J. Rakuno Gakuen Univ., 24 (1) : 89~94 (1999)

Cytokine mRNA Expression in Bovine Alveolar Macrophages

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(June 1999)

Abstract

The expression of mRNAs for Interleukin (IL)-1α, IL-1β, IL-6, IL-8, Interferon (IFN)-γ, Tumor necrosis factor (TNF)-α, and Granulocyte macrophage-colony stimulating factor (GM-CSF) in bovine alveolar macrophages (BAMs) stimulated with bovine parainfluenza virus type 3 (BPIV3) *in vitro* was analyzed by reverse transcription-polymerase chain reaction. Results indicate two different expression patterns. One was that in the BAMs from two thirds of the cattle examined, several cytokine mRNAs clearly expressed between 4 hr and 24 hr post-culture without viral stimulation. Another was that cytokine mRNAs except for IL-6 and IFN-γ were expressed within 4 hr and decreased by 24 hr post-stimulation. Degree of expression was comparable between stimulation with live and inactivated viruses. The expression of lL-6 and IFN-γ mRNA was not detectable.

Key Words: bovine alveolar macrophage, cytokine mRNA, parainfluenza virus.

Introduction

Alveolar macrophages (AMs) offer the first line of host defense against invading microorganisms in the lung and participate in local humoral and cellular immune responses[19]. Up to 90% of bacterial respiratory diseases develop after a primary viral respiratory tract infection[11]. Therefore, it is very important to elucidate the interaction between AMs and viruses. While, studies concerning the expression of cytokine mRNA or the synthesis of proteins in AMs after stimulation with viruses and bacterial endotoxin have been undertaken[1-3,9,12,15-18], there is still insufficient knowledge on the interaction between BAMs and viruses. In this paper, we will describe cytokine mRNA expression in BAMs stimulated with BPIV3.

Materials and Methods

BAMs were obtained from the healthy lungs of 1.5 to 2-year-old Holstein cattle, slaughtered at an abattoir, by bronchial lavage with phosphate-buffered saline (Ca^{2+} and Mg^{2+} free, pH 7.2) according to the method of Fox[8]. Lavage fluids were centrifuged at 2,000 rpm for 10 min at 4℃ after the removal of tissue debris by filtration through sterile stainless mesh. The pelleted cells were washed three times with RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing penicillin G potassium (250 U/ml; Banyu Pharmaceutical, Tokyo, Japan), kanamycin sulfate (25 μg/ml; Meiji Seika, Tokyo, Japan), streptomycin sulfate (250 μg/ml; Meiji Seika) and fungizone (1 μg/ml; Bristol-Myers Squibb, Tokyo, Japan). The cells were finally resuspended at a concentration of $4x10^6$/ml in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, U.S.A.) One milliliter of the cell suspension was seeded into each well of 24-well tissue culture plates (Corning, New York, U.S.A.) and incubated at 37℃ for 4 hr in air containing 5% CO_2. The wells were washed with RPMI 1640 medium to remove non-adherent cells. About 95% of the cells obtained were non-specific esterase positive with a viability of more than 98% as determined by trypan blue exclusion.

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Adherent cells were kept in RPMI 1640 mediurn containing 10% FBS for 24 hr before experiments.

Two strains of BPIV3, the K-5 provided by the National Institute of Animal Health Japan and the 663-2 isolated from a pneumonic cow in our laboratory, were propagated in Madin-Darby Bovine Kidney (MDBK) cells. Their titer was 10^6 median tissue culture infective dose ($TCID_{50}$)/25 μl. Live or ultraviolet light (UV) -inactivated viruses of each strain were used to stimulate the BAMs. The UV-inactivated BPIV3 was prepared as follows. One milliliter of live BPIV3 in a 6-cm Petri dish was irradiated for 10 min at a distance of 12 cm from a 15-W UV light while being constantly stirred. Inactivation was confirmed by the absence of CPE in the MDBK cells inoculated with the UV-treated virus. Cultured BAMs were washed with RPMI 1640 medium and were infected with live or UV-inactivated BPIV3 at an MOI of 10 ($TCID_{50}$/cell) in 0.1 ml serum-free RPMI 1640 medium at 37℃. Non-infected control BAMs were incubated with RPMI 1640 medium alone. After incubation for 1 hr, the inoculum was carefully removed and washed twice with RPMI 1640 medium, and then 1 ml of RPMI 1640 medium containing 1% FBS was added. After incubation for a proper period at 37℃ in air containing 5% CO_2, the culture supernatants were harvested and stored at-80℃ until virus titration. Virus titration was performed using MDBK cells in a microtitre assay. The remaining adherent cells were used for determining cytokine mRNA expression.

Total cellular RNA was isolated from the BPIV3 infected or non-infected control BAMs using TRIzol reagent (Gibco), and the yield was quantitated by measuring optical density at 260 nm. First-strand cDNA was synthesized at 42℃ for 50min using superscript preamplification system (Gibco), from 1 μg of total RNA in a 40-μl reaction mixture containing 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, 25 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTP mix, random hexamers (50 ng), and 200U superscript II RT. After incubation, the solution was heated to 70℃ for 15 min and then cooled to 4℃. Specific oligonuclcotide primers for cytokines and β-actin (as a control) listed in Table. 1, were commercially synthesized. The prepared cDNA was amplified by PCR in a volume of 50 μl using the primer pairs (0.2 μM each), Expand High-Fidelity PCR System enzyme mix (1.8 units) composed of Taq polyrnerase and Pwo DNA polymerase (Boe-

Table 1 Oligonucleotides of upstream (sense) primers and downstream (antisense) primers of target genes.

Gene		Primers	Expected fragment size(bp)	References
IL-1α	sense antisense	5'-CTCTCTCAATCAGAAGTCCTTCTATG-3' 5'-CATGTCAAATTTCACTGCCTCCTCC-3'	424	10, 13
IL-1β	sense antisense	5'-AAACAGATGAAGAGCTGCATCCAA-3' 5'-CAAAGCTCATGCAGAACACCACTT-3'	394	10, 13
IL-6	sense antisense	5'-GACGGATGCTTCCAATCTG-3' 5'-ACCCACTCGTTTGAAGACTGCATCTT-3'	251	7
IL-8	sense antisense	5'-ATGACTTCCAAACTGGCTGTTGC-3' 5'-TCATGGATCTTGCTTCTCAGCTCTCTTCA-3'	306	15
TNF-α	sense antisense	5'-CAGAGGGAAGAGTCCCCAGG-3' 5'-CCTTGGTCTGGTAGGAGACT-3'	325	5
GM-CSF	sense antisense	5'-ATGTGGCTGCAGAACCTGCTTCTCC-3' 5'-CTTCTGGGCTGGTTCCCAGCAGTCA-3'	429	14
TFN-γ	sense antisense	5'-AATGCAAGTAGCCCAGATG-3' 5'-GATCTGCAGATCATCCACCGG-3'	270	4
β-actin	sense antisense	5'-ACGTCGCCTTGGACTTCGAGCAGG-3' 5'-GCTGGAAGGTGGACAGGGAGGCCAGGA-3'	405	6

hringer Mannheim, Tokyo, Japan), and the reagents recommended by the manufacturer. Amplification was performed by 30 cycles in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). Each cycle consisted of denaturation at 95℃ for 30 sec, primer annealing at 55℃ for 30 sec and extension at 72℃ for 1 min. An aliquot (5 μl) or each PCR product was electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and photographed.

Results and Discussion

BAMs obtained from 6 cattle indicated two different expression of patterns. One pattern was found in the BAMs obtained from 4 cattle. The mRNAs for IL-1α, IL-1β, IL-6, IL-8, TNF-α and GM-CSF were clearly detected without any viral stimulation at both 4 and 24 hr post-culture. The degree of mRNA expression in live or inactivated K-5 strain-stimulated BAMs was found not to differ from that in non-stimulated control (Fig. 1). Similar results were also obtained for the 663-2 strain-stimulated BAMs. The mRNA for IFN-γ was only slightly, if at all, detectable in each of the cows. Other investigators have also reported that BAM culture without any stimulation expressed detectable levels of mRNAs for IL-1β, IL-6, TNF-α, GM-CSF[3,9], and IL-8[15], although there is no report describing IL-1α gene expression. These data suggest the possibility that the BAMs were already activated in vivo and expressed a higher level of cytokine mRNA.

Another pattern was observed in the BAMs from the other 2 cattle, in which the expression or mRNAs was only faintly, if at all, detectable in unstimulated controls (Fig. 2). Expression of mRNAs for IL-1α, IL-1β, IL-8, TNF-α and GM-CSF was induced or enhanced after stimulation with both live and inactivated K-5 strain at 4 hr post-stimulation. However, these mRNA expressions faded out or became undetectable by 24 hr post-stimulation. The expression of IL-6 mRNA in virus-stimulated BAMs was obscure. No expression of IFN-γ mRNA was observed. Similar results were obtained from 663-2 strain-stimulated BAMs. The induction of mRNA expression by virus stimulation, irrespective of the virus being live or inactivated, was also reported in respiratory syncytial virus-infected human AMs and murine AMs[1,17]. Therefore, it is suggested that the proliferation of a virus is not

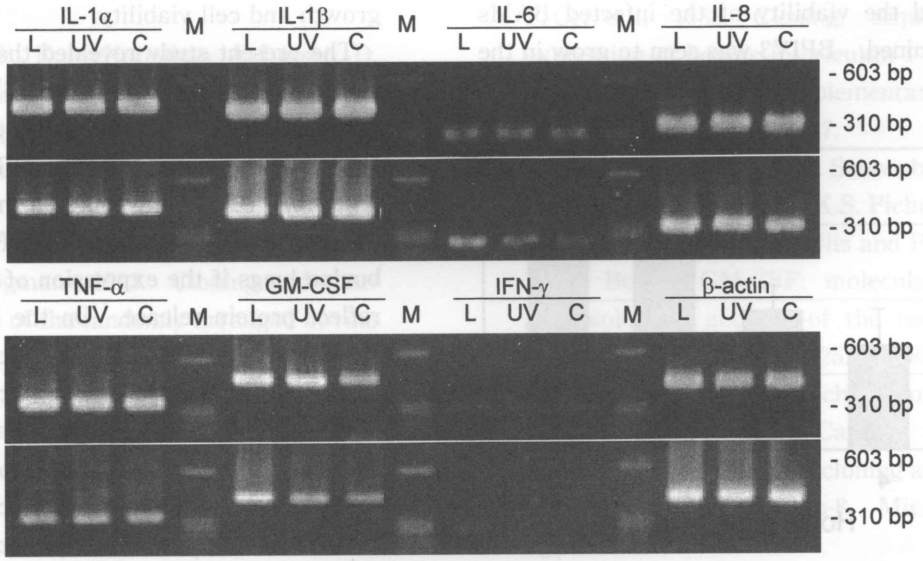

Fig. 1 Expression of multiple cytokine mRNAs in BAMs stimulated with live (lane L) or UV-inactivated (1ane UV) BPIV3 (K-5 strain), and non-stimulated control (lane C) BAMs showing higher mRNA expression without viral infection. Lane M shows DNA size markers. The RT-PCR-amplified mRNA for IL-1a (424 bp), IL-1b (394 bp), IL-6 (251 bp), IL-8 (306 bp), TNF-a (325 bp), GM-CSF (429 bp), and b-actin (405 bp) at 4 and 24 hr after stimulation are shown.

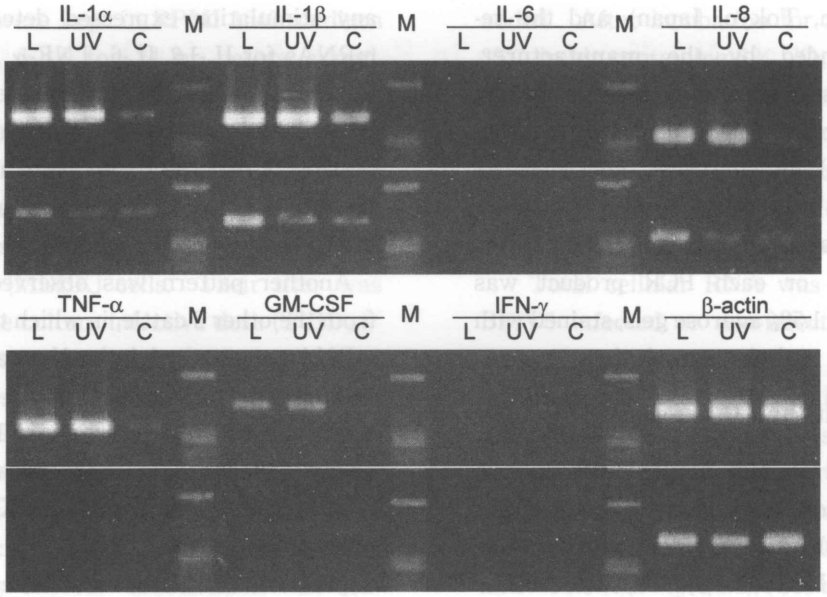

Fig. 2 Expression of multiple cytokine mRNAs in BAMs stimulated with live (lane L) or UV-inactivated (1ane UV) BPIV3 (K-5 strain), and non-stimulated control (lane C) BAMs showing low or no mRNA expression without viral infection.

always necessary for the expression of cytokine genes and that the expression may be stimulated by the first stage of infection such as adsorption or penetration.

Since most of the mRNAs detected at 4-hr post-stimulation faded out or became undetectable by 24 hr post-stimulation, viral growth in the BAMs and the viability of the infected BAMs were examined. BPIV3 was seen to grow in the BAMs from these cattle (Fig. 3). CPE was not observed at any time during the observation period. The viability of infected BAMs was more than 90% during the period from 4 to 24 hr post-infection and 85 to 90% at 72 hr post-infection. Accordingly, reduction of mRNA expression is indicated to be independent of viral growth and cell viability.

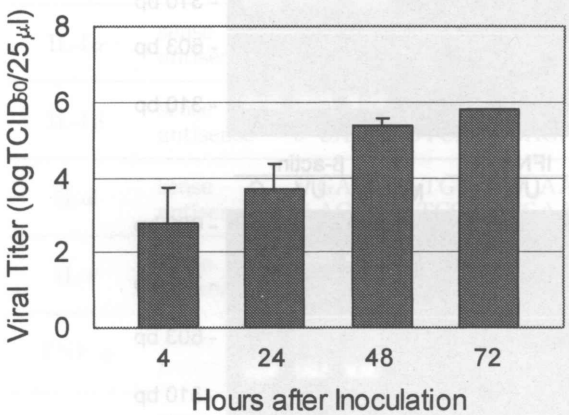

Fig. 3 Virus growth in BAM from 2 cows. Virus titer ($TCID^{50}/25\mu l$) of BAM culture supernatants inoculated with the K-5 strain was determined on MDBK cell monolayer by the Behrens-Kärber method. Each bar represents the mean and standard deviation.

The present study revealed that expression of IL-1α, IL-1β, IL-8, TNF-α and GM-CSF mRNAs in BAMs was induced after stimulation with BPIV3 without viral growth. This result suggests that BAMs may play an important role in the first line of defense against BPIV3 infection in bovine lungs if the expression of these cytokines reflect protein release. On the other hand, the BAMs from two thirds of the cattle examined expressed mRNAs without viral stimulation. This result suggests that the isolated BAMs may have already expressed a higher level of cytokine mRNA due to microbial and chemical agents inhaled into the respiratory tract of the cattle. Indeed, broncho-alveolar lavage fluids were found to contain a few to several score of bacteria in this study (data not shown). These results may help to clarify the role of BAMs in protecting cattle against respiratory viral infection.

Acknowledgements

We thank the Hokkaido Ebetsu Meat Inspection Center for supplying the bovine lungs. We also thank Prof. Naoya Kikuch, the Department of Veterinary Epizootiology, Rakuno Gakuen University, for the bacterial examination of the bovine lungs. This study was partially supported by a Grant-in-Aid for Gakujutsu Frontier Cooperative Research in Rakuno Gakuen University.

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要　約

屠場で得たホルスタイン種の健康な雄ウシの肺から分離した肺胞マクロファージをウシパラインフルエンザウイルス3型で刺激し4および24時間後のIL-1 α, IL-1 β, IL-6, IL-8, IFN-γ, TNF-α, GM-CSFのmRNA発現をPCRで検索した．用いた肺胞マクロファージによりサイトカインmRNAの発現パターンは大きく二つに分かれた．一つはウイルスの刺激により，接種後4時間目までにIL-6とIFN-γ以外のmRNA発現が誘導あるいは増強され，24時間目までに減弱ないし消失し，IL-6とIFN-γのmRNAの発現が認められないパターンである．このような発現動態は刺激ウイルスの感染性の如何にかかわらず認められた．第二のパターンはウイルス刺激の有無にかかわらず，IFN-γを除くサイトカインmRNAの発現が4～24時間を通して減弱することなく認められるものである．このパターンは調べたウシの2/3に認められた．