THE RELEVANCE OF IMMUNOGLOBULIN IN THE PREVENTION OF NECROTIZING ENTEROCOLITIS

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe acquired gastrointestinal disease and an important cause of morbidity and mortality among stressed low-birth-weight infants in neonatal intensive care units. Although prematurity is the primary risk factor for NEC, it is generally accepted that the pathogenesis of the disease is multifactorial, and a combination of immaturity of the host's gastrointestinal defense mechanisms, intestinal ischemia, and colonization of the gut by infectious agents leads to the development of the disease[1]. NEC develops in 2 to 7% of premature infants admitted to a neonatal intensive care unit[2]. Early abdominal signs indicative of NEC include abdominal distension, diarrhea, gastric retention, emesis, and macroscopic or occult gastrointestinal bleeding. When the disease progresses to unstable stage with vital signs that resemble sepsis, perforation of the intestine or an obstruction pattern on abdominal radiograph, patients require aggressive medical and/or surgical therapy. Histologically the disease, affecting primarily the terminal ileum and ascending colon, is characterized by coagulation necrosis of mucosa and submucosa, inflammation, ulceration, peritonitis, and intramural gas-filled cysts (i.e. pneumatosis intestinalis)[3].

The mortality rate approaches 40-50%, depending on birth weight, maturity and coexisting medical problems[4,5]. Prevention of NEC would therefore substantially contribute to a decrease in morbidity and mortality rates among premature infants. In a recent controlled, randomized clinical trial published in detail[6], we evaluated whether supplementation of feeding of low-birth-weight infants with an oral IgA-IgG preparation would reduce the incidence of NEC.

MATERIALS AND METHODS

Study Design and IgA-IgG Preparation

A detailed description of the study design has been published elsewhere[6]. In brief, a total of 434 infants with birth weights between 800 and

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2000 g, for whom breast milk from their mothers was not available, were prospectively enrolled in the study and randomly assigned to one of two groups. For the first 4 weeks of life infants in group A (N=211) received 600 mg per day of an oral IgA-IgG preparation as a supplement to their regular feeding (infant formula with or without pasteurized, pooled human milk), whereas 223 infants in group B received their regular feeding only (control group).

Two hundred thirty-four infants (123 in the IgA-IgG treatment group and 111 in the control group) were withdrawn during the first week of the study because breast milk from their mothers became available, and 21 control infants were excluded during weeks 2 to 4 of the study because of violations of the study protocol or because breast milk from their mothers became available.

The oral IgA-IgG preparation used in our study was made from human serum, Cohn fraction II, by ion-exchange chromatography (Igabulin, kindly supplied by Immuno AG, Vienna, Austria)[6]. Nine different lots contained 66-84% IgA, 15-33% IgG and <1.5% IgM. As determined using standard techniques (hemagglutination inhibition, neutralization, radioimmunoassay, indirect immunofluorescence, bacterial agglutination), the preparation contained high titers of antibodies against a multitude of infectious pathogens (bacterial toxins such as pertussis, tetanus, diphtheria; viruses such as poliovirus, coxsackievirus, rotavirus and echovirus)[6].

Examination of Fecal Immunoglobulins

Stool samples were collected over a period of 24 hours up to twice a week during the four-week study period. The feces were lyophilized, dissolved in PBS, and immunoglobulin concentrations were determined by single radial immunodiffusion (for a detailed description see Ref. 6).

Immunofluorescence Analysis of Anti-Bacterial Antibodies

IgA- and IgG-antibody titers against enteropathogenic (*Escherichia coli* 125, *Salmonella typhimurium, Shigella sonnei, Klebsiella pneumoniae, Clostridium difficile,* a kind gift of Prof. Rotter, Institute of Hygiene, University of Vienna) and nonpathogenic (*E. coli* 089.H10) bacteria were investigated using indirect immunofluorescence and evaluated with a cytofluorograph.

In brief, 50 µl of a suspension of heat-inactivated bacteria in PBS (approximately 10^7 - 10^8 bacteria/ml) were added to 50 µl of serial two-fold dilutions of the IgA-IgG preparation (one representative lot) and incubated for 60 min at 37°C. The bacteria were then washed twice at 2500 rpm (10 min). Fifty µl of the second step reagent (FITC-conjugated goat anti-human IgA [α chain-specific] or goat anti-human IgG [H+L chain-specific], Jackson Laboratories; final dilution 1:40) were added and the mixture was further incubated for 30 min at 4°C. After washing, the bacteria-associated fluorescence was examined using a cytofluorograph (FACS 440, Becton Dickinson, San Jose, CA). Bacteria and debris were separated by setting a gate on the side scatter histogram (log-amplified with maximal gain). Results are depicted as percent specific fluorescence reactivity, i.e., the percentage of fluorescence-positive bacteria in the experimental sample minus the percentage of background positivity of bacteria stained with the second-step reagent only (mean of quadruplicate samples). For each two-fold dilution of the IgA-IgG preparation the mean of two to three determinations is given.

RESULTS

Prophylaxis of NEC by Oral IgA-IgG

No untoward effects of the immunoglobulin feeding were observed in the treated infants. In contrast, feeding of the oral IgA-IgG preparation significantly reduced the incidence of NEC in our study population (Table 1A). One hundred seventy-nine infants who completed the study were evaluated in great detail. No case of NEC occurred among 88 infants receiving oral IgA-IgG, compared to 6 cases among the 91 control infants (i.e. 6.59%). The clinical diagnosis of NEC was confirmed by abdominal X-ray (N=2), histopathologic examination of specimens obtained during surgery (N=2) or autopsy (N=2). Of the infants withdrawn from the study, two

Table 1. Effect of Oral IgA-IgG Treatment on the Incidence of Necrotizing Enterocolitis (NEC)

A Incidence of NEC during the randomized trial:

	N	Controls	Treatment group
Cases of NEC among the total number of infants enrolled in the study	434	8/223 (3.59%) p=0.0055[a]	0/211
Cases of NEC among the infants who completed the study and had never received any breast milk	179	6/91 (6.59%) p=0.0143[a]	0/88

[a]statistically significant difference between controls and IgA-IgG-treated infants as determined by Chi-square analysis

B Incidence of NEC among low-birth-weight infants (<2500g) - - admitted to the Glanzing Children's Hospital after the randomized trial from March 1989 to August 1990, who all received oral IgA-IgG for the first ten days of life

Total number of infants admitted (N):	606
Cases of NEC:	4 (0.66%)
birth weight (g)	1090,1180,1410,1500
onset of disease	day 2,4,8,10
confirmation of diagnosis at	operation: 2 autopsy: 2

assigned to the control group developed NEC. The different incidence of NEC in the two groups is most likely due to the oral IgA-IgG treatment in group A, since the distribution of several risk factors for NEC (e.g., birth weight, incidence of perinatal complications, choice and date of onset of enteral alimentation) was comparable between IgA-IgG-treated and control infants.

In addition, IgA-IgG feeding seemed to have a beneficial effect on the occurrence of pneumonia in infants who completed the study, since the number of days with clinical symptoms of pneumonia in infants who survived was significantly lower in IgA-IgG-treated group[6]. IgA-IgG feeding also seemed to have a beneficial effect on thriving in low-birth-weight infants. In infants with a birth weight between 1300 and 1700 g, the time required to regain birth weight was significantly lower in IgA-IgG-treated infants (11.3 $\pm$ 0.7 days) compared to the controls (14.6 $\pm$ 1.1 days, mean $\pm$ SEM, $P < 0.02$).

Further support for the beneficial effect of oral IgA-IgG on the incidence of NEC comes from the analysis of the frequency of the disease at our institution after the clinical trial. Since March 1989, all low-birth-weight infants (<2500 g) who are admitted to the Glanzing Children's Hospital neonatal intensive care unit receive 600 mg of oral IgA-IgG per day for the first ten days of life. Until August 1990, 4 cases of NEC were observed among a total of 606 infants admitted (i.e., 0.66%) (Table 1B). The birth weight of the affected infants was between 1090 and 1500 g, the onset of the disease between day 2 and 10 of life. The diagnosis was confirmed in 2 infants by histologic examination of surgical specimens, in two cases at autopsy. We feel that the incidence of NEC observed in infants receiving oral IgA-IgG after the clinical trial is lower than the incidence of the disease in the control group during the study, at it also seems to be lower than the average incidence reported in the literature.

Recovery of Immunoglobulin in the Stool

We found no evidence for resorption of oral immunoglobulin through the gastrointestinal tract of the neonates. As can be seen in Table 2, the number of infants with detectable serum IgA (i.e., IgA above 3 mg/dl) was comparable in IgA-IgG-treated and control infants; serum levels of IgG and IgM tended to be higher in the control group, which might reflect the higher exposure of control infants to environmental antigens through the intestinal tract[6]. However, intact immunoglobulin could be recovered in the stool of IgA-IgG-treated infants. When fecal immunoglobulin was studied in 24 h stool samples obtained from 7 infants treated with oral IgA-IgG and from 6 controls, concentrations of IgA and, to a lesser extent, IgG were significantly higher in stool samples collected from IgA-IgG-treated infants compared to fecal samples from control infants (median [range] mg/g dry feces, first week: IgA-IgG-treated: IgA 7.3 [1.9 - 34.1], IgG 1.3 [0.1 - 12.9]; controls: IgA 0.0 [0.0 - 0.3], IgG 0.4 [0.1 - 3.1]; results for weeks 2-4, see Ref. 6). Fecal IgM levels were comparably low in both groups. In addition, fecal immunoglobulin levels were examined in 3 breast fed infants (Fig. 1). Levels of IgA were comparable in stool samples from IgA-IgG-treated and breast-fed infants. Only small amounts of IgG could be found in the stool of breast-fed infants (fecal immunoglobulin levels in breast-fed infants during the first 2 months of life: IgA 5.8 [3.5 - 24.5], IgG 0.1 [0.02 - 0.6] mg/g dry feces, median and range of 14 determinations).

Table 2. Number of Infants with Detectable Serum IgA Levels During the Study Period

Weeks	IgA-IgG-treatment	Number of infants with detectable serum IgA (>3mg/dl) / total number of infants examined (percent)
1	–	2/82 (2.4%)
	+	3/88 (3.4%)
2	–	7/74 (9.5%)
	+	3/80 (3.8%)
3	–	10/72 (13.9%)
	+	4/72 (5.6%)
4	–	13/65 (20.0%)
	+	14/57 (24.6%)

Serum immunoglobulin levels were determined once a week by single radial immunodiffusion.

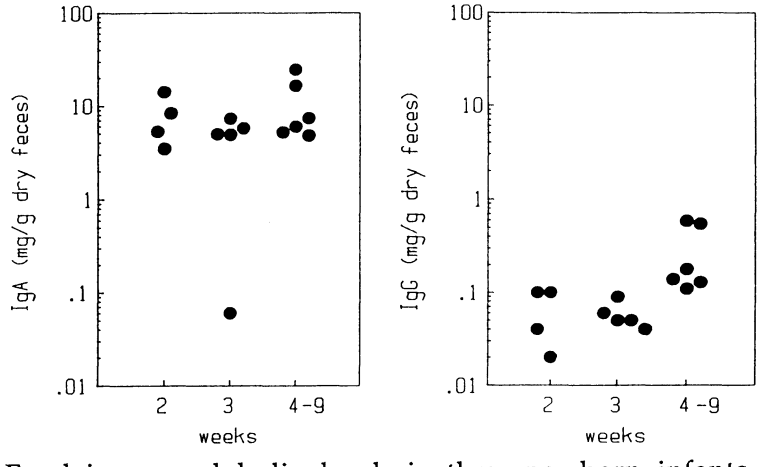

Figure 1. Fecal immunoglobulin levels in three newborn infants receiving breastfeeding.

Antibacterial IgA and IgG Titers of the Immunoglobulin Preparation

Since the oral immunoglobulin preparation contained both IgA and IgG, we were interested in the isotypes of antibodies against enteropathogenic (*E. coli* 125, *Salmonella typhimurium, Shigella sonnei, Klebsiella pneumoniae, Clostridium difficile*) and nonpathogenic (*E. coli* 089.H10) bacteria. Determination of the respective titers of IgA and IgG antibodies of one representative lot of the immunoglobulin preparation was performed using isotype-specific anti-human-immunoglobulin in indirect immunofluorescence, evaluated with a cytofluorograph (Fig. 2). Whereas antibodies against *Salmonella, Shigella, Klebsiella* and *E. coli* 125 were of both the IgA and IgG isotype, predominantly IgA antibodies bound to *Clostridium difficile.*

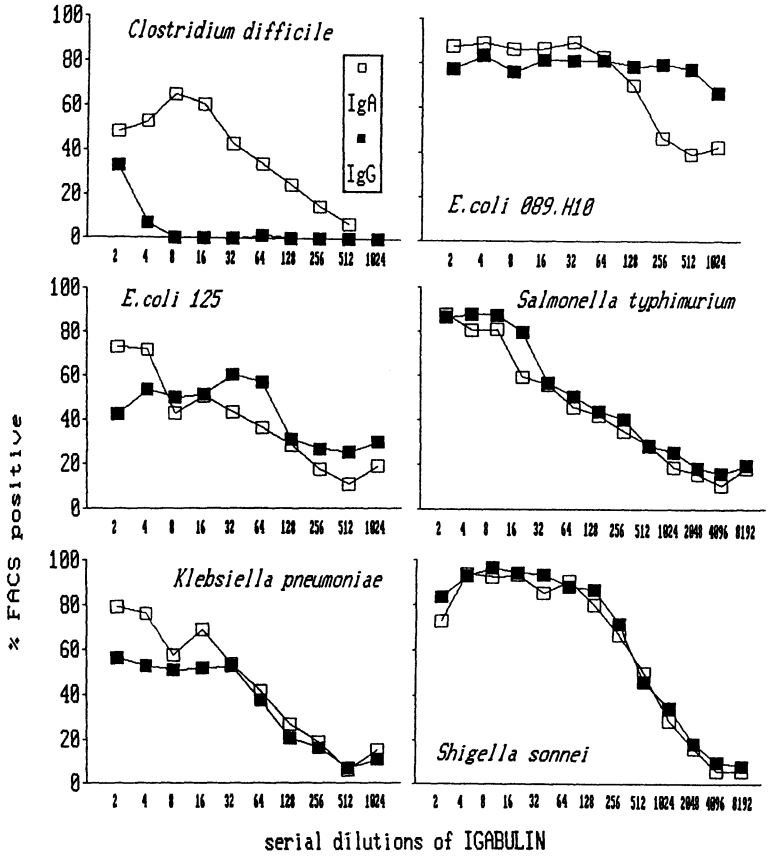

Figure 2. Examination of antibacterial IgA and IgG antibodies of the oral immunoglobulin preparation by indirect immunofluorescence evaluated with a cytofluorograph

In contrast, slightly higher titers of IgG antibodies against *E. coli* 089.H10 were found.

DISCUSSION

Multiple factors play a role in the pathogenesis of NEC. Colonization and invasion of the intestinal mucosa by pathogenic microorganisms as well as the presence of excess protein substrate in the lumen (due to formula feeding) might be essential in the development of NEC. In stressed low-birth-weight infants, clinical conditions associated with perinatal hypoxia might result in ischemia of the gastrointestinal mucosa, which facilitates the invasion of infectious pathogens[7,8]. A variety of infectious organisms by their nature are likely to invade susceptible damaged bowel and/or produce large amounts of toxin. This probably accounts for the general experience that different infectious agents (e.g. *Klebsiella, Salmonella, Clostridia, E. coli,* human enteric coronavirus, rotavirus) have been associated with the disease[9].

Studies performed on the gastrointestinal immunologic defense mechanisms of human neonates generally describe a lack of locally produced antibodies (secretory IgA, i.e. dimeric IgA covalently linked to secretory component) in the gastrointestinal tract of full-term as well as premature neonates[10]. In this condition of inadequate local immunoprotection, alternative mechanisms must function to inhibit overgrowth of potentially pathogenic intestinal flora and prevent invasion. Strong evidence has accumulated for the anti-infectious effect of breastfeeding[11,12]. Due to the immunoprotection provided by breastfeeding, the intestinal flora of breast-fed and formula-fed infants differ with a prevalence of non-pathogenic bacteria in the intestine of breast-fed neonates[13,14]. However, to prevent transmission of infectious agents, pooled human breast milk must be pasteurized, a treatment that significantly reduces not only its content of functional IgA but also its immunoprotective effect[15,16].

The effective prophylaxis of NEC by oral IgA-IgG in our study can best be explained by the well-established immunoprotective effect of orally administered antibodies against infection of the gastrointestinal mucosa in children and adults[17-20]. Results of our study indicate that oral immunoglobulin acts at the level of the intestinal mucosal surfaces. Examination of fecal immunoglobulin in IgA-IgG-treated infants demonstrated that substantial amounts of orally administered IgA and IgG lacking a secretory component can resist proteolytic degradation in the gastrointestinal tract of low-birth-weight infants[6]. Furthermore, the finding of comparable concentrations of fecal IgA in the feces of IgA-IgG-treated and breast-fed infants suggests that we administered physiologic amounts of IgA-IgG as a substitution for the immunoglobulin normally provided by breastfeeding. These data confirm and extend previous reports from others who noted the survival of oral IgG in stool samples of low-birth-weight infants or children with primary immunodeficiency. As assessed by its antigen-binding capacity[21] and the ability to opsonize group B streptococci[22], the fecal immunoglobulin recovered in these studies was functionally intact.

It is interesting to note that different titers of IgA and IgG antibodies against a bacterial strain frequently associated with NEC (*Clostridium difficile*) can be found in our oral immunoglobulin preparation. Whereas the preparation contained comparable amounts of IgA and IgG antibodies against a variety of pathogenic bacterial strains (*Salmonella, Shigella, Klebsiella* and *E. coli* 125), titers of *Clostridium difficile*-specific IgA were significantly higher than IgG titers. Studies performed *in vitro* and in suckling mice prove that secretory IgA from human colostrum can neutralize the cytopathic effect of *Clostridium difficile* toxins A and B[23]. Although both IgA and IgG in our preparation are monomeric, it could be speculated that for at least one bacterial pathogen (*Clostridium difficile*) the IgA antibodies are more effective in protecting from gastrointestinal infection than IgG antibodies.

Analogous to the function of antibodies normally provided by breast-feeding, the immunoprotective effect of oral immunoglobulin (IgA and/or IgG) on the intestinal mucosa can best be explained by the formation of antigen-antibody complexes in the bowel lumen or on the mucosal surface. This hypothesis is supported by the finding of immune complexes formed between orally administered human serum immunoglobulin and endogenous rotavirus in immunodeficient patients with viral gastroenteritis[21]. Binding of functionally intact oral immunoglobulin (IgA and/or IgG) to the antigen (e.g., a bacterial or alimentary constituent) may

cause intraluminal agglutination of potentially pathogenic microorganisms, thereby interfering with the bacterial colonization of the intestinal epithelial surface and by neutralizing bacterial virulence factors or preventing toxic effects of an excess of alimentary protein (i.e. formula feeding) on the intestinal mucosa.

CONCLUSIONS

1. Feeding of human serum IgA and IgG was shown to prevent the development of necrotizing enterocolitis in low-birth-weight infants.

2. The immunoglobulin preparation contained high titers of antibodies against a battery of bacterial and viral pathogens, and comparable amounts of IgA and IgG antibodies against several pathogenic bacteria (e.g., *Klebsiella, Salmonella, Shigella, E. coli*).

3. The preparation's IgA titer against *Clostridium difficile*, a bacterium frequently associated with NEC, was much higher than the IgG titer.

4. Both the inhibition of bacterial colonization and the neutralization of bacterial toxins and/or viral pathogens by IgA and/or IgG antibodies might be responsible for the protective effect of the oral immunoglobulin preparation.

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