Arch Virol (1997) 142: 1491–1498

Archives of Virology
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Printed in Austria

Neutralization of rotavirus and recognition of immunologically important epitopes on VP4 and VP7 by human IgA

Brief Report

K. Johansen[1,2] and **L. Svensson**[2]

[1]Department of Women and Child Health, St. Göran and Karolinska Hospitals, Karolinska Institute, Stockholm, Sweden, [2]Department of Virology, Swedish Institute for Infectious Disease Control, Karolinska Institute, Stockholm, Sweden

Accepted January 22, 1997

Summary. Rotavirus is an important cause of gastroenteritis in young children. Locally produced antibodies in the intestinal mucosa are proposed to play an important role in the defence against rotavirus infection, but it is not established whether IgA alone can neutralize rotavirus, nor if IgA antibodies recognize epitopes involved in protective immunity. To evaluate whether human IgA plays a role in virus neutralization, serum IgA was purified from nineteen rotavirus seropositive individuals and examined for its neutralizing capacity by a peroxidase focus reduction test. In all nineteen sera IgA neutralizing antibodies against serotype 3 (rhesus rotavirus) were demonstrated. Purified IgA was further investigated and shown not only to neutralize rotavirus in solution but also to neutralize rotavirus already pre-bound to epithelial cells (MA-104). IgA epitope blocking assays with monoclonal antibodies directed against heterotypic epitopes on VP4 and VP7, revealed that IgA antibodies from 4/16 sera recognized epitopes on VP4, while 5/16 sera recognized a VP7 epitope. When whole sera were investigated for comparison 7 and 9/16 sera recognized epitopes on VP4 and VP7 respectively.

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Rotaviruses remain an important cause of severe diarrheal disease in infants and young children throughout the world. It is generally assumed that locally produced antibodies in the intestinal mucosa play a key role in protective immunity against rotavirus-induced diarrhea. Rotavirus-specific IgA antibodies have been identified in serum and local secretions, i.e. breast milk, duodenal fluid and fecal material [2, 4, 5, 14, 16, 20, 23]. Milder symptoms in young children exposed to a natural infection have been shown to correlate better with

serum IgA than serum IgG [6, 11]. Inspite of this there is no direct proof that human IgA is involved in rotavirus neutralization nor if IgA antibodies recognize epitopes involved in protective immunity. Monoclonal and polyclonal antibodies of the IgA isotype have previously been shown to protect in in vitro and in vivo models against Sendaivirus, influenza virus and rotavirus infections [1, 3, 12, 13, 15]. IgA is believed to prevent attachment of virus to epithelial cells by cross-linking and aggregation of virus [1] but recently also other modes of action have been suggested such as intracellular neutralization [3, 12, 13] and transport of IgA-antigen complexes from lamina propria to the lumen [8]. To characterize the qualitative IgA responses after a rotavirus infection, we recently developed a method to examine human serum IgA antibody responses to individual rotavirus polypeptides [7]. The study established that serum IgA antibodies after a primary infection mainly are directed against the inner capsid proteins VP2 and VP6. This method has now enabled us to continue to study whether human IgA antibodies alone are capable of neutralizing rotavirus in vitro. The role of human serum IgA in virus neutralization has so far only been investigated in HIV-infected patients [9].

Sera from rotavirus antibody positive adult blood donors (n=19) with varying neutralizing titres (40–5 120) against rhesus rotavirus (RRV), G type 3, by a peroxidase focus reduction assay in whole sera, were included in the study. Three sera from IgA-deficient patients (serum IgA $\leqslant$ 0.1 g/l) served as controls as well as two rotavirus-negative sera. All samples were stored at $-70\,^{\circ}$C until analyzed.

Serum IgA was purified as previously described with a few changes [7]. Briefly, 200 μl of jacalin/agarose beads (Vector, Burlingame, CA, USA) were added to 200 μl of serum diluted in 600 μl of RIPA-buffer (2% v/v Triton X-100, 150 mM Nacl, 600 mM KCl, 5 mM disodium EDTA, 3 mM PMSF, 1 μg aprotinin per ml in 10 mM Tris-HCl, pH 7.8). The mixture was incubated end-over-end (room temperature, 2 h) and the jacalin-agarose beads with bound IgA were carefully washed six times with RIPA-buffer and twice with PBS. The bound IgA was then eluted by adding 2 ml of 0.8 M d-galactose (pH 7.5) to the jacalin-agarose beads and incubated end-over-end (room temperature, 15 h). The eluate was absorbed with 100 μl of Protein-G Sepharose B beads (room temperature, 1 h) to remove residual contaminating IgG. The residual total IgG after absorption was quantified with ELISA. The amount of eluted IgA was also determined with ELISA to enable the expression of IgA neutralizing titer per mg total IgA.

Before the peroxidase focus reduction assay was performed the IgA eluates (2 ml) were concentrated to 200 μl on Centricon 30 filters (Amicon Inc, Beverly, MA, USA). Two-fold serial dilutions starting from undiluted were then made and mixed with an equal volume of 1×10^3 p.f.u. of trypsin-activated (5 μg/ml) RRV. After incubation (room temperature, 1 h) the mixture was added in duplicates to washed monolayers of MA 104 cells in 96-well plates (Nunc, Roskilde, Denmark) followed by incubation (37 °C, 1 h). Cells were then washed again and incubated in serum-free Eagles MEM, 5% CO_2 (37 °C, 15 h).

After fixation of cells with 2% paraformaldehyde in PBS (4 °C, 24 h) they were made permeable with 1% Triton X-100 in PBS (room temperature, 15 min). Staining was then performed with a monoclonal antibody directed against VP6 (255) diluted 1:1 000 (37 °C, 1 h), followed by a peroxidase-labeled goat-anti-mouse (Bio-Rad, Hercules, CA, USA) diluted 1:2 000 (37 °C, 1 h). The reaction was developed at room temperature with 0.3 g 3-amino-ethylcarbazole (Sigma, St Louis, MO, USA) per 100 ml of dimethylformamide (BDH Laboratory Supplies, Poole, England) as substrate and the residual infectivity was recorded by a reduction in peroxidase stained foci. A 60% reduction of the number of infected cells was considered a significant reduction.

After examining the IgA neutralizing capacity in three IgA deficient (s-IgA $\leqslant$ 0.1 mg/ml) and two seronegative donors, the neutralizing cut off titer was set to $\geqslant$4. With this cut-off value, neutralizing IgA antibodies in titers varying from 4–512 were identified in 19/19 investigated sera obtained from rotavirus antibody positive and IgA competent donors (Table 1). To compensate for the variation in serum IgA concentration and subsequently in the amount of IgA obtained during purification, rotavirus neutralizing IgA titers were also expressed as rotavirus IgA titer per mg total IgA (Table 1). The amount of total IgA eluted from 200 µl serum varied from 80–750 µg (median 433 µg, ±2 SD 356). In the eluate from donor no. 12 a low recovery of IgA was repeatedly found (160 µg/200 µl serum). The low recovery resulted in a low IgA neutralization titer but when this was compensated for, the IgA neutralization was shown to correlate well with the IgG titer as in most other samples. To confirm purification of IgA and rule out the possibility of neutralization by residual IgG, total IgG concentrations were determined in the eluates by ELISA (not shown) and was found to vary between 0.5–4.3 µg per 200 µl eluate before the dilutions were made.

To explore the possibility that serum IgA also could neutralize already cell-bound rotavirus, we adopted an approach by Ruggeri and Greenberg [17] and allowed trypsin-activated RRV (1×10^3 p.f.u.) to bind to prechilled MA-104 cells in 96-well plates (on ice, 1 h). Cells were then carefully washed three times with ice-cold serum-free Eagle's MEM to remove unbound virus followed by the addition of ice-cold IgA eluates at serial two-fold dilutions and incubated (on ice, 1 h). Monolayers were then washed twice with ice-cold serum-free Eagle's MEM, before incubation in Eagle's MEM, 5% CO_2 (37 °C, 15 h). In this experiment five sera were included (# 4, 7, 20–22). Sera #4 and 7 proved to be highly effective in neutralizing cellbound RRV, both with neutralizing titers of 256 (Table 2). The two rotavirus antibody negative sera (# 20, 21) and the IgA deficient serum (#22) did not show any neutralizing capacity (titer<2).

Rotavirus can be neutralized by at least two distinct pathways; VP7-specific Mabs of the IgG isotype neutralize virus efficiently with a sharp decline in infection, while Mabs of the IgG isotype to VP4 proved less efficient in neutralization [17]. To study whether human serum IgA antibodies recognize these epitopes involved in protective immunity, we established two epitope

Table 1. Neutralizing serum IgA antibody titers to rotavirus (RRV)

Blood donor no.	Whole serum NT-titer[a]	IgA eluate NT-titer	Rotavirus-specific IgA NT-titer/mg total IgA
1.	5 120	512	1 182
2.	80	16	47.2
3.	160	32	74.9
4.	640	256	436
5.	160	8	15.5
6.	80	8	98.8
7.	1 280	64	216.9
8.	80	4	9.6
9.	80	4	7.1
10.	1 280	32	97.3
11.	640	16	35.2
12.	2 560	4	25.0
13.	1 280	64	98.8
14.	5 120	16	29.1
15.	1 280	64	107.0
16.	40	32	42.7
17.	80	4	27.4
18.	640	16	34.7
19.	160	16	55.2
Negative control donors[b]			
20.	<40	<2	0
21.	<40	<2	0
IgA deficient donors[c]			
22.	80	<2	0
23.	80	<2	0
24.	160	<2	0

[a]Neutralizing antibody titers were defined as the reciprocal of the highest dilution that showed more than 60% peroxidase focus inhibition

[b]Rotavirus antibody (IgG, IgA) negative individuals

[c]IgA-deficiency defined as $\leqslant$0.1 mg/ ml serum

blocking assays with monoclonal antibodies directed against epitopes on VP4 and VP7. Without knowledge of the investigated individuals' past exposure to various rotavirus serotypes, we decided to examine epitopes on VP4 and VP7, known to induce heterotypic rather than homotypic neutralization [18]. For this purpose two epitopes recognized by the Mabs 2G4 and 159 were selected [18]. In order to study whether purified human serum IgA could block these epitopes blocking assays were performed essentially as previously described for whole serum [10, 19]. Briefly, polyvinyl microtiter plates (Costar, Cambridge, MA, USA) were coated with partially purified RRV, backcoated with 1% BSA-PBS and then jacalin-purified-serum IgA (serial two-fold dilutions starting at undiluted) or whole serum (serial two-fold dilutions starting at 1/20) were

Table 2. Five IgA-eluates were tested for neutralizing capacity post-attachment of rotavirus to Ma-104 cells at +4 °C

Blood donor no.	IgA-titer	
	+4 °C	37 °C
4.	256	256
7.	256	64
20.	<2	<2
21.	<2	<2
22.	<2	<2

added and incubated (4 °C, 15 h and 37 °C, 1 h respectively). Monoclonal antibodies directed against epitopes on either VP4 (2G4) or VP7 (159) were then added. The monoclonal antibodies were kindly provided by H. Greenberg and have previously been characterized and used in epitope blocking experiments [19]. Horseradish-peroxidase conjugated goat-anti-mouse (BioRad, Hercules, CA, USA) diluted 1/5 000 was used as conjugate, and the reaction was developed with TMB (ICN Biochemicals, Cleveland, OH, USA). The titer was expressed as the reciprocal of the highest dilution which gave an optical density value equal to or less than 50% of the value of control wells, to which no test sera were added [10].

The epitope-specific responses are summarized in Table 3 and shows that 4/16 examined IgA-competent and rotavirus-antibody positive blood donors exhibited a serum IgA antibody response to an epitope on VP4 and 5/16 to an epitope on VP7, while 7 and 9/16 were recognized to exhibit an antibody response when whole sera were investigated. It is interesting to note that all sera that recognized the heterotypic neutralizing epitope on VP4, not only recognized the VP7 epitope identified by Mab 159, but also contained IgG antibodies which recognized the same epitopes. One individual (#15) possessed both IgG and IgA antibodies against the same VP7 epitope but lacked both IgG and IgA antibodies against the VP4 epitope recognized by Mab 2G4.

We have previously focused our interest on the qualitative characterization of the IgG response to rotavirus in humans after a natural infection [21], after vaccination [22] and more recently found that the IgA response as well as the IgG response mainly is directed against the two inner capsid proteins VP2 and VP6 [7]. In this study we have demonstrated that human serum IgA possesses the capacity to neutralize rotavirus in vitro and that a natural rotavirus infection stimulates production of circulating IgA antibodies, as well as IgG antibodies that recognize immunologically important epitopes on VP4 and VP7. Future studies will focus on whether these neutralizing IgA antibodies can protect in vivo and the significance of this observation merits further investigation and may be relevant for future vaccine development.

Table 3. Serum IgA epitope-specific antibody responses[a] to rotavirus (RRV)

	Serum IgA titer		Whole serum titer	
Blood donor no.	Mab 159 VP7	Mab 2G4 VP4	Mab 159 VP7	Mab 2G4 VP4
1.	16	4	640	160
2.	<2	<2	20	20
3.	<2	<2	40	40
4.	32	8	160	80
5.	<2	<2	40	20
6.	<2	<2	<20	<20
7.	nd[b]	nd	nd	nd
8.	<2	<2	<20	<20
9.	<2	<2	<20	<20
10.	<2	<2	<20	<20
11.	nd	nd	nd	nd
12.	32	4	160	160
13.	<2	<2	80	<20
14.	nd	nd	nd	nd
15.	4	<2	40	<20
16.	<2	<2	<20	<20
17.	<2	<2	<20	<20
18.	8	4	80	80
19.	<2	<2	<20	<20
Negative control sera				
20.	<2	<2	<20	<20
21.	<2	<2	20	<20
IgA-deficient sera				
22.	<2	<2	<20	<20
23.	<2	<2	<20	<20
24.	<2	<2	80	20

[a]Antibody titers were defined as the reciprocal of the highest dilution at which 50% inhibition of the absorbance value (450 nm) was observed

[b]*nd* Not done. Sera from donors 7, 11 and 14 were not available for the epitope-blocking assay

Acknowledgements

Monoclonal antibodies 255, 2G4 and 159 was a generous gift by Harry Greenberg. This research project was supported by funds from Swedish Medical Reasearch Council grants K92-16P-01A and B93-16X-10392-01A, Swedish Society for Medical Research and Stiftelsen Samariten.

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Authors' address: Dr. K. Johansen, Department of Virology, Swedish Institute for Infectious Disease Control, 105 21 Stockholm, Sweden.

Received October 26, 1996