Katedra parasitologie a hydrobiologie
Přírodovědecká fakulta University Karlovy v Praze

DISERTAČNÍ PRÁCE

VÝVOJ LEISHMANIÍ V PŘENAŠEČÍCH RODU *PHLEBOTOMUS*

JOVANA SÁDLOVÁ

Školitel: Doc. RNDr. Petr Volf, CSc.
Praha 1999

PODĚKOVÁNÍ

Ráda bych poděkovala všem, kteří jakkoli přispěli ke vzniku této práce. Především je mi milou povinností poděkovat doc. Petrovi Volfovi, Csc., který mi umožnil zabývat se významnou a krásnou skupinou hmyzích přenašečů flebotomů. Jeho zásluhou mohl začít výzkum této pro nás exotické skupiny v podmínkách pražské laboratoře. Dále mu děkuji za odborné vedení práce i ochotné předávání praktických zkušeností s flebotomy. Dr. Mileně Svobodové bych ráda poděkovala za zaučení v práci s leishmaniemi a za ochotné konzultace experimentálních problémů.

Dr. Jean-Claudovi Dujardinovi vděčím za příležitost naučit se aktuální techniky molekulární biologie v příjemném prostředí jeho laboratoře v Institutu tropického lékařství Prince Leopolda v Antverpách. Za poskytování cenných rad i literatury jsem zavázána prof. Jiřímu Vávrovi, DrSc., doc. Jaroslavu Kuldovi, CSc. a doc. Liborovi Grubhofferovi, CSc. Za konzultace biochemických metod děkuji Dr. Ivanovi Hrdému, za konzultace metod statistických Dr. Danielu Fryntovi. Celému kolektivu katedry, především všem „flebotomařům“ a helmintologům, děkuji za přátelskou a podnětnou atmosféru, bez které si nedovedu svou práci představit. Velmi cenná byla pro mne i nevšední „všední“ práce Dr. Heleny Kulíkové a Dušana Eremiáše.

Tato disertační práce by nemohla vzniknout bez trpělivosti a všestranné podpory mé rodiny, která mi vytvářela potřebné zázemí po celou dobu postgraduálního studia. Zejména děkuji svému muži Jirkovi, kterému jsem občas pila krev a vylévala adrenalin nejen já, ale i moji flebotomové.

OBSAH

ČLENĚNÍ A CÍLE DISERTAČNÍ PRÁCE

Práce se skládá z těchto částí: Za úvodním **výčtem cílů** následuje **literární přehled**, vypracovaný ve formě review (přijato pro publikaci v Acta Soc. Zool. Bohem.). **Výsledková část** je tvořena pěti původními vědeckými pracemi, z nichž čtyři jsou již publikované a jedna připravena k publikaci. V těchto publikacích jsou řešena obě hlavní témata disertační práce: (1) faktory ovlivňující vývoj leishmanií v přenašeči a (2) mechanismus přenosu leishmanií flebotomy. Práce je zakončena **shrnutím** a **závěry**.

HLAVNÍ CÍLE PRÁCE:

(1) Faktory ovlivňující vývoj leishmanií v přenašeči.

VÝCHOZÍ STAV: V trávicím traktu přenašeče se leishmanie setkávají s prostředím tvořeným mnoha faktory, například proteolytickými enzymy a lektiny flebotomů či složkami krevní a cukerné potravy, kterou samice přijímají. Toto prostředí je do jisté míry druhově specifické a určitý druh leishmanie může být adaptován na konkrétní podmínky v trávícím traktu určitého druhu flebotoma (či spíše několika blízce příbuzných druhů z téhož podrodu). Adaptace leishmanií pro vývoj v přenašeči jsou však relativně málo probádány a není jasné, do jaké míry se zde uplatňují i hlavní povrchové molekuly ovlivňující vývoj parasita v hostiteli (virulenční faktory). Nejasná je například role povrchové metaloproteázy gp63 v přenašeči.

Tato práce vychází z předpokladu, že existují rozdíly ve vývoji různých kmenů jednoho druhu parasita v jeho přenašeči. Podrobnější charakterizace takto se lišících kmenů může napovědět, které vlastnosti parasita jsou pro vývoj v přenašeči klíčové a které hrají méně významnou roli.

Cílem práce bylo tedy porovnat vývoj několika geograficky odlišných kmenů a linií *L. major* v jejich přirozených přenašečích *P. papatasi* a *P. duboscqi*. U těchto kmenů pak otestovat virulenci a infektivitu pro hostitele (myš), a studovat virulenční faktory, především expresi lipofosfoglykanu (LPG) a aktivitu povrchové proteázy gp63.

Druhým metodickým přístupem bylo zjistit změny všech těchto parametrů po několikanásobné pasáži atenuované linie kmene LV561 přes flebotomy nebo myši BALB/c. Srovnáním výsledných linií (navzájem, vůči výchozí atenuované linii a vůči virulentní linii téhož kmene) pak opět získat informaci o roli hlavních povrchových molekul parasita v přenašeči/hostiteli a o reverzibilitě změn souvisejících s dlouhodobou kultivací *in vitro*.

DÍLČÍ CÍLE:

(a) Srovnat vývoj pěti kmenů a linií *L. major* ve dvou druzích flebotomů (*P. papatasi* a *P. duboscqi*), t.j.:

- porovnat intensitu a lokalizaci infekcí v trávicím traktu flebotomů.
- zjistit rozdíly v zastoupení jednotlivých morfologických forem leishmanií.
- pokusit se o experimentální přenos na hostitele (křečka).

(b) Porovnat infektivitu a virulenci kmenů a linií v myších BALB/c.

(c) Zjistit rozdíly ve virulenčních faktorech kmenů a linií, t.j. studovat
- expresi metacyklického LPG.
- gp63 na genové úrovni (počet genových kopií, struktura gp63 lokusu a jeho lokalizace na chromosomech).
- gp63 na proteinové úrovni (množství exprimovaného proteinu a jeho proteázová aktivita).

(d) Atenuovanou linii kmene LV561 pasážovat 5x přes flebotoma (*P. duboscqi*) a 5x přes hostitele (myš BALB/c) a porovnat vlastnosti výsledných isolátů s původní linií, především studovat jejich:
- vývoj ve flebotomech, virulenci a infektivitu pro hostitele.
- povrchové molekuly LPG a gp63 (viz bod c).
- hemaglutinační aktivitu.

(2) Mechanismus přenosu leishmanií flebotomy.

VÝCHOZÍ STAV: Přesný mechanismus přenosu leishmanií na hostitele nebyl jednoznačně objasněn. Předpokládá se buď inokulace promastigotů přítomných v okamžiku sání v proboscis flebotoma, nebo regurgitace parasitů způsobená obtížemi při sání krve. Žádná novější studie nevěnovala pozornost možnosti kontaminativního přenosu; ve starších publikacích je uvažováno buď pozření infikovaného flebotoma nebo jeho rozmáčknutí na kůži během sání. Naše pozorování, že samice flebotomů vypuzují již během sání na hostiteli kapičky moči (prediurese), nás vedlo k myšlence prozkoumat podrobněji tento fyziologický proces a zhodnotit jeho význam pro možný kontaminativní přenos leishmanií.

DÍLČÍ CÍLE

(a) Popsat základní charakteristiky prediurese u nenakažených samic dvou druhů flebotomů (*Phlebotomus papatasi, P. duboscqi*).

(b) Pomocí kapilárového sání zjistit přítomnost či absenci leishmanií v moči flebotomů (*P. papatasi, P. duboscqi*) infikovaných *L. major*. V případě positivního výsledku:
- srovnat četnost nálezů leishmanií vyloučených v moči a vypuzených do obsahu kapilár.
- charakterizovat morfologicky leishmanie nalezené v moči.
- ověřit přítomnost metacyklických promastigotů v moči pomocí monoklonální protilátky proti metacyklickému lipofosfoglykanu (LPG).
- ověřit kultivací životaschopnost vyloučených leishmanií.

Acta Soc. Zool. Bohem. **63**: 331–366, 1999
ISSN 1211-376X

The life history of *Leishmania* (Kinetoplastida: Trypanosomatidae)

Jovana SÁDLOVÁ

Department of Parasitology, Charles University, Viničná 7, CZ–128 44 Praha 2, Czech Republic;
e-mail: josadlo@natur.cuni.cz

Received May 15, 1999; accepted June 3, 1999
Published August 22, 1999

Abstract. This paper summarizes the current state of research concerning protozoan parasites of the genus *Leishmania* Ross, 1903 and its vectors, phlebotomine sandflies. The leishmanias, causative agents of leishmaniasis, a medically important disease, are an excelent example of a successful adaptation to parasitic life. In the present review, both parts of the *Leishmania* life-cycle, i. e. development of the parasite in the sandfly and the parasite-macrophage interaction in the vertebrate host, are analyzed. Special attention is given to factors influencing susceptibility of sandflies to *Leishmania*, and factors influencing *Leishmania* virulence. The role of lipophosphoglycan and gp63, two main cell-surface molecules, in parasite survival is discussed.

Leishmaniasis, sandfly, *Phlebotomus*, life-cycle, transmission, virulence, macrophage, lipophosphoglycan, gp63

INTRODUCTION

Leishmania spp. are causative agents of leishmaniasis, one of the six most important human parasitic diseases in the world. The medical importance of this genus stimulates medical and highly diversified basic biological research. Most frequent are biochemical, genetical and immunological studies (hundreds of papers published each year). Intensive research concerns virulence factors and drug targets. Less abundant, but equally important, is the research in the field of epidemiology, vector biology and vector-parasite interactions.

Whereas most recent reviews concentrate on particular problems, the present review attempts to inform on the main lines of the current leishmania research ranging from molecular biology to ecology. Equal attention is given to both the vector and host parts of the *Leishmania* life-cycle. Factors influencing *Leishmania* development in the vector, as well as *Leishmania* interactions with the defense system of host cells, are analyzed. The importance of main surface molecules of the *Leishmania*-cell during the life-cycle is discussed.

Leishmania is an excelent example of a successful adaptation to parasitic life. The intensive research on this parasite gives us the chance to recognize many details of the parasite – host – vector interactions.

PART I.
Human leishmaniasis: a current status of the disease

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted by the bite of an insect vector, the phlebotomine sandfly (Diptera: Psychodidae). The life cycle of *Leishmania* includes morphologically and physiologically distinct forms in the sandfly and in the vertebrate host (Fig. 1). Parasites taken with a blood meal by female

sandflies transform into flagellated promastigote stages and undergo multiplication and differentiation from procyclic forms into infective, metacyclic forms in the sandfly gut. Metacyclic promastigotes are transmitted to the host during the next blood meal. In its tissue, they are phagocytosed by macrophages and, subsequently, survive and multiply in macrophage phagolysosomes as non-flagellated amastigote stages.

A number of *Leishmania* spp. cause disease in animals, and humans become infected incidentally when they enter an area of endemicity. Rarely humans serve as a reservoir. The clinical manifestation of the disease depends on complex interactions between the virulence characteristics of the parasite and the immune response of its host. Generally, the disease takes four main forms. **Visceral leishmaniasis (VL)** is the most serious form, fatal if untreated. **Cutaneous leishmaniasis (CL)** is the most common infection, causing simple or a limited number of skin lesions which usually heal in few months, but leave scars. **Mucocutaneous leishmaniasis (MCL)** begins with simple skin ulcers, which can spread and cause destruction of the nose, mouth, pharynx, or larynx tissues. **Diffuse cutaneous leishmaniasis (DCL)** is characterized by disseminated chronic lesions, difficult to treat. A single *Leishmania* species can produce more than one clinical syndrome and each syndrome is caused by multiple species (Pearson & de Queiroz Sousa 1996).

Leishmaniasis currently affects about 12 million people, the global annual incidence is estimated at 1–1.5 million new cases of CL and 0.5 million new cases of VL per year. These official data are strongly underestimated as they are obtained almost exclusively through a passive case detection. Leishmaniasis is endemic in 88 countries of all the continents except Australia and Antarctica, 350 million people being at the risk of infection. More than 90% of VL cases in the world are reported from Bangladesh, Brazil, India and Sudan and more than 90% of CL cases came from Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru. MCL is an important problem in Brazil and Latin America. An outbreak of VL in Southern Sudan involved 100.000 deaths over the past five years in a population of less than one million. The importance of leishmaniasis increases today with expansion of AIDS: in southern Europe, high number of adult VL cases is related to HIV infection (data obtained from WHO web page; http://www.who.int/ctd/html).

PART II.
The systematic position and phylogeny of the genus *Leishmania*

Leishmania is a monophyletic genus (Thomaz-Soccol et al. 1993a) belonging to the family Trypanosomatidae (order Kinetoplastida). This ancient family involves species which are morphologically very similar, but their adaptations to many different life strategies led to a great heterogeneity in physiological and biochemical traits.

There are two opinions on the origin of digenetic trypanosomatids: 1. origin from monogenetic parasites of invertebrates, with subsequent adaptation to vertebrates, and 2. origin in vertebrates, with secondary transmission by blood-sucking invertebrates (reviewed by Lainson & Shaw 1987). The first hypothesis is supported mainly by (a) a common occurrence of monogenetic flagellates in various ancient invertebrate host groups, (b) hindgut development and contaminative transmission with the excreta in such parasites, (c) sexual reproduction of some trypanosomes in the insect vector, (d) more complicated development of trypanosomatids in invertebrates as compared to the development in vertebrates. The strongest argument for the second hypothesis is that monogenetic insect flagellates are more frequently found in invertebrate orders and families that feed on blood than in other taxa.

The origin of *Leishmania* from monogenetic insect flagellates is generally more widely accepted. According to this hypothesis, the origin of the genus *Leishmania* depends on the evolutional history of sandflies. Ancestors of modern sandflies probably existed as early as in the Jurassic era

and ancestors of Phlebotominae *sensu stricto* were found in Cretaceous deposits (Hennig 1972). From the Jurassic period, ancestral parasites might get established in vertebrate hosts. It is suggested that the haematophagous habit of sandflies was primarily associated with mammals, which is in agreement with the absence of *Leishmania* spp. in birds and their relative rarity in reptiles (Shaw 1997).

The *Leishmania* adaptation to mammals has run for about 90 million years, i. e., approximately, from the period when mammals were diversifying into different orders. When the African and South American continents divided, the subgenera *Leishmania* and *Viannia* became separated together with their hosts and vectors. Indeed, the genetic distance between the species of the New World subgenus *Viannia* and the subgenus *Leishmania* (Beverley et al. 1987) is of the same order as that of mammalian orders that separated some 85 million years ago (reviewed by Shaw 1997). The ancient division and the subsequent independent evolution of the two subgenera were confirmed also by a cladogram based on isoenzyme analysis (Thomaz-Soccol et al. 1993a). The subgenus *Leishmania* contains two sister groups of the Old World and the New World species complexes, respectively (Thomaz-Soccol et al. 1993a). The authors suggest that New World members of the subgenus *Leishmania* were introduced into American continent secondarily during early Cenozoic by migration of rodents from the Old World (Fig. 2). The subgenus *Sauroleishmania* containing reptile parasites diverged from the lineage leading to the subgenus *Leishmania* after this group separated from the *Viannia* subgenus (Noyes et al. 1998).

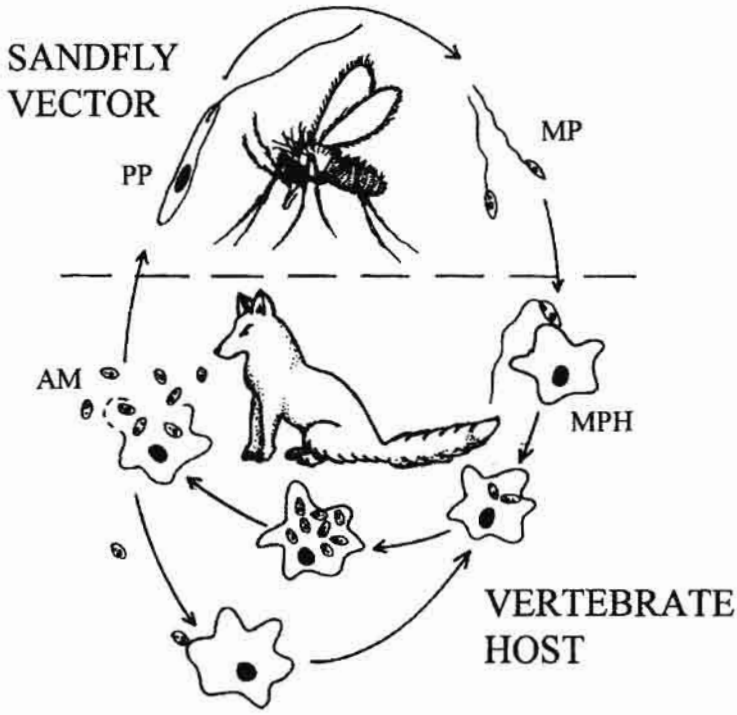


Fig. 1. Life-cycle of *Leishmania* parasites. PP, procyclic promastigotes; MP, metacyclic promastigotes; AM, amastigotes; MPH, macrophages.

The criteria used for the classification of leishmanial parasites have changed in time from primarily epidemiological, behavioral and clinical characters (influenced also by host and vector characters) to more objective methods of molecular biology, biochemistry, immunology, serology and ultrastructural morphology (reviewed by Schnur & Greenblatt 1995). After Shaw (1994) there are today 30 named species of *Leishmania* from mammals, 10 in the Old World and 20 in the New World (Tab. 1). Many of the recognized species share characters which allow to group them into species complexes (Lainson & Shaw 1987). The isoenzyme analysis of Thomas-Soccol et al. (1993a, b) support the classification of Lainson & Shaw (1987) and defines the phylogenetic hierarchy of the complexes (clusters of related zymodemes).

The classification of the *Leishmania*-like parasites of Old World reptiles is still controversial. Saf'janova (1982) originally used the term *Sauroleishmania* for the subgenus which groups the reptilian *Leishmania* spp. This classification is consistent with recent DNA-based evidence of a close relatedness of these parasites to the subgenus *Leishmania* (Noyes et al. 1998). However, the generic rank *Sauroleishmania* established by Killick-Kendrick et al. (1986) is still accepted (Lainson & Shaw 1989, Telford 1995).

PART III.
The taxonomy and biology of sandflies (Diptera: Phlebotominae)

Phlebotomine sandflies are small (1.5–3.5 mm) hairy flies characterized by erect narrow wings, slender bodies, long legs, black eyes and long mandibles (Fig. 3). There are about 700 recognized species grouped in several genera within the subfamily Phlebotominae of the family Psychodidae. Sandflies are widely distributed in tropics and other warm climate mainland areas. Northwards they extend to the latitude 48–50°, southwards to about 40°. Species in three genera, *Phlebotomus* Rondani, *Lutzomyia* França and *Sergentomyia* França et Parrot, suck blood from vertebrates. The former two genera are medically important as they contain disease vectors. The genus *Phlebotomus* occurs in the Old World, mostly in semiarid and savannah areas. *Lutzomyia* species are found only in New World tropics, where they prefer forested areas. *Sergentomyia* representatives are Old World sandflies feeding mainly on reptiles; some species bite humans but do not transmit *Leishma-*

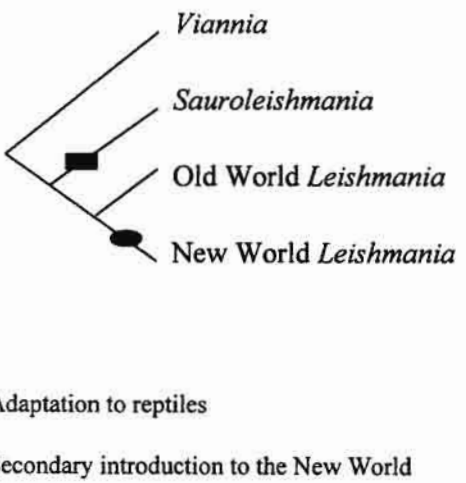


Fig. 2. Phylogeny of the genus *Leishmania*.

Table 1. Classification and basic characteristics of named *Leishmania* species. Based on Lainson & Shaw (1987), Lewis & Ward (1987), Killick-Kendrick (1990), Lawyer et al. (1991), Lane (1993), Thomaz-Soccol et al. (1993a, b), Shaw (1994), Jaramillo et al. (1994), Schnur & Greenblat (1995), Telford (1995) and Strelkova (1996)

	Reservoir hosts[a]	Geographical distribution	Disease in man [b]	Vector species [c]
Genus *Leishmania* Ross, 1903				
Subgenus *Sauroleishmania*[d] Ranque, 1973				
L. agamae David, 1929	*Agama stellio*	Mediterranean and the Middle East		(*P. papatasi*)
L. ceramodactyli Adler et Theodor, 1929	*Ceramodactylus doriae*	Mediterranean and the Middle East		(*P. papatasi*)
L. tarentolae Wenyon, 1921	*Tarentola mauritanica* *T. annularis* *Cyrtodactylus kotschyi*	Mediterranean and the Middle East		(*Se. antennata*) (*P. papatasi*)
L. gymnodactyli Khodukin et Sofiev, 1940	*Crossobamon eversmanni* *Gymnodactylus caspius* *Agama sanguinolenta* *Eremias intermedia*	Southern Asia		(*Se. arpaklensis*) (*P. papatasi*)
L. hemidactyli Mackie, Gupta et Swaminath, 1923	*Hemidactylus gleadovii*	Southern Asia		
L. nicollei Khodukin et Sofiev, 1940	*Agama sanguinolenta* *Phrynocephalus helioscopus* *Ph. mystaceus* *Ph. interscapularis*	Southern Asia		
L. gulikae Ovezmukhammedov et Saf'janova, 1987	*Agama caucasica*	Southern Asia		
L. adleri Heisch, 1958	*Latastia longicaudata*	Africa		(*Se. clydei*)
L. hoogstraali McMillan, 1965	*Hemidactylus turcicus*	N Africa		
L. senegalensis Ranque, 1973	*Tarentola annularis*	Africa		
Subgenus *Leishmania* Saf'janova, 1982				
Old World species complexes				
***L. donovani* complex**				
L. donovani (Laveran et Mesnil, 1903)	humans	E Pakistan, India, Nepal, China	VL, PKDL	***P. argentipes*** ***P. alexandri*** *P. caucasicus*
L. archibaldi Castellani et Chalmers, 1919	rodents, carnivores	Sudan, Ethiopia, Djibouti, Somalia, Chad, Niger, Congo, Central African Republic	VL, CL, OL	***P. martini***, ***P. orientalis*** *P. celiae* *P. vansomerenae*

***L. infantum* complex**				
L. infantum Nicolle, 1908	wild canids, (dogs)	Central and SW Asia, NW China, Middle East, Balkans, Mediterranean littoral, N and sub-Saharan Africa,	VL, CL, AL	***P. ariasi***
				P. perniciosus
				P. perfiliewi
				P. neglectus
				P. chinensis
				P. langeroni
				P. longiductus
				P. kandelaki
				P. longicuspis
				P. smirnovi
				P. tobbi
				P. transcaucasicus
L. chagasi Cunha et Chagas, 1937*	wild canids, (dogs)	From Argentina to Mexico	VL, CL, AL	***Lu. longipalpis***
***L. tropica* complex**				
L. tropica (Wright, 1903)	?, (humans, dogs, rodents)	Mediterranean and neighboring countries, N Africa (?), Middle East, Central Asia, India	CL, VL, LR, PKDL, OL	***P. sergenti***
L. killicki Rioux, Lanotte et Pratlong, 1986	hyraxes	Kenya, Namibia, Tunisia	CL	***P. guggisbergi***
				P. saevus
***L. major* complex**				
L. major Yakimoff et Schokhor, 1914	desert rodents, (*Hemiechinus auritus*, *Lepus tolai*, mustelids, dogs)	Middle East, Africa N of equator, Central Asia, India, Pakistan, NW China	CL, DCL, (VL)	***P. papatasi***
				P. duboscqi
				P. salehi
				P. alexandri
				P. ansarii
L. turanica Strelkova et Le Blancq, 1990	*Rhombomys opimus*	Middle Asia, Mongolia	–	(*P. papatasi*)
				(*P. andrejevi*)
				(*P. caucasicus*)
				(*P. mongolensis*)
***L. gerbilli* complex**				
L. gerbilli Wang, Qu et Guan, 1964	*Rhombomys opimus*	Middle Asia, S Mongolia	–	(*P. mongolensis*)
***L. aethiopica* complex**				
L. aethiopica Bray, Ashford et Bray, 1973	hyraxes, (*Cricetomys*)	Ethiopian highlands, Kenya, S Yemen (?)	CL, DCL, OL	***P. longipes***
				P. pedifer
***L. arabica* complex**				
L. arabica Peters et al., 1987	*Psammomys obesus*, (dogs)	Saudi Arabia	–	(*P. papatasi*)
New World species complexes:				
***L. mexicana* complex**				
L. mexicana Biagi, 1953	*Ototylomys phyllotis*, (other forest rodents)	Yucatan, Belize, Guatemala, N Mexico, S Texas	CL, (DCL)	***Lu. olmeca olmeca***
				Lu. ylephiletor

***L. enriettii* complex**				
L. enriettii Muniz et Medina, 1948	*Cavia porcellus*	Paraná State, Brazil	–	(*Lu. monticola*)
***L. amazonensis* complex**				
L. amazonensis Lainson et Shaw, 1972	*Proechimys* spp., (other forest rodents, marsupials, *Cerdocyon thous*)	The Amazon Basin, Brazil	CL, DCL, VL, PKDL, MCL	***Lu. flaviscutellata***, ***Lu. olmeca nociva*** *Lu. ylephiletor*
L. aristidesi Lainson et Shaw, 1979	forest rodents, marsupials	Panama	–	(*Lu. olmeca bicolor*)
Venezuelan species**				
L. venezuelensis Bonfante-Garrido, 1980	?	Venezuela	CL	*Lu. olmeca bicolor*
L. garnhami Scorza et al., 1979	?, (*Didelphis marsupialis*)	Venezuelan Andes	CL	*Lu. townsendi* *Lu. youngi*
L. pifanoi Medina et Romero, 1959	?	Venezuela	DCL, (CL)	*Lu. flaviscutellata*
L. hertigi* complex**				
L. hertigi Herrer, 1971	*Coendou rothschildi*	Panama, Costa Rica	–	?
L. daenei Lainson et Shaw, 1977	*Coendou prehensilis*	Brazil	–	?
Newly described species				
L. forattinii Yoshida et al., 1993	*Didelphis marsupialis*, *Proechimys iheringi*	Brazil	–	(*Lu. ayrozai*) (*Lu. yuilli*)
Subgenus *Viannia* Lainson et Shaw, 1987				
***L. braziliensis* complex**				
L. braziliensis Vianna, 1911	rodents, *Didelphis marsupialis*, *Choloepus didactylus*, (dogs, humans)	Brazil, Venezuela	CL, MCL	***Lu. wellcomei*** ***Lu. carerrai***, ***Lu. intermedia*** ***Lu. whitmani*** ***Lu. trapidoi*** ***Lu. gomezi*** *Lu. spinicrassa* *Lu. migonei* *Lu. pessoai* *Lu. amazonensis* *Lu. ayrozai* *Lu. complexa* *Lu. yucumensis* *Lu. paraensis* *Lu. llanosmartinsi* *Lu. panamensis*
L. peruviana Velez, 1913	?, (dogs)	Peruvian Andes	CL	***Lu. verrucarum*** *Lu. peruensis* *Lu. aracuchensis*

***L. guyanensis* complex**				
L. guyanensis Floch, 1954	*Choloepus didactylus*, *Tamandua tetradactyla*, (marsupials, rodents)	Guyana, Surinam, N Amazonian basin	CL	***Lu. umbratilis*** ***Lu. anduzei*** *Lu. whitmani*
L. panamensis Lainson et Shaw, 1972	Sloths, (*procyonids*, *monkeys*)	Panama, Costa Rica, Colombia	CL	***Lu. trapidoi*** ***Lu. gomezi*** ***Lu. ylephiletor*** ***Lu. panamensis*** *Lu. hartmanni* *Lu. sanguinaria* *Lu. ovallesi* *Lu. shannoni*
L. shawi Lainson et al., 1989	monkeys, sloths, procyonids	Amazonian Brazil	?	(*Lu. whitmani*)
***L. naiffi* complex**				
L. naiffi Lainson et Shaw, 1989	*Dasypus novemcinctus*	Amazonian Brazil	?	(*Lu. paraensis*) (*Lu. ayrozai*) (*Lu. squamiventris*)
***L. lainsoni* complex**				
L. lainsoni Silveira et al., 1987	*Agouti paca*	Brazil	CL	***Lu. ubiquitalis***
Newly described species				
L. colombiensis Kreutzer et al., 1991	*Choloepus hoffmani*	Colombia, Panama	CL	***Lu. hartmani***
L. equatorensis Grimaldi et al., 1992	*Choloepus hoffmanni*, *Sciurus granatensis*	Pacific coast of Equator	–	?

**Leishmania chagasi* is *L. infantum* introduced into the New World in the Pliocene-Pleistocene era in infected canids or humans (Thomaz-Socccol et al. 1993, Eisenberger & Jaffe 1999).

***Leishmania venezuelensis* not mentioned by Thomaz-Soccol et al. 1993a, b; *L. pifanoi* synonymized with *L. mexicana* (Thomaz-Soccol et al. 1993b), *L. garnhami* synonymized with *L. amazonensis* (Thomaz-Soccol et al., 1993b), all the species members of the *L. mexicana* complex after Lainson & Shaw 1987.

*** would be more appropriately classified as species of the genus *Endotrypanum* Mesnil et Brimont, 1908 (Croan & Ellis 1996).

[a] primary reservoir-host; (secondary or accidental host); ?, primary host unknown or unproven.

[b] key to clinical manifestations of diseases: CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; LR, leishmaniasis recidivans; MCL, mucocutaneous leishmaniasis; OL, oronasal or nasopharyngeal leishmaniasis; VL, visceral leishmaniasis; PKDL, post-kala-azar dermal leishmaniasis; AL, asymptomatic leishmaniasis; ( ), uncertain existence.

CL: chronic, painless, localized, single or multiple ulcerating "wet" or nodular nonulcerating "dry" lesions. Self-limiting and self-curing, can leave disfiguring scars.

DCL: chronic, painless, fleshly, lepromatouslike nodules spreading locally and metastatically. No self-cure, difficult to treat, tends to relapse.

LR: a sequel of CL; chronic, painless, discrete or coalescing, tuberculoidlike lesions spreading peripherally. No self-cure, difficult to treat.

MCL: a sequel of CL; primary lesions, later metastatic spread to oronasal, pharyngeal, and anal mucosae with ulceration and progressive erosion of the soft tissue and cartilaginous structures. No self-cure, difficult to treat, can relapse.

OL: reminiscent of MCL in the Old World

VL: chronic fever, splenomegaly, hepatomegaly, lymphadenopathy, malaise, wasting, darkening of the skin, anemia, leucopenia, thrombocytopenia. Death

can occur if untreated, spontaneous self-cure rarely.
PKDL: a sequel of VL appearing 6 months to one or more years after cure of VL. Chronic dermal lesions without ulceration, either hypopigmented or erythematous macules at any part of body, which can become nodular. Tendency to self-cure variable.
AL: asymptomatology based on parasitological evidence, the presence of parasites at some time either in the past or present .
[c]: **proven vector**, parasites isolated and typed several times, man-vector and reservoir-vector contact established, experimental transmission in some cases; **strongly suspected vector**, anthropophilic species, but only a few parasite isolations have been made and typed or parasites observed in the wild-caught sandfly and not typed.
suspected vector, females morphologically indistinguishable from closely related species or parasites only observed in a blood-meal, experimental transmission in some cases;
( ), no transmission to man or transmission can occur, but the parasite is no-infective or non-pathogenic to man.
P. = *Phlebotomus*, Se. = *Sergentomyia*, Lu. = *Lutzomyia*.
[d]: The species *Leishmania chamaeleonis* Wenyon, 1921, *L. davidi* Strong, 1924, *L. henrici* Leger, 1918, *L. zmeevi* Andrushko et Markov, 1955, *L. sofieffi* Markov et al., 1964, *L. phrynocephali* Khodukin et Sofiev, 1940 and *L. helioscopi* Khodukin et Sofiev, 1940 were excluded from the subgenus *Sauroleishmania* as their life cycles (intestinal location in reptiles) do not correspond to the development of the *Leishmania* species from reptiles. These species should be compared biochemically or genetically with those from the bloodstream and with accepted species of *Herpetomonas* Kent, 1880 and *Leptomonas* Kent, 1880 before considering them to be congeneric with *Leishmania* species (Telford 1995).

nia. The most important disease vectors are listed in Tab. 1. The other three recognized genera are *Chinius* Leng in the Old World and *Warileya* Hertig and *Brumptomyia* França et Parrot in the New World (Lane 1993).

Females lay eggs in moist soil, among leaf litter, in the animal burrows and termite hills, in the bark of old trees, soil or wall cracks, ruined buildings and household rubbish or stable floors. In such habitats, larvae find food (various kinds of organic matter), heat and humidity necessary for development. Similarly to other Nematocera, larvae are eucephalic and apodal. There are four larval instars. In temperate or arid areas, the fourth instar larvae can undergo diapausis. Adult sandflies of both sexes feed on plant sugars, females, in addition, need a blood meal for ovarian development. They suck blood from a variety of vertebrates, mainly from mammals but also from reptiles and amphibians, a few species feed on birds. Biting is usually restricted to crepuscular and nocturnal periods, although in forests or darkened rooms females bite throughout the day. Sandflies are weak fliers, they fly in a series of characteristic hops. Their flight range is limited to a few hundred meters from the breeding site.

*P. papatasi** and some other species can be a nuisance pests owing to irritation from their bites. Besides of *Leishmania* parasites, sandflies are vectors of a kinetoplastid protozoan *Endotrypanum*, a parasite of sloths. They transmit the bacterium *Bartonella baciliformis*, agent of the Oroya fever (also called Carrion's disease) in Peru, Ecuador and Colombia (transmitted by *Lu. verrucarum**) and several viruses (phleboviruses, vesiculoviruses). The most important are phleboviruses causing sandfly fevers (papataci fever), common in the Mediterranean region and the Middle East and transmitted principally by *P. papatasi* (Lewis 1973, Lane 1993, Service 1996).

PART IV.
Geographical distribution: vector-parasite-host association

The distribution of *Leishmania* spp. is a result of long-termed complex interaction between the parasite, the vector and the host. Therefore, the ecology and epidemiology of the leishmaniases are extremely diverse. The most detailed reviews of the epidemiology of New World and Old World leishmaniases were published by Shaw & Lainson (1987) and Ashford & Bettini (1987), respectively.

Leishmania spp. are essentially parasites of wild animals. In the Old World, various species of rodents, carnivores and hyraxes are involved. In the New World, the spectrum of reservoirs is enlarged with marsupials, edentates, procyonids and monkeys (Tab. 1). Most natural hosts tolerate infections, that often remain benign and inapparent. This is advantageous for *Leishmania* spp. in countries in which the climate is not suitable for sandflies during all year and, therefore, the overwintering of the parasite must occur in the reservoir hosts. Survival of *Leishmania* in their hosts for such a long period suggests minimal antileishmanial activity against the parasite by these hosts (Schnur & Greenblat 1995).

The habitat preference of vectors and hosts and a degree of overlap of their habitats leads to a range of various parasite strategies seen in nature. The Fig. 4 is a diagrammatic summary adopted from Shaw & Lainson (1987) which shows how the relationships of habitats determine the possibility of disease transmission. The first three situations (a–c) represent enzootic cycles and the following three diagrams are zoonotic cycles in which man can be an accidental host (R_2). The situation (d) possibly applies to *L. amazonensis* and the situation (e) to *L. guyanensis* and *L. panamensis*: man becomes infected at ground level, while the enzootics among sloths occur in the canopy. The

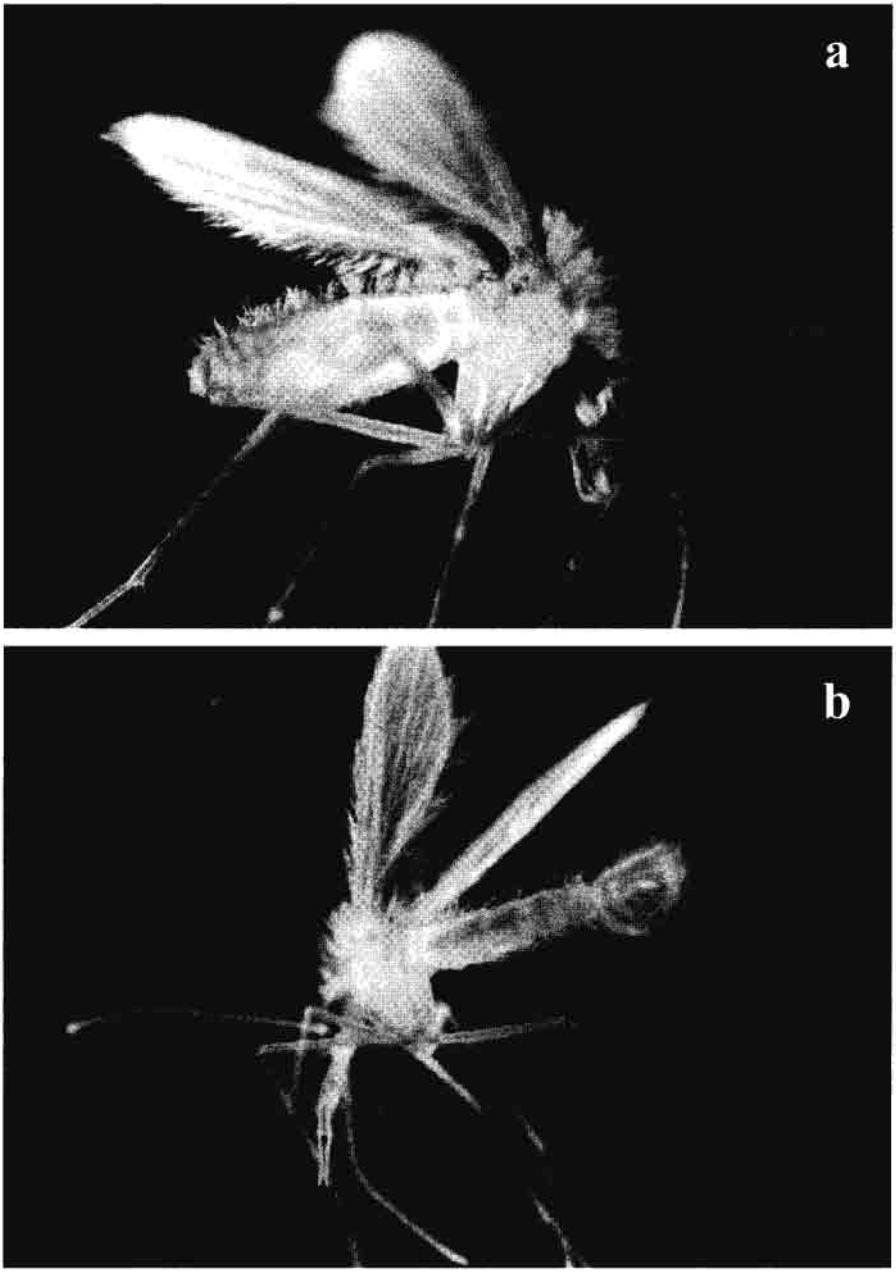


Fig. 3. Female (a) and male (b) of the sandfly *P. papatasi*, a vector of *L. major*. Typical appearance of abdomen distinguishes the sexes. Male terminal abdominal segments with external genitalia are important taxonomical characters.

last possibility is supposed to occur in *L. guyanensis* as anteaters are probably infected by the bite of a secondary vector.

Shaw (1997) believes that the major factor controlling the distribution of *Leishmania* spp. in mammals is probably the biting habit of sandflies. He argued that vectors exert greater selective pressure on the parasite than mammalian hosts: only a few *Leishmania* species, like *L. enriettii* restricted to guinea pigs, developed a high degree of host specificity. On the other hand, there are hosts (e. g. man), which are susceptible to infections by many species of *Leishmania* whose natural hosts range from edentates to rodents. Similarly, the golden hamster is not a natural reservoir of any *Leishmania* species but is susceptible to most of the species that infect man. In some cases, however, the distribution of a suitable host rather than that of a vector governs the parasite (Schnur & Greenblat 1995). In Israel, *P. papatasi* is widely distributed, but CL is contracted only by people visiting very specific areas, those in which *Psammomys obesus* and *Meriones crassus* occur (Schle-in et al. 1984).

The example of *L. major* was used by Schnur & Greenblat (1995) to show that both host and vector specificity does not occur at the species level. This parasite is distributed over a wide geographical range with at least 15 reservoir rodent species and 3 vector species in different parts of the range. However, a detailed study on Central Asian foci of *L. major* (reviewed by Strelkova 1996) showed that the two reservoir host species present in this foci highly differ in the susceptibility to the infection. In *Rhombomys opimus* ulceration and visceralization never developed and infections lasted on average for 7 months with self healing in nearly 90% cases. However, in *Meriones libycus* the infection led to a rapid ulceration and lasted, on average, for five months. Also, the infection rate of *M. libycus* in nature is low (3–4%). Similar situation can be expected in other parts of the *L. major* range: despite the parasite being isolated from a broader spectrum of species, the number of true reservoirs can be far lower.

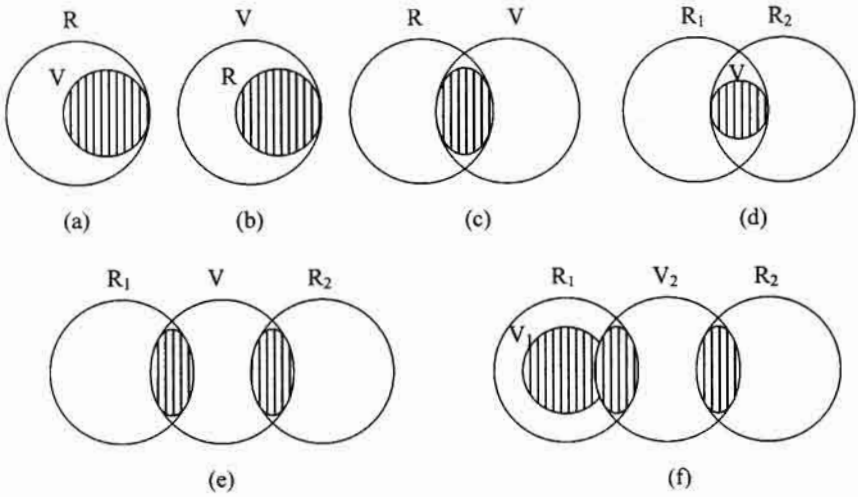


Fig. 4. The relation of vector (V) and reservoir (R) habitats to transmission (shared areas). Adopted from Shaw & Lainson (1987). (a) transmission throughout vector habitat; (b) transmission throughout reservoir habitat; (c) transmission limited to a small area of habitat overlap; (d) transmission to two hosts with a little different habitat preferences throughout the habitat of the vector; (e) transmission outside the habitat of primary host by the same vector, (f) transmission by a secondary vector to a secondary host outside the habitat of the primary hosts and vector. R_1, primary reservoir; R_2, secondary reservoir; V_1, primary vector; V_2, secondary vector.

Table 2. Suggested functions of gp63 and LPG in the host

	Gp63	LPG
Inhibition of chemotaxis of monocytes and neutrophils	(Sorensen et al. 1994)	(Lo et al. 1998, Frankenburg et al. 1990, 1992)
Direct binding to CR3 receptor, mannose-fucose receptor and fibrinogen receptor	(Russell & Wright 1988, Wilson & Pearson 1986, Rizvi et al. 1988)	(Talamas-Rohana et al. 1990, Kelleher et al. 1995, Wilson & Pearson 1986)
A C3 acceptor in the uptake of *Leishmania* by macrophages via the CR3 receptor	(Russell 1987)	(Da Silva et al. 1989)
Conversion of C3b to iC3b and, therefore, exploitation of opsonic properties of complement while avoiding its lytic effects	(Brittingham et al. 1995)	
A barrier preventing the insertion of MAC complexes		(Puentas et al. 1990)
Protection from intracellular degradation in the phagolysosome	(Chaudhuri et al. 1989, Seay et al. 1996, McGwire & Chang 1994, Sorensen et al. 1994)	(McNeely et al. 1989, McNeely & Turco 1990, Proudfoot et al. 1995, 1996)
Lesion formation in the host via a degradation of the extracellular matrix	(McMaster et al. 1994)	
Cleavage of CD4 on T cells	(Hey et al. 1994)	
Suppression of cytokine expression in monocytes		(Frankenburg et al. 1990, Hatzigeorgiou et al. 1996)
Inhibition of phagosome-lysosome fusion		(Desjardin & Descoteaux 1997)

Also in other aspects, these Central Asian foci are a good example of a complicated epidemiological situation. *Rhombomys opimus* in this area is infected by three *Leishmania* spp.: *L. major*, *L. turanica* and *L. gerbilli*; the only species pathogenic for humans being *L. major*. *L. turanica* is widespread in *Rhombomys opimus* throughout the region. In southern part of *Rhombomys opimus* range, *L. turanica* existed sympatrically with *L. major* and *L. gerbilli*. The rate of *Rhombomys opimus* infection by *L. turanica* is stably very high (50–100%). The infection rate of *Rhombomys opimus* by *L. major* is very low in the beginning of the transmission season, but in early autumn it attains 50% or more. *L. gerbilli* is ever rarer in *Rhombomys opimus* as compared with *L. major;* this species is restricted to the region of the Emba river. High proportions of *Rhombomys opimus* are infected with more than one *Leishmania* species. In fact, no pure *L. major* isolates could be obtained from the host, but only *L. major–L. turanica* mixtures and, in two cases, the presence of all three parasite species in the same individual rodent was described (Strelkova 1996).

Humans and dogs are found infected in many regions but they are only secondary hosts of most leishmania (Lainson & Shaw 1987). The only *Leishmania* spp. for which humans are considered to be primary reservoir hosts are *L. donovani* in India and, less certainly, *L. tropica* in different regions. Dogs are not considered to be reservoirs of *L. tropica* and the presence of *L. major* in dogs is only incidental. They are important domestic (secondary) hosts of *L. infantum* and *L. chagasi*, and, maybe, also of *L. braziliensis* and *L. peruviana*. Primary hosts in rural foci of *L. infantum* and

L. chagasi are wild canids: the jackal *Canis aureus*, the wolf *Canis lupus* and the fox *Vulpes vulpes* in the Old World and *Lycalopex vetulus* and *Cerdocyon thous* in the New World (Ashford & Bettini 1987, Schnur & Greenblat 1995). It should be noted that *L. chagasi* was introduced into the New World in infected humans or dogs during historic times (Schnur & Greenblat 1995, Eisenberger & Jaffe 1999) and, therefore, the adaptation to New World canids must be a recent event.

PART V.
Leishmania life-cycle

A. Development in the sandfly vector

1. Mode of development – implications for Leishmania *taxonomy*

Leishmania spp. undergo development in the gut of the sandfly. This development (a) is restricted to the hindgut causing contaminative transmission, or (b) the hindgut development is followed by migration of promastigotes to the midgut and foregut causing transmission by bite or (c) the development takes place only in the midgut and foregut with transmission by bite. Lainson & Shaw (1979) placed emphasis on the mode of development and divided phlebotomine species into three sections (Fig. 5): Hypopylaria, Peripylaria and Suprapylaria, respectively, these sections having no taxonomic status. Lainson & Shaw (1987) later erected the subgenus *Viannia* for the species of the Section Peripylaria, retaining the subgenus *Leishmania* Saf'janova, 1982 for the species of the Section Suprapylaria. The reptilian parasites with both hypopylarian and peripylarian development were included in the genus *Sauroleishmania*.

The same authors argued that the mode of development enables a natural grouping of the leishmanial parasites and perhaps, therefore, reflects their phylogenetic relationships (Lainson et al. 1977, Lainson & Shaw 1987). The hindgut development was considered to be the ancient mode while the parasites from the section Suprapylaria were interpreted like the most derived ones, which have lost the primitive hindgut development. This hypothesis is logically consistent with the assumption that *Leishmania* parasites evolved from monogenetic parasites of invertebrates, which also undergo hindgut development with passing of resistant, infective forms with excreta. Also the primitive nature of the peripylarian parasites (subgenus *Viannia*) in comparison with the suprapylarian ones (subgenus *Leishmania*) is in accordance with the assumed evolution of the genus (see the Part II). Peripylarian parasites are restricted to the Neotropics, a suggested area of the origin of the genus, and they are often found in edentates, the ancient group of eutherian mammals which evolved at the turn of Mesosoic/Cenosoic period with the highest species richness in Pliocene and Pleistocene, representing possibly the earliest hosts of *Leishmania*.

In view of current information, however, the evolutionary significance of the development in sandfly should be interpreted with caution. Firstly, the mode of development can be influenced by a number of extrinsic factors and is more flexible, than was suggested (Schlein 1986, Añez 1989, Walters 1993). Secondary, the application of modern taxonomic methods leads to the revision of the phylogenetic relationships between the members of the genus *Leishmania.* Molecular analyses showed, for example, that *L. hertigi* with the suprapylarian development is the most divergent of all *Leishmania* species studied by Croan & Ellis (1996), and is close to the *Endotrypanum* lineage. However, this argument can not be as strong in view of the fact, that natural vector of *L. hertigi* is still unknown and experimental infections were done with *Lu. longipalpis* and *Lu. truncata* (Lainson & Shaw 1987). Additionally, a significant DNA-based evidence (reviewed by Noyes et al. 1998) established the subgenus *Sauroleishmania* with hindgut development to be a sister group of the subgenus *Leishmania*, separated after the divergence of the subgenus *Viannia*. Therefore, it seems that several transitions between the modes of development have occurred during the phylogeny of

the genus. The simplest possible scenario is shown in Fig. 6, but additional studies on natural combinations of vectors and parasites and detailed phylogenetic trees are necessary to obtain plausible information about the evolutionary significance of this trait.

The following text concerns only on the parasites with suprapylarian and peripylarian patterns of development which are infective for mammals.

2. Midgut infections

The complex development of *Leishmania* spp. in the vector was described only in some natural parasite-vector combinations, mainly because to find naturally infected flies is rare and the establishing and raising of sandfly colonies is difficult. Most valuable studies were done on natural systems: *L. panamensis* in *Lu. gomesi* (Walters et al. 1989a), *L. chagasi* in *Lu. longipalpis* (Walters et al. 1989b, Elnaiem et al. 1992) and *L. major* in *P. papatasi* (Warburg et al. 1986, Killick-Kendrick et al. 1988). A general pattern of the development was established as follows (reviewed by Killick-Kendrick 1979, Walters 1993, Schlein 1993, Schnur & Greenblat 1995).

A bloodmeal containing **amastigotes** (Fig. 7) is quickly surrounded by the peritrophic matrix, a chitinous framework with a protein-carbohydrate matrix, which is secreted by epithelial cells of the abdominal midgut. In ingested bloodmeal, amastigotes usually divide and then differentiate into

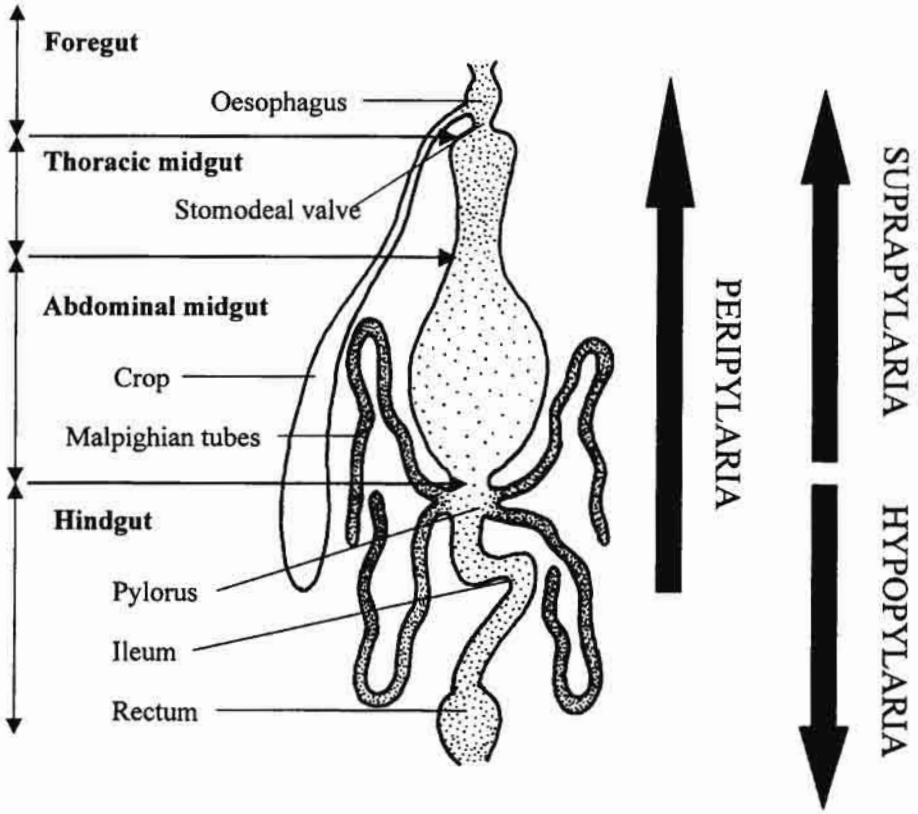


Fig. 5. Gut morphology of sandflies with illustration of three recognised modes of development in the genus *Leishmania* (according to Lainson & Shaw 1987).

flagellated promastigotes, called by Walters (1993) **stumpy promastigotes**, which then transform or enlarge into long, slender **nectomonad promastigotes**. After the breakdown of the peritrophic matrix (about 3 days post the meal), nectomonads escape into the ectoperitrophic space of the midgut lumen. Schlein et al. (1991) found in *L. major- P. papatasi* system, that the disintegration of the anterior end of the peritrophic matrix is catalyzed by chitinolytic enzymes of *Leishmania*. Once inside the midgut, promastigotes attach by the tip of flagellum among the microvilli of epithelial cells. The attachment is reversible and it prevents the promastigotes from being expelled with undigested bloodmeal remnants. The peripylarian parasites do not attach in the abdominal midgut but migrate straight out to the hindgut. In suprapylarian spp., promastigotes repeatedly attach and detach, and rapidly multiply in the abdominal midgut, and afterwards move anteriorly. Walters (1993) described that the elongated nectomonads transform into **short nectomonad promastigotes**, which are predominant forms in the thoracic part of the midgut, called also cardia, 5–7 days post bloodmeal. Short promastigotes were proposed by the author to be precursors to **metacyclic promastigotes**, small, highly motile forms with a long flagellum, which are infective for the mammalian host. In culture, these forms are produced in the stationary phase of growth.

The described morphogenesis is coincident with developmentally regulated changes in expression of main surface glycoconjugates of the parasite, the **lipophosphoglycan (LPG)** and the **surface protease gp63** (Davies et al. 1990). There are two hypotheses concerning the role of leishmanial LPG in the vector. The excreted form of LPG (EF; excreted factor) was described to be involved in **a protection from hydrolytic damage by the gut proteases** by modulating their activity during bloodmeal digestion (Schlein et al. 1990). However, results of Saraiva et al. (1995) argued against this role of LPG: they reported a delay in LPG expression on the surface of *L. major* in *P. papatasi* until the appearance of nectomonads on day 3, while the peak of protease activity in this sandfly species occur at 24–34h post bloodmeal (Dillon & Lane 1993a). The expression, however, coincides with the time of escape of promastigotes from the peritrophic matrix, which supports the second proposed role of LPG in the sandfly, **the attachment to the midgut wall** (Pimenta et al. 1992, Sacks et al. 1994). Dillon & Lane (1999) described a 65 kDa microvillar protein which probably acts as a ligand for the parasite's LPG. The attachment is developmentally regulated by a modification of LPG which accompanies the transformation of promastigotes from procyclic to metacyclic forms. Particularly, it is the substitution of terminally exposed galactose residues by arabinose in *L. major* (Pimenta et al. 1992) and folding and clustering of the extended phosphoglycan chains in *L. donovani* (Sacks et al. 1995). As a result, the infective metacyclic promastigotes lost the property to bind to the midgut epithelium and are selectively released for subsequent transmission by bite.

The role of gp63 in the sandfly vector is probably the acquisition of nutrients from the bloodmeal. Proteolytical degradation of haemoglobin could provide both amino acids (especially prolin) used as the primary energy source of promastigotes (Mukkada 1985) and haem, which they cannot synthesize (Chang & Chang 1985). The nutritional role of gp63 in the vector is indirectly supported by the fact that surface metalloproteases similar to leishmanial gp63 were found in monoxenous insect trypanosomatids *Crithidia* and *Herpetomonas* (Etges 1992). The digestion of haemoglobin in the vicinity of the parasites inside the peritrophic matrix may have an additional effect: the presence of haemoglobin inhibits a secretion of parasite chitinases (Schlein & Jacobson 1994a). Therefore, its break-down is a prerequisite for the function of these enzymes and, consequently, for lysis of the peritrophic matrix (Schlein 1993).

3. Foregut and/or hindgut infection

Suprapylarian parasites transform in the vicinity of a stomodeal valve (Fig. 7) from free-swimming nectomonads to non-motile shorter and broader **haptomonad stages**. Some haptomonads colonize the oesophagus and pharynx, where are found also attached **paramastigotes** (the forms distin-

guished from promastigotes by a juxtanuclear kinetoplast; the free-swimming ‚nectomonad' phase of paramastigotes could be seen already in the midgut). Both haptomonads and paramastigotes attach to the cuticle lining of stomodeal valve and foregut by the flagellum which forms broad, hemidesmosome-like plagues. Lumenal free-swimming slender promastigotes are most likely metacyclic forms. From the pharynx, the infection may spread forwards to the cibarium and in some systems, metacyclic promastigotes are found even in the proboscis (Killick-Kendrick 1979, Schlein 1993, Walters 1993).

The initial establishment of infection in peripylarian spp. is in the pylorus and the ileum of the fly. In these parts of the hindgut, haptomonads and paramastigotes attach to the cuticular surface by hemidesmosomes and multiply. Motile promastigotes which migrate to the thoracic midgut are produced. Some parasites migrate straight to the thoracic midgut without going through the hindgut phase. Further development is similar like in the suprapylarian spp. (Killick-Kendrick 1979, Walters 1993).

4. Transmission

The location of parasites in the sandfly digestive tract is of crucial importance for the transmission to the vertebrate host. Transmission by bite takes place in suprapylarian and peripylarian species, which colonize the foregut. In this respect, two main hypotheses have been suggested: either the parasites emerge from an infected sandfly during feeding only if the proboscis itself is infected (Adler & Theodor 1935) or they are regurgitated with a backflow of ingested blood. Originally, the regurgitation was supposed to result from a mechanical block of the foregut (Shortt & Swaminath 1928) or stomodeal valve (Warburg & Schlein 1986) by the parasites. More recently, the damage to the chitin layer of the stomodeal valve by leishmanial chitinolytic enzymes was proposed to be the cause of regurgitation of parasites from the thoracic midgut (Schlein et al. 1991, 1992). This hypothesis also offers the explanation of multiple probing by infected flies and the difficulty they

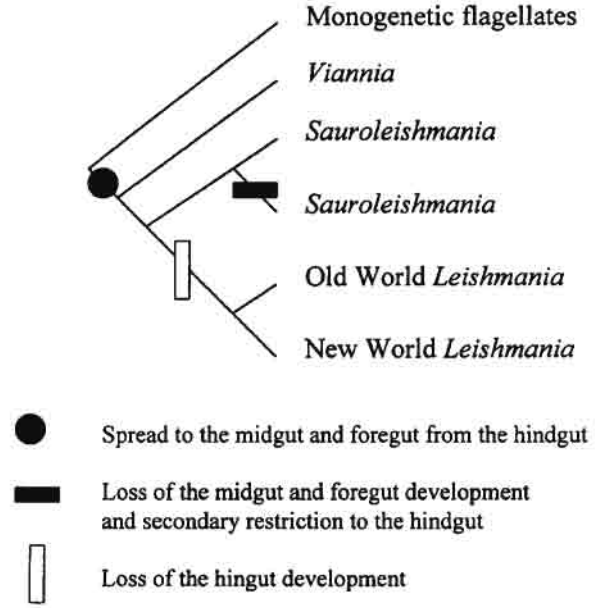


Fig. 6. Suggested evolution

have in obtaining a full blood meal (Killick-Kendrick et al. 1977). A third possible way of transmission, the inoculation of parasites into the host skin with sandfly saliva, is supported by the study of Killick-Kendrick et al. (1996), who found metacyclic promastigotes of *L. tropica* invading salivary glands of *P. duboscqi*. Therefore, there is perhaps more than one mechanism of transmission by bite (Killick-Kendrick et al. 1996).

Contaminative transmission by ingestion of infected sandfly takes place in the genus *Sauroleishmania* (Lainson & Shaw 1987). In mammals, the contaminative route of transmission is probably an occasional, less frequent alternative. It probably occurs in transmission of *Leishmania* to dogs which regularly lick off the flies engorging on their nose and breathe with the mouth open. In man, contaminative transmission may occur when a heavily infected biting fly is crushed on the skin (reviewed by Killick-Kendrick 1979).

5. Factors affecting susceptibility of sandflies to Leishmania

The *Leishmania*-sandfly interaction is affected by many factors, which have either adverse or beneficial effects on the course of infection (for review see Killick-Kendrick 1979). Intrinsic factors, such as proteolytic enzymes (Dillon & Lane 1993a) and sandfly lectins (Wallbanks et al. 1986, Volf et al. 1994) make each sandfly species a unique habitat for leishmania parasites. This habitat is further influenced by many exogenous factors, particularly by temperature (Leaney 1977) and the two types of food, blood and sugar solutions, taken by sandfly females.

The main sources of sugars in nature are nectar, fruit and plant saps and, above all, honeydew. This is a sugary solution containing also amino acids, excreted from the anus of aphids, coccids and other plant-sucking insects. The beneficial effect of sugars on the parasite development is evidenced by the fact that experimental transmissions were not successful until sugars were offered to infected females (reviewed by Killick-Kendrick 1979). As shown later (Schlein et al. 1993), the sugarmeal modifies the functioning of parasite chitinases which damage the stomodeal valve and, therefore, facilitate the *Leishmania* transmission. However, sandflies feed selectively on different sugar sources (Schlein and Warburg 1986), some of which, on the contrary, may impair leishmanial infections. In the experiments of Schlein & Jacobson (1994b), feeding on *Malva nicaeensis* and on the honeydew excreted by *Icerya purchasi* produced vital and thriving infections, whereas diets of *Ricinus communis*, *Capparis spinosa* or *Solanum luteum* reduced the numbers of parasites.

Sugar diet can alter the gut environment also indirectly, by bacterial or fungal contamination, which negatively influence not only the parasite, but also the fly (Killick-Kendrick 1979, Schlein et al. 1985). Therefore, it was suggested that defence mechanisms evolved in sandflies to decrease the probability of intensive gut contamination. Sugar meals taken from leaves and stems by piercing (‚sterile' meal) directly enter the midgut like the blood (also ‚sterile'), while meals taken from the plant surface (possibly contaminated) are located in the crop. The crop contains an antibacterial agent which influences the meal before its gradual release to the midgut (Schlein & Warburg 1985, Schlein & Warburg 1986). This hypothesis was not supported by the study of Añez et al. (1997) who used capillary feeding (mimicking the piercing) and found that the destination of a meal depends on the salt concentration of the fluid rather than the feeding mode of the fly. However, the number of flies in their experiments were not sufficient for a definite conclusion.

The development of leishmania in the sandfly is also considerably affected by the blood source. Schlein et al. (1983) described inhibition of experimental leishmanial infection in *P. papatasi* fed on turkey blood. Flies inhabiting burrows of *Psammomys obesus* near turkey sheds were uninfected in a strong contrast with females from burrows in other sites (Schlein & Jacobson 1994c). The same lethal effect was reported by the same authors for chicken blood. In this respect, there is also a strong difference among different mammalian species: the percentage of heavy infections of *L. major* in *P. papatasi* is considerably decreased in flies fed on human blood in comparison with

those fed on the blood of *Psammomys obesus* (Schlein & Jacobson 1996). Blood from different mammals may cause different effects on the secretion of sandfly midgut proteases (Schlein & Jacobson 1998). In addition, the bloodmeal might affect the parasite indirectly through the lectin-like activity in the sandfly gut. These lectin-like molecules bind to LPG (Palánová & Volf 1997) and agglutinate *Leishmania* promastigotes (Wallbanks et al. 1986, Svobodová et al. 1996). Secretion of the midgut agglutinin is increased after bloodfeeding (Volf & Killick-Kendrick 1996, Volf & Palánová 1996) and specific inhibition of lectin activity increases leishmanial infection (Volf et al. 1998).

As shown above, the habitat inside the sandfly gut undergoes temporal changes during the life of the individual fly, and it differs also between populations of one sandfly species with variable feeding preferences and food sources in various geographical areas. Despite of this variability, there is more or less close fit between *Leishmania* spp. and their vectors, which is presumably the result of their coevolution. Proven vector of one parasite species is often unable to support the full development of another species. The taxonomic relationships of Old World vectors suggest that the coevolution results in a restriction of the range of sandflies in which the parasite species can readily develop. For example, *L. major* is transmitted by species of the subgenus *Phlebotomus* Rondani, *L. tropica* by *Paraphlebotomus* Theodor, *L. aethiopica* and *L. infantum* by *Larrousius* Nitzulescu, *L. archibaldi* by *Synphlebotomus* Theodor and *L. donovani* by *Paraphlebtomus* and *Euphlebotomus* Theodor (Killick-Kendrick 1985, Killick-Kendrick 1990).

The characters which determine the susceptibility or refractoriness of sandflies to leishmanial infection are controlled genetically, but multiple genes are involved. A completely refractory lines of *P. papatasi* to infection with *L. major* were not obtained despite 17 consecutive generations of selection (Wu & Tesh 1990a, b). Similar results were obtained by Killick-Kendrick on *P. duboscqi* infected with *L. tropica* (pers. comm.). There are now two hypotheses concerning the mechanism of **species specificity in the parasite-vector relationship**, both involving the surface LPG (see also Part VA2).

Schlein (1986) suggested that the mechanism favouring the successful growth of *Leishmania* in the gut of sandflies is the suppression of digestive enzymes by the parasite in its natural vector. The level of proteolytic enzymes was reduced in *P. papatasi* infected with promastigotes of the natural parasite, *L. major*, and elevated by the unnatural parasite, *L. donovani* (Borovsky & Schlein 1987). Similar results were obtained with amastigote-initiated infections (Dillon & Lane 1993b). Further search for the mechanism of protection led to the discovery of the role of species-specific surface LPG in its excreted form (EF). The addition of EF of *L. major* significantly increased survival of LPG-defective strain of *L. major*, while addition of EF of *L. donovani* did not promote the survival (Schlein et al. 1990).

The second hypothesis is based on the role of LPG as a ligand to sandfly midgut epithelium. Due to the extensive interspecific polymorphism of LPG, the attachment might contribute to species-specific differences in vectorial competence (Pimenta et al. 1994, Sacks et al. 1994). It was shown that only *L. major* strains having the complete procyclic LPG could attach to the midgut of their natural vector, *P. papatasi*. The second sandfly tested, *P. argentipes*, was permissive not only to *L. donovani* (natural vector) but also to *L. major*, *L. tropica* and *L. amazonensis* (unnatural combinations). Similar preliminary results are reported also for *Lu. longipalpis*. The authors suggest that *P. argentipes* (and *Lu. longipalpis*) midguts possess a receptor, lacking in *P. papatasi*, for a relatively conserved oligosaccharide on procyclic LPGs. The natural selection in *L. major* led to the expression of an unusual, galactose-substituted LPG able to bind to the midgut of *P. papatasi* through an other, unique receptor. It provides them with the advantage of a ‚free niche': a colonization of the widely distributed sandfly species, *P. papatasi*, which is refractory to other parasites (Pimenta et al. 1994, Sacks et al. 1994).

In this respect, it would be interesting to characterize the LPG of other *Leishmania* species known to be transmitted by *P. papatasi* (*L. turanica*, *L. arabica*, *L. tarentolae*) and assess the vector competences of the other two natural vectors of *L. major*, *P. duboscqi* and *P. salehi* (Jacobson 1995). The susceptibility of *P. duboscqi* to *L. tropica* was already shown by Killick-Kendrick et al. (1994). Schlein & Jacobson (1998) disagree with the interpretation of Pimenta et al. (1994). In their experiments (Schlein & Jacobson 1998) with *L. donovani* and *P. papatasi*, the crucial period for the success of the infections was during blood digestion. Afterwards, the attachment of *L. donovani* to the epithelium was indistinguishable from that of *L. major*.

Regardless of the exact mechanism, the vectorial competence is often tested in laboratories. In this respect, it should be mentioned that experimental infections with unnatural vector-parasite combinations are often more successful, than expected (Killick-Kendrick et al. 1977, Lawyer et al. 1987, Ryan et al. 1987, Walters et al. 1987, Rangel et al. 1993, etc.). It may be an artifact of the laboratory conditions including an abnormally large numbers of parasites offered to sandflies. Therefore, the assessment of vector competence should always combine experimental and field data like geographical distribution and association with endemic regions, habitat preference, biting behavior and host preferences (anthropophily vs. zoophily).

B. Development in the vertebrate host

1. The parasite-macrophage interaction

The most intriguing aspect of *Leishmania*-host association is the ability of amastigotes to live in mononuclear phagocytes, mammalian cells specialized in defence against invaders. Although macrophages and monocytes are well equipped to internalize, kill and digest invading pathogens, *Leishmania* spp. are able to counteract their degradation activities. During the adaptation to the intracellular life, these parasites developed multiple strategies how to evade host's defence system. Four sequential events are essential for the establishment of the intracellular life: recognition, entry, survival and multiplication (Chang 1983).

1.1. Recognition and entry

After deposition in the skin by the bite of sandflies, promastigotes face two effective immune mechanisms of the mammalian host: lysis by complement and destruction by phagocytes. In a blood pool, induced by the sandfly bite, promastigotes immediately encounter host serum and activate complement via the classical pathway, alternative pathway or lectin pathway (reviewed by Mosser & Brittingham 1997). **Complement activation** leads to the formation of the complement cleavage product C5a which attracts macrophages by chemotaxis. Monocytes from the blood move in the direction of the chemotaxin gradient to the area of infection and start to interact with promastigotes (Bray 1983). The other effect of complement activation is the opsonisation of the parasite by complement protein C3b. Surface bound C3b can act as a ligand for macrophage complement receptors (CR) which results in enhanced uptake of the parasite into phagocytic cells. On the other hand, C3b can also initiate the formation of membrane attack complex (MAC) mediating parasite cell lysis (Pearson & Steigbigel 1980, Mosser & Edelson 1984).

Leishmania promastigotes developed a unique defensive mechanism how to avoid the destruction by complement, while exploiting its opsonic properties. Metacyclic promastigotes resist the complement-mediated lysis due to the function of two main surface molecules, promastigote surface protease gp63 and lipophosphoglycan LPG. It was shown that C3b is converted by gp63 to inactive form iC3b, which remains opsonic, but is unable to support the formation of MAC (Brittingham et al. 1995, Brittingham & Mosser 1996). The role of LPG in serum resistance is most likely mechanical; elongated LPG in metacyclic promastigotes may act as a barrier preventing the inser-

tion of MAC complexes (Puentas et al. 1990). In addition, leishmanial protein kinases have been reported to inactivate complement components by phosphorylation (Hermoso et al. 1991).

Phagocytosis of *Leishmania* by macrophages is a receptor-mediated event which involves multiple macrophage receptors, parasite surface proteins and host serum factors (reviewed by Mosser & Brittingham 1997). **Serum dependent adhesion** consists in binding of C3b opsonised promastigotes to macrophage complement receptors. Da Silva et al. (1989) identified in *L. major* the CR1 receptor for C3b as the principal molecule involved in the binding of serum opsonised metacyclic promastigotes. In their experiments, CR1-mediated uptake of metacyclic parasites did not generate respiratory burst in the macrophage whereas CR3-mediated uptake of promastigotes from log-phase cultures triggered a respiratory burst. More recently it was demonstrated that CR1 and CR3 cooperate in a unique manner in binding both procyclic and metacyclic promastigotes of *L. major* and that not CR1 but CR3 is the primary receptor involved in the complement-mediated phagocytosis of *Leishmania* (Sutterwala et al. 1996, Rosenthal et al. 1996). The benefit of utilizing both CR1 and CR3 receptors is that they do not elicit respiratory bursts.

Besides the opsonin facilitated, i. e. serum dependent binding, there is also a serum independent **direct binding** of promastigotes to macrophages that contributes to parasite attachment and internalization. Direct binding involves interactions of parasite carbohydrates and macrophage lectin-like receptors, mainly mannose/fucose receptor, receptor for advanced glycosylation end products (AGE) and β-glucan receptor (Blackwell 1985, Wilson & Pearson 1986, Mosser et al. 1987, Mosser &

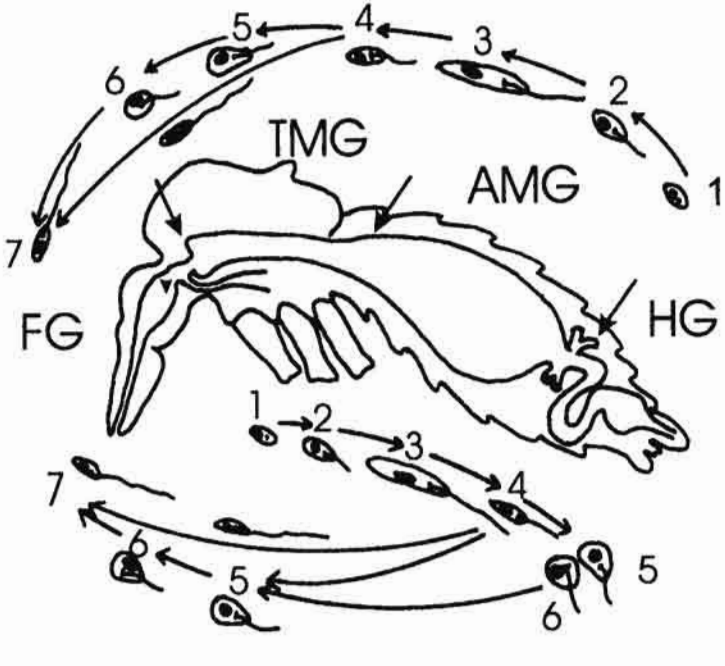


Fig. 7. Generalized scheme of the morphological development and localization of suprapylarian (above) and peripylarian (below) *Leishmania* spp. in sandflies. Arrows divide the gut to: FG, foregut; TMG, thoracic midgut; AMG, abdominal midgut; and HG, hindgut. The morphological forms are: 1, amastigotes; 2, stumpy promastigotes; 3, elongated nectomonad promastigotes; 4, short nectomonad promastigotes; 5, haptomonad promastigotes; 6, paramastigotes; 7, metacyclic promastigotes.

Handman 1992). On the other hand, both promastigotes and amastigotes possess their own lectin-like receptor which is, in some way, involved in the attachment (Hernández 1986, Svobodová et al. 1997a, b). Early studies emphasized also the role of direct binding of purified LPG and gp63 to CR3 receptor (Talamas-Rohana et al. 1990, Russell & Wright 1988). However, this hypothesis was not confirmed using living promastigotes (Brittingham et al. 1995, Rosenthal et al. 1996). Similarly, direct binding of gp63 to fibronectin receptor also remains ambiguous (Mosser & Brittingham 1997). Although the biological significance of direct binding seems to be rather low in comparison with the efficient serum-dependent attachment, the observation that mice lacking C3 can be infected with *Leishmania* supported the role of direct binding *in vivo* (Mosser & Brittingham 1997).

The attachment is followed by phagocytosis: the macrophage encloses the parasite cell and forms a parasitophorous vacuole where parasite surface membrane is surrounded by a host cell membrane which was originally on the surface of the phagocyte (reviewed by Bray & Alexander 1987, Chang 1983).

1.2. Survival and multiplication

During classical phagocytosis, lysosomes containing hydrolases fuse with phagosome containing the parasite (i. e., parasitophorous vacuole) to form a phagolysosome. The entry of *Leishmania* promastigotes is a somewhat different event; recent studies showed that promastigotes could inhibit phagosome-lysosome fusion due to an effect of LPG (Desjardin & Descoteaux 1997). Phagosome-lysosome fusion is re-established during the transformation of promastigotes to amastigotes, which is associated with the loss of LPG expression. Then, amastigotes appear inside the hostile environment of the acid parasitophorous vacuole and have to survive macrophage microbicidal equipment including the lethal products of oxygen metabolism, lysosomal hydrolases and low pH.

The survival of amastigotes is enabled not only by passive resistance to macrophage cell actions, but also by active influence on the host cell. One of these parasite-induced changes is an impairment of macrophage Ca^{2+} homeostasis. *Leishmania* spp, like other intracellular pathogens, can induce the elevation of Ca^{2+} in the host cell (Eilam et al. 1985). Therefore, the parasite alters the macrophage Ca^{2+}-dependent signaling mechanisms and the response to extracellular stimuli, which affects macrophage functions like the respiratory burst and the MHC class II expression (reviewed by Olivier 1996). *Leishmania* spp. can also enhance the viability of the host cell: it was shown that *L. donovani* infection prevents macrophage apoptosis. The enhanced macrophage viability facilitates the spread of infection as well as the uptake by the sandfly vector by increasing number of host cells available for parasitation (Moore & Matlashewski 1994)

The main killing mechanism of macrophage which threaten leishmanias is the **oxidative burst**, i. e., the generation of reactive oxygen intermediates (O_2^- and H_2O_2) by the NADPH oxidase system. *Leishmania* spp. posses only negligible amounts of enzymes catalase and glutathione peroxidase which can inactivate the resultant elements of the burst (Meshnick & Eaton 1981). However, oxidative burst can be impaired in another fashion. Firstly, as mentioned above, *Leishmania* can enter macrophages without eliciting oxidative burst. Secondly, the parasite LPG is a powerful inhibitor of the activation of protein kinase C (PKC) (McNeely et al. 1989, McNeely & Turco 1990). PKC is involved in the regulation of several cellular functions, including the enzymatic induction of oxidative burst. As the consequence of a signal (rapid increase of intracellular Ca^{2+} levels), PKC is redistributed from the macrophage cytosol to the plasma membrane where it is further activated to its enzymatic function. LPG was shown to inhibit both of these events. It prevents the binding of PKC to membranes as well as inhibits the activity of membrane-bound form of this enzyme (Giorgione et al. 1996). Thirdly, as shown by Sørensen et al. (1994), purified gp63 also strongly inhibits the oxidative burst in monocytes. And additionally, if the oxidative burst for all that is induced, the

glycan part of *Leishmania* LPG (McNeely & Turco 1990) and proteophosphoglycan (Ilg et al. 1995) function as oxygen metabolites scavengers in promastigotes and amastigotes, respectively.

The other recognized effector mechanism for the killing of *Leishmania* spp. inside macrophages is the **production of nitric oxide (NO)** by the enzyme NO synthase (NOS). The cellular targets of NO toxicity are various enzymes of glycolysis and respiratory metabolism as well as trans-membrane transport systems of parasites (Mauel & Ransijn 1997). Proudfoot et al. (1995, 1996) described that the synthesis of NO can be inhibited by *Leishmania* glycoinositolphospholipids (GIPLs) and phosphoglycan part of LPG. GIPLs, the major glycolipid class and prominent surface antigens of *Leishmania* parasites, are structurally related to the anchor of LPG and are expressed at constant levels in both developmental stages (McConville & Blackwell 1991, Schneider et al. 1993).

Both GIPLs and LPG can also provide a surface shield against **lysosomal hydrolases** (Chang 1993, Opat et al. 1996). Alternatively, the resistance to lysosomal enzyme degradation is due to a direct inactivation of enzymes by excreted factors, i. e. LPG and phosphoglycan released by parasites (El-On et al. 1980) or by the change of the intralysosomal pH (Coombs 1982).

Amastigotes do not proliferate immediately after the entry into macrophages. It was shown *in vitro* that the proliferation of *L. donovani* amastigotes do not start during the first 3–5 days of the infection (Pearson et al. 1983, Eslami & Tanner 1994). The multiplication is followed by rupture of parasitized cell and the liberated amastigotes are ingested by other macrophages. In comparison with wealth of information about promastigotes binding to macrophages, very little is known about the mechanism of entry of amastigote stages. During macrophage attachment, amastigotes encounter different environment than did promastigotes before (early vs. acute inflammation). It is, therefore, not surprising that amastigotes generally require for entry other receptors than promastigotes, mainly the receptor for Fc domain of immunoglobulins (Guy & Belosevic 1993, Peters et al. 1995), although the involvement of LPG was also described for *L. major* (Kelleher et al. 1995).

1.3. Modulation of the host immune response

The *Leishmania* – macrophage interactions influence the host immune response and have a strong impact on either the spread or the suppression of the disease. Activated macrophages, natural killer (NK) cells and T lymphocytes play a crucial role in the host defence against *Leishmania* spp. The activation state of these effector cells is dependent on the availability of stimulatory and inhibitory cytokines. Not surprisingly, *Leishmania* are able to alter their production and, in this way, affect a protective immune response of the host.

The fundamental point to establish control of intracellular infection is the macrophage activation by cytokines derived from $CD4^+$T helper (Th) lymphocytes. This event has been intensively investigated in experimental infections of inbred strains of mice with *L. major* (reviewed by Reiner & Locksley 1995). The central finding evolved from the model was that resistance to the disease (spontaneous healing of the lesions) is related to the preferential expansion of Th1 cells while susceptibility involves the preferential expansion of Th2 cells. Th1 and Th2 cells are differentiated from naive Th precursors in response to cytokines such as IL-12 and IL-4, respectively. Th1 and Th2 cell subsets differ in the production of cytokines and, subsequently, in their functions. Th1 cells mediate cellular immunity, effective against intracellular pathogens (activation of macrophages and cytotoxic $CD8^+$T-cells), while Th2 cells are responsible for humoral immunity, unprotective in this case (reviewed by Lohoff et al. 1998, Lehmann & Alber 1998, Bogdan & Röllinghof 1999).

Leishmania spp. counteract the development of Th1 immune response by modulation of the macrophage cytokine production. In particular, they suppress IL-12 production which negatively affects Th1-cell expansion. In addition, they stimulate macrophages for the induction of transforming growth factor-β (TGF-β) and IL-10 which suppress NK cells and macrophage effector functions (reviewed by Bogdan & Röllinghof 1998, 1999).

Leishmania spp. can also circumvent the induction of a protective Th1-cell response by impeding the presentation of antigens by MHC (major histocompatibility complex) class II. Without signals from antigen-presenting cells, Th1 cells could not be efficiently activated. De Souza-Leao et al. (1995) found that *L. amazonensis* amastigotes are able to internalize MHC class II molecules into parasite lysosomes (called megasomes) where they are degraded by cysteine proteases. However, it is not known yet whether this mechanism is sufficient enough to protect parasites. The interaction between antigen-presenting cells and Th cells is stabilized by CD4 molecules on the surface of Th cells. It was shown that gp63 from *L. major* and *L. donovani* cleaves CD4 molecules (Hey et al. 1994). This protective mechanism, however, is still ambiguous as it is not clear how gp63 could reach the surface of Th cells. The other factor impeding the host immune defence is a complex glycocalyx of amastigotes (containing mainly GIPLs) which protects the parasites from proteolytic attack as well as it masks the surface peptides of intact parasites. Therefore, only very few peptide epitopes are available for loading on the MHC molecules.

2. Factors affecting Leishmania *virulence*

In parasitic diseases, virulence is not the own characteristic of the parasite, but it is a complex process determined by the interactions of multiple parasite and host factors. According to Chang (1993), virulence is caused by **immunopathogenic determinants** of the parasite on one side and host **immunologic determinants** on the other side. Chang (1993) also distinguished the term 'pathogenicity' (or infectivity) which denotes an ability of the parasite to invade host using its various intracellular and surface molecules (**invasive determinants**). Therefore, pathogenicity is a prerequisite for leishmanial virulence. It is assumed that parasite invasive and immunopathogenic determinants are different molecules, regulated independently, and the disease symptoms as a manifestation of virulence depend on up- or down-regulation of all the three mentioned determinants.

The virulence of *Leishmania* isolates is, conventionally, compared by inoculation of animals with the same parasite numbers and by evaluation of disease symptoms. Most often, skin lesions and hepatosplenomegaly are checked in cutaneous and visceral disease forms, respectively. The assessment of virulence is based on the number of parasites found in infected organs and/or the size of the organs measured at different periods of the infection.

As the virulence is strongly influenced by host immunity and genetic susceptibility (Liew & O'Donnell 1993, Tanner 1996, Blackwell 1996), the animals used for experimental infections should be inbred, of the same age and sex (Giannini 1974, Sempervivo et al. 1981a, Neal 1984). Evenly important is the site of inoculation (Nabors et al. 1995) and conditions of *Leishmania* cultivation, like the medium and the growth phase of parasites used for inoculation (Giannini 1974, Neal 1984). It is generally believed that only the metacyclic promastigotes from stationary-phase cultures are able to infect vertebrates (Franke et al. 1985, Mallinson & Coombs 1986, Da Silva & Sacks 1987, Sacks & Da Silva 1987, Grimm et al. 1991). Thus, the actual assessment of *Leishmania* infectivity and virulence could be made, and difference among various strains and even cloned lines of the same strain could emerge, only if the above experimental conditions are controlled.

The diversity in *Leishmania* virulence in nature has not yet been elucidated. First reports concerning this topic came from field studies carried out in Central Asia (former USSR), where foci of zoonotic cutaneous leishmaniasis are caused by *L. major* with *Rhombomys opimus* as the main reservoir (see the Part IV). Kellina et al. (1981) found that field isolates from gerbils and humans were composed of clones displaying various degrees of virulence for mice and hamsters. Later, however, the avirulent forms have been identified as two separate species, *L. turanica* and *L. gerbilli* (reviewed by Strelkova 1996). More recently, different strains and clones of *L. peruviana* and *L.*

braziliensis which show reproducible differences in virulence have been obtained from Peru and Colombia (Dye 1992, Blackwell 1992, Dujardin et al. 1995, Davies et al. 1997). *L. peruviana* is the causative agent of Andean cutaneous leishmaniasis ("uta"). It is endemic in Andean valleys, where it forms small populations isolated by numerous natural barriers. Dujardin et al. (1995) found that the insulated nature of the habitat and decreased gene flow between separated populations contributed to formation of strong chromosomal polymorphism which is linked with phenotype variability, particularly the lesion type in patients and the virulence *in vitro*. Also the experiments with *L. tropica* suggest a role for natural parasite-related virulence determinants. Isolates from patients with cutaneous lesions displaying different clinical patterns differed similarly in mice infections (Ebrahimzadeh & Jones 1983) and strains from cutaneous, viscerotropic and visceral human isolates differed in disease progression in mice and hamsters (Lira et al. 1998).

In laboratories, parasites maintained by serial transfers in axenic cultures are under a strong artificial selective pressure. In such conditions the quickly dividing forms of less virulent parasites are favoured and mutations affecting virulence are tolerated. It is, therefore, suggested that a common mechanism occurring in laboratory strains *in vitro* is a gradual adaptation to culture conditions and/or selection for certain subpopulations that outgrow others present in the original heterogeneous population (Handman et al. 1983, Segovia et al. 1992, Camara et al. 1995).

The loss of infectivity and/or virulence for host in long-term cultures was frequently observed (Adler 1961, Neal 1964, Ebert et al. 1979, Giannini et al. 1981, Kutish & Janovy 1981, Grimaldi et al. 1982, Sempervivo et al. 1981b, Handman et al. 1983, Nolan & Herman 1985, Grimm et al. 1991, Segovia et al. 1992). Avirulence of parasites attenuated in the process of cultivation correlates with the loss of their ability to transform into metacyclic forms (Kallinikova et al. 1992). On the other hand, promastigotes from long-term cultures could differ from virulent first-passage promastigotes in larger size and vigorous growth *in vitro* (Nolan & Herman 1985, Sempervivo et al. 1981b, Grimm et al. 1991).

It must be admitted that parasites maintained exclusively as amastigotes by serial passages in laboratory animals (or in macrophage-like cell cultures) are not threatened by avirulence, but they have a decreased capacity to function as promastigotes and to grow in culture. Therefore, only frequent alternate passages in mammalian host and cultures could yield *Leishmania* capable of morphological and physiological transformations known to occur in the natural life cycle (Sempervivo et al. 1981b).

In some cases, the loss of virulence is reversible. Katakura & Kobyashi (1985) obtained a more virulent line of promastigotes from a less infective original strain after 15 serial passages in mice. A single passage through susceptible animals resulted in increased virulence of parasites in less virulent clones producing delayed footpad lesions (Da Silva & Sacks 1987, Marchand et al. 1987, Shankar et al. 1993).

Avirulent parasites obtained by prolonged *in vitro* cultivation or produced on purpose by mutagen treatment are often used in research. A comparison of these avirulent variants with the virulent ones is a useful method to study the phenotypic repertoire associated with the character of virulence. In this way, one can obtain information about factors necessary for *Leishmania* survival in macrophages (Nolan & Herman 1985, Katakura 1986, Marchand et al. 1987), about *Leishmania* surface antigens (Handman et al. 1983, Ayesta et al. 1985, Saraiva & Andrade 1986, Greenblatt et al. 1985, Sanyal et al. 1994) and about important **molecular determinants of virulence** like LPG and gp63 (see the Part VI), cysteine proteinases (Mottram et al. 1996), GIPLs (McConville & Blackwell 1991), heat shock proteins Hsp100 (Hübel et al. 1997) and Hsp70 (Kantengwa et al. 1995) or Ca^{2+}-ATPase (Lu et al. 1997).

PART VI.

The role of main surface molecules of *Leishmania* in the life-cycle

1. The structure and developmental regulation of LPG and gp63

As was shown above, molecules on the cell surface are crucial for the survival of *Leishmania* parasites both within the host and the vector. This chapter briefly summarizes main results of investigations concerning lipophosphoglycan (LPG) and the glycoprotein of 63 kDa (gp63). Although there are other important surface and excreted molecules like the gene B protein, GIPLs, proteophosphoglycan, acid phosphatase or protein kinases, it is clear that LPG and gp63 are exceptionally significant (reviewed by Turco & Descoteaux 1992, Moody 1993, Coombs & Mottram 1997). Both molecules are glycosylphosphatidylinositol (GPI)-anchored glycoconjugates constituting a major part of the dense glycocalyx covering the entire surface of promastigotes.

LPG is composed of four distinct structural domains: GPI-anchor, a conserved hexasaccharide core, a linear array of phosphorylated oligosaccharide repeat units and a saccharide cap (for details see Turco & Descoteaux 1992). LPGs from different *Leishmania* species and different developmental stages exhibit structural variations. During metacyclogenesis, the development of infective metacyclic forms is accompanied by about two-fold elongation of the molecule due to the increase in the number of repeat units, and concomitantly, a subtle compositional changes in these units (Sacks et al. 1990). These changes are suggested to be involved in the regulation of the attachment and detachment of promastigotes in the sandfly gut. The resulting metacyclic promastigotes display markedly enhanced resistance to both complement-mediated lysis and macrophage killing mechanisms (reviewed by Sacks 1992). Amastigote stages generally do not express LPG, the only described exception is *L. major*. However, the amastigote LPG of *L. major* is structurally and antigenically different from the LPG of promastigotes (Glaser et al. 1991, Moody et al. 1993)

The interspecific polymorphism in LPG structure is expressed in the type and number of oligosaccharide side chains branching from the conserved backbone repeat units. The most complex LPG with high number of side chains was found in *L. major* (McConville et al. 1990), while little or no substitutions occur in *L. donovani*, *L. mexicana* and in the *Viannia* subgenus (Thomas et al. 1992, Ilg et al. 1992, Muskus et al. 1997).

Gp63 has been identified as a zinc metalloproteinase, active on a wide range of protein substrates (Etges et al. 1986, Chaudhuri & Chang 1988, Bouvier et al. 1989) and with a substrate-dependent pH optimum (Ip et al. 1990, Tzinia & Soteriadou 1991). This molecule is ubiquitous among *Leishmania* promastigotes (Bouvier et al. 1987, Etges 1992) and is also present in amastigotes, although in lower levels (Button et al. 1989, Schneider et al. 1992, Bahr et al. 1993, Streit et al. 1996). In *L. mexicana*, most amastigote gp63 is confined to the flagellar pocket (Medina-Acosta et al. 1989) or to amastigote lysosomes called megasomes (Bahr et al. 1993, Ilg et al. 1993). On the other hand, surface location of the gp63 was found in amastigotes of *L. major* and *L. chagasi* (Pimenta et al. 1991, Streit et al. 1996).

2. LPG and gp63 as a virulence factors

Association of gp63 with parasite virulence has been reported by various authors. The increase of gp63 expression correlates with the development of infective metacyclic promastigotes (Kweider et al. 1987). Decreased amount and proteolytic activity of gp63 was found in avirulent parasites in comparison with the virulent ones (Chaudhuri & Chang 1988, Wilson et al. 1989, Santos-Gomes & Abranches 1996, Seay et al. 1996) and the transfection of avirulent strains with a gp63 gene has increased parasite infectivity for macrophages (Liu & Chang 1992, McGwire & Chang 1994, Chakrabarty et al. 1996). In other systems, however, the main role of LPG in parasite virulence was highlighted (Handman et al. 1986, McNeely & Turco 1990, Frankenburg et al. 1992). Decreased

virulence of *L. major* and *L. donovani* after chemical mutagenesis was found to correlate with the decrease in expression and change of LPG structure, while the expression and proteolytic activity of gp63 remained unchanged (Elhay et al. 1990, Shankar et al. 1993, Cappai et al. 1994).

This controversy may reflect the natural variability and plasticity in the relative importance of these two virulence factors. Indeed, Chakrabarty et al. (1996) showed that in two virulent strains of *L. donovani* the contributory roles of gp63 and LPG differ either in recognition or in the rate of internalization into macrophages. In their experiments, preblocking of macrophage receptors with either gp63 or LPG affected the entry of the one or the other virulent strain, respectively.

The cross-substitution of these two virulence factors can be imagined with respect to their suggested roles in the vertebrate host (summarized in Table 2). Both LPG and gp63 have been implicated in crucial steps of *Leishmania*-host interactions, i. e., the influence on monocyte migration, avoidance of complement attack, attachment to macrophages and protection from degradation in macrophage phagolysosomes. Some of the functions remain to be confirmed and some may be species-specific, however, it is clear that these molecules accomplish very similar functions in a different manner. Such diversity in evading strategies and multiplicity in molecules which are involved in the evading mechanisms may be, in fact, a main qualification for a successful survival of the parasite.

Acknowledgements

I would like to thank Prof. Jiří Vávra, Dr Petr Volf and Dr Milena Svobodová (all from Charles University) for reading the manuscript.

REFERENCES

ADLER S. 1961: Infectivity of a strain of Leishmania infantum after prolonged culture. *Bull. Res. Counc. Isr. Section E: Exp. Med.* **9**: 166.

ADLER S. & THEODOR O. 1935: Investigations on mediterranean kala azar. IX – Feeding experiments with Phlebotomus perniciosus and other species on animals infected with Leishmania infantum. *Proc. Roy. Soc. Series* B **116**: 516–542.

AÑEZ N., NIEVES E. & CAZORLA D. 1989: The validity of the developmental pattern in the sandfly gut for classification of Leishmania. *Trans. Roy. Soc. Trop. Med. Hyg.* **83**: 634–635.

AÑEZ N., TANG Y., KILLICK-KENDRICK R. & KILLICK-KENDRICK M. 1997: The use of microcapillary feeding system to determine the fate of a meal in phlebotomine sand flies (Diptera: Psychodidae). *Bol. Dir. Malar. Saneam. Amb.* **37**: 1–6.

ASHFORD R. W. & BETTINI S. 1987: Ecology and epidemiology: Old World. Pp.: 365–424. In: PETERS W. & KILLICK-KENDRICK R. (eds): *The Leishmaniases in Biology and Medicine*. London: Academic Press, 550 pp.

AYESTA C., ARGÜELLO C. & HERNÁNDEZ A. G. 1985: Leishmania braziliensis: cell surface differences in promastigotes of pathogenic and nonpathogenic strains. *Exp. Parasitol.* **59**: 185–191.

BAHR V., STIERHOF Y.-D., ILG T., DEMAR M., QUINTEN M. & OVERATH P. 1993: Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of Leishmannia mexicana. *Mol. Biochem. Parasitol.* **58**: 107–122.

BEVERLEY S. M., ISMACH R. B. & MCMAHON PRATT D. 1987: Evolution of the genus Leishmania as revealed by comparison of nuclear DNA restriction fragment patterns. *Proc. Natl. Acad. Sci. USA* **84**: 484–488.

BLACKWELL J. M. 1985: Role of macrophage complement and lectin-like receptors in binding Leishmania parasites to host macrophages. *Immunol. Letters* **11**: 227–232.

BLACKWELL J. M. 1992: Leishmaniasis epidemiology: all down to the DNA. *Parasitology* **104**: 19–34.

BLACKWELL J. M. 1996: Genetic susceptibility to leishmanial infection: studies in mice and man. *Parasitology* **112**: 67–74.

BOGDAN C. & RÖLLINGHOFF M. 1998: The immune response to Leishmania: mechanisms of parasite control and evasion. *Int. J. Parasitol.* **28**: 121–134.

BOGDAN C. & RÖLLINGHOFF M. 1999: How do protozoan parasites survive inside macrophages? *Parasitol. Today* **15**: 22–28.

BOROVSKY D. & SCHLEIN Y. 1987: Trypsin and chymotripsin-like enzymes of the sandfly Phlebotomus papatasi infected with Leishmania and their possible role in vector competence. *Med. Vet. Entomol.* **1**: 235–242.

BOUVIER J., ETGES R. & BORDIER C. 1987: Identification of the promastigote surface protease in seven species of Leishmania. *Mol. Biochem. Parasitol.* **24**:73–79.

BOUVIER J., BORDIER C., VOGEL H., REICHELT R. & ETGES R. 1989: Characterization of the promastigote surface protease of Leishmania as a membrane-bound zinc endopeptidase. *Mol. Biochem. Parasitol.* **37**: 235–246.

BRAY R. S. 1983: Leishmania: Chemotaxic responses of promastigotes and macrophages in vitro. *J. Protozool.* **30**: 322–329.

BRAY R. S. & ALEXANDER J. 1987: Leishmania and the macrophage. Pp.: 211–233. In: PETERS W. & KILLICK-KENDRICK R. (eds): *The Leishmaniases in Biology and Medicine*. London: Academic Press, 550 pp.

BRITTINGHAM A. & MOSSER D. M. 1996: Exploitation of the complement system by Leishmania promastigotes. *Parasitol. Today* **12**: 444–447.

BRITTINGHAM A., MORRISON C. J., MCMASTER W. R., MCGWIRE B. S., CHANG K.-P. & MOSSER D. M. 1995: Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistence to complement-mediated lysis. *J. Immunol.* **155**: 3102–3111.

BUTTON L. L., RUSSELL D. G., KLEIN H. L., MEDINA-ACOSTA E., KARESS R. E. & MCMASTER W. R. 1989: Genes encoding the major surface glycoprotein in Leishmania are tandemly linked at a single chromosomal locus and are constitutively transcribed. *Mol. Biochem. Parasitol.* **32**: 271–284.

CAMARA M., NAVARRO M. & SEGOVIA M. 1995: Evidence from genotypic and phenotypic markers that an attenuated line outgrows a virulent one in a mixed population of Leishmania major promastigotes cultured in vitro. *Ann. Trop. Med. Parasitol.* **89**: 477–484.

CAPPAI R., MORRIS L., AEBISCHER T., BACIC A., CURTIS J. M., KELLEHER M., MCLEOD K. S., MOODY S. F., OSBORN A. H. & HANDMAN E. 1994: Ricin-resistant mutants of Leishmania major which express modified lipophosphoglycan remain infective for mice. *Parasitology* **108**: 397–405.

CHAKRABARTY R., MUKHERJEE S., LU H.-G., MCGWIRE B. S., CHANG K.-P. & BASU M. K. 1996: Kinetics of entry of virulent and avirulent strains of Leishmania donovani into macrophages: a possible role of virulence molecules (gp63 and LPG). *J. Parasitol.* **84**: 632–635.

CHANG K. P. 1983: Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. *Int. Rev. Cytol.*, Suppl. **14**: 267–305.

CHANG K. P. 1993: Evolution and regulation of Leishmania virulence. Pp.: 15–28. In: BHADURI A. N., BASU M. K., SEN A. K. & KUMAR S. (eds.): *Current Trends in Leishmania Research*. New Delhi, India: CSIR Publications & Information Directorate.

CHANG C. S. & CHANG K. P. 1985: Heme requirement and acquisition by extracellular and intracellular stages of Leishmania mexicana amazonensis. *Mol. Biochem. Parasitol.* **16**: 267–276.

CHAUDHURI G. & CHANG K. P. 1988: Acid protease activity of a major surface membrane glycoprotein (gp63) from Leishmania mexicana promastigotes. *Mol. Biochem. Parasitol.* **27**: 43–52.

CHAUDHURI G., CHAUDHURI M., PAN A. & CHANG K. P. 1989: Surface acid proteinase (gp63) of Leishmania mexicana. *J. Biol. Chem.* **264**: 7483–7489.

COOMBS G. H. 1982: Proteinases of Leishmania mexicana and other flagellate protozoa. *Parasitology* **84**: 149–155.

COOMBS G. H. & MOTTRAM J. C. 1997: Parasite proteinases and amino acid metabolism: possibilities for chemotherapeutic exploitation. *Parasitology* **114**: 61–80.

CROAN D. & ELLIS J. 1996: Phylogenetic relationships between Leishmania, Viannia and Sauroleishmania inferred from comparison of a variable domain within the RNA polymerase II largest subunit gene. *Mol. Biochem. Parasitol.* **79**: 97–102.

DA SILVA R. P. & SACKS D. L. 1987: Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. *Inf. Immunol.* **55**: 2802–2806.

DA SILVA R. P., HALL B. F., JOINER K. A. & SACKS D. L. 1989: CR1, the C3b receptor, mediates binding of infective Leishmania major metacyclic promastigotes to human macrophages. *J. Immunol.* **143**: 617–622.

DAVIES C. R., COOPER A. M., PEACOCK C., LANE R. P. & BLACKWELL J. M. 1990: Expression of LPG and gp63 by different developmental stages of Leishmania major in the sandfly Phlebotomus papatasi. *Parasitology* **101**: 337–343.

DAVIES C. R., LLANOS-CUENTAS E. A., SHARP S. J., CANALES J., LEON E., ALVAREZ E., RONCAL N. & DYE C. 1997: Cutaneous leishmaniasis in the Peruvian Andes: factors associated with variability in clinical symptoms, response to treatment, and parasite isolation rate. *Clinic. Inf. Dis.* **25**: 302–310.

DE SOUZA-LEAO S., LANG T., PRINA E., HELLIO R. & ANTOINE J. C. 1995: Intracellular Leishmania amazonensis amastigotes internalize and degrade MHC class II molecules of their host cells. *J. Cell Sci.* **108**: 3219–3231.

DESJARDINS M. & DESCOTEAUX A. 1997: Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *J. Exp. Med.* **185**: 2061–2068.
DILLON R. J. & LANE R. P. 1993a: Bloodmeal digestion in the midgut of Phlebotomus papatasi and Phlebotomus langeroni. *Med. Vet. Parasitol.* **7**: 225–232.
DILLON R. J. & LANE R. P. 1993b: Influence of Leishmania infection on blood-meal digestion in the sandflies Phlebotomus papatasi and P. langeroni. *Parasitol. Res.* **79**: 492–496.
DILLON R. J. & LANE R. P. 1999: Detection of Leishmania lipophosphoglycan binding proteins in the gut of the sandfly vector. *Parasitology* **118**: 27–32.
DUJARDIN J. C., BAÑULS A. L., VICTOIR K., DE DONCKER S., AREVALO J., LLANOS-CUENTAS A., TIBAYRENC M. & LE RAY D. 1995: From population to genome: ecogenetics of Leishmania (Viannia) braziliensis and L. (V.) peruviana. *Ann. Trop. Med. Parasitol.* **89**: 45–53.
DYE C. 1992: Leishmaniasis epidemiology: the theory catches up. *Parasitology* **104**: 7–18.
EBERT F., BUSE E. & MÜHLPFORDT H. 1979: In vitro light and electron microscope studies on different virulent promastigotes of Leishmania donovani in hamster peritoneal macrophages. *Ztschr. Parasitenkd.* **59**: 31–41.
EBRAHIMZADEH A. & JONES T. C. 1983: A comparative study of different Leishmania tropica isolates from Iran: correlation between infectivity and cytochemical properties. *Amer. J. Trop. Med. Hyg.* **32**: 694–702.
EILAM Y., EL-ON J. & SPIRA D. T. 1985: Leishmania major: excreted factor, calcium ions, and the survival of amastigotes. *Exp. Parasitol.* **59**: 161–168.
EISENBERGER C. L. & JAFFE C. L. 1999: Leishmania: Identification of Old World species using a permissively primed intergenic polymorphic-polymerase chain reaction. *Exp. Parasitol.* **91**: 70–77.
ELHAY M., KELLEHER M., BACIC A., MCCONVILLE M. J., TOLSON D. L., PEARSON T. W. & HANDMAN E. 1990: Lipophosphoglycan expression and virulence in ricin-resistant variants of Leishmania major. *Mol. Biochem. Parasitol.* **40**: 255–268.
ELNAIEM D. A., WARD R. D. & YOUNG P. E. 1992: An ultrastructural study on the early development of Leishmania chagasi (Kinetoplastida: Trypanosomatidae) in its vector Lutzomyia longipalpis (Diptera: Psychodidae). *Ann. Parasitol. Hum. Comp.* **67**: 3–8.
EL-ON J., BRADLEY D. J. & FREEMAN J. C. 1980: Leishmania donovani: action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. *Exp. Parasitol.* **49**: 167–174.
ESLAMI Z. & TANNER C. E. 1994: Time course and intensity of infection in vitro in the resident peritoneal macrophages of resistant and susceptible mice exposed to different doses of Leishmania donovani promastigotes. *Int. J. Parasitol.* **24**: 743–747.
ETGES R. 1992: Identification of a surface metalloproteinase on 13 species of Leishmania isolated from humans, Crithidia fasciculata, and Herpetomonas samuelpessoai. *Acta Tropica* **50**: 205–217.
ETGES R., BOUVIER J. & BORDIER C. 1986: The major surface protein of Leishmania promastigotes is a protease. *J. Biol. Chem.* **261**: 9098–9101.
FRANKE E. D., MCGREEVY P. B., KATZ S. P. & SACKS D. L. 1985: Growth cycle-dependent generation of complement-resistant Leishmania promastigotes. *J. Immunol.* **134**: 2713–2718.
FRANKENBURG S., LIEBOVICI V., MANSBACH N., TURCO S. J. & ROSEN G. 1990: Effect of glycolipids of Leishmania parasites on human monocyte activity. *J. Immunol.* **145**: 4284–4289.
FRANKENBURG S., GROSS A. & LEIBOVICI V. 1992: Leishmania major and Leishmania donovani: effect of LPG-containing and LPG-deficient strains on monocyte chemotaxis and chemiluminescence. *Exp. Parasitol.* **75**: 442–448.
GIANNINI M. S. 1974: Effects of promastigote growth phase, frequency of subculture, and host age on promastigote-initiated infections with Leishmania donovani in the golden hamsters. *J. Protozool.* **21**: 521–527.
GIANNINI M. S. H., D'ALESANDRO P. A., GARCIA C. R. & SARAIVA E. M. B. 1981: Failure of hamster macrophages to discriminate between infective and noninfective promastigotes of Leishmania donovani during attachment in vitro. *Inf. Immunol.* **34**: 629–632.
GIORGIONE J. R., TURCO S. J. & EPAND R. M. 1996: Transbilayer inhibition of protein kinase C by the lipophosphoglycan from Leishmania donovani. *Proc. Natl. Acad. Sci. USA* **93**: 11634–11639.
GLASER T. A., MOODY S. F., HANDMAN E., BACIC A. & SPITHILL T. W. 1991: An antigenically distinct lipophosphoglycan on amastigotes of Leishmania major. *Mol. Biochem. Parasitol.* **45**: 337–344.
GREENBLATT C. L., HANDMAN E., MITCHELL G. F., BATTYE F. L., SCHNUR L. F. & SNARY D. 1985: Phenotypic diversity of cloned lines of Leishmania major promastigotes. *Ztschr. Parasitenkd.* **71**: 141–157.
GRIMALDI G., MOMEN H., SOARES M. J. & MORIEARTY P. L. 1982: Enzyme variation and differences in infectivity within a single strain of Leishmania mexicana mexicana. *Int. J. Parasitol.* **12**: 185–189.
GRIMM F., BRUN R. & JENNI L. 1991: Promastigote infectivity in Leishmania infantum. *Parasitol. Res.* **77**: 185–191.

GUY R. A. & BELOSEVIC M. 1993: Comparison of receptors required for entry of Leishmania major amastigotes into macrophages. *Inf. Immunol.* **61**: 1553–1558.
HANDMAN E., HOCKING R. E., MITCHELL G. F. & SPITHILL T. W. 1983: Isolation and characterisation of infective and non-infective clones of Leishmania tropica. *Mol. Biochem. Parasitol.* **7**: 111–126.
HANDMAN E., SCHNUR L. F., SPITHILL T. W. & MITCHELL G. F. 1986: Passive transfer of Leishmania lipophosphoglycan confers parasite survival in macrophages. *J. Immunol.* **137**: 3608–3613.
HATZIGEORGIOU D. E., GENG J., ZHU B., ZHANG Y., LIU K., ROM W. N., FENTON M. J., TURCO S. J. & HO J. L. 1996: Lipophosphoglycan from Leishmania suppresses agonist-induced interleukin 1b gene expression in human monocytes via a unique promoter sequence. *Proc. Natl. Acad. Sci. USA* **93**: 14708–14713.
HENNIG W. 1972: Insektenfossilien aus der unteren Kreide. IV. Psychodidae (Phlebotominae), mit einer kritischen Übersicht über das phylogenetische System der Familie und die bisher beschriebenen Fossilien (Diptera). *Stuttgart. Beitr. Naturk.* **241**: 1–69.
HERMOSO T., FISHELSON Z., BECKER S., HIRSCHBERG K. & JAFFE C. L. 1991: Leishmanial protein kinases phosphorylate components of the complement cascade. *EMBO J.* **10**: 4061–4067.
HERNÁNDEZ A. G., RODRIGUEZ N., STOJANOVIC D. & CANDELLE D. 1986: The localization of a lectin-like component on the Leishmania cell surface. *Mol. Biol. Rep.* **11**: 149–153.
HEY A. S., THEANDER T. G., HVIID L., HAZRATI S. M., KEMP M. & KHARAZMI A. 1994: The major surface glycoprotein (gp63) from Leishmania major and Leishmania donovani cleaves CD4 molecules on human T cells. *J. Immunol.* **152**: 4542–4548.
HÜBEL A., KROBITSCH S., HÖRAUF A. & CLOS J. 1997: Leishmania major Hsp100 is required chiefly in the mammalian stage of the parasite. *Mol. Cell. Biol.* **17**: 5987–5995.
ILG T., ETGES R., OVERATH P., MCCONVILLE M. J., THOMAS-OATES J., THOMAS J., HOMANS S. W. & FERGUSON M. A. J. 1992: Structure of Leishmania mexicana lipophosphoglycan. *J. Biol. Chem.* **267**: 6834–6840.
ILG T., HARBECKE D. & OVERATH P. 1993: The lysosomal gp63-related protein in Leishmania mexicana amastigotes is a soluble metalloprotease with an acidic pH optimum. *FEBS Let.* **327**: 103–107.
ILG T., STIERHOF Y. D., MCCONVILLE M. J. & OVERATH P. 1995: Purification, partial characterization and immunolocalization of a proteophosphoglycan secreted by Leishmania mexicana amastigotes. *Eur. J. Cell Biol.* **66**: 205–215.
IP H. S., ORN A., RUSSELL D. G. & CROSS G. A. M. 1990: Leishmania mexicana mexicana gp63 is a site-specific neutral endopeptidase. *Mol. Biochem. Parasitol.* **40**: 163–172.
JACOBSON R. L. 1995: Leishmania, LPG and the sandfly connection. *Parasitol. Today* **11**: 203–204.
JARAMILLO C., TRAVI B. L. & MONTOYA J. 1994: Vector competence of some neotropical sandflies for the Leishmania (Viannia) braziliensis complex. *Med. Vet. Entomol.* **8**: 1–7.
KALLINIKOVA V. D., NASYROVA R. M., NASYROV F. S. & SAF'JANOVA V. M. 1992: Morphogenesis and virulence of Leishmania major in the process of long-term cultivation. *Arch. Protistenkd.* **141**: 327–334.
KANTENGWA S., MÜLLER I., LOUIS J. & POLLA B. S. 1995: Infection of human and murine macrophages with Leishmania major is associated with early parasite heat shock protein synthesis but fails to induce a host cell stress response. *Immunol. Cell Biol.* **73**: 73–80.
KATAKURA K. 1986: Studies on infectivity of Leishmania donovani promastigotes: isolation, intracellular behavior and biological characterisation of virulent lines. *Jikeikai Med. J.* **33**: 157–173.
KATAKURA K. & KOBYASHI A. 1985: Enhancement of infectivity of Leishmania donovani promastigotes by serial mouse passages. *J. Parasitol.* **71**: 393–394.
KELLEHER M., MOODY S. F., MIRABILE P., OSBORN A. H., BACIC A. & HANDMAN E. 1995: Lipophosphoglycan blocks attachment of Leishmaia major amastigotes to macrophages. *Inf. Immunol.* **63**: 43–50.
KELLINA O. I., PASSOVA O. M. & ALEKSEEV A. N. 1981: [Experimental proof of heterogeneous composition of natural populations of Leishmania major with regard to virulence]. *Med. Parasitol.* **1**: 4–12 (In Russian.).
KILLICK-KENDRICK R. 1979: Biology of Leishmania in phlebotomine sandflies. Pp. 396–460. In: LUMSDEN W. H. R. & EVANS D. A. (eds): *Biology of the Kinetoplastida. Vol. 2.* Academic Press: London, New York.
KILLICK-KENDRICK R. 1985: Some epidemiological consequences of the evolutionary fit between leishmaniae and their phlebotomine vectors. *Bull. Soc. Path. Exp.* **78**: 747–755.
KILLICK-KENDRICK R. 1990: Phlebotomine vectors of leishmaniases: a review. *Med. Vet. Entomol.* **4**: 1–24.
KILLICK-KENDRICK R., LEANEY A. J., READY P. D. & MOLYNEUX D. H. 1977: Leishmania in phlebotomid sandflies. IV. The transmission of Leishmania mexicana amazonensis to hamsters by the bite of experimentally infected Lutzomyia longipalpis. *Proc. Roy. Soc. Lond. B* **196**: 105–115.
KILLICK-KENDRICK R., LAINSON R., RIOUX J. A. & SAF'JANOVA V. M. 1986: The taxonomy of leismania-like parasites of reptiles. Pp. 143–148. In: RIOUX J. A. (ed.) *Leishmania: Taxonomie et Phylogénèse: Applications Eco-Epidémiologiques.* Montpellier: Colloq. Int. CNRS/INSERM 1984, IMEEE.

KILLICK-KENDRICK R., WALLBANKS K. R., MOLYNEUX D. H. & LAVIN D. R. 1988: The ultrastructure of Leishmania major in the foregut and proboscis of Phlebotomus papatasi. *Parasitol. Res.* **74**: 586–590.

KILLICK-KENDRICK R., KILLICK-KENDRICK M. & TANG Y. 1994: Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: the low susceptibility of P. papatasi to Leishmania tropica. *Trans. Roy. Soc. Trop. Med. Hyg.* **88**: 252–253.

KILLICK-KENDRICK R., KILLICK-KENDRICK M., TANG Y. & BASTIEN P. 1996: Metacyclic promastigotes of Leishmania in the salivary glands of experimentaly infected phlebotomine sandflies. *Parasite* **3**: 55-60.

KUTISH G. F. & JANOVY J. 1981: Inhibition of in vitro macrophage digestion capacity by infection with Leishmania donovani (Protozoa: Kinetoplastida). *J. Parasitol.* **67**: 457–462.

KWEIDER M., LEMESRE J. L., DARCY F., KUSNIERZ J. P., CAPRON A. & SANTORO F. 1987: Infectivity of Leishmania braziliensis promastigotes is dependent on the increasing expression of a 65.000-dalton surface antigen. *J. Immunol.* **138**: 299–305.

LAINSON R. & SHAW J. J. 1979: The role of animals in the epidemiology of South American leishmaniasis. Pp.: 1–116. In: LUMSDEN W. H. R.& EVANS D. A. (eds.): *Biology of the Kinetoplastida. Vol. 2.* London, New York: Academic Press.

LAINSON R. & SHAW J. J. 1987: Evolution, classification and geographical distribution. Pp.: 2–120. In: PETERS W. & KILLICK-KENDRICK R. (eds.). *The Leishmaniases in Biology and Medicine.* London: Academic Press, 550 pp.

LAINSON R., WARD R. D. & SHAW J. J. 1977: Leishmania in phlebotomid sandflies: VI. Importance of hingut development in distinguishing between parasites of the Leishmania mexicana and L. braziliensis complex. *Proc. Roy. Soc. Lond. B* **199**: 309–320.

LANE R. P. 1993: Sandflies (Phlebotominae). Pp.: 78–119. In: LANE R.P. & CROSSKEY R.W. (eds.): *Medical Insects and Arachnids.* London: Chapman & Hall, 723 pp.

LAWYER P. G., YOUNG D. G., BUTLER J. F. & AKIN D. E. 1987: Development of Leishmania mexicana in Lutzomyia diabolica and Lutzomyia hannoni (Diptera: Psychodidae). *J. Med. Entomol.* **24**: 347–355.

LAWYER P. G., MEBRAHTU Y. B., NGUMBI P. M., MWANYUMBA P., MBUGUA J., KILU G., KIPKOECH D., NZOVU J. & ANJILI C. O. 1991: Phlebotomus guggisbergi (Diptera: Psychodidae), a vector of Leishmania tropica in Kenya. *Amer. J. Trop. Med. Hyg.* **44**: 290–298.

LEANEY A. J. 1977: The effect of temperature on Leishmania in sandflies. *Parasitology* **75**: 28–29.

LEHMANN J. & ALBER G. 1998: Murine leishmaniosis: a paradigm for the importance of T helper 1 and T helper 2 Cells. *Rev. Sci. Tech. OIE* **17**: 176–187.

LEWIS D. J. 1973: Phlebotomidae and Psychodidae (Sand-flies & Moth-flies). Pp.: 155–179. In: SMITH K. G. V. (ed.): *Insects and Other Arthropods of Medical Importance.* London: British Museum, 561 pp.

LEWIS D. J. & WARD R. D. 1987: Transmission and vectors. Pp.: 235–262. In: PETERS W. & KILLICK-KENDRICK R. (eds): *The Leishmaniases in Biology and Medicine.* London: Academic Press, 550 pp.

LIEW F. Y. & O'DONNELL C. A. 1993: Immunology of leishmaniasis. *Adv. Parasitol.* **32**: 161–259.

LIRA R., MÉNDEZ S., CARRERA L., JAFFE C., NEVA F. & SACKS D. 1998: Leishmania tropica: the identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. *Exp. Parasitol.* **89**: 331–342.

LIU X. & CHANG K. P. 1992: Extrachromosomal genetic complementation of surface metalloproteinase (gp63)-deficient Leishmania increases their binding to macrophages. *Proc. Natl. Acad. Sci. USA* **89**: 4991–4995.

LO S.K., BOVIS L., MATURA R., ZHU B., HE S., LUM H., TURCO S. J. & HO J. L. 1998: Leishmania lipophosphoglycan reduces monocyte transendothelial migration: modulation of cell adhesin molecules, intercellular junctional proteins, and chemoattractants. *J. Immunol.* **160**: 1857–1865.

LOHOFF M., GESSNER A., BOGDAN C. & RÖLLINGHOFF M. 1998: The Th1/Th2 paradigm and experimental murine leshmaniasis. *Int. Arch. Allergy Immunol.* **115**: 191–202.

LU H.-G., ZHONG L., CHANG K.-P. & DOCAMPO R. 1997: Intracellular Ca^{2+} pool content and signaling and expression of a calcium pump are linked to virulence in Leishmania mexicana amazonensis amastigotes. *J. Biol. Chem.* **272**: 9464–9473.

MALLINSON D. J. & COOMBS G. H. 1986: Molecular characterisation of the metacyclic forms of Leishmania. *IRCS Med. Sci.* **14**: 557–558.

MARCHAND M., DAOUD S., TITUS R. G., LOUIS J. & BOON T. 1987: Variants with reduced virulence derived from Leishmania major after mutagen treatment. *Parasite Immunol.* **9**: 81–92.

MAUEL J. & RANSIJN A. 1997: Leishmania spp.: mechanisms of toxicity of nitrogen oxidation products. *Exp. Parasitol.* **87**: 98–111.

MCCONVILLE M. J., THOMAS-OATES J. E., FERGUSON M. A. J. & HOMANS S. W. 1990: Structure of the lipophosphoglycan from Leishmania major. *J. Biol. Chem.* **265**: 19611–19623.

MCCONVILLE M. J. & BLACKWELL J. M. 1991: Developmental changes in the glycosylated phosphatidylinositols of Leishmania donovani. *J. Biol. Chem.* **266**: 15170–15179.

McGwire B. & Chang K. P. 1994: Genetic rescue of surface metalloproteinase (gp63)-deficiency in Leishmania amazonensis variants increases their infection of macrophages at the early phase. *Mol. Biochem. Parasitol.* **66**: 345–347.

McMaster W. R., Morrison C. J., McDonald M. H. & Joshi P. B. 1994: Mutational and functional analysis of the Leishmania surface metalloproteinase gp63: similarities to matrix metalloproteinases. *Parasitology* **108**: 29–36.

McNeely T. B. & Turco S. J. 1990: Requirement of lipophosphoglycan for intracellular survival of Leishmania donovani within human monocytes. *J. Immunol.* **144**: 2745–2750.

McNeely T. B., Rosen G., Londner M. V. & Turco S. J. 1989: Inhibitory effects on protein kinase C activity by lipophosphoglycan fragments and glycosylphosphatidylinositol antigens of the protozoan parasite Leishmania. *Biochem. J.* **259**: 601–604.

Medina-Acosta E., Karess R. E., Schwartz H. & Russell D. G. 1989: The promastigote surface protease (gp63) of Leishmania is expressed but differentially processed and localized in the amastigote stage. *Mol. Biochem. Parasitol.* **37**: 263–273.

Meshnick S. R. & Eaton J. W. 1981: Leishmanial superoxide dismutase: a possible target for chemotherapy. *Biochem. Biophys. Res. Commun.* **102**: 970–976.

Moody S. F. 1993: Molecular variation in Leishmania. *Acta Tropica* **53**: 185–204.

Moody S. F., Handman E., McConville M. J. & Bacic A. 1993: The structure of Leishmania major amastigote lipophosphoglycan. *J. Biol. Chem.* **268**: 18457–18466.

Moore K. J. & Matlashewski G. 1994: Intracellular infection by Leishmania donovani inhibits macrophage apoptosis. *J. Immunol.* **152**: 2930–2937.

Mosser D. M. & Edelson P. J. 1984: Activation of the alternative complement pathway by Leishmania promastigotes: parasite lysis and attachment to macrophages. *J. Immunol.* **132**: 1501–1505.

Mosser D. M. & Handman E. 1992: Treatment of murine macrophages with interferon-γ inhibits their ability to bind Leishmania promastigotes. *J. Leucocyte Biol.* **52**: 369–376.

Mosser D. M. & Brittingham A. 1997: Leishmania, macrophages and complement: a tale of subversion and exploitation. *Parasitology* **115**: 9–23.

Mosser D. M., Vlassara H., Edelson P. J. & Cerami A. 1987: Leishmania promastigotes are recognized by the macrophage receptor for advanced glycosylation endproducts. *J. Exp. Med.* **165**: 140–145.

Mottram J. C., Souza A. E., Hutchison J. E., Carter R., Frame M. J. & Coombs G. H. 1996: Evidence from disruption of the lmcpb gene array of Leishmania mexicana that cysteine proteinases are virulence factors. *Proc. Natl. Acad. Sci. USA* **93**: 6008–6013.

Mukkada A. J. 1985: Energy metabolism in Leishmania. Pp. 49–64. In: Chang K. P. & Bray R. S. (eds.): *Human Parasitic Diseases. Vol. 1. Leishmaniasis*. Amsterdam, New York: Elsevier.

Muscus C., Segura I., Oddone R., Turco S. J., Leiby D. A., Toro L., Robledo S. & Saraiva N. G. 1997: Carbohydrate and LPG expression in Leishmania Viannia subgenus. *J. Parasitol.* **83**: 671–678.

Nabors G. S., Nolan T., Croop W., Li J. & Farrell J. P. 1995: The influence of the site of parasite inoculation on the development of Th1 and Th2 type immune responses in (BALB/c x C57BL/6) F1 mice infected with Leishmania major. *Parasite Immunol.* **17**: 569–579.

Neal R. A. 1964: Chemotherapy of cutaneous leishmaniasis: Leishmania tropica infections in mice. *Ann. Trop. Med. Parasitol.* **58**: 420–430.

Neal R. A. 1984: Leishmania major: culture media, mouse strains, and promastigote virulence and infectivity. *Exp. Parasitol.* **57**: 269–273.

Nolan T. J. & Herman R. 1985: Effects of long-term in vitro cultivation on Leishmania donovani promastigotes. *J. Protozool.* **32**: 70–75.

Noyes H. A., Chance M. L., Croan D. G. & Ellis J. T. 1998: Leishmania (Sauroleishmania): a comment on classification. *Parasitol. Today* **14**: 167.

Olivier M. 1996: Modulation of host cell intracellulat Ca^{2+}. *Parasitol. Today* **12**: 145–150.

Opat A., Ng K., Currie G., Handman E. & Bacic A. 1996: Characterization of lipophosphoglycan from a ricin-resistant mutant of Leishmania major. *Glycobiology* **6**: 387–397.

Palánová L. & Volf P. 1997: Carbohydrate-binding specificities and physico-chemical properties of lectins in various tissue of phlebotomine sandflies. *Folia Parasitol.* **44**: 71–76.

Pearson R. D. & Steigbigel R. T. 1980: Mechanism of lethal effect of human serum upon Leishmania donovani. *J. Immunol.* **125**: 2195–2201.

Pearson R. D. & De Queiroz Sousa A. 1996: Clinical spectrum of leishmaniasis. *Clin. Inf. Dis.* **22**: 1–13.

Pearson R. D., Harcus J. L., Roberts D. & Donowitz G. R. 1983: Differential survival of Leishmania donovani amastigotes in human monocytes. *J. Immunol.* **131**: 1994–1999.

PETERS C., AEBISCHER T., STIERHOF Y. D., FUCHS M. & OVERATH P. 1995: The role of macrophage receptors in adhesion and uptake of Leishmania mexicana amastigotes. *J. Cell Sci.* **108**: 3715–3724.

PIMENTA P. F. P., SARAIVA E. M. B. & SACKS D. L. 1991: The comparative fine structure and surface glycoconjugate expression on three life stages of Leishmania major. *Exp. Parasitol.* **72**: 191–204.

PIMENTA P. F. P., TURCO S. J., MCCONVILLE M. J., LAWYER P. G., PERKINS P. V. & SACKS D. L. 1992: Stage-specific adhesion of Leishmania promastigotes to the sandfly midgut. *Science* **256**: 1812–1815.

PIMENTA P. F. P., SARAIVA E. M. B., ROWTON E., MODI G. B., GARRAWAY L. A., BEVERLEY S. M., TURCO S. J. & SACKS D. L. 1994: Evidence that the vectorial competence of phlebotomine sand flies for different species of Leishmania is controlled by structural polymorphism in the surface lipophosphoglycan. *Proc. Natl. Acad. Sci. USA* **91**: 9155–9159.

PROUDFOOT L., O'DONNELL C. A. & LIEW F. Y. 1995: Glycoinositolphospholipids of Leishmania major inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. *Eur. J. Immunol.* **25**: 745–750.

PROUDFOOT L., NIKOLAEV A. V., FENG G. J., WEI X. Q., FERGUSON M. A. J., BRIMACOMBE J. S. & LIEW F. Y. 1996: Regulation of the expression of nitric oxid synthase and leishmanicidal activity by glycoconjugates of Leishmania lipophosphoglycan in murine macrophages. *Proc. Natl. Acad. Sci. USA* **93**: 10984–10989.

PUENTES S. M., DA SILVA R. P., SACKS D. L., HAMMER C. H. & JOINER K. A. 1990: Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. *J. Immunol.* **145**: 4311–4316.

RANGEL E. F., TRAVI B. L., BARBOSA A. F. & MONTOYA J. 1993: Development of colombian isolates of Leishmania (Viannia) panamensis, Lc. (V.) guyanensis and Lc. (V.) braziliensis in the sandfly Lutzomyia intermedia (Lutz & Neiva, 1912) under experimental conditions. *Mem. Inst. Oswaldo Cruz* **88**: 513–515.

REINER S. L. & LOCKSLEY R. M. 1995: The regulation of immunity to Leishmania major. *Annu. Rev. Immunol.* **13**: 151–177.

RIZVI F. S., OUAISSI M. A., MARTY B., SANTORO F. & CAPRON A. 1988: The major surface protein of Leishmania promastigotes is a fibronectin-like molecule. *Eur. J. Immunol.* **18**: 473–476.

ROSENTHAL L. A., SUTTERWALA F. S., KEHRLI M. E. & MOSSER D. M. 1996: Leishmania major-human macrophage interactions: cooperation between Mac-1 (CD11B/CD18) and complement receptor type 1 (CD35) in promastigote adhesion. *Inf. Immunol.* **64**: 2206–2215.

RUSSEL D. G. 1987: The macrophage-attachment glycoprotein gp63 is the predominant C3-acceptor site on Leishmania mexicana promastigotes. *Eur. J. Biochem.* **104**: 213–221.

RUSSELL D. G. & WRIGHT S. D. 1988: Complement receptor type 3 (CR3) binds to an arg-gly-asp-containing region of the major surface glycoprotein, gp63, of Leishmania promastigotes. *J. Exp. Med.* **168**: 279–292.

RYAN L., LAINSON R., SHAW J. J. & WALLBANKS K. R. 1987: The transmission of suprapylarian Leishmania by the bite of experimentally infected sand flies (Diptera: Psychodidae). *Mem. Inst. Oswaldo Cruz* **82**: 425–430.

SACKS D. L. 1992: The structure and function of the surface lipophosphoglycan on different developmental stages of Leishmania promastigotes. *Inf. Agents Dis.* **1**: 200–206.

SACKS D. L. & DA SILVA R. P. 1987: The generation of infective stage Leishmania major promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *J. Immunol.* **139**: 3099–3106.

SACKS D. L., BRODIN T. N. & TURCO S. J. 1990: Developmental modification of the lipophosphoglycan from Leishmania major promastigotes during metacyclogenesis. *Mol. Biochem. Parasitol.* **42**: 225–234.

SACKS D. L., SARAIVA E. M., ROWTON E., TURCO S. J. & PIMENTA P. F. 1994: The role of the lipophosphoglycan of Leishmania in vector competence. *Parasitology* **108**: 55–62.

SACKS D. L., PIMENTA P. F. P., MCCONVILLE M.J., SCHNEIDER P. & TURCO S. J. 1995: Stage-specific binding of Leishmania donovani to the sandfly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. *J. Exp. Med.* **181**: 685–697.

SAF'JANOVA V. M. 1982: The problem of taxonomy with Leishmania. *Ser. Protozool. Sov. Acad. Sci.* **7**: 5–109.

SANTOS-GOMES G. M. & ABRANCHES P. 1996: Comparative study of infectivity caused by promastigotes of Leishmania infantum MON-1, L. infantum MON-24 and L. donovani MON-18. *Folia Parasitol.* **43**: 7–12.

SANYAL T., GANGOPADHYAY P., GHOSH D. K. & SARKAR D. 1994: Expression of antigens in virulent and avirulent indian strains of Leishmania donovani. *J. Biosci.* **19**: 291–299.

SARAIVA E. M. B. & ANDRADE A. F. B. 1986: Cell surface carbohydrate of Leishmania mexicana amazonensis: differences between infective and non-infective forms. *Europ. J. Cell Biology* **40**: 219–225.

SARAIVA E. M. B., PIMENTA P. F. P., BRODIN T. N., ROWTON E., MODI G. B. & SACKS D. L. 1995: Changes in lipophosphoglycan and gene expression associated with the development of Leishmania major in Phlebotomus papatasi. *Parasitology* **111**: 275–287.

SCHLEIN Y. 1986: Sandfly diet and Leishmania. *Parasitol. Today* **2**: 175–177.

SCHLEIN Y. 1993: Leishmania and sandflies: interactions in the life cycle and transmission. *Parasitol. Today* **9**: 255–257.
SCHLEIN Y. & JACOBSON R. L. 1994a: Haemoglobin inhibits the development of infective promastigotes and the secretion of chitinases in L. major cultures. *Parasitology* **109**: 23–28.
SCHLEIN Y. & JACOBSON R. L. 1994b: Mortality of Leishmania major in Phlebotomus papatasi caused by plant feeding of the sand flies. *Amer. J. Trop. Med. Hyg.* **50**: 20–27.
SCHLEIN Y. & JACOBSON R. L. 1994c: Some sandfly food is a Leishmania poison. *Bull. Soc. Vector Ecol.* **19**: 82–86.
SCHLEIN Y. & JACOBSON R. L. 1996: Why is man an unsuitable reservoir for the transmission of Leishmania major. *Exp. Parasitol.* **82**: 298–305.
SCHLEIN Y. & JACOBSON R. L. 1998: Resistance of Phlebotomus papatasi to infection with Leishmania donovani is modulated by components of the infective bloodmeal. *Parasitology* **117**: 467–473.
SCHLEIN Y. & WARBURG A. 1985: Feeding behaviour, midgut distention and ovarian development in Phlebotomus papatasi (Diptera: Psychodidae). *J. Insect Physiol.* **31**: 47–51.
SCHLEIN Y. & WARBURG A. 1986: Phytophagy and the feeding cycle of Phlebotomus papatasi (Diptera: Psychodidae) under experimental conditions. *J. Med. Entomol.* **23**: 11–15.
SCHLEIN Y., WARBURG A., SCHNUR L. F. & SHLOMAI J. 1983: Vector compatibility of Phlebotomus papatasi dependent on differentially induced digestion. *Acta Tropica* **40**: 65–70.
SCHLEIN Y., WARBURG A., SCHNUR L. F., LE BLANCQ S. M., GUNDERS A. E. 1984: Leishmaniasis in Israel: reservoir hosts, sandfly vectors and leishmanial strains in the Negev, Central Arava and along the Dead Sea. *Trans. Roy. Soc. Trop. Med. Hyg.* **78**: 480–484.
SCHLEIN Y., POLACHECK I. & YUVAL B. 1985: Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology* **90**: 57–66.
SCHLEIN Y., SCHNUR L. F. & JACOBSON R. L. 1990: Released glycoconjugate of indigenous Leishmania major enhance survival of foreign L. major in Phlebotomus papatasi. *Trans. Roy. Soc. Trop. Med. Hyg.* **84**: 353–355.
SCHLEIN Y., JACOBSON R. L. & SHLOMAI J. 1991: Chitinase secreted by Leishmania functions in the sandfly vector. *Proc. Roy. Soc. Lond. B* **245**: 121–126.
SCHLEIN Y., JACOBSON R. L. & MESSER G. 1992: Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proc. Natl. Acad. Sci. USA* **89**: 9944-9948.
SCHLEIN Y., JACOBSON R. L. & MESSOR G. 1993: Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission. *Parasitol. Today* **9**: 6.
SCHNEIDER P., ROSAT J. P., BOUVIER J., LOUIS J. & BORDIER C. 1992: Leishmania major: differential regulation of the surface metalloprotease in amastigote and promastigote stages. *Exp. Parasitol.* **75**: 196–206.
SCHNEIDER P., ROSAT J. P., RANSIJN A., FERGUSON M. A. J. & MCCONVILLE M. J. 1993: Characterization of glycoinositol phospholipids in the amastigote stage of the protozoan parasite Leishmania major. *Biochem. J.* **295**: 555–564.
SCHNUR L. F. & GREENBLAT C. L. 1995: Leishmania. Pp.: 1–160. In: KREIER J. P. (ed.): *Parasitic Protozoa. Vol. 10.* London: Academic Press, 430 pp.
SEAY M. B., HEARD P. L. & CHAUDHURI G. 1996: Surface Zn-proteinase as a molecule for defense of Leishmania mexicana amazonensis promastigotes against cytolysis inside macrophage phagolysosomes. *Inf. Immunol.* **64**: 5129–5137.
SEGOVIA M., ARTERO J. M., MELLADO E. & CHANCE M. L. 1992: Effects of long-term in vitro cultivation on the virulence of cloned lines of Leishmania major promastigotes. *Ann. Trop. Med. Parasitol.* **86**: 347–354.
SEMPERVIVO L. H., DETOLLA L. J., PASSMORE H. C. & PALCZUK N. C. 1981a: Spectral model of leishmaniasis in congenic strains of mice. *J. Parasitol.* **67**: 8–14.
SEMPERVIVO L. H., YUSUF J. N. & HONIGBERG B. M. 1981b: Changes in growth rate and infectivity of Leishmania donovani subjected to various laboratory procedures. *Ztschr. Parasitenkd.* **65**: 43–51.
SERVICE M. W. 1996: Phlebotomine sandflies (Phlebotominae). Pp.: 95–103. In.: *Medical Entomology for Students*. London: Chapman & Hall.
SHANKAR A., MITCHEN T. K., HALL L. R., TURCO S. J. & TITUS R. G. 1993: Reversion to virulence in L. major correlates with expression of surface lipophosphoglycan. *Mol. Biochem. Parasitol.* **61**: 207–216.
SHAW J. J. 1994: Taxonomy of the genus Leishmania: present and future trends and their implications. *Mem. Inst. Oswaldo Cruz* **89**: 471–478.
SHAW J. J. 1997: Ecological and evolutionary pressures on Leishmanial parasites. *Brazil. J. Genetics* **20**: 123–128.
SHAW J. J. & LAINSON R. 1987: Ecology and epidemiology: New World. Pp.: 291–363. In: PETERS W. & KILLICK-KENDRICK R. (eds): *The Leishmaniases in Biology and Medicine*. London: Academic Press, 550 pp.
SHORTT H. E. & SWAMINATH C. S. 1928: The method of feeding of Phlebotomus argentipes with relation to its bearing on the transmission of Kala-Azar. *Ind. J. Med. Res.* **15**: 827-836.

SØRENSEN A. L., HEY A. S. & KHARAZMI A. 1994: Leishmania major surface protease gp63 interferes with the function of human monocytes and neutrophils in vitro. *APMIS* **102**: 265–271.

STREIT J. A., DONELSON J. E., AGEY M. W. & WILSON M. E. 1996: Developmental changes in the expression of Leishmania chagasi gp63 and heat shock proteins in a human macrophage cell line. *Inf. Immunol.* **64**: 1810–1818.

STRELKOVA M. V. 1996: Progress in studies on Central Asia foci of zoonotic cutaneous leishmaniasis: a review. *Folia Parasitol.* **43**: 1–6.

SUTTERWALA F. S., ROSENTHAL L. A. & MOSSER D. M. 1996: Cooperation Between CR1 (CD35) and CR3 (CD11B/CD18) in the binding of complement-opsonized particles. *J. Leukocyte Biol.* **59**: 883–890.

SVOBODOVÁ M., VOLF P. & KILLICK-KENDRICK R. 1996: Agglutination of Leishmania promastigotes by midgut lectins from various species of phlebotomine sandflies. *Ann. Trop. Med. Parasitol.* **90**: 329–336.

SVOBODOVÁ M., BATES P. A. & VOLF P. 1997a: Detection of lectin activity in Leishmania promastigotes and amastigotes. *Acta Tropica* **68**: 23–35.

SVOBODOVÁ M., CAPO C. & MEGE J. L. 1997b: A biological role for haemaglutination activity of Leishmania promastigotes and amastigotes. *Parasite* **4**: 245–251.

TALAMAS-ROHANA P., WRIGHT S. D., LENNARTZ M. R. & RUSSELL D. G. 1990: Lipophosphoglycan from Leishmania mexicana promastigotes binds to members of the CR3, P150,95 and LFA-1 family of leukocute integrins. *J. Immunol.* **144**: 4817–4824.

TANNER C. E. 1996: Immunobiology of visceral leishmaniasis. *Clinic. Immunol. Immunopathol.* **78**: 105–111.

TELFORD S. R. 1995: The kinetoplastid hemoflagellates of reptiles. Pp.: 161–223. In: KREIER J. P. (ed.): *Parasitic Protozoa. Vol. 10.* London: Academic Press, 430 pp.

THOMAS J. R., MCCONVILLE M. J., THOMAS-OATES J., HOMANS S. W., FERGUSON M. A. J., GORIN P. A. J., GREIS K. D. & TURCO S. J. 1992: Refined structure of the lipophosphoglycan of Leishmania donovani. *J. Biol. Chem.* **267**: 6829–6833.

THOMAZ-SOCCOL V., LANOTTE G., RIOUX J.A., PRATLONG F., MARTINI-DUMAS A. & SERRES E. 1993a: Monophyletic origin of the genus Leishmania Ross, 1903. *Ann. Parasitol. Hum. Comp.* **68**: 107–108.

THOMAZ-SOCCOL V., LANOTTE G., RIOUX J.A., PRATLONG F., MARTINI-DUMAS A. & SERRES E. 1993b: Phylogenetic taxonomy of the New World Leishmania. *Ann. Parasitol. Hum. Comp.* **68**: 104–106.

TURCO S. J. & DESCOTEAUX A. 1992: The lipophosphoglycan of Leishmania parasites. *Annu. Rev. Microbiol.* **46**: 65–94.

TZINIA A. K. & SOTERIADOU K. P. 1991: Substrate-dependent pH optima of gp63 purified from seven strains of Leishmania. *Mol. Biochem. Parasitol.* **47**: 83–89.

VOLF P. & KILLICK-KENDRICK R. 1996: Post-engorgement dynamics of hemagglutination activity in the midgut of six species of phlebotomine sandflies. *Med. Vet. Entomol.* **10**: 247–250.

VOLF P. & PALÁNOVÁ L. 1996. Relationship between protein content of meal and lectin secretion in Phlebotomus duboscqi midgut. *Ann. Trop. Med. Parasitol.* **90**: 567–570.

VOLF P., KILLICK-KENDRICK R., BATES P. A. & MOLYNEUX D. H. 1994: Comparison of the haemaglutination activities in gut and head extract of various species and geographical populations of phlebotomine sandflies. *Ann. Trop. Med. Parasitol.* **88**: 337–340.

VOLF P., KIEWEGOVÁ A. & SVOBODOVÁ M. 1998: Sandfly midgut lectin: effect of galactosamine on Leishmania major infections. *Med. Vet. Entomol.* **12**: 151–154.

WALLBANKS K. R., INGRAM G. A. & MOLYNEUX D. H. 1986: The agglutination of erythrocytes and Leishmania parasites by sandfly gut extracts: evidence for lectin activity. *Trop. Med. Parasitol.* **37**: 409–413.

WALTERS L. 1993: Leishmania differentiation in natural and unnatural sand fly hosts. *J. Euk. Mikrobiol.* **40**: 196–206.

WALTERS L. L., MODI G. B., TESH R. B. & BURRAGE T. 1987: Host-parasite relationships of Leishmania mexicana mexicana and Lutzomyia abonnenci (Diptera: Psychodidae) *Amer. J. Trop. Med. Hyg.* **36**: 294–314.

WALTERS L. L., CHAPLIN G. L., MODI G. B. & TESH R. B. 1989a: Ultrastructural biology of Leishmania (Viannia) panamensis in Lutzomyia gomezi (Diptera: Psychodidae): a natural host-parasite association. *Amer. J. Trop. Med. Hyg.* **40**: 19–39.

WALTERS L. L., MODI G. B., CHAPLIN G. L. & TESH R. B. 1989b: Ultrastructural development of Leishmania chagasi in its vector, Lutzomyia longipalpis (Diptera: Psychodidae). *Amer. J. Trop. Med. Hyg.* **41**: 295–317.

WARBURG A. & SCHLEIN Y. 1986: The effect of post-bloodmeal nutrition of Phlebotomus papatasi on the transmission of Leishmania major. *Amer. J. Trop. Med. Hyg.* **35**: 926–930.

WARBURG A., HAMADA G. S., SCHLEIN Y. & SHIRE D. 1986: Scanning electron microscopy of Leishmania major in Phlebotomus papatasi. *Ztschr. Parasitenkd.* **72**: 423–431.

WILSON M. E. & PEARSON R. D. 1986: Evidence that Leishmania donovani utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. *J. Immunol.* **136**: 4681–4688.

WILSON M. E., HARDIN K. K & DONELSON J. E. 1989: Expression of the major surface glycoprotein of Leishmania donovani chagasi in virulent and attenuated promastigotes. *J. Immunol.* **143**: 678–684.

WU W. K. & TESH R. B. 1990a: Genetic factors controlling susceptibility to Leishmania major infection in the sandfly Phlebotomus papatasi (Diptera: Psychodidae). *Amer. J. Trop. Med. Hyg.* **42**: 329–334.

WU W. K. & TESH R. B. 1990b: Selection of Phlebotomus papatasi (Diptera: Psychodidae) lines susceptible and refractory to Leishmania major infection. *Amer. J. Trop. Med. Hyg.* **42**: 320–328.

VÝSLEDKY

Výsledky disertační práce jsou předloženy ve formě pěti původních vědeckých prací. Čtyři z nich jsou již publikovány, jedna je k publikaci připravena.

Faktory ovlivňující vývoj leishmanií v přenašeči.

(1) Čiháková J. & Volf P. (1997): Development of different *Leishmania major* strains in the vector sandflies *Phlebotomus papatasi* and *P. duboscqi*. Ann. Trop. Med. Parasitol. 91: 267-279.

(2) Sádlová J., Svobodová M. & Volf P. (1999): *Leishmania major*: effect of repeated passages through sandfly vector or murine hosts. Ann. Trop. Med. Parasitol. 93: 599-611.

(3) Sádlová J., Votýpka J., Victoir K., Dujardin J.C. & Volf P. : The surface metalloproteinase gp63 in virulent and attenuated lines of *Leishmania major*. Zasláno do časopisu Int. J. Parasitol.

Mechanismus přenosu leishmanií flebotomy.

(4) Sádlová J., Reishig J. & Volf P. (1998): Prediuresis in female *Phlebotomus* sandflies (Diptera: Psychodidae). Eur. J. Entomol. 95: 643-647.

(5) Sádlová J. & Volf P. (1999): Occurrence of *Leishmania major* in sandfly urine. Parasitology 118: 455-460.

Část výsledků byla také presentována na třech českých a pěti mezinárodních odborných konferencích:

(1) Čiháková J., Svobodová M. & Volf P. (1995): Infectivity of various *Leishmania major* strains for two Phlebotomine species. In: ISOPS II, September 4 – 6, Merida, Venezuela. Abstr. No. 18.

(2) Čiháková J., Svobodová M. & Volf P. (1996): The development of *Leishmania major* in vectors and mice. In: Joint meeting of Parasitology, May 13.-15., Brussels, Belgium. Abstr. P.11.

(3) Čiháková J. & Volf P. (1996): Differences in development of various *Leishmania major* strains in mice and phlebotomine sandlfiese. In: VII European Multicolloquium of Parasitology, September 2-6, Parma, Italy. Parassitologia 38, p. 111.

(4) Čiháková J. & Volf P. (1996): Srovnání vývoje kmenů *L. major* v přenašečích a myších. České a slovenské parasitologické dny, Měřín, 11.-13. Července, str.26.

(5) Sádlová J., Svobodová M. & Volf P. (1998): *Leishmania major* in sandflies and mice: virulence factors and parasite development. In: 2nd European Congress on Tropical Medicine, September 14-18, Liverpool, UK. Abstr. P. 145.

(6) Sádlová J., Svobodová M., Votýpka J. & Volf P. (1998): Virulenční faktory *Leishmania major:* srovnání pěti kmenů vyvíjejících se odlišně v přenašeči a hostiteli. 28. Jírovcovy protozoologické dny. Uhelná 4.-7. Května.

(7) Svobodová M., Sádlová J., Votýpka J. & Volf P. (1998): Virulenční faktory *Leishmania major:* změny vlivem pasážování přes hostitele. 28. Jírovcovy protozoologické dny. Uhelná 4.-7. Května.

(8) Sádlová J. & Volf P. (1999): Prediuresis in female sandflies: consequences for *Leishmania* transmission. In: ISOPS III, August 23-27, Montpellier, France. Abstr. No O47.

Annals of Tropical Medicine & Parasitology, Vol. 91, No. 3, 267–279 (1997)

Development of different *Leishmania major* strains in the vector sandflies *Phlebotomus papatasi* and *P. duboscqi*

BY J. ČIHÁKOVÁ* AND P. VOLF

Department of Parasitology, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

Received 17 December 1996, Accepted 14 February 1997

Five lines of four *Leishmania major* strains, which differ in geographical origin and virulence for mice, were used for experimental infections of *Phlebotomus papatasi* and *P. duboscqi*. Differences between the lines, which became evident 6 and 9 days after the infective feed, were more pronounced in *P. papatasi*. The highest infection rates were found for the more virulent line of strain LV561, while the lowest rates were recorded for strains L119 (low-virulence for mice) and Neal (avirulent for mice). Infection rates depended significantly on the *Leishmania* strain/line, but not on the vector species. Anterior migration and colonization of the stomodeal valve were observed in flies infected with LV561 and FV1 but infections with other strains were restricted to the whole midgut (L119) or to the abdominal midgut only (Neal). The proportions of the different morphological forms of *Leishmania* seen in gut smears of infected flies varied considerably with the parasite strain/line. In general, vector forms of LV561 and FV1 were characterized by relatively long flagella and bodies. The strains developing less successfully in vectors tended to have a relatively broad body (L119) or short flagellum (Neal). Transmission experiments were successful with *P. duboscqi* females infected with the virulent line of LV561.

Within the sandfly gut, *Leishmania* promastigotes encounter a number of factors which affect their development. Intrinsic factors, such as proteolytic enzymes (Borovsky and Schlein, 1987; Dillon and Lane, 1993) and sandfly lectins (Wallbanks *et al.*, 1986; Volf *et al.*, 1994), contribute to a unique habitat in each sandfly species. This habitat is further affected by extrinsic factors, particularly by the two types of food, blood and sugar solutions, taken by female sandflies (Killick-Kendrick, 1979; Molyneux and Killick-Kendrick, 1987; Schlein, 1993). Sugarmeals were found necessary for *Leishmania* differentiation into forms infective for vertebrates, while the presence of protein in sugarmeals enhanced transmission of the parasites (Warburg and Schlein, 1986).

The development of *Leishmania major* in phlebotomine sandflies is relatively well described, including parasite ultrastructural characters in both unnatural (Walters *et al.*, 1993) and natural vectors (Shatova *et al.*, 1984; Warburg *et al.*, 1986; Killick-Kendrick *et al.*, 1988). Also, *Le. major* and *Phlebotomus papatasi* have been used together, in a traditional, model, parasite–vector combination, for several biochemical and physiological studies. Although the high susceptibility of *P. papatasi* and *P. duboscqi* to *Le. major* is established, differences in the courses of infections with various lines of *Le. major* in *P. papatasi* were recently reported by Pimenta *et al.* (1994) and Camara *et al.* (1995).

The aim of the present study was to extend these findings by comparing the vectorial competence of both natural vectors, *P. papatasi* (Scopoli, 1786) and *P. duboscqi* Neveu-Lemaire, 1906, to five *Le. major* lines which differ in their geographical origin, virulence for mice and the structure of their lipophosphoglycan (LPG), the main surface glycoconjugate of promastigotes.

Preliminary experiments on the role of

* E-mail: volf@beba.cesnet.cz; fax: + 420 2 299713.

0003-4983/97/030267-13 $9.00
Carfax Publishing Company

sandfly midgut lectin in *Leishmania* development (unpubl. obs.) showed striking differences between various *Le. major* strains. In the present study, localization of infection in the sandfly gut and morphology of a highly virulent line of *Le. major* (LV561/V) were compared with those of strains/lines that appeared to be of low virulence or non-virulent to Balb/c mice. One of the strains used, L119, has a defect in synthesis of the disaccharide backbone of the repeat units of LPG (McConville and Homans, 1992).

MATERIALS AND METHODS

Leishmania Lines

The five lines of *Le. major* used belonged to four strains: LV561 (MHOM/IL/67/LRC-L137 Jericho-II); FV1 (MHOM/IL/80/Friedlin); Neal (MRHO/SU/59/P); and L119 (MTAT/KE/00/T4). Two different lines of the strain LV561 were studied: the virulent line, LV561/V, was used for sandfly infections after two in-vitro subcultures; the avirulent line, LV561/AV, attenuated by long-term cultivation *in vitro*, was kindly provided by Dr E. Nohýnková (Faculty of Medicine, Department of Tropical Medicine, Prague). Parasites were maintained on SNB-9 blood agar (Diamond and Herman, 1954) at 25°C and subcultured every 3–5 days.

The lines used differ highly in virulence for Balb/c mice. When mice were each inoculated in the rump with 10^7 promastigotes, all mice given LV561/V developed lesions in <6 weeks, whereas not all mice given FV1 and L119 developed lesions and any lesions only developed after 12 weeks, and no lesions developed in mice given Neal or LV561/AV within 6 months of the inoculations (M. Svobodová and J. Čiháková, unpubl. obs.).

Sandfly Infections

Colonies of *Phlebotomus papatasi* (Cyprus strain) and *P. duboscqi* (Senegal strain) were obtained from Professor R. Killick-Kendrick (Imperial College at Silwood Park, Ascot, U.K.) and maintained on 50% sucrose at 27–28°C and $>65\%$ relative humidity, with a 14-h light/10-h dark photoperiod

As some of the parasitic lines investigated were non-infective for mice, cultured promastigotes and membrane feeding were used for sandfly infections throughout. Female, 5–7-day-old sandflies were fed through a chick-skin membrane on heat-inactivated human blood containing 5×10^5 promastigotes (from 4-day-old cultures)/ml. Each possible combination of *Leishmania* line and vector species was produced in two to four separate experiments. Engorged females were separated, maintained as described above, and dissected 3, 6 or 9 days after the infective feed. The location and number of promastigotes in the midgut were estimated under a light microscope. If infection had reached the stomodeal valve, the anterior part of the digestive tract was examined.

Morphometry

Gut smears were fixed with methanol, stained with Giemsa and examined under the light microscope with an oil-immersion objective. For each sampling time post-infection, for each parasite–vector combination, 60 randomly selected promastigotes were measured (parasites from six flies were measured whenever possible). As the infections were promastigote-initiated, 60 cultured promastigotes of each line (the inoculum for sandfly feeding on day 0) represented the initial morphological state. The position of the kinetoplast in relation to the nucleus was examined and body length, body width and flagellar length were measured.

Parasite stages were identified according to descriptions given by Killick-Kendrick (1979) and Walters (1993). In the absence of accurate information about the attachment of the measured parasites before gut dissection, no comment on their motility can be made. Six categories were distinguished:

(1) Elongated nectomonads (EN; body length >14.3 μm, unattached).
(2) Short promastigotes (SP; flagellum <1.8 times body length and body length ≤ 13 μm. (Seven promastigotes

with body length of 14.3 μm found associated with gut debris in smears were also included as SP). SP were subdivided into slender forms (SP1; body width < 2.8 μm) and broad forms (SP2; body width ≥ 2.8 μm).

(3) Metacyclic promastigotes (MP; flagellum ≥ 1.8 times body length and body width < 2.8 μm).

(4) Rounded promastigotes (RP; flagellum at least 1.8 times body length and body width ≥ 2.8 μm).

(5) Paramastigotes (PM; kinetoplast lateral to the nucleus).

Infection of Hamsters

Ten days after the infective meal, sandflies were allowed to feed on the hind legs of uninfected hamsters immediately before the flies were dissected to check for the presence of parasites. Hamsters were then examined for leishmanial lesions weekly for 5 months. If no lesions appeared, attempts to isolate parasites from the skin of the biting site and from the spleen were made.

Statistical Analysis

Multifactorial analysis of variance (ANOVA) was used for the evaluation of factors influencing the infection rates. Numbers of promastigotes were log-transformed and the mean value from each experiment was used for the analysis. The compositions of each infection, in terms of morphological forms present and the actual measurements of the parasites present, were compared using χ^2 tests and analysis of variance, respectively. All analyses were made with commercial software (Statgraphics version 5.0; Manugistics, Rockville, MD).

RESULTS

Sandfly Infections

DAY 3

On day 3 after the infective feeds, midgut infection rates were high for all parasite–vector combinations except those with strain L119; only 31% of the *P. papatasi* and 39% of the *P. duboscqi* fed on blood with L119 appeared infected on day 3. Heavy infections (> 500 parasites/gut) of all the *Le. major* strains/lines studied were observed (see Fig.).

Although most parasites in the infections were in the residual bloodmeal within the peritrophic matrix in the posterior midgut, a ruptured peritrophic matrix and free-swimming parasites in the abdominal midgut were frequently observed in both vector species (Table 1). Of all the strains/lines investigated, LV561/V was the one that developed most successfully in both sandfly species and that escaped from the peritrophic matrix in *P. papatasi* most rapidly. The Neal strain was the slowest to escape from the peritrophic matrix of *P. papatasi*.

DAY 6

The proportions of females of each sandfly species found infected on day 6 were considerably lower than those on day 3, dramatically so for strains L119 and Neal (see Fig.). The highest infection rates were with LV561/V.

By this time the bloodmeal had been digested and the remnants defacated. In most of the parasite–vector combinations, the parasites had migrated anteriorly and colonized the thoracic midgut. Colonization of the stomodeal valve was observed in flies infected with LV561; parasites of the virulent line (LV561/V) reached the stomodeal valve in both sandfly species but those of the avirulent line (LV561/AV) only reached the valve in *P. duboscqi*. Parasites of the Neal strain remained localized to the abdominal midgut in both sandfly species.

DAY 9

On day 9, the highest infection rates were found with LV561/V in *P. papatasi* and for both LV561 lines in *P. duboscqi*. Heavy infections (> 500 parasites/gut) of both of the LV561 lines and of the FV1 strain, but not of L119 or Neal, were detected (see Fig.).

In *P. papatasi*, intensive infections of the stomodeal valve developed when LV561/V was used, whereas parasites of the other

TABLE 1

Location of parasites in infected P. papatasi *and* P. duboscqi

Sandfly	*Parasite strain*	*Days post-bloodmeal*	*No. of flies*	*No. and (%) of flies infected*	*% of positive flies infected in:**			
					PS	*AMG*	*TMG*	*SV*
Phlebotomus duboscqi	LV561/V	3	32	27 (84)	96	4	0	0
		6	35	19 (54)	0	26	53	21
		9	26	13 (50)	0	31	38	31
	LV561/AV	3	36	20 (55)	65	30	5	0
		6	30	7 (23)	0	14	57	29
		9	30	14 (47)	0	14	64	22
	FV1	3	34	24 (70)	75	25	0	0
		6	36	8 (22)	0	63	37	0
		9	52	6 (12)	0	0	34	66
	L119	3	31	12 (39)	67	33	0	0
		6	39	3 (8)	0	33	67	0
		9	32	9 (28)	0	56	44	0
	Neal	3	30	22 (73)	91	9	0	0
		6	32	1 (3)	0	100	0	0
		9	52	6 (12)	0	33	67	0
Phlebotomus papatasi	LV561/V	3	29	26 (90)	31	69	0	0
		6	26	22 (85)	0	9	73	18
		9	25	19 (76)	0	5	53	42
	LV561/AV	3	29	21 (72)	57	38	5	0
		6	34	11 (32)	0	45	55	0
		9	33	6 (18)	0	50	50	0
	FV1	3	29	22 (76)	55	45	0	0
		6	46	10 (22)	0	60	40	0
		9	54	7 (13)	0	57	43	0
	L119	3	26	8 (31)	88	12	0	0
		6	35	0 (0)	0	0	0	0
		9	38	4 (11)	0	50	50	0
	Neal	3	26	18 (69)	89	11	0	0
		6	27	2 (7)	0	100	0	0
		9	40	2 (5)	0	100	0	0

* PS, Peritrophic sac; AMG, abdominal midgut; TMG, thoracic midgut; SV, stomodeal valve.

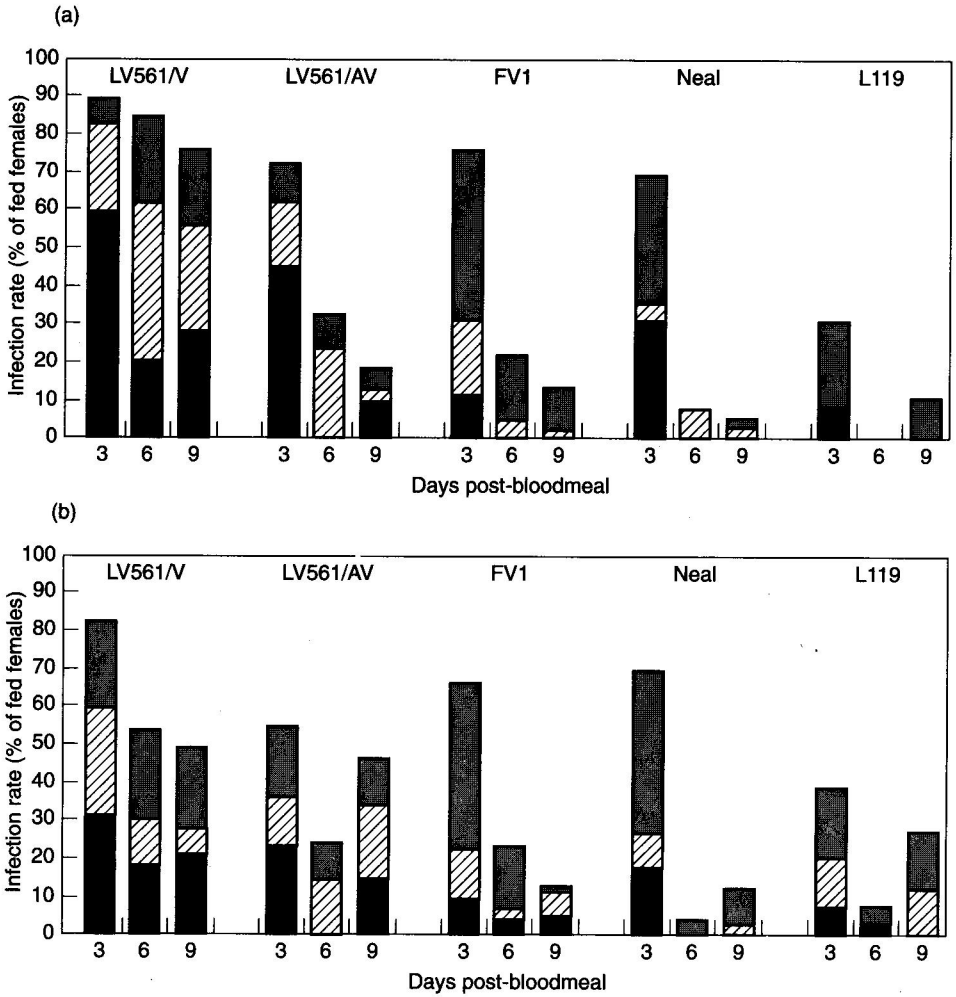


Fig. Rates and intensities of infections with one of five *Le. major* strains/lines in (a) *P. papatasi* and (b) *P. duboscqi*. Infections were low (▦; ≤ 100 parasites/gut), medium (▨; 100–500 parasites/gut) or heavy (■; > 500 parasites/gut).

strains/lines were restricted to the thoracic midgut (LV561/AV, FV1, L119) or even the abdominal midgut (Neal) of the sandfly. In *P. duboscqi*, colonization of the stomodeal valve was observed with both LV561 lines and the FV1 strain, parasites of the Neal and L119 strains being limited to the midgut. In one *P. duboscqi* female, two metacyclic promastigotes of the LV561/V line were found in the proboscis. ANOVA showed that differences in *Leishmania* development between the two sandfly species was not significant ($P = 0.3755$; $F = 0.828$). However, infection rates did depend significantly on the *Leishmania* strain used ($P < 0.0001$; $F = 7.825$) and duration of infection ($P < 0.0001$; $F = 14.783$). Multiple-range tests revealed that both LV561 lines achieved significantly higher ($P < 0.05$) infection rates than the Neal and L119 strains. The FV1 strain developed better than Neal

and L119, resembling the avirulent line LV561/AV, but was significantly less successful than the virulent LV561/V line.

Morphological Transformations

With no evidence that the vector species significantly influenced sandfly infections, morphological measurements were evaluated without consideration of this factor. The relative proportions of six morphological forms of parasite varied considerably between *Leishmania* lines (Table 2). χ^2 tests revealed that the differences in this parameter were most pronounced in the inocula (day 0) ($P < 0.0001$; 20 degrees of freedom; $\chi^2 = 76.48$). They remained highly significant on days 3 ($P < 0.0001$; 20 degrees of freedom; $\chi^2 = 64.97$) and 6 ($P < 0.0001$; 20 degrees of freedom; $\chi^2 = 58.92$) but were less significant on day 9 ($P = 0.0293$; 15 degrees of freedom; $\chi^2 = 26.93$). In inocula of all the *Leishmania* lines studied, EN and SP1 were the prevailing forms, SP2 becoming common on days 3 and 6 (Table 2).

The morphometrics of the distinct forms are given in Table 3. The strongest interstrain variability occurred in all characters of the EN measured and the greatest morphological uniformity was found in MP.

When temporal changes in the parasites' morphology during the course of infection were evaluated (ignoring the six morphological categories), significant differences were found between various lines (Table 4). The most pronounced morphological transformations were found in LV561/V, the body and flagellar lengths of which changed from relatively small values in culture (day 0) to high values during development in the sandfly gut (days 3–9). The FV1 strain exhibited a similar trend on days 6 and 9. In contrast, the flagellar and body lengths of the Neal strain were both significantly shorter in sandflies (days 3 and 6) than in culture (day 0).

The body width of culture forms was small for both LV561 lines, intermediate for FV1 and large for the L119 and Neal strains. During development in the sandfly (days 3 and 6), the L119 strain was significantly broader than other strains (Table 4).

Overall (ANOVA, multiple-range test), compared with the other lines, the Neal strain had significantly shorter flagella and the LV561/V line significantly longer flagella. LV561/V and FV1 had the longest bodies and L119 the broadest (Table 4).

Infections of Hamsters

Hamsters 1–4 were bitten by female sandflies heavily infected with LV561/V (hamster 1 by three *P. duboscqi*, hamster 2 by two *P. papatasi*, and hamsters 3 and 4 by one *P. papatasi* each). Hamsters 5 and 6 were bitten by females heavily infected with LV561/AV (by five *P. duboscqi* and two *P. papatasi*, respectively). Five weeks after the bites, lesions appeared on both hind legs of hamster 1. No lesions developed on any other hamster within 5 months of the bites and attempts to isolate parasites from skin and spleen samples from hamsters 2–6 were not successful.

DISCUSSION

The present results reveal remarkable differences in the development of various *Le. major* strains in *P. papatasi* and *P. duboscqi*. Two strains which were avirulent (Neal) or of low-virulence (L119) in mice grew poorly in the sandflies, indicating that virulence in the mammalian host may be related to that in the insect host. However, LV561/AV, a line which is avirulent in mice, developed more-or-less successfully in both fly species, indicating that loss of virulence in mice did not greatly affect this lines' development in the vectors.

In terms of infection rates, the difference between lines was most pronounced on day 6, when $\leq 10\%$ of flies fed on Neal and L119 appeared to be infected. That the proportion of flies infected with these two strains should fall dramatically between days 3 and 6 is probably connected with the defacation of the bloodmeal residues between these two times. Parasites that have not penetrated the per-

TABLE 2

Morphological forms of Le. major *in inocula (day 0) and in the guts of* P. papatasi *and* P. duboscqi

Days post-bloodmeal	*Morphological form**	*No. counted*	*% of population of strain:*				
			LV561/V	*LV561/AV*	*FV1*	*L119*	*Neal*
0	EN	132	16.7	45.0	38.3	58.3	61.7
	SP1	134	65.0	51.7	51.7	26.7	28.3
	SP2	12	1.7	0	3.3	6.7	8.3
	PM	6	0	1.7	5.0	3.3	0
	RP	2	0	0	0	3.3	0
	MP	14	16.7	1.7	1.7	1.7	1.7
3	EN	227	41.7	30.0	46.7	34.2	36.7
	SP1	177	33.3	40.0	21.7	15.0	37.5
	SP2	164	22.5	23.3	30.8	40.0	20.0
	PM	17	0	5.8	0.8	3.3	4.2
	RP	6	1.7	0.8	0	2.5	0
	MP	9	0.8	0	0	5.0	1.7
6	EN	256	55.8	62.3	76.5	60.0	54.5
	SP1	82	18.3	23.7	8.8	13.3	34.5
	SP2	58	19.2	10.5	14.7	20.0	1.8
	PM	15	5.8	2.6	0	0	9.1
	RP	4	0.8	0	0	5.0	0
	MP	2	0	0.9	0	1.7	0
9	EN	185	51.7	42.5	65.6	48.1	NPS
	SP1	93	20.8	37.5	16.7	29.6	NPS
	SP2	51	18.3	14.2	10.0	11.1	NPS
	PM	15	5.0	4.2	2.2	7.4	NPS
	RP	6	3.3	0	2.2	0	NPS
	MP	7	0.8	1.7	3.3	3.7	NPS

* EN, Elongated nectomonads; SP1, short slender promastigotes; SP2; short broad promastigotes; PM, paramastigotes; RP, rounded promastigotes; MP, metacyclic promastigotes.

NPS, No parasites seen on smears.

TABLE 3
Measurements of the six morphological forms identified

		Body length			*Body width*			*Flagellar length*		
Form†	Leishmania *strain*	*No. of measurements*	*Mean and (S.D.) (μm)*	*Range (μm)*	*No. of measurements*	*Mean and (S.D.) (μm)*	*Range (μm)*	*No. of measurements*	*Mean and (S.D.) (μm)*	*Range (μm)*
EN	LV561/V	189	17.5 (3.5)	14.3–31.4	185	1.8 (0.6)	0.7–2.9	184	16.4 (3.1)	7.1–22.9
	LV561/AV	185	16.1 (2.1)	14.3–22.9	189	1.7 (0.6)	0.7–4.3	189	17.9 (4.6)	7.1–35.7
	FV1	190	16.7 (2.7)	14.3–25.7	190	1.9 (0.7)	1.4–4.3	187	15.8 (4.3)	5.7–28.6
	L119	125	15.9 (2.1)	14.3–24.3	125	2.2 (0.7)	1.4–4.3	116	15.5 (2.9)	10.0–22.9
	Neal	111	15.5 (1.5)	14.3–21.4	111	1.9 (0.7)	1.4–4.3	111	13.5 (2.9)	5.7–20.0
	All	800	16.4 (2.7)***	14.3–31.4	800	1.9 (0.7)***	0.7–4.3	777	16.1 (4.0)***	5.7–35.7
SP1	LV561/V	126	10.7 (1.8)	7.1–14.3	127	1.6 (0.3)	0.7–2.1	122	12.6 (3.1)	5.7–24.3
	LV561/AV	151	10.3 (1.8)	5.7–12.9	151	1.6 (0.3)	0.7–2.1	151	11.1 (3.0)	4.3–21.4
	FV1	78	10.7 (1.9)	5.7–12.9	78	1.7 (0.4)	0.7–2.1	78	11.7 (3.1)	4.3–21.4
	L119	50	10.1 (2.2)	5.7–12.9	50	1.6 (0.3)	1.4–2.1	47	11.4 (3.0)	5.7–17.1
	Neal	81	9.9 (2.1)	5.7–12.9	81	1.6 (0.3)	1.4–2.1	81	10.7 (2.5)	4.3–18.6
	All	486	10.4 (1.9)*	5.7–14.3	487	1.6 (0.3)	0.7–2.1	479	11.6 (3.0)**	4.3–24.3
SP2	LV561/V	73	10.1 (2.5)	4.3–17.1	73	3.1 (0.7)	2.9–5.7	59	10.9 (4.0)	4.3–20.0
	LV561/AV	57	9.6 (2.0)	4.3–12.9	57	3.0 (0.4)	2.9–4.3	57	9.8 (3.8)	2.9–20.0
	FV1	58	10.5 (1.8)	7.1–12.9	58	3.0 (0.4)	2.9–4.3	58	11.7 (3.6)	5.7–21.4
	L119	67	10.6 (1.8)	7.1–12.9	67	3.0 (0.4)	2.9–4.3	65	11.7 (3.2)	4.3–20.0
	Neal	30	9.4 (1.8)	7.1–12.9	30	3.0 (0.4)	2.9–4.3	30	8.8 (3.6)	1.4–14.3
	All	285	10.1 (2.1)*	4.3–17.1	285	3.0 (0.5)	2.9–5.7	269	10.8 (3.7)**	1.4–21.4
PM	LV561/V	13								

PM	LV561/V	13	8.5 (2.6)	7.1–15.7	13	3.6 (0.7)	2.9–4.3	9	15.2 (3.0)	10.0–18.6
	LV561/AV	16	7.3 (1.5)	5.7–10.0	16	2.7 (0.6)	1.4–4.3	16	7.7 (2.8)	2.9–11.4
	FV1	6	8.3 (2.6)	5.7–12.9	6	2.9 (0.8)	2.1–4.3	6	11.9 (2.3)	8.6–11.3
	L119	8	7.5 (1.3)	5.7–8.6	8	2.8 (0.3)	2.1–2.9	6	11.2 (5.1)	7.1–20.0
	Neal	10	8.6 (3.3)	4.3–14.3	10	2.6 (1.1)	1.4–4.3	10	8.3 (1.8)	5.7–11.4
	All	53	8.0 (2.3)	4.3–15.7	53	2.9 (0.8)**	1.4–4.3	47	10.2 (4.1)***	2.9–20.0
RP	LV561/V	6	7.6 (1.2)	5.7–8.6	6	2.9 (0.0)	2.9–2.9	6	17.9 (3.0)	14.3–21.4
	LV561/AV	1	7.1 (0.0)	7.1–7.1	1	2.9 (0.0)	2.9–2.9	1	12.9 (0.0)	12.9–12.9
	FV1	2	9.3 (3.0)	7.1–11.4	2	3.6 (1.0)	2.9–4.3	2	22.1 (1.0)	21.4–22.9
	L119	8	9.6 (2.0)	7.1–12.9	8	2.9 (0.0)	2.9–2.9	8	19.6 (3.9)	14.3–25.7
	Neal	0	–	–	0	–	–	0	–	–
	All	17	8.8 (1.9)	5.7–12.9	17	2.9 (0.4)*	2.9–4.3	17	18.8 (3.6)	12.9–25.7
MP	LV561/V	12	8.7 (1.6)	7.1–11.4	12	1.5 (0.2)	1.4–2.1	12	17.5 (3.0)	12.9–21.4
	LV561/AV	4	6.1 (2.1)	4.3–8.6	4	1.6 (0.4)	1.4–2.1	4	13.9 (2.9)	10.0–17.1
	FV1	4	8.2 (0.7)	7.1–8.6	4	1.4 (0.6)	0.7–2.1	4	17.1 (2.3)	14.3–20.0
	L119	9	7.8 (1.5)	5.7–10.0	9	1.8 (0.4)	1.4–2.1	9	15.9 (3.1)	11.4–21.4
	Neal	3	7.1 (0.0)	7.1–7.1	3	1.7 (0.4)	1.4–2.1	3	14.3 (0.0)	14.3–14.3
	All	32	7.9 (1.6)	4.3–11.4	32	1.6 (0.4)	0.7–2.1	32	16.2 (3.0)	10.0–21.4

*, **, *** Significant difference between strains/lines of parasites in measurements of morphological stage: *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$.

† EN, Elongated nectomonads; SP1, short slender promastigotes; SP2; short broad promastigotes; PM, paramastigotes; RP, rounded promastigotes; MP, metacyclic promastigotes.

TABLE 4

Dimensions of Le. major *present in inoculum (day 0) and developing in the guts of* P. papatasi *or* P. duboscqi *3, 6 and 9 days after the bloodmeal*

		Body length			*Body width*			*Flagellar length*		
Days post-bloodmeal	Leishmania *strain*	*No. of measurements*	*Mean and (S.D.) (μm)*	P*	*No. of measurements*	*Mean and (S.D.) (μm)*	P*	*No. of measurements*	*Mean and (S.D.) (μm)*	P*
0	LV561/V	60	10.7 (0.33)		60	1.6 (0.048)		60	13.4 (0.42)	
	LV561/AV	60	13.0 (0.42)		60	1.4 (0.032)		60	14.7 (0.42)	
	FV1	60	12.5 (0.37)		60	1.7 (0.068)		60	12.9 (0.34)	
	L119	60	13.4 (0.44)		60	1.8 (0.076)		60	14.0 (0.40)	
	Neal	60	13.4 (0.42)		60	1.8 (0.075)		60	13.3 (0.40)	
				<0.001			<0.001			<0.05
3	LV561/V	120	12.6 (0.39)		120	2.0 (0.069)		119	13.5 (0.43)	
	LV561/AV	120	11.5 (0.28)		120	2.1 (0.067)		120	12.0 (0.32)	
	FV1	120	12.7 (0.31)		120	2.3 (0.073)		120	12.4 (0.37)	
	L119	120	11.4 (0.28)		120	2.6 (0.062)		109	12.9 (0.36)	
	Neal	120	11.7 (0.32)		120	2.0 (0.067)		120	10.8 (0.30)	
				<0.001			<0.0001			<0.0001
6	LV561/V	120	15.4 (0.48)		120	2.1 (0.082)		98	17.3 (0.57)	
	LV561/AV	114	14.3 (0.38)		114	2.0 (0.057)		114	15.1 (0.42)	
	FV1	68	16.2 (0.51)		68	2.1 (0.082)		65	16.5 (0.59)	
	L119	60	14.5 (0.53)		60	2.6 (0.069)		58	15.2 (0.57)	
	Neal	55	12.8 (0.47)		55	2.1 (0.10)		55	11.9 (0.42)	
				<0.001			<0.0001			<0.0001
9	LV561/V	120	13.8 (0.38)		120	2.2 (0.084)		111	15.7 (0.40)	
	LV561/AV	120	12.0 (0.35)		120	1.9 (0.059)		119	11.9 (0.42)	
	FV1	90	15.0 (0.41)		90	2.0 (0.085)		90	16.0 (0.41)	
	L119	27	13.4 (0.59)		27	1.8 (0.128)		24	14.0 (0.68)	
	Neal	0	–		0	–		0	–	
				<0.0001			<0.05			<0.0001
0, 3, 6 and 9	LV561/V	420	13.5 (0.22)		420	2.0 (0.040)		388	15.1 (0.25)	
	LV561/AV	414	12.6 (0.19)		414	1.9 (0.032)		413	13.2 (0.21)	
	FV1	338	13.9 (0.21)		338	2.1 (0.041)		335	14.2 (0.24)	
	L119	267	12.7 (0.22)		267	2.3 (0.044)		251	13.8 (0.24)	
	Neal	235	12.4 (0.23)		235	2.0 (0.046)		235	11.7 (0.22)	
				<0.0001			<0.0001			<0.0001

* Significance of differences between *Leishmania* strains/lines.

itrophic membrane and attached to the midgut when their sandfly hosts defecate are likely to be eliminated. The extent of midgut attachment appears directly related to the level of subsequent infection (Molyneux and Killick-Kendrick, 1987; Schlein, 1993).

Penetration of the peritrophic matrix and attachment to the vector midgut is mediated by *Leishmania* chitinase and LPG, respectively (Schlein, 1993). If parasites of the Neal strain have defective chitinase activity, this would account for their slow escape from the peritrophic membrane and their subsequent failure to establish infections in the vectors. The LPG deficiency of L119 strain is thought to be responsible for this strain's avirulence (McConville and Homans, 1992) or low virulence in mice (Cappai *et al.*, 1995). It may also explain the strain's low infectivity to both *P. papatasi* and *P. duboscqi*, as LPG modifications are known to control midgut binding of promastigotes (Pimenta *et al.*, 1992). Defects in LPG synthesis may also be responsible for the low infection rates seen in sandflies fed on promastigotes of L119 3 days earlier, as LPG promotes parasite survival by modulating the activity of sandfly proteolytic enzymes during bloodmeal digestion (Schlein *et al.*, 1990; Dillon and Lane, 1993).

The present results are in partial agreement with those of other authors, who demonstrated that LV561 and other virulent strains of *Le. major* survived well in *P. papatasi*, whereas the infection rate with L119 was low on day 4 (Schlein *et al.*, 1990) or completely lost on days 6–8 (Pimenta *et al.*, 1994). Although Camara *et al.* (1995) achieved higher infection rates in sandflies when they used the avirulent clone of LV561 than when they used the virulent one, they used inocula which were extremely large (3×10^6 promastigotes/ml) and much higher than those in the present experiments (5×10^5/ml).

For any strain/line of *Leishmania*, infection rates did not differ significantly between the two sandfly species. Strain LV561 grew successfully in both fly species, confirming the vectorial competence of *P. duboscqi* for *Le. major* strains, even those of non-African origin. Although there were profound inter-strain differences in the locations of the infections in *P. papatasi*, the differences were less pronounced in *P. duboscqi*, where infection with the LV561/V line was similar to those with LV561/AV and FV1. This observation may be related to the differences in vector competence of these two sandfly species, *P. duboscqi* supporting the development of both *Le. tropica* and *Le. major* whereas *P. papatasi* is only susceptible to *Le. major* (Shatova *et al.*, 1984; Killick-Kendrick *et al.*, 1994).

Although the present sandfly infections were promastigote-initiated, the pattern of parasite development from day 3 was generally similar to that described for amastigote-initiated *Le. major* infections in *P. papatasi* and *P. duboscqi* (Warburg *et al.*, 1986; Killick-Kendrick, 1988; Lawyer *et al.*, 1990): elongated nectomonads and short slender promastigotes were the predominant forms after rupture of the peritrophic matrix and evacuation of the bloodmeal residues. However, infection rates seen in the present studies were lower than those reported in amastigote-initiated infections and metacyclic promastigotes were only found in the proboscis of a single fly in the present study. This scarcity of foregut infections was probably caused by the present mode of infection and relatively high temperatures (27–28°C). The absence of foregut migration in promastigote-initiated infections, and slower development in comparison with amastigote-initiated infections, was reported for *Le.* (*Viannia*) *braziliensis* in *Lutzomyia gomezi* (Jaramillo *et al.*, 1994). The negative effect of relatively high temperature on foregut migration was described by Leaney (1977) for *Le.* (*Le.*) *amazonensis* in *Lu. longipalpis*. Similarly, in *Le. major* infections, promastigotes were found in the pharynx (Walters *et al.*, 1993) and even in the mouthparts (Lawyer *et al.*, 1990) of flies maintained at 25°C, whereas migration to the pharynx and more anteriorly did not occur in flies maintained at 28 ± 2°C (Warburg *et al.*, 1986). Moreover, human blood, as used in the present experiments, is known to decrease infection rates with *Le. major* significantly and the number of parasites in individual flies

(Schlein and Jacobson, 1996), and may be responsible for general loss of fly infections with time.

The scarcity of metacyclics in the mouthparts of infected flies did not preclude transmission. One hamster, bitten by three infected *P. duboscqi*, developed two lesions. Both lesions may well have been produced following the bites of a single 'blocked' fly (Warburg and Schlein, 1986; Warburg *et al.*, 1986).

ACKNOWLEDGEMENTS. We would like to thank Professor R. Killick-Kendrick, M. Killick-Kendrick and Dr Y. Tang (all of Imperial College at Silwood Park) for providing the sandfly colonies and for their help. We also thank Dr E. Nohýnková (Faculty of Medicine, Prague) for supplying the avirulent line of *Leishmania major* LV561, Professor J. Vávra (Faculty of Science, Prague) for reading the manuscript and Dr R. Jacobson (Hebrew University, Jerusalem) for his advice on sandfly infections and critical comments. The study was supported by the Ministries of Health (IGA MZCR 3191-3) and Education (FDR 0272) of the Czech Republic.

REFERENCES

BOROVSKY, D. & SCHLEIN, Y. (1987). Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. *Medical and Veterinary Entomology*, **1**, 235–242.

CAMARA, M., ORTIZ, G., VALERO, P. L., MOLINA, R., NAVARRO, I., CHANCE, M. L. & SEGOVIA, M. (1995). Complement-mediated lysis and infectivity for mouse macrophages and sandflies of virulent and attenuated *Leishmania major* promastigotes varying in expression of the major surface protease and lipophosphoglycan. *Annals of Tropical Medicine and Parasitology*, **89**, 243–251.

CAPPAI, R., MORRIS, L., AEBISCHER, T., BACIC, A., CURTIS, J. M., KELLEHER, M., MCLEOD, K. S., MOODY, S. F., OSBORN, A. H. & HANDMAN, E. (1995). Ricin resistant mutants of *Leishmania major* which express modified lipophosphoglycan remain infective for mice. *Parasitology*, **108**, 397–405.

DIAMOND, L. S. & HERMAN, C. M. (1954). Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. *Journal of Parasitology*, **40**, 195–202.

DILLON, R. J. & LANE, R. P. (1993). Influence of *Leishmania* infection on blood-meal digestion in the sandflies *Phlebotomus papatasi* and *P. langeroni*. *Parasitology Research*, **79**, 492–496.

JARAMILLO, C., TRAVI, B. L. & MONTOYA, J. (1994). Vector competence of some Neotropical sandflies for the *Leishmania* (*Viannia*) *braziliensis* complex. *Medical and Veterinary Entomology*, **8**, 1–7.

KILLICK-KENDRICK, R. (1979). Biology of *Leishmania* in phlebotomine sandflies. In *Biology of the Kinetoplastida*, Vol. 2, eds Lumsden, W. H. R. & Evans, D. A. pp. 395–460. London: Academic Press.

KILLICK-KENDRICK, R., WALLBANKS, K. R., MOLYNEUX, D. H. & LAVIN, D. R. (1988). The ultrastructure of *Leishmania major* in the foregut and proboscis of *Phlebotomus papatasi*. *Parasitology Research*, **74**, 586–590.

KILLICK-KENDRICK, R., KILLICK-KENDRICK, M. & TANG, Y. (1994). Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: the low susceptibility of *Phlebotomus papatasi* to *Leishmania tropica*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 252–253.

LEANEY, A. J. (1977). The effect of temperature on *Leishmania* in sandflies. *Parasitology*, **75**, 27–29.

LAWYER, P. G., NGUMBI, P. M., ANJILI, C. O., ODONGO, S. O., MEBRAHTU, Y. B., GITHURE, J. I., KOECH, D. K. & ROBERTS, C. R. (1990). Development of *Leishmania major* in *Phlebotomus duboscqi* and *Sergentomyia schwetzi* (Diptera: Psychodidae). *American Journal of Tropical Medicine and Hygiene*, **43**, 31–43.

MCCONVILLE, M. & HOMANS, S. W. (1992). Identification of the defect in lipophosphoglycan biosynthesis in a non-pathogenic strain of *Leishmania major*. *Journal of Biological Chemistry*, **267**, 5855–5861.

MOLYNEUX, D. H. & KILLICK-KENDRICK, R. (1987). Morphology, ultrastructure and life cycles. In *The Leishmaniases in Biology and Medicine*, Vol. 1., eds Peters, W. & Killick-Kendrick, R. pp. 121–176. London: Academic Press,

PIMENTA, F. P., TURCO, S. J., MCCONVILLE, M. J., LAWYER, P. G., PERKINS, P. V. & SACKS, D. L. (1992). Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science*, **256**, 1812–1815.

PIMENTA, P. F. P., SARAIVA, E. M. B., ROWTON, E., MODI, G. B., GARRAWAY, L. A., BEVERLEY, S. M., TURCO, S. J. & SACKS, D. L. (1994). Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 9155–9159.

SCHLEIN, Y. (1993). *Leishmania* and sandflies: interactions in the life cycles and transmission. *Parasitology Today*, **9**, 255–258.

SCHLEIN, Y. & JACOBSON, R. L. (1996). Why is man an unsuitable reservoir for the transmission of *Leishmania major*? *Experimental Parasitology*, **82**, 298–305.

SCHLEIN, Y., SCHNUR, L. F. & JACOBSON, R. L. (1990). Released glycoconjugate of indigenous *Leishmania major* enhances survival of a foreign *L. major* in *Phlebotomus papatasi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 353–355.

SHATOVA, S. M., SHULGA, M. A., SAFIANOVA, V. M. & AVAKIAN, A. A. (1984). Sravnitelnoe elektronnomikroskopicheskoe izuchenie *Leishmania major* i *L. tropica* pri eksperimentalnem zarazhenii moskita *Phlebotomus papatasi*. *Parazitologiya*, **18**, 154–159.

VOLF, P., KILLICK-KENDRICK, R., BATES, P. A. & MOLYNEUX, D. H. (1994). Comparison of the haemaglutination activities in gut and head extracts of various species and geographical populations of phlebotomine sandflies. *Annals of Tropical Medicine and Parasitology*, 88, 337–340.

WALLBANKS, K. R., INGRAM, G. A. & MOLYNEUX, D. H. (1986). The agglutination of erythrocytes and *Leishmania* parasites by sandfly gut extracts: evidence for lectin activity. *Tropical Medicine and Parasitology*, **37**, 409–413.

WALTERS, L. L. (1993). *Leishmania* differentiation in natural and unnatural sand fly hosts. *Journal of Eukaryotic Microbiology*, **40**, 196–206.

WALTERS, L. L., IRONS, K. P., CHAPLIN, G. & TESH, R. B. (1993). Life cycle of *Leishmania major* in the Neotropical sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae). *Journal of Medical Entomology*, **30**, 699–718.

WARBURG, A. & SCHLEIN, Y. (1986). The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *American Journal of Tropical Medicine and Hygiene*, **35**, 926–930.

WARBURG, A., HAMADA, G. S., SCHLEIN, Y. & SHIRE, D. (1986). Scanning electron microscopy of *Leishmania major* in *Phlebotomus papatasi*. *Zeitschrift für Parasitenkunde*, **72**, 423–431.

Annals of Tropical Medicine & Parasitology, Vol. 93, No. 6, 599–611 (1999)

Leishmania major: effect of repeated passages through sandfly vectors or murine hosts

By J. SÁDLOVÁ, M. SVOBODOVÁ and P. VOLF*
Department of Parasitology, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

Received 14 April 1999, Accepted 6 May 1999

Virulence for BALB/c mice, infectivity for *Phlebotomus papatasi*, haemagglutination activity and expression of metacyclic lipophosphoglycan (LPG) were studied in four strains of *Leishmania major* (LV561, FV1, L119 and Neal) and various lines of the LV561 strain. Attenuated line LV561/AV was passaged five times through sandflies or mice and the resulting lines (AVS5 and AVM5, respectively) and two of the earlier sandfly passages (AVS1 and AVS2) were used for further study. The highly virulent line LV561/V served as a control.

Virulence for mice was not regained during passaging of LV561/AV in sandflies or mice (none of the mice infected with AVM5, AVS1, AVS2 or AVS5 displayed overt lesions) and the success rate in cultivating parasites, from lymph-node samples from inoculated mice, was not significantly higher for any of these lines than for the original line (LV561/AV). However, AVM5 and AVS5 developed better than LV561/AV in *P. papatasi* and the intensity and localisation of their infections were similar to those of the virulent control. In smears of the infected guts of *P. papatasi*, the AVS5 parasites resembled the virulent line (LV561/V) morphologically whereas the AVM5 parasites were similar to the avirulent LV561/AV.

Haemagglutination activity increased as a result of passaging, the most pronounced difference being observed in AVM5, which had 60-fold higher activity than LV561/AV. Expression of metacyclic LPG was not increased by passaging. The proportion of forms reacting positively with 3F12 antibodies was high (about 17%) in the virulent LV561/V but low (2%–3%) in the avirulent lines LV561/AV, AVS5 and AVM5. A defect in LPG is not, however, likely to be the only reason for the avirulence observed, as the avirulent lines of LV561 still produced about 10 times as many metacyclic promastigotes as the strain L119, which caused delayed lesions in mice.

Leishmanial parasites encounter two distinct environments during their life-cycle. In the sandfly midgut, procyclic promastigotes differentiate into the metacyclic forms that are highly infective for mammalian hosts. In the vertebrate hosts' tissues, the parasites are phagocytosed by macrophages and multiply as amastigotes in macrophage phagolysosomes.

One of the molecules considered to play an important role in both parasite–sandfly and parasite–mammal interactions is lipophosphoglycan (LPG), the major surface glycoconjugate of *Leishmania*. In the sandfly, during the process of metacyclogenesis, LPG undergoes an extensive and developmentally regulated, structural modification (Sacks, 1992) and this may control attachment of the promastigotes to the sandfly midgut epithelium (Pimenta *et al.*, 1992, 1994). In the vertebrate host, LPG plays a role in the parasites' resistance to complement-mediated lysis, their internalisation by macrophages and their protection within the phagolysosomes (Turco and Descoteaux, 1992).

Other types of molecules involved in leishmanial pathogenesis are the carbohydrate-binding receptors and lectin-like components present on the parasite surface (Bray, 1983; Hernández *et al.*, 1986; Love *et al.*, 1994). One of these components, a haemagglutinin, can be found in the promastigotes of various species of *Leishmania* and in the axenic amastigotes of *L. mexicana*. The haemagglutination (HA) ac-

* Author to whom correspondence should be addressed.
E-mail: volf@cesnet.cz; fax: + 420 2 299713.

ISSN 0003-4983 (print) ISSN 1364-8594 (online)/99/060599-13
Carfax Publishing

tivity is higher in metacyclic promastigotes and amastigotes than in log-phase promastigotes (Svobodová *et al.*, 1997*a*) and appears to participate in the adhesion of amastigotes and promastigotes to human macrophages (Svobodová *et al.*, 1997*b*).

Various strains of the same species of *Leishmania* and even various lines of the same strain frequently differ in their infectivity for the mammalian host. Long-term cultivation *in vitro*, for example, often results in the parasites losing their ability to develop into metacyclic promastigotes and to infect vertebrate hosts (Grimm *et al.*, 1991; Segovia *et al.*, 1992; Kallinikova *et al.*, 1992). In some cases, this loss of virulence is reversible, passaging through susceptible mice resulting in increased virulence of the parasite (Katakura and Kobyashi, 1985). In *L. major*, such a reversion to virulence appears to be correlated with the level and type of LPG expressed by the parasite (Da Silva and Sacks, 1987; Shankar *et al.*, 1993). The effect on virulence of passaging the parasites through sandflies has not been studied, although clones and lines of *L. major* that are virulent in mice are known to grow better in *P. papatasi* than avirulent ones (Strelkova *et al.*, 1981; Pimenta *et al.*, 1994; Čiháková and Volf, 1997).

The parasites used in the present study were of four geographically distinct strains of *L. major* that display different levels of virulence in mice. Čiháková and Volf (1997) described the varied abilities of these strains to develop in the gut of sandflies. LV561/AV, a line that has been attenuated by long-term maintenance *in vitro* and is avirulent for BALB/c mice, was chosen for serial passage through mice or sandflies. The aim of this study was to determine the effect of serial passage *in vivo* on the parasite's virulence and infectivity for mice and sandflies. In addition, HA activity and metacyclic LPG were investigated in the strains and passaged lines.

MATERIALS AND METHODS

Parasites

Four strains of *Leishmania major* were used: LV561 (MHOM/IL/67/Jericho-II LRC-L137), FV1 (MHOM/IL/80/Friedlin), Neal (MRHO/SU/59/P) and L119 (MTAT/KE/??/T4).

The two Israeli strains (LV561 and FV1) were isolated originally from patients with cutaneous lesions. Two different lines of the strain LV561 were studied: the virulent line LV561/V, which has been passaged no more than five times *in vitro*; and the avirulent LV561/AV, which has been attenuated by >40 in-vitro passages. The avirulent LV561/AV, provided by Dr E. Nohýnková (Medical Faculty, Charles University, Prague), was used for serial passages in *P. papatasi* or mice.

The L119 strain was isolated in Kenya from the spleen of a 'healthy' gerbil, *Tatera nigricauda* (Heisch *et al.*, 1959). After long-term, serial passage *in vitro*, it had lost its infectivity to hamsters (Schnur, 1979; L. F. Schnur, unpubl. obs.) and was found to be a defective in its synthesis of the disaccharide backbone of the repeat units of LPG (McConville and Homans, 1992). However, despite this defect, it could still infect inbred mice and their isolated macrophages (L. F. Schnur, unpubl. obs.).

The Neal strain was isolated in Uzbekistan from *Rhombomys opimus*. The line of this strain used for the present study was provided by Dr V. M. Safjanova (Gamaleya Institut, Moscow). It had diminished virulence for mice, probably because of its long-term maintenance *in vitro*.

All the parasites were maintained at 23°C, on SNB-9 blood agar (Diamond and Herman, 1954) or in RPMI 1640 medium (Sigma) supplemented with 10% foetal calf serum and 45 μg gentamicin/ml, and subcultured at 4-day intervals. The identity of each strain or line used was checked by pulsed-field gel electrophoresis throughout the experiment; each strain or line had a distinct karyotype (data not shown).

Sandflies and Mice

Colonies of *P. duboscqi* (from Senegal) and *P. papatasi* (from Cyprus), obtained from Professor R. Killick-Kendrick (Imperial

College in Ascot), were maintained on 50% sucrose at 25–26°C on a 14-h light/10-h dark photoperiod. BALB/c mice, 8–12 weeks old, were used in all the experiments.

Repeated Passages of LV561/AV in Mice

Ten male mice were each inoculated in the rump with 10^7 promastigotes from 6-day old cultures and killed 3 weeks later. Parasites re-isolated from the inguinal nodes of these mice were cultured briefly (through two subcultures) and then inoculated in another 10 mice. This process was repeated until the line had gone through five passages in mice, when it was called AVM5.

Repeated Passages of LV561/AV in Sandflies

Female *P. duboscqi* were fed through a chick-skin membrane on heat-inactivated human blood containing 1×10^6 promastigotes (from 4-day-old cultures)/ml and then maintained as described above. Engorged females were dissected in sterile saline 10 days after they had been offered an infective bloodmeal. The midguts of the infected flies were inoculated into blood-agar medium and, after one to three in-vitro subcultures, the promastigotes were used for the next sandfly infection. The lines AVS1, AVS2 and AVS5 were obtained from the sandflies after one, two or five passages, respectively.

Infectivity of the *L. major* Strains and Lines for Mice

Strains FV1, Neal, L119 and six lines of the LV561 strain (LV561/V, LV561/AV, AVS1, AVS2, AVS5, and AVM5) were compared in four experiments. Female mice were inoculated as described above and lesion diameters were measured weekly. After either 6 months (experiments 1–3) or 9 months (experiment 4), mice without apparent lesions were killed and attempts made to culture parasites from their inguinal nodes.

Development of the LV561 Lines in Sandflies

The development of LV561/AV, AVM5 and AVS5 in *P. papatasi* was compared in separate experiments, using the virulent LV561/V as a control. Sandflies were fed on heat-inactivated rabbit blood containing 5×10^5 promastigotes (4-day-old cultures)/ml. Engorged females were dissected at 6 or 10 days post-bloodmeal. The location and intensity of any gut infections and parasite morphometry were evaluated as described previously (Čiháková and Volf, 1997). One hundred parasites were measured from each line at each sampling time.

Detection of Metacyclic LPG

Metacyclic LPG was detected by indirect immunofluorescence, using the 3F12 monoclonal antibody. Mouse ascitic fluid containing the 3F12 antibody, which is specific for metacyclic-stage LPG (Sacks and Da Silva, 1987), was kindly provided by Dr D. L. Sacks (National Institutes of Health, Bethesda, MD). Preliminary experiments showed that this metacyclogenesis marker gave more interpretable results than did agglutination by PNA lectin (Sacks *et al.*, 1985), as the PNA-negative promastigotes were not only metacyclic but also non-metacyclic parasites with defective LPG, like most promastigotes of the L119 strain. Stationary-phase promastigotes (from a 6-day culture) were washed and resuspended at 1×10^7/ml in PBS. Small, 10-μl drops of this suspension were air-dried on glass slides and fixed with methanol. Subsequently, they were incubated for 30 min with the mouse ascites diluted 1:20 in Tris-Tw buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% foetal calf serum. After washing, the slides were incubated for 30 min with swine anti-mouse IgG conjugated to fluoroscein isothiocyanate (SEVAC, Prague), diluted 1:20 in Tris-Tw, and then re-washed and stained for 2 min with 0.01% Evans Blue. Fifteen oil-immersion fields were examined for each line, so that the proportion of parasites reacting with the monoclonal antibody (and therefore fluorescing) could be determined. Each experiment was repeated once.

Haemagglutination (HA) Tests

The HA activity of each line or strain was determined as described previously (Svo-

TABLE 1
The infectivity and virulence of Leishmania major *strains and lines in BALB/c mice*

Experiment	*Parasites*	*No. of mice inoculated*	*No. of mice and (% of inoculated mice)*	
			Developing lesions	*Culture-positive post-mortem*
1	LV561/V	7	7 (100)	ND
	LV561/AV	7	0 (0)	4 (57)
	FV1	7	2 (29)	3 (43)
	L119	3	3 (100)	ND
	Neal	7	0 (0)	5 (71)
2	LV561/V	10	10 (100)	ND
	AVM5	10	0 (0)	4 (40)
3	AVS1	9	0 (0)	0 (0)
	AVS2	9	0 (0)	0 (0)
	AVS5	9	0 (0)	2 (22)
4	LV561/AV	9	0 (0)	3 (33)
	AVM5	9	0 (0)	6 (67)
	AVS5	9	0 (0)	2 (22)

ND, Not determined.

bodová *et al.*, 1997*a*). Briefly, a suspension of washed promastigotes (from 6-day-old cultures) was adjusted to give 10^8 cells/ml, lysed by freezing and thawing and the supernatant solutions, after centrifuging at $2000 \times g$ for 3 min, were used for the HA tests. Doubling dilutions of the lysates were prepared in HA buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) in the U-shaped wells of microtitre plates to give final dilution ranges of 1:10–1:20 480. A 2% suspension of native rabbit erythrocytes was then added to each well. The HA was scored after 1 h, the end-point titre being defined as the last dilution causing visible HA.

RESULTS

Infectivity of the *L. major* Strains and Lines for BALB/c Mice

The lines/strains differed considerably in their virulence for BALB/c mice (Table 1). In each of the seven mice infected with LV561/V line, lesions appeared 4–6 weeks post-infection and grew rapidly (Fig.1). Lesions only developed in two of the seven mice infected with the FV1 strain and these took 16 weeks to appear. Another three mice infected with FV1 strain still had viable parasites in their inguinal nodes when they were killed. In mice inoculated with the L119 strain, lesion development was even more delayed (starting 16, 24 and 27 weeks post-inoculation) but the lesions grew rapidly once they had appeared. The Neal and LV561/AV strains failed to cause any lesions within 6 months of the inoculations but most of the post-mortem cultures of inguinal nodes were positive (Table 1).

Virulence was not regained by passage through mice (AVM5) or through sandflies (AVS1, AVS2 and AVS5): none of the mice infected with these lines developed lesions (Table 1). Infectivity, as assessed by the number of positive cultures of inguinal nodes, appeared to decrease during passage through sandflies, although passage through mice had no such effect (Table 1). The infectivities of AVM5 and the original LV561/AV line were similar ($\chi^2 = 2.000$; $P = 0.1573$).

Development of the LV561 Lines in *P. papatasi*

On both days 6 and 10 post-bloodmeal, the intensity of infections produced by LV561/AV was significantly lower than that of the

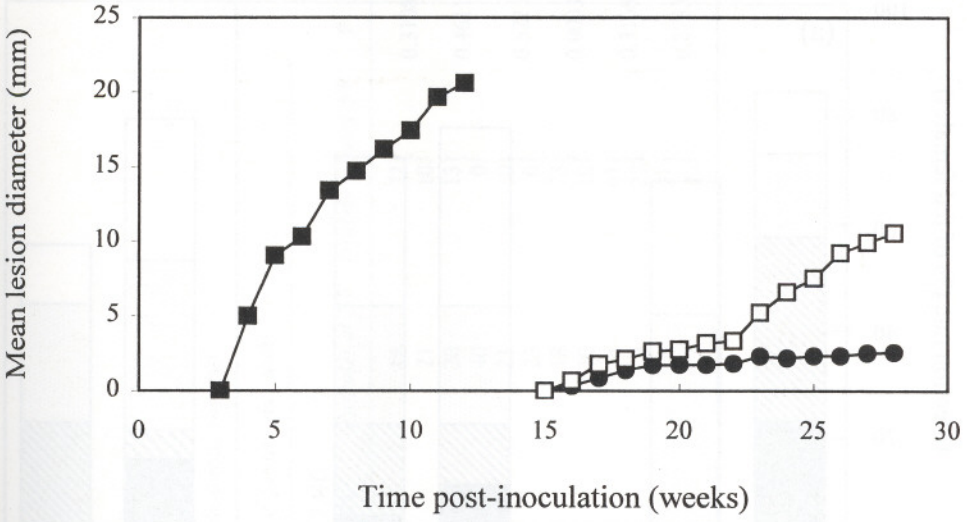


Fig. 1. Development of the LV561/V (■), FV1 (•) and L119 (□) strains of *Leishmania major* in BALB/c mice.

infections produced by the virulent LV561/V ($P < 0.001$). However, the infections produced by AVS5 and AVM5 were as intense as those produced by LV561/V (Fig. 2).

On day 6 post-infection, the localisation and morphology of the parasites of each of the lines tested were similar (Tables 2–4). In most of the infected *P. papatasi*, the parasites had migrated anteriorly from the abdominal midgut and had colonised the thoracic midgut and even the stomodeal valve by day 6. On day 10 post-infection, most parasites had left the abdominal midgut and intensive infections of the stomodeal valve had often developed. A much higher proportion of the flies infected with the virulent control, LV561/V, had developed stomodeal-valve infections than of the flies infected with LV561/AV (Table 2; $P = 0.0003$).

Promastigotes of the avirulent lines (LV561/AV, AVM5 and AVS5) recovered from the sandfly infections were generally larger than those of the virulent control, particularly on day 10 post-infection, when promastigotes of all three avirulent lines had significantly larger bodies and longer flagella than those of the virulent control (Table 3). A more detailed morphological analysis, in which parasite populations were broken down into six morphological forms, showed that AVS5 (but not LV561/AV or AVM5) was very similar to the virulent line in this respect (Table 4).

Expression of Metacyclic LPG by Stationary-phase Promastigotes

The proportion of promastigotes reacting with 3F12 antibodies differed with the strain or line investigated (Table 5). In comparison with the LV561/V line, the proportion of metacyclic forms was significantly lower in LV561/AV, AVM5 and AVS5. Although a entirely negative result was expected with L119, a very small number (0.2%) of the promastigotes of this strain were found to be positive. The same result (i.e. 0.2% positivity) was also observed with the Neal strain (Table 5).

Haemagglutination

Among the four lines of strain LV561 investigated, the highest titres of HA activity

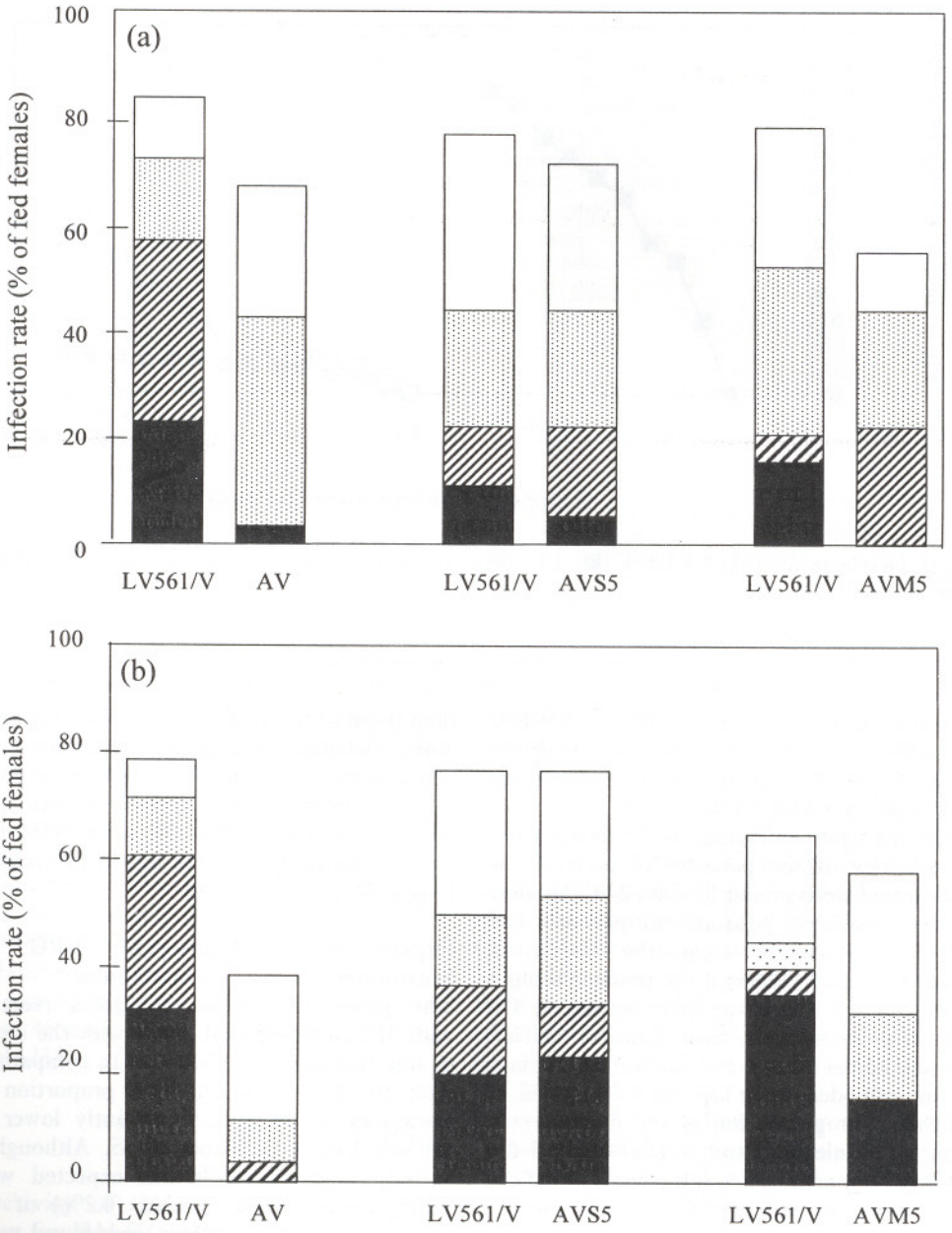


Fig. 2. Rates and intensities of infections with lines of *Leishmania major* LV561 in *Phlebotomus papatasi* 6 days (a) and 10 days (b) after the infective bloodmeal. Infections were light (□; < 100 parasites/gut), moderate (▤; 100–500 parasites/gut), heavy (▨; 500–1000 parasites/gut) or very heavy (■; > 1000 parasites/gut).

(20 480–40 960) were observed in the virulent line LV561/V and the lowest (40) in the avirulent line LV561/AV. HA activities in AVS5 and AVM5 were higher than in LV561/AV, with titres of 160 and 2560, respectively.

TABLE 2

Development of the lines of Leishmania major *LV561 in* Phlebotomus papatasi

Days post-bloodmeal	*Parasites*	*No. of flies fed*	*No. and (%) of flies infected*	*% of positive flies with:*				P
				Infected AMG only	*Infected TMG and AMG*	*Parasites at SV*	*Heavily infected SV*	
6	LV561/V	26	22 (85)	5	45	18	32	0.3108
	LV561/AV	28	19 (68)	16	53	21	10	
6	LV561/V	19	15 (79)	13	53	20	13	0.4611
	AVM5	18	10 (56)	20	40	40	0	
6	LV561/V	18	14 (78)	43	36	21	0	0.5225
	AVS5	18	13 (72)	23	54	23	0	
10	LV561/V	28	22 (79)	0	9	18	73	0.0003
	LV561/AV	26	10 (38)	0	80	10	10	
10	LV561/V	20	13 (65)	0	31	8	61	0.3168
	AVM5	19	11 (58)	9	55	9	27	
10	LV561/V	30	23 (77)	0	39	4	57	0.2753
	AVS5	30	23 (77)	0	44	17	39	

AMG, Abdominal midgut; TMG, thoracic midgut; SV, stomodeal valve.

TABLE 3

Means (S.D.) and [ranges] for the dimensions of lines of Leishmania major *LV561 developing in the guts of* Phlebotomus papatasi *on days 6 and 10 post-infection*

	Lines compared			*Lines compared*			*Lines compared*		
Dimension	*LV561/V*	*LV561/AV*	P	*LV561/V*	*AVM5*	P	*LV561/V*	*AVS5*	P
BODY LENGTH (μm)									
Day 6	14.9 (5.0) [5.7–28.6]	14.9 (4.1) [4.3–25.7]	>0.05	15.9 (4.8) [2.8–30.0]	12.8 (3.2) [4.3–21.4]	<0.0001	16.1 (3.8) [7.1–24.3]	17.6 (5.8) [7.1–30.0]	<0.05
Day 10	11.3 (3.6) [2.9–18.6]	13.7 (3.9) [2.9–22.8]	<0.0001	10.7 (3.2) [2.9–15.7]	13.4 (2.9) [2.9–18.6]	<0.0001	12.4 (3.1) [4.3–18.6]	15.5 (3.6) [7.1–24.3]	<0.0001
BODY WIDTH (μm)									
Day 6	1.4 (0.4) [0.7–2.1]	1.5 (0.4) [0.7–2.9]	<0.05	1.3 (0.4) [0.7–2.9]	1.7 (0.6) [0.7–4.3]	<0.0001	1.3 (0.4) [0.7–2.9]	1.6 (1.5) [0.7–5.7]	>0.05
Day 10	1.4 (0.5) [0.7–2.9]	1.6 (0.4) [0.7–2.9]	<0.001	1.4 (0.5) [0.7–2.9]	1.6 (0.4) [0.7–2.9]	<0.001	1.5 (0.5) [0.7–2.9]	1.6 (0.6) [0.7–5.7]	>0.05
FLAGELLAR LENGTH (μm)									
Day 6	16.0 (3.4) [7.1–24.3]	16.0 (3.4) [8.6–28.6]	>0.05	15.6 (3.7) [4.3–24.3]	14.5 (2.9) [8.6–21.4]	<0.05	16.1 (3.2) [8.6–22.8]	16.0 (3.4) [5.7–28.6]	>0.05
Day 10	13.7 (3.3) [2.9–22.8]	15.9 (4.1) [7.1–25.7]	<0.001	12.8 (2.4) [7.1–18.6]	15.4 (2.5) [8.6–22.8]	<0.0001	14.4 (2.5) [7.1–20.0]	15.4 (2.6) [8.6–21.4]	<0.05

TABLE 4

The relative representation of six morphological forms of the parasite in three lines of Leishmania major *LV561, in 6– and 10–day–old infections in* Phlebotomus papatasi

		% of parasites in infection						
Days post-infection	*Parasite line*	*Elongated nectomonads*	*Short, slender promastigotes*	*Short, broad promastigotes*	*Paramastigotes*	*Rounded promastigotes*	*Metacyclic promastigotes*	P
6	LV561/V	62	29	0	9	0	0	>0.05
	LV561/AV	72	22	1	4	0	1	
6	LV561/V	73	24	0	2	1	0	>0.05
	AVM5	49	39	3	6	0	3	
6	LV561/V	74	24	1	1	0	0	>0.05
	AVS5	84	15	0	1	0	0	
10	LV561/V	29	54	2	11	1	3	<0.001
	LV561/AV	60	37	2	0	1	0	
10	LV561/V	25	59	3	8	1	4	<0.001
	AVM5	61	35	1	2	1	0	
10	LV561/V	42	49	0	6	0	3	>0.05
	AVS5	78	16	0	3	0	3	

TABLE 5

The proportions of stationary-phase promastigotes of Leishmania major *reacting specifically with the monoclonal antibody 3F12*

Parasite strain or line	*No. of parasites observed*	*No. and (%) of parasites reacting*
LV561/V	1306	220 (16.8)
LV561/AV	1130	24 (2.1)
FV1	1280	65 (5.1)
L119	1324	3 (0.2)
Neal	1852	4 (0.2)
AVM5	1718	54 (3.1)
AVS5	1522	42 (2.8)

DISCUSSION

Prolonged cultivation of the virulent strain LV561 in SNB-9 blood-agar medium yielded an avirulent line LV561/AV with decreased infectivity for mice. The infectivity and virulence of this line for mice were not regained after repeated passages through either sandflies or mice.

Strain LV561, as demonstrated by Handman *et al.* (1983), is a mixed population of more and less virulent phenotypes and, as Segovia *et al.* (1992) and Camara *et al.* (1995) showed, the non-infective clones of *L. major* tend to outgrow the infective ones during in-vitro cultivation. Although it was thought that serial passage through mice would, in contrast, favour the virulent subpopulation, selection through five passages in the present study failed to yield a line that was virulent for the vertebrate host. Presumably, the proportion of virulent parasites in the LV561/AV population was too small and/or the deviation(s) occurring in the absence of selective pressure had been too large. The results of studies on strains of *Leishmania* that have undergone prolonged cultivation *in vitro* should therefore be interpreted with extreme caution. This is true not only for studies on infectivity and virulence but also in studies on the efficacy of new drugs for the treatment of leishmaniases.

The present failure to observe a reversion to virulence conflicts with the results of earlier studies, where such reversion was obtained after either one (Da Silva and Sacks, 1987; Marchand *et al.*, 1987; Shankar *et al.*, 1993) or 15 passages through mice (Katakura and Kobyashi, 1985). However, the clones used in these earlier investigations were not avirulent, like LV561/AV, but displayed only decreased virulence, producing delayed footpad lesions.

Development in the sandflies, in terms of the intensity of the infections and anterior migration, was improved by passaging either through sandflies or mice. Selection by five passages (through invertebrate or vertebrate hosts) was presumably sufficient to favour a subpopulation of the parasites with characters beneficial to their development in sandflies.

The present haemagglutination tests confirmed the observations of Svobodová *et al.* (1997*a*): strains infective to mice possessed higher HA activity than uninfective strains and HA activity was considerably decreased by long-term cultivation *in vitro*. Such changes in HA activity appear to be reversible, AVM5 and AVS5 having higher activities than the original avirulent line (LV561/AV). Serial passage through mice had the most dramatic effect on HA activity, with a 60-fold increase over five passages.

The highest proportion of stationary-phase promastigotes with metacyclic LPG (16.8%) was produced by the highly virulent line LV561/V. The FV1 strain had, proportionally, about three times fewer promastigotes with metacyclic LPG; this corresponds with the lower virulence and infectivity of this

strain for mice. All three lines of LV561 that were avirulent for BALB/c mice (LV561/AV, AVS5 and AVM5) produced few metacyclics (2%–3%). Defectiveness in LPG is not, however, likely to be the only reason for the avirulence of these lines as they still produced about 10 times as many metacyclic promastigotes as L119, a strain that was able to cause (delayed) lesions in mice.

For all of the strains/lines used, the proportion of promastigotes with metacyclic LPG coincided well with the ability of the strain/line to develop in sandflies (see also Čiháková and Volf, 1997). Decreased production of metacyclic LPG may reflect a deficiency in the production of a procyclic form of LPG. If LPG does have a crucial role in *Leishmania*–sandfly interactions, defective LPG might be lethal for promastigotes in the vector.

The role of LPG in leishmanial virulence for mice has been studied using clones and strains defective in LPG expression. Clones of *L. major* with LPG modified by chemical mutagenesis were not able to survive in macrophages *in vitro* and were either avirulent for mice or caused delayed and small lesions (Elhay *et al.*, 1990). Promastigotes of the non-infective strain L119 were phagocytosed rapidly by macrophages *in vitro* but, in contrast to virulent strains, they were then killed (over a period of 18 h). Transfer of purified LPG from a virulent clone of *L. major* into cultures of L119 promastigotes confered on them the ability to survive *in vitro* (Handman *et al.*, 1986).

In the present study, the promastigotes of the L119 strain did cause lesions in BALB/c mice, although the time required for their detection was much longer than that for the virulent LV561/V; lesion development resembled that described by Cappai *et al.* (1994) in mutant clones of *L. major* LV561, which also had defective LPG. Development of the lesions was rapid once the infection had established. Delayed lesion development following inoculation with L119 has been observed before (Blackwell, 1992; D. Rangarajan, unpubl. obs.). Selection and multiplication of genetically fully competent individuals present in the inoculum may have to occur before any lesions develop.

The present results support the hypothesis that leishmanial virulence for mice is a complex phenotypic trait and that LPG is only one of the important molecules involved. To try to elucidate the mechanisms responsible for virulence in *L. major*, the activity and expression of the main surface metalloprotease, gp63, in the strains and lines used in this study are currently under investigation.

ACKNOWLEDGEMENTS. We thank Dr D. Sacks and Professor R. Killick-Kendrick for providing the monoclonal antibodies and sandfly colonies, respectively, and Dr L. F. Schnur for advice and comments. The study was supported by the Ministry of Education of the Czech Republic (projects FRVS1401, 13/981131-B4 and VS 96/142).

REFERENCES

BLACKWELL, J. M. (1992). Leishmaniasis epidemiology: all down to the DNA. *Parasitology*, **104**, 19–34.

BRAY, R. S. (1983). *Leishmania mexicana mexicana*: attachment and uptake of promastigotes to and by macrophages *in vitro*. *Journal of Protozoology*, **30**, 314–322.

CAMARA, M., NAVARRO, M. & SEGOVIA, M. (1995). Evidence from genotypic and phenotypic markers that an attenuated line outgrows a virulent one in a mixed population of *Leishmania major* promastigotes cultured *in vitro*. *Annals of Tropical Medicine and Parasitology*, **89**, 477–484.

CAPPAI, R., MORRIS, L., AEBISCHER, T., BACIC, A., CURTIS, J. M., KELLEHER, M., MCLEOD, K. S., MOODY, S. F., OSBORN, A. H. & HANDMAN, E. (1994). Ricin-resistant mutants of *Leishmania major* which express modified lipophosphoglycan remain infective for mice. *Parasitology*, **108**, 397–405.

ČIHÁKOVÁ, J. & VOLF, P. (1997). Development of different *Leishmania major* strains in vector sandflies *Phlebotomus papatasi* and *P. duboscqi*. *Annals of Tropical Medicine and Parasitology*, **91**, 267–279.

DA SILVA, R. & SACKS, D. L. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infection and Immunity*, **55**, 2802–2806.

DIAMOND, L. S. & HERMAN, C. M. (1954). Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. *Journal of Parasitology*, **40**, 195–202.

ELHAY, M. J., KELLEHER, M., BACIC, A., MCCONVILLE, M. J., TOLSON, D. L., PEARSON, T. W. & HANDMAN, E. (1990). Lipophosphoglycan expression and virulence in ricin-resistant variants of *Leishmania major*. *Molecular and Biochemical Parasitology*, **40**, 255–268.

GRIMM, F., BRUN, R. & JENNI, L. (1991). Promastigote infectivity in *Leishmania infantum*. *Parasitology Research*, **77**, 185–191.

HANDMAN, E., HOCKING, R. E., MITCHELL, G. F. & SPITHILL, T. W. (1983). Isolation and characterisation of infective and non-infective clones of *Leishmania tropica*. *Molecular and Biochemical Parasitology*, **7**, 111–126.

HANDMAN, E., SCHNUR, L. F., SPITHILL, T. W. & MITCHELL, G. F. (1986). Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. *Journal of Immunology*, **137**, 3608–3613.

HEISCH, R. B., GRAINGER, W. E. & HARVEY, A. E. C. (1959). The isolation of a *Leishmania* from gerbils in Kenya. *Journal of Tropical Medicine and Hygiene*, **62**, 158–159.

HERNÁNDEZ, A. G., RODRÍGUEZ, N., STOJANOVIC, D. & CANDELLE, D. (1986). The localisation of a lectin-like component on the *Leishmania* cell surface. *Molecular Biology Reports*, **11**, 149–153.

KALLINIKOVA, V. D., NASYROVA, R. M., NASYROV, F. S. & SAFJANOVA, V. M. (1992). Morphogenesis and virulence of *Leishmania major* in the process of long-term cultivation. *Archiv für Protistenkunde*, **141**, 327–334.

KATAKURA, K. & KOBYASHI, A. (1985). Enhancement of infectivity of *Leishmania donovani* promastigotes by serial mouse passages. *Journal of Parasitology*, **71**, 393–394.

LOVE, D., ESKO, J. D. & MOSSER, D. M. (1994). A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. *Journal of Cell Biology*, **123**, 759–766.

MARCHAND, M., DAOUD, S., TITUS, R. G., LOUIS, J. & BOON, T. (1987). Variants with reduced virulence derived from *Leishmania major* after mutagen treatment. *Parasite Immunology*, **9**, 81–92.

MCCONVILLE, M. J. & HOMANS, S. W. (1992). Identification of the defect in lipophosphoglycan biosynthesis in a non-pathogenic strain of *Leishmania major*. *Journal of Biological Chemistry*, **267**, 5855–5861.

PIMENTA, P. F. P., TURCO, S. J., MCCONVILLE, M. J., LAWYER, P. G., PERKINS, P. V. & SACKS, D. L. (1992). Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science*, **256**, 1812–1815.

PIMENTA, P. F. P., SARAIVA, E. M. B., ROWTON, E., MODI, G. B., GARRAWAY, L. A., BEVERLEY, S. M., TURCO, S. J. & SACKS, D. L. (1994). Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 9155–9159.

SACKS, D. L. (1992). The structure and function of the surface lipophosphoglycan on different developmental stages of *Leishmania* promastigotes. *Infectious Agents and Disease*, **1**, 200–206.

SACKS, D. L. & DA SILVA, R. P. (1987). The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *Journal of Immunology*, **139**, 3099–3106.

SACKS, D. L., HIENY, S. & SHER, A. (1985). Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. *Journal of Immunology*, **135**, 564–569.

SCHNUR, L. F. (1979). Leishmanial excreted factors in establishing infections *in vivo* and *in vitro*. *Israel Journal of Medical Science*, **15**, 626.

SEGOVIA, M., ARTERO, J. M., MELLADO, E. & CHANCE, M. L. (1992). Effects of long-term *in vitro* cultivation on the virulence of cloned lines of *Leishmania major* promastigotes. *Annals of Tropical Medicine and Parasitology*, **86**, 347–354.

SHANKAR, A., MITCHEN, T. K., HALL, L. R., TURCO, S. J. & TITUS, R. G. (1993). Reversion to virulence in *Leishmania major* correlates with expression of surface lipophosphoglycan. *Molecular and Biochemical Parasitology*, **61**, 207–216.

STRELKOVA, M. V., ALEKSEEV, A. N. & PASSOVA, O. M. (1981). Changes in the virulent properties of the strains of ZCL pathogen as passaged through *Phlebotomus papatasi*. *Meditsinskaya Parazitologiya i Parazitarnye Bolezni*, **55**, 19–23.

SVOBODOVÁ, M., BATES, P. A. & VOLF, P. (1997*a*). Detection of lectin activity in *Leishmania* promastigotes and amastigotes. *Acta Tropica*, **68**, 23–35.

SVOBODOVÁ, M., CAPO, C. & MEGE, J. L. (1997*b*). A biological role for haemagglutination activity of *Leishmania* promastigotes and amastigotes. *Parasite*, **4**, 245–251.

TURCO, S. J. & DESCOTEAUX, A. (1992). The lipophosphoglycan of *Leishmania* parasites. *Annual Review of Microbiology*, **46**, 65–94.

The surface metalloproteinase gp63 in virulent and attenuated lines of *Leishmania major*.

J. Sádlová*, J. Votýpka*, K. Victoir**, J.C. Dujardin** and P. Volf*

* Department of Parasitology, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

** Laboratory of Protozoology, Institute of Tropical Medicine "Prince Leopold",

155 Nationalestraat, B-2000 Antwerpen, Belgium.

Corresponding author:

Dr. Petr Volf, Department of Parasitology, Charles University, Viničná 7, 128 44 Prague 2,

Czech Republic

Fax: 420 2 299713

E-mail: volf@cesnet.cz

Phone: 420 2 21953196

Sádlová J., Votýpka J., Victoir K., Dujardin J.C. and Volf P.: The surface metalloproteinase gp63 in virulent and attenuated lines of *Leishmania major*. *Int. J. Parasitology*

ABSTRACT

The *Leishmania* metalloproteinase gp63 is known as a parasite virulence factor, while its importance for promastigote survival in the sandfly is less clear. To understand better the role of gp63 during the whole *Leishmania* life-cycle, the amount and enzymatic activity of gp63 were compared in several strains and lines of *L. major* differing in virulence for BALB/c mice and ability to develop in sandflies. Possible changes in the structure of gp63 genes and/or gene dosage were also investigated. The chromosomal location of the gp63 gene locus was conserved among the strains: gp63 probe hybridised to one chromosome-sized band of about 570 kb in all the strains and lines studied. Hybridisation of gp63 probe to genomic DNA cleaved by various restriction enzymes and to Southern blots from PFGE revealed no significant differences among strains/lines in the structure and dosage of gp63 genes, respectively. However, marked differences were observed in amount and proteolytical activity of the gp63 protein. Both expression and proteolytical activity of gp63 were very low in attenuated LV561/AV line and slightly increased after five serial passages of this line through sandflies (AVS5 line) and mice (AVM5 line). Parasites of all three lines developed heavy infections in sandflies, but were avirulent for BALB/c mice. On the other hand, overexpression of gp63 was found in two strains (L119, Neal), which were defective in metacyclic LPG and unable to survive and multiplicate in sandflies, although they caused lesions in some of mice. The possibility that overexpression of gp63 may overcome the LPG defect is discussed. The results do not confirm a notable role of the surface protease in the vector part of *Leishmania* life-cycle while they support a significance of this molecule as a virulence factor.

Key words: virulence, *Phlebotomus*, sandflies, proteolytical activity, genomic organisation..

INTRODUCTION

A major promastigote surface glycoprotein, gp63, has been identified to be a zinc metalloproteinase active on a wide range of protein substrates (Etges et al., 1986, Chaudhuri & Chang, 1988, Bouvier et al., 1989) with a substrate-dependent pH optimum (Ip et al., 1990, Tzinia & Soteriadou, 1991). This molecule is ubiquitous among *Leishmania* promastigotes (Bouvier et al., 1987, Etges, 1992) and is present also in amastigotes, although in lower levels (Button et al., 1989, Schneider et al., 1992, Bahr et al.,1993, Streit et al., 1996).

The genes encoding gp63 are arranged as multicopy tandem arrays in all *Leishmania* species studied to date with large differences in copy numbers among the species (Button et al., 1989, Webb et al., 1991, Roberts et al., 1993, Medina-Acosta et al., 1993a, Steinkraus et al., 1993, Victoir et al., 1995). The number of gene copies can, to a certain extent, vary among various strains of the same species (Murray et al., 1990) and there is also molecular heterogeneity between the different gene copies in the gene array (reviewed by Medina Acosta et al., 1993b). In *L. major*, the seven genes exhibit stage-specific differences in their expression: five homologous tandemly repeated gp63 genes 1-5 are expressed in promastigotes only, a separated less conserved gp63 gene 6 is expressed in both promastigotes and amastigotes, while a gp63 gene 7, located downstream from the gene 6, is expressed in stationary phase promastigotes and amastigotes (Voth et al., 1998). Similar developmental changes in both gene expression and protein isoforms occur also in *L. mexicana* and *L. chagasi* gp63 during promastigote growth from logarithmic to stationary phase *in vitro* and during transformation into amastigote stage (Ramamoorthy et al., 1992, Medina-Acosta et al., 1993a, Roberts et al., 1995, Streit et al., 1996).

The high level of gp63 genes conservation between pathologically and geographically diverse *Leishmania* species (Webb et al., 1991) imply a functional significance of this molecule. The suggested function of gp63 in the host is multiple: it is involved in the uptake of *Leishmania* by macrophages (Russell, 1987, Russell & Wright, 1988, Wilson & Hardin, 1990,

Liu & Chang, 1992), in exploitation of the opsonic properties of complement while avoiding its lytic effects (Brittingham et al., 1995) and in protection from intracellular degradation in the phagolysosome (Chaudhuri et al., 1989, Seay et al., 1996, McGwire & Chang, 1994, Sorensen et al., 1994). It was also demonstrated that gp63 inhibits chemotaxis of monocytes and neutrophils (Sorensen et al., 1994). In addition, the protease activity of gp63 is supposed to be involved in the pathology of lesion formation in the host via a degradation of the extracellular matrix (McMaster et al., 1994).

The role of gp63 in the sandfly vector (promastigote stage) is less clear, the most probable function would be the acquisition of nutrients from the blood meal. Proteolytical degradation of haemoglobin could provide both amino acids (especially prolin) used as an primary energy source of promastigotes (Mukkada, 1985) and haem, which they cannot synthesise (Chang & Chang, 1985). The nutritional role of gp63 in a vector is indirectly supported by the fact that surface metalloprotease similar to leishmanian gp63 were found in monoxenous insect trypanosomatids *Crithidia* and *Herpetomonas* (Etges, 1992).

Association of leishmanian gp63 with parasite virulence has been reported by various authors. In *L. braziliensis*, the increase of gp63 expression correlates with a development of infective metacyclic promastigotes (Kweider et al., 1987). Decreased amount and proteolytic activity of gp63 was found in avirulent parasites in comparison with virulent ones in *L. mexicana*, *L. infantum*, *L. chagasi* and *L. amazonensis* (Chaudhuri & Chang, 1988, Wilson et al., 1989, Santos-Gomes & Abranches, 1996, Seay et al., 1996) and transfection of avirulent strains with a gp63 gene increased parasite infectivity for macrophage in *L. amazonensis* and *L. donovani* (Liu & Chang, 1992, McGwire & Chang, 1994, Chakrabarty et al., 1996).

The study presented here was aimed at a characterisation of gp63 in four geographically distinct strains of *L. major;* gene structure and dosage, protein amount and proteolytical activity of gp63 was ascertained. In addition, changes in these gp63 characters associated with serial passage of an avirulent parasite line through mice or sandflies were investigated. The

used strains displayed various degree of virulence for mice (Sádlová et al., 1999) and different abilities to develop in the vector sandflies *P. papatasi* and *P. duboscqi* (Čiháková & Volf, 1997). Strain-specific differences were recently described also in expression of metacyclic LPG and heamagglutination activity (Svobodová et al., 1997, Sádlová et al., 1999).

MATERIALS AND METHODS

Leishmania strains and culture. Four strains of *Leishmania major* were used: LV561 (MHOM/IL/67/LRC-L137 Jericho-II), FV1 (MHOM/IL/80/Friedlin), Neal (MRHO/SU/59/P) and L119 (MTAT/KE/00/T4). The two Israeli strains (LV561, FV1) originate from human cutaneous lesions, the L119 and the Neal strains were isolated in Kenya from *Tatera nigricauda* (Heisch et al., 1959) and in Uzbekistan from *Rhombomys opimus*, respectively. The line of Neal strain used for the present study was provided by Dr. Safjanova, Gamaleya Institut, Moscow. Four different lines of the strain LV561 were studied: the virulent line (LV561/V) was used after fresh reisolation (< 5 *in vitro* subcultures) from BALB/c mice; the avirulent line (LV561/AV) was attenuated by >40 *in vitro* passages and the lines LV561/AVM5 and LV561/AVS5 were obtained from the LV561/AV line by five passages through mice and sandflies, respectively. All the strains and lines were tested previously for the infectivity and virulence in BALB/c mice (Sádlová et al., 1999). High virulence (rapid lesions development) was observed in LV561/V line, while delayed lesions development was found in both FV1 and L119 strains. The line of L119 reisolated from a mouse lesion is denominaed as L119V. The Neal strain and LV561/AV, LV561/AVM5 and LV561/AVS5 lines were avirulent (no lesions appeared throughout experiments lasting 6 or even 9 months). However, they retain the infectivity for mice as the parasites were reisolated from inguinal nodes in a part of inoculated animals. Contrary to the LV561/AV line, freshly reisolated parasites of the Neal strain were able to cause lesions after next inoculation into mice

(Svobodová and Sádlová, unpublished observation). Parasites were maintained at 25°C on SNB-9 blood agar (Diamond and Herman 1954) or RPMI 1640 medium (SIGMA) supplemented with 10% foetal calf serum (FCS) and Gentamicin, and subcultured at four-days intervals.

Immunodetection of gp63. SDS-PAGE was performed according to Leammli (1970) on 10% gel (thickness 0.75 mm) under reduced conditions. Lanes were loaded with proteins from equal numbers (10^7) of stationary phase (day 6) parasites. After separating the proteins, gels were stained with Coomassie Brilliant blue R-250 or proteins were transferred to nitrocellulose membrane according to Towbin et al. (1979). The membrane was rinsed in TRIS-Tw buffer (20mM TRIS, 150 mM NaCl, 0.1% TWEEN 20, pH 7.6), blocked overnight in 5% milk, washed in TRIS-Tw and incubated for 1h with rabbit anti-gp63 antiserum (1:100 dilution in 5% FCS). Polyclonal anti-gp63 antiserum obtained from rabbit injected with the bacterial lipoprotein/*L. major* gp63 fusion protein (Cornelis et al., 1996) was kindly provided by Prof. P. Cornelis, Vrije University of Brussels. After washing in TRIS-Tw, the membrane was incubated for 45 min with peroxidase-conjugated swine anti-rabbit immunoglobulins (SEVAC, Prague) diluted 1: 500 in TRIS-Tw and washed in TRIS-Tw. The peroxidase reaction product was developed in PBS buffer with 4-chloro-1-naphtol and 30% H_2O_2.

Protease activity of gp63 on gelatine gels. SDS-PAGE was carried out on 10% gels co-polymerised with 0.1% gelatine under nonreduced conditions. Gels were soaked three times for 10 minutes in 0.5% Nonidet in TRIS-NaCl, incubated in TRIS-NaCl at 37 °C overnight and then stained with Coomasie blue. Proteolytic activity was detected as a clear band on a blue-stained gelatine background.

The azocasein assay. Enzymatic activity was assessed according to Etges et al. (1986). Stationary-phase promastigotes were washed twice in PBS (pH 7.4), resuspended (4 x 10^8 cells ml^{-1}) in PBS containing 20 mg ml^{-1} azocasein (Sigma) and incubated at room temperature with agitation. At time intervals, aliquots of the reaction mixtures were mixed with an equal volume of 5% TCA on ice. After centrifugation, the absorbance of the supernatant was determined at 440 nm.

Molecular karyotyping (PFGE) and hybridisation. Agarose blocks containing chromosomal DNA were prepared as described by Van Der Ploeg et al. (1984). OFAGE electrophoresis using equipment described by Dujardin et al. (1987) were performed on 1.5% agarose gels in 0.4 x TBE running buffer at 7-12 °C for 23 hours. Resolution of the whole karyotype was achieved by three distinct runs with 45-, 65- and 125-s pulses. The karyotype of reference strain *L. braziliensis* M2903 was used to estimate the sizes of chromosomal bands. Chromosomal bands resolved by OFAGE using 125-s pulses were transferred onto nylon membrane and hybridised according to manufacturer's instructions (Amersham). Three different hybridisation probes were used: pLb-134Sp (most conserved part of *L. braziliensis* gp63 gene, Dujardin *et al.,* 1994), pLb-22 (putative serine/threonine protein kinase from *L. braziliensis* M2904, Dujardin et al., 1993, and unpublished data), and SSUcl79 (a highly conserved fragment of the 18S rDNA gene, Van Eys et al., 1992); the two last probes are linked on chromosome 27 (Inga et al., 1998).

DNA isolation and RFLP. Genomic DNA was isolated as described elsewhere (Sambrook et al., 1989) and digested to completion with restriction enzymes (Pst I, Sal I, Alu I and Bgl I) according to manufacturer's instructions (BRL). Electrophoresis was performed overnight, at a low voltage, in a 10 x 14 cm agarose (0.7%) gel. Lambda Hind III DNA ladder (BRL) was used to size DNA molecules.

RESULTS

Expression and protease activity of gp63.

Immunoblothing with polyclonal rabbit antiserum showed that expression of surface metalloprotease gp63 differed among strains (Table 1, Fig. 1b). In a comparison with the virulent line LV561/V, expression of gp63 was reduced in LV561/AV, whereas it was considerably elevated in both L119 and Neal strains. Similar differences among the strains were apparent using the gp63 protease activity assay (Fig. 1c) on gelatine gels. In lines of the LV561 strain passaged through mice and sandflies (LV561/AVM5, LV561/AVS5), both expression and protease activity of gp63 was increased in comparison with original avirulent line LV561/AV, but did not reach the level observed in LV561/V line (Fig. 2).

Differences among strains/lines in the amount and enzymatical activity of gp63 were confirmed by loading of higher amount of the LV561/AV sample and lower amounts of both L119 and Neal samples. This rough quantitative estimation revealed that both intensity of gp6 bands on immunoblots as well the digestion area on gelatine gels were equal when loaded fiv times higher amount of LV561/AV and 30 times lower amounts of both L119 and Neal strain (not shown).

The ability of a given strain/line of *Leishmania* to digest gelatine co-polymerised in polyacrylamide gel correlates with its ability to degrade azocasein in solution. The azocasein assay showed considerably higher degradation of azocasein in L119 and Neal strains than in the other strains and lines. However, the method was not sufficiently sensitive to reveal differences between lines/strains with lower proteolytical activity (Fig. 3).

Karyotype analysis.

Molecular karyotyping of strains here used was analysed to check the identity of the different lines maintained in parallel. Karyotypes of strains LV561, FV1, L119 and Neal were

significantly different; differences between strains are apparent in the range of both the high as well as the low molecular weight chromosome-sized bands (Fig. 4a). Within strain LV561 (Fig. 5a), the 4 lines presented the same karyotype profile with the exception of one band corresponding to small linear DNA of about 200 kb (LD elements, known to be fluctuating; Segovia, 1994). No karyotype differences are apparent between L119 promastigotes used for mice infection and those reisolated from the mouse lesion (L119V).

Genomic organisation of gp63 genes.

The chromosomal location of the gp63 gene locus was determined to ascertain possible interchromosome translocations. Hybridisation of gp63 probe to Southern blots from PFGE showed that one chromosome-sized band of about 570 kb bears the gp63 genes in all the strains and lines (Figs. 4, 5).

The structure of the gp63 locus was examined by hybridisation of gp63 probe to genomic DNA cleaved with various restriction enzymes. The most representative patterns were obtained using SalI enzyme (Fig. 6), which cuts within the intergenic region of the repeats (Button et al. 1989, Murray et al. 1990). All isolates showed identical restriction fragment lengths for the gp63 gene with a small exception in Neal strain (1 band in SalI restriction pattern is smaller). This suggests conservation of gp63 genomic organisation among strains and lines here analysed. Densitometrical scanning of SalI patterns allowed to estimate a copy number of 7 gp63 genes: for a relative intensity of 1 for each weakly hybridising bands (separated genes), an intensity of 5 was calculated for the strongly hybridising band (cluster of tandemly repeated genes).

Quantitative analysis of gp63 genes.

Differences in hybridisation intensities of gp63 probe to chromosome 8 was observed in some strains. In order to check if this was due to gene dosage or the quantity of loaded DNA, the

same PFGE blots were rehybridised with probes cl79 and pLb-22, both linked to chromosome 27 (Inga *et al.*, 1998). Hybridisation intensities with the respective probes were quantified by densitometrical scanning. This indicated that differences in gp63 hybridisation intensity was accompanied by a similar difference with other probes (Fig. 7). Therefore, the differences among strains and lines should be attributed to the quantity of the DNA present on the gel. The comparison with other chromosomes also showed that there are no differences like aneuploidy.

DISCUSSION

In the present study, marked differences among *L .major* strains/lines were observed in amount and proteolytical activity of the gp63 protein, although high similarity was found in the structure of gp63 genes and in the gene dosage. Therefore, differences found originate probably on the level of transcription, translation or post-translational regulation of the gp63 gene product. This finding is in accordance with results of another authors; Wilson et al. (1989) observed reduced amount of gp63 protein in attenuated *L. chagasi* parasites in comparison with the virulent line, but they did not find genomic changes in the gp63 locus. Similarly, in clones derived from L119 strain of *L. major*, Murray et al. (1990) described widely fluctuating gp63 protein levels without apparent changes in the gp63 gene locus. This does not exclude the possibility that in some cases, genomic changes could affect gp63 gene expression; in Neotropical *Leishmania*, the lower pathogenicity of *L peruviana* was correlated with strong reduction in copy number of gp63 genes (Victoir et al., 1998), and the absence of a specific gene family (Victoir and Dujardin, unpublished data).

Information about gp63 genes and gp63 expression complete characterisation of *L. major* strains and lines recently published by our group, namely the infectivity and virulence for BALB/c mice, development in *Phlebotomus papatasi* and *P. duboscqi* and expression of metacyclic LPG in promastigotes (Čiháková & Volf, 1997, Sádlová et al., 1999). Data are

summarised in Table 1. We believe that a comparison of the relative representation of gp63 in individual strains may led to the recognition of a functional significance of this molecule in the host and/or in the vector part of the *Leishmania* life-cycle.

Both expression and proteolytical activity of gp63 was very low in LV561/AV line and slightly increased in passaged AVS5 and AVM5 lines. Reduced gp63 level is evidently a frequent phenomenon in *Leishmania* promastigotes which underwent a long-termed maintenance *in vitro*. It was found in attenuated variants compared to virulent parasites in *L. mexicana*, *L. chagasi*, *L. infantum* and *L. amazonensis* (Chaudhuri & Chang, 1988, Wilson et al., 1989, Santos-Gomes & Abranches, 1996, Seay et al., 1996).

In the present case, parasites of all the three lines were able to develop heavy infections in sandflies with colonisation of the stomodeal valve (Čiháková & Volf, 1997, Sádlová et al., 1999), but were completely avirulent for BALB/c mice. In these lines, metacyclic LPG was present in 6-8 times less amount than in virulent line, but still in 10-15 times higher amount in comparison with L119 and Neal strains (Sádlová et al., 1999). It seems that low amount of gp63 does not prevent the parasites from infecting sandflies, but it is incompatible with successful infections of the vertebrate host. Inverse results observed in L119 and Neal strains (see Table 1) suggest similar interpretation: gp63 overexpression and higher proteolytical activity in these strains did not enhance the infections of sandflies, however, it probably contributed to the ability of these strains to cause lesions in some infected mice, despite their defective LPG.

In conclusion, our results do not support a notable role of gp63 in the vector part of *Leishmania* life-cycle while they prove a significance of this molecule as a virulence factor. Low role of gp63 for the development in sandflies was indicated previously by Joshi et al. (1998). They used gp63 deficient mutants to show that promastigote form of gp63 encoded by genes 1-6 do not appear to be required for this part of *Leishmania* life-cycle. On the other hand, Camara et al. (1995) found that the amount and enzymatic activity of surface gp63 was

relatively high in the attenuated promastigotes of *L. major*, which were unable to survive in macrophages but were successful in sandflies infections.

The most intriguing finding of this study is that both strains defective in production of metacyclic LPG (L119, Neal) overexpressed the metalloprotease gp63. Previously also Murray et al. (1990) observed higher level of gp63 expression in L119 strain, comparing to virulent LV561 strain, and suggested that this phenomenon may be associated with lacks of detectable levels of LPG in this strain. It can be a regular contra-strategy of the parasite how to avoid the defect, otherwise lethal. Both these surface molecules seems to be crucial for the survival in the vertebrate host. The requirement of gp63 for parasite interaction with macrophages was confirmed by genetic approaches (Liu & Chang, 1992, McGwire & Chang, 1994, Chakrabarty et al., 1996). In other systems, however, the main role of LPG in determination parasite virulence was highlighted. The decreased virulence of *L. major* and *L. donovani* following to chemical mutagenesis was found to be correlated with decreased expression and changed structure of LPG while expression and proteolytical activity of gp63 remained unchanged (Elhay et al., 1990, Shankar et al., 1993, Cappai et al., 1994).

These results are not contradictory and may reflect a natural variability and plasticity in the relative importance of these two virulence factors. In fact, Chakrabarty et al. (1996) showed that in two virulent strains of *L. donovani* the contributory roles of gp63 and LPG differ either in recognition or in the rate of internalisation into macrophages. In their experiments, preblocking of macrophage receptors with either gp63 or LPG affect the entry of the one or the another virulent strain, respectively.

The cross-substitution of these two virulence factors can just be imagined with respect to their suggested roles in the vertebrate host. Both LPG and gp63 have been implicated in crucial steps of *Leishmania*-host interactions, i.e., influence of monocyte migration (Frankenburg et al., 1990, 1992, Sorensen et al., 1994), attachment to macrophages (Da Silva et al., 1989, Talamas-Rohana et al., 1990, Russell & Wright, 1988, Russell, 1987), avoiding complement

attack (Puentas et al., 1990, Brittingham et al., 1995) and protection from degradation in macrophage phagolysosomes (McNeely & Turco, 1990, Proudfoot et al., 1996, Seay et al., 1996). Some of the functions remain yet to be confirmed and some may be species-specific, however, it is clear that these molecules accomplish in a different manner very similar functions. Such multiplicity in molecules which are involved in the evading mechanisms may be, in fact, one of the main qualifications for a successful survival of the parasite.

Acknowledgements: We thank Prof. P. Cornelis, Vrije University of Brussels for providing anti-gp63 antibodies, Dr. M. Svobodová, Dr. I. Hrdý and Mrs S. De Doncker for help during experiments. This research was supported by the Ministry of Education of the Czech Republic (projects VS 96/142 and J13/981131-B4), and by a grant of the Fund for Scientific Research-Flanders (KV.E 192/1997). J.S. and J.V. were supported by TEMPUS mobility grant S-JEP-07281/94

REFERENCES

Bahr V, Stierhof YD, Ilg T, Demar M, Quinten M, Overath P. Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmannia mexicana*. Mol Biochem Parasitol 1993; 58: 107-122.

Bouvier J, Etges R, Bordier C. Identification of the promastigote surface protease in seven species of *Leishmania*. Mol Biochem Parasitol 1987; 24: 73-79.

Bouvier J, Bordier C, Vogel H, Reichelt R, Etges R. Characterization of the promastigote surface protease of *Leishmania* as a membrane-bound zinc endopeptidase. Mol Biochem Parasitol 1989; 37: 235-246.

Brittingham A, Morrison ChJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistence to complement-mediated lysis. J Immunol 1995; 155: 3102-3111.

Button LL, Russell DG, Klein HL, Medina-Acosta E, Karess RE, McMaster WR. Genes encoding the major surface glycoprotein in *Leishmania* are tandemly linked at a single chromosomal locus and are constitutively transcribed. Mol Biochem Parasitol 1989; 32: 271-284.

Camara M, Ortiz G, Valero PL et al. Complement-mediated lysis and infectivity for mouse macrophages and sandflies of virulent and attenuated *Leishmania major* promastigotes varying in expression of the major surface protease and lipophosphoglycan. Ann Trop Med Parasitol 1995; 89: 243-251.

Cappai R, Morris L, Aebischer T et al. Ricin-resistant mutants of *Leishmania major* which express modified lipophosphoglycan remain infective for mice. Parasitology 1994; 108: 397-405.

Chang CS, Chang KP. Heme requirement and acquisition by extracellular and intracellular stages of *Leishmania mexicana amazonensis*. Mol Biochem Parasitol 1985; 16: 267-276.

Chakrabarty R, Mukherjee S, Lu HG, McGwire BS, Chang KP, Basu MK. Kinetics of entry of virulent and avirulent strains of *Leishmania donovani* into macrophages: a possible role of virulence molecules (gp63 and LPG). J Parasitol 1996; 84: 632-635.

Chaudhuri G, Chang KP. Acid protease activity of a major surface membrane glycoprotein (gp63) from *Leishmania mexicana* promastigotes. Mol Biochem Parasitol 1988; 27: 43-52.

Chaudhuri G, Chaudhuri M, Pan A, Chang KP. Surface acid proteinase (gp63) of *Leishmania mexicana*. J Biol Chem 1989; 264: 7483-7489.

Čiháková J, Volf P. Development of different *Leishmania major* strains in the vector sandflies *Phlebotomus papatasi* and *P. duboscqi*. Ann Trop Med Parasitol 1997; 91(3): 267-279.

Cornelis P, Sierra JC, Lim Jr A et al. Development of new cloning vectors for the production of immunogenic outer membrane fusion proteins in *Escherichia coli*. Biotechnology 1996; 14: 203-208.

Da Silva RP, Hall BF, Joiner KA, Sacks DL. CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. J Immunol 1989; 143: 617-622.

Diamond LS, Herman CM. Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. J Parasitol 1954; 40: 195-202.

Dujardin JC, Gajendran N, Hamers R et al. Leishmaniasis in the lowlands of Bolivia. VIII. Characterization and identification of Bolivian isolates by PFG karyotyping. In.: Leishmaniasis: the First Centenary (1885-1985). New Strategies for Control, NATO ASIŤ series A, Vol. 163, ed. Hart D., pp. 137-148. 1987; New York: Plenum Press.

Dujardin JC, Gajendran N, Arevalo J et al. Karyotype polymorphism and conserved characters in the *Leishmania (Viannia) braziliensis* complex explored with chromosome-derived probes. Ann Soc Belge Méd Trop 1993; 73: 101-118.

Dujardin JC, De Doncker S, Victoir K, Le Ray D, Hamers R, Arevalo J. Size polymorphism of chromosomes bearing gp63-genes in *L. braziliensis* and *L. peruviana*. Ann Trop Med Parasitol 1994; 88: 445-448.

Elhay M, Kelleher M, Bacic A et al. Lipophosphoglycan expression and virulence in ricin-resistant variants of *Leishmania major*. Mol Biochem Parasitol 1990; 40: 255-268.

Etges R. Identification of a surface metalloproteinase on 13 species of *Leishmania* isolated from humans, *Crithidia fasciculata*, and *Herpetomonas samuelpessoai*. Acta Tropica 1992; 50: 205-217.

Etges R, Bouvier J, Bordier C. The major surface protein of *Leishmania* promastigotes is a protease. J Biol Chem 1986; 261: 9098-9101.

Frankenburg S, Liebovici V, Mansbach N, Turco SJ, Rosen G. 1990: Effect of glycolipids of *Leishmania* parasites on human monocyte activity. J Immunol 1990; 145: 4284-4289.

Frankenburg S, Gross A, Leibovici V. *Leishmania major* and *Leishmania donovani*: effect of LPG-containing and LPG-deficient strains on monocyte chemotaxis and chemiluminescence. Exp Parasitol 1992; 75: 442-448.

Heisch RB, Grainger WE, Harvey AEC. The Isolation of a *Leishmania* from Gerbils in Kenya. J Trop Med Hyg 1959; 62: 158-159.

Inga R, De Doncker S, Gomez J et al. Relation between variation in copy number of ribosomal RNA encoding genes and size of harbouring chromosomes in *Leishmania* of subgenus *Viannia*. Mol Biochem Parasitol 1998; 92: 219-228.

Ip HS, Orn A, Russell DG, Cross GAM. *Leishmania mexicana mexicana* gp63 is a site-specific neutral endopeptidase. Mol Biochem Parasitol 1990; 40: 163-172.

Joshi PB, Sacks DL, Modi G, McMaster WR. Targeted gene deletion of *Leishmania major* genes encoding developmental stage-specific leishmanolysin (GP63). Molec Microbiol 1998; 27: 519-530.

Kweider M, Lemesre JL, Darcy F, Kusnierz JP, Capron A, Santoro F. Infectivity of *Leishmania braziliensis* promastigotes is dependent on the increasing expression of a 65.000-dalton surface antigen. J Immunol 1987; 138: 299-305.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Liu X, Chang KP. Extrachromosomal genetic complementation of surface metalloproteinase (gp63)-deficient *Leishmania* increrases their binding to macrophages. Proc Natl Acad Sci USA 1992; 89: 4991-4995.

McGwire B, Chang KP. Genetic rescue of surface metalloproteinase (gp63)-deficiency in *Leishmania amazonensis* variants increases their infection of macrophages at the early phase. Mol Biochem Parasitol 1994; 66: 345-347.

McMaster WR, Morrison CJ, Macdonald MH, Joshi PB. Mutational and functional analysis of the *Leishmania* surface metalloproteinase gp63: similarities to matrix metalloproteinases. Parasitology 1994; 108: 29-36.

McNeely TB, Turco SJ. Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. J Immunol 1990; 144: 2745-2750.

Medina-Acosta E, Karess RE, Russell DG. Structurally distinct genes for the surface protease of *Leishmania mexicana* are developmentally regulated. Mol Biochem Parasitol 1993a; 57: 31-46.

Medina-Acosta E, Beverley SM, Russell DG. Evolution and expression of the *Leishmania* surface proteinase (gp63) gene locus. Inf Agents Dis 1993b; 2: 25-34.

Moody SF. Molecular variation in *Leishmania*. Acta Tropica 1993; 53: 185-204.

Mukkada AJ. Energy metabolism in *Leishmania*. In: Human Parasitic Diseases, Vol. 1. Leishmaniasis (Chang KP and Bray RS, eds.). pp. 49-64, 1985; Elsevier, Amsterdam, New York.

Murray PJ, Handman E, Glaser TA, Spithill TW. *Leishmania major*: Expression and gene structure of the glycoprotein 63 molecule in virulent and avirulent clones and strains. Exp Parasitol 1990; 71: 294-304.

Proudfoot L, Nikolaev AV, Feng GJ et al. Regulation of the expression of nitric oxid synthas and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. Proc Natl Acad Sci USA 1996; 93: 10984-10989.

Puentes SM, Da Silva RP, Sacks DL, Hammer CH, Joiner KA. Serum resistance of metacycli stage *Leishmania major* promastigotes is due to release of C5b-9. J Immunol 1990; 145: 4311-4316.

Ramamoorthy R, Donelson JE, Paetz KE, Maybodi M, Roberts SC, Wilson ME. Three distinc RNAs for the surface protease gp63 are differentially expressed during development of *Leishmania donovani chagasi* promastigotes to an infectious form. J Biol Chem 1992; 267: 1888-1895.

Roberts SC, Swihart KG, Agey MW, Ramamoorthy R, Wilson ME, Donelson JE. Sequence diversity and organization of the msp gene family encoding gp63 of *Leishmania chagasi*. Mol Biochem Parasitol 1993; 62: 157-171.

Roberts SC, Wilson ME, Donelson JE. Developmentally regulated expression of a novel 59-kDa product of the major surface protease (msp or gp63) gene family of *Leishmania chagasi* J. Biol Chem 1995; 270: 8884-8892.

Russell DG. The macrophage-attachment glycoprotein gp63 is the predominant C3-acceptor site on *Leishmania mexicana* promastigotes. Eur J Biochem 1987; 104: 213-221.

Russell DG, Wright SD. Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein, gp63, of *Leishmania* promastigotes. J Exp Med 1988; 168: 279-292.

Sádlová J, Svobodová M, Volf P. *Leismania major*: effect of repeated passages through sandfly vectors or murine hosts. Ann Trop Med Parasitol 1999: 599-611.

Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular cloning*, 2nd Edn. New York: Cold Spring Harbor Laboratory Press.

Santos-Gomes GM, Abranches P. Comparative study of infectivity caused by promastigotes of *Leishmania infantum* MON-1, *L. infantum* MON-24 and *L. donovani* MON-18. Folia Parasitol. 1996; 43: 7-12.

Schneider P, Rosat JP, Bouvier J, Louis J, Bordier C. *Leishmania major*: differential regulation of the surface metalloprotease in amastigote and promastigote stages. Exp Parasitol 1992; 75: 196-206.

Seay MB, Heard PL, Chaudhuri G. Surface Zn-proteinase as a molecule for defense of *Leishmania mexicana amazonensis* promastigotes against cytolysis inside macrophage phagolysosomes. Infect Immun 1996; 64: 5129-5137.

Segovia M. *Leishmania* gene amplification: a mechanism of drug resistance. Ann Trop Med Parasitol 1994; 88: 123-130.

Shankar A, Mitchen TK, Hall LR, Turco SJ, Titus RG. Reversion to virulence in *Leishmania major* correlates with expression of surface lipophosphoglycan. Mol Biochem Parasitol 1993; 61: 207-216.

Sorensen AL, Hey AS, Kharazmi A. *Leishmania major* surface protease gp63 interferes with the function of human monocytes and neutrophils in vitro. APMIS 1994; 102: 265-271.

Steinkraus HB, Greer JM, Stephenson DC, Langer PJ. Sequence heterogeneity and polymorphic gene arrangements of the *Leishmania guyanensis* gp63 genes. Mol Biochem Parasitol 1993; 62: 173-185.

Streit JA, Donelson JE, Agey MW, Wilson ME. Developmental changes in the expression of *Leishmania chagasi* gp63 and heat shock proteins in a human macrophage cell line. Inf Immun 1996; 64: 1810-1818.

Svobodová M, Bates PA, Volf P. Detection of lectin activity in *Leismania* promasigotes and amatigotes. Acta Tropica 1997; 68: 23-35.

Talamas-Rohana P, Wright SD, Lennartz MR, Russell DG. Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocute integrins. J. Immunol. 1990; 144: 4817-4824.

Towbin H, Steahlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350-4354.

Tzinia AK, Soteriadou KP. Substrate-dependent pH optima of gp63 purified from seven strains of *Leishmania*. Mol Biochem Parasitol 1991; 47: 83-89.

Van der Ploeg LHT, Cornelissen WCA, Michels PAM, Borst P. Chromosome rearrangements in *Trypanosoma brucei*. Cell 1984; 39: 213-221.

Van Eys GJM, Schoone GJ, Kroon NCM, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 1992; 51: 133-142.

Victoir K, Dujardin JC, De Doncker S et al. Plasticity of gp63 gene organization in *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) peruviana*. Parasitology 1995; 111: 265-273.

Victoir K, Bañuls AL, Arevalo J et al. The gp63 gene locus, a target for genetic characterisation of *Leishmania* belonging to subgenus *Viannia.* Parasitology 1998; 117:1-13.

Voth BR, Kelly BL, Joshi PB, Ivens AC, McMaster WR. Differentially expressed *Leishmania major* gp63 genes encode cell surface leishanolysin with distinct signals for glycosylphosphatidylinositol atachment. Mol Biochem Parasitol 1998; 93: 31-41.

Webb JR, Button LL, McMaster WR. Heterogeneity of the genes encoding the major surface glycoprotein of *Leishmania donovani*. Mol Biochem Parasitol 1991; 48: 173-184.

Wilson ME, Hardin KK. The major *Leishmania donovani chagasi* surface glycoprotein in tunicamycin-resistant promastigotes. J Immunol 1990; 144: 4825-4834.

Wilson ME, Hardin KK, Donelson JE. Expression ot fhe major surface glycoprotein of *Leishmania donovani chagasi* in virulent and attenuated promastigotes. J Immunol 1989; 143: 678-684.

TABLES

Table 1. Correlation of amount and enzymatic activity of gp63 with previously described characters of the *L. major* strains and lines (Čiháková & Volf, 1997, Sádlová et al., 1999).

	Virulence in BALB/c mice	Development in sandflies	Expression of metacyclic LPG	Gp63 amount and proteolytical activity
LV561/V	+++	+++	+++	++
LV561/AV	-	++	++	-
LV561/AVS5	-	+++	++	+
LV561/AVM5	-	+++	++	+
FV1	++	++	++	++
L119	++	+	+	+++
Neal	+	+	+	+++

LEGEND TO FIGURES

Fig. 1. Comparison of amount and enzymatic activity of gp63 in various *L. major* strains. (A) SDS-PAGE stained with Coomassie blue. (B) Immunoblot with. antibodies against gp63. (C) Protease activity of gp63 on gelatine gels.

Fig. 2. Comparison of amount and enzymatic activity of gp63 in various lines of LV561 strain. (A) SDS-PAGE stained with Coomassie blue. (B) Immunoblot with antibodies against gp63. (C) Protease activity of gp63 on gelatine gels.

Fig. 3. Degradation of azocasein by promastigotes of different strains and lines of *L. major*. Each point is the average of two experiments.

Fig. 4. Chromosomal location of gp63 genes in *L. major* strains. (A) Chromosome-sized bands resolved by OFAGE using 125-s pulses. (B) Hybridisation with gp63 probe pLb-134Sp. Lanes: 2, LV561/V; 3, LV561/AV; 4, FV1; 5, L119; 6, L119V; 7, Neal; 1 and 8, *L. braziliensis* M2903.

Fig. 5. Chromosomal location of gp63 genes in various lines of LV561 strain. (A) Chromosome-sized bands resolved by OFAGE using 125-s pulses. (B) Hybridisation with gp63 probe pLb-134Sp. Lanes: 2, LV561/V; 3, LV561/AV; 4, AVS5; 5, AVM5; 1 and 6, *L. braziliensis* M2903.

Fig. 6. Hybridisation of gp63 probe pLb-134Sp to genomic DNA cleaved with the restriction enzyme SalI. (A) Various lines of the strain LV561: 1, LV561/V; 2, LV561/AV; 3, AVS5; 4, AVM5. (B) Various strains of *L. major*: 1, LV561/V; 2, LV561/AV; 3, FV1; 4, L119; 5, L119V; 6, Neal. The molecular weight of each band is given in kb.

Fig. 7. Quantification of gp63 gene dosage in various *L. major* strains (A) and in various lines of LV561 strain (B). Reaction of probes pLb-134Sp (♦), pLb-22 (▲) and SSUcl79 (□) with the same PFGE blots. Hybridisation intensity was quantified by densitometrical scanning (in both L119 lines the result of SSUcl79 probe was excluded because of signal saturation).

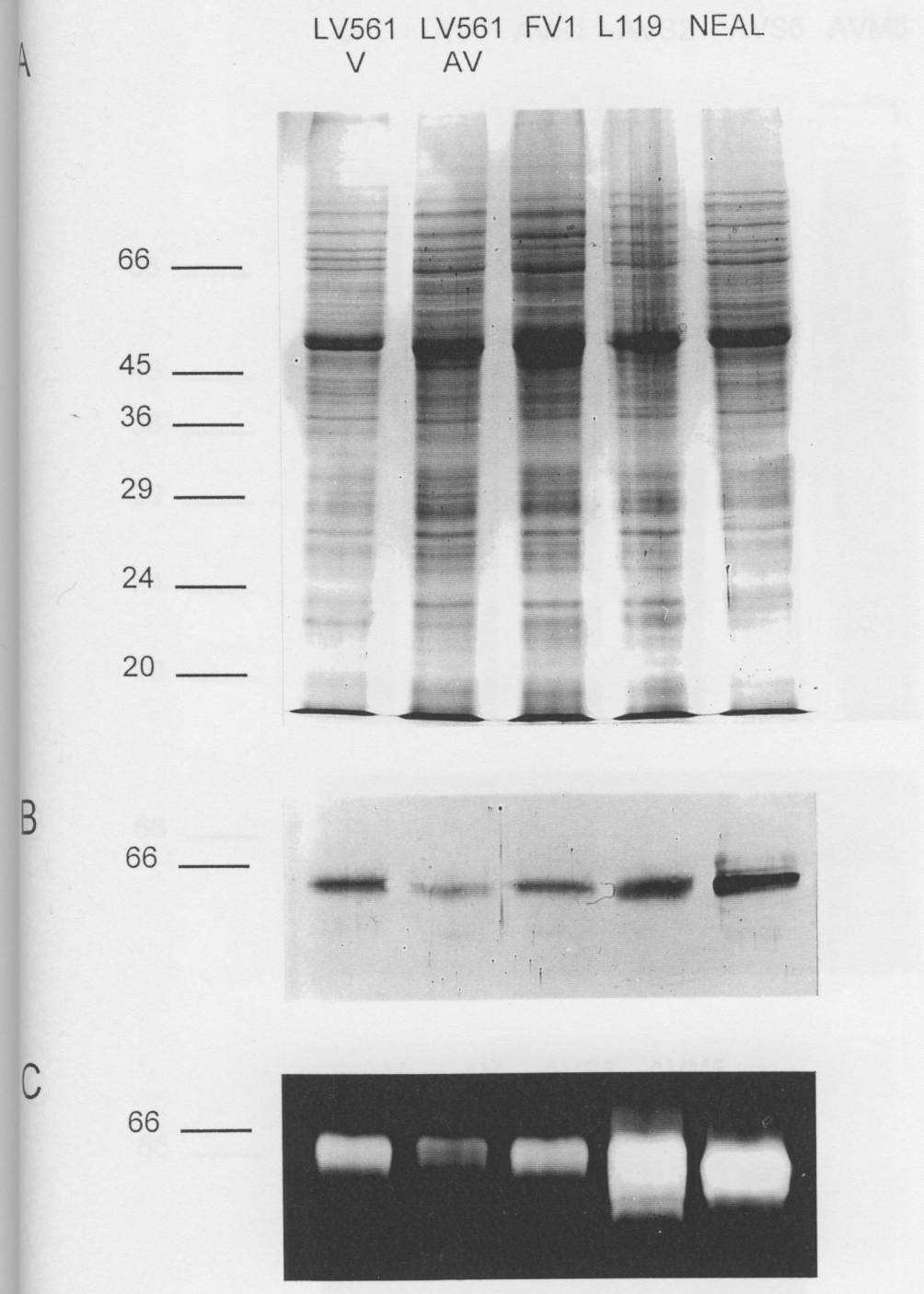


Fig. 1

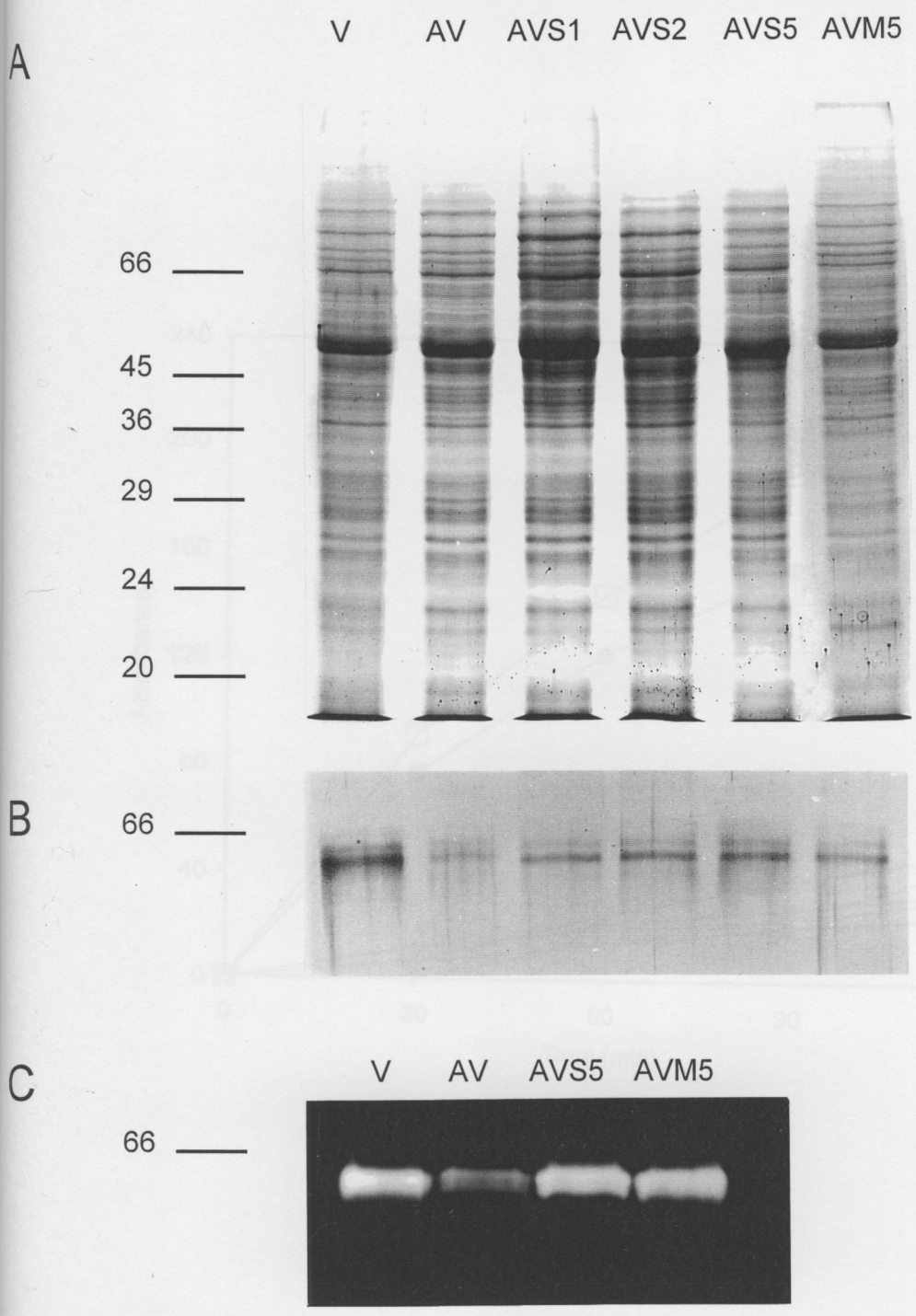


Fig 2

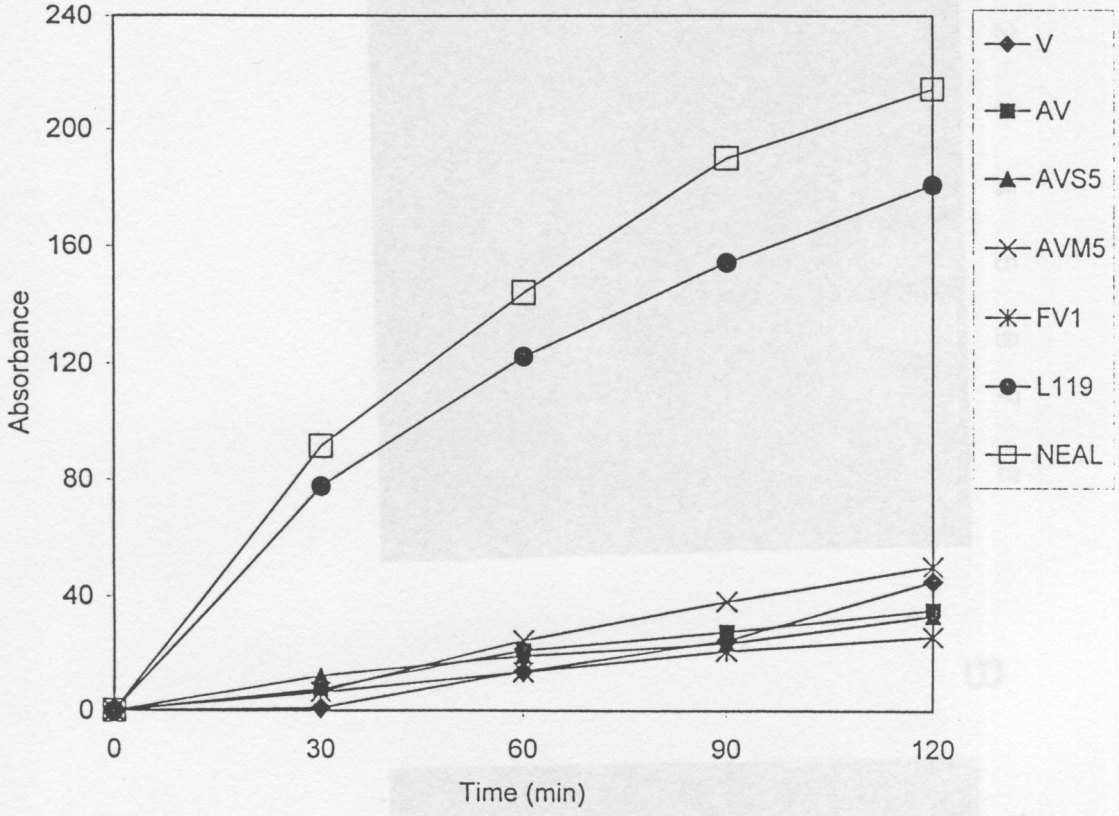
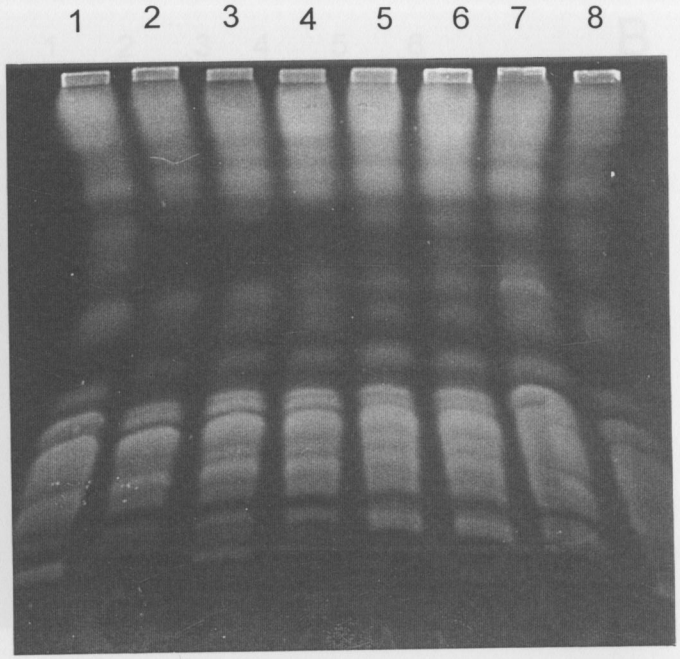

240
200
160
120
80
40
0
Absorbance
0
30
60
90
120
Time (min)
V
AV
AVS5
AVM5
FV1
L119
NEAL

Fig. 3

A



B

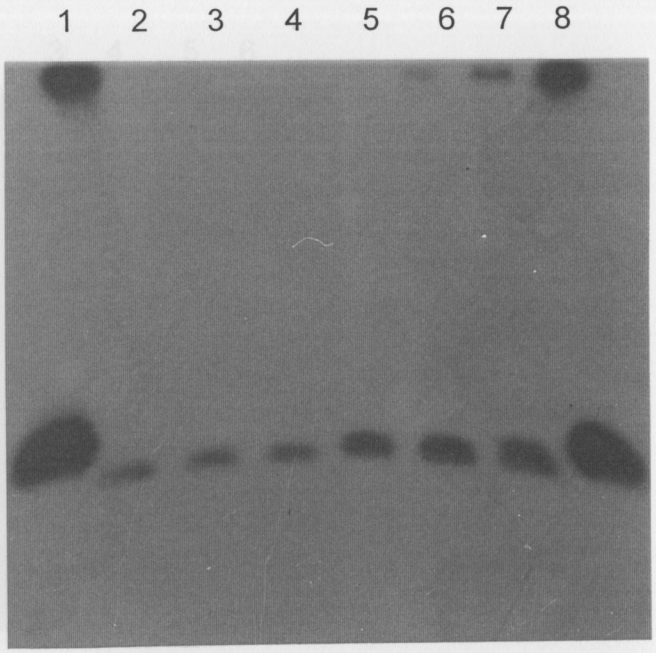
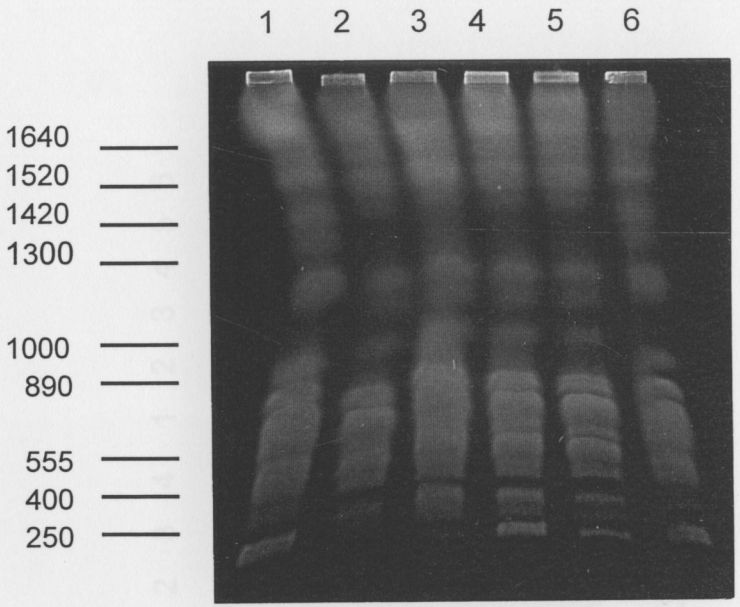


Fig. 4

93

A



B

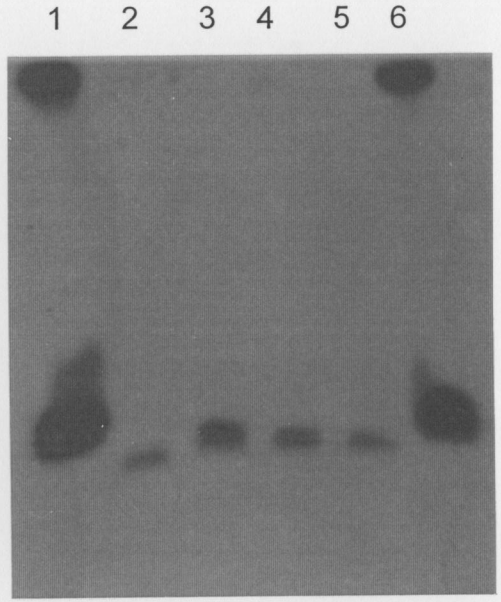


Fig. 5

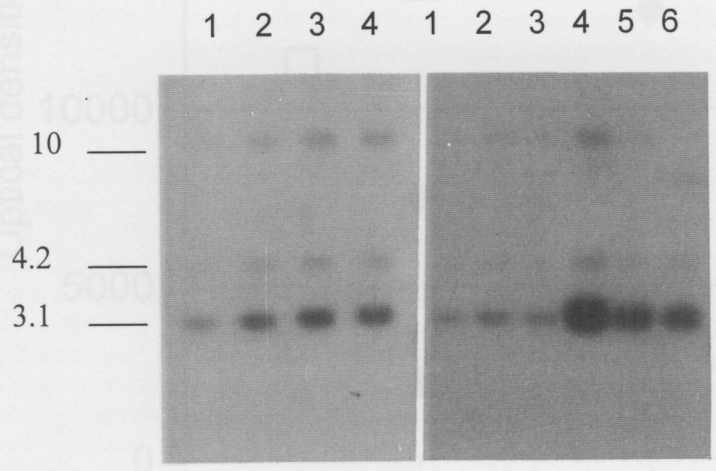


Fig. 6

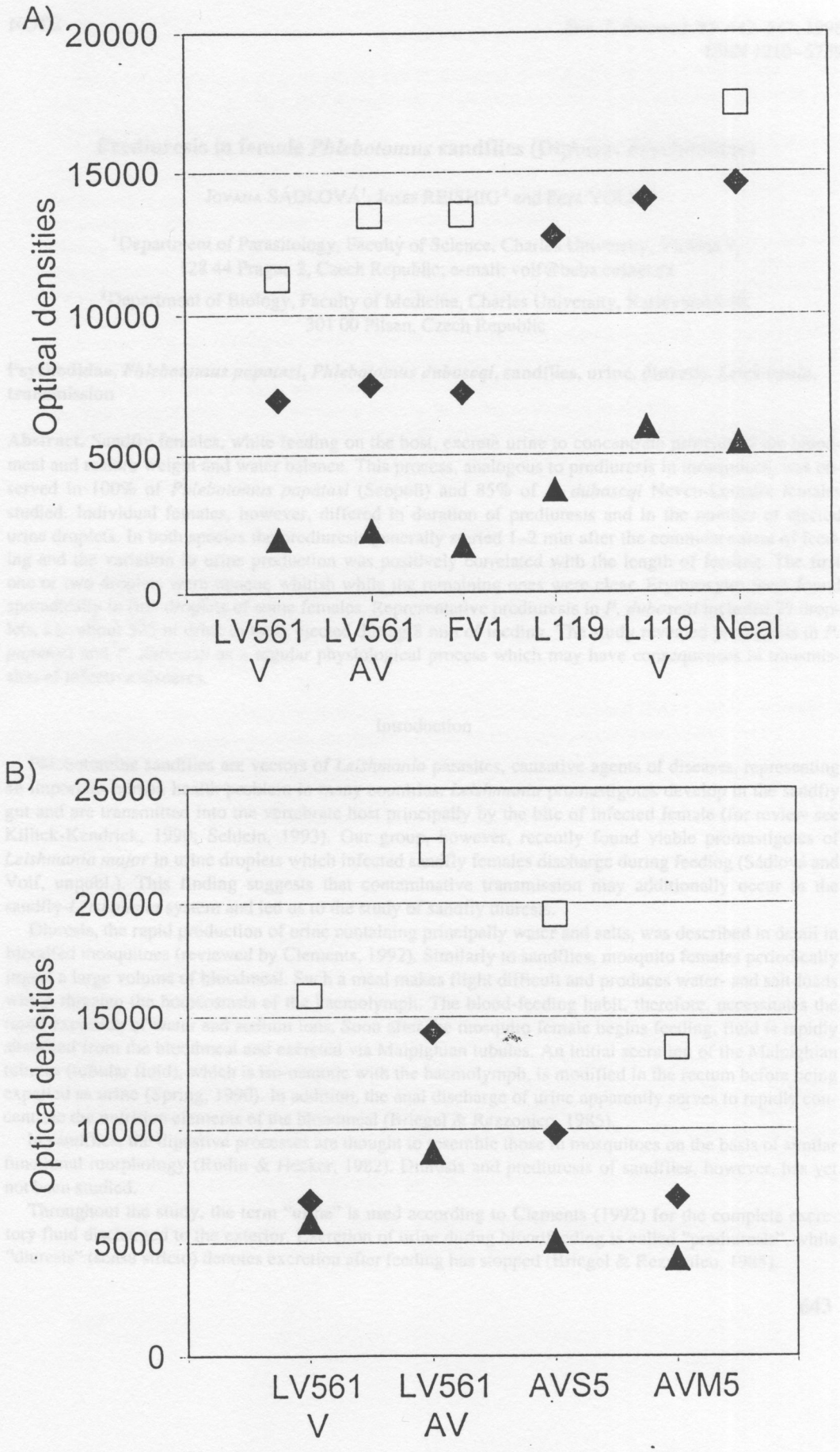

A)
20000
15000
10000
5000
0
Optical densities
LV561 V
LV561 AV
FV1
L119
L119 V
Neal
B)
25000
20000
15000
10000
5000
0
Optical densities
LV561 V
LV561 AV
AVS5
AVM5

Fig. 7

NOTE

Eur. J. Entomol. **95**: 643–647, 1998
ISSN 1210–5759

Prediuresis in female *Phlebotomus* sandflies (Diptera: Psychodidae)

JOVANA SÁDLOVÁ[1], JOSEF REISHIG[2] and PETR VOLF[1]

[1]Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic; e-mail: volf@beba.cesnet.cz

[2]Department of Biology, Faculty of Medicine, Charles University, Karlovarská 48, 301 00 Pilsen, Czech Republic

Psychodidae, *Phlebotomus papatasi*, *Phlebotomus duboscqi*, sandflies, urine, diuresis, *Leishmania*, transmission

Abstract. Sandfly females, while feeding on the host, excrete urine to concentrate proteins of the bloodmeal and restore weight and water balance. This process, analogous to prediuresis in mosquitoes, was observed in 100% of *Phlebotomus papatasi* (Scopoli) and 85% of *P. duboscqi* Neveu-Lemaire females studied. Individual females, however, differed in duration of prediuresis and in the number of ejected urine droplets. In both species the prediuresis generally started 1–2 min after the commencement of feeding and the variation in urine production was positively correlated with the length of feeding. The first one or two droplets were opaque whitish while the remaining ones were clear. Erythrocytes were found sporadically in first droplets of some females. Representative prediuresis in *P. duboscqi* included 27 droplets, i.e., about 325 nl urine in total, ejected during 8 min of feeding. The study revealed prediuresis in *P. papatasi* and *P. duboscqi* as a regular physiological process which may have consequences in transmission of infective diseases.

Introduction

Phlebotomine sandflies are vectors of *Leishmania* parasites, causative agents of diseases, representing an important human health problem in many countries. *Leishmania* promastigotes develop in the sandfly gut and are transmitted into the vertebrate host principally by the bite of infected female (for review see Killick-Kendrick, 1990; Schlein, 1993). Our group, however, recently found viable promastigotes of *Leishmania major* in urine droplets which infected sandfly females discharge during feeding (Sádlová and Volf, unpubl.). This finding suggests that contaminative transmission may additionally occur in the sandfly-*Leishmania* system and led us to the study of sandfly diuresis.

Diuresis, the rapid production of urine containing principally water and salts, was described in detail in bloodfed mosquitoes (reviewed by Clements, 1992). Similarly to sandflies, mosquito females periodically ingest a large volume of bloodmeal. Such a meal makes flight difficult and produces water- and salt-loads which threaten the homeostasis of the haemolymph. The blood-feeding habit, therefore, necessitates the rapid excretion of water and sodium ions. Soon after the mosquito female begins feeding, fluid is rapidly absorbed from the bloodmeal and excreted via Malpighian tubules. An initial secretion of the Malpighian tubules (tubular fluid), which is iso-osmotic with the haemolymph, is modified in the rectum before being expelled as urine (Spring, 1990). In addition, the anal discharge of urine apparently serves to rapidly concentrate the nutritive elements of the bloodmeal (Briegel & Rezzonico, 1985).

In sandflies, the digestive processes are thought to resemble those in mosquitoes on the basis of similar functional morphology (Rudin & Hecker, 1982). Diuresis and prediuresis of sandflies, however, has yet not been studied.

Throughout the study, the term "urine" is used according to Clements (1992) for the complete excretory fluid discharged to the exterior. Excretion of urine during bloodfeeding is called "prediuresis", while "diuresis" (sensu stricto) denotes excretion after feeding has stopped (Briegel & Rezzonico, 1985).

Material and methods

Phlebotomus papatasi (Cyprus strain) and *P. duboscqi* (Senegal strain) were maintained on 50% sucrose at 25–26°C and a 14L : 10D photoperiod. Flies used to establish colonies were received from the Imperial College in Ascot.

Twenty females of each species, 5–6 days post-emergence, were allowed to feed on a human finger. The feeding process was observed with a stereomicroscope, recorded and analyzed afterwards. Urine droplets were caught on a piece of coverslip held with forceps behind the anus. Air dried drops were fixed by methanol, stained with Giemsa and subsequently examined for the presence of erythrocytes. Total feeding time was measured from insertion of the fascicle into the skin (if multiple probing occurred, the last penetration was measured) to female take off. Excretion time designates the length of prediuresis; i.e., the period from appearance of the first urine droplet to female take off (they flew away immediately after withdrawing their fascicles from the skin).

Additionally, the feeding of sandfly females on anaestetized animals (hamsters, mice) or a human arm was repeatedly videorecorded in order to evaluate exact time course of excretion and the volume of droplets. Volume of urine droplets was determined by measuring the diameter of an apparently spherical droplet. This method gave data on the actual volume flow rates expressed in nl/min.

Statistical evaluations were made using the PC program Statgraphics, version 5.0. Non-parametric tests were used for the analysis of droplets numbers, excretion time and feeding time where data distribution were significantly different from the normal distribution.

Results

The basic characteristics of prediuresis for both species are shown in Table 1. *P. papatasi* had a significantly longer excretion time and produced significantly higher number of droplets than *P. duboscqi* (Fig. 1). Prediuresis was observed in all *P. papatasi* flies. However, in one female, droplets were not ejected away and urine accumulated on the anus. In *P. duboscqi*, three females did not expel urine and flew away after a very rapid engorgement.

TABLE 1. The comparison of prediuresis characteristics in *P. papatasi* (PAP) and *P. duboscqi* (DUB) females. The difference between species was tested by non-parametric (Mann-Whitney) test.

	Droplets number		Excretion time (s)		Feeding time (s)	
	PAP	DUB	PAP	DUB	PAP	DUB
Median	20.5	6	193	96	254	206
Lower quartile	13	1.5	138	69	214	137
Upper quartile	33	12.5	224	159	338	255
No.	20	20	20	17	20	20
Difference between species	P = 0.0007 Z = –3.3725		P = 0.0044 Z = –2.8498		P = 0.0498 Z = –1.9617	

Besides interspecific differences, considerable intraspecific variations in urine production were observed, especially in *P. papatasi* (Fig. 1). In both species, the variation in droplet numbers coincided with feeding times. Generally, the longer the total feeding time, the higher the number of droplets (Spearman test; coefficient 0.5912, P = 0.0100 for *P. papatasi* and coefficient 0.6055, P = 0.0083 for *P. duboscqi*).

The first droplet appeared most often within the range 50–92 s and 70–125 s (lower and upper quartiles) with median 69 and 92 s in *P. papatasi* and *P. duboscqi*, respectively. The later start of prediuresis in *P. papatasi*, the longer was the feeding time (Spearman test, coefficient 0.7245, P = 0.0016). In *P. duboscqi*, however, a correlation between the start of prediuresis and the length of feeding was not found (coefficient 0.3899, P = 0.1188).

A time course of prediuresis based on videorecorded *P. duboscqi* included twenty seven droplets ejected during 8 min of feeding (Fig. 2). The first droplet was released after 91 s and than the time

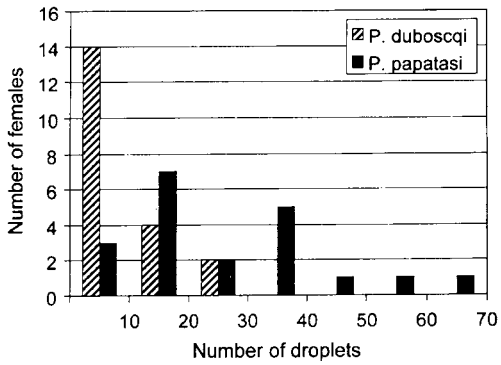


Fig. 1. Frequency histogram depicting urine production of *P. papatasi* and *P. duboscqi* females feeding on human finger.

intervals shortened to the rate of about one droplet per 10 s. The highest urine flow rate (8 droplets/min) was reached in the 7th min of feeding. The volumes of urine droplets in this female ranged from 2.7 nl (the first one) to 21.9 nl with the average 12.1 (±2) nl. In total, the female excreted about 325 nl of urine during bloodfeeding.

Extrusion of a droplet of urine takes about a second and involves a slow tilting upwards of the terminal abdominal segments. The droplet is then very quickly and forcefully ejected, often over 5 mm (Fig. 3). After this, the terminalium quickly returns to its original position. The first 1–2 droplets were often opaque whitish, the following ones were clear. Sporadically, the first droplets were reddish with blood; erythrocytes were found in the first droplets of five (25%) *P. papatasi* and two (10%) *P. duboscqi* females.

Discussion

Prediuresis in sandfly females starts when the midgut is partially filled by blood. We suppose that, similarly to mosquitoes, abdominal distension could be the initial stimulus that leads to the induction of prediuresis. Correlation between total feeding time and the start of diuresis was found in *P. papatasi* but not in *P. duboscqi*. The latter resembles the situation reported for *Aedes aegypti* (L.) by Mellink et al. (1982).

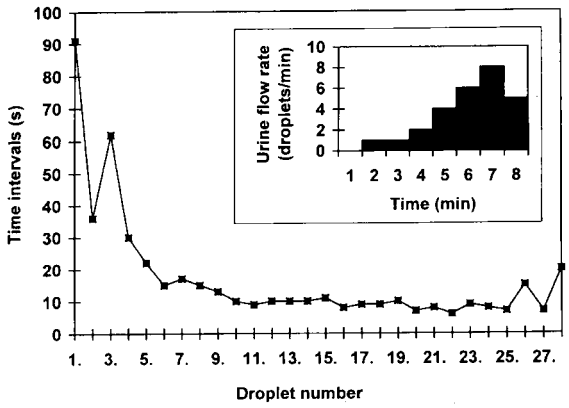


Fig. 2. Time course of urine excretion of *P. duboscqi* female feeding on human arm.

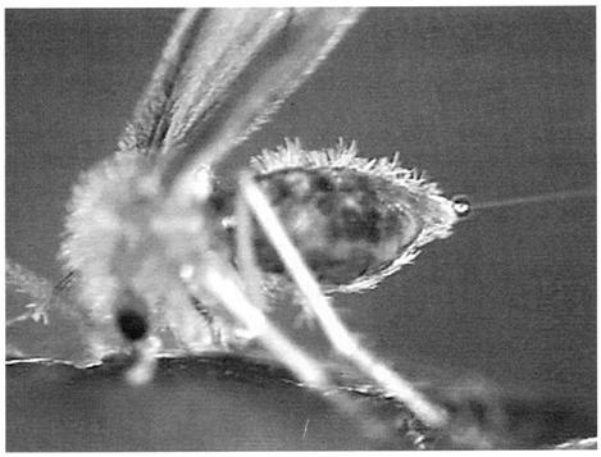


Fig. 3. Ejection of the urine by *P. duboscqi* female during blood-feeding. A picture taken from a video recording.

In mosquitoes, the urine excretion usually begins in less than 2 min after the commencement of feeding (i.e., while the insect is still engorging) (Williams et al., 1983; Spring, 1990) and passes through three phases which differ in the rate of flow and urine composition. The first phase with a high rate of urine flow takes the first few minutes (peak phase) and is followed by a steady decline (post peak phase) to a relatively stable low flow rate (late phase) (Williams et al., 1983; Clements, 1992). In our experiments, the high urine production within the first minutes of feeding coincides probably with the peak phase of urine production described for mosquitoes.

Certain species/strains of mosquitoes discharge clear fluid while others dispose a reddish fluid containing erythrocytes. High variability was described also for excretion rates, number of droplets, and their size (Nijhout & Carrow, 1978; Jones & Brandt, 1981; Briegel & Rezzonico, 1985; Vaughan et al., 1991). In our experiments, *P. papatasi* and *P. duboscqi* sandflies produced a predominately clear fluid, only the first urine drops contained blood in some cases. This indicates efficient filtration and resembles the situation in *Anopheles albimanus* Wiedemann and *A. quadrimaculatus* Say (Briegel & Rezzonico, 1985) and in *Anopheles freeborni* Aitken (Nijhout & Carrow, 1987).

Anopheline mosquitoes, which are unable to distend the midgut with bloodmeal efficiently, use prediuresis to accumulate nutritions and significantly increase protein intake. This, in consequence, leads to enhanced fecundity (Briegel & Rezzonico, 1985). A similar role of prediuresis can be predicted for sandflies. Because of the correlation between feeding time and the number of urine droplets (i.e., the volume of urine excreted) sandfly females which feed slowly may withdraw relatively larger amounts of blood from the host. On the other hand, a longer feeding time increases the probability to evoke host defensive behaviour. As indicated by great intra-species variations in the feeding habits of *P. papatasi* and *P. duboscqi*, this trade-off might be solved individually by each of the females within a species.

ACKNOWLEDGEMENTS. We thank R. Killick-Kendrick (Imperial College, Ascot) for providing *Phlebotomus* flies used to establish our colonies. The study was supported by the Ministry of Education of the Czech Republic (project VS96/142).

References

BRIEGEL H. & REZZONICO L. 1985: Concentration of host blood protein during feeding by anopheline mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* **22**: 612–618.

CLEMENTS A.N. 1992: *The Biology of Mosquitoes. Vol. 1. Development, Nutrition and Reproduction.* Chapman and Hall, London, 509 pp.

JONES J.C. & BRANDT E. 1981: Fluid excretion by adult Aedes aegypti mosquitoes. *J. Insect Physiol.* **27**: 545–549.

KILLICK-KENDRICK R. 1990: The life-cycle of Leishmania in the sandfly with special reference to the form infective to the vertebrate host. *Ann. Parasitol. Hum. Comp.* **65**: 37–42.

MELLINK J.J., POPPE D.M.C. & VAN DUIN G.J.T. 1982: Factors affecting the blood-feeding process of laboratory strain of Aedes aegypti on rodents. *Entomol. Exp. Appl.* **31**: 229–238.

NIJHOUT H.F. & CARROW G.M. 1978: Diuresis after a bloodmeal in female Anopheles freeborni. *J. Insect Physiol.* **24**: 293–298.

RUDIN W. & HECKER H. 1982: Functional morphology of the midgut of a sandfly as compared to other hematophagous Nematocera. *Tissue Cell* **14**: 751–758.

SCHLEIN Y. 1993: Leishmania and sandflies: Interactions in the life cycle and transmission. *Parasitol. Today* **9**: 255–257.

SPRING J.H. 1990: Endocrine regulation of diuresis in insects. *J. Insect Physiol.* **36**: 13–22.

VAUGHAN J.A., NODEN B.H. & BEIER J.C. 1991: Concentration of human erythrocytes by anopheline mosquitoes (Diptera: Culicidae) during feeding. *J. Med. Entomol.* **28**: 780–786.

WILLIAMS J.C., HAGEDORN H.H. & BEYENBACH K.W. 1983: Dynamic changes in flow rate and composition of urine during the post-bloodmeal diuresis in Aedes aegypti (L.). *J. Comp. Physiol.* **153**: 257–265.

Received July 21, 1998; accepted September 25, 1998

Occurrence of *Leishmania major* in sandfly urine

J. SÁDLOVÁ *and* P. VOLF*

Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

(*Received 15 October 1998; revised 2 December 1998; accepted 3 December 1998*)

SUMMARY

Promastigotes of *Leishmania major* were frequently detected in the urine droplets discharged by infected *Phlebotomus papatasi* and *P. duboscqi* females during feeding. Parasites were present in the urine of 37·5% *P. papatasi* and 16·1% *P. duboscqi* females, even in those with low intensity gut infections. Free-swimming forms (elongated nectomonads, short slender promastigotes and metacyclic forms) predominated in excreted droplets. Viability of excreted parasites was proved by cultivation on blood agar, and the presence of metacyclic forms in urine droplets was confirmed by specific fluorescence assay with 3F12 antibodies. While the release of promatigotes from the anus of the sandfly was frequent, these were rarely egested from the mouth-parts of sandfly females (1·3% for *P. duboscqi* and 0% for *P. papatasi*) fed on microcapillaries, even if the females were heavily infected. The possible role and significance of the discharge of parasites in sandfly urine are discussed.

Key words: *Phlebotomus* spp., transmission, urine, prediuresis, capillary feeding, metacyclic promastigotes.

INTRODUCTION

In the suprapylarian *Leishmania* spp., including *L. major*, part of the life-cycle takes place both in the midgut and foregut of the sandfly vector. Amastigotes ingested with a bloodmeal transform into promastigotes which attach to the midgut wall and subsequently migrate to the stomodeal valve, oesophagus, pharynx and, eventually, to the mouth-parts (for review see Killick-Kendrick, 1990; Schlein, 1993).

The location of parasites in the sandfly digestive tract is of crucial importance for the transmission to the vertebrate host. In this respect, 2 main hypotheses have been suggested: either parasites emerge from an infected sandfly during feeding only if the proboscis itself is infected (Adler & Theodor, 1935) or they are regurgitated with a backflow of ingested blood. Originally, the regurgitation was supposed to result from a mechanical block of the foregut (Shortt & Swaminath, 1928) or stomodeal valve (Warburg & Schlein, 1986) by the parasites. More recently, the damage to the chitin layer of the stomodeal valve by chitinolytic enzymes produced by the parasites was proposed to be the cause of the regurgitation of parasites from the thoracic midgut (Schlein, Jacobson & Shlomai, 1991; Schlein, Jacobson & Messer, 1992). A third possible way of transmission, the inoculation of parasites into the host skin with sandfly saliva, is supported by the study of Killick-Kendrick *et al.* (1996), who found metacyclic promastigotes of *L. tropica* invading salivary glands of *P. duboscqi*. Contaminative transmission by ingestion of infected sandfly takes place in the genus *Sauroleishmania* (Lainson & Shaw, 1987) and probably occurs in transmission of *Leishmania* to dogs (reviewed by Killick-Kendrick, 1979). In man, contaminative transmission may occur occasionally when an infected biting fly is crushed on the skin (Adler, 1929).

In the present work we studied another possible route of contaminative transmission: the discharge of parasites in sandfly urine. The term 'urine' is used here according to Clements (1992) for the excretory fluid discharged to the exterior during female's feeding on the vertebrate host. In mosquitoes the rapid anal excretion of urine during feeding is called prediuresis (Briegel & Rezzonico, 1985). This phenomenon enables the female to concentrate proteins of the bloodmeal and both physical (pyloric armatures) and metabolic (active transport into Malpighian tubules) filtrations are probably involved (Vaughan, Noden & Beier, 1991). In sandflies, prediuresis was observed in the majority of blood-feeding females studied (Short & Swaminath, 1928; Sádlová, Reishig & Volf, 1999) and previous study of *P. papatasi* and *P. duboscqi* (Sádlová *et al.* 1999) showed that first droplets ejected contained erythrocytes in 25% and 10% of females, respectively.

MATERIALS AND METHODS

Sandfly infections

Phlebotomus papatasi (Cyprus strain) and *P. duboscqi* (Senegal strain) obtained from Professor Killick-

* Corresponding author. Tel: +4202 21953195. Fax: +4202 299713. E-mail: volf@beba.cesnet.cz

Kendrick, Imperial College in Ascot, were maintained on 50% sucrose at 25–26 °C and 14/10 LD photo-period.

Leishmania major, LV 561 strain (MHOM/IL/67/LRC-L 137 Jericho-II) was maintained *in vitro* at 23 °C on SNB-9 blood agar (Diamond & Herman, 1954) and, concurrently, in BALB/c mice. Parasites isolated from the mouse were used for sandfly infection after 1–3 *in vitro* subcultures.

P. duboscqi and *P. papatasi* females were fed through a chick-skin membrane with infective food containing either 2×10^6 promastigotes or 1×10^6 amastigotes/ml. Promastigotes from 4-day-old cultures were resuspended in heat-inactivated human blood. Amastigotes derived from mouse lesions were resuspended in human erythrocytes mixed (1:1 v/v) with Schneider's medium supplemented with gentamicin (45 μg/ml).

Examination of urine droplets and capillary contents

Microcapillary feeding (Hertig & McConnell, 1963) was used for testing whether parasites are ejected from mouthparts. Simultaneously, the urine droplets discharged during the feeding were examined. In the first series of experiments, 235 flies were fed on microcapillaries containing SNB-9 (over-lay medium for blood agar) 10 or 14 days after an infective bloodmeal. During capillary feeding, the excreted droplets were caught on a piece of a cover-slip. Immediately after feeding, contents of capillaries were blown onto slides and flies were dissected for detection of *Leishmania* infections. Infected flies (150 females) were the subjects of further analysis.

In the second series of experiments, 100 females were allowed to feed on the capillary for 1·5–2 min only, starting from the first sucking movements of the clypeus. Dissection revealed that 53 females were infected. While during such short feeding time urine excretion usually did not start, only capillary contents were examined.

Amastigote-infected females were fed on capillaries 14 days after infection and excreted droplets were examined for parasite presence as described above.

Parasite morphometry

Capillary contents and urine droplets from infected flies were air dried, fixed with methanol and stained with Giemsa. The body length and width and the flagellum length of parasites were measured under the light microscope with an oil-immersion objective. However, the length of flagellum and the position of the kinetoplast were not visible in some parasites due to the dense background produced by the excreted urine. Therefore, out of the 6 morphological categories defined by Čiháková & Volf (1997) three (short broad promastigotes, rounded promastigotes and paramastigotes) were lumped together into one designated as 'broad forms' (BF). The other 3 categories, i.e. elongated nectomonads (EN), short slender promastigotes (SP1), and metacyclic promastigotes (MP) remained as described previously. Parasites with flagellum which cannot be measured were not classified. Data from gut smears (day 9 after the infective meal; Čiháková & Volf, 1997) were used for comparison of *Leishmania* morphometry in the midgut versus that excreted in droplets.

Leishmania viability test

To examine the viability of discharged parasites, infected *P. duboscqi* females were placed for capillary feeding on the top of a multiwell plate (GAMA n.p. České Budějovice). During capillary feeding, the urine was directed into the wells filled with blood agar and an over-lay medium. The contents of the wells were examined daily for parasites.

Immunofluorescence detection of metacyclic LPG

Excreted droplets of the urine were dried, fixed with methanol and incubated for 30 min with the monoclonal antibody 3F12 specific for the metacyclic stage lipophosphoglycan (Sacks & Da Silva, 1987). Mouse ascitic fluid was diluted 1:20 in 20 mM Tris buffer with 0·1% Tween 20 (Tris–Tw) and 5% fetal calf serum. After washing, slides were incubated for 30 min with FITC-conjugated swine anti-mouse IgG (SEVAC, Prague) diluted 1:20 in Tris–Tw, washed and stained for 2 min with Evans Blue. Positive and negative promastigotes were then detected by fluorescence microscopy using an oil immersion objective. Smears made from 6-day-old cultures of LV561 were used as a positive control. In negative controls, the antibody-incubation step was omitted.

Statistical analysis

All the statistical evaluations were made using the Statgraphics 5.0 programme.

RESULTS

Detection of Leishmania *parasites in urine*

Urine droplets of promastigote-infected sandflies contained promastigotes in 37·5% and 16·1% females of *P. papatasi* and *P. duboscqi*, respectively (Table 1). The difference between these two species is significant after log-linear analysis ($\chi^2 = 4{\cdot}2594$, D.F. = 1, $P = 0{\cdot}0390$) even when the results of individual experiments in one series also differed significantly ($\chi^2 = 34{\cdot}6375$, D.F. = 11, $P = 0{\cdot}0003$). On the other hand, the difference among the actual

Table 1. Representation of *Leishmania* parasites in urine droplets of *Phlebotomus papatasi* and *P. duboscqi*

Vector species	Time post-infective bloodmeal		
	10th day	14th day	Total
P. papatasi			
Number of infected flies examined	27	13	40
Number (%) of flies with positive droplets	10 (37)	5 (38·5)	15 (37·5)
P. duboscqi			
Number of infected flies examined	31	31	62
Number (%) of flies with positive droplets	8 (25·8)	2 (6·5)	10 (16·1)

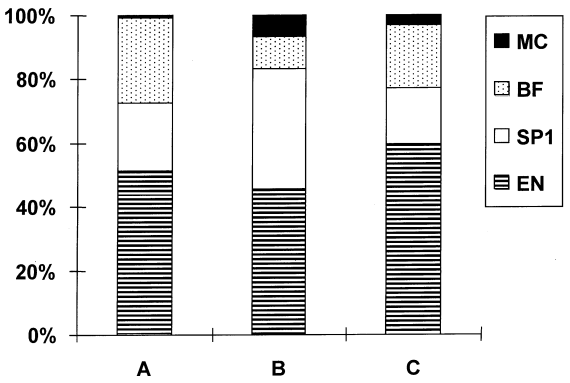


Fig. 1. Relative proportions of morphotypes from smears of gut contents (A) and from excreted droplets 10th (B) and 14th (C) day post-infection in both vector species. EN, elongated nectomonads; SP1, short slender promastigotes; BF, broad forms; MC, metacyclic promastigotes.

numbers of excreted parasites on the 10th and 14th days after infection was not statistically significant ($\chi^2 = 2{\cdot}7924$, D.F. = 1, $P = 0{\cdot}0947$).

In *P. papatasi*, the number of excreted parasites varied from 2 to 125 per female (average 21, median 8 parasites). The range of *P. duboscqi* was 1–260 (average 44, median 11 parasites per female). Parasites were concentrated in the first one or the first few ejected droplets.

Although the majority of females excreting parasites showed heavy (more than 500 parasites per gut) or medium (100–500 parasites per gut) infection, we detected parasites in urine droplets of 2 *P. papatasi* females with as few as 2 or 20 visible parasites in the gut.

Parasites were also detected in the urine of females infected by amastigotes on day 14 after the infective bloodmeal.

Capillary contents

In the first series of experiments parasites were ejected into a capillary by 1 *P. duboscqi* female (1·3 % of infected flies, $n = 79$) and no parasites were detected in capillary contents of infected *P. papatasi* females ($n = 71$). In the second series of experiments, when females were capillary fed for 1·5–2 min only, no parasites were found in 34 infected females of *P. duboscqi* and 19 infected females of *P. papatasi*.

Morphometrical analysis

All parasites found in capillaries ($n = 9$) were metacyclic promastigotes (MP). On the other hand, different morphological forms were detected in urine droplets. The relative proportion of individual forms in urine droplets (Fig. 1) was analysed by log-linear analysis. It differed significantly between the 10th and 14th day of examination ($\chi^2 = 15{\cdot}7854$, D.F. = 3, $P = 0{\cdot}0003$). Differences between the two *Phlebotomus* species were not significant ($\chi^2 = 4{\cdot}0705$, D.F. = 3, $P = 0{\cdot}3814$). The morphometric data of distinct forms are given in Table 2.

Comparison of morphological forms excreted in urine 10 days post-infection versus that in females midgut shows that MP and SP1 were significantly more represented in urine (SP1: $\chi^2 = 7{\cdot}7038$, D.F. = 1, $P = 0{\cdot}0055$; MP: $\chi^2 = 4{\cdot}2458$, D.F. = 1, $P = 0{\cdot}0393$). In contrast, BF were more represented in gut smears ($\chi^2 = 10{\cdot}5090$, D.F. = 1, $P = 0{\cdot}0012$) and proportions of EN differed non-significantly ($\chi^2 = 0{\cdot}7154$, D.F. = 1, $P = 0{\cdot}3976$).

Corresponding morphological differences were obtained when measurements were evaluated for the total material (ignoring morphological categories) using a *t*-test. Excreted promastigotes were shorter ($t = 3{\cdot}357$, $P = 0{\cdot}0008$) and more slender than gut forms: the difference in body width was non-significant in *P. duboscqi* ($t = 1{\cdot}551$, $P = 0{\cdot}1236$) while highly significant in *P. papatasi* ($t = 5{\cdot}309$, $P < 0{\cdot}0001$). Almost no difference was found in flagellar lengths ($t = 0{\cdot}238$, $P = 0{\cdot}8116$).

Cultivation of excreted parasites

The cultivation confirmed that parasites excreted by *P. duboscqi* were viable. The portion of positive

Table 2. Dimensional morphology of four *Leishmania major* developmental stages excreted in droplets and emerged into the capillary (*)

(EN, elongated nectomonads; SP1, short slender promastigotes; BF, broad forms; MC, metacyclic promastigotes.)

Morphological forms	10 days post-infection					14 days post-infection				
	n	Mean	S.D.	Min	Max	*n*	Mean	S.D.	Min	Max
Body width (μm)										
EN	62	1·8	0·77	0·7	4·3	61	2·3	0·86	1·4	4·3
SP1	51	1·5	0·28	0·7	2·1	18	1·5	0·23	1·4	2·1
BF	14	3·6	1·34	2·9	7·1	20	3·4	0·70	2·9	4·3
MC	9	1·3	0·31	0·7	1·4	3	1·4	0·00	1·4	1·4
MC*	9	0·7	0·00	0·7	0·7	—	—	—	—	—
Body length (μm)										
EN	62	15·7	2·29	14·3	22·8	61	15·6	1·74	14·3	20·0
SP1	51	10·8	1·88	7·1	12·9	18	10·3	2·05	5·7	12·9
BF	14	8·4	2·56	4·3	12·9	20	11·6	3·68	4·3	20·0
MC	9	8·4	1·51	5·7	10·0	3	7·6	1·65	5·7	8·6
MC*	9	9·0	1·01	7·1	10·0	—	—	—	—	—
Flagellar length (μm)										
EN	62	17·6	4·14	7·1	28·6	61	15·2	2·64	10·0	22·8
SP1	51	13·4	3·58	7·1	21·4	18	11·3	2·21	7·1	14·3
BF	14	12·6	4·19	4·3	17·1	20	9·5	2·33	5·2	12·9
MC	9	18·2	4·39	12·9	25·7	3	16·7	1·65	15·7	18·6
MC*	6	20·2	2·1	17·1	22·8	—	—	—	—	—

cultures from sandfly urine (8·3 %, $n = 12$) was lower than the percentage of females with positive urine droplets after Giemsa staining (16·1 %). However, fungal contamination occurred in some cultures and this probably contributed to the underestimation of the presence of viable flagellates.

Detection of metacyclic LPG

The presence of metacyclic promastigotes in the sandfly urine was confirmed by the positive reaction of monoclonal antibodies 3F12 with some parasites. Exact counting of metacyclic forms was, however, hampered in some preparations by non-specific reaction of fluorescein conjugates with excreted liquid. In the control, monoclonal antibodies reacted positively with 17 % of promastigotes from 6-day-old cultures.

DISCUSSION

Recent knowledge implies that not only one, but several modes could be involved in *Leishmania* transmission by sandflies. These may act simultaneously or may be specific for individual vector–parasite species combinations.

From the evolutionary point of view the contaminative route of transmission (passing of infective forms in the excreta) is probably the original route of transmission occurring in monogenetic flagellates. It takes place also in some digenetic trypanosomatids, like in *Trypanosoma lewisi* transmitted by the rat flea or *T. cruzi* transmitted by reduviids. In these cases, the development of parasites in the hindgut enables the contaminative transmission. In 'peripylarian' *Leishmania* species, colonization of the hindgut is maintained prior to migration of promastigotes to the midgut and foregut, which has been interpreted as a recapitulation of the primitive hindgut development of ancestral flagellates (Lainson & Shaw, 1987). In suprapylarian spp., the hindgut development was completely lost. Therefore, the contaminative transmission was thought to be non-functional in the *Leishmania*–sandfly system.

Transmission of *Leishmania* species by sandfly excreta was rejected by Adler (1929) as '*P. papatasi* very seldom defecates during the act of feeding'. However, our recent study showed that 100 % of *Phlebotomus papatasi* and 85 % of *P. duboscqi* females discharged urine during the feeding on human arm (Sádlová *et al*. 1999). Shortt & Swaminath (1928) first described frequent discharge of droplets from the anus of blood-feeding *P. argentipes*. They, however, failed to demonstrate *L. donovani* in droplets and concluded: 'It seems unlikely that the habit of the fly here being discussed can have any bearing on the transmission of this parasite'. On the other hand, living promastigotes were occasionally found in urine droplets of *Lutzomyia youngi* infected by peripylarian species *L. braziliensis* (Y. Tang, personal communication).

In the present study, parasites were found more often in urine than in capillary contents. They were never ejected into the capillary by *P. papatasi*, and only once by *P. duboscqi*. This may correspond with the previously described absence of parasites of LV561 line in the foregut and proboscis of *P. papatasi* and their exceptional occurrence in the

proboscis of *P. duboscqi* (data shown in Čiháková & Volf, 1997). Similarly, Adler & Theodor (1935) found that *L. infantum* parasites emerge from an infected *P. perniciosus* into the capillary only if the distal part of the proboscis is infected (2 from 52 heavily infected flies in their experiment). An alternative explanation for the low percentage of females ejecting promastigotes into the capillary might be the fact that gorging females only were studied. As described by Warburg & Schlein (1986), *P. papatasi* females which failed to engorge, regurgitated fluid containing *L. major* parasites into capillaries more often than did the feeding ones.

Parasites ejected by *P. duboscqi* into the capillary were morphologically classified as metacyclic forms which corresponds with the data obtained in other parasite–vector combinations. These forms, highly infective for vertebrate host, were selectively egested during the 'forced feeding' also in *L. infantum* – *P. perniciosus* (Adler & Theodor, 1931) and *L. major* – *P. papatasi* (Warburg & Schlein, 1986; Saraiva *et al*. 1995).

Metacyclics were shown to represent an abundant form of promastigotes in the midgut content (Davies *et al*. 1990; Saraiva *et al*. 1995). The present study showed that free-swimming developmental stages including metacyclic promatigotes were even more frequently represented in excreted droplets than in the midgut. On the contrary, broad forms, likely to be attached to the gut by hemidesmosomes were found in urine droplets in lower proportion than in the midgut.

Our findings indicate a certain potential for the contaminative transmission of *Leishmania* and further study is needed to evaluate this possibility. Transmission by bite is certainly the predominant route by which promastigotes enter the host. However, experiments with capillary feeding suggest that it could be successful only in heavily infected flies when the stomodeal valve is heavily colonized by promastigotes and the foregut infection is established. Contaminative transmission, on the other hand, would not be so much conditioned by the number or the localization of parasites. Parasites occurred in urine even in females with low intensity of midgut infections.

We would like to thank Dr Y. Tang and Professor Killick-Kendrick for advice in microcapillary feeding, Dr Sacks for kindly providing us monoclonal antibodies and Professor Ashford and Professor Vávra for their helpful comments. The study was supported by the Ministry of Education of the Czech Republic (project VS96/142).

REFERENCES

ADLER, S. (1929). An analysis of the *Leishmania* sandfly problem. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **23**, 289–300.

ADLER, S. & THEODOR, O. (1931). Investigations on mediterranean kala azar. IV. Infection of sandflies with *Leishmania infantum*. Natural parasites of wild sandflies in Catania. Observations on *Trypanosoma ptyodactyli* Catouillard. *Proceedings of the Royal Society*, *B* **108**, 481–493.

ADLER, S. & THEODOR, O. (1935). Investigations on mediterranean kala azar. IX. Feeding experiments with *Phlebotomus perniciosus* and other species on animals infected with *Leishmania infantum*. *Proceedings of the Royal Society*, *B* **116**, 516–542.

BRIEGEL, H. & REZZONICO, L. (1985). Concentration of host blood protein during feeding by anopheline mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* **22**, 612–618.

CLEMENTS, A. N. (1992). *The Biology of Mosquitoes*, Vol. 1. *Development*, *Nutrition and Reproduction*. Chapman and Hall, London.

ČIHÁKOVÁ, J. & VOLF, P. (1997). Development of different *Leishmania major* strains in the vector sandflies *Phlebotomus papatasi* and *P. duboscqi*. *Annals of Tropical Medicine and Parasitology* **91**, 267–279.

DAVIES, C. R., COOPER, A. M., PEACOCK, C., LANE, R. P. & BLACKWELL, J. M. (1990). Expression of LPG and GP63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitology* **101**, 337–343.

DIAMOND, L. S. & HERMAN, C. M. (1954). Incidence of trypanosomes in the Canada goose as revealed by bone marrow culture. *Journal of Parasitology* **40**, 195–202.

HERTIG, M. & McCONNELL, E. (1963). Experimental infection of panamanian *Phlebotomus* sandflies with *Leishmania*. *Experimental Parasitology* **14**, 92–106.

KILLICK-KENDRICK, R. (1979). Biology of *Leishmania* in phlebotomine sandflies. In *Biology of Kinetoplastida*, vol. 2 (ed. Lumsden, W. H. R. & Evans, D. A.), pp. 396–460. Academic Press, London.

KILLICK-KENDRICK, R. (1990). The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. *Annales de Parasitologie Humaine et Comparée* **65**, 37–42.

KILLICK-KENDRICK, R., KILLICK-KENDRICK, M., TANG, Y. & BASTIEN, P. (1996). Metacyclic promastigotes of *Leishmania* in the salivary glands of experimentally infected phlebotomine sandflies. *Parasite* **3**, 55–60.

LAINSON, R. & SHAW, J. J. (1987). Evolution, classification and geographical distribution. In *The Leishmaniases in Biology and Medicine*, Vol. 1 (ed. Peters, W. & Killick-Kendrick, R.), pp. 1–120. Academic Press, London.

SACKS, D. L. & DA SILVA, R. P. (1987). The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *Journal of Immunology* **139**, 3099–3106.

SÁDLOVÁ, J., REISHIG, J. & VOLF, P. (1999). Prediuresis in female sandflies (Diptera: Psychodidae). *European Journal of Entomology* **95**, 643–647.

SARAIVA, E. M. B., PIMENTA, P. F. P., BRODIN, T. N., ROWTON, E., MODI, G. B. & SACKS, D. L. (1995). Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* in *Phlebotomus papatasi*. *Parasitology* **111**, 275–287.

SCHLEIN, Y. (1993). *Leishmania* and sandflies: Interactions in the life cycle and transmission. *Parasitology Today* **9**, 255–257.

SCHLEIN, Y., JACOBSON, R. L. & MESSER, G. (1992). *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proceedings of the National Academy of Sciences, USA* **89**, 9944–9948.

SCHLEIN, Y., JACOBSON, R. L. & SHLOMAI, J. (1991). Chitinase secreted by *Leishmnia* functions in the sandfly vector. *Proceedings of the Royal Society, B* **245**, 121–126.

SHORTT, H. E. & SWAMINATH, C. S. (1928). The method of feeding of *Phlebotomus argentipes* with relation to its bearing on the transmission of kala-azar. *Indian Journal of Medical Research* **15**, 827–836.

VAUGHAN, J. A., NODEN, B. H. & BEIER, J. C. (1991). Concentration of human erythrocytes by anopheline mosquitoes (Diptera: Culicidae) during feeding. *Journal of Medical Entomology* **28**, 780–786.

WARBURG, A. & SCHLEIN, Y. (1986). The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *American Journal of Tropical Medicine and Hygiene* **35**, 926–930.

SHRNUTÍ

(1) Faktory ovlivňující vývoj leishmanií v přenašeči.

(a) Srovnáním vývoje pěti kmenů a linií *L. major* ve dvou druzích flebotomů (*P. papatasi* a *P. duboscqi*) bylo zjištěno, že nejúspěšněji se vyvíjela virulentní linie kmene LV561 (nejvyšší procento infikovaných samic, nejvyšší podíl silných infekci, silné kolonizace stomodeální valvy, v jednom případě i metacykličtí promastigoti v proboscis), relativně dobře také avirulentní linie kmene LV561 a kmen FV1, nejméně úspěšně pak kmeny L119 a Neal (malé procento infikovaných samic, převažující slabé infekce, které nikdy nedosáhly k stomodeální valvě). Procento samic infikovaných jednotlivými kmeny a liniemi nebylo signifikantně ovlivněno druhem přenašeče (*P. papatasi* vs. *P. duboscqi*), avšak rozdíly v lokalizaci infekcí byly výraznější u *P. papatasi.*

Kmeny a linie leishmanií se signifikantně lišily v relativním zastoupení jednotlivých morfologických forem. Kmeny, které se vyvíjely ve flebotomech úspěšněji (LV561, FV1), vykazovaly výraznější prodloužení těla a bičíku oproti stavu v kultuře, zatímco špatně se vyvíjející kmeny měly tendenci ke zkrácení těla a bičíku (Neal) či větší šířce těla (L119).

Kmen LV561 (virulentní linie) byl úspěšně přenesen na křečka sáním tří infikovaných samic *P. duboscqi*.

(b) Studované kmeny a linie *L. major* se výrazně lišily také infektivitou a virulencí pro myši BALB/c. U všech myší infikovaných virulentní linií kmene LV561 se brzy vyvinuly rychle rostoucí léze. Kmeny FV1 a L119 způsobily tvorbu lézí o 12 týdnů později a pouze u části infikovaných zvířat. Kmen Neal a atenuovaná linie kmene LV561 byly avirulentní, ale neztratily zcela infektivitu pro hostitele: kultivace z uzlin byly z větší části pozitivní. Oba tyto kmeny byly předmětem dalších pokusů: reisolovaní parasiti kmene Neal byly již schopni tvořit v BALB/c myších léze, avirulentní linie kmene LV561 byla studována podrobněji (viz bod d).

(c) Exprese metacyklického LPG ve stacionární růstové fázi kultur byla nejvyšší u virulentní linie kmene LV561 (16,1%), nižší u kmene FV1 (5,1%) a atenuované linie LV561 (2,1%) a velmi nízká u kmenů L119 a Neal (0,2%). Velmi nízká tvorba metacyklického LPG pravděpodobně souvisí s defekty v tvorbě procyklického LPG. V případě kmene L119 je defekt v procyklickém LPG prokázán, u kmene Neal o něm svědčí dosud nepublikované výsledky jiných autorů (Prof. Turco, osobní sdělení).

Studiem povrchové proteázy gp63 bylo zjištěno, že gp63 lokus je u všech kmenů a linií lokalizován na stejném chromosomu. Hybridizační pokusy s gp63 sondou neprokázaly rozdíly mezi kmeny a liniemi ve struktuře genů kódujících gp63 ani v jejich kvantitě. Značné rozdíly byly naopak zjištěny v množství exprimovaného proteinu a jeho proteázové aktivitě. Uvažujeme-li virulentní linii kmene LV561 jako standart pro kvantifikaci, pak kmen FV1 produkuje nepatrně menší množství tohoto proteinu, atenuovaná linie kmene LV561 je v tomto směru značně defektní a naopak u kmenů L119 a Neal je tvorba gp63 výrazně zvýšena. Obdobné rozdíly byly pozorovány v proteázové aktivitě gp63.

(d) Atenuovaná linie kmene LV561 pasážovaná 5x přes flebotomy byla označena AVS5, linie pasážovaná 5x přes myši AVM5. V *P. papatasi* se obě tyto linie vyvíjely

prokazatelně lépe (kvantitou i lokalizací infekcí), než linie výchozí. Naopak v myši BALB/c nebyla u pasážovaných linií zvýšena ani virulence (tvorba lézí) ani infektivita (procento pozitivních kultivací z uzlin).

Exprese metacyklického LPG byla u pasážovaných linií jen slabě zvýšena oproti výchozí atenuované linii. Exprese a aktivita gp63 a haemaglutinační aktivita vzrostly u obou linií, nedosáhly však úrovně zjištěné u virulentní linie.

(2) Mechanismus přenosu leishmanií flebotomy.

(a) Prediurese je fyziologický proces, detailně popsaný u komárů, který spočívá v rychlé filtraci přijaté krve a vylučování moči již během sání na hostiteli. Tím se samice rychle zbaví vody a solných iontů, které by jinak bránily letu a narušily homeostázi organismu, a zároveň přijme větší koncentrace proteinů. V této práci je poprvé popsána prediurese také u samic flebotomů. Byla pozorována u 100% samic *P. papatasi* a 85% samic *P. duboscqi* sajících na lidské ruce.

Prediurese začínala 1-2 min po začátku sání vyloučením několika bělavých kapek moči, další kapky byly čiré. Erytrocyty byly nalezeny jen výjimečně, což svědčí o velmi důkladné filtraci vylučované moči. Kapky byly vystřikovány velkou rychlostí na vzdálenost přes 5 mm. Analýza videozáznamu sající samice *P. duboscqi* ukázala, že samice vyloučila celkem 325 nl moči (v 27 kapkách o průměrné velikosti 12 nl) během osmi minut sání na lidské ruce.

Mezidruhové rozdíly byly zaznamenány v celkové délce sání, v délce prediurese a počtu vyloučených kapek moči. Výrazná byla i vnitrodruhová variabilita v produkci moči; přitom čím delší byla délka sání, tím více moči samice vyloučila. Zřejmě tedy samice může buď sát déle a akumulovat více živin, ale vystavit se přitom vyšším riziku hostitelovi obranné reakce, nebo sát rychle s nižším příjmem bílkovin, ale také s menším rizikem (trade-off). Přes tuto variabilitu v jednotlivých parametrech prediurese jde zřejmě o běžný fyziologický jev, pravidelně přítomný u této skupiny krevsajícího hmyzu.

(b) Pomocí kapilárového sání flebotomů infikovaných *L. major* bylo zjištěno, že leishmanie byly přítomny v moči 37,5% *P. papatasi* a 16,1% *P. duboscqi*, zatímco do kapilár byli promastigoti vypuzeni jen jednou samicí *P. duboscqi* (n = 79). Počet promastigotů v moči jedné samice se pohyboval v rozmezí 2 – 125 (medián 8) u *P. papatasi* a 1 – 260 (medián 11) u *P. duboscqi*. Leishmanie byly přítomné i v moči samic se slabou infekcí mesenteronu.

Zatímco v obsahu kapiláry byly nalezeni pouze metacykličtí promastigoti, moč flebotomů obsahovala směs různých morfologických forem. Oproti mesenteronu byla v moči relativně více zastoupena volně plovoucí morfologická stádia (včetně metacyklických promastigotů), naopak méně zastoupeny byly široké haptomonády. Přítomnost metacyklických promastigotů v moči byla potvrzena pozitivní reakcí části promastigotů s monoklonální protilátkou proti metacyklickému LPG. Positivní kultivace prokázala životaschopnost vyloučených leishmanií.

ZÁVĚRY

- Schopnost leishmanií vyvíjet se úspěšně v přenašeči nekorelovala zcela s jejich virulencí pro myš (viz kmen L119 a atenuovaná linie kmene LV561). **V přenašeči a v hostiteli se tedy zřejmě uplatňují různé vlastnosti parasita nebo tytéž vlastnosti v různé míře.**

- **Schopnost jednotlivých kmenů a linií infikovat flebotomy korelovala dobře s jejich schopností produkovat metacyklický LPG**, což je v souladu s hypotézou, že LPG hraje důležitou roli ve vývoji leishmanií v trávícím traktu přenašeče. Konkrétní funkce LPG v přenašeči je vysvětlována dvěma odlišnými způsoby (viz literární přehled). Zajímavé je, že zatímco slabé infekce kmene Neal tři dny po infekčním sání by svědčily o úloze LPG již v peritrofickém prostoru (rezistence vůči trávícím enzymům flebotoma), výrazný pokles procenta infikovaných samic u kmene L119 nastal až mezi třetím a šestým dnem po infekčním sání (v době, kdy samice defekuje zbytky potravy), což odpovídá spíše hypotéze o roli LPG v přichycení k střevnímu epitelu. Naopak, **pro vývoj v hostiteli nebyl stav LPG rozhodujícím faktorem**: i kmen L119 s defektním LPG byl částečně virulentní a naopak atenuovaná linie kmene LV561 byla avirulentní, přestože tvorba metacyklického LPG zde byla desetkrát vyšší než u kmene L119. **Virulence je tedy komplexní fenotypický znak a LPG je v tomto ohledu pouze jedním z důležitých faktorů.**

- **Získané výsledky nesvědčí pro zásadní význam gp63 v přenašeči, ale spíše potvrzují roli této povrchové proteázy v hostitelském organismu**: snížené množství gp63 nebránilo parasitům atenuované linie LV561 v úspěšné infekci flebotomů, linie však byla avirulentní pro myš. Naopak, kmeny L119 a Neal s vysokou proteázovou aktivitou gp63 sice byly do určité míry virulentní, ale neschopné úspěšného vývoje v přenašeči.

- To, že u obou kmenů s defektním LPG (L119, Neal) byla zjištěna nadprodukce gp63 zároveň naznačuje, že může jít o adaptivní odpověď parasita na jinak letální defekt. Obě tyto povrchové molekuly plní velmi podobné funkce v interakci s imunitním systémem hostitele (viz literární přehled). Je tedy možné, že **funkce LPG a gp63 se mohou v hostitelském organismu doplňovat a v případě defektu vzájemně nahrazovat**. Tento mechanismus může zvyšovat šanci parasita na přežití. Jak ale vyplývá z literárních dat i z výše uvedených výsledků s kmeny L119 a Neal, **v přenašeči zřejmě plní obě molekuly natolik odlišné funkce, že zde gp63 roli LPG nenahradí**.

- Tendence k růstu hemaglutinační aktivity u obou pasážovaných linií svědčí o jisté roli tohoto faktoru v obou částech životního cyklu leishmanií.

- Dlouhodobá kultivace organismů *in vitro* přináší riziko, že bude ztracena část jejich genetické výbavy kódující vlastnosti, které tyto organismy v umělém prostředí nutně nepotřebují. Výsledky této práce svědčí o tom, že **ztráta virulence může být u atenuovaných linií leishmanií definitivní**. Při opakovaném vystavení atenuovaných parasitů přirozenému selekčnímu tlaku se zvýšila schopnost vyvíjet se v přenašeči, neobnovila se však virulence pro hostitele.

- Proces prediurese a přítomnost živých promastigotů (včetně metacyklických stádií) v moči flebotomů představují možnost dosud neuvažovaného způsobu přenosu leishmanií na hostitele. Nakolik se tato potenciální cesta přenosu opravdu uplatňuje, je však nutno ověřit dalšími experimenty. Z evolučního hlediska je zajímavý už samotný fakt, že přítomnost infekčních stádií v exkrementech, tedy znak považovaný za fylogeneticky původní a přítomný již u monogeneticých trypanosomatidů, zůstal zachován u suprapylariálního druhu *L. major*.