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THE FML (FUCOSE MANNOSE LIGAND) OF *LEISHMANIA DONOVANI*. A NEW TOOL IN DIAGNOSIS, PROGNOSIS, TRANSFUSIONAL CONTROL AND VACCINATION AGAINST HUMAN KALA-AZAR

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The Fucose-Mannose Ligand (FML) of Leishmania donovani *is a complex glycoproteic fraction. Its potential use as a tool for diagnosis of human visceral leishmaniasis was tested with human sera from Natal, Rio Grande do Norte, Brazil. The FML-ELISA test, showed 100% sensitivity and 96% specificity, identifying patients with overt kala-azar ($p < 0.001$, when compared to normal sera), and subjects with subclinical infection. More than 20% apparently healthy subjects with positive reaction to FML developed overt kala-azar during the following 10 months. In the screening of human blood donnors, a prevalence of 5% of sororeactive subjects was detected, attaining 17% in a single day. The GP36 glycoprotein of FHL is specifically reconized by human kala-azar sera. The immunoprotective effect of FML on experimental* L. donovani *infection was tested in swiss albino mice. The protection scheemes included three weekly doses of FML, supplemented or not with saponin by the subcutaneous or intraperitoneal routes and challenge with 2×10^7 amastigotes of* Leishmania donovani. *An enhancement of 80.0 % in antibody response ($p < 0.001$) and reduction of 85.5 % parasite liver burden ($p < 0.001$) was detected in animals immunized with FML saponin, unrespectively of the immunization route.*

Key-words: Glycoconjugate. Leishmania donovani. *Diagnosis. Prognosis. Kala-azar. Visceral leishmaniasis. Blood transfusion. Leishmanial antigens.*

Visceral leishmaniasis or kala-azar is a chronic and frequently lethal disease, caused by parasites of the *Leishmania donovani* complex. The disease is characterized by fever, malaise, loss of weight, hepatomegaly, splenomegaly, anemia, leukopenia, hypergammaglubulinemia and progressive suppression of the cellular immune response. Kala-azar is often fatal if untreated after the onset of symptoms. Furthermore, chemoterapy shows several undesirable colateral effects. The total number of *Leishmania* infected people all over the world is being estimated in twelve millions. Five hundred thousand of these cases, each year, correspond to visceral leishmaniasis[25]. This number is probably underestimate since an accurate and uniform early diagnose of disease has not yet been established. Visceral leishmaniasis is spreading out in the World due to the increased resistance of parasites to chemoterapy and of insect vectors to insecticides[41]. In Brazil, the disease is disseminating from the North and North-East regions of the country and has recently been described in the State of Rio de Janeiro, previously considered as not endemic[20].

The current diagnostic in endemic and epidemic areas is still based on clinical analysis and identification of parasites in bone-marrow aspirates. This very invasive method can give positive results in cases of advanced kala-azar. The cure of the disease cannot be monitored, and preclinical patients are not detected, their clinical evolution is not followed-up, and they are a potential reservoir of the disease in endemic areas. Anti-*Leishmania* circulating antibodies can be detected in human serum soon after infection: they attain high titers with the evolution of the disease and progressively

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disappear after its treatment. The sorologycal diagnosis of kala-azar has been proposed as an alternative by several works, based on antigens that show different degrees of purification, and developping assays with diverse sensitivity and speciffìcity ranges[5 32 35 38]. The stability of these antigens has also been discussed[32]. Nevertheless, a non invasive method of monitorization of the disease was not yet not available in Brazilian Public Health Services.

Infected monocytes, as well as, polymorphonuclear neutrophiles[7 19] and eosinophiles[9] were described in the blood, as potential carriers of *L. donovani* parasites from the skin to viscera and bone marrow. In older studies[19 7], and in some recent reports on human kala-azar[22], the presence of infective parasites in the secreta of the upper respiratory and digestive tracts, as well as in urine and faeces were cited. Only occasionally, inoculation of leishmaniasis by the transfusional via were cited[10]. Recent indirect evidences support the need of the study of the blood mediated contamination with kala-azar in humans. *Leishmania tropica* was identified in bone marrow of american soldiers returning from Operation Desert Storm with viscerotropic leishmaniasis, and for this reason all individuals that traveled to that region were recommended to be deferred as blood donors[12]. Notwithstanding, the existence of a blood-mediated contamination is up to now not admitted as a potential danger for leishmanial infections, and no control of blood is performed for the detection of leishmaniasis in blood banks, even in regions reported to be endemic for kala-azar, and expected to harbor a large number of subjects with subclinical infection.

The analysis of the protective potential of *Leishmanial* antigens to cutaneous leishmaniasis in murine models has been the focus of detailed studies. The World Health Organization encouraged the development of vaccines for leishmaniasis, using total or partially identified parasite lysates and crude antigens, that could induce a clear prophylactic immune response[24]. This kind of vaccine has shown to be effective against cutaneous leishmaniasis[11 21]. The vaccines derived from whole killed *Leishmania* are in study in Brazil (*L. amazonensis*), Iran (*L. major*) and Venezuela (*L. mexicana and L. braziliensis*)[25]. However, for human visceral leishmaniasis, no vaccine is up to now available. Only few studies on immunization against *Leishmania donovani* have been undertaken in the murine model, using total parasites[26 18 14] or purified antigens[17].

In recent studies, we described the isolation of the FML (Fucose Mannose Ligand) of *L. donovani* promastigotes, a complex glycoproteic fraction that strongly inhibits promastigote[27] and amastigote infection of murine macrophages[29]. Its proteic moiety is mainly composed of acidic and apolar aminoacid residues. Among its neutral sugar components, fucose (10%), mannose (47%), galactose (12%) and glucose (30%) were described[27]. FML developped a species-speciffic effect on the inhibition of promastigote uptake by macrophages *in vitro*[28]. This is a potent antigen for rabbits, hamsters and mice[28 31 30]. Its main antigenic fraction is a 36kD glycoprotein, recognized by most mouse IgG anti-FML monoclonal antibodies, and present at the surface of both the promastigote and amastigote forms of *L. donovani* parasites[29]. The protective potential of FML on visceral leishmaniasis was analyzed in the isogenic CB-hamster model[31]. We studied the effect of three intraperitoneal weekly doses of FML ($100\mu g$) in saponin ($100\mu g$), followed by an intracardiac injection of 10^7 amastigotes. Protection was speciffic and highly significant (87.7%, $p<0.01$) in the enhancement of anti-FML antibodies titers, of the splenocyte proliferative response, and the intradermal delayed hypersensitivity reaction to antigen, as well as in the decrease of the parasite burden in spleen and of splenomegaly[31]. An analogous study in the inbred balb/c mice model showed an average of 84.2% ($p<0.001$) of protection in antibody titers, splenocyte *in vitro* proliferation and liver parasitic load[30].

In the present study we report the use of the FML antigen in diagnosis, prognosis[32] and transfusional control of human visceral leishmaniasis, monitoring the epidemic and endemic disease in Public Health Services of Natal, Rio Grande do Norte, Brazil. This work shows the results obtained with the implementation of the FMLELISA assay in the routine of the laboratory sorologycal screening of the Hospital de Doenças Infecciosas Giselda Trigueiro and the Centro de Hematologia e Hemoterapia-HEMONORTE, with

the support of the Fundação Nacional de Saúde, FNS. Furthermore, preliminary results of the analysis of the FMLvaccine in the outbred swiss albino mouse model, are described.

MATERIAL AND METHODS

Human sera

A total of 462 sera was analyzed in this study. A series of 149 sera was collected by the Fundação Nacional de Saúde do Rio Grande do Norte, in the peri-urban area of Natal, RN (North East Brazil), a focus of a recent outbreak of kala-azar. Ten of the sera in this series came from subjects that had a patent kala-azar at the time of serum collection. A series of 26 sera of kala-azar patients, before and during their treatment, was obtained at the Hospital de Doenças Infecciosas Giselda Trigueiro, Natal. All the 36 patients with clinical symptoms of acute kala-azar (loss of weight, lymphadenopathy, hepatomegaly and/or splenomegaly, and fever) were submitted to bone marrow aspiration. Parasitologic analysis confirmed the presence of *Leishmania* amastigotes in the Giemsa-stained smears. After the serum collection, patients were submitted to anti-*Leishmania* treatment. Thirty five sera of patients that have had kala-azar were collected at least three months after the end of the treatment with antimonials or amphotericin-B, during their clinical and serological follow-up[32].

Twenty one persons with no patent signs of disease, neighbors and relatives of kala-azar patients, or owners of dogs with *L. donovani* infection, were also included in the study. We also analyzed the reactivity with FML of 21 sera of patients with cutaneous or mucocutaneous leishmaniasis from Rio de Janeiro and Minas Gerais states, obtained from Dr. S.M. Coutinho, Fundação Oswaldo Cruz, (Rio de Janeiro), 22 sera of fully characterized patients with chronic Chagas disease from Bambuí, Minas Gerais, and 18 sera from healthy adult blood donors from the Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, that were screened and considered negative for Chagas disease, HIV I and II, hepatitis, and syphilis[32]. For differential control diagnosis, sera of patients with fever and/or hepatosplenomegaly due to *Paracoccidioides brasiliensis* infections (1), lymphoma (2), typhus (1) and hemophagocytic disease (1) were obtained from the Departamento de Doenças Infecciosas, UFRJ, and Departamento de Pediatria UERJ, Rio de Janeiro, RJ.

A series of 171 sera of volunteer blood donors was obtained from the blood bank Centro de Hematologia e Hemoterapia-HEMONORTE, and tested for their reactivity with FML. Informed consent for this study was obtained from all the subjects included in this study.

FML-ELISA qualitative assay

The FML complex was obtained from *Leishmania donovani* (LD-1S/MHOM/SD/00-strain 1S) promastigotes as previously described[29]. Titration of normal human, kala-azar, Chagas' disease and cutaneous leishmaniasis patient's sera pools against FML antigen was performed in order to establish the optimal antigen concentration. The micro-ELISA method was done as described[32], using FML diluted in carbonate buffer (pH 9.6), to sensitize flat-bottom 96-well plates (Haemobag, Ribeirão Preto, SP, Brazil). Antibodies were detected by peroxidase-labeled protein-A (Sigma, St Louis, MO). Reaction was developed with O-phenyldiamine (Sigma), interrupted with 1N sulfuric acid, and monitored at 492nm. Sera were analyzed by double blind tests, in triplicates, and results expressed as mean values. Positive and negative control sera were included in each test. The absorbance values of sera were compared at 1:100. The *cut off* limit between the normal sera from a non-endemic area and sera of patients with parasitologically confirmed kala-azar, was identified using the Youden's J index[43]. Signifficance of the differences between groups of sera was established by a standard *t* test.

Western Blot analysis

FML was submitted to SDS-PAGE under reducing conditions, in 10% slab "baby" gels and transferred to nitrocellulose. Strips were incubated with human sera diluted in blocking buffer, overnight at 4°C, washed and developed with peroxidase-labeled protein-A followed by diaminobenzidine (Sigma).

Vaccination assays

2,5 month old swiss albino female mice were obtained from the Biotério Central, Instituto de Microbiologia da UFRJ. Groups of 6-8 females were immunized with three either subcutaneous or intraperitoneal doses of 150μg FML and 100 μg saponin (Riedel-de Haën, Seelze, Germany) in 0.2ml sterile 0.95% saline, with weekly interval. Saline and saponin treated controls were included. Seven days after immunization, sera were collected and animals were challenged by intravenous injection of 2×10^7 *L. donovani* amastigotes, obtained from infected hamster's spleens[6]. Animals were sacrificed by etherization, 15 days after infection, and their liver and spleen parasite-load was monitored in Leishman-Donovan Units of Stauber of Giemsa stained imprintings (LDU = number of amastigotes/ 1000 cell nuclei x mg organ weight). Sera of animals before and after infection were analyzed by ELISA using 2μg FML/well and reacted with a Peroxidase labeled anti-mouse IgG (Sigma, Co.). ELISA results were expressed as scores (sum of all absorbances at 492nm, up to the end-point titer)[32]. Statistical comparison of the groups was done by the Student *t* test.

RESULTS

Standardization of ELISA experiments was done using FML in dilutions from 0.03 to 2μg per well, reacted with pools of normal human sera from a non-endemic area, sera of patients with kala-azar, tegumentar leishmaniasis and Chagas disease, in dilution 1:2000. The results presented in the Figure 1, indicated the antigen concentration of 0.125μg / well as the highest that gave no unspecific reaction. All the studied samples were titered and scored in this condition.

The individual absorbance values in FML-ELISA assay for 1:100 diluted sera are represented in Figure 2A. The mean value of Abs 1:100 ($X \pm SE = 0.452 \pm 0.038$) of patients with acute kala-azar was significantly different from the one of the normal subjects of a nonendemic area ($p << 0.001$). Eight standard error values separated

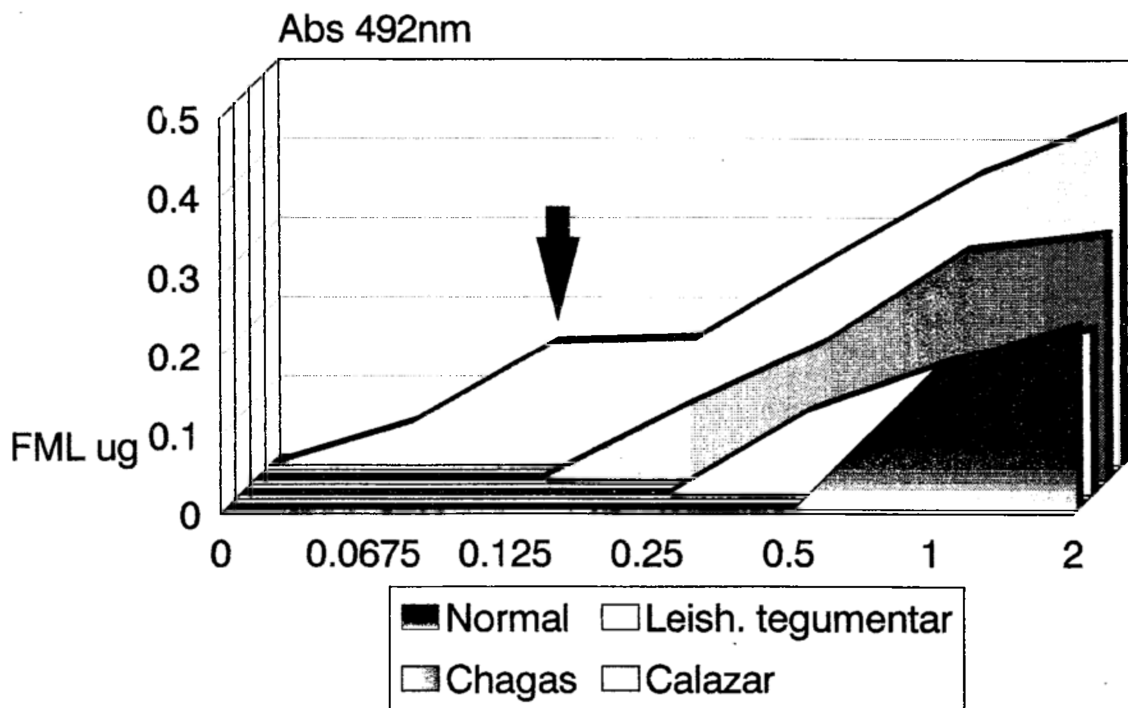

Figure 1 - Determination of FML optimal concentration for ELISA test.
ELISA test of FML reactivity with a normal serum, and sera from patients with tegumentar leishmaniasis, kala-azar or Chagas disease. The arrow indicates the concentration used in ELISA assay standardization.

the two means. The use of mean value + 2SD in the FML-ELISA assay attain the maximal Youden value=1, corresponding to the absence of *false positive* or *false negative* results (cut off value = 0.204). Sera of patients with chronic Chagas disease showed, as expected from standardization experiments, a very reduced reactivity (Abs 1:100 mean value 0.149). In the endemic area, (Figure 2B). the FML-ELISA assay functioned as a highly discriminating tool. It allowed to distinguish the non-reactive sera from the endemic area (NE) (n =86), from the group of subjects without any sign of leishmanial infection but reactive with FML (RE) (n = 59). We interpret these subjects as a subclinical but active *Leishmania* infection, in view of their clinical evolution. During the ten months following the first study of 41 sera from the kala-azar outbreak region, nine of the inhabitants of the studied endemic area spontaneously searched medical treatment for the severe disease (NKA). Kala-azar was diagnosed by identification of amastigotes in the bone-marrow aspirate. Among these, six had had high titers of anti-FML antibodies and the highest titers had been observed in two patients that eventually died of severe kala-azar. During the same period, at our knowledge, no

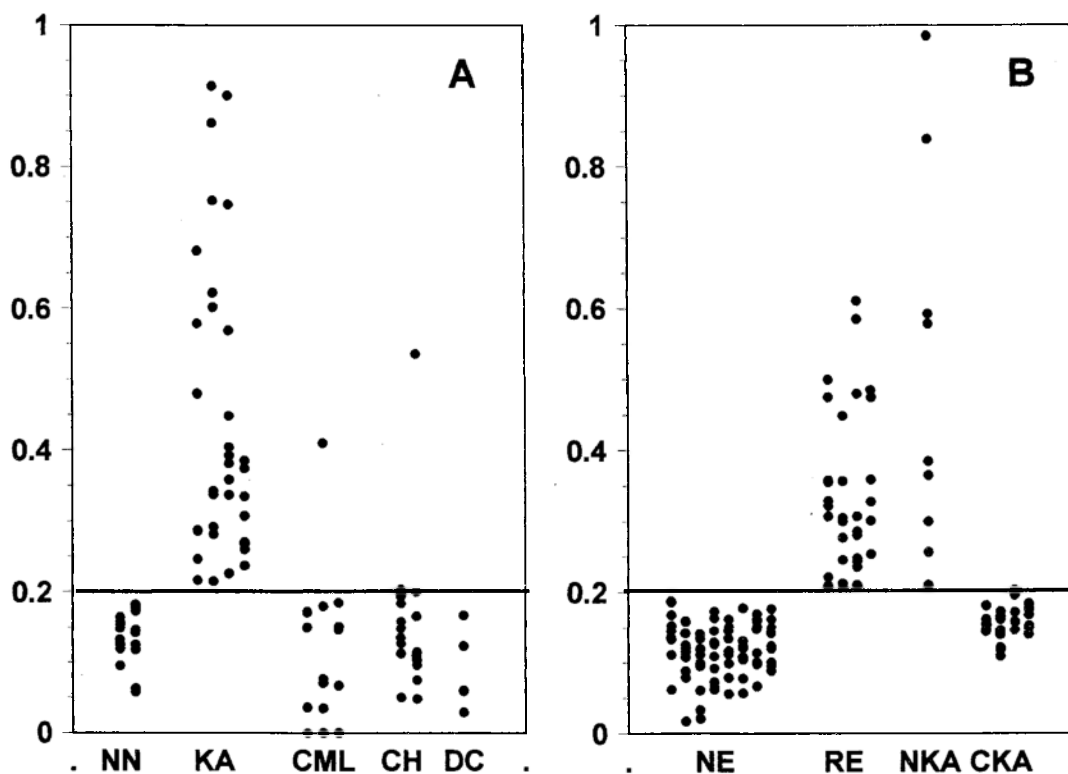

Figure 2 - The FML-ELISA assay in an endemic and non-endemic region for human visceral leishmaniasis.
*In A: FML-ELISA reactivity with sera of normal subjects from a non-endemic area (NN), patients with kala-azar diagnosed by the parasitological analysis of bone marrow aspirates (KA), patients with cutaneous or muco-cutaneous leishmaniasis (CML), patients for differential diagnosis (DD) (*Paracoccidioides brasiliensis, *lymphoma, typhous and hemophagocytic disease), patients with chronic Chagas' disease (CH). In B: normal subjects from an area endemic for kala-azar (NE), subject from the endemic area without overt disease, but reactive with FML (RE), subjects from the endemic area, that had been reactive for FML in the first screening of sera, but did not present at the time overt disease, which developed severe kala-azar in the subsequent ten months period (NKA), and kala-azar patients that have been submitted to anti-parasite treatment and have been cured from the leishmanial infection (CKA). Dots represent absorbance values at sera dilution of 1:100; the horizontal line represents the* cut off *value (0.204).*

subjects with negative reaction to FML developed kala-azar. Consequently, among 41 sera that had shown high or low reactivity with FML but had not clinical signs of kala-azar, more than 20% developed the overt disease in the ten month period. This number is probably underestimated, since a full clinical and serological survey of the same area has not been completed[32]. Sera of patients with fever and hepatosplenomegaly due to *Paracoccidioides brasiliensis* infection (1), lymphoma (2) and hemophagocytic disease (1) gave also negative results in the FML-ELISA assay. These results determined 100% sensitivty and 96 % specificity.

A random monitoring of anti-FML reactivity among 171 volunteer blood donors at the blood bank Centro de Hematologia e Hemoterapia-HEMONORTE, Natal, was performed, testing an average of 24.4 diary donor samples, for a period of seven consecutive days. The results obtained are summarized in Table 1. The FML-ELISA test showed the clearly positive reaction for 8 donors. The other 163 samples showed no reactivity (X+SE= 0.138+ 0.002). This values represent a total of 5 % of samples and were distributed attaining even 17% in a single day, no one of which had a positive reaction for Chagas's disease, hepatites, HIV nor syphilis or a decreased hematocrite value. Conversely, among the nonreactive donors, two cases of hepatites B were detected indicating that also in this assay the FML-ELISA showed to be highly specific.

Since the FML components can be separated by electrophoresis, we have analyzed by Western Blot the reactivity of human sera with FML (Figure 3).

Table 1 - Blood donor samples reactive in the FML-ELISA assay.

Donor number	FML-ELISA reactivy at 492nm	Date
462.	.506	30.8.94
463.	.291	31.8.94
464.	.231	1.9.94
465.	.271	1.9.94
466.	.250	1.9.94
467.	.232	1.1.94
468.	.220	1.9.94
469.	.232	5.9.94

All the samples were tested at 1/100 sera dilution. Results represent the absorbency values at 490nm. Normal sera gave values below 0.204.

The 55kDa component of FML was recognized by normal sera from kala-azar endemic and non-endemic area, and sera from patients with tegumentar leishmaniasis, kala-azar or Chagas disease. This antigen is thus non-specific. Conversely the GP36 glycoprotein band was only detected in Western Blots by kala-azar patients' sera. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas disease, or normal sera. The GP36 antigen of FML shows to be a marker of human kala-azar.

Finally the analysis of the protective potential of FML in experimental vaccination against visceral leishmaniasis was performed in the outbred swiss albino model. We have tested the potential activity

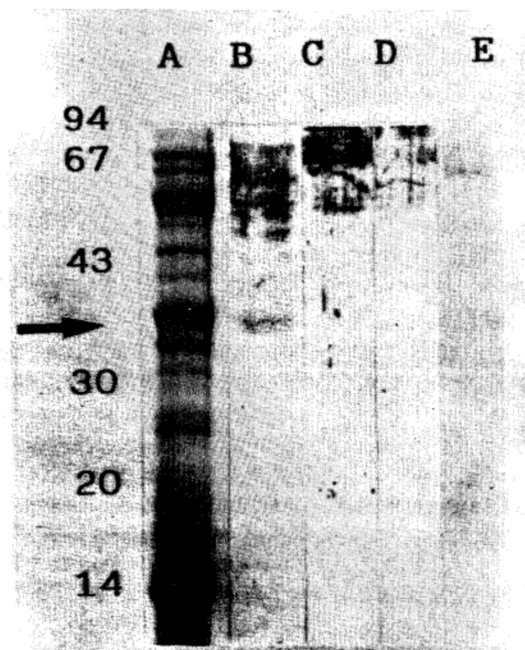

Figure3 - SDS-PAGE and Western Blot analysis of FML antigen of L. donovani using human sera. Molecular weight standards and FML (150µg) analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R250, are indicated on the left side. Blotts containig 1-3ug FML were treated with: a: serum of a patient with confirmed kala-azar (11/11), b: serum of a normal donor from endemic area, c: serum of a patient with tegumentar leishmaniasis (5/5), d: serum of a patient with Chagas' disease (5/5). e: serum of normal heathy donor from nonendemic area (2/8). The arrow indicates the GP36 band. The number in brackets represent the samples assayed that showed the same profile of reactivity.

of FML in protection of outbred swiss albino mice against the infection with *L. donovani*. We compared the administration of FML with Saponin through the intraperitoneal or subcutaneous route. Results of anti-FML antibodies are summarized in Figure 4A. Before the infection a pronounced speciffic response to FML ($p < 0.001$) was detected in animals immunized with FML and saponin, higher by the intraperitoneal than the subcutaneous route. After the infection, both group of mice showed an increased response whereas no response was detected in any control group. Taken together, these results show the slow establishment of immune response in mice, even when infected by this relatively high dose of *L. donovani* amastigotes. Indeed, no immunoglobulin secretion to FML was detected in untreated infected animals, at 15 days of infection. Noteworthy, the specific pre-stimulation with FML and saponin triggered the anti-FML antibody synthesis. The protective effect of this response is shown by the diminished parasite burden in host's tissues. Indeed, the parasite counts in liver were significantly decreased ($p<0.001$), in the same order of magnitude as the immunoglobulins to FML expanded Figure 4B. At this stage of experimental murine infection, parasites are found only in liver, and they infect the spleen only 30 days later (data not shown). No significant differences were detected between the saline and the saponin treated animals, in any parameter ($p>0.3$). Taken

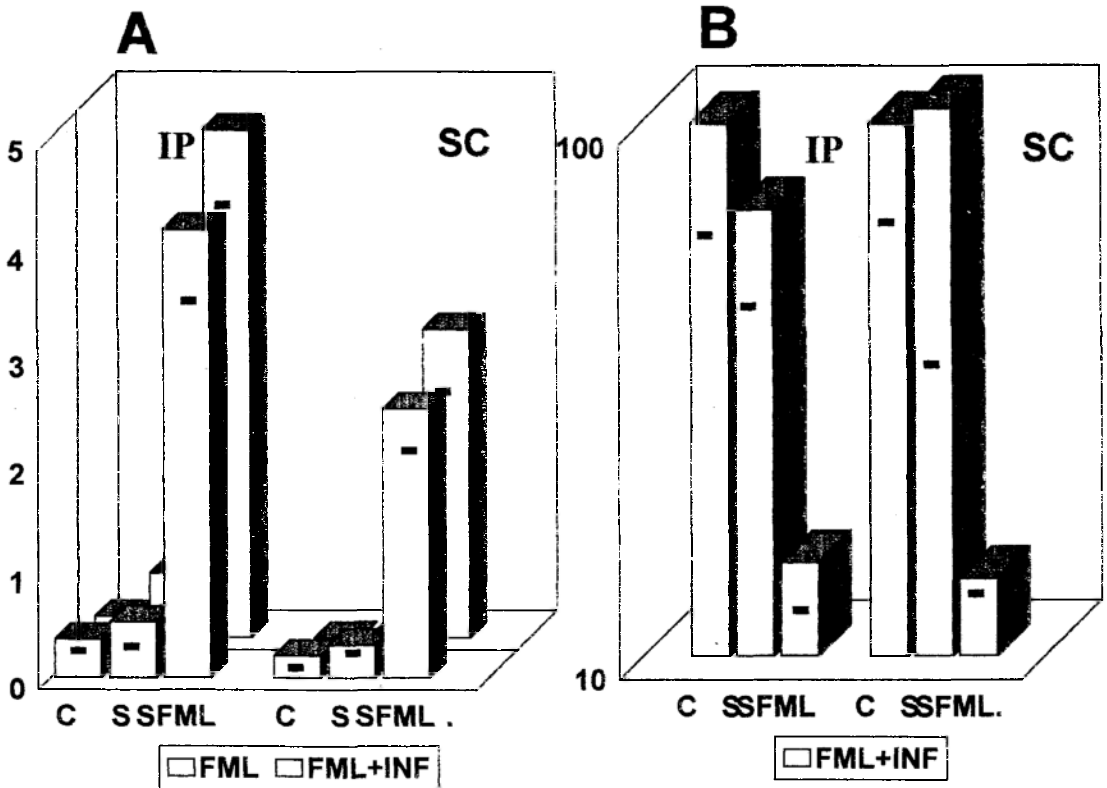

Figure 4 - Effect of FML on Leishmania donovani infection in swiss albino mice. In A: antibody response:open columns: seven days after three intraperitoneal (IP) or subcutaneous (SC) injections of control saline solution (C), 100µg saponin (S), or 150µg FML combined to100µg saponin (SFML). Shaded columns: idem, 15 days after infection with L. donovani. *Results represent score mean values of the pool of sera of two independent experiments done in triplicates, using 4 -5 mice in each group. In B: liver parasitic burden., of the same groups in A, after 15 days infection with* L. donovani. *Results are expressed as percents of the average of LDU values (counts in 1000 cells) of liver imprints of infected mice, of two independent experiments, using 4 to 5 mice in each group. Horizontal bars represent standard errors. FML saponin treated mice, after infection, gave significantly different results ($p < 0.001$) when compared to all other groups.*

toguether these results represent an average of 80.0 % increase in speciffic antibody response and a 85.5% protection in reduction of parasite liver burden. Although the humoral response was higher in the case of intraperitoneal administration, no signifficant difference between the vias was shown in what concern to protective effect.

DISCUSSION

ELISA tests using crude native antigens are usually done in dilutions of 2 to 5μg/well[15 16]. In an improved ELISA assay, the recombinant GP63 antigen of *L. chagasi* was used in concentration of 1 μg/well[35 39]. The FML is thus a very sensitive antigen, and standard ELISA tests can be done in dilutions increased by an order of magnitude. This sensitivity can be probably advantageously used in development of rapid and automated tests for kala-azar. This property of FML can be possibly attributed to its glycoproteic nature. Previous studies have attributed the species-specific characteristics of *Leishmania* glycoconjugates to their glycidic moiety[28].

The presented results indicate that assays using FML can be highly sensitive and specific in diagnosis of kala-azar. This antigen can clearly separate visceral leishmaniasis from all the other closely related tegumentar infections caused by parasite of the genus *Leishmania*, with the sensitivity comparable to those reported for recombinant antigens[35 39]. Moreover, this antigen can identify inhabitants of endemic areas with a potential subclinical infection, and point out those with a high risk of evolution towards the overt severe disease. It can be also used to follow-up of the parasitologic cure. Different form previously reported data[2], the FML antigen isolated from an African strain of *L. donovani* was accurate in diagnosis of South American visceral leishmaniasis, representing apparently a tool that can be used throughout the diagnostics of diseases caused by the *L. donovani* complex.

Two cases of a positive reaction of FML with sera of a patient without overt kala-azar were observed. The first was a patient with chronic disseminated muco-cutaneous leishmaniasis. This serum had high titers of IgG and IgM antibodies against *L. braziliensis*, but the parasite could not be isolated nor characterized. The second was from a patient with Chagas's disease. In both examples the possibility of a concomitant infection with *L. donovani* could not be ruled out since visceral leishmaniasis was also endemic in these regions. These two positive reactions are at present, of difficult interpretation. Their presence has decreased the specificity of the assay to 96%. The FML antigen developed equal levels of sensitivity and specificity than the recombinant antigen used by Burns et al (1993)[8]. However, different from the recombinant antigen, FML is a a glycoproteic complex and its long lasting stability makes of it a good candidate for work in field assays. We were able to use the same batch for period over two years with no signifficant changes in its performance. The FML-ELISA test is now being used as a new tool for diagnosis, prognosis, cure survey of visceral leishmaniasis and it is used as an alternative to bone marrow punction in several human cases with no false negative or positive results. The test is performed routinely in the cited Hospital and Blood bank services. Furthermore a clinical and epidemical survey of perypherical areas of Natal, using the FML-ELISA test is underway.

In this study we described the presence of *L. donovani* sororeactive individuals among spontaneous blood donors in the kala-azar endemic area. This prevalence attained up to 17% of diary donors. These values are higher than those usually detected for chagasic donors in Rio de Janeiro. Recent results from our laboratory demostrated that blood transfusion is an efficient via of transmission of experimental kala-azar. No one of the 8 FML-ELISA positive sera was reactive in the tests for Chagas' disease or other diseases, performed in the blood bank and therefore the use of these blood package units would be considered possible, since the presence of antibodies against *Leishmania donovani* would not be detected by the present used control tools. Since these specific antibody titers could be a signal of active infection and of a potential source of blood borne visceral leishmaniasis, the extension of the FML-ELISA screening in blood banks and the detailed study of each sororeactive case is extremely necessary.

We further analyzed by Western Blot the reactivity of human sera with FML. Isolated GP36 was only detected in Western Blots by kala-azar

patients' sera. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas disease, or normal sera. The GP36 has shown to be the most specific fraction of the FML antigen. The GP36 component of FML could correspond in living parasites to a lower molecular weight component since its extraction procedure, favours the glycidic enrichment that could retard ist migration in SDS PAGE. The specificity of a 32-35 kDa band for kala-azar was suggested by Reed et al. (1987)[34], who used a total soluble extract of *L chagasi* with sera of patients from Brazil. Circulating antigens of 30-35 kDa were detected in sera of human patients by monoclonal specific antibodies[42] and were recovered from kidneys of *L. donovani* infected hamsters[36]. The GP36 may be thus shed in vivo by parasites, representing a circulating antigen. The isolation of GP36 antigen of FML is in progress.

Finally, the reported results indicate that FML may be a potential candidate for vaccination in visceral leishmaniasis. Both intraperitoneal or subcutaneous immunizations were effective. These results are particularly signifficant in view of the outbred nature of the experimental model. Indeed a high intragroup variation was expected. This protective effect of FML and saponin is in agree to previous evidences of the protective potential against experimental visceral leishmaniasis in CB hamsters and Balb/c mice[30 31]. In our conditions, saponin showed nor toxic neither unspeciffic effects. Its use as adjuvant was compared to *Corynebacterium parvum*, Freund Adjuvant, Alhydrogel, *Bordetella pertussis* and Muramyl dipeptide and considered to be advantageous[4]. In agreement with our results, saponin has been previously shown to be a good potentiator of anti-protozoan immunity in experimental vaccines against *T cruzi*[37]. However, saponins are discriminated as adjuvant because of their hemolytic properties[3]. For this reason, we started the comparative analysis of the hemolytic and ajuvant potential of different kind of saponins. Using the purified antigen DP72 for immunization of balb/c mice against *L. donovani* infection, a protective effect of the same order of magnitude was reported[16 33]. However, in those works, for protection or lymphocyte proliferation assays, respectively, after sensitization with the antigen, the animals received different doses of promastigotes. It is consequently not clear if the described effects were due only to DP72 administration or to promastigote injection or to both of them. Two other antigens of *Leishmania*, extensively studied were candidates for vaccination against cutaneous leishmaniasis in mice, however, it was recently shown that GP63 is unable to induce lymphocyte proliferation in human leishmaniasis[23] and that the ability of LPG to induce a protective response in mice is due to contaminant proteins or glycoproteins, called LPG associated proteins (LPGAP)[17]. Furthermore subcutaneous LPG vaccination against murine tegumentar leishmaniasis by the subcutaneous route showed to induce an increase of lesion size, instead of protection[13]. Recently, the immunization with one of the LPGAP flagellar proteins, present in a wide range of *Leishmania* species and in African trypanosomes, in the recombinant form, stimulates primed lymphocytes to proliferate *in vitro*[40]. The use of FML of *Leishmania donovani* and of its components in sorologycal screening for visceral leishmaniasis shows high sensitivity, specificity, stability and comparatively easier performance. Its use in Blood banks would improve the quality control to prevent blood borne infections. Furthermore it showed a signifficant potential on experimental vaccination against kala-azar. The study of the molecular aspects related to the described FML potentials is under progress.

RESUMO

O FML (Ligante de Fucose-Manose) de Leishmania donovani *é una fração glicoproteica complexa. O seu potencial no diagnóstico da leishmaniose visceral humana foi testado com soros provenientes de Natal, Rio Grande do Norte, Brasil. O teste de FML-ELISA mostrou 100% de sensibilidade e 96% de especificidade, identificando pacientes com calazar declarado (p < 0.001, comparados com soros normais) e indivíduos com infecção subclínica. Mais de 20% dos sororreativos assimptomáticos desenvolveram a doença no prazo de 10 meses. Na análise de doadores de sangue, 5% de sororeativos, atingindo até 17% num único dia foram detectados. A glicoproteína GP36 do FHL é reconhecida especificamente por soros de pacientes com calazar. O potencial imunoprotetor do FML no calazar experimental foi testado no modelo swiss albino em combinação com saponina pelas vias subcutâneas e/ou intraperitoneal seguido de desafio com 2x 10⁷ amastigotas de* Leishmania donovani. *Um aumento de 80.0% na resposta de anticorpos específicos (p < 0.001) e a redução de 85.5%*

da carga parasitária no fígado ($p < 0.001$) foi detectado nos animais vacinados com FML e saponina, independentemente da via de administração.

Palavras-chaves: Glicoconjugado. Leishmania donovani. *Calazar. Transfusão de sangue. Diagnóstico.*

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