Improvement of *Leishmania major* Amastigote ELISA Test Using Various Antigen-Antibody Dissociating Agents and Pepsin Digestion

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Summary

Amastigotes are main pathogenic stage of *Leishmania* parasite in mammalian host. During the course of leishmania infection the amastigote surface would be covered by host proteins like immunoglobulins and complement components. These proteins produce false positive reaction and low signal/noise ratio in evaluation of antibody raising against lesion-derived amastigote by indirect ELISA. To overcome this problem we were treated lesion-derived amastigotes with several known antigen-antibody dissociating agents like glycine, potassium thiosufate, chloroquine, guanidine HCl, urea, etc. Our results indicated that these agents were ineffective in ELISA results and could not produce a clear discrimination between positive and negative controls. We were also studied the effect of pepsin digestion on amastigotes and coated these pepsin-treated amastigotes to check the ELISA results. Our results revealed combination of tests including pepsin treated amastigotes coated in alkaline pH and protein A-peroxidase conjugates as secondary antibody in an indirect ELISA design, possess high signal/noise ratio i.e. low background color.

Keywords: *Leishmania major*, amastigote, antigen-antibody dissociating agent, pepsin, ELISA

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Introduction

Leishmania infection mainly affects people in tropical and subtropical regions of the world and approximately 12 million individuals infected annually (Chang *et al* 1990, Hepburn 2000, Gupta 2001). The parasite presents in two morphological forms; promastigote and amastigote, which could be found in sand fly and mammalian host respectively. When metacyclic promastigotes were entered into the mammalian body, host antibodies and complement components especially C3 attached to the promastigote surface (Birttingham *et al* 1995, Green *et al* 1994). Promastigotes initially employed these proteins for binding and invading the macrophage, the natural host cell of parasite (Rittig & Bogdan 2000, Resenthal *et al* 1996, Guy 1993). In macrophage the parasite exploits their virulent factors to resist against killing power of macrophage and gradually transformed into amastigotes, which is different from promastigote in terms of morphology, some metabolic pathway and their special antigens (Gupta *et al* 2001). Amastigotes proliferate inside the macrophage and ultimately rupture them. They are released into the extra-cellular environment and perhaps in this stage they are exposed again to abovementioned host proteins and covered by them (Guy 1993). Studies on amastigote showed that immunoglobulins, complement components especially C3, C4 and other host proteins like fibronectins are present on amastigote surface (Guy & Belosevic 1993). Since these proteins especially Igs produce false positive reaction in serological tests, it is essential to remove these proteins from amastigote surface (Guy & Belosevic 1993). For experimental work amastigotes have been obtained from laboratory animal lesions (lesion-derived amastigote) or from culture of those in cell-free media or various cell-lines (axenic amastigote) (Jaffe & Rachamim 1989, Gupta *et al* 2001). Since a reliable and reproducible technique for axenic culture of *L.major* amastigote has not been introduced, the *in vivo* source is remained on lesion-derived amastigotes which are covered by host proteins (Gupta *et al* 2001). To overcome the problem of attached host proteins lesion-derived amastigote was

isolated and frequently passed through the J774.1 macrophage cell line (Jaffe & Rachamim 1989) or was isolated from SCID mice which has no immune response against amastigote (Guy & Belosevic 1993). In a similar circumstances to dissociate antibody from red blood cells, some chemical and physical agents have been used (Revig *et al* 1977, Landsteiner & Miller 1990, Rubin 1963, Edwards *et al* 1982). In this study we were tested several antigen -antibody dissociating agents to detach antibody from amastigote surface followed by assessing the results by ELISA. We were also examined effect of pepsin digestion on lesion-derived amastigote followed by coating the pepsin-treated amastigotes in different pH buffers to prevail on host proteins issues and obtain a clear cut-off value between positive and negative serum controls. Eventually to increase the sensitivity of ELISA, two kind of secondary antibody conjugates including mouse IgG whole molecule and protein A peroxidase conjugates were also tested.

Materials and Methods

Parasite strain. *L.major* strain (MRHO/IR/75/ER) was donored by Dr. E.Javadian, School of Public Health, Tehran University of Medical Sciences, Iran. Pathogenicity of parasite was maintained by regular passage through BALB/c mice.

Experimental infection and isolation of lesion-derived amastigote. Female BALB/c mice (6-8 weeks old) were obtained from Animal Breeding Division of the Pasteur Institute of Iran. Mice were inoculated with 10^6 *L.major* promastigotes in the stationary phase (a 7–day-old culture after changing the color) intradermally on the hind leg. Two months later, the mice were bled and sacrificed. Sera were collected and used as positive control in ELISA test and the lesions were removed to isolate amastigotes according to Glaser *et al* (1990) method. Sera from normal mice were used as negative control.

Preliminary ELISA test. The ELISA was performed as described by Jaffe and Rachamim (1989) with a few modifications. A suspension of 10^7 lesion-derived

amastigotes/ml was prepared in carbonate buffer (pH9.4) and 100µl of it used for coating 96-well microplate (GRIENERS). The microplates were incubated for 1h at 37°C and washed three times with PBS+0.05%Tween20 (Sigma). Then they were blocked by 100µl PBS+1%BSA (Sigma) for 30min at room temperature and washed again as above. A total of 8 replicates mice sera (positive and negative controls) were used. They were diluted 1:100 with PBS (pH7.4) and 100µl of each serum was added into the duplicate wells. Sera were incubated for 1h at 37°C followed by washing as above. After that, 100µl of 1:4000 anti-mouse IgG (whole molecule)-HRP conjugate (Sigma) diluted with PBS+0.05%Tween20 was added and the microplates were leave for 1h at 37°C to complete the reaction. After washing away unbound conjugates, 100µl of *O*-Phenylenediamine (Sigma) prepared as 1mg/ml in 0.1M citrate-phosphate buffer pH5.0 containing 0.03% hydrogen peroxide was added into each well and the plates were incubated for 15min in room temperature. The reaction was stop by adding 100µl of 2N H_2SO_4 and optical density (OD) at 492nm was read by microplate reader (ICN FLOW).

Treatment of lesion-derived amastigote with antigen-antibody dissociating agents. The amastigotes suspension (10^7 cells/ml) was washed three times with PBS+2mM EDTA and the final pellet was treated with heat, ether, chloroquine, glycin, potassium thiocyanate 3.5M, guanidine hydrochloride 6M, urea 8M and $MgCl_2$ 5M elutions according to the Landsteiner and Miller (1990), Rubin (1963), Edwards *et al* (1982), Revig *et al* (1977), Goding (1986) and Hausen *et al* (1982) methods, respectively. Citric acid elution was performed according to the technique introduced by Burich *et al* (1986) on the amastigote suspension. The pepsin proteolytic digestion was used based on the Coligan *et al* (1994) technique. To coat pepsin-treated amastigotes in different pH buffering systems the following buffers were used: HCl/KCl (pH1.4), glycine/HCl (pH2.4), citrate/Na_2HPO_4 (pH3.2), acetate (pH4.3 and pH5), PBS (pH6.4 and pH7.2), Tris (pH8.4), sodium carbonate (pH9.5), carbonate (pH10.2), phosphate (pH11.3) and hydroxide chloride (pH12.0)

(all chemicals from Merck). The untreated amastigotes were also coated in the same buffers as control. At last ELISA test was accomplished as explained before.

Changing the secondary antibody conjugate. To increase signal/background ratio in ELISA anti-mouse IgG (whole molecule)-HRP conjugate (Sigma) was substituted with protein A-HRP conjugate (Sigma) in pepsin-treated amastigote ELISA test.

Statistical analysis. To achieve acceptable cut off value and to find that the difference between positive and negative controls, paired *t*-test was done (Sigma STAT) and the p-value of <0.05 ($P=0.001$) was obtained.

Results

Preliminary ELISA test for lesion-derived amastigotes. The results showed that differentiation between positive and negative controls were not possible by routine ELISA test. Both normal and infected mice sera as well as control (amastigote coated well without serum) have high OD values approximately 1, and cannot distinguish from each other by ordinary ELISA design.

Treatment of lesion-derived amastigotes with antigen-antibody dissociating agents. The results of various antigen-antibody dissociating agents effects on lesion-derived amastigotes are shown in Table 1.

Table 1. *Effect of various antigen-antibody dissociating agents on lesion-derived amastigotes by ELISA test*

Control	Heat	Diethyl ether	Chloroquine	Citric acid	Glycine	Potassium thiocyanate	Guanidine HCl	Urea	$MgCl_2$
Positive	0.118	0.505	0.380	0.985	0.300	1.060	0.640	0.520	0.505
Negative	0.670	0.350	0.200	0.820	0.250	0.870	0.534	0.525	0.470
Without serum	0.570	0.220	0.160	0.790	0.224	0.860	0.520	0.490	0.470

Pepsin Treatment: Preliminary study using pepsin treatment showed some promising results, which revealed that infected mice serum has higher OD value (0.960) than both normal mice serum (0.480) and control without serum (0.480). The results showed pepsin-treated amastigote as antigenic source in ELISA cause increase discrimination between positive and negative controls. The estimated cut off value which obtained by negative control mean+3SD was 0.377. Anti-mouse IgG-HRP conjugate was used as secondary antibody and the estimated signal/ratio was 2.

Coating pepsin-treated amastigotes in different pH buffering systems. The pepsin treated amastigotes were mixed in various buffers and each separately coated in appropriate wells. The untreated amastigotes also used as control for each buffering system. The results are shown in table 2 and figure 1.

Table 2. *ELISA results of various buffering systems used for coating pepsin-treated lesion derived amastigotes*

Buffer	Without treatment				Pepsin treatment			
	Normal	Infected	Control	S/N	Normal	Infected	Control	S/N
HCl/KCl (pH1.4)	1.17	1.20	1.19	1.008	0.13	0.29	0.09	3.2222
Glycine/HCl (pH2.4)	0.33	0.51	0.29	1.758	0.15	0.30	0.08	3.7500
Citrate/Na_2HPO_4 (pH3.2)	0.35	0.42	0.24	1.750	0.16	0.30	0.05	6.0000
Acetate (pH4.3)	0.29	0.46	0.27	1.703	0.24	0.44	0.30	1.4667
Acetate (pH5.0)	0.32	0.49	0.26	1.884	0.07	0.15	0.03	5.0000
PBS (pH6.4)	0.46	0.68	0.40	1.700	0.06	0.18	0.04	4.5000
PBS (pH7.2)	0.38	0.57	0.31	1.838	0.09	0.22	0.05	4.4000
Tris (pH8.4)	0.39	0.59	0.43	1.372	0.11	0.20	0.09	2.2222
Sodium carbonate (pH9.5)	0.52	0.70	0.46	1.521	0.20	0.35	0.21	1.6667
Carbonate (pH10.2)	0.68	0.83	0.61	1.360	0.28	0.87	0.25	3.4800
Phosphate (pH11.3)	0.79	0.93	0.69	1.347	0.45	1.08	0.45	2.4000
Hydroxic acid (pH12.0)	0.73	0.91	0.63	1.444	0.48	0.81	0.50	1.6200

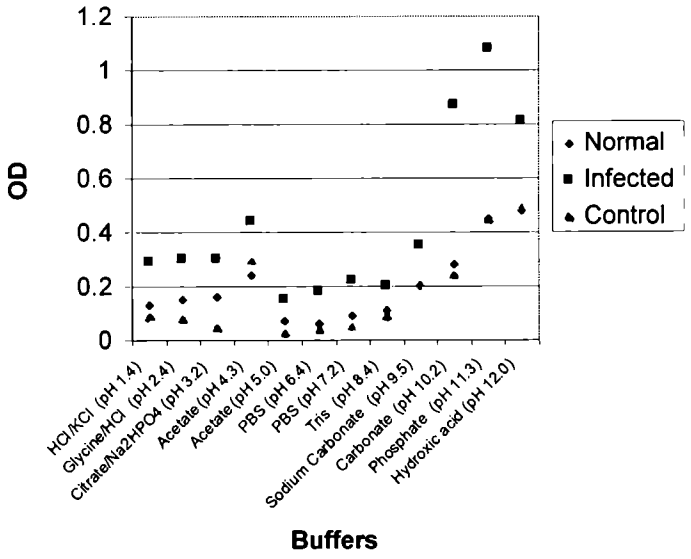

Figure 1. *ELISA results of pepsin digested amastigotes coated in various pH*

Changing the secondary antibody conjugate. To increase signal/background ratio of ELISA anti-mouse IgG-HRP conjugate was substituted with protein A-HRP conjugate. The result showed higher OD value of infected mice serum (0.575) than both normal mice serum (0.170) and control (0.165) indicating employment of protein A HRP-conjugate improved S/N ratio to 3.48 which is higher than other conjugate (S/N:2).

Discussion

Amastigotes of *Leishmania* are main pathogenic stage of parasite by which the protozoa can survive inside its relevant mammalian host. Inside the host, once the metacyclic promastigotes entered by sandfly inoculation, as a part of nonspecific immune response, antibodies and complement system are initially provoked against the invading parasite (Alexander 1999, Peters 1995) and covered the parasite

surfaces. Recent study has been shown not only the parasites do not prevent by this defense mechanism but also they use them as an effective tool for exerting their pathogenicity (Kane & Mosser 2001). Guy (1993) has been shown that these proteins also present on amastigotes surface which is proliferate inside the parasitophorous vacuole of macrophage.

Presence of these proteins especially Igs on amastigotes surface lead to false positive reaction in ELISA test due to their direct reaction with secondary antibody conjugate. To eliminate or reduce false positive reaction and achieve better S/N ratio in ELISA we used several agents including those known as antigen-antibody dissociating agents and enzyme digestion. Shi *et al* (1997) applied heat treatment, 50-60°C for 2h, to retrieve their studied antigens but this treatment has not considerable effect on the antigens. Rubin (1963), Edwards *et al* (1982) and Burich *et al* (1986) successfully used ether, chloroquine and citric acid treatments to elute attached antibodies especially IgG class on red blood cells surface. Revig *et al* (1977), Goding (1986), Ezaki (1996 and 2000) and Hausen *et al* (1982) had same experiments with potassium thiocyanate, urea, $MgCl_2$, etc. Although these techniques were successfully used in antibody elution from red blood cells surface but they were not efficient in our study on amastigotes. The main cause of this drawback might be due to the nature of the antigens and it is highly supposed that amastigotes need special condition to unmask their antigens. It also seems that combination of several elution methods to create a tests battery for retrieving amastigotes antigens would be a valuable suggestion. Shi *et al* (1997) concluded that combination of tests has greater value to unmask antigens.

Pepsin, an enzymatic agent, has dual biological effects. It splits antibody molecules and also can be use as antigen recovering or retrieval agent. Ezaki (1996 and 2000) and Shi *et al* (1997) introduced pepsin as effective agent for antigen retrieval. Our results on pepsin treatment of lesion-derived amastigotes indicate that pepsin digestion reveal clear discrimination between positive and negative mice

sera. Hazelbag (1995), Frost *et al* (2000) and Bahn (1988) have similar results on pepsin treatment of antigens. Alkaline pH enhanced differentiation effect on positive and negative controls of pepsin treatment. Carbonate buffer (pH10.2) especially has highest advantage to distinguish positive and negative controls (Figure 1). Shi *et al* (1997) suggested alkaline pH is more useful for antigen recovery.

Another factor which increase S/N ratio and raise sensitivity of ELISA is type of secondary antibody conjugate. In this study replacement of anti-mouse IgG-HRP conjugate with protein-A-HRP conjugate increased S/N ratio from 2 to 3.48, the lower background color. The same studies have been showed that protein-A-HRP conjugate is a valuable tool for the localization of antigens,which improves serodiagnosis of leishmaniasis especially visceral type (Dubois-Dalcq 1977, Reed 1990). In conclusion, employing pepsin-treated lesion-derived amastigote in alkaline pH as antigen source and using protein A HRP-conjugate as secondary antibody conjugate improve the ELISA test for diagnosis of *L.major* amastigote and achieved acceptable S/N ratio.

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