ULTRASTRUCTURAL CHANGES IN PARASITES INDUCED BY NANOPARTICLE-BOUND PENTAMIDINE IN A *LEISHMANIA MAJOR*/MOUSE MODEL

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Summary :

Drug targeting enhances drug efficacy. This principle was tested in the treatment of an experimental visceral leishmaniasis. Using transmission electron microscopy (TEM) we localized pentamidine-loaded polymethacrylate nanoparticles in the liver of mice infected with *Leishmania major* and compared the ultrastructural changes in the parasites of these mice when they were treated with bound *versus* free pentamidine. Between days 13 and 17 after infection, loaded nanoparticles treated group were injected i. v. with 3 doses of 0.17 mg/kg bound pentamidine loaded on 2×10^{11} nanospheres; control groups received 2×10^{11} unloaded nanospheres. Drug reference control groups received five doses of 200 mg/kg pentavalent antimony (Glucantime®) or three doses of free pentamidine (0.17 mg/kg or 2.28 mg/kg). Mice treated with bound pentamidine displayed a 77 % reduction in their parasite burden *versus* the untreated controls. Nanoparticles were located by TEM inside parasitized Küpffer cells, in the phagolysosomes without entering the *Leishmania*. The low dose of 0.17 mg/kg bound pentamidine damaged the *Leishmania* to the same extent as 2.28 mg/kg of free pentamidine (the usual dose in human chemotherapy). In the parasites inside the Küpffer cells, TEM showed a swollen mitochondrion with loss of cristae, destruction or fragmentation of the kinetoplast, loss of ribosomes and destruction of parasite structures except for the subpellicular microtubules. This study therefore shows that a dose of bound pentamidine 13 times smaller than the usual dose of free pentamidine has a similar effect on the parasite.

KEY WORDS : murine visceral leishmaniasis, pentamidine, drug carrier, electron microscopy.

Résumé : MODIFICATIONS ULTRASTRUCTURALES DES LEISHMANIES INDUITES PAR PENTAMIDINE VECTORISÉE SUR UN MODÈLE DE LEISHMANIOSE VISCÉRALE MURINE

La vectorisation facilite l'accès de certaines drogues aux parasites intra-cellulaires. Ce concept a été appliqué à un modèle de leishmaniose expérimentale murine au cours duquel des souris BALB/c infectées par une souche de Leishmania major *ont été traitées par pentamidine vectorisée sur nanoparticules de polyméthacrylate. Du 13ème au 17ème jours les souris ont reçu par voie i. v. 0,17 mg/kg de pentamidine chargée sur* 2×10^{11} *nanoparticules. Les souris des groupes témoins ont reçu soit* 2×10^{11} *nanoparticules non chargées, soit 2.28 mg/kg ou 0,17 mg/kg de pentamidine libre, soit un traitement de référence (200 mg/kg de glucantime®). Les souris traitées par pentamidine vectorisée ont présenté une réduction de leur charge parasitaire de 77 % par rapport au groupe témoin non traité. Les nanoparticules ont été mises en évidence par microscopie électronique dans les cellules de Küpffer. Certaines étaient à l'intérieur des vacuoles parasitophores au contact des leishmanies mais aucune n'a été vue dans les parasites. Les altérations des leishmanies traitées par 0,17 mg/kg de pentamidine vectorisée étaient identiques à celles des leishmanies traitées par 2,28 mg/kg de pentamidine libre (dose habituelle chez l'homme). Les parasites présentaient une mitochondrie dilatée ayant perdu ses crêtes, un kinétoplaste partiellement détruit, une diminution des ribosomes, seuls les microtubules restaient intacts. Au cours de cette étude la pentamidine vectorisée a produit les mêmes modifications ultrastructurales qu'une dose de pentamidine libre 13 fois supérieure.*

MOTS CLÉS : *leishmaniose viscérale murine, pentamidine, vectorisation, microscopie électronique.*

INTRODUCTION

Drug distribution can be controlled in different ways, one of which is the drug carrier system. This system can improve drug efficacy. This principle was first applied successfully with antineoplastic drugs (Astier *et al.*, 1988, Chiannikulchaï *et al.*, 1989). To date, drug carriers have proved to be of potential interest in the treatment of intracellular macrophage infections (Fattal *et al.*, 1989), including visceral leishmaniases.

To improve the condition of patients with a chronic or relapsing course following drug therapy, more efficient and easily administrable drugs are required (Croft *et al.*, 1991). Treatment with pentamidine is not fully effective and has toxic side effects, nevertheless it is used against species such as *Leishmania braziliensis* (Soto *et al.*, 1994) or after failure of treatment with other drugs (Croft & Davidson, 1993). Pentamidine bound to a colloidal drug carrier might enhance its efficacy and reduce its toxicity. This procedure was tested

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in our laboratory in an *in vitro* model, using the monohistiocytic U 937 cell line and *L. major* amastigotes. In this model, pentamidine loaded polymethacrylate nanoparticles were twenty times more active than free pentamidine (Deniau *et al.*, 1993). *In vivo*, with a leishmaniasis/BALB/c mouse model, these results were confirmed by light microscopy (Fusai *et al.*, 1994).

The aims of the present study were to localize the nanoparticles in the liver by transmission electron microscopy (TEM), to confirm that they are harmless to the host cells, to examine the parasite changes in the Küpffer cells of mice treated with bound pentamidine and to compare the changes in groups which were untreated or treated with free pentamidine.

MATERIALS AND METHODS

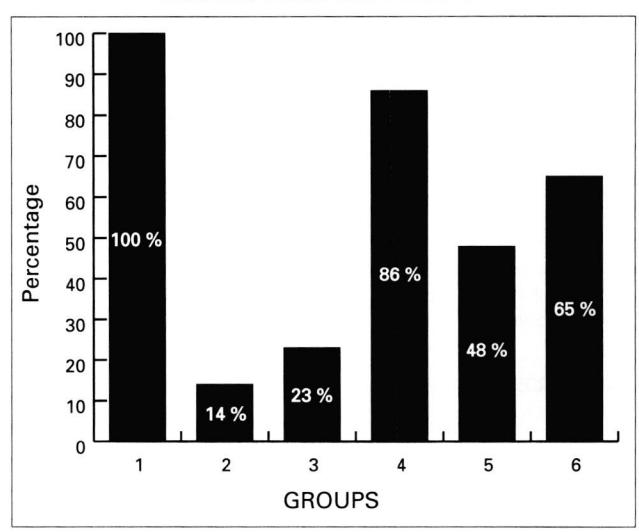
Slowly biodegradable polymethacrylic nanoparticles, with diameter of up to about 300 nm were used as drug carriers. The number of nanoparticles per ml was estimated in function of polymeric density and of the volume of one nanoparticle. They were loaded with pentamidine by ionic binding on free carboxylic groups, as previously described (Paul *et al.*, 1997).

Pentamidine bound nanoparticles were assayed on the murine model of visceral leishmaniasis as previously described (Fusai *et al.*, 1993). Briefly, promastigotes of *L. major* MON 74, cryopreserved or maintained in golden hamsters were cultivated for this study on RPMI medium. On day 0, 4×10^7 infective promastigotes were injected via the tail vein. The mice were then randomly divided into six groups of ten animals (Table I). Group 1 received isotonic sodium chloride. Group 2 received Glucantime® as drug reference treatment. Group 3 received 0.17 mg/kg bound pentamidine loaded on 2×10^{11} nanoparticles (i. e. 13.6 µg of pentamidine per mg of polymere). Group 4 received 2×10^{11} unloaded nanoparticles. Group 5 and 6 received free pentamidine. Twelve days after infection, severe visceralization was obtained, with an average liver parasite burden of 12.9×10^8. Treatments were given from day 13 to 17 and, on day 21 the animals were killed and autopsied. Drug efficacy was determined by evaluating the liver parasite burden according to Stauber (Stauber *et al.*, 1958).

Histological sections were prepared for electron microscopy as follows: pieces of liver, roughly 1 mm^3, were fixed for one hour at 4 °C in 2 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), rinsed three times in this buffer at 4 °C, and post-fixed in 2 % osmium tetroxide in the same buffer. 0.5 % potassium ferricyanide was added to both fixatives. The samples were rinsed three times in distilled water, kept in 0.5 % uranyl acetate water solution (v/v) for 12 hours, at 4 °C and dehydrated in graded ethanol solution. The samples were then washed twice with propylene oxide and embedded in a 1:1 mixture of araldite with epon. Fifty nm sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. The fine structure was examined with a Phillips EM 201 electron microscope, at the CIME (Centre Interuniversitaire Jussieu de Microscopie Électronique, quai Saint-Bernard, 75252 Paris Cedex 05).

- Group 1: Untreated control mice
- Group 2: Drug reference control mice (i. e. 200 mg/kg Glucantime® × 5, i. p.)
- Group 3: Loaded nanoparticle-treated mice (0.17 mg/kg of pentamidine base × 3, i. v.)
- Group 4: Unloaded nanoparticle-treated mice (0.1 ml × 3, i. v.)
- Group 5: Free pentamidine-treated mice (2.28 mg/kg of pentamidine base × 3, i. v.)
- Group 6: Free pentamidine-treated mice (0.17 mg/kg of pentamidine base × 3, i. v.)

Table 1. — The different groups of treated or untreated mice.



Group 1: Untreated control mice: the parasite burden, considered as 100 %, reached 12×10^8.

Group 2: Glucantime treated mice: the residual burden percentage dropped dramatically. The difference between Glucantime-treated and the bound pentamidine-treated mice was small.

Group 3: Bound pentamidine-treated mice: the residual parasite burden fell to 23 %.

Group 4: Unloaded nanoparticle treated mice: nanoparticles were ineffective in reducing the burden.

Group 5: With the usual dosage of 2.28 mg/kg × 3 free pentamidine, the burden of 48 % was the average one expected.

Group 6: At the low dosage of 0.17 mg/kg × 3 free pentamidine did not reduce the burden effectively.

Fig. 1. — The residual Leishmania burden in different groups of treated mice compared to the untreated controls.

RESULTS

Electron microscopy study revealed the following features:

CONTROL GROUPS

- Untreated mice

In the Küpffer cells of untreated mice (Fig. 2), amastigotes exhibited their well known characteristic structures: a condensed kinetoplast, a mitochondrion with a double wall membrane and clearly delineated cristae. The ribosomes, nucleus, microtubular and flagellar structures were intact. They had the morphology reported by different authors (Langreth *et al.*, 1983, Molyneux & Killick-Kendrick, 1987).

- Mice treated with free pentamidine

Free pentamidine did not alter the kinetoplast (Fig. 5) at the dose of 0.17 mg/kg, i. e. the low dosage targeted in nanoparticles. However, at the usual dose of 2.28 mg/kg, it caused the typical ultrastructural changes induced by pentamidine (Croft & Brazil, 1982, Langreth *et al.*, 1983), i. e. the mitochondrion was greatly distended, the matrix contained fragments of mitochondrial membrane and the kinetoplast was disrupted and electron-dense. The ribosomes were segregated and depleted (Fig. 6).

MICE TREATED WITH BOUND-PENTAMIDINE

Effective phagocytosis of nanoparticles was frequently observed, as demonstrated in Figure 3. On this section, up to 300 nanoparticles were concentrated around the nucleus of a Küpffer cell. Nanoparticles were also present in the lysosomial compartment of the parasitized cell but did not enter the parasite, although they were located close to it (Fig. 4).

Amastigotes displayed various ultrastructural changes, especially in the kinetoplast which was more dense and seemed free in a ruptured mitochondrion. Moreover, the mitochondrion lost its cristae and a part of its matrix. The ribosomes were segregated and depleted (Fig. 8 and 9). Other views showed profiles of swollen mitochondrion, but the microtubule of the flagellum and those of subpellicular structures were unchanged (Fig. 10). The proliferation of pseudomyelinic structures was frequently observed in these treated parasites (Fig. 11).

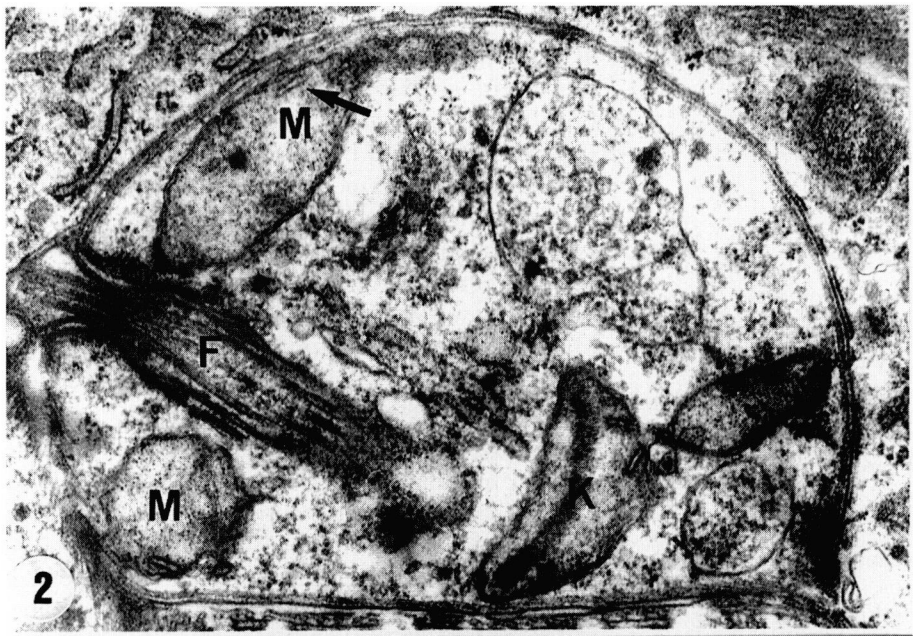
In the liver of mice treated with unloaded nanoparticles (Fig. 7) and loaded nanoparticles (Fig. 8), the host cells were not altered.

DISCUSSION

The ultrastructural changes induced by bound pentamidine that we observed in our study were similar to those described by Croft & Brazil (1982) of *L. amazonensis* after treatment with free pentamidine, and by Hentzer & Kobayasi (1977) in *L. tropica*. There is no study concerning the ultrastructural changes induced by pentamidine in *L. major*. Most publications concern other species (Croft & Brazil, 1982; Langreth *et al.*, 1983; Hentzer & Kobayasi, 1977). In their review, Molyneux & Killick-Kendrick (1987) hypothesized that *Leishmania*, whatever the species, could have a similar configuration and ultrastructural morphology.

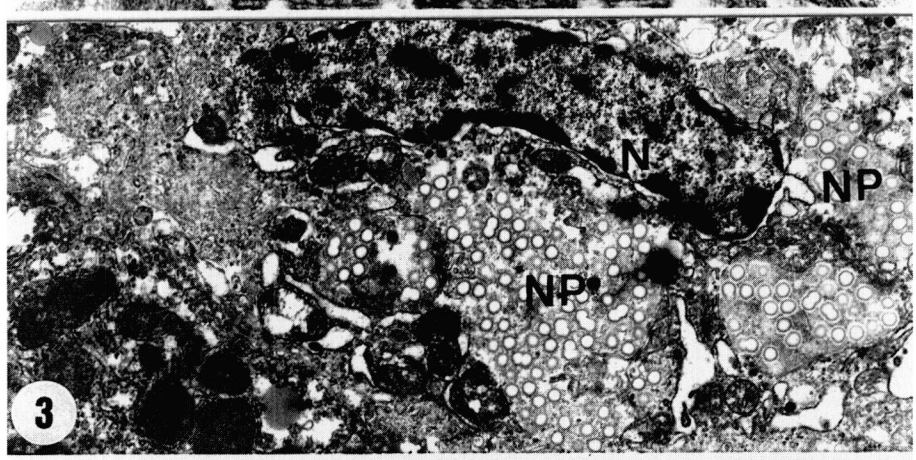
In this study, the percent of decrease in the parasite burden in the mice treated with bound pentamidine was established by the Stauber method, which is limited to a light microscopy examination. The ultrastructural study confirmed that, in these mice, the alterations were indeed due to the action of pentamidine, in this group. It confirmed also the good penetration of the carrier into Küpffer cells, the amount of drug released *in situ* increasing with the number of phagocytized particles. Furthermore, morphologically the action of 0.17 mg/kg bound pentamidine was similar to that of 2.28 mg/kg free pentamidine. In the present experiment, the mice treated with bound pentamidine exhibited more numerous pseudo-myelinic bodies than did control mice. This probably reflects alterations in the phospholipidic system which appear to have been unnoticed by other authors. Hentzer & Kobayasi (1977) observed multivesiculate bodies that we did not see.

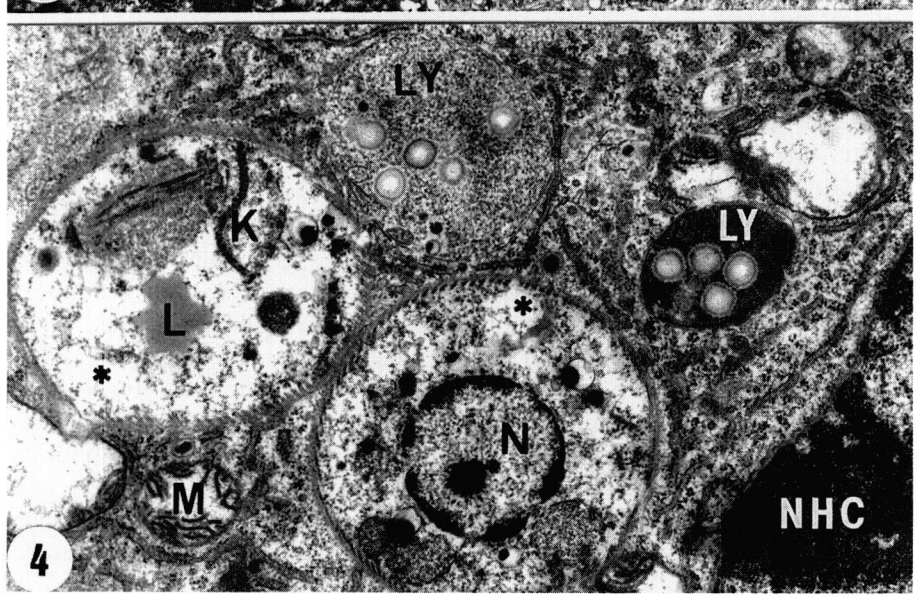
The mode of action of pentamidine is not quite clear and the images we obtained did not allow us to propose other hypothesis than those already suggested by different authors (Calonge *et al.*, 1996, Kandpal *et al.*, 1996). However, there is a reason to believe that the similarity of the ultrastructural changes in the group of mice treated with free and the group treated with bound pentamidine is due to a similar mode of action. The close contact between parasitophorous vacuole (PV) and phagolysosomes containing nanoparticles may be of crucial importance. Most of our micrographs show nanoparticles inside lysosomes, sometimes, in the PV, but we also observed free nanoparticles in the cell cytoplasm (as observed in the Figure 7), this could represent the last step of intracellular trafficking. An hypothesis proposed about the turn over of lysosome could explain this aspect: after neutralization of lysosomial enzymes, the membrane of lysosome has been disrupted, then the nanoparticles are in close contact to the cytoplasm. Our images did not show fusion between secondary lysosomes and the PV membrane.



Untreated control groups

Fig. 2. — *Leishmania* in a Küpffer cell of an untreated mouse: the cell displays the normal ultrastructural features of a leishmania amastigote control, a mitochondrion (M) within a double membrane, a normal dense matrix containing cristae (arrow) and a prominent kinetoplast (*k*). The flagellar pocket (F) is apparent. Parasitophorous vacuole membrane is close to the parasite (× 40,000).

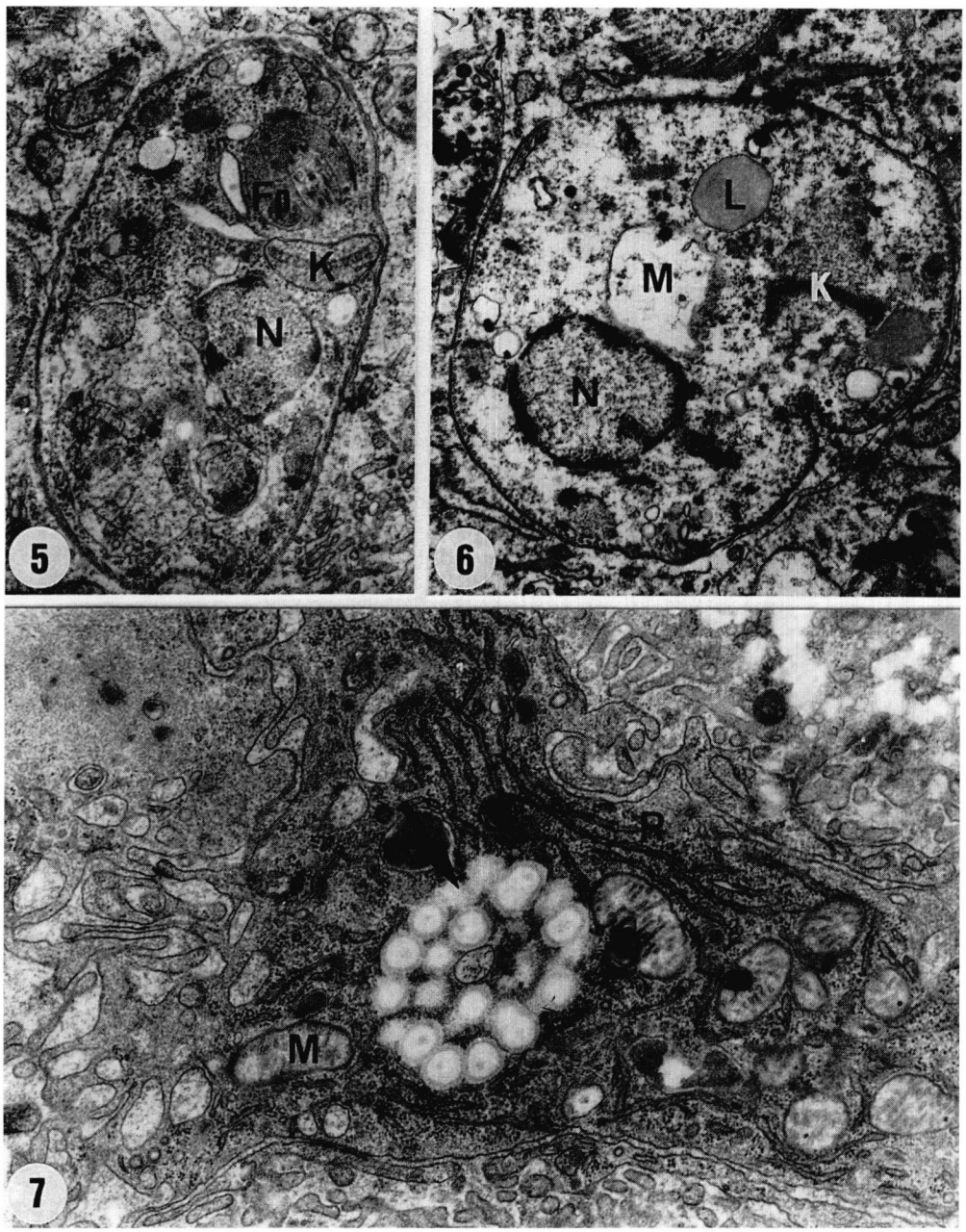




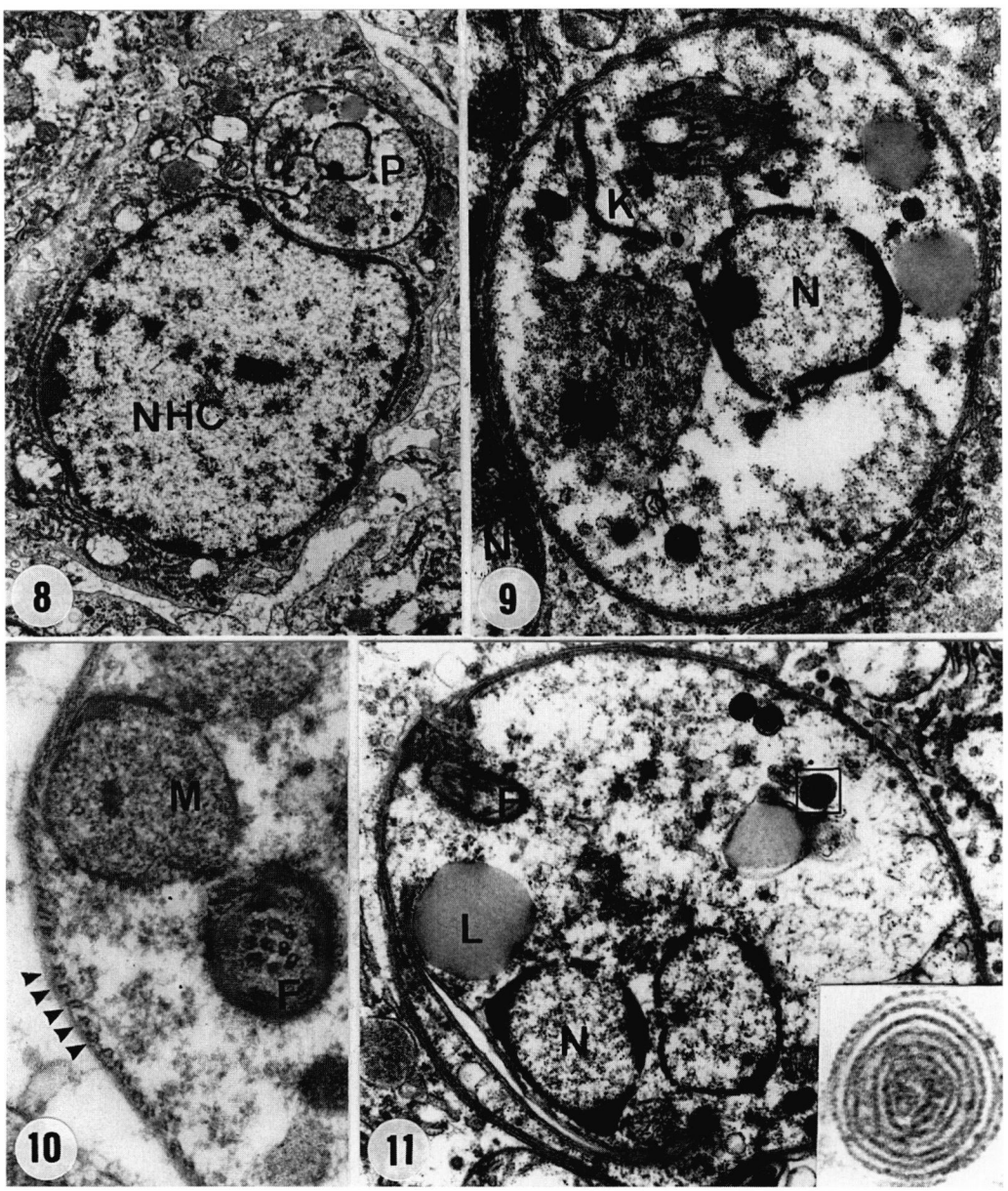
Localization of nanoparticles (figures 3 and 4)

Fig. 3. — Küpffer cell of a mouse treated with bound pentamidine: The abundance of nanoparticles phagocytized by this Küpffer cell must be noted: nanoparticles (NP) are clustered in large vacuoles identified by their clearer aspect than the surrounding cytoplasme (N: nucleus of the Küpffer cells) (× 7,000).

Fig. 4. — *Leishmania* in a Küpffer cell of a mouse treated with bound pentamidine. The nanoparticles are localized in secondary lysosomes (LY) and seem to remain outside the parasites (*). One parasite shows alteration of the kinetoplast (K) and of the lipid droplets (L) (NHC: nucleus of the host cell) (× 14,000).



Figs. 5 to 7. — Controls of free pentamidine and unloaded nanoparticles treated groups: Fig. 5. — *Leishmania* in a Küpffer cell of a mouse treated with 0.17 mg/kg × 3 of free pentamidine. At this low dosage, similar to its concentration in nanoparticles, injection of free pentamidine did not alter the structures (Fp: Flagellar pocket, K: kinetoplast, N: nucleus) (× 16,000). Fig. 6. — *Leishmania* in a Küpffer cell of a mouse treated with 2.28 mg/kg × 3 of free pentamidine, *Leishmania* structures show the same alterations as with bound pentamidine treatment (0.17 mg/kg): the kinetoplast (*k*) shows disruption of the mitochondrion membrane and is more electrondense; the mitochondrion (M) is empty because of the loss of the matrix; ribosomes are segregated and depleted (× 20,000). Fig. 7. — Küpffer cell of a mouse treated with unloaded nanoparticles, the host cell, observed 3 days after the last injection, is not affected by the drug carrier (arrow) (M: mitochondrion, R: rough endoplasmic reticulum) The nanoparticles are clustered (× 20,000).



Bound-pentamidine treated group: Figs 8 to 11

Figs. 8 and 9. — *Leishmania* in a Küpffer cell of a mouse treated with bound pentamidine (concentration: 0,17 mg/kg). The host cell does not exhibit alteration (Fig. 8). The parasite (P) is shown with a higher magnification in Figure 9; the kinetoplast and mitochondrion (M) exhibit major changes: the kinetoplast (K) is very electrondense and its membrane is disrupted, a part of the matrix is lost. In a profile of the mitochondrion, the membrane is also disrupted and if small cristae are still visible, the matrix seems being dispersed. Ribosomes are segregated and depleted. Nucleoplasma of the nucleus is clear (× 6,000, and × 19,500). Fig. 10. — *Leishmania* in a Küpffer cell of a mouse treated with bound pentamidine, a swollen mitochondrion (M) and segregated ribosomes contrast with the intact appearance of flagellar structures (F) and subpellicular microtubules (arrow-head) (× 45,000). Fig. 11. — *Leishmania* in a Küpffer cell of a mouse treated with bound pentamidine, besides the usual changes due to pentamidine, more numerous pseudomyelinic multilamellar structures (□) than in controls can be observed (× 22,000); insert: a higher magnification of one of these structures (× 90,000).

As far as we know, the type of carrier used here is the first to be loaded with pentamidine. Methacrylate polymer nanoparticles, which allow the ionic binding of drugs, have the advantage of releasing drugs at a low pH, as encountered in the lysosomes. The release of the drug *in situ* close to the target, may reduce drug toxicity to the host. The lack of immediate side effects in the mice treated with bound pentamidine was evident. Pentamidine tolerance and toxicity could be improved by the use of drug carriers. Evaluation of these nanoparticles showed that the carrier was not toxic either, *in vitro,* for rat liver cells or, *in vivo,* in rabbit (Rolland, 1988). However a long time period of follow-up is required to assess the inocuity of these nanoparticles after chronic treatment in man.

These results, with bound pentamidine, show that drug carriers are of interest for the treatment of leishmaniasis. Other studies with a panel of drug carriers loaded with different drugs are now in progress.

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