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Humoral and Cellular Immune Responses in Gluten-Treated Suckling or Hand-Fed Rats

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Summary

We analyzed the immune response to gliadin in suckling rats and rats hand-fed with an artificial milk formula, an animal model of gluten enteropathy. Animals of both groups were intragastrically given either gliadin or albumin (control animals) or gliadin from birth till day 55. When compared to the controls, spleen lymphocytes from both groups of gliadin-treated rats cultivated *in vitro* exhibited a significant increase of spontaneous ^{3}H-thymidine incorporation. Moreover, the proliferation of spleen and mesenteric lymph node (MLN) lymhocytes from both groups of gliadin-treated suckling and hand-fed rats was specifically increased by the *in vitro* gliadin challenge. Spleen B cells from gliadin-treated rats spontaneously produced higher amounts of gliadin-specific antibodies than those from the controls, however, *in vitro* stimulation by gliadin caused no further increase in antibody production. Apoptotic DNA fragmentation in MLN cells was higher in gliadin-treated rats than in albumin-treated ones, independently of the milk diet during the suckling period.

Key words

Model of coeliac disease • Rats • Proliferation • Antigliadin antibodies • Apoptosis

Introduction

Coeliac disease (CD) is characterized by active inflammation and cell damage in the small intestine mucosa, resulting in a loss of villous height, enlarged crypts, increased crypt mitotic figures and infiltration of epithelial layer and the lamina propria by mononuclear cells (Kagnoff *et al.* 1996). It is generally accepted that CD belongs to immunologically mediated diseases with autoimmune features. Morphological changes of intestinal mucosa are thought to be initiated by a T-cell-mediated reaction to dietary gluten in genetically susceptible individuals (Goggins and Kelleher 1994). Gliadin-specific T-cell clones from the small intestinal mucosa of patients with coeliac disease are predominantly restricted by the coeliac disease-associated HLA-DQ2 and HLA-DQ8 molecules, suggesting a link between the HLA association and immunopathogenesis (Lundin *et al.* 1997). Immunological markers used for screening and diagnosis of coeliac disease are circulating antigliadin antibodies, antiendomysium, antijejunal, antireticulin and anticalreticulin autoantibodies (Sategna-Guidetti *et al.* 1995, Tučková *et al.* 1995, 1997, Ascher *et al.* 1996).

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Animal models represent a powerful tool for elucidating the pathogenetic mechanisms involved in the development of human diseases. Experimental enteropathy induced in rats by long-term feeding with gliadin (a major protein of gluten) has many features in common with human CD (Štěpánková *et al.* 1989, 1996, Tlaskalová-Hogenová *et al.* 1995a). A two-month intragastric administration of gliadin starting at birth to germ-free Wistar AVN rats fed an artificial milk diet caused mucosal lesions (shortening of jejunal villi, crypt hyperplasia, increased number of mitoses in the crypt epithelium, and enhanced the number of intestinal $CD8\alpha\beta^+$ and $CD4^+$ lymphocytes) (Štěpánková *et al.* 1995). The changes of the intestinal mucosa were less pronounced in AVN rat pups suckled with mother's milk (Štěpánková *et al.* 1997a). While gliadin administered similarly to immunocompetent mice of various strains did not induce morphological changes of the intestinal mucosa (Tlaskalová-Hogenová *et al.* 1995a, 1995b), immunodeficient athymic (nude) mice revealed signs of enteropathy (Tlaskalová-Hogenová *et al.* 1997, Kozáková *et al.* 1998).

It seems to be of importance to analyze the potential effect of mother's milk in the protection against mucosal damage after gliadin and to compare the immune response of suckling and artificially fed rats. The aims of our study were to compare the effect of long-term gliadin feeding on rats nourished from birth to weaning with either an artificial milk diet or those maternally fed: (1) to analyze the proliferative response to gliadin of lymphocytes isolated from the spleen and mesenteric lymph nodes, (2) the level of antigliadin antibodies in supernatants from a 7-day spleen lymphocyte culture, and (3) to detect spontaneous apoptosis of mesenteric lymph node cells.

Material and Methods

Animals

55-day-old inbred rats of the Wistar AVN strain were used in our experiments. Some of the rats were delivered in the natural way and suckled till weaning by their mothers. Half of the rats were Cesarean-delivered and deprived of mother's milk, receiving a milk diet through a silicone tubing 0.2 mm in diameter. The tube was introduced into the esophagus and the animals were fed every four hours until the stomach was full (0.15 ml on the first day and up to 1 ml). At day 16, the groups of artificially fed young were put on spontaneous food intake, using the same liquid diets as before, but adding 10 g rice starch per 100 ml. On 21 days, all the groups – both artificially fed and suckled – were fed the same granulated pellet food *ad libitum* (Štěpánková *et al.* 1985, 1990, 1997b). Animals from both groups were split into two groups. One group of suckling rats and one group of hand-fed rats were given gliadin intragastrically (from 0.5% to 10% in 0.02 M acetic acid, Sigma) according to their body weight (Table 1). The animals from the second group serving as controls were given human serum albumin in the same concentrations.

Table 1. Experimental design

Day of life	Concentration of gliadin or albumin (%)	Volume administered (ml)
0	0.5	0.1
3	0.5	0.2
7	0.5	0.3
10	0.5	0.3
14	5.0	0.7
20	5.0	0.7
44	10.0	1.0
46	10.0	2.0
48	10.0	5.0
52	10.0	5.0

Preparation of cells

Cell suspensions of lymphocytes from the spleen or mesenteric lymph nodes were prepared by careful teasing with two forceps in RPMI 1640 (Sigma) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Inst. Sera and Vaccines, Prague) and 2 % (heat-inactivated) fetal calf serum (Sigma), buffered by 10 mM HEPES (Serva), pH 7.3. Cell suspensions were washed twice and filtered for removing clumps using Nybolt bolting cloth (pore 40 μm) (Seidengazfabrik, Switzerland).

Lymphocyte proliferation

Isolated cells were washed out into RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin supplemented with 2 mM L-glutamine (Serva), 5 mM sodium pyruvate (Lachema, Brno) and $5x10^{-5}$ M mercaptoethanol (Sigma) and 5 % (heat-inactivated) fetal calf serum, buffered by 10 mM HEPES, pH 7.3. The cells, in a concentration of $5x10^5$/well, were

seeded in a 96 flat-bottomed microwell plate (Costar, Cambridge MA, USA) and incubated with or without 100 μg/ml gliadin (Sigma) in a final volume of 250 μl. Incubation proceeded in a humified atmosphere (37 °C, 5 % CO_2) for 54 h, at which time each well was pulsed with 10 μl (37 kBq) ^{3}H-thymidine (UVVR, Czech Republic) and the cultivation was carried on for another 18 h. The cells were filtered onto GF/C filters (Whatman, Great Britain) and washed with water. The filters were counted in 2 ml scintillation fluid OptiPhase HiSafe II (LKB Wallac England) using a Rackbeta 1214 (LKB) beta-counter. The results are expressed as mean counts per minute (cpm). A computer program was used for planning and evaluating these microplate experiments (Šiman 1992).

In vitro antigliadin antibody response and ELISA of antigliadin antibodies

Cells isolated from spleens were cultivated in RMPI 1640 medium in a concentration of 10^6/ml in a 24- well plate (Costar, Cambridge MA, USA). Cell suspensions were cultured with or without gliadin (100 μg/ml) in a final volume of 1 ml. Incubation proceeded in humidified atmosphere (37 °C, 5 % CO_2) for 7 days. Cell-free supernatants were collected and the levels of antigliadin antibodies were evaluated by modified Enzyme Linked Immunosorbent Assay (ELISA) method (Tučková *et al.* 1995), using peroxidase-labeled sheep anti-rat Ig antibodies.

Gel electrophoresis DNA fragmentation assay

A modified method of Newell *et al.* (1990) was used. Briefly, the pellet of 2.10^6 cells isolated from mesenteric lymph nodes (described above) was resuspended in 400 μl of lysis buffer (0.2 % Triton X-100, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, all from Sigma). Cell lysates were immediately spun down and the supernatants containing small DNA fragments were immediately separated from the pellet. DNA fragments were precipitated from the supernatants using 50 % isopropanol (Sigma) and 0.5 M NaCl and then collected by centrifugation, washed with 70 % ethanol, air-dried, and dissolved in Tris-EDTA buffer. The loading buffer containing 10 mM EDTA, 50 % glycerol, and 0.25 % bromophenol blue (Sigma) was added to the samples and electrophoresis was performed on 1 % agarose slab gels containing 1 μg/ml ethidium bromide (Molecular Probes). DNA ladders were visualized by UV light and photographed.

Statistical analysis

The results were expressed as means ± S.D. from 8-16 animals for each experimental group. Statistical analyses were performed using SigmaStat (Jandel Corporation). Multiple comparison procedures were made by one way analysis of variance (ANOVA), Student-Newman-Keuls method. $P<0.05$ values were considered significant.

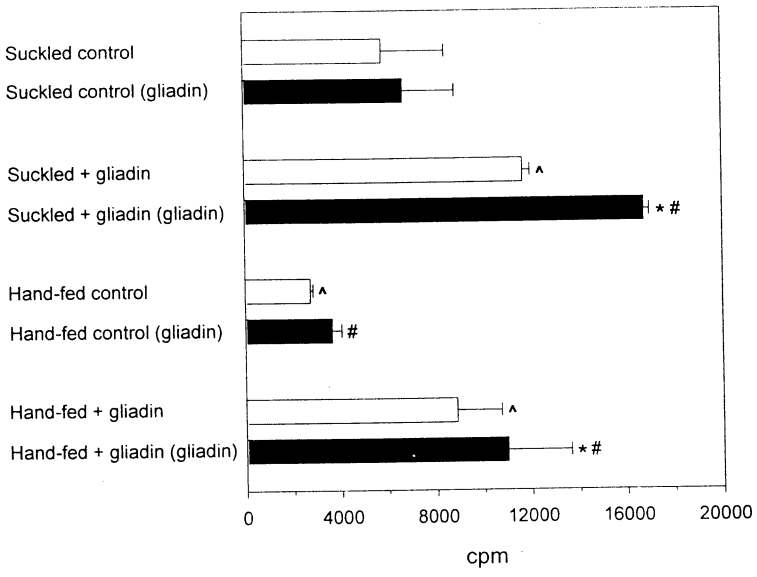

Fig. 1. *Effect of gliadin feeding on spleen lymphocyte proliferation. Cells isolated from each group of gliadin-treated and control rats were equally divided, half of the cells were cultivated in vitro with or without gliadin (as shown in brackets). Data were expressed as ^{3}H-thymidin incorporation (cpm) ± S.D. * indicates a significant effect on proliferation ($P<0.05$) following in vitro gliadin addition, ^ indicates a significant effect ($P<0.05$) on the proliferative response of cells cultivated without gliadin when data were compared with the response of cells from suckled controls (1st column on the top), # indicates a significant effect ($P<0.05$) on the proliferative response of cells cultivated with gliadin when data were compared with the response of cells from suckled controls (2nd column on the top).*

Results

Effect of gliadin feeding on proliferation of spleen or mesenteric lymph node lymphocytes

The spontaneous proliferation was significantly enhanced in spleen cell cultures isolated from both groups of gliadin-treated rats (suckled, hand-fed) in comparison with control cells from albumin-treated animals. The highest proliferative response was detected in spleen cell suspensions from suckled gliadin-treated rats stimulated *in vitro* with gliadin (Fig. 1). Proliferative responses after gliadin stimulation *in vitro* were also significantly increased in spleen lymphocytes isolated from hand-fed gliadin-treated rats.

Spontaneous proliferation of mesenteric lymph node cells isolated from hand-fed gliadin-treated rats was higher than that in hand-fed controls, since the *in vitro* gliadin treatment increased the proliferative responses slightly, but significantly, in all the experimental groups (Fig. 2).

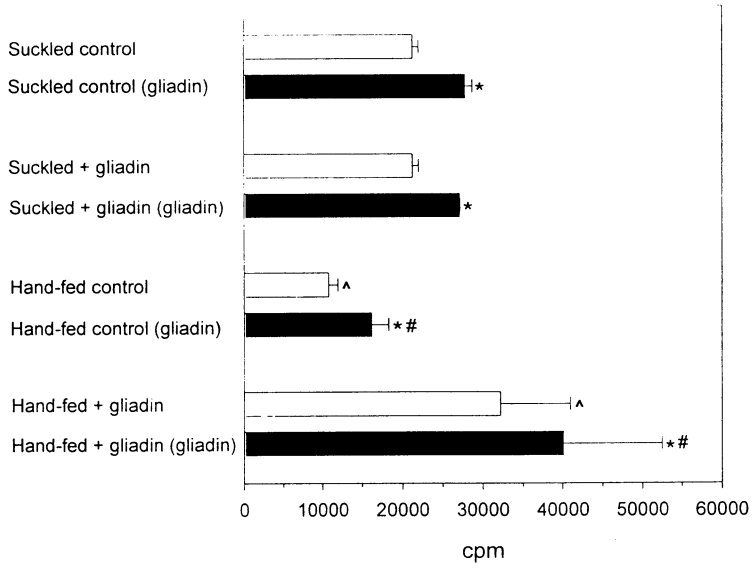

Fig. 2. *Effect of gliadin feeding on mesenteric lymph node cell proliferation. Cells isolated from each group of gliadin-treated and control rats were equally divided, half of the cells were cultivated in vitro with or without gliadin (as shown in brackets). Data were expressed as ^{3}H-thymidin incorporation (cpm) ± S.D. * indicates a significant effect on proliferation (P<0.05) following in vitro gliadin addition, ^ indicates a significant effect (P<0.05) on the proliferative response of cells cultivated without gliadin when data were compared with the response of cells from suckled controls (1st column on the top), # indicates a significant effect (P<0.05) on the proliferative response of cells cultivated with gliadin when data were compared with the response of cells from suckled controls (2nd column on the top).*

Effect of gliadin feeding on production of antigliadin antibodies by spleen lymphocytes

The levels of antigliadin antibodies measured by ELISA was very low in supernatants obtained from spleen lymphocytes isolated from both suckled and hand-fed albumin-treated control animals. The significantly increased spontaneous response in suckling gliadin-treated rats, considered as 100 %, was not enhanced by *in vitro* gliadin stimulation. Moreover, the highest antibody production by spleen cells isolated from gliadin-treated hand-fed animals was reduced after *in vitro* gliadin stimulation (Fig. 3).

Effect of gliadin feeding on apoptosis of mesenteric lymph node cells

Freshly isolated mesenteric lymph node cells from hand-fed or suckled rats with both the albumin or gliadin diet were cultured and harvested after 12 h for DNA fragmentation assay. Agarose gel electrophoresis indicated no sign of programmed cell death in freshly isolated cells or cells kept on ice for 12 h (Fig. 4, I and J, respectively). However, samples cultured at 37 °C for 12 h exhibited an evidence of progressive DNA fragmentation manifested as multiple 180 to 200-bp units of DNA (Fig. 4, A-H). In this respect, the level of apoptosis was independent of the manner of feeding; the

same level of apoptosis was found in hand-fed and suckled animals (Fig. 4, A-B and C-D versus E-F and G-H, respectively). On the other hand, the level of apoptosis of MLN lymphocytes from albumin-treated animals (Fig. 4, A-B and E-F) was significantly lower than that in gliadin-treated animals (Fig. 4, C-D and G-H).

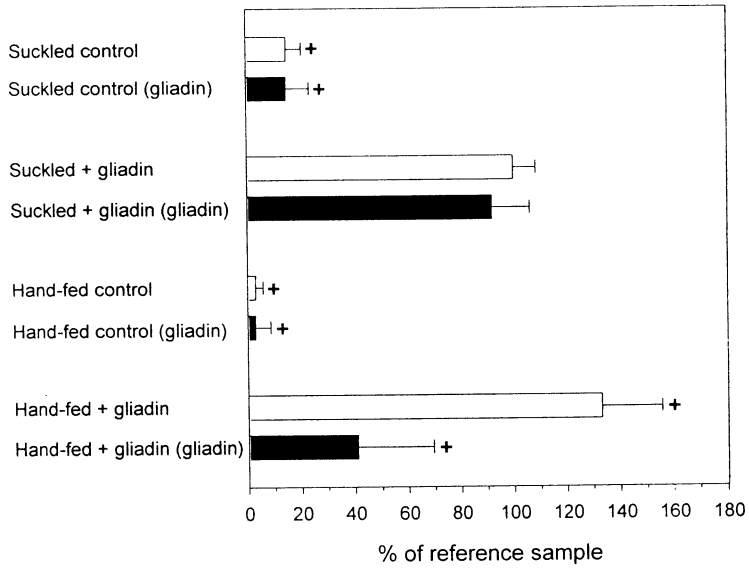

Fig. 3. *Levels of antigliadin antibodies secreted in vitro from splenic lymphocytes of gliadin-fed rats compared to the internal control (the level of antigliadin antibody produced by suckled gliadin-fed rats: 100 %).* $^{+}$ *indicates a significant changes ($P<0.05$) in levels of antigliadin antibodies compared to internal control.*

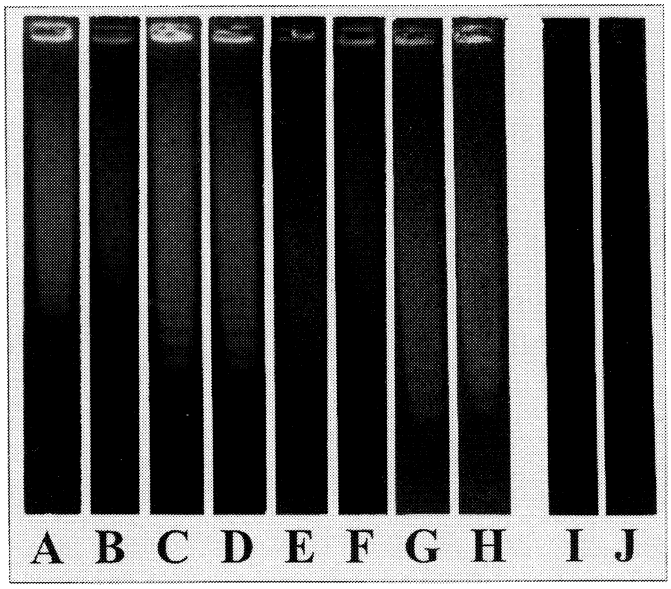

Fig. 4. *Agarose gel electrophoresis ladders (DNA fragments) of fresh MLN cells (I), MLN cells incubated at 4 °C for 12 h (J) and MLN cells incubated at 37 °C for 12 h: A & B – MLN cells from albumin-treated hand-fed rats; C & D – MLN cells from gliadin-treated hand-fed rats; E & F – MLN cells from albumin-treated suckled rats; G & H – MLN cells from gliadin-treated suckled rats.*

Discussion

Using the experimental model of gluten-induced enteropathy, we tried to analyze the immune responses to gluten at the cellular and humoral levels. Systemic immune responses were evaluated by determining the proliferative response of spleen and mesenteric lymph node cells and the *in vitro* production of antigliadin antibodies. Spleen lymphocytes isolated from gliadin-treated rats exhibited higher spontaneous proliferation than lymphocytes isolated from their control counterparts. However, the proliferative responses increased when lymphocytes, isolated from both suckled and hand-fed gliadin-treated rats, were stimulated *in vitro* with gliadin. The level of antigliadin antibodies, determined in culture media after 7-day cultivation of spleen lymphocytes

isolated from 55-day-old suckled and hand-fed control (without gliadin) rats, was very low even when the cells were cultivated with gliadin. There was, however, a significantly higher release of antigliadin antibodies in culture media of lymphocytes isolated from animals intragastrically treated with gliadin than in that of the controls. Interestingly, additional gliadin stimulation *in vitro* had no effect on the production of antigliadin antibodies in suckling animals or even decreased their production.

Oral tolerance breakdown was suggested as one of the possible mechanisms causing coeliac disease (Troncone 1992). The aim of our study was to analyze the immune responses in an effort to elucidate the mechanisms involved in experimentally induced gluten enteropathy which resembles human coeliac disease. Our results concerning the proliferative responses suggest that the suppressive mechanisms responsible for oral tolerance induction were not operating in our experimental model (proliferative response after *in vitro* gluten challenge was not inhibited). On the other hand, no increase of the level of specific antibodies was found after *in vitro* gluten application. These results could be explained as being due to a suppression of B-cell activity or by their non-reactivity caused by exhaustion (Weiner 1996).

Apoptosis, programmed cell death or "cell suicide", as distinct from passive cell death or necrosis, is an active physiological process. In tissues that undergo continual cellular turnover, such as the gut, homeostasis is dependent upon the balance between cell proliferation and apoptosis (Thompson 1995). Previous results from *in vivo* experiments with untreated coeliac patients showed that the majority of cells which undergo apoptosis in the gut are epithelial cells whereas apoptotic lymphocytes were seen only occasionally (Moss *et al.* 1996). This is in accordance with our findings where few if any apoptotic cells were detected in freshly isolated MLN lymphocytes. Such results seem to indicate that apoptotic lymphocytes *in vivo* are probably cleared from the organism by macrophages before any DNA-fragmentation can be detected. However, the same lymphocytes cultured at 37 °C for 12 h showed evidence of progressive DNA fragmentation. In this respect, the level of apoptosis did not depend on whether the animals were hand-fed or suckled, but the apoptosis of MLN lymphocytes in animals with gliadin diet was significantly higher than in control ones. These results indicate that some lymphocytes are preprogrammed for cell death in animals with enteropathy. It follows that the increased apoptosis of these lymphocytes could indicate their inability to mount a normal immune response and therefore to participate in coeliac disease.

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