Iranian J Publ Health, Vol. 36, No.1, 2007, pp.55-61

Original Article

Tc1 Cells Percentage in Patients with Cutaneous Leishmaniasis before and after Treatment with Glucantime

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(Received 15 Apr 2006; accepted 30 Sep 2006)

Abstract

Background: Various responses and different prognosis to specific treatment in different patients from one hand, and importance of IFN-γ producer cells on the other hand impressed us to study Tc1. **Methods:** The study was conducted in Ghaem Medical Center and Bu-Ali Research Institute, Mashhad University of Medical Sciences, Iran from 2001 to 2002. Lymphocytes of 36 patients were counted and cultured. Percentage of different responsible immunity cells in 29 patients, were determined by Flow Cytometry System before and after medication with glucantime (IM). Patients who showed improvement after the treatment were put into group 1 and those who did not recover were labeled group 2. In this self-control clinical trial, sampling method was consecutive non-probability and the results were analyzed by *t*-test consequently. **Results:** The percentage of Tc1 cells showed a significant increase despite of being stimulated with Phorpol-Mristate-Acetate (PMA) among the whole studied patients and group 1 (P= 0.069 and P= 0.040, respectively). While no significant change was observed among patients in group 2. **Conclusion:** This verifies the influence of Tc1 cells for the treatment of patients with CL and perhaps the role of glucantime in improving the cell immunity response through increasing such cells.

Keywords: *Cutaneous leishmaniasis, Flow cytometry, Glucantime and lymphocyte, Tc1*

Introduction

Leishmaniasis is a wide spectrum disease, from a self-healing skin lesion, Cutaneous Leishmaniasis (CL) to a severe visceral form with high rate of mortality, Visceral Leishmaniasis (VL). Every year two million people are affected and ten percent of world human population is at risk of being infected by *Leishmania* parasites. CL is widely spread in four continents and in eighty-eight countries; however, more than ninety percent of its cases are reported from Iran, Afghanistan, Syria, Saudi Arabia, Brazil, and Peru (1).

Khorasan, a province in northeast of Iran, and its capital Mashhad is recognized as one of the important foci of this disease. There are some reports on epidemics of CL in Mashhad in recent years. Resistance to pentavalent antimonial drugs, the main medication for CL in developing countries, in some patients and different levels of response to this treatment in different patients even in the members of the same family have been reported (2). Previous studies, these observations and the importance of the investigation on the effects of epidemiologic factors in treatment of patients and the key role of T-lymphocytes and their cytokines in the fate of infection led us to analyze the lymphocyte subtypes and the pattern of their responses in such patients, before and after medical treatment, by flow cytometry (3) which is a smart method for the study of intra cellular antigens as well as superficial antigens.

Study on cytokines in human and laboratory animals by flow cytometry have been improved recently (4). For instance, flow cytometry was applied to compart and analyze white blood cells from CL in ears of infected mice successfully (5).

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Materials and Methods

Thirty-six new cases of CL were studied, which was confirmed by direct parasitological techniques. None of these cases received any previous treatment. The control group consisted of twenty members of their family who had no history of infection prior to. Overall, three groups were studied:

Patients who were sensitive to treatment and after one month of follow up, their skin lesions were improved clinically and became negative by parasitological exams (group 1).

Those who were resistant-to-treatment, consist of all who did not respond to medical treatment despite one month of glucantime regimen (group 2).

Healthy people who had never been infected, with no systemic and/ or immune compromised disease. This group gave a negative response to *Leishmanin* test (group 3).

Patients with the clinical signs of CL, primarily diagnosed by dermatologists, were referred to Parasitology laboratory of Ghaem Medical Center, where direct parasitological smears of these lesions were used to prove the infection.

For those who were parasitologically positive, physical examination took place and questionnaires were filled out, then ten-milliliter venous blood sample was taken from all patients and controls and immediately divided into three tubes. The first tube was sent to Bu-Ali Research Center for flow cytometry, the second tube and some of patients' serum from third tube was sent for CBC, HIV and HTLV-1 testes to Academic Center of Education, Culture and Research (A.C.E.C.R.) Central Lab. and the rest of the serum samples were kept in -20 ºC. Those who had a history of immunocompromised disease or their HIV and/ or HTLV-1 tests were positive were excluded.

The study took place from August 2001 until October 2002. Simple and non-randomized sampling was done from all different parts of Mashhad.

In this study, Flow cytometry system (Facs Cilibur, BD Company, USA) and specific conjugated monoclonal antibodies for measurement of cytokines (Anti-IFN$_\gamma$-PE, IQ Product, the Netherlands) were utilized.

Patients were referred to Ghaem Medical Center Dermatology Clinic for medical treatment with Glucantime (Specia Co., France).

Local injection in the periphery of the lesion, five injections in five weeks, was prescribed for patients with one or two lesions. Systemic treatment, 20 mg/ kg, daily in twenty days- was ordered for the patients with more than two lesions. Each glucantime ampoule has 1500 mg of effective substance (6).

From the completely 22 patients of the first group, 16 were treated locally and 6 were given systemic treatment and from 7 patients of the second group, 5 patients got local treatment while 2 remaining had systemic injections.

One month after completion of treatments, lesions were investigated for Leishman body. Blood samples were taken in this stage from all patients for flow cytometry and CBC.

The sampling method in this self-controlled clinical trial was consecutive non-probability sampling, and the results were analyzed by *t*-test consequently.

Results

IFN$_\gamma$ producer cells were studied by flow cytometry in 36 patients with CL, before and after treatment with glucantime. However, twenty-nine patients, thirteen males, and sixteen females stayed in the study up to the end. Seven patients were excluded for different reasons such as positive HTLV-1 test, side effects of using drug including urticaria, muscle pain; diffuse erythema, bruising of the injection site, incomplete treatment and migrating to another area.

Patients after primary diagnosis and treatment period were studied for Leishman body and clinical aspects of lesion. Results divided the patients in two groups.

Patients who were improved clinically and in laboratory examination showed no parasite or a few, but they were clinically healed (Group 1).

Patients who showed no improvement clinically and their direct smears were positive for parasite (Group 2).

None of the treated patients was obviously complicated by cardiac or kidney diseases. Almost all of the patients were in middle and low socioeconomic level and their regular diet is full of carbohydrate. None of them had previous history of autoimmune disease or other infections involving cellular immune system.

Comparing patients with the control group, the difference between percentage of Th1 cells and IFN-γ producing cell was significant before treatment, with $P= 0.007$ and $P= 0.080$, respectively. However, there was no significant difference between the other cells ($P> 0.1$). In addition, the difference of Th1 and Tc1 cells percentage in patients, before and after treatment, was significant with $P= 0.063$ and $P= 0.069$, respectively, on the other hand there was no significant difference in Th2 cells, IFN-γ and IL-4. There was significant difference between the first group of patients and the control group in Th1 cells percentage before treatment ($P= 0.016$). The difference of Th1 and Tc1 cells percentage in 22 patients of first group, before and after treatment was significant with $P= 0.039$ and $P= 0.040$ respectively, (Table 1), while it was not significant in other cells. The maximum level of significance was considered as 10 percent.

When patients of the second group compared with the normal group, the percentage of the cells producing IL-4 showed a significant difference just before treatment with $P= 0.043$, however evaluation of immune cells of patients of this group, before and after treatment, did not show any significant difference.

Table 1: The mean percentage of different cells and cytokines in the three groups of study before and after treatment

	Before treatment					After treatment				
	IFN-γ	**IL_4**	**Th2**	**Th1**	**Tc1**	**IFN-γ**	**IL_4**	**Th2**	**Th1**	**Tc1**
Control group	32.32	24.30	7.75	16.35	18.05	32.32	24.30	7.75	16.35	18.05
Group 1	38.95	32.09	8.50	15.95	20.27	50.14	29.82	8.73	15.27	26.18
Group 2	34.86	27.86	15.00	26.71	25.00	36.29	29.57	11.00	20.29	29.43

The histograms belong to one of the patients in the study and show the detection of the lymphocytes with CD3, CD4, CD8 and IFNγ markers (Fig. 1, 2).

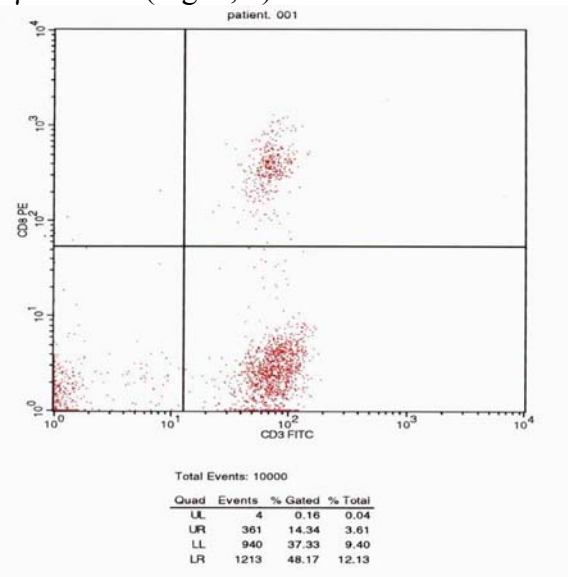

Fig. 1: The evaluation of lymphocytes with CD_8 and CD_3 markers in one of the patients involved in the study after treatment

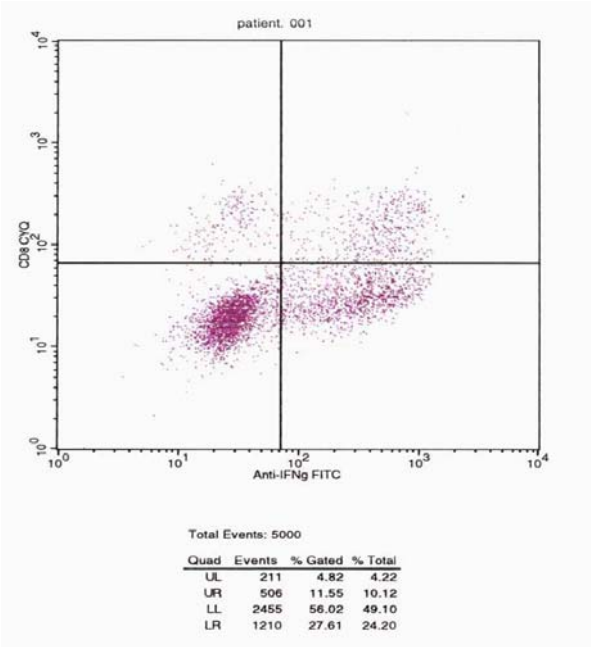

Fig. 2: The evaluation of lymphocytes with CD_8 and IFNγ markers in one of the patients involved in the study after treatment

Discussion

CL is a chronic disease caused by *Leishmania* parasite, which transmits to man thorough infected sand fly (7).

In Iran, CL is caused by *Leishmania tropica* and *Leishmania major* in urban areas and rural areas, respectively. Most of the mononuclear phagocytes, which are the first line of defense against infections, especially intracellular infections, will be infected by these monocellular parasites (8, 9). *Leishmania* species do not prevent from phagolysosyme formation for escaping from mechanisms of intracellular death; however, they enter into inactive macrophages and are able to resist against lysosomal enzymes. This mechanism leads to the parasite division inside the macrophages and do not influenced by killing mechanisms of macrophages and replicate inside the vacuoles (10).

Heinzel and colleagues found the relationship between infection and Th1 cells' response in *Leishmania* infection in mice (11) .Resistance and sensitivity to *L. major* is related to Th1 and Th2 response, respectively on the basis of released cytokines T-helper cells are divided into Th1 and Th2 (12). Variation of cytokines causes activity and different effects on immune cells participating in the disease (13). Although cytokines can cause extensive specific response, this could be the way of understanding the level of resistance or sensitivity of different persons to infection.

Manifestations of *Leishmania* infections depend on parasitic species and the condition of the immune system. Although, general health and physiologic condition of host may affects the process of developing the disease, but the genetic background inevitably plays the major role in defining the manifestation of disease.

In the present study, the percentage of Tc_1 cells increased in all participants and first group of patients, by using of PMA stimulator, before and after treatment with $P= 0.069$ and $P= 0.040$ and revealed the significant difference, while there was no significant difference in the patients of second group.

It has been recognized that subgroup of Th1 cells producing IFN-γ is necessary for healing in leishmaniasis (14), but the role of TC cells (T_{CD8}^{+}) is always controversial. Some scientists believe in their necessity for cleaning the infection and some believe in their inefficiency (15, 16).

It is clear that TC cells have an important role in healing of lesions and the difference between the results of studies is due to the difference in methods (16).

It has been recognized that NK cells and specific activated TC cells are found in cellular infiltration of the site of lesion (17).

Although the role of IL-12 and IFN-γ in formation of immune responses in mice models have been revealed (18) and designing the accurate models for studying of the disease have been shown that TC cells have the most important role in controlling of lesions and healing the primary infections. Mice with lack of T_{CD8}^{+} cells are not able to control the growth of *Leishmania* and in the resistant mice, TC cells accumulate in the site of parasite and involve in the local, systemic, or visceral form of disease interface with formation of different clinical forms of infection (19, 20).

It has also been shown that expansion of this cell population (TC) is possible in the presence of IL-12 (16). Recently, Scott has proved that production of IL-12 is necessary for activating the cellular immune response and removal of the infection (21) .Many of the CD8+ cytotoxic T cells contain proteins in their granules that if released directly in front of plasma membrane of target cell, they could destroy the cell (22).

We know that T_{CD8}^{+} cells protect the mammals against viruses and intracellular pathogens and cancers. These cells proliferate 2 to 3 days after stimulation by MHC dependent class I antigen and find killing activation potential (23). Although TC cells have cellular storage equals to 5×10^{11} cells, only a small fraction of them activate and replicate in the infection, but this small fraction extent and replicate as well during a short period that they will be the large force for destroying a considerable number of the infected cells and removing the infection (19).

The activated cells could be followed after removing the infection and later amount of them does not depend on antigenic stimulation. However, each cell is programmed in a way that when it activates, divides 7 times and increases the activated cell population for at least 128 times. Simultaneously, the number of infected cells decreases greatly resulting from its killing activation. Therefore, the number of T_{CD8}^{+} cells depends greatly on level of antigenic stimulation through MHC class I associated antigen presenting cells. Fatigue resulting from persistent stimulation of T_{CD8}^{+} cell, makes it to lose its killing activation, in spite of developing to activation (replication) stage.

These factors including persistent antigenic stimulation through the infected cells by *leishmania* might be one of the reasons that TC cells increased in the present study. However, based on a recent study (23), such increasing should be seen even in the first week of infection.

Vieira and colleagues showed that T_{CD8}^{+} cells in biopsy samples were much more than T_{CD4}^{+} cells (24). In addition, in another study, it was revealed that T_{CD8}^{+} cells were increased in individuals vaccinated against leishmaniasis (25) especially when the amount of activated T_{CD8}^{+} cells in individuals who respond rapidly to vaccine was much more than volunteers who respond slowly (26). In addition, the specific T_{CD8}^{+} cells were also seen in peripheral blood of the individuals affected by mucocutaneous leishmaniasis (MCL) (27).

Therefore, as a conclusion and according to these studies, we could get two interesting points of increasing T cell.

It was said that the production of IL-12 is necessary for causing the cellular immune response and removing the infection (21). By considering the fact that IL-12 is a facilitating factor for cellular immune response and if we remind the previous study (24), maybe IL-12 makes TC cells change to activated TC cells and increases them more than any other cells,

which are the most important arm of the cellular immune response for removing the intracellular infections and this could control the primary infection.

The mechanism of increasing T_{CD8}^{+} cells in infected individuals by leishmaniasis that is the pattern of the study of cellular responses should be studied more in vitro, because based on the most recent study by Wong (23), it could be the way of opening the new fields in producing and defining the time of the injection of efficient vaccines on cellular immunity.

It is also possible that the increase of T_{CD8}^{+} cells in the present study is the result of drug intervention, if this come to truth, the mechanism of this phenomenon should be assessed more. In other diseases that cellular immune system especially TC cells are greatly weakened, drugs may affect the stimulation of this part of immune system.

In conclusion, increasing of Tc1 cells population in this study is either due to the effect of IL-12 and activation of Tc1 cells or perhaps this is the drug intervention, which may cause Tc1 cells population growth.

Acknowledgements

The authors wish to thanks the Vice Chancellor for Research, Mashhad University of Medical Sciences for his constant financial support.

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