NOTE *Immunology*

Anti-Influenza A Virus Activities of Mannan-Binding Lectins and Bovine Conglutinin

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ABSTRACT. Mannan-binding lectin (MBL) and bovine conglutinin (BKg) belong to the collectin family, which is involved in first-line host defense against various infectious agents. We have previously reported that human MBL inhibited type A influenza viral hemagglutination, infection and spreading to adjacent cells without complement activation. In this study, we investigated the direct antiviral activities of bovine MBL, rabbit MBL and BKg. All collectins used in this study inhibited viral infectivity and hemagglutination at concentrations of 0.02–0.3 μg/m*l*. They also demonstrated inhibitory activity against viral spreading. Like human MBL, bovine MBL and BKg showed antiviral activities at their physiological concentrations. These results suggest that mammalian MBLs and BKg may inhibit the spread of influenza A virus through the bloodstream.

KEY WORDS: conglutinin, influenza A virus, mannan-binding lectin.

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Collectins are a family of C-type lectins that are structurally characterized by a collagen-like region and a carbohydrate recognition domain [6]. Serum collectins, mannan-binding lectin (MBL) and bovine conglutinin (BKg), and pulmonary collectins, surfactant protein A and D (SP-A and SP-D), play important roles in first-line host defense against a variety of microorganisms, including bacteria, fungi, and viruses by binding to sugars on their surfaces [10]. Although these collectins have different structures and molecular weights [17], they can bind to mannose and inhibit type A influenza viral hemagglutination and infectivity [2, 4, 14, 16]. Guinea pig MBL can neutralize viral infectivity and lyse influenza virus-infected cells by activating complement [3, 27]. In addition, human MBL, human SP-D and BKg act as opsonins, enhancing the activation of neutrophils by influenza A virus and protecting them from deactivation by the virus [14–16]. Human SP-D and BKg, but not human MBL, can induce the formation of viral aggregates and increase the internalization of viruses by neutrophils [12].

Recently, we have focused on the direct anti-influenza virus activities of human MBL independent of complement activation and opsonization, and showed that human MBL had an ability to inhibit viral spreading from primary infected cells to noninfected cells, which was designated as viral growth inhibition (GI) activity, as well as inhibit viral hemagglutination and infectivity directly [19]. However, whether other animal serum collectins exert direct inhibitory effects against influenza A virus has not been definitely determined. We have previously determined the primary structure and the complement activating ability of bovine MBL and rabbit MBL [20, 21], and characterized the biochemical and biological activities of BKg [29, 30]. In this study, we investigated the direct antiviral activities of bovine MBL, rabbit MBL and BKg against influenza A virus.

Bovine MBL, rabbit MBL and human MBL were purified from each serum sample using mannan-agarose (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) as previously described [19–21]. BKg was purified from heat-inactivated (56°C, 30 min) bovine serum [29]. The identities of purified collectins were confirmed by N-terminal amino acid sequence analyses and their protein concentrations were quantified by SDS-PAGE, followed by Coomassie brilliant blue staining (data not shown).

The hemagglutination inhibition (HI) test was done by a standard microtiter method [19]. Influenza A virus A/Ibaraki/1/90 (H3N2) used was sensitive to human MBL [19]. Viral solution (4 HA units) was incubated with serial dilutions of collectins for 30 min at 37°C, and then mixed with 0.5% chicken erythrocytes. The HI activity of collectins was determined after incubation for 1 hr at room temperature.

To examine the neutralizing activity of collectins, the rapid focus reduction neutralization test was performed as described before [19]. Briefly, Madin-Darby canine kidney (MDCK) cells were cultured as a monolayer in a 96-well microtiter plate (Asahi Techno Glass Corp. Tokyo, Japan). Influenza A virus A/Ibaraki/1/90 (H3N2) (about 100 focus forming units; FFU) was mixed with each dilution of collectins at 37°C for 1 hr, inoculated onto the MDCK cell monolayer washed with phosphate-buffered saline (PBS), and incubated at 37°C in 5% CO_2 for 1 hr. After washing with PBS, cells were cultured with viral growth medium containing 0.5% tragacanth gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The growth medium was composed of modified Eagle's medium (MEM) (Invitrogen Corp., Carls-

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bad, CA, U.S.A.) supplemented with 0.2% bovine serum albumin (Fraction V) (Sigma-Aldrich Corp.), 0.1% glucose, 3% tissue culture vitamins (MP Biomedicals, LLC., Irvine, CA, U.S.A.), 2 μg/m*l* acetyltrypsin (Sigma-Aldrich Corp.) and 5 μg/m*l* amphotericin B (Bristol-Myers K. K., New York, U.S.A.). After 24 hr, the cells were washed with PBS and fixed with absolute ethanol. The infected cells were immunostained using the monoclonal antibody against influenza A virus, subsequently rabbit anti-mouse IgG, goat anti-rabbit IgG and peroxidase-rabbit anti-peroxidase complex. All the antibodies used for staining were purchased from MP Biomedicals, LLC. The color reaction was conducted by incubating with 0.3 mg/m*l* 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd.) and 0.01% H_2O_2 in PBS. The numbers of stained foci were counted under a light microscope. The neutralizing activity was presented as the percent reduction of FFU compared with the FFU in samples not incubated with collectins.

The GI test was performed by the immunohistochemistry assay as described previously [19]. Influenza A virus A/Ibaraki/1/90 (H3N2) (about 50 FFU) was inoculated onto monolayers of MDCK cells in 24-well microplates and incubated at 37°C in 5% CO_2 for 1 hr. After washing with PBS, cells were incubated with viral growth medium containing 0.5% tragacanth gum and several dilutions of collectins for 3 days. The virus-infected areas were immunostained as described above, scanned using HP DeskScan II software and a Scanjet II cx (Hewlett-Packard Co., Palo Alto, CA, U.S.A.), traced and estimated quantitatively using Color-it (MicroFrontier Inc., Winterset, Iowa, U.S.A.) and NIH Image 1.60 software. The GI activity was determined as the percentage of viral infected areas compared to those without collectins.

The HI activity of bovine MBL, rabbit MBL, human MBL and BKg against influenza A virus A/Ibaraki/1/90 (H3N2) was examined. ALL MBLs and BKg inhibited viral hemagglutination (Table 1). The minimum concentration of collectins causing HI was within the range of 0.02–0.15 μg/m*l*. BKg showed more potent HI activity than MBLs. The HI activity was inhibited in the presence of 10 mM EDTA, 100 mM D-mannose or 50 mM *N*-acetyl-D-glucosamine (GlcNAc) (data not shown).

To measure the neutralizing activity of collectins, the rapid focus reduction assay was done. Bovine MBL and rabbit MBL had the ability to neutralize the infectivity of the influenza A virus in a dose-dependent manner, as human MBL and BKg (Fig. 1 and Table 1). The range of collectin concentrations required for 50% FFU reduction was 0.08–0.3 μg/m*l*. These neutralization tests were designed to prevent contamination of any serum complement components. Therefore, these results show that the neutralizing activity of both bovine MBL and rabbit MBL is independent of complement activation.

To investigate whether bovine MBL, rabbit MBL and BKg could inhibit viral spreading to adjacent cells, the GI test was performed. The areas infected with influenza A virus were reduced when medium containing collectins was overlaid on MDCK cells after viral infection (Fig. 2). Among the investigated collectins, bovine MBL and human MBL at a concentration of 1.0 μg/m*l* showed the most potent GI activity, reducing the infected areas to less than 5% of the controls. Rabbit MBL inhibited viral growth moderately (reduction to 16% of control growth) at 1.0 μg/m*l* and BKg showed the lowest GI activity (32% of control growth) at 2.0 μg/m*l*. When 100 mM mannose or 50 mM GlcNAc was added to the overlay medium containing 1.0 μg/m*l* of MBL or 2.0 μg/m*l* of BKg, respectively, viral infected areas recovered to 40–90% of the control area. These results indicate that the GI activity of MBLs and BKg is also dependent on their lectin activity.

Table 1. The HI and neutralizing activities of collectins against influenza A virus A/Ibaraki/1/90 (H3N2)

	HI activity[a)]	Neutralizing activity[b)]
Human MBL	0.15	0.3
Rabbit MBL	0.08	0.2
Bovine MBL	0.08	0.08
BKg	0.02	0.2

a) The minimum concentration showing HI activity (μg/m*l*).
b) The minimum concentration causing 50% FFU reduction (μg/m*l*).

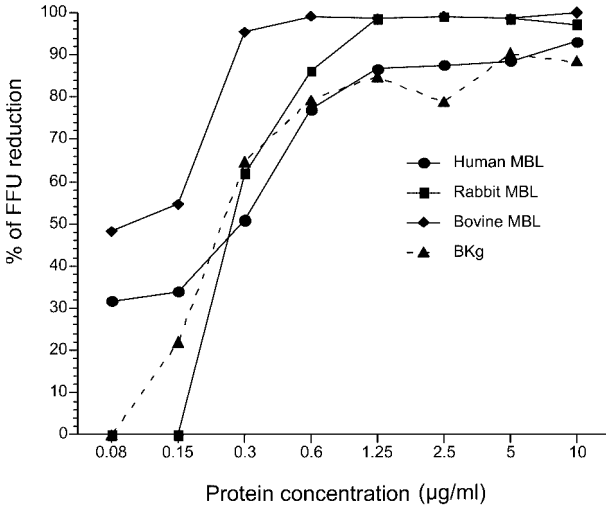

Fig. 1. The neutralizing activity of human MBL, bovine MBL and rabbit MBL and BKg against influenza A virus A/Ibaraki/1/90 (H3N2). The activity was estimated by the rapid focus reduction neutralization test and expressed as the percentage reduction of FFU compared with FFU from cells infected in the absence of collectins. FFU is the mean of triplicate results in a single experiment.

Binding of human MBL, human SP-D and BKg to viral hemagglutinin (HA), which is one of major envelope glycoproteins of influenza A virus, is important for viral neutralization and inhibition of viral hemagglutination activity [2, 13]. On the other hand, antibody against viral neuraminidase (NA), which is another envelope glycoprotein, is known to inhibit viral release from infected cells [22]. Human MBL and rat SP-D inhibit viral NA activity by bind-

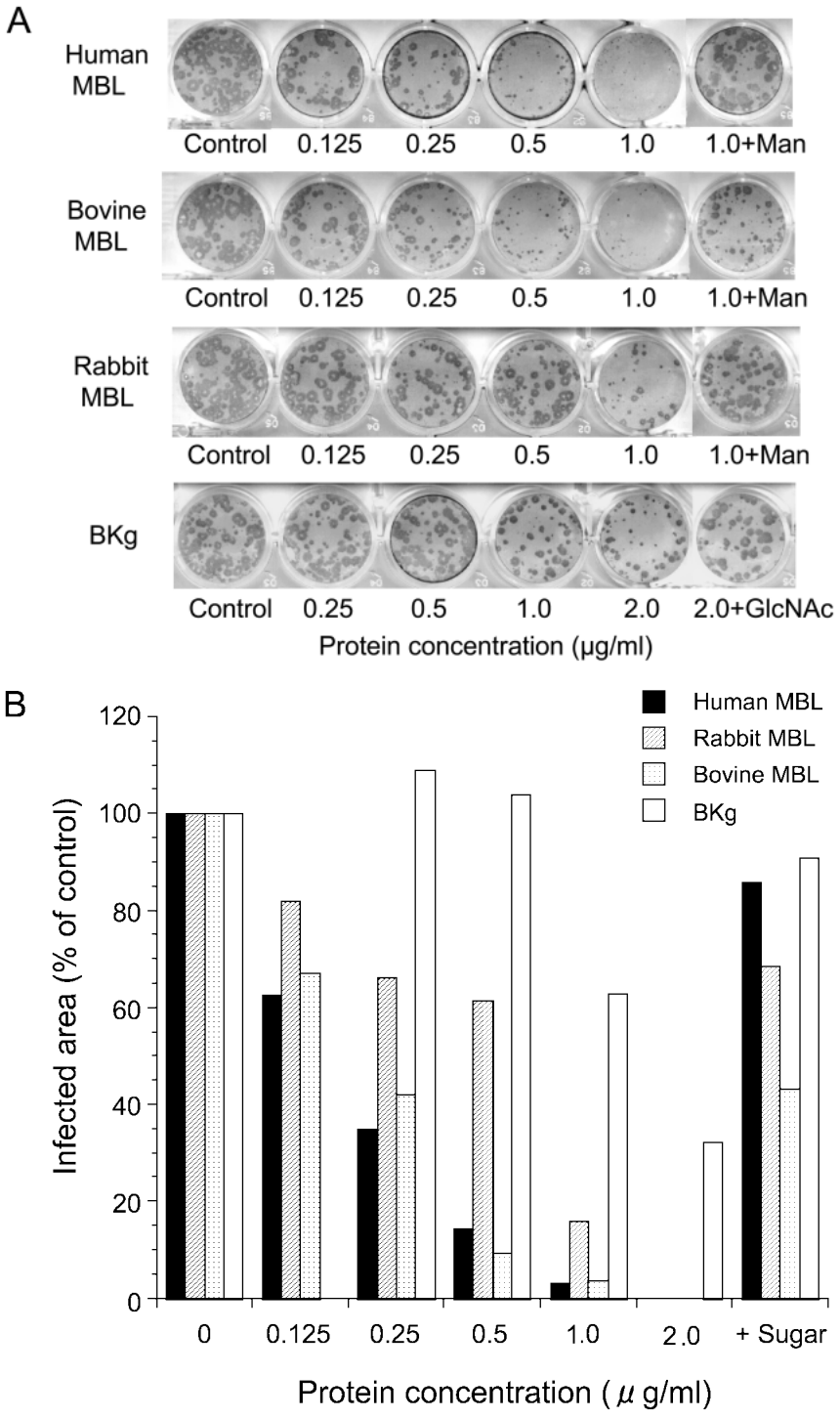

Fig. 2. The viral growth inhibition (GI) activity of human, bovine and rabbit MBL and BKg. MDCK cells were incubated with influenza A virus A/Ibaraki/1/90 (H3N2) (50 FFU/well) for 60 min, washed with PBS, and then incubated with viral growth medium containing 0.5% tragacanth gum and diluted collectins (0–2 μg/m*l*) with or without 100 mM mannose (Man) or 50 mM GlcNAc for 3 days. The infected foci were immunostained using the monoclonal antibody against influenza A virus (A). The infected area is depicted as a histogram of percentages of the control (B). Values are expressed as the mean of duplicate results in a single experiment.

ing to NA [26, 28]. Our recent lectin blot studies using native and recombinant truncated human MBL and human SP-D lacking the collagen-like region indicated that they bound to both viral HA and NA of A/Ibaraki/1/90 (H3N2), whereas BKg bound only to HA [7–9, 19]. Although human SP-D has a size and oligomeric structure similar to those of BKg [17], it strongly reduced the infected areas to less than 5% of the control areas at 2.0 μg/m*l* (data not shown). Therefore, it is considered that binding of collectins to both HA and NA might be important for their GI activity, where binding to NA might be involved in the enhancement. NA has many N-glycosylation sites, like HA [31]. The N-glycosylation site at residue 165 of HA is conserved in all H3 subtype influenza viruses and its high-mannose sugar chain at this site is essential for viral neutralization by collectins [2, 28]. Therefore, further studies will be required to identify the N-glycosylation site playing a crucial role in the GI activity of collectins.

BKg showed potent neutralizing activity, like MBLs, but required a higher concentration for inhibition of viral spreading to adjacent cells. The concentrations of MBL and BKg in the serum of healthy cows were reported to be 2.37 $\pm$ 0.87 and 56.5 $\pm$ 14.4 μg/m*l*, respectively [1]. Therefore, bovine MBL and BKg were thought to exert both neutralizing and GI activities under physiological conditions. It has been reported that cattle can be infected with both swine and human influenza A viruses in experimental studies [5, 25]. However, influenza A virus has rarely been isolated from cattle infected naturally [11, 24]. In addition, low levels of BKg were reported to be associated with increased susceptibility to respiratory infection [18]. In the case of MBLs, MBL-insufficient children have an increased risk for acute respiratory tract infection, including viral infections [23]. MBL is also detected in the bronchoalveolar lavage from mice infected with influenza A virus that is highly virulent for mice [28]. Therefore, these serum collectins may act as one of the first line host defense agents against influenza virus infection in cattle.

In this study, we investigated the direct anti-influenza virus activities of bovine MBL, rabbit MBL and BKg. Bovine MBL and BKg, like human MBL, could neutralize viral infectivity and inhibit viral spreading at their physiological concentrations in serum. These results suggest that mammalian MBLs and BKg may inhibit the spread of influenza A virus via the bloodstream by direct viral neutralization and inhibition of viral spreading to adjacent cells.

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