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Effects of Interleukin-10 on Chemokine KC Gene Expression by Mouse Peritoneal Macrophages in Response to *Candida albicans*

Chemokine KC has been considered to be a murine homologue of human GRO/MGSA and was identified as chemoattractant for monocytes and neutrophils. This study examined the expression of *KC* mRNA in thioglycollate-elicited mouse peritoneal macrophages that were stimulated in vitro with *Candida albicans* (CA). Also examined were the inhibitory effects of IL-10 on the CA-induced expression of *KC* gene by Northern blot analysis. CA was found to induce chemokine gene expression in a gene-specific manner, CXC chemokine IP-10 mRNA expression was not detected in CA-stimulated macrophages. Maximum *KC* mRNA expression was observed approximately 2 hr after adding CA. The inhibitory action of IL-10 to CA-induced *KC* mRNA expression on mouse peritoneal macrophages was independent on concentration and stimulation time of IL-10 and was observed approximately one hour after adding IL-10 and CA simultaneously. IL-10 produced a decrease in the stability of *KC* mRNA, and CA-stimulated macrophages with cycloheximide blocked the suppressive effect of IL-10. These results suggest that CA also induces chemokine KC from macrophages, and IL-10 acts to destabilize CA-induced *KC* mRNA and de novo synthesis of an intermediate protein is a part of the IL-10 suppressive mechanism.

Key Words: *Chemokines; Candida albicans; Gene expression*

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INTRODUCTION

Candida albicans (*C. albicans*, CA) is a ubiquitous opportunistic yeast that causes candidiasis and exerts many effects on macrophage function. Much of these effects depend on alterations in gene expression. The production of chemokines (chemotactic cytokines) and proinflammatory cytokine by macrophages in response to fungal infection is thought to be critical during the course of candidiasis (1-3). This pro-inflammatory response can be suppressed when macrophages are exposed to antiinflammatory cytokine, interleukin-10 (IL-10). However, the mechanisms of chemokine synthesis by macrophages in response to fungal infection are not well understood.

Interleukin-10 (IL-10) has an important regulatory role in limiting the duration and extent of acute inflammatory response. It was initially described as a cytokine synthesis inhibitory factor produced by TH2 cells (4, 5). It has a broad spectrum of functions, and display inhibitory as well as stimulatory effects on diverse cells. Subsequent studies have shown that IL-10 is produced in various cells and acts on a number of cell types (6, 7). It has been shown to have profound effects on monocytes and macrophages down-regulating the expression of a number of cytokine genes. The molecular mechanism through which this inhibitory function is achieved has been examined in a number of experimental models and the results are highly diverse (8-11).

The modulation of chemokine gene expression occurs in response to various types of effector cells from a variety of sources under different stimulatory conditions, and these chemokines include KC and interferon-γ (IFN-γ)-inducible protein 10 kDa (IP-10) (12). IP-10 which exhibits chemoattractant activity for activated T cells can be induced in macrophages in response to multiple stimuli and the mechanisms involoved in controlling induced expression are well characterized. KC is a chemoattractant for monocytes and neutrophils, but the physiological significance of KC gene expression is not fully understood. Lipopolysaccharide (LPS) has been known as the only inducer of KC gene expression in macrophages (13, 14).

Chemokines may play a beneficial role as mediators of host resistance to *Candida* infections. However, overproduction can lead to local and systemic toxicity. One potential approach would be to administer IL-10 to control

the inflammatory response caused by candidiasis. In view of the potential for *KC* mRNA induction of *C. albicans* and inhibitory effect of IL-10 on *C. albicans*-induced KC gene expression, we investigated the ability of *C. albicans* to stimulate expression of KC mRNA, and possible inhibitory mechanism of IL-10 on the *C. albicans*-induced expression of KC gene in thioglycollate-elicited mouse peritoneal macrophages.

MATERIALS AND METHODS

Materials

Brewer's thioglycollate broth and Sabrouaud's dextrose agar were purchased from Difco Laboratories (Detroit, U.S.A.). RPMI 1640, Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS) and L-glutamin, trypsin, agarose were purchased from Life Techologies Inc. (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS), phenol, guanidine isothiocyanate, cesium chloride, and formamide were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). Magna nylon transfer membrane was obtained from Micron Separation Inc. (Westboro, U.S.A.). High prime kits were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Dupont-New England Nuclear (Boston, MA, U.S.A.) was where [α-^{32}P] dCTP was purchased. Trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), and *Escherichia coli* LPS (0111:B4) were obtained from Sigma Chemical Co. (St. Louis). Recombinant mouse IL-10 (5×10^5 units/mg) was purchased from Genzyme (Cambridge, MA, U.S.A.). The plasmid encoding genes KC, IP-10, and GAPDH were kindly provided by Dr. Hamilton, Department of Immunology, Lehner Research Institute, Cleveland Clinic Foundation, U.S.A..

Mouse

Specific pathogen free, inbred BALB/c mice nine to 12 weeks of age were purchased from Hyeuchang Sci, Corp, (Taegu, Korea). Utmost precautions were taken to ensure that the mice remained free from infection by environmental pathogens guaranteeing that the degree of spontaneous activation of tissue macrophages would be minimal.

Methods

C. albicans preparation

Candida albicans (typeA, B311) was cultured on Sabouraud's dextrose agar for 76 hr at 37℃ and harvested with PBS. After washing the culture with PBS three times, the concentration of yeast cells in RPMI 1640 containing L-glutamine, penicillin, streptomycin, and 5% FBS (complete medium) was adjusted to 2×10^6/well in 60 mm plates for the experiments. The yeast form was observed with light microscope and present throughout the experiments.

Cell culture

Thioglycollate (TG)-elicited macrophages were obtained by Tannenbaum's method (15). Peritoneal lavage was performed using 10 mL of cold HBSS (Hank's balanced salt solution) containing 10 U/mL heparin. Macrophages in complete medium were plated in 60 mm tissue culture dishes, incubated for 2 hr at 37℃ in an atmosphere of 5% CO_2, and then washed three times with HBSS to remove nonadherent cells. The macrophages were cultured overnight in complete medium at 37℃ in 5% CO_2, and then cultured in the presence or absence of stimuli for the indicated times.

Preparation of RNA and Northern hybridization analysis

Total cellular RNA was extracted by the guanidine thiocyanate-cesium chloride method (16). Equal amount of RNA (10 μg/mL) was used in each lane of the gel. The RNA was denatured, separated by electrophoresis in a 1% agarose-formaldehyde gel, and transferred to nylon membrane as previously described (17). The blots were prehybridized for 6 hr at 42℃ in 50% formamide, 1% SDS, 5X saline sodium citrate, 1x Denhardt's (0.02% bovine serum albumin and 0.02% polyvinylpyrrolidone), 0.25 mg/mL denatured herring testis DNA, and 50 mM sodium phosphate buffer, pH 6.5. Hybridization was carried out at 42℃ for 18 hr with 1×10^7 CPM of denatured plasmid DNA containing appropriate specific cDNA inserts. The blots were rinsed with a solution of 0.1% SDS-0.2X SSC, washed at 42℃ for 1 hr and at 65℃ for 15 min. The blots were dried and exposed using XAR-5 X-ray film (Eastman Kodak Co. Rochester U.S.A.) at -70℃. Blots were quantified with computer analysis using BIO-ID version 6.

RESULTS

C. albicans (CA) induces chemokine KC gene expression in mouse peritoneal macrophages

Initially, the study was planned to assess the relative capacity of CA inducing IP-10 and KC mRNA expression in mouse peritoneal macrophages. While LPS markedly induced IP-10 and KC mRNA expression, CA was not able to induce IP-10 mRNA expression in mouse

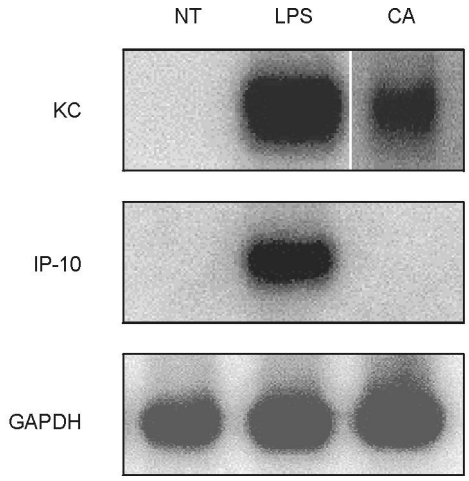

Fig. 1. *C. albicans* selectively induces chemokine gene expression. Thioglycollate (TG)-elicited macrophages (2×10^6 cells) were untreated (NT) or treated with *C. albicans* (2×10^7 cells, CA) or lipopolysaccharide (10 ng/mL, LPS) for 2 hr. Total RNA was prepared and the levels of KC, IP-10 and GAPDH mRNA were analyzed by Northern hybridization. Similar results were obtained in three separate experiments.

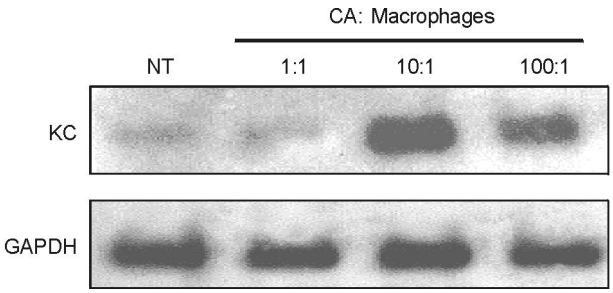

Fig. 2. Effects of various doses of LPS on expression of KC mRNA in mouse peritoneal macrophages. TG-elicited macrophages (2×10^6 cells) were stimulated with various amounts (1:1, 10:1, 100:1) of *C. albicans* (CA) for 2 hr. Total RNA was prepared from each sample and analyzed for KC and GAPDH mRNA level as described in materials and methods. Similar results were obtained in three separate experiments.

peritoneal macrophages. CA could only induce KC mRNA. Although LPS and CA were stimuli for KC mRNA expression, LPS was a much more effective stimulus of KC mRNA expression in mouse peritoneal macrophages (Fig. 1).

Induction of KC mRNA was not dependent upon the dose of CA. A low dose of CA (CA to Macrophage ratio, 1:1) did not induce KC mRNA; the CA to macrophage ratio of 10:1 showed a greater induction of KC mRNA expression than the ratio of 100:1 (Fig. 2).

IL-10 suppresses KC mRNA expression and IL-10-mediated suppression of CA-induced KC mRNA expression does not depend on the dose of IL-10

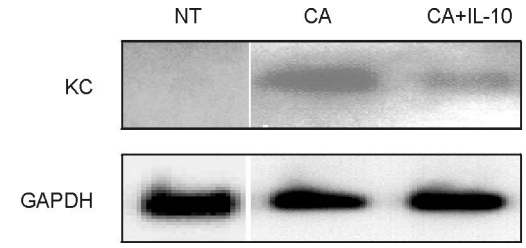

Fig. 3. IL-10 inhibits *C. albicans*-induced KC gene expression. TG-elicited macrophages (2×10^6 cells) were stimulated with *C. albicans* (2×10^7 cells) in the presence or absence of IL-10 (25 ng/mL) for 2 hr. Total RNA was isolated from each sample and analyzed for KC and GAPDH mRNA level as described in materials and methods. Similar results were obtained in three separate experiments. NT, non-treated; CA, *C. albicans*

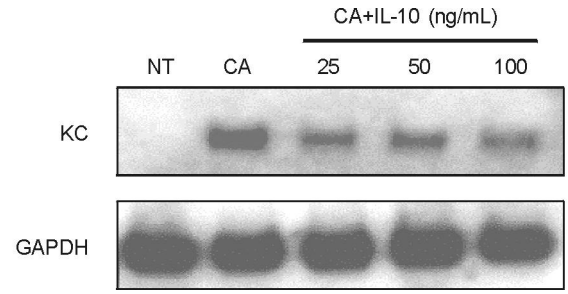

Fig. 4. Dose independent suppression of *C. albicans*-induced KC gene expression by IL-10. TG-elicited macrophages (2×10^6 cells) were untreated (NT) or treated with *C. albicans* (2×10^7 cells, CA) for 2 hr. Some samples were also exposed to IL-10 with either 25 ng/mL, 50 ng/mL, or 100 ng/mL concentration. After stimulation, total RNA was isolated from each sample and analyzed for KC and GAPDH mRNA level as described in materials and methods. Similar results were obtained in three separate experiments.

Many studies have demonstrated that IL-10 is a potent suppressor of induced gene expression in mononuclear phagocytes. The mechanisms through which such inhibitory effects are achieved are diverse. To evaluate IL-10 mediated suppression of CA-induced chemokine mRNA expression in mouse peritoneal macrophages, we have examined the effects of IL-10 on the induced expression of chemokine gene KC. Mouse peritoneal macrophages were treated with CA for 2 hr in the presence or absence of IL-10 and the expression of mRNA encoding KC was analyzed with Northern hybridization. The expression of CA-induced KC mRNA was reduced in macrophages treated simultaneously with IL-10 (Fig. 3).

In order to determine whether the inhibition of KC expression by IL-10 was dependent on the dose of IL-10, we experimented with various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL) of IL-10 by adding each concentration to macrophages simultaneously with CA. The suppression of CA-induced KC mRNA expression was

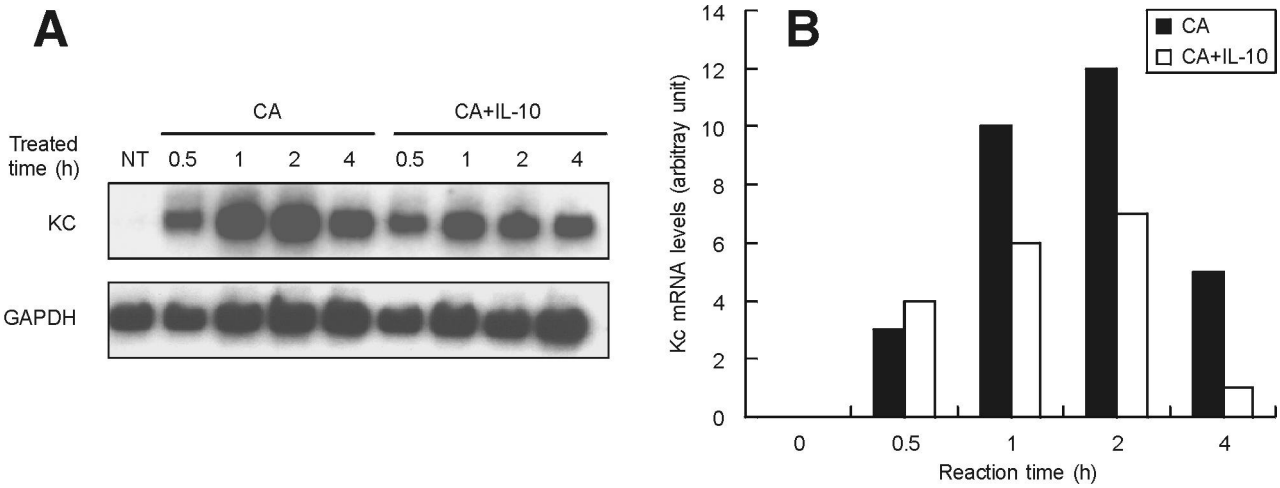

Fig. 5. Time dependence of IL-10-mediated suppression of *C. albicans*-induced KC mRNA expression. A: TG-elicited macrophage were treated with CA (1×10^7) for indicated times. Total RNA was prepared and levels of KC and GAPDH mRNA were analyzed by Northern hybridization. B: The levels of specific mRNA on the blot shown in panel A were quantified by computer analysis using BIO-ID version 6. Levels of KC mRNA in each sample were normalized for levels of GAPDH mRNA. Similar results were obtained in three separate experiments.

not dependent upon the dose of IL-10. Almost the same inhibitory effects of IL-10 were seen between 25 to 100 ng/mL of IL-10 (Fig. 4).

IL-10 acts to suppress KC mRNA in early response to CA

The time dependence for IL-10-mediated suppression of CA-induced KC mRNA expression was determined in mouse peritoneal macrophages at various times (Fig. 5). The maximum CA-induced KC mRNA expression occurred at 2 hr after adding CA. When KC mRNA level was measured in cells treated with *C. albicans* and IL-10 for 0.5 hr, no suppression was evident. However, KC mRNA was reduced by 1 hr and the suppression became more evident over the 4 hr compared with cells treated with CA alone.

These results suggest that at least 0.5 hr of exposure to IL-10 may be required to induce the necessary component of the inhibitory mechanism, or the step that is sensitive to IL-10 occurs after 0.5 hr in the response of macrophages to CA. In order to determine whether the timing in the inhibition of KC gene expression by IL-10 was dependent on the time of macrophage exposure to IL-10, macrophages were treated with IL-10 either 1 hr before, or 1 hr after, or at the same time as CA, and the KC mRNA level was measured 2 hr after adding CA. IL-10 produced equivalent suppression of KC mRNA levels independently of the exposure time for IL-10 (Fig. 6). The length of time for IL-10 added to CA did not alter the pattern of KC mRNA expression.

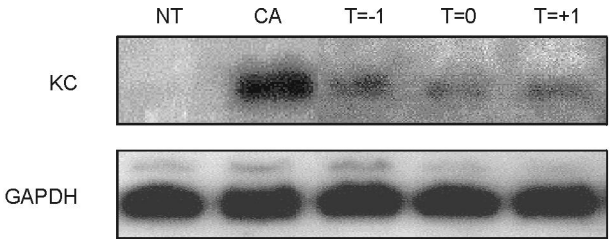

Fig. 6. Time of treatment with IL-10 does not alter the pattern of *C. albicans*-induced KC mRNA expression. TG-elicited macrophages (2×10^6 cells) were untreated (NT) or treated with *C. albicans* (2×10^7 cells) for 2 hr. Samples were also exposed to IL-10 (25 ng/mL) either 1 hr prior (T=−1) to or after (T=+1), or simultaneously (T=0) with addition of *C. albicans*. Total RNA was isolated from each sample and analyzed for KC and GAPDH mRNA levels as described in materials and methods. Similar results were obtained in three separate experiments.

Mechanisms of IL-10-mediated suppression of CA-induced KC mRNA expression

Rate of gene transcription and/or mRNA degradation may cause alterations in KC mRNA levels. To determine whether IL-10 reduces the stability of CA-induced KC mRNA, macrophages were treated with CA in the presence or absence of IL-10 for 2 hr before treating the cells with actinomycin D (ActD) to prevent further transcription. After an additional incubation period of up to 1 hr, KC mRNA levels were assessed by Northern hybridization and quantified by BIO-ID version six analysis (Fig. 7). In the absence of IL-10, KC mRNA levels declined very slowly with about 6% reduction at 30 min, 40% reduction at 60 min after adding ActD. In IL-10 and CA-stimulated cells, KC mRNA levels decreased

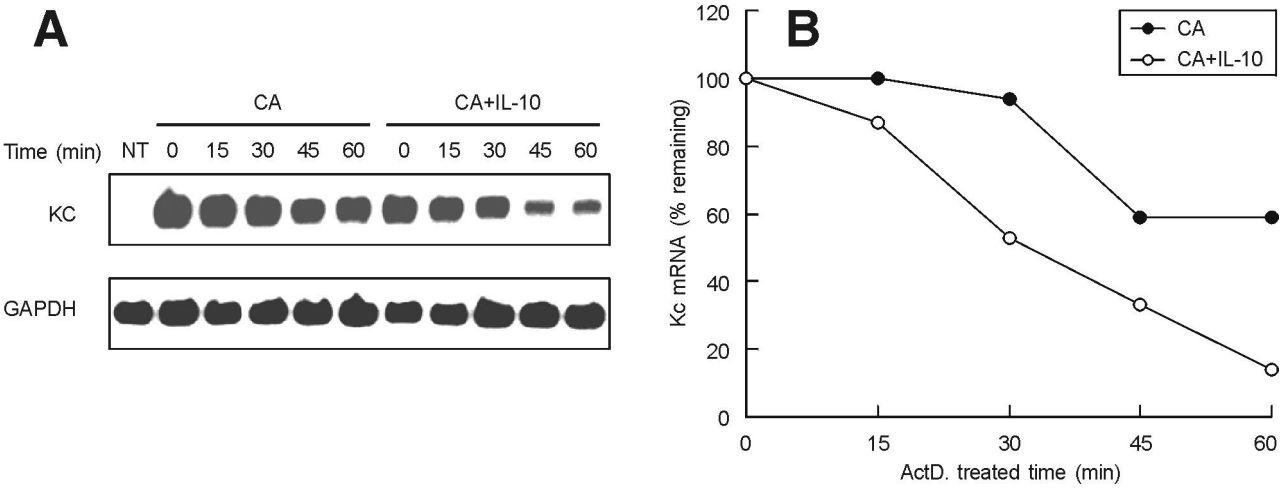

Fig. 7. IL-10 reduces the stability of KC mRNA. A: TG-elicited macrophages were treated with *C. albicans* (CA, 1×10^7) in the absence or presence of IL-10 (25 ng/mL) for 2 hr. Actinomycin D (ActD, 5 μg/mL) was added to all cultures and incubation continued for the indicated times before analysis of KC and GAPDH mRNA levels by Northern hybridization. B: The blots were quantified by computer analysis using BIO-ID version 6, KC mRNA levels were normalized for GAPDH content for each sample, and plotted as percent mRNA remaining versus time. Similar results were obtained in three separate experiments.

more rapidly, resulting in an estimated half-life of less than 45 min.

The time at which IL-10 showed activity could reflect the need for an inducible intermediate gene product. To determine the possibility of a role for one or more IL-10/CA-inducible proteins, we determined the requirement for continuous protein synthesis in the suppressive action of IL-10. Peritoneal macrophages were stimulated with CA with or without IL-10 in the absence or presence of cycloheximide (CHX) as an inhibitor of protein synthesis. CA stimulated the expression of KC mRNA, which was effectively suppressed when IL-10 was included in the treatment protocol. When protein synthesis was inhibited with CHX, the suppressive effect of IL-10 was fully abrogated (Fig. 8).

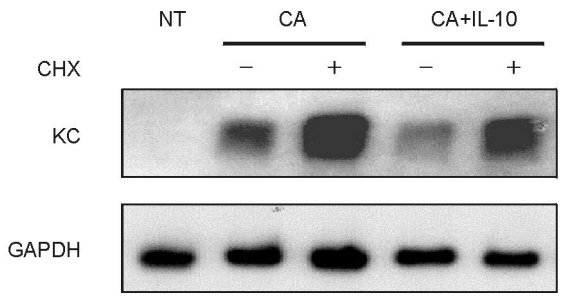

Fig. 8. IL-10 mediated suppression of KC mRNA expression is prevented by inhibitor of protein synthesis. TG-elicited macrophages (2×10^6 cells) were untreated (NT) or treated with *C. albicans* (CA, 2×10^7 cells) alone, with IL-10 (25 ng/mL) and/or with cycloheximide (CHX, 10 μg/mL) for 2 hr. Total cellular RNA was prepared and used to determine levels of KC and GAPDH mRNA by Northern hybridization as described in materials and methods. Similar results were obtained in three separate experiments.

DISCUSSION

Chemokine KC is thought to be a murine counterpart of human groα, which shares a 65% sequence identity. It is intimately involved in murine inflammation, and is now widely regarded as a neutrophil attractant and activator (18). *KC* gene is induced by LPS, polyIC, TNF-α, and IL-1α in a number of cell types (12, 19). However, the actual role of KC in the inflammatory process remains to be established. IL-10 has been shown to have profound effects on monocytes and macrophages, causing inhibition of a number of LPS-induced proinflammatory cytokines and some chemokines, and has been reported to inhibit stimulus-induced monocyte/macrophage gene expression by blocking transcription, by altering the stability of mRNAs, and by reducing the translation of mRNAs (8, 10, 11, 20-22). But studies of KC gene expression induced by CA and inhibitory action of IL-10 on CA-induced KC gene expression have not been reported.

In this study, CA was found to induce chemokine gene expression in gene-specific manner and IL-10 inhibits CA-induced KC gene expression by reducing KC mRNA stability. IP-10 gene expression can be induced by various stimulants, although IFN-γ has been known to be a main inducer of IP-10 (23); however, in the case of CA as a stimulant of macrophages, it was not able to induce IP-10 mRNA expression in mouse peritoneal mac-

rophages. Schall (24) reported that the pattern of chemokine genes expression was diverse in a stimulus-, cell type-, and gene-dependent fashion. An effective dose of CA to cause reaction with macrophages was a 10:1 (CA to macrophages) ratio. Studies (25, 26) of TNF in the reactions of macrophages with CA have reported that the concentration range between 10:1 to 50:1 was effective for studies of yeast as a stimulant. The time kinetics of KC mRNA expression in this study was not identical with that from a previously reported expression of KC gene in murine peritoneal macrophages treated with LPS (17). The study reported that maximum KC mRNA expression occurred at 1 hr after adding of LPS. In this study, however, the maximum KC mRNA occured at 2 hr after adding CA. Also, the inhibitory action of IL-10 on CA-induced KC gene expression was relatively weak compared to that of LPS-induced KC gene expression. The expression of CA-induced KC mRNA was suppressed at 1 hr after adding IL-10, but the inhibitory action of LPS-induced KC gene expression occurred relatively late, at least 2 hr of exposure to IL-10 was required to induce the inhibitory action of IL-10. Namely, IL-10 did not act to suppress the expression of CA-induced KC mRNA which was late compared to the suppression of KC expression induced by LPS. In previous unpublished data, IL-10 inhibited a gene expression through a mechanism which depended on the stimulus. IL-10 selectively reduces LPS-induced but not IFN-γ or IFN-β-induced IP-10 mRNA levels. It was suggested that different stimuli which may utilize distinct signaling pathways may contribute to the different result. Although mannan protein, a cell wall component of CA, has been known to have selective inducibility of cytokine gene expression (1), Yamamoto et al. (3) demonstrated that the mannan component was not involved in the induction of mRNA for the chemokines MIP-1β, MIP-2, and KC. The precise mechanism of induction of KC synthesis induced by CA has not still been demonstrated.

Our result that IL-10 produces a substantial decrease in the stability of CA-induced KC mRNA is consistent with earlier reports showing IL-10-mediated destabilization of mRNAs encoding TNF-α, IL-1α, IL-1β, GM-CSF, IL-6, and MIP-1 and IL-8 (8, 11, 22). The pathway through which IL-10 enhances mRNA degradation is currently unknown. Repeated AU-rich sequence elements (AREs) in the 3'-untranslated region (3'-UTR) of several mRNAs have been shown to be responsible for a short mRNA half-life (27, 28). KC mRNA possesses an ARE that is closely related to that of the TNFα gene (29, 30). Indeed, ARE sequences are present in the 3'UTR of a variety of cytokine mRNAs that is sensitive to suppression by IL-10 (31, 32). Thus, ARE motif might be a common target of IL-10, through which this cytokine controls the inflammatory gene expression.

In conclusion, chemokine KC gene expression may be concern with the macrophage inflammatory function in CA infection in the tissue microenviroment, and the precise mechanism for IL-10 mediated suppression of CA-induced KC gene expression requires additional analyses.

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