Pediatr. Res. *16:* 1004–1010 (1982)

Effects of Gliadin-Derived Peptides from *Bread* and *Durum* Wheats on Small Intestine Cultures from Rat Fetus and Coeliac Children

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Summary

Peptic-tryptic-cotazym (PTC) digests were obtained, simulating *in vivo* protein digestion, from albumin, globulin, gliadin and glutenin preparations from hexaploid (*bread*) wheat as well as from diploid (*monococcum*) and tetraploid (*durum*) wheat gliadins. The digest from *bread* wheat gliadins reversibly inhibited *in vitro* development and morphogenesis of small intestine from 17-day-old rat fetuses, whereas all the other digests (obtained both from nongliadin fractions and from gliadins from other wheat species) were inactive.

The PTC-digest from *bread* wheat gliadins was also able to prevent recovery of and to damage the *in vitro* cultured small intestinal mucosa from patients with active coeliac disease (gluten-induced entheropathy). The PTC-digest from *durum* wheat gliadins caused a much less adverse effect on this human pathologic tissue culture system.

Speculation

When tested with different *in vitro* systems, *bread* wheat gliadin peptides active in coeliac disease displayed several peculiar biologic activities including an immunogenic character in susceptible individuals (2), a probable immunomediated cytotoxic activity on small intestinal mucosa specimens from coeliac patients (6–10, 13–15, 17), and a probable direct cytotoxic activity on developing rat fetal intestine (5). In fact, these peptides induce the following: (1) proliferation of peripheral blood lymphocytes from coeliac patients as well as from many first degree relatives of coeliacs and from only a few normal controls; (2) damage of the *in vitro* cultured small intestinal mucosa of patients with active coeliac disease; and (3) reversible inhibition of the *in vitro* development of fetal rat intestine.

Although no one of the mentioned *in vitro* systems can be considered a model fully representative of coeliac disease, it is likely that each one of them highlights a different important feature underlying the appearance of the small intestine lesion in coeliac patients ingesting *bread* wheat gliadin peptides.

Peptide mixtures obtained by enzymic digestion of the gliadin fraction from hexaploid (*bread*) wheats significantly differ from those from tetraploid (*durum*) wheat gliadins for their higher toxicity toward cultures of intestine from rat fetuses or coeliac children. However, gliadin peptides from both *bread* and *durum* wheats are capable of inducing proliferation of peripheral blood lymphocytes from coeliac patients thus suggesting a similar immunogenic character (2).

We suggest that *durum* wheat gliadins peptides are *in vitro* less toxic than *bread* wheat gliadins peptides for the small intestinal mucosa of coeliacs as they have less direct cytotoxic effect on the enterocyte; moreover, *durum* wheat products (*e.g.*, spaghetti), as compared to *bread* wheat products (*e.g.*, bread and biscuits), might present a lower risk for patients suffering for coeliac disease or other wheat intolerances.

The detection and characterization of wheat components that are toxic in coeliac disease and in other forms of wheat intolerance (5) is very difficult because of the lack of suitable *in vitro* methods for toxicity testing.

Falchuk *et al.* (6, 7) have proposed the organ culture of human small intestinal biopsies as an *in vitro* model of coeliac disease. Jejunal specimens obtained from patients with active enteropathy shows morphological and biochemical improvement when cultured in a gluten-free medium. No improvement occurs when the tissue is cultured in the presence of gluten peptides. Several other authors (10, 13, 14, 15) have confirmed Falchuk *et al.*'s findings, although Haury *et al.* (11) were not able to show a cytotoxic effect of various gluten preparations on organ culture of small intestinal mucosa from coeliac children. Jejunal mucosa from coeliac patients in remission and from normal subjects is not affected by the presence of gluten in the culture medium. These findings led Falchuk *et al.* to suggest that gluten must first initiate a set of events *in vivo*, possibly related to the immune system (8, 9), before the cytotoxic effect on enterocytes can be displayed *in vitro*.

In 1976 we undertook a research program to evaluate whether the *in vitro* developing intestine from rat fetus may offer a model for the study of proteins and peptides toxic for the intestinal mucosa in some pathologic conditions resulting in the presence of immature enterocytes on the surface of the human mucosa. This typical feature also characterizes some stages underlying maturation of rat fetal intestine, which takes place *in vitro,* in a way comparable to what happens *in vivo* (4). We have previously demonstrated (5) that a peptic-tryptic-pancreatic digest of hexaploid (*bread*) wheat gliadin, obtained by stimulating *in vivo* protein digestion, was very active in inhibiting *in vitro* development and morphogenesis of small intestine from 17- and 18-day old rat fetuses. It had no effect on the culture of jejunum from 21-day old fetuses or from newborn rat; moreover, this digest induced extensive tissue degeneration and necrosis of *in vitro* cultured small intestinal mucosa from patients with active coeliac disease, but did not cause any detectable effect on normal human small intestinal mucosa.

This paper deals with further investigations we have carried out on this topic. As a first point we studied the reversibility of the inhibiting effect of the PTC-digest of *bread* wheat gliadin on *in vitro* development and morphogenesis of small intestine from rat fetus. We tested peptides obtained with an identical digestion procedure by several other protein fractions from *bread* wheat (including albumins, globulins and glutenins) as well as a tryptic fragment of Hekkens' α-gliadin to see whether they display similar toxic effects. Wheat seed endosperm contains heterogeneous protein classes usually divided, according to their extractability and

solubility in different solvent systems, into albumins, globulins, gliadins and glutenins. Albumins and globulins are extracted in neutral salt solutions of low and high ionic strengths respectively, whereas gliadins are alcohol-soluble and glutenins require acidic or alkaline solutions of low ionic strength to be extracted (16). Gliadins are most deficient in the essential amino acid lysine and are characterized by a very high content of glutamine (one residue each three amino acid residues) and proline (one residue each seven amino acid residues).

Finally, we have continued previous experiments with both cultured fetus rat jejunum and small intestinal mucosa from coeliac children with active disease in order to assess whether wheat species other than *bread* hexaploid wheats (*e.g.,* tetraploid and diploid wheats) also contain toxic amino acid sequences in their gliadin fractions.

MATERIALS AND METHODS

A tryptic fragment of purified α-gliadin prepared according to Hekkens *et al.* (12) was generously supplied by Dr. W.Th.J.M. Hekkens. Pure *bread* (Mentana and San Pastore), *durum* (Azizia and Cappelli) and *monococcum* varieties were kindly supplied by the Istituto Sperimentale per la Cerealicoltura in Roma. *Bread* (*Triticum aestivum*) wheat varieties are hexaploid (AABB DD), *durum* (*Triticum durum*) wheat varieties tetraploid (AA BB) and *monococcum* (*Triticum monococcum*) varieties are diploid (AA) (18).

For preparation of the globulin fraction, 100 g of finely ground whole wheat flour was extracted at 4°C for 3 h in a shaker with 1 liter of a 0.04 M Na_2HPO_4 (pH 7) buffer containing 1.8 M $(NH_4)_2SO_4$. After extraction, the suspension was centrifuged for

Table 1. *Morphologic features in maturation of rat fetal jejunum cultured in vitro in the presence of peptides from wheat*

	Villi			Epithelium			Goblet Cells	Degenerative[2] changes	
				Stratified	Monostratified				
Sample[1]	Absent	Rudimentary	Present		Cuboidal	Columnar		±	++
Uncultured 17-day-old fetuses (50)	47	3		47	2	1			
After 48 h culture:									
a) without peptides (50)	2	5	43		1	49	22	12	
b) PTC gliadin from hexaploid wheat (Mentana)[3]									
0.1 mg/ml (10)	9	1			3	7		5	
0.5 mg/ml (8)	8				3	5		5	
c) PTC gliadin from hexaploid wheat (San Pastore)									
0.1 mg/ml (6)	6				2	4		2	
0.5 mg/ml (6)	6				3	3		3	
d) PTC gliadin from tetraploid wheat (Azizia)[4]									
0.1 mg/ml (10)		1	9			10	4	3	
0.5 mg/ml (8)		2	6		1	7	3	4	
e) PTC gliadin from tetraploid wheat (Cappelli)									
0.1 mg/ml (6)		1	5			6	2	1	
0.5 mg/ml (6)			6			6	1	2	
f) PTC gliadin from diploid wheat (Monococco)									
0.1 mg/ml (10)		2	8			10	3	3	
0.5 mg/ml (10)	1	2	7		1	9	2	3	
PTC albumins from hexaploid wheat (Mentana)									
0.1 mg/ml (2)			2			2			
0.5 mg/ml (2)		1	1			2		1	
PTC globulins from hexaploid wheat (Mentana)									
0.1 mg/ml (2)			2			2			
0.5 mg/ml (2)			2			2			
PTC glutenins from hexaploid wheat (Mentana)									
0.1 mg/ml (2)			2			2		1	
0.5 mg/ml (2)			2			2		1	

[1] Number of fetuses examined or number of fetal jejunums cultured in parenthesis.

[2] ± Indicated slight degenerative changes; ++ indicates large necrotic areas.

[3] The following peptides mixtures were used: (total) PTC protein digest in 12 cultures and peptide fraction with molecular weight 2,000–10,000 in six cultures.

[4] The following peptide mixtures were used: (Total) PTC protein digest in 8 cultues, peptide fraction with molecular weight 2,000–10,000 in six cultures and with molecular weight 5,000–10,000 in four cultures.

15 min at 16,000 × *g*. The supernatant was dialyzed against distilled water for 48 h at 4°C to obtain the globulin fraction as a precipitate. Identical experimental conditions were applied with other solvents for the consecutive extractions of the albumin, gliadin and glutenin fractions from the residue from the first extraction. The extraction solvent of albumins was 0.04 M Na_2HPO_4(pH 7) containing 0.4 M $(NH_4)_2SO_4$, that for extraction of gliadins was ethanol: water (70:30 v/v), and for the extraction of glutenin 0.1 M acetic acid was used. Gliadin and glutenin fractions were extracted at room temperature using 300 ml of solvent for 100 g of flour.

The procedure for gliadin preparation used here is significatively different from that previously used by de Ritis *et al.* (5) who extracted gliadins from commercial wheat gluten instead of from wheat flour.

Peptic-tryptic-cotazym (PTC) digests were prepared from wheat protein fractions following the three step procedure of Bronstein *et al.* (3) as reported by de Ritis *et al.* (5). One hundred g of protein fraction was digested in 1 liter of 0.2 N HCl (pH 1.8) with 2 g of purified pepsin at 37°C for 2 h. The peptic digest was further digested by addition of 2 g of purified trypsin after pH adjustment to 8.0 with 2 N NaOH. The reaction mixture was vigorously stirred at 37°C for 4 h at pH 8.0. Then, the peptic-tryptic digest was treated with 2 g of purified cotazym and mechanically stirred for 2 h at pH 8.0. During all the digestion procedure the pH was checked periodically and, when needed, adjusted with HCl or NaOH. At the end of the whole digestion procedure, the digest was submitted to gel filtration and the peptide fractions eluted after cytochrome *c* were collected and freeze-dried. These enzyme-free low-molecular-weight peptide pools have been coded as PTC protein digest.

Lyophilized PTC from Mentana hexaploid wheat and Azizia tetraploid wheat, were redissolved in water and submitted to ultrafiltration in an Amicon 400 milliliter cell equipped with UM 2 Amicon membrane to remove compounds with molecular weights lower than 2,000 and to obtain a peptide fraction with molecular weight in the range 2,000–10,000. Then this fraction was submitted to ultrafiltration using a DM5 Amicon membrane to prepare peptide subfraction with molecular weights in the range 5,000–10,000.

For *in vitro* culture of fetal jejunum, time-pregnant Wistar rats were anesthetized with ether and 17-day-old fetuses were removed at laparatomy. Fetal jejunum segments were isolated and cultured *in vitro* for 48 h in a serum-free medium, according to the method described by de Ritis *et al.* (4). Jejunal segments from the same fetus were cultured in the absence and in the presence of tested

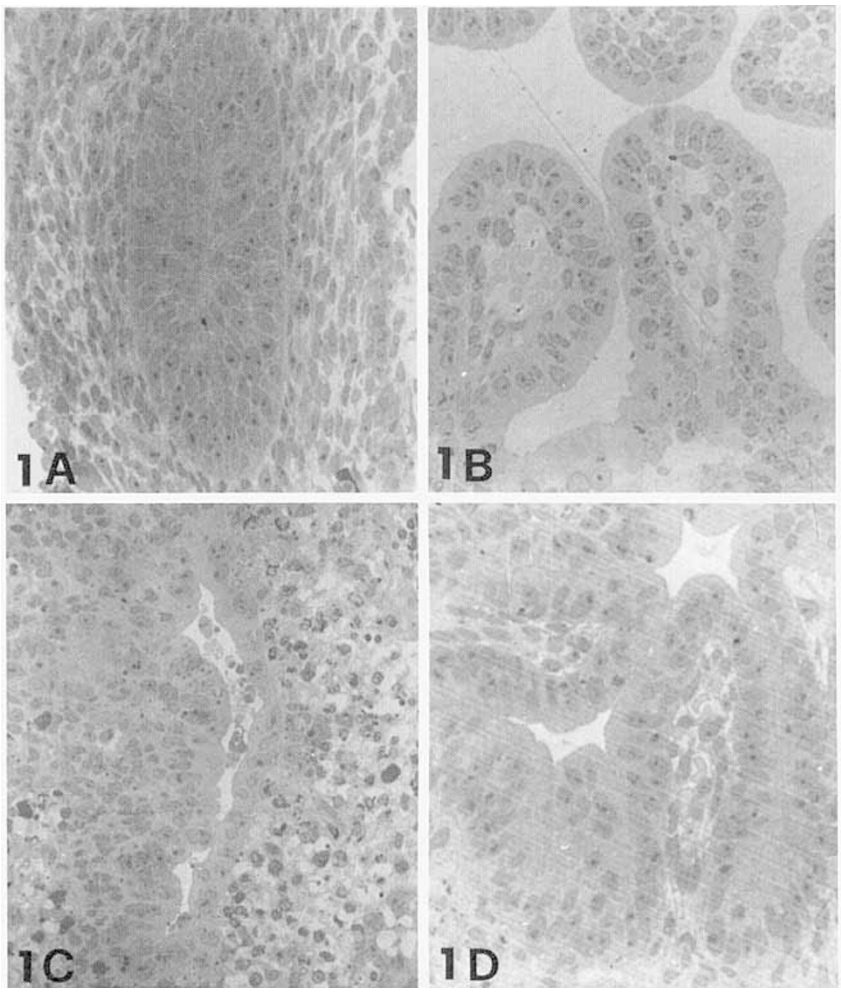

Fig. 1. Effect of peptic-tryptic-cotazym digest of gliadins from *bread* wheat (var. Mentana) and *durum* wheat (var. Azizia) on *in vitro* development and morphogenesis of jejunum from 17-day-old-rat fetus. (*A*) Jejunal mucosa before culture. The epithelium consists of stratified undifferentiated cells and no villi are present. (*B*) Jejunal mucosa after 48-h culture. Well developed villi and an epithelium consisting of a single layer of columnar cells more differentiated than those observed in uncultured jejunum are evident. Some differentiated goblet cells are also present. (*C*) Jejunal mucosa after 48-h culture in the presence of the PTC gliadins digest (0.5 mg/ml) from *bread* wheat (var. Mentana). No villi developed on the surface, lined primarily by columnar epithelial cells. Large lysosome-like inclusions are present and no goblet cells could be identified. (*D*) Jejunal mucosa after 48-h culture in the presence of the PTC gliadins digest (0.5 mg/ml) from *durum* wheat (var. Azizia). Well formed villi lined by a single layer of relatively differentiated columnar epithelial cells are evident. Some goblet cells are also present.

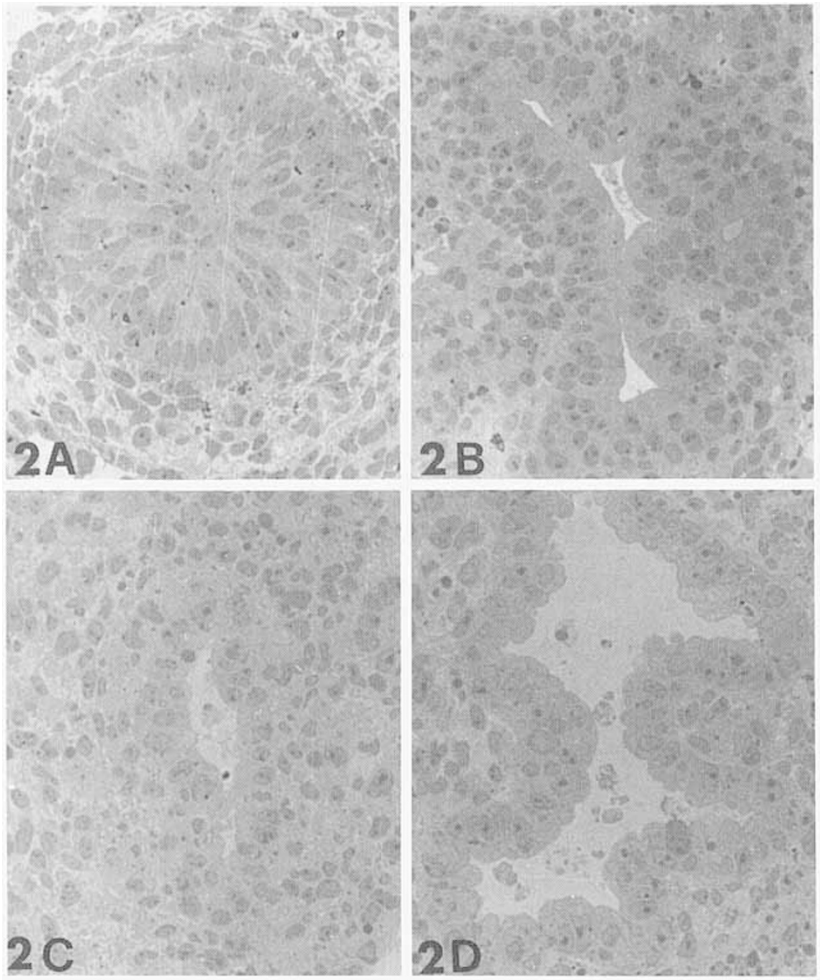

Fig. 2. Reversibility of the inhibitory effect of the peptic-tryptic-cotazym digest of gliadins from *bread* wheat (var. Mentana) on *in vitro* development and morphogenesis of jejunum from 17-day-old rat fetus. (*A*) Jejunal mucosa before culture, showing no villi and an epithelium consisting of stratified undifferentiated cells. (*B*) Jejunal mucosa after 24-h culture in the absence of gliadins peptides showing rudimentary villi lined by an epithelium consisting of a single layer of columnar cells. (*C*) Jejunal mucosa after 24-h culture in the presence of the PTC gliadins digest (0.5 mg/ml) from *bread* wheat showing a flat mucosal surface. The epithelial lining consists of both stratified and single cuboidal cells. (*D*) Jejunal mucosa cultured for 24-h in the presence of the PTC gliadins digest (0.5 mg/ml) from *bread* wheat and then for 24 h in a peptide free medium showing that maturation of the mucosa takes places under these conditions with the appearance of villi lined by columnar epithelium.

peptides. Differentiation of the fetal rat jejunum was followed morphologically by light microscopy as reported by de Ritis *et al.* (4).

For *in vitro* culture of human small intestinal mucosa, intestinal biopsies were obtained at the duodenojejunal flexure from 37 children affected by active coeliac disease, with subtotal mucosal atrophy; of these 26 had never been treated before, 11 were in relapse after the reintroduction of gluten in the diet since at least 5 months. Diet of biopsied children contained both *durum* (spaghetti and other pasta products) and *bread* wheat products (bread and biscuits). The biopsies were cultured for 48 h in a serum containing medium, with or without added peptides and then processed for morphological examination by light microscopy, as reported by de Ritis *et al.* (5). Seven out the 37 children with active coeliac disease were also examined for the histocompatibility antigens. Four were cultured with hexaploid and three with tetraploid gliadin peptides: they were either HLA-DR W7 or HLA-DR W3 positive.

RESULTS

Culture of fetal rat intestine. Before *in vitro* culture, jejunal segments from 47 17-day-old rat fetuses did not show any villus. Only undifferentiated cuboidal stratified epithelia lining the lumen were present: in three other fetuses few very rudimentary villi with monostratified epithelial cells were observed (Table 1 and Fig. 1a). No goblet cells could be detected at this stage of tissue development. After 48-h of *in vitro* culture without added wheat peptides (Table 1 and Fig. 1b), there was clear morphologic evidence of tissue maturation in 48 cultured fetal jejunum out of a total of 50. Well differentiated villi were present in cultured jejunal segments from as many as 43 fetuses and rudimentary villi were observed in the specimens from five other fetuses. In 49 fetuses, the epithelial lining consisted exclusively of simple columnar epithelium and in 22 cases goblet cells were identified. Patchy, slightly degenerative changes characterized by a decreased cytoplasmic staining, nuclear pycnosis and large lysosomelike inclusions were observed only in a few cultured jejunal segments from 12 fetuses. These findings confirm previous results by de Ritis *et al.* (4, 5), showing that differentiation and maturation of small intestinal mucosa from rat fetuses takes place *in vitro* in a way comparable to what happens *in vivo*.

The peptic-tryptic-cotazym digests of the gliadin fractions from two varieties (*Mentana and San Pastore*) of hexaploid *bread* wheat, and the 2,000–10,000 peptide fraction of the PTC digest from Mentana gliadins were very active in slowing down *in vitro* development of fetal rat intestine and in increasing occurrence and severity, especially at the mesenchyme level, of degenerative

changes sporadically observed in the control tissue (Table 1 and Fig. 1c). Such effects were also clearly observed at the lowest peptide concentration tested (0.1 mg/ml of incubation medium). After 48 h of culture, well differentiated villi were absent in all cultures. Villi were rudimentary in one case and absent in 29 cases; moreover, the epithelium was monostratified in all the cases, but it was cuboidal in 11 cases and goblet cells were absent in all cultures. These results are very similar to those obtained by de Ritis *et al.* (5) who tested a PTC digest of the gliadin fraction prepared with a different procedure from commercial gluten. When tested under identical experimental conditions, the tryptic fragment of α-gliadin fraction prepared according to Hekkens *et al.* (12) showed, at a concentration of 0.1 mg/ml of culture medium, a comparable activity in interfering with the process of *in vitro* maturation and differentiation of fetal rat intestine. On the contrary PTC digests from *bread* wheat albumins, globulins, and glutenins showed no one of the effects induced by gliadin digest from *bread* wheat and were practically inactive (Table 1).

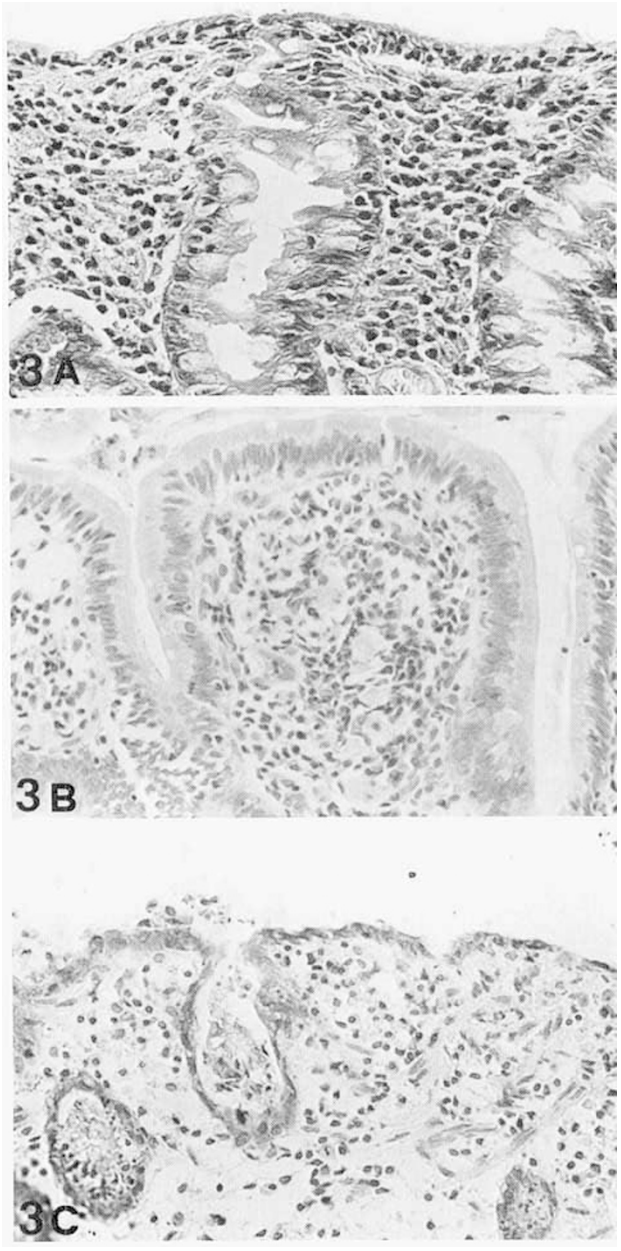

Fig. 3. Effect of *in vitro* culture in the absence or presence of gliadin peptides from *bread* (var. Mentana) wheat on jejunal biopsy specimens from children with untreated coeliac disease. (*A*) Jejunal biopsy before culture. A flat mucosa with elongated crypts and damaged surface epithelium is evident. (*B*) Jejunal biopsy after 48-h culture in the absence of gliadin peptides. Some recovery has occurred with respect to the uncultured mucosa: the morphologic abnormalities of the surface epithelium have almost completely disappeared. The epithelial cells are taller and more regular. (*C*) Jejunal biopsy after 48-h culture in the presence of PTC gliadins digest (0.5 mg/ml) from *bread* wheat (var. Mentana). The surface epithelium was injured and some degenerative changes and necrotic areas were evident.

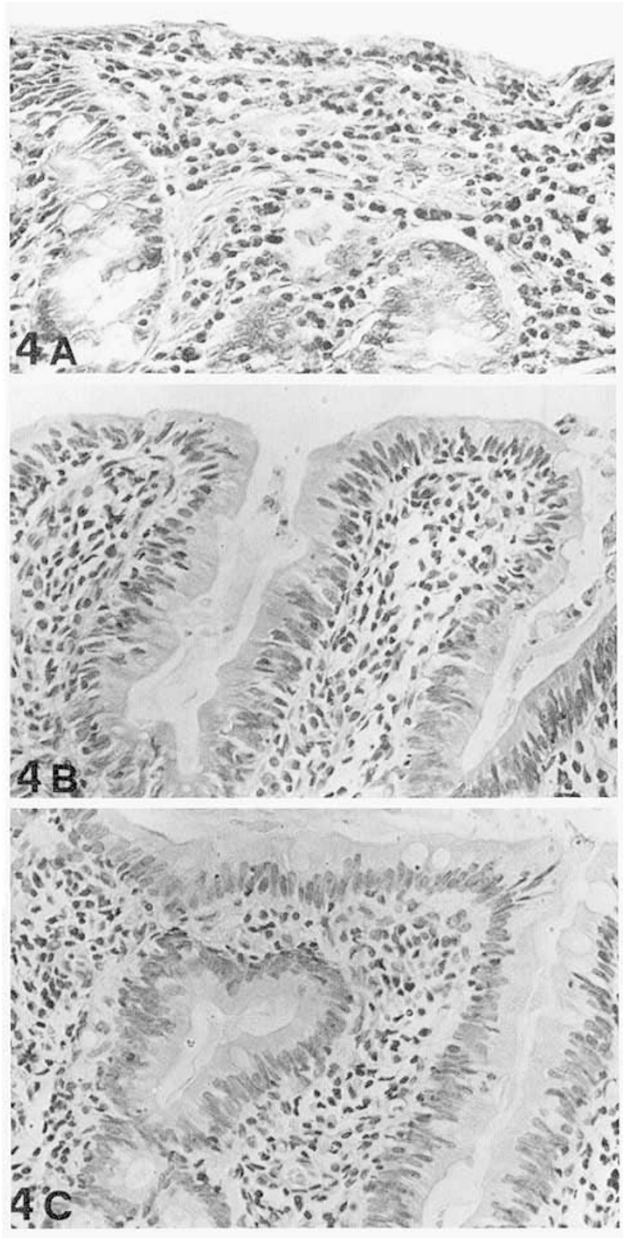

Fig. 4. Effect of *in vitro* culture in the absence or presence of gliadin peptides from *durum* (var. Azizia) wheat on jejunal biopsy specimens from children with untreated coeliac disease. (*A*) Jejunal biopsy before culture. A total loss of villous pattern, with cuboidal and irregular epithelial cells, is evident. (*B*) Jejunal biopsy after 48-h culture in the absence of gliadin peptides. The morphologic abnormalities of the surface epithelium have almost completely disappeared. (*C*) Jejunal biopsy after 48-h culture in the presence of the PTC gliadins digest (0.5 mg/ml) from *durum* wheat (Var. Azizia). The appearance is similar to that of the mucosal specimen cultured in the absence of gliadin peptides, with recovery of the surface epithelial cells.

Table 2. *Effect of peptic-tryptic-cotazym (PTC) digests of gliadins from bread and durum wheats on in vitro cultures of small intestinal biopsy specimens from coeliac children with subtotal mucosal atrophy*

	Number of culture					
				With PTC-digest of gliadins from *durum* wheat (var. Azizia)		
Effect	Without peptides	With PTC-digest of gliadins from *bread* wheat (var. Mentana) (0.5 mg/ml)	Without peptides	(0.5 mg/ml)	(1 mg/ml)	2.5 mg/ml)
Recovery	13		24	12		
No change		1		6	2	
Impairment		7		1		3
Extensive necrosis		5				

The reversibility of the effect of PTC gliadin digest from *bread* wheat was shown by the fact that, after an initial 24 h culture in a medium added with the digest of *bread* wheat gliadin, the intestinal mucosa underwent a normal morphogenesis when transferred to a gliadin peptide-free culture medium. In fact, when jejunal segments from three fetuses were cultured in the presence of peptides (0.5 mg/ml of culture medium), villi were absent. The villi developed in all cases with a cylindrical epithelium after 24 h when the culture medium had been substituted with a medium free from wheat peptides (Fig. 2).

In contrast to what was observed with *bread* wheat, the PTC digests of gliadins from two varieties (Azizia and Cappelli) of *durum* tetraploid wheat as well as the 2,000–10,000 and 5,000–10,000 peptide fractions did not affect *in vitro* development and morphogenesis of fetal rat jejunum, even when they were tested at a concentration as high as 0.5 mg/ml (Table 1 and Fig. 1d). After 48-h culture, villi were present in all 30 cases: they were rudimentary in only four cases and well developed in all other cultures, with a differentiated columnar epithelium in all cases except one and goblet cells in 10 cases. Slight degenerative changes were present in only few cultures. Similar results were obtained with PTC digest of gliadins from diploid (*Triticum monococcum*) wheat: villi were absent only in one case, rudimentary in four and well developed in 15 cases with a differentiated columnar epithelium in all cases except one, and goblet cells in five cases out of 20 total.

Durum wheat gliadin peptides did not afford any protection of rat fetal intestine against toxicity of *bread* wheat gliadin peptides. In fact, no maturation and differentiation was observed when intestine specimens from two rat fetuses were cultured in the simultaneous presence of equal concentrations (0.25 mg/ml of medium) of peptides from both *bread* (Mentana) and *durum* (Azizia) gliadins.

Culture of small intestinal biopsies of children with active coeliac disease. Before *in vitro* culture, all the small intestinal biopsy specimens from 37 children showed a flat mucosa with total loss of villous pattern and elongated hypertrophic crypts. The surface epithelium showed cuboidal cells with irregular nuclei and increase of intraepithelial lymphocytes (Fig. 3a). After 48 h culture with no added wheat peptides, all these biopsies showed significant recovery with an almost complete disappearance of the morphologic abnormalities of the surface epithelium (Fig. 3b). Enterocytes were taller with basally oriented oval nuclei and the lymphocytes were reduced. Moreover, in some areas, very short villi could be observed after culture.

Biopsy specimens from children with coeliac disease did not recover when cultured for 48 h in the presence of 0.5 mg of PTC gliadin digest from *bread* wheat per ml culture medium. Furthermore, a severe mucosal damage was evident in 12 out of 13 cultures tested including five cultures with extensive necrotic areas (Fig. 3c). Impairment was also observed in two biopsies cultured in the presence of 0.1 mg/ml of medium of the tryptic fragment of α-gliadin fraction prepared according to Hekkens (12). On the contrary, at the concentration of 0.5 mg/ml of culture medium, the PTC digest of gliadin fraction from *durum* (var. Azizia) wheat, did not inhibit *in vitro* occurring epithelial recovery in 12 out of 19 flat biopsies and caused no change in six other cases (Fig. 4). In only one case a damaging effect of these peptides on flat coeliac mucosa was observed. Even when tested at a concentration of 1 mg/ml, the PTC digest of *durum* gliadins caused no change in two small intestinal biopsies, but, at a concentration of 2.5 mg/ml, this digest induced a significant mucosal damage in all the three biopsies tested (Table 2).

DISCUSSION

The data reported in this paper confirm previous findings by de Ritis *et al.* (5) showing that gliadins from *bread* (hexaploid) wheats contain amino acid sequences capable of inhibiting *in vitro* development and morphogenesis of jejunum from the 17-day-old rat fetus as well as epithelial recovery of cultured specimens of flat intestinal mucosa from active coeliac patients. These amino acid sequences persist in peptides formed during *in vitro* digestion of *bread* wheat gliadins under experimental conditions simulating *in vivo* protein digestion. The inhibitory effect of the PTC gliadin digest from *bread* wheat on rat fetal intestine is reversible and not common to any other protein fraction from *bread* wheat.

It was somewhat surprising to find out that, under *in vitro* conditions, peptides from *durum* wheat gliadins, although not harmless, are much less toxic than those from *bread* wheat gliadins for both human and rat intestines. This was shown by the absence of adverse effects of *durum* wheat peptides on rat fetal intestine at the highest tested concentration and by the fact that, as compared to *bread* wheat peptides, a 5-fold higher concentration of *durum* wheat peptides is necessary to induce impairment, but not yet extensive necrosis, in cultures of mucosa specimens from coeliac children. If these *in vitro* findings also apply to *in vivo* conditions, they would indicate that *durum* wheat, although not harmless, could be much better tolerated than *bread* wheat in coeliac disease. As a matter of fact preliminary clinical data concerning a few coeliac children in remission who ingested for several months *durum* wheat products, indicate that *durum* wheat is also able to cause atrophy of duodenal mucosa (1). However, only a very extensive clinical trial of *durum* wheat foods (spaghetti and other pasta products) can show whether *durum* wheat food present, as compared to *bread* wheat foods (*bread*, biscuits and other products), a lower risk for coeliac patients or for patients suffering for other wheat intolerances. In our opinion, the possibility that *durum* wheat is better tolerated than *bread* wheat by humans under some special circumstances deserves thorough attention.

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19. Preliminary data were presented at the 13th Annual Meeting of the European Society for Paediatric Gastroenterology, Capri, May 27–29, 1980.
20. Received for publication Dr. Salvatore Auricchio, Clinica Pediatrica, II Facoltà di Medicina e Chirurgia, Via Sergio Pansini, 5-80131, Naples Italy.

0031-3998/82/1612-1004 $0.200/0

Printed in U. S. A.