ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2021; 20(2):188-197.
Doi: 10.18502/ijaai.v20i2.6052

Effects of Gliadin on Autoimmune Responses of Central Nervous System of C57BL/6 Mice

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Received: 1 September 2019; Received in revised form: 17 September 2020; Accepted: 27 September 2020

ABSTRACT

Gluten sensitivity contributes to various degrees of neurological manifestations and neurodegenerative immunological changes. We investigated the experimental features of anti-gliadin immune responses in the central nervous system (CNS) of mice.

Female C57BL6 mice were divided into three groups. Mice immunized with complete Freund's adjuvant (CFA) or gliadin emulsified in CFA, and the control group received phosphate-buffered saline (PBS). Immunohistochemistry, hematoxylin-eosin, and Luxol fast blue staining were performed on the sections. The serum levels of interleukin (IL)-17 and interferon-gamma (IFN-γ) were measured using enzyme-linked immunosorbent assay (ELISA). Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to assess the mRNA levels of chemokine (C-X-C motif) ligand-2 (CXCL-2), C-C motif chemokine ligand-2 (CCL-2), and CXCL-10.

In gliadin+CFA immunized mice, the microscopic lesions included perivascular edema, focal-microgliosis, and acute neuronal necrosis in the cortex, subcortical, Purkinje cell layer, and ventral horn of the spinal cord. While extravasation of anti-IgG antibodies and selective targeting of Purkinje cells were observed in gliadin+CFA immunized mice. A significant increase in serum IL-17 and IFN-γ levels ($p<0.05$), as well as expression of CXCL-2, CCL-2, and CXCL-10 in mice immunized with gliadin+CFA, were monitored versus controls.

Our findings indicated that the immune responses directed against gliadin peptides might

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contribute to blood-brain barrier breakdown, extravasation of serum anti-IgG, gliosis, and acute neuronal necrosis in the cortex and cerebellar Purkinje cells. Anti-IgG antibodies may cause extravasation of blood-born anti-gliadin antibodies and selective targeting of Purkinje cells observed in mice immunized with peptide tryptic (pt) -gliadin in CFA.

Keywords: Central nervous system; Gliadin; Immunity; Neurological disorder

INTRODUCTION

Tolerance toward nutritional antigens is considered a complicated process related to various human disease states. Accordingly, celiac disease (CD) as enteric intolerance to a dietary antigen is accompanied by enteropathy following gluten ingestion. Glutensarea group of proteins that originate in wheat, barley, and rye.[1-3] Gluten contains diverse proteins (comprising gliadins and glutenins) and immunogenic amino acid sequences that are pathogenic in the context of CD.[4,5] Previous studies have mainly confirmed the nervous system (NS) as a complication of pre-diagnosed CD. Other reports have indicated that a wide range of neurological disorders might present gluten intolerance's extraintestinal manifestation.[6,7] Besides, a high incidence of other autoimmune diseases has been recognized among CD individuals. Neurological complications, including demyelinating disorders such as multiple sclerosis (MS), have also been reported to contribute to the CD.[8,9] In this regard, T helper 1 (TH1) cells, with their main cytokine, interferon-gamma (IFN-γ), and TH17 cells with interleukin-17 (IL-17) have vital roles in experimental autoimmune encephalomyelitis (EAE) pathogenesis,[10-13] which is an experimental model for the human inflammatory demyelinating disorder, MS.[14]Moreover, chemokines, and their receptors play essential roles in leukocyte trafficking and are engaged in immune responses provoked in the central nervous system (CNS). Indeed, their roles are associated with the large numbers of neuro-pathologies comprising MS.[15]

IL-17, as an inflammation inducer, has regulatory effects on chemokine (C-X-C motif) ligand-2 (CXCL-2) production.[16] Since T-cell accumulation in the CNS has a crucial role in MS pathogenesis, and large numbers of T cells within the cerebrospinal fluid (CSF) section express the CXCR3, the role of prolonged $CXCR3^+$ T cells accumulation in the MS lesion development was proposed. It has been shown that CXCR3 ligand expression, IFN-γ -inducible protein/CXCL-10, has increased in MS, contributing to demyelination. CXCL-10 provokes T cells accumulation in MS pathogenesis.[17,18] Another chemokine that recruits immune cells in the CNS during EAE is C-C motif chemokine ligand-2 (CCL2), which has been proposed as a new target for medical intervention in neuroinflammatory disorders.[19]

Gluten sensitivity contributes to various degrees of neurological manifestations associated with an immunological attack on the CNS and neurodegenerative changes[20]. Studying molecular mechanisms of a potential linkage between neurological manifestations and gluten sensitivity could significantly affect sufferers. Thus, the present experimental study was conducted to investigate the histopathological and biochemical features of anti-gliadin immune responses in mice CNS.

MATERIALS AND METHODS

Preparation of Peptic-tryptic Digests of Gliadin

The peptic-tryptic (pt) digest of gliadin was prepared according to the previously published protocol[21]. Briefly, 100 mg of gliadin (Sigma-Aldrich, Canada) was suspended in 2 mL HCl (0.1 N, pH: 1.8) (Merck, Germany) and then incubated with 3 mg pepsin (Bio Basic, Canada Inc; specific activity: 3000-3500 U/mg) for 4 hours at 37°C in shaking incubator. After adjusting pH to 7.8, further, incubation was performed for 4 hours with 3 mg trypsin (Sigma Chemical, St. Louis, MO, USA; specific activity: 1000-1500 U/mg). The activity of digestive enzymes was stopped by incubating in boiling water for 10 minutes.

Animals

Sixto eight weeks-old female C57BL/6 mice were selected. All mice were kept under controlled conditions with ad libitum access to food and water. This experimental study was approved at Kurdistan University of Medical Sciences (grant number: 14/30408/414) (Ethic code: IR. MUK. REC.1393. 414).

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Experimental Protocol

The mice were randomly divided into three groups (10 mice in each group). In group 1, the mice received subcutaneously (SC) phosphate-buffered saline (PBS) (400 µL). In group 2, the mice have immunized SC with complete Freund's adjuvant (CFA) (400 µL), and in group 3, the mice have immunized SC with pt-gliadin (300 µg) emulsified in CFA (400 µL). Boosters containing the same amount of antigen were injected on days 7 and 14.

Histopathological Analysis

After 28 days, the mice were euthanized, and their brain and spinal cord tissues were removed. All tissue samples were initially fixed in buffered formalin (not exceeding 10% formaldehyde) before embedding in paraffin. After preparing tissues, each group's sections were stained with hematoxylin-eosin (H&E) and Luxolfast blue (LFB). The histopathological changes were considered the main neuro-histopathological manifestations.

Immunohistochemistry

Briefly, 5 µm sections prepared from the tissues were deparaffinized in xylene and then were rehydrated in graded ethanol. After rinsing in distilled water, antigen retrieval was performed by heat treatment at 98°C in citrate buffer (pH: 6.0). Neutralization of endogenous peroxidases was carried out by incubating the sections in 3% H_2O_2 in PBS. Subsequently, the sections were detected for extravasated IgG using incubation with a sheep anti-mouse IgG antibody (Avicenna Research Institute, Iran) at a 1:100 dilution ratio for one hour. After rinsing with 0.01M PBS, samples were incubated with peroxidase-conjugated rabbit anti-sheep antibody (Avicenna Research Institute, Iran) at a 1:100 dilution ratio as the secondary antibody. The sections were then rinsed again and incubated in 100 µL diaminobenzidine (DAB) (Bio Basic Inc, Canada) for 10 minutes. After rinsing with 0.01 M PBS four times, the sections were air-dried, dehydrated in graded ethanol, and cleared in xylene. The sections were examined with Olympus BX-41 microscope.[22]

Serum Levels of IFN-γ and IL-17

Twenty-eight days following injection, retro-orbital blood was drawn from the eyes of the mice. The blood sample was immediately centrifuged at 4°C for 5 min at 4000 rpm, and serum samples were stored at -20°C until enzyme-linked immunosorbent assay (ELISA) tests were performed. During the experiment, the serums were melted, and IL-17 and INF-ɣ were measured by the ELISA method utilizing commercial kits (eBioscience, USA) according to the kit instructions (sensitivity of IFN-gamma 15 pg/mL, the sensitivity of IL-17; 4 pg/mL).

Reverse Transcription-polymerase Chain Reaction Assay (RT-PCR)

RNA was extracted from frozen brain and spleen tissue; using a total RNA extraction kit (ParsTous, Iran), based on the manufacturer's instructions. Then, the equal amounts of RNA were reverse-transcribed into cDNA with the cDNA synthesis kit (ParsTous, Iran), containing random hexamer.

An RT-PCR was carried out to investigate the expression levels of CXCL-10, CCL-2, and CXCL-2 in the spinal cord and brain samples. According to the manufacturer's protocol, RT-PCR examinations were performed with PCR master mix (ParsTous, Iran). The primer set is presented in Table 1. Thermocycling of each reaction was performed with each primer at a concentration of 1 µL. The following protocols were

Table 1. The primer sequences for reverse transcription-polymerase chain reaction (RT-PCR) examinations

Gene	Sequence	Product Size (bp)
ß-actin	**F**:5-CTTGGGTATGGAATCCTGTG-3	97
	R:5-ACTGTTGGCATAGAGGTC-3	
CCL-2	**F**:5-TCAGCCAGATGCAGTTAACG-3	117
	R:5-TCTTTGGGACACCTGCTGC-3	
CXCL-2	**F**:5-TCATAGCCACTCTCAAGGG-3	151
	R:5-TTGGTTCTTCCGTTGAGGG-3	
CXCL-10	**F**:5-ATCATCCCTGCGAGCCTATC	202
	R:5-GGTAAAGGGGAGTGATGGAG-3	

used: denaturation program (94℃ for 10 min), followed by the annealing at 56˚C (β-actin) and 66˚C (CCL-2), 58˚C (CXCL-2) and 60˚C (CXCL-10) for the 30 s and amplification at 72˚C for 45s. The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. A β-actin expression normalized the difference in the initial amount of total RNA among the samples.

Statistical Analysis

Statistical analyses were carried out by Graph Pad Prism software, data are shown as means ± SEM, and statistical analyzes were performed using statistical software R version three. One-way variance analysis was used to compare chemokine gene expression and cytokine concentration changes between different groups. Tukey test was also used for post hoc comparisons. Fisher's exact test was used to correct the histopathological data. The significance level was considered $p<0.05$.

RESULTS

To evaluate the histopathological changes in the brain and spinal cord tissues and IgG infiltration into the brain parenchyma, hematoxylin-eosin, Luxol fast blue, and immunohistochemical methods were carried out. Also, serum levels of IL-17, IFN-γ, and mRNA expression of CXCL-10, CCL-2, and CXCL-2 were measured by ELISA and RT-PCR methods.

Immunohistochemical and Histopathological Results

Immunohistochemical analysis revealed a significant difference in anti-IgG antibody infiltration rate into the perivascular brain parenchyma. Acute neuronal necrosis was monitored in the cortex subcortical, cerebellum, Purkinje cell layer, and spinal gray matter in the gliadin+CFA immunized group versus control groups. Likewise, there were significant differences in perivascular edema and glial cell proliferation in the gliadin+CFA treated group compared to CFA and PBS control groups (Figures 1 and 2). Results of hematoxylin-eosin staining demonstrated that there was acute neuronal necrosis associated with mild Purkinje cell injury in cerebellar tissues of gliadin+CFA treated mice. Moreover, neuronal necrosis, pre-vascular edema, microgliosis, and cavitation underlying the brain cortex's injury sites were observed (Figures 1 to 2).

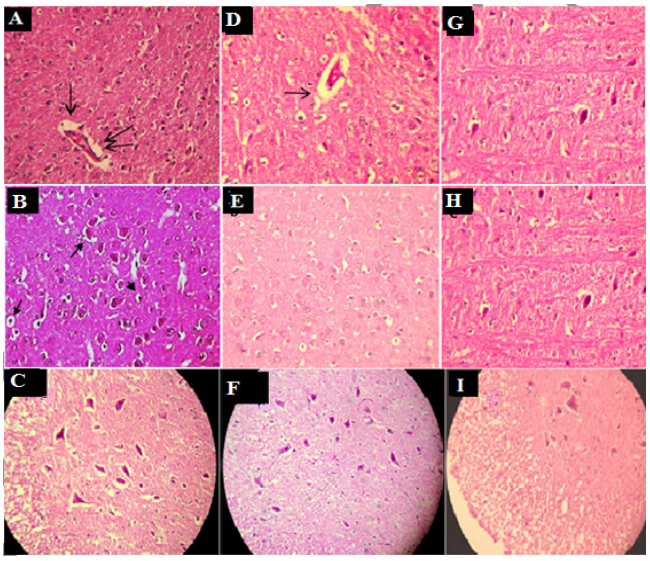

Figure 1. Hematoxylin-eosin staining of the brain and spinal cord sections. A, B and C (the mouse immunized with gliadin+ incomplete Freund's adjuvant (CFA)); (A) microscopic lesions include the foci of perivascular edema surrounding blood vessels, (B) the necrotic neurons (polygonal to round cells), (C) diffuse patterns in the cerebral cortex and ventral horn of the spinal cord in the mouse immunized with gliadin+ incomplete Freund's adjuvant (CFA). D, E and F (the mice treated with CFA); perivascular edema was only seen in D. G, H and I (the mice treated with phosphate-buffered saline (PBS)); necrotic neurons and perivascular edema not seen. (400X magnification)

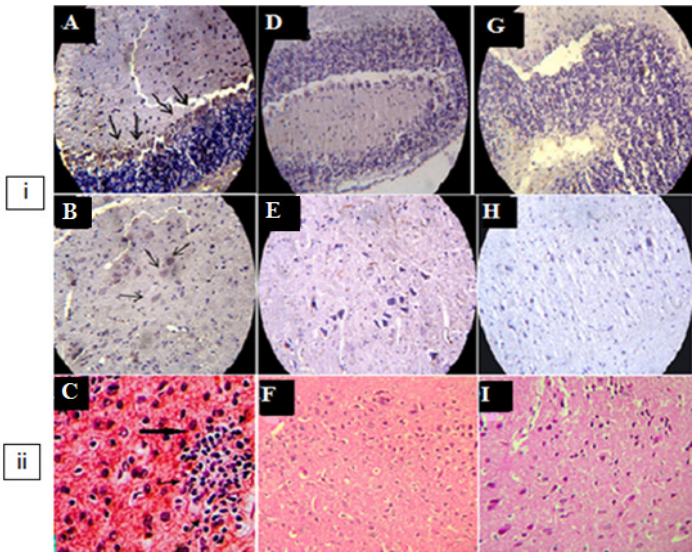

Figure 2. (i) Immunohistochemistry staining of the cerebellar cortex sections. (ii) Hematoxylin-eosin staining of brain sections. Photomicrographs of the cerebellar cortex tissue sections of gliadin+complete Freund's adjuvant (CFA) treated mice (A, B, and C). Extravasation of antibodies along with Purkinje cells (A) and cerebral cortex (B) Brown spots indicate extravasation of anti- IgG Antibodies. Arrows show selective targeting of the Purkinje cell layer by anti-IgG antibodies. Targeting Purkinje cells by anti-antibodies demonstrates the possible cross-reaction between epitopes of gliadin and Purkinje cells. (C) Microscopic lesions included the photomicrograph of focal microgliosis in mice treated with pt-gliadin emulsified in CFA. (D, E, and F) Photomicrographs of the cerebellar cortex in a mouse treated with CFA. (G, H and I) Photomicrographs of the cerebellar cortex in a mouse treated with phosphate-buffered saline (PBS). The mice treated with CFA and PBS, respectively, do not show the extravasation of anti-IgG and its reaction to cells and none of the experimental groups showed microscopic microgliosis. (400X magnification)

Immunological Results

The pattern and serum levels of IFN- γ and IL-17 cytokines secreted by TH1 and TH17 lymphocytes were measured to evaluate gliadin peptides administration effects. As displayed in Table 2 and Figure 3, in gliadin treated mice, serum levels of IL-17 and IFN-γ levels (520±202 and 242±166.5 pg/mL, respectively) were remarkably enhanced compared to the control group CFA (437.5±110 and 292.5±116 pg/mL, respectively) and PBS treated control group (70±42.70 and 87.5±30) (p<0.05).

There were significant differences in serum levels of IL-17 and IFN-γ cytokines in the gliadin peptide treated mice compared to the CFA and PBS groups (p<0.05).

Table 2. Statistical analysis of interleukin (IL)-17 and interferon-gamma(IFN-γ) cytokines

	Sum of sequences	Df	Mean square	F	*p*
IL-17					
Between Groups	81424.150	3	27141.383	87.292	**0.000**
Within Groups	4974.800	16	310.925		
Total	86398.950	19			
IFN-γ					
Between Groups	74653.750	3	24884.583	70.972	**0.000**
Within Groups	5610.0000	16	350.625		
Total	80263.750	19			

Df; degrees of freedom, F; frequency

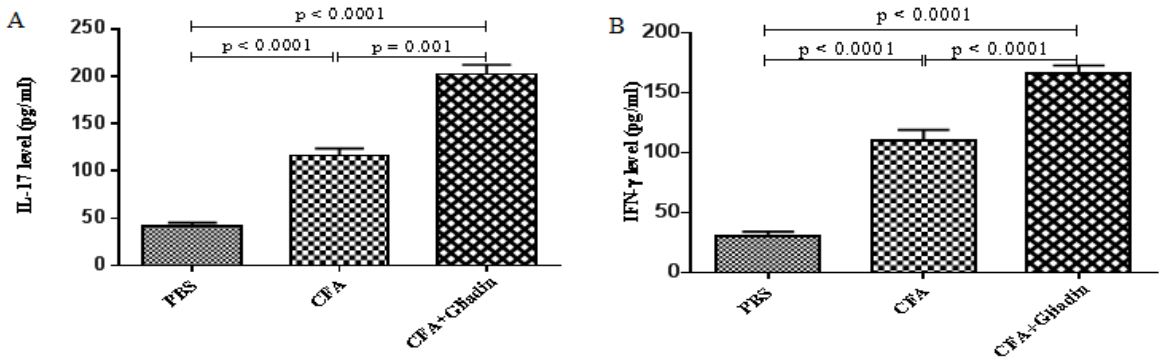

Figure 3. The serum level of interleukin (IL)-17 and interferon-gamma (IFN-γ) in three experimental groups: Data analysis showed an increase in serum levels of IL-17 (A) and IFN-γ (B) cytokines in the gliadin+complete Freund's adjuvant (CFA) versus phosphate-buffered saline (PBS) and CFA recipient groups (p<0.05). One-way variance analysis was used to compare cytokine concentration changes between different groups. Statistical analyses were carried out by Graph Pad Prism software version six, data are shown as means ± SEM, and statistical analyzes were performed using statistical software R version 3.

Gene Expression Results

RT-PCR assessed the mRNA expression levels of CCL-2, CXCL-2, and CXCL-10 chemokines engaged in inflammatory processes, neuronal degeneration, and immune cells infiltration into the CNS. According to the results of RT-PCR analysis, there was a significant increase in the gene expression levels of CCL-2 and CXCL-10 chemokines in the gliadin+CFA treated group compared to CFA and PBS treated mice (p<0.05) (Figure 4). Besides, there was a meaningful increase in CXCL-2 chemokine expression levels in the gliadin+CFA recipient group versus CFA and PBS control groups (p<0.001) (Figure 4, Table 3).

Table 3. Statistical analysis of mRNA expression of C-C motif chemokine ligand-2 (CCL-2), chemokine (C-X-C motif) ligand-2 (CXCL-2), and chemokine (C-X-C motif) ligand-10 (CXCL-10) chemokines

	Sum of sequences	Df	Mean square	F	*p*
CXCL-10					
Between Groups	0.427	2	0.214	15.401	**0.001**
Within Group d	0.125	9	0.014		
Total	0.552	11			
CXCL-2					
Between Groups	0.233	2	0.117	35.463	**0.000**
Within Group d	0.030	9	0.003		
Total	0.263	11			
CCL-2					
Between Groups	0.656	2	0.328	358.064	**0.000**
Within Group d	0.008	9	0.001		
Total	0.665	11			

Df; degrees of freedom, F; frequency

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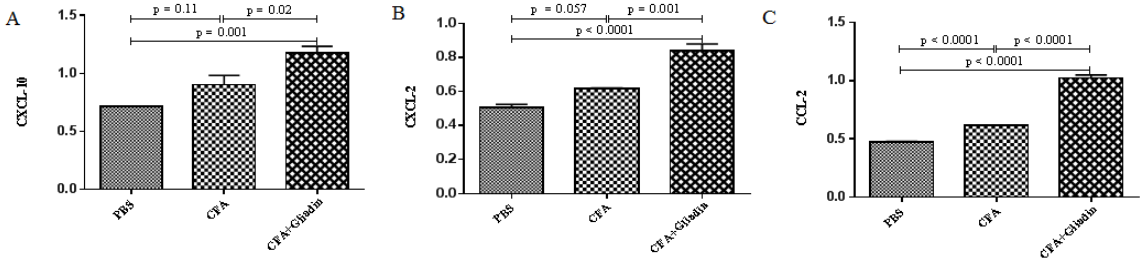

Figure 4. The mRNA expression patterns of chemokine (C-X-C motif) ligand-10 (CXCL-10) , chemokine (C-X-C motif) ligand-10 (CXCL-2), and C-C motif chemokine ligand-2 (CCL-2) chemokines in experimental groups. There were significant differences in CXCL-10 (A), CXCL-2 (B), and CCL2 (C) gene expression in the gliadin+complete Freund's adjuvant(CFA) recipient group compared to CFA and phosphate-buffered saline (PBS) treated groups(p<0.05). One-way variance analysis was used to compare chemokine gene expression concentration changes between different groups. Statistical analyses were carried out by Graph Pad Prism software version six, data are shown as means ± SEM, and statistical analyzes were performed using statistical software R version3.

DISCUSSION

The present study indicates that histopathological changes in the brain, increased IFN- γ and IL-17 serum levels, and the upregulation of inflammatory chemokines of CXCL-2, CCL-2, and CXCL-10 might initiate inflammatory processes and leukocyte trafficking and could be considered as related immune-mediated responses against gliadin.

Celiac disease (CD) is a gluten-related autoimmune disorder triggered by poorly ingestion of gluten proteins, especially those derived from gliadins in genetically susceptible individuals.[23] Furthermore, there is increasing evidence that gluten is also associated with the development of numerous extraintestinal autoimmune complications, such as MS.[9,23] In 2017, an epidemiological study showed that the prevalence of MS in CD patients was 0.1%.[24] Neurological symptoms of CD have been documented in neuroimaging scans, include seizures, cerebellar ataxia, myelopathy, dementia, and progressive leukoencephalopathy. A possible association between CNS related diseases and CD has been suggested through cross-reacted with neural proteins, production of cytokines, increased blood-brain barrier permeability, and the activation of autoreactive T cells.[9,23,25] In the present study, we aimed to examine the immune responses in neural tissues after immunization with gliadin and CFA. Based on our obtained results, perivascular edema was the histopathological manifestation in CFA treated mice; nevertheless, in mice immunized with gliadin+CFA, the microscopic lesions were included the perivascular edema, focal microgliosis, acute neuronal necrosis in the cortex, subcortical, cerebellum, Purkinje cell layer, and ventral horn of the spinal cord. Moreover, extravasation of anti-IgG antibodies and Purkinje cells were observed in mice immunized with gliadin in CFA. In this regard, it has been reported that the CNS may be a proposed site of gluten-mediated pathogenesis involving cross-reacting antibodies and further immune-mediated factors.[26]

In the previous studies, the cross-reaction between α-gliadin and various neural antigens has been confirmed.[27] Obviously, gliadin fragment can activate the innate immune system and affects the in situ T-cell recognitions of dominant gliadin epitopes.[28,29,30] Moreover, circulating IgG may interact with the brain parenchyma structures by crossing the blood-brain barrier once its penetrability is altered. This phenomenon suggests a probable pathogenic brain disease mechanism mediated by immune responses.[31,32,33] Similarly, the previous study displayed that IgG uptake in the rat neurons was related to eosinophilia and degenerative ultrastructural alteration. It could be indicated that IgG leakage is associated with neuronal impairment and might be a pathogenic mechanism in epileptogenesis.[34] Following our results, previous data exhibit that histopathological changes of the CNS in gluten-sensitive cases comprise inflammation in the dorsal root ganglia, degeneration of the posterior columns of the spinal cord, and focal inflammatory cell infiltration in the epineurium and around a small endoneurial blood vessel.[35,36] In a

similar study, to investigate cross-reactivity between α-gliadin antibody and various tissue antigens, Vojdani et al measured the antibody binding to the body antigens, and the most meaningful binding had appeared with neural antigens.[27] The previous investigations indicate that anti-gliadin-antibody in the autoimmune cerebellar ataxia case is related to the loss of Purkinje cells.[37,38] Therefore, it is suggested that the neurological alterations during CD are associated with inflammation and cross-reaction of immune responses against gliadin peptide. Indeed, gliadin may induce immune responses, which are related to autoimmunity.[9,38, 39]

Our results showed the elevated levels of IFN-γ and IL-17 and upregulation of inflammatory chemokines CXCL-2, CCL-2, and CXCL-10 in further analysis for evaluating the possible mechanism of neurological complications after gliadin immunization, which played essential roles in leukocyte trafficking. These data could be considered as possible immune-mediated responses against gliadin.

TH1 and TH17 produce IFN-γ and IL-17, respectively, by crossing the blood-brain barrier and encounter CNS antigen-presenting cells. Indeed, they secrete inflammatory mediators, which disrupt the myelin and axons. Likewise, they activate the inhabitant microglia and produce factors that persuade the inflammatory process.[14] It could be proposed that IFN-γ had a notable role in increasing the epithelial permeability during inflammation[40] since T-cell mediated permeability-increasing activity is impeded by blocking antibodies against INF-γ and effects are reversed by recombinant INF-γ. So, it could be indicated that TH17, through the intermediary role of IL-17A, interrupts the blood-brain barrier and this effect is promoted by oxidative stress induction, which consequently led to down-regulation of the tight junction molecule occluding.[41] In agreement with our data, Tanuma et al proposed that monocyte chemotactic protein-2 (MCP-1)/CCL2 and interferon gamma-induced protein-10 (IP-10)/CXCL-10, secreted by astrocytes, might stimulate astrocyte activities and lead to reactive gliosis after migration and activation of microglia/macrophages that are the main effectors in the lesional expansion.[42] According to the present knowledge, there are no further similar studies that evaluate the examined chemokine systems after gliadin immunization. Although there was no evidence of CNS demyelination in the three examined groups in this study, further studies should be conducted to evaluate the possible relationships between immunization with gliadin and demyelinating disorders of the CNS. Due to the lack of time and funding, we were not able to identify anti-gliadin antibodies; therefore, a more detailed study is required. Evaluation of the neurotoxicity effect of gliadin peptide in transgenic animals (transgenic mice with celiac-related alleles and multiple sclerosis) is an attractive target, and tracing of gliadin peptides to track its pathway to the central nervous system in laboratory animals to be placed.

Our findings demonstrate that the immune responses against gliadin peptides may contribute to blood-brain barrier breakdown, extravasation of serum anti-IgG antibody, Purkinje cell targeting, gliosis, and acute neuronal necrosis in the cortex, subcortical and cerebellar Purkinje cells. Gliadin peptides, probably causing extravasation of blood-born anti-gliadin antibodies and selective targeting of Purkinje cells were observed in mice immunized with pt-gliding in CFA. Also, elevated levels of IFN-γ and IL-17 and upregulation of inflammatory chemokines CXCL-2, CCL-2, and CXCL-10, which played an essential role in leukocyte trafficking, could be considered as related immune-mediated responses against gliadin.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Ashkan Faridi, Dr. Mojtaba Abbasi, and Dr. Asadollah Mohammadi for their constructive comments and help prepare the manuscript and format it.

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