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Cationic Liposomes Target Sites of Acute Neuroinflammation in Experimental Autoimmune Encephalomyelitis

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Abstract: The binding selectivity of charged liposomes to the spinal cord of rats affected by experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, was investigated. Positively and negatively charged liposomes were injected into the tail vein of rats, and blood/brain barrier (BBB) targeting was determined by confocal microscopy as a function of the temporal evolution of the inflammatory response. Accumulation in spinal cord endoneural vessels was observed for cationic, but not for anionic, liposomes, and only in EAE but not in healthy rats. The overall binding efficacy paralleled the severity of the clinical score, but targeting was observed already before clinical manifestation of inflammation. Preferential binding of positively charged liposomes in the course of acute EAE can be ascribed to subtle changes of BBB morphology and charge distribution in a similar way as for the binding of cationic particles to proliferating vasculature in chronic inflammation and angiogenesis. Our findings suggest that vascular changes related to increased binding affinity for cationic particles are very early events within the inflammatory reaction in acute EAE. Investigation of cationic vascular targeting can help to shed further light on these occurrences, and, potentially, new diagnostic and therapeutic options may become available. In neuroinflammatory diseases, cationic colloidal carrier particles may enable intervention at affected BBB by an approach which is independent from permeability increase.

Keywords: Multiple sclerosis, MS; experimental autoimmune encephalomyelitis, EAE; blood−brain barrier, BBB; angiogenesis; inflammation; cationic liposomes; drug delivery; cancer

Introduction

Multiple sclerosis (MS) is a chronic multifocal inflammatory disease of the central nervous system (CNS) characterized by inflammatory changes, blood/brain barrier (BBB) breakdown, perivascular inflammation, axonal and oligodendrocyte loss, and demyelination.[1]

BBB is the complex cerebral vascular endothelium, and its surrounding astrocyte foot processes and pericytes, at the interface between the CNS and systemic blood circulation.

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It restricts permeability to most hydrophilic solutes and is crucial for CNS protection and the maintenance of the homeostasis of the CNS environment. BBB changes dramatically during the early stages of MS: it not only shows an enhanced permeability but appears to be also actively involved as mediator of neuroinflammatory processes.[2,3]

Magnetic resonance imaging (MRI) and pathological studies have shown that MS lesions are predominantly distributed around venules, and that the inflammatory damage to blood vessels and disruption of the BBB is an early event in the pathogenesis of MS and in the formation of new focal lesions.[4]

The aim of the current study was to investigate if sites of neuroinflammation can be targeted by electrostatically charged colloidal carriers such as cationic (CL) and anionic (AL) liposomes.[5,6] CL have been shown to bind and internalize at angiogenic endothelial cells in tumor growth and chronic inflammation,[7−9] which has been attributed to the specific pathological changes that become manifest in angiogenic vasculature.[10−17] As a result of this finding, new therapeutic approaches for treatment of solid tumors through delivery of drugs by CL have emerged,[9,18−26] and a CL formulation of the anticancer drug paclitaxel (EndoTAG-1, MediGene AG, 82152 Martinsried, Germany)[27] is undergoing clinical trials for tumor therapy. Besides tumor growth, angiogenesis plays a key role in a variety of other pathological situations

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of chronic inflammation,[10] and recently it has been hypothesized that angiogenesis is also involved in later stages of MS.[28,29]

In the present study, we investigated the binding characteristics of CL and AL in a situation of nonchronic neuroinflammation, namely in experimental autoimmune encephalomyelitis (EAE), a MS model with a clear temporal profile of acute inflammation.[30,31] The binding to the spinal cord of rats was determined at different time points after induction of EAE. As a positive control for binding to angiogenic vasculature, ovarian targeting was monitored, based on the high physiologic rate of angiogenesis in the normal ovary.[32]

The studies consisted of 2 separate experiments performed in sequence. In the first study, the temporal evolution of CL binding in comparison to clinical signs was explored. In the second study targeting of CL and AL was directly compared at selected time points. Laser scanning confocal microscopy (LSCM) was used to determine binding of fluorescence labeled liposomes in histological samples.

Materials and Methods

Studies were performed after protocol revision by the *ad hoc* committee of the University of Milan "Bicocca". Care and husbandry of animals were in conformity with the institutional guidelines and in compliance with national (D.L. n. 116, *Gazzetta Ufficiale della Repubblica Italiana,* Suppl. 40, Feb 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Materials. Dioleoyl trimethylammonium propane chloride (DOTAP), dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylglycerol (DOPG) and rhodamine-dioleoyl phosphatidylethanolamine (Rho-DOPE) were from Avanti Polar Lipids, Alabaster, AL. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and cholesterol (Chol) were from Sigma, St. Louis, MO; glucose was from Merck, Darmstadt, Germany; and glucose solution was from Braun, Melsungen, Germany.

Liposomes. Cationic (CL) and slightly anionic (AL) liposome preparations had a total lipid concentration of 10 mM. In each case the liposomes contained 5 mol % of the fluorescence label Rho-DOPE. CL consisted of a mixture of cationic DOTAP and zwitterionic DOPC (molar composition: DOTAP/DOPC/Rho-DOPE = 50/45/5). The targeting specificity to angiogenic endothelial cells of this formulation has been confirmed in a number of preclinical experiments and in clinical trials.[9,27] For AL a relatively low fraction of the negatively charged lipid DOPG was selected (DOPC/Chol/DOPG/Rho-DOPE = 45/45/5/5) in order to minimize electrostatic repulsion by the negatively charged biomembranes.

Manufacturing was performed according to established protocols.[33] Either the "film method" or "ethanol injection" was used for liposome preparation. For the film method, the lipids were dissolved in chloroform and put in appropriate amounts into a round-bottom flask. The organic solvent was evaporated in a rotary evaporator, and the dry film was reconstituted with a 5% (w/w) glucose solution in water to the total lipid concentration of 10 mM by gently shaking the flask. For ethanol injection, the lipids were dissolved at suitable molar ratios in ethanol to a total concentration of about 200 mM. The ethanol solution was injected by a pump system under stirring into a 5% (w/w) glucose solution to obtain a lipid concentration of 10 mM in the aqueous phase. Subsequently, the ethanol was removed by cross-flow diafiltration (Vivaflow 50 casettes, MWCO 50,000, polyether sulfonic acid, Vivasciences, Sonehouse, U.K.). The equivalence of the liposome characteristics for the preparations as manufactured by the two methods was proven by internal tests.

The polydisperse liposome preparations were extruded five times across polycarbonate membranes of 200 nm pore size (GE Osmonics, Minnetonka, MN); subsequently they were sterile filtrated (Millipore, Molsheim, France) two times, and aliquoted in suitable vials (typically, 2 mL GeNunc storage vials from Nalge Nunc International, Rochester, NY).

Liposome size was determined by photon correlation spectroscopy using a Malvern Zetasizer 3000 (Malvern Instruments Ltd., Worcestershire, U.K.). The intensity weighted average size (Z_{ave}) and the polydispersity index (PI) were as follows: CL, Z_{ave} = 195 nm, PI = 0.11; AL, Z_{ave} = 183 nm, PI = 0.2.

The lipid composition of the formulation was controlled by HPLC analysis. The concentration of the fluorescent marker was controlled by HPLC and by fluorescence spectroscopy.

Study 1. A total of 40 adult female Lewis rats (Harlan Italy, Correzzana, Italy) were used for this study. Animals were randomly divided into 2 groups: healthy controls (n = 6) and EAE rats (n = 34). EAE was actively induced by subcutaneous inoculation into both hind limb footpads of

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50 μg of purified guinea pig myelin basic protein (MBP)[34] in 100 μL of complete Freund's adjuvant with 3 mg/mL of inactivated *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI).[30,31] The observation was protracted up to 21 days postimmunization (pi), and the assessment of the clinical score was performed on days 7, 10, 12, 14, 17, and 21 pi. The score was assessed by two independent examiners according to a scale ranging from 0 to 5 as follows: 0 = normal; 1 = limp tail; 1.5 = as 1 plus unable to turn over when placed on back; 2 = mild paraparesis; 2.5 = moderate paraparesis; 3 = paraplegia; 3.5 = as 3 plus upper limb paresis; 4 = quadriplegia; 5 = moribund or death. When there was disagreement, in order to reach a consensus, a further evaluation was performed by a third examiner who was unaware of the scores assigned by the two first-line examiners.[35,36] At fixed dates after immunization rats were sacrificed according to the following plan: day 7 (1 healthy rat + 4 EAE), day 10 (1 healthy rat + 6 EAE), day 12 (1 healthy rat + 8 EAE), day 14 (1 healthy rat + 8 EAE), day 17 (1 healthy rat + 4 EAE), day 21 (1 healthy rat + 4 EAE). At each time point the healthy and EAE rats received CL at a dose corresponding to 5 mg of total lipid/kg of body weight iv as slow bolus into the tail vein (injection volume 0.617 μL/g). The EAE rats were divided at each administration in 2 groups of equal size and received CL 10 min (EAE-10m group) or 2 h (EAE-2h group) before sacrifice obtained by means of CO_2 inhalation followed by exsanguination. Healthy rats received CL only 10 min before sacrifice. At the sacrifice the spinal cord (the site where inflammatory changes are more prominent in this EAE model) was removed from EAE and healthy rats (negative controls), fixed in 4% paraformaldehyde for 2 h and OCT embedded. For the histological examination, 8 μm thick cryosections were stained with hematoxylin-eosin, while LSCM examination was performed on unstained sections within 24 h from sacrifice. LSCM examination was carried out on a Radiance 2100 microscope (Biorad Laboratories, Hercules, CA) equipped with a krypon/argon laser; noise reduction was achieved by Kálmán filtering during aquisition.

Ovary specimens were obtained from healthy and EAE rats, fixed in 4% paraformaldehyde for 2 h, and OCT embedded, and cryosections were processed exactly as spinal cord specimens and used as reference positive control.

No systematic investigation of targeting to other organs has been performed, because the particular pattern of CL biodistribution has been determined earlier[37] and the present study was focused primarily on binding to BBB as compared to AL.

Study 2. A total of 36 adult female Lewis rats from Harlan were used for this study. EAE was induced as previously described in 27 rats, while 9 healthy rats were used as controls. Based on the results obtained in study 1, in this experiment rats were sacrificed on days 7, 14, and 21 pi. At each of these time points 9 EAE rats and 3 healthy controls were sacrificed and received 10 min before sacrifice one of these administrations: CL (n = 6) or AL (n = 3). All liposome formulations had a total lipid concentration of 10 mM and were delivered iv via the tail vein as slow bolus in order to administer in all cases the same total molar amount of total lipid and the same amount of dye. The injection volume was 0.617 μL/g, corresponding to a dose of 5 mg of total lipid/kg of body weight for CL and 3.9 mg of lipid/kg of body weight for AL.

The severity of the clinical signs of EAE was assessed as described in study 1. At the end of the experiment, the selected animals were sacrificed by means of CO_2 inhalation followed by exsanguination and used for biological sampling.

At the sacrifice the spinal cord was removed from CL and AL EAE and healthy rats (negative controls), fixed in 4% paraformaldehyde and OCT embedded. For the histological examination, 8 μm thick cryosections were stained with hematoxylin-eosin, while LSCM examination was performed on unstained sections as previously described. Ovary specimens from EAE rats were fixed in 4% paraformaldehyde and OCT embedded, and the obtained cryosections were used as reference positive control. After comparison with anti-CD31 and anti RECA-1 antibody staining, that resulted only in a faint contrast in our experimental conditions, endoneural vessels were stained with an antilaminin Ab (1:25, rabbit antilaminin Ab, Sigma-Aldrich Italy, Milan, Italy) which was labeled with a FITC-conjugated goat antirabbit Ab (1:200, Invitrogen, S. Giuliano Milanese, Italy) for observation at the LSCM. For this part of the study, sections were treated briefly with 0.1 M glycin in PBS (pH 7.4) followed by 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer (pH 7.4). Sections were then incubated 2 h at room temperature with the primary Ab, washed and glass slides were mounted with glycerol.

Results

The summary of the median clinical scores is reported in Table 1. As observed in the first study, after hematoxylin-eosin staining and histological examination, the extent of inflammatory infiltrate in the spinal cord of EAE rats steadily increased from day 10 pi (when it was located predominantly in the subarachnoid space), to days 12−14 pi (when it peaked

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Table 1. Clinical Scoring at Each Time Point in EAE Rats[a]

	7[b]	10	12	14	17	21
study 1						
clinical score (median)	0	1	2	3	3	0
number of rats	34	30	24	16	8	4
study 2						
clinical score (median)	0			3		0
number of rats	27			18		9

[a] Clinical severity is reported as median value scored as described in the *Results* section.[28,29] [b] Days after immunization = 7, 10, 12, 14, 17, or 21.

and it was evident around endoneural vessels and also in the parenchyma). The infiltrate was still marked on day 17 pi but negligible on day 21 pi.

Accumulation of CL in the ovary (positive control) was observed by LSCM for healthy controls and EAE rats at each time point of examination (Figure 1A), thus confirming the effectiveness of angiogenic targeting and demonstrating that the experimental protocol was appropriate.

No CL staining was found in the spinal cord of healthy rats at each time (Figure 1B). In contrast, in the spinal cord of EAE rats, already on day 7 occasionally rhodamine spots could be seen. Clear CL fluorescence was found on day 10 pi, even before a massive perivascular inflammatory infiltrate was present (Figure 1C). At this time point rhodamine staining was predominantly localized at the meningeal level and in the white matter of the spinal cord. The extent of the signal increased until day 14 pi (Figure 1D), when it was visible still 2 h after injection and involved the white and gray matter endoneural vessels. The signal was rather stable on days 14 and 17 pi, and it was clearly evident still on day 21 (although infiltrate was virtually no longer present in the spinal cord at this time point, Figure 1E,F). Serial reconstructions at the LSCM strongly suggested that CL was strictly confined within endoneural vessels, where it had frequently the appearance of discrete spots localized at the vessel wall (Figure 1F).

Our findings were corroborated by the second study, in which at day 7 (before manifestation of clinical signs), day 14 (maximum inflammation), and day 21 (after decline of clinical signs), CL binding and AL binding were directly compared. As shown in Table 1, EAE was effectively induced and the time course of the clinical impairment reflected that observed in study 1.

As before, CL staining was observed in the ovary of both healthy and EAE rats at each time point of examination, while for AL only rare spots with markedly reduced overall extent were found. No CL or AL staining was found in the spinal cord of healthy rats at each time point, and no AL staining was observed in EAE rats.

CL signal within the spinal cord of EAE rats was even slightly stronger than in study 1. Mild but clearly observable labeling was present in some endoneural vessels in EAE rats already on day 7 pi (Figure 2A). At day 14 pi, the signal was markedly higher (Figure 2C), and it was still evident (although reduced vs day 14 pi) on day 21 pi (Figure 2E), when no infiltrate was present in the spinal cord.

In the sections costained also for laminin, the intensity of the CL signal was markedly reduced by the mild detergent pretreatment necessary to allow an optimal laminin staining. However, CL staining confined within endoneural vessels was evident in most cases (Figure 2B,D,F). Anti-CD31 and anti-RECA-1 staining, which was also tested to colocalize the blood vessels, was found too faint to detect reliably small endoneural vessels (see Materials and Methods).

Discussion

In this study we have shown that very early changes of the CNS vasculature occur in the course of acute EAE as evidenced by binding of CL. The accumulation was directly correlated to the positive charge of the liposomes and the extent of inflammation: CL but not AL accumulated at spinal cord endoneural vessels of EAE rats, while binding to BBB was absent in healthy rats for both, CL and AL. In the ovaries (positive control) in all cases only CL and no AL binding was found, thus underlining the selectivity of CL for angiogenic vessels.

It is noteworthy that, for the size range of the liposomes used here (smaller than 200 nm), also passive accumulation, due to the increase of BBB permeability in the course of the neuroinflammatory response, should have been possible.[38] Most likely, we did not observe AL accumulation because passive infiltration is much slower compared to active CL binding, and the time interval between liposome application and euthanization was too short to enable sufficient accumulation of AL. In contrast, CL binding was demonstrated to be a rather fast process, as already 10 min after injection efficient staining was observed.

The temporal evolution of CL binding after EAE induction corresponded to the profile of inflammation: the intensity of the signal increased along with EAE severity and finally tapered when rats recovered from the acute phase of the disease. Accordingly, at the peak of inflammation the staining remained for longer time after application of the liposomes. Furthermore, the finding that CL staining was present (although less marked) even before the rats had signs of EAE (day 7) clearly suggests that CL targeting marked very early events in the acute neuroinflammatory processes in EAE.

CL are established for targeting endothelial cells in tumor growth and chronic inflammation,[7−9,18−25] and tumor vasculature targeting with CL formulations as used in the present study has been demonstrated in preclinical studies[9] and clinical phase I trials.[27] The evident similarities between the present findings and the binding preference of CL to tumor vasculature suggest that certain attributes of activated endothelial cells in tumor angiogenesis are represented at the BBB in early development of acute neuroinflammation.

The association between neovascularization and MS lesions has recently been hypothesized, but this referred only to later stages of the disease,[29] and an increase of vascular endothelial growth factor (VEGF) expression and number

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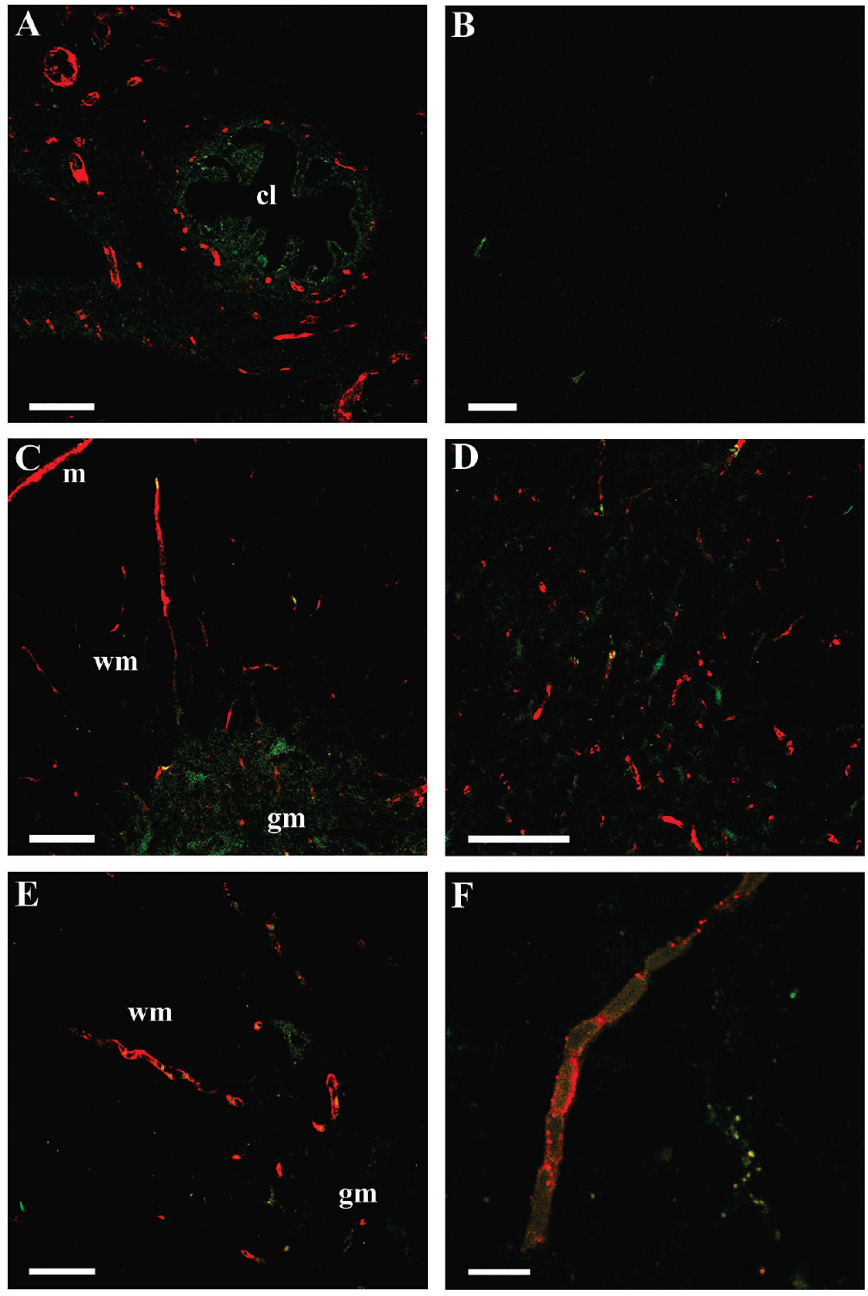

Figure 1. Cryosection images observed at the LSCM injection of CL from study 1. (A) Ovary: Staining is clearly evident, indicating ovarian targeting of CL to the positive control; cl = corpus luteum, bar = 100 μm. (B) Spinal cord of healthy rat: Absence of rhodamine staining indicates that no binding occurred. Results were equivalent at all time points after immunization; bar = 150 μm. (C) Spinal cord of EAE rat at day 10 after immunization, sacrificed 10 min after application of CL: Rhodamine staining was clearly evident, particularly in the perimenningeal space; *m* = meninges, wm = white matter, gm = gray matter, bar = 100 μm. (D) Spinal cord of EAE rat at day 14 after immunization, sacrificed 10 min after application of CL: The distribution of rhodamine was more widespread compared to day 10, involving the white (wm) and gray matter (gm); bar = 50 μm. (E) Spinal cord of EAE rat at day 21 after immunization, sacrificed 10 min after application of CL: Staining was similar to that at day 14; bar = 50 μm. (F) Spinal cord of EAE rat at day 21 after immunization, sacrificed 10 min after application of CL: Staining was still evident, the intensity of the intraluminal signal was reduced, and the staining appeared as discrete spots at the vessel wall; bar = 10 μm.

of vessels has been observed in chronic-progressive EAE.[28] However, here, we found elevated CL binding in a model of acute neuroinflammation, already before the clinical signs of EAE, and certainly in the absence of marked angiogenesis and chronic inflammation. As a consequence, the range of pathological situation where cationic vascular targeting can be applied might not be limited to tumor growth and chronic inflammation.

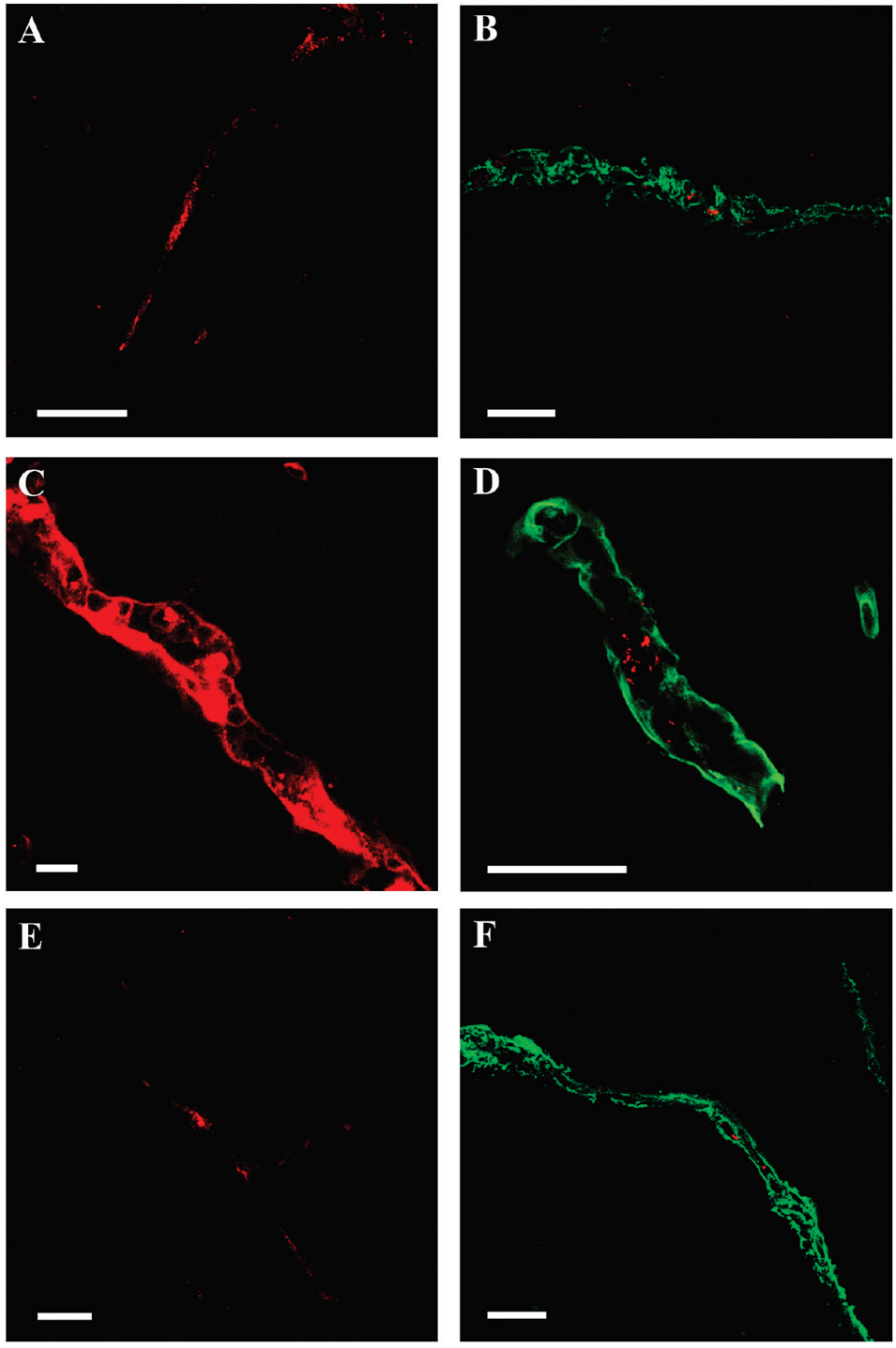

Figure 2. Cryosection images of the white matter spinal cord from EAE rats from study 2 observed at the LSCM. The left panels refers to days 7 (A, bar = 50 μm), 14 (C, bar = 10 μm) and 21 after immunization (E, bar = 150 μm) and confirm the results obtained in study 1. The sections reported in the right panels have been stained with an antilaminin Ab, and allow demonstration that the CL signal is confined within spinal cord vessels 7 (B, bar = 100 μm), 14 (D, bar = 50 μm) and 21 days after immunization (F, bar = 150 μm).

In the context of MS and neuroinflammatory diseases, CL can be of interest for early and sensitive intervention at affected BBB sites. Determination of BBB permeability for hydrophilic solutes is important for diagnostic purposes.[3,39−41] Examination of CL targeting could provide valuable supplementary information on early signs of BBB dysfunction, independent from and potentially preceding the breakdown of its function as a barrier. This could contribute to clarify unknown aspects of the precise sequence of the events in the course of neuroinflammation and their role for BBB lesion formation and disease progression.

The main focus of current MS therapy is on the immune response through systemic administration of drugs.[42] Drug delivery to inflammatory sites of BBB could enable local interference in the inflammatory process, allowing administration of lower doses of drugs compared to systemic administration, and thus reduction of side effects. Delivery of colloidal particles by passive infiltration across leaky BBB has already been demonstrated,[43,44] however, long circulating times are necessary to obtain sufficient uptake. In contrast, binding of CL is very fast and does not require elevated permeability. CL loaded by cytotoxic or anti-inflammatory compounds, as well as agents able to modulate the activity of endothelial cells, may be of interest for such local intervention. Physiological effects of CL lipids, in particular their anti-inflammatory activity,[16,45] might be an additional benefit for this treatment strategy.

Conclusions

We have demonstrated that, in the course of acute EAE inflammation, BBB becomes susceptible to binding by positively charged liposomes. CL displayed sensitive, fast and efficient accumulation at affected BBB. The apparent coherencies to cationic tumor vascular targeting suggest that similar processes are involved at the molecular level. In light of these observations and successful clinical trials with cationic liposomes for tumor therapy, new options for treatment of neuroinflammatory diseases based on CL may be considered.

Abbreviations Used

AL, anionic liposomes; CL, cationic liposomes; DOTAP, dioleoyl trimethylammonium propane chloride; DOPC, dioleoyl phosphatidylcholine; DOPG, dioleoyl phosphatidylglycerol; Rho-DOPE, rhodamine-dioleoyl phosphatidylethanolamine; Chol, cholesterol; LSCM, laser scanning confocal microscopy; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MRI, magnetic resonance imaging; VEGF, vascular endothelial growth factor.

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