Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 8(2):202-207

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

The effect of inhibited *Leishmania tropica* by mitomycine\c in combination with DNA of parasites as vaccine against *Leishmania tropica* in Balb\c mice

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ABSTRACT

Leishmania tropica is the causative agent of cutaneous Leishmaniasis in mammalian. Previous studies showed that vaccination Balb\c mice with inhibited Leishmania tropica by mitomycine\c induced production of interferon gamma in the serum of mice, but does not protect it from developing a lesion where it was mixture of Th1 and Th2. To enhance immune response, we evaluate the role of the genomic DNA of Leishmania tropica as adjuvant in combination with inhibited Leishmania tropica promastigotes by mitomycine\c in the immune response as a vaccine by inoculation the right footpads of Balb\c mice. After 4 weeks from vaccination, the results showed absence of any dermal lesions, absence of parasites in the inoculation site, high gene expression of interleukin -12, and interferon gamma, low gene expression of interleukin -4 in the draining lymph nodes of the vaccinated footpads. These results indicate that the dominant immune response it could be Th1.

Key words: *Leishmania tropica*, DNA of parasites, vaccine, mitomycine\c, immune response.

INTRODUCTION

Leishmania are obligated intracellular protozoa parasites causes Leishmaniasis diseases [1,2]. Cutaneous leishmaniasis (CL) is world widely distributed. 70 to 75% of global estimated CL incidence occurs in ten countries including Syria. *Leishmania tropica* (*L. tropica)* is the major causative agent of the human CL in Syria and in the old word [3,4]. Leishmaniasis prevention requires the presence of an effective vaccine, which is still not available yet [5,6]. Successful vaccine needs to cause a controlled infection and stimulate a long lasting Th1 protective immunity [7]. Previous works showed that, vaccination Balb\c mice with inhibited *Leishmania tropica* promastigotes by mitomycine\c, which is a DNA replication inhibitor, resulted in an increase in IFN-γ and IL-12 values in mice serum [8], but this vaccination didn't protect these mice from infection after challenge test (lesion appearance), which suggest presence of mixture of Th1 and Th2 immune response. Many studies indicate that, bacterial DNA stimulates myeloid dendritic cells (mDC) and plasmacytoid DC (pDCs) by toll-like receptor 9 (TLR9), and induces Th1 immune response in mice [9,10,11]. Therefore, DNA could be a good adjuvant in *Leishmania* vaccine.

In this study, we have investigated the effect of vaccination Balb\c mice with inhibited *L. tropica* promastigotes by mitomycine\c (inh-L) in combination with genomic DNA of *L. tropica* promastigotes (Lt.DNA) itself as an adjuvant, after 4 weeks from vaccination. Our results showed that, protection from infection, clear the parasites completely in the site of inoculation, high gene expression of IL-12 and IFN-γ, low gene expression of IL-4, which indicated that, the resulted dominant immune response could be Th1 after four weeks of vaccination.

EXPERIMENTAL SECTION

Mice

Female BALB/c mice were 6 to 8 wk-old at the onset of the experiments. Mice Purchased from Scientific Research Center-Damascus-Syria. During experimentation, mice kept under conventional conditions in an isolation facility. Mice were divided into 4 groups each one contained 9 mice.

Mice vaccination

Mice were vaccinated in the right footpads dermis with 10^4 inhibited promastigotes of *L. tropica* by mitomycine\c with or without 80 µg of Lt.DNA by using a 27.5-gauge needle in a volume of 50 µl. Mice were vaccinated with phosphate buffer saline (PBS) as control.

Parasites

Local *Leishmania tropica* strain LCEBS-1 from of *Leishmaniasis* Center for Epidemological and Biological Studies (LCEBS) in Damascus University-Syria, was used in this study. The parasites were grown at 26℃ in 50-ml disposable flasks containing 5 ml of culture medium RPMI-1640 (Sigma, Germany) supplemented with 20 Mm HEPES, 2 Mm L- glutamine and10% fetal bovine serum. Penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added as antibiotics. Promastigotes reached stationary phase after inoculation culture medium with 10^6 promastigotes per milliliter. Parasites were harvested in stationary phase, centrifuged (2,500 × g, 10 min, 4°C) and washed 3 times with PBS before being counted and treated with mitomycine\c before inoculation in mice.

Inhibition by Mitomycine\c

In order to get inhibited parasites, 10^4 oflive *L. tropica* promastigotes in stationary phase treated with increasing concentrations of mitomycine\c (Sigma, Germany) which avoid DNA replication.Cultures incubated in 26°C for 24 hours. Parasites counted, the lowest concentration, which inhibited the parasites but not killed it and kept them alive and motile has used.

Parasite DNA isolation

We isolate the genomic DNA from *L. tropica* promastigotes by using QlAamp Blood MiniKit (QIAGEN, Germany). Genomic DNA extraction was verified by electrophoresis on agarose 1%. DNA separated by migration through agarose gel detected by staining with ethidium bromide dye. Bands of DNA stained with these dye are visualized by illuminating the gel with UV light at one wavelength and recording at another. To determine DNA yield and purity, measurement of absorbance has done by spectrophotometer. Absorbance readings are performed at 260nm and 320nm.

To evaluate DNA purity calculation is the ratio of the absorbance at 260 nm divided by the reading at 280nm. Good-quality DNA will have an A260/A280 ratio of 1.7–2.0.

Monitoring of Infection

Vaccinated right footpad thicknesses were measured by caliper (Mitutoyu, Kawasaki Kanagawa, Japan), after 4 weeks from the vaccination. The increase due to the infection has defined as the difference between the thickness of the vaccinated footpads and the thickness of the unvaccinated contralateral footpads. The difference between both measurements corresponded to the size of the lesion. Comparison has done between the vaccinated mice groups and the control group.

Parasite load assay

The parasite load assay has in the inoculation site of each mice group after 4 weeks from the vaccination, where determined by limiting dilution assay method as previously describe [1]. Parasite burdens were determined in the vaccinated footpads and in dLNs of these footpads. Dermis of inoculated footpad and dLNs tissues have homogenized separately in 500 µl of RPMI 1640. 48 well cell culture plates were used. Each homogenate has diluted by 10-fold serial dilutions in consecutive wells. After 7 days of incubation at 26^o C with CO_2 5%, the wells were examined for motile promastigotes using an inverted microscope. The last dilution was positive for parasites, was considered to be the number of parasites in the tissue.

Real-time RT-PCR

To investigate gene expressions of IL-12, IFN-ɣ and IL-4 after 4 weeks from vaccination, RNA has extracted from dLNs of each mice group using a Gene JET RNA Purification extraction kit (Qiagen). A trace of genomic DNA has removed with an RNase free-DNase set. RNA (2 µg) was reverse transcribed using reverse transcriptase (200 U). Subsequent real-time PCR was performed on Step One real- time PCR system (Applied Biosystems) using Taq polymerase (Taq-Man Universal master mix; Applied Biosystems/Roche), 20 ng of cDNA as template (cDNA

Omniscript RT kit; Qiagen). The following are the forward and reverse primers, and the FAM-labeled probe for the target gene.

IFN-γ-RV	5-TGGCTCTGCAGGATTTTCATG-3
IFN-γ-FW	5-TCAAGTGGCATAGATGTGGAAGAA-3
IFN-γ- prope	5-TCACCATCCTTTTGCCAGTTCCTCCAG-3
IL-4-RV	5-GAAGCCCTACAGACGAGCAGCTCA-3
IL-4-FW	5-ACAGGAGAAGGGACGCCAT-3
IL-4- prope	5-TCCTCACAGCAACGAAGAACACCACA-3
HPRT-RV	5-CCAGCAAGCTTGCAACCTTAACCA-3
HPRT-FW	5-GTAATGATCAGTCAACGGGGGAC-3
HPRT-prope	5-TGTTGGATACAGGCCAGACTTTGTTGG AT-3
IL-12 p75-RV	5- AAC TTG AGG GAG AAG TAG GAA TGG-3
IL-12 p75-FW	5- GGA AGC ACG GCA GCA GAA TA-3
IL-12p40- prope :	5- CAT CAT CAA ACC AGA CCC GCC CAA-3

The mRNA expression levels were normalized to the hypo-xanthine phosphor ribosyltransferase (HPRT) gene and calculated as the n-fold difference of the expression in activated cells compared with its naive counterpart. Estimation of relative expressions of the respective mRNA was made by RT-PCR and primers of IL-12, IL-4, IFN-γ. RT-PCR conditions were as follows: 94 °C for initial denaturation (2 min), amplification with 35 cycles of 94°C (1 min) for denaturation, 58℃ (1 min) for annealing, 72°C (1 min) for elongation.

Statistical analysis:

The experiments were carried out three times in order to evaluate the accuracy of the results. The values have expressed by average X and SD, and were compared by the Student's t-test. P-valuesless than 0.05 were considered significant.

RESULTS

The inhibited concentration of mitomycine\c:

The relationship between the concentrations of mitomycine\c and numbers of parasites after 24 hours of incubation was shown in figure 1. The curve confirm the existence of a strong inverse correlation r = -0.987.

y = -0.643x + 18.17, where this equation calculates the inhibitory dose for the proliferation of 10^4 of parasite, which was 12.7 µg\ml. Consequently, we have used this concentration to inhibit parasite growth.

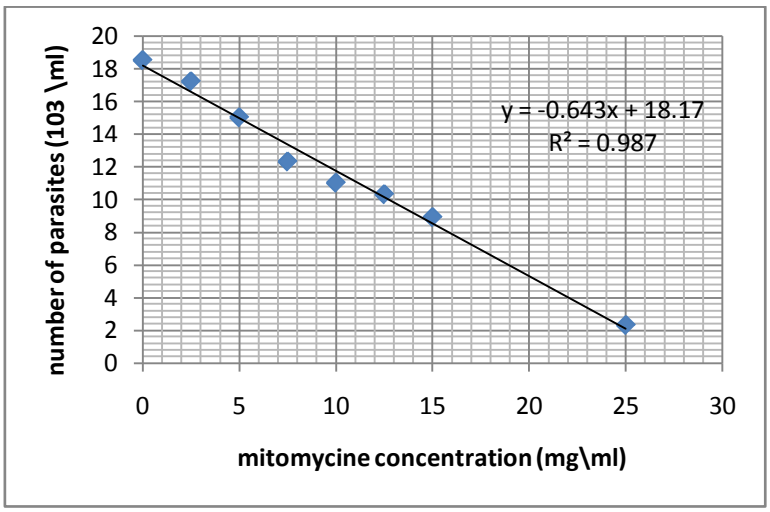


Figure.1. 10^4 of *L. tropica* promastigotes incubated in increasing concentration of mitomycine \c to determine the concentration that inhibit the proliferation of parasites. Curved shows the relationship between concentrations of mitomycine\c and parasites number.

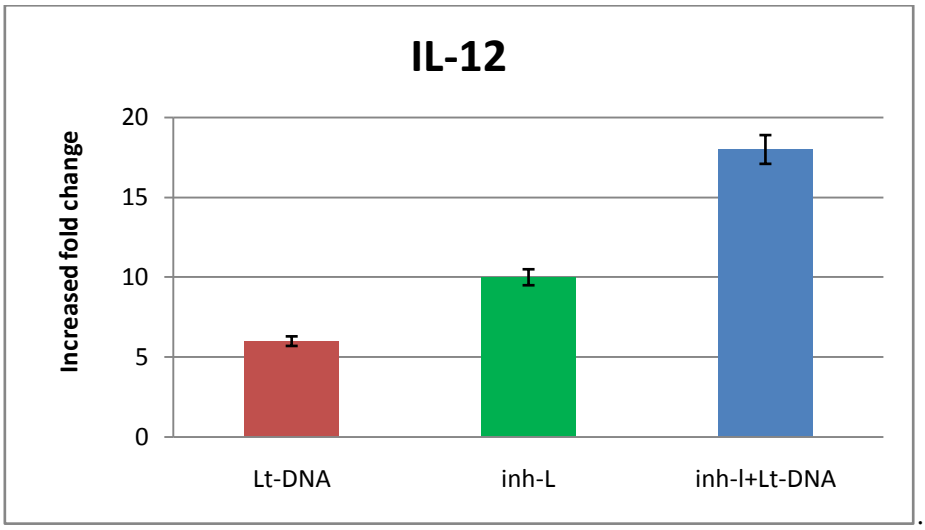
The effect of mice vaccination on infection development and parasite load:

To check the efficacy of Lt.DNA as an adjuvant with inh-L, we vaccinated mice with inh-L in the presence or absence of Lt.DNA. Footpads swelling in vaccinated mice were compared with that of control, footpads thickness have measured, and parasite load analysis has done in the inoculation site. No lesions have observed in the three vaccinated mice groups after 4 weeks from vaccination, but there were parasites (10^3) in the inoculation site of mice

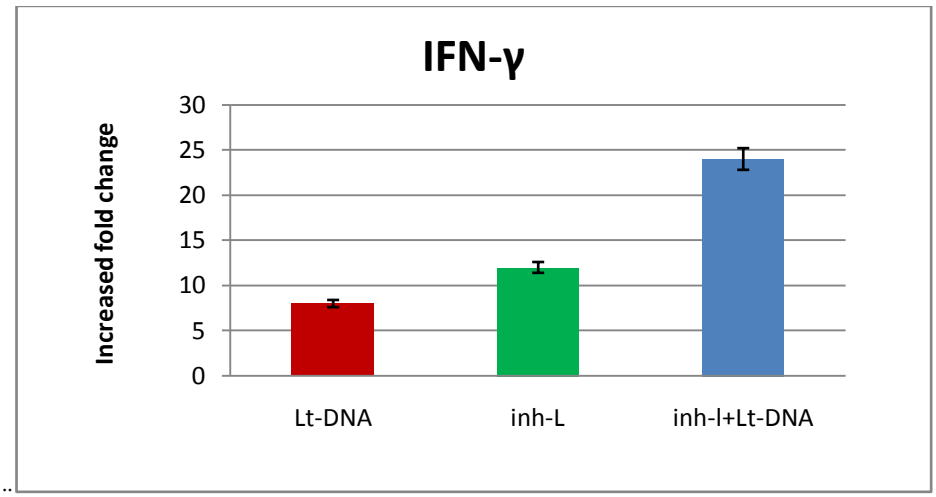
vaccinated with inh-L alone. These data showed that combination of Lt.DNA with inh-L help mice to control the infection.

The effect of vaccination on the gene expressions of IL -12, IFN-γ and IL-4

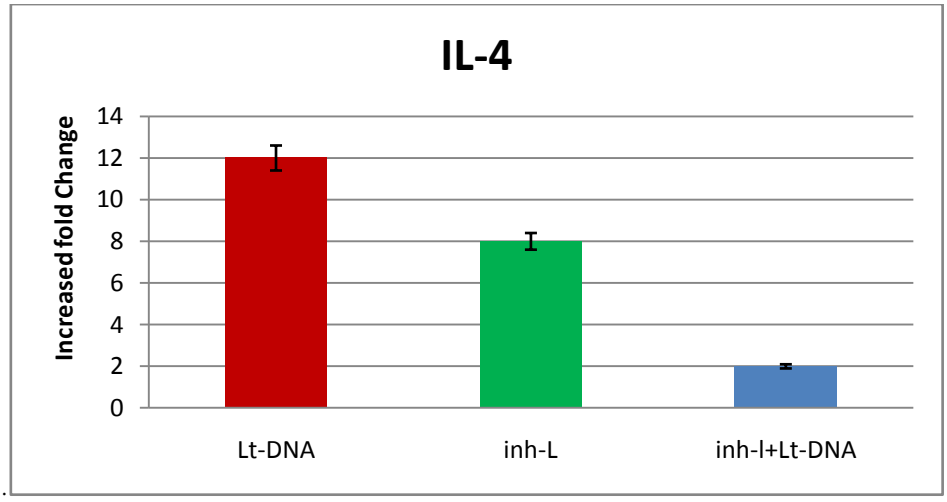
In order to determine whether the cellular immune response induced by vaccination mice with inh-L+Lt.DNA is associated with a cytokine expression, the gene expressions of interleukin -12 (IL-12), interferon gamma (IFN-γ) and interleukin -4 (IL-4) were assessed in the dLNs of each of mice group after 4 weeks of vaccination. As expected, vaccination mice with inh-L + Lt.DNA led to increase the expression of mRNA of IL-12 about 2 fold (P= 0.001) and 3 fold (P= 0.001) in comparison with either mice vaccinated with inh-L alone or with Lt.DNA alone respectively. Whereas, vaccination mice vaccinated with inh-L alone resulted in increasing the expression of mRNA of IL-12 about 2 fold (P= 0.0132) in comparison with mice vaccinated with Lt.DNA alone. Figure.2-A. Vaccination mice with inh-L + Lt.DNA led also to increase the expression of mRNA of IFN-γ about 2 fold (P= 0.003) and 3 fold (P= 0.005) in comparison with either mice group vaccinated with inh-L alone, or with Lt.DNA alone respectively. Whereas, the expression of mRNA of IFN-γ in mice group vaccinated with inh-L increased 1.5 fold (P= 0.02). Figure.2-B. In another hand, the expression of mRNA of IL-4 mice group vaccinated with inh-L + Lt.DNA decreased 4 fold (P= 0.005) and 6 fold (P= 0.015) in comparison with either mice vaccinated with inh-L alone, or with Lt.DNA alone respectively. Whereas the expression of mRNA IL-4 in mice group vaccinated with inh-L alone decreased 1 fold (P= 0.013) in comparison with mice vaccinated with Lt.DNA alone. Figure.2-C. Mice vaccinated with Lt.DNA alone showed expressions of mRNA of IL-12, IFN-γ and IL-4 same to control where the differences were insignificant.



2-A



2-B



2-C

Figure. 2.IL-12 (fig. 2-A), IL-4 (fig. 2-B), IFN-γ (fig. 2-C) expression in the draining lymph nodes obtained from Balb\c mice inoculated with 10^4 of mitomycine\c treated *leishmaina* (inh-L) alone or in combination with 80 µg of DNA of *leishmania tropica* promastigotes (Lt.DNA), or with Lt.DNA alone. The messenger RNA (mRNA) for IL-12, IL-4, IFN-γ genes were determined by RT-PCR. As geometric mean± SD (three dLNs). Data are representative of three independent experiments

DISCUSSION

Leishmania are obligate parasites, so cellular immune response mediated with T cells, play a critical role in control the parasite proliferation. T cells divided into two types Th1 and Th2 according to types of produced cytokines. IL-12 secrated from DCs cells elicits Th1 cells to produce IFN-γ and IL-2 that associated with cell mediated immune response including macrophage activation and kill the parasites by nitrite oxide NO [12]. Within IL-4 secreted from DCs cells elicits Th2 cells to produce different cytokines IL-4 and IL-10 that inhibit the macrophages to kill the parasites conversely, disease progression is generally associated with a T-helper-2 response [13]. The predominance of these cell types determines the outcomes of infection [13]. Thus the effectiveness of a vaccine study requires determination of the immune response type Th1 or Th2 [4]. After 4 weeks from balb\c mice vaccination, we made a several important observations which are contribute to our understanding of requirements for successful immune interventions against cutaneous leishmaniasis. First, we were able to demonstrate that vaccination balb\c mice with Lt.DNA alone couldn't modulate the immune response since the gene expressions of cytokines were same to control which, could be understood by the necessity of parasites present to stimulate effective immune response. Second, it was obvious to us that, vaccination mice with inh-L alone was insufficient for eliminate all the parasites in both inoculation site, where the parasites survive, which may due to the T regulatory cells ($CD4^+CD25^+$) which are a major source of IL-10 and crucial for maintenance of parasites persistence in macrophage cells [7], besides, the gene expression of IL-12 was high, but the ratio of IFN-γ to IL-4 was lower than 2 (IFN-γ\IL-4=1.5). According to these data,we suggest that, the immune response of mice vaccinated with inh-L alone is a mixture response of Th1 and Th2, and there is a need to adjuvant effect to shift this response to Th1 type. Third, it was clear to us that, combination of Lt.DNA with inh-L in vaccination mice resulted in more powerful immune response presented in absence of parasites completely in inoculation site, in addition to more increasing of IL-12 and IFN-γ gene expressions and decreasing of gene expression of IL-4, which could be due to the role of DNA in activation DC cells by - like receptor 9 (TLR-9) [6,9]. This activation leeds to activate T cells to produce IFN-γ which in turn induces macrophages to secret IL-12 and kill the parasites and control the infection. Our results in this study showed more increasing of IL-12 and IFN-γ gene expressions, besides more decreasing of IL-4 gene expression in mice vaccinated with Lt.DNA + inh-L, that made the ratio IFN-γ\IL-4 more than 2 (IFN-γ\IL-4=12), that allowed us to suggest that, the immune response resulted from this vaccination is Th1 type. Teis result is consistent with study by Zhang et al. [14] who showed that presenting a high IFN-γ expression and low IL-4 expression indicates that the mice developed an immune response type Th1 mice which protect from infection.

CONCLUSION

Finally, The absence of dermal lesion and clearance the parasites in the inoculation site and the high expressions of mRNA of IL-12 and IFN-γ and the weak expression of mRNA of IL-4, enable us to suppose that vaccination of Balb\c mice with Lt.DNA+inh-L could stimulate a Th1 immune response which could protect Balb\c mice from *L. tropica* infection .

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