Immunology 1989 **66** 394–397

Comparison of concentration and avidity of specific antibodies to *E. coli* in breast milk and serum

F. H. SENNHAUSER,* R. A. MACDONALD,† D. M. ROBERTON*† & C. S. HOSKING† *Department of Paediatrics, University of Melbourne and †Department of Immunology, Royal Children's Hospital, Melbourne, Australia

Accepted for publication 11 November 1988

SUMMARY

To investigate the relationship between mucosal and systemic immunity we analysed the specific anti-*Escherichia coli* antibody concentration and avidity of IgA in colostrum and IgG in paired blood samples from 47 mothers giving birth to premature neonates. The avidity of each sample, expressed as an avidity index, was determined using a novel enzyme immunoassay (EIA)-based procedure, while specific antibody determinations were performed by means of conventional sandwich EIA techniques. All subjects had detectable antibody to *E. coli* in serum and breast milk. The median avidity index for specific IgA antibody in breast milk (3·53 M NH_4SCN, range 2·77–4·90) was significantly higher ($P<0·0001$) than that for specific IgG antibody in serum (median 2·03 M NH_4SCN, range 1·15–3·65). Using Spearman correlation analysis, a weak but significant association was found between the avidity of colostral IgA antibody and the avidity of systemic IgG antibody to pooled *E. coli* polysaccharides ($r_s=0·29$, $P=0·02$). There was also a weak correlation between the concentrations of specific serum IgG antibody and of specific colostral IgA antibody ($r_s=0·36$, $P=0·006$). There was no correlation between the concentration of IgA anti-*E. coli* antibody in colostrum and the avidity of colostral IgA antibody ($r_s=0·14$, $P<0·05$). Similarly, there was no correlation between the concentration and the avidity of serum IgG anti-*E. coli* antibody ($r_s=0·23$, $P<0·05$). The findings of this study suggest independent regulation of concentration and avidity of specific IgA antibody in preterm breast milk. Similar results were seen for specific IgG antibody in serum. The correlations between systemic and mucosal antibody with respect to both concentration and avidity were significant, but are relatively weak and therefore suggest that there also may be independent factors which afford differential regulation of systemic and mucosal antibody responses.

INTRODUCTION

The independence of the mucosal antibody response from the systemic immune system was first demonstrated early this century when orally presented *Shigella* sp. produced a mucosal antibody response which preceded the serum antibody response. In the same study, mucosal and serum responses were shown not to be significantly correlated (Besredka, 1919). Further evidence was provided by studies employing parenteral immunization with various antigens that did not stimulate an IgA response in external secretions (Ogra *et al.*, 1968, 1976; Svennerholm *et al.*, 1980), although prior mucosal exposure has been shown to markedly influence the outcome of subsequent systemic immunization (Svennerholm *et al.*, 1980).

Antibody avidity, which may be described as the summation of forces maintaining the integrity of antigen–antibody interactions, reflects the biological efficacy of antibody function. Using a novel EIA technique to measure the avidity of serum and secretory antibodies (Jones *et al.*, 1987; Macdonald, Hosking & Jones, 1988), we have further investigated the relationship between the mucosal and systemic immune responses. As IgA and IgG are the predominant isotypes in secretions and serum, respectively, we have compared specific mucosal IgA and systemic IgG responses to an antigen common to both systems.

Correspondence: Dr D. M. Roberton, Dept. of Paediatrics, Royal Children's Hospital, Flemington Rd, Parkville, Victoria 3052, Australia.

MATERIALS AND METHODS

Paired serum and colostral samples were obtained 1–6 days (median 2 days) after delivery from 47 mothers giving birth to premature neonates (25–33 weeks of gestation, median 28 weeks). Colostral samples were centrifuged after collection to remove fat and cells and all samples were stored in polypropylene containers at −70° until required.

Measurement of antigen-specific IgA and IgG

Sandwich enzyme immunoassays (EIAs) were used to measure the amount of specific IgA antibody to *E. coli* O polysaccharide in all breast milk samples. A pool of eight somatic antigens from

Table 1. Concentration and avidity of anti-*E. coli* IgA and IgG in paired samples of colostrum and serum. Where appropriate, Spearman correlation analysis was used to determine associations between the various parameters

	Conc. (relative units × 10^5/ml) median (range)	Avidity index (M NH_4SCN) median (range)	Spearman (r_s)
Colostral *E. coli* IgA antibody	1·04 (0·45–10·8)	3·53 (2·77–3·65)	0·14 (NS)
Serum *E. coli* IgG antibody	2·1 (0·5–5·8)	2·03 (1·15–3·65)	0·23 (NS)
Spearman correlation $r_s(P)$	0·36 (0·006)	0·29 (0·020)	—

r_s = Spearman coefficient of correlation.
NS = $P > 0·05$.

E. coli serotypes (serotypes O1, O2, O4, O7, O16, O18 and O75) commonly causing infection (Lidin-Janson *et al.*, 1977) was prepared by heat extraction (Turck, Petersdorf & Fournier, 1962), diluted to 0·09 μg/ml in 0·05 M bicarbonate buffer (pH 9·5) and coated onto 96-well microtitre plates (Nunc, Roskilde, Denmark) overnight at 4°. Four different dilutions of each colostral sample were then added and incubated for 2 hr at 37°. Peroxidase-conjugated anti-human IgA and o-phenylenediamine (OPD; BDH Chemicals, U.K.) substrate were then used to quantify the amount of bound IgA. The plates were washed three times with phosphate-buffered saline containing 0·05% Tween 20 between each step. Serial three-fold dilutions (1/20 to 1/43740) of a pooled ($n = 5$) colostral reference preparation, containing 141 IU of IgA/ml (= 203·1 mg IgA/dL) were included on each test plate and a standard curve was constructed with an arbitrary value of 1 unit of anti-*E. coli* IgA/ml assigned at a dilution of 1/43740. The amount of *E. coli*-specific IgG in each serum sample was measured using a similar EIA to that for IgA, with sample values being determined using a pooled reference serum sample (Roberton *et al.*, 1986) containing 100 IU of IgG/ml (= 806 mg IgG/dL). Again, an arbitrary value of 1 unit of anti-*E. coli* IgG/ml was assigned at a dilution of 1/43740.

Avidity of colostral IgA and serum IgG antibodies to E. coli *polysaccharide*

The avidity of IgA and IgG antibodies to pooled *E. coli* antigens was estimated using a modified EIA incorporating thiocyanate elution as published previously (Jones *et al.*, 1987; Macdonald *et al.*, 1988). Briefly, the method involves a similar EIA to that described above. Sample dilutions were chosen to ensure antigen excess for all isotypes. Following the sample incubation step, 100 μl of NH_4SCN in 0·1 M phosphate buffer, pH 6·0, were added to appropriate wells in duplicate in concentrations ranging from 0 to 5·0 M. The NH_4SCN was washed out after 30 min and the EIA continued as above. Results from wells incubated with phosphate buffer alone represented 100% antibody binding, and the avidity index was defined as the molarity of NH_4SCN required to reduce this absorbance by 50% (Pullen, Fitzgerald & Hosking, 1986).

Adsorption of IgA and IgM

To ascertain the possible effects of *E. coli*-specific antibodies of serum IgA or IgM isotypes competing for antigen-binding sites in the above EIA for serum IgG antibody, immunoglobulins of these classes were removed from a serum sample by adsorption. Affinity-adsorbed goat anti-human IgA and IgM (Kallestad, Austin, TX) were coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) using conventional techniques (Williams & Barclay, 1986). Serum was obtained from an individual with an intermediate titre of *E. coli*-specific IgG antibodies and was mixed with Sepharose anti-human IgM or Sepharose anti-human IgA in PBS/Tween + 0·5% BSA. An identical sample was mixed with uncoupled Sepharose 4B and used as a control. Supernatants were collected after centrifugation. Total IgM or IgA assays (Roberton *et al.*, 1986) and avidity determinations were then performed on each supernatant as described above.

Statistics for data analysis

Spearman rank order correlation analysis was used to determine associations between concentration (in relative units/ml for specific colostral IgA and for specific serum IgG) and avidity index for specific *E. coli* antibodies in paired breast milk and serum samples. The Wilcoxon matched-pairs signed-ranks test was used for comparison of colostral IgA antibody avidity results and serum IgG antibody avidity results.

RESULTS

The concentrations and avidity indices of *E. coli*-specific IgA and IgG in colostrum and serum samples are shown in Table 1. All samples had detectable anti-*E. coli* antibodies. The median avidity index of colostral IgA antibodies was higher than that for serum IgG antibodies (Wilcoxon matched-pairs signed-ranks test, $P < 0·0001$) and there was no significant correlation between concentration and avidity for colostral IgA anti-*E. coli* antibody ($r_s = 0·14$) or serum IgG anti-*E. coli* antibody ($r_s = 0·23$). Spearman correlation analyses of the characteristics of colostral and serum anti-*E. coli* antibodies are also shown in Table 1. At a significance level of $P < 0·05$, a weak rank order correlation exists between the avidity indices for colostral IgA and serum IgG anti-*E. coli* antibodies ($r_s = 0·29$, $P = 0·02$). A weak correlation ($r_s = 0·36$, $P = 0·006$) was also seen between the concentrations of colostral IgA anti-*E. coli* antibodies and serum IgG anti-*E. coli* antibodies. To correct for the effect of the rapidly decreasing IgA concentration in colostrum during the first days of lactation (McClelland, McGrath & Samson, 1978), colostral IgA anti-*E. coli* antibody concentrations were calcu-

lated as a proportion of the total colostral IgA concentration for each sample. The rank-order correlation coefficient for the colostral IgA anti-*E. coli* antibody:total IgA ratio and paired serum IgG anti-*E. coli* antibody concentrations ($r_s = 0{\cdot}33$, $P = 0{\cdot}01$) were slightly lower than the coefficient of 0·36 obtained using the absolute colostral IgA antibody concentrations.

Complete adsorption of IgM or IgA from serum, as shown by the relevant total IgM or IgA EIA, had no effect on the determination of the avidity of *E. coli*-specific IgG antibodies, with similar avidity values recorded for adsorbed and non-adsorbed samples.

DISCUSSION

Enzyme immunoassays have been reported to be a measure of the avidity of antibodies rather than strictly the concentration of antibody (Ahlstedt, Holmgren & Hanson, 1974; Butler *et al.*, 1978). The EIA-based technique used for evaluating avidity employs concentrations of serum or colostrum such that further dilution does not influence the measurement of the avidity index (Jones *et al.*, 1987). Thus this experimental technique is able to demonstrate that there is no significant correlation between concentration and avidity of *E. coli* antibodies either in breast milk or in serum (Table 1). Independent genetic control of the amount and avidity of antibodies to particular antigens has been described previously in mice and in rats (Soothill & Steward, 1971; Katz & Steward, 1976; Kim & Siskind, 1978), with avidity particularly being dependent upon antigen processing (Soothill & Steward, 1971).

The presence of four antigen-binding sites on secretory IgA most probably accounts for the higher avidity index measured for colostral IgA antibodies to *E. coli* in comparison with serum IgG antibodies in which there are only two binding sites per molecule. Almost all IgA in mature milk is present in dimeric form, and Goldman *et al.* (1982) have shown that milk from mothers of preterm infants also has an IgA content that is almost entirely secretory IgA. For immunoglobulins of the same isotype (viz. IgG), however, other workers also have found mucosal antibodies to have higher average avidity than antibodies in serum (Cox, Furman & Muench, 1985).

The present study has demonstrated a weak positive correlation between the characteristics of antibodies of different classes in different tissue fluids but produced in response to the same antigens. Thus, there is a weak tendency in an individual who produces high avidity IgG anti-*E. coli* antibodies in serum to also produce high avidity secretory IgA antibodies in breast milk. The immunological and biological significance of this observation can not be determined from the present data, although it is suggestive of avidity control mechanisms which have some features in common at least for IgA and IgG. From a different perspective, Katz & Steward (1975) in their studies of inbred mice showed that strains of mice selected according to their ability to produce high affinity antibodies to human serum albumin also produced high affinity antibodies to human serum transferrin. Although no correlation was found between concentration and avidity for either anti-*E. coli* IgG in serum or anti-*E. coli* IgA in colostrum in this study, there was nevertheless an association between the concentration of specific IgA antibodies in breast milk and of specific IgG antibodies in serum. Thus, as is the case with avidity, in any particular individual there is some propensity to produce an amount of specific IgA which has a relationship to the amount of specific IgG produced.

The nature of exposure to *E. coli* polysaccharides will vary from colonization of the gastrointestinal tract, where *E. coli* is part of the normal flora, to possible pathological systemic exposure. Similarly, the mode of antigen presentation will differ, thereby introducing the potential for variable antigen recognition and in turn variable avidity. Another factor which may influence the quality and quantity of the response is systemic tolerance induced by mucosal immunization (Slade & Schwartz, 1987), in this case with *E. coli* polysaccharides.

The predominant antibody fractions in serum and at mucosal surfaces are of different isotypes, i.e. IgG in serum and secretory IgA at mucosal surfaces. Therefore several factors may influence comparisons of avidity of mucosal and serum immunoglobulins. Further studies with purified Fab fragments might overcome the problems of different numbers of antigen-binding sites, as is the case with IgA and IgG.

Our findings indicate independent control mechanisms of concentration and avidity of antibodies within serum samples in humans as has been demonstrated in animal models, and also in mucosal secretions as represented by colostral breast milk. Further analysis of avidity of mucosal and serum antibodies for different antigens might clarify the functional relationships of secretory and humoral antibodies.

ACKNOWLEDGMENTS

Dr F. H. Sennhauser was supported by grants from the Swiss National Research Foundation (No. 83.337.0.86) and the Swiss Academy of Medical Sciences. Dr R. A. MacDonald was supported by grants from the Royal Children's Hospital Research Foundation. The assistance of Mr M. Shelton in the statistical analysis of data is gratefully acknowledged. We thank Mrs B. Clarke for typing the manuscript.

REFERENCES

AHLSTEDT S., HOLMGREN J. & HANSON L.A. (1974) Protective capacity of antibodies against *E. coli* antigen with special reference to the avidity. *Int. Arch. All. Appl. Immunol.* **46,** 470.

BESREDKA A. (1919) De la vaccination contre les etats typhoids par la vois buccale. *Ann De L'Inst. Pasteur,* **33,** 882.

BUTLER J.E., FELDBUSH P.L., MCGIVERN P.L. & STEWART N. (1978) The enzyme-linked immunosorbent assay (ELISA): a measure of antibody concentration or affinity? *Immunochemistry,* **15,** 131.

COX D.S., FURMAN S. & MUENCH D. (1985) Affinity of antibody at a secretory site in the rat. *Immunol. Investigations,* **14,** 151.

GOLDMAN A.S., GARZA C.G., NICHOLS B., JOHNSON C.A., SMITH E.O. & GOLDBLUM R.M. (1982) Effects of prematurity on the immunologic system in human milk. *J. Pediatr.* **101,** 901.

JONES C.L., MACDONALD R.A., HOSKING C.S. & ROBERTON D. (1987) Estimating the relative avidity of mucosal IgA for antigen. *J. immunol. Meth.* **105,** 111.

KATZ F.E. & STEWARD M.W. (1975) The genetic control of antibody affinity in mice. *Immunology,* **29,** 543.

KATZ F.E. & STEWARD M.W. (1976) Studies on the genetic control of antibody affinity: the independent control of antibody levels and affinity in Biozzi mice. *J. Immunol.* **177,** 477.

KIM Y.T. & SISKIND G.W. (1978) Studies of the control of antibody synthesis. XII. Genetic influences on antibody affinity. *Immunology,* **34,** 669.

LIDIN-JANSON G., HANSON L.A., KAYSER B., LINCOLN K., LINDBERG V., ORLING S. & WEDEL H. (1977) Comparison of *E. coli* from bacteriuric patients with those from faeces of healthy school children. *J. Infect. Dis.* **136,** 346.

MACDONALD R.A., HOSKING C.S. & JONES C.L. (1988) Measurement of relative antibody affinity by ELISA using thiocyanate elution. *J. immunol. Meth.* **106:** 191.

MCCLELLAND D.B.L., MCGRATH J. & SAMSON R.R. (1978) Antimicrobial factors in human milk. Studies of concentration and transfer to the infant during the early stages of lactation. *Acta Paediatr. Scand. Suppl.* **271,** 3.

OGRA P.L., CHIA Y., BEUTNER K.R. & MORAG A. (1976) Vaccination by non-parenteral routes: characterization of immune response. *Dev. Biol. Stand.* **33:** 19.

OGRA P.L., KARZON D.T., RIGHTHAND F. & MACGILLIVRAY M. (1968) Immunoglobulin response in serum and secretions after immunization with live and inactivated polio vaccine and natural infections. *N. Engl. J. Med.* **279,** 893.

PULLEN G.R., FITZGERALD M.G. & HOSKING C.S. (1986) Antibody avidity determination by ELISA using thiocyanate elution. *J. immunol. Meth.* **86,** 83.

ROBERTON D.M., FORREST P.J., FRANGOULIS E., JONES C.L. & MERMELSTEIN N. (1986) Early induction of secretory immunity in infancy: specific in neonatal breast milk. *Arch. Dis. Child,* **61,** 489.

SLADE H.B. & SCHWARTZ S.A. (1987) Mucosal immunity: the immunology of breast milk. *J. Allergy Clin. Immunol.* **80,** 348.

SOOTHILL J.F. & STEWARD M.W. (1971) The immunopathological significance of the heterogeneity of antibody affinity. *Clin. exp. Immunol.* **9,** 193.

SVENNERHOLM A.M., HANSON L.A., HOLMGREN J., LINDBLAD B.S., NILSSON B. & QUERESHI F. (1980) Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. *Infect. Immun.* **30,** 427.

TURCK M., PETERSDORF R.G. & FOURNIER M.R. (1962) The epidemiology of non-enteric *E. coli* infections: prevalence of serological groups. *J. clin. Invest.* **41,** 1760.

WILLIAMS A.F. & BARCLAY A.N. (1986) Glycoprotein antigens of the lymphocyte surface and their purification by antibody affinity chromatography. In: *Handbook of Experimental Immunology* (Ed. D. M. Weir), Vol. I, pp. 22.1–22.24. Blackwell, London.