Oral Passive Immunization Effect of Anti-Human Rotavirus IgY and Its Behavior against Proteolytic Enzymes

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The neutralization titer of anti-human rotavirus (HRV) IgY was completely inactivated by pepsin at pH 2.0. However, it was not significantly affected by trypsin or chymotrypsin under certain conditions. The immunological activity of the IgY was observed in the intestine of suckling mice for 2 h after oral administration and the activity rapidly decreased thereafter. The effects of oral supply of IgY were thus estimated for HRV-induced diarrhea in suckling mice and it was found that a previous supply of the IgY (1 h before HRV infection) completely prevented the HRV-induced diarrhea. The preventive effect was decreased as the time gap between IgY administration and HRV infection was longer. However, the oral supply of the IgY within 24 h after HRV infection was still effective and decreased the incidence of HRV diarrhea in suckling mice.

Human rotavirus (HRV) was found in 1973 by Bishop et al.1) and is a major pathogen of infectious gastroenteritis in infants and young children. HRV infection is characteristically localized to epithelial cells of the intestinal tract, and causes severe diarrhea with vomiting. It has been presumed that more than 2 million infant deaths annually are due to HRV diarrhea disease, mainly in developing countries.2)

Vaccination trial for HRV infection has remained unsuccessful because it is difficult to introduce the active antibody into the intestinal tract of the infants whose immunity generally has not developed yet.3) As an alternative to vaccination, oral passive immunization has been investigated for prevention of HRV infectious disease. Several researchers demonstrated that oral administration of anti-rotavirus IgG or egg yolk IgG (called IgY4) was effective in controlling rotaviral diarrhea using several animal models.5—10) However, for the practical application by oral administration a relatively large amount of active antibody will be necessary. In addition, information about stability of the antibody against heat, pH, digestive enzymes, etc. are also necessary for practical applications of this strategy.

We have previously reported a large-scale purification method of IgY from egg yolk by using λ-carrageenan, as a lipoprotein precipitant.11) We have found that the productivity of anti-HRV IgY by a hen immunized with HRV Mo strain (one year) was 120 times as more as that of an immunized rabbit with regard to the neutralization titer of the antibodies.12) This potential productivity of antibody of hen egg suggests that the anti-HRV IgY might be practical for prevention of HRV infection by its oral administration.

This paper deals with the behavior of anti-HRV IgY against several digestive enzymes. Also, the study of the effects of oral passive immunization of the IgY on suckling mice infected with HRV is described in relation to the anti-HRV IgY activity in the digestive tract.

Materials and Methods

Materials. Human rotavirus Mo strain (serotype 3) and MA104 cell (an established cell line derived from rhesus monkey kidney) were of those preserved at the Department of Bacteriology, Tohoku University School of Medicine. Anti-HRV(Mo) IgY was purified from yolk of eggs laid by hens immunized with HRV (Mo strain) as described in a previous paper.12) Pregnant BALB/c mice were purchased from the Shizuoka Breeding Farm for Laboratory Animals, Hamamatsu, Japan. Pepsin (from porcine stomach mucosa, 2 × crystallized, 3,700 units/mg protein), trypsin (from bovine pancreas, 2 × crystallized, 7,710 BAEE units/mg protein), and a-chymotrypsin (from bovine pancreas, 3 × crystallized, 54 units/mg protein) were obtained through Sigma Chemical (U.S.A.). All the reagents used were of chemical grade.

ELISA and neutralization titer measurement. The methods of ELISA and neutralization titer measurement were described previously.12)

Protein measurement and SDS—PAGE. The protein content of IgY solution was determined by measuring the absorbance at 280 nm, assuming its absorption constant to be 13.5 for 1% protein at 280 nm.13) The IgY incubated with proteolytic enzymes was electrophoresed on a linear-gradient (4—20%) polyacrylamide gel plate (Daichi pure chemicals) by the method of Laemmli.14)

Examination of the effects of proteolytic enzymes. Pepsin was dissolved in 0.07 M sodium acetate buffer (pH 2.0 or 4.0, pH was adjusted by 1 N HCl) at a concentration of 5 μg/ml. Immediately after dissolving the enzyme, the solution was mixed with anti-HRV(Mo) IgY solution (10 mg/ml distilled water). The mixture (weight ratio of pepsin to IgY, 1/200) was incubated at 37°C at pH 2.0 or 4.0. After appropriate incubation periods for 0—4 h, a 0.45-ml sample of the mixture was mixed with 0.05 ml of 5.0% sodium carbonate (incubated mixture at pH 2.0) or with that of 1.0% solution (incubated mixture at pH 4.0) for inactivation of the enzyme. The remaining anti-HRV IgY activity was measured by the neutralization titer method.

For the examination of the effects of trypsin and chymotrypsin, the anti-HRV(Mo) IgY was dissolved in 50 mM Tris buffer containing 10 mM CaCl2, pH 8.0, at a concentration of 2 mg/ml. Trypsin or chymotrypsin was dissolved at a concentration of 2 mg/ml of the Tris buffer, and immediately mixed with the IgY solution in the ratio of one enzyme to 20 of IgY by weight. After the mixtures were incubated at 37°C for appropriate periods between 0—8 h, a 0.45-ml sample of the incubation
mixture was mixed with 0.05 ml of phenylmethyl sulfonyl fluoride solution (40 mm in isopropanol) to inactivate the enzyme. The remaining anti-HRV IgY activity of the mixture was measured by the neutralization titer method.

Measurement of anti-HRV IgY activity in intestinal tract. Four suckling mice (6 days old) of each group, which were orally administered with 50 μl of anti-HRV(Mo) IgY solution (225 μg IgY/mouse), were dissected at the desired time, and the whole intestinal tract of each mouse was removed. Two ml of cold phosphate buffered saline (PBS: 10 mm phosphate, 0.15 mm NaCl, pH 7.2) and 2 ml of 1,1,2-trichloro-1,2,2-trifluoroethane were added to each intestinal tract. The mixture was homogenized and centrifuged at 10,000 × g for 10 min. The resulting supernatant was filtered through a membrane filter of 0.45 μm pore size and the filtrate was serially diluted (×10) with the PBS. Anti-HRV IgY activities of the diluted samples were measured by the ELISA end point test. The anti-HRV IgY activity was expressed as the value of the highest dilution rate that was measured by the intensity of the color developed at 405 nm (values > 0.2) by the ELISA end point test.

Observation of dye transit in digestive tract. Two suckling mice (6 days old) of each group were orally administered with 100 μl of 0.1% trypan blue in PBS. Mice were dissected at the desired time after administration of the dye. Stomach and intestinal tract of each mouse were removed to inspect the portions colored in the digestive tract.

HRV infection procedure and anti-HRV IgY treatment. The HRV challenge test developed by Ebina et al.10 was used with a slight modification using suckling mice. Six-day-old mice (8–16 mice/group) were inoculated orally with 50 μl of 1.8–3.5 × 10² fluorescent cell focus forming units (FCFU) of HRV Mo strain. Anti-HRV(Mo) IgY solution (50 μl) was also orally administered to the mice at the desired time before or after HRV inoculation. The mice were inspected daily for diarrhea by gentle palpation of the abdomen, and the incidence of diarrhea was analyzed using the results 48 h after HRV inoculation.

Histopathological experiment. Fresh specimens of small intestine of suckling mice were fixed in 10% formalin, and embedded in paraffin wax. Sections (3 μm) were cut with a microtome and stained with hematoxylin and eosin. The small intestinal sections thus stained were inspected under a microscope (50 ×).

Results

Behavior of IgY against pepsin

The value of neutralization titer of IgY was almost lost on incubation with pepsin at pH 2.0, while 91% of the activity remained after 1 h of incubation with the enzyme at pH 4.0. Even after 4 h of incubation under these conditions, 63% remaining activity was found by neutralization titer (Table 1). SDS-PAGE profiles of IgY after incubation with pepsin showed that IgY at pH 2.0 was digested to small peptides and no bands corresponding to IgY were detected after 1 h of incubation in the test by 4–20% linear-gradient polyacrylamide gel electrophoresis (Fig. 1). On the contrary, the incubation with pepsin at pH 4.0, heavy(H)- and light(L)-chain of IgY were clearly observed after 4 h of incubation, although some new bands appeared between H- and L-chains.

Behavior of IgY against trypsin and chymotrypsin

The changing patterns of neutralization titer of the IgY were almost the same when incubated with trypsin and chymotrypsin. After 8 h of incubation, 39% and 41% of the activity of IgY by neutralization titer remained for the mixture with trypsin and chymotrypsin, respectively (Table II). SDS-PAGE profiles of IgY incubated with trypsin or chymotrypsin are shown in Fig. 2. On incubation with trypsin, the IgY H-chain disappeared, and several bands between the H- and L-chains on SDS-PAGE appeared. In the case with chymotrypsin, both H- and L-chains of IgY remained unchanged, although a small band below the H-chain was observed.

IgY activity in intestinal tract

The anti-HRV(Mo) IgY activity in the intestinal tract of

![Fig. 1. SDS-PAGE Profiles of Anti-HRV(Mo) IgY Incubated with Pepsin at pH 2.0 and 4.0. H and L show heavy and light chains of IgY, respectively. See Table I.](image)

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* Enzyme: anti-HRV(Mo) IgY by weight = 1:200, see the text.

![Table II. Changes in Neutralization Titer of Anti-HRV(Mo) IgY during Incubation (37°C) with Trypsin or Chymotrypsin](image)

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* Enzyme: anti-HRV(Mo) IgY by weight = 1:200, see the text.
suckling mice after oral administration of the IgY was measured by the ELISA end point method (positive reaction, absorbance at 405 nm > 0.2, Fig. 3). The reciprocal value of the maximum dilution for the test with the homogenate of intestinal tract was $10^6$ either immediately after (indicated as 0 h in Fig. 3) or 1 h after administration of IgY. The value at 2 h after the administration, however, decreased significantly, and it was $10^2$ after 24 h.

**Transit of dye to intestinal tract**

The color intensity by staining of the digestive tract after administration of dye to suckling mice is shown in Table III. A strong color was observed in both stomach and upper intestinal tract portions immediately after the dye administration (indicated as 0 h in Table III). The zones stained were found to be in the upper intestinal tract at 1 and 2 h, and in the lower intestinal tract at 4 and 8 h after the dye administration, respectively.

**Passive immunization with IgY against HRV infection**

Suckling mice (11–16 mice/group) were inoculated with HRV Mo strain (3.5 × 10^7 FCFU/mouse) at 1, 3, 9, and 24 h after oral administration of anti-HRV(Mo) IgY (225 μg IgY/mouse). No incidence of diarrhea were observed in all the mice that were administrated with IgY at 1 h before HRV inoculation (Fig. 4). However, the mice administrated with...
IgY at 3, 9, and 24 h before HRV inoculation suffered from diarrhea with incidence of 27.3, 41.7, and 93.3%, respectively. In the positive control group, the incidence of diarrhea was 83.3%.

Anti-HRV(Mo) IgY (225 μg/mouse) was orally administered to suckling mice (8–12 mice/group) at 1, 3, 10, and 24 h after inoculation of HRV Mo strain (3.0 ± 10^7 FCFU/mouse). All the mice of the positive control group suffered from diarrhea. On the other hand, the incidence of diarrhea of the mice which were given the IgY at 1, 3, 10, and 24 h after HRV inoculation, were 37.5, 45.5, 41.2, and 70.0%, respectively (Fig. 5).

**Histopathological view of intestinal section**

Photographs of sectioned small intestine of suckling mice are shown in Fig. 6. Morphologically, expansion and vacuolation of cells and destruction of villus tips were observed in the sectioned small intestine of the suckling mice that severely suffered from diarrhea caused by HRV infection (1.8 ± 10^7 FCFU/mouse). However, the sections of those of mice which were prevented from HRV diarrhea by oral administration of anti-HRV IgY (225 μg IgY/mouse) 1 h before HRV inoculation (1.8 ± 10^7 FCFU/mouse), were morphologically the same as those of non-inoculated control mice.

**Discussion**

Several researchers demonstrated that rotaviral diarrhea was prevented by administration of IgY specific to rotavirus using the HRV infection model in suckling mice.\(^7\)\(^-\)\(^10\)

However, to our knowledge, no information is available about the stability of IgY, especially of that in digestive tract in regard to the activity of antibody.

Shimizu et al.\(^{15}\) reported that the activity of IgY specific to *E. coli* examined by competitive ELISA was quite stable on incubation with trypsin or chymotrypsin but it was sensitive to pepsin especially at pHs lower than 4.5. Ohtani et al.\(^{16}\) also reported that IgY specific to αs1-casein was relatively resistant to trypsin and chymotrypsin while it was quite sensitive to pepsin. We previously reported the pH stability of an anti-HRV IgY. About 85% of the initial neutralization titer remained at pHs between 4.0 to 9.0, but about 10% of the titer remained at pH 2.0 after 8 h of incubation.\(^{12}\) In this investigation, stability of the anti-HRV IgY against digestive enzymes were estimated by neutralization titer.

The activity measured by neutralization titer of anti-HRV IgY was completely lost by peptic digestion at pH 4.0 in the same digestive conditions (enzyme/substrate = 1/20, 37°C, 4 h) as those of Shimizu et al.\(^{15}\) (data not shown). However, a considerable extent of neutralization titer of anti-HRV IgY (63% of the initial value) remained on incubation with pepsin (E/S = 1/200, 37°C, 4 h) at pH 4.0, although the titer decreased to almost zero at pH 2.0 (Table I). Therefore, the stability of IgY against pepsin seems to be highly dependent on pH and the enzyme/substrate ratio.

Nakai\(^{16}\) reported that the pH values of gastric juice of infants of less than 5 months old generally are between 4–5 during 2–3 h after intake of milk. Therefore, it is likely that anti-HRV IgY administrated orally to infants reaches the small intestine without severe damage by gastric enzymes, if the pepsin concentration against the IgY (E/S ratio) in the stomach is lower enough not to severely digest the IgY.

The neutralization titer of the IgY on incubation with trypsin or chymotrypsin was found to gradually decrease with both the enzymes, though SDS–PAGE profiles of the IgY incubated with trypsin or chymotrypsin were significantly different. H-chain of IgY was degraded to peptides when incubated with trypsin, while there was not a similar type of digestion with chymotrypsin (Fig. 2). These results are coincident with those reported by Shimizu et al.\(^{15}\). Therefore, it is noted that trypsin and chymotrypsin do not completely degrade the active site of the IgY. The difference in the digestion pattern of IgY found between the two enzymes could be due to the difference in their specificities on the IgY H-chain, although more evidence...
and clarification are necessary.

The neutralization titer of anti-HRV IgY in the intestinal tract of suckling mice could not be measured in this report because of the extract of the intestinal tract had cytotoxicity to MA 104 cells. We have previously reported that the stability of anti-HRV IgY under various pH values measured by ELISA is slightly more sensitive than that measured by neutralization titer.12) Therefore, in this report, the ELISA activity of anti-HRV IgY in the intestinal tract of suckling mice was measured. It was found that the ELISA activity of IgY was retained for 1 h after the IgY administration (Fig. 3). This together with the result of the transit of dye through the intestinal tract (Table III) indicate that the IgY administered orally swiftly reaches the intestine of suckling mice, retaining the anti-HRV activity. It is likely that rotaviral diarrhea induced by HRV in suckling mice is almost completely prevented by the oral supply of IgY 1 h before the infection by the virus (Figs. 4, 6).

The neutralization titer of anti-HRV IgY incubated with intestinal enzymes was retained at nearly a half of the initial at even 8 h later (Table II). However, the anti-HRV activity by ELISA found in the intestinal tract of suckling mice was only $10^{-1}$, $10^{-3}$, and $10^{-4}$ of the initial activity at 2, 8, and 24 h after the IgY administration, respectively (Fig. 3). This decrease of the activity seems to correlate with observation of dye transit (Table III) in which the color intensity of dye swiftly moved toward the lower part of the intestine. There may be a possibility that a substantial portion of IgY administrated orally had been excreted out of the intestinal tract of suckling mice, retaining its activity.

Formerly, we reported that oral administration of IgY (225 μg/mouse) 1 h before viral inoculation completely prevented HRV diarrhea. However, a dose having the concentration of $10^{-2}$ and $10^{-3}$ of that of IgY resulted in incidence of diarrhea of 25.0 and 42.9%, respectively.10) In this investigation, the preventive effect from HRV diarrhea of suckling mice was found to decrease as the time gap between IgY administration and HRV inoculation was prolonged (Fig. 4). This result together with that of decrease of IgY activity in intestinal tract (Fig. 3) seems to be in accordance with our former result10) in which the effect of dose-response of anti-HRV(Mo) IgY on infection by HRV was examined. Both the results indicate that the effectiveness of IgY for prevention from HRV diarrhea depends very much on the time that IgY is administered before infection by HRV.

There seem to be no reports published on the therapeutic effects of IgY on HRV diarrhea induced in suckling mice. This paper demonstrated that oral administration of anti-HRV(Mo) IgY within 24 h after HRV infection was very effective to decrease the number of animals suffering from HRV-induced diarrhea in suckling mice (Fig. 5).

Ebina et al.11) have reported that oral administration of anti-HRV antibodies (skinned colostrum) protected several infants from rotavirus-induced gastroenteritis. This strongly suggests that HRV infection of infants can be prevented by oral administration of anti-HRV IgY, if the IgY is introduced into the small intestine without a significant loss of activity caused by gastric enzymes. This investigation showed that anti-HRV IgY reached the small intestine of suckling mice a short time after oral administration. Therefore, oral administration of anti-HRV IgY at certain times may lead to the practical application for prevention from HRV-induced diarrhea in human infants.

References