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Program: Immunology

Methods for *Leishmania* parasite detection and quantification as a tool for study of the pathogen-vector-host interactions

Summary of the PhD thesis

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Leishmaniasis in human is caused by total 21 species of the intracellular protozoan parasite *Leishmania*, which are transmitted by about 30 species of phlebotomine sand flies. Besides human, *Leishmania* can infect a number of vertebrate hosts. The major host cell is the macrophage, in which parasites multiply, eventually rupturing the cell and spreading to uninfected cells. Infected monocytes and macrophages circulating in the peripheral blood are thought to be carriers of the parasite to distal sites. Depending on the infected sites of the body, there are three forms of leishmaniasis: cutaneous, mucocutaneous and visceral. Leishmaniasis is a disease for which we still lack effective, affordable and easy to use drugs. In addition, surveillance and control are also neglected.

This thesis summarizes the results of several projects using different approaches for parasite load measurement in the mouse model of leishmaniasis, including two methods that were developed and optimized in our laboratory. Detection and quantification of pathogens belongs to the major topics of the research of various infectious diseases. This parameter is necessary for confirmation of the diagnosis, characterization of the host defense, complex pathological changes in the infected organisms, and for the evaluation of the effectiveness of therapy. We use quantification of *Leishmania* parasites to study influence of the genotype on susceptibility to the disease and to estimate the effectiveness of immunization with sand fly saliva.

Application of various parasite measurement assays revealed gene- and sex-dependent differences in susceptibility to *Leishmania.* Two genetic loci that control parasite dissemination in the internal organs were first detected by our laboratory in genome-wide screening using a mouse model of leishmaniasis. The obtained knowledge brings more complex understanding of the pathogen-vector-host interactions.

Metody detekce a kvantifikace parazitů rodu *Leishmania* jako nástroj pro studium vzájemného působení patogenu, vektoru a hostitele

Leishmaniáza je u lidí působena 21 druhem vnitrobuněčného jednobuněčného parazita rodu *Leishmania*, který je přenášen přibližně 30 druhy flebotomů. Kromě člověka jsou leishmánie schopné infikovat celou řadu obratlovců. Hlavní hostitelskou buňkou, v níž se parazit množí, je makrofág; posléze leishmánie buňku ničí a infikují další buňky. Nakažené monocyty a makrofágy cirkulují v periferní krvi a roznášejí parazita do dalších orgánů a tkání. V závislosti na infikované část těla jsou rozlišovány tři typy leishmaniázy: kožní, kožně-slizniční a viscerální. Pro léčbu leishmaniázy stále ještě chybí účinné, dostupné a jednoduše aplikovatelné léky. Zanedbaná je také diagnostika a monitorování onemocnění.

Tato disertační práce shrnuje výsledky několika projektů, ve kterých byly použity různé přístupy k určení množství parazitů v myším modelu leishmaniázy, včetně dvou metod, které byly vyvinuty a optimizovány v naší laboratoři. Detekce a kvantifikace patogenů patří k hlavním tématům výzkumu různých infekčních chorob. Tento parametr je nezbytný pro potvrzení diagnózy, charakterizaci imunitní odpovědi hostitele a komplexních patologických změn v nakaženém organismu, a také pro posouzení účinnosti léčby.

Kvantifikaci leishmanií jsme použili pro studium vlivu genotypu na vnímavost k onemocnění a pro určení efektivity imunizace hostitele slinami flebotomů. Využití různých metod měření množství parazitů vedlo k nalezení rozdílů ve vnímavosti k leishmaniím, které závisely na genotypu a na pohlaví. Citlivost použité metody vedla k prvnímu zmapování dvou genů, které kontrolují rozšíření parazitů do vnitřních orgánů, v celogenomovém skríningu. Získané znalosti přinášejí lepší porozumění interakce mezi patogenem, vektorem a hostitelem.

Introduction

Various intracellular pathogens, which cause a wide spectrum of diseases, live and replicate within endosomal compartments or the cytosol of diverse host cells. Leishmaniasis is a vector-borne protozoan infection, caused by the intracellular parasite of the genus *Leishmania*. Parasites reside in neutrophils, monocytes and macrophages, as well as in dendritic cells and fibroblasts (Lipoldová and Demant 2006, Bogdan 2008). The disease remains a public health problem worldwide, affecting approximately 12 million people in 88 countries; 50 000 die of this disease each year. Leishmaniasis is endemic in areas of tropics, subtropics, and southern Europe, in setting ranging from rain forests in the Americas to desert western Asia (Herwaldt 1999). Leishmaniasis includes asymptomatic infection and three main clinical syndromes. In the dermis, parasites cause the cutaneous form of the disease (CL), which can be localized or diffuse; in the mucosa, they cause mucocutaneous leishmaniasis (ML), and the metastatic spread of infection to the spleen and liver leads to visceral leishmaniasis (VL; also known as "kala-azar"). Parasites can also enter other organs, such as lymph nodes, bone marrow and lungs, and in rare cases, can even reach the brain (Lipoldová and Demant 2006, Pavli et al. 2010). The control of intercellular bacteria and protozoa, including *Leishmania*, usually requires $CD4^+$ T cells and IFNγ and/or TNF-dependent activation of macrophages. The key effector pathways of macrophage against the intracellular parasite stage during acute phase of infection include IFNγ and iNOS, which converts arginine into citruline and leishmanicidal NO. However, it is important to note that the expression of inducible nitric oxide synthase (iNOS) alone is not necessary sufficient to control *Leishmania* (Bogdan 2008). A range of studies revealed a complexity of responses in relation to susceptibility or resistance to leishmaniasis, which are not easily integrated into a simple functional model and are influenced by multiple genetic factors (Lipoldová and Demant 2006).

Scientists that perform human studies of complex diseases meet significant complications due to the genetic heterogeneity of outbred human populations, extensive gene interactions, variations in allelic frequencies and the incomplete penetrance of disease-causing alleles (Weiss and Terwilliger 2000). Animal models help to overcome problems of human studies and understand the genetic basis of polygenic diseases. Use of recombinant congenic mouse strains (RCS) made a great contribution to the study of complex diseases (Demant 2003), including leishmaniasis. RC strains carry 12.5% genes from the donor inbred strain, the remaining 87.5% from the background inbred strain (Demant and Hart 1986).

As with any infectious disease, spread and load of the pathogen during leishmaniasis is an important parameter, as well as precise diagnostics of *Leishmania* species. Until recently, this task was a significant complication for practical doctors and researchers. The optimal methods should be fast, simple and price-worthy. Easy-to-use techniques for rapid pilot tests during field application in the absence of the equipped laboratory are also highly necessary. The main approaches for parasite detection include DNA-based techniques, microscopy assays, cell cultures, and immunodetection (Kobets et al. 2010). Microscopy detection of parasites in Giemsa or hematoxylin and eosin (H&E) stained samples considered to be the classical standard from the beginning of *Leishmania* studies (Borovsky 1898, Donovan 1903). Though these methods are laborious, time consuming and lack sensitivity, they are highly specific, visualize the parasite directly, and remain a perfect tool for the result illustration. Until recent, the limiting dilution assay (Titus et al. 1985) was commonly used for parasite quantification. This method has similar limitations as microscopy, requiring a lot of time and work; moreover, all culture-based techniques are highly dependent on sterile conditions. Nevertheless, culturing of parasites can reflect their viability and ability to multiply. At the present, the PCR -based methods create the most popular and intensively developing group of assays. They detect parasite DNA and were proved to be reliable, time-saving, and most suitable for the purposes of diagnostics and research, especially for analysis of large numbers of samples. These significant advantages over the other methods make them a powerful tool for *Leishmania* parasites detection, quantification and identification of species and subspecies. In comparative studies that used different DNA regions as targets for PCR, the kinetoplast DNA (kDNA) PCR was the most sensitive diagnostic assay and should be employed as gold standard against which all new techniques should be compared (Bensoussan et al. 2006, Nasereddin et al. 2008, de Lima et al. 2010). Noninvasive rapid tests (latex agglutination test (LAT), direct agglutination test (DAT), and rK39 dipstick test) deserve special attention, because they are commercially available, easily applicable and give fast results (Bern et al. 2000, Vilaplana et al. 2004).

As we can conclude, there is no one universal method that could be used in any case when the parasite load is necessary to be measured. Due to their different advantages and disadvantages, all approaches have different perspectives of use. PCR-based techniques are the most sensitive at the present moment, so we highly concentrated our attention on them. PCR-ELISA is robust, relatively simple, and it is a cheaper method in comparison with other quantitative assays. Taking into account these facts, we decided to design an improved PCR-ELISA. Rising the advantages and reducing disadvantages of the method, we made it more

optimal for the purposes of our study, and also increased its potential of application by other laboratories. In addition, we continue using different, non-PCR-based techniques, as we use multiple approaches for the research of leishmaniasis.

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Aims of the study

I. Design, optimization and application of different assays for detection and quantification of *Leishmania* parasites

1. Development of the improved PCR-ELISA method for quantitative measurement of parasite load in tissues of the infected organism.

2. Modification of the limiting dilution assay that would allow processing of a large number of samples in one experiment.

3. Using of microscopy assays as a classical standard for validation of novel methods and a visible illustration of presence of *Leishmania* in organs and tissues.

II. Estimation of parasite burden in the infected mice for characterization of the immune response during leishmaniasis

1. Detection of genetic loci that control *Leishmania major* dissemination in the infected organism.

2. Establishment of the mouse model of *Leishmania tropica* infection using genetically different RC strains.

3. Study of the influence of sand fly saliva on the development of leishmaniasis in a host.

Materials and Methods

I. Animals

Males and females of strains BALB/c, STS, selected RC strains and F_2 hybrids were tested. When used for these experiments, RC strains were in more than 38 generation of inbreeding and therefore highly homozygous. The parts of their genome inherited from the

BALB/c or STS parents were defined (Stassen et al. 1996). The mice were 8 to 24 weeks old at the time of infection.

II. Infection

Experimental animals were infected with *Leishmania major* LV 561 (MHOM/IL/67/LRCL137 JERICHO II) or *Leishmania tropica* from Urfa, Turkey (MHOM/1999/TR/SU23). Parasites were inoculated into the tail base or ear. For immunization with sand fly saliva, mice were exposed to *Phlebotomus duboscqi* females (colony originating from Senegal) following four different exposure schemes (Rohoušová et al. 2011, Fig. 1A).

III. Disease phenotype

To determine disease phenotype, size of the skin lesions was measured weekly using a Vernier caliper gauge. Mice were sacrificed from 7 to 43 weeks after infection. Inguinal lymph nodes, and also blood, spleen and liver were collected for the further analysis. In the experiment with sand fly saliva, blood, infected ears and lymph nodes were isolated. Splenomegaly (enlargement of the spleen) and hepatomegaly (enlargement of the liver) were calculated as organ-to-body weight ration × 1000.

IV. Quantification of parasite load in spleens and inguinal lymph nodes

In total, three different approaches were used to detect and quantify *Leishmania* parasites in the infected mice tissues: light and fluorescent microscopy, modified limiting dilution assay and PCR-ELISA.

Parasites were counted in **Giemsa-stained tissue smears** as the mean amastigote number from 100 microscopy fields observed.

Inguinal lymph nodes and spleens were fixed in 4% formaldehyde and embedded to parafin. Sections were stained with standard **H&E procedure** or processed for **immunohistochemical parasite detection** using anti-*Leishmania* lipophosphoglycan monoclonal mouse IgM antibody (CLP003A, Cedarlane, Canada) and TRITC-conjugated goat anti-mouse IgM (Code Nr. 115-025-020, Jackson Immunoresearch, USA). Samples were examined using a microscope Leica DM 6000 (Leica, Germany) under x1000 magnification.

For **quantification of *Leishmania* by PCR-ELISA**, DNA from parasites and from analyzed tissues was isolated with TRI Reagent® solution (Molecular Research Center, USA) according to the manufacturer's protocol or by a standard proteinase procedure (Laird et al.

1991). Two labeled primers were used in PCR: digoxigenin-labeled F 5'-ATT TTA CAC CAA CCC CCA GTT-3' and biotinlabeled R 5'-GTG GGG GAG GGG CGT TCT-3' (VBC-Genomics, Biosciences Research, Austria); the sequences were described in Rodgers et al. 1990. For ELISA, plates were coated with streptavidin solution at 4°C overnight. The positive PCR control with amplified *Leishmania* DNA was used as a standard for ELISA. After incubation with labeled amplicon, anti-digoxigenin detecting Fab fragments, conjugated with peroxidase, were added (Roche Diagnostic GmbH, Germany). The parasite load was determined using the ELISA reader after the substrate was applied. Concentration of *L. major* DNA in the samples was calculated using the curve fitter program KIM-E for Windows (Schoeller Pharma, Czech Republic) by least squares–based linear regression analysis.

Modified semi quantitative culture technique for measurement of the parasite burden is based on the limited dilution assay (Titus et al. 1985). We used only one pre-selected cell concentration of the infected cells and counted parasites after incubation with a Coulter Counter (Beckman Coulter Inc., USA). In comparison with the original limited dilution technique, this modified culture method is less laborious and allows rapid estimation of parasite number.

V. Estimation of cytokine, chemokine and immunoglobulin levels

ELISA procedure was performed similarly as described above for PCR-ELISA. IFNγ, IL-4, IL-12, and IgE levels in serum were determined using the primary and secondary monoclonal antibodies (Kurey et al. 2009). Specific anti-*P. duboscqi* saliva IgGs in serum were measured by immunoblot and ELISA as previously described (Rohoušová et al., 2005). For ELISA tests, wells were coated with *P. duboscqi* salivary gland homogenate in 0.01 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. Incubation of 1:200 diluted sera in the coated plates was followed by incubation with goat anti-mouse IgG-peroxidase conjugate. Orthophenylendiamine and H_2O_2 in McIlwein phosphate-citrate buffer (pH 5.5) were used as the substrate solution. The absorbance was measured at 492 nm wavelengths. Immunoblots were performed on salivary gland homogenate separated by SDS–PAGE on 12.5% gel under non-reducing conditions. An equivalent of 50 gland pairs was loaded. Separated proteins were electro-transferred onto nitrocellulose membrane. After transfer, the membrane was cut into strips and incubated with mouse sera diluted 1:100 in Tris buffer and goat anti-mouse IgG peroxidase-conjugate. The color reaction was developed using substrate solution containing diaminobenzidine and H_2O_2. Levels of GM-CSF, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-

1β , CCL5/RANTES and CCL7/MCP-3, in serum were determined using antobody-coated beads (Mouse chemokine 6-plex kit, Bender MedSystems, Austria).

VI. Genotyping of F_2 hybrids

DNA was isolated from mouse tails using a standard proteinase procedure (Laird et al. 1991). The strain CcS-11 differs from BALB/c at STS-derived segments on eight chromosomes, respectively (Stassen et al. 1996). These differential segments were typed in the F_2 hybrid mice between CcS-11 and BALB/c using 16 microsatellite markers (Research Genetics, USA): D1Mit403, D3Mit45, D7Mit54, D7Mit18, D7Nds1, D7Mit282, D7Mit67, D7Mit259, D8Mit85, D10Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, and D19Mit46 (all sequences can be found at http://www.informatics.jax.org). Each PCR product was electrophoresed in 2–3% agarose gel with ethidium bromide or in 6% acrylamide gel (for [γ-^{32}P]ATP end-labeled primers) (Kurey et al. 2009).

VII. Statistical analyses

Differences in parasite load between BALB/c and STS mouse strains were estimated by the Mann–Whitney *U*-test in Statistica (Kobets et al.2010). NCSS and SAS programs were used to detect the linkage of genes that control parasite spread, organ pathology, and immune parameters with microsatellite markers by analysis of variance (ANOVA). The differences between CcS/Dem strains in parasite numbers in lymph nodes were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison using Statistica (Kobets et al., in preparation). Lesion size development was analyzed by general linear models ANOVA and Scheffe's Multiple Comparison Procedure. Other data (parasite load and antibody production) were subjected to non-parametric Wilcoxon tests. For correlation tests we used the non-parametric Spearman Rank Correlation Matrix in NCSS (Rohoušová et al. 2011).

Results and Discussion

Detection and quantification of different parasites belongs to the hot topics of the tropical medicine. *Leishmania* is among the first targets on the list of interest. The present work proves how different approaches can be used to obtain valuable scientific information. The experiments included to this thesis estimated various disease manifestations, such as skin lesion size; parasite load in spleen, lymph nodes and infected ears; levels of cytokines, chemokines and immunoglobulins; splenomegaly and hepatomegaly. Full detailed discussion, involving all obtained results, is given in each publication or manuscript. Here the attention

will be predominantly concentrated on the data concerning importance of estimation of the parasite load in the *Leishmania* research. However, all other results could not be omitted since leishmaniasis is a complex disease and it is absolutely necessary to have in mind a complete overview of the question.

I. Development and optimization of the methods

Despite of the abundance of the methods for measurement of the parasite burden, most of them are problematic to use if more than one hundred samples has to be tested fast, in one run. In addition, many of them have significant drawbacks.

As mentioned above, we were first to introduce two labeled primers in PCR-ELISA test developed for leishmaniasis research. All other similar methods include only one labeled primer and label the product with an additional probe before the ELISA step. Each additional step in the reaction itself increases the possibility of distortion of the result. In a case of non-complete or non-specific binding of the labeled probe to the PCR product, it is impossible to detect the presence of the technical mistake, because there is no method to find out if such hybridization was absolutely successfull. As a consequence, it increases the possibility to obtain false positive, as well as false negative, results. Such discrepancies may play especially dramatic role during examination of clinical samples. When two primers are labeled in advance and the conditions of PCR are carefully optimized, the method can give a guaranteed result. A particular advantage is the ability to make an additional control procedure and check the PCR product by agarose gel electrophoresis (see Kobets et al. 2010) to ensure that the synthesis was specific and the result is reliable.

The second method that was modified and applied in our laboratory was a protocol for measurement of parasite load using tissue cultures. This method is based on the limiting dilution assay (Titus et al. 1985, Lima et al. 1997) and targets only viable parasites that are able to multiply. This specific feature makes cell culture based techniques still useful for various purposes, such as antileishmanial drugs development, in which distinction between dead and alive parasites is important. The most significant change introduced to the method was cultivation of cells in only one concentration, chosen according to the results of the previous experiments. This modification brought a big advance and allowed testing of more than a hundred samples in one experiment, what would be extremely laborious and technically very difficult with classical limiting dilution assay (Kobets et al., in preparation).

II. *Leishmania* and different host genotypes

As described above, genetically different mice from the experiments with *Leishmania major* (BALB/c, CcS-11 and F_2 hybrids between these strains) were tested for parasite load with modified PCR-ELISA protocol. Parasite presence in secondary lymphoid organs is controlled by two loci, *Lmr20* and *Lmr5* (Kurey et al. 2009, table 3). In addition to parasite load in draining lymph nodes, *Lmr20* influenced levels of serum IgE and IFNγ; whereas *Lmr5* was also involved in control of lesion size, parasite load in spleen, splenomegaly, serum IgE, serum IFNγ, and serum IL-4 (Kurey et al. 2009, table 2).

On the basis of the obtained results, we can conclude that parasite numbers in lymph nodes and in spleen are controlled independently. *Lmr20* is not involved in regulation of skin lesion size, splenomegaly, or hepatomegaly, but it influences systemic immunological changes along with parasite numbers in lymph nodes. Control of spread of parasites to the spleen is exercised by another locus - *Lmr5* along with pathology of another organ (the skin lesions) and systemic immune responses (Kurey et al. 2009).

In leishmaniasis research, *Lmr20* on the chromosome 1 and *Lmr5* on the chromosome 16 were the first genetic loci that control parasite dissemination in *Leishmania major* infection detected in genome-wide study.

The importance of involvement of *Leishmania tropica* in human leishmaniasis has been recognized only recently. Similarly as *L. major, L. tropica* causes cutaneous leishmaniasis in humans, but was also reported to visceralize and cause systemic illness (Magill et al. 1993, Alborzi et al. 2008, Weiss et al. 2009). Genetic basis of susceptibility or resistance to *L. tropica* infection has been poorly studied because of the absence of the suitable animal models.

Similarly to the experiments of other groups (Lira et al. 1998), the disease progress was very slow. In comparison with the results of our previous studies with *L. major* infection in RC strains (Lipoldová et al. 2000, Lipoldová et al. 2002, Havelková et al. 2006), all disease manifestations were less progressed in *L. tropica* infected mice. Moreover, genotype effects in RC strains were different after *L. tropica* and *L. major* infection (Kobets et al., in preparation).

Parasite load in the inguinal lymph nodes did not correlate with development of skin lesions after *L. tropica* infection. Such existence indicates independent genetic control of different disease manifestation is similar to the experiment with *L. major* infected F_2 hybrids between BALB/c and CcS-11, described above (Kurey et al. 2009). Microscopy examinations of histological sections from the spleen confirmed the presence of parasites, previously

detected with culture method. Thus, we first report visceralization of *L. tropica* in mice. Taking into account the information that *L. tropica* was able to cause viscerotropic leishmaniasis in human (Magill et al. 1993, Alborzi et al. 2008, Weiss et al. 2009), the mouse model of visceral pathology could contribute to understanding of genetical background of this phenomenon. In the *L. tropica* experiment parasite load in spleens and inguinal lymph nodes was measured with tissue culture method.

Our studies show that comparison of *L. tropica* and *L. major* infections indicate existence of the species-specific patterns of response to the certain pathogen, with different sex effects and different host susceptibility genes (Kobets et al., in progress). In human population, the influence of genotype (Lipoldov á and Demant 2006, Sakthianandeswaren et al. 2009) and sex on response to different parasite infections was also observed (Jones et al. 1987, Weigle et al. 1993, Shlagenhauf et al. 2010). The data, coming from different populations, varied. This fact illustrates the complex influence of genetic and environmental factors. There is relatively few data derived from human studies due to the general complications of human genetic research. However, such information is remarkably valuable and may confirm the theory that leishmaniasis in heterogeneous human population is influenced by multiple factors, such as genotype of the host, sex, environmental factors and *Leishmania* species.

III. Immunization with sand fly saliva

It was hypothesized that the enhancing effect on *Leishmania* infection could be neutralized in humans vaccinated against vector saliva, thereby preventing the establishment of infection. This concept was proven in various animal models, though such level of protection has not been reported from the field. Despite of continuous exposure of the individuals to the insect bites, leishmaniasis still persists in the endemic areas (Rohoušová et al. 2011). In our present study, *Leishmania* infection was monitored in BALB/c mice exposed to *Phlebotomus duboscqi* bites following four different schemes, mimicking short- and long-term exposure to the saliva during different sand fly seasons (Rohoušová et al. 2011, fig. 1A). In the group of mice that got 2 days exposure to sand fly bites right before the infection (group 2), the cumulative parasite load in immunized mice was significantly reduced, as well as lesion development. Moreover, only the protected group (group 2) had significantly lower anti-*L. major* IgG levels compared with non-immunized mice (Rohoušová et al. 2011, fig. 1F), correlating with the lowest parasite load in this group. The protective effect was lost in all other groups. On the other hand, no correlation was found between anti-*P. duboscqi* IgG

and parasite load in the draining lymph node, indicating that preexposure to *P. duboscqi* bites could completely prevent parasite dissemination, though higher parasite load in the ears indicate that in a bitten hosts *L. major* preferentially multiplies in the skin tissue.

Our experiments proved that the immune response resulting from this immunization scheme have certain protective effects, however the history of exposure may significantly change the character of anti-saliva immune response with substantial consequences for *Leishmania* infection development. This assumption is in agreement with field results from an endemic area of cutaneous leishmaniasis caused by *Leishmania braziliensis* (de Moura et al. 2007, Rohoušová et al. 2011).

This is one more study in which we used PCR-ELISA (Kobets et al. 2010). Measurement of the parasite load helped to reveal different aspects of the influence of sand fly saliva on the development of leishmaniasis. Our results might help to explain the persistence of *Leishmania* infection in endemic areas and should be taken into account when designing and testing accines based on vector salivary proteins (Rohoušová et al. 2011).

IV. Microscopy as a visual proof

Detection of *Leishmania* parasites both with light and fluorescent microscopy remains an informative and irreplaceable tool for visual illustration of the experimental results. Using histological examination, we confirmed presence of parasites in inguinal lymph nodes in *L. major* study (Kurey at al. 2009) and proved the fact of penetration of the parasite into the spleen in *L. tropica* project (Kobets et al., in preparation). A classical method of parasite count in tissue smears was applied for validation of the improved PCR-ELISA (Kobets et al. 2010).

The present work shows the importance of parasite detection and quantification as a parameter that can significantly contribute to understanding of the mechanism of leishmaniasis.

Conclusions

I. We designed and optimized two assays for detection and quantification of *Leishmania* parasites and used classical microscopy for validation.

1. The improved PCR-ELISA method uses two labeled primers and allows measurement of parasite load in tissues of the infected organism with high specificity and sensitivity.

2. Modified culture-based method enables processing of much larger numbers of samples than classical limiting dilution assay and detects viable parasites in tissue samples.

3. Using of microscopy assays served as a classical standard for validation of novel methods, as well as illustrated the obtained results.

II. Measurement of parasite load revealed important aspects of the disease development, and showed genotype-dependent variations of the immune response during leishmaniasis. Estimation of parasite numbers also helped to characterize effects of sand fly saliva on the pathological changes in the infected organism.

1. Two novel loci that control parasite load were first detected in genome-wide study.

2. Parasite dissemination to the spleen and lymph nodes is controlled independently by the genetic loci situated on two different chromosomes.

3. We first established the mouse model of *Leishmania tropica* infection using RC strains.

4. In *L. tropica* infection, parasite numbers were much lower in comparison with *L. major* experiments. This data confirms the fact that, in addition to genetic and environmental factors, immune response of the organism strongly depends on the *Leishmania* species.

5. *L. tropica* parasites were present in lymph nodes of all tested strains, and some strains penetrated to the spleen; however, no splenomegaly was observed. Parasite load also did not correlate with skin lesion that suggests independent genetic regulation of these disease parameters.

6. Measurement of the parasite load helped to reveal various types of the influence of sand fly saliva on development of leishmaniasis. Protective effect of saliva is limited and depends on the history of exposure to the insect bites.

7. Immunization with sand fly saliva does not completely prevent dissemination of *Leishmania major* parasite to the lymph nodes; however it can reduce pathology in case of short-term exposure before the infection. This data should be taken into account during development of saliva-based vaccines.

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Curriculum vitae

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Citizenship / nationality: Ukraine

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Degrees

2002 – **BSc** (Biochemistry, Graduated with Honors): Karazin Kharkiv National University, Kharkiv, Ukraine

2003 – **MSc** (Biochemistry, Graduated with Honors): Karazin Kharkiv National University, Kharkiv, Ukraine

Affiliations

2001-2002 – BSc Diploma Student, State Scientific Center of Drugs of the Ministry of Health, Academy of Sciences of Ukraine, Kharkiv, Ukraine

2002-2003 – MSc Diploma Student, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Puschino, Russian Federation

2004–2011 – PhD Student, Institute of Molecular Genetics Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic

Special courses

April 14-21, 2007 – ENII Summer School in Advanced Immunology, Capo Caccia, Sardinia, Italy

May 12-16, 2008 - Acquisition and Processing of the Image in Microscopy, Prague, Czech Republic

Main research interests

Infectious diseases and immune response, various approaches for detection and quantification of pathogens, mapping and functional analysis of genes that control susceptibility and resistance to infections.

Experience with methods

PCR, PCR-ELISA, light and fluorescent microscopy, histological analysis of tissue sections, immunohistochemistry, ELISA, DNA/RNA isolation, reverse transcription, restriction analysis, electrophoresis, preparation of organs from animals, statistical analysis, sequencing, cell cultures, isolation of cells from blood.

Current participation in research projects

1. Mapping of genes that control parasite numbers in *Leishmania major* and *Leishmania tropica* infection.

2. Development of drugs for treatment of leishmaniasis.

3. Histopathological changes after *Leishmania* infection.

4. Immunohistochemical detection of molecules that take part in host-parasite interactions.

Main international conferences

September 6-9, 2006 – 16th European Congress of Immunology, Paris, France

T. Kobets, H. Havelková, I. Kurey, I. Grekov, J. Badalová, M. Svobodová, K. Trtková, M. Lipoldová. Mapping of genes that control *Leishmania* parasite number. Book of abstracts, p. 382.

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August 21-25, 2007 – 13th International Congress of Immunology, Rio de Janeiro, Brazil.

I. Kurey, **T. Kobets**, H. Havelková, M. Slapničková, K. Trtková, I. Grekov, P. Demant, M. Lipoldoavá. Genetic control of parasite dissemination in *Leishmania major* infection – specificity and complex relation to local and systemic disease. On CD.

February 3-7, 2009 – 4th World Congress of Leishmaniasis Lucknow, India.

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Publications

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In preparation...