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The Effects of Allitridin on the Expression of Transcription Factors T-bet and GATA-3 in Mice Infected by Murine Cytomegalovirus

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ABSTRACT This study was designed to investigate the effects of allitridin on the expression of transcription factors T-bet and GATA-3 in mice infected by murine cytomegalovirus (MCMV). A BALB/c mouse model system of MCMV infection was established. Twenty mice were allocated randomly into an allitridin-treated group ($n = 10$) and a placebo control group ($n = 10$). The same dose (25 mg/kg/day) and regimen of allitridin were used in the treated group in the 24 hours after virus infection; the same volume of saline solution was injected in placebo control mice. In an additional blank control group ($n = 10$), the same volume of saline solution was injected. The expression levels of the transcription factors T-bet and GATA-3 were measured by reverse transcription–polymerase chain reaction. The expression levels of the T helper (Th) 1 cytokine interferon-γ (IFN-γ) and the Th2 cytokine interleukin (IL)-10 in supernatant of spleen cell culture were measured by enzyme-linked immunosorbent assay. MCMV infection markedly down-modulated the expression of IFN-γ and T-bet and significantly up-modulated the expression of IL-10 and GATA-3. Allitridin induced significantly ($P < .01$) increased expression of the transcription factor T-bet and the Th1 cytokine IFN-γ and markedly ($P < .01$) decreased expression of the transcription factor GATA-3 and the Th2 cytokine IL-10. Thus MCMV infection could lead to disequilibrium of Th1/Th2 cytokine expression: The level of the Th1 cytokine IFN-γ was decreased significantly, and Th2 cytokine IL-10 was overexpressed markedly. Allitridin could up-regulate the expression of T-bet and IFN-γ and inhibit the expression of GATA-3 and IL-10 in MCMV-infected mice, indicating a Th1 dominant state, which should enhance the specific cellular immune reactions against cytomegalovirus (CMV) and be helpful for clearance of CMV from the host.

KEY WORDS: • ***allitridin*** • ***cytokine*** • ***cytomegalovirus*** • ***transcription factor***

INTRODUCTION

HUMAN CYTOMEGALOVIRUS (HCMV) is a widely distributed species-specific β-herpesvirus that causes severe and fatal diseases. In China, the HCMV seropositivity rate in pregnant women has reached 90–96%.[1] Indeed, most HCMV infections are subclinical, and they can cause substantial morbidity and mortality following infection or reactivation, especially for immunosuppressed or immunocompromised individuals. Currently, there is no effective safe vaccine for cytomegalovirus (CMV), as the vaccines studied so far only provide partial protective immunity upon challenge with infectious virus.

A number of studies have demonstrated that *Allium sativum* (garlic) exhibits a striking antiviral effect against CMV infection.[2,3] *A. sativum* is among the oldest of all cultivated plants and has multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, anti-arthritic, hypoglycemic, and antitumor activity. Allitridin (diallyl trisulfide, $CH_2{=}CH\text{-}CH_2\text{-}S\text{-}S\text{-}S\text{-}CH_2\text{-}CH{=}CH_2$), obtained in extracts from *A. sativum*, is the main organic compound responsible for its antiviral efficacy. At present, the mechanism of action of allitridin against CMV is unclear. However, some studies have shown that the immunomodulatory properties of allitridin from *A. sativum* were elucidated in terms of shifting the cytokine response to a T helper (Th) 1-type pattern and therefore causing the protective response.[4] In this study we investigate the effects of allitridin on expression of the Th1/Th2-related transcription factors T-bet and GATA-3 in mice infected by murine CMV (MCMV) and analyze its role in anti-CMV mechanisms.

MATERIALS AND METHODS

Mice

Specific pathogen-free inbred female BALB/c mice (4 weeks old, weighing 10–12 g, seronegative for MCMV) were purchased from the Purebred Animal Room, Hubei Academy of Medical Science (Hubei, China) and maintained under minimal disease conditions. BALB/c mice were

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housed under a 12-hour light–dark cycle and allowed unrestricted access to food and water at least 7 days before use.

Drug

Allitridin was extracted from *A. sativum* by distilling at the Hefeng Pharmaceutial Co., Ltd. (Shanghai, China).

Cells

Primary murine embryofibroblasts (MEFs) were isolated from 17–19 day post-coitus mouse embryos. Embryos were surgically removed, minced finely, and then incubated in a solution of trypsin/EDTA [0.05% trypsin (Sigma Chemical Co., St. Louis, MO), 1 m*M* EDTA, glucose, and 1× phosphate-buffered saline (PBS)] with shaking at 37°C for 15–30 minutes. The solution was allowed to settle for 2 minutes, and the supernatant was centrifuged for 3 minutes at 1,000 rpm. The resulting pellet was resuspended in culture medium, and cells were plated at 10^4 cells/cm^2. Attachment of cells then constituted passage. All primary MEFs were maintained in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Zhejiang, China), 100 units of penicillin, and 100 μg/mL streptomycin. Cultured cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Filial generation 5–9 MEFs were used in all experiments.

Virus

MCMV Smith strain (ATCC VR.19) was a kind gift from the American Type Culture Collection (Manassas, VA). The stock of MCMV was prepared by salivary gland passage in BALB/mice and stored in gas-phase liquid nitrogen.

Procedure for preparing MCMV Smith strain from salivary gland

The female BALB/c mice (6–8 weeks of age, weighing 18–22 g) were intramuscularly injected with methylprednisolone acetate (2 mg per mouse) every 4 days. Two days after the first injection, the mice were innoculated intraperitoneally with virus [10^4 plaque-forming units (PFU) per mouse]. Fourteen days after innoculation, the mice were sacrificed, and the salivary glands were removed and put into serum-free Minimum Essential Medium culture medium (with HEPES and antibiotics, 1 mL per salivary gland) (Gibco BRL). The salivary glands were homogenized, and the supernatant was collected after centrifugation (3,000 rpm, 20 minutes). We store the virus in plastic vials (0.1 mL per vial) in a −80°C freezer and use one vial each time.

MCMV infection of mice and viral plaque assay

MCMV Smith strain was used in all experiments. Infectious MCMV was quantified by plaque assay using MEF monolayers in 24-well tissue culture plates.[5] Briefly, cells were first infected with the virus at 10-fold serial dilutions. After 90 minutes of incubation with the homogenates diluted in 1 mL of complete medium at 37°C, the cells were overlaid with fresh complete medium containing 1% agarose and cultured for 4–5 days before the plaques were counted (Fig. 1). Viral titers for each sample were determined in triplicate. MCMV Smith strain titers were 1×10^6 PFU/mL. Experimental mice were infected with 5×10^3 PFU of MCMV by the intraperitoneal route.[6]

Dosage of allitridin

The dosage of allitridin for mice was determined by the following equation[7]: $D_B = D_A \times R_B/R_A \times (W_A/W_B)^{2/3}$, where W is body weight, D is dosage, A is human, B is mouse, and R is the build coefficient. The dosages of allitridin, 25 mg/kg/day (equal to the mean dosage for a human, 2 mg/kg/day), were given by intraperitoneal injection once a day in our experiments.

Treatment of mice with allitridin

Thirty mice were randomly divided into three groups: allitridin therapy group ($n = 10$), infected control group ($n = 10$), and normal control group ($n = 10$). For the allitridin therapy group, allitridin diluted in pyrogen-free saline was given to mice via the intraperitoneal route once a day with the general dosage (25 mg/kg/day) at 24 hours after infection with MCMV; the infected control group was given the same volume of 0.89% sodium chloride, and the normal control group was only given the same volume of 0.89% sodium chloride but was not infected with MCMV.

Sample collection

All experimental mice were sacrificed at 14 days postinfection. Spleens were removed under aseptic conditions. Spleen cells were obtained by squeezing the organ in the culture medium (RPMI 1640, containing 5% fetal bovine serum, 100 IU of penicillin, and 100 μg/ml of streptomycin) (Gibco BRL) using frosted slide glasses aseptically. After centrifugation (800 rpm, 5 minutes, 4°C), erythrocytes in the pellet were removed by washing the pellet with a solution containing 0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.367% disodium EDTA. After the centrifugation, the pellet was washed two times using PBS and was allowed to settle at 37°C for 90 minutes. Attached cells were monocytes. The supernatant cells were washed two times using

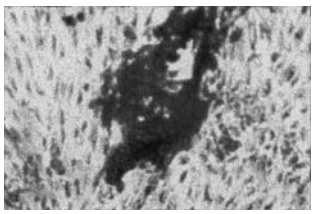

FIG. 1. Plaques of MCMV Smith strain in MEFs. ×100.

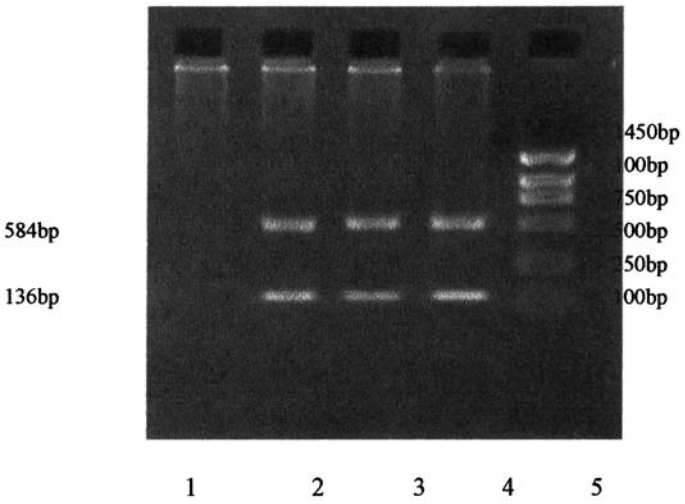

FIG. 2. T-bet mRNA (136 base pairs) expression in BALB/c mouse spleen: lane 1, blank control; lane 2, normal control group; lane 3, infected control group; lane 4, allitridin therapy group; lane 5, markers.

PBS, which then constituted passage. The spleen lymphocyte cell count was adjusted to 2×10^6/mL with culture medium and used as the spleen cell suspension. Phytohemagglutinin (150 μg/mL) (Gibco BRL) was poured and cultured at 5% CO_2 and 37°C for 48 hours. The spleen cell suspension was collected for cytokine analysis. A second part of each spleen was immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed.

Reverse transcription–polymerase chain reaction (PCR)

Total RNA was isolated from spleen with TRIZOL reagent according to the instruction manual of the kit. RNA was reverse-transcribed using an RNA PCR Kit (AMV, Takara Shuzo, Tokyo, Japan) for PCR with the supplied oligo d(T) primer using a thermal program of 42°C for 30 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. PCR procedures were done with *Taq* polymerase (Takara Shuzo) using the following primers: mouse T-bet (forward, 5′-GCCAGGGAACCGCTTATATG-3′; reverse, 5′-GACGATC ATCTGGGTCACATTGT-3′), GATA-3 (forward, 5′-TCTGGAGGAGGAACGCTA ATGG-3′, reverse, 5′-GAACTCTTCGCACACTTGGAGACTC-3′), and β-actin (forward, 5′-GTGGGCCGCTCTAGGCACCA-3′; reverse, 5′-CGGTTGGCCTTAGGGTTC AGGGGGG -3′), generating fragments of 136 base pairs, 408 base pairs, and 584 base pairs, respectively. Reaction conditions were as follows: pre-denaturation for 4 minutes at 94°C and then 30 cycles of (denaturing for 45 seconds at 94°C; annealing for 45 seconds at 58°C; extension for 50 seconds at 72°C). PCR products were resolved on 2% agarose gels and visualized with ethidium bromide under ultraviolet light. Relative levels of T-bet and GATA-3 expression were expressed using the optical density ratio (T-bet/β-actin, GATA-3/β-actin), as determined by a Kodak Digital Science (Rochester, NY) scanning system.

Cytokine enzyme-linked immunosorbent assay (ELISA)

The cytokines interferon-γ (IFN-γ) and interleukin (IL)-10 in the supernatant of spleen cell culture were quantitated by ELISA (Jingmei, China). Three samples were titrated in duplicate, and data are expressed as mean ± standard error of the mean values.

Statistical analysis

Student's *t* test was used to assess differences between groups of data. *P* values of .05 or less were considered to be statistically significant.

RESULTS

The expression of the transcription factors T-bet and GATA-3 gene with allitridin treatment is given in Figures 2 and 3 and Table 1.

Levels of IFN-γ and IL-10 in the spleen cell suspension with allitridin treatment are shown in Table 2.

DISCUSSION

Antigen-stimulated naive $CD4^+$ Th cells differentiate into two distinct subsets of effector cells, Th1 and Th2, that are defined by their distinct cytokine profiles and their immune regulatory functions. Th1 cells provide protection against intracellular pathogens and viruses and produce predominantly IFN-γ, IL-2, and tumor necrosis factor-β. Th2 cells

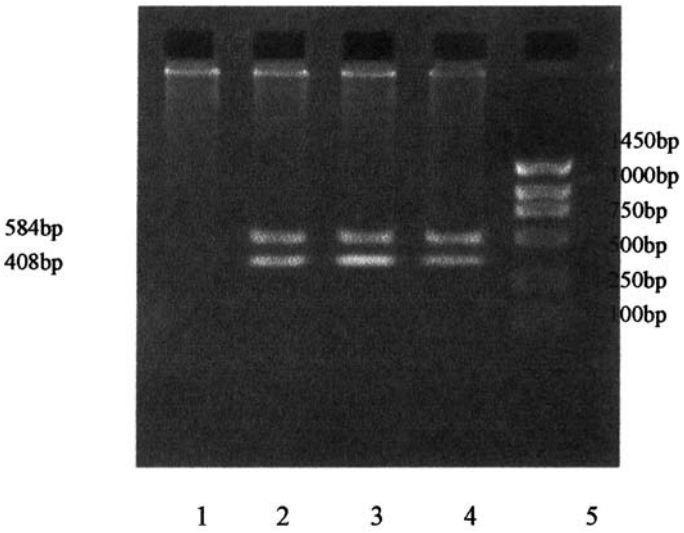

FIG. 3. GATA-3 mRNA (408 base pairs) expression in BALB/c mouse spleen: lane 1, blank control; lane 2, normal control group; lane 3, infected control group; lane 4, allitridin therapy group; lane 5, markers.

Table 1. Expression of Transcription Factors T-bet and GATA-3 mRNA with Allitridin Treatment

Group (n)	*T-bet mRNA*	*GATA-3 mRNA*
Allitridin therapy (10)	0.37 ± 0.03[a]	0.22 ± 0.02[b]
Infected control (10)	0.15 ± 0.03	0.46 ± 0.04
Normal control (10)	0.12 ± 0.02	0.17 ± 0.01

Data are mean ± standard error values.

[a]Semiquantitative analysis showed that the very strong expression of T-bet mRNA was detected in the allitridin therapy group on day 14 post-infection (versus infected control group and normal control group, $P < .01$).

[b]The expression of GATA-3 mRNA in the allitridin therapy group on day 14 post-infection was significantly lower than expression levels in the infected control group on day 14 post-infection ($P < .01$).

mainly produce IL-4, IL-5, IL-9, IL-10, and IL-13 and are involved in humoral immunity, immunity to parasites, and the allergic response in the presence of antigen.[8] It has been widely accepted that the balance between Th1 and Th2 is critical for various diseases in terms of immunological status. Previous studies have shown[9] that the type of immune response (humoral or cellular) is important in determining whether a given infection leads to pathogen-induced disease. The humoral immune response functions mainly to prevent infection by extracellular agents, whereas the cell-mediated immune response is more critical for elimination and control of intracellular pathogens. CMV is an intracellular pathogen. That the humoral immune response functions do not effectively clear the virus implies that the cellular response is primary in determining outcome.

Major efforts have been made to elucidate the molecular basis of Th1 versus Th2 cell subset differentiation and to identify transcription factors controlling Th1 and Th2 cytokine expression. The T-box transcription factor T-bet, a newly discovered Th1-specific transcription factor, is involved in the commitment of Th1 cells by inducing IFN-γ synthesis and repressing IL-4 and IL-5 production.[10] The protein T-bet appears to function by initiating Th1 differentiation while repressing Th2 differentiation.[11] Another key cytokine regulating Th1 differentiation is IFN-γ. Beside being the signature cytokine of Th1 effector cells, IFN-γ is important for the stabilization of the Th1 phenotype itself. In addition, Th1-associated cytokine IFN-γ is also important for MCMV clearance early or late in infection.[12] GATA-3 is a T and natural killer cell–specific transcription factor that is essential for Th2 cell development and involved in the regulation of Th2 cytokine expression.[13] In the acute phase of CMV infection, expression of the Th2 cytokine IL-10 was significantly increased. IL-10 has been reported to play an important role in CMV infection: CMV can down-regulate major histocompatibility class II expression on macrophages by the induction of IL-10.[14] In addition, the Th2 cytokine IL-10 is an important inhibitor of IL-12 and plays a negative adjustment role in Th1 cell differentiation,[15] indicating a Th2 dominant response state, which may be one of the mechanisms by which CMV can evade specific cellular immune reactions in the host. In the present study BALB/c mice were inoculated intraperitoneally with 5×10^3 PFU of MCMV on day 0. The expression of transcription factor T-bet mRNA and levels of Th1-associated cytokine IFN-γ had returned to basal levels on day 14 post-infection (versus normal controls, $P > .05$), whereas the expression of transcription factor GATA-3 mRNA and levels of Th2-associated cytokine IL-10 rose significantly (versus normal controls, $P < .01$), potentially providing the lack of a persistent Th1 dominant state and suppressing the cellular immune response functions in the early phase of CMV infection. However, allitridin significantly up-regulated the expression of IFN-γ and T-bet mRNA on day 14 post-infection and down-regulated the expression of IL-10 and GATA-3 mRNA (versus normal controls, and infected controls, $P < .01$), suggesting that allitridin may promote secretion of the Th1 cytokine IFN-γ through up-regulating the expression of Th1-specific transcription factor T-bet mRNA and reduce secretion of the Th2 cytokine IL-10 through down-regulating the expression GATA-3 mRNA, and reverse the Th2 dominant response state to a Th1 dominant response state, which should enhance the specific cellular immune reactions against CMV and be helpful for clearance of CMV in the host.

In addition, we previously found that allitridin with a 14-day course of treatment in MCMV hepatitis models significantly improved hepatic function and reduced histopathological lesions and the MCMV DNA load in liver tissues as well. Moreover, the efficacy of allitridin was similar to ganciclovir at the dose employed.[16] At present, the mechanism of action of allitridin against CMV is unclear. This study suggest that allitridin may up-regulate the expression of Th1-specific transcription factor T-bet mRNA to promote secretion of the Th1 cytokine IFN-γ and down-regulate the expression of GATA-3 mRNA to reduce secretion of the Th2

Table 2. Levels of IFN-γ and IL-10 in Spleen Cell Suspension with Allitridin Treatment

	Level (pg/mL)	
Group (n)	*IFN-γ*	*IL-10*
Allitridin therapy (10)	254.46 ± 29.12	40.68 ± 4.32
Infected control (10)	152.08 ± 1.55	63.3 ± 11.27
Normal control (10)	148.36 ± 1.49	35.97 ± 1.32

Data are mean ± standard error values. Levels of IFN-γ significantly rose in the allitridin therapy group compared with the infected control group and the blank control group on day 14 post-infection ($P < .01$), and levels of IFN-γ in infected controls group returned to levels similar to those in uninfected mice by day 14 post-infection ($P > .05$). However, after day 14 post-infection animals in the allitridin therapy group produced significantly lower levels of IL-10 compared with the infected control group ($P < .01$).

cytokine IL-10, indicating a Th1 dominant response state and promoting the innate cellular response and the specific cellular responses to CMV infection, thus resulting in a protective effect from the CMV-associated pathogenicities in the host.

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