

IgA cross-reactivity between a nuclear autoantigen and wheat proteins suggests molecular mimicry as a possible pathomechanism in celiac disease

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Celiac disease patients display IgA antibody reactivity to wheat as well as to human proteins. We used serum IgA from celiac patients and, for control purposes, from patients with Crohn's disease, ulcerative colitis and from healthy individuals to identify celiac disease-specific IgA autoantigens in nitrocellulose-blotted extracts from various human cell types (epithelial, endothelial, intestinal cells, fibroblasts). The pattern, recognition intensity and time course of IgA autoreactivity was monitored using serial serum samples obtained from celiac children before and under gluten-free diet. By immunoblot inhibition and subcellular (cytosolic, nuclear) cell fractionation we identified a 55 kDa nuclear autoantigen expressed in intestinal, endothelial cells and in fibroblasts which was recognized by IgA antibodies of approximately half of the celiac disease patients and cross-reacted with wheat proteins. IgA reactivity to the 55 kDa autoantigen disappeared during gluten-free diet and was inhibited after pre-absorption of sera with wheat proteins but not with tissue transglutaminase, previously reported as the unique celiac disease-specific autoantigen. In conclusion, we defined a novel 55 kDa celiac disease-specific nuclear IgA autoantigen which shares epitopes with wheat proteins and which is different from tissue transglutaminase and calreticulin. Although the newly defined autoantigen was recognized much less frequently than tissue transglutaminase, our data suggest molecular mimicry between wheat and human proteins as a possible pathomechanism for the induction and/or maintenance of mucosal tissue damage in celiac disease.

Key words: Celiac disease / IgA autoreactivity / Wheat / Cross-reactivity / Molecular mimicry

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1 Introduction

In celiac disease (gluten-sensitive enteropathy) the characteristic lesion of the small bowel mucosa (villous atrophy, crypt hyperplasia and increased number of intraepithelial lymphocytes) is induced by dietary gliadin from wheat or by analogous proteins from rye, barley and less by oats in genetically susceptible individuals [1]. Symptomatic gluten-sensitive enteropathy affects approximately 1 out of 1,000 individuals in Europe [2–4]. A greater proportion of individuals has clinically silent disease [5, 6]. Most evidence suggests that the mucosal lesion represents an immunologically-mediated injury triggered by gluten in the context of a particular assort-

ment of major histocompatibility complex genes [7–9]. Celiac disease is frequently associated with a range of autoimmune diseases [10, 11]. Celiac patients show T cell-mediated immunity against gliadin [12], and develop high titers of IgA antibodies against gliadin [13–15] and components of extracellular matrix of primate [16, 17] and human tissue [18, 19]. The measurement of IgA anti-gliadin antibodies by enzyme-linked immunosorbent assay (ELISA) and of IgA anti-endomysial antibodies on monkey esophagus by indirect immunofluorescence has therefore become an important procedure in screening, diagnosing and monitoring celiac disease [20].

The association of immune responses to adenoviruses [21] with celiac disease together with the observation that mouse monoclonal antibodies raised against gliadin and patients' isolated antigliadin antibodies cross-reacted with epitopes present on rat enterocytes [22] made it tempting to speculate that immunological and

[1 21134]

Abbreviations: EMA: Anti-endomysial antibody AGA: Anti-gliadin antibody

structural similarities between environmental and endogenous antigens may be a factor for chronic tissue damage. Several attempts have been made to characterize and isolate the targets for autoantibodies. One of the autoantigens possessing common epitopes with gliadin was identified as calreticulin [23]. IgG- and IgM-recognition of a 90-kDa glycoprotein [24], IgA-reactivity to non-collagenous polypeptides [25], jejunal antigens [18], and fibroblast-derived antigens [26] were reported. A 85-kDa protein was immunoprecipitated from the IgA fractions of celiac disease patients and identified as tissue transglutaminase by protein sequencing [27]. Although pre-incubation of sera with tissue transglutaminase significantly inhibited endomysial antibody labeling, other authors suggest the occurrence of several antigens as targets for IgA autoantibodies of celiac disease patients [28–30].

In the present study we used sera from celiac patients and, for control purposes, from patients with other

inflammatory bowel diseases (Crohn's disease, ulcerative colitis) as well as from healthy persons to search for, and characterize IgA-defined autoantigens in human intestinal cell extracts by immunoblotting. We compared the pattern of IgA-reactive moieties defined by celiac patients in the human intestinal cell extract with that detected in endothelial cell and fibroblast extracts. The time course of IgA reactivity (recognition pattern and intensity) to nitrocellulose-blotted human proteins was analyzed with sera obtained from celiac children before and during gluten-free diet. By immunoblot inhibition experiments IgA autoantigens were identified which shared IgA epitopes with wheat proteins. Cell fractionation and ELISA competition experiments revealed that the most frequently detected cross-reactive autoantigen represented a 55-kDa nuclear antigen which is different from calreticulin and tissue transglutaminase. Our findings are discussed regarding the relevance of cross-reactivity between endogenous and wheat antigens for the pathogenesis of celiac disease.

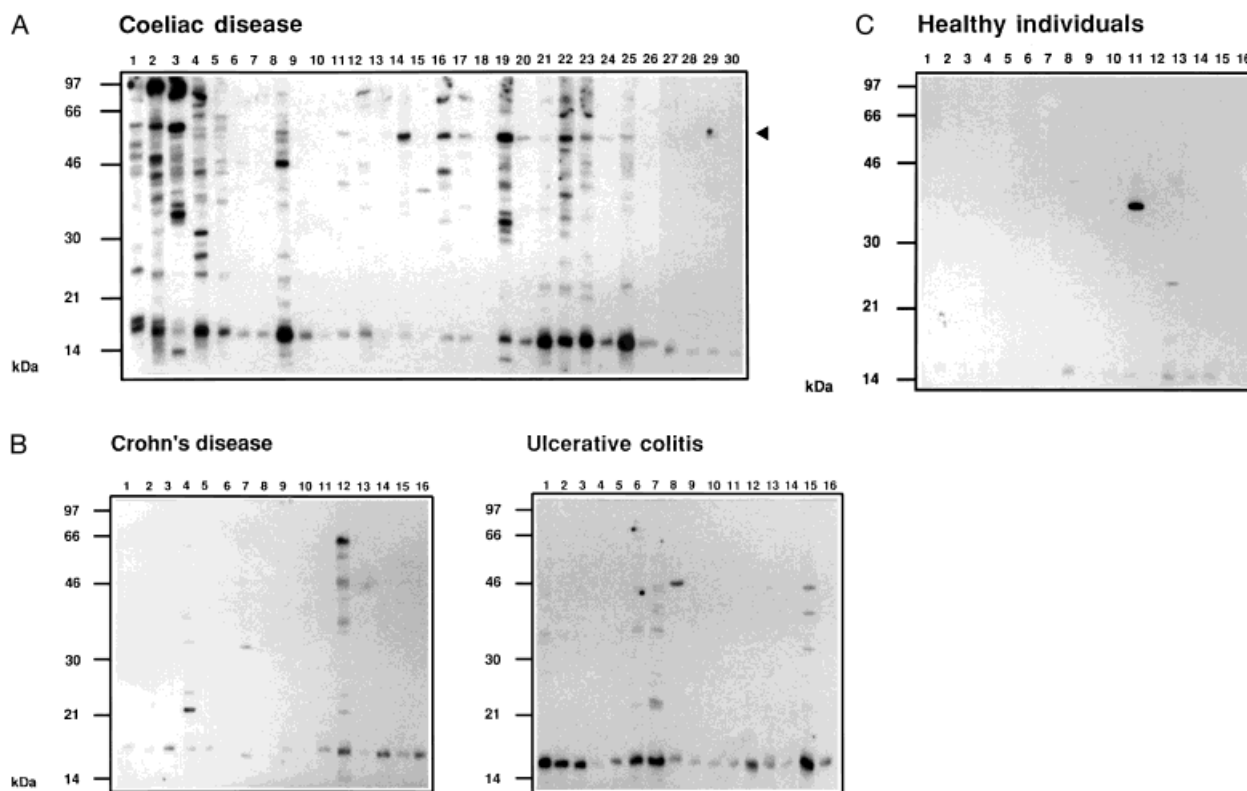


Fig. 1. IgA autoantigens detected in nitrocellulose-blotted human intestinal cell extracts. Nitrocellulose-blotted protein extract from the human colon cancer cell line CCL27 was exposed (A) to 30 sera from celiac disease patients (lanes 1–30), (B) to 16 sera from patients with Crohn's disease (lanes 1–16) and to 16 sera from patients with ulcerative colitis (lanes 1–16) as well as (C) to sera from 15 healthy persons (lanes 1–15) and buffer (lane 16) without addition of serum. Molecular masses (kDa) are displayed on the left side. The arrow on the right side indicates the position of the 55-kDa celiac disease-specific antigen.

Table 1. Demographic and laboratory parameters of patients with celiac disease in Fig. 1^{a)}

Patient	Age (years)	Sex	Total serum IgA levels (mg/ml)	EMA IIF	AGA IgG	AGA IgA	Transglutaminase
1	0.8	m	20.4	+++	136.4	153.4	1.521
2	0.9	f	5.84	–	> 200	> 200	n. d.
3	1	f	20.6	+++	145.5	> 200	1.264
4	0.9	m	30.1	–	176.7	> 200	–
5	32	f	29.2	++++	–	106.1	1.218
6	3	f	10.7	+++	33.5	–	0.715
7	3	m	13.5	++++	86.5	126.0	1.392
8	51	f	49.1	+++++	35.0	> 200	1.232
9	15	f	13.9	+++	–	–	–
10	27	f	16	++	–	–	–
11	64	f	19.3	++++	–	–	0.627
12	9	f	31.2	+++	–	–	1.395
13	1	m	4.8	+++++	188.7	121.9	0.956
14	7	f	10.5	++++	142.8	113.4	0.954
15	1	f	8.6	++++	171.0	> 200	0.664
16	1	f	11.6	+++	120.0	181.0	1.457
17	3	f	15	++++	143.1	> 200	1.554
18	13	m	7.9	++	–	–	0.839
19	1	f	28.2	++++	154.4	> 200	1.486
20	1	m	11.8	++++	0.96	0.82	n. d.
21	7	f	18.4	+++++	0.92	0.77	1.275
22	1	m	21.1	++++	0.91	0.90	n. d.
23	1	f	18.4	+++	0.92	1.02	n. d.
24	3	f	n. d.	++++	78.1	37.5	1.248
25	1	m	n. d.	++++	> 200	> 200	1.433
26	1	f	3.5	+	89.7	–	–
27	5	m	13.3	++++	–	–	1.220
28	1	f	6.6	++++	–	–	1.111
29	2	f	11.9	++++	> 200	200.0	1.412
30	1	f	15.1	+++	> 200	200.0	n. d.

a) Except for celiac disease patients 20–23, AGA levels were measured by Pharmacia EIA and are expressed as % of a positive reference serum. For patients 20–23, AGA levels were measured by ELISA and are expressed as optical density (OD). Dashes indicate negative results, n. d. = not done. AGA IgG and IgA are considered positive OD > 0.4 (> 30 % Pharmacia) and OD > 0.3 (> 25 % Pharmacia), respectively. IgA anti-transglutaminase levels OD > 0.3 were considered to be indicative for celiac disease.

2 Results

2.1 A 55-kDa antigen represents the most frequently detected celiac disease-specific IgA-autoantigen in nitrocellulose-blotted human intestinal cell extracts

Exposure of nitrocellulose-blotted human intestinal cell extract to sera showed that 28 out of 30 sera from patients with celiac disease, 10 out of 16 sera from Crohn's disease, 13 out of 16 sera from ulcerative colitis patients and 5 out of 15 sera from healthy persons contained Western blot-detectable IgA autoantibodies (Fig. 1A–C). While sera from celiac patients displayed IgA reactivity to a great variety of bands ranging from 14 kDa – 90 kDa (Fig. 1A), sera from Crohn's disease and ulcerative colitis patients and healthy individuals reacted only with a few bands. They included bands of 45 kDa, 42 kDa and 25 kDa detected by serum from healthy persons (Fig. 1C: lanes 11 and 13), and moieties of 65 kDa, 60 kDa, 46 kDa, 42 kDa, 35 kDa, 24 kDa, 21 kDa and 16–17 kDa recognized by the Crohn's disease and ulcerative colitis patients (Fig. 1B). The 16–17-kDa band was also frequently detected by serum IgA from celiac disease patients (Fig. 1A). The most frequently detected celiac disease-specific antigen was a 55-kDa band recognized strongly by sera 1, 2, 3, 8, 14, 16, 19, 22, 23 and to a lower degree by sera 4, 5, 11, 17, 20, 24, 25 and 29 and, very weakly, by serum 21 (Fig. 1A: arrow). Other bands less frequently recognized by serum IgA from celiac disease patients included a 40-kDa band identified by serum 2 and 19 and a 35-kDa band bound by serum 3 and 19. The 55-kDa antigen, thus, represented the celiac disease-specific IgA autoantigen most frequently detected in nitrocellulose-blotted intestinal cell extracts. When we tested, for control purposes, sera from additional patients with immunologically-mediated disorders (Crohn's disease: 14 sera; ulcerative colitis: 10 sera; rheumatic disease: 12 sera) and healthy persons (10 sera from adults and 10 sera from children under the age of 1 year), we found no IgA antibodies against the 55-kDa autoantigen (data not shown).

IgA recognition of the 55-kDa antigen did not depend on elevated levels of total serum IgA (Table 1) and was not always in parallel with anti-endomysial, anti-gliadin or anti-transglutaminase immunoreactivity (Fig. 1A; Table 1). IgA anti-transglutaminase antibody levels were frequently associated with IgA anti-endomysial (EMA) and IgA anti-gliadin (AGA) reactivity but several patients (e. g., Table 1, patients 13, 21) with strong EMA reactivity had rather low IgA anti-transglutaminase levels while other patients with high IgA anti-transglutaminase titers showed a less pronounced EMA reactivity (e. g., Table 1, patients 1, 3).

Patients with Crohn's disease, ulcerative colitis and healthy persons showed neither EMA reactivity nor IgA reactivity to gliadin or transglutaminase. Only 3 out of the 16 Crohn's disease patients but none of the 16 ulcerative colitis patients or 15 healthy individuals contained elevated AGA IgG (data not shown).

2.2 The 55-kDa IgA-defined autoantigen occurs also in fibroblasts and endothelial cells

To investigate whether IgA autoreactivity may vary depending on the cell source for human proteins, we probed nitrocellulose-blotted fibroblast and endothelial cell extracts with sera from celiac patients which contained IgA antibodies to intestinal cell-derived proteins (Fig. 2). The obtained results showed that certain IgA autoantigens were not expressed in all cell types (e. g., a 32-kDa component detected in fibroblasts was not recognized in endothelial cells) (Fig. 2, lanes 21, 22). However, most of the sera reacted with moieties of comparable molecular mass in all three cell extracts (fibroblasts, endothelial cells, intestinal cells). These components included e. g., a 46-kDa band (Fig. 2, lanes 2, 3, 8, 16) and the celiac disease-specific band of 55 kDa (Fig. 2, lanes 22, 2, 3 and 16).

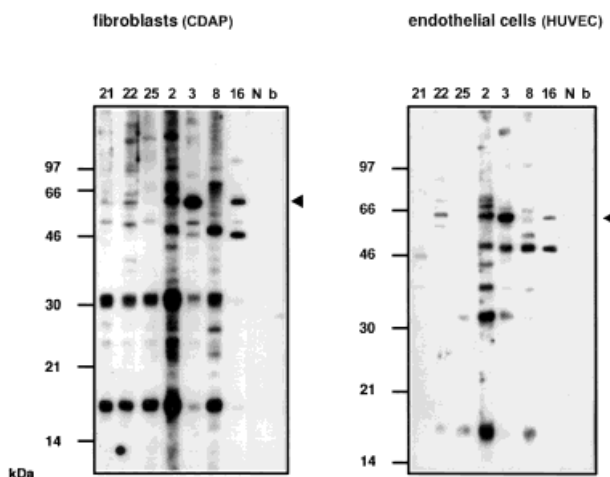


Fig. 2. IgA autoantigens detected in human fibroblasts and in human endothelial cells. Nitrocellulose-blotted fibroblast cell line and umbilical vein endothelial cell (HUVEC) extracts were exposed to sera from seven celiac disease patients (lanes 21, 22, 25, 2, 3, 8, 16), from a healthy person (lane N) and buffer without serum (lane b). The molecular masses (kDa) are displayed on the left side. The arrow on the right side indicates the position of a 55-kDa cross-reactive component.

2.3 IgA-reactivity to the 55-kDa autoantigen decreases under gluten-free diet

Celiac disease mostly affects patients early in childhood. We have therefore analyzed sera obtained from six children (mean age 2 years and 8 months) before and under gluten-free diet for IgA-reactivity to nitrocellulose blotted human intestinal cell-derived proteins (Fig. 3). In addition, IgA EMA levels and IgA as well as IgG AGA levels were determined (Table 2). Serum from all six children (no. 20–25) contained IgA antibodies against the 55-kDa celiac disease-specific autoantigen (Fig. 3, arrow) and against a 16–17-kDa component. Components of 100 kDa, 90 kDa, 80 kDa, 46 kDa, 40 kDa, 35 kDa and 12–14 kDa were detected by certain sera (e. g., serum 22, 23 and 25). Sera obtained during gluten-free diet showed a prompt reduction/abolishment of IgA reactivity to nitrocellulose-blotted intestinal cell extract which was in parallel to the decrease of IgA and IgG AGA levels determined by ELISA and the decrease of EMA reactivity (Table 2). In the first two serum samples obtained from patient 20 before gluten-free diet we noted increases in IgA anti-gliadin levels as well as in IgA reactivity to nitrocellulose-blotted intestinal cell extract. A prompt abolishment of IgA reactivity to blotted proteins and reduction of anti-endomysial as well as anti-gliadin immunoreactivity was observed in response to gluten-free diet. Taken together, our results indicate that IgA reactivity to blotted human intestinal proteins, in particular to the celiac disease-specific 55-kDa autoantigen, decreased/disappeared under gluten-free diet.

2.4 The 55-kDa celiac disease-specific autoantigen shares IgA epitopes with wheat proteins

The decrease of IgA reactivity to nitrocellulose-blotted human intestinal proteins in response to gluten-free diet and its association with the parallel reduction of IgA AGA responses led us to investigate the possible presence of common IgA epitopes in wheat and human proteins. Sera from seven celiac patients (no. 21, 22, 25, 2, 3, 8, 16) and, for control purposes, from a healthy individual (N) and buffer alone (b) were pre-incubated with wheat proteins (lanes 1), BSA (lanes 2) or buffer without competitor (lanes 3) prior to exposure to nitrocellulose-blotted human intestinal proteins (Fig. 4). Pre-incubation with wheat proteins abolished (sera no. 21, 22, 25, 2, and 16) or substantially reduced (sera no. 3, 8) IgA reactivity to the 55-kDa celiac disease-specific IgA autoantigen while IgA recognition of other moieties (e. g., the IgA-reactive 35–46-kDa moieties recognized by serum no. 2) was not affected. Likewise, we observed that IgA reactivity of sera no. 21, 22, 25 and 3 to a 32-kDa component and IgA binding of sera 22, 25 and 3 to the 16–17-kDa band were greatly reduced after pre-absorption with wheat proteins (Fig. 4). Pre-incubation of sera with tissue transglutaminase did not inhibit IgA binding to the nitrocellulose-blotted human cell extracts (data not shown).

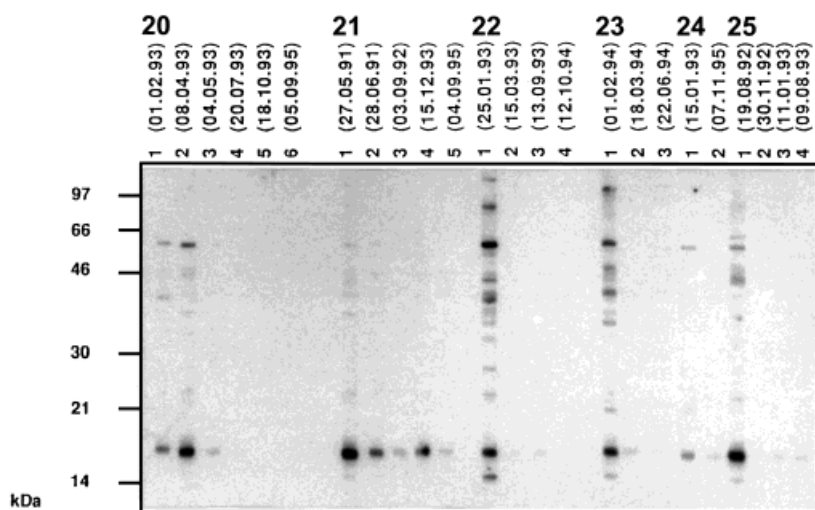


Fig. 3. Serum IgA reactivity of the celiac children described in Table 2 with nitrocellulose-blotted human intestinal cell-derived proteins. Nitrocellulose strips containing blotted human proteins were incubated with sera obtained at different time points (indicated on top of the strips) from celiac children (no. 20–25) before and during gluten-free diet. Molecular masses are indicated on the left side. The arrow indicates the position of the 55-kDa cross-reactive component.

Table 2. Demographic and laboratory parameters of six children suffering from celiac disease^{a)}

Patient	Serum sample	Exposure to gliadin (days)	Diet (days)	Total serum IgA levels (mg/ml)	EMA IIF	AGA IgG	AGA IgA
20 (m) 24.02.1992	01.02.1993	78	0	11.8	++++	0.90	0.71
	08.04.1994	144	0	n. d.	++++	0.96	0.82
	20.07.1993	145	24	n. d.	+++	0.91	0.61
	18.10.1993	145	101	n. d.	+++	0.87	–
	05.09.1995	145	191	n. d.	+	0.79	–
		145	878	10.9	–	n. d.	n. d.
21 (f) 08.09.1983	27.05.1991	2681	0	18.4	+++++	0.92	0.77
	28.06.1991	2693	20	15.5	+++	0.99	0.63
	03.09.1992	2693	453	11.9	++	0.57	0.31
	15.12.1993	2693	921	12.4	++	0.57	–
	04.09.1995	2693	1459	12.4	–	n. d.	n. d.
22 (m) 30.10.1991	25.01.1993	257	0	n. d.	++++	0.91	0.90
	15.03.1993	259	47	13.2	+++	0.82	0.55
	13.09.1993	259	623	n. d.	+/-	0.77	–
	12.10.1994	259	652	n. d.	–	n. d.	n. d.
23 (f) 18.07.1992	01.02.1994	359	0	18.4	+++	0.92	1.02
	18.03.1994	359	34	4.6	++	0.91	0.56
	22.06.1994	359	130	2.4	–	0.78	–
24 (f) 26.11.1989	15.01.1993	965	0	n. d.	++++	0.69	0.59
	07.11.1995	966	1025		–	n. d.	n. d.
25 (m) 19.07.1991	19.08.1992	283	0	n. d.	++++	0.95	0.91
	30.11.1992	295	89	n. d.	+	0.87	–
	11.01.1993	295	131	n. d.	–	0.84	0.39
	09.08.1993	295	341	n. d.	–	0.83	–

a) Twenty-four serum samples were obtained from these children (no. 20–25) before and under gluten-free diet. AGA levels were measured by ELISA and are expressed as optical density (OD). Dashes indicate negative results, n. d. = not done. AGA IgG and IgA are considered positive OD > 0.4 and OD > 0.3, respectively.

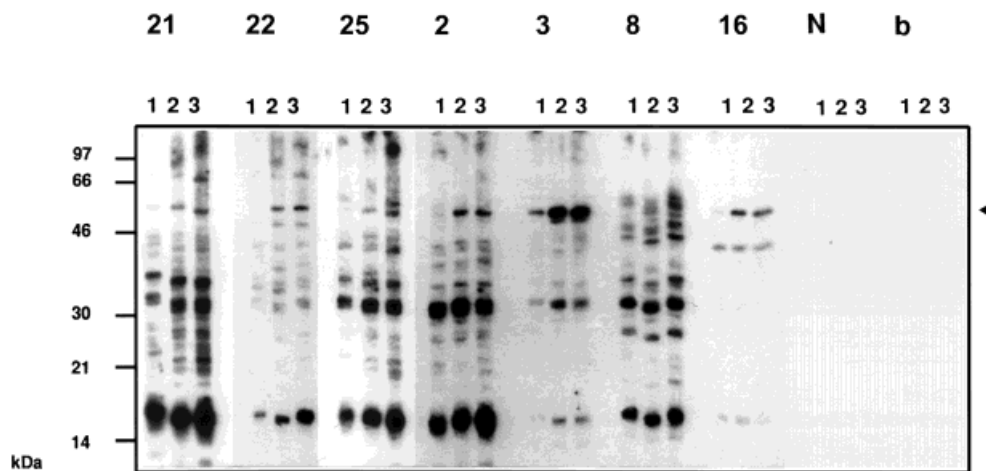


Fig. 4. Cross-reactivity of IgA autoantibodies with wheat proteins. Sera from seven celiac children obtained before gluten-free diet (no. 21, 22, 25, 2, 3, 8, 16), a healthy person (N) and buffer (b) were pre-incubated with wheat proteins (lane 1), BSA (lane 2) or with buffer alone (lane 3). After pre-incubation sera were probed for IgA reactivity to nitrocellulose-blotted human intestinal cell extracts. Molecular masses are indicated on the left side. The arrow (right side) indicates the position of the major cross-reactive component at 55 kDa.

2.5 The cross-reactive 55-kDa autoantigen is a nuclear antigen different from calreticulin

It is well established that calreticulin, a previously published cross-reactive autoantigen [23], is a protein of the endoplasmic reticulum which does not occur in the cell nucleus. Cell fractionation experiments showed that the cross-reactive 55-kDa autoantigen could be detected in total cell extract and in the nuclear fraction but was absent from the cytosolic fraction (Fig. 5A). IgA binding to the 55-kDa autoantigen in total and nuclear extracts was inhibited by pre-incubation of sera from celiac disease patients (Fig. 5A, lanes 16 versus 16*; lanes 3 versus 3*). Serum from a healthy individual (Fig. 5A, lanes 1 and 1*) and buffer (Fig. 5A, lanes b) showed almost no reactivity. Besides the 55-kDa cross-reactive antigen several other IgA-reactive nuclear autoantigens were separated from the cytosolic fraction. They included antigens unrelated to wheat proteins (e. g., 17-kDa components detected by sera no. 16 and 3) and cross-reactive components (e. g., a 29-kDa moiety detected by serum 3). IgA binding of serum 1 and 2 to the cytosolic IgA autoantigens (e. g., 46 kDa) was not inhibited by pre-incubation with wheat proteins (Fig. 5A). The successful separation of the nuclear and cytosolic fraction is demonstrated by the fact that a rabbit anti-histone H2B antiserum reacted with total cell extracts (lane t) and nuclear fractions (lane n) but not with the cytosolic fraction (lane c) (Fig. 5B). The cell fractionation experiments thus identify the 55-kDa cross-reactive autoantigen as a

nuclear component which is absent from the cytosol and, therefore, must be different from calreticulin.

3 Discussion

Although IgA-auto/alloantibody reactivity is meanwhile routinely used to diagnose and monitor the course of celiac disease [19, 20], little is known about the precise targets for the IgA antibodies and how IgA auto/alloantibody production is linked to gluten-induced intestinal tissue damage. In a recent study human transglutaminase was found in IgA immunoprecipitates of sera from celiac disease patients [27]. Based on the fact that sera from most celiac patients displayed IgA reactivity to ELISA plate-coupled guinea pig and human transglutaminase and that pre-incubation of sera with this protein reduced EMA reactivity, transglutaminase was proposed as the perhaps exclusive autoantigen of celiac disease [27, 31, 32]. However, despite the frequent and prominent IgA reactivity to transglutaminase, other studies suggested that also other autoantigens may play a role in celiac disease [28–30]. The occurrence of IgA-reactive autoantigens other than tissue transglutaminase would also explain the incomplete association of anti-endomysium and anti-transglutaminase reactivity observed in our study.

In this study we aimed to characterize IgA-defined autoantigens in human cellular extracts from intestinal and

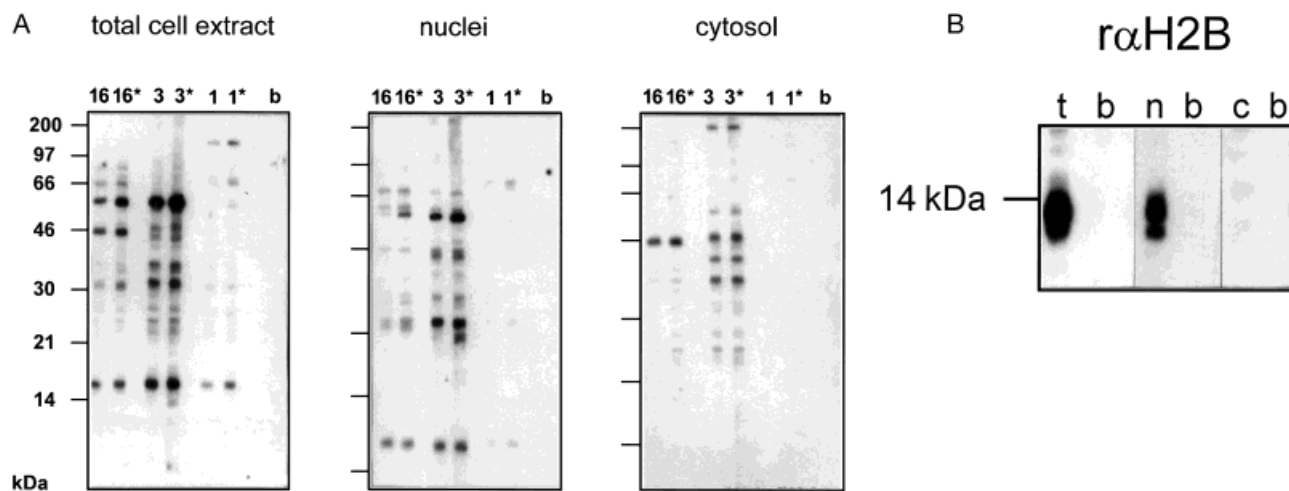


Fig. 5. Detection of IgA-reactive autoantigens in subcellular fractions of human intestinal cells. (A) Sera from two celiac patients (no. 16, 3) and a healthy individual (no. 1) were tested for IgA reactivity to nitrocellulose-blotted total cell extracts, nuclear or cytoplasmic cell fractions. Sera were pre-incubated with wheat proteins (lanes 16, 3, 1) or buffer alone (lanes 16*, 3*, 1*). In lanes b nitrocellulose strips were incubated with buffer alone. Serum 1 corresponds to serum 1 in Fig. 1C. (B) Nitrocellulose-blotted total cell extract (lane t), nuclear (lane n), and cytoplasmic fractions (lane c) were probed with a rabbit anti-human histone H2B antiserum or buffer alone (lanes b).

endothelial cells as well as fibroblasts by immunoblotting. We identified a 55-kDa antigen localized within the nuclear fraction of intestinal cells which was strongly recognized by 30% of sera from celiac disease patients but not from patients who suffered from other chronic inflammatory bowel diseases (Crohn's disease, ulcerative colitis) or healthy individuals. The antigen described by us is much less prominent than tissue transglutaminase because it is recognized by fewer patients and in part with low intensity. As yet, we also do not have evidence that IgA recognition of the newly defined autoantigen is linked to certain clinical manifestations. Extensive clinical studies will therefore be required to correlate the presence of IgA reactivity to the 55-kDa autoantigen with clinical characteristics or other parameters. Nevertheless, at least two arguments support the assumption that IgA recognition of the 55-kDa autoantigen is specific for celiac disease. First, we found that only sera from celiac disease patients but not from healthy persons or patients suffering from other immunologically-mediated diseases (Crohn's disease, ulcerative colitis, rheumatoid diseases) reacted with the 55-kDa cross-reactive autoantigen. Secondly, and perhaps most importantly, IgA reactivity to the 55-kDa autoantigen decreased in response to gluten-free diet and could be inhibited by pre-incubation of sera with wheat proteins.

We believe that the 55-kDa component represents a newly defined celiac disease-specific autoantigen because several observations suggest that it is different from calreticulin and tissue transglutaminase. In contrast to calreticulin which represents a protein of the endoplasmic reticulum and which is absent from the nucleus [33], we found that the 55-kDa cross-reactive autoantigen is a nuclear antigen. Furthermore, the molecular mass of the 55-kDa autoantigen in our study is different from the previously described 90-kDa glycoprotein [24] and the 85-kDa tissue transglutaminase [27] and it has been reported that immunoblotted transglutaminase does not react with IgA antibodies from celiac patients [27]. Finally we found that serum IgA reactivity to the 55-kDa antigen and to ELISA plate-coupled tissue transglutaminase were not always in agreement and that IgA reactivity to the 55-kDa autoantigen could not be inhibited by pre-incubation of sera with tissue transglutaminase. We thus must assume that the cross-reactive 55-kDa nuclear autoantigen represents an as yet undescribed celiac disease-specific autoantigen different from calreticulin and tissue transglutaminase. Our observations that celiac disease sera which had reacted with the 55-kDa autoantigen in a human intestinal cell line also reacted with the 55-kDa moiety in cells of different histogenetical origin (endothelial cells, fibroblasts) and that the 55-kDa autoantigen is a component of the nucleus suggest that it may represent a rather ubiquitous and conserved antigen.

The fact that IgA reactivity to the 55-kDa autoantigen disappeared under gluten-free diet indicates that IgA recognition of this autoantigen may play a role in the course and, perhaps, pathogenesis of celiac disease or at least be useful as a parameter for monitoring gluten-induced tissue damage.

The frequent association of immune responses against wheat proteins (IgA anti-gliadin production) and autoimmunity as evidenced by EMA reactivity and IgA recognition of the 55-kDa autoantigen raises questions regarding pathomechanisms which could be operative in celiac disease. Concerning transglutaminase, the hypothesis was spelled that endogenous transglutaminase released after wounding of enterocytes may attach to gliadin, a known substrate of this enzyme, and thus create new antigenic complexes/epitopes [27, 34]. Another mutually not exclusive mechanism for the association of anti-gliadin immunoreactivity and autoreactivity may be molecular mimicry between exogenous (e. g., wheat) and human antigens. In this context it was demonstrated that the 54-kDa human adenovirus type 12 E1b protein shared a homologous epitope with α -gliadin [21] and it was reported that mouse monoclonal anti-gliadin antibodies and patients' isolated anti-gliadin antibodies cross-reacted with rat and human enterocytes. The corresponding rat protein was identified as calreticulin [35]. Other authors reported sequence identities of a 180-kDa basement membrane protein from mouse and man with α -gliadin [36]. With the exception of one study reporting that gliadin-specific patients antibodies precipitated two enterocyte components of 66 and 62 kDa of which the latter was identified as calreticulin [23], no distinct cross-reactivities of IgA AGA from celiac patients with human proteins were reported. We demonstrate by immunoblot inhibition experiments that the 55-kDa celiac disease-specific autoantigen and certain other autoantigens (32 kDa, 16–17 kDa) shared epitopes with wheat proteins and thus propose that cross-reactivity of IgA between wheat and human proteins may play a pathogenic role in celiac disease. The structural and immunological similarity of wheat and human IgA-defined antigens revealed by our experiments would explain the rapid and parallel onset of IgA anti-gliadin and autoantibody (EMA) production observed in 24–48 h culture supernatants of gluten-exposed intestinal biopsies from celiac patients [37]. Taken together, these findings and our data would suggest that local production of IgA AGA which cross-react with human proteins may indeed contribute to the induction and/or maintenance of mucosal tissue damage in at least a certain subset of celiac disease patients. In these patients, IgA reactivity against the 55-kDa autoantigen may serve as a useful parameter for the monitoring of gluten-induced tissue damage.

4 Materials and methods

4.1 Characterization of patients

The demographic, clinical and serological parameters of the studied individuals are displayed in Table 1 and 2. The study groups consisted of 30 patients with celiac disease (Table 1) and, for control purposes, of 16 patients with Crohn's disease, 16 patients with ulcerative colitis and 15 healthy persons. Throughout the manuscript (figures and tables) celiac disease patients were numbered as in Table 1. Twenty-four serum samples were obtained from six celiac children before and under gluten-free diet to analyze the time course and recognition pattern of IgA autoreactivity by immunoblotting. Demographic data, dates of serum sampling, exposure time to gliadin, duration of gluten-free diet as well as serological parameters (total IgA levels, presence of IgA EMA and AGA) of the latter group are displayed in Table 2. In all celiac patients, history and clinical signs were indicative for celiac disease. Small bowel suction biopsies were performed in order to confirm the diagnosis. Before gluten-free diet the typical flat mucosa was demonstrated by dissecting microscopy, histological analysis revealed villous atrophy, crypt hyperplasia and increased numbers of intraepithelial lymphocytes. All celiac patients showed prompt and unequivocal clinical improvement after introduction of gluten-free diet.

4.2 Determination of IgA EMA, IgG and IgA AGA levels and IgA anti-transglutaminase reactivity

Blood was collected by venipuncture and sera were stored at -20°C until analysis. The same serum samples were used for EMA, AGA and transglutaminase measurements as well as for immunoblotting experiments. IgA-EMA were estimated by indirect immunofluorescence using fixed cryostat sections of monkey esophagus (INOVA, San Diego, CA) as a substrate. Patients sera were screened at a dilution of 1:10 in PBS. Polyclonal FITC-labeled anti-human IgA antibodies were used for detection of tissue-bound IgA as recommended by the manufacturer. Fluorescence patterns were analyzed on a Leitz Orthoplan fluorescent microscope (Leitz, Marburg, Germany) by one investigator, the reactivity was scored from negative to +++++ positive. AGA levels were determined by ELISA as described previously [38] with some modifications: Serum samples were diluted 1:360 for IgG- and 1:80 for IgA-assays. Peroxidase-conjugated rabbit anti-human IgG and anti-human IgA (Dako, Glostrup, Denmark) were used for detection in a dilution of 1:200. The optical densities corresponding to the levels of bound antibodies were determined in an "EIA Reader" (Bio-Rad, Richmond, CA) at 492 nm (Table 1: patients 20–23). AGA levels in all other serum samples were determined with a commercial gluten EIA (Pharmacia Diagnostics, Uppsala, Sweden).

IgA anti-tissue transglutaminase reactivity was measured by ELISA using a calcium-containing buffer (200 mM Tris, 150 mM NaCl, 5 mM CaCl_2 , pH 7.5) for coating as recommended [31]. Purified transglutaminase (15 $\mu\text{g}/\text{ml}$) (Sigma, St. Louis) was coated to ELISA plates (Nunc, Maxisorb, Roskilde, Denmark) and exposed to 1:50 diluted sera as described [38]. Bound human IgA antibodies were detected as described for the AGA determinations. The OD values displayed in Table 1 represent means of duplicate determinations.

4.3 Cell lines and protein extracts

The human colon cancer cell line CCL 227 [39] was obtained from the American Type Culture Collection (Rockville, MD). A human fibroblast cell line as described in [26] was kindly provided by Pharmacia Diagnostics AB, Uppsala, Sweden. Human umbilical vein endothelial cells were cultured as described [40]. Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μM β -mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco, Gaithersburg, MD) and 10% fetal bovine serum (FBS) (Sera Lab, Crawley Down, GB). After culture all cells were washed twice in PBS to remove culture medium-derived proteins and cell pellets were stored at -70°C in SDS-lysis buffer [41] until use.

4.4 Characterization of IgA-defined autoantigens by immunoblotting

Cellular pellets containing approximately 5×10^6 cells each were lysed in SDS-sample buffer (1 ml/cell pellet), boiled for 10 min and subjected to denaturing 12.5% preparative SDS-polyacrylamide gel electrophoresis (20 cm width) [41]. Sections from the preparative gels were stained with Coomassie Blue to ensure that comparable amounts of intact proteins from each cell type were analyzed. Gel-separated proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) [42]. Nitrocellulose strips (0.5 cm width) were cut from the membranes and were blocked twice for 5 min and once for 30 min in buffer A (50 mM sodium phosphate buffer, pH 7.4, containing 0.5% BSA, 0.5% Tween 20, and 0.05% NaN_3) [43]. Strips were probed overnight with patients sera (1:1,000 diluted in this buffer) at 4°C . Strips were then washed as described above and incubated overnight at room temperature with a mouse monoclonal anti-human IgA antibody (M1265, CLB, Amsterdam, The Netherlands) which had been ^{125}I -labeled using the Chloramine-T method. After washing and drying, membranes were exposed to Kodak X-OMAT S films using intensifying screens (Kodak, Heidelberg, Germany) for approximately 4 h. To visualize weak IgA reactivity in certain sera from celiac disease patients exposure times were increased up to 8 h.

4.5 IgA-inhibition experiments

To investigate the presence of common IgA epitopes among wheat and human proteins, immunoblot inhibitions were performed as described [43]. In brief, sera were pre-incubated with nitrocellulose strips containing blotted wheat proteins (100 µg), and for control purposes, BSA (100 µg), or without addition of membrane-bound proteins overnight at 4°C as described [43]. In addition, sera were also pre-incubated with dissolved native tissue transglutaminase overnight at 4°C. The pre-adsorbed sera were recovered and exposed to nitrocellulose-blotted human intestinal cell extract. Bound IgA antibodies were detected with the ¹²⁵I-labeled mouse monoclonal anti-human IgA antibody (CLB) and visualized by autoradiography.

4.6 Cell fractionation experiments

Cultured cells (10⁸) of the human colon cancer cell line CCL27 were harvested and washed twice in PBS to remove culture medium components. The cytosolic and nuclear fraction was prepared as described [44]. In brief, the cell pellet was resuspended in 3 ml RSB medium (10 mM Tris/Cl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM PMSF) and incubated on ice for 10 min. Cells were broken by adding detergents NP-40 and Na-Deoxycholate to a final concentration of 0.5% and 0.15%, respectively. After incubation on ice for 30 min, cells were homogenized with a glass-glass potter and the homogenate was centrifuged for 10 min at 4°C, 1,500×g. The supernatant representing the cytosolic fraction was stored at -20°C until use. The pellet containing the crude nuclear fraction was resuspended in 3 ml RSB medium and centrifuged through a sucrose cushion (30% sucrose in RSB medium) at 4°C and 2,000×g for 30 min. The pellet was resuspended in 500 µl extraction buffer (10 mM Tris/Cl pH 7.4, 3 mM MgCl₂, 2 mM mercaptoethanol, 1 mM PMSF) containing 0.4 M NaCl and incubated on ice for 20 min. After centrifugation at 4°C, 8,000×g for 5 min the pellet was resuspended in 500 µl extraction buffer containing 1 M NaCl, incubated (20 min, ice) and centrifuged (8,000×g, 4°C, 5 min). The supernatant which was obtained after the last centrifugation represented the nuclear fraction and was stored at -20°C until use. The successful separation of the nuclear and cytosolic fraction was controlled by exposing the nitrocellulose-blotted fractions to a rabbit anti-human histone H2B antiserum (Chemicon, Temecula, CA).

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