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Effect of γ-interferon on binding of gliadin and other food peptides to the human intestinal cell line HT-29

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Abstract

γ-Interferon is one of the main cytokines released during activation of intestinal lymphocytes in coeliac patients. The question has never been addressed whether γ-interferon influences binding of gliadin and other food peptides to human enterocytes. Therefore, the human intestinal epithelial cell line HT-29 was cultured with gliadin, casein, β-lactoglobulin and ovalbumin, with or without γ-interferon, and peptide binding to cells was determined by flow cytometry and fluorescence microscopy. γ-Interferon stimulated gliadin binding by a factor of 4. Binding was saturable with half maximal binding at 0.15 mg/ml. For maximal binding, an incubation of at least 24 h was necessary. γ-Interferon increased binding of β-lactoglobulin and casein, too, but inhibited that of ovalbumin. Binding of gliadin was inhibited by the other peptides. Under the conditions of ongoing mucosal inflammatory reactions and release of γ-interferon, enhanced binding may trigger intestinal lymphocytes, increase secretion of cytokines and thus induce a vicious circle. © 1997 Elsevier Science B.V.

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1. Introduction

The epithelial lining of the small intestine represents a barrier shielding intestinal immune cells from foreign food proteins. However, very low amounts of immunogenic peptides may escape digestion, penetrate the epithelium and elicit a pathological immune response [1,2]. Wheat gliadins and prolamins from related cereals increase lymphocytic infiltration of the epithelium and the lamina propria in coeliac patients, releasing cytokines [3–5] and inducing damage of the mucosa. One of the reasons for up-regulation of the immune response in coeliac disease may be enhanced delivery of antigenic peptides due to increased binding of gliadins to enterocyte membranes. Increased binding of other food peptides may have pathological consequences, too.

Food peptide binding to different cells has already been investigated often. Concerning intestinal epithelial cells, binding of food peptides, including gliadin, was demonstrated to rat microvillus membrane proteins [6–9], of gliadin peptides to isolated glycoproteins of rat brush border membranes [10], to the intestinal epithelial cell line HT-29 [11], to isolated rat enterocytes and human enterocytes from coeliac patients in remission as well as from controls [12], and to crypt cells of human biopsies from patients with active coeliac disease but not from disease controls or patients in remission [13]. Further data demonstrate uptake of gliadin into enterocytes [14,15], suggesting prior binding of gliadin to cell membranes.

From the cytokines released in coeliac disease, interferon-γ (γ-IFN) has been shown to play an important role [4,16]. Until now, the influence of γ-IFN on binding of food peptides has not been addressed. Using the human intestinal epithelial cell line, HT-29, we demonstrate that gliadin and other food peptides are bound significantly to enterocytic cell membranes and that binding is strongly influenced by γ-IFN.

2. Materials and methods

2.1. Food proteins and antibodies

Gliadin was obtained by ethanolic extraction of flour of wheat cv. "Kanzler" [17] and purified by ion-exchange chromatography on DEAE–cellulose [18]. Gliadin was partially hydrolysed by trypsin as described previously [19]. According to SDS–polyacrylamide gel electrophoresis, the molecular weight of the digested gliadin (Gli) was between 12.5 and 29 kDa. Ovalbumin (Ova) from chicken egg, β-lactoglobulin (Blg) and casein (Cas) from bovine milk were obtained from Sigma (A-7641, L-0130, C-4032).

Antisera against Gli were obtained by immunisation of rabbits with gliadin obtained from wheat cv. Kanzler. Rabbit anti-chicken egg albumin was from ICN Biochemicals (65-115), rabbit anti-bovine β-lactoglobulin was from Bibby Dunn (APBK 2905) and sheep anti-bovine casein was from Biozol (J030). FITC-conjugated swine anti-rabbit immunoglobulins (DAKO; F205) and FITC-conjugated rabbit anti-sheep immunoglobulins (Dako, F135) were used as secondary antibodies.

2.2. Cell culture

The human colonic adenocarcinoma cell line, HT-29, was maintained at 37°C in a humidified atmosphere of 5% CO_2 in DMEM (Serva 15604) supplemented with 10% foetal calf serum, HEPES (10 mmol/l), non-essential amino acids (1%), glutamine (2 mmol/l), $NaHCO_3$ (10 mmol/l), mercaptoethanol (50 μmol/l) and gentamycin (200 mg/l). After trypsinisation, the cells were transferred to tissue culture flasks (Greiner, 25 cm^2, $5 \cdot 10^5$ cells/12 ml of medium). After 24 h, fresh medium (±γ-IFN, human, recombinant, Boehringer Mannheim, 15.6 U/ml) was added and the cells were cultured for a further 120 h according to one of the 2 protocols outlined below. After culture, the cells were harvested by trypsin treatment, washed and resuspended in phosphate buffered saline (PBS) for investigation by flow cytometry or in PBS plus NaN_3 for fluorescence microscopy.

2.2.1. Culture protocol I

Gli was present for 120 h or was added in a time-dependent manner. Control experiments were performed without Gli.

2.2.2. Culture protocol II

48 h before harvesting, the medium was renewed. Either Gli and other food peptides were added only 24 h before the end of culture or Gli was added in a time-dependent manner from time zero. When Gli and/or γ-IFN were already present before medium change, the fresh medium was supplied with Gli and/or γ-IFN again. Control experiments were performed without Gli.

2.3. Fluorescence analysis

2.3.1. Flow cytometry

A 50-μl volume of a suspension containing 10^6 cells was incubated for 30 min at 4°C with 50 μl of antibodies against food peptides (anti-Gli 1:5, v/v; anti-Ova, anti-Blg and anti-Cas, 1:2, v/v), washed 3 times in PBS and incubated for a further 30 min at 4°C with 50 μl of FITC-conjugated secondary antibody (1:25, v/v). Then the cells were fixed for 10 min in the dark in 1 ml of fixing buffer (1% formalin in PBS), washed 3 times in PBS, and resuspended in 175 μl of fixing buffer. Fluorescence intensity was measured using a FACSscan analyser (Becton Dickinson). The cells were acquired and analysed using LYSIS II software. The density of expression of binding sites for the rabbit and sheep antibodies was taken as a measure for binding of the food peptides and was given as the mean fluorescence intensity.

2.3.2. Fluorescence microscopy

Cells were processed respectively but fixed cells were mixed 1:1 (v/v) with PBS buffered glycerol.

2.3.3. Fluorescence analysis on dot blots

Defined quantities of food peptides (32 ng–32 μg) were applied to nitrocellulose sheets in an Easy Titer ELIFA system (Pierce). The sheets were blocked (50 mmol/L Tris, 150 mmol/L NaCl, pH 10.2, 2% Tween 20), washed (50 mmol/L Tris, 150 mmol/L NaCl, pH 10.2, 0.05% Tween 20), and incubated with antibodies under the conditions used for flow cytometric analysis. Controls were performed without peptides. Fluorescence intensity was measured by means of a plate reader connected to a Luminescence Spectrophotometer LS 50B (Perkin Elmer) and analysed using GRAMS/386TM-Software (Galactic Industries). The analysis was kindly performed in the Environmental Research Centre Leipzig (Dr. Geyer).

2.4. Expression of results

Results concerning food protein binding are expressed as an increase in mean fluorescence intensity (ΔMFI) above control experiments (means±S.E.M.). Statistical differences ($P \leq 0.05$) were evaluated by the 2-tailed rank sum test according to Wilcoxon for paired (*) or for unpaired experiments (°).

3. Results

After 5 days of culture (protocol I) in the presence of Gli (0.25 mg/ml), FACSscan analysis revealed a significant binding of Gli to HT-29 cells. The binding was enhanced considerably in the presence of γ-IFN. With rabbit control sera, no difference could be found between experiments with and without Gli (Fig. 1). In fluorescence microscopy, Gli produced a weak surface fluorescence only in the presence of γ-IFN.

Binding of Gli was dependent on Gli concentration and a saturable binding curve was obtained (Fig. 2). Binding was not saturated at the highest concentration investigated (0.25 mg/ml). According to the Scatchard plot, binding

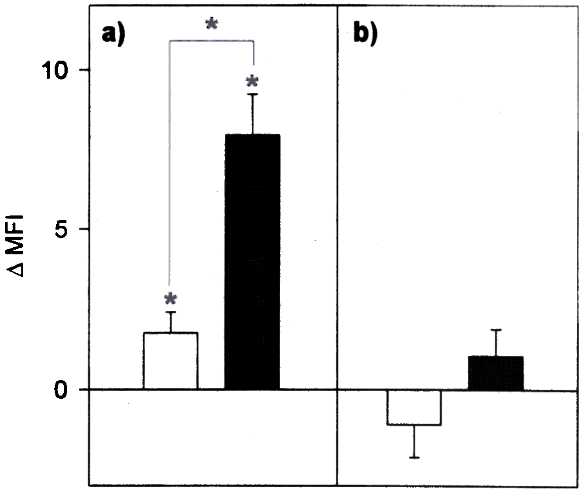

Fig. 1. Effect of γ-IFN on binding of Gli to HT-29 cells (culture protocol I). Concentration of Gli, 0.25 mg/ml. Open columns = experiments without γ-IFN; shadowed columns = experiments with γ-IFN. (a) Gli binding determined using Gli antibodies, means±S.E.M. of 14 experiments; (b) controls performed with control sera, means±S.E.M. of at least 2 experiments with 6 different sera. * = Significant binding and significant difference between experiments with and without γ-IFN. ΔMFI = increase in mean fluorescence intensity compared with that of controls.

was half-maximal at 0.15 mg/ml. Significant binding was still demonstrable at 0.0625 mg/ml.

For maximal binding of Gli, an incubation time of at least 24 h was necessary (Fig. 3). When old culture medium was replaced by fresh medium 48 h before

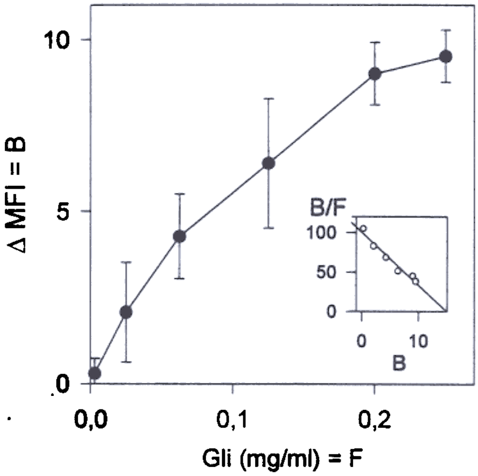

Fig. 2. Concentration dependence of binding of Gli to HT-29 cells in the presence of γ-IFN (culture protocol I). Means±S.E.M. of at least 3 (only 1 at 0.2 mg/ml) experiments. Inset: Scatchard plot of the data.

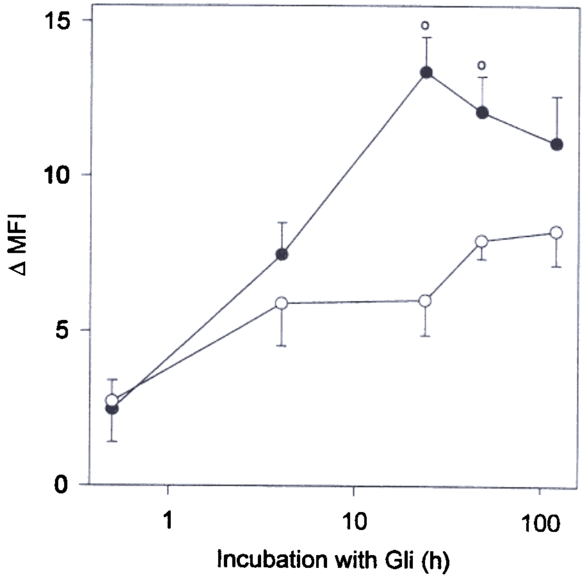

Fig. 3. Time course of binding of Gli to HT-29 cells. Concentration of Gli, 0.25 mg/ml. Means±S.E.M. of at least 5 experiments. Open circles = without medium change (protocol I); closed circles = with change of medium 48 h before harvesting (protocol II). ° = significant difference between cultures with and without medium change.

the end of culture (protocol II), binding was significantly enhanced. Therefore, in the following experiments, medium was renewed 48 h before the end of culture and food peptides were added 24 h before harvesting.

Other food peptides (Ova, Blg, Cas) bound to HT-29 cells as well (Fig. 4). However, whereas γ-IFN elevated binding of Gli, Blg and Cas, binding of Ova was inhibited. Ova, Blg and Cas produced typical surface fluorescence. Binding of Gli (0.05 mg/ml) was inhibited by the presence of Ova, Blg and Cas in 20-fold excess (Fig. 5).

To compare binding of the different peptides quantitatively, defined peptide amounts were applied to nitrocellulose and incubated with primary and secondary antibodies. The same amount of the various peptides produced different fluorescence signals (see inset in Fig. 4). The signals for Blg or Cas were 7- or 9-times higher, respectively, than that for Gli, indicating different sensitivities of the assays of the 3 peptides. The sensitivities of the assay of Ova and Gli were nearly identical. If the fluorescence signals were corrected for the different sensitivities, binding of Gli, Ova, Blg and Cas could be compared quantitatively. Thus, in the presence of γ-IFN, binding of Gli was in the range of that of the other food peptides.

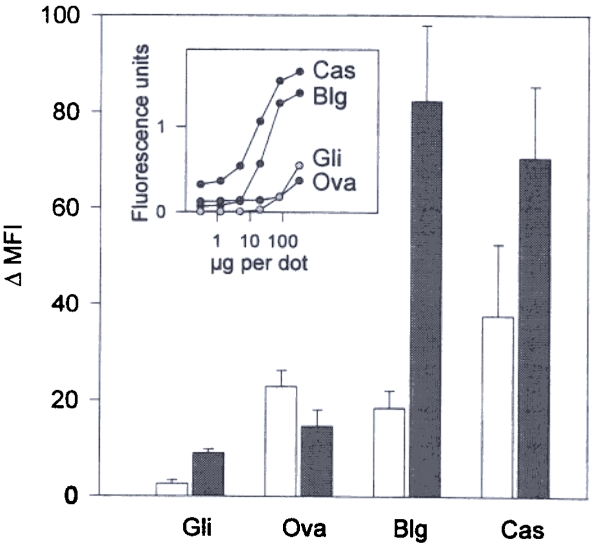

Fig. 4. Binding of Ova, Blg and Cas to HT-29 cells in comparison to the binding of Gli (protocol II). Concentration of food peptides, 0.25 mg/ml. Open columns = experiments without γ-IFN; shadowed columns = experiments with γ-IFN. Means±S.E.M. of at least 6 experiments. Binding and difference between binding in the presence and absence of γ-IFN is significant in each case. Inset: Comparison of fluorescence signals when defined amounts of different food peptides were applied to nitrocellulose. Means of 3 experiments.

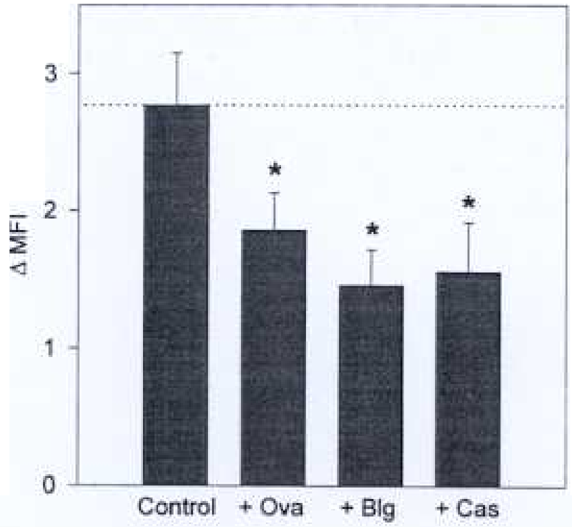

Fig. 5. Inhibition of binding of Gli to HT-29 cells by other food peptides in the presence of γ-IFN (protocol II). Concentration of Gli, 0.05 mg/ml; of the other food peptides, 1 mg/ml. Control: culture with Gli but without competitive food peptides. Means±S.E.M. of at least 6 experiments.

4. Discussion

γ-IFN is one of the important cytokines released during activation of intestinal lymphocytes. Production of mRNA for γ-IFN is increased in the lamina propria of untreated coeliac patients [4] and in cultured biopsies of treated patients after stimulation with gliadin peptides [5]. The deleterious effect on cultured biopsies of supernatants of HLA-DQ2-restricted T-cell clones derived from coeliac patients and stimulated with gliadin could be mimicked by γ-IFN and blocked by antibodies against this cytokine [14]. Besides γ-IFN, which represented the main secretion product, interleukins 4, 5, 6 and 10, tumor necrosis factor α and transforming growth factor β were found in cell culture supernatants of gliadin-stimulated mucosal [3] and peripheral [20] T-cells.

γ-IFN acts via gene activation and the effects observed until now are diverse. Concerning intestinal epithelial cells, γ-IFN has been shown to induce expression of MHC class-II molecules on the surface of enterocytes of biopsies cultured in vitro [21,22] and on HT-29 cells [4,23,24] as well as on other enterocytic cell lines [25]. Furthermore, in HT-29 and other colon epithelial cell lines, the cytokine elicited the expression of carcinoembryonic antigen [26–28], of intercellular adhesion molecule-1 [29–31], of biliary glycoprotein [28], of glutaminase [32], stimulated the secretion of interleukin 8 [33] and down-regulated cystic fibrosis transmembrane regulator mRNA [34]. Moreover, γ-IFN increased the tight junctions' permeability [35,36] and influenced neutrophil migration across intestinal epithelial monolayers [37] and the binding of intraepithelial lymphocytes to colon cancer cells [38]. Our results demonstrated for the first time that γ-IFN was also able to affect the binding of food peptides to enterocytes.

The binding curve of Gli indicates that, in the presence of γ-IFN, the number of binding sites is limited, suggesting specific (selective) receptor sites, however, the concentration necessary for half maximal binding is high. The competition of other food peptides with gliadin for binding argues for common binding sites. Until now, only speculation existed about the mechanism of binding. The initial concept of a lectin-like binding of gliadin to enterocytes or brush border membrane glycoproteins was questioned [12,13,39]. Instead, binding may be mediated by hydrophobic interactions between apolar peptides and hydrophobic membrane areas [40] to which other food peptides may also be bound. In contrast to Gli, Blg and Cas, γ-IFN decreases the binding of Ova, demonstrating the different requirements of the peptides for binding to enterocytes.

Until now, there were no studies on the kinetics of gliadin binding. The long time necessary for maximal binding may reflect either a slow process of association, or metabolic events must first be initiated in the enterocytes by gliadin so that binding can take place. Recently, binding of peptides to isolated MHC class-II molecules has been shown to occur within several days [41]. The effect of changing the medium 48 h before harvesting the cells may be due mainly to the fresh γ-IFN that was supplied with the medium. However, the renewed supply of substrates and growth factors might also be important for keeping the cells in a metabolic or differentiation state that is susceptible to the binding of Gli.

Binding of food peptides may represent an important step in the induction of food intolerances. The binding capacity of the cell membranes may contribute to regulation of the delivery of antigens to intestinal T-cells. Under the conditions of on-going inflammatory reactions, γ-IFN is secreted and enhances binding of Gli. At the same time, γ-IFN induces MHC class-II molecules and this process is enhanced in the presence of Gli [19]. Increased MHC-expression would trigger T cells which, in turn, would release more γ-IFN, thus initiating a vicious circle. Of the other food peptides, only binding of Blg and Cas was stimulated by γ-IFN. Cas has been shown to increase the γ-IFN-induced expression of MHC class-II molecules, too [19]. The capacity of enterocytes to bind these peptides is in the same range as that of Gli. However, when the different degrees of digestibility of the food peptides is taken into account, with proline-rich gliadins representing poorly digestible substrates [42–44], and when the limited capacity of coeliac patients in remission is considered to hydrolyse gliadin peptides [45,46], the effective concentration of gliadins in the proximity of the enterocytes and, thus, their effectiveness may even be higher than that of other peptides.

Thus, HT-29 cells from colon cancer represent a useful tool for the study of food peptide binding. Even if difficult to obtain and to use, however, non-malignant primary enterocytes should be applied in future investigations to verify the findings.

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