IJMS
Vol 27, No. 2, June 2002

Original Article

Down-Regulation of T Cell Function by Heat Shock-Induced Excretory Factor of *Leishmania Major*

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Abstract

Background: Despite demonstration of molecular and biochemical changes induced by heat shock on *Leishmania*, the immunological importance of such changes has not been elucidated.

Objective: Studying the effect of two excretory factors prepared under heat shock and ambient temperature from *Leishmania major* on Balb/c splenocytes function.

Methods: The parasites were cultured at 25°C and then subjected to heat shock by overnight incubating at 35°C. Both 25°C (R25) and 35°C (R35) supernatants were collected and used for biochemical analysis and as culture media for T cell activation. The protein profile of two supernatants was studied by SDS-PAGE. Splenocytes of Balb/c mice were stimulated by concavalin-A in the presence of RPMI, R25 and R35. Consequently, CD25 expression was studied by means of flow cytometry and the production of IFN-γ and IL-4 was determined by ELISA.

Results: SDS-PAGE analysis showed distinct protein profile for R25 and R35. Significant decrease in IFN-γ and IL-4 production by cells cultured in R35 was noticed ($p<0.001$). CD25 expression was also down-regulated ($p<0.01$). In contrast, cytokine production in the supernatants of the cells cultured in R25 demonstrated suppression on IL-4 ($p<0.01$) but not on IFN-γ production. In parallel, CD25 expression was not down-regulated in the presence of R25 medium.

Conclusion: The data suggest that heat shock-induced excretory factor of *L. major* is capable of inhibiting T cell function by decreasing IFN-γ production and down-regulating CD-25 expression which might be involved in the induction of immunosuppression in this susceptible murine strain. Furthermore, we suppose that the supernatant of *L. major* culture in ambient temperature have an intrinsic ability to promote Th_1 response.
Iran J Med Sci 2002; 27(2):70-74

Keywords • *Leishmania major* • heat shock • excretory factor • IFN-γ • IL-4 • CD25

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Introduction

Leishmania causes a spectrum of diseases which affect more than 12 million people world-wide.[1] Therefore, understanding mechanism(s) involved in the pathogenesis would help to combat this disease.

The parasite alternates between two developmental stages; a flagellated promastigote stage in the digestive tract of the sand fly and a non-motile amastigote stage marked by proliferation within macrophages of the mammalian host.[1] The parasite is transmitted by the sand fly bite. Consequently, promastigotes are exposed to elevated temperature, which in turn results in an over-expression of the heat-shock genes (HSG). The products of HSG help the survival of the parasites in mammalian host. In addition, *Leishmania* undergoes other changes that increase its infectivity.[2,3] However; the molecular change(s) involved in augmentation of *Leishmania* virulence has not been fully recognized.

As in other intracellular infections, help from T cell is decisive for activating infected macrophages and inducing intracellular killing of invading parasites.

In genetically resistant mice, C57BL/6, Th1 response is dominated during the infection and IFN-γ, the potent activator of macrophage is involved in elimination of parasite. However, in genetically susceptible mice, Balb/c, Th2 response dominates and high IL-4 production is involved in dissemination and susceptibility while the underlying mechanism is still obscure.[4]

Secretion of different components of *Leishmania* is well documented and their biochemical properties have been explained. Some of these excretory components are recognized by sera of infected patients.[5] In addition, it has been reported that *Leishmania* parasites excreted factor is capable of down-regulating IL-2 receptor expression on T cells. However, cytokine production has not been studied.[6] Therefore, we investigated the effect of heat shock excretory factor of *Leishmania major* (*L. major*) on Balb/c splenocytes function. We studied IFN-γ and IL-4 production in addition to IL-2R expression in response to concavalin A in the presence of different *L. major* excretory factors prepared under heat shock (35°C) and ambient temperature (25°C).

Materials and Methods

Animals

Four to six-week-old female Balb/c mice were purchased from Pasteur Inst. of Iran (Tehran, Iran).

Parasites

The strain of *L. major* was MRHO/IR/75/ER. Promastigotes were cultured at 23-25°C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 292 µg/ml L-glutamine, 4.5 mg/ml glucose, 100 µg/ml streptomycin and 100 IU/ml penicillin (Sigma Chemical Co., St. Louis) as previously described.[7]

Preparation of media under different conditions

The organisms at the late log phase of culture were washed by centrifugation ($400 \times g$, 10 min, 4°C) and resuspended in the above medium without FBS at 2×10^7 organisms/ml and incubated at 35°C (R35) and/or 25°C (R25) with 5% CO_2 for 15 hrs. The parasites were then removed by filtration through a sterile 0.22 µm pore filter. Filtrate aliquots were stored at -20°C until used. RPMI 1640 was also used as control.[8]

SDS-PAGE and Western-blotting analysis of R25 and R35 excretory factors

The protein content was determined by the method of Lowry et al[16] with bovine serum albumin as standard for both excretory factors. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide gels stained with a modified silver staining method based on the standard Lammeli method[17]. Since we found a band around 60 Kd for R25 and R35 in SDS-PAGE, western blot analysis was performed by mAb specific for gp63. As positive control we used recombinant gp63, (a gift from Dr. Mahboudi, Pasteur Institute of Iran WHO/TDR grant for vaccine development against cutaneous leishmaniasis). The mAb against gp63 was also a gift from Dr. Mahboudi. The isolated proteins on SDS-PAGE were electrotransferred onto PVDF membrane. The strips were blocked by bovine serum albumin and then let react with anti-gp63 mAb. After washing, the strips were then soaked in buffer containing appropriate dilution of anti-mouse conjugated with horseradish peroxidase. At the end of washing steps, the strips were soaked in substrate solution, di-aminobanzidine and left to develop the color.

Culture and stimulation of mononuclear cells

Five mice were sacrificed by cervical dislocation. The spleens were removed and meshed to produce single cell suspension. Red blood cells were lysed by the addition of a lysis buffer (0.16 M ammonia chloride Tris buffer, pH 7.2) at 37°C for 3 min. Then the cells were washed twice with RPMI and the Cell concentration was counted and adjusted to 1×10^6 per

Table 1: The effect of the different media on CD25 expression induced by Con-A on BALB/c splenocytes. Results showed the absolute number of $CD3^+CD25^+$ cells $\times 10^{-4}$. Cells were cultured in the different culture media, with or without Con-A stimulation. After 48 hr incubation the cells were harvested and stained with appropriate conjugated mAb. * significantly different from the control.

Con-A	RPMI	R25	R35
–	10.8±1.3	13.3±1.4	12.9±1.8
+	54.3±4.4	51.6±6	20.1±2.1*

ml in different media including RPMI, R25 and R35 supplemented with 2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin and 10% heat-inactivated FBS. Splenocytes viability was consistently more than 99% as determined by trypan blue exclusion. 0.2 ml/well of splenocytes were cultured in triplicate on 96-well flat-bottom culture plates (Falcon 3072, Lincoln Park, NJ). Splenocytes were then stimulated with concavalin-A (Con-A, 10 µg/ml, 0.1 ml/well, Sigma) and incubated for 48 hrs. The supernatants were then collected and stored at -70°C until used and the cells were harvested and stained for flow cytometry analysis.

Cytokine determination

For cytokine analysis specific sandwich ELISA were used (Biosource International Inc. California, USA) and the assays performed based on manufacturer's instructions. The lowest amount of IL-4 and IFN-γ determined by the kits was 10 pg/ml.

Flow cytometric analysis

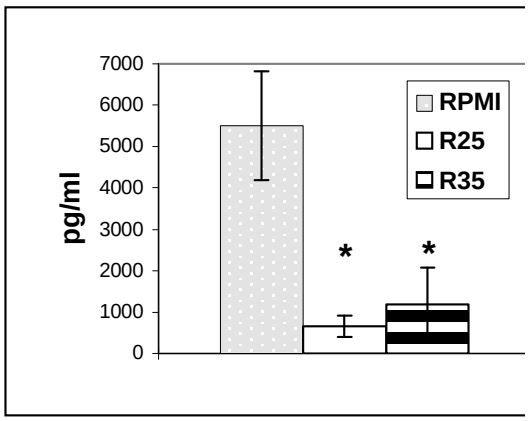
The cells were washed with staining buffer (Dulbecco's PBS containing 1% Bovine serum albumin (Sigma) and 0.1% sodium azide (DPBS-AA). The cells were resuspended in 50µl of the same buffer. Cell surface CD3 molecules were stained by incubation with the specific FITC-labeled mAb (Sigma) at 0°C for 30 min. The cells were subsequently washed by DPBS-AA buffer and stained with specific PE-labeled mAb to CD25 (IL-2R, Sigma) at 0°C for 30 min. The cells were washed again and resuspended in 0.5% paraformaldehyde in the same buffer and analyzed by Becton Dickinson FACScan flow cytometer (Flow cytometrylysis II software, FACS can, Becton Dickinson, USA). Non-lymphoid cells were excluded from analysis by setting an appropriate gate on the forward *vs* side scatter parameters. Ten thousand cells were collected for each sample and the percentage of $CD3^+CD25^+$ cells was determined.

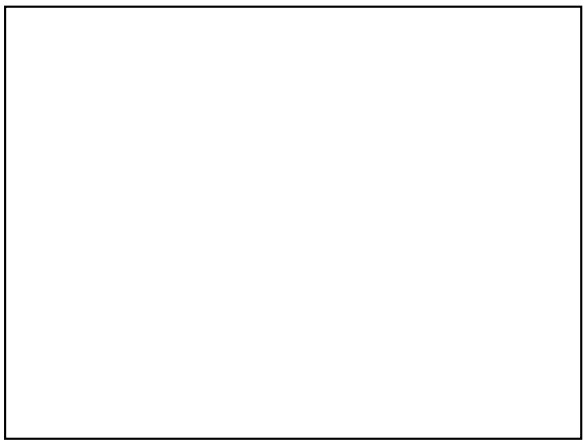
Figure 2: IFN- γ production by BALB/c splenocytes cultured in the pre
were stimulated by
supernatants were
* Significantly differ

Figure 3: Production of IL-4 by BALB/c splenocytes cultured in different media. The cells were stimulated by Con-A incubated for 72 hrs and the IL-4 secretion the supernatants were measured by ELISA.
* Significantly different from the control cells, RPMI.

Results

Protein profiles of the excretory factors showed distinct pattern:





As seen in Fig.1 there was a single band around 60Kd for R25 and R35. However, two other bands were observed in R35, which were absent in R25.

Since gp63 is reported to be released from the surface of *Leishmania* species,[18] we set up western-blot analysis using recombinant gp63 as positive control and specific anti-gp63 mAb in order to define the bands found in SDS-PAGE analysis of R25 and R35. As shown in Fig. 1, no reactivity to anti-gp63 mAb was observed by R25 and R35. However, positive control showed good reactivity with anti-gp63 mAb.

Down-regulation of cytokines production is induced by heat-shock excretory factor of L. major

As shown in Fig.2, a significant decrease of IFN-γ was observed in supernatant of cells cultured in R35 (159±54 pg/ml) but cells cultured in RPMI and R25 produced a high amount of IFN-γ (2238±1339 and 2375±415 pg/ml, respectively). In parallel, IL-4 secretion was down-regulated in supernatants of cells cultured in R35 (1188±895 pg/ml) and R25 (663±256 pg/ml) in contrast to the control, which produced high amount of IL-4 (5500±1323 pg/ml, Fig.3). The low level of cytokines in supernatant of cells cultured in R35 was not due to cell death since cell viability after 48 hr incubation time was checked and all wells had comparable cell density (data not shown). In addition, cytokine level in supernatants of cells cultured in R25 without con-A was very low and no induction of cytokine production was seen and non-specific cytokine production was not observed (data not shown).

CD25 expression is also down-regulated in the presence of heat-shock excretory factor of L. major

Table 1 shows the percentage of $CD3^+CD25^+$ cells in the presence of each media. Consistently, R35 down-regulated the expression of CD25 (20.1±2.1%) as compared to R25 (51.6±6%, $p<0.05$) and RPMI (54.3±4.4, $p<0.01$). There was no significant difference between RPMI and R25 in terms of CD25 expression.

CD25 expression was induced by none of the media in the absence of con-A (Fig.2) and there was no significant difference between percentage of $CD3^+CD25^+$ cells cultured in RPMI, R25 and R35.

Discussion

It has been demonstrated that *L. major* infection in Balb/c mice down-regulates specific cellular immune response during the first few weeks after infection which finally causes a general unresponsiveness, the so called anergy.[9,10] Such anergic state is also seen in human mucocutaneous leishmaniasis.[11] Moreover, it was shown that excreted factor of *L. major* suppressed mouse T cells antigen specific response.[6] The mechanism of such modulation was shown to be due to inhibition of transcription and expression of IL-2R.[6]

In the present study we demonstrated that *L. major* promastigotes subjected to heat shock released immunosuppressive factor(s) capable of directly down-modulating T cell immune response by affecting cytokine production (IFN-γ and IL-4) and cytokine receptor (CD25) expression. It is worth mentioning that the parasites incubated in ambient temperature did not suppress IFN-γ production and CD25 expression but decreased IL-4 production. Therefore, the possibility of nutrient deficiency due to the density of the parasite in the cultures may be ruled out since there were equal numbers of parasites in both conditions. There are some reports on immunomodulation induced by some of the secreted components of *L. major*, including glycoinositolphospholipids which inhibit nitric oxide synthesis, lipophosphoglycan which inhibits protein kinase C activity in macrophages and gp63 which cleaves CD4 molecules on human T cells, interfering with macrophage-T cell interaction.[12-14] However, it is not clear whether these molecules are immediately released when the parasite enters into the skin of mammalian host.

Suppression of IL-4 but not IFN-γ production by *L. major* excreted factor in ambient temperature may be the most interesting finding of this study. It has been demonstrated that down-regulation of IL-4 ameliorates the disease in Balb/c mice.[15] Therefore, characterization and isolation of IL-4 suppressive component of R25 would be intriguing.

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