RESEARCH ARTICLE

Establishment of a quantitative bovine CXCL8 sandwich ELISA with newly developed monoclonal antibodies

Jiro Hirota[1], Shinya Shimizu[1], Atsushi Watanabe[2], Fumiko Suzuta[3], Kazue Yajima[4], Kumiko Kimura[5], Makoto Haritani[5], Shigeki Inumaru[1], Yukio Yagi[6]

[1] The Research Team for Advanced Biologicals, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki
[2] The Research Team for Environmental/Enzootic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Sapporo, Hokkaido
[3] Nagasaki South Livestock Hygiene Service Center, Shimabara, Nagasaki
[4] Hyogo Himeji Livestock Hygiene Service Center, Himeji, Hyogo
[5] The Research Team for Bacterial/Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba Ibaraki
[6] National Institute of Animal Health, National Agriculture and Food Research Organization Tsukuba, Ibaraki, Japan

Correspondence: Dr S. Shimizu, Research Team for Advanced Biologicals National Institute of Animal Health, National Agriculture and Food Research Organization 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
<shimizux@affrc.go.jp>

Accepted for publication October 12, 2010

Pour citer cet article : Hirota J, Shimizu S, Watanabe A, Suzuta F, Yajima K, Kimura K, Haritani M, Inumaru S, Yagi Y. Establishment of a quantitative bovine CXCL8 sandwich ELISA with newly developed monoclonal antibodies. *Eur. Cytokine Netw.* 2011; 22(1) : 73-80 doi:10.1684/ecn.2011.0275

ABSTRACT. Three IgG class anti-bovine CXCL8 (bCXCL8) monoclonal antibody (mAb)-secreting hybridomas, SH8-8D7, SH8-12A5 and SH8-2A1, were developed. SH8-8D7 was IgG2a, and SH8-12A5 and SH8-2A1 were IgG1. All three mAbs detected recombinant bCXCL8 (rbCXCL8) by immunoprecipitation and Western blotting. SH8-2A1 could neutralise the chemotactic activity of rbCXCL8 towards neutrophils. The quantitative bCXCL8 ELISA was constituted by the combination of SH8-12A5 and biotin-SH8-2A1. The detection range was 20-1000 pg/mL. A sandwich ELISA was used to measure native bCXCL8 derived from the supernatant of cultured bovine peripheral blood mononuclear cells stimulated with ConA, LPS or PHA. Furthermore, SH8-2A1 could detect bCXCL8 in formalin-fixed, paraffin-embedded, pneumonic calf tissues. These findings indicate that the newly developed anti-bCXCL8 mAbs could contribute to research on bovine inflammatory responses and immunology.

Key words: bovine, CXCL8, monoclonal antibody, immunohistochemistry, sandwich ELISA

CXCL8 was originally reported as a neutrophil chemotactic cytokine [1]. CXCL8 also has a wide range of activities towards neutrophils, T cells [2], B cells [3] and basophils [4]. CXCL8 is secreted by various types of cells, including T cells, neutrophils, endothelial cells, fibroblasts and epithelial cells, as a result of contact with pro-inflammatory cytokines, viruses, bacteria or bacterial products [4]. In fact, CXCL8 is frequently observed in tissues infected with viruses or bacteria [4]. On the other hand, CXCL8 also plays important roles in diseases, such as re-perfusion injury, glomerulonephritis and rheumatoid arthritis [4]. CXCL8 has been observed in several species of mammals, including human, monkey, rabbit, pig, dog, sheep, cattle, horse and rat. Bovine CXCL8 (bCXCL8) was first isolated and characterised by Hassfurther in 1994 [5]. CXCL8 is secreted in body fluids of cattle suffering from bovine pneumonic pasteurellosis [6], mastitis [7-9] and of those infected with *Fasciola gigantica* [10]. CXCL8 concentrations in body fluids correlate with disease severity [11-13]. A convenient quantitative system for CXCL8 needs to be developed in order to diagnose diseases and analyse the role of CXCL8 in the immune system. Monoclonal antibodies (mAbs) are applied in various fields, such as immunology, biology and medicine, because they exhibit highly specific reactivities. In the present study, we developed three anti-bCXCL8 mAbs and used them for a quantitative bCXCL8 sandwich ELISA as well as immunohistochemical staining of bCXCL8 in tissue sections.

METHODS AND MATERIALS

Recombinant bovine CXCL8

Recombinant bovine CXCL8 (rbCXCL8) was produced in a *Brevibacillus choshinensis* expression system [14]. Crude rbCXCL8 was purified using ultrafiltration membranes (MW 100,000 and 3,000) and ion exchange chromatography (Tosoh, Tokyo, Japan). According to SDS-page analysis, the molecular weight of rbCXCL8 was 10 kDa. The purity of 10 kDa rbCXCL8 was approximately 84%. The rbCXCL8 showed chemotactic ability in the chemotaxis assay performed using bovine peripheral neutrophils. The rbCXCL8 was used as an antigen for mAb development and characterisation, and as a standard sample for sandwich ELISA.

doi: 10.1684/ecn.2011.0275

Development of anti-bovine CXCL8 monoclonal antibodies

Anti-bCXCL8 mAbs were developed according to a method published in a previous report [15] with minor modification. Briefly, nine-week-old female BALB/c mice were immunised three to four times intraperitoneally with 20 μg of purified rbCXCL8 mixed with aluminium hydroxide gel. The mice were anaesthetised and exsanguinated three days after the final immunisation. Spleen cells were fused with P3-X63-Ag8-U1 myeloma cells using 50% polyethylene glycol (Hampton Research, California, USA). Fused cells were dispersed in HAT selection medium and incubated for 7-10 days in a CO_2 incubator. Hybridomas reacting to bCXCL8 were screened by indirect ELISA using rbCXCL8 as the antigen. After selecting positive cells, hybridomas were cloned by limiting dilution. Finally, three hybridoma cell lines that produced IgG class mAbs were established. Immunoglobulin isotypes of mAbs were identified using mouse mAb isotyping kit (Roche, Mannheim, Germany). To obtain sufficient amounts of each mAb, hybridoma cells were inoculated intraperitoneally into the BALB/c mice or cultured in serum-free medium. Anti-bCXCL8 mAbs from ascitic fluids were purified by ammonium sulphate precipitation and DEAE ion exchange chromatography, and anti-CXCL8 mAb from serum-free medium was purified by ammonium sulphate precipitation. Purified mAbs were labelled with horseradish peroxidase (HRP) or biotin using commercially available conjugation kits (Dojin Laboratories, Kumamoto, Japan).

Immunoprecipitation

Anti-CXCL8 mAbs were immobilised using an immunoprecipitation kit (Pierce, Illinois, USA) following the manufacturer's instructions. Following the reaction of immobilised mAbs with purified rbCXCL8, the mAb bound proteins were analysed by SDS-PAGE. The *SDS-PAGE* gels *were stained with CBB* R-250.

Western blot analysis

The rbCXCL8 was electrophoresed on a 4-12% Bis-Tris gradient gel (Invitrogen, California, USA), followed by electroblotting onto a polyvinylidene difluoride membrane using a gel transfer system (Invitrogen). The membrane was blocked using non-fat milk in TBS for overnight at 4°C. The membrane was cut into strips, and each strip was immersed in diluted mAb at room temperature for 1 h with occasional agitation. After incubation, each strip was washed with TBS containing 0.02% Tween 20 (TBST) and incubated with HRP-conjugated anti-mouse IgGAM (Zymed Laboratories, San Francisco, USA). After washing with TBST, the strips were immersed in 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma, St. Louis, USA).

Monoclonal antibody cross-reactivity with human CXCL8

Anti-bCXCL8 mAbs were tested for their cross-reactivity against recombinant human CXCL8 (rhCXCL8) by indirect ELISA. A 96-well ELISA plate (Nunc, Roskilde, Denmark) was coated with 100 ng/well of rhCXCL8 72 amino acid (PeproTech, New Jersey, USA) or rbCXCL8 diluted in carbonate-bicarbonate buffer (50 mM, pH 9.6), and the plate was incubated for 1 h at 37°C. The plate was then washed three times with 350 μL of PBS containing 0.02% Tween20 (PBST). At each step, incubation was performed for 1 h at 37°C and washing was performed three times using 350 μL of PBST. The plate was blocked with 20% Block Ace (Dainihon Seiyaku, Osaka, Japan) in PBST, and 100 μL of diluted HRP-labelled mAb in PBST containing 2% Block Ace (PBST-BA) was added to each well. The plate was then filled with 100 μL of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution in sodium citrate-buffered saline (50 mM, pH 4.0). After 1 h of incubation at 37°C, the absorbance values of each well were measured at 405 nm.

Preparation of bovine peripheral blood neutrophils

Bovine peripheral blood neutrophils were separated from citrated blood. The citrated blood was mixed with 0.83% ammonium chloride and 0.02% EDTA in distilled water. The haemolysed blood was centrifuged at 190 × g for 10 min and re-suspended in PBS containing 5% GIT (Nihon Pharmaceutical, Tokyo, Japan). The suspension of blood cells was then overlayed on 60% Percoll (GE Healthcare UK Ltd., Little Chalfont, England) and centrifuged at 2,300 ×g for 20 min. The sediment containing the neutrophils was washed with RPMI1640 medium containing 5% GIT (GIT-RPMI1640), centrifuged twice at 190 × g for 10min and re-suspended in GIT-RPMI1640. The purity of the neutrophils was above 90%. The isolated neutrophils appeared morphologically intact under the light microscope.

Recombinant bovine CXCL8 neutralising assay

The neutralising abilities of mAbs were determined by the chemotaxis assay. Briefly, purified mAbs were diluted to 10, 1 and 0.1 μg/mL in GIT-RPMI 1640. Identical volumes of 40 ng/mL of rbCXCL8 in GIT-RPMI 1640 and diluted mAbs were mixed and maintained for 1 h at 37°C. As a control mAb, 8A3B.6 (anti-VP7 for bluetongue virus; ATCC), not related to CXCL8, was used. Microtitre plates (96-well) were filled with the mixed solution and transwell inserts of 3 μm pore size (Kurabo, Tokyo, Japan) were applied. Immediately, 2×10^6 cells/mL of the bovine peripheral neutrophils in GIT-RPMI 1640 were poured into the transwell inserts. The plates were incubated for 1 h in the CO_2 incubator, and the transwell inserts were removed from the plates. Subsequently, to count the penetrated neutrophils, 10 μl of AlamarBlue solution (TREK Diagnostic Systems, West Sussex, England) were added to each well and incubated overnight in the CO_2 incubator, then the fluorescence of the wells in the plates was determined. Serial dilutions of the bovine peripheral neutrophils were used to plot a standard curve for the cell count. The number of cells in each well was calculated using the standard curve.

Selection of monoclonal antibody combinations for quantitative bovine CXCL8 sandwich ELISA

Nine combinations of mAbs for the sandwich ELISA were tested using the chequerboard ELISA assay; 10 μg/mL of each mAb in carbonate-bicarbonate buffer (50 mM, pH 9.6) was dispensed into the 96-well ELISA plate (Nunc), and the plate incubated for 1 h at 37°C. The plate was then washed three times with 350 μL of PBST. At each step,

incubation was performed for 1 h at 37°C and washing was performed three times using 350 μL of PBST. The plate was blocked with 20% Block Ace in PBST, and 100 μL of 100 ng/mL rbCXCL8 in PBST-BA was added to each well. This was followed by the addition of 100 μL of 10 μg/mL HRP-labelled mAbs in PBST-BA to each well. The plate was washed and filled with 100 μL of ABTS solution in sodium citrate-buffered saline (50 mM, pH 4.0). After 1 h of incubation at 37°C, the absorbance values of each well were measured at 405 nm.

Quantitative bovine CXCL8 sandwich ELISA

The 96-well ELISA plate (Nunc) was coated with 100 μL/well of purified SH8-12A5 diluted to 5 μg/mL in carbonate-bicarbonate buffer (50 mM, pH 9.6), incubated for 1 h at 37°C and washed three times with 350 μL of PBST. At each step, incubation was performed for 1 h at 37°C and washing was performed three times using 350 μL of PBST. The plate was blocked with 20% Block Ace in PBST, followed by the addition of 100 μL of serial diluted purified rbCXCL8 in PBST-BA (10-1,000 pg/mL) and serial diluted samples, to each well. The plate was filled with 100 μL of biotin-labelled SH8-2A1 in PBST-BA followed by the addition of 100 μL of HRP conjugated streptavidin (Jackson Immuno-Research Laboratories, Pennsylvania, USA), diluted 8000 times in PBST. After five more washes, each well was filled with 100 μg/mL of 3,3',5,5'-tetramethylbenzidine solution (Sigma). After 1 h of incubation at 37°C, 50 μL of 2M H_2SO_4 were added to each well, and the absorbance values of each well were measured at 450 and 620 nm.

Cross-reactivity of the quantitative bovine CXCL8 sandwich ELISA

Cross-reactivity of the quantitative bCXCL8 sandwich ELISA was tested against rhCXCL8 and bovine recombinant IL-1β, IL-6, IL-21, GM-CSF, M-CSF, TNF-α, TNF-γ and IFN-τ. Each recombinant protein was tested using the sandwich ELISA developed, at a concentration of 1 ng/mL.

Native bovine CXCL8 preparation

Bovine peripheral blood mononuclear cells (PBMC) were separated from the citrated blood using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Separated PBMC were washed twice in PBS, re-suspended in GIT-RPMI 1640 and diluted at a concentration of 2×10^6 cells/mL. The PBMC were stimulated with three types of mitogens, including 10 μg/mL of LPS (Difco Laboratories, Sparks, Michigan, USA), 5 μg/mL of Con-A (Boehringer Mannheim Biochemica, Mannheim ,Germany), and 10 μg/mL of PHA-P (Difco), and then incubated in the CO_2 incubator. The supernatants were harvested after 24 and 48 h. Finally, the culture supernatant was separated by centrifugation and maintained at -20°C until use.

Recovery of recombinant bovine CXCL8 from whey

The rbCXCL8 was mixed with the whey obtained using milk collected from 12 clinically healthy cows, and detected by the quantitative bCXCL8 sandwich ELISA. The average somatic cell count of the milk collected was 58,000 cells/mL, and the standard deviation was ± 48,000 cells/mL. The milk was centrifuged twice at 1,500 × g for 20 min at 4°C to remove milk fat, and subsequently centrifuged twice at 39,000 × g for 30 min at 4°C. After separating the supernatant, the whey was frozen at -20°C until use. A 200 pg/mL solution of rbCXCL8 was prepared using 50, 25, 12.5, 6.3 and 0% whey in PBS. These samples were measured using the sandwich ELISA, and the recovery rate was calculated using the following formula: recovery rate (%) = (observed CXCL8 − base CXCL8) × 100/added CXCL8.

Immunohistochemistry

Formalin-fixed, paraffin-embedded pneumonic calf tissues were immunostained with the developed mAbs using a polymer method kit (Nichirei, Tokyo, Japan). In addition, anti-bluetongue virus VP7 mAb 8A3B.6 (ATCC) was used as the control. The pneumonic tissues were collected from calves experimentally infected with *Mannheimia haemolytica*.

Animal welfare

All experiments using live animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan.

RESULTS

Characterisation of anti-bovine CXCL8 monoclonal antibodies

Three hybridomas (SH8-8D7, SH8-12A5 and SH8-2A1) secreting anti-rbCXCL8 mAb were obtained. The immunoglobulin class of SH8-8D7 was IgG2a, that of SH8-12A5, and SH8-2A1 was IgG1.

All three mAbs had immunoprecipitated 10kD protein, which is a major component of rbCXCL8 *(figure 1A)*. Western blot analysis indicated that all mAbs had reacted with rbCXCL8 *(figure 1B)*. All mAbs reacted with rhCXCL8 in indirect ELISA, but total optical density (OD) values of mAbs were 50-70% lower than those of rbCXCL8 determined by indirect ELISA *(table 1)*. More than 1 μg/mL of SH8-2A1 could neutralise the chemotactic activity of 40 ng/mL of rbCXCL8, but the other two hybridomas did not show any neutralising activity against rbCXCL8 *(figure 2)*.

Quantitative bovine CXCL8 sandwich ELISA

The chequerboard sandwich ELISA assay demonstrated that four combinations of capture (c)/detector (d) – c: SH8-12A5/d: SH8-2A1; c: SH8-12A5/d: SH8-8D7; c: SH8-8D7/d: SH8-12A5 and c: SH8-2A1/d: SH8-12A5 – could detect rbCXCL8. The combination of c: SH8-12A5 and d: biotin-SH8-2A1 was the most sensitive for bCXCL8 detection. The standard curve for the bCXCL8 sandwich ELISA is shown in *figure 3*.

T2 Cross-reactivity of the quantitative bovine CXCL8 sandwich ELISA

Bovine recombinant IL-1β, IL-6, IL-21, GM-CSF, M-CSF, TNF-α, TNF-γ and IFN-τ were not detected in the sandwich ELISA developed *(table 2)*. Recombinant human CXCL8 (1,000 pg/mL) was weakly detected in the sandwich ELISA, but the OD value was below the detection range.

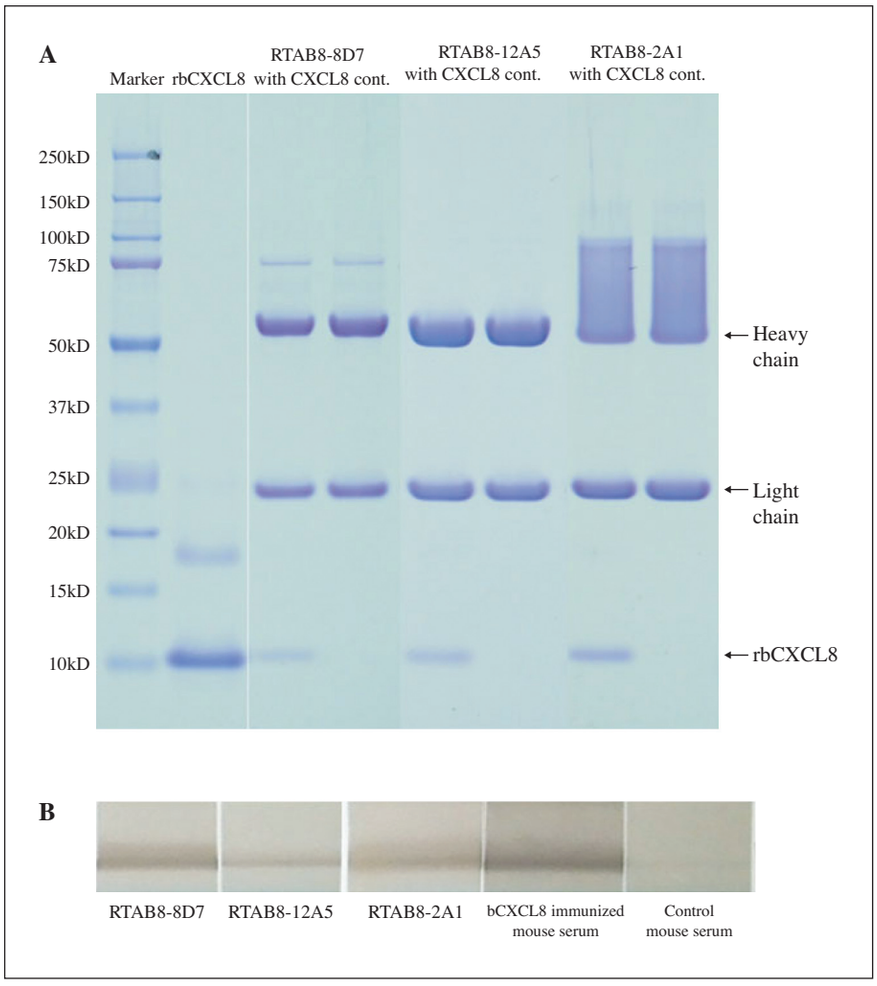

Figure 1
A) Immunoprecipitation pattern of developed mAbs. **B)** Western blot pattern of developed mAbs.

Recovery of recombinant bovine CXCL8 from whey

CXCL8 levels of whey samples tested were below detectable levels, apart from one sample. The recovery rate of rbCXCL8 decreased in the presence of a high concentration of whey; a 38.5% decrease was observed for 50% whey, 18.1% decrease for 25% whey, 10.3% decrease for 12.5% whey and 1.3% decrease for 6.3% whey.

Detection of native bovine CXCL8 by the sandwich ELISA

The SH8-12A5/biotin-SH8-2A1 sandwich ELISA system detected native bCXCL8 derived from bovine PBMC. The mean ± SD for detected native bCXCL8 was 25.4 ± 6.6 ng/mL in the LPS-stimulated supernatant, 43.8 ± 15.7 ng/mL in the ConA-stimulated supernatant, 29.9 ± 9.6 ng/mL in the PHA-stimulated supernatant, and 1.2 ± 1.5 ng/mL in control medium after 24 h of incubation. The mean ± SD for native bCXCL8 detected was 41.6 ± 17.8 ng/mL in the LPS-stimulated supernatant, 69.9 ± 12.5 ng/mL in the ConA-stimulated supernatant, 59.2 ± 32.7 ng/mL in the PHA-stimulated supernatant, and 3.9 ± 6.0 ng/mL in the control medium after 48 h of incubation *(figure 4)*.

Table 1
OD values obtained in indirect ELISA.

	Recombinant bovine CXCL8 (A)	**Recombinant human CXCL8 (B)**	**B/A**
SH8-8D7	0.783	0.417	0.532
SH8-12A5	0.960	0.522	0.543
SH8-2A1	1.933	0.551	0.285

Immunohistochemistry

In the immunohistochemical staining of formalin-fixed, paraffin-embedded pneumonic calf tissues, SH8-2A1 reacted with the neutrophils near necrotic regions *(figure 5A, C)*. On the other hand, no reaction was observed in pneumonic tissues immunostained with SH8-8D7, SH8-12A5 or control mAb *(figure 5B, D)*. In addition, SH8-2A1 did not react to tissue components in normal lung tissues (data not shown).

DISCUSSION

CXCL8 is secreted in body fluids of mammals suffering from various infections and diseases [16-20], and CXCL8 concentrations in human body fluids frequently correlate

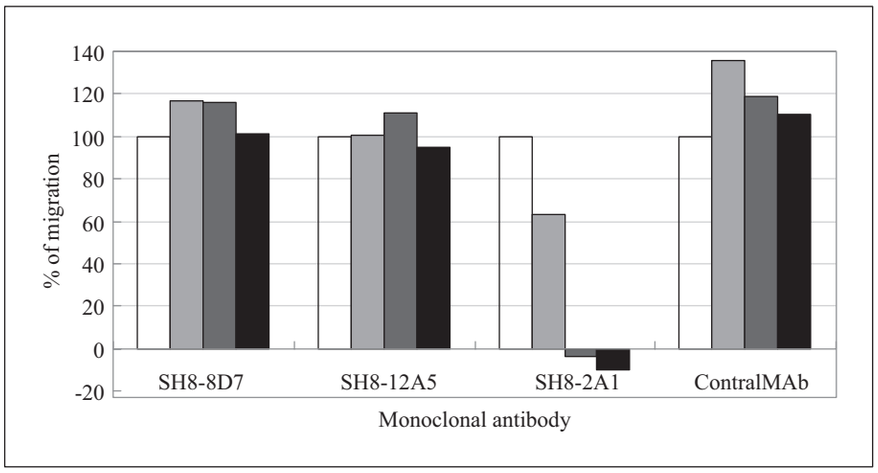

Figure 2

rbCXCL8-neutralising assay.
White bar: CXCL8 only (no mAb); stippled bar: mAb conc. (0.1 μg/mL); striped bar: mAb conc. (1 μg/mL) and black bar: mAb conc. (10 μg/mL). Percentage of migration = {(MCN* of mAb-containing chamber) − (MCN* of the chamber with no CXCL8)} × 100/{(MCN* of 40 ng/mL CXCL8-containing chamber) − (MCN* of the chamber with no CXCL8)}.
* MCN: migrated cell number.

with disease severity [11-13]. Measurement of CXCL8 concentrations in body fluids is important to observe the progress of various types of diseases or infections.

CXCL8 can be quantified to determine its chemotactic effect [21], release of intracellular enzymes [22], and production of reactive oxygen [23]. However, these assays require considerable time, and are comparatively complex. In contrast, the measurement of CXCL8 concentrations by quantitative sandwich ELISA is easier than the above-mentioned assays. In addition, ELISA can rapidly detect CXCL8, and needs only small amounts of samples and a short detection time. Sandwich ELISA using mAbs is a more convenient, sensitive and specific technique that provides reliable results.

We developed three mAbs that reacted with bCXCL8. Homologous mAb combinations could not sandwich rbCXCL8, and therefore, each epitope is thought to be single. The combination of capture antibody SH8-12A5 and detector antibody SH8-2A1 was most sensitive for rbCXCL8 detection. The 20 pg/mL mean OD value was higher than the 0 pg/mL mean OD value plus three standard divisions at 0 pg/mL. Thus, as per this calculation, the detection range of the sandwich ELISA developed was 20-1,000 pg/mL *(figure 3)*. The sandwich ELISA could detect native bCXCL8 derived from the mitogen-activated bovine PBMC culture supernatant. The sandwich ELISA did not cross-react with bovine IL-1β, bIL-6, bIL-21, bGM-CSF, bM-CSF, bTNF-α, bTNF-γ or bIFN-τ, and thus, the specificity of this sandwich ELISA to bCXCL8 was thought to be high. Moreover, SH8-12A5 had selectively immunoprecipitated bCXCL8 from LPS-stimulated PBMC supernatant (data not shown). From these results, the sandwich ELISA developed was shown to be suitable for bCXCL8 detection in several specimens of bovine origin, because the technique was not influenced by other cytokines.

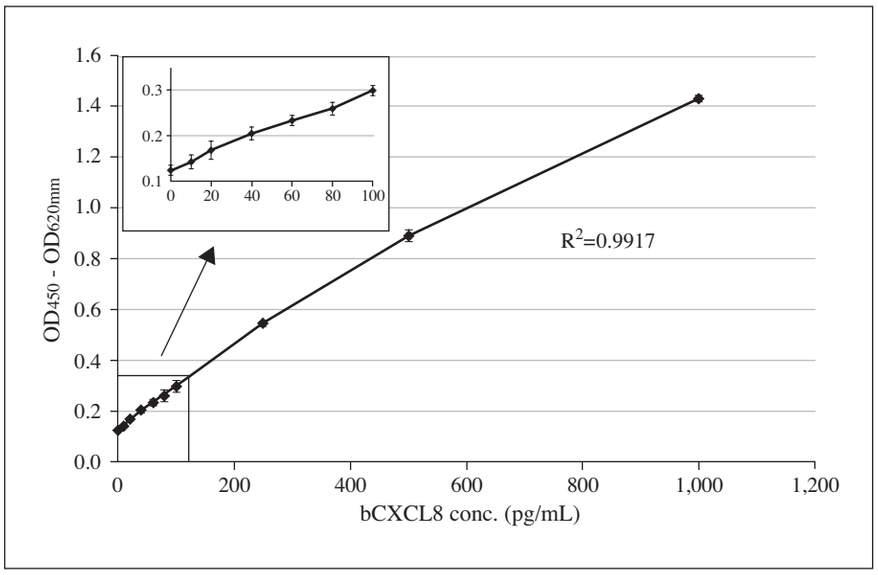

Figure 3

Standard curve for the bCXCL8 sandwich ELISA. Each point represents mean ± SD values of six standard curves. The coefficient of determination, R^2 was 0.9917 in the range 0-1,000 pg/mL.

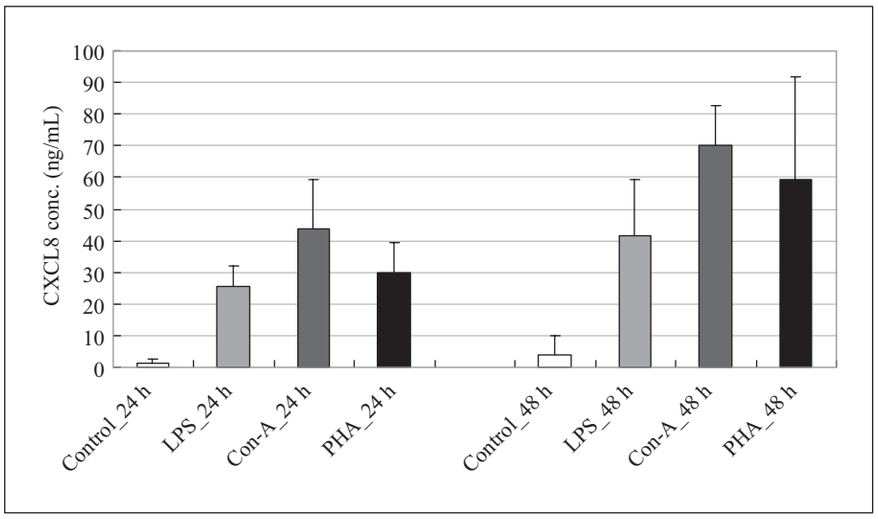

Figure 4
Detection of bCXCL8 in cell culture supernatant. "xx_24h" or "xx_48h" means incubation hour. Data represent mean ± SD (n=5).

Mastitis is an economically important disease affecting dairy cattle. CXCL8 is detected in the milk of cows suffering from mastitis caused by *Streptococcus uberis* [24], *Pseudomonas aeruginosa* [25], *Staphylococcus aureus* or *Escherichia coli* [7, 9]. Furthermore, it is reported that rbCXCL8, injected into the mammary glands, is effective in improving the symptoms of mastitis [26]. These findings suggest that CXCL8 is an important cytokine present in cows with mastitis. Certain components of the sample solution (*e.g.* anti-CXCL8 auto-antibodies in serum [28]), may influence the accuracy of the ELISA used for measuring CXCL8 [27]. Thus, it is important to determine the influence of sample components while developing sandwich ELISA. We determined the recovery rate of rbCXCL8 in the presence of whey to estimate the influence of whey on bCXCL8 detection. The presence of whey influenced the recovery rate, especially at 50% concentration. Some components of whey are thought to influence the mAb binding ability of bCXCL8. However, the influence of whey at 25% concentration was limited to a 20% reduction in the recovery rate, and moreover, 6.25% whey did not influence the recovery rate at all. Thus, the bCXCL8 sandwich ELISA developed can be used to measure bCXCL8 in milk.

Table 2
Cross-reactivity of cytokines observed in bCXCL8 sandwich ELISA.

Cytokine	Concentration (ng/mL)	OD_{450} - OD_{620} nm Mean ± SD
bCXCL8	1	1.074 ± 0.032
hCXCL8	1	0.115 ± 0.005
bIL-1β	1	0.063 ± 0.002
bIL-6	1	0.064 ± 0.005
bIL-21	1	0.057 ± 0.002
bGM-CSF	1	0.057 ± 0.004
bM-CSF	1	0.056 ± 0.002
bIFN-τ	1	0.059 ± 0.003
bIFN-γ	1	0.060 ± 0.003
bTNF-α	1	0.061 ± 0.003
Diluent	-	0.073 ± 0.006

Data shown are mean ± SD values of five wells.

In a preliminary experiment, we tried to measure recombinant and native bCXCL8 using a commercially available human CXCL8-measuring kit. However, no rbCXCL8 was detected, and the native bCXCL8 concentration determined using the kit was one twentieth of that determined by the bCXCL8 sandwich ELISA developed (data not shown). On the other hand, in this study, although both capture and detector mAbs reacted with rhCXCL8 in an indirect ELISA *(table 1)*, the concentration of 1,000 pg/mL of rhCXCL8 was estimated to be less than 20 pg/mL using bCXCL8 sandwich ELISA. The reason for this is believed to depend on mAb affinity. On comparing amino acid sequences of hCXCL8 with bCXCL8, it was found that one of the CXCL8 receptor-binding regions and some regions of amino acids are different. These differences affected mAb affinity towards bCXCL8 and hCXCL8. In fact, this difference in affinity was observed in the ELISA; OD values in the hCXCL8 ELISA were lower than those in the bCXCL8 ELISA *(table 1)*. As a result of the combination of these two mAbs, sensitivity to hCXCL8 decreased. The difference in sensitivity between hCXCL8 and bCXCL8 observed in the assay performed using the hCXCL8 quantitative ELISA kit, is believed to occur for the same reason. One should be wary however, while measuring CXCL8 using techniques based on antibody cross-reactions between different species, especially in the case of a sandwich ELISA.

Immunohistochemistry is a useful technique for analysing pathological and pathophysiological features of diseases. Some studies have reported on the immunohistochemical analysis of inflammatory cytokines, including CXCL8, in pulmonary diseases [29], skin diseases [30] and corneal diseases [31]. For such purposes, highly specific antibodies for each cytokine are required, particularly those that can be used for formalin-fixed, paraffin-embedded tissues. One of the developed mAbs SH8-2A1 could detect bCXCL8 in the necrotic regions of pneumonic tissues *(figure 5)*. In addition, SH8-2A1 was used to visualise bCXCL8

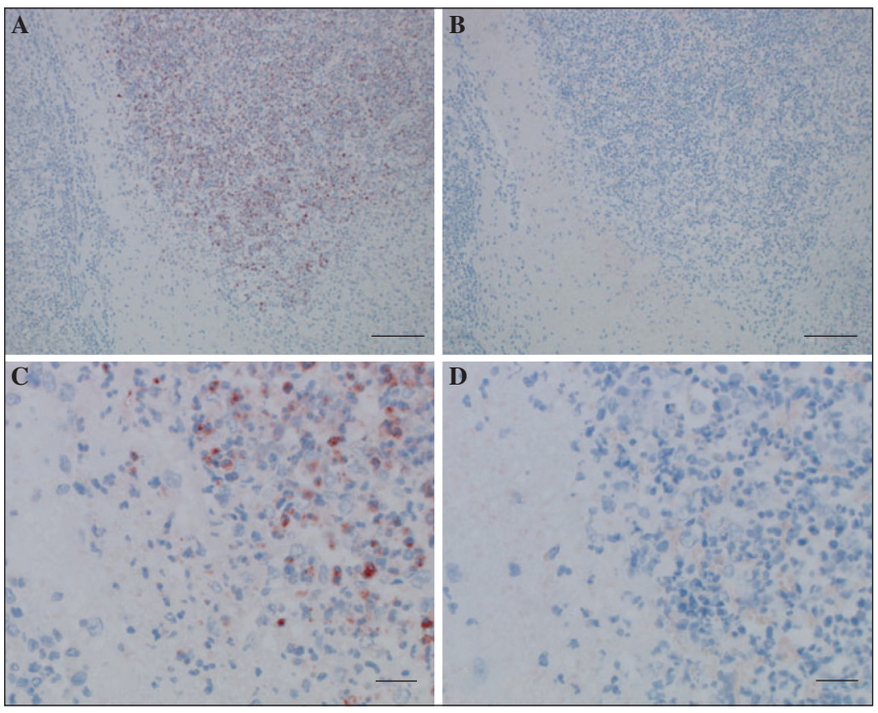

Figure 5
Immunohistochemistry of bovine pneumonic lung tissues using SH8-2A1 and control mAb. SH8-2A1-stained neutrophils around the necrotic lesion **(A, C)**, but control mAb did not stain any cells **(B, D)**. Bars = 100 μm **(A, B)** and 20 μm **(C, D)**.

in formalin-fixed, paraffin-embedded tissues. This mAb could help in the pathological and pathophysiological analyses of many bovine diseases.

In conclusion, a quantitative sandwich ELISA for bCXCL8 was established using newly developed mAbs; one of these mAb was also useful for immunohistochemistry. These mAbs could help reveal the role of bCXCL8 in several infections and diseases, and in bovine immunity.

Acknowledgments. *Recombinant bovine IL-6 was kindly provided by Dr Miyako YOSHIOKA (NIAH, Naro, Japan). Recombinant bovine IL-21 was kindly provided by Dr Yoshihiro MUNETA (NIAH, Naro, Japan). Recombinant bovine M-CSF was kindly provided by Dr Kazuhiro YOSHIHARA (NIAH, Naro, Japan).*

Disclosure. *This study was partially supported by a Grant-in-Aid from the "National Agriculture and Food Research Organization" and the "Japan Livestock Technology Association".*

None of the authors has any conflict of interest to disclose.

REFERENCES

1. Yoshimura T, Matsushima K, Tanaka S, *et al*. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci USA* 1987; 84: 9233.

2. Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* 1989; 243: 1464.

3. Jinquan T, Møller B, Storgaard M, *et al*. Chemotaxis and IL-8 receptor expression in B cells from normal and HIV-infected subjects. *J Immunol* 1997; 158: 475.

4. Mukaida N., Ketlinsky SA. and Matsushima K. Interleukin-8 and other CXC chemokines. In: Angus WT, Michael TL, eds. *The Cytokine Handbook*. 4th ed., California: Academic Press, 2003; 1049

5. Hassfurther RL, Canning PC, Geib RW. Isolation and characterization of an interleukin-8-like peptide in the bovine species. *Vet Immunol Immunopathol* 1994; 42: 117.

6. Caswell JL, Middleton DM, Sorden SD, Gordon JR. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. *Vet Pathol* 1998; 35: 124.

7. Bannerman DD, Paape MJ, Lee JW, Zhao X, Hope JC, Rainard P. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin Diagn Lab Immunol* 2004; 11: 463.

8. Lee JW, Paape MJ, Elsasser TH, Zhao X. Elevated milk soluble CD14 in bovine mammary glands challenged with Escherichia coli lipopolysaccharide. *J Dairy Sci* 2003; 86: 2382.

9. Riollet C, Rainard P, Poutrel B. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin Diagn Lab Immunol* 2000; 7: 161.

10. Molina EC. Serum interferon-gamma and interleukins-6 and -8 during infection with Fasciola gigantica in cattle and buffaloes. *J Vet Sci* 2005; 6: 135.

11. Biffl WL, Moore EE, Moore FA, Carl VS, Franciose RJ, Banerjee A. Interleukin-8 increases endothelial permeability independent of neutrophils. *J Trauma* 1995; 39: 98.

12. Hsu CD, Meaddough E, Aversa K, *et al*. Elevated amniotic fluid levels of leukemia inhibitory factor, interleukin 6, and interleukin 8 in intra-amniotic infection. *Am J Obstet Gynecol* 1998; 179: 1267.

13. Miller EJ, Cohen AB, Nagao S, *et al*. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am Rev Respir Dis* 1992; 146: 427.

14. Mizukami M, Yashiro K, Ronnanhays P, *et al*. The efficient production of some livestock cytokines by protein-producing Brevibacillus choshinensis. *Int Vet Cyto Vac Conf* 2000; 1: 212.

15. Yokoyama WM. Production of Monoclonal Antibodies. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. *Current Protocols in Immunology*. New York: John Wiley and Sons, 1991; Unit 2.5.: 2.5.1.

16. Friedland JS, Suputtamongkol Y, Remick D G, *et al*. Prolonged elevation of interleukin-8 and interleukin-6 concentrations in plasma and of leukocyte interleukin-8 mRNA levels during septicemic and localised Pseudomonas pseudomallei infection. *Infect Immun* 1992; 60: 2402.

17. Hack CE, Hart M, van Schijndel RJ, *et al*. Interleukin-8 in sepsis: relation to shock and inflammatory mediators. *Infect Immun* 1992; 60: 2835.

18. Donnelly SC, Strieter RM, Kunkel SL, *et al*. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 1993; 341: 643.

19. Endo H, Akahoshi T, Takagishi K, Kashiwazaki S, Matsushima K. Elevation of interleukin-8 (IL-8) levels in joint fluids of patients with rheumatoid arthritis and the induction by IL-8 of leukocyte infiltration and synovitis in rabbit joints. *Lymphokine Cytokine Res* 1991; 10: 245.

20. Puchner T, Egarter C, Wimmer C, Lederhilger F, Weichselbraun I. Amniotic fluid interleukin-8 as a marker for intraamniotic infection. *Arch Gynecol Obstet* 1993; 253: 9.

21. Schröder JM, Mrowietz U, Morita E, Christophers E. Purification and partial biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. *J Immunol* 1987; 139: 3474.

22. Sklar LA, McNeil VM, Jesaitis AJ, Painter RG, Cochrane CG. A continuous, spectroscopic analysis of the kinetics of elastase secretion by neutrophils. The dependence of secretion upon receptor occupancy. *J Biol Chem* 1982; 257: 5471.

23. Thelen M, Peveri P, Kernen P, von Tscharner V, Walz A, Baggiolini M. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. *FASEB J* 1988; 2: 2702.

24. Rambeaud M, Almeida RA, Pighetti GM, Oliver SP. Dynamics of leukocytes and cytokines during experimentally induced Streptococcus uberis mastitis. *Vet Immunol Immunopathol* 2003; 96: 193.

25. Bannerman DD, Chockalingam A, Paape MJ, Hope JC. The bovine innate immune response during experimentally-induced Pseudomonas aeruginosa mastitis. *Vet Immunol Immunopathol* 2005; 107: 201.

26. Takahashi H, Komatsu T, Hodate K, Horino R, Yokomizo Y. Effect of intramammary injection of RbIL-8 on milk levels of somatic cell count, chemiluminescence activity and shedding patterns of total bacteria and S. aureus in Holstein cows with naturally infected-subclinical mastitis. *J Vet Med B Infect Dis Vet Public Health* 2005; 52: 32.

27. Fledkamp CS. Practical guide to immunoassay method evaluation. In: Chan DW, ed. Immunoassay: A Practical Guide. Orlando: Academic Press, 1987 ; 49.

28. Sylvester I, Yoshimura T, Sticherling M, *et al*. Neutrophil attractant protein-1-immunoglobulin G immune complexes and free anti-NAP-1 antibody in normal human serum. *J Clin Invest* 1992; 90: 471.

29. Allen TC, Fudala R, Nash SE, Kurdowska A. Anti-interleukin 8 autoantibody:interleukin 8 immune complexes visualized by laser confocal microscopy in injured lung. *Arch Pathol Lab Med* 2007; 131: 452.

30. Abd El All HS, Shoukry NS, El Maged RA, Ayada MM. Immunohistochemical expression of interleukin 8 in skin biopsies from patients with inflammatory acne vulgaris. *Diagn Pathol* 2007; 2: 4.

31. Shoshani Y, Pe'er J, Doviner V, Frucht-Pery J, Solomon A. Increased expression of inflammatory cytokines and matrix metalloproteinases in pseudophakic corneal edema. *Invest Ophthalmol Vis Sci* 2005; 46: 1940.