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IL-10-driven immunoglobulin production by B lymphocytes from IgA-deficient individuals correlates to infection proneness

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SUMMARY

In search for a possible explanation of the phenotypic heterogeneity in IgA deficiency, we studied the function of B cells from IgA-deficient (IgAd) individuals. Two groups of IgAd individuals, one frequently infected and one clinically apparently healthy, as well as normal controls, were studied. Peripheral blood mononuclear cells (PBMC) and B cells from IgAd individuals and controls were cultured with *Staphylococcus aureus* Cowan I strain and with anti-CD40 MoAb presented on the CD32-transfected fibroblast cell line in the presence of IL-10. In this experimental system PBMC and B cells from the infection-prone IgAd individuals produced only minute amounts of IgA. In contrast, PBMC and B cells from healthy IgAd subjects secreted significantly more IgA1 and IgA2 in comparison with infection-prone IgAd patients ($P < 0{\cdot}05$). These data suggest that the abnormalities of B cell differentiation in IgAd could be of heterogeneous origin. Thus, whereas in healthy IgAd subjects IgA production may be efficiently up-regulated *in vitro* by addition of IL-10 to CD40-activated B cell culture, the corresponding B cell differentiation does not occur in infection-prone IgAd patients. These observations provide a conceptual framework for phenotypic heterogeneity in IgAd subjects.

Keywords IgA deficiency B lymphocytes CD40 IL-10 immunoglobulin production

INTRODUCTION

IgA deficiency (IgAd) is the most common form of primary immunodeficiency and has been associated with an increased frequency of infections and autoimmune diseases [1,2]. However, approximately two-thirds of IgAd individuals remain healthy [3]. The reason for this difference in susceptibility to infections, as well as to other diseases, is not well understood. It has been suggested that the difference in infection rate between IgAd individuals is due to variable patterns of compensatory rise of secretory IgM in the nasal and oral mucosa [4,5]. On the other hand, other studies failed to demonstrate higher levels of IgM in the saliva [6] or of IgM antibody-secreting cells in the intestinal mucosa [7] in healthy compared with infection-prone individuals with IgAd.

IgAd is a heterogeneous syndrome, and therefore various pathogenic mechanisms have been suggested, such as arrest of B cell differentiation [8], including impaired B cell switching [9,10] and/or T cell immunoregulatory abnormalities with defective helper cell activity or increase of suppressor T cell function [11–13].

A unique model for the study of isotype regulation is the B cell culture system, in which B cells are activated with an anti-CD40 MoAb presented on a CD32-transfected fibroblast cell line ('CD40 system') [14]. In this system, certain cytokines were shown to enhance B cell proliferation and to induce B cell differentiation and immunoglobulin production [14,15]. In particular, addition of IL-10 to CD40-activated B lymphocytes resulted in secretion of considerable amounts of IgM, IgG and IgA [16,17]. Furthermore, costimulation of B lymphocytes via their CD40 antigen using anti-CD40 MoAb and through their antigen receptor using *Staphylococcus aureus* Cowan I strain (SAC) results in increased proliferation [18] and intense differentiation leading to large amounts of secreted immunoglobulins [19]. Recent studies have shown that B cells from most patients with common variable immunodeficiency (CVID) can be induced to secrete IgG, IgM and IgA in the presence of anti-CD40 MoAb and IL-10 [20–22]. Both CVID and IgAd represent heterogeneous syndromes with a similar genetic background characterized by defective antibody production. It has been suggested that IgAd and CVID may represent the extremes of the spectrum of a common B cell defect. In a recent study we showed that, in the CD40 system with SAC (CD40/SAC system), B cells from infection-prone IgAd patients were capable of producing IgA when cultured in the presence of IL-10 [23].

The present study aimed at disclosing possible differences between healthy and infection-prone IgAd individuals with respect to the ability of their B cells to produce immunoglobulin in the CD40/SAC system in the absence and presence of IL-10.

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SUBJECTS AND METHODS

Subjects

Peripheral blood mononuclear cells (PBMC) were obtained from eight IgAd patients with frequent upper and/or lower respiratory tract infections as defined below (all of them females, mean age 39·7 years, range 28–58 years) and nine healthy IgAd individuals (seven males and two females, mean age 39·0 years, range 26–65 years). Seven age-matched healthy individuals with a normal level of immunoglobulins (five females and two males, mean age 38·4 years, range 24–53 years) served as controls. B cells were purified, as described below, from four IgAd individuals in each IgAd group (mean age 34·2 years, range 27–47 years for infection-prone IgAd patients; two females and two males, mean age 43·5 years, range 29–63 years for healthy IgAd individuals) and from four healthy controls (all females, mean age 36·8 years, range 30–48 years).

IgA deficiency was defined as serum IgA levels $< 0{\cdot}05$ g/*l* with IgM, IgG and IgG1–4 above the lower limit of the normal range, i.e. 0·5, 7·0, 4·22, 1·17, 0·41 and 0·01 g/*l*, respectively [2,24]. All IgAd individuals displayed also lack of secretory IgA production measured either in saliva by an ELISA, or in the intestinal mucosa by the enzyme-linked immunospot (ELISPOT) assay [7].

All IgAd individuals included in the study registered all their infections as well as any antibiotic treatment during a period of 2 years before the study started. Patients with at least three respiratory tract infections per year requiring antibiotic treatment, and/or with respiratory tract infection symptoms for more than 100 days per year, were defined as infection-prone IgAd individuals. All of these patients had been referred to the hospital because of their multiple infections. The healthy IgAd individuals were blood donors who had been diagnosed at screening. None of the IgAd individuals defined as healthy had more than one antibiotic treated infection episode per year, and none of them had symptoms of infection for more than 15 days per year. Most of the healthy IgAd individuals had no antibiotic treatment at all. At the time of blood sampling, there was no apparent infection in any of the investigated individuals.

The study was approved by the Ethic's Committee of the Medical Faculty of Göteborg University.

Reagents

The anti-CD40 MoAb 89 was described elsewhere [18]. The CDw32/FcγRII-transfected Ltk cell line (CDw32 L cells) was described earlier [25]. SAC was obtained as Pansorbin from Calbiochem-Behring (San Diego, CA). Purified human rIL-10 (1×10^7 U/ml) was provided by the Shering-Plough Research Institute (Kenilworth, NJ).

PBMC and B cell preparation

PBMC were isolated from the peripheral blood by centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway). The proportion of B cells in the PBMC suspension as determined by flow cytometry on previously frozen samples varied between 12% and 20% with no differences between the three groups. For purification of the B lymphocytes, the PBMC were incubated with MoAb 89 anti-CD40 (for 30 min at 4°C in the medium with fetal calf serum (FCS) and gentamicin), washed, and submitted to positive selection with magnetic beads coated with sheep anti-mouse IgG (Dynabeads; Dynal, Oslo, Norway) [26]. For the detaching of the Dynabeads from positively selected cells we used DetacheBeads (Dynal). After one round of purification procedure 50–60% of the cells expressed CD20 as determined by flow cytometry, and there was no difference with regard to the frequency of B cells between the three experimental groups. The cells were stored at -135°C until assayed.

Cell cultures

For immunoglobulin production, 5×10^4 PBMC or purified B cells were cultured in flat-bottomed 96-well plates in the presence of 5×10^3 irradiated CDw32 L cells, SAC at a final concentration of 0·005%, MoAb 89 (0·5 μg/ml), and in the presence or absence of IL-10 (200 ng/ml) in a final volume of 200 μl. The cultures were carried out in modified Iscove's medium [27].

Immunoglobulin determination in cell culture supernatants

Culture supernatants were harvested after 10 days and levels of IgG, IgA and IgM were measured by ELISA as described previously [27]. For detection of IgA subclasses in the supernatants an ELISA was established. Flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-IgA (Behring, Marburg, Germany) diluted 1:10 000 in a bicarbonate buffer pH 9·6. After incubation for 18 h at 4°C, the plates were washed with PBS with 0·05% Tween (Merck, Darmstadt, Germany) and incubated with RPMI 1640/10% FCS to saturate protein binding sites for 1 h at room temperature. Culture supernatants diluted to the appropriate concentrations in PBS/0·05% Tween were added to the plates and incubated for 2 h at room temperature. The plates were washed and monoclonal anti-IgA1 (Nordimmune, Tilburg, The Netherlands) or anti-IgA2 (Nordimmune) in the form of ascites were diluted at 1:1000 or 1:100, respectively, and incubated for 2 h at room temperature. After washing, the plates were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase diluted at 1:2000 (Sigma Chemical Co., St Louis, MO). Finally, the plates were washed and the enzyme substrate *p*-nitrophenylphosphate (Sigma), in 1 mol/*l* of diethanolamine buffer pH 9·8, was added. Human IgA1 or IgA2, λ and κ, myelomas were used as standard and were provided by The Binding Site (Birmingham, UK). The limit of sensitivities of the assays were 20 and 40 ng/ml for IgA1 and IgA2, respectively.

Quantification of serum immunoglobulins

The concentration of IgM, IgG, and IgG subclasses in serum was determined by single radial immunodiffusion according to Mancini *et al.* [28]. Since the sensitivity of immunodiffusion methods for determination of IgA is 0·01 g/*l*, there is a risk of omitting certain subjects with minute amounts of circulating IgA. Thus, we have employed a sensitive ELISA system to detect low IgA levels. The microtitre plates were coated with 2·8 μg/ml rabbit anti-human IgA (Dako A/S, Glostrup, Denmark) and incubated with serum samples appropriately diluted in PBS/0·05% Tween 20. Swine anti-human IgA conjugated to alkaline phosphatase (Orion Diagnostica AB, Trosa, Sweden) diluted 1:1000 in PBS/0·05% Tween 20 was used for detection and *p*-nitrophenylphosphate in diethanolamine buffer pH 9·8 was used for development of the reaction. The enzyme–substrate reaction was recorded at 405 nm and calculations were made from the standard curve using standard human serum (Behring) with a known concentration of IgA. The sensitivity of this method for IgA detection is 0·0001 g/*l*.

Statistical analysis

Kruskal–Wallis K-sample test followed by the Mann–Whitney *U*-test

were used for comparison of the extent of immunoglobulin synthesis in the various groups.

RESULTS

Serum immunoglobulin levels

The levels of serum IgG, IgA, IgM and IgG subclasses were measured in all individuals participating in the study (Table 1). No significant differences in IgA levels were found between healthy and infection-prone IgAd individuals (Table 1). Three of nine healthy IgAd individuals had detectable levels of IgA in serum, measured by ELISA, but the levels were extremely low (0·0004 g/*l* at highest level). The healthy IgAd individuals had significantly higher levels of total IgG in serum compared with infection-prone IgAd individuals and controls ($P < 0·05$, Table 1). Both IgAd groups had significantly higher serum IgG1 compared with controls ($P < 0·05$, Table 1).

IgG, IgA and IgM synthesis by PBMC

PBMC from IgAd individuals and controls were cultured in the CD40 system together with SAC, with and without IL-10, and subsequent immunoglobulin production was analysed in the culture supernatants by ELISA. In the absence of IL-10, there was a low immunoglobulin production by PBMC from all study subjects, and no significant differences could be seen, with the exception of significantly higher IgA synthesis by PBMC from controls compared with IgAd individuals with a history of frequent infections (Fig. 1a–c).

In the presence of IL-10, PBMC from controls and healthy IgAd individuals showed a marked increase in the synthesis of IgG, IgA and IgM (Fig. 1a–c), and there were no significant differences between these two groups. A clearly different pattern emerged when PBMC from infection-prone IgAd patients were cultured in the presence of IL-10. Thus, incubation of PBMC from infection-prone IgAd individuals in the CD40 system with SAC and IL-10 led to significantly lower production of IgA compared with PBMC from healthy IgAd individuals ($P < 0·05$) and from controls ($P < 0·05$) (Fig. 1a). There was also significantly lower IgG production by PBMC obtained from infection-prone IgAd individuals compared with healthy IgAd individuals ($P < 0·05$) (Fig. 1b). No significant differences were found in IgM production between the three groups (Fig. 1c).

IgG, IgM and IgA subclass synthesis by purified B cells

B cells were purified from four individuals in each of the two IgAd groups and from four controls. B cells were then cultured in the CD40/SAC system with or without the presence of IL-10. Anti-CD40 MoAb was used in both the purification and

Table 1. Immunoglobulin levels (g/*l*) in serum of IgA-deficient individuals with frequent infections, healthy IgA-deficient individuals and controls

	Infection-prone IgAd ($n = 8$)	Healthy IgAd ($n = 9$)	Controls ($n = 10$)
IgA			
Median	0	0	2·9*†
Range	0–0	0–0·0004	1·3–> 3·8
IgG			
Median	16·0	20·0‡	14·5§
Range	14·0–21·6	15·2–24·4	11·8–20·0
IgM			
Median	1·6	1·3	2·5
Range	0·9–1·8	0·7–4·0	1·1–8·0
IgG1			
Median	9·4	11·3	7·9¶**
Range	8·4–13·3	5·6–15·8	4·9–13·3
IgG2			
Median	5·0	6·7	3·9
Range	4·1–10·5	3·6–11·1	2·7–11·1
IgG3			
Median	0·8	1·0	0·7
Range	0·4–1·2	0·4–1·3	0·4–1·4
IgG4			
Median	0·3	1·0	0·4
Range	0·1–0·8	0·1–1·5	0·2–1·8

* Infect. IgAd *versus* controls, $P < 0·001$.
† Healthy IgAd *versus* controls, $P < 0·001$.
‡ Infect. IgAd *versus* healthy IgAd, $P < 0·05$.
§ Healthy IgAd *versus* controls, $P < 0·05$.
¶ Infect. IgAd *versus* controls, $P < 0·05$.
** Healthy IgAd *versus* controls, $P < 0·05$.

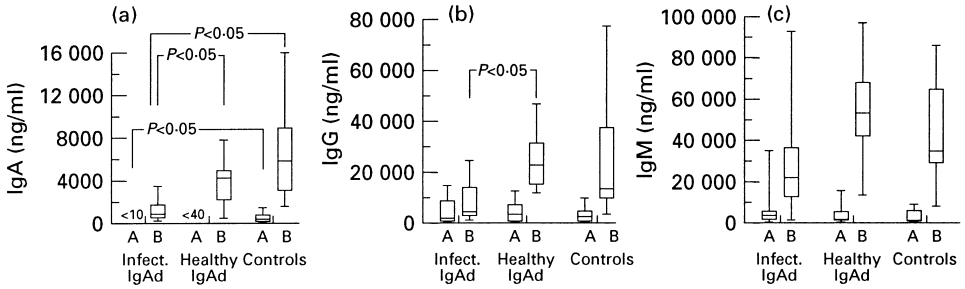

Fig. 1. *In vitro* IgA, IgG, and IgM production by peripheral blood mononuclear cells (PBMC) from IgA-deficient individuals with frequent infections (infect. IgAd) ($n = 8$), healthy IgA-deficient individuals (healthy IgAd) ($n = 9$), and healthy controls (controls) ($n = 7$). PBMC (5×10^4) were cultured on 5×10^3 irradiated CDw32 L cells with 0·5 μg/ml MoAb 89 and *Staphylococcus aureus* Cowan I (SAC; 0·005% v/v) without (A) and with (B) 200 ng/ml IL-10. Culture supernatants were harvested after 10 days of culture. Box plots indicate range (whiskers), 25–75% interval (box), and median value (horizontal line).

activation of B cells. Potentially, this approach might give rise to lower subsequent immunoglobulin responses, when CD40 antigen is ligated with anti-CD40 MoAb during *in vitro* culture. However, since this procedure was used in all three groups in the study, its potential bias should affect the immunoglobulin responses in infection-prone IgAd patients, healthy IgAd individuals and controls in a similar manner.

In the absence of IL-10, B cells obtained from infection-prone IgAd patients did not produce significant amounts of immunoglobulins (Fig. 2a–c). The B cells from healthy IgAd individuals and controls secreted significantly higher amounts of IgG and IgA than the B cells obtained from infection-prone IgAd patients in the absence of IL-10. IgA production by B cells obtained from controls was significantly higher compared with that from the healthy IgAd group (Fig. 2a–c).

The addition of IL-10 enhanced IgG, IgA and IgM production in all three groups (Fig. 2d–f). There was a significantly higher synthesis of IgA by B cells from healthy IgAd individuals compared with B cells from infection-prone IgAd patients ($P < 0{\cdot}05$). IgA production by B cells from control individuals was significantly higher than that by B cells from both IgAd groups ($P < 0{\cdot}05$) (Fig. 2d). There was a tendency towards higher IgG production in healthy IgAd individuals compared with infection-prone IgAd patients, but it did not reach statistical significance (Fig. 2e). IgM synthesis did not differ significantly between the three groups (Fig. 2f). In infection-prone IgAd patients we found a broad range of IgM levels, and there was a close relationship between the individual IgM and IgG levels.

Next, IgA subclass production was evaluated in the supernatants of the B cell cultures. As in the case of total IgA, in the absence of IL-10, IgA1 production by B cells obtained from infection-prone IgAd individuals was under the limit of detection. Also, B cells from healthy IgAd subjects produced very little, if any, IgA1 in the absence of IL-10 (Fig. 3a). However, when stimulated with IL-10, B cells from healthy IgAd individuals secreted significantly higher amounts of IgA1 compared with infection-prone IgAd patients ($P < 0{\cdot}05$, Fig. 3b). B cells obtained from controls synthesized significantly more IgA1 compared with both IgAd groups ($P < 0{\cdot}05$, Fig. 3b). IgA2 synthesis by B cells obtained from infection-prone IgAd individuals was under the limit of detection, and IL-10 did not apparently enhance IgA2 production (Fig. 3c,d). IL-10-stimulated B cells from healthy IgAd individuals produced low amounts of IgA2, significantly lower compared with controls ($P < 0{\cdot}05$, Fig. 3d), yet more than infection-prone IgAd patients ($P < 0{\cdot}05$).

DISCUSSION

Our results suggest that the abnormalities of B cell differentiation in IgA deficiency are of heterogeneous origin, since IgA production by B cells from apparently healthy IgAd subjects may be efficiently up-regulated by IL-10 *in vitro*, whereas a corresponding up-regulation is not achieved in infection-prone IgAd patients. Furthermore, we have demonstrated that PBMC from healthy IgAd individuals produced significantly higher levels of IgG when stimulated with IL-10 compared with infection-prone IgAd patients.

Although IgAd is the most common form of primary immunodeficiency, its pathogenesis is still largely unknown. There is considerable heterogeneity in IgAd as to clinical presentation, and presumably also as to pathogenesis. Especially striking is the fact that most IgAd individuals remain healthy, while others are affected by various disorders such as frequent respiratory tract infections, autoimmune and gastrointestinal diseases, suggesting that the condition is not due to a single defect, but rather to a number of different immunopathological abnormalities, all possibly leading to diminished IgA levels. Certain HLA antigens are found at an increased frequency in IgAd individuals [29–31], but no correlation was seen between proneness to infections and HLA antigens [6].

Several authors have shown normal numbers of B cells with surface IgA in IgAd individuals [30,32,33]. In contrast, Conley & Cooper [34] reported reduced numbers of surface IgA-positive B cells in IgAd patients. In an early study Wu *et al.* [35] suggested that B cells from IgAd individuals had the ability to differentiate into IgA-producing and -secreting plasma cells when stimulated with pokeweed mitogen (PWM). Later studies of IgAd using PWM as mitogen have shown only minute if any production of IgA [10–13,36]. Islam *et al.* [9,10] have recently shown a significant decrease in germ-line αmRNA expression and Cα membrane mRNA expression in PBMC from patients with IgAd.

In our study, PBMC and B cells from control individuals displayed some IgA production when cultured in the CD40 system together with SAC, even in the absence of IL-10. However, addition of IL-10 appeared to be critical in IgAd individuals for triggering the terminal differentiation of IgA-committed B cells, as also has been shown previously in a group of infection-prone IgAd children [23]. Notably, we found a distinct pattern where healthy IgAd individuals displayed a higher IgA and also IgG production in response to IL-10 compared with infection-prone IgAd patients. The difference in responsiveness to IL-10 between these two

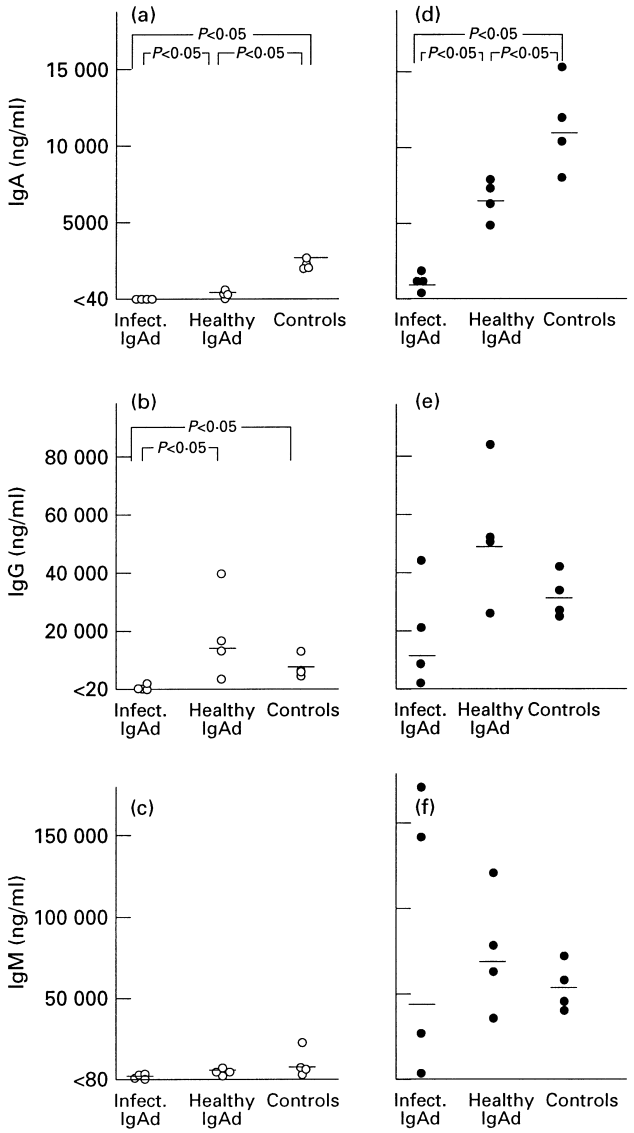

Fig. 2. *In vitro* IgA, IgG, and IgM production by B cells from IgA-deficient individuals with frequent infections (infect. IgAd) ($n = 4$), healthy IgA-deficient individuals (healthy IgAd) ($n = 4$), and healthy controls (controls) ($n = 4$). B cells (5×10^4) were cultured on 5×10^3 irradiated CDw32 L cells with 0·5 μg/ml MoAb 89 and *Staphylococcus aureus* Cowan I (SAC; 0·005% v/v) with (●) and without (○) 200 ng/ml IL-10. Culture supernatants were harvested after 10 days of culture. The bar indicates the geometric mean.

clinically distinct entities might account for defects in resistance to infectious agents. At present, we cannot provide a clear explanation for these differences in the two IgAd groups. There are several potential explanations for the differences in the IL-10-driven IgA and IgG responses seen between infection-prone and healthy IgAd individuals. One possible explanation is that the infection-prone IgAd patients have lower density or deviant expression of IL-10 receptor on their B lymphocytes than healthy IgAd individuals. Another explanation could be that infection-prone IgAd patients might have a lower density of CD40 on B cells than healthy IgAd individuals. However, the high IgM response in some infection-prone IgAd patients does not support these hypotheses. Infection-prone IgAd patients could also have a defective B cell differentiation in response to IL-10 compared with healthy IgAd individuals. Another factor involved in the terminal differentiation of B cells is IL-6 [37]. Defective IgA and IgG responses by infection-prone IgAd individuals in the CD40/SAC/IL-10 system could potentially be due to decreased triggering of IL-6 production. This is, however, less likely since in the CD40/SAC/IL-10 system the synthesis of IL-6 is down- rather than up-regulated [37,38]. Furthermore, *in vitro* blockade of IL-6 receptors in CD40-activated B cells did not affect IL-10-induced B cell differentiation [37]. The defective IgG response in infection-prone IgAd patients in this *in vitro* study is supported by a recent finding of a lower specific antibacterial antibody response (IgG2 antibodies to meningococcal polysaccharide A) in infection-prone IgAd patients compared with healthy IgAd individuals (Friman *et al.*, unpublished observations). A similar difference has also been shown in specific antibody response to pneumococcal polysaccharides [39].

A minute production of IgA by PBMC and B cells was seen in the healthy IgAd group, even in the absence of exogenous IL-10. This low IgA production could depend on endogenous IL-10 production by B cells. It has been recently shown that B cells triggered via CD40 will secrete IL-10, and furthermore, SAC up-regulates this endogenous IL-10 production [37].

In the infection-prone IgAd group, IgA2 production was more affected than IgA1. This hierarchy represents the order of heavy-chain constant region genes on chromosome 14, since α_1 is located upstream of α_2. This result is consistent with Nonoyama *et al.* [20], who demonstrated that in CVID patients IgA2 production from B cells, cultured with anti-CD40 and IL-10, was more frequently affected than IgA1 production.

There is a sex difference between the two IgAd groups. All the infection-prone IgAd patients were female, due to the fact that most sick IgAd patients in our clinic are female, for unknown reasons. Nearly all the healthy IgAd subjects were male, which is a sampling artefact, since they were found when screening blood donors, and most blood donors are male. We do not believe that the lower IgA and IgG production by PBMC and B cells obtained from infection-prone IgAd individuals depend on this material skewness. It is well established that females display stronger, rather than weaker, B cell responses than males [40].

In conclusion, the present study demonstrates that the combination of anti-CD40 MoAb, SAC and IL-10 induces B cells from healthy IgAd individuals to produce considerable amounts of IgA. In contrast, in the same system B cells from infection-prone IgAd patients synthesize only minute levels of IgA. Further studies are required to explain the difference in response to the addition of IL-10 between the two IgAd groups.

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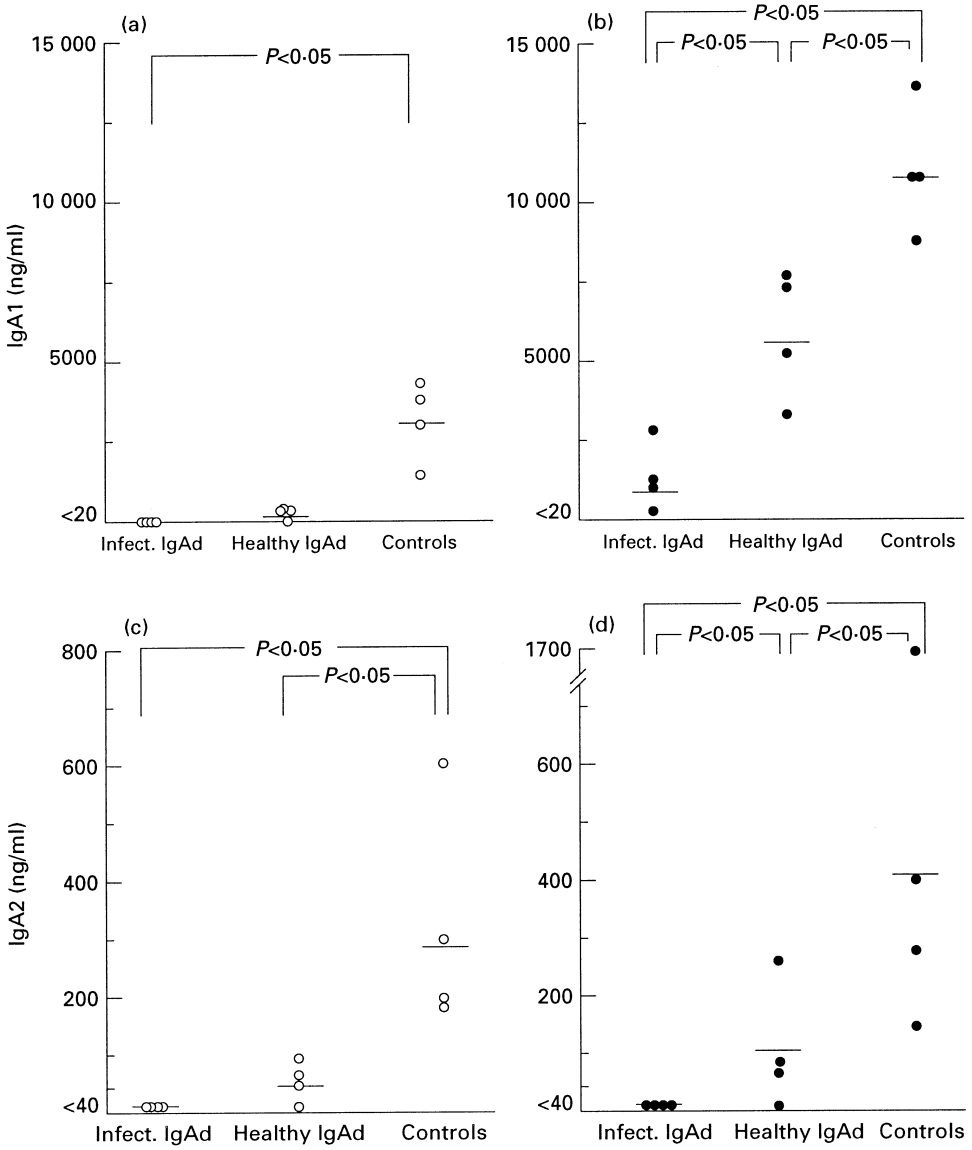

Fig. 3. *In vitro* IgA subclass production by B cells from IgA-deficient individuals with frequent infections (infect. IgAd) ($n = 4$), healthy IgA-deficient individuals (healthy IgAd) ($n = 4$), and healthy controls (controls) ($n = 4$). B cells (5×10^4) were cultured on 5×10^3 irradiated CDw32 L cells with 0·5 μg/ml MoAb 89 and *Staphylococcus aureus* Cowan I (SAC; 0·005% v/v) with (●) and without (○) 200 ng/ml IL-10. Culture supernatants were harvested after 10 days of culture. The bar indicates the geometric mean.

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