An In Vitro Model of Gluten-Sensitive Enteropathy

EFFECT OF GLIADIN ON INTESTINAL EPITHELIAL CELLS OF PATIENTS WITH GLUTEN-SENSITIVE ENTEROPATHY IN ORGAN CULTURE

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ABSTRACT Jejunal biopsy specimens from patients with gluten-sensitive enteropathy (GSE) (obtained during gluten challenge) as well as from normal individuals and patients with other gastrointestinal abnormalities were cultured in vitro for 48 h in the presence or absence of a peptic-tryptic digest (P-T digest) of gliadin.

In the absence of gliadin the alkaline phosphatase activity in the biopsy specimens obtained from normal control individuals increased from an initial value of 384± 83 U to a 48 h value of 561±151 U (mean±SD) (difference significant at $P < 0.01$). The initial alkaline phosphatase activity of specimens obtained from patients with GSE was strikingly lower than that of normals, 117±79 U, and increased to a 48 h value of 399± 203 U (difference significant at $P < 0.01$). The biochemical change in cultured biopsy specimens of GSE patients correlated with increases in the length and regularity of brush borders of epithelial cells as seen with the electron microscope.

In the presence of a P-T digest of gliadin, the alkaline phosphatase activity of biopsy specimens of control individuals increased from an initial value of 384±83 U to a 48 h value of 578±156 U. In contrast, the alkaline phosphatase activity of biopsy specimens of patients with GSE in exacerbation showed a markedly diminished increase in activity during 48 h of culture; in this case the initial activity was 117±79 U and the final activity was 203±93 U. This inhibitory effect on increase of alkaline phosphatase activity during organ culture was specific in that a P-T digest of casein (a protein not toxic in vivo to patients with GSE) had no effect on alkaline phosphatase increases in culture. Finally, these results obtained with biopsy specimens taken from patients with GSE in exacerbation were compared with results obtained from patients with GSE in remission. Alkaline phosphatase activity of specimens obtained from the latter group of patients also increased during culture but in this instance P-T digest of gliadin in the culture medium had no significant inhibitory effect.

In conclusion, the inhibitory effect of gliadin on intestinal epithelial cells in organ culture represents an in vitro model of gluten-sensitive enteropathy. Inasmuch as this effect of gliadin is not seen in cultures of specimens taken from patients in remission, it appears that gliadin is not directly toxic to GSE jejunal mucosa per se, but rather toxicity requires the participation of an endogenous effector mechanism which must first be stimulated in vivo.

INTRODUCTION

Gluten-sensitive enteropathy (GSE)[1] is a disease of uncertain etiology. It has been postulated that the disease is due to the lack of an intestinal enzyme, allowing gliadin or degradation products of gliadin to accumulate in the intestinal lumen or epithelial cell and produce direct cellular damage (1). Alternatively, it has been suggested that the disease is primarily due to an immuno-

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[1] *Abbreviations used in this paper:* GSE, gluten-sensitive enteropathy; P-T digest, peptic-tryptic digest.

logic dysfunction of the intestinal mucosa and gliadin plays the role of an antigen which evokes an injurious local immune response (2). We have previously obtained data which support the second hypothesis. Thus, we have shown that in patients with GSE, but not in normal individuals, gluten challenge induces an increase in the production of immunoglobulins within the gastrointestinal mucosa (3). Furthermore, a major part of the immunoglobulin increase is composed of anti-gliadin antibodies (4). These studies establish that immunologic events occur in patients with GSE at the site of tissue injury but do not provide information as to whether this mechanism is a primary or secondary factor in the pathogenesis of disease or even if these events are related at all to the tissue injury seen.

Further analysis of the pathologic process responsible for gluten-sensitive enteropathy clearly awaits development of in vitro systems which will allow one to control the various possible pathogenetic elements. In this paper we describe the behavior of intestinal biopsy specimens obtained from patients with GSE, normal individuals, and patients with other diseases of the gastrointestinal tract, when placed into organ culture with and without gliadin in the culture medium. We demonstrate that jejunal epithelial cells from normal controls and patients with GSE display increased activities of alkaline phosphatase (and other brush border enzymes) and improve in ultrastructural appearance when maintained in organ culture free of gliadin. In contrast, jejunal epithelial cells from patients with GSE do not show increases of alkaline phosphatase activity when cultured in the presence of gliadin. The inhibition of rise of enzyme activity in organ culture by gliadin represents an in vitro model of gluten-sensitive enteropathy. Using this model we have studied the in vitro behavior of biopsy specimens obtained from patients with GSE in exacerbation and obtained from patients with GSE in remission. In these studies we have obtained evidence that gluten is not directly toxic to the epithelial cells of patients with GSE, but must first activate an endogenous mechanism in vivo to produce a toxic effect.

TABLE I

Change in Alkaline Phosphatase Activity in Intestinal Biopsy Specimens in Organ Culture with Time*

Patient	Hour			
	0	24	48	72
Normal				
I. J.	422	501	528	452
R. W.	306	520	637	539
A. M.	369	501	576	468
C. G.	353	442	449	680
M. S.	305	361	654	588
W. G.	278	590	714	410
GSE				
C. G.	47	266	483	320
C. G.	47	639	710	531
B. N.	61	234	346	223
F. F.	163	885	1,239	1,130

* 1 unit = 1 μmol of PNP liberated per gram protein per minute. Each point is the mean of duplicate cultures.

METHODS

General outline of study. Intestinal (jejunal) biopsy specimens were obtained from various patient groups and controls. These specimens were placed into organ culture (in duplicate) for periods ranging from 24 to 72 h. Initially (at zero time) and during the course of organ culture, the specimens were examined ultrastructurally as well as by assay of disaccharidase and alkaline phosphatase activities. The biopsy specimens in organ culture were studied in the presence or absence of a peptic-tryptic digest (P-T digest) of gliadin, a purified fraction of gliadin (α-gliadin), or a P-T digest of casein. Using this system biopsy specimens obtained from GSE patients after prolonged periods on a gluten-free diet, biopsy specimens obtained from GSE patients after a period of in vivo gluten challenge, and, lastly, biopsy specimens obtained from control patients were studied.

Patients. 11 patients with GSE (ages 9–68 yr) were studied. The diagnosis of GSE was made in each case by a positive history of malabsorption syndrome, documented malabsorption of fat and D-xylose, clinical response to gluten restriction, a characteristic rise of IgA and IgM synthesis by jejunal biopsy specimens after oral challenge with gluten (3, 4), and a typical histologic picture of flattening of the intestinal villi and infiltration of the submucosa by plasma cells and lymphocytes. Eight patients were biopsied during remission and again during exacerbation after 7–14 days on an unrestricted (gluten-containing) diet. Two patients were studied only during remission and one patient only during exacerbation. Disease activity was defined using clinical and laboratory criteria. 10 patients were defined as patients with GSE in remission if, after a prolonged period on a gluten-free diet, they had minimal or no symptoms. 8 of 10 patients so defined had normal fecal fat, serum carotene, or D-xylose tolerance tests. The remaining two patients had fecal fat excretion values greater than 5 g/day which, however, increased markedly after gluten challenge. Patients were defined as patients with GSE in exacerbation if they had been on a gluten-containing diet and, as a result, had shown deterioration of at least one test of absorptive function (D-xylose absorption, 72-h stool fat excretion, or serum carotene); most of these patients also developed gastrointestinal symptoms. Histologic abnormalities were noted in both patients with GSE in remission and patients with GSE in exacerbation and were not used in grouping the patients.

Two groups of control individuals were used. One "normal" control group consisted of 11 patients without malabsorption or abnormalities of jejunal histology. Three were normal volunteers, one had myotonia dystrophica, one had diabetes mellitus, one had Milroy's disease, two had dysgammaglobulinemia, two had irritable bowel syndrome, and one had healed Whipple's disease. Eight of these patients were biopsied once while on an unrestricted (gluten-con-

TABLE II

*Effect of Puromycin on Brush Border Enzyme Activity during Organ Culture**

	Sucrase‡			Lactase‡			Trehalase‡			Alkaline phosphatase§		
	0 time	48 h	48 h + puromycin	0 time	48 h	48 h + puromycin	0 time	48 h	48 h + puromycin	0 time	48 h	48 h + puromycin
N. G. (GSE)	10	14	8.4	0	0	0	1.0	3.8	0	108	498	143
A. N. (normal)	52	58	49	26	25	20	14	20	11	183	269	155

* Concentration of puromycin in culture medium: 10^{-3} M.

‡ Disaccharidase units = micromoles glucose liberated per gram protein per minute.

§ Alkaline phosphatase units = micromoles PNP liberated per gram protein per minute.

taining) diet. Three were biopsied twice, once after a 3-wk period on a gluten-free diet and once after a 2-wk period on a gluten-containing diet.

The second or "disease" control group consisted of three individuals with malabsorption and histologic abnormalities of the jejunal mucosa (not gluten-related). This group was made up of one female patient with abetalipoproteinemia, one male patient with adult-onset agammaglobulinemia, and one female patient with radiation-induced gastroenteropathy. The jejunal epithelium of the patient with abetalipoproteinemia showed the characteristic vacuolization of the epithelial cells associated with normal villous architecture, whereas the two other patients had marked villous atrophy. This control group was utilized to ensure that a preexistent abnormal state of the jejunum does not account in a nonspecific way for the findings in patients with GSE.

The nature of the study was explained to each participant and informed consent was obtained in each case.

Clinical studies. Fecal fat excretion was evaluated by collecting a 72-h stool sample from patients on a 100 g fat diet and measuring stool fat according to the method of van der Kamer et al. (5). D-Xylose absorption was evaluated by administering 25 g of the pentose and subsequent measurement of xylose by the method of Roe and Rice (6) in a 5-h urine collection and a 2-h blood specimen. Serum carotene was determined by extraction of serum with alcohol and petroleum ether (7).

Intestinal biopsy. Intestinal biopsies were obtained with a four hole Rubin tube biopsy instrument positioned fluoroscopically at the ligament of Treitz (8). An average of three specimens was obtained with each biopsy. The biopsy specimens were placed immediately into Krebs-Ringer bicarbonate buffer (pH 7.3) (4°C), rinsed three times in the same buffer, and cut into small pieces, each approximately 1–2 mm in diameter. Several of the resulting bits of tissue were processed as described below to obtain zero time values for enzyme activity and for study of morphology; the remaining bits of tissue were placed into organ culture.

Preparation of proteins. Peptic-tryptic digests of gliadin and casein were prepared according to the method of Frazer et al. (9). Gliadin treated in this manner has been shown by Frazer et al. to cause exacerbation of jejunal disease in GSE patients in vivo. A water-soluble α-gliadin was prepared according to the method of Bernardin et al. (10).

Organ culture. Organ cultures were performed using a modification of the methods of Browning and Trier (11). Cultures were carried out in plastic culture dishes (Falcon Plastics, Los Angeles, Calif.) with central wells containing suspended steel grids. Biopsy tissue was placed on the grids (villous side up) and the wells were filled with media, allowing the media to bathe the tissue by capillary action. Medium consisted of RPMI tissue culture medium 1640 enriched with 10% fetal calf serum (previously heated to 56°C for 1 h), insulin (0.5 mg/100 ml medium), penicillin (6.2 mg/100 ml), streptomycin (13.5 mg/100 ml), neomycin (7.3 mg/100 ml), glutamine (30 mg/100 ml), and glucose (366 mg/100 ml). The medium was sterilized before use by Millipore filtration using a 0.45-μm filter. The outside ring of the culture dishes was lined with absorbent paper saturated with sterile Dulbecco's solution. The culture dishes were placed into a closed plexiglass box which was then thoroughly gassed with a 95% O_2–5% CO_2 gas mixture. Incubation was carried out at 37°C for 24, 48, or 72 h. At the end of the incubation period the tissue was removed, rinsed in cold 0.1 M NaCl (4°C), and either processed for electron microscopy as described below or homogenized in 1 ml of 0.85% NaCl solution using a glass homogenizer. The homogenates were frozen with dry ice-acetone, stored at −25°C, and assayed within 5 days for enzyme activity. Aliquots of homogenate were also taken for protein determinations which were performed using a modification of the method of Lowry et al. (12). On the average, 0.1 mg of tissue protein was present in each culture dish.

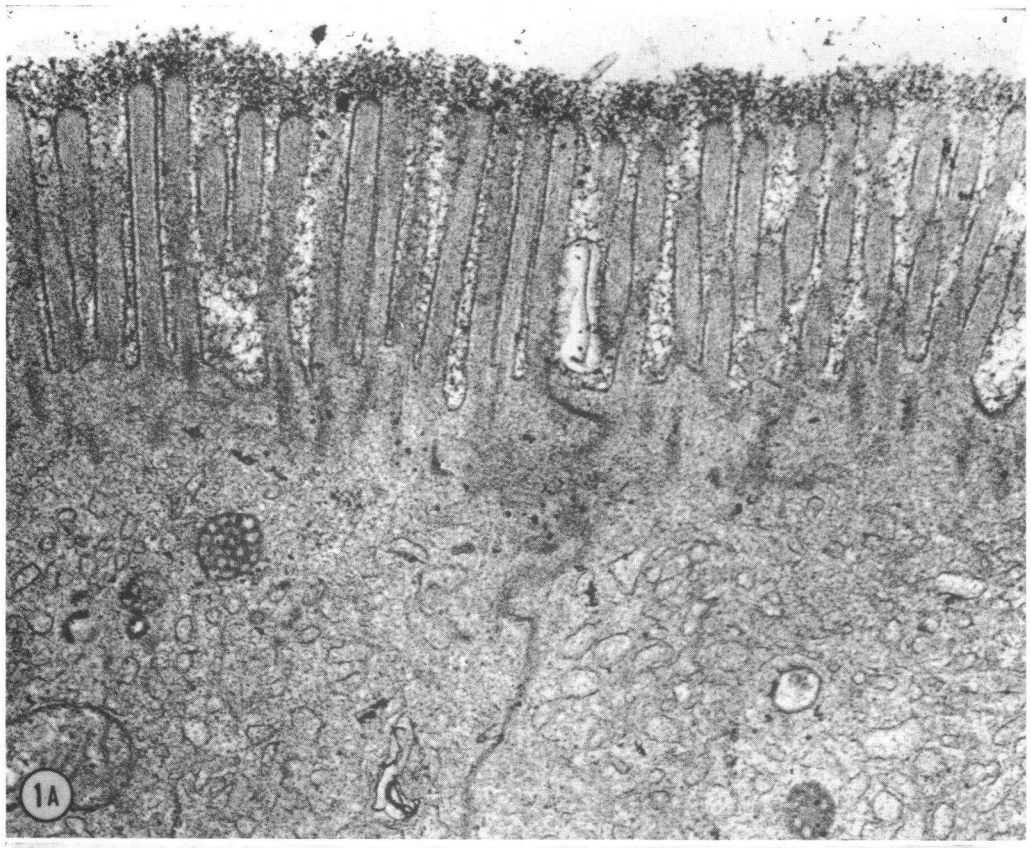
Electron microscopy. Specimens were fixed for electron microscopy immediately after being obtained or after a

TABLE III

Total Alkaline Phosphatase Activity in Intestinal Biopsy Specimens in Patients with GSE in Exacerbation

	Activity*		
Patient	0 h	48 h	48 h + gliadin
F. F.	49	58	24
L. H.	19	56	23
M. N.	26	83	24

* 1 unit = 1 μmol PNP liberated per total protein per minute. Each point is the mean of three separate studies, each done in duplicate, thus minimizing random variation in biopsy specimen size.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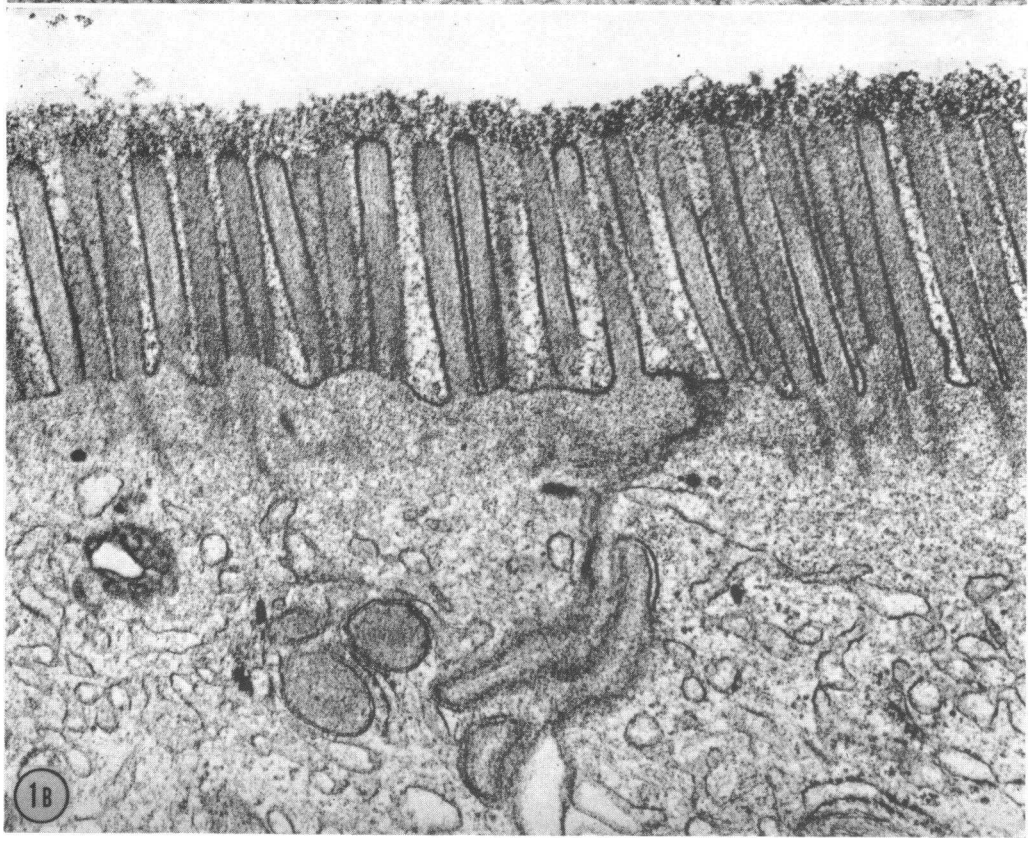
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period of time in organ culture. The tissue was placed in cold (4°C) 1.25% glutaraldehyde solution in a Michaelis buffer (pH 7.2). It was then postfixed in 1% OsO_4 in the same buffer, dehydrated in ethanol, stained *en bloc* with 5% uranyl acetate in alcohol, and embedded in Epon-Araldite mixture. The diamond knife ultrathin sections were stained with 1% silicotungstate and electron microscopy was performed at 3,000 × to 100,000 × initial magnification. Magnification of the transmission electron microscope was calibrated with a carbon diffraction grating replica. All electron micrographs were prepared by Dr. A. J. Tousimis of the Biodynamics Research Corporation, Rockville, Md. The specimens were examined by Dr. Tousimis without prior knowledge of the source of the biopsy specimens.

Enzyme assays. Biopsy homogenates were assayed for sucrase, lactase, and trehalase activity by a modification of the method of Dahlqvist (13). Substrates (sucrose, lactose, and trehalose [Sigma Chemical Co., St. Louis, Mo.]) were prepared at a concentration of 0.056 M in 0.1 M maleate buffer, pH 6.0. 0.1 ml of uncentrifuged, dilute, or undiluted intestinal homogenate was incubated with 0.1 ml of substrate at 37°C in a shaking water bath for 1–3 h. The reaction was stopped by placing the tubes into a boiling water bath for 2 min. The liberated glucose was measured by the glucose oxidase method (13), using appropriate dilutions of glucose to obtain a standard curve. The values obtained using boiled homogenates served as blanks and were subtracted from values obtained using unboiled homogenates. A unit of disaccharidase activity represented 1 μmole of glucose liberated per gram tissue protein per minute.

Alkaline phosphatase was assayed according to the method of Bessey, Lowry, and Brock (14). The standard reaction mixture contained 50 μl of diluted or undiluted homogenate and 200 μl of 0.15 mM *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) in glycine buffer (0.0625 M, pH 10.3). The glycine buffer contained magnesium chloride (0.625 mM) and zinc acetate (0.125 mM). After 10–30 min incubation at 37°C in a shaking water bath, the reaction was stopped by the addition of 1.25 ml of 0.02 N NaOH. The values obtained using homogenates first inactivated with 0.02 N NaOH and incubated at 37°C for the same time were subtracted from the values obtained using active homogenate. A unit of phosphatase activity represented 1 μmole of *p*-nitrophenol liberated per minute per gram tissue protein.

In both disaccharidase and alkaline phosphatase assays specimens were measured at dilutions which provided values falling on linear portions of the standard curves.

Statistical analysis. Statistical analysis of data was done with Student's *t* test and the paired *t* test (15).

RESULTS

Electron microscopy of biopsy specimens obtained from normal individuals. Examination of specimens obtained from normal individuals cultured for 24 and 48 h (Fig. 1) showed that the epithelial cells of the specimens maintained their integrity and orientation, and that the brush border and glycocalyx were well preserved. After 48 h of culture, mitochondrial swelling as well as separation of the epithelial cells from the lamina propria was noted. In addition, occasional nests of necrotic cells were visible. After 72 h of organ culture there was evidence of more widespread cellular necrosis.

TABLE IV

Ratio of Alkaline Phosphatase Activity to Sucrase Activity in Biopsy Specimens from Patients with Active GSE

Patient	Ratios	
	At zero time (just after biopsy)	After 48 h in organ culture
S. B.	5.2	9.1
F. F.	9.7	22
C. C.	8.8	29
G. K.	9.4	10.6
M. N.*	34	37.3
	5.7	16.5
B. N.	6.1	104
N. G.	18.4	99.6

* Patient M. N. studies on two occasions.

Electron microscopy of biopsies obtained from patients with gluten-sensitive enteropathy. Specimens from three patients with gluten-sensitive enteropathy were examined with electron microscopy. Specimens fixed immediately after biopsy were characteristic in all three: the epithelial cell brush borders were irregular and shortened (measuring an average of 0.4–0.5 nm in length as compared to 1.0 nm in normal individuals). In addition, the glycocalyces were nearly absent and mitochondria were in varying states of degeneration; finally, numerous lysosomes and lipid lakes were seen within the epithelial cells. After 24 h of culture, specimens obtained from two patients (M. N. and F. F.) revealed several dramatic changes. In these cases, the epithelial cell brush borders were more regular and longer than those of zero time specimens (measuring an average of 0.9 nm in length) (Figs. 2 and 3). Intracellular lipid lakes, previously noted, were small or absent, and the terminal bar areas and golgi zones showed more normal organization. Mitochondrial abnormalities as well as cuboidal cell shapes persisted. In the cultured biopsy specimens of a third patient (C. C.), microvilli were morphologically unchanged during the 24 h of culture, remaining 0.6 nm in length; however, some improvement in subcellular architecture

FIGURE 1 Electron micrograph of an epithelial cell of a normal control individual (K. D.). (A) Brush border region examined just after intestinal biopsy; (B) brush border region after 24 h in organ culture. × 30,000. Brush border morphology is unchanged during the period of organ culture.

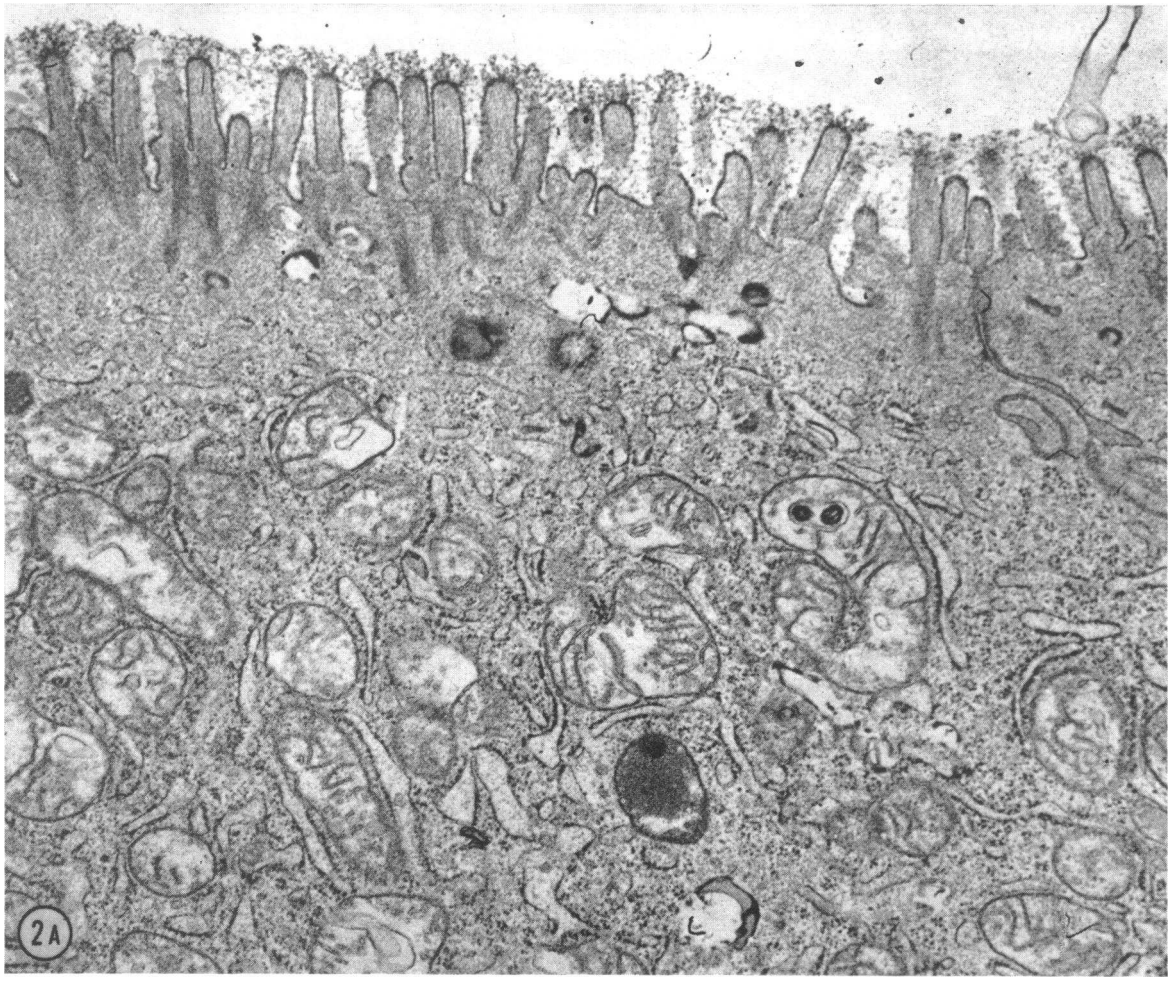

FIGURE 2A Electron micrograph of epithelial cell obtained from patient with gluten-sensitive enteropathy (M. N.). Brush border region of epithelial cell just after intestinal biopsy.

was evidenced by reduced penetration of the terminal bar area by endoplasmic reticulum.

Intestinal alkaline phosphatase activities during organ culture. Alkaline phosphatase activities of biopsy specimens changed significantly during the course of organ culture. Initial studies were undertaken to demonstrate the time course of these changes. As shown in Table I, alkaline phosphatase activity normalized for protein in biopsy specimens underwent an increase which was apparent at 24 h of culture, peaked at 48 h of culture, and was declining at 72 h of culture. Because the peak increase was seen at 48 h, most studies were performed at this time interval.

In specimens obtained from normal control individuals (11 studies in 11 individuals) alkaline phosphatase activity increased from a mean initial (zero time) value of 384±83 U[2] to a mean 48 h value of 561±151 U (mean±1 SD) (Fig. 4, left panel). This increase was statistically significant ($P < 0.01$) and represented a fractional increase of 46%. In specimens obtained from patients with GSE (15 studies in nine patients), alkaline phosphatase activities were initially much lower than those of specimens from normal controls and increased during culture to levels normally found in normal specimens at zero time. In this case, the enzyme value increased from a mean initial value of 117±79 U to a mean 48 h value of 399±203 U (Fig. 4, middle panel). This increase was again statistically significant at $P <$ 0.01 and represented a fractional increase of 241%. The fractional increase in the alkaline phosphatase value in

[2] Units (U) = micromoles of *p*-nitrophenol liberated per minute per gram tissue.

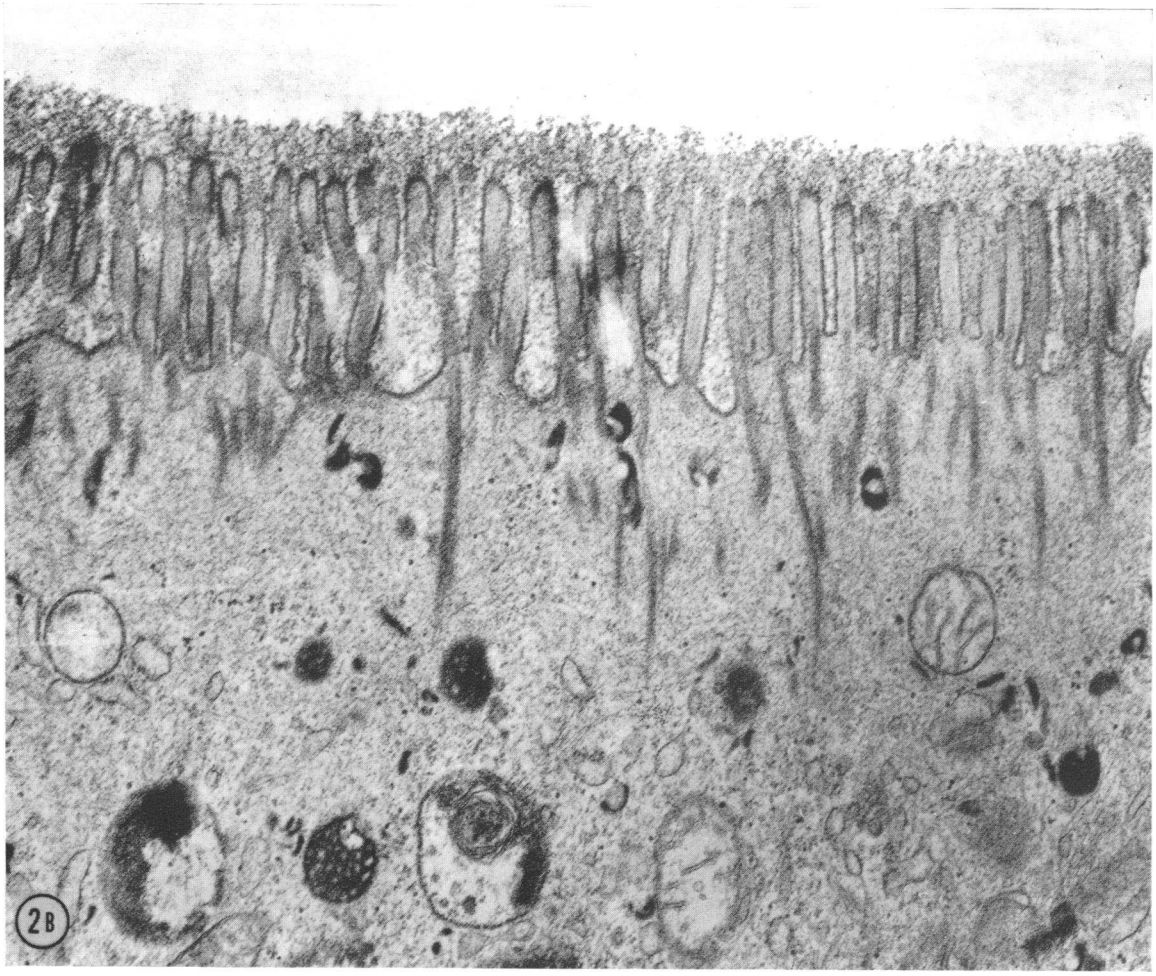

FIGURE 2B Brush border region of epithelial cell after culture for 24 h. The length and regularity of microvilli are increased after 24 h in culture, and, in addition, the glycocalyx is wider. × 30,000.

GSE specimens is significantly greater than that of the control group at the $P < 0.01$ level, whereas the absolute increase is numerically greater for the GSE specimens but not to a level that achieves statistical significance.

Nature of alkaline phosphatase activity increases during organ culture. The alkaline phosphatase activities reported above are normalized for tissue protein. Thus, the increase in alkaline phosphatase activity noted during culture could result factitiously from a general decrease in tissue protein during the period of culture. To rule this out, the following studies were done. (*a*) In two experiments, one involving a normal control individual and another involving a patient with gluten-sensitive enteropathy, cultures were set up in the presence of puromycin (10^{-3} M). Puromycin at this concentration is regularly associated with the inhibition of new protein synthesis in vitro (16). In each case the presence of puromycin substantially inhibited the increase in alkaline phosphatase activity (Table II) suggesting that the rise in alkaline phosphatase activity is at least in part due to new protein synthesis. (*b*) In three patients in whom studies were done on three separate occasions, mean values for total alkaline phosphatase content of biopsy specimens were determined (Table III); in each case, total alkaline phosphatase content increased during the period of culture, again supporting the contention that the increase in enzyme activity was due to protein synthesis. (*c*) Disaccharidase enzymes were measured independently (see below); a decrease in tissue protein would affect these enzymes in a fashion similar to the effect on alkaline phosphatase so that the ratios of these enzymes at the start and end of culture would remain the same if the effects seen were explainable only on the basis of a general loss in tissue protein; as shown in Table IV, this did not prove to be the case and instead

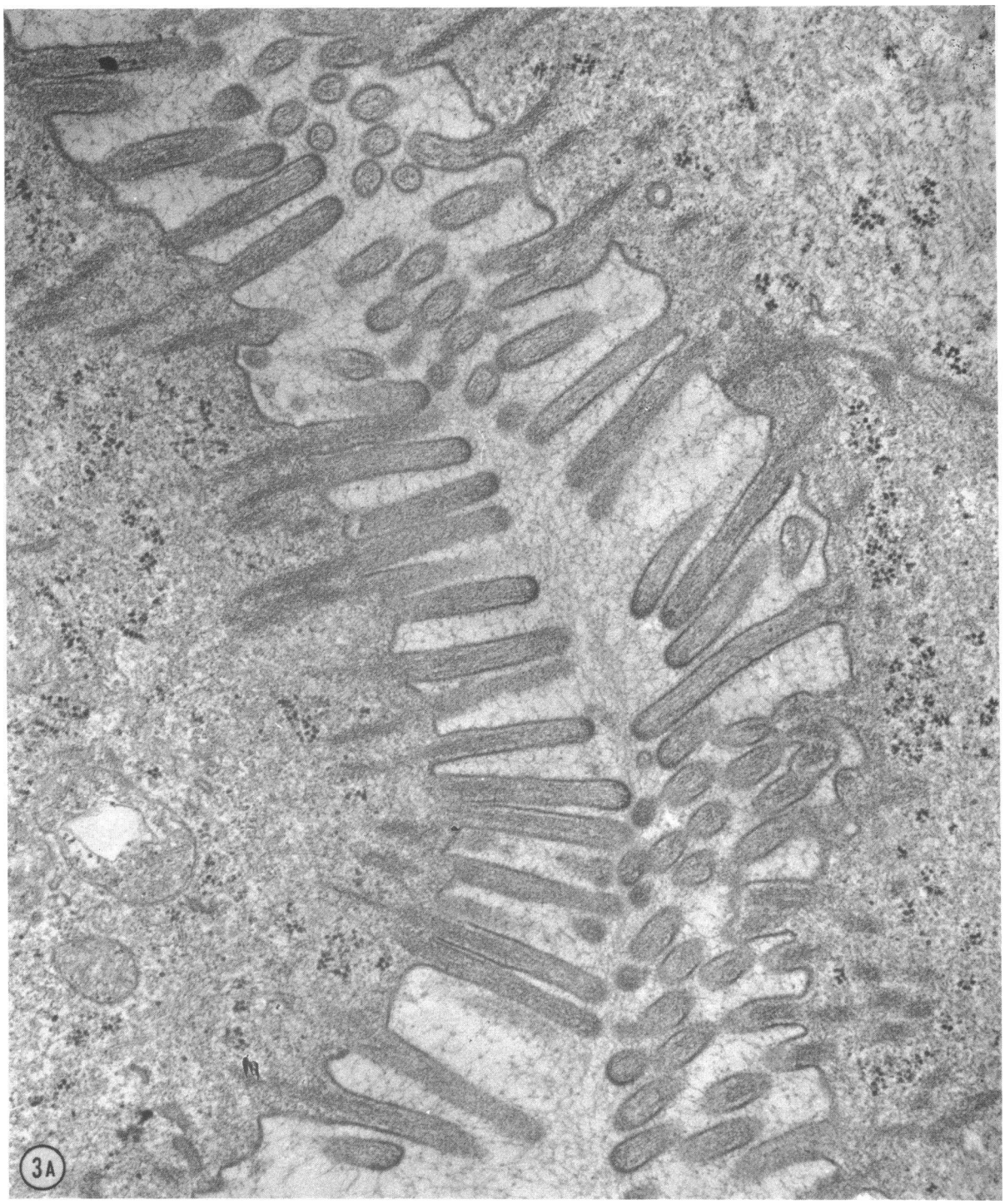

FIGURE 3A Electron micrographs of epithelial cell from patient with gluten-sensitive enteropathy (F. F.). Brush border region of specimen taken just after intestinal biopsy.

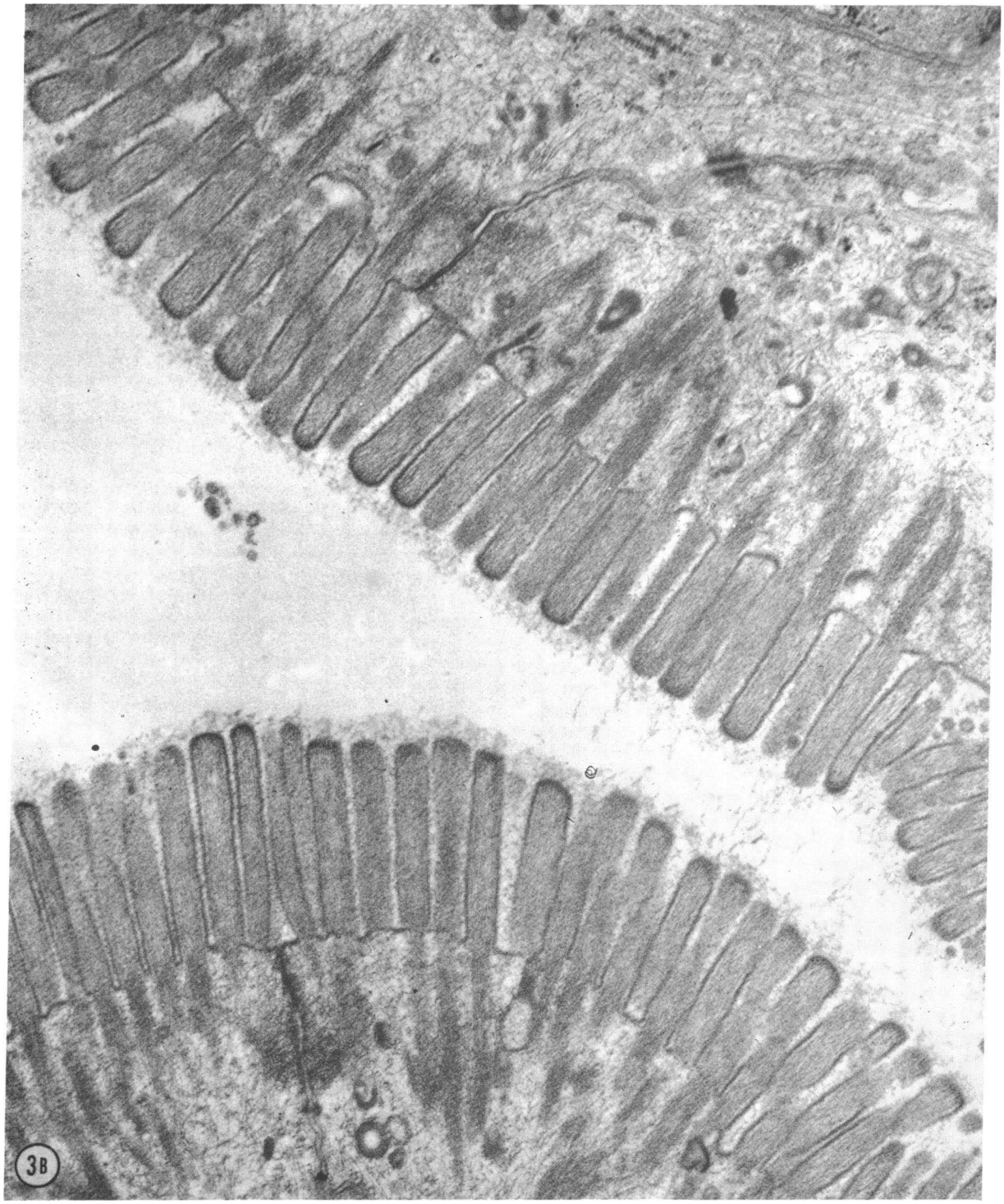

FIGURE 3B Brush border region of cell after 24 h in organ culture. × 28,000. After 24 h in culture the average length of the microvilli is increased and, in addition, the number and regularity of the microvilli are improved.

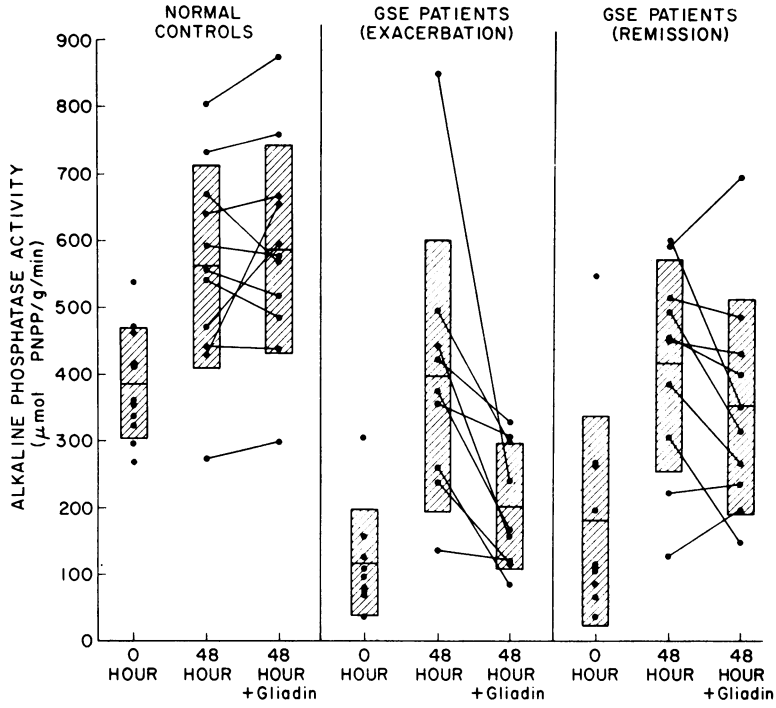

FIGURE 4 Alkaline phosphatase activity during jejunal tissue culture of biopsies from normal controls ($n = 11$) (left panel), patients with GSE in exacerbation ($n = 9$) (middle panel), and patients with GSE in remission ($n = 10$) (right panel). In exacerbation, six of nine data points represent the mean of two separate studies. In remission, three of ten data points represent the mean of three separate studies. The shaded areas represent the mean±1 SD. Lines connect parallel 48-h cultures with or without the presence of a P-T digest of gliadin.

the ratio of alkaline phosphatase to sucrase increased during the culture period.

In vitro effect of gliadin: normal controls and patients with GSE in exacerbation. As noted above, in the absence of gliadin, alkaline phosphatase activity in biopsy specimens obtained from normal individuals increased from an initial value of 384±83 U to a 48 h value of 561±151 U, a 46% increase. Addition of the P-T digest of gliadin (1.2 mg/ml) to parallel cultures had no effect on the increase in alkaline phosphatase activity of cultured tissue (48-h activity was 578±156 U) (Fig. 4, left panel).

TABLE V

Reproducibility of Alkaline Phosphatase Changes in Jejunal Biopsy Specimens of Three Patients with GSE

Patient	Alkaline phosphatase activity		
	0 h	48 h	48 h + gliadin
F. F.	183	325	150
	29	243	29
	24	219	81
L. H.	60	312	112
	112	299	168
	54	107	73
M. N.	78	362	129
	87	361	264
	47	403	81

* 1 unit = 1 μmol PNP liberated per gram protein per minute. Each point is the mean of duplicate cultures. All patients were studied after gluten challenge.

In similar studies of cultured jejunal tissue from patients with GSE in exacerbation (15 studies in nine patients), alkaline phosphatase activity also increased during 48-h culture. In this case, however, P-T digest of gliadin inhibited the alkaline phosphatase increase. In the absence of the P-T digest of gliadin, enzyme activity increased 241% from an initial value of 117± U to a 48 h value of 399±203 U (difference significant at $P < 0.01$), whereas in the presence of the P-T digest of gliadin, the 48 h mean value increased only 73.5% to 203±93 U (Fig. 4, middle panel). The activity after 48 h culture with gliadin was not significantly different from the initial activity, but was significantly lower than the activity after 48 h culture without gliadin ($P < 0.02$). This effect of gliadin on the behavior of biopsy specimens

was quite consistent in that, as indicated in the middle panel of Fig. 4, final alkaline phosphatase activity was always lower in biopsies cultured in the presence of gliadin than in biopsies cultured in its absence. Similarly when multiple studies were performed at different times on the same patient, the direction and magnitude of the changes were quite similar (Table V).

In vitro effect of gliadin: patients with GSE in exacerbation vs. patients with GSE in remission. 16 organ culture studies from 10 patients with GSE in remission (eight of these patients were studied both in remission and exacerbation) were performed. When cultured in the absence of gliadin, tissue from patients with GSE in remission underwent a 130% increase in alkaline phosphatase during 48 h of culture: the initial alkaline phosphatase activity was 181±157 U and increased significantly to a value of 418±156 U ($P < 0.02$) (Fig. 4 right panel). However, unlike the enzyme activity in tissue from patients with GSE in exacerbation, the enzyme activity in tissue from patients with GSE in remission increased significantly even in the presence of gliadin. Alkaline phosphatase activity increased 96% from 181± 157 U to 354±161 U ($P < 0.02$), and the activity after 48 h of culture with a P-T digest of gliadin was not significantly different from the activity after 48 h of culture without gliadin. This result is especially significant in that many of the patients in remission as defined herein may not have been in complete remission (e.g., the mean initial alkaline phosphatase of the remission group was lower than the mean of the normal group), a fact which would tend to bias the study against the results obtained.

Three normal individuals were studied twice, once after a 3-wk period on a gluten-free diet and again after a 2-wk gluten challenge. No effect of gliadin on increase of jejunal tissue enzymes was observed either before or after the gluten challenge (Table VI). Thus, dietary manipulation per se does not appear to account for the results obtained with GSE patients.

In vitro effect of gliadin: Patients with gastrointestinal disease unrelated to gluten sensitivity. Jejunal biopsy specimens obtained from three patients with malabsorption and villous abnormalities but without sensitivity to gluten were cultured in the presence and absence of a P-T digest of gliadin and studied as above with regard to enzyme changes. The mean initial alkaline phosphatase activity of 139±102 U was significantly lower than normal and was comparable to the mean value observed in biopsies from GSE patients. An increase in activity was seen in all instances after 48-h culture which was unaffected by the presence of gliadin in the medium (Table VII). Thus, an in vitro effect of gliadin is not seen in any patient with a low initial level of alkaline phosphatase in the mucosa and appears to be an effect specific for patients with GSE.

TABLE VI

Effect of Diet on Intestinal Alkaline Phosphatase Activity during Organ Culture of Jejunal Biopsies of Normal Control Individuals

Normal control individuals (3)	Alkaline phosphatase activity*		
	0 h	48 h	48 h + gliadin
Gluten-free diet	454±55	607±141	614±127
Unrestricted diet	470±63	679±121	659±191

* 1 unit = 1 μmol PNP liberated per gram protein per minute.

TABLE VII

Intestinal Alkaline Phosphatase Activity during Organ Culture of Jejunal Biopsies of Disease Control Patients

Patients	Alkaline phosphatase activity*		
	0 h	48 h	48 h + gliadin
Patient 1 (Abetalipoproteinemia)	252±18	438±16	517±3
Patient 2 (Agammaglobulinemia)	55±0	113±32	135±0
Patient 3 (Radiation enteritis)	109±38	291±35	267±18

* 1 unit = 1 μmol PNP liberated per gram protein per minute.

In vitro effects of α-gliadin and a peptic-tryptic digest of casein. Specimens from two patients with active GSE, three patients with GSE in remission, and two control patients were cultured in the presence of α-gliadin, a water-soluble derivative of gluten prepared without the use of proteolytic enzymes (10). α-Gliadin inhibited the increase in alkaline phosphatase occurring in cultures of patients with active GSE, but had no effect on the rise in enzyme activity in tissue of control subjects or patients in remission (Table VIII).

As a further control, the effect of a P-T digest of casein (1. 2 mg/ml) on cultured jejunal tissue was studied. The P-T digest of casein did not affect 48-h enzyme increases in four out of five of the active GSE patients' biopsies, whereas parallel cultures demonstrated the inhibitory effect of a P-T digest of gliadin (Table IX). In the one instance where an effect of casein was observed, the protein concentration used was 10 times that normally used for gliadin (i.e., 12 mg/ml). No effect of casein was noted in cultures of seven control patients' biopsies or biopsies of three GSE patients in remission. These experiments with casein and α-gliadin demonstrate that the inhibitory effect of the P-T digest of

TABLE VIII

Effect of α-Gliadin on Intestinal Alkaline Phosphatase Activity during Organ Culture of Jejunal Biopsies of GSE Patients and Normal Controls

Patients	Alkaline phosphatase activity*			
	0 h	48 h	48 h + P-T gliadin	48 h + α-gliadin
GSE in exacerbation (2)‡	122±88	319±8	131±27	108±45
GSE in remission (3)	324±237	537±143	591±162	797±232
Normals (2)	414±131	628±54	582±14	539±8

* 1 unit = 1 μmol PNP liberated per gram protein per minute.
‡ Number in parentheses indicates number of patients studied.

TABLE IX

Effect of P-T Digest of Casein on Intestinal Alkaline Phosphatase Activity during Organ Culture of Jejunal Biopsies of GSE Patients and Controls

Patients*	Alkaline phosphatase activity‡			
	0 h	48 h	48 h + gliadin	48 h + casein
Control patients (7)	261±102	375±71	448±148	403±113
GSE in remission (3)	108±80	376±75	336±121	413±106
GSE in exacerbation				
D. G.	103±34	278±115	172±192	268±223
F. F.	29±5	243±147	29±3	253±4
D R.	58±7	507±120	370±114	669±25
D. W.	37±8	138±68	119±27	138±25
L. H.	62±46	185±101	111±50	89±71

* Number in parentheses indicates number of patients studied.
‡ 1 unit = 1 μmol PNP liberated per gram protein per minute.

gliadin cannot be attributed to its content of pepsin and trypsin and appears to be specific for gliadin.

The in vitro effect of gliadin on disaccharidase activity before and after gluten challenge. Disaccharidase activities in biopsy specimens were measured for all patients and controls participating in the study. Initial disaccharidase activities in specimens obtained from normal control patients were comparable to those reported by others (17–19). In general disaccharidase activity increased during organ culture although not to the same degree as alkaline phosphatase activities. Thus, jejunal sucrase and trehalase activities of normal control tissue were higher after culture ($P < 0.05$ by paired t test) with or without gliadin (Fig. 5).

In tissue obtained from GSE patients and cultured for 48 h, only trehalase increased significantly ($P \leq 0.01$ by paired t test). In tissue obtained from patients with GSE in remission, gliadin had no effect on sucrase or trehalase activity at 48 h. However, in tissue obtained from patients with GSE in exacerbation, the mean absolute increase in activity for both enzymes was less after culture in the presence of gliadin than in its absence (for sucrase, $P < 0.05$; for trehalase, $P \leq 0.01$). Lactase levels were low to absent in all biopsies obtained from GSE patients. Thus it appears that brush border enzyme changes during organ culture are a general phenomenon, although alkaline phosphatase activity undergoes the most easily discerned changes.

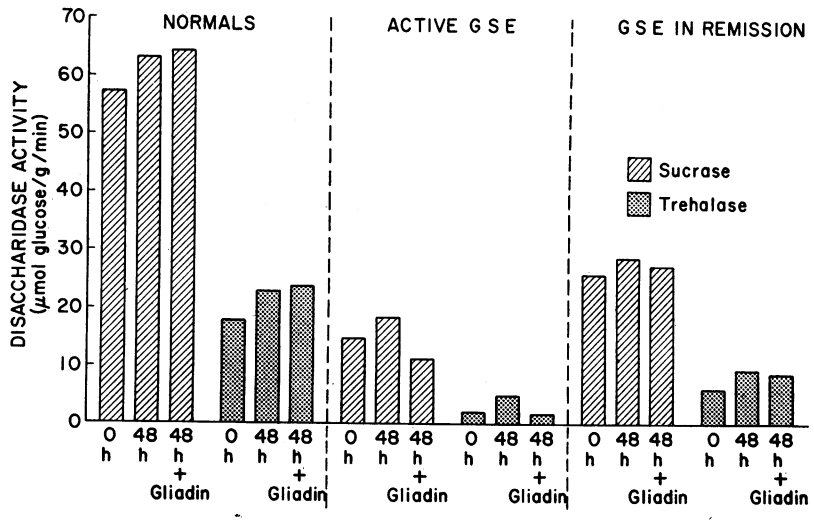

FIGURE 5 Disaccharidase activities during jejunal tissue culture of biopsies from normal controls (left panel), patients with GSE in exacerbation (middle panel), and patients with GSE in remission (right panel). Mean values for sucrase and trehalase are shown initially and after 48 h of culture with or without gliadin.

DISCUSSION

Organ culture of intestinal biopsy tissue has previously been described by Browning and Trier (11). These investigators showed that intestinal epithelial cells can be placed into organ culture and maintained with near normal morphology and functional activity for at least 24 h. In another study, Trier and Browning showed that intestinal biopsy specimens from three patients with active gluten-sensitive enteropathy demonstrated increased incorporation of tritiated thymidine, but showed more normal rates of incorporation when studied after a gluten-free period, suggesting a normalization of cellular turnover upon cessation of exposure of gluten peptides (20). Finally, Trier and Browning showed that, in at least one instance, epithelial cells of a specimen taken from a patient with GSE underwent morphologic improvement during the period of culture (20).

In the present investigation we have used the organ culture methodology to develop an in vitro model of gluten-sensitive enteropathy. This model has, as its basis, the finding that epithelial cells from all patient groups increase their content of alkaline phosphatase as well as at least one disaccharidase (sucrase) during organ culture. However, in the presence of gliadin this increase in enzyme activity is inhibited in specimens obtained from patients with active gluten-sensitive enteropathy, whereas specimens from controls are not so inhibited. In providing an explanation of these biochemical effects during organ culture, it must first be pointed out that changes in the enzyme content of epithelial cells are a normal accompaniment of the maturation of cells as they emerge from intestinal crypts and ascend the intestinal villi. Thus Moog, Etzler, and Grey (21) have shown, in a series of studies, that cells in crypts contain less alkaline phosphatase than cells at villous tips. In addition, Brown (22) has shown that microvilli are poorly developed in crypts and become progressively longer and more numerous as the epithelial cells move up the crypts and villi as a result of differential proliferation. Thus, it is reasonable to assume that the changes observed during organ culture in specimens from all groups of patients, e.g., the increase in alkaline phosphatase activities, are the result of the normal maturational process so that the population of cells in the organ culture at the end of the cultural period contain a greater number (on a weight basis) of more mature, alkaline phosphatase-rich, villus-like cells.

This brings us to the fact that the mean initial alkaline phosphatase content of specimens obtained from GSE patients was far lower than the mean initial alkaline phosphatase content of normal control specimens and that GSE specimens underwent an increase in alkaline phosphatase content once they were placed into organ culture, which apparently was not possible in vivo. An explanation for this phenomenon which is consistent with the maturation concept mentioned above is that, in vivo, gliadin prevents the differentiation of immature intestinal epithelial cells or else is toxic only to more differentiated cells. In the gliadin-free environment of the organ culture, cells are free to differentiate and reach maturity and then synthesize normal amounts of enzyme. In this view, gliadin ingestion in GSE leads to the development of a population of immature, but mitotically active cells that have a restricted capacity to synthesize certain enzymes. A second possibility is that gliadin exerts a toxicity on epithelial cells of GSE patients regardless of their state of differentiation and that in the gliadin-free environment of organ culture, cells can develop normally or even recover from damage inflicted during their in vivo life. However, this latter possibility is less consistent with the idea put forth above that epithelial cells mature in organ culture and, furthermore, does not take into account the fact that normal specimens undergo an increase in alkaline phosphatase content during the culture period.

In any case, culture of biopsy tissue from patients with active GSE in the presence of gliadin is accompanied by a greatly diminished or even nil increase in alkaline phosphatase activity of biopsy specimens. Thus, the effect of gliadin in organ culture is similar to its effect in vivo and the in vitro prevention of a rise in alkaline phosphatase activity by gliadin constitutes an in vitro model of gluten-sensitive enteropathy.

Several inferences concerning the mechanism of gluten-sensitive enteropathy can be drawn from this model. In the first place, gliadin appears to be necessary to the cellular toxicity presumed to occur in GSE, making it unlikely that GSE is mediated by autoantibodies as in the autoimmune diseases. If such were the case, autoantibodies elaborated by the gastrointestinal mucosa would be capable of destructive interaction with self-components in the absence of gliadin and the requirement for the presence of gliadin for cell toxicity demonstrated in these experiments would not be necessary.

In the second place, the in vitro "toxicity" of gliadin P-T digest was observed in biopsy tissue obtained from patients with GSE in exacerbation but not from patients with GSE in remission. This strongly implies that gliadin does not adversely affect tissue directly, but must first activate or trigger a host (endogenous) effector mechanism of toxicity. In the present studies, this activation was presumably brought about by the ingestion of gluten in vivo, i.e., by the gluten challenge, and the toxic effect was seen only after the gluten challenge (or in an attenuated form in patients with partial remissions).

A prime candidate for such an endogenous effector mechanism is the local immunologic mechanism. In this

regard, we have previously shown that anti-gliadin responses are evoked at local mucosal sites after gluten challenge (4). These responses were demonstrated in jejunal biopsy specimens maintained in short-term culture in vitro using tissue obtained from patients with GSE in exacerbation but not from those with GSE in remission. Thus the effect of gliadin peptides on cultured biopsy specimens obtained from patients with GSE in exacerbation could well have been mediated by antibody produced in vitro and, by the same token, the lack of this effect on specimens obtained from patients with GSE in remission could have been due to the specific inability of remission tissue to produce sufficient anti-gliadin antibody. This construct of the mechanism underlying gluten-sensitive enteropathy is susceptible to experimental test using the system described in this report. Namely, it seems feasible to culture jejunal specimens under a variety of conditions which allow one to identify some of the factors or combination of factors necessary to produce injury in tissue from patients with gluten-sensitive enteropathy.

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