Europe PMC Funders Group
Author Manuscript
Int J Parasitol. Author manuscript; available in PMC 2010 March 16.

Published in final edited form as:
Int J Parasitol. 2007 May ; 37(6): 589–593. doi:10.1016/j.ijpara.2007.02.002.

Increased transmission potential of *Leishmania major/ Leishmania infantum* hybrids

Petr Volf[a,*], **Ivana Benkova**[a], **Jitka Myskova**[a], **Jovana Sadlova**[a], **Lenea Campino**[b], and **Christophe Ravel**[c]

[a]Department of Parasitology, Faculty of Science, Charles University, Vinicna 7, Prague 2, 128 44, Czech Republic

[b]Unidade de Leishmanioses/CMDT, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal

[c]Laboratoire de Parasitologie, Centre National de Reference des Leishmania, 163 rue Broussonet, F34090 Montpellier, France

Abstract

Development of *Leishmania infantum/Leishmania major* hybrids was studied in two sand fly species. In *Phlebotomus papatasi*, which supported development of *L. major* but not *L. infantum*, the hybrids produced heavy late-stage infections with high numbers of metacyclic promastigotes. In the permissive vector *Lutzomyia longipalpis*, all *Leishmania* strains included in this study developed well. Hybrids were found to express *L. major* lipophosphoglycan, apparently enabling them to survive in *P. papatasi* midgut. The genetic exchange of the hybrids thus appeared to have enhanced their transmission potential and fitness. A potentially serious consequence is the future spread of the hybrids using this peridomestic and antropophilic vector.

Keywords

Leishmania transmission; Parasite-vector interaction; Emerging diseases

Recently, Ravel et al. (2006) described natural genetic hybrids between *Leishmania infantum* and *Leishmania major*. These two *Leishmania* species are transmitted by different sand fly vectors to different mammalian reservoir hosts. Human *Leishmania infantum* infection is a zoonosis with dogs acting as the main reservoir. It causes potentially fatal visceral disease and in Southern Europe it has been associated with human immunodeficiency virus (HIV)-positive patients (for review see Gramiccia and Gradoni, 2005). Throughout the Mediterranean, it is transmitted by about a dozen sand fly species of the subgenus *Larroussius* (Killick-Kendrick, 1999). In contrast, *Leishmania major* circulates in arid and semi-arid areas between rodents, causing self-healing cutaneous lesions in humans (for review see Gramiccia and Gradoni, 2005). In the Middle East and Magreb area it is transmitted by *Phlebotomus (Phlebotomus) papatasi* (Killick-Kendrick, 1999). The discovery of natural hybrids between these very divergent *Leishmania* species raised many questions about the genetic exchange processes of *Leishmania* and possible circulation of hybrids under natural conditions (Ravel et al., 2006). This led to the current study on experimental infections of *L. infantum/major* hybrids in sand flies.

*Corresponding author. Tel.: +420 2 2195 1815; fax: +420 2 2491 9704. volf@cesnet.cz.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Laboratory studies examining the development of different *Leishmania* strains in a range of sand fly species showed that these vectors fall into two groups. Few sand fly species are specific vectors as they display remarkable specificity for the *Leishmania* they transmit. For example, *P. papatasi* supports the development of *L. major* but not other parasite species tested (Killick-Kendrick et al., 1994; Pimenta et al., 1994). In contrast, most of the other sand fly species examined to date support the development of a broad range of *Leishmania* species and fall into the second group called permissive vectors (Volf and Myskova, 2007; Myskova et al., 2007). These include *Phlebotomus* species transmitting parasites of the *Leishmania donovani* complex (Pimenta et al., 1994; Myskova et al., in press) and *Lutzomyia longipalpis*, the New World vector of *Leishmania infantum* (Walters et al., 1993). We have chosen one member of each sand fly group to compare their susceptibility to infection by the hybrids and their presumed parental species, to evaluate their differences in fitness.

Differences were found between hybrids and parental *Leishmania* species in their development in *P. papatasi*. This led us to study lipophosphoglycan (LPG), the major surface molecule of the parasite, which is critical for *L. m*ajor to establish infection in *P. papatasi* (Pimenta et al., 1992). Terminal galactosyl residues of *L. major* LPG are recognized by the *P. papatasi* midgut receptor PpGalec (Kamhawi et al., 2004). The specific LPG-dependent attachment of *Leishmania* promastigotes to the *P. papatasi* midgut receptor enables the parasite to avoid expulsion when the sand fly defecates (Kamhawi et al., 2004). We found that the hybrids were recognized by the mAbs specific to β1,3-galactosyl-residues unique for LPG and secreted proteophosphoglycan of *L. major* (Kelleher et al., 1994; Ilg et al., 1996).

Four *Leishmania* strains were used: *L. major* LV561 (MHOM/IL/67/LRC-L137 Jericho II), *L. infantum* OG-VL (MHOM/TR/2000/OG-VL) and two hybrid strains isolated in the Leishmanioses Unit, Lisbon, from HIV-infected patients: LEM4833 (MHOM/PT/94/IMT208) and LEM4891 (MHOM/PT/2004/IMT367). Parasites were maintained on 199 medium (Sigma) supplemented with 20% FCS (Gibco) and gentamycin (50 μg/ml). Laboratory colonies of *P. papatasi* (originating from Sanliurfa, Turkey) and *Lutzomyia longipalpis* (Jacobina, Brazil) were maintained at 25-26 °C under a photoperiod of 14 h light/10 h dark. Female sand flies were fed through a chick-skin membrane with 5-day-old promastigotes (density of 10^6 cells per ml) in heat-inactivated rabbit blood. Blood-engorged females were separated, maintained at a constant temperature of 25 °C and sacrificed on days 2, 7 and 10 after the blood meal. The location and number of promastigotes in the midgut was estimated under a light microscope. Parasite density was graded according to accepted criteria (Cihakova and Volf, 1997), i.e. < 100, 100-1,000 and > 1,000 parasites/gut were graded as low, medium and heavy infection, respectively. Data were statistically evaluated using the Statgraphics 4.2 programme (Manugistics, Rockville, MD). Numbers of sand flies from four experiments were pooled and infection rates were statistically compared using a $\underline{X}^2$ test. In addition, *P. papatasi* midgut smears were fixed with methanol, stained with Giemsa and microscopically examined as described by Cihakova and Volf (1997). Briefly, the position of kinetoplast in relation to the nucleus was examined and body length, body width and flagellar length were measured. Parasite stages were classified according to descriptions given by Walters (1993). Metacyclic promastigotes were distinguished by flagellum at least 2.0 times the body length.

In *L. longipalpis* all parasite strains used developed well by producing high infection rates and a high percentage of heavy infections in all intervals studied (Fig. 1A). Infection rates of hybrids varied from 58 to 82% on days 2, 7 and 10 p.i. In general, both hybrid development in *L. longipalpis* was similar to both parental controls, i.e. *L. infantum* and *L. major*. On day 10, infection rates of hybrid H3 resembled those of *L. major* while hybrid H1 produced

Europe PMC Funders Author Manuscripts

infection rates similar to *L. infantum* (Fig. 1A). Colonization of the stomodeal valve was observed in the majority of infected females in all *Leishmania* strains used.

In *P. papatasi* (Fig. 1B) the highest infection rates were observed with *L. major*. In this positive control, the infection rate was about 70% and heavy infection 50% on day 10 p.i. On day 10 the colonization of the stomodeal valve was observed in 91% of infected females and metacyclic promastigotes represented 21% of morphological stages found in smears from late-stage infections. In contrast, *L. infantum* parasites were only detectable on day 2 and were absent in *P. papatasi* females examined on days 7 and 10.

Hybrid strains produced late-stage infections (on days 7 and 10 p.i.) in 35-58% of *P. papatasi* females, reaching heavy infections in about 15% on day 10 (Fig. 1B). The two different hybrids did not differ significantly in their infection rates until day 7 ($P < 0.001$). Importantly, both hybrids developed significantly better than *L. infantum* ($P < 0.001$ on every recorded day), although less efficiently than *L. major* ($P < 0.05$ on day 2, $P < 0.001$ on days 7 and 10) in *P. papatasi* (Fig. 1B). By day 10, hybrids had reached the stomodeal valve in half of infected females (50% and 59% for LEM4891 and LEM4833, respectively) heavily colonizing this part of the gut on some occasions (38% for LEM4891 and 18% for LEM4833). Metacyclic promastigotes represented about 11% of morphological stages found in smears from late-stage infections (9.3% for LEM4891 and 13.3% for LEM4833).

Mouse mAb WIC 79.3 (Kelleher et al., 1994) was used for detection of *L. major* LPG by indirect immunofluorescent microscopy and by direct agglutination. For immunofluorescent studies, promastigotes from 4-day-old culture (log phase) were washed in PBS (150 mM NaCl, pH 7.4) and adjusted to 10^7 cells per ml. Drops (10 μl) were air-dried on slides and fixed in methanol. The slides were washed in PBS and incubated for 30 min at room temperature with ascites fluid from hybridoma-bearing mice diluted to 1:500 in PBS. The promastigote-bound antibodies were detected with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) diluted to 1:500 in PBS. Parasites were then mounted in Vectashield mounting medium with propidium iodide (Vector Laboratories) and checked under an Olympus BX51 fluorescent microscope. In negative controls the diluted ascites fluid was replaced with PBS.

Indirect fluorescence (Fig. 2) revealed themAbs had a high reactivity with promastigotes of *L. major* (positive control) and no reaction with those of *L. infantum* (negative control). Intermediate fluorescence intensity was observed in hybrids. Their reaction with WIC 79.3 antibodies was weaker than that seen in the positive control. In most cases, a “patchy” reaction was observed in the hybrids (Fig. 2). No reaction was observed in negative controls where ascites fluid was replaced with PBS (data not shown).

In agglutination assays, suspension of promastigotes (2×10^7 cells per ml) was mixed with a equal volume of WIC 79.3 antibodies in PBS containing 5% FCS and incubated at room temperature for 1 h. After gentle mixing, the number of single, unagglutinated promastigotes was determined in a hemocytometer. Monoclonal antibodies agglutinated promastigotes of both hybrid strains. Agglutination profiles confirmed that the amount of surface LPG expressed by hybrids was lower than in the control *L. major* strain. In contrast, *L. infantum* promastigotes were not agglutinated by a high concentration of mAbs (Fig. 3).

Experimental infection of different sand fly species with hybrids and their presumed parental parasites revealed striking differences. While all *Leishmania* strains tested developed well in the permissive vector *L. longipalpis*, not all were able to do so in *P. papatasi*. In this specific vector, *L. major* and both hybrid strains produced late-stage infections (7 and 10 days p.i.) and colonized the stomodeal valve, while *L. infantum* was defecated from the midgut with blood meal remains and did not develop further. Results

obtained with the *L. infantum-P. papatasi* pair confirmed that the specificity of parasite-vector interaction is confined to late-stage development when parasites must attach to the midgut epithelium of the vector (review by Kamhawi, 2006). Our study further supports the hypothesis that LPG has a crucial role in the attachment of *L. major* to *P. papatasi* midgut. Both hybrid strains posses *L. major* LPG, as shown by indirect immunofluorescence and agglutination with WIC 79.3 antibodies. The presence of this LPG appears to enable them to attach to the *P. papatasi* midgut, apparently via PpGalec receptors.

During their life cycle, *Leishmania* parasites adapt themselves to varied and heterogeneous environments; those of insect vectors and vertebrate hosts differ in temperature, pH and many other parameters. Sexual recombination is conventionally believed to play a major role in organismal adaptive evolution. Therefore, one might expect such an event to occur in parasites, such as *Leishmania* spp., to ensure their fitness for survival in varying environments. In *Leishmania*, however, details of sexuality and genetic exchange remain elusive. Genetic exchange seems to be extremely rare under natural conditions (Tibayrenc and Ayala, 1999) and it was not possible to obtain hybrids by experimental crossings. As reviewed by Victoir and Dujardin (2002), *Leishmania* strains have developed non-sexual mechanisms for generating the large repertoire of genotypes and these asexual mechanisms contribute efficiently to parasite fitness. This postulation was made in light of the paradigm that parasites are more successful without sex (Ayala, 1998; Victoir and Dujardin, 2002). However, our study showed that fitness of *L. major/L. infantum* hybrids was increased when compared with *L. infantum*. Genetic exchange appears to have conferred on the latter a certain level of *L. major* LPG that enables the hybrid parasites to survive in a specific vector, *P. papatasi.* This means that sex may have increased the transmission potential of hybrids and positively affected parasite fitness.

The finding of *Leishmania* hybrids' ability to develop in *P. papatasi* may have important epidemiological implications. *Phlebotomus papatasi* is wide-spread in Europe, Africa and Asia, particularly in those areas that lie between 20 and 45 degrees in the northern latitudes. It breeds as far west as Portugal, throughout the Mediterranean littoral, the Adriatic region, the Balkans, the Middle East, North Africa, Central Asia and India. It is a peridomestic and antropophilic sand fly, reaching high densities in many places. In addition to its well-known aggressive behaviour in biting humans, *P. papatasi* transmits phleboviruses and zoonotic cutaneous leishmaniases caused by *L. major* (review by Lewis and Ward, 1987; Lane, 1993). On the other hand, this sand fly is not a vector of visceral leishmaniases because it is refractory to infection by *L. infantum* and *L. donovani* (Pimenta et al., 1994; Myskova et al., in press). Our findings revealed that *L.infantum/L. major* hybrids causing visceral disease in HIV-positive patients develop heavy late-stage infections in *P. papatasi.* This suggests that in nature, and particularly in Europe, hybrid strains may circulate by using this sand fly vector, thereby increasing the risk of their spreading into new foci throughout the broad range of *P. papatasi* distribution.

Acknowledgments

We would like to thank Anna Svarovska for help during the experimental work and K.P. Chang for the *L. infantum* isolate and critical reading of the manuscript. We also acknowledge M. Svobodova and S. Turco, who kindly provided us with the antibodies. This work was supported by the Ministry of Education of the Czech Republic (VZ 21620828, LC06009), Czech Science Foundation (206/06/0967), Wellcome Trust (PV and JP) and INCO-CT-2004-509086.

References

Ayala F. Is sex better? Parasites say “no”. Proc. Natl. Acad. Sci. USA. 1998; 95:3346–3348. [PubMed: 9520366]

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Cihakova J, Volf P. Development of different *Leishmania major* strains in vector sandflies *Phlebotomus papatasi* and *P. duboscqi*. Ann. Trop. Med. Parasitol. 1997; 91:267–279. [PubMed: 9229020]

Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. Int. J. Parasitol. 2005; 35:1169–1180. [PubMed: 16162348]

Ilg T, Stierhof YD, Craik D, Simpson R, Handman E, Bacic A. Purification and structural characterization of a filamentous mucin-like proteophosphoglycan secreted by *Leishmania* parasites. J. Biol. Chem. 1996; 271:21583–21596. [PubMed: 8702946]

Kamhawi S, Ramalho-Ortigao M, Pham VM, Kumar S, Lawyer PG, Turco SJ, Barillas-Mury C, Sacks DL, Valenzuela JG. A role for insect galectins in parasite survival. Cell. 2004; 119:329–341. [PubMed: 15543683]

Kamhawi S. Phlebotominae sand flies and *Leishmania* parasites: friends or foes? Trends Parasitol. 2006; 22:439–445. [PubMed: 16843727]

Kelleher M, Curtis JM, Sacks DL, Handman E, Bacic A. Epitope mapping of monoclonal antibodies directed against lipophosphoglycan of *Leishmania major* promastigotes. Mol. Biochem. Parasitol. 1994; 66:187–200. [PubMed: 7808469]

Killick-Kendrick R, Killick-Kendrick M, Tang Y. Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: the low susceptibility of *Phlebotomus papatasi* to *Leishmania tropica*. Trans. R. Soc. Trop. Med. Hyg. 1994; 88:252–253. [PubMed: 8036692]

Killick-Kendrick R. The biology and control of phlebotomine sand flies. Clin. Dermatol. 1999; 17:279–289. [PubMed: 10384867]

R.P., Lane. Sandflies (Phlebotominae). In: Lane, RP.; Crosskey, RW., editors. Medical Insects and Arachnids. Chapman and Hall; London: 1993. p. 78-119.

Lewis, DJ.; Ward, RD. Transmission and vectors. In: Peters, W.; Killick-Kendrick, R., editors. The Leishmaniases in Biology and Medicine. Vol. 1. Academic Press; London: 1987. p. 235-262.

Myskova J, Svobodova M, Beverley SM, Volf P. A lipophosphoglycan - independent development of *Leishmania* in permissive sand flies. Microb. Infect. 2007; 9 doi: 10.1016/j.micinf.2006.12.010.

Pimenta PF, Saraiva EMB, Rowton E, Modi GB, Garraway LA, Beverley SM, Turco SJ, Sacks DL. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc. Natl. Acad. Sci. USA. 1994; 91:9155–9159. [PubMed: 8090785]

Pimenta PF, Turco SJ, Mcconville MJ, Lawyer PG, Perkins PV, Sacks DL. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science. 1992; 256:1812–1815. [PubMed: 1615326]

Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. Int. J. Parasitol. 2006; 36:1383–1388. [PubMed: 16930606]

Tibayrenc M, Ayala FJ. Evolutionary genetics of *Trypanosoma* and *Leishmania*. Microb Infect. 1999; 1:465–472.

Victoir K, Dujardin JC. How to succeed in parasitic life without sex. Asking *Leishmania*. Trends Parasitol. 2002; 18:81–85. [PubMed: 11832299]

Volf P, Myskova J. Sand flies and *Leishmania*: specific versus permissive vectors. Trends Parasitol. 2007; 23 doi:10.1016/j.pt.2006.12.010.

Walters LI. Leishmania differentiation in natural and unnatural sand fly hosts. J. Eukaryot. Microbiol. 1993; 40:196–206. [PubMed: 8461893]

Walters LI, Irons KP, Chaplin G, Tesh RB. Life cycle of *Leishmania major* (Kinetoplastida: Trypanosomatidae) in the neotropical sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae). J. Med. Entomol. 1993; 30:699–718. [PubMed: 8360894]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

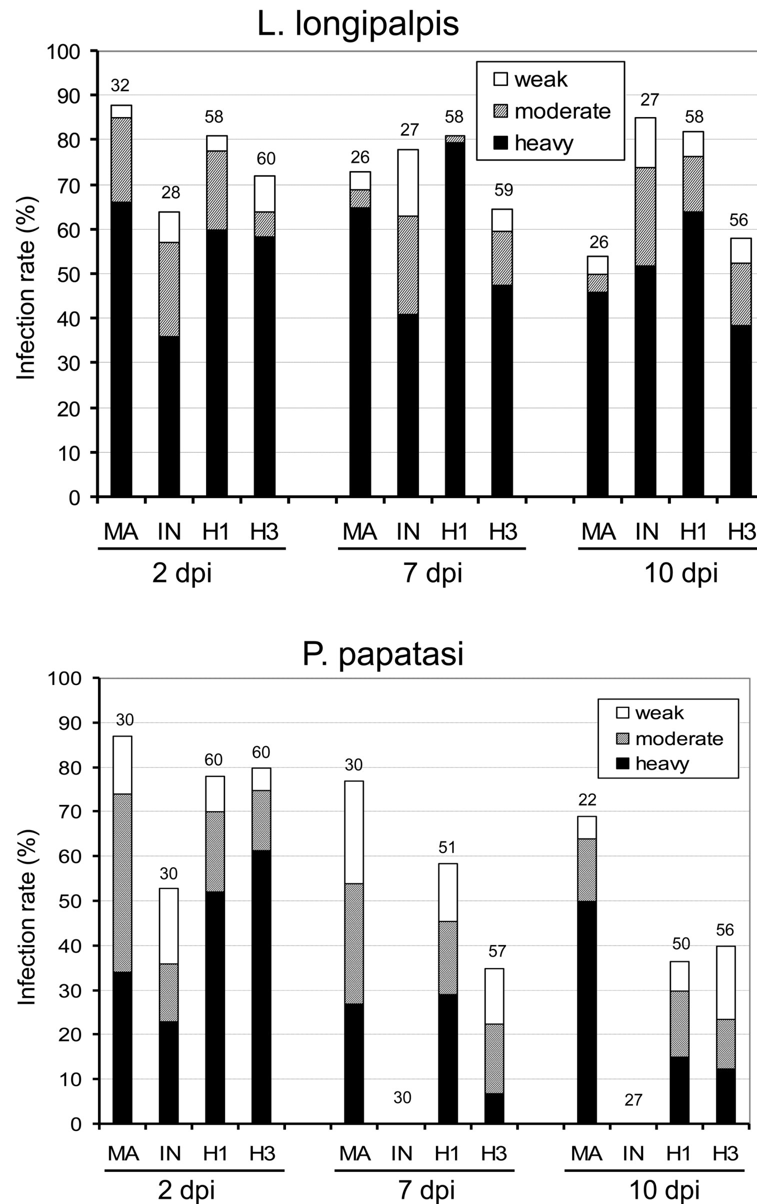

Fig. 1.

Development of *Leishmania* hybrids in *Lutzomyia longipalpis* and *Phlebotomus papatasi*. Infection rates and density of *Leishmania major* (MA), *Leishmania infantum* (IN), hybrid LEM4891 (H1) and hybrid LEM4833 (H3) in sand fly midgut on days 2, 7 and 10 p.i. Infections were classified into three categories: heavy (more than 1,000 promastigotes per gut) - black bars, moderate (100-1,000) - grey bars, light (1-100) - white bars. Numbers above the bars indicate the number of dissected females. A) Development in *L. longipalpis*: the infection rate and the intensity of infection did not differ between *Leishmania* strains studied. B) Development in *P. papatasi*: on days 7 and 10 p.i., the infection rate and the intensity of infection significantly differed between *L. major*, hybrids and *L. infantum*.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

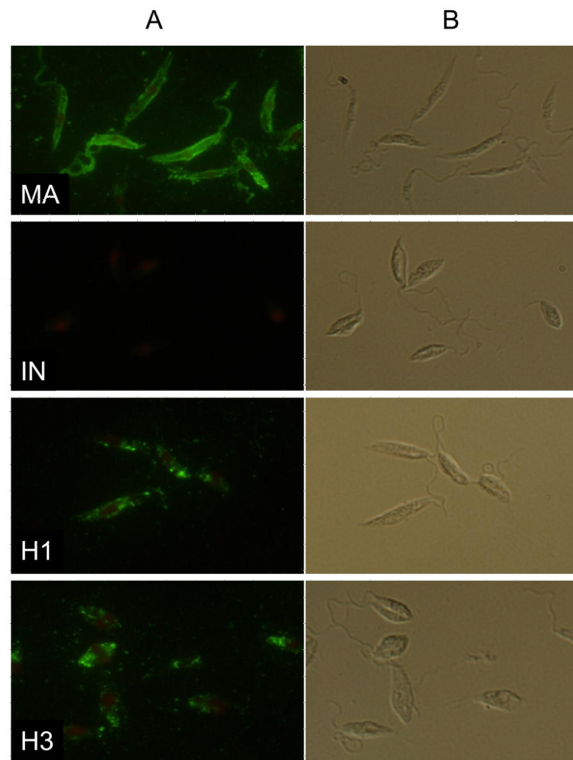

Fig. 2.

Detection of *Leishmania major* lipophosphoglycan using specific mAbs. A) Indirect immunofluorescence of *L. major* (MA), *Leishmania infantum* (IN), hybrid LEM4891 (H1) and hybrid LEM4833 (H3) with WIC 79.3 antibodies. Note the intermediate phenotype of hybrids. B) Same by differential interference contrast (Nomarski).

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

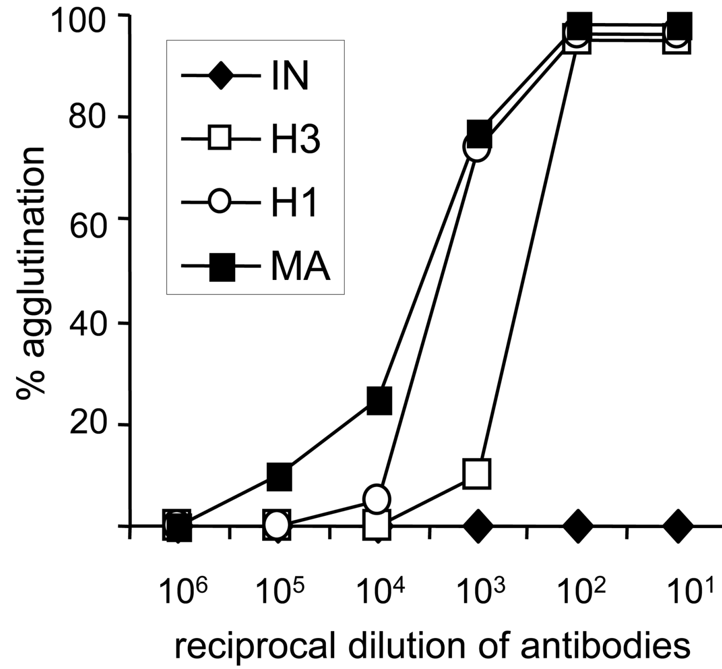

Fig. 3.

Agglutination profiles of *Leishmania* hybrids with mAbs. *Leishmania major* (MA), *Leishmania infantum* (IN), hybrid LEM4891 (H1) and hybrid LEM4833 (H3) were incubated with WIC 79.3 to detect the localization of *L. major* lipophosphoglycan on parasite surface.