Meeting Report

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Report of the Fourth TDR/IDRI Meeting on Second-Generation Vaccines against Leishmaniasis

1-3 May 2001

Universidad Autónoma de Yucatán, Mexico

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FOURTH TDR[1]/IDRI[2] MEETING ON SECOND-GENERATION VACCINES AGAINST LEISHMANIASIS

Universidad Autónoma de Yucatán, Mexico, 1-3 May 2001

Eric Dumonteil,[3] Diane McMahon-Pratt[4] and Virginia L. Price[5]

Executive Summary

Introduction

The development of a *Leishmania* vaccine has been part of TDR activities for several years. Starting in 1996, during an initial meeting on Second-generation *Leishmania* Vaccines, TDR decided to organize a comparative study of several of the leading recombinant antigens identified at that time. A total of nine antigens were chosen, coded randomly and tested in two independent laboratories. The fourth meeting on Second-generation Vaccines against Leishmaniasis was held in Mérida, Yucatán, Mexico, in May 2001. The goal of this meeting was to evaluate the results of these studies and discuss current trends in *Leishmania* vaccine development.

Evaluation of recombinant antigens as vaccine candidates

A comparative study of recombinant antigens was perfomed in Denmark (T. Theander, University of Copenhagen, Denmark) and Brazil (M. Barral-Netto, FIOCRUZ, Salvador, Brazil). The evaluation consisted of testing the antigens for human peripheral blood mononuclear cell (PBMC) stimulation and challenge experiments in immunized mice. Mice were immunized with two doses of 25µg of recombinant antigen plus either recombinant interleukin-12 (rIL-12, donated by Genetics Institute) or monophospholipid A (MPL, donated by Corixa) as adjuvant. Table I shows the list of antigens. Soluble *Leishmania* antigen (SLA) was also included in the study as a positive control. Inhibition of footpad swelling following challenge with *Leishmania* promastigotes was assessed in the vaccinated animals as a protective response.

PBMC proliferation experiments showed that most antigens induced some proliferative responses in naive controls as well as in *Leishmania major* and *L. donovani* infected patients, except flagellar pocket antigen (FPA, Theander's laboratory). Most antigens also induced interferon-gamma (INF-γ) production in control and infected patients (Barral-Netto's laboratory). These data suggest that the selected antigens had some degree of immunogenicity in humans. Table II summarizes the results of mouse challenge experiments. The only antigens that showed protection against infection with *L. major* in BALB/c mice were the antigens MIX (contains several antigens – see Table 1), LACK (*Leishmania* homologue of receptors for activated C kinase), and 4H6 (an *L. major* antigen). Both adjuvant systems (rIL-12 and MPL) provided comparable results against *L. major*. In challenge experiments with *L. amazonensis*, none of the antigens induced significant protection with MPL as adjuvant. With rIL-12, both LACK and FPA induced significant protection in C57BL/6 mice.

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Based on these results, the participants agreed that specific recommendations could not be made regarding potential use of the antigens as vaccine candidates. Several technical problems (including inadequate information about the quality, stability and potency of the antigens) made the results of the comparison hard to draw solid conclusions from. Because of this issue, TDR has recommended stricter guidelines for the characterization and quality control testing of recombinant antigens before they are used for pivotal animal studies. It was also pointed out that heterologous challenge, i.e. *L. amazonensis* challenge in mice immunized with *L. major* antigens, was not optimal. Finally, differences in the route and times of challenge may have been important, although the procedures used were common to both laboratories.

Table I: List of recombinant antigens tested

Code #	Antigen	Description
1	MIX	Containing LACK, Hsp80, TSA, GP63, FPA
2	TSA	*L. major* thio specific oxidant (S. Reed)
3	GP63	*L. major* leishmanolysin (F. Mahboudi/R. McMaster)
4	Hsp80	Heat shock protein 83 *L. braziliensis* (S. Reed)
5	SLA	*L. major* soluble antigen (promastigote cell extract, P. Scott)
6	LACK	*L. major* (N. Glaichenaus)
7	FPA	Flagellar pocket antigen *Trypanosoma brucei rhodesiense* (C. Powell)
8	1G6	*L. major* (S. Reed)
9	4H6	*L. major* (S. Reed)
10	GBP	*L. major* gene B protein (D. Smith)
11	CP	*L. mexicana* cysteine proteinase (J. Mottram)

Table II: Results of antigen screening

Antigen	Theander's Lab*		Barral-Netto's Lab**	
	With rIL-12	With MPL	With rIL-12	With MPL
1. MIX (LACK, Hsp80, TSA, GP63, FPA)	++	++	-	ND
2. TSA	-	ND	-	ND
3. GP63	-	-	-	-
4. Hsp80	-	-	-	ND
5. SLA (non-recombinant)	-	ND	-	ND
6. LACK	+	++	+++	+/-
7. FPA	-	ND	+++	ND
8. 1G6	ND	-	ND	-
9. 4H6	ND	++	ND	-
10. GBP	ND	-	ND	-
11. CP	ND	-	ND	+/-

* site of injection of *L. major*: base of tail, BALB/C mice, challenge performed 20 days after last immunization.

** site of injection of *L. amazonensis*: foot, C57BL/6 and BALB/C mice, challenge performed 15 days after last immunization

ND: not done

Summary of First-Generation (Killed *Leishmania*) Vaccines

Modabber F, Infectious Disease Research Institute, USA

The first-generation vaccine against leishmaniasis consists of autoclaved *Leishmania* promastigotes with or without BCG (bacillus of Calmette and Guerin) as adjuvant. This vaccine has been tested in Phase III field efficacy trials against cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). In a randomized, BCG-controlled, double-blind trial in Sudan by Khalil et al.[6] against VL, no evidence was found that two injections of the vaccine (autoclaved *L. major* [ALM] with BCG) resulted in significant protection against VL when compared to BCG alone.

However, the vaccine did induce significantly higher rates of leishmanin skin test (LST) conversion relative to BCG alone (30% vs. 7% respectively.). LST-converted individuals had a significantly lower incidence of disease compared to LST non-responders (7.2% of LST responders vs. 12.7% of LST non-responders, a 42% reduction).

Similarly, the single injections and triple injection of ALM+BCG against CL resulted in reduced incidence of CL in LST-converted individuals but no significant overall protection compared to BCG alone.[7]

The safety and immunogenicity of Mayrink's vaccine (killed *L. amazonensis*) produced by Biobras, with and without BCG as adjuvant, was tested by Velez et al. in Colombia.[8] Due to BCG-associated lesions, the trial for efficacy is being continued without adjuvant. This same vaccine, with BCG, is being tested against CL in Ecuador by Armijos.

The ALM vaccine has been reformulated with alum and BCG as adjuvants, and preliminary results from studies in Sudan show strong LST conversion in every recipient. Studies in monkeys using rIL-12 and alum as adjuvants gave good protection. New phase I/II studies are planned for this year in the Sudan and Iran.

Current Trends in Second-Generation *Leishmania* Vaccine Development

Product profile for second-generation vaccine: characterization and testing of recombinant vaccines

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The immunology surrounding *Leishmania* infection is complicated both from the standpoint of the host response to a given *Leishmania* species and the fact that different species can elicit very different responses. Particularly difficult from a product development standpoint is the fact that it is not entirely understood what constitutes a protective response in humans. The animal models currently available are perhaps somewhat, but not entirely, predictive of how effective a vaccine candidate will be in

6 Khalil EA et al. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. *The Lancet*, 2000, 356 (9241): 1565-69

7 Sharifi I et al. Randomised vaccine trial of single dose of killed *Leishmania major* plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. *The Lancet*, 1998, 351: 1540-1543

8 Velez ID et al. Safety and immunogenicity of a killed *Leishmania (L.) amazonensis* vaccine against cutaneous leishmaniasis in Colombia: a randomized controlled trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2000, 94: 698-703

humans. In general, the lack of easy assays to define potency of a vaccine candidate makes progress in this area difficult.

The lack of strong correlates of protection, similar to the field of malaria vaccine development, makes it all the more important to test candidate antigens as soon as possible in human phase I/II trials to obtain the necessary information. The decision to proceed to phase I trials in humans is, however, costly and requires rigorous quality control and protocol development. For this reason, pivotal animal studies (usually challenge studies in animal models that determine whether or not a vaccine candidate should proceed to human trials) must also be conducted with adequately characterized antigen. With adequate characterization, problems such as reproducibility of the process and stability of the antigen are minimized when moving towards Phase I studies.

Differences in IFN-γ production in vitro predict the pace of in vivo anti-leishmania responses in normal volunteers

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A priming in vitro (PIV) system was used to evaluate the immune response of naive individuals before and after in vivo vaccination against *Leishmania*. Interferon-gamma (IFN-γ) levels were followed as an indicator of type of response to *Leishmania* antigen. PBMC from volunteers that were delayed type hypersensitivity (DTH) negative and anti-leishmania antibody negative were used for in vitro stimulation with *Leishmania* antigen. IFN-γ levels were then monitored. This in vitro stimulation showed two different types of responders: those that produced low amounts of IFN-γ and those that produced high levels of IFN-γ. The IFN-γ levels were directly proportional to tumour necrosis factor-alpha (TNF-α) and IL-10 levels but not to IL-5 levels. Those volunteers who produced low amounts of IFN-γ in vitro remained low producers 40 days after vaccination, whereas high producers exhibited increased IFN-γ production after vaccination. However, at six months post-vaccination, all individuals tested were producing similarly high levels of IFN-γ upon stimulation of their PBMC with *Leishmania* promastigotes, indicating that the low in vitro producers are slow in vivo responders to vaccination. High IFN-γ producers exhibited an increased frequency of activated CD8+T lymphocytes both in vitro and in vivo, as compared to low IFN-γ producers. Additionally, neutralization of either IL-10 or transforming growth factor-beta (TGF-β) led to increased IFN-γ production in some low responders. Such findings suggest that PIV response is able to predict the rate of post-vaccination response. Although all vaccinated individuals eventually present a potent anti-leishmania cell-mediated immune (CMI) response, a delay in the rate of mounting the CMI response may influence resistance against leishmaniasis.

The unreliable paradigm of Th1/Th2 immune responses as criteria for antigen selection in vaccine development against leishmaniasis

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For the past 20 years, it has been generally accepted that immunity against experimental infection of mice with *Leishmania* species is strictly dependent on the generation of a Th1-type of immune response against the parasite's antigens. However, it has been observed that several leishmanial recombinant antigens, selected on this criteria and expressed as recombinant proteins, do not necessarily turn out to be protective antigens. For example, BALB/c mice infected with *L. major* develop low antibody titres to the

antigen Ldp23, and to many others tested. In addition, Ldp23 preferentially induces a Th1 response in lymph node cells from infected mice. However, in combination with adjuvants that preferentially induce Th1 responses such as IL-12 and MPL-SE, and despite stimulating a strong antigen specific Th1 response in the absence of any detectable Th2 response, Ldp23 does not confer protection. In contrast, the antigen LACK stimulates strong and preferentially Th2 responses in lymph node and spleen cells from *L. major*-infected mice. The sera of these animals also contain high titres of IgG1 anti-LACK antibodies. In spite of this, LACK induces substantial protection in BALB/c mice if administered in conjunction with adjuvants that stimulate Th1 responses. In another situation, antigens like LmSTI1 stimulate strong and preferentially Th1 responses in lymph node cells of BALB/c mice infected with *L. major*, and the sera of these animals contain high titres of both IgG1 and IgG2a anti-LmSTI1 antibodies. Moreover, LmSTI1 induces excellent protection in these animals if used with IL-12 or MPL-SE as adjuvants.

Therefore, for antigen selection purposes in vaccine development against leishmaniasis, these results do not support the Th1 paradigm, although a Th1 response may well be essential for protection against leishmaniasis. In conclusion, for vaccine development against leishmaniasis, the polarization to Th1/Th2 antigen-specific immune response that is developed against the parasite antigens during the infectious process may be irrelevant. Immunogenicity, and perhaps the amount of antigen expressed or secreted by the parasite in vivo, are more important factors reflecting protective anti-parasite immune response. Antigens that fulfill these criteria should therefore be more successful in inducing protection as long as they are administered with adjuvants that, in combination with them, modulate a strong Th1 response.

Evaluation of single and combination DNA vaccines against *Leishmania mexicana* in mice and hamsters

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DNA vaccines have been shown to be a promising strategy against *L. major*. DNA vaccines encoding *L. mexicana* GP63 and CPb, *L. amazonensis* GP46, and *L. major* LACK, were tested against *L. mexicana*. cDNAs encoding these antigens were subcloned into the VR1012 plasmid (Vical Inc., USA), and susceptible BALB/c mice were immunized with two intramuscular injections of 100µg of DNA. All mice immunized with VR1012-GP46, VR1012-CPb, VR1012-GP63 and VR1012-LACK showed elevated IgG titres against *L. mexicana* as determined by enzyme-linked immuno-sorbent assay (ELISA). T-cell proliferation assays indicated that all four DNA vaccines induced some T-cell responses to soluble *L. mexicana* antigen. Overall, there was no significant difference between the immunogenicity of the four DNA vaccines tested.

Mice immunized with VR1012-GP46, VR1012-GP63 and VR1012-CPb, and challenged with 2 x 10^6 parasites in the footpad, had significantly smaller lesions and reduced parasite burdens relative to controls, as assessed by limiting dilution assays. VR1012-LACK had no effect. In the hamster, preliminary results suggest that the plasmids VR1012-GP63 and VR1012-CPb could also induce some protection against infection, as assessed by both footpad lesion and parasite burden.

Mice immunized with a high dose of the plasmid pool (50µg of each plasmid VR1012-GP46, VR1012-GP63 and VR1012-CPb) had higher levels of protection than mice immunized with the individual plasmids alone. Mice immunized with a low dose of the plasmid pool (20µg each) were poorly protected. These data suggest that further optimization is required for the development of a DNA vaccine of high efficacy against *L. mexicana*.

Immunogenicity and protective capacity of histone H1 against leishmaniasis

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A vaccination study against the cutaneous *L. major* was performed in African green monkeys. This monkey model is thought to mimic human cutaneous leishmaniasis quite well in that the lesions are self-healing and the infection elicits a similar CMI response.

The antigen used was *L. major* histone H1, a nuclear antigen that has no homology to human H1. Antigen was made either as a recombinant glutathione-S-transferase (GST)-fusion protein in *E. coli* or as a long synthetic peptide representing the complete histone H1 sequence. Montanide ISA 721 was used as adjuvant. Antibodies, IFN-, and DTH responses were monitored. ELISA data showed that both antigens elicited an increase in specific antibodies after vaccination, some (but not all) monkeys had increased IFN-γ levels, and all exhibited a positive DTH response. At the time of writing, results of a subsequent live parasite challenge were not in.

A DNA vaccine encapsulated in microspheres (small particles of poly-lactide-co-glycolide) was also prepared. The DNA encoded antigens were histone H1, cysteine proteinase B (CPB), and cysteine proteinase A (CPA), alone or in combination. A single injection of 20μg DNA was used for vaccination of mice. Only the cocktail containing CPA and CPB showed protection.

A vaccine trial against visceral leishmaniasis in dogs is in progress using a cocktail of long synthetic peptides derived from *L. infantum*: Li H1, LiCPb, LipapLe22, and LiKmp-11, using Montanide ISA 720 as adjuvant.

Cysteine proteinases of leishmanias: a putative vaccine candidate

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As an approach to developing a second-generation vaccine against leishmaniasis, type I and II cysteine proteinases (CPs) of *L. major* (IR 75) were studied. Previous investigations had led to the reporting of a new type of cysteine proteinase in *L. major* called type II or cpa gene (Accession No. AJ130942), and it had also been possible to isolate type I or cpb gene from *L. major*. Analysis of cysteine proteinases a and b in three different strains of *L. major* revealed that *cpb* is a multi-copy gene and *cpa* a single-copy gene. Recombinant *L. major* CPA and CPB were made in a bacterial expression vector, and 40μg of each recombinant antigen was used to vaccinate BALB/c mice using poloxamer as adjuvant. CPB as antigen resulted in high IFN-γ levels >8 weeks after infection with no increase in IgG (any isotype). CPA vaccination did not result in increased IFN-γ levels but did result in an increase in both IgG1 and IgG2a type antibodies. CPB as antigen resulted in partial protection (reduction in footpad swelling after parasite challenge) and CPA was not protective.

DNA vaccinations were also performed in BALB/c mice using constructs encoding type I (CPA) and type II (CPA) cysteine proteinases. Alum and IL-12 were used separately as adjuvant. The CPA DNA vaccination showed no protection; CPB DNA showed a small amount of reduction in footpad swelling compared to controls following challenge; and the combination of the two also showed partial reduction in footpad swelling up to 14 weeks after challenge. Adding the adjuvants did not increase the antigenicity or protection of any of the DNA vaccines.

The PBMC response in 21 individuals recovered from cutaneous leishmaniasis was examined using both CPA and CPB to stimulate lymphocytes. Both cysteine proteinases could stimulate IFN-γ production but CPA resulted in much higher IFN-γ production. Neither CPA nor CPB induced the production of IL-4.

Type I and II cysteine proteinases were also isolated from *L. infantum* (and show high similarity to the *L. major* proteins), and recombinant protein was prepared in *E. coli*. The immunoreactivity of sera from 15 kala-azar patients showed that sera from the active phase of disease could recognize both types of CPs. The immunogenicity of *L. infantum* CPs will be evaluated in both infected humans and dogs with the intention of vaccinating mixed-breed dogs with a cocktail of *cpa* and *cpb* genes.

The vaccine potential of *Leishmania mexicana* cysteine proteinases

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The ability of vaccination with enzymatically active and inactive recombinant *L. mexicana* cysteine proteinases B (CPB), both with and without IL-12 as adjuvant, to confer protection against subcutaneous infection with *L. mexicana* has been studied. Vaccination was tested in CBA/Ca, BALB/c and C57BL/6 mice. Five experimental groups of each strain, each consisting of 5 mice, were vaccinated (on 2 occasions, 2 weeks apart and 2 weeks before infection with 2×10^5 promastigotes) subcutaneously with either (1) 5μg CPB, (2) 5μg CPB + 0.5μg IL-12, (3) 5μg inactivated CPB, (4) 5μg inactivated CPB + 0.5μg IL-12, or (5) phosphate buffered saline. In all three strains of mice, vaccination with CPB, both active and inactive, without adjuvant, resulted in more rapid lesion growth following challenge infection than in unvaccinated animals. Significantly, inoculation of CPB (both active and inactive) into the footpad was subsequently shown, using the competitive reverse transcriptase polymerase chain reaction (RT-PCR), to upregulate IL-4 mRNA at least 4-fold in the draining lymph nodes. These results may, in part, explain the previously identified role of *L. mexicana* CPs as virulence factors.

Although vaccination with CPB alone may exacerbate infection, the addition of IL-12 to the active and inactive CPB recombinant antigens resulted in significant inhibition of lesion growth in all strains of mice examined following challenge infection. Both active and inactive CPB were effective under such circumstances, and splenocytes from vaccinated animals had upregulated IFN-γ production and downregulated IL-4 production compared with non-vaccinated animals at the termination of the experiments.

Investigations on the vaccine potential of cysteine proteinase deficient mutants are continuing.

Rhesus macaques vaccinated with recombinant *Leishmania major* antigens are protected against homologous challenge infections

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Simian species most closely related to humans should be more attractive than rodents as animal models for evaluating the safety and immunogenicity of vaccines. Class I and class II major histocompatibility complex (MHC) products in Old World monkeys

resemble closely their human counterparts. The rhesus macaque (*Macaca mulatta*) was used as an experimental model for the study of CL induced by *L. major*, *L. amazonensis* or *L. braziliensis* infection. The outcome of *Leishmania* infection in primates is similar to that in humans in that it depends largely on the virulence of the infecting parasite strain and the immune responsiveness of the host. Lesion development, histopathology of cutaneous lesions, and the Th1-type profile are similar to the human disease.

Several antigen/adjuvant combinations were tested for protection in both mice and monkeys. The *L. major* antigens tested were: LeIF (ribosomal initiation factor), STI1 (a stress and temperature inducible protein), and TSA (a thiol-specific antioxidant).

Mouse studies involving challenge showed that LmSTI1+IL-12 and TSA+IL-12 inhibited footpad swelling. These two antigens tested as a DNA vaccine showed high protection with TSA and partial protection with LmSTI1. (Expression from a transfected cell line showed higher expression of TSA, so there may have been more antigen present.)

Mice were also immunized with a di-fusion comprised of TSA-LmSTI1 and a tri-fusion comprised of TSA-LmSTI1-LeIF. Protection was seen with either the di-fusion or the tri-fusion with IL-12 as adjuvant, but not with several other adjuvants tested.

Monkey immunizations were performed with TSA and LmSTI1 as antigens and IL-12+alum as the adjuvant. (Alum with IL-12 does not appear to change the Th1 pattern normally seen with IL-12 as adjuvant but seems to make the antigen more immunogenic.) Three immunizations with 25μg each antigen were given, with a challenge 6 weeks after the last immunization. Protective immunity followed immunization – no lesions developed in any of the vaccinated monkeys (n = 6) after infection, whereas all controls developed lesions. Rhesus monkeys vaccinated with LmSTI1-TSA+IL-12+alum were protected against rechallenge with *L. major* (4 months following the first challenge), but not cross-protected against infection 2 months following the second challenge with *L. braziliensis*. These data point to a long-lasting anti-*L. major* immunity induced in primates by the recombinant antigens LmSTI1 and TSA.

Protection against experimental *L. donovani* infection by immunization with the A2 gene or protein

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The A2 genes encode proteins of unknown function that are part of a multigene family of at least 11 genes. The protein is composed largely of multiple copies of a 10 amino acid repeat sequence. It is abundant in amastigotes and absent in promastigotes. It is present in *L. infantum*, *L. chagasi*, *L. mexicana*, *L. amazonensis*, and *L. donovani* but not in *L. tropica* and *L. major*. (*L. major* has been shown to have the A2 genes but in truncated form.)

The role of the A2 proteins has been studied both by creating knock-out strains (with 8 of the 11 A2 genes inactivated) and by using antisense technology. A2-deficient strains of *L. donovani* were shown to be avirulent in mice. Thus, A2 can be considered an amastigote specific virulence factor and a good vaccine candidate for *L. donovani*.

A2 was tested as a vaccine (recombinant protein or DNA vaccine) in a murine model of visceral leishmaniasis. Mice vaccinated with A2 protein showed greatly reduced numbers of parasites in the spleen and a mixed Th1/Th2-type of response (increased IFN-γ

by spleen cells and no increase of IL-4 but increased IgG2a and IgG1 levels). A2 as a DNA vaccine gave partial protection (measured by numbers of parasites in the spleen).

It appears that A2 represents a potential vaccine candidate due to the fact that it can protect against experimental visceral leishmaniasis, is highly expressed in an amastigote specific manner, and is essential for survival of amastigotes in visceral organs.

Considerations in the development of a leishmanial vaccine

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The mechanism of protection against leishmaniasis was studied in a vervet monkey model in Kenya, at the Institute for Vaccine Research. The antigen used was autoclaved *Leishmania major* (ALM) with IL-12 as adjuvant. The immunizations (three injections at four-week intervals) resulted in increased specific IFN-γ levels and a typical Th1-type response. Specific IFN-γ levels were as high as in the positive controls, which were animals that had already healed and were resistant. However, the vaccine did not protect. The conclusion from this study was that IFN-γ production by peripheral blood lymphocytes to crude leishmanial antigen does not always correlate with immunity.

To date, the best protection is induced by infection and healing. The role of IL-12 was analysed as one of several parameters that might be required for acquiring and maintaining resistance to *Leishmania*.

It was shown that IL-12 knockout mice, if treated with IL-12 exogenously, are resistant to *L. major* infection. However, upon rechallenge after cessation of IL-12 treatment, they are susceptible. In addition, adoptive transfer of Th1 cells from healed mice into IL-12 knockout mice failed to protect against challenge and the IFN-γ response was not recovered in the IL-12 knockout mice. Thus, IL-12 seems to be needed continuously to maintain resistance. This might be due to a requirement for IL-12 to: optimize IFN-γ production from Th1 cells, protect Th1 cells from apoptosis, promote Th1 cell survival, and/or recruit additional Th1 cells from a naive or undifferentiated T cell population.

Microarray analysis of the *Leishmania* life cycle: identification of new vaccine candidates

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As genomic sequencing of pathogenic organisms proceeds, advanced technologies will need to be applied to obtain a global genetic perspective on the biological processes important in parasite survival and host-parasite interactions. The driving force behind the genome effort is the identification of new drug targets and vaccine candidates. For *Leishmania*, this means identifying the genes that are expressed in forms of the parasite that invade and survive in the vertebrate host. In the sandfly vector, *Leishmania* undergoes developmental changes that lead to the generation of infective metacyclic forms transmitted to the vertebrate host. This can be mimicked in culture as promastigote forms of the parasite pass through logarithmic to stationary phases of the growth cycle. In the vertebrate host, metacyclic promastigotes rapidly enter macrophages and transform to amastigote forms. DNA microarrays were used to simultaneously monitor the expression profiles for 2183 unique *Leishmania* genes as the parasite undergoes developmental transition from the logarithmic promastigote

to the metacyclic form, and in the host-derived amastigote form. From this analysis, over 100 previously unknown genes were identified that are upregulated in expression in amastigotes. These are now being tested as new vaccine candidates; some cocktails of them appear to be effective as DNA vaccines in mice.

Leishmania hydrophilic acylated surface antigens: candidate antigens for vaccination against New World and Old World *Leishmania* species

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The family of surface proteins (HASPA, HASPB, SHERP) encoded at the LmcDNA16 locus on chromosome 23 of *L. major* represents potential vaccine candidate molecules. Hydrophilic acylated surface proteins (HASPs) are dually acylated at their N-termini, a modification required for subcellular targeting of the proteins to the parasite's membrane. Although the precise function(s) of these proteins is(are) not known, genetic experiments using knock-out and over-expression mutants clearly indicate a role in the virulence of *L. major*. Although all of the protein family members are expressed in metacyclic promastigotes, only HASPs A1 and B are detectable in amastigotes.

Recombinant (unmodified) HASP proteins expressed in *E. coli* were employed for immunization of BALB/c mice (with or without IL-12). Immunization resulted in protection against infection with *Leishmania donovani*.[9] Reduction in parasite burden was significant in both the liver and spleen and, surprisingly, did not require the presence of IL-12 as adjuvant. HASPB1 induces a low level of IL-12 production in murine dendritic cells. Protection correlates with the level of IFN-γ producing CD8+ T cells and IgG1 production. Interestingly, rHASPB1 fails to confer protection in IL-4 and IL-4Rα deficient mice. These data suggest further complexity in the regulation of vaccine-induced immunity, and support the view that, at least for visceral leishmaniasis, vaccination to induce non-polarized immune responses may be optimally protective.

The FML vaccine: a second-generation candidate for vaccination against murine and canine visceral leishmaniasis

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The fucose mannose ligand (FML) antigen is a complex glycoprotein fraction isolated from *L. donovani* promastigotes. This antigen was used to immunize mice using the adjuvants IFA, alum, BCG, IL-12, saponin and QuilA. Antibody responses were primarily IgG1, IgG2a and IgG2b. The highest titres were found for mice receiving FML+QuilA, followed by FML+saponin, FML+IL-12, and FML+BCG and FML+alum; no Ig2a response was observed for mice receiving FML together with either alum or BCG. All vaccine groups showed an enhanced DTH response. Significant protection was observed for mice receiving FML+QuilA (93%), and FML+saponin (73%). Recent studies employing saponin and the GP36 component of the FML antigen complex indicate that this molecule can provide significant protection in the murine model (BALB/c) against *L. donovani* infection (reduction of parasite burdens of 68%). Further, the GP36 vaccinated group had significantly higher survival rates: 6/20 control mice and 3/20 mice receiving the adjuvant alone died, whereas 20/20 vaccinated mice survived.

9 Stager S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *Journal of Immunology,* 2000, 165: 7064-71

The protection provided by vaccination with FML vaccine was examined in dogs naturally exposed in an endemic area in Natal, Brazil. Ninety-seven per cent of the vaccinated dogs produced antibodies and 100% demonstrated a DTH response to the FML antigen seven months post-vaccination. After two years of monitoring the dogs, data indicate that protection was achieved: only 8% of vaccinated dogs showed mild signs of visceral leishmaniasis, with no deaths, while in the control group, 33% of the dogs developed clinical symptoms or fatal disease. Thus, the FML vaccine induced significant protection against canine visceral leishmaniasis in the field.

Subunit vaccination against cutaneous leishmaniasis: what is to be learned from murine models

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Experiments were designed to examine the ability of *L. mexicana* parasites genetically deficient in a gene encoding a homologue for a mitogen-activated kinase (LMPK) to serve as a vaccine candidate for leishmaniasis. Vaccination using *lmpk* knockout *L. mexicana* parasites failed to provide significant protection.[10] Subsequent studies focused on determining the properties of protective antigens. Comparative studies of the proteophosphoglycan (PPG), cysteine proteinase B, the metalloproteinase-gp63, and membrane-bound acid phosphatase (MBAP)[11] indicate that protective antigens require a minimal level of expression of 10^{5-6} molecules per organism throughout the parasite life cycle. In addition, protection is dependent upon antigen accessibility, i.e. a molecule expressed on the surface membrane or secreted.

Studies of the kinetics of antigen presentation in infected mice, examined using green fluorescence protein-labelled parasites, confirms these observations. An early wave of antigen into the draining lymph node, apparently the result of lysed organisms, is observed 4-16 hours post-infection. Early lymph node antigen is associated with dendritic cells, and IL-12 production is observed in both C57BL/6 and BALB/c mice. The initial pulse of antigen clears and at later stages/times post-infection, infected cells are found. Therefore, protective antigens, most likely secreted or surface components, must be accessible throughout infection. These results strengthen the conclusion that, in the rational search for candidate vaccine antigens to be included in future subunit vaccines, there should be abundant, preferably secreted or surface-exposed, protein molecules of *Leishmania*.

Vaccination of rhesus monkeys (*Macaca mulatta)* against *L. major:* a genetically attenuated (DHFR-TS deficient) *Leishmania* vaccine line

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The vaccine potential of dihydrofolate thymidylate synthase (DHFR-TS) gene locus deficient *L. major* organisms was evaluated in the rhesus monkey model. Previous murine model studies had indicated that protection against infection occurs. The effectiveness of the attenuated *L. major* (DHFR-TS) vaccine was evaluated in monkeys receiving

10 Wiese M. A mitogen-activated protein (MAP) kinase homologue of *Leishmania mexicana* is essential for parasite survival in the infected host. *The EMBO Journal,* 1998, 17:2619-28

11 Aebischer T et al. Subunit vaccination of mice against new world cutaneous leishmaniasis: comparison of three proteins expressed in amastigotes and six adjuvants. *Infection and Immunity,* 2000, 68:1328-36

3 vaccinations of killed parasite lysates, BCG alone, or a placebo. Although the mutant parasites were found to persist for three months post-infection, no significant protection was observed. The lesion progression and time to healing were similar in the naive and vaccinated groups: in 75% of naive monkeys, an immune proliferative response was observed but no DTH response; similarly, in vaccinated monkeys, although 79% had proliferative responses at 4 to 18 weeks post-infection, no significant levels of IFN-γ or DTH responses were observed in response to leishmanial antigen. In both vaccinated groups, very low levels of antibody were found before challenge, which increased after the infective challenge. These results indicate that the vaccine protocol used was apparently safe and immunogenic in primates, but requires further improvement for application.

Mechanisms of pathogenesis and vaccines

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One of the features of the genus *Leishmania* is the diversity of tropism/disease resulting from infection. With notable exceptions, the form (visceral, cutaneous, diffuse cutaneous, mucocutaneous) and severity of disease is a function of the infecting *Leishmania* species together with host genetics and consequent inflammatory and immune responses. It has become evident from genetic and immunologic studies using the murine model, that the various members of the genus *Leishmania* differ in their approach to the host immune system. In the case of cutaneous murine leishmaniasis, infection caused by *L. amazonensis* appears to be readily distinguished from that induced by *L. major* in a number of significant immunologic features. For *L. amazonensis*, the mechanisms involved in pathogenesis in the murine model appear to singularly involve immune subversion and require the participation of both CD4+ T cells and B cells (immunoglobulin). A low level of IFN-γ (with little or no IL-4) production seems to be a feature of disease. Antigen presentation and monocyte recruitment at the site of infection appears to be critical for the development of pathology. The roles of immune T and B cells in pathology and in resolution of disease could affect the efficacy of vaccines against New World leishmaniasis, especially when the immune response wanes after vaccination.

CONCLUSIONS AND RECOMMENDATIONS

Based on the discussions of these results, the participants reached the following conclusions:

1. Pivotal animal studies (those studies in animal models used to determine whether a candidate should move forward to monkey or human trials) should be performed with antigen(s) that have met minimal quality control standards. This should include: sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, reduced and non-reduced), Western blot analysis, endotoxin levels, sterility and amino acid analysis for determination of protein concentration. High pressure liquid chromatography (HPLC) profile and mass spectrometry are also recommended. Antigen prepared for testing in monkey and human trials will also need to undergo such quality control tests.
   Dr Nicolas Fasel of the Institute of Biochemistry, University of Lausanne, Switzerland, has generously offered to assist laboratories in quality control testing of selected *Leishmania* antigens. It is believed that collaboration and coordination of efforts in the testing of vaccine candidate antigens in more than one laboratory is warranted.

2. The minimum criteria for moving a candidate vaccine to phase I clinical trial must include the consistent induction of significant protection in an animal model, i.e. protection studies must be repeated in more that one laboratory. In addition, the antigen(s) must be formulated with an adjuvant that can currently be used in human clinical trials. Based on these criteria, several promising antigens exist as potential *Leishmania* vaccine candidates.

3. Even though there have been important developments in this field, we still do not understand completely what constitutes a protective response in humans. The animal models currently available are perhaps somewhat, but not entirely, predictive of how efficacious a vaccine candidate will be in humans. It is thus considered important to encourage continued effort towards understanding the human immune response and correlates of protection, and developing predictive assays for the evaluation of vaccine candidates.

4. Additional fundraising activities will require a coordinated, credible plan for selecting and bringing forward candidate antigens.

In view of these conclusions, the group made the following recommendations:

1. Although the development of a *Leishmania* vaccine appeared to be within close reach a few years ago, is is now clear that it will not be an easy task. WHO/TDR should thus devote more resources to *Leishmania* vaccine research and development to really drive the field forward.

2. The group should continue as a consortium to coordinate testing and advancement of candidate antigens (according to the criteria set forth above). Obviously, the consortium remains open to all interested laboratories willing to contribute to this effort.

3. Meetings/workshops aimed at fostering collaborative efforts towards vaccine development should continue to be supported by WHO/TDR, as this will greatly facilitate the evaluation of antigens and the identification of possible candidates for further development.

4. TDR should put together a list of adjuvants that are currently acceptable for use in human clinical studies to assist laboratories in designing their testing in agreement with the criteria mentioned above.

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