

Variable increases of IgG and IgM antibodies in milk of IgA deficient women

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Serum, milk and saliva from seven IgA deficient mothers were studied for the presence of IgA, IgG and IgM antibodies to *Escherichia coli* and poliovirus antigens. Different variable patterns were obtained. One mother had very much increased IgM and IgG antibodies in milk and saliva against both antigens; the milk IgG antibodies were 11–14 times higher than the reference milk pool. Another mother showed also striking increases of both IgM and IgG antibodies in milk, as well as in saliva where the increases were much higher for the poliovirus than the *E. coli* antibodies. Yet another mother showed a certain increase of IgM but not of IgG antibodies in the milk.

The uneven appearance of IgG and IgM antibodies in serum and secretions suggests local production. So do the differences of antibody avidities, the variations in IgG subclass distribution of antibodies and different patterns after isoelectric focusing (IEF) /immunoblotting analysis of antibody spectrotypes in secretions and serum.

The study illustrates the variable patterns of compensatory increases of IgG and IgM antibodies which may occur in IgA deficiency. It also shows that the milk from IgA deficient mothers can still be rich in antibodies, in spite of the lack of secretory IgA.

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IgA in humans occurs in two independent pools in serum and secretions, which are produced at separate sites in the body (1, 2). The dimeric secretory IgA (SIgA) is the predominant antibody in mucosal secretions such as saliva, tears, colostrum, milk, tracheobronchial and gastrointestinal secretions and is the major antibody which protects against bacteria and viruses on mucosal surfaces (3, 4, 5). Moreover, human milk provides the breast-fed infant with large amounts of SIgA antibodies against a huge number of bacterial, viral, parasite and food antigens (6). As much as 0.5–1 g/day of milk SIgA protects the fully breast-fed infant against various infections (7).

Selective IgA deficiency (IgAd), which is the most common primary immunodeficiency, occurring in about 1/600, usually involves both serum and SIgA (8). An increased incidence of malabsorption, infections, allergy and autoimmune diseases is associated with IgAd, but at the same time many IgAd individuals remain apparently healthy. Compensatory mechanisms, such as increases in

other immunoglobulins (9), including secretory IgM (SIgM) (10, 11, 12), might be responsible for their good health.

In the present study we investigated total immunoglobulins and specific antibody levels in serum and secretions, e.g. saliva and milk of IgA-deficient mothers against *Escherichia coli* resulting from natural exposure and against poliovirus type 1 resulting from vaccination, to search for possible patterns of compensatory mechanisms.

Material and methods

Samples from seven healthy mothers with selective IgA deficiency were studied. Serum was obtained at delivery, while the whole saliva and milk samples were collected during the first few days post partum. Milk was not available from one mother (no. VI) and no saliva from mother no. II. All samples were immediately frozen and stored at –20°C until tested. IgA-deficiency was previously diagnosed by the cri-

teria: serum IgA ≤ 0.05 g/l and normal levels of other immunoglobulins (8).

Total serum IgG, IgM and IgA immunoglobulin levels were quantified by radial immunodiffusion (13).

Specific antibodies were determined in a modified enzyme-linked immunosorbent assay (ELISA) (14, 15) in hard polystyrene microtiter plates (Dynatech, Alexandria, VA, USA, or NUNC Immunoplate I, Roskilde, Denmark) coated either with a pool of 10 common *E. coli* O antigens, or with a concentrated inactivated poliovirus type 1 vaccine, produced at the Rijksinstitute voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands. Alkaline phosphatase (AP) (Boehringer Mannheim, Mannheim, Germany) -conjugated rabbit antisera against human IgA (α -chain specific), IgM (μ -chain specific) and IgG (γ -chain specific) (Dakopatts, Copenhagen, Denmark), as well as sheep anti-human secretory component (SC) (Seward Lab., London, UK) were used as second antibody. The reaction between enzyme and substrate (1 g/l of p-nitro-phenyl-phosphate disodium, Sigma, St. Louis, MO, USA, in 1M diethanolamine buffer, pH 9.8 containing 0.001M MgCl_2) was read in a Titertek Multiscan (Flow Labs, Ayrshire, Scotland) at 405 nm after appropriate reaction time (while the enzyme-substrate reaction was linear) and extrapolated to 100 minutes. The sensitivity of this method allows detection of <1 $\mu\text{g/l}$ of specific antibody. All the samples were run in 4 ten-fold dilutions, saliva starting from 1/5, milk from 1/10 and serum from 1/100. Antibody levels were expressed as percent of a reference, which was an early milk pool diluted 1/10 for milk and saliva samples and a high titred serum standard diluted 1/100 for serum samples. The specific antibody levels in the serum standard used were previously compared to the commercial Standard Human Serum (Behring, Behringwerke AG, Marburg, Germany). Since the antibody levels in saliva are known to be very low compared to those in early milk, a saliva pool from ten healthy adults was also used as reference for saliva samples and the specific antibody levels were compared to those of the milk pool. The standards were run on each plate to control the inter- and intra-assay reproducibility ($<10\%$ and $<5\%$ respectively).

Avidity of antigen-specific antibody in serum, milk and saliva of five mothers (nos III, IV, V, VI and VII) was determined by elution of antibody from solid phase immobilized antigen using sodium thiocyanate (KSCN). In this modified ELISA (16), the molarity of KSCN required to elute 50% of bound antibody was used as a measure of relative avidity. After overnight incubation with antigen and washings of the plates, previously titrated samples (as described above) were added to the 16 wells in one appropriate

dilution each. The 4 hours' incubation and washings were followed by the elution of bound antibody with different molarities of KSCN (0.25, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0M) in PBS. Each molarity was added to two wells and an additional two wells contained of PBS alone without KSCN. After 30 minutes' incubation and extensive washings (six times), AP-conjugated swine antisera to human IgG and IgM (Orion) were added and incubated overnight. After washing, the enzyme-substrate was added and the amount of residual bound antibody was determined. The mean absorbances of the duplicate wells with various molarities of KSCN and PBS only were plotted after subtraction of the sample-free background (PBS instead of the sample). The interpolated point at which the absorbance was 50% of the absorbance achieved in the absence of KSCN (100%) was determined and the relative avidity index for each sample was expressed as the molarity of KSCN equivalent to the 50% elution point.

Antibodies of *IgG1-4 subclasses* in serum, milk and saliva were quantified in ELISA. As standard was used Human Immunoglobulin Calibrator (The Binding Site, Birmingham, UK) and for coating mouse monoclonal antibodies against human IgG1-4 subclasses designated HP 6069, HP 6002, HP 6047 and HP 6025 respectively. Mouse anti-human IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., Penn., USA) was used as secondary antibody and the AP substrate was used for visualization of the reaction. The ratios between the 4 subclasses were determined within each sample and then compared between serum and secretions of each mother.

Isoelectric focusing (IEF) combined with affinity immunoblotting (17) was performed on serum, milk and saliva available in sufficient amounts from only one mother (no. IV). The milk sample was first centrifuged at 20 000 g to remove lipids, then the pH was lowered to 4.5 with HCl for precipitation of casein overnight, followed by another centrifugation at 20 000 g. In the whey fraction left, pH was brought back to 7.0 with NaOH. IgG from defatted milk or native saliva was purified by affinity chromatography at Prosep®-A High Capacity column (Bioprocessing Inc., USA) with elution by 0.1 M glycine-HCl solution, pH 3.0. Eluate was dialysed immediately against PBS, pH 7.2–7.4 and concentrated (milk 25 times, saliva 40 times). The final protein concentration was determined by photometry at 280 nm. The serum for IEF was diluted 10 times prior to the run. The samples were separated on agarose gel according to their isoelectric points and blotted to nitrocellulose membrane coated with a pool of 10 common *E. coli* O antigens. As secondary antibodies monoclonal mouse anti-human IgG1

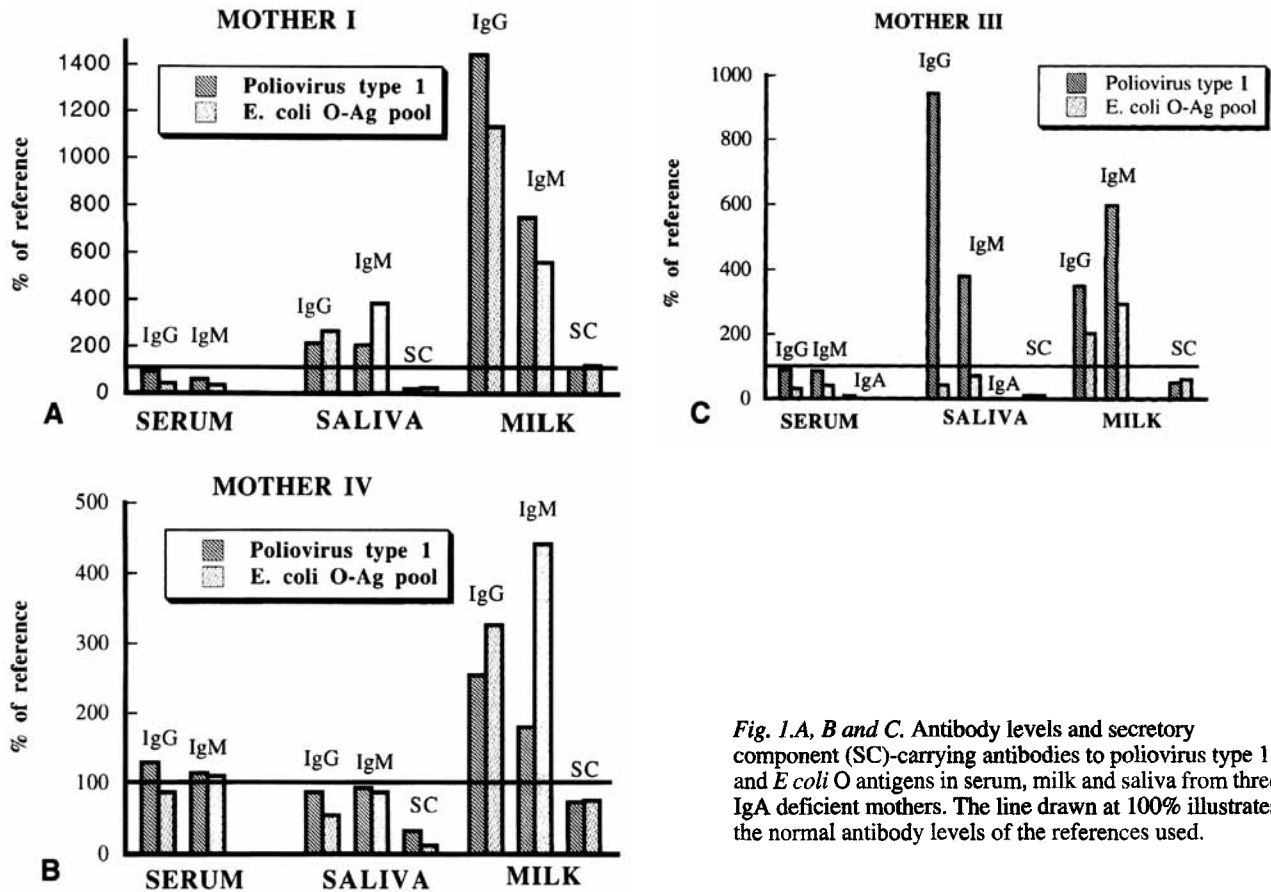


Fig. 1. A, B and C. Antibody levels and secretory component (SC)-carrying antibodies to poliovirus type 1 and *E. coli* O antigens in serum, milk and saliva from three IgA deficient mothers. The line drawn at 100% illustrates the normal antibody levels of the references used.

and IgG2 (clones HP 6012 and HP 6014) were used. Rabbit anti-mouse Igs (Dako) conjugated to AP were used and after reaction with AP-substrate the spectrotypes/clonotypes were visualised and compared in serum and secretion samples.

Because of the lack of or insufficient amounts of some samples, certain analysis described above could not be done on all the material.

Results

IgA was undetectable by radial immunodiffusion in the serum of all women.

Specific antibody levels against the poliovirus type 1 and the *E. coli* O antigen pool were determined by ELISA and are shown in Figs 1A–C for the three most complete set of samples (mothers nos I, III and IV). All seven mothers had specific serum IgG and IgM antibodies against the two antigens. Mother no. III had a very low level of IgA anti-poliovirus in serum (Fig. 1B).

Salivary antibodies

Mothers no. III and VII had a minimal level of IgA antibodies in the saliva against both antigens.

Saliva was available from six mothers. Two mothers (nos I and III) had massively increased levels of both IgM and IgG antibodies in the saliva. Mother no. I had elevated specific IgM and IgG antibody titres against both antigens, 2–4 times as compared to the milk pool reference (Fig. 1A), while mother no. III had 4 times higher IgM and 9 times higher IgG antibody levels against poliovirus type 1 antigen when compared to the milk pool reference (Fig. 1B). Antibody levels against *E. coli*, however, were not increased in the saliva of this mother. In mother no. VI levels of the salivary IgM antibodies against poliovirus type 1 were 1.5 times increased and in three remaining mothers (nos V, IV and VII), IgM and IgG antibody levels in saliva were at the level of the milk reference pool. Levels of SC-containing antibodies against both antigens were not increased in the saliva of either of the mothers.

Milk antibodies

Five out of six IgA-deficient mothers had no detectable IgA antibodies against poliovirus type 1 and *E. coli* O antigens in their milk, while milk of one mother (no. II) contained low levels of IgA antibodies against both antigens (Figs 1A–C).

IgG antibody levels in the milk of mother no. I were much increased - antibodies to poliovirus type 1 were 14 times and against *E. coli* 11 times higher than the milk reference pool (Fig. 1A). Mother no. III also had elevated levels of milk IgG against both antigens: 3.5 times and twice respectively (Fig. 1B). Mother no. IV had 2.5 and 3.5 times increased levels of milk IgG against the two antigens and mother no. VII had 1.5 and 1.2 times increase of the milk IgG antibody levels. Only mother no. II did not have increased levels of IgG antibodies in the milk.

Furthermore, IgM antibody levels in the milk of all mothers were elevated, ranging from 1.5-7.5 times (median 1.8 times) against poliovirus type 1 and from 3.0-5.6 times (median 4.4 times) of the reference against *E. coli* O antigens (Figs 1A-C).

Levels of SC-carrying antibodies against both antigens were only slightly elevated as compared to the reference in the milk of mother no. I (1.1 and 1.2 times) and against *E. coli* in mother no. II (1.3 times), while all other mothers had normal levels of antibodies containing SC in the milk (Figs 1A-C).

The *avidities* of specific IgG and IgM antibodies to the two antigens were determined in five mothers as a possible indicator of their different or similar origin in serum and secretions. In four mothers (nos II, IV, V and VII) there was a clear difference in antibody avidity against one or both antigens between serum and milk, but in some cases also saliva (Table 1). For example, in mother no. III there was a striking difference between the avidity of the naturally acquired antibodies to *E. coli* O antigens compared to the avidity of antibodies to a vaccine, i. e. poliovirus type 1 antibodies. The difference was both in the isotype response and the serum vs. secretory response. Thus, the avidity of the IgG antibodies to *E. coli* in serum was more than twice the avidity of the same isotype in milk and saliva (2.1 compared to

1.0 and 1.0 respectively), while the avidity of serum IgM was almost two times lower than that of milk and salivary antibodies (0.5 compared to 1.0 and 0.9 respectively). However, the avidity of poliovirus type 1 antibodies of both isotypes did not show any greater differences between different compartments, but a striking difference in avidity levels between two isotypes: IgG antibody avidity in serum was 3.5 compared to 3.0 in milk and 3.7 in saliva; IgM antibodies had an avidity of 0.5 in serum, 0.9 in milk and 0.5 in saliva.

The *IgG subclass distribution* in serum and secretions of five mothers is shown in Table 2. The levels of IgG subclasses 1-4 were expressed in g/L and the total IgG as the sum of 4 subclasses. The ratios between IgG1 : IgG2 : IgG3 : IgG4 were calculated in % of the contribution to the total IgG in the sample. The pattern of the subclass distribution (expressed as mean) in sera was: 63% (IgG1) : 32% (IgG2) : 4% (IgG3) : 1% (IgG4), in milk (available from only two mothers) the proportions were similar to those in serum for IgG1 and IgG2, but not for IgG3 and IgG4: 57 : 31 : 9 : 3% and in saliva the distribution of the four subclasses differed from both serum and milk: 19 : 74 : 5 : 2%.

The *spectrotypic/clonotypic patterns* of IgG1 and IgG2 antibodies specific to a pool of *E. coli* O antigens in serum, saliva and milk of one IgAd mother are shown in Fig. 2. It is clearly seen that serum and saliva in this particular case show the same isoelectric properties, while milk differs.

Discussion

Since IgA is the primary immunoglobulin of secretory surfaces, it is not surprising that selective IgA deficiency can be associated with various abnormalities on mucosal surfaces including an increased incidence of infections. What is surprising is that most IgAd individuals are asymptomatic. It has been well known that the absence of IgA in secretions is usually compensated by an increased production of IgM (10, 11, 12, 16), but rarely by IgG (18, 19). A recent study showed, however, that oral immunization of IgAd individuals with cholera vaccine resulted in a predominant IgG response in the gut mucosa (20, 21).

In our study, determinations of IgM and IgG antibodies against poliovirus type 1 and *E. coli* O antigens in saliva and milk of IgAd mothers showed variable compensation in the lack of IgA. While there was no compensation with specific antibodies in the serum of these women, they all compensated in one way or the other in their milk and/or saliva. Not only that, but in the milk of one mother, who had no detectable IgA in her serum determined even with as sensitive a method as ELISA, low levels of

Table 1. Avidity of IgG and IgM antibodies in serum, saliva and milk of IgA deficient mothers (expressed as the molarity of KSCN equivalent to the 50% elution point)

Mother no.	Ig	Poliovirus type 1			<i>E. coli</i> O 10 pool		
		Serum	Saliva	Milk	Serum	Saliva	Milk
III	IgG	3.5	3.7	3.0	2.0	1.0	1.0
	IgM	0.5	0.5	0.9	0.5	0.9	1.0
IV	IgG	3.6	0.8	n.d.**	1.7	0.8	n.d.**
	IgM	0.5	0.7	n.d.**	0.7	0.8	n.d.**
V	IgG	1.7	0.5	1.3	1.9	1.6	1.7
	IgM	0.4	0.4	0.2	1.2	1.8	2.7
VI	IgG	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
	IgM	0.3	0.3	n.d.*	2.3	2.8	n.d.*
VII	IgG	2.5	<0.25	n.d.**	1.7	<0.25	n.d.**
	IgM	0.6	<0.25	n.d.**	0.8	0.3	n.d.**

n.d. not done. * sample not available. ** not enough sample.

Table 2. IgG subclass distribution in serum and secretions of IgA deficient mothers

Mother no.	Sample	IgG1	IgG2	IgG3	IgG4	Sum IgG 1+2+3+4	Ratio (%) of IgG G1:G2:G3:G4
III	serum	14.8 g/L	7.2 g/L	0.8 g/L	0.7 g/L	23.5 g/L	63 : 31 : 3 : 3
	milk	2.6 mg/L	2.6 mg/L	0.7 mg/L	0.3 mg/L	0.006 g/L	42 : 42 : 11 : 5
	saliva	0.03 mg/L	2.4 mg/L	0.008 mg/L	0.03 mg/L	0.003 g/L	1 : 97 : 1 : 1
IV	serum	15.3 g/L	7.7 g/L	1.3 g/L	0.04 g/L	24.3 g/L	63 : 31 : 5 : 1
	saliva	4.1 mg/L	7.4 mg/L	1.3 mg/L	0.04 mg/L	0.013 g/L	32 : 57 : 10 : 1
V	serum	12.2 g/L	3.3 g/L	0.3 g/L	0.07 g/L	15.9 g/L	77 : 20 : 2 : 1
	milk	6.2 mg/L	1.8 mg/L	0.6 mg/L	0.04 mg/L	0.009 g/L	72 : 20 : 7 : 1
	saliva	1.2 mg/L	1.2 mg/L	0.3 mg/L	0.007 mg/L	0.003 g/L	44 : 44 : 11 : 1
VI	serum	25.6 g/L	17.2 g/L	1.5 g/L	1.1 g/L	45.4 g/L	57 : 38 : 3 : 2
	saliva	1.9 mg/L	>9 mg/L*	0.1 mg/L	0.8 mg/L	>0.012 g/L	16 : 76 : 1 : 7
VII	serum	9.2 g/L	5.7 g/L	1.1 g/L	0.09 g/L	16.1 g/L	57 : 35 : 7 : 1
	saliva	0.1 mg/L	5.9 mg/L	0.03 mg/L	0.1 mg/L	0.006 g/L	2 : 96 : 1 : 1

* above the detection limits of the assay.

SIgA antibodies against both antigens could be detected. Although the SIgA deficiency occurs - except in rare instances - concomitantly with serum IgA deficiency, it is apparent that in this case there was some local specific SIgA production in the mammary glands. If this only applies to the production in the mammary gland, or if it could be alike in the salivary gland we cannot say, because the saliva from this mother was not available.

As to the compensatory mechanisms in the secretions, all mothers had increased IgG and/or IgM antibodies in both milk and/or saliva, but in variable ways. Mother no. I compensated more or less equally well with both specific IgG and IgM antibodies in her saliva, while in the milk, although the IgM anti-

body levels were also increased, IgG antibody levels were extremely high. Mother no. II, compensated only with milk IgM, but not IgG antibodies. Mother no. III, who had very low levels of IgA antibodies against poliovirus type 1 in her serum and saliva, compensated in the saliva with both IgM and IgG antibodies against poliovirus, but much more pronounced with IgG antibodies. In the milk it was opposite - although both IgG and IgM antibody levels against two antigens were elevated, IgM anti-poliovirus levels were increased the most. Mothers nos IV, V, and VII did not have increased levels of antibodies in their saliva, but elevated levels of both IgG and IgM antibodies against both antigens in milk, except for mother no. V, who had only IgM antibody levels increased in her milk. Mother no. VI, with no available milk sample and highly elevated serum IgM antibodies, had a slight increase of salivary IgM antibody levels against both antigens.

It cannot be excluded that some or all of the IgG and IgM antibodies in the milk came from serum. If this was the case, however, it then seems strange that antibodies to *E. coli* and poliovirus were not transferred to the same extent, and a similar transfer into saliva and milk would have been expected. On the other hand, SC-carrying antibodies to the two antigens had not increased to the same extent as the total amount of IgM antibodies detected. This could signify that a local production, possibly with an addition of some serum antibody, could overrun the SC transfer mechanism so that some of the IgM appeared in the saliva and milk without SC. We have previously noted increased IgM antibodies with and without SC in nasal secretions of IgAd individuals (11).

A local production of IgM and IgG in exocrine glands would agree with our previous results from

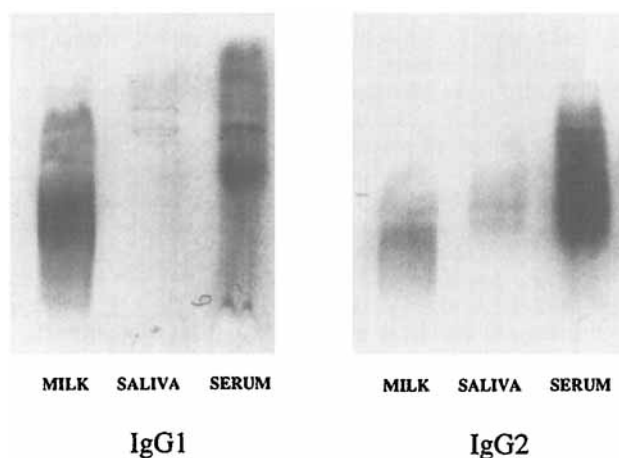


Fig. 2. Spectrotypes of IgG1 and IgG2 antibodies against a pool of 10 common *E. coli* O antigens in serum and secretions of an IgA deficient mother (no. IV). Difference in IEF spectrotypes of antibodies in serum and milk and similarity of the antibody clonotypes in serum and saliva are illustrated.

cell transfer experiments in rats showing selective homing of not only IgA, but also IgM and IgG immunocytes e.g. to the mammary glands (22).

The big difference between serum and secretory antibody avidity, seen in this study, especially in the case of the antibodies acquired by natural exposure, speaks in favour of the presence of antibodies with different properties, which are produced at the different sites in the body.

It was previously suggested that IgG4 may be locally produced in the mammary gland and that both the overall levels of IgG4 and IgG4 antibodies to mucosal antigens were enhanced in milk (23). The different distribution of 4 IgG subclasses in serum compared to those in milk and/or saliva which we show in this study might be another indication that at least partially these antibodies are produced locally at different rate in separate compartments.

The different IEF spectrotypes of antibodies in serum and milk from one mother points as well towards a local production of IgG antibodies in the milk. The similarity of the antibody clonotypes in serum and saliva might indicate leakage or selective transport of serum antibodies into saliva, instead of the local production, at least in the only case tested.

In conclusion, our study has illustrated more dramatically than previously, by investigating milk also, that the increase in IgM antibody production locally on the mucosal surfaces is one of the compensatory mechanisms for the lack of SIgA. Moreover, it also shows a very effective compensation in the secretions with increased levels of IgG antibody which is normally predominant in serum, but scarce in mucosal secretions. This also speaks in favour of local mucosal IgG production, since the increase was not parallel in serum. Thirdly, it appears that, in some cases, there is a local SIgA production in the mammary gland, when IgA is absent in the serum.

Furthermore, this study shows a striking compensatory IgG and IgM antibody increase in the milk of IgAd women, which most likely is of importance for the well-being of their suckling offspring.

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