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The Serologic Screening for Celiac Disease in the General Population (Blood Donors) and in Some High-Risk Groups of Adults (Patients with Autoimmune Diseases, Osteoporosis and Infertility) in the Czech Republic

Preliminary Report

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ABSTRACT. The prevalence of celiac disease (CD) was determined in healthy blood donors and in high-risk groups of adults (a total of 1835 adults – randomly selected 1312 healthy blood donors, 102 patients with primary osteoporosis, 58 patients with autoimmune diseases and 365 infertile women). It was calculated on the basis of a two-step serologic screening method – in the first step IgA and IgG antigliadin antibodies (AGA) and IgA anti-γ-glutamyltransferase ('transglutaminase') antibodies (ATG) were estimated, in the second step sera positive for IgA AGA and/or IgA ATG were examined for antiendomysial IgA (AEA) antibodies. Immunoenzymic assay (ELISA) was used for determining of AGA and ATG antibodies; immunoflurescence method, performed on human umbilical cord tissue, was used for assaying of AEA antibodies. Total serum IgA level in only IgG AGA positive subjects was measured by routine turbidimetric method. 0.45 % of healthy blood donors, 0.98 % of osteoporotic patients, 2.7 % of patients suffering from autoimmune disease and 1.13 % of women with infertility considered as immunologically mediated were found to be positive in both steps of serologic screening (AGA and/or ATG and antiendomysium positive). The presumed high prevalence of seropositivity for CD in apparently healthy Czech adult population was confirmed. In the high-risk groups, the prevalence of seropositivity for CD was approximately 2–4 times higher than in healthy blood donors. The real prevalence of CD in the tested groups, however, can be estimated after performing small intestinal biopsy in the seropositive patients.

Celiac disease (CD) is an inflammatory disease of the upper small intestine that results from gluten ingestion in susceptible individuals. Intestinal inflammation leads to malabsorption of important nutrients; institution of a gluten-free diet results in mucosal recovery (Marsh 1992).

Our knowledge of CD pathogenesis has recently made rapid progress. The disorder is now considered to be the result of a complex interplay of genetic and environmental factors that explain the wide spectrum of clinical manifestations ranging from asymptomatic to severe forms with the consequences of malabsorption. The major mechanism underlying intestinal damage in CD is a T-cell driven autoimmune inflammation, which is triggered by presentation of gluten peptides by HLA-DQ2 and HLA-DQ8 positive antigen presenting cells (Fasano and Catassi 2001). The immune response occurs predominantly in the connective tissue of lamina propria. Tissue γ-glutamyltransferase (EC 2.3.2.13; 'transglutaminase'), which has been identified as one of the highly specific endomysial autoantigens in CD (Dieterich *et al.* 1997), is released from the cells during inflammation. It may potentiate antigen presentation by HLA-DQ2 and HLA-DQ8 by deaminating or cross-linking gluten peptides. Another defined autoantigen in CD is calreticulin – a calcium binding protein with multiple biological functions, localized mainly in the endoplasmatic reticulum and

translocated and/or released under stress conditions. We suggest it has an important role in CD pathogenesis (Tučková *et al.* 1997, 1998; Sánchez *et al.* 2000).

The clinical manifestations of CD are protean in nature and vary markedly with the age of the patient, duration and extent of the disease. A range of symptoms may be associated with untreated CD, and these can be divided into intestinal features and those caused by malabsorption. It should be emphasized, however, that many patients – especially those with disease manifested in adulthood – have minimal or atypical, extra-intestinal symptoms. Many diseases have been associated with CD. Recent screening studies have shown an increased prevalence of CD in type 1 diabetes (Cronin and Shanahan 1997) and other autoimmune diseases (Collin *et al.* 1994), in IgA deficiency, undefined neurological disorders or epilepsy (Feighery 1999). The major complications of untreated disease are osteoporosis and intestinal lymphoma (for review *see* Tlaskalová *et al.* 2000). Dietary compliance was shown to reduce the risk of malignancy (Holmes *et al.* 1989) and reduce bone loss in adults (Valdimirasson *et al.* 1994).

The prevalence of CD in Europe was quoted to be 1 : 1000 a decade ago (Logan 1992). However, with the advent of sensitive serological tests for population screening, several European countries have shown that the true prevalence of CD may be 1 in 200 or even greater (Catassi *et al.* 1994; Johnston *et al.* 1998). From the data given above it is obvious that the screening of silent CD is important since these patients have an increased risk of gluten-dependent morbidity caused mainly by higher incidence of malignities in untreated CD (Tlaskalová *et al.* 2000).

The aim of our study was to investigate the seroprevalence of CD in healthy Czech blood donors, in patients with osteoporosis, patients with various autoimmune diseases and with infertility, *i.e.* in patients with known higher risk of developing CD.

SUBJECTS AND METHODS

Subjects. The study was carried out in 1999–2001. The following groups were analyzed: 1312 healthy blood donors (789 males, 523 females) from the *Institute of Hematology and Blood Transfusion* (Prague, Czechia) mean age 35 years, (range 18–60 years); 102 patients with primary osteoporosis as documented by dual energy X-ray absorptiometry at the femur neck and lumbar spine (5 males, 97 females), mean age 64 (range 45–85); 75 patients with clinically and laboratory-proven autoimmune diseases (14 with systemic lupus erythematodes, 7 with Sjögren's syndrome, 2 with chronic glomerulonephritis, 3 with rheumatoid arthritis, 1 polyarteritis nodosa, 1 dermatomyositis, 1 sclerodermia, 1 Wegener's granulomatosis, the rest of the patients with yet unclassified connective tissue diseases (8 males, 67 females), mean age 30 (range 15–80) (Table I); 88 women with immunological infertility: 30 with positive antiphospholipid antibodies (APL), 28 with positive antispermatozoal antibodies (ASP), 30 with positive anti-zona pellucida antibodies (AZP) and 275 with idiopatic infertility, mean age 30 (range 25–40).

Table I. Spectrum of patients with autoimmune diseases tested for CD serology

Diagnosis	Number of patients
Systemic lupus erythematodes	14
Sjögren's syndrome	7
Chronic glomerulonephritis	2
Rheumatoid arthritis	3
Polyarteritis nodosa	1
Dermatomyositis	1
Systemic sclerodermia	1
Wegener's granulomatosis	1
Connective tissue diseases, not classified	45

Serological markers used in screening for CD. We analyzed the blood donors and patients cohort by a 2-step procedure. In the first step, all individuals were analyzed for IgA and IgG AGA and IgA anti-γ-glutamyltransferase antibodies (ATG). In the second step, all patients manifesting either IgA AGA or IgA ATG or both were analyzed for antiendomysial IgA (AEA). The diagnostic procedure is outlined in Fig. 1.

Gliadin and γ-glutamyltransferase antibody analysis. Serum gliadin IgA and IgG antibodies were analyzed by ELISA (Stoyanov *et al.* 1988). Briefly: 96-well polystyrene microtiter plates (*Gamma*, Czechia) were filled with 50 μL of crude gliadin (*Sigma-Aldrich*) diluted in 70 % ethanol (concentration 0.1 g/L). After overnight incubation, a 5-min fixation with 10 % glutaraldehyde solution was done. After repeated washing with water and PBS, non-specific binding was blocked by 1 % BSA in PBS for 1 h. After further repeated washing (PBS, PBS–Tween 20, PBS), the sera, diluted in 1 % BSA in PBS to a final concentration of 1 : 20 and 1 : 100 for IgA and 1 : 100 and 1 : 500 for IgG estimation, were added in triplicates and incubated for 2 h. The plates were then washed (PBS, PBS–Tween 20, PBS) and pig antihuman immunoglobulin peroxidase conjugates (SwaHU IgAPx, SwaHU IgG Px; *Sevac*, Czechia) diluted 1 : 1000 in 10 % normal pig serum in PBS were added.

After a 1-h incubation at room temperature, the plates were washed with PBS and PBS–Tween 20 and the enzyme reaction was developed by adding a solution containing 1,2-benzenediamine (0.7 g/L) and 60 ppm H_2O_2. The reaction was stopped by 2 mol/L H_2SO_4 and A_{492} was read on Titertek Multiscan MCC/340 (*Flow Lab*, UK). The values were expressed as percent of absorbance of the simultaneously tested standard serum.

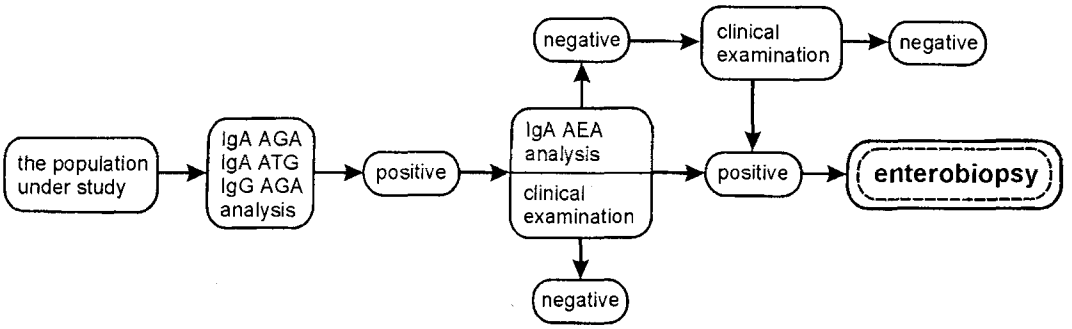

Fig. 1. Screening protocol for CD employed.

Five-hundred blood donor's sera were examined by a commercial anti-gliadin IgG and IgA ELISA kit (*Immunotech*, Prague) according to the manufacturer's instructions.

The detection of γ-glutamyltransferase antibodies was done by a modified ELISA according to Dieterich *et al.* (1997) and Sánchez *et al.* (2000). The coating concentration of guinea-pig tissue γ-glutamyltransferase (*Sigma–Aldrich*) was 5 mg/L PBS, the developing solution was diluted in 0.1 mol/L sodium citrate (pH 4.2).

Endomysial antibody analysis. An indirect immunofluorescence (IF) method using human umbilical cord tissue sections was used for AEA determination (Landiser *et al.* 1994). Cryostat sections of human umbilical cord (6 μm thick) were cut and stored at –20 °C until use. Thawed sections were fixed in cold acetone for 10 min before transferring to cold chloroform for 20 min. The next step was pretreatment with 1 % BSA in PBS for 20 min. After washing three times for 5 min with PBS, 100 μL of test serum samples, diluted at 1 : 20 and 1 : 50 in 1 % BSA in PBS were added to each section and incubated for 30 min. A positive and a negative control were included in each batch. Sections were rinsed in PBS and then 100 μL of fluorescein–isothiocyanate (FITC)-conjugated sheep anti-human IgA (α-chain specific; product no. AF0 10.M; *The Binding Site*, UK) was added to each section and slides incubated for 30 min. After washing in PBS and mounting in glycerol, slides were examined using the Fluoval immunofluorescence microscope (*Carl Zeiss Jena*, Germany). A positive result was recorded if the connective tissue surrounding the muscle cells fluoresced brightly in a honeycomb pattern.

Total serum IgA analysis. Total IgA was analyzed in patients positive only for AGA IgG, in a routine immunological or biochemical laboratory.

Calculations. We calculated the percentage of individuals seropositive for CD in the sample under study. As a cut-off value, the mean values of the estimation in 1312 healthy blood donors plus 2 × SD were taken for all ELISA tests.

RESULTS

All the AEA positive patients were positive for at least two other serologic markers: in 2 cases IgA AGA and IgA ATG, in 8 cases IgA AGA and ATG and IgG AGA. No IgA deficient patient was found in the tested groups (Table II).

Prevalence of AGA, ATG and AEA positivity in healthy blood donors. A total of 1312 healthy blood donors were screened for CD. One-hundred and one (7.7 %) had positive IgA AGA. 93 (7.1%) had positive IgG AGA, 92 (7 %) had positive IgA ATG. Positivity for both IgA and IgG AGA was found in 29 (2.2 %) and positivity for IgA AGA and ATG together in 11 (0.83 %) of blood donors. Combination of positivity for three markers – IgG and IgA AGA and IgA ATG – was detected in 9 (0.68 %) persons. Positive IgA AEA was detected in 6 (0.45 %) persons.

Prevalence of AGA, ATG and AEA positivity in high-risk groups of patients. The prevalence of seropositivity in these groups was higher than in blood donors (*see* Table II).

The highest seropositivity rate was in patients with autoimmune diseases: IgA AGA was positive in 6 (8.0 %) patients, IgG AGA in 5 (6.7) patients, IgA ATG in 4 (5.3) patients, IgA AGA and ATG in 2 (2.7) patients, IgA AEA in 2 (2.7) patients (unclassified connective tissue disease, systemic lupus erythematodes). No combined positivity in all the three serological markers together was detected.

Table II. Seropositivity for CD in blood donors' and high-risk patients' sera

Subjects	Blood donors[a]		Primary osteoporosis[a]		Autoimmunity[a]		Immunologic infertility[a]		Idiopatic infertility[a]	
Number of tested individuals	1312	100	102	100	75	100	88	100	275	100
ELISA positivity for										
IgA AGA	101	7.7	2	1.96	6	8.0	6	6.8	19	6.9
IgG AGA	93	7.1	2	1.96	5	6.7	12	13.6	33	12.0
IgA ATG	92	7.0	7	6.9	4	5.3	1	1.13	3	1.09
IgA + IgG AGA	29	2.2	0	0	0	0	1	1.13	2	0.72
IgA AGA + IgA ATG	11	0.83	10	9.8	2	2.7	1	1.13	3	1.09
IgA + IgG AGA + IgA ATG	9	0.68	0	0	0	0	1	1.13	0	0
IF positivity for										
IgA AEA[b]	6	0.45	1	0.98	2	2.7	1	1.13	0	0

[a]*First columns* – number, *second columns* – percentage.
[b]Only sera positive for one or more of the above mentioned CD markers tested.

In the group of women with infertility, which is considered as immunologically mediated, the positivity rates were also relatively high: IgA AGA 6 (6.8), IgG AGA 12 (13.6), IgA ATG 1 (1.13), IgA and IgG AGA 1, IgA AGA and ATG 1, IgA and IgG AGA and IgA ATG 1, IgA AEA 1 (all 1.13) (given in absolute numbers and as a percentage of the total number of patients in the group). In the group of women with idiopathic infertility, no IgA AEA positive patient was detected, but the positivity for other CD markers was remarkable: IgA AGA 19 (6.9), IgG AGA 33 (12.0), IgA ATG 3 (1.09), IgA and IgG AGA 2 (0.72), IgA AGA and ATG 3 (1.09) (given in absolute numbers and as a percentage of the total numberof patients).

Patients with primary osteoporosis had lower prevalence of AGA and ATG antibodies, but higher of IgA AEA than blood donors: IgA AGA 2 (1.96), IgG AGA 2 (1.96), IgA ATG 7 (6.86), IgA AGA and ATG 10 (9.80), IgA AEA 1 (0.98) (given in absolute numbers and as a percentage of the total number of patients in the group).

DISCUSSION

We used the positivity of AEA as the most specific marker for CD. In the two-step screening procedure used, the ideal is to optimize sensitivity in the first step and specificity in the second, thus enabling identification of the majority of patients with CD with minimum of falsely seropositive individuals. Accordingly, we screened the population with AGA and ATG by ELISA and in the second step we used AEA analysis with specificity close to 100 %. Using these criteria, the prevalence of seropositivity for CD in Czech blood donors was calculated to be 0.45 %, in patients with autoimmune diseases 2.0 %, in osteoporotic patients 0.89 % and in infertile women with immunological cause of infertility 1.13 %. These figures probably represent a minimum, since the sensitivity in both the first step (AGA and ATG) and the second step is around 90 % for the detection of CD. In the following part of this study, we are going to contact all the patients with positive IgA AEA, IgA AGA, IgG AGA and IgA ATG for further clinical and laboratory examination and, based on the outcome, selected patients will be advised to undergo small intestinal biopsy. Using this approach, we expect to diagnose also AEA negative CD patients.

The prevalence of CD in adults in Czechia based on AEA positivity is comparable to figures reported from other European and American countries (Catassi *et al.* 1996; Trevisiol *et al.* 1999; Carlsson *et al.* 2001). The higher prevalence in high-risk groups is not surprising, particularly in patients with autoimmunity where immunoregulatory defects and epitope spreading could explain the coexistence of these disorders.

Our recent study (Ashabani *et al.* 2002) concerned with the prevalence of CD in diabetic children in Libya showed figures higher (10.3 %) than those given in studies performed in Europe (De Vitis *et al.* 1996). Prevalence of CD in Czech diabetic children (IDDM) was found to be 4.1 % and routine serologic examination of these children for CD was suggested (Šumník *et al.* 2000). On the basis of our preliminary results, we could recommend routine screening for CD in high-risk groups of patients and in all infants at the age of 9–18 months.

Various methods of serologic screening for CD have been used and recommended (Ascher *et al.* 1996; Stern 2000). Some authors found that a two-step screening (AGA and AEA) is the most cost-effective procedure. However, for its sensitivity and above all specificity some researchers consider the determination of AEA in one-step screening to be an alternative option. Substrates such as human umbilical cord decrease the cost of the technique and avoid the ethical problems related to the use of organs of animal origin for human research. The results with both substrates – monkey esophagus and human umbilical cord – are almost identical (our own as well as another author's observation (Volta *et al.* 1995). On the other hand, Dickey *et al.* (2000) warn against the use of AEA as a single serological test for CD, because it underestimates the true prevalence of CD by one fifth.

The recent identification of tissue γ-glutamyltransferase as the autoantigen against which the AEA are directed and the development of ELISA for the detection of ATG led to its use for the early detection of cases of CD in oligosymptomatic or at-risk groups. However, the published data about its validity for CD screening are discrepant and the use as a single marker of CD cannot be therefore recommended.

In our study, the positivity for a combination of IgA AGA and ATG was more frequent than for the IgA AEA. Better correlation was achieved when IgA AGA + IgG AGA + ATG were compared to AEA. Seven out of the 10 AEA positive sera were highly positive in all tested antibodies. The remaining three were positive in IgA AGA and ATG antibodies.

The ELISA tests have the advantage of possible automation, the indirect immunofluorescence on human umbilical cord is cheaper but more time-consuming and needs a skilled person. The choice of the best method for CD serologic testing depends on the equipment and skills of the laboratory. To reach the highest sensitivity, a combination of several tests is the best, but serologically negative and bioptically positive patients with normal serum IgA were also reported (Dickey *et al.* 2000). Hence, if the clinical presentation is typical, negative serology should be disregarded and the small intestinal biopsy performed. Questionnaire would be appropriate to add to the screening protocol of the general population. Another possibility is to add another laboratory parameter – HLA testing – which, on the other hand, has the disadvantage of adding also significant financial expenses and is thus recommended only in some high-risk groups or very selected patients.

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