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کارکده آنلاین پروپوزال نویسی
DNAs from *Brucella* Strains Activate Efficiently Murine Immune System with Production of Cytokines, Reactive Oxygen and Nitrogen Species

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**ABSTRACT**

Brucellosis is an infectious disease with high impact on innate immune responses which is induced partly by its DNA. In the present study the potential differences of wild type and patients isolates versus attenuated vaccine strains in terms of cytokines, ROS and NO induction on murine splenocytes and peritoneal macrophages were investigated.

This panel varied in base composition and included DNA from *B. abortus*, *B. melitensis*, *B. abortus* strain S19 and *melitensis* strain Rev1, as attenuated live vaccine. Also we included *Escherichia coli* DNA, calf thymus DNA (a mammalian DNA), as controls. These DNA were evaluated for their ability to stimulate IL-12, TNF-α, IL-10, IFN-γ and ROS production from spleenocytes as well as NO production from peritoneal macrophages. Spleen cells were cultured in 24 well at a concentration of 10⁶ cells/ml with subsequent addition of 10 μg/ml of *Brucella* or *Ecoi* DNAs.

These cultures were incubated at 37°C with 5% CO2 for 5 days. Supernatants were harvested and cytokines, ROS and NOx were evaluated. It was observed that TNF-α was induced in days 1,3,5 by all *Brucella* strains DNAs and *E. coli* DNA, IL-10 only was induced in day 1, IFN-γ was induced only in day 5 and IL-12 not induced. ROS and NOx were produced by all strains; however, we observed higher production of NOx which were stimulated by DNA of *B. melitensis*.

**Key words:** Cytokines; Genomic DNAs from *Brucella* strains; Nitrogen species; Reactive oxygen

**INTRODUCTION**

Optimal immunity to intracellular bacteria is typically mediated by the Th1 subset of CD4⁺ T lymphocytes. Upon activation of macrophages,
proinflammatory cytokines are produced by these cells, thus the effector cells of the innate immunity are mobilized. Subsequently the effector cells of the adaptive immunity are activated.\(^1\)

Intensive interest is being directed towards the use of bacterial derivatives which promote Th1-like responses. DNA is an essential macromolecule whose immunologic properties vary with sequence heterogeneity.

While mammalian DNA is immunologically inert, bacterial DNA has potent immunological properties. It appears to function as one of the "danger signals" to trigger innate immunity against infection as well as triggering a specific adaptive immune response.\(^2\) Recent evidence has demonstrated that toll-like receptor-9 (TLR9) mediates CpG-ODN immunostimulatory activity in murine and human immune cells.\(^3\) A number of studies in mammalian cells concerning the in vitro and in vivo immunostimulatory effects of CpG-ODN have previously been reported. These studies have shown that bacterial DNA and synthetic CpG-ODN stimulate variety of cells including B lymphocytes, natural killer cells, macrophages and dendritic cells which result in production of cytokines, including IFN-\(\gamma\), interferon –\(\alpha\) (IFN-\(\alpha\)), tumor necrosis factor –\(\alpha\) (TNF-\(\alpha\)), IL-1\(\beta\), IL-6, IL-12 and IL-18.\(^6,7\) B cells, macrophages and dendritic cells express TLR-9 which makes them capable of recognizing these differences between prokaryotic and mammalian DNA based on the presence of unmethylated CpG dinucleotids. Few researchers have shown recently that the CpG motifs with special base composition which flank the CpG dinucleotide confer immunostimulatory potential in some species. The most active CpG motif for murine immune cells is GACGTT.\(^2,8\)

There are also other factors such as the sequence and the number of motifs and their locations in DNA which may influence the activity of CpG-ODN. Among these factors the CpG-ODN which contain two or more CpG motifs have a higher stimulatory activity.\(^6\) The uptake of the CpG DNA is increased in presence of poly (dG) sequence.\(^9,10\)

This study was conducted to determine the responses of the immune system to four different DNA Brucella stains and to evaluate the immunomodulatory effects of these bacterial DNA. Escherichia coli DNA and calf thymus DNA were included in the study as controls.

### MATERIALS AND METHODS

#### Bacterial Strains

*B. melitensis* biotype 1 and *B. abortus* biotype 3, the pathogenic isolates, *B. melitensis Rev1* and *B. abortus* S19, the vaccine strains were kindly given by Faculty of Veterinary Medicine, University of Tehran, Iran. *E. coli* was obtained from the Department of Biology, Faculty of Sciences, University of Tehran.

#### Extraction of Genomic DNA from Bacterial Cultures

The extraction of genomic DNA was achieved as described by Romeo et al. and Johns, Jr. et al.\(^11,12\).

Briefly, following lyophilization of each strain of bacteria, one gram of each bacteria was suspended in 15ml of NET buffer (50mM NaCl, 125mM EDTA, 50mM Tris–HCl), then SDS with final concentration of 3.4% was added to each suspension of bacteria followed by incubation of each mixture at 80 ºC for 10 min. Then, RNase A (Sigma, USA) 75 mg/ml was added to the lysate and incubated at 50 ºC for 2 h. Then, proteinase K (300μg/ml) was added and incubated at 42ºC for one h. 5 M NaCl and CTAB-NaCl solution were added to the above mentioned mixture and incubated at 65ºC for 10 min in order to precipitate the denatured proteins, polysaccharides and cell wall debris. DNA extraction was achieved by phenol-chloroform-isoamyl alcohol with the ratio of 25:24:1 and then centrifuged at 10,000g for 15 min. To get rid of unwanted materials in the supernatant, it was dialyzed against water for 24 h with three changes.

DNA in supernatant was precipitated with a mixture of two volumes of absolute ethanol and 3M sodium acetate (pH 5.2). The precipitate was then suspended in distilled water. The quantity and quality of DNA were determined by evaluation of the ratio at OD 280 to OD 260. The DNA concentration also was determined by reading the OD at 280nm.\(^13\) To obtain single stranded DNA, the DNA solution was boiled for 10 min until it was melted and then it was rapidly cooled. The endotoxin concentration of isolated DNA was assayed using Limulus Amebocyte Lysate EndochromeTM (Charles River).

#### Animals

BALB/c mice were obtained from Razi research institute (Karaj- Iran). Female mice were used at 6-8 weeks of age.
Spleocyte Preparation and Cell Culture

Spleens were removed from mice, and single cell suspensions were prepared by gentle teasing through cell strainer. Erythrocytes were lysed by AKC lysis buffer and spleen cells were washed three times in phosphate buffered saline (PBS). These cells were cultured in 24 well flat-bottom plates at a concentration of 10⁶ cells/ml in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100μg/ml of streptomycin and 10% heat-inactivated FBS. bDNA was added to each well at the same time that we cultured the cells. Preliminary dose response analyses were conducted with bDNA concentration ranging from 0.05μg/ml to 10μg/ml. Each experiment was done on three BALB/c mice and spleen cells were cultured triplicate. Optimal cytokine producing concentration was 10μg/ml for Brucella and Ecoli DNAs. This concentration was used for all experiments. Cultures were incubated at 37ºC with 5% CO₂ for 5 days. Supernatants were harvested on day1, 3, and 5 after culturing and were frozen at -70ºC for the cytokine assays. The medium which contained calf thymus DNA, served as control.

Cytokine Assay

Cytokine secretion in the supernatants was determined by enzyme linked immuosorbent assay (ELISA) using by R&D systems, Inc. kits for IFN-γ, IL-10, IL-12p70 and TNF-α.

Reactive Oxygen Species (ROS) Assay

Production of ROS by cultured spleen cells was quantitated by the 2’′, 7’′-dichlorofluorescin-diacetate (DCF-DA) method14. In 24-well plate, 1×10⁶ cells were seeded in a final volume of 1 ml PBS in each well. Then 25μg DCF-DA (final concentration 0.2 M) in DMSO was added to each well and incubated in CO₂ incubator. After 30-min pre-incubation with DCF-DA, the cells were incubated with aqueous solutions of 10μg/ml bDNAs in duplicate wells for 1h. Fluorescence was measured (Ex=485, Em=530) using Cary eclips, Varian Fluorospectrometer.

Macrophage Preparation

Resident peritoneal macrophages were obtained by lavage of the peritoneal cavity of mice with 10 ml of cold sterile PBS. Cells were centrifuged at 300g for 5 minutes at 4ºC, washed twice with serum free DMEM, and resuspended at 10⁶ cells/ml in DMEM medium supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 24- well plates and allowed to adhere to tissue culture plates for 2h at 37ºC in a humidified incubator in 95% air 5% CO₂. Non adherent cells were removed by washing with serum free DMEM. Cells were cultured (1×10⁶/ml) in the presence of 10 μg/ml single-stranded DNA.

Measurement of NO

Nitric oxide (NO) production was determined by Griess assay. The peritoneal macrophages were cultured and stimulated with 10μg/ml of Brucella strains and control DNAs, for 24h at 37ºC. The supernatant of each cultured plate was centrifuged at 200g for 10 min at room temperature and an aliquot of 100 μl of the supernatant from each well was taken and added into a new flat bottom 96-well plate. For nitrite determination, an aliquot of 50 μl of %0.1 sulfanilamide(Sulf) in 5% phosphoric acid solution and 50 μl of %0.1 N-(1-Naphthyl) ethylenediamine dehydrochloride (NEDD) were added to each well sequentially. The plates were allowed to stand for 10 min at room temperature before the absorbance at 595 nm was read using the Asys/Hitech Expert 96. Since NO is unstable and is rapidly converted to nitrate and nitrite, it was necessary to determine both nitrate and total nitrite concentrations in samples. In order to convert nitrates to nitrites in a sample solution, 100μl of culture supernatant was added to 100μl vanadium chloride (400 mg were prepared in 50 ml HCl1M) and aliquot of 25 μl 2% Sulf in H3PO4 and 25 μl 0.1% NEDD were added to each well. The plates were allowed to stand for 45 min at 37ºC. The absorbance at 595 nm was read.15

Statistical Analysis

Statistical analysis was performed by Student t-test.

RESULTS

DNA from Brucella Strains and E. Coli Induced High Levels of TNF-α

Induction of TNF- α by DNAs extracted from all Brucella strains (pathogenic isolated and vaccine strains) was quite significant and showed significant difference with medium (P <0.05).TNF-α production remained high up to 5 days without any reduction (Figure 1). There was no significant difference among different Brucella strains DNAs in terms of TNF-α. Also, there was no significant difference between Brucella strains DNA and E. coli DNA in terms of TNF-α.
Induction of IL-10 by *Brucella* Strains and *E.Coli* DNAs

In response to DNAs of all *Brucella* strains, the production of IL-10 reached the highest levels after twenty-four hours incubation in comparison with negative control (P <0.05). However, thereafter the concentration of IL-10 decreased substantially (Figure 2). The production of IL-10, upon induction by *Brucella* strains DNAs and *E. coli* DNA did not differ significantly.

IFN-γ Production was Increased by *Brucella* Strains and *E. Coli* DNAs

Figure 3 shows the results of IFN-γ concentrations in the splenocytes stimulated by different bacterial DNAs. All DNAs, induced significant quantities IFN-γ on day 5 in comparison to control (P <0.05). There is no significant difference among different *Brucella* strains DNAs in terms of IFN-γ production. Also, there is no significant difference between *Brucella* strains DNAs and *E. coli* DNA in terms of IFN-γ.

**IL-12 Induction by Brucella Strains DNAs**

None of the *Brucella* DNAs induced production of IL-12 (data not shown).
Response to Genomic DNA from *Brucella* Strains

![Graph showing IFN-γ induction by *B. strains* DNAs and *E. coli* DNA. BALB/c spleen cells were cultured with 10μg/ml DNA; supernatants were harvested on day 1, 3 and 5. Supernatants were assayed for IFN-γ. P value was <0.05 on fifth day whereas no difference on first and third days.](image)

**Figure 3.** IFN-γ induction by *B. strains* DNAs and *E. coli* DNA. BALB/c spleen cells were cultured with 10μg/ml DNA; supernatants were harvested on day 1, 3 and 5. Supernatants were assayed for IFN-γ. P value was <0.05 on fifth day whereas no difference on first and third days.

**Induction of Nitrate and Total Nitrite**

In peritoneal macrophages stimulated by *Brucella* strains DNAs and control DNA after 24h incubation, nitrate and total nitrite production reached maximum levels in response to all *Brucella* strains DNAs (P <0.01, compared with the medium and *E. coli* DNA). *B. melitensis* DNA showed the most potent stimulatory effect on nitrite production (Table 1).

**ROS was also Induced**

ROS production showed significant difference with medium by all *B. strains* DNAs (P<0.01-0.005).

![Graph showing detection of ROS induced by *B. strains* DNAs and *E. coli* DNA. BALB/c spleen cells were cultured with DCF-DA for 30 min then 10μg/ml DNA was added and incubated for 1 h; then the production of ROS was determined, (* means P value was <0.05; ** means P value was <0.01; *** means P value was <0.005).](image)

**Figure 4.** Detection of ROS induced by *B. strains* DNAs and *E. coli* DNA.BALB/c spleen cells were cultured with DCF-DA for 30 min then 10μg/ml DNA was added and incubated for 1 h; then the production of ROS was determined, (* means P value was <0.05; ** means P value was <0.01; *** means P value was <0.005).

*B. melitensis* DNA showed the most potent stimulatory effect on ROS secretion by spleenocytes (Fig. 4).

**Specificity of Cytokine Production**

DNAase treated DNA, iNOS inhibitor, NG -Methyl-L-Arginine (NMMA), were added to some of the culture wells. It was found that DNAase and iNOS inhibitor completely abolished the stimulatory effect of DNA on cytokine and NO production respectively (data not shown).
Nucleic acids were considered for years not to be immunogenic. However, there have recently been reports indicating that DNA from prokaryotes especially from bacteria have the potential to stimulate the immune system. It has been shown that the innate immune system of the mammalian species can discriminate between DNA from eukaryote and prokaryote by pattern recognition. It also has been demonstrated that production of proinflammatory cytokines in macrophages can be induced by CpG DNA and synthetic CpG-ODN.

The immunostimulatory properties of CpG-ODN were also used to enhance innate immune responses against tumor. CpG-ODN can also induce production of cytokines including IFN-γ and TNF-α, moreover it enhances NK activity in mice. TNF-α is a cytokine which initiates activation of cells and is involved in production of inflammatory mediators. In the current study we have shown that treatment of spleenocytes with Brucella strains DNAs and E. coli DNA induced the production of TNF-α. Jian jun Gao et al. reported that E. coli DNA induced TNF-α in the Raw264.7 macrophage – like cell line. We could not detect IL-12 production from cells stimulated with Brucella DNAs strains on days 1, 3, 5, while TNF-α was produced by day 1 until day 5. TNF-α which is produced after macrophage activation, can inhibit IL-12 expression selectively. This phenomenon is part of self-limiting modulations. The inhibition of IL-12 by TNF-α is beneficial for the host since it could down regulate the inflammatory response. This may of course pave the way for the evasion of intracellular pathogens from the immune system. Chronic infections are manifested by TNF-α expression such as in HIV-1 infection which sustains immune activation. Expression of TNF-α is associated with a decrease in IL-12 production and cell-mediated responses. Xioajing Ma also reported that TNF-α is a potent inhibitor and selective for IL-12 p40 and p70 production from human macrophages induced by LPS. High level of TNF-α in our study could have inhibited IL-12 production. We demonstrated that Brucella strains DNAs were able to activate spleenocyte inducing IL-10 on day 1 then a decreased production on day 3 and day 5. In contrast, IFN-γ production was gradually increased and reached the maximum level on the fifth day. These data are consistent with the results of a recent in vitro study by Lashkarbolouki et al. They showed induction of IL-10 by Brucella DNA in human peripheral blood mononuclear cells (PBMC) after 24 h incubation and its gradual decrease while IFN-γ increased with time and reached the maximum level on fifth day.

In the current study we have shown that treatment of spleenocytes with E. coli DNA induced IFN-γ. Jian jun Gao et al. reported that E. coli DNA induced TNF-α in the Raw264.7 macrophage – like cell line. We could not detect IL-12 production from cells stimulated with Brucella DNAs strains on days 1, 3, 5, while TNF-α was produced by day 1 until day 5. TNF-α which is produced after macrophage activation, can inhibit IL-12 expression selectively. This phenomenon is part of self-limiting modulations. The inhibition of IL-12 by TNF-α is beneficial for the host since it could down regulate the inflammatory response. This may of course pave the way for the evasion of intracellular pathogens from the immune system. Chronic infections are manifested by TNF-α expression such as in HIV-1 infection which sustains immune activation. Expression of TNF-α is associated with a decrease in IL-12 production and cell-mediated responses.

Table 1. Nitrate/nitrite induction by B. strains DNAs and E. coli DNA. BALB/c peritoneal macrophages cells were cultured with 10μg/ml DNA; supernatants were harvested at 24h and supernatants were assayed for nitrate/nitrite.

<table>
<thead>
<tr>
<th>DNA</th>
<th>SI9</th>
<th>Rev1</th>
<th>Abortus</th>
<th>Melitensis</th>
<th>Ecoli</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct nitrite</td>
<td>2.78±0.29**</td>
<td>2.46±0.4*</td>
<td>3.12±0.2**</td>
<td>4.2±0.4**</td>
<td>1.8±0.4</td>
<td>1.63±0.36</td>
</tr>
<tr>
<td>Total nitrite</td>
<td>9.4±1.1**</td>
<td>9.4±0.65**</td>
<td>10.4±0.91**</td>
<td>12.3±1.19**</td>
<td>3.6±0.5</td>
<td>5.7±1.36</td>
</tr>
<tr>
<td>Nitrate</td>
<td>6.58±1**</td>
<td>6.91±0.5**</td>
<td>7.31±0.78**</td>
<td>8.16±0.8**</td>
<td>1.8±0.87</td>
<td>4.1±1.1</td>
</tr>
</tbody>
</table>

* means P value was <0.05
** means P value was <0.01

DISCUSSION

Nitric oxide (NO) is an important bioregulator molecule. Inducible NO has an antimicrobial activity
similar to that of reactive oxygen species and plays a vital role in host defense and immunity, including the modulation of inflammatory responses.\textsuperscript{34} LPS and Lipid derived from \textit{Brucella} strains induce in rats a minute amount of NO in comparison to production of NO by other bacteria such as \textit{E.Coli}.\textsuperscript{35} However, we showed \textit{Brucella} strains DNAs directly induced NO production without priming with IFN-\textgamma or LPS, but \textit{E. coli} DNA could not induce NO production. Utaiisincharoen P, et al. and Gao JJ, et al. showed \textit{E.Coli} DNA and \textit{S. aureus} DNA by itself did not induce production of NO by Raw 264.7 macrophages,\textsuperscript{35,36} however, all \textit{Brucella} strains DNAs induced NO in peritoneal macrophages of BALB/c mice.

In conclusion, DNA from \textit{Brucella} strains, in particular from \textit{Brucella melitansis} accommodate a number of CpG with special locations which makes these DNA, a proper adjuvant for DNA vaccines.

DNA from \textit{Brucella} has also the potential to induce NO and ROS, thus recruits the innate immunity. Moreover, it stimulates acquired immune system by inducing IFN-\gamma that is Th1 response while as the response progresses it down regulates the IL-10 production.

In preparation of DNA from bacteria, often a minute amount of LPS contaminates the final yield. The LPS from Gram negative bacteria such as \textit{Ecoli} on inoculation therefore, induce fever and septic shock. Whereas, the LPS from \textit{Brucella} does not bear these unwanted properties.

This study was conducted in BALB/c mice. Such an study in future should be conducted in human cell lines to evaluate the usefulness of \textit{Brucella} DNA for human applications.

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