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ORIGINAL ARTICLE

# Humoral immunity to cow's milk proteins and gliadin within the etiology of recurrent aphthous ulcers?

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**OBJECTIVES: The goal of this study was to determine the incidence of serum antibodies to gliadin and to cow's milk proteins (CMP) using ELISA test, within patients who have recurrent aphthous ulcers (RAU).**

**SUBJECTS AND METHODS: Fifty patients with recurrent aphthous ulcers and fifty healthy people were included in this research. Levels of serum IgA and IgG antibodies to gliadin and IgA, IgG and IgE to CMP were determined using ELISA.**

**RESULTS: The levels of serum antigliadin IgA and IgG antibodies were not significantly higher in patients with RAU in comparison with the controls ($P = 0.937$ and $P = 0.1854$ respectively). The levels of serum anti-CMP IgA, IgG and IgE antibodies were significantly higher in patients with RAU in comparison with the controls ($P < 0.005$, $P < 0.002$ and $P < 0.001$ respectively). In general, the increased humoral (IgA or IgG) immunoreactivity to CMP was found in 32 of 50 patients, while 17 of them showed the increased levels of both IgA and IgG immunoreactivity to CMP. At the same time, 16 out of 50 patients had IgA, IgG and IgE immunoreactivity to CMP.**

**CONCLUSION: These results indicate the strong association between high levels of serum anti-CMP IgA, IgG and IgE antibodies and clinical manifestations of recurrent aphthous ulcers.**

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**Keywords:** cow's milk proteins; gliadin; IgA; IgE; IgG; immunoreactivity; recurrent aphthous ulcers

## Introduction

Recurrent aphthous ulcers (RAU) are currently one of the most common oral disorders, with a worldwide distribution (Scully, 2006). The relationship between RAU and some precipitating factors such as trauma, anxiety, stress, hormonal changes and hypersensitivity to some nutritive ingredients has also been found. (Wright *et al*, 1986; Piskin *et al*, 2002).

The impact of food intolerance on human health has been very extensively studied, especially the intolerance to antigens such as gliadin and cow's milk proteins. Although some patients with recurrent oral ulcers are gluten sensitive but without gastrointestinal abnormalities (Wray, 1981), there might be a possibility that some of them subsequently develop gluten enteropathy. However, this has not been confirmed yet. In addition, antibodies against α-gliadin, a wheat protein fraction, have been demonstrated in patients with RAU (O'Farrelly *et al*, 1991).

Taylor *et al* (1964) investigated the incidence of serum antibodies to milk proteins and gluten in patients with diseases of the gastrointestinal tract. They noted an increased incidence in patients with major aphthous ulcerations of the mouth, but this observation has not been pursued further.

Using passive hemagglutination technique, Thomas *et al* (1973) found statistically significant presence of antibodies (the classes of immunoglobulins were not determined) to cow's milk proteins in patients with minor RAU and in patients with other acute and chronic ulcerative lesions of the mouth as well.

Saarinen *et al* (1999) reported that feeding with cow's milk during the first days of life increased the risk of allergy to cow's milk, in comparison with feeding with other supplements. However, mere breastfeeding did not eliminate the risk either. One retrospective analysis provides evidence that breastfeeding causes decreased incidence of RAU (McCullough *et al*, 2007), and one review group concluded that breastfeeding seems to protect from the development of atopic disease (van Odijk *et al*, 2003).

As a result of the innate increased permeability of mucosa that some people have, the milk proteins could penetrate into deeper strata of the epithelia and induce the major immune disorder – stimulation of the humoral

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immune response – synthesis of anti-CMP (IgE and/or IgG and/or IgA) antibodies. The anatomical and functional arrangement of the gastrointestinal tract suggests that this organ, besides its digestive and absorptive functions, regulates the trafficking of macromolecules between the environment and the host through a barrier mechanism (Fasano, 2008). The zonulin pathway has been used to deliver drugs, macromolecules, or vaccines that are not normally absorbed through the mucosal barrier. However, in the case of prolonged zonulin up-regulation, the excessive flow of non-self antigens in the intestinal submucosa can cause both intestinal and extraintestinal autoimmune disorders in genetically susceptible individuals (Fasano *et al*, 2000; Fasano, 2008).

The goal of this study was to determine whether patients with RAU have an increased levels of serum IgG and IgA antibodies to gliadin and serum IgA, IgG and IgE antibodies to cow's milk proteins in comparison with the healthy people without recurrent aphthous ulcerations.

## Subjects and methods

Fifty patients (19 males and 31 females; average age 39.4 years) with RAU were examined at the Clinics of Periodontology and Oral Medicine, Faculty of Stomatology, University of Belgrade. Patients with RAU did not have any gastrointestinal or other systemic disease or abnormalities, but they often had recurrent oral ulcerations, manifesting as small areas of erosions in the mucosa of the mouth and tongue causing a painful, shallow lesions (Scully, 2006). Fifty healthy people (13 males and 37 females; average age 43 years) without diseases and RAU were included in this research as a control group (C). All the patients as well as the control group are Serbian inhabitants and their sera were collected during the period of 2 years.

Ethical permission was obtained from the Ethical Committee of Faculty of Stomatology, University of Belgrade. The experiments were carried out with the comprehension and written consent of each subject.

*Enzyme-linked immunosorbent assay (ELISA) for antibodies*

Polystyrene microtiter wells were coated with skimmed cows milk pasteurized powder (ICN Biomedicals Inc., Cosa Mesa, CA, USA.) and crude gliadin (Fluka, Buchs, Switzerland). Determination of IgA and IgG serum's immunoreactivity to gliadin or IgA, IgG and IgE to cow's milk protein (CMP) has been performed using ELISA, using sheep antihuman IgA, IgG (Binding Site, Birmingham, England) and IgE (Sigma Chemicals Co, Saint Louis, MO, USA), horseradish peroxidase (HRP) labeled antibodies as secondary antibodies. Blocker was 1% bovine serum albumin (BSA – Sigma Chemical Co). The peroxidase activity was assayed using colorimetry as follows. A substrate solution *o*-phenylenediamine (OPD – Sigma Chemical Co) was added to the wells, and the plates were incubated at 20°C for 15 min. The enzyme reaction was terminated by addition of $H_2SO_4$. The absorbance of developed color was measured at 492 nm *vs* 630 nm, with a plate reader. The absorbance of blank (the sample with primary and secondary antibodies but without tested antigens) and absorbance of sample with antigen and secondary antibodies were always subtracted from the absorbance of tested sample. Serum immunoreactivity to CMP in arbitrary units (AU/ml) was determined by using the serum from one of the tested patient, which showed high immunoreactivity (positive control), while immunoreactivity to gliadin (IU/ml) was determined by using commercial calibrators from antigliadin ELISA kit (Binding Site). The cut off value for each test was evaluated as the Mean + 2 s.d. The statistical analysis of data was performed using Student's *t*-test.

## Results

Cut off values of anti-gliadin reactivity obtained by analyzing 50 healthy sera were 3.89 IU $ml^{-1}$ for IgA and 13.03 IU $ml^{-1}$ for IgG. The increased immunoreactivity of serum IgA with gliadin was found in three out of 50 patients with RAU and in two out of 50 healthy persons from the control group (C) (Figure 1), and the increased immunoreactivity of serum IgG with gliadin, was found in four out of 50 patients with RAU and in two out of 50 healthy persons from the control group (Figure 2).

Statistical analysis of obtained data reveals that the level of anti-gliadin IgA and IgG immunoreactivities estimated using Student's *t*-test were not significantly higher in RAU patients than the one determined

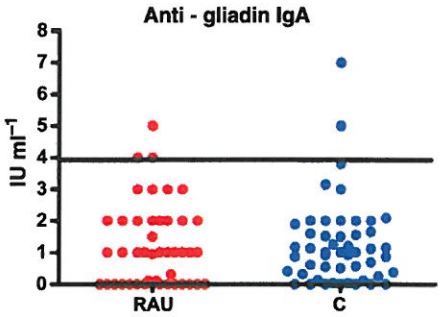


**Figure 1** Serum IgA immunoreactivity (IU $ml^{-1}$) with crude gliadin determined for patients with RAU and healthy controls (C)

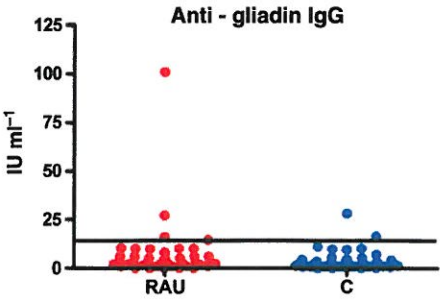


**Figure 2** Serum IgG immunoreactivity (IU $ml^{-1}$) with crude gliadin determined for patients with RAU and healthy controls (C)

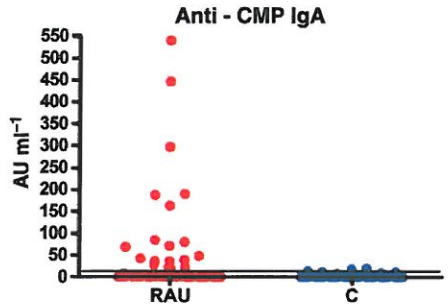


**Figure 3** Serum IgA immunoreactivity (AU $ml^{-1}$) with cow's milk proteins determined for RAU and for healthy controls (C)

in the control group ($P = 0.937$ and $P = 0.1854$ respectively).

Cut off values of anti-CMP immunoreactivity obtained analyzing 50 healthy sera were 12.17 AU $ml^{-1}$ for IgA, 35.29 AU $ml^{-1}$ for IgG and 10.18 AU $ml^{-1}$ for IgE.

The data in Figure 3 showed the increased immunoreactivity of serum IgA with cow's milk proteins higher than 12.17 AU $ml^{-1}$ in 19 out of 50 examined patients with RAU and in four out of 50 healthy persons. The increased immunoreactivity of serum IgG with cow's milk proteins higher than 35.29 AU $ml^{-1}$ was found in 30 out of 50 patients with RAU and in three out of 50 healthy people (Figure 4), and the increased immunoreactivity of serum IgE with cow's milk proteins higher than 10.18 AU $ml^{-1}$ was found in 28 out of 50 patients with RAU and in four out of 50 healthy people (Figure 5).

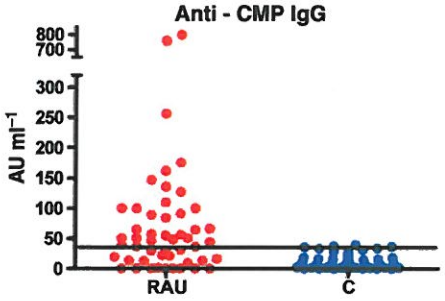


**Figure 4** Serum IgG immunoreactivity (AU $ml^{-1}$) with cow's milk proteins determined for RAU and for healthy controls (C)

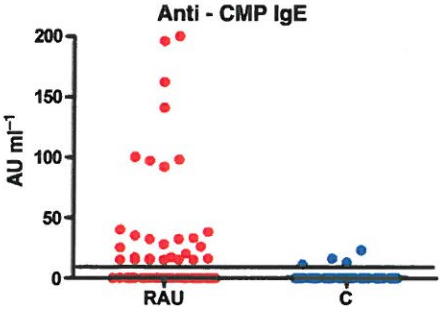


**Figure 5** Serum IgE immunoreactivity (AU $ml^{-1}$) with cow's milk proteins determined for RAU and for healthy controls (C)

**Table 1** Number of patients with increased anti-CMP immunoreactivity

| *Immunoglobulin Class* | n | *% of patients* |
|---|---|---|
| IgE and IgG and IgA | 16/50 | 32 |
| IgE and IgG | 6/50 | 12 |
| IgE and IgA | 2/50 | 4 |
| IgG and IgA | 17/50 | 34 |
| IgE | 4/50 | 8 |
| IgG | 7/50 | 14 |
| IgA | 0/50 | 0 |

Statistical analysis of obtained data shows that the levels of anti-CMP IgA and IgG immunoreactivities estimated using Student's *t*-test were significantly higher in RAU patients in comparison with the control group ($P < 0.005$ and $P < 0.002$ respectively).

Levels of anti-CMP IgE immunoreactivities estimated by using Student's *t*-test were significantly higher in RAU patients in comparison with the control group ($P < 0.001$).

The increased humoral (IgA, or IgG) immunity to CMP has been found in 32 out of 50 patients with RAU, while 17 of them showed the enhanced both IgA and IgG immunoreactivity to CMP (Table 1.).

The increased humoral (IgA, IgG and IgE) immunity to CMP has been found in 16 out of 50 patients with RAU (Table 1.).

## Discussion

Many patients with lesions of recurrent aphthous stomatitis (RAS) believe that foods are causative. Often the association represents irritating established ulcers rather than provoking their development. Numerous studies with small samples of patients with RAS have implicated a large number of foods, preservatives, and dyes, including gluten, cinnamaldehyde, benzoic acid, sorbic acid, and azo dyes (Rogers, 1997).

However, the results from this study have not showed the increased humoral immunity to gliadin in patients with RAU, although it was published that severe recurrent oral ulcerations (in the absence of gastrointestinal abnormalities) which were associated with a humoral response to α-gliadin, a wheat protein fraction, remitted in three out of four patients after gluten-free diet (Nolan *et al*, 1991).

The levels of anti-CMP IgA, IgG and IgE antibodies were significantly higher in patients with RAU in comparison with the healthy controls which confirmed the findings of Taylor *et al* (1964)and Thomas *et al* (1973)whose patients with recurrent oral ulcerations had an abnormally high incidence of antibodies against milk antigens. RAU were not strictly connected with the presence of the high level of anti-CMP IgE, as 34% of patients had only anti-CMP IgG and IgA imunoglobulins enhanced.

The data from this work showed that the humoral immunity to CMP is not strictly typical for RAU because a few healthy people also had increased IgA, and/or IgG immunity to CMP. The above mentioned

results are in accordance with the data of Thomas *et al* (1973) who reported that immunoreactions to CMP was found in patients with aphthous and other acute and chronic ulcerative lesions affecting the mouth, and that the presence of antibody to food antigens is merely an indication of increased mucosal permeability.

Cow's milk proteins is a mixture of many different constituents such as α-casein, β-casein, α-lactalbumin and many others, and the immunity to these proteins or to their immunogenic epitopes, may be different in people with RAU and those without RAU. As result of the innate increased permeability of mucosa in some people, initiated by the action of zonulin, a protein implicated in the opening of tight junctions (Fasano *et al*, 2000; Fasano, 2008; Lammers *et al*, 2008), the milk proteins may penetrate into deeper strata of the epithelia and could induce the profound immune disturbance, stimulation of the humoral immune response and synthesis of all the three main anti–CMP (IgE and/or IgG and/or IgA) antibodies. As result of the insufficient local recruitment of mast cells, or CD16, or CD89 positive cells in cases of IgE or IgG or IgA positivity, especially in healthy people, subsequent antibody-dependent cell mediated reaction in the form of RAU could not be developed.

Three patients decided to change their diet regime *i.e.* they chose not to drink cow's milk and eat dairy products – cow's milk protein free diet (CMFD) for at least 2 or 3 months. The sera from one of the patients screened for immunity to cow's milk proteins were analyzed for five times over the period of 12 months. A constant decrease in the concentration of anti-CMP IgA and IgG immunoglobulins was found in four out of five instances during 1 year (from 190 AU $ml^{-1}$ to 36 AU $ml^{-1}$ for IgA and from 800 to 172 AU $ml^{-1}$ for IgG), and there were no reappearance of clinical manifestation of RAU within this year. The level of patient sera anti-CMP IgE immunoglobulins decreased in the second and fourth analysis but increased in the third and fifth. RAU re-appeared again after introducing milk in the diet, before the fifth analysis. This was accompanied by a significant increase of the concentration of IgA, IgG and IgE anti-CMP immunoglobulins in its serum.

The sera of the second patient were analyzed two times. A significant decrease in the concentration of IgA and IgE anti-CMP immunoglobulins was noticed after eight months of CMFD, but at the same time, an increase in the concentration of IgG anti-CMP immunoglobulin and IgG anti-gliadin antibodies was noted. The re-appearance of one or two ulcerations was observed only after some ice cream had been consumed by the patient.

A decrease in the concentration of anti-CMP IgG and IgE immunoglobulin in the serum of the third patient was found 2.5 months after starting the CMFD, but at the same time, it was accompanied by the increase in the concentration of anti-CMP IgA immunoglobulin. The periods between exacerbations of ulcerations were longer than before the CMFD.

In conclusion, this study points out the strong association between high levels of serum anti-CMP IgA, IgG and IgE antibodies and clinical manifestations of RAU. The results obtained indicate the need for further investigations with the aim to light up the mechanism of the pathological role of complex, immune mediated reactions induced by CMP constituents in RAU and for the new clinical trials aimed to decrease clinical manifestation of RAU by CMP free diet.

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## Author contributions

Irina Besu designed the study, analysed the data and drafted the paper. Ljiljana Jankovic analysed the data. Ileana Ursu Magdu analysed the data. Aleksandra Konic-Ristic analysed the data. Sanvila Raskovic analysed the data. Zorica Juranic designed the study, analysed the data and drafted the paper.

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