



Wheat gliadin promotes the interleukin-4-induced IgE production by normal human peripheral mononuclear cells through a redox-dependent mechanism

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Abstract

Increased levels of serum IgE have been described in gliadin-intolerant patients; however, biological mechanisms implicated in this immunoglobulin production remained unknown. In this study, we demonstrated that *in vitro* crude gliadins and gliadin lysates (Glilys) promoted the IL-4-induced IgE production by human peripheral blood mononuclear cells (PBMC), indicating that the biological process related to gliadin intolerance and/or allergy may lead to IgE production *in vivo*. It was found that crude gliadin and Glilys potentiated, after 13 days of culture in a dose-dependent manner, IL-4-induced IgE production and, to a lesser extent, the IgG production, while they did not affect IgA or IgM productions. This promoting effect of gliadin and Glilys on the IL-4-induced activation of normal human PBMC was also observed on the early release (2 days) of the soluble fraction of CD23, suggesting its possible involvement in IgE potentiation. The promoting effect of crude gliadin and Glilys appeared to be indirect because they did not modify purified B-lymphocytes IgE production after IL-4 and anti-CD40 monoclonal antibody stimulation.

In addition, as revealed by luminol-dependent chemiluminescence, we demonstrated that crude gliadin and Glilys promoted a substantial production of free radicals by normal human PBMC, treated or not with IL-4. This redox imbalance associated with an increased IgE production led us to evaluate the effect of pharmacological antioxidants (*N*-acetyl-cysteine (NAC) and Cu/Zn-superoxide dismutase (SOD1)) on IgE production by human PBMC. The NAC and the intracellularly delivered SOD1 were found to suppress the IL-4 ± crude gliadin or Glilys-induced IgE production by normal human PBMC. Taken together, these data indicated that gliadin specifically enhanced IL-4-induced IgE production by normal human PBMC, probably by the regulation of redox pathways, and that this 'pro-allergenic' effect could be counteracted by natural antioxidants: thiols and/or vectorized SOD1. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Gliadin; IgE; Thiol; Superoxide dismutase; Allergy

Abbreviations: Gli, gliadin; Glilys, lysate of gliadin; NAC, *N*-acetyl-cysteine; PBMC, peripheral blood mononuclear cells; SOD1, Cu/Zn-superoxide dismutase

1. Introduction

Although recent data have indicated an elevation of IgE in gliadin-intolerant patients [1,2], little is known

about mediators and cellular mechanisms involved in such IgE over-production. The generation of specific IgE against gliadin in atopic patients and the absence of correlation between the IgE over-expression and symptomatic status of patients suggested that IgE dysregulation is rather the consequence of gliadin intolerance than the real inducing effect [3–5]. The generation of IgE production by human B-lymphocytes is mainly controlled by interleukin (IL)-4 [6] produced by TH₂-lymphocytes,

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mast cells or basophils [7,8] and by CD40–CD40 ligand interactions between B and T-lymphocytes [9]. IL-4, a TH₂ cytokine, that is up-regulated during gliadin intolerance [10], modulates the phenotype and function of B- and T-lymphocytes and monocytes/macrophages [11,12]. On B cells and monocytes/macrophages, IL-4 induces CD23-expression and release [13,14], which amplifies IL-4-induced IgE production [15]. In addition to these immunological mechanisms, it has been recently shown that cellular redox status also controlled this production [16–20]. Indeed, the redox metabolism is essential for controlling dendritic cell functions [21–23], and thus it controls the protective immunity/immunopathology balance [24]. Recent data suggested that gliadin induced a redox imbalance in gastro-intestinal epithelial cells and macrophages probably by an over-production of free radicals [25–27], suggesting a possible link between elevated IgE production and the redox imbalance in gliadin-intolerant patients.

The aim of the present study was to evaluate: (i) the regulatory effects of native gliadin and gliadin lysates (Gliyls) on the spontaneous and the IL-4-induced IgE production by normal human peripheral blood mononuclear cells (PBMC); (ii) the possible involvement of free radicals in the regulation of IgE production; and (iii) the potent pharmacological role of enzymatic (Cu/Zn-superoxide dismutase (SOD1)) and non-enzymatic (thiols) antioxidants.

2. Materials and methods

2.1. Reagents

Recombinant human IL-4 from *Escherichia coli* (5×10^6 U/mg) was purchased from Immugenex (Los Angeles, CA, USA). All culture reagents and the Ficoll–Hypaque density gradient were from Seromed (Biochrom, Berlin, Germany). Human and bovine SOD1 were obtained from Sigma (St. Louis, MO, USA). Gliadin (prolamine extracted from wheat) batches were from two different sources: the crude Gliadin product was from Sigma, and Gliamine® and crude zein (another class of vegetal prolamine extracted from maize) were from Lavipharm SA (Paris, France). Anti-CD19 antibody-coupled magnetic beads used to purify human B-lymphocytes were purchased from Dynal (Compiègne, France). Specific ELISA kits for human sCD23 were from Medgenix (Liège, Belgium), and sCD23 determinations were conducted according to the manufacturer's instructions, the threshold sensitivity of this test being 0.2 ng/ml. The rabbit polyclonal antibody against human IgE used for the coating of the ELISA plates was from Dako (Les Ulis, France), the monoclonal mouse anti-human IgE used to reveal IgE (I27) was kindly provided by Dr F. Brière (Schering Plough, Dardilly, France) and

the peroxidase-coupled polyclonal antibody (goat anti-mouse) was from Biosys (Compiègne, France). Standard Phadexact total IgE serum used to calibrate the ELISA was from Pharmacia (Les Ulis, France). Monoclonal peroxidase-coupled and polyclonal antibodies used for the detection of IgA, IgM and IgG and human standard proteins, IgG, IgA, IgM, were from Sigma. The mouse anti-human CD40 monoclonal antibody (mAb) was purchased from Diaclone (Besançon, France).

2.2. Gliadin lysates

The pepsin/trypsin digests of gliadin (Gliyls-1) and gliamine® (Gliyls-2) were prepared as follows. Gliadin or Gliamine® (1 g) was solubilized in 10 ml of 1 M acetic acid and was boiled for 10 min. Pepsin (10 mg; Sigma P-6887, purified, activity 3200–4500 U/mg) was added, and the mixture was incubated for 4 h at 37°C. Subsequently, the pH was adjusted to 7.8 with NaOH, followed by the addition of 10 mg of trypsin (Sigma T-8253, type III, activity 10,000–13,000 benzoyl L-arginine ethyl ester U/mg). The mixture was then incubated for another 4 h at 25°C. Finally, 10 mg of trypsin inhibitor (Sigma) was used to stop further trypsin activity, followed by dialysis of the mixture against a large volume of water. Protein concentration was determined by using a bicinchoninic acid protein assay (Pierce).

2.3. Cell preparations and cultures

Normal human PBMC were isolated from heparinized blood obtained after cytopheresis from non-infected (by type B or C hepatitis or HIV-1), non-allergic donors. Mononuclear cells were separated by centrifugation on Ficoll–Hypaque density gradients and were seeded at 2×10^6 cells/ml in a 24-well plastic culture plate (Nunc) in complete Iscove medium (with 0.25% BSA, 11.8 µg/ml ethanolamine, 40 µg/ml apo-transferrin, 5 µg/ml insulin, 2 µg/ml linoleic acid, 2 µg/ml palmitic acid, 2 µg/ml oleic acid from Sigma supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin). For immunoglobulin productions, cells were treated or not for 13 days by different doses of IL-4 (10, 25, 50 ng/ml) and/or Gliadin, Gliamine®, Gliyls-1 or Gliyls-2 (0.25–1 µg/ml) or zein (1 µg/ml) at 37°C under a 5% CO₂-humidified condition. Normal human B-lymphocytes were positively purified, as described by the manufacturer, with magnetic beads coupled to anti-human CD19 antibody, adjusted at 10^6 cells/ml in complete Iscove medium, seeded in 48-well cell culture plates (600 µl/well) and were treated or not for 13 days by IL-4, anti-human CD40 antibody and Gliadin, Gliamine®, Gliyls-1 or Gliyls-2 (0.25–1 µg/ml). After a 13-day incubation period, cell-free supernatants of PBMC and B-lymphocytes were collected after centrifugation at $500 \times g$ for 10 min and were stored

at -20°C prior to IgE, IgG, IgA, IgM measurements. For pharmacological experiments using the redox drugs (*N*-acetyl-cysteine (NAC) and SOD1 from human and bovine origins), similar culture conditions were used except that the drugs were added to the culture 6 h before stimulation with IL-4. For soluble CD23 determination, cells were treated for 7 days and sCD23 concentration in supernatant was determined by ELISA as per manufacturer's instructions.

2.4. Protein transduction by the Tat–Chariot[®] system in normal human PBMC

The Tat–Chariot[®] protein-transfection system (Active Motif, Belgique) is derived from the HIV-1 Tat protein [28]. This Tat–Chariot[®] system is functionally tested by intracellular delivery of protein and antibody in different cell lines [29,30]. The transduction of normal human PBMC by human or bovine SOD1 was done according to the manufacturer's instructions. Briefly, the mixture of 1 μg of either hSOD1 or bSOD1 and 500 ng of the Tat–Chariot[®] peptide was prepared in phosphate-buffered saline (PBS; final volume of 200 μl) at room temperature for 30 min, this incubation period being necessary to make the Chariot–macromolecule complex. This concentrated solution is then added to the normal human PBMC (2×10^6 cells preferably washed in PBS) and were incubated for 2 h at 37°C under a humidified condition of 5% CO_2 to a final volume of 200 μl . After the end of the incubation period, cells were washed in PBS and were then resuspended in complete medium before stimulation by IL-4 $\pm$ gliadin and/or Gliyls for the different time periods (day 1 to day 13).

2.5. Free-radical production by luminol-dependent chemiluminescence

Free-radical production was evaluated, as already described [31], by the luminol-dependent chemiluminescence (LDCL) method using a Berthold 095 chemiluminometer. Briefly, at different time-points (day 1 to day 5), PBMC (10^6 cells/ml), treated or not with IL-4 $\pm$ gliadin, Gliyls or zein, were resuspended in 700 μl of PBS in the presence of 200 μl of luminol (2 mM; Sigma) and 100 ng/ml of phorbol-myristate-acetate (PMA; Sigma). The LDCL was then recorded over a period of 10 min, and the data were expressed in cpm at the peak of the reaction. A cellular free-radical production was assessed using the xanthin/xanthin oxidase (X/XO) system, as already described.

2.6. ELISA for IgE, IgG, IgA and IgM

IgE, IgG, IgA and IgM measurements were made as previously described [32–34]. Briefly, flat-bottomed microtiter plates (Nunc) were coated with 100 μl of

rabbit anti-human IgE (1/2000), anti-human IgG (1/200), anti-human IgA (1/1000) or anti-human IgM (1/1000) in 10 mM bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C , the plates were washed three times with PBS containing 0.05% Tween 20 (Sigma) and were incubated for 2 h at 37°C with PBS–1% FCS to saturate non-specific protein-binding sites. After washing, 100 μl of serial dilutions of standard IgE, IgG, IgA, IgM in complete Iscove medium or dilutions of the culture supernatants to be tested were added. For IgG, IgA and IgM determinations, the plates were then incubated for 2 h at 37°C , washed with PBS–Tween, and 100 μl of the corresponding peroxidase-conjugated antibody (final dilutions: 1/2000, 1/3000, 1/3000, respectively) were added and incubated for 1 h at 37°C . After washing, 100 μl of *o*-phenylenediamine (OPD) solution (5 mg/ml) was added. Plates were incubated for 2–5 min at room temperature, the reaction was stopped by 50 μl of 20% H_2SO_4 , and optical densities (OD) were measured at 405 nm using an autoreader (Multiscan 340, Labsystem, Eragny, France). For determination of IgE concentrations, standard and supernatants were incubated overnight at 4°C , plates were washed and incubated for 4 h at room temperature with mouse anti-human IgE mAb (I27), washed again and incubated for 2 h with the peroxidase-conjugated goat anti-mouse mAb. After three washes, 100 μl of 2, 2'-azino-bis-(3-ethylbenzthiazoline 6-sulfonic acid) (ABTS) and substrate solution (1 mg/ml) were added and the OD was measured at 405 nm after a 30 min incubation period. The threshold sensitivities of these assays were 0.16 ng/ml for IgE, 1 ng/ml for IgG, IgA and 2 ng/ml for IgM. The variation between duplicate determinations of samples never exceeded 10%. In all the experiments, the effects of residual trypsin and pepsin have been evaluated, which appeared to interfere with the IgE-producing system.

3. Results

3.1. Effect of gliadin, Gliyls and zein on IgE production by normal human PBMC

As the recent reports suggested that intolerance to gliadin was associated with the development of various allergic manifestations [35], we evaluated the effect of different sources of gliadin and Gliyls on IgE production by normal human PBMC, treated or not by IL-4. PBMC were stimulated with different doses (from 0.1 to 3 $\mu\text{g}/\text{ml}$) of gliadin (Gli-1), gliamine[®] (Gli-2), Gliyls-1 and Gliyls-2 or zein, in the presence or absence of a sub-optimal (10 ng/ml) and an optimal (25 ng/ml) dose of IL-4 (Fig. 1) added simultaneously. As shown in Fig. 1A, B, both crude gliadin batches and lysates also promoted IgE production induced by the sub-optimal dose of

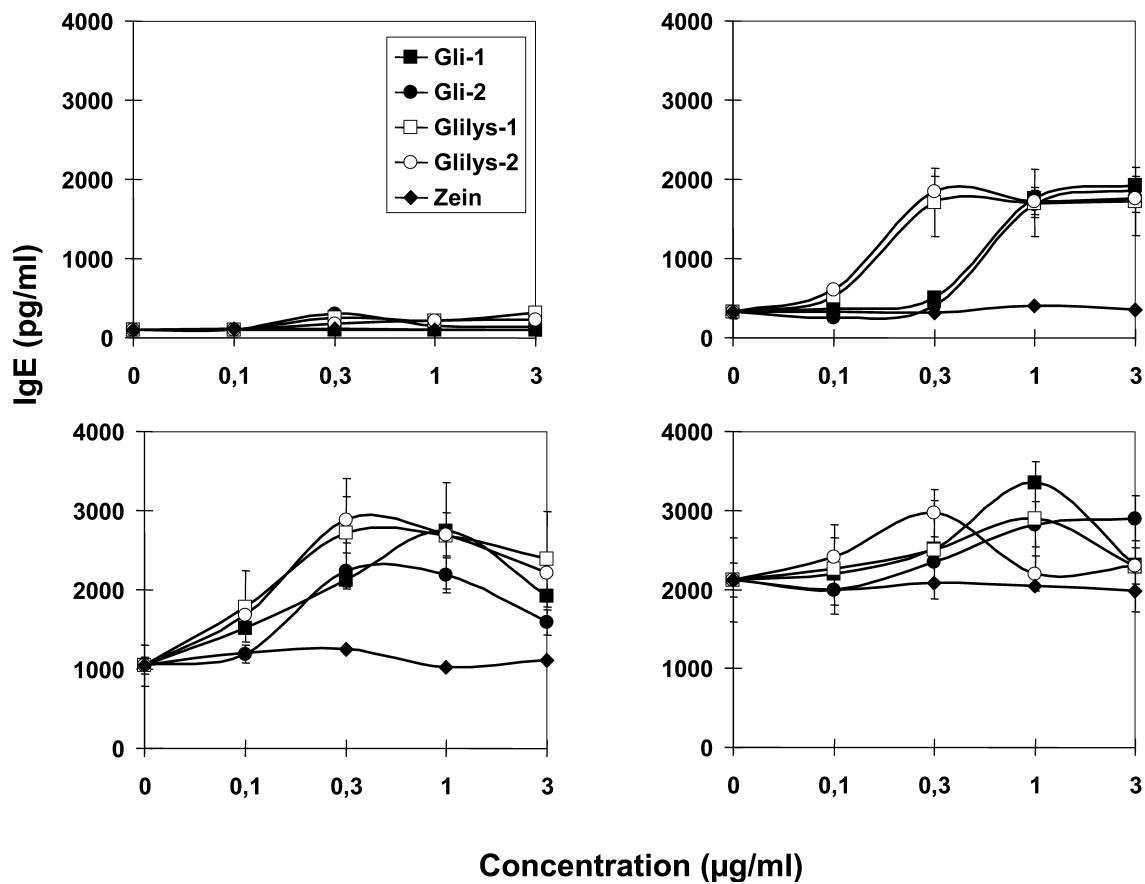


Fig. 1. Dose effect of gliadin and Gli-lys on IL-4-induced IgE production by normal human PBMC. Human PBMC (2×10^6 cells/ml) were treated either in the presence or absence of two different gliadin batches (Gli-1 and Gli-2) and Gli-lys-1 and Gli-lys-2 at different concentrations (0.1–3 µg/ml) in the absence (A) or in the presence of a sub-optimal (B, 10 ng/ml), an optimal (C, 25 ng/ml) and a supra-optimal (D, 50 ng/ml) dose. After 13 days, cell-free supernatants were harvested and IgE production was determined as described in Section 2. Cellular count was determined by trypan blue exclusion method. Data represent the mean $\pm$ SEM of six different experiments.

IL-4, the maximal effect being observed in the presence of 1 µg/ml of Gli-1 or Gli-2 and 0.3 µg/ml of Gli-lys-1 and Gli-lys-2. This promoting effect is also observed for the optimal dose of IL-4, but to a lower extent. Simultaneously, the zein preparation was unable to promote IgE production in both situations: in the presence of either a sub-optimal or an optimal dose of IgE. In these experiments, we verified that the potent residual protease present in the lysates preparations did not interfere with the IgE production (data not shown).

The determination of viable cell count was also done by trypan blue exclusion. Crude and lysates of gliadins at high concentration (1 µg/ml) reduced the viability of PBMC, stimulated or not by IL-4 (Table 1). However, at doses in which Gli-1 and Gli-2 promoted the IL-4-induced IgE production, the percentage of cell viability was relatively high as compared with control cells.

Taken together, these data suggested that in the presence of IL-4, the crude gliadin products and the enzymatically processed products were able to promote IgE production by normal human PBMC.

Table 1

Effect of gliadin preparations on the viability of normal human PBMC stimulated or not by IL-4

Gliadin preparations (µg/ml)	Viable cell count ($\times 10^6$ cells/ml)	
	Medium	+ IL-4
Control	1.2 $\pm$ 0.2	2.5 $\pm$ 0.3
Gli-1		
0.1	0.85 $\pm$ 0.06	2.12 $\pm$ 0.2
0.3	0.78 $\pm$ 0.02	2.32 $\pm$ 0.2
1	0.60 $\pm$ 0.06	1.78 $\pm$ 0.1
3	0.54 $\pm$ 0.01	1.45 $\pm$ 0.2
Gli-2		
0.1	1.15 $\pm$ 0.1	2.20 $\pm$ 0.1
0.3	1.05 $\pm$ 0.2	1.99 $\pm$ 0.2
1	1.20 $\pm$ 0.2	2.12 $\pm$ 0.2
3	1.20 $\pm$ 0.1	2.17 $\pm$ 0.1

Human PBMC (2×10^6 cells/ml) were treated in the presence or absence of two different gliadin preparations (Gli-1 and Gli-2) at different concentrations (0.1–3 µg/ml) in the presence or absence of IL-4 (25 ng/ml). After 13 days, cellular count was determined by trypan blue exclusion method. Data represent the mean $\pm$ SD of triplicate values of one representative experiment of the four.

3.2. Effect of crude gliadin and Gliyls on CD23 release by normal human PBMC

As the low affinity receptor for IgE, CD23, plays an important regulatory role in IgE production [36], we evaluated the effect of the different gliadin preparations on the production of the soluble form of this receptor by PBMC. Normal human PBMC were stimulated for 7 days with or without IL-4 (10 ng/ml) and/or Gli-1 or Gli-2 (1 µg/ml), or Gliyls-1 or Gliyls-2, and sCD23 was quantified at different time-points in the cell-free supernatants (Fig. 2). In the presence of IL-4, the soluble fraction of CD23 was released after 2 days of culture, reached a maximal effect between 5 and 7 days and then progressively diminished until day 13. The gliadin preparations increased the early release of CD23 on day 2 ($p < 0.01$), but no significant effect was observed for the other kinetics time-points. These data suggested that even gliadin preparations could increase the initial activation process induced by IL-4 by increasing the release of the soluble fraction of CD23.

3.3. Effect of crude gliadin and Gliyls on IgM, IgG and IgA production by normal human PBMC

As gliadin intolerance causes severe dysfunction of cellular immunity, we evaluated the effect of the different gliadin preparations on IgM, IgG and IgA production by PBMC. Cells were stimulated with or without optimal concentrations of IL-4 (25 ng/ml) and/or Gli-1 or Gli-2 (1 µg/ml), Gliyls-1 or Gliyls-2 (0.3 µg/ml) and, as presented in Table 2, gliadin preparations significantly ($p < 0.01$) up-regulated the IL-4-induced

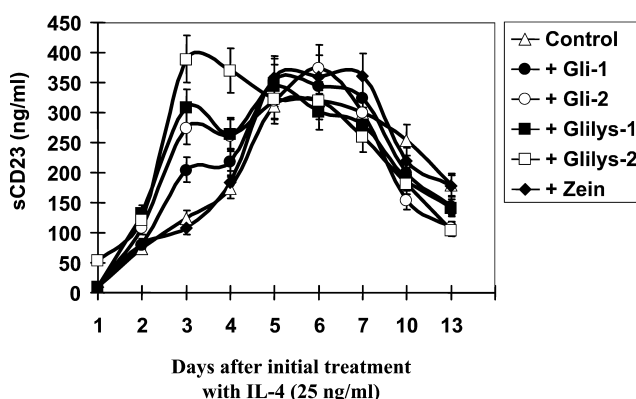


Fig. 2. Effect of crude gliadin and Gliyls on IL-4-induced soluble CD23 release by normal human PBMC. Human PBMC (2×10^6 cells/ml) were incubated in the presence or absence of two different gliadin batches (Gli-1 and Gli-2) and Gliyls (Gliyls-1 and Gliyls-2) at different concentrations (0.1–3 µg/ml) in the absence (A) or presence of a sub-optimal dose (10 ng/ml) of IL-4. Cell-free supernatants were harvested at different time-points, and sCD23 release was determined as described in Section 2. Data represent the mean $\pm$ SEM of three different experiments.

Table 2

Effect of crude gliadin and lysates on IL-4-induced IgG, IgA, IgM production by normal human PBMC

Challenge	IgG (µg/ml)	IgA (µg/ml)	IgM (µg/ml)
Medium	1.54 $\pm$ 0.2	0.5 $\pm$ 0.02	1.21 $\pm$ 0.03
IL-4	5.67 $\pm$ 0.2	1.1 $\pm$ 0.03	1.30 $\pm$ 0.10
Gli-1	1.76 $\pm$ 0.5	0.5 $\pm$ 0.01	1.15 $\pm$ 0.05
Gli-1 + IL-4	6.50 $\pm$ 0.9	0.9 $\pm$ 0.02	1.20 $\pm$ 0.04
Gli-2	1.46 $\pm$ 0.2	0.3 $\pm$ 0.01	1.50 $\pm$ 0.03
Gli-2 + IL-4	6.75 $\pm$ 0.6	1.0 $\pm$ 0.03	1.30 $\pm$ 0.10
Gliyls-1	1.76 $\pm$ 0.5	0.7 $\pm$ 0.01	1.25 $\pm$ 0.02
Gliyls-1 + IL-4	7.25 $\pm$ 0.2	1.2 $\pm$ 0.02	1.22 $\pm$ 0.02
Gliyls-2	1.62 $\pm$ 0.2	0.7 $\pm$ 0.03	1.34 $\pm$ 0.01
Gliyls-2 + IL-4	7.05 $\pm$ 0.1	1.3 $\pm$ 0.02	1.32 $\pm$ 0.10

Normal human PBMC were plated at a cell concentration of 2×10^6 cells/ml in complete Iscove medium and treated or not for 13 days by IL-4 (25 ng/ml) and/or Gli-1, Gli-2, Gliyls-1 or Gliyls-2 (0.3 µg/ml). Cell-free supernatants were then harvested and assayed for IgG, IgA, IgM productions as described in Section 2. The data represent the mean $\pm$ SD of duplicate values of one representative experiment of the four.

IgG production by PBMC, and did not significantly modulate the IgA and IgM productions.

3.4. Effect of gliadin and Gliyls on IgE production by purified B-lymphocytes

The capacity of gliadin product to recognize the HLA antigens at the cell surface of lymphoid cell [37] led us to evaluate the effect of different gliadin preparations on IgE production by purified B-lymphocytes. Highly purified B-lymphocytes (96% CD19 positive cells as determined by flow cytometry) were stimulated for 13 days by IL-4 and anti-human CD40 antibody to induce IgE production, and the effect of different concentrations of Gli-1, Gli-2, Gliyls-1 or Gliyls-2 was evaluated. In the presence of IL-4 plus an anti-CD40 mAb, purified B-lymphocytes produced IgE (Table 3), and in the presence of gliadin this production was not affected even when sub-optimal doses of IL-4 and anti-CD40 mAb were used, thus suggesting an indirect effect on IgE synthesis.

3.5. Effects of gliadin and Gliyls on the free-radical production by normal human PBMC stimulated or not by IL-4

Considering that gliadin was able to provoke an important oxidative stress in targeted cells [25,26], we evaluated the capacity of normal human PBMC to produce free radicals in the presence of the different gliadin preparations and in the presence or absence of IL-4. As shown in Fig. 3 the different gliadin preparations stimulated the spontaneous (Fig. 3A) and the PMA-induced (Fig. 3B) free-radical production. In the presence of IL-4, the spontaneous LDCL was suppressed

Table 3
Effect of crude gliadin and lysates on IgE production by normal human purified B-lymphocytes

Challenge	IL-4 (ng/ml)	Anti-CD40 mAb (0.1 µg/ml)	IgE (pg/ml)
Medium	10	—	<100
Medium	10	+	1025 ± 72
Gli-1	10	—	<100
Gli-1	10	+	999 ± 88
Gli-2	10	—	<100
Gli-2	10	+	1001 ± 32
Glilys-1	10	—	<100
Glilys-1	10	+	1121 ± 101
Glilys-2	10	—	<100
Glilys-2	10	+	1093 ± 19
Medium	25	—	<100
Medium	25	+	7500 ± 100
Gli-1	25	—	<100
Gli-1	25	+	7240 ± 200
Gli-2	25	—	223 ± 96
Gli-2	25	+	7845 ± 200
Glilys-1	25	—	155 ± 35
Glilys-1	25	+	6999 ± 100
Glilys-2	25	—	143 ± 78
Glilys-2	25	+	7320 ± 123

Purified normal human B-lymphocytes were plated at a cell concentration of 10^6 cells/ml in complete Iscove medium and treated or not for 13 days by a sub-optimal (10 ng) or an optimal dose (25 ng/ml) of anti-human CD40 (0.1 µg/ml) and/or Gli-1, Gli-2, Glilys-1 or Glilys-2 (0.3 µg/ml). Cell-free supernatants were then harvested and assayed for IgE production as described in Section 2. The data represent the mean ± SD of duplicate values of one representative experiment of the four in which the statistical analyses were similar.

(Fig. 3C), while the PMA-induced LDCL was only partially suppressed (Fig. 3D). Using the acellular X/XO system, we verified that the gliadin preparations did not interfere with the LDCL test (data not shown).

3.6. Effects of thiols and vectorized SOD1 on the promoting effect of gliadin and Glilys on the IL-4-induced IgE production by human PBMC

The data presented in this study, on the induction of free radical by gliadin, agree with our previously published data, indicating that redox alterations at the level of phagocytes regulate the production of IgE by normal human PBMC in response to IL-4 [16–18], and with data published by Yanagihara et al. that have shown that the activation of transcriptional factors such as NF-κB, which is activated by free radicals, is required to obtain IgE production [38]. Because gliadin induced redox imbalance [25,26], we evaluated the pharmacological effect of two different antioxidant molecules that have been extensively used in therapeutics [39,40]—(i) a thiol derivative (NAC) and (ii) an enzymatic antioxidant molecule, SOD1—on the IL-4-induced IgE production. Normal human PBMC were pre-incubated for 2 h in the presence or absence of NAC, free bSOD1 or hSOD1 and Tat-Chariot®-transfected bSOD1 or hSOD1, and were then stimulated for 13 days by IL-4 (10 ng/ml) and/or Gli-1, Gli-2, Glilys-1 or Glilys-2 (0.3 µg/ml). Prior to this effect on IgE production, we verified that these antioxidant molecules inhibited the pro-oxidant properties of crude gliadin and peptides (Fig. 4). In addition, and in accordance with the literature [41], NAC inhibited IL-4-induced IgE production by PBMC (Fig. 5A) and significantly decreased the IL-4 plus gli (1 or 2) or Glilys (1 or 2) IgE production, demonstrating that redox mechanisms are involved during IgE production. In the same experiments, and as already described, when PBMC were pre-incubated in the presence of free bovine or human SOD1, no significant inhibitory effect was observed (Fig. 5B).

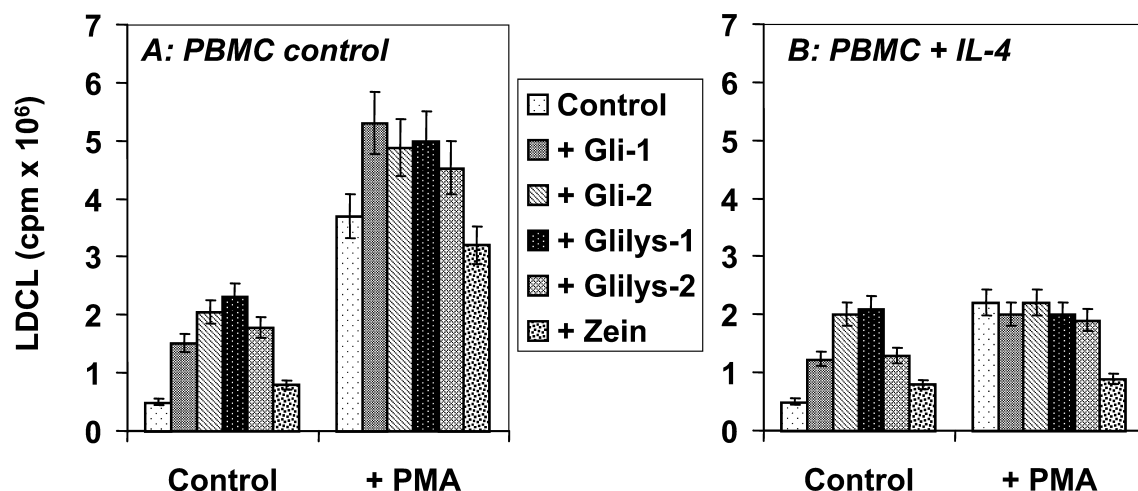


Fig. 3. Effect of crude gliadin, Glilys and zein on the spontaneous and the PMA-induced LDCL by normal human PBMC activated or not with IL-4. PBMC (10^6 cells/ml) treated or not with IL-4 ± gliadin, glilys or zein for 5 days were resuspended in PBS in the presence of luminol (and 100 ng/ml of PMA). The LDCL was then recorded over a period of 10 min and the data were expressed in cpm at the peak of the reaction and represent the mean ± SD for the triplicate samples of one representative experiment of the three.

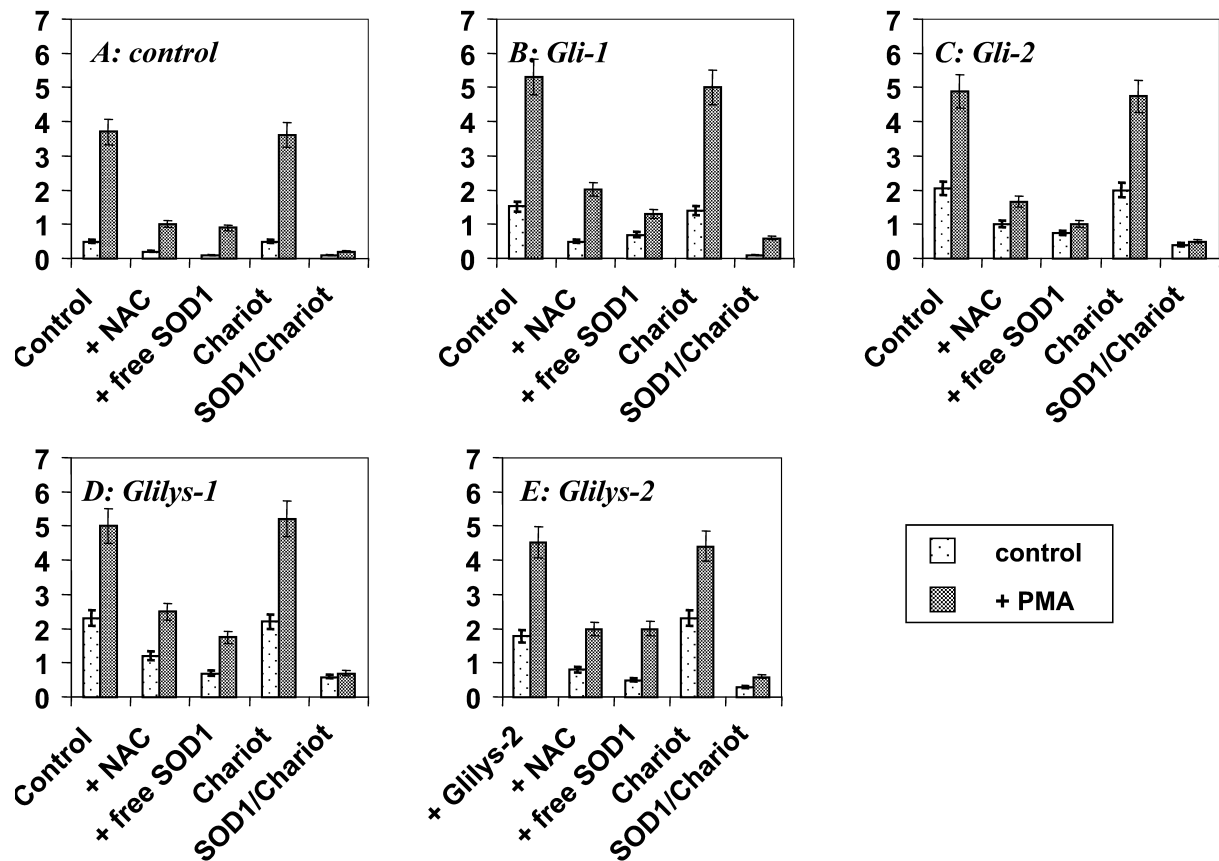


Fig. 4. Effect of NAC, free SOD1 and intracellularly delivered SOD1 on the gliadin- and the Gliyls-induced oxidative stress in human PBMC. PBMC were plated at the concentration of 2×10^6 cells/ml in complete Iscove medium and were pre-incubated for 2 h in the absence or presence of NAC (50 μ M), free bovine SOD1 (3 μ g/ml), Chariot® system (3 μ g/ml) or the SOD1–Chariot® mixture that promote the intracellular delivery of SOD1. Then these cells were stimulated for 5 days by Gli-1 (B), Gli-2 (C), Gliyls-1 (D) and Gliyls-2 (E) at the final concentration of 0.3 μ g/ml, and the LDCL was then evaluated as described in Section 2. The data represent the mean $\pm$ SD of triplicate samples of one representative experiment of the three.

However, when bovine or human SOD1 were transported in the cytoplasm of PBMC using the HIV-1–Tat–Chariot® system [42], the production of IgE by autologous PBMC stimulated with IL-4 was significantly inhibited ($p < 0.001$) for both bovine and human SOD1 (Fig. 5C). In these experiments, the inhibitory effect of bovine SOD1 on IgE production was more pronounced than that observed with human SOD1, these difference in results being probably explained by the immunoregulatory properties of heterologous SODs [43,44].

4. Discussion

The results of the present study clearly showed that gliadin biopolymers and lysates that are implicated in the development of gluten intolerance [45], potentiated the IL-4-induced early release of the soluble fraction of CD23 (2–5 days) and the IgE production after 13 days of culture. In addition, and in accordance with the literature [25,26], we demonstrated that gliadin (crude and lysates)

induced an important oxidative stress in normal human PBMC activated or not by IL-4, suggesting a possible role of this oxidative metabolism on the pro-IgE effect of gliadin preparations. It has been previously shown that antioxidant molecules favour the Th1-dependent immune responses by down-regulating the production of Th2-derived cytokines [20,38,41,46,47], while gliadin is known to promote a Th2-dependent reaction [10,48,49]. In keeping with these findings, we evaluated the pharmacological effect of NAC and SOD1 on both the gliadin-induced oxidative stress (revealed by LDCL) and on the promoting effect of this vegetal prolamine on the IL-4-induced IgE production. Our data clearly indicated that while reducing the pro-oxidative effects of gliadin preparations, we significantly reduced the pro-IgE effects of gliadin on normal human PBMC. These data could explain, at least in part, the ‘pro-allergenic’ profile of this vegetal prolamine family [50] and provide useful tools for the biological evaluation of this dramatic coeliac disease.

As gliadins and gliadin peptides can recognize HLA antigens at the cell surface of lymphoid cells [51], we

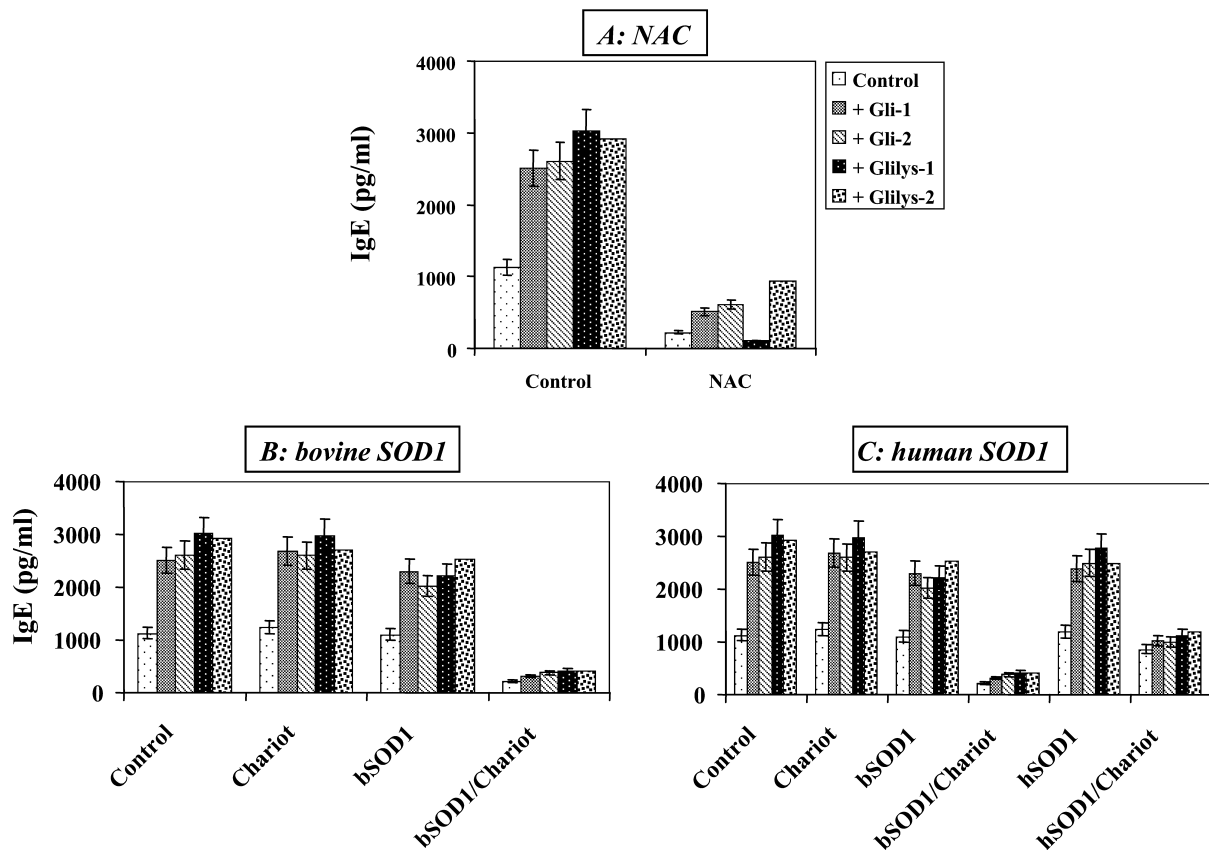


Fig. 5. Effect of NAC, free SOD1 and intracellularly delivered SOD1 on IL-4 ± gliadin or Gliyls-induced IgE production by normal human PBMC. PBMC were plated at the concentration of 2×10^6 cells/ml in complete Iscove medium and were pre-incubated for 2 h in the presence or absence of NAC (50 μ M), free bovine or human SOD1 (3 μ g/ml), of the Chariot[®] system (3 μ g/ml) or of the SOD1–Chariot[®] mixture that promote the intracellular delivery of SOD1. These cells were then stimulated or not for 13 days by IL-4 (25 ng/ml) and/or gliadin or Gliyls (0.3 μ g/ml). Cell-free supernatants were then harvested and assayed for IgE production as described in Section 2. The data represent the mean $\pm$ SEM of four different experiments.

tested their effects on IgE synthesis by highly purified B cells. Indeed, in the presence of IL-4 plus anti-CD40 mAb, purified B cells produce high amounts of IgE independent of any cell–cell interactions [18,41], and in this case, crude gliadins and/or lysates did not affect the IgE production. Taken together, these results suggested that the modulation of IgE production by gliadin is indirect and requires the presence of different cell types. Cellular mechanisms implicated in this promoting effect could be related to a stimulation of expression of receptor/ligand molecules, implicated in IgE production, by monocytes/macrophages and lymphocytes, or to an enhanced production by these cells of different factors affecting finally IgE production (cytokines, chemokine, etc.).

The main hypothesis is that gliadin and gliadin-derived peptides could act at the level of antigen-presenting cells probably by affecting their activations states and promoting the differentiation of highly specific dendritic cells. Indeed, the induction of oxidative metabolism in these cells not only controls the production of pro-inflammatory cytokines and/or chemokines [52], but

also contributes to dendritic cell differentiation, thus affecting immune polarization [21–23].

This hypothesis corresponds well with data of the literature, which showed that: (i) the oxidative metabolism and, more specifically, nitric oxide controls most of the IgE-dependent processes [16–18,53–56] and (ii) gliadin stimulated these same biochemical metabolisms in the antigen-presenting cells [25,26,57].

Based on these lines of evidence that the oxidative stress could control the ‘pro-allergenic’ effect of gliadin, we have tested the pharmacological effects of SOD1. Indeed, this detoxifying enzyme that controls the cellular respiration and stress [58] has been widely used in therapeutics [39,59–61] in the treatment of immune-dependent pro-inflammatory chronic diseases. As a previous work [41] has suggested that while NAC suppressed the IL-4-induced IgE production, the free SOD1 surprisingly was unable to elicit this effect. Considering that the SOD1 molecule is a protein localized in the cytoplasm of mammalian cells, which normally do not have receptors at the cell surface of immunocompetent cells, we suggested that the lack of

efficacy of free SOD1 was related to its incapacity to translocate the cytoplasm of the targeted cells. In order to circumvent this problem of low-diffusibility of SOD1 through the cytoplasmic membrane, we have used the HIV-1–Tat-mediated transduction system [62] to transduce the SOD1 protein in the cytoplasm of normal human PBMC. This protein-transduction system not only promoted SOD1 transduction, but also did not induce non-specific activation processes that are usually observed with other kind of trans-membrane transporters [63]. In this study, we demonstrated that the intracellular supplementation of normal human PBMC not only reduced the gliadin-induced oxidative stress, but also inhibited the IL-4 $\pm$ gliadin-induced IgE production. These data agree with a previous work [64], demonstrating that superoxide dismutase supplementation inhibited the oxidative stress-induced biological perturbations.

Taken together, these data indicated that gliadin and gliadin peptides enhanced the IL-4-induced IgE production probably by inducing an oxidative stress response at the level of antigen-presenting cells, and thus favouring a Th2-dependent immune response. In addition, we demonstrated that antioxidant products (NAC and vectorized SOD1) could not only limit the gliadin pro-oxidative effects, but also reduce the ‘pro-allergenic’ profile of this vegetal prolamine, thus providing new tools for the treatment of coeliac disease. In addition to presenting the immunopharmacological profile of antigenic SODs [43,44,65–67], we suggested that this detoxifying enzyme could be used as therapeutic support [68,69] in many pro-inflammatory diseases.

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