Abstract  Blood-brain barrier (BBB) permeability increases prior to the development of clinical signs in early-stage multiple sclerosis (MS). Detection of subtle changes would thus be helpful for diagnostic purposes and rapid therapeutic decisions before new episodes. Since multiple sclerosis and experimental allergic encephalomyelitis (EAE) have numerous common features, in particular BBB-permeability characteristics, and since we have previously shown that BBB localization is disturbed by tumors, embolism, and mannitol injection, we investigated BBB-liposome permeability in an EAE rat model. Twenty young male Lewis rats received a single intradermal inoculation of guinea-pig spinal cord. The effect of the Freund’s adjuvant and spinal cord alone on brain permeability were also assessed. In order to compare solution permeability and liposome localization, radioactive liposomes and, 1 h later, $^{99m}$Tc-DTPA were injected intravenously. Scintigraphic acquisitions were obtained to follow the biodistribution of radioactivity in the whole body. Each rat was subjected to a first examination before inoculation and then every two days until completion and may be considered as its own control.

EAE induced a previously unreported increase in global-body permeability, probably due to inflammation. Liposome brain localization and brain/heart ratio were significantly different between normal animals and those with early-stage EAE (before appearance of clinical signs) and distinguished between different disease stages in clinically patent EAE. The index of disease progression was modified earlier than with $^{99m}$Tc-DTPA injection. One explanation may be particle pick-up by circulating macrophages, which cross the BBB during this pathology. For clinical applications, experiments must be confirmed on models more reliable for human multiple sclerosis.

Key words  Blood-brain barrier · Experimental allergic encephalomyelitis · Multiple sclerosis · Liposomes · $^{99m}$Tc · Brain/heart ratio

Introduction  Diagnosis of multiple sclerosis (MS) is largely based on clinical evidence of white-matter dysfunction. Magnetic resonance imaging (MRI) is widely used to study the morphological basis of the disease, allowing objective assessment of progression in both clinic settings and treatment trials (Duckers et al. 1997; Verhoye et al. 1996). Blood-brain barrier (BBB) breakdown due to MS may be precisely visualized with Gd-DTPA MRI (Hawkins et al. 1990; Newcombe et al. 1991). Nevertheless, the examination remains non-specific since the observed signal-intensity changes may also occur for other tumor or inflammatory pathologies (Owens and Sriram 1995). Furthermore, images cannot be correlated with clinical signs and, in acute stage diseases, MRI fails to detect pathological changes (Chambron et al. 1994; Kendall et al. 1987).

It has been shown that increased BBB permeability is an early manifestation of MS (Juhler et al. 1984; Kato and Nakamura 1989). BBB permeability precedes the appearance of the first clinical signs (D’Amelio et al. 1990; Benveniste 1997) and would thus be very helpful for early diagnosis or rapid therapeutic decision making. We have shown that nanoparticles and liposomes localize in the brain when the BBB has been altered (Rousseau et al. 1997). Early detection of subtle changes in the BBB permeability during early stage MS would be useful. As MS and experimental allergic encephalomyelitis (EAE) have numerous common features, in particular BBB permeability characteristics (Juhler 1988; Wekerle et al. 1994), we used this model to investigate liposome-permeability changes. The aim of this study was to compare liposome localization during EAE and intracarotid mannitol injection, creating a disturbed BBB model, to better understand the permeability changes.
Materials and methods

All animal experiments were performed following the recommended French procedures by authorized technicians.

Experimental allergic encephalomyelitis

Experimental allergic encephalomyelitis was induced by intradural injection of a homogenate of guinea-pig spinal cord (Daniel et al. 1981; Wiesniewski and Keith 1977). Guinea-pig spinal cord (10 mg) was homogenized in 10 ml physiological saline solution and 20 ml complete Freund’s adjuvant (CFA) containing 10 mg/ml M. tuberculosis (Sigma, Saint Quentin-Fallavier, France). Twenty young male Lewis rats were given intradural inoculations of 0.5 ml homogenate at several sites on the back. The animals were weighed and clinically scored daily after inoculation. The severity of clinical signs was assessed by establishing a six-level score in order to follow the animal’s clinical state (Daniel et al. 1981; Leibowitz and Kennedy 1972; Namen et al. 1992).

Spinal-cord and Freund’s-adjuvant influences

The effects of the Freund’s adjuvant and spinal cord alone on the brain permeability were assessed. In the first experiment, ten Lewis rats were intradermally injected with 0.5 ml of a solution containing 10 ml physiological saline solution and 20 ml Freund’s adjuvant for CFA evaluation. After complete remission, the ten Lewis rats from the first experiment were intradermally re-injected with 0.5 ml of a solution containing 30 ml physiological saline and 10 mg guinea pig spinal cord, but without Freund’s adjuvant.

Mannitol-disturbed BBB model

Injection of hyperosmolar solutions into the carotid induces a transitory opening of the BBB to small molecules (Rapoport et al. 1980), but a delayed localization of nanoparticles (Rousseau et al. 1997). After anesthesia with halothane, osmotic opening was induced in 10 Lewis rats by intracarotid injection of 25% mannitol (Sigma, Saint Quentin-Fallavier, France) at a dose of 10 mg/kg in 30 s in a volume of approximately 3 ml.

Liposome preparation

The lipid formulation of liposomes was 70% M/M fresh egg lecithin and 30% cholesterol (Sigma, Saint Quentin-Fallavier, France). Lipid suspension was extruded through a 100-nm pore-diameter calibrate polycarbonate filter (Hope et al. 1985). They were labeled by 99mTcO4- incorporation in the native aqueous solution. The bulk excess was removed by cation-exchange resin (Dowex HCR-S Sigma, Saint Quentin-Fallavier, France).

Biodistribution assessment

In order to compare solution permeability and liposome pickup, we first injected radioactive liposomes (5.08×105 Bq/injected dose) via the penis vein and then, 1 h later 99mTc-DTPA, a complex known to cross injured BBB (Marchal et al. 1993), at a level of 2.6×105 Bq/injected dose. To follow the liposome biodistribution in the whole rat body, we made scintigraphic acquisitions on a Gammatome-2 gamma camera (Sopha, Buc, France). We acquired 60 images of 1 min, the first image starting at liposome injection. Each animal was followed throughout disease development, and the first examination, performed before BBB breakdown or spinal-cord homogenate injection, was taken as the reference.

Radioactivity measurements were made with free NIH-Image software (freely available at http://rsb.info.nih.gov). We selected a region of interest on the first image for computerized analysis of subsequent images and activity measurements. Biodistribution was expressed as a percentage of the radioactive-injection dose. The radioactivity measurement in hind leg muscle gave the blood-radioactivity background, and the radioactivity of the liposomes remaining in the vascular area was corrected by subtraction of this background noise. Radioactivity in the spleen and liver was studied to assess liposome elimination from blood flow. Any free 99mTc in the bladder, kidneys, salivary glands, and thyroid was detected. Values were compared with those in the same organ in normal rats.

Statistical methods

The non-parametric Mann and Whitney U test was used to analyze the data. A P<0.01 significance threshold was chosen.

Results

Experimental allergic encephalomyelitis

During the first ten days after spinal-cord-homogenate/CFA injection, none of the rats exhibited clinical signs (clinically silent step). At day 11, three rats were scored level 1 (step 1) and two level 2 (step 2). As we were interested in early stage EAE, we did not image rats scored level 3 or more. Fourteen days after spinal-cord-homogenate injection, some rats recovered partially (step 3, recovery step). At day 16, no clinical signs were exhibited (step 4, rescue). All rats were studied from day 0 to 10 (n=20). One rat died during step 1. There were no further deaths.

After liposome injection, muscle radioactivity increased at day 6 (1.41±0.16 compared to 1.18±0.1 60 min after liposome injection in normal rats) and later from day 10 until step 3. Mean increase was 1.4% and normal levels were recovered in step 4. After 99mTc-DTPA injection, muscle radioactivity showed no significant difference to the normal value, except at day 4. Radioactivity rate decreased to 0.84±0.59 compared with 1.27±0.29 60 min after 99mTc-DTPA injection in normal rats. For step 2, the radioactivity rate remained unchanged during the entire experiment at a mean value of 1.34±0.16 during the hour of scintigraphic acquisition.

In the spleen and liver, liposome localization decreased at day 6 and 8. In the spleen, the radioactivity rate 60 min after liposome injection was 9.69±0.97 compared with 12.75±2.89 in normal rats (13.13±1.42 compared with 14.24±1.96 in the liver). However, at day 10, macrophage activity increased. The radioactivity rate was 15.98±2.06 compared with 12.75±2.89 in the spleen (16.87±2.70 compared to 14.24±1.96 in the liver). The radioactivity rate returned to normal at step 4. No significant effect was observed after 99mTc-DTPA injection. No modification was observed in the salivary glands and thyroid.

In kidneys and bladder, liposome localization was observed from the first day after spinal-cord-homogenate injection until step 3 (Fig. 1a, dotted line). For example, at day 8, the radioactivity rate in the bladder 60 min after