

CONCISE REPORT

Humoral and cell mediated immune response to cow's milk proteins in Behçet's disease

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Objective: To investigate the humoral and cellular immune response against cow's milk proteins in Behçet's disease and to distinguish any behaviour during active or inactive disease.

Methods: Peripheral blood mononuclear cells from 16 patients and from eight normal controls were cultured in the presence of phytohaemagglutinin (PHA), β -casein, β -lactoglobulin, or chicken egg albumin. Interferon γ (IFN γ) and interleukin 4 (IL4) were measured in the culture supernatants by enzyme linked immunosorbent assay (ELISA). Serum samples from 46 patients with Behçet's disease and from 37 healthy subjects were also studied for antibody detection. Antibodies to β -casein, β -lactoglobulin, and chicken egg albumin were determined by ELISA.

Results: High IFN γ but not IL4 levels were found in the supernatants of lymphocytes from patients with active disease cultured in the presence of cow's milk proteins. Levels were comparable with those obtained in cultures stimulated with PHA. A significantly higher level of anti- β -casein and anti- β -lactoglobulin IgG and IgA antibodies was found in patients with active Behçet's disease. No relation was found between their occurrence and the age of the patients, the duration of disease, or the presence of gastrointestinal abnormalities. Antibodies to chicken albumin were detected at low levels and with a prevalence similar to that of healthy subjects.

Conclusion: The results indicate that an active immune response occurs in Behçet's disease. This response involves an increased frequency of antibodies to cow's milk protein and a strong Th1 polarisation after exposure to these antigens. The occurrence of these abnormalities supports a putative role for cow's milk protein immune response in the pathogenesis of Behçet's disease.

Behçet's disease (BD) is a chronic multisystemic inflammatory disorder of unknown cause characterised by recurrent oral and genital ulcers together with ocular, arthritic, vascular, and neurological involvement. A number of immunological variables have been studied by Triolo *et al*,¹ but there are insufficient data to support an autoimmune pathogenesis. Gastrointestinal involvement may be part of the spectrum of the disease and an association between BD and coeliac disease (CD) has also been considered to be possible.² Together these data suggest a putative role for the gut immune system in the pathogenesis of the disease.

There is increasing awareness that early consumption of cow's milk may be a risk factor for the development of autoimmune disease such as multiple sclerosis, mild rheumatoid arthritis in rabbits, and type 1 diabetes (reviewed by Kolb and Pozzilli³). Thus studies on the humoral and cellular immune response against cows' milk protein may be of interest in BD.

PATIENTS AND METHODS

Participants

We studied 46 patients (26 men, 20 women) with BD who fulfilled the diagnostic criteria of the International Study Group for Behçet's disease.⁴ The mean (SD) duration of the disease was 12.5 (9.7) years (range 2–40); the patients' ages ranged from 14 to 68 years (mean 40). Criteria for exclusion from the study were coexistent autoimmune disorders and gastrointestinal manifestation fulfilling the diagnosis of inflammatory bowel disease. Twenty eight patients had at least two of the major manifestations in the active stage at the time of blood withdrawal. Disease activity was assessed by the 1994 criteria of disease activity for BD, which were proposed by the BD Research Committee of Japan.⁵ Thirty seven healthy subjects of equivalent age, six patients with Crohn's disease, and 16 adult patients with CD were included in the study.

Preparation of human peripheral blood mononuclear cells and culture conditions

Peripheral blood mononuclear cells (PBMC) were obtained by separating heparinised venous blood on Ficoll-Hypaque (Euroclone, UK), as previously described.⁶ Viable mononuclear cells which excluded trypan blue were counted with a haemocytometer (viability always >90%) and then diluted in RPMI medium to a concentration of 1×10^6 cells/ml. All experiments were performed in multiwell tissue culture plates, and cultures were set up in quadruplicate. Cultures of PBMC were stimulated by the addition of phytohaemagglutinin (PHA, 10 μ g/ml; Sigma) or treated with various doses of β -casein (β C) or β -lactoglobulin (β LG), or chicken egg albumin (OA); (1, 10, 100 μ g; final concentration; Sigma). Cultures were incubated for 72 hours in 5% CO₂ at 37°C. After incubation, viability was assessed again and supernatants stored for cytokine assay at –70°C.

Interferon γ (IFN γ) and interleukin 4 (IL4) assay

Cytokine levels in culture supernatants were measured using an enzyme immunoassay (EuroClone, Devon, UK) according to the procedure suggested by the manufacturers. A standard curve was used to quantify the levels of both IFN γ or IL4. The lowest sensitivity was <5 pg/ml for IFN γ and <0.5 pg/ml for IL4 assay.

Assay for cow's milk protein antibodies

Duplicate serum samples were tested for antibodies to β C and β LG by solid phase enzyme immunoassay. Wells of flat bottom microtitre plates (Costar, Cambridge, MA) were coated with 100 μ l of the antigens at a concentration of 1 μ g/well in

Abbreviations: β C, β -casein; BD, Behçet's disease; β LG, β -lactoglobulin; CD, coeliac disease; ELISA, enzyme linked immunosorbent assay; IFN, interferon; IL, interleukin; OA, chicken egg albumin; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin

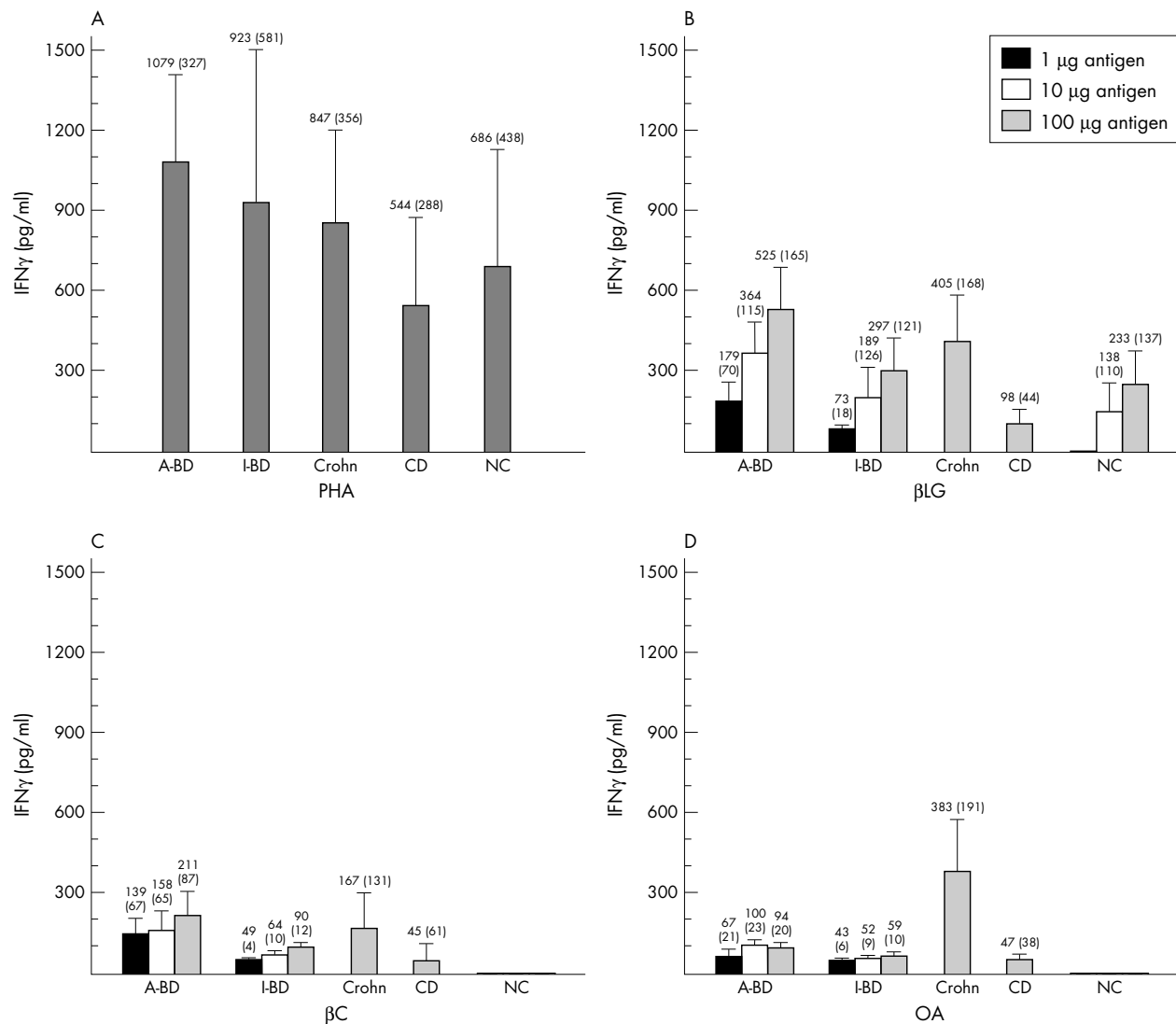


Figure 1 IFN γ levels in supernatants of PBMC from patients with active or inactive Behçet's disease (A-BD, I-BD), from patients with Crohn's disease and coeliac disease (CD), and from normal controls (NC) cultured in the presence of (A) PHA or of increasing amounts of (B) β -lactoglobulin (β LG), (C) β -casein (β C), and (D) chicken egg albumin (OA).

carbonate-bicarbonate buffer, pH 9.6. Additional wells were coated with OA at a concentration of 1 μ g/well by the same procedure and used to investigate the prevalence of antibodies against other unrelated food antigens. Results were expressed as standard deviation units according to the formula $(X-M)/SD$, where X is the absorbance of the test specimen, M is the mean absorbance of a basic set of 20 blood donor serum samples, and SD their standard deviation. A result above three standard deviation units was considered to be positive. The enzyme linked immunosorbent assay (ELISA) was reproducible with a coefficient of variation within plates of 5% and between plates of 9% for low and medium antibody levels and 8% and 11%, respectively, for high antibody levels. Specificity in the binding was assessed by incubating positive and negative sera with different concentration (2, 5, 10 μ g) of the antigens or phosphate buffered saline for one hour at 37°C before testing in the specific antibody assay. Total serum IgG and IgA were also measured by standard radial immunodiffusion assay (Istituto Behring, Italy).

RESULTS

Th1/Th2 cytokine levels

Figures 1 and 2 show the results obtained. Cytokines were measured in supernatants of PBMC cultures from 16 patients

with active BD, 12 patients with inactive BD, 10 healthy subjects, and from a group of six patients with Crohn's disease and 16 patients with CD. Quantitative determination of Th1/Th2 cytokines in supernatants of PBMC cultured in the presence of PHA or various concentrations of β C or β LG clearly showed a large production of IFN γ in patients with BD and Crohn's disease compared with normal donors and patients with CD. In active BD, IFN γ secretion progressively increased as the concentration of the culture medium β C or β LG, but not OA, increased. PBMC from patients with Crohn's disease cultured with both cow's milk proteins and OA showed significantly higher IFN γ secretion than the other groups. No significant changes were seen in controls and in patients with CD when the OA concentration was increased. Statistical analysis was carried out by analysis of variance and by Student-Neuman-Keuls' *t* test, and significance defined as $p < 0.05$. IL4 was not detectable or detected at low levels in all culture conditions. No significant differences were found for IL4 between the groups.

Cow's milk proteins antibodies

Table 1 shows the number of subjects with antibody positive results, their binding reactivity, and statistics. The IgG and IgA binding to β LG and β C was significantly higher in patients

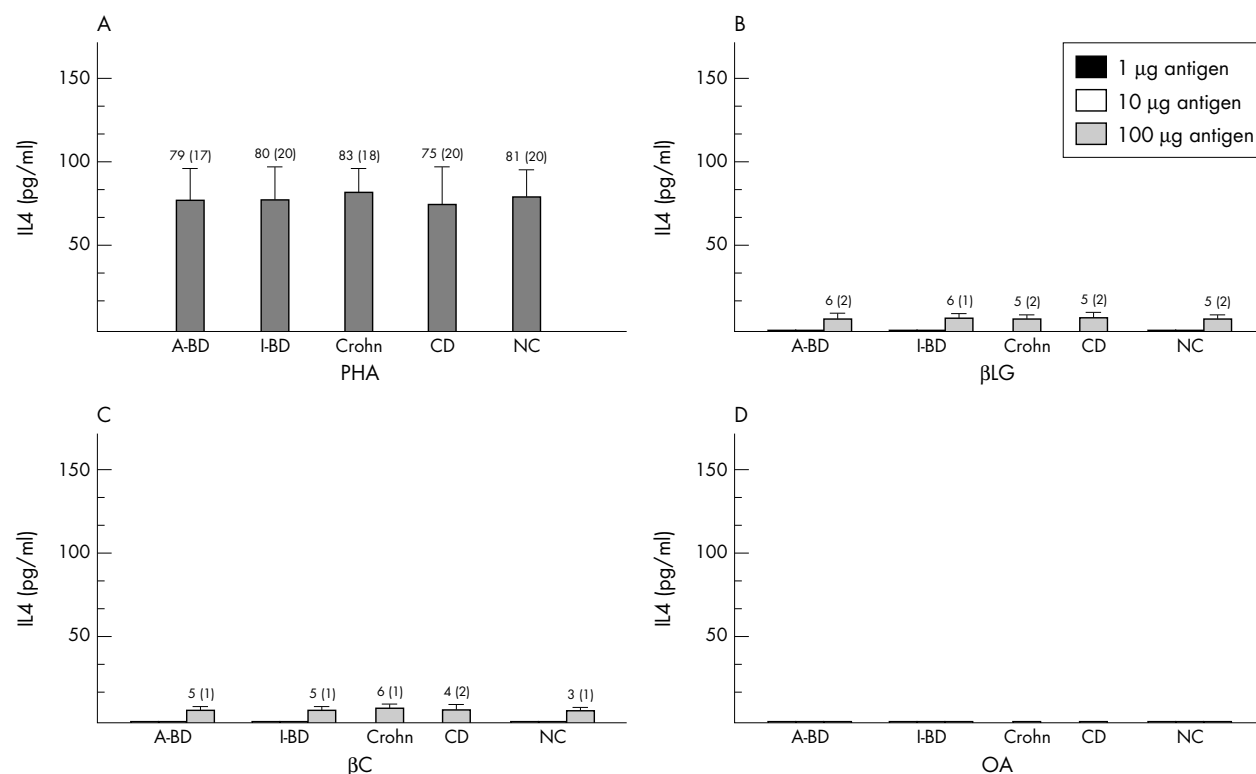


Figure 2 IL4 levels in supernatants of PBMC from patients with active or inactive Behçet's disease (A-BD, I-BD), from patients with Crohn's disease and coeliac disease (CD), and from normal controls (NC) cultured in the presence of (A) PHA or of increasing amounts of (B) β -lactoglobulin (β LG), (C) β -casein (β C), and (D) chicken egg albumin (OA).

Table 1 Serum levels (mean (SD)) of IgG and IgA anti- β -lactoglobulin (β LG), anti- β -casein (β C), and anti-chicken egg albumin (OA) from patients with active or inactive Behçet's disease (BD), Crohn's disease, coeliac disease, and normal controls. Results are expressed as standard deviation units. The final parentheses give the number of positive results for each determination.

Subjects	n	Binding reactivity against					
		β LG		β C		OA	
		IgG	IgA	IgG	IgA	IgG	IgA
Active BD	28	1.72 (2.70) (8)	2.07 (2.5) (7)	3.64 (5.93) (9)	1.89 (1.2) (9)	0.81 (0.67) (0)	0.62 (0.48) (0)
Inactive BD	18	0.37 (0.65) (0)	0.7 (1.12) (1)	0.83 (1.26) (2)	0.68 (0.92) (1)	0.72 (0.76) (0)	0.50 (0.40) (0)
Crohn's disease	6	1.86 (1.48) (2)	1.53 (1.48) (2)	2.1 (1.8) (2)	1.38 (1.48) (2)	1.6 (1.4) (2)	1.2 (1.5) (2)
Coeliac disease	16	5.8 (2.10) (16)	4.2 (1.3) (16)	3.8 (1.5) (16)	3.5 (0.8) (16)	4.8 (1.6) (16)	3.6 (0.9) (16)
Normal controls	37	0.59 (0.07) (0)	0.47 (0.58) (0)	0.83 (1.10) (1)	0.48 (0.70) (1)	0.6 (0.3) (0)	0.5 (0.3) (0)

with active BD than in either patients with inactive BD ($p < 0.05$) or healthy controls ($p < 0.01$). No relation was seen between the occurrence of anti- β LG and the age of the patients, the duration of disease, the presence of gastrointestinal abnormalities, or the severity score of the disease (not shown). Binding to OA was low in patients both with active and inactive BD, and no positive results were found in either patients with BD or controls. Increased antibody levels against all dietary antigens (OA included) were seen in all the patients with CD and in two of the patients with Crohn's disease. To determine whether the increase in β C and/or β LG binding activity was specific the inhibition tests were performed with a group of positive and negative serum samples. Sera were tested in the enzyme immunoassay before and after incubation with different amounts of purified antigens, phosphate buffered saline, or an unrelated protein (human albumin). No inhibitory effect was noticed when human albumin was used in the inhibition study. Low concentrations

of β C were sufficient to inhibit anti- β C in their binding to β C coated solid phase in two of the serum samples studied. Medium/large amounts of β C or β LG were necessary to obtain an inhibition in the other studied sera (four).

Immunoglobulin levels

Levels of total IgG were 13.28 (2.44) g/l in the anti-cow's milk positive and 1.285 (3.28) g/l in the anti-cow's milk negative sera (NS, Student's t test). Levels of total IgA were 2.88 (0.32) g/l in positive and 2.67 (0.54) g/l in negative sera (NS, Student's t test).

DISCUSSION

Our study shows that exposure to cow's milk proteins elicits the in vitro production of the Th1 cytokine IFN γ by peripheral blood mononuclear cells from patients with active BD. Similar results were obtained in Crohn's disease (also considered a Th1 disease) but not in CD and healthy subjects.

Type 1 helper T cells and type 2 helper T cells are well known to be two extremely polarised forms of the effector specific immune response, based on a distinctive profile of cytokine production; the importance of Th1/Th2 balance in the emergence of autoimmunity has been also emphasised.

To protect the host from undesired immune response against proteins derived from food, oral tolerance must be induced by the intestinal immune system.⁷ Although intestinal intraepithelial lymphocytes expressing T cell receptor α/β or γ/δ produce a similar array of cytokines of the Th1 and the Th2 type, evidence has been presented that γ/δ intraepithelial lymphocytes in the gut abrogate oral tolerance.⁸ In most studies Th1 cytokines, in particular IFN γ , have been shown to be produced by stimulated γ/δ T lymphocytes.⁹ An increased number of activated circulating γ/δ T lymphocytes are present in most patients with active BD included in this study,¹⁰ but at present the role of γ/δ T cells in cow's milk immune response is under investigation. Meanwhile, the data reported here support the recent hypothesis by Frassanito *et al* of a Th1 polarisation in the pathogenesis of BD.¹¹

Patients in an active stage of the disease have an enhanced frequency of antibodies directed against cow's milk proteins (β LG and β C) not correlated with the severity score of disease, but not against other dietary antigens.

The reason for these immunological abnormalities in BD is still unclear. The presence of anti- β C or anti- β LG may reflect the loss of self tolerance to cow's milk proteins or be the result of gastrointestinal abnormalities. Increased uptake of dietary antigens may be a putative factor in the emergence of auto-reactivity in CD and Crohn's disease.¹² On the other hand, in BD, antibodies to cow's milk protein occurred in the absence of antibodies directed against other dietary antigens—namely, OA, rendering the last hypothesis unlikely. Large amounts of free antigens were necessary to obtain an inhibitory effect in ELISA competition studies, suggesting that reactivity between anti- β C or anti- β LG and their antigens is at low affinity. In particular, low levels were found not only in normal subjects in our study but also in various autoimmune diseases,³ suggesting that they may represent a component of the normal immune repertoire. Natural autoantibodies have an important regulatory role in the maintenance of immune homeostasis. Increased titres of such antibodies are present in several autoimmune disorders, both systemic and organ-specific, usually with clonal expansion confined to a few auto-specificities. Immune complexes formed with these autoantibodies may also have a physiological role in the immune system, delivering activation or tolerisation signals to autoreactive cells (reviewed by Guarnotta *et al*).¹³

An intriguing concept may also be that cow's milk components exert detrimental effects on gut or on systemic immunoreactivity, leading to disturbed peripheral tolerance mechanisms or enhanced susceptibility to viral infections, or both. Upon digestion, several caseins may give rise to opioid peptides, which bind to opiate receptors on immune cells leading to modulation of T cell and macrophage reactivity,¹⁴ a further destabilising oral tolerance mechanism. A diet rich in cow's milk might provide also antigens that elicit an immune response cross reacting with self, thereby overcoming peripheral self tolerance. Such mimicry of the peptides would also be available from intestinal flora, in particular the highly immunogenic microbial heat shock proteins that have high relevance in the pathogenesis of BD.¹⁵

In conclusion, the occurrence of antibodies to cow's milk protein in BD, and studies on the cellular immune response with Th1 cytokine secretion, reported in this paper may support the putative role of cow's milk proteins immune response in the pathogenesis of BD.

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