STUDIES ON THE BOVINE INTERLEUKIN 1

- KINETICS OF ITS PRODUCTION AS AN EARLY PHASE CYTOKINE -

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Cytokine is a general term for certain high-molecule peptides produced and secreted by cells, mainly by immune system cells, and it has an important function in living organisms as a messenger between various cells. To be more specific, when a foreign object enters a living body, cytokine acts as a non-specific protective factor in preventing infection, in immune response, and in inflammatory reactions. It also plays an important role as an initiator of humoral immunity and cellular immunity.

Interleukin (IL)-1 is one cytokine and has been reported in such animal species as human, mice, rabbits, cats, pigs and cows. In particular, human IL-1 has been studied in detail. According to such study, IL-1 is made up of proteins whose molecular mass are approximately 17 kDa and that are mainly secreted by monocytes and macrophages, and there exist two kinds of IL-1 with different isoelectric points: IL-1α (isoelectric point: approx. pI≒5) and IL-1β (isoelectric point: approx. pI≒7). It is said that both IL-1α and IL-1β combine with the same IL-1 receptor and have the same biological functions. For human and mice, a kit to measure IL-1α and β with ELISA (Enzyme linked immunosorbent assay) using monoclonal antibodies has already been developed and used. Also, with regard to IL-1 gene expression and their regulation mechanism, detailed studies on regulatory gene and intranucleus trsanscription factor are being carried out. Like other cytokines, IL-1 has a wide range of bioactivities. Its most important function is inducing the production of IL-2 from helper T cells and promoting division and propagation of T cells through this IL-2. It

also influences the division and propagation of B cells. IL-1 acts not only on immune system cells but also is involved in inflammatory reactions. For example, in the brain, it works as an endogeneous pyrogen; in hepatic cells, it promotes production of acute phase proteins which reveal inflammatory reaction; and in synovial cells, it induces production of collagenase and prostaglandins.

As for bovine IL-1, Maliszewski et al. succeeded in cDNA cloning and determined its sequence in 1988. Protein expression by E. coli was also reported. Their report, however, is limited to molecular biological study of IL-1 as a substance and dose not touch upon the dynamic state of cytokine regarding protective reactions in living bodies, i.e., from gene expression in the producing cell to secretion and its kinetics. Also, bioactivity of IL-1 in both human and mice is measured using an index a propagation of reinforcing action in the presence of mitogen in mice thymocytes. But it is impossible to distinguish IL-1α from β with this method, which means that comprehensive activities of IL-1α and β is measured. Thus it is impossible to determine, with a biological measuring method, whether IL-1 activity is caused by α or β.

This study was conducted to clarify the action of bovine IL-1 as an immunological initiator (the significance of IL-1 production during illness in particular) and the relation between individual differences of IL-1 production and the induction of immune response. For this purpose, I established a method to detect small amounts of bovine IL-1 mRNA by using RT-PCR (Reverse transcription-polymerase chain reaction) and ELISA, the latter of which uses monoclonal antibody as a method to measure IL-1β which is said to be produced

in large amount. I used these methods to examine IL-1 gene expression and productivity.

The results of this study are summarized as follows.

1. Separation of bovine peripheral mononuclear cells, monocytes and alveolar macrophages

Bovine peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Conray (s.g.=1.081) gradient centrifugation of heparinized peripheral blood. As a result, about 1×10^7 mononuclear cells were obtained from 10 ml of the heparinized blood, and a two-hour adhesive operation yielded about $1\text{-}1.5\times10^6$ monocytes. To prepare the bovine alveolar macrophages (AMs) the lungs of healthy cow were lavaged using sterile phosphate-buffered saline (PBS) and AMs were separated by an adhesive method. As a result, about $1\text{-}2\times10^8$ alveolar macrophages per adult cow were separated. Both PBMCs and AMs were cultured in RPMI-1640 medium supplemented with 2mM of L-glutamine, 25mM of HEPES, 5×10^{-5}M of 2ME, 100IU/ml of penicillin, 100μg/ml of streptomicin, and 10% heat inactivated fetal calf serum (FCS). These cells were proven to be macrophages, because of their phagocytosis of fixed sheep erythrocytes, positive stain of non-specific esterase and acid-phosphatase.

2. Detection of bovine IL-1α and IL-1β mRNA by RT-PCR method

Poly(A)$^+$RNA from PBMCs and AMs stimulated with 20μg/ml lipopolysaccharide (LPS) were isolated using a mRNA purification kit. Using poly(A)$^+$RNA as a template, RT-PCR method to detect IL-1α and IL-1β mRNA was established. RT reaction was employed at 42℃ for 15 min and then at 99℃ for 5 min to inactivated reverse transcriptase. The following PCR reaction was employed as following condition: 1) denaturation: 95℃, 1 min. (except for the 1st denaturation, which was 3 min), 2) primer annealing: 55℃, 1 min, and 3) extension: 72℃, 1 min. PCR reaction was coducted generally in cycles of 35. For a primer at RT reaction, generally 3 kinds can be used: 1) random hexamers, 2) oligo d(T)16, and 3) specific downstream primer. In this experiment also, each of the primers produced good results. That is, the fragments of predicted size (IL-1α: 424bp, and IL-1β: 394bp) were detected as a result of 4% agarose gel electrophoresis. These RT-PCR products were identified by restriction enzyme analysis. The enzymes used and diagnostic fragments were 317/107bp; (IL-1α, HindⅢ), 154/146/124bp; (IL-1α, HinfⅠ), 301/93bp; (IL-1β, HindⅢ), 326/68bp; (IL-1β, PstⅠ). These results were in agreement with the sequence data of bovine IL-1α and IL-1β by Maliszewski et al. To evaluate the sensitivity of RT-PCR, serial ten-fold dilutions (0.1 ng to 1 fg) of poly(A)$^+$RNA isolated from PBMCs stimulated with LPS for 24 hours were amplified by using the IL-1α and IL-1β specific primers. After 35 cycles of PCR, IL-1α specific fragment was detected at 0.01ng of template poly(A)$^+$RNA, whereas, IL-1β specific fragment was detected at 0.1 pg. Furthermore, to evaluate the efficiency of poly(A)$^+$RNA

isolation, poly(A)$^+$RNA was isolated from serial ten-fold dilutions (1×10^7 to 1×10^2) of PBMCs and subjected to RT-PCR using the IL-1α specific primer. After 35 cycles of PCR, IL-1α specific fragment could be detected in as few as ten PBMCs. Furthermore, for detection sensitivity by the number of PCR cycles, where 5 ng of poly(A)$^+$RNA was used as template, the IL-1α specific fragment was detected using more than 25 cycles of PCR. Also, the RT-PCR method was about 10^6 times more sensitive than the norther blotting method. Evaluation of application of RT-PCR products proved them quite useful as a probe in the northern blotting, dot blotting and in situ hybridization methods.

Evaluation of the dynamic expression state of IL-1 mRNA in vitro using the RT-PCR method confermed that, in peripheral blood mononuclear cells, the expression of IL-1α and IL-1β mRNA is seen structurally (continuously) even in the so-called resting situation, although it is quite low in level. And also, expression of IL-1α and IL-1β mRNA is increased in a shorter time period (within 3 hours) due to the LPS stimulation, and the expression of IL-1α mRNA is transient in nature. As a result of consideration of the dynamic expression state of IL-1 mRNA in cattle administered LPS, it was indicated that, also in vivo, mRNA was found in quite a short time period after LPS was administered, and that it is deeply involved in inflammation as so-called early-phase cytokine. Also, as in the case in vitro, the transient expression of IL-1α mRNA was found. Forther examination about individual differences in the expression of IL-1β mRNA demonstrated individual differences in its expression level.

3. Measerement of bovine IL-1 activity and monoclonal antibody against IL-1β

A series of monoclonal antibodies was producted by an in vitro stimulation method with IL-1β refined from a supernatant of the macrophage culture by purified by isoelectric focusing as antigen. To measure IL-1β, ELISA using this monoclonal antibody was established and be compared the measurement with result of bioassay using mouse thymocytes. As a result, IL-1 in the supernatant of the culture with almost the same level of sensitivity as the bioassay. IL-1 produced by peripheral mononuclear cells was measured both by bioassay using mouse thymocytes and by ELISA, and activity was detected in 3 hours after the stimulation, and high activity in 24 and 48 hours.

As explained above, expression of IL-1α and IL-1β mRNA was found in quite a short time (30 minutes-3 hours) after LPS stimulation. This indicates that IL-1 α and IL-1β are deeply involved in early phase, non-specific inflammation of protective reaction of living bodies as so-called "early phase cytokines". That is, protective reaction of living bogies following infection can be divided into an early phase, non-specific inflammation reaction and a late phase, specific immune response. Non-specific inflammation reaction such as fever in the early reaction, production of acute phase protein, and infiltraion of phagocytes involve so-called early cytokines, of which IL-1 is typical. Antigens processed by macrophages and B cells early in reaction are presented to corresponding T

cells along with class II molecules of MHC (Major histocompatibility complex), and the stimulated T cells produce late-phase cytokines such as IL-2, IL-4, IL-5 and IFNγ. I also detected the expression of IL-1β mRNA in peripheral mononuclear cells in so-called resting state of healty cows, and confermed individual differences. This result, though it is not clear whether it is simply due to differences in situatioin including the breeding environment of individual cows or to immunogenetic individual differences, is interesting. In other words, if the difference in expression of IL-1β at a time of infection appears as an individual difference in the immune response which takes part afterward and it can be analyzed as one of the things regulating the sensitivity and resistance to disease, it is significant as a direction of future cattle clinical immunological study.

The report on the detection of the expression of bovine IL-1α and IL-1β by the RT-PCR method follows that of on IL-2 and IL-6, will be applied to many cytokins, and will help clarify details of the cytokine network. The IL-1β measurement method by ELISA using monoclonal antibodies is also useful as an easy measurement method of bovine IL-1β.

Recently, cDNAs such as bovine IL-1, IL-2, IFN, TNFα and CSF have been isolated, which has made possible studies on clarification of immune system using recombinant cytokine. Clinical application of cytokine to cattle is designed to prevent or cure infections. In particular, cytokine can be used for

calves which have a high risk of becoming unusable due to infection in order to improve the protective ability of their immature bodies. It can also be used to restore immune systems which have deteriorated during the perinatal period or times of stress. Furthermore, the adjuvant effect of cytokine to intensify the immune effect of recombinant vaccines has also been indicated. However, in order to utilize cytokines as medicine for preventing or curing infections, it is necessary to clarify each one's effect and network in detail. With the production of many recombinant cytokines, and the establishment of a detection method of the expression of cytokine genes and a cytokine activity measurement method, there will be remarkable progress in the clarification of the cattle immune system.