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Avidity progression of dietary antibodies in healthy and coeliac children

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SUMMARY

In most individuals minute amounts of food proteins pass undegraded across the intestinal mucosa and trigger antibody formation. Children with coeliac disease have enhanced antibody production against gliadin as well as other dietary antigens, e.g. β-lactoglobulin, in cow's milk. Antibody avidity, i.e. the binding strength between antibody and antigen, often increases during antibody responses and may be related to the biological effectiveness of antibodies. The aim of the present study was to determine the avidity of serum IgG antibodies against β-lactoglobulin and gliadin in healthy children during early childhood and compare these avidities to those found in children with coeliac disease. The average antibody avidity was analysed using a thiocyanate elution assay, whereas the antibody activity of the corresponding sera was assayed by ELISA. The avidity of serum IgG antibodies against β-lactoglobulin as well as gliadin increased with age in healthy children, even in the face of falling antibody titres to the same antigens. Children with untreated coeliac disease had IgG anti-β-lactoglobulin antibodies of significantly higher avidity than healthy children of the same age, and the same trend was observed for IgG antigliadin antibodies. The present data suggest that the avidities of antibodies against dietary antigens increase progressively during early childhood, and that this process seems to be accelerated during active coeliac disease.

Keywords antibody avidity children coeliac disease dietary antibodies

INTRODUCTION

A small fraction of food proteins passes undegraded across the gut mucosa into the circulation [1], which enables antibodies to be produced against protein antigens in the diet. This is probably a physiological phenomenon, as most exposed individuals have low levels of antibodies in serum against cow's milk proteins, including β-lactoglobulin, as well as cereal proteins such as gliadin [2–4].

Certain diseases are characterized by enhanced antibody formation against dietary antigens, e.g. coeliac disease [5,6], cow's milk protein intolerance of delayed onset [7,8] and inflammatory bowel disease [9,10]. T cells are held to play a central role in these conditions. Thus, cell-free supernatants from gluten-specific T cell clones can cause an enteropathy identical to coeliac disease in normal small intestine *in vitro*, and this can be blocked by anti-interferon (IFN)-γ [11]. Further, a case report of coeliac disease in a patient with panhypogammaglobulinaemia suggests that antibodies are not necessary to trigger the disease process [12]. On the other hand, IgG antibodies might activate complement [13] or trigger cytolysis of antigen-coated cells via an ADCC (antibody-dependent cell-mediated cytotoxicity) reaction [14,15], thereby contributing to mucosal damage.

An important characteristic of an antibody is affinity, the binding strength of a single antigen-binding site of an antibody to the corresponding antigenic epitope. A closely related measure is avidity, or functional affinity, which is the overall strength of the interaction between an antibody and its antigen [16] that depends on both antibody affinity and valency [17]. A progressive rise in antibody avidity, termed avidity maturation [18], usually characterizes an immune response. This results from point mutations in the variable region of the immunoglobulin genes of B cells participating in the response, followed by selection of high-affinity B cells by competition for antigen [19,20]. However, the affinity may also remain constant during an antibody response [21,22], oscillate after an initial increase [23] or even decrease. For example, hyposensitization against bee venom by repeated injections leads to decreased antibody avidity [24]. Thus, constant exposure to dietary antigens might result either in increased or decreased antibody avidity.

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High-avidity antibodies are more effective than those of low avidity in a number of immune reactions [25,26]. Antibodies of high avidity directed to dietary antigens could be protective by their superior capacity to eliminate food antigens from the circulation compared to low-avidity antibodies. Conversely, they might be pathogenic, e.g. by effectively fixing complement. In autoimmune diseases high-avidity antibodies may play a pathogenic role; in patients with systemic lupus erythematotosus, appearance of high-avidity antibodies against double-stranded DNA heralds disease exacerbations and prognosticate a severe disease course [27], and in autoimmune thyroiditis symptoms appear when IgG1 antithyroglobulin antibodies progress from low to high affinity[28].

The aim of the present study was to measure the avidity of serum IgG antibodies against the dietary antigens β-lactoglobulin and gliadin in early childhood, both in healthy children and in children with coeliac disease.

PATIENTS AND METHODS

Patients

Sera from healthy children, as well as from children with coeliac disease, were studied with respect to the avidity of antibodies against β-lactoglobulin and gliadin. Some children yielded more than one sample, permitting longitudinal analyses of avidity progression, whereas only one sample was obtained from others. If the serum quantity was large enough, antibodies to both antigens were assayed. In some cases, especially for the coeliac children, this was not possible.

The initial feeding pattern of the children was uniform, following the recommendations of the Swedish National Board of Health and Welfare. Thus, after initial exclusive breast feeding, cow's milk was introduced into the diet at 3–6 months of age and gluten-containing food at the age of 4–6 months. The diagnostic groups had the following characteristics.

Healthy children. The children were followed because of asymptomatic bacteriuria, but at the time of serum sampling they were healthy and abacteriuric. All children were fed a diet containing cow's milk protein as well as gluten.

Thirty-three individuals were examined for their serum IgG anti-β-lactoglobulin antibody levels and avidities. Their median age was 11 months (range 6–36). From 21 of the 33 children a second sample was collected at a median age of 35 months (range 26–48). Thirty children were examined for activities and avidities of serum IgG antigliadin antibodies. Their median age at initial sampling was 12 months (range 7–37). A second sample was taken from 17 of the 30 children at a median age of 36 months (range 26–48). Twenty-nine of the children were analysed for both antigens, 16 of whom yielded two consecutive serum samples.

Children with coeliac disease. The diagnosis of coeliac disease was based on the criteria of European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) [29]. All coeliac children were on a cow's milk-containing diet. Serum samples were obtained when the first biopsy was taken (untreated coeliac disease), in remission on a gluten-free diet (treated), and/or at relapse after reintroduction of gluten (challenged). Anti-β-lactoglobulin antibody levels and avidities were analysed in sera from 16 children with untreated disease (median age 12 months, range 10–25) and nine with treated disease (median age 35 months, range 17–48). Antigliadin antibody levels and avidities were measured in 16 untreated coeliac children (median age 15 months, range 10–24), 11 treated cases (median age 32 months, range 17–57) and in eight coeliac children at relapse on gluten challenge (median age 45 months, range 32–53). In many coeliac patients the small amount of sera permitted analysis of antibodies to only either of the antigens. However, 12 coeliac children were analysed for antibodies to β-lactoglobulin as well as gliadin.

Determination of IgG antibody levels against β*-lactoglobulin or gliadin*

Serum IgG antigliadin and anti-β-lactoglobulin antibody levels were analysed by enzyme-linked immunosorbent assay (ELISA) as described previously [14,15]. Briefly, microtitre plates [Enzyme Immunoassay (EIA)/radioimmunoassay (RIA), Costar Corp., Cambridge, MA, USA] were coated overnight at room temperature with 5 μg/ml gliadin (crude, Sigma Chemicals, St Louis, MO, USA) or 5 μg/ml of equal proportions of β-lactoglobulin A and B (Sigma). Serum samples, diluted in PBS-Tween, were added and incubated overnight. A peroxidase-conjugated rabbit antihuman IgG antibody (Dakopatts A/S, Copenhagen, Denmark), diluted 1/500, was used for detection. The antibody levels were expressed either in relation (%) to a reference serum (for IgG anti-β-lactoglobulin antibodies) or as arbitrary ELISA units calculated with the aid of a standard curve derived from a high titred serum (for IgG antigliadin antibodies).

Measurement of antibody avidity

The average avidity of serum IgG anti-β-lactoglobulin and antigliadin antibodies was assessed using a thiocyanate elution enzyme immunoassay, used previously in a number of studies [30–33]. In principle, wells containing antibody bound to antigens are exposed to various concentrations of the chaotropic agent potassium thiocyanate (KSCN) and resistance to elution is taken as a measure of avidity. Microtitre plates (Costar EIA/RIA plates) were coated at room temperature with 5 μg/ml gliadin (crude, Sigma) or 5 μg/ml of equal quantities of β-lactoglobulin A and B (Sigma). After three washes in phosphate buffered saline (PBS) with 0·05% Tween (Kebo Laboratory AB, Stockholm, Sweden), 100 μl of each serum sample, diluted to give an absorption of 0·6 in the ELISA, were added and incubated for 3 h at room temperature. After three washes, 100 μl of KSCN (Kebo Laboratory AB) ranging from 0·1 M to 4 M were added and the plates were incubated for another 30 min at room temperature, whereupon the wells were washed six times with PBS-Tween. The amount of residual bound antibody was determined by the addition of 100 μl of rabbit antihuman IgG conjugated to alkaline phosphatase (diluted 1/500 in PBS-Tween). After incubation overnight at room temperature and three washes with PBS-Tween, 1 g/l of *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer (pH 9·8) was added and the absorbance at 405 nm was read in a spectrophotometer (Titertek Multiscan MCC/340, Flow Laboratories, Solna, Sweden) after 100 min. The molar KSCN concentration, giving an absorbance of 50% of that in the absence of KSCN, was used as a relative measure of average antibody avidity.

Ethics

Informed consent was obtained from the parents. The study was approved by the Ethics Committee of Göteborg University.

Statistical analysis

In order to evaluate the influence of age on antibody avidity, linear regression analyses were performed separately for each diagnostic group, with avidity as the dependent variable and age as the

independent one. The slopes of the regression lines were compared using Student's *t*-test. Most of the healthy children contributed with more than one serum sample. The degrees of freedom in the analyses were, however, based on the number of individuals and not on the number of observations. The avidity values of different diagnostic groups were compared using Fisher's permutation test [34]. A comparison where the influence of age was eliminated was performed with Mantel's test [35]. Correlations were assessed using Spearman's rank correlation test.

RESULTS

Avidity of serum IgG antibodies against β*-lactoglobulin in relation to age and diagnostic group*

Twenty-one healthy children contributed two consecutive serum samples during their first years of life; serum-IgG anti-*β*-lactoglobulin antibody activity in these samples was measured by ELISA and the avidity of the antibodies was assessed by KSCN elution. In these children, the avidity of IgG anti-*β*-lactoglobulin antibodies increased with time ($P < 0{\cdot}001$) (Fig. 1a), despite the fact that their IgG antibody levels did not rise during the same period (Fig. 1b). An increased avidity with age was also observed using linear regression on data from all 33 children ($P < 0{\cdot}001$, cross-sectional data) (Fig. 2).

A significant age-dependent increase of the avidity of anti-*β*-lactoglobulin antibodies was also seen in the untreated coeliac children (cross-sectional data, linear regression analysis, $P = 0{\cdot}02$) (Fig. 2). Children with untreated coeliac disease tended to have a more rapid progression of avidity with time than healthy children ($P = 0{\cdot}059$ for the difference in regression line slopes, Fig. 2). Accordingly, the IgG anti-*β*-lactoglobulin antibodies in untreated coeliac children were of significantly higher avidity than the corresponding antibodies in healthy children of the same age ($P < 0{\cdot}05$, Mantel's test). In remission, induced by a gluten-free diet, the avidity of anti-*β*-lactoglobulin antibodies remained at the same level as during the active state of disease, despite decreasing antibody levels (data not shown).

In healthy children, IgG anti-*β*-lactoglobulin antibody levels and avidities were significantly positively correlated ($r = 0{\cdot}47$, $P < 0{\cdot}05$). No such relationship was demonstrated in the groups of children with coeliac disease.

Avidity of serum IgG antibodies against gliadin in relation to age and diagnostic group

Serum IgG antigliadin antibodies were assessed for antibody activity and avidity. In healthy children, from whom two consecutive serum samples were obtained ($n = 17$), avidity tended to increase with time, although not significantly (Fig. 3a). However, in a cross-sectional linear regression model including data from all 30 healthy children, a significant rise in avidity with age could be demonstrated ($P < 0{\cdot}05$) (Fig. 4). Serum antigliadin antibody activity as measured by ELISA also increased with age in most children (Fig. 3b).

In the nine coeliac children followed consecutively, there was also a trend of increasing antigliadin antibody avidity (Fig. 5a). When in remission on a gluten-free diet (treated) the avidity seemed to be at least preserved, despite a marked decrease in IgG antigliadin antibody activity as measured with ELISA (Fig. 5a,b). Further, when coeliac children relapsed after gluten reintroduction, most showed a marked increase in avidity, in parallel with elevated IgG antigliadin antibody activity (Fig. 5a,b).

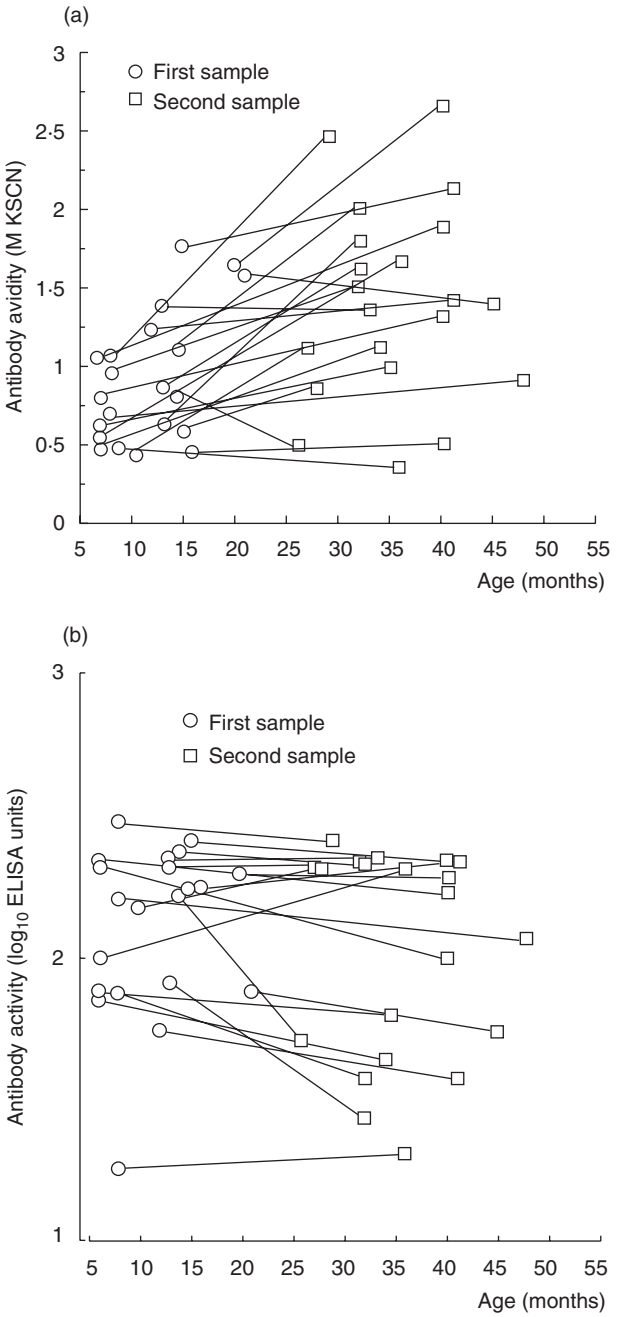

Fig. 1. (a, b) IgG anti-*β*-lactoglobulin antibodies in sera from healthy children followed with two consecutive samples. (a) Avidity as determined by KSCN elution. (b) Activity as measured by ELISA.

The avidity seemed to rise more rapidly in children with untreated coeliac disease than in healthy children, although the slope of the regression line did not reach significance in coeliac children ($P = 0{\cdot}10$, cross-sectional data) (Fig. 4). In accordance, coeliac children at relapse had significantly higher avidity of their IgG antigliadin antibodies than healthy children of the same age ($P < 0{\cdot}03$, Mantel's test).

A weak positive correlation between serum IgG antigliadin antibody activities and avidities was found in the healthy children ($r = 0{\cdot}35$, $P < 0{\cdot}05$), but not in any of the groups of coeliac children.

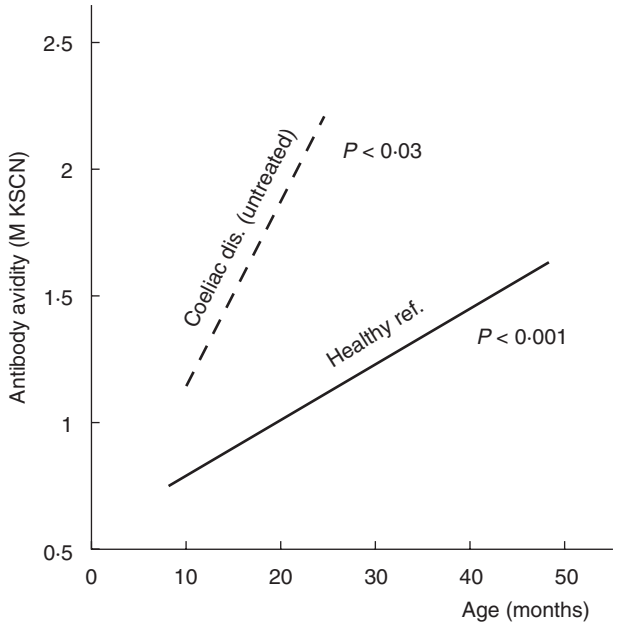

Fig. 2. Linear regression lines of IgG anti-β-lactoglobulin antibodies as a function of age in healthy and coeliac children. The *P*-values indicate the significance of the slope of respective regression line.

Covariation of dietary antibody avidities

The relationship between the avidities of antibodies directed to β-lactoglobulin and gliadin was studied in the 29 control children whose sera were analysed for both antigens. A significant correlation was found between these food-specific antibody avidities ($r = 0{\cdot}47$, $P < 0{\cdot}02$). In the 12 coeliac children investigated, the avidities of the two antibody specificities also correlated, although not significantly ($r = 0{\cdot}51$, $P = 0{\cdot}09$).

Sixteen control children yielded two consecutive serum samples of sufficient quantity to permit analysis of antibodies to both β-lactoglobulin and gliadin. In 14 of these children, the avidities of both antibody types changed in parallel (in 11 children both increased while in three children both decreased between the first and second sample). Only in two children did the avidity of antibodies to β-lactoglobulin increase, while the avidity of antibodies to gliadin decreased. Thus, avidity progression between antibodies against the two dietary antigens was significantly associated ($P < 0{\cdot}02$, Fisher's exact test).

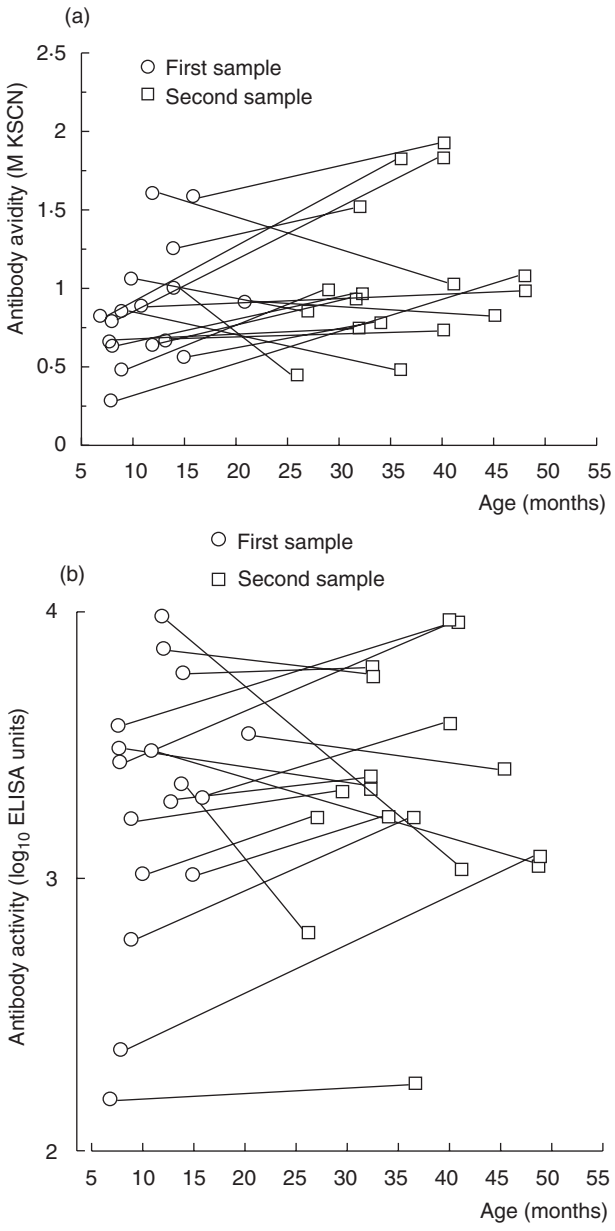

Fig. 3. (a, b) IgG antigliadin antibodies in sera from healthy children followed with two consecutive samples. (a) Avidity as determined by KSCN elution. (b) Activity as measured by ELISA.

DISCUSSION

In the present study the avidity of serum IgG antibodies against two common food antigens, β-lactoglobulin and gliadin, was studied in healthy children as well as in children with coeliac disease. In both groups of children, antibody avidity progressed with age. This suggests that persistent exposure to dietary antigens during the first years of life drives avidity maturation. Accordingly, 1-year-old bottle-fed infants have IgG1 anticasein antibodies of higher avidity than breast- or mixed-fed infants [36]. The increasing avidity of IgG antibodies against β-lactoglobulin and gliadin with age in healthy children may be a physiological process that could be beneficial by enabling the immune system to eliminate these antigens effectively.

Persistent exposure to dietary antigens is considered to result in oral tolerance, a process that has been documented mainly in animals, but also occurs in humans [37]. Oral tolerance manifests in decreasing antibody levels and a paralysis of the T helper cell response to the antigen in question. Whether such tolerance results only in lower antibody levels, with persistence or even increased avidity, or if the avidity will eventually fall following decreasing antibody titres can only be speculated upon. Our results demonstrate clearly that antibody avidity could increase in the face of constant or decreasing antibody activity. This was particularly well demonstrated in healthy children whose anti-β-lactoglobulin antibodies increased in avidity with age but decreased in antibody activity. Similarly, patients with coeliac disease exhibited increased or preserved avidity of IgG antigliadin antibodies during remission on a gluten-free diet, in parallel with falling antibody levels. In accordance, a number of studies carried out previously have shown

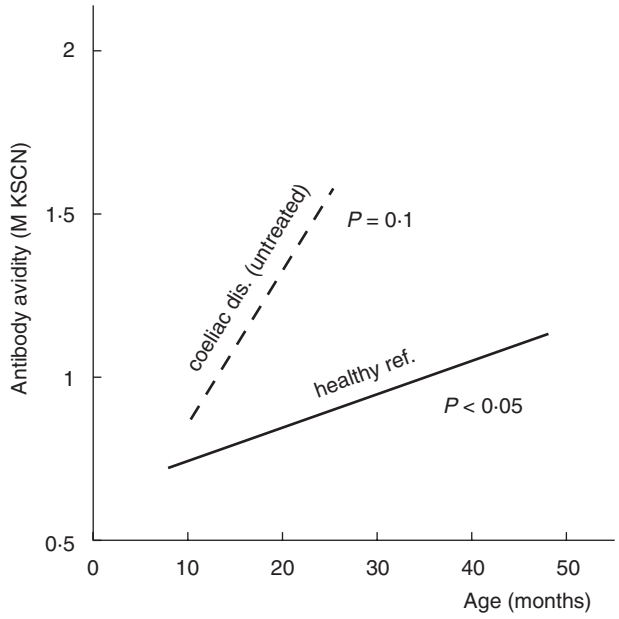

Fig. 4. Linear regression lines of IgG antigliadin antibodies as a function of age in healthy and coeliac children.The *P*-values indicate the significance of the slope of respective regression line.

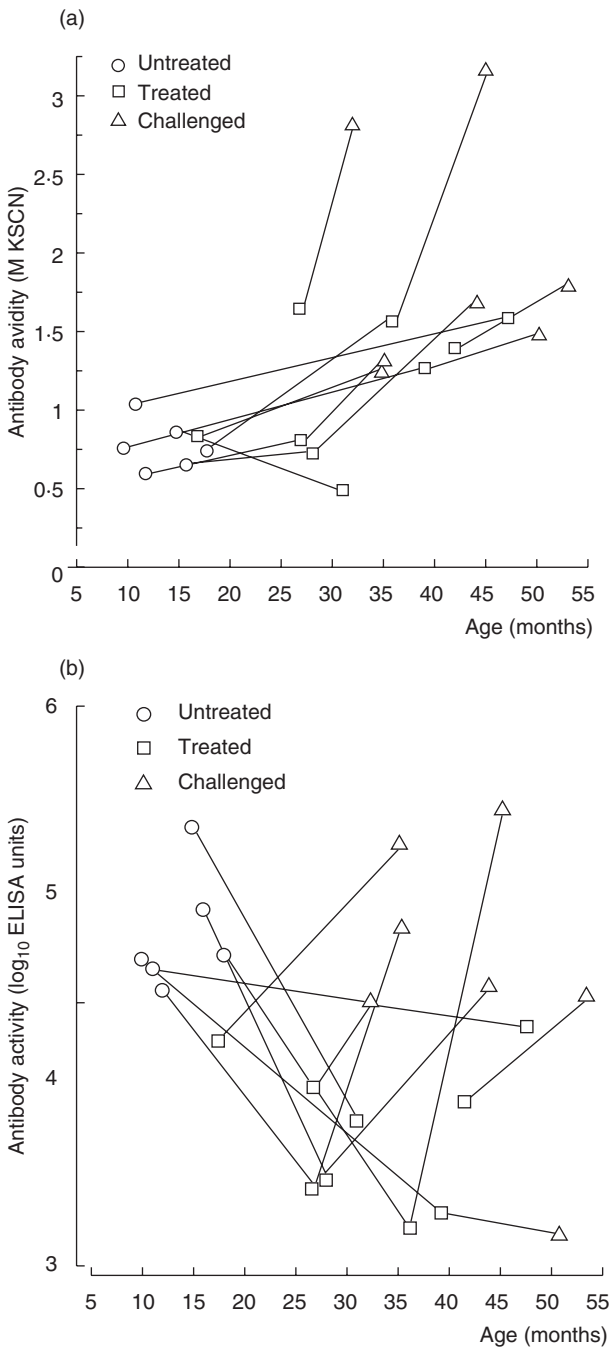

Fig. 5. (a, b) IgG antigliadin antibodies in sera from children with coeliac disease followed longitudinally. (a) Avidity as determined by KSCN elution. (b) Activity as measured by ELISA.

no relation between antibody levels and affinity [30,38]. In animal models, antibody affinity is controlled by mechanisms independent of those governing antibody levels [39].

The avidity progression of both anti-β-lactoglobulin and antigliadin antibodies seemed to be enhanced during active coeliac disease. Thus, coeliac children not yet treated had anti-β-lactoglobulin antibodies of higher avidity than healthy children of the same age. There are several alternative explanations of this phenomenon. The enhanced avidity may be the result of increased intestinal permeability to food antigens, which accompanies the mucosal damage [1,40]. Theoretically, however, an increased antigen load is not linked directly to avidity progression; affinity maturation results from preferential selection of high-affinity B cells by antigen, a process promoted by low rather than high concentrations of antigen [18,20,41]. Another possibility is that, because avidity maturation is a T cell-driven process, the abundance of activated T cells directed to food antigens in children with coeliac disease [42] might promote avidity maturation, e.g. by secreting large amounts of IFN-γ, a cytokine proposed to be involved in affinity maturation [43]. Besides, avidity may be linked to the IgG antibody subclass [44,45]. Patients with active coeliac disease have antigliadin antibodies predominantly of the IgG1 and IgG3 subclass [6,46]. However, this would not explain the increased avidity of anti-β-lactoglobulin antibodies in coeliac children, as their anti-β-lactoglobulin antibodies have a subclass pattern similar to that found in healthy children [47]. Finally, there is a possibility that coeliac patients could be inherently predisposed to produce high-avidity antibodies to dietary antigens, as antibody avidity is genetically influenced [48].

High-avidity antibodies against dietary antigens may be more effective than those of low avidity in different immune reactions. In coeliac disease the humoral response is commonly thought to be less important than the cell-mediated one. However, to speculate, high-avidity IgG antibodies against gliadin may contribute to the disease process by participating in harmful antibody-mediated immune reactions. Accordingly, T cells might not only cause damage to the mucosa by, e.g. activating tissue-destructive macrophages, but also by helping B cells to produce high-avidity antibodies with pathogenic potential.

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