

Immunoglobulin concentrations in nasopharyngeal secretions

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SUMMARY Sequential nasopharyngeal secretions were collected from 36 breast fed and 14 bottle fed babies followed from birth to 7 weeks of age. Secretions free of covert blood contamination were obtained from only 12 breast fed and 5 bottle fed infants aged less than 1 week. In secretions from all bottle fed babies negative for blood contamination and from 8 bottle fed babies positive for blood contamination no IgA was detected by enzyme-linked immunosorbent assay. All secretions contained IgG and four contained IgM. In contrast, IgA could be shown in the nasopharyngeal secretions of 6 of 12 breast fed babies. All breast fed babies had detectable concentrations of IgG and in two, IgM was detectable. By 7 weeks of age all babies had detectable nasal IgA, IgG, and IgM and there were no differences between breast fed and bottle fed babies. At this time IgG concentrations were low in both groups, having halved since birth. The origins of nasopharyngeal IgA and IgG in infant secretions and their possible role in protection against respiratory virus infection is discussed.

Epidemiological studies over the years have consistently indicated that breast feeding offers infants protection against infection in the first months of life.¹ In infections of the gut, recent studies have confirmed this impression with defined bacterial and viral pathogens and have indicated a central protective role for passively acquired maternal IgA antibody.²⁻⁴ There is also considerable evidence of reduced morbidity and mortality from respiratory disease among breast fed infants.^{5,6} Although it is clear that infant mucosal cells are bathed in colostral IgA along the whole length of the gut, passive transfer to the respiratory mucosa is in doubt. Downham *et al* have suggested that IgA is inhaled during feeding,⁷ and aspiration of radioopaque feed in normal infants has been shown by radiography.⁸

Because of the low concentrations of immunoglobulin present and the difficulty of obtaining adequate specimens from infants it has not been possible until recently to test this hypothesis directly by comparing immunoglobulin concentrations in secretions from breast fed and bottle fed babies. The development of radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs) for class specific immunoglobulins at nanogram concentrations has enabled this comparison. The purpose of this study was to determine IgA, IgG, and IgM immunoglobulin concentrations per unit total protein in the nasopharyngeal secretions of bottle fed and breast fed babies by ELISA.

Materials and methods

Fifty pregnant women were recruited at antenatal clinics. After delivery 14 mothers exclusively bottle fed and 36 breast fed their babies. Nasopharyngeal secretions were collected from each infant at delivery, between two and five days postpartum, and again 7 weeks postpartum with an Argyle feeding tube (Sherwood Medical Industries) attached to a mucous trap (Henleys Medical Supplies). At the same time as the postpartum nasopharyngeal secretions were collected mothers were requested to express by hand 5 ml of colostrum or breast milk.

Sample preparation. Secretions were flushed out of the feeding tube in phosphate buffered saline (0.5 ml) and stored at -70°C until required. Thawed nasopharyngeal secretions were diluted one in 8 in phosphate buffered saline containing 0.05 Tween 20 (PBS/Tween), and mucous was broken up by repeatedly passing through a 25 g needle. Protein content of the preparation was determined by the method of Hartree.¹¹ In some cases, where insufficient material was available for the Hartree method, an estimate of protein content was made by ultraviolet absorption using the formula, protein (mg/ml) = $(E_{215} - E_{225}) 144/1000$.¹² Five specimens were tested in parallel by both methods, with good agreement. Each specimen was tested for haemogenous contamination with Hemastix (Miles

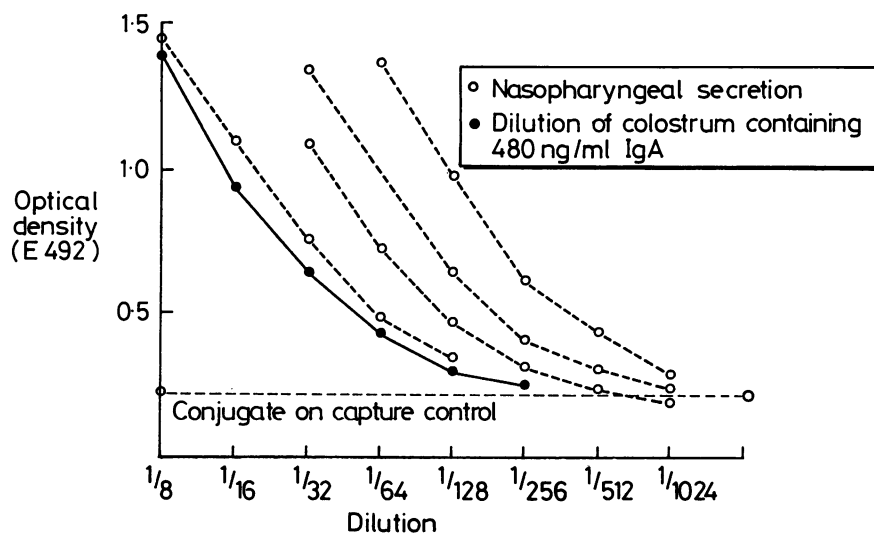


Fig. 1 Titration of infant nasopharyngeal secretions and reference colostrum for IgA by ELISA.

Laboratories). Taking the sensitivity of Hemastix as 150 $\mu\text{g/l}$ haemoglobin as indicated by the manufacturer, the maximum degree of contamination of a Hemastix negative secretion with IgA from maternal blood may be calculated as approximately 1.5 ng/ml; and of IgG as 15 ng/ml. As the limit of sensitivity of ELISA for IgA was mean (SD) 26 (18) ng/ml and IgG values greater than 146 ng/ml were found in all but four secretions we concluded that blood contamination could not have affected appreciably our results in Hemastix negative secretions.

The whey fractions of colostrums and breast milks were diluted one to 2.5 in RPMI medium containing 20 mmol Hepes buffer (pH 7.2), 2 mmol glutamine, penicillin at 500 IU/ml concentration, and streptomycin at 250 $\mu\text{g/ml}$ concentration and spun at 400 g for 10 minutes at 4°C to deposit cells and partially defat for other studies. They were then stored at -70°C until required.

ELISA for total IgA, IgG, and IgM. ELISAs for IgA, IgG, and IgM were carried out according to the method described by Voller *et al.*¹³ Briefly, the wells of flat bottomed microELISA plates (Dynatech) were each coated with 200 μl of capture antibody—namely, goat anti-human IgA or IgG (Hyland Laboratories) or sheep anti-human IgM (Seward Laboratories) diluted in carbonate/bicarbonate buffer. In each test, wells coated with an equivalent concentration of normal goat or sheep serum were included, as appropriate. After overnight incubation at 4°C the plates were washed three times in

PBS/Tween. Twofold dilutions of sample secretions were prepared in 100 μl volumes of PBS/Tween in coated wells. A reference serum or colostrum was titrated in parallel with test samples in every plate, and control coated wells received diluent instead of sample to assess non-specific binding of conjugate to capture antibody. After a further two hours at 27°C the plates were again washed three times as above and 100 μl of an appropriate dilution of rabbit anti-IgA, anti-IgG, or anti-IgM conjugated to horseradish peroxidase (Orion Diagnostica) in PBS/Tween containing 1% normal goat serum was added to each well. After incubation for one hour at 37°C the plates were washed as above and 100/ μl of freshly prepared substrate solution (10/ μg orthophenylenediamine (Sigma Chemical Company) in 25 ml citrate phosphate buffer pH 5.0 and 10 μl of 30% hydrogen peroxide) was added to each well. The reaction was stopped after a further 30 minutes at 37°C by adding 200 μl of three molar sulphuric acid, and optical densities were measured at 492 nm in an automatic spectrophotometer (Multiscan: Flow Laboratories). The curves of optical density against dilution were plotted for each test specimen and for the reference serum or colostrum. In all cases these were roughly parallel (Fig. 1). The concentration of immunoglobulin in the dilution of test specimen giving an optical density of 1.0 was then read off the standard curve. Immunoglobulin concentrations in the reference serum and colostrum were determined by radial immunodiffusion against a calibrated standard serum (Seward Laboratories).

The reproducibility of the assay was tested by titrating a pool of nasopharyngeal secretions taken from infants and frozen in aliquots at -70°C on five different occasions. The immunoglobulin titres of the secretion were mean (SD) $32.7 (5.9) \mu\text{g IgA/ml}$, $33.2 (3.5) \mu\text{g IgG/ml}$, and $13.75 (0.4) \mu\text{g IgM/ml}$.

To assess the mean sensitivity of the assay, the immunoglobulin concentration giving an optical density (OD) 0.35 above that of capture antibody on conjugate background level was chosen. This reproducibly fell near the bottom of the straight line section of the curve—for example, circa OD 0.5 in Fig. 1. The sensitivity varied from day to day and with changes in batches of reagents and plates. For the experiments described here the sensitivities of the assays were mean (SD), IgA 26 (18) ng/ml, IgG 15.4 (7) ng/ml, and IgM 45 (26) ng/ml.

Specificity of ELISA. ELISAs for IgA, IgG, and IgM were carried out on purified IgA and IgM from patients with myeloma and IgG from normal human serum purified by affinity chromatography. The percentage cross reaction was of the order of 1% or less in all cases. IgA, IgG, and IgM concentrations were determined in parallel by radial immunodiffusion (RID)¹⁴ and ELISA in 6 human serums. The ratios of ELISA to RID in the serums were mean (SD), 0.92 (0.05), 1.4 (0.3), and 0.98 (0.16) for IgA, IgG, and IgM. IgA concentrations were determined by both methods in three samples of adult nasal secretions and in three samples of breast milk where IgA is predominantly 11S. The ratio of ELISA to RID was mean (SD) 1.5 (0.2).

Results

Blood contamination of nasopharyngeal secretions.

All nasopharyngeal secretions collected at delivery were either overtly contaminated with blood or gave a strong positive reaction when tested with Hemastix. Of the 46 secretions collected between two and five days postpartum, none was overtly blood stained but 26 gave a positive reaction with Hemastix. Of 27 secretions collected in the 7th week postpartum, only four gave a positive reaction with Hemastix.

Immunoglobulin concentrations in nasopharyngeal secretions collected between two and five days postpartum. The total IgA, IgG, and IgM contents of all Hemastix negative samples were determined by ELISA and standardised against their total protein content determined by ultraviolet absorption. Fig. 2 compares IgA and IgG concentrations in the secretions of breast fed and bottle fed babies. Of the 14

secretions collected from exclusively bottle fed babies, only five were free of blood contamination. None of these contained detectable IgA, and so a further 8 Hemastix positive secretions were also tested. These too were found to be IgA negative. Six of 12 secretions from breast fed babies that gave a negative reaction with Hemastix contained IgA concentrations ranging from 0.03% to 6% of total protein. IgG, at a mean concentration of 1.3% of total protein, was present in all tested secretions from breast fed babies. Concentrations in the five samples from bottle fed babies that gave negative reactions to Hemastix were lower (mean 0.44% total protein), but the difference was not significant. The mean concentration of IgG in samples from bottle fed babies that gave positive reactions with Hemastix was 1.4%. This higher concentration may have been due to blood contamination. IgM was detected in only four Hemastix negative secretions—namely, two from breast fed and two from bottle fed babies—at concentrations between 0.1 and 0.5% total protein. The Table shows IgA and IgG concentrations in nasal secretions of breast fed infants that gave negative reactions with Hemastix together with the time postpartum when

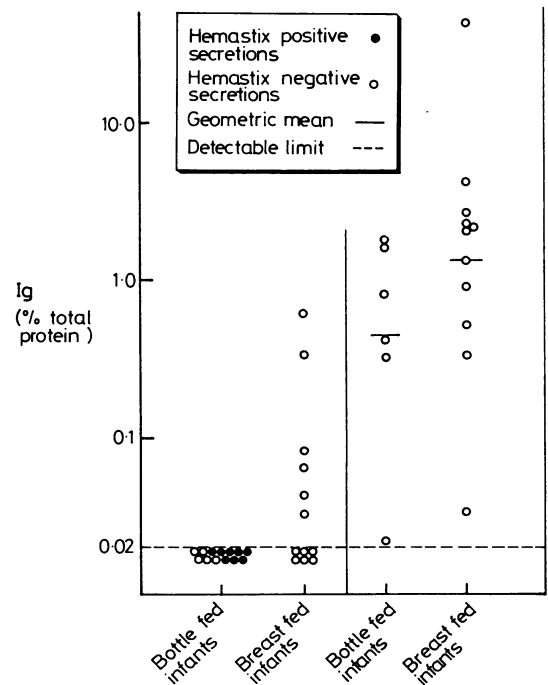


Fig. 2 IgA (left) and IgG (right) concentrations in nasopharyngeal secretions of infants in the first week of life.

collected, the length of time elapsing between collection and the last breast feed, and the immunoglobulin concentrations in colostrum collected on the same day. No clear correlation with any of these factors emerges. This is perhaps emphasised by consideration of cases 1 and 2. These were twins, fed by the same mother; one had the highest detected concentration of IgA in her secretions, but her sister had an undetectable concentration of less than 0.005%.

Immunoglobulin concentrations in nasopharyngeal secretions collected in the 7th week postpartum. Fig. 3 shows the IgA, IgG, and IgM concentrations in nasopharyngeal secretions as a percentage of total protein content, determined by the Hartree method. No significant differences between breast and bottle fed infants were found in the concentrations of any of the three immunoglobulin classes. IgA is the predominant immunoglobulin, with samples obtained at 7 weeks showing a 10-fold increase in concentrations of this class over the concentrations found in breast fed babies during the first week of life. This rise undoubtedly represents the onset of infant IgA synthesis. IgM was also consistently found in these samples suggesting an increase in mean concentrations.

Fall in nasal IgG concentrations over the first 7 weeks of life. IgG concentrations in Hemastix negative secretions collected from 7 infants (6 of whom were breast fed) in weeks 1 and 7 of life were compared. In 6 of the 7 (including the bottle fed infant) a considerable fall off in nasal IgG was observed over this time and mean concentrations were more than halved from 1.4% to 0.6% ($P < 0.025$).

Discussion

The ELISA for IgA described here detected reproducibly nanogram quantities of IgA in expressed breast milk and in nasal secretions from adults. Immunoglobulin concentrations in nasopharyngeal secretions are most satisfactorily expressed in relation to an external marker of the quality of the specimen. In preliminary studies we assessed albumin concentrations and total protein concentrations in secretions as markers. Both showed close correlation of concentrations, but we chose total protein because leakage of fluids from the blood will raise albumin concentrations and dilute any passively acquired IgA. Such an error in total protein concentrations, which represent the sum of transuded, locally secreted, and passively acquired protein, will be less likely. Care was taken to exclude any infant

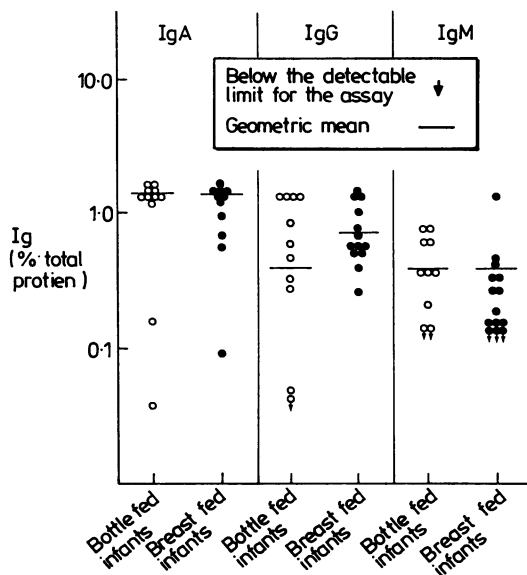


Fig. 3 IgA, IgG, and IgM concentrations in nasopharyngeal secretions of infants at 7 weeks of age.

with overt signs of nasal inflammation from the study.

With this system we were unable to detect any IgA in the secretions of 13 bottle fed infants during their first week of life. In contrast, Roberts and Freed have shown relatively high concentrations (circa 1000 ng/ml) IgA by radioimmunoassay in nasal secretions washed from filter paper plugs inserted into the nares of bottle fed infants of similar age.⁹ This discrepancy may result in part from differences in the efficiency of sampling nasal secretions, but contamination of early secretions with maternal blood may also contribute. Thus, we were unable to collect secretions free of blood from newborn babies and from more than half of 46 babies tested at 2 to 5 days old. Whether this is maternal blood is unknown, but only four of 27 babies aged 7 weeks, sampled in the same way, gave positive reactions with Hemastix, suggesting that the procedure of collection is not unduly traumatic.

Against this background of negative results for bottle fed infants, the finding of IgA in nasopharyngeal secretions of 6 out of 12 breast fed infants is significant. Roberts and Freed reported an increased concentration of IgA in both nasal secretions and saliva of breast fed infants. Subsequent work by Gross and Buckley, however, failed to confirm this result for saliva.¹⁰ Again discrepancies may result from the variable presence of maternal blood in secretions. In neither of these studies were

Table IgA and IgG concentrations in nasopharyngeal secretions (NPS) from breast fed infants and in colostrum collected on the same day

Case No	Day postpartum	Hour after feed	IgA		IgG	
			NPS % total protein	Colostrum IgA concentration (ng/100 ml)	NPS % total protein	Colostrum IgG concentration (ng/100 ml)
1*	2	4	<0.02	60	1.9	0.2
2*			0.6		0.3	
3	4	3	0.08	157	2.2	0.7
4	4	0	0.04	39	42.3	0.2
5	3	4	<0.02	UD	0.03	2.2
6	2	4	<0.02	28	2.7	0.3
7	2	6	0.3	195	0.3	1.3
8	2	4	<0.22	91	2.3	0.3
9	2	2	0.06	37	0.9	0.2
10	2	1	0.03	4	4.1	0.05
11	5	2	0.1	32	1.2	0.4

* Twins.

UD=undetectable.

IgA concentrations standardised against total protein, nor were concentrations of IgG in the secretions or immunoglobulin cross reactions in the assay quoted. For the assay reported here the reactivity of the IgA assay system with purified IgG was 1%. At this concentration the IgG present in secretions could not have affected significantly the IgA titres. Maternal blood contamination was excluded by prior screening with Hemastix.

Our results throw no light on the origin of nasal IgA immunoglobulin in breast fed babies. Perhaps the most likely source would be by inhaled or regurgitated colostrum as has been previously suggested.⁷ If this were the case, then nasal concentrations would be expected to be at their peak just after or during a feed and some correlation between nasal and colostrum IgA values might be expected. Unfortunately, the number of infants giving negative reactions with Hemastix and with detectable IgA in their secretions was too low to allow determination of reliable correlation coefficients, but the indications are that such a correlation would not be perfect. In particular, IgA was found in the nasal secretions of an infant whose mother had abnormally low colostrum concentrations (case 10, Table) and twins receiving colostrum from the same mother showed widely discrepant concentrations of nasal IgA (cases 1 and 2, Table).

Further possibilities include gut absorption of IgA from colostrum and transmission via the blood to the respiratory mucosa,¹⁵⁻¹⁸ or early maturation of IgA secreting plasma cells in the infant mucosa induced by some product in colostrum. The latter has been proposed by Pittard who showed that colostrum cell culture supernatants stimulate IgA but not IgG or IgM production of peripheral blood lymphocytes *in vitro*.¹⁹

Whatever the origin of the IgA in the secretions of breast fed neonates, by 7 weeks postpartum IgA has become the predominant immunoglobulin in both bottle and breast fed babies. Most of the IgA seen at this time is almost certainly a product of the infants' immune response to environmental antigens. A possible functional role, however, of low level passive acquisition of maternal IgA in breast fed infants should not be discounted, even though this may have a negligible effect on total IgA concentrations. Such acquired immunoglobulin would be directed against a wider antigenic repertoire than that of the infant immunoglobulin and could give a valuable prophylactic effect against infection.

In contrast to the concentrations of both IgA and IgM, nasal IgG concentrations fell significantly over the first 7 weeks of life. This probably reflects declining concentrations of transplacentally acquired maternal IgG and may indicate a temporary gap in the infant's respiratory immune defences. It is noteworthy that infections with respiratory syncytial virus, though mild in the first weeks of life, peak in severity at 2 months of age.²⁰

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