初乳抗体の腸管内での作用に関する研究

Bovine immune colostral antibody against verotoxin 2 derived from Escherichia coli O157:H7-Resistance to proteases and effects on verotoxin 2 in small intestine of beagle dogs

山本静雄[1]，栗林尚志[1]，清田哲郎[1]，福山正文[2]，古畑勝則[2]

麻布大学生命・環境科学部[1]免疫学研究室，[2]微生物学研究室

Shizuo Yamamoto [1], Takashi Kuribayashi [1], Tetsuro Seita [1], Katsunori Furuhata [2] and Masafumi Fukuyama [2]

Laboratories of [1] Immunology and [2] Microbiology, School of Life and Environmental Science, Azabu University

Abstract: Resistance to intestinal proteases and efficacy against verotoxin (VT) 2 of a colostral antibody administered orally via catheter were investigated in beagle dogs. Bovine colostral antibody remained in the small intestine for 2 hours, whereas little serum antibody remained at 1.5 hours after administration. The bovine colostral antibody was not inactivated by proteases in the small intestine. Furthermore, the antibody activity of S-IgA did not change until 2 hours after administration; however, the activity of IgG and IgM antibodies decreased by two-thirds and two-fifths at 2 hours after administration, respectively.

Seven beagle dogs inoculated with *Escherichia coli* (*E. coli*) O157:H7 producing VT2 were administered bovine colostral antibody or bovine colostral whey without antibody. With administration of bovine colostral whey without antibody, the amount of VT2 in feces decreased gradually after administration and increased again at 5 days after inoculation, while bovine colostral antibody significantly reduced the amount of VT2 in feces the day after administration. In addition, 9 beagle dogs were administered bovine colostral antibody, bovine plasma antibody or saline. The amount of VT2 in feces also significantly reduced rapidly after administration of bovine colostral antibody than administration of bovine plasma antibody or saline.

Key words: colostral antibody, protease, EHEC, Verotoxin, dogs

Introduction

In Japan, a diffuse outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection occurred among schoolchildren in Sakai city, Osaka prefecture, in July 1996.[4, 18] In that year, numerous outbreaks of food poisoning due to *Escherichia coli* O157:H7 occurred in various localities throughout Japan, and such outbreaks became a social problem.[18] *Escherichia coli* (*E. coli*) O157:H7 infection is monitored in Japan, in accordance with the Infection Diseases Control Law, and in 2005, 3,589 cases were reported.[10] Furthermore, incidents of EHEC infection occur in many industrialized nations.20 Thus, EHEC infection is an emergent infectious disease of significant clinical importance.[11, 12, 22]

The therapeutic approaches for EHEC infection are the subject of widespread discussion.[9, 24, 30] Generally, the treatment for bacterial food poisoning is the administration of antibiotics; however, administration of antibiotics is not recommended for food poisoning caused by EHEC infection, as this increases the risk of serious complications, such as hemolytic uremic syndrome (HUS), due to verotoxin (VT) released from EHEC killed by antibiotics. Appropriate therapeutic approaches, such

as inhibiting VT activity or absorption from the intestine, are thus required. The authors previously obtained a bovine colostral antibody against VT2 from cows immunized with VT2.[14] Furthermore, we confirmed the neutralization efficacy of this bovine colostral antibody against VT2 in mice.[14] However, if this bovine colostral antibody is administered orally to patients infected with *E. coli* O157, its resistance to decomposition by intestinal proteases must be investigated. Although neutralization efficacy against VT2 has been verified in mice, resistance to proteases could not be confirmed. Moreover, many studies of proteolytic degradation of immunoglobulin (Ig) *in vitro* have been performed, and each Ig class reportedly has different resistances to protease degradation.[3, 21, 25, 26] However, Ig resistance to proteases has not been clarified *in vivo*. Furtheremore, few appropriate experimental animals are able to evaluate for *E. coli* O157:H7 infection. The weaned immature mouse model was used for *E. coli* O157:H7 infection and VT(Iwakura *et al.* The 77th Annual Meeting of the Japanese Association for infectious Diseases. 2003. 238).[14] Nothing is model of beagle dogs for *E. coli* O157:H7 infection. However, diarrhea was shown in beagle dogs inoculated of *E. coli* O157:H7 by pre-treatment of fradiomycin. Then, this model was used in our study.

In this study, the resistance of bovine colostral antibody to proteases in the small intestine of beagle dogs, as well as that of each Ig class isolated from intestinal fluid after administration of bovine colostral antibody, was investigated. Furthermore, efficacy against VT2 in beagle dogs was also investigated.

Materials and Methods

Microorganisms and VT2

E. coli O157:H7 producing VT2 isolated from humans was used in this study. *E. coli* O157:H7 was cultured using brain heart infusion broth (Difco, Becton, Dickinson and Company, NJ, USA) for 48 hours, and culture supernatant was obtained by centrifugation (1,600 × g, 20 minutes). Microorganisms were suspended in sterile saline and diluted to 1×10^9 CFU/ml for administration to beagle dogs.

VT2 in culture medium and feces was measured using a commercial kit (VTEC RLPA, DENKA SEIKEN Co., Ltd., Tokyo, Japan) based on reversed passive latex agglutination.

Animals

Nine beagle dogs aged 1 year (8 males and 1 female; Saitama Experimental Animals Supply Co., Ltd., Saitama, Japan) and twenty-three male beagle dogs aged 1 year (AQS Co., Ltd., Chiba, Japan) were used. Four male Japanese white rabbits were obtained from Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan), and three 6- to 8-year-old dairy cows (3 to 4 months prior to delivery) bred at a cattle farm in Shimane prefecture, Japan, were used.

Dogs and rabbits were reared individually in cages housed in an animal room maintained at a temperature of 22 ± 2°C and humidity of 60 ± 10% with a 12-h light/dark cycle (07:00-19:00), and the air was changed 15 times/h. Dogs were fed solid stock food (Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed free access to water.

All experiments conformed to Japanese regulations concerning animal care and use, as specified in the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, JALAS, 1987), and were approved by the Animal Research Committee of Azabu University.

Immunization to cows with VT2

Two cows were immunized in order to obtain sufficient bovine colostral antibody for the study. Immunization of 6-year-old cows with VT2 was as described by Kuribayashi *et al.*[14] Eight-year-old cows were intradermally immunized with the supernatant of culture medium containing VT2 mixed with (1:1) Freund's complete adjuvant (Difco Laboratories, Detroit, USA) initially, and with supernatant of culture medium containing VT2 thirteen times at 7-day intervals.

Preparation of colostral whey without antibody

Colostrum was treated to obtain colostral whey in the

same manner as bovine colostral antibody against VT2.15 Thus, skim milk was obtained by centrifuging the colostrum at 1,600 × g for 15 minutes in order to remove fat. One liter of skim milk was then mixed with 100 mg of rennet (ICN Biochemicals Inc., OH, USA) and incubated overnight at 22°C. This skim milk was centrifuged at 2,200 × g for 20 minutes. Bovine colostral antibody was obtained by filtering the supernatant using a membrane filter (pore size: 22 μm).

Preparation of bovine plasma, bovine serum and rabbit serum antibodies against VT2

Bovine plasma antibody was obtained from 6-year-old cows immunized with VT2. Bovine serum antibody was obtained from 8-year-old cows.

Japanese white rabbits were immunized with culture medium containing VT2 suspended in Freund's complete adjuvant (Difco Laboratories, Detroit, USA), followed by the same culture medium 22 times. Rabbits were sacrificed under pentobarbital anesthesia and blood was collected. Antisera were obtained by centrifugation at 2,200 × g for 20 minutes.

Measurement of neutralization titer

Titers of bovine colostral antibody, bovine serum antibody and rabbit serum antibody were tested by neutralization test according to standard methods using vero cells.12 Colostral antibody (40 μl) diluted from 2- to 2048-fold was mixed with 40 μl of culture medium (MEM culture medium; Nissui Pharmaceutical Co., Ltd., Tokyo) containing VT2. This was incubated overnight at 37°C, after which 100 μl of vero cells were added and cultured for 2 days. Neutralization titer was measured based on the number of dead vero cells.

Estimation of resistance to intestinal proteases in beagle dogs

Comparison of resistance of bovine colostral antibody and rabbit serum antibody to intestinal proteases in beagle dogs

Six beagle dogs were divided into two groups for administration of bovine colostral antibody or rabbit serum antibody. Each beagle dog was fasted for 18 hours and was orally administered 50 ml of bovine colostral antibody or rabbit serum antibody. Dogs were sacrificed at 1.5, 2, 3 or 4 hours after administration under anesthesia by administration of pentobarbital and small intestinal fluid including bovine colostral antibody or rabbit serum antibody was collected. Nothing was present in the small intestine at 4 hours after administration and small intestinal fluid could not be removed. Small intestinal fluid was filtrated with a membrane filter (0.20 μm) and was stored under −80°C until measurement.

Measurement of activities of bovine colostral and serum antibodies by enzyme-linked immnosorbent assay (ELISA)

VT2 was adjusted with 0.05 mol/L sodium hydrogencarbonate buffer (pH 9.6), and was transferred to immunoplates (Nunc, DK4000 Roskilde, Denmark). To all wells, 1% gelatin in 0.05 mol/L sodium hydrogencarbonate buffer (pH 9.6) was added at 200 μl/well in order to block non-binding sites. After washing, small intestinal fluid including bovine colostral antibody or rabbit serum antibody was added at 100 μl/well. Antibodies against to bovine immunoglobulin (Monosan, Uden, Netherlans) were added at 100 μl/well to wells only containing wash samples from small intestine fluid after administration of bovine colostral antibody. Antibodies against rabbit immunoglobulin-coupled peroxidase (ICN Biomedicals, Costa Mesa, Canada) were added to all wells at 100 μl/well. After washing, 2,2-aminodi(3-ehtylbenzthiazoline sulphonic acid (ABTS) (Invitrogen Corporation, CA, USA)) in citrate buffer was added at 100 μl/well. Absorbance at 415 nm and 492 nm was then measured with an immunoplate reader.

Comparison of resistance of each Ig class to intestinal proteases in beagle dogs

Fifty milliliters of bovine colostral antibody or bovine serum antibody was administered orally to four beagle dogs in each group. Beagle dogs were then sacrificed at

1.5, 2, 3 or 4 hours after administration under anesthesia by administration of pentobarbital and intestinal fluid was removed. Intestinal fluid could not be collected at 4 hours after administration. Small intestinal fluid was filtrated with a membrane filter (0.20 μm) and was stored under −80°C until measurement.

Measurement of activity of each Ig class in small intestinal fluid by ELISA

Horseradish peroxidase-conjugated antibodies, antibody against bovine IgA (α) (VMRD, Inc., WA, USA), antibody against to bovine IgM (μ) (VMRD, Inc.) and antibody against IgG (γ) (VMRD, Inc), were used to measured the activity of each Ig class in small intestine by ELISA. Horseradish peroxidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan)-conjugated antibodies were prepared using the method of Nakane and Kawaoi.[19]

VT2 was adjusted with 0.05 mol/L sodium hydrogencarbonate buffer (pH 9.6) and transferred to immunoplates (Nunc). To all wells, 1% gelatin in 0.05 mol/L sodium hydrogencarbonate buffer (pH 9.6) was added at 200 μl/well to block non-binding sites. After washing, small intestinal fluid collected from beagle dogs was added at 100 μl/well. Horseradish peroxidase-coupled antibody against bovine IgA (α) (VMRD, Inc.), antibody against bovine IgM (μ) (VMRD, Inc.) or antibody against IgG (γ) (MP Biomedical Inc.) were added at 100 μl/well to wells. After washing, ABTS (Invitrogen Corporation) in citrate buffer was added at 100 μl/well. Absorbance at 415 nm and 492 nm was then measured with an immunoplate reader.

Estimation of neutralization efficacy of bovine colostral antibody against VT2 in beagle dogs inoculated with *E. coli* O157:H7

Comparison between bovine colostral antibody and colostral whey

Seven beagle dogs were divided into two groups. Four beagle dogs were administered bovine colostral antibody and three beagle dogs were administered bovine colostral whey without antibody. Beagle dogs were administered fradiomycin sulfate (FRM; Nippon Kayaku Co. Ltd., Tokyo, Japan) at 50 mg/kg for 3 days prior to inoculation with *E. coli* O157:H7 in order to eliminate native enterobacterial flora. Beagle dogs were fasted for 18 hours prior to inoculation, and were then inoculated orally with a feeding tube containing 5 ml of *E. coli* O157:H7 suspended in physiological saline. From the next day, fosfomycin sodium (FOM; Meiji Seika Kaisha, Ltd., Tokyo, Japan) at 50 mg/kg was administered. Fecal samples were collected daily after *E. coli* O157:H7 inoculation and fecal characteristics were noted. One hundred milliliters of bovine colostral antibody or bovine colostral whey without antibody was administered orally following confirmation of increased VT2 in feces. Titers in feces are shown as geometric means. Unpaired Student's *t*-test was used to compare titers before and on the day after administration of bovine colostral antibody or colostral whey without antibody.

Comparison of bovine colostral antibody, serum antibody, saline

Nine beagle dogs were divided into three groups and were used for *E. coli* O157:H7 inoculation. Each group of beagle dogs was administered bovine colostral antibody, plasma antibody or saline. The experimental procedures, pretreatment of FRM, inoculation of *E. coli* O157:H7 and administration of FOM, were similar to those in the previous experiment. One hundred milliliters of bovine colostral antibody, plasma antibody or saline was administered orally following confirmation of increased VT2 in feces. Titers in feces are shown as geometric means. Unpaired Student's *t*-test was used to compare titers before and the day after administration of bovine colostral antibody, plasma antibody or saline.

Results

Neutralization titer of bovine, plasma and serum antibody

The neutralization titer of colostral antibody obtained from 6-year-old cows against VT2 was 1:32 by neutralization test according to standard methods using vero cells. Bovine plasma antibody obtained from the same cows was 1:8. The neutralization titer of colostral

antibody obtained from 8-year-old cows against VT2 was 1:128. Bovine serum antibody obtained from the same cows was 1:32. The neutralization activities against VT2 were not confirmed in bovine colostral whey obtained from non-immunized cows or in rabbit serum antibody.

Comparison of antibody activities between bovine colostral antibody and rabbit serum antibody recovered from small intestines of beagle dogs

A comparison of the activity of bovine colostral antibody and rabbit serum antibody in the small intestine is shown in Figure 1. Activity of the bovine colostral antibody did not change until 2 hours after administration. At 3 hours after administration, activity had decreased by 20-fold. Activity of rabbit serum antibody decreased immediately after administration, giving values of one-tenth at both 1.5 and 2 hours after administration. The activities of both bovine colostral antibody and rabbit serum antibody were very low at 3 hours after administration.

Comparison of antibody activity among each Ig class in small intestinal fluid recovered from beagle dogs

The activities of each antibody class in small intestinal fluid of beagle dogs administered bovine colostral antibody, bovine serum antibody are shown in Figures 2. IgG antibody activity decreased by two-thirds, while IgM antibody decreased by two-fifth at 2 hours after administration of bovine colostral antibody; however, that of S-IgA scarcely changed until 2 hours after administration. At 3 hours after administration, the activity of secretory IgA (S-IgA) antibody decreased by half, while that of IgG and IgM antibodies decreased to

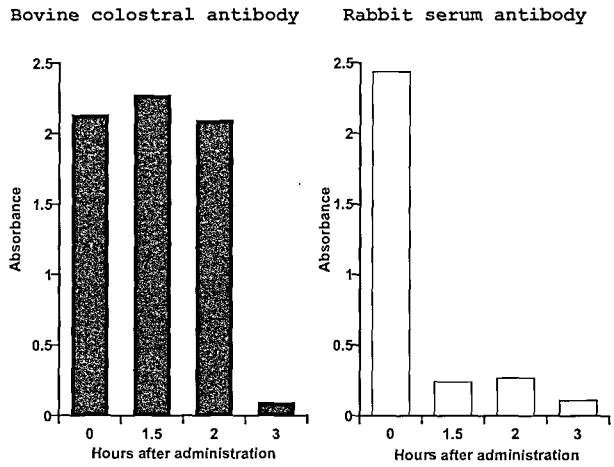

Fig 1. Comparison of antibody activities between bovine colostral antibody and rabbit serum antibody recovered from small intestine. Fifty milliliters of bovine colostral antibody or rabbit serum antibody were administered to beagle dogs. Small intestinal fluid was collected at 1.5, 2 or 3 hours after administration. Antibody activities are given in terms of absorbance, as measured by ELISA. Time zero represents the antibody activity before administration.

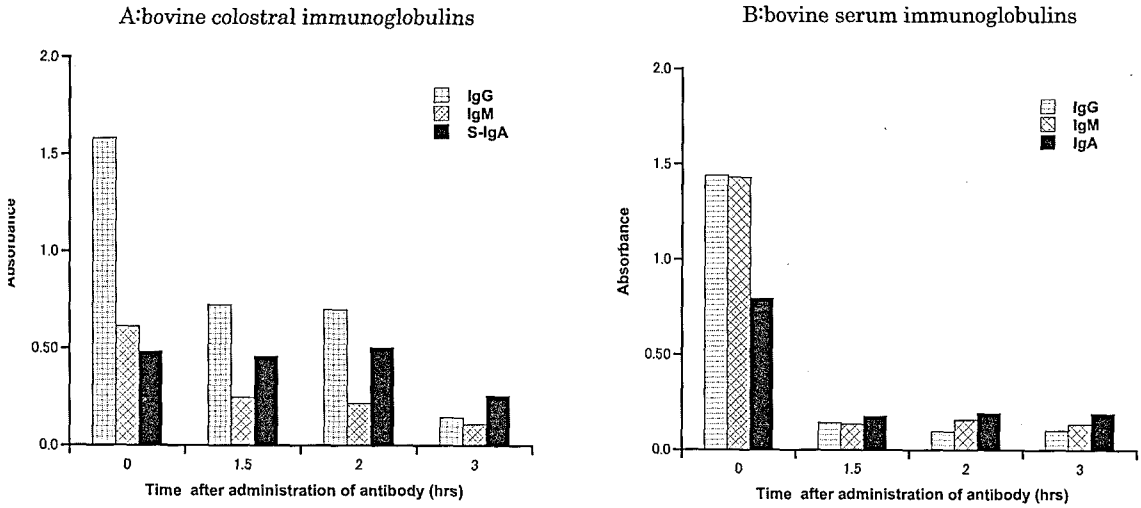

Fig 2. Comparison of antibody activities among each immunoglobulin recovered from the small intestine of beagle dogs after administration of 50 ml of bovine colostral antibody or serum antibody. Small intestinal fluid was collected at 1.5, 2 or 3 hours after administration. Antibody activities are given in terms of absorbance, as measured by ELISA. Time zero represents the antibody activity before administration.

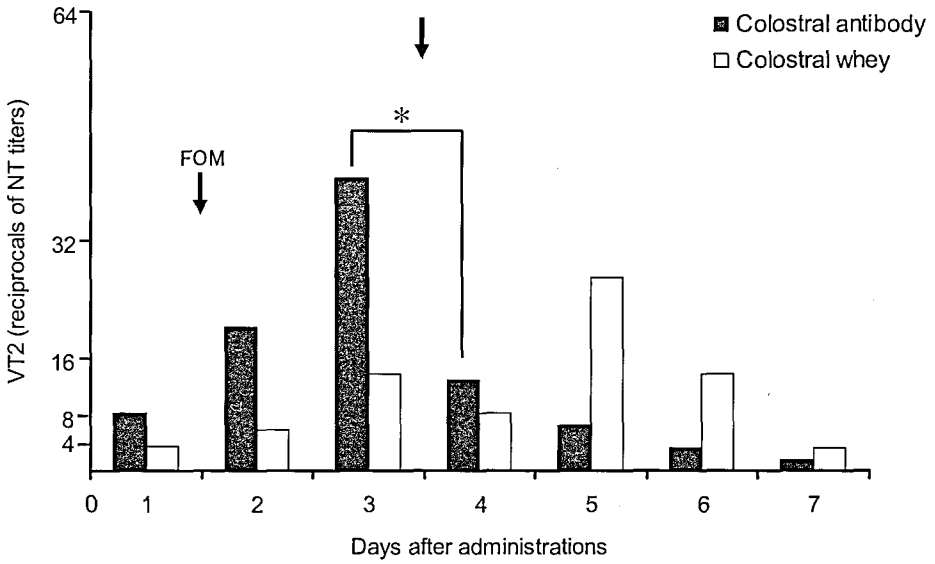

Fig 3. Changes in fecal VT2 levels after administration of bovine colostral antibody and bovine colostral whey in beagle dogs inoculated with *Escherichia coli* O157:H7.Fosfomycin (50 mg/kg) was administered next day of inoculation. Vertical arrow indicates the timing of administration of 100 ml of bovine colostral antibody or bovine colostral whey. The reciprocals of neutralizing test (NT) titers to VT2 are plotted on the ordinate. Significant difference obtained by unpaired Student's *t*-test is shown by * ($p < 0.05$).

one-tenth and one-sixth, respectively. For bovine serum antibodies, IgG and IgM activities decreased to one-fifth, while IgA activity fell to after administration decreased to half at 1.5 hours after administration.

Neutralization efficacy of bovine colostral antibody and bovine colostral whey in beagle dogs inoculated with *E. coli* O157:H7

Changes in fecal VT2 levels after administration of bovine colostral antibody or bovine colostral whey without antibody in beagle dogs inoculated with *E. coli* O157:H7 are shown in Figure 3.

Bovine colostral antibody was administered to beagle dogs when the amount of VT2 in feces increased 1:40.7, and the amount decreased significantly to 1:12.6 by the next day ($p<0.05$). The amount of VT2 in feces decreased from 1:13.5 to 1:7.9 on the day after administration of colostral whey without antibody. Furthermore, the amount of VT2 in feces increased to 1:26.9 at 5 days after inoculation.

VT2 neutralization efficacy of bovine colostral and plasma antibodies in beagle dogs inoculated with *E. coli* O157:H7

Changes in fecal VT2 levels after administration of bovine colostral antibody, bovine plasma antibody or saline in beagle dogs inoculated with *E. coli* O157:H7 are shown in Figure 4. The amount of VT2 in feces decreased significantly from 1:40.8 to 1:3.2 on the day after administration of bovine colostral antibody ($p<0.05$), and reached 1:1.3 at 7 days after inoculation. The amount of VT2 in feces also decreased on the day after administration of bovine plasma antibody and saline (from 1:20.0 to 1:6.3, and from 1:20.0 to 1:10.0, respectively), and gradually continued to decrease.

Discussion

We previously obtained a bovine colostral antibody against VT2 from cows immunized VT2 and confirmed its neutralization efficacy against VT2 in mice administered VT2 or inoculated with *E. coli* O157:H7 producing VT2.[14] If this bovine colostral antibody is to be administered orally to patients infected *E. coli* O157:H7, it must be resistant to protease degradation in the digestive tract. We

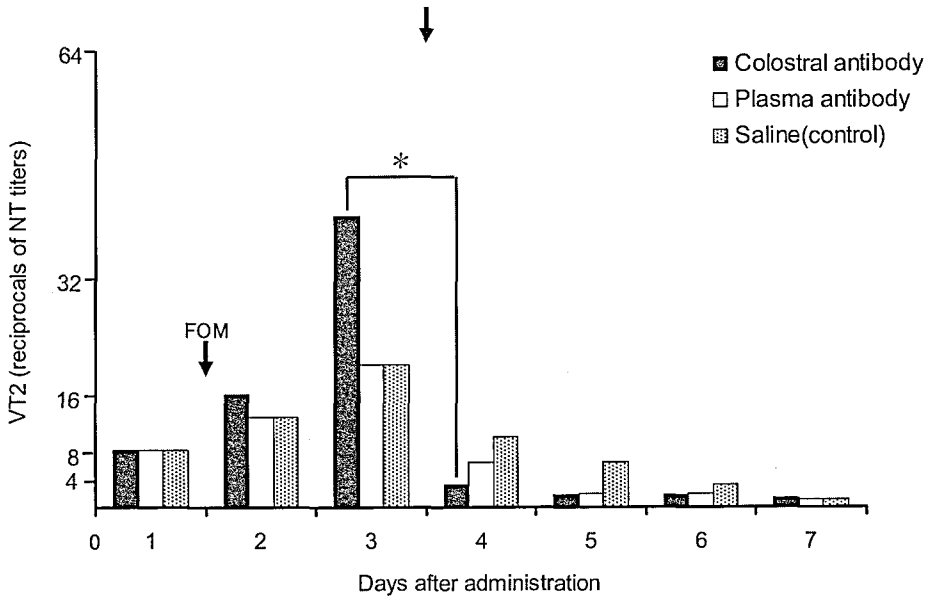

Fig 4. Changes in fecal VT2 levels after administration of bovine colostral and plasma antibodies, and saline in beagle dogs inoculated with *Escherichia coli* O157:H7. Fosfomycin (50 mg/kg) was administered on the day after inoculation. Vertical arrow indicates the timing of administration of 100 ml of bovine colostral antibody, bovine plasma antibody or saline. The reciprocals of neutralizing test (NT) titers to VT2 are plotted on the ordinate. Significant difference obtained by unpaired Student's *t*-test is shown by * ($p < 0.05$).

thus investigated the resistance of this bovine colostral antibody to intestinal proteases in beagle dogs, which was presumed to secrete proteases more abundantly than mice and be able to extrapolate to human.

The beagle dogs inoculated live *E. coli* O157:H7 (1×10^9 CFU/ml) without pre-treatment of antibiotics did not show any symptoms as a diarrhea. For this reason, these healthy dogs could not use in our study (data not shown). The beagle dogs with normal enterobacterial flora may not sensitive to *E. coli* O157:H7. *E. coli* O157:H7 may remain and/or grow in intestine of beagle dogs with pre-treated of antibiotics. It was important to note that the experimental beagle dogs in our study were pre-treated with antibiotics to alter their indigenous enterobacterial flora. The beagle dogs with pre-treated of FRM had slight or severe diarrhea after inoculation *E. coli* O157:H7. These beagle dogs with such pre-treatment could use in our study as an experimental animal model for *E. coli* O157:H7 infection. Furthermore, aminoglycoside antibiotics, such as FRM, are poorly absorbed and largely pass through the intestine.[8, 9] One day after administration was allowed for excretion of FRM from the intestine prior to inoculation with *E. coli* O157:H7. Therefore, FRM did not influence the elimination of *E. coli* O157:H7.

The colostral antibody remained until 2 hours after administration, while the rabbit serum antibody was significantly reduced at 1.5 hours after administration. Furthermore, S-IgA class did not decrease as much and IgG class remained at two-thirds until 2 hours after administration. These results suggest that bovine colostral antibody is more resistant to proteases when compared with serum antibody *in vivo*.

S-IgA is known to be more resistant to pepsin when compared with serum IgA, as the presence of the secretory component (SC) helps resist proteolysis *in vitro* experiments.[16, 23, 26-29] Less than 15% of S-IgA was digested during in vitro experiment with papain.[28] In this study, S-IgA antibody activity in the beagle dog intestine remained for 2 hours after administration of bovine colostral antibody, and thus S-IgA showed similar resistance as *in vitro* experiment.[3, 25, 26] On the other hand, IgG1 was digested completely *in vitro* experiment[28], while IgG activity in beagle dog intestine remained at approximately two-thirds of pretreatment values until 2

hours after administration. These results suggested that both S-IgA and IgG antibodies are thought to contribute to the resistance of bovine colostral antibody to intestinal proteases. Thus, the bovine colostral antibody against VT2 appears to be resistant to intestinal proteases, and is able to maintain its neutralizing activity against VT2 in the digestive tract.

As the bovine colostral antibody was confirmed to be resistant to intestinal proteases, the efficacy of bovine antibody against VT2 in beagle dogs was then investigated in additional studies.

The amount of VT2 in feces decreased rapidly after administration of bovine colostral antibody. The titer decreased significantly the day after administration of bovine colostral antibody. On the other hand, the amount of VT2 in feces decreased gradually after administration of colostral whey. Furthermore, the amount of VT2 in feces increased at 2 days after administration of bovine colostral whey without antibody. It has been reported that *E. coli* O157:H7 does not flourish in the mouse intestine due to commensal flora.[18] This suggests that VT2 is not neutralized by colostral whey without antibody and that *E. coli* O157:H7 is able to survive and reproduce. The bovine colostrum obtained from non-immunized cows with antibodies against numerous pathogens showed efficacy in mice infected with *E. coli* O157:H7.[6, 15] However, these results suggest that bovine colostral antibody obtained from cows immunized with VT2 are more effective at neutralizing VT2 than colostral whey.

Next, we compared efficacy against VT2 among bovine colostral antibody, plasma antibody and saline in beagle dogs. The amount of VT2 in feces decreased significantly the day after administration of bovine colostral antibody than administration of plasma antibody or saline. This result suggested that plasma antibody was digested by proteases, similarly to rabbit serum, and decreased more rapidly than bovine colostral antibodiy in the beagle dog intestine. Therefore, the plasma antibody could not neutralize VT2 effectively due to rapid degradation by proteases. On the other hand, colostral antibody including S-IgA and IgG was resistant to protease degradation and showed sufficient neutralization efficacy against VT2 in the intestine.

Bovine colostrum containing antibodies has already been applied against human Rotavirus infection.[2, 5, 7] However, bovine colostrum against human Rotavirus was not investigated with regard to degradation in the small intestine. Resistance to proteases in the small intestine and efficacy against VT2 were thus investigated for the present bovine colostral antibody. Based on the present results, bovine colostrum obtained from cows immunized with pathogens is useful for neutralizing toxins and pathogens. Little is available on comparisons of protease activity in the small intestines of human and beagle dogs; however, we used beagle dogs, as protease secretion is thought to be comparable to that in humans. Bovine colostral antibody against VT2 including S-IgA and IgG showed resistance to proteases in the small intestine of beagle dogs, thus suggesting the possibility of resistance to proteases in the human intestine. Treatment with both FOM and bovine colostral antibody has also been confirmed to improve the survival of mice infected by *E. coli* O157:H7.[14] In the future, combination therapy with antibiotics and bovine colostral antibody against VT2 may prevent serious complications, such as HUS, in EHEC infection.

In summary, the efficacy of bovine colostral antibody against VT2 was verified in digestive organs abundantly secreting proteases. These results suggest that it is possible for patients infected with *E. coli* O157:H7 to be treated with oral bovine colostral antibody. Furthermore, when administered with antibiotics, bovine colostral antibody may effectively inhibit the activity of VT2 released from *E. coli* O157:H7.

References

1) Butler JE. Bovine immunoglobulins :A review. J. Dairy Sci.52:1895-1909.
2) Brussow H., Hilpert H, Walther I, Sidoti J, Mietens C. 1987. Bovine milk immunoglobulins for passive immunity to infantile rotavirus gastroenteritis. J. Clin. Microbiol. 25:982-986.
3) Brown WR, Newcomb RW, Ishizuka K. 1970. Proteolytic degradation of exocrine and serum immunoglobulins. J. Clin. Invest. 49:1374-1380.

4) Cleray TG. 2004. The role of shiga-toxin-producing *Escherichia coli* in hemorrhagic colitis and hemolytic uremic syndrome. Semin. Pediatr. Infect. Dis. 15: 260-265.

5) Ebina T, Ohta M, Kanamaru Y, Yamamoto-Osumi Y, Baba K. 1992. Passive immunization of suckling mice and infants with bovine colostrum containing antibodies to human rotavirus. J. Med. Virol. 38:117-123.

6) Funatogawa K, Ide T, Kirikae F, Saruta K, Nakano M, Kirikae T. 2002. Use of immunoglobulin enriched bovine colostrum against oral challenge with enterohaemorrhagic *Escherichia coli* O157:H7 in mice. Microbiol. Immunol. 46:761-766.

7) Hilport H, Brussow H Mietens C, Sidoti J, Lerner L, Bachman P. 1987. Use of bovine milk concentrate containing antibody to rotavirus to treat rotavirus gastroenteritis in infants. J. Infec. Dis. 156:158-166.

8) Hombach J, Hoyer H, Berkop-Schnurch A. 2007. Thiolated chitosans: development and *in vitro* evaluation of an oral tobramycin sulphate delivery system. Eur. J. Pharm. Sci. 33:1-8.

9) Horspool LJ, Taylor DJ, Mckellar QA. 1994. Plasma disposition of amikacin and interactions with gastrointestinal microflora in Equidae following intravenous and oral administration. J. Vet. Pharmacol. Ther. 17:291-298.

10) Infection Disease Surveillance Center [Internet]. Surveillance Data Table. 2005. Catagoly III. Available at http://www.nih.go.jp.

11) Karch H., Tarr PI, Bielaszeska. 2005. Enterohaemorrhagic Escherichia coli in human medicine. Int. J. Med. Microbiol. 295:405-418.

12) Karmali MA. 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. 2:15-38.

13) Konowalchuk J, Sepeirs JI, Stavric S. 1977. Vero response to a cytotoxin of *Escherichia coli*. Infection and Immunity. 18:775-779.

14) Kuribayashi T., Seita T, Fukuyama M, Furuhata K, Honda M, Matsumoto M, Seguchi H, Yamamoto S. 2006. Neutralizing activity of bovine colostral antibody against verotoxin derived from enterohemorrhagic *Escherichia coli* O157:H7 in mice. J. Infect. Chemother. 12:251-256.

15) Lissner RH, Karch H. 1996. A standard immunoglobulin preparation produced from bovine colostra shows antibody reactivity and neutralization activity against Shiga-like toxins and EHEC-hemolysin of *Escherichia coli* O157:H7. Infection. 24:378-383.

16) Mach JP, Pahud JJ. 1971. Secretory IgA, A major immunoglobulin in most bovine external secretions. J. Immunol. 106:552-563.

17) Mcnabb PC. 1981. Host defense mechanisms at mucosal surfaces. Ann. Rev. Microbiol. 35:477-496.

18) Michino H, Araki K, Minami S, Takaya S, Sakai N, Miyazaki M, Ono A, Yazagawa H. 1999. Massive outbreak of Escherichia coli O157:H7 infection in schoolchildren in Sakai City, Japan, Associated with consumption of white radish sprouts. Am. J. Epidemiol. 150:787-796.

19) Nakane JP, Kaper JB. 1974. Peroxidasae-labelled antibody. A new method of conjugation. J. Histochem. 22:1084-1091.

20) Nataro JP, Kaper JB. 1998. Diarrheagenic Escherichia coli. Clin. Microbiol. Rev. 11:142-201.

21) Newby TJ, Bourne FJ. 1976. Relative resistance of bovine and porcine immunoglobulins to proteolysis. Immunol. Commun. 5:631-635.

22) Paton JC, Paton AW. 1998. Pathogenesis and diagnosis Shiga toxin-producing Escherichia coli infections. Clin. Microbiol. Rev. 11:450-479.

23) Plaut AG. 1972. A review of secretory immune mechanisms. Am. J. Clin. Nutr. 25:1344-1350.

24) Tarr PI, Gordon CA, Chandler WL. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet. 365:1073-1086.

25) Tax A, Korngold L. 1971. Comparison of the effect of elasatase on human secretory IgA and serum IgA. J. Immunl. 107:1189-1191.

26) Tomasi TB, Tan EM, Solomon A, Prendergast RA. 1965. Characteristics of an immune system common to certain external secretions. J. Exp. Med. 1:101-124.

27) Tomasi TB, Grey HM. 1972. Structure and function of immunoglobulin A. Prog. Allergy. 16:106-133.

28) Underdown BJ, Dorrington KJ. 1974. Studies on the structural and conformational basis for the relative resistance of serum and secretory immunoglobulin a to proteolysis. J. Immunol. 112:949-959.

29) Yurchak AM, Bulter JE, Tomasi TB.1971. Fluorescent localization of immunoglobulins in the tissue of the cow. The J. Dairy Sci. 54:1324-1325.

30) Wong CA, Jelacic S, Hareeb RL, Watkins SL, Tarr PI. 2000. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N. Engl. J. Med. 342:1930-1936.