells staining positively for IgG, IgE and IgD are also present in the lamina propria of the alimentary tract (Brandtzaeg & Baklien, 1976a; Savilahti, 1972) and small amounts of these immunoglobulins can be found in gastrointestinal secretions (Savilahti, 1972; Hanson & Brandtzaeg, 1980; Belut, Moneret-Vautrin, Nicholas & Grilliat 1980). Total IgE in the duodenal fluid is increased during inflammatory processes (Hanson & Brandtzaeg, 1980) and in children suffering from food allergy (Belut *et al.*, 1980). The specificity of intestinal IgE and IgD against food antigens has not previously been studied, nor is it known whether the pancreatic secretagogue hormones, pancreozymin-cholecystokinin (PZ-CCK) and secretin, have any effect on IgE and IgD antibody levels in duodenal fluids. Recently we demonstrated the presence of antibodies of the IgG, IgA, and IgM classes in duodenal fluids of

* Visiting Professor from the Shaare-Zedek Medical Centre, Jerusalem, Israel, and recipient of the Buswell Award from the Dean of the School of Medicine, State University of New York at Buffalo.

Correspondence: Dr Emanuel Lebenthal, Division of Gastroenterology, Children's Hospital of Buffalo, 219 Bryant Street, Buffalo, New York 14222, U.S.A.

0019-2805/83/0500-0069$02.00

children (Lebenthal, Clark & Kim, 1980; Lebenthal & Clark, 1981). In addition, we have shown that the antibodies specific against five cow's milk protein changed their levels in response to PZ-CCK and secretin (Shah, Freier, Park, Lee & Lebenthal, 1982). In this specific study we extend our investigation to that of IgE and IgD antibodies specific to four milk proteins and one soy protein in duodenal fluids and measured the effect of PZ-CCK and secretin on their activities.

MATERIALS AND METHODS

Individuals

The pediatric group comprised thirteen patients, aged 4 months to 13·5 years (mean 4·5 years), none of whom had proven milk allergy (Table 1). Duodenal intubation in these children was done as a part of a diagnostic investigation after informed consent was obtained. None was initiated for research purposes. After an overnight fast, duodenal intubation was performed under fluoroscopic control using a double lumen Anderson tube. The tip of the tube was positioned at the ligament of Treitz and the position was checked periodically during the test. Following the collection of the basal sample, PZ-CCK (GIH Research Unit, Chemistry Department, Karolinska Institute, Stockholm and distributed by Kabi Diagnostica, Stockholm, Sweden) was injected slowly, intravenously in a dose of 2 Ivy units/kg body weight and fluid collected in three sequential 10 min samples. Secretin (also from GIH Research Unit) was then injected intravenously in a dose of 2 clinical units/kg body weight and a similar collection was made.

Thirteen adult volunteers who had no evidence of an immuno-deficiency disease, nor recurrent symptoms related to the gastrointestinal tract were also studied. All consumed milk without untoward reactions. The investigation was approved by the Institutional Review Board of the Children's Hospital of Buffalo. An informed consent was obtained from all subjects. After an overnight fast, intubation was performed as described above. In fourteen individuals duodenal fluid was collected for 20 min into flasks

Table 1. Details of patient group

Patients	Age (yrs)	Diagnosis	Weight (percentile)	Height (percentile)	Intestinal histology (atrophy grades)*	IgE antibodies†	IgE antibodies†
MP	4/12	Gastroschisis and surgical short bowel	< 5th	< 5th	III–IV	+	0
AM	2 6/12	S.G.A., Gastroschisis, FTT	< 3rd	< 5th	normal	+	0
RC	12 6/12	Acute vomiting, FTT	< 5th	< 5th	II	0	0
RJ	1 8/12	Chronic diarrhoea, Giardia	< 15th	< 30th	N.D.	+	+
JA	1	Chronic diarrhoea	< 3rd	< 3rd	normal	+	0
NR	1 8/12	Acute diarrhoea	97th	80th	normal	+	N.D.
PB	1	Acute diarrhoea	50th	30th	IV	+	+
SM	1	Chronic diarrhoea	25th	25th	II–III	+	+
RJ	10/12	Abdominal distention	40th	90th	normal	+	+
OR	10/12	Chronic diarrhoea	3rd	3rd	II	+	+
WD	13 6/12	Celiac disease	< 5th	< 5th	III–IV	+	+
JS	9 6/12	Diabetes and celiac disease	50th	50th	IV	0	+
PJ	10	Pancreatic insufficiency, Sweat test normal	10th	25th	normal	+	+

SGA = small for gestational age; FTT = failure to thrive; N.D. = not done; 0 = levels not detectable.

* Grade I—normal; Grade II—moderately abnormal; Grades III & IV—severely abnormal. (Rossi, Lebenthal, Nord & Fazili, 1980.)

† The presence of antibodies referred to here applies to resting and stimulated duodenal fluid.

placed on ice (basal sample). Eight of the volunteers were then tested with PZ-CCK and secretin. Six volunteers received an intravenous injection of PZ-CCK (1 Ivy unit/kg) and six sequential 5 min collections were made. Secretin (1 clinical unit/kg) was then injected intravenously and six serial 5 minute collections of duodenal fluid were obtained. In the other two volunteers, the order of the injections was reversed, secretin being given before PZ-CCK. All specimens were stored at −20°.

Duodenal fluid was assayed for protein according to Lowry, Rosebrough, Farr & Randall (1951). Trypsin and chymotrypsin were determined by the methods of Erlanger, Kowsky & Cohen (1961) and Hummel (1959) respectively. Immunoglobulin E and D antibodies to α-casein, β-lactoglobulin A (BLG-A), α-lactalbumin (ALA), bovine serum albumin (BSA) and soy bean agglutin were estimated by an enzyme-linked immunosorbent assay (ELISA) (Park & Lebenthal, 1981).

Enzyme linked immunosorbent assay (ELISA): Milk α-casein, BLG-A, ALA, BSA and soy bean agglutinin were obtained from Sigma Co., St. Louis, MO. They were dissolved in sodium carbonate buffer (0·1 M Na_2CO_3 and 1 mM $MgCl_2$ pH 9·8) at a concentration of 0·1 mg of protein per 100 ml. Reaction wells of microtitration plate (polystyrene, 96 well, falt-bottomed, Dynatech Labs, Alexandria, Virginia) were filled with 100 μl of the milk or soy protein solutions and control wells with 100 μl of phosphate-buffered saline (PBS pH 7·2). Positive control wells were filled with IgE or IgD standards of known concentrations. The tray was incubated at 37° for 3 hr and then kept overnight at 4°.

The wells with and without adsorbed milk or soy protein were washed three times with 0·2–0·4 ml of PBS-Tween '20' (0·5%) and aspirated to dryness. One hundred microlitres of homogenized, undiluted duodenal fluid were introduced into each well and incubated for 16 hr at 25°. Subsequently the wells were washed with PBS-Tween '20' as before and dried. Peroxidase-conjugated IgG fractions of sheep anti-human IgE (epsilon-chain-specific) and of goat anti-human IgD (delta-chain-specific) (both from Cappel Laboratories, Inc., Cochranville, PA) were prepared in 1:100 dilution. (Preliminary experiments had shown this to be the optimal dilution for our assay procedure). One hundred microlitres of these antibodies were added to each well and incubated at 37° for 2 hr.

The plates were washed and dried as before. A substrate solution was prepared by adding 5 μl of 50% H_2O_2 to 50 ml of PBS (pH 6) containing 40 mg 5-aminosalicylic acid. One hundred microlitres of the substrate solution were added to each well and incubated at 25° for 1 hr. The reaction was stopped by adding 50 μl of IN NaOH to each well. The optical density was read at 488 nm. Preliminary experiments using serial dilution of duodenal fluid were carried out to establish the linearity of the assay. Subsequently, undiluted duodenal fluid samples were used since they were found to lie on the linear portion of the curve. All experiments were run in duplicate.

The rate of change in antibody activity per ml was expressed as $(O.D. \times V \times 10)/T$, where O.D. is the optical density reading of each well, V is the total volume in ml of duodenal fluid collected within a period of T min.

In order to test the sensitivity of the assay, we had access to human IgE standard (obtained from NIAID-NIH/BOB-FDA) containing 2250 ng/ml (900 iu/ml) and to a human serum containing IgD in a concentration of 40 mg/dl (kindly supplied by Dr Douglas Heiner). An ELISA assay was run by coating the wells with the above standards in dilutions of 1:1600 to 1:819200 for IgE and 1:20 to 1:10240 for IgD. The corresponding conjugated antisera were used in duplicate in dilutions of 1:100 and 1:200 for each standard.

Immunological specificity of the ELISA reaction was checked by preabsorption of duodenal fluid with casein. This treatment reduced the optical density to background level. As additional controls, the sera from rat, rabbit and dog were used to substitute for the duodenal fluid in the ELISA procedure. Results showed complete absence of reaction. Since duodenal fluid contains proteases which may lead to antibody degradation during sample storage and antibody assay, we also performed ELISA in the presence and absence of soy bean trypsin inhibitor and/or aprotinin. Results showed no significant difference in the O.D. of the duodenal fluids tested whether the protease inhibitor was present or absent. Subsequent assays were performed in the absence of protease inhibitors.

For statistical evaluation we used the Spearman's coefficient of rank correlation for estimating all correlations, and the χ^2 test for measuring the differences of duodenal antibody concentration between patients and controls.

RESULTS

Using the IgE and IgD standards in serial dilution we

Table 2. Spearman coefficient of rank correlation for antibodies to α-casein (α-cas) and to α-lactalbumin (α-LA) to β-lactoglobulin A (β-LG-A), bovine serum albumin (BSA), and soy bean agglutinin (soy)

		n*	*P* values
IgE	α-cas -- αLA	37	<·01
	α-cas -- β-LG-A	139	<·01
	α-cas -- BSA	36	<·01
	α-cas -- soy	197	<·01
	α-LA -- β-LGA	35	<·01
	α-LA -- BSA	34	<·01
	α-LA -- soy	35	<·01
	β-LGA -- BSA	33	<·01
	β-LGA -- soy	136	<·01
	BSA -- soy	33	<·01
IgD	α-cas -- β-LG-A	133	<·01
	α-cas -- soy	166	<·01
	β-LGA -- soy	98	<·01

*n = number of duodenal fluids studied.

found that the lower limits of detection for our antisera were 0·18 ng/ml for IgE and 39 ng/ml for IgD.

Immunoglobulin E antibody activity against α-casein showed positive correlation with antibody activity against BLG-A, ALA, BSA and soy agglutinin. The same was true for IgD antibodies against α-casein, BLG-A and soy agglutinin. We therefore give results for α-casein only (Table 2).

Of the thirteen children, eight (61%) had IgE antibodies against α-casein in their basal duodenal fluid as compared to only one (8%) of the adult volunteers. This difference is statistically significant ($P < 0{\cdot}01$) (Table 3). Following PZ-CCK the prevalence of IgE and IgD antibodies rose from 8% to 50%. After secretin stimulation only one of ten children (10%) had detectable IgE antibody activity.

Immunoglobulin D antibodies in basal duodenal fluid against α-casein were present in five of thirteen children (38%) but only in one of thirteen adult volunteers (8%). The difference between these two groups was not significant. The administration of PZ-CCK resulted in a rising prevalence of IgD antibodies in the adults followed by a decrease after secretin.

Mean basal IgE and IgD antibody activities in the children were higher than in the adults (Figs 1a, 2a). Following stimulation with PZ-CCK these activities rose four- to five-fold in the children, and even more so in the adults, and returning to basal levels within 30 min (Figs 1a, 2a). Following stimulation by secretin a further drop in mean IgE antibody activity was observed in the children. The profile of antibody activity was similar to the changes in protein content and trypsin and chymotrypsin activity after PZ-CCK in both children and sults (Figs 1, 2).

It should be noted that only one-half of the adult volunteers produced IgE and IgD antibodies following PZ-CCK. The increase in activity in adults, when present, was clearly detected.

DISCUSSION

The ELISA technique is a sensitive method of detection of IgE and IgD specific antibodies to α-casein, BLG-A, ALA, BSA and soy agglutinin. Presence of antibodies to the five antigens correlated well. In resting duodenal fluid, antibodies were absent in all but one of the adults, but present in 61% of the children. This difference is possibly due to the fact that all the children had gastrointestinal disease, as high IgE levels have been described in this clinical situation (Brandtzaeg, 1977). However, the possible influence of age must also be taken into consideration.

Table 3. Prevalence of antibodies to α-casein in duodenal fluids of adult and children group

	IgE						IgD					
	Adult			Children			Adult			Children		
	N	Positive	%	N	Positive	%	N	Positive	%	N	Positive	%
Basal	13	1	8	13	8	61*	13	1	8	13	5	38
PZ-CCK	8	4	50	6	4	66	8	4	50	6	4	66
Secretin	8	2	23	10	1	10	8	1	12·5	13	6	46

* Significantly different from adult group with $P < 0{\cdot}01$

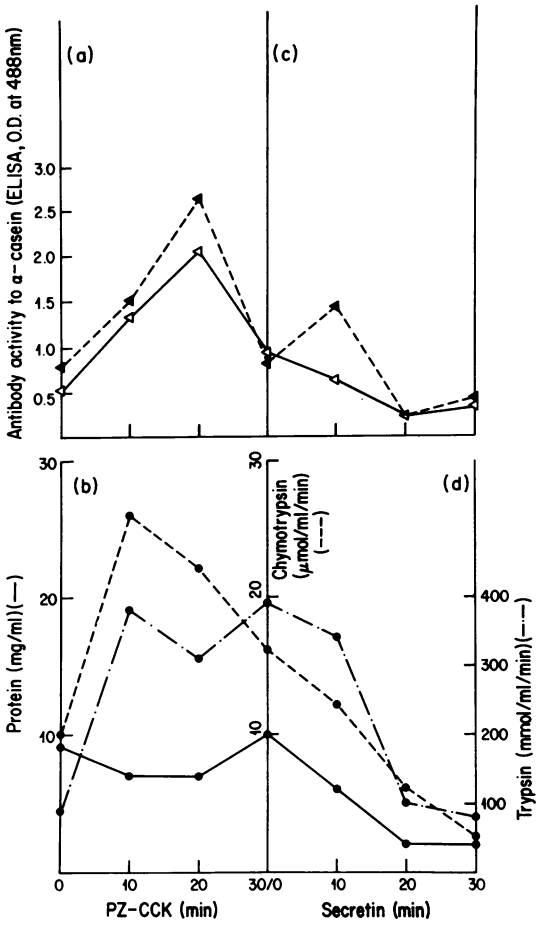

Figure 1. Mean intestinal secretion rate of IgE and IgD antibodies to α-casein in thirteen children following stimulation with PZ-CCK (a) and secretin (c). The corresponding changes in trypsin, chymotrypsin and proteins in the same duodenal fluid are shown in (b) and (d); The child with pancreatic insufficiency was not included in (b) and (d). To enhance visual clarity, statistical variations were not included. Arrows on the x-axis represent the time when PZ-CCK or secretin was administered. (△——△) IgE; (▲——▲) IgD; (●——●) protein; (●- - -●) chymotrypsin; (●—·—●) trypsin.

IgE is known to be present in pancreatic juice (Bresher, Dyck, Hall & Spiekerman, 1975) and PZ-CCK is a potent stimulator of zymogen secretion by the pancreas (Rehfeld, 1980). The administration of PZ-CCK resulted in a marked rise of specific antibody activity in the duodenal fluid concomitant with the rise of the trypsin and chymotrypsin levels. It is possible, therefore, that not only the proteolytic enzymes but also the antibodies which we measured were discharged from the pancreas. Secretin, with its known efect in the selective outpouring of fluid, bicarbonate and electrolytes from the pancreas (Hacki, 1980), presumably dilutes the luminal contents of pancreatic enzymes and IgE and IgD.

In addition, to its effect on the pancreas, PZ-CCK also results in contraction of the gall bladder (Rehfeld, 1980). Thus it is possible that the IgE and IgD antibodies following PZ-CCK may therefore have come from this source. The IgE and IgD content of human biliary fluid has not been reported to date. Another potential source would be the lamina propria. It is believed that during inflammatory processes, locally formed exudates in the lamina propria lead to increased diffusion of IgE and IgD together with IgG into the lumen of the intestine (Hanson & Brandtzaeg, 1980). The rise in IgE and IgD levels in the intestinal lumen following PZ-CCK stimulation raises the possibility that this hormone may have an effect on translocation of these antibodies through the epithelium. As yet, no specific transport mechanisms for IgE and IgD from the lamina propria to the intestinal lumen have been described (Nakejima, Gillespie & Gleich, 1975). Against this mechanism is the fact that neither IgE nor IgD are regarded as true secretory immunoglobulins. IgE and IgD containing plasma cells are found in the lamina propria (Brandtzaeg & Baklien, 1976b), but they do not polymerize in the presence of 'J' chains produced by the same cells (Hanson & Brandtzaeg, 1980) nor do they combine with secretory component (Brandtzaeg, 1977), the glycoprotein which endows IgA with its resistance to proteolysis. In these respects they differ from the true secretory immunoglobulins IgA and IgM.

Immunoglobulin E plasma cell precursor cells are found in rat Peyer's patches (Durkin, Bazin & Waksman, 1981). It is possible, therefore, that like IgA, they become primed with antigen at this site in order, ultimately, to become seeded along all the mucosal surfaces of the body. Indeed, rat Peyer's patches contain cells which stain doubly positive for IgA and IgE (Durkin *et al.*, 1981). These cells therefore may be the source of specific intestinal IgE antibodies.

We have recently described the increase in IgA and IgM antibodies specific against milk proteins following PZ-CCK stimulation (Shah, Freier, Park, Lee & Lebenthal, 1982). As this hormone is believed to be released during normal digestion, the antibodies which appear as a result of this hormonal stimulation

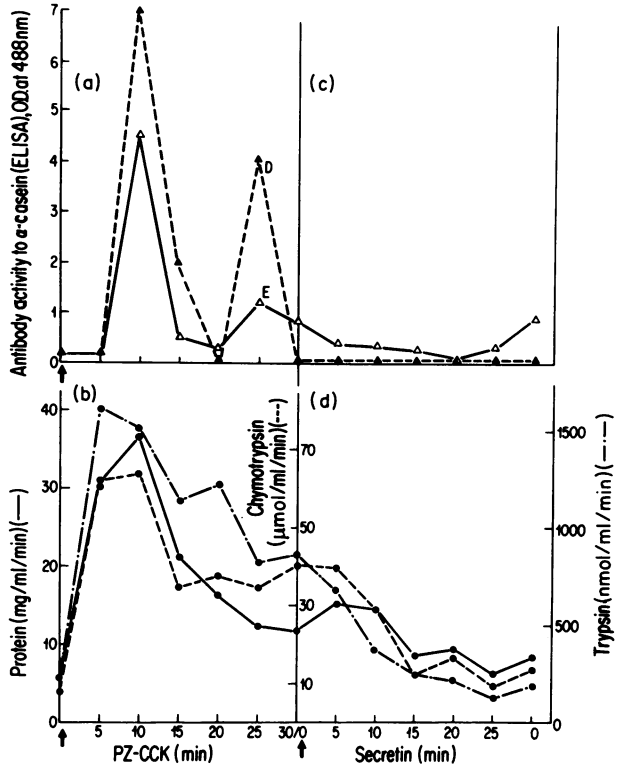

Figure 2. Mean intestinal secretion rate of IgE and IgD antibodies to α-casein in eight adult volunteers following stimulation with PZ-CCK (a) and secretin (c). The corresponding changes in trypsin, chymotrypsin and proteins in these duodenal fluids are shown in (b) and (d). To enhance visual clarity, statistical variations have been omitted. All other symbols are similar to those in Fig. 1.

may have a physiological role in the processing of food proteins. In the human gastrointestinal tract under physiological conditions IgE lacks a demonstrable function. In view of the known effects of IgE, it is possible that it induces localized histamine release as a result of mast-cell degranulation. This would enlarge the adjacent intestinal vascular bed and increase vascular permeability. Such a mechanism, if it exists, may also be operative to a greater degree in pathological states as the duodenal fluid content of total IgE in food allergy has been found to be raised (Belut *et al.*, 1980).

In the animal model, intestinal reaginic antibodies equivalent to human IgE have been studied more intensively than in the human. In the rat, reaginic antibodies to egg albumin have been found to correlate with gastrointestinal hypersensitivity to this protein (Bloch, Lake, Sinclair & Walker, 1981). The local hypersensitivity response in the intestine is accompanied by enhanced mucus secretion and by an increase in vascular permeability (Bloch *et al.*, 1981). Mucus may then protect the mucosal surface against penetration by microorganisms and by toxins, and limits the access of soluble antigens to the absorptive surface of intestinal cells.

In conclusion, we have found IgE and IgD antibodies in resting duodenal fluid of children with gastrointestinal disease. PZ-CCK enhances antibody activity in children with gastointestinal disease and in healthy adults. We speculate that these antibodies may play an active role in normal digestive processes, possibly by enlarging the local vascular bed and by inducing mucus release.

ACKNOWLEDGMENTS

This study was supported in part by a Buswell Grant,

USPH grants DE-005505 and AG-02417 and by National Dairy Council grant-in-aid.

REFERENCES

BELUT D., MONERET-VAUTRIN D.A., NICHOLAS J.P. & GRILLIAT J.P. (1980) IgE levels in intestinal juice. *Dig. Dis. Sci.* **25,** 323.

BLOCH K.J., LAKE A.M., SINCLAIR K. & WALKER A.W. (1981) IgE mediated alterations in intestinal permeability. In: *The Mucosal Immune System in Health and Disease. Proc. Eight-First Ross Conference of Pediatric Research* (eds P. L. Ogra and J. Bienenstock), p. 273 *et. seq.* Ross Laboratories, Columbus, Ohio.

BRANDTZAEG P. (1977) Human secretory component. VI. Immunoglobulin binding properties. *Immunochemistry*, **14,** 179.

BRANDTZAEG P. & BAKLIEN K. (1976a) Immunohistochemical studies of the formation and epithelial transport of immunoglobulins in normal and diseased human intestinal mucosa. *Scan. J. Gastroenterol.* suppl 36, 1045.

BRANDTZAEG P. & BAKLIEN K. (1976b) Inconclusive immunohistochemistry of human IgE in mucosal pathology. *Lancet*, **1,** 1297.

BRESHER G.W., DYCK W.P., HALL F.F. & SPIEKERMAN A.M. (1975) Immunoglobulin characterization in human pancreatic fluid. *Am. J. dig. Dis.* **20,** 454.

DURKIN H.G., BAZIN H. & WAKSMAN B.H. (1981) Origin and fate of IgE-bearing lymphocytes. I. Peyer's patches as differentiation site of cells simultaneously bearing IgA and IgE. *J. exp. Med.* **154,** 640.

ERLANGER B.F., KOWSKY N. & COHEN W. (1961) The preparation and properties of two chromogenic substrates of trypsin. *Archs Biochem.* **95,** 271.

HACKI W.H. (1980) Secretin. *Clin. Gastroenterol.* **9,** 609.

HANSON L. & BRANDTZAEG P. (1980) The mucosal defense system. In: *Immunologic Disorders In Infants and Children* (E. R. Stiehm and V. A. Fulginiti), p. 137 *et seq.* W. B. Saunders and Co., Pennsylvania.

HUMMEL B.C.W. (1959) Modified spectrophotometric determinations of chymotrypsin, trypsin and thrombin. *Can. J. Biochem. Physiol.* **37,** 1393.

LEBENTHAL E. & CLARK B. (1981) Immunoglobulin concentrations in duodenal fluids in infants and children. II. The effect of pancreozymin and secretin. *Am. J. Gastroenterol.* **75,** 436.

LEBENTHAL E., CLARK B. & KIM O. (1980) Immunoglobulin concentrations in duodenal fluid of infants and children. *Am. J. Dis. Child.* **134,** 834.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J. (1951) Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265.

NAKEJIMA S., GILLESPIE D.N. & GLEICH A.J. (1975) Differences between IgA and IgE as secretory proteins. *Clin. exp. Immunol.* **21,** 306.

PARK B.H. & LEBENTHAL E. (1981) Age related changes in the levels of antibodies to cow's milk proteins as measured by enzyme linked immunosorbent assay (ELISA). *IRCS Medical Science*, **9,** 866.

REHFELD J.F. (1980) Cholecystokinin. *Clin. Gastroenterol.* **9,** 595.

ROSSI T.M., LEBENTHAL E., NORD K.S. & FAZILI, R.R. (1980) Extent and duration of small intestinal mucosal injury in intractable diarrhea of infancy. *Pediatrics*, **66,** 730.

SAVILAHTI E. (1972) Immunoglobulin-containing cells in the intestinal mucosa and immunoglobulins in the intestinal juice in children. *Clin. Exp. Immunol.* **11,** 415.

SHAH P.C., FREIER S., PARK B.H., LEE P.C. & LEBENTHAL E. (1982) Detection of antibodies to cow's milk protein in duodenal fluid. *Gastroenterology*, **83(4),** 916.