Mechanism of Suppression by Bovine Serum on Growth of Infectious Bursal Disease Virus

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ABSTRACT. The mechanism of suppression of plaque formation of infectious bursal disease virus (IBDV) by calf serum (CS) was investigated. Suppressor in CS was absorbed by incubation of the serum with chicken embryo fibroblast (CEF), indicating the attachment of suppressor to CEF. The amount of IBDV that was able to adsorb to CEF decreased when the cells were pretreated with CS. Suppression of IBDV plaque formation was also noted when CS was added to agar overlay medium, indicating that the serum suppressed the spread of the virus infection to the surrounding cells. The suppressive effect of CS was not due to a direct inactivation (neutralization) of IBDV. The results indicated that the suppressor in CS attaches to the surface of CEF and probably occupies the virus receptors on the cells, therefore blocking the adsorption of the IBDV and consequently, leading to the suppression of plaque formation by the virus.—**KEY WORDS:** IBDV, plaque formation, serum suppressor.

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Infectious bursal disease (IBD) is an acute disease of young chickens caused by infectious bursal disease virus (IBDV), and characterized chiefly by degenerative changes in lymphoid tissues, especially the bursa of Fabricius. In the previous study, we found that the growth of IBDV was significantly suppressed by incorporation of certain lots of bovine serum in tissue culture system [9]. When the suppressive rate of the serum was examined by plaque assay, all of the sera collected from adult dairy cattle or commercially purchased as calf serum (CS) suppressed the plaque formation of IBDV by more than 90%. In the agar gel precipitation test, the suppressor-active substances in the sera were found either not to be specific antibody-reactive or cross-reactive to IBDV, but rather considered as non-specific

suppressor(s) present in the bovine sera.

It is well known that the virus growth in cell cultures can be prevented by the non-specific inhibitors or by the specific antibodies existing in animal sera. The inhibitory effect could be caused by several mechanisms including 1) direct inactivation of virus, 2) formation of virus-inhibitor complex which prevents virus adsorption to cells or interferes with the expression of biological activities of the virus such as hemagglutination, 3) attachment of inhibitor to cells and occupation of virus receptor on the cell surface, or 4) inhibition of virus multiplication after the uptake of the virus by cells [7].

The aim of the present study is to elucidate the mechanism involved in the growth suppression of IBDV by bovine serum in chicken embryo fibroblast (CEF) cultures. We describe that the suppressive effect might be due to the coverage of the IBDV

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receptor sites by non-specific suppressor(s) and thereby suppressing the absorption and/or penetration of the virus.

MATERIALS AND METHODS

Cell cultures: The CEF cultures were prepared from 9–10 days-old specific pathogen-free chicken embryos by the method described by Hitchiner *et al.* [1]. The cells were grown in Eagle's minimal essential medium (MEM, Nissui Co., Ltd. Tokyo, Japan) supplemented with 10% tryptose phosphate broth (Difco, Detroit, Michi.), 10% fetal calf serum (FCS) or 10% CS and antibiotics [3] in 35 mm plastic dishes (Falcon, Oxnard, Calif.). For plaque assay, MEM containing 1% special agar noble (Difco) without the serum was used as an agar overlay medium (AOM).

Virus: The RF-1 strain of IBDV used was a generous gift from Dr. Y. Eiguchi (Research Center for Veterinary Science, Kitasato Institute, Kashiwa, Japan) and passaged three times in CEF cultures in our laboratory [4]. The culture fluid from the virus-infected CEF was used as the virus inoculum.

Test serum samples: FCS-20, FCS-14, CS-19, CS-27 and IgG or other fractions obtained from the CS-27 were used as test serum samples. FCS and CS used had weak and strong suppressive activity to IBDV growth, respectively. Both FCS and CS were inactivated at 56°C for 30 min prior to use. For fractionation of the CS-27, the serum precipitated with 50% saturated ammonium sulfate was applied to a DEAE-cellulose column and the IgG and other fractions were eluted by 0.5 M NaCl and 0.1 M tris-phosphate-buffered saline (PBS), respectively [8]. The protein amount in each test serum sample was determined by the method of Lowry *et al.* [2] and adjusted to 10 mg per ml for the inhibition test.

Inhibition test: The suppressive activity of

the test serum samples of the virus growth was examined by plaque reduction test [9]. Half ml of each sample (diluted 10 times with medium) was mixed with an equal volume of IBDV suspension containing 500 plaque forming units (PFU) and incubated for 30 min at 37°C. And then either 0.2 ml of the mixture or two-fold serial dilutions of the mixture was inoculated onto three CEF monolayer cultures. The dishes were allowed to stand for one hour at 37°C for virus absorption, washed with medium and then overlaid with the AOM. After a 48-hr incubation, the AOM containing 0.01% neutral red was added for the plaque count. A diluent, replacing the test serum samples, was used as the control inoculum. The suppressive effect of the test serum samples was shown either as percent plaque reduction or as blocking activity. The former was expressed as the relative PFU given as a percentage of the control while the latter was expressed as the reciprocal of the dilution at which 50% of the plaque formation was inhibited.

In one experiment, the effect of various treatments of CEF monolayers with CS in IBDV plaque formation was examined. CEF monolayers prepared with a medium containing CS-27 or FCS-20 were washed three times with MEM and then 1 ml of either CS-27 or medium was added. after incubating at 37°C for 30 min, monolayers were washed three times and infected with 100 PFU/0.2 ml of the virus. In one case, a mixture of 0.1 ml of CS-27 and 0.1 ml of a virus suspension (100 PFU) was added to the culture. After absorption of the virus, monolayers were washed once and AOM containing 10% CS-27 or without serum was overlaid on the dishes. The dishes were cultivated for 48 hours for observation of plaque formation.

Absorption test: The serum suppressor was absorbed with CEF. After adjusting the protein content to 10 mg/ml, 0.1 ml of the

Table 1. Absorption of the virus suppressor in bovine serum with CEF

Serum	Absorption with CEF ^{a)}	Blocking activity ^{b)}	Reactivity of CEF with anti-bovine IgG serum ^{c)}
CS-27	+	<20	—
	—	160	+
CS-27 IgG fraction	+	<20	—
	—	160	++
CS-27 fraction except IgG	+	<20	—
	—	80	+
FCS-20	+	<20	—
	—	<20	—

a) Serum (0.1 ml) absorbed with 1×10^7 CEF was centrifuged and the supernatant was used in the test. All the CEF preparations that absorbed either CS-27, the IgG fraction of CS-27 or the fraction other than IgG of CS-27 reacted with anti-bovine IgG rabbit serum. However, CEF that absorbed FCS-20 did not react with the anti-bovine IgG serum.

b) Expressed as the reciprocal of serum dilution which inhibits 50% plaque formation.

c) Examined by the FA test.

test serum samples was mixed with the dispersed CEF (1×10^8), incubated at 37°C for 30 min and centrifuged at 2,000 rpm for 10 min. The supernatants were assayed for the suppressive activity and the cells were examined for the presence of the serum component by the FA test.

Attachment of IBDV to CEF absorbed the bovine serum was examined. CEF (1×10^7) were mixed with 1 ml of the test serum samples or MEM as the control and incubated at 37°C for 30 min. The mixtures were centrifuged at 2,000 rpm for 5 min. The sedimented cells were washed three times with PBS, after which 0.1 ml of the virus (6.8×10^5 PFU) was added, mixed and incubated at 37°C for 30 min. After centrifugation at 2,000 rpm for 5 min, the supernatant was serially diluted from 10^{-1} to 10^{-7} and assayed for the residual infectivity in the CEF monolayers. The cells were also examined for the presence of IBDV antigen by the FA test.

FA test: The attachment of serum to CEF was examined by the FA test [9]. Smears of CEF suspension on a glass slide were fixed

in cold acetone and then incubated with the test serum samples for 30 min at 37°C in a humidified chamber. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-bovine IgG rabbit serum for 30 min at 37°C .

The presence of IBDV antigen on CEF was also examined. Smears of CEF pretreated with bovine serum and then infected with the virus were incubated with anti-IBDV chicken serum for 30 min at 37°C . After washing with PBS, the cells were stained with FITC-conjugated anti-chicken immunoglobulin rabbit serum for 30 min at 37°C .

RESULTS

The effect of absorption of virus suppressor in serum with CEF was examined. As shown in Table 1, the blocking activity of the absorbed CS-27 and its IgG or other fractions reduced the IBDV plaque formation from 160 or 80 to less than 20. Further, the reactivities of these test samples against CEF monolayers were completely dimi-

nished after the absorption with CEF as determined by the FA test (data not shown).

Table 2. Attachment of IBDV to CEF pretreated with bovine serum^{a)}

Serum ^{b)}	Residual virus titer ^{c)} (PFU/0.1 ml)	Reactivity of CEF to anti-IBDV serum ^{d)}
CS-19	1.3×10^5	±
FCS-14	2.3×10^4	+
MEM	1.5×10^4	+

- a) CEF was pretreated with serum as indicated in the text.
 b) CS-19 possessed very high suppressor activity for virus plaque formation, but the activity of FCS-14 was rather weak.
 c) Residual virus titer after absorption to CEF pretreated with bovine serum.
 d) The presence of IBDV antigen on CEF pretreated with bovine serum was examined by the FA test.

To explain the possible mechanism(s) through which the factor suppresses the virus growth, three experiments were carried out.

In the first experiment, we examined whether or not, the attachment of IBDV to pretreated CEF by CS or FCS, was interfered with. As shown in Table 2, the virus fluid absorbed with the dispersed cells which had been pretreated with CS-19 had a titer of 1.3×10^6 PFU, whereas those pretreated with FCS-14 or MEM were 2.3×10^5 and 1.5×10^5 PFU, respectively, indicating the suppression of virus absorption to CEF pretreated with CS-19. Furthermore, the cells pretreated with CS-27 gave a negative or a weak positive FA reaction for IBDV antigen whereas those pretreated with FCS-20 or MEM gave a marked positive reac-

Table 3. The Effect of treatment of CEF monolayers with bovine serum on IBDV plaque formation

Serum used to prepare CEF monolayer	Serum used to treat CEF monolayer	Inoculum	Serum contained in AOM	Blocking activity (%) ^{a)}
A. CS-27	CS-27	Virus only	Nil	91.2
B. FCS-20	CS-27	Virus only	Nil	68.7
C. FCS-20	MEM	Virus only	10% CS-27	81.8
D. FCS-20	MEM	Virus+CS-27	Nil	100.0
E. FCS-20	MEM	Virus only	Nil	0.0

a) Average value of three experiments.

Fig. 1. Recovery of virus infectivity with serial dilution of virus-serum mixture.

tion.

In the second experiment, we examined the effect of various treatments of CEF monolayers with the test serum samples on IBDV plaque formation. As shown in Table 3, the percentage plaque reduction of cultures A and B was 91.2 and 68.7, respectively. In culture C overlaid with AOM containing 10% CS-27, the percentage plaque reduction was 81.8 and the plaque size was about one-fourth of the normal size observed in culture E. On the other hand, the plaque formation in culture D was completely inhibited. It is interesting to note that the number of plaques in culture A was similar to those of culture C.

In the third experiment, mixtures of IBDV and test serum samples were two-fold serially diluted and inoculated onto CEF monolayer to observe whether the suppressor in serum attaches to the virus and therefore suppresses the plaque formation. As shown in Fig. 1, no plaque was formed on the CEF monolayer inoculated with serial dilutions of the CS-27-IBDV mixture below 1:160. The plaque formation was observed on the CEF monolayer inoculated with the FCS-20-IBDV mixture of 1:40 dilution. The infectivity of the virus gradually recovered in parallel with the dilution of the virus-CS or virus-FCS mixture up to 1:640 dilution.

DISCUSSION

Normal animal serum contains various substances which nonspecifically inhibit virus replication. For instance, mucoprotein has affinity to influenza virus and causes interference in the hemagglutination by the virus [6, 7]. In the same way, the infection of cells with the virus is also inhibited. We have reported on the substance in bovine serum which suppresses the formation of the plaques of IBDV [9]. The suppressor was inactivated by heating at 65°C for 30 min or

by trypsin treatment; it was resistant to the treatment with KIO_4 or to a receptor destroying enzyme. The suppressor mainly existed in the globulin fraction of the serum. Though the suppressor seemed to be similar to the β type of inhibitory substance of the influenza virus [5, 7] from the results of the previous study, further experiment should be required to determine whether the suppressor is included in the category of the β inhibitor.

Several mechanisms can be proposed for the suppression of IBDV plaque formation by bovine serum as described in the introduction. Firstly, the suppressor in serum attaches to the virus particles and thereby the infectivity of the virus is inhibited (neutralized). Secondly, there is a possibility that the suppressor adheres to the surface of CEF (occupies the virus receptors on the cells) and therefore suppressing both the absorption of IBDV to CEF and penetration of the virus.

The suppressive activity was absorbed by the cells (Table 1), indicating the attachment of suppressor to CEF. In the previous study [9], the presence of a serum component has been demonstrated on cells treated with CS by the FA test. Suppression of virus absorption to CEF pretreated with bovine serum was ascertained in another experiment. As shown in Table 2, the amount of virus absorbed on the CEF pretreated with CS was far less than that of the virus absorbed to CEF which was treated with either FCS or culture medium. These results indicated that the suppressor attaches to CEF and therefore the virus can not adsorb to the cell receptor sites which are covered by the suppressor.

In order to examine another possibility that the suppressor attaches to the virus and neutralize its infectivity, a virus suspension incubated with bovine serum was serially diluted and then its infectivity was titrated on CEF monolayers. If the suppressor

attaches to the virus and neutralize it, the virus infectivity should not be recovered even when the serum-virus mixture is diluted. As shown in Fig. 1, however, the infectivity of the virus pretreated with CS recovered gradually with the dilution of the virus-CS mixture, indicating that the suppressive effect by bovine serum was not due to the neutralization of the virus by the suppressor.

The present study also showed that CS suppresses the formation of IBDV plaques even when the serum was added to the cultures after the cells had been infected with the virus. More than 80% of the plaque formation was suppressed when 10% CS was added in AOM, and the size of the plaques formed was about one-fourth of the normal size (Table 3). The results indicated that the virus propagated and released from cells did not re-infect other cells since the uninfected cells had already been covered with the suppressor in serum, and therefore the spread of virus infection was suppressed.

As shown from the results of the present study and by the FA test in the previous report [9], the suppressor in bovine serum could adsorb onto the surface of CEF. As a result, the virus failed to attach to the surface of CEF and therefore suppressing the propagation of the virus. The suppressor probably occupies the virus receptors on CEF.

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要 約

仔牛血清の伝染性ファブリキウス嚢病ウイルス増殖抑制機序：于 大海，伊澤久夫，児玉 洋，小沼 操，¹⁾ 東京朋子，²⁾ 見上 彪（北海道大学獣医学部家畜伝染病学講座，¹⁾ 酪農学園大学農学部家畜微生物学教室，²⁾ 帝京大学医学部第二外科学教室）——仔牛血清（CS）による伝染性ファブリキウス嚢病ウイルス（IBDV）プラーク形成抑制機序を調べた。CS中の抑制物質は，CSを鶏胚線維芽細胞（CEF）とともに保温することで吸収され，抑制物質のCEFへの付着が示唆された。CEFに吸着するIBDV量は細胞をあらかじめCSで処理すると減少した。また，プラーク観察用重層寒天にCSを加えた場合にもIBDVプラーク形成は抑制され，CSは周囲細胞への感染拡大を阻止した。さらにCSはIBDVを直接不活化（中和）するのではないことが示された。これらの成績から，CS中の抑制物質はCEF表面に付着して，おそらくは細胞表面のウイルスレセプターをおおうことにより，IBDVの細胞吸着を阻止することが示唆された。