Suppression of Experimental Allergic Encephalomyelitis in Lewis Rats After Elimination of Macrophages

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Summary

Almost 50% of the cells infiltrating the central nervous system (CNS) of animals with experimental allergic encephalomyelitis (EAE) are macrophages (Mφ). To investigate the role of the Mφ in the pathogenesis of EAE, we eliminated Mφ by means of mannosylated liposomes containing dichloromethylene diphosphonate (Cl_2MDP). Cl_2MDP-containing liposomes injected intravenously eliminate Mφ in spleen and liver. Incorporation of mannose into the lipid layers enables the liposomes to pass the blood-brain barrier (BBB). Injections of Cl_2MDP-containing mannose liposomes intravenously shortly before the appearance of clinical signs, markedly suppressed the expression of clinical signs of EAE. This suppression was accompanied by a marked reduction of infiltrated Mφ in the CNS. Cl_2MDP-containing liposomes without mannose incorporated had no effect. Cl_2MDP-containing mannosylated liposomes had no effect on plasma corticosterone levels compared with injections of saline; thus, the suppression of expression of EAE was not corticosterone mediated. These results show that the Mφ within the CNS play an important role in the pathogenesis of EAE.

Experimental allergic encephalomyelitis (EAE)[1] is an inflammatory demyelinating disease of the central nervous system (CNS), which can be induced in genetically susceptible animals by an injection of whole nervous tissue homogenate emulsified in CFA. On clinical and pathological grounds EAE is considered to represent an animal model for immune-inflammatory diseases of the CNS in general, and for multiple sclerosis (MS) in particular (1). About 10–12 d after sensitization the animal starts to exhibit neurological signs caused by the effects of mononuclear cells infiltrating the CNS, forming perivascular cuffs. The invading cells mainly consist of T cells and macrophages (Mφ) and to a lesser extent of B cells (2–5).

EAE is considered to be a T cell–dependent, delayed type of hypersensitivity reaction. It is not clear, however, by which mechanism cell-mediated immunity initiates clinical and pathological expression of EAE. In a previous study we have shown that Mφ are present in large numbers in the CNS of EAE animals (6). It is very likely that these cells play a role in the development of tissue damage in the CNS and subsequent neurological disorders. Mφ have been demonstrated to strip off myelin from axons (7) and to phagocytize myelin in a receptor-mediated way (8). Furthermore, activated Mφ secrete numerous products that can play multiple roles in nonspecific inflammatory reactions underlying breakdown of the blood-brain barrier (BBB), the attraction of immunocompetent cells into the CNS, the activation of immunocompetent cells, the generation of edema, and degradation of myelin (9). In vitro degradation of myelin by Mφ products such as neutral proteinases (10), oxygen radicals (11), and TNF (12) has been demonstrated. Blood monocytes of MS patients show an increased oxidative burst activity (13). Increased levels of proteinases are found in lesions of MS brains (14) and EAE CNS (15). Indeed, administration of both proteinase inhibitors (16, 17) and oxygen radical scavengers (18), suppresses the expression of EAE or experimental allergic neuritis (EAN), the peripheral counterpart of EAE.

Depletion of macrophages after injection of silica dust shortly before and during the appearance of clinical signs in EAE (19) and EAN (20–22), remarkably attenuates the de-

A report of part of this work will be published in abstract form (*Ann. NY Acad. Sci.* 1990. 594:458).

[1] *Abbreviations used in this paper:* BBB, blood-brain barrier; Cl_2MDP, dichloromethylene diphosphate; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; EAN, experimental allergic neuritis; GSC, guinea pig spinal cord; Mφ, macrophages; MS, multiple sclerosis; PC-liposomes, liposomes constructed of phosphatidylcholine and cholesterol; PCMAN-liposomes, mannosylated PC-liposomes; p.i., post-immunization.

velopment of the disease, which suggests that Mφ play a crucial role in the effector phase of the disease. Silica however, is known to exert additional effects on other immunocompetent cells (23–25), and there is no evidence that silica is capable of passing the BBB. To bypass the side effects of silica we used an in vivo Mφ elimination method, described by van Rooijen and van Nieuwmegen (26). Mφ were eliminated by intravenous injections of liposomes constructed of phosphatidylcholine and cholesterol and containing dichloromethylene diphosphonate (Cl_2MDP) (Cl_2MDP PC-liposomes). Cl_2MDP PC-liposomes injected intravenously eliminate almost all Mφ in spleen and liver (26, 27). Free Cl_2MDP is not toxic; it needs enclosure in liposomes and subsequent phagocytosis is necessary for its toxic effect. Therefore, only phagocytes are susceptible (26). To reach the CNS, we used mannosylated Cl_2MDP PC-liposomes (Cl_2MDP PCMAN-liposomes). Mannosylated PC-liposomes are, in contrast with non-mannosylated PC-liposomes, capable of passing the BBB (28). To cause the liposomes to reach the CNS at the moment the Mφ start to infiltrate, we injected the liposomes just before the expected expression of clinical signs. We found that Cl_2MDP PCMAN-liposomes injected on days 8 and 10 post-immunization (p.i.) completely suppressed or markedly attenuated the expression of EAE. Non-mannosylated Cl_2MDP PC-liposomes had no effect on expression of EAE. Timing of the liposome injections appeared to be critical.

Materials and Methods

Animals. Male Lewis rats were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, FRG), housed two to three animals per plastic cage, and kept under standard conditions, water and food available *ad libitum*. The animals had a weight of ~180–200 g at the time of inoculation.

Induction of EAE. Acute EAE was induced by a single subcutaneous injection under Hypnorm anesthesia (Janssen Pharmaceutica, Beerse, Belgium, i.m., 0.5 ml/kg bodyweight) of 50 μl guinea pig spinal cord (GSC) homogenate in one hind food pad. This emulsion consisted of 1 g GSC in 1 ml saline (0.9% NaCl), to which 10 mg *Mycobacterium tuberculosis* H37 RA (Difco Laboratories, Detroit, MI) in 1 ml CFA (Difco Laboratories) was added.

Preparation of Liposomes. Multilamellar liposomes were prepared as described (29). Briefly, to prepare the PC-liposomes, 86 mg phosphatidylcholine and 8 mg cholesterol (Sigma Chemical Co., St. Louis, MO) (molar ratio phosphatidylcholine/cholesterol = 6:1, with a total amount of 140 μmol of lipids) were dissolved in 10 ml chloroform in a 500-ml, round-bottomed flask, dried in vacuo on a rotary evaporator to form a film. Subsequently the film was dispersed into liposomes after the addition of PBS (0.15 M NaCl in 10 mM phosphate buffer, pH 7.4). To enclose Cl_2MDP into PC-liposomes, 1.89 g Cl_2MDP (maximum soluble amount) was added to 10 ml PBS. the preparations were kept for 2 h at room temperature (RT) and sonicated for 3 min at 20°C in a sonicor (50 Hz) and kept at RT for another 2 h. The liposomes were centrifuged at 100,000 *g* for 30 min and finally resuspended in 4 ml PBS. To prepare PCMAN-liposomes, 70.9 mg phosphatidylcholine and 10.8 mg cholesterol were dissolved in 8 ml chloroform and added to 3.6 mg *p*-aminophenyl-α-D-mannopyranoside (Sigma Chemical Co., St. Louis, MO), dissolved in 2 ml methanol, dried as described above. The molar ratio of phosphatidylcholine/cholesterol/mannoside of 7:2:1 was chosen according to Umezawa and Eto (28). The total amount of lipids was 140 μmol. The dried lipid film was dissolved in chloroform and dried once again before the aqueous phase, with or without Cl_2MDP, was added. Liposomes were sonicated and centrifuged as described above.

Treatment. Two experiments are described. In Exp. A timing of liposome injections is based on data on kinetics of Mφ movement into the CNS post-infection. Mφ start to infiltrate ± 8–10 d p.i. (6). Mannosylated liposomes are detected in the CNS 24–48 h post-injection (28). Therefore, we gave a first injection of liposomes 8 d p.i., followed 2 d later by a second injection. Three groups of eight rats each were treated with either 0.9% NaCl, PBS PCMAN-liposomes (two control groups), or with Cl_2MDP PCMAN-liposomes 8 and 10 d p.i. (2 ml i.v.). On day 10 p.i. some blood samples were taken to study the effect of the mannose liposomes on the circulating monocytes. On days 15–17 p.i. the animals were killed for histological examination. At this moment infiltrated Mφ are abundantly present in the CNS of EAE rats.

Exp. B was constructed to study (*a*) effects of Mφ elimination at different stages during the induction phase and early effector phase of the disease, and (*b*) effects on the expression of EAE after elimination of Mφ in spleen and liver by Cl_2MDP PC-liposomes, which were not mannosylated and not capable of passing the BBB (30). This experiment consisted of four groups of five rats each which received two intravenous injections of 2 ml Cl_2MDP PCMAN-liposomes on days 6 and 8, 7 and 9, and 8 and 10 p.i., or two intravenous injections of 2 ml Cl_2MDP PC-liposomes on days 8 and 10 p.i. The control group in this experiment consisted of six rats and received no treatment post-immunization. On day 50 p.i. the animals were killed for histological evaluation.

Clinical Assessment. The rats were weighed and investigated daily to score the development of neurological signs. Clinical signs were scored from 0 to 4: 0, no clinical signs; 1, loss of tail tonus and unsteady gait (partial loss of tail tonus was scored only when registered on two consecutive days); 2, paresis of the hind legs; 3, complete paralysis of the hind legs or complete lower part of the body; 4, death due to EAE. *2* and *3* are often accompanied by urinary and fecal incontinence (31).

Histological Techniques. Animals were anesthetized with hypnorm (i.m., 1 ml/kg bodyweight) and killed by an intracardial injection of 0.5 ml nembutal (Algin B.V., Maassluis, The Netherlands). Brain, optic nerves, spinal cord, liver, and spleen were dissected and frozen in liquid nitrogen and stored at −20 or −70°C. Cryostat sections of 8 μm were cut serially, picked up on glass slides, and dried in a container with silica gel. Immunocytochemistry was applied to examine cellular infiltrates in the CNS and efficacy of Mφ elimination by the Cl_2MDP-liposomes in spleen and liver. The following mAbs were used: mouse anti-pan-T cell (OX-19), mouse anti-Ia (OX-4), both from Serotec, Oxford, UK; mouse anti-rat macrophages (ED1 and ED3) (32); and the mAb ED8 which recognizes rat Mφ as well as glial cells (33, 34). ED8 shows a staining pattern comparable with OX-42, which recognizes the iC3b receptor (35). As conjugate a rabbit anti-mouse IgG~peroxidase (Dako, Tilburg, The Netherlands) was used. Antibodies and conjugate were diluted in 0.01 M PBS (pH 7.4) with 0.2% BSA (Organon Technika, Oss, The Netherlands) and used in dilutions between 1:100 and 1:400. All incubations were carried out horizontally at RT. After incubation with the first antibody for 45 min, the slides were rinsed in PBS, incubated in conjugate with 1% normal rat serum for 30 min, and washed again in PBS. Peroxidase activity was demonstrated after a 10-min incubation with 0.5 mg/ml 3,3'-diaminobenzidine-tetra-hydrochloride (DAB; Sigma

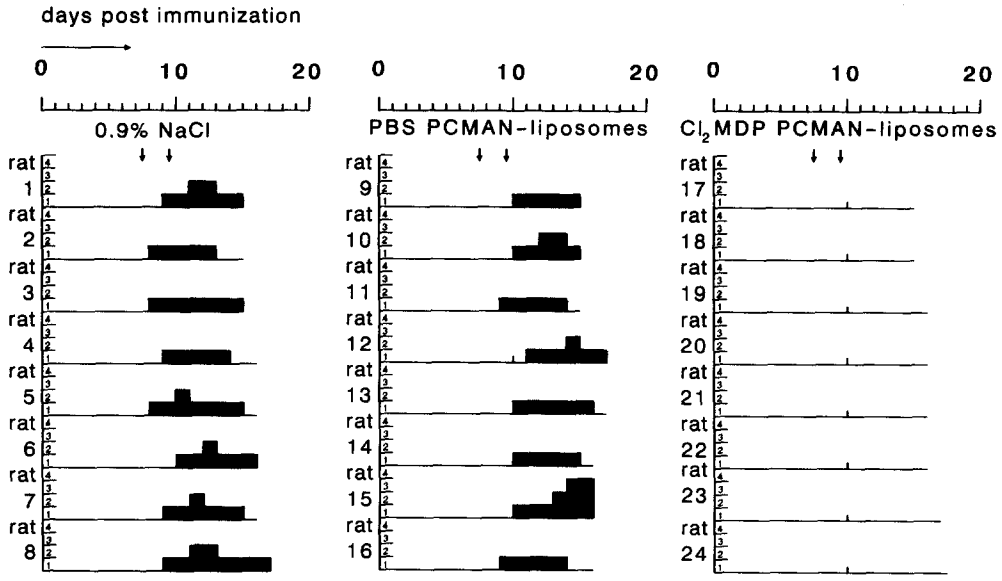

Figure 1. Exp. A: Effect of injections of 0.9% NaCl, PBS-containing PCMAN-liposomes, and Cl_2MDP-containing PCMAN-liposomes on EAE. Injections contained 2 ml volume and were given intravenously on days 8 and 10 p.i. Scoring of clinical signs of EAE: 0, no clinical signs; 1, paralysis of tail; 2, incomplete paralysis of the hindlegs; 3, complete paralysis of the hindlegs; and 4, death due to EAE.

Chemical Co.) in 0.05 M Tris buffer, pH 7.6, containing 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin.

Bloodsmears of animals treated with liposomes were air dried and stained with May Grünwald–Giemsa dyes (Merck, Darmstadt, FRG), according to standard histological staining methods.

Determination of Effects of Cl_2MDP PCMAN-Liposomes on Plasma Corticosteroid Levels. To exclude the possibility that the effect of treatment of EAE was caused by the induction of a stress response in the CNS by the PCMAN liposomes and a concomitant rise in plasma corticosterone levels, we studied the effect of mannosylated liposomes on plasma corticosterone levels. Lewis rats were injected intravenously with either 2 ml 0.9% NaCl or 2 ml Cl_2MDP PCMAN-liposomes. Blood was collected from the tail at 6, 24, 48, and 72 h after the injections, kept on ice in heparin-containing tubes, centrifuged (100 *g*, 15 min; 4°C) and stored at −20°C until use. Blood was collected within 5 min after the beginning of an ether anesthesia to prevent elevation of corticosterone levels due to ether stress. Ether causes plasma corticosterone levels to rise up to 60 μg/100 ml plasma after 20 min (36, 37). Plasma corticosterone was measured using an extraction step with dichloromethane followed by a fluorometric determination as described (38).

Results

EAE–Experiment A

Clinical Data. Effects of 0.9% saline, PBS PCMAN-, and Cl_2MDP PCMAN-liposomes, all injected 8 and 10 d p.i. (2 ml, i.v.), on clinical signs and weight are shown in Figs. 1 and 2, respectively. 0.9% NaCl and PBS PCMAN-liposome–treated animals expressed EAE as normal, showing the first neurological signs on day 9.8 ± 0.7 and day 10.8 ± 0.6 p.i. respectively. Clinical signs were spotted until days 14–17 p.i.; the exact duration can not be given, since 50% of the animals were still having clinical signs when animals were killed for histological evaluation. Clinical signs included flaccid tail and paresis of the hindlegs. In contrast, all animals that received Cl_2MDP PCMAN-liposomes showed a striking absence of neurological disorders. Weight loss was apparent in all three groups, though animals in the Cl_2MDP- and PBS PCMAN-liposome group lost a little less weight than animals in the saline-treated group (difference not statistically significant) (Fig. 2).

Histological Evaluation. Nervous tissue collected 15–17 d p.i. revealed severe lesions in saline-treated animals. Lesions contained high numbers of T cells and Mφ, infiltrating the CNS. The majority of the infiltrating cells were Ia^+. Throughout the CNS glial cells were also reacting with the anti-Ia mAb. Lesions were dominant in submeningeal areas,

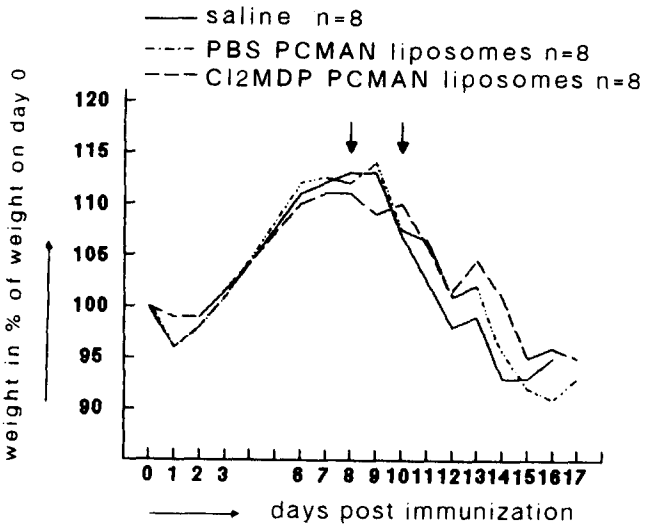

Figure 2. Exp. A: Effect of injections of 0.9% NaCl, PBS-containing PCMAN-liposomes, and Cl_2MDP-containing PCMAN-liposomes on bodyweight of EAE rats. Injections contained 2 ml volume and were given intravenously on days 8 and 10 p.i. Weight is given in percentage of weight as it was during immunization. On days 4 and 5 p.i. animals were not weighed. Each group consists of eight rats.

in regions around the ventricles and areas where nerves leave or enter the brain and spinal cord. Serial blocks from these areas of the different experimental groups were compared by two independent observers. A clear reduction of the number of lesions in PBS- as well as in the Cl_2MDP PCMAN-liposome treated groups was observed. As a consequence the amount of infiltrated cells was reduced in both groups, although Ox-19$^+$ cells (T cells) and ED1$^+$ and ED3$^+$ cells (Mφ) were clearly present. The Mφ in the Cl_2MDP PCMAN-liposome treated group however, showed a restricted infiltration into the parenchyma. In these animals ED1$^+$ and ED3$^+$ cells were spotted in the lumen of the blood vessels or sticking between endothelial cells; in many lesions they were only sparsely seen outside the blood vessels (Fig. 3 *b*). This phenomenon was not observed in lesions of saline and PBS PCMAN-liposome treated animals (Fig. 3 *a*). No changes in glial cell populations could be observed; in particular no differences were observed in the morphology and number of ED8$^+$ glial cells of the different experimental groups.

Spleen and liver sections showed efficient elimination of the Mφ by the Cl_2MDP PC- and Cl_2MDP PCMAN-liposomes as described before (27). Blood samples collected on day 10 p.i. (2 d after the first injection), showed an increase in the ratio of PMN to lymphocytes. Percentages PMN ranged from normally 11% in healthy Lewis rats to 39% in the saline treated, 22% in the PBS PCMAN-liposome, and

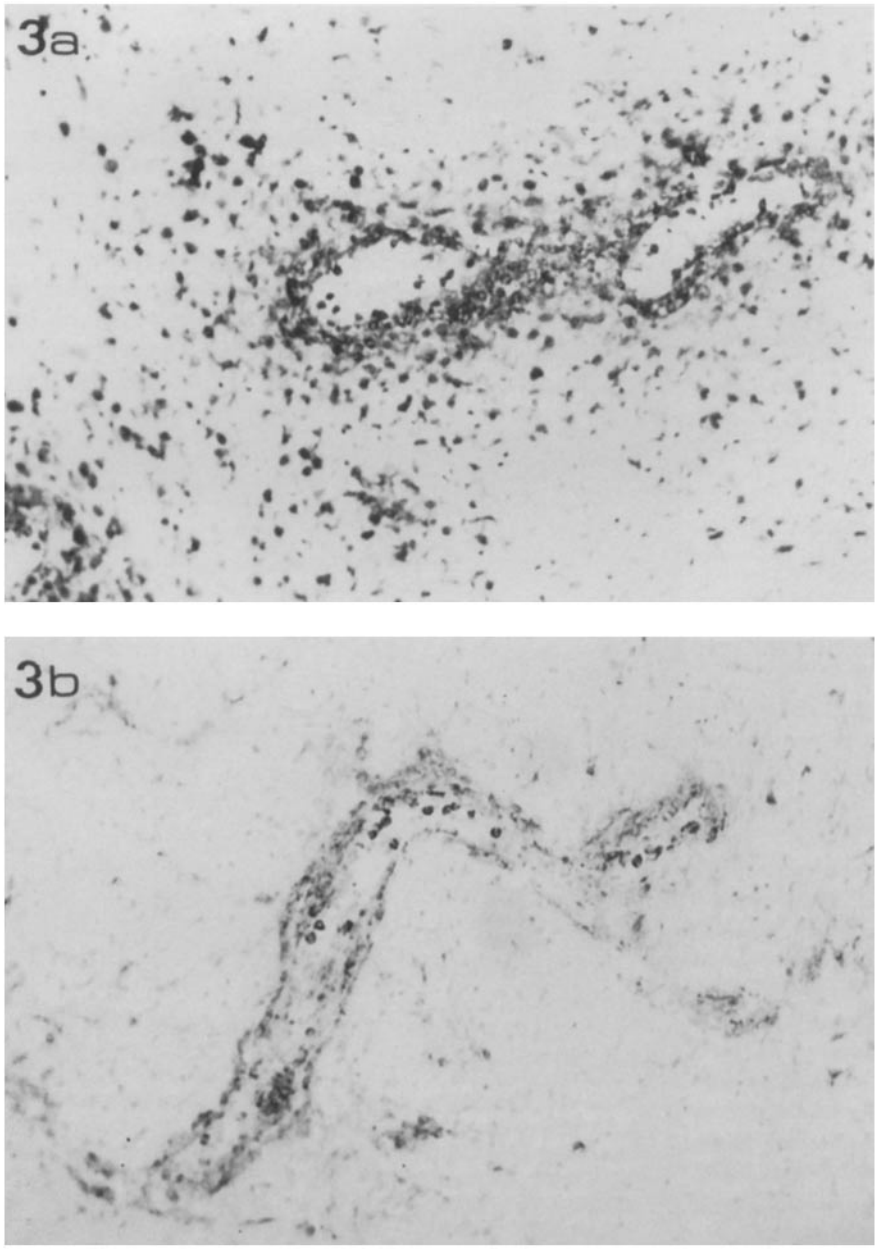

Figure 3. Exp. A: Representative micrographs of 8-μm cryostat sections incubated with the mAb ED1 which specifically recognizes rat Mφ, at the medulla oblongata of animals that had received 0.9% NaCl (*a*) or Cl_2MDP containing PCMAN-liposomes (*b*) on day 8 and 10 p.i. (both same magnification, ×250). Note extensive infiltration of the CNS parenchyma by the Mφ in the saline-treated animals, whereas in the CNS of the Cl_2MDP-containing PCMAN-liposome– treated animals no Mφ can be spotted outside the lumen of the blood vessel. Tissue was collected 16 d p.i.

34% in the Cl_2MDP PCMAN-liposome treated group (Fig. 4). Percentages are given of counts of 200 white blood cells per animal. Expanded and vacuolized monocytes made up 8% of the Cl_2MDP PCMAN-liposome and 2.5% of the PBS PCMAN-liposome–treated animals. Such enlarged and sometimes disintegrating monocytes were not spotted in healthy Lewis rats or saline-treated EAE rats. Percentages of healthy looking monocytes varied between 2% in healthy Lewis rats, to 3% in the saline-, 7% in the PBS PCMAN-liposome–, and 2.5% in the Cl_2MDP PCMAN-liposome–treated EAE group.

EAE–Experiment B

Clinical Data. The effect of Cl_2MDP PCMAN-liposomes injected at three different time points (days 6 and 8, 7 and 9, and 8 and 10 p.i.) and of Cl_2MDP PC-liposomes (days 8 and 10 p.i.) on the expression of clinical signs of EAE is shown in Fig. 5 and Table 1. Animals were observed until day 50 p.i. The control group in this experiment received no treatment (2-ml i.v. injections of saline at days 8 and 10 p.i. have no effect on expression of EAE; unpublished data).

First signs of EAE (flaccid tail) were spotted 9.4 ± 1.3 d p.i. in the Cl_2MDP PC-liposome–treated group, subsequently followed by the control animals (10.2 ± 0.4 d), and the Cl_2MDP PCMAN-liposome groups treated 6 and 8 d p.i. (10.8 ± 1.0 d), 7 and 9 d p.i. (12.7 ± 0.6 d), and finally

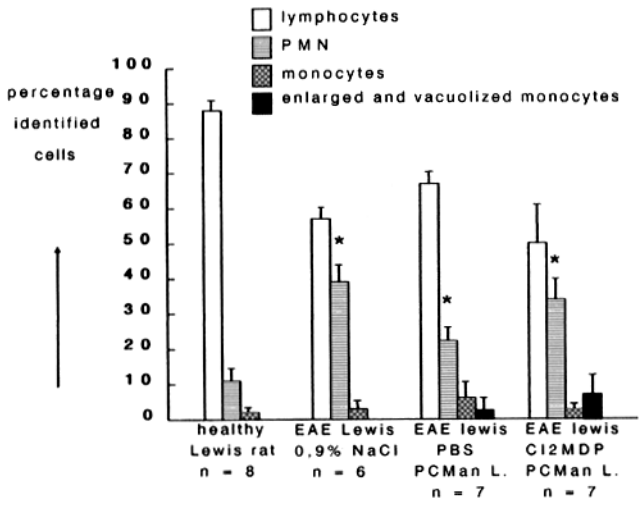

Figure 4. Experiment A: Effect of EAE, 2 ml 0.9% NaCl, 2 ml PBS containing PCMAN-liposomes, and 2 ml Cl_2MDP containing PCMAN-liposomes, injected i.v., days 8 and 10 p.i., on the ratio lymphocytes:PMN:monocytes of the Lewis rat. Blood samples were taken 10 days p.i., this was two days after a first i.v. injection of 2 ml saline or liposomes. Blood smears were stained with May Grünwald-Giemsa and 200 leucocytes per smear were identified on morphological features and counted. The average ratio of each experimental group ± SD is represented. Note the increase in ratio PMN:lymphocytes in EAE animals and the increase in ratio monocytes:lymphocytes in the PBS PCMAN-liposomes treated animals, and the appearance of the group enlarged and vacuolized monocytes. Difference with healthy Lewis rats is statistical significant: * = $p < 0.01$ (Wilcoxon Rank sum Test).

8 and 10 d p.i. (13.0 ± 0.0 d) (Table 1). Thus, there was a correlation between the timing of the injections of Cl_2MDP PCMAN-liposomes and the day of onset of clinical disease. Severity of the disease was analyzed based on the number of animals that had at least paresis of hindlegs (score 2). It appeared that Cl_2MDP PCMAN-liposomes injected 7 and 9 d or 8 and 10 d p.i. had the best suppressive effects on expression of clinical signs of EAE ($p < 0.05$). Incidence of clinical disease was 60% in these groups, but signs were restricted to flaccid tails for only a maximum of 3 d. Based on the incidence of one relapse in the days 7 and 9 group and on the total number of days during which flaccid tails (score 1) were scored, EAE in the days 8 and 10 group was even better suppressed than in the days 7 and 9 group. EAE was also suppressed in animals that received Cl_2MDP PCMAN-liposomes 6 and 8 d p.i., but two relapses occurred and paresis of the hindlegs occurred during 10 d against 1 d in each of the other two Cl_2MDP PCMAN-liposome–treated groups (Fig. 5). In contrast, animals that were treated with Cl_2MDP PC-liposomes all developed EAE. Mean day of onset of clinical disease was, as already mentioned, one day before that of the controls. Two of five animals did not recover as quickly as animals in the other experimental groups. One relapse occurred and one animal died due to EAE. Weight loss was apparent in all five experimental groups and comparable to the weight loss seen during EAE in Exp. A (Fig. 2).

Histological Evaluation. Nervous tissue collected 50 d p.i. showed dim remainders of multiple lesions. Lesions in animals of all experimental groups showed only a very small amount of infiltrating cells. Pan-T cells were present in some lesions as were ED1 and very sparse numbers of $ED3^+$ cells. Comparison of central nervous tissue of untreated and liposome-treated groups revealed no difference in the amount of lesions and infiltrating pan-T cells. $ED1^+$ and $ED3^+$ cells, however, showed a tendency to be present in higher numbers in animals in which the disease had been most strongly suppressed. Histological evaluation of spleen sections did not reveal signs of Mφ eliminating activities 6 wk before dissection. $ED1^+$ and $ED3^+$ cells were present as normal. Recovery within 2 mo of Mφ in the spleen after depletion by Cl_2MDP-containing liposomes has been described (27).

Effect of Cl_2MDP PCMAN-Liposomes on Plasma Corticosterone Levels

No difference was observed between the effect of injection of Cl_2MDP PCMAN liposomes and injection of saline on the plasma corticosterone levels (Fig. 6). Plasma corticosterone levels were in both groups slightly elevated up to 10–18 μg/100 ml plasma until 3 d after the injections. Basic levels were 4.0 ± 1.1 and 4.5 ± 1.4 μg corticosterone/100 ml plasma, respectively. The elevation after the injections is probably caused by stress reactions due to the injections and repetition of blood sampling, and is similar in both groups.

Discussion

The results clearly demonstrate that Lewis rats can be protected against development of neurological signs of EAE by

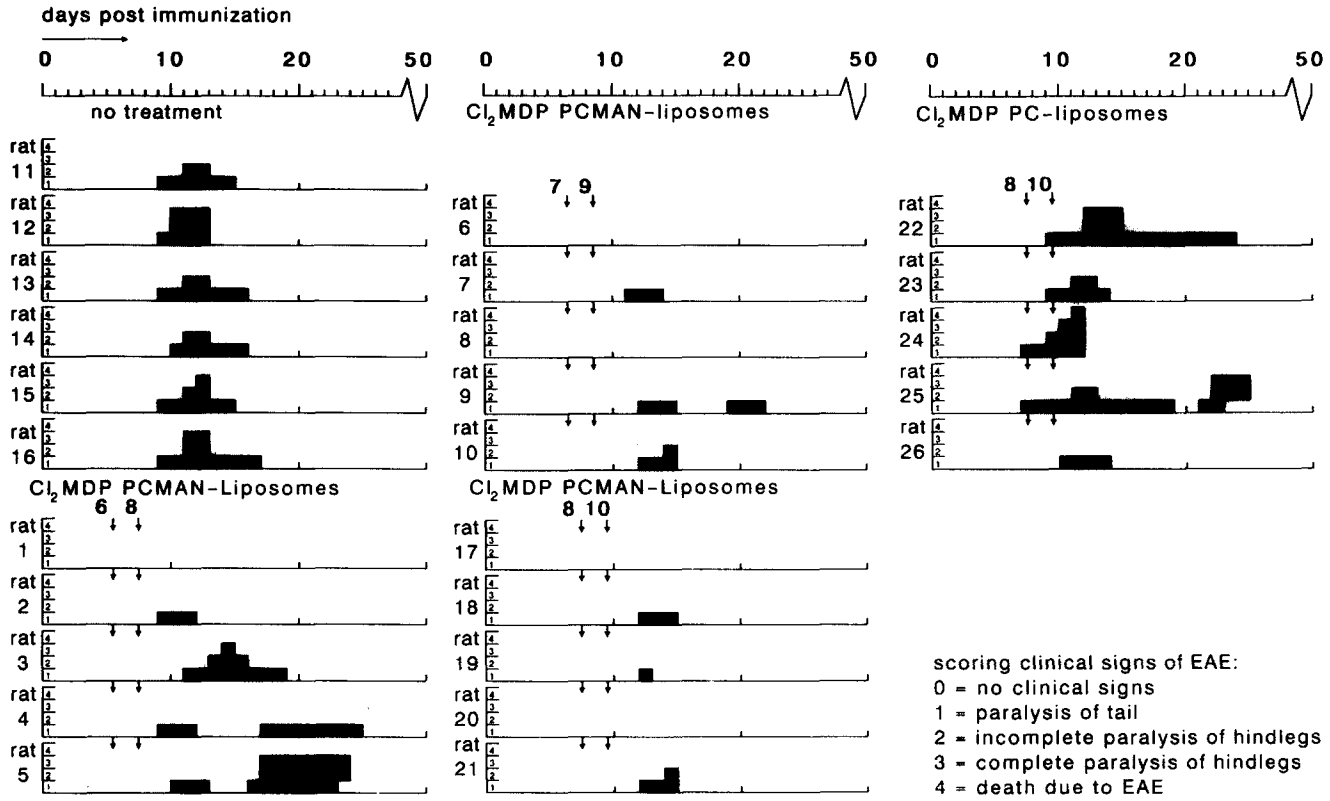

Figure 5. Exp. B: Effect of 2 ml Cl_2MDP-containing PCMAN-liposomes injected intravenously at different timepoints post-immunization (days 6 and 8, 7 and 9, and 8 and 10) and Cl_2MDP-containing PC-liposomes (days 8 and 10 p.i.), on the expression of clinical signs of EAE.

Table 1. *Effect of Different Liposomes Administered at Different Timepoints on Expression of EAE*

Experimental group	Average day of clinical onset ± SD	Incidence	Average duration of clinical signs ± SD	Number of animals with at least paresis of hindlegs (score 2)	Relapses
		%	*d*		
No treatment (n = 6)	10.2 ± 0.4	100	6.2 ± 1.4	6/6	0
days 6 and 8 p.i. CL_2MDP MANL (n = 5)	10.8 ± 1.0	80	4.3 ± 2.5	2/5	2
days 7 and 9 p.i. Cl_2MDP MANL (n = 5)	12.7 ± 0.6	60	3.0 ± 0.0	1/5*	1
days 8 and 10 p.i. Cl_2MDP MANL (n = 5)	13.0 ± 0.0	60	2.3 ± 1.2	1/5*	0
days 8 and 10 p.i. Cl_2MDP L (n = 5)	9.4 ± 1.3	100	9.0 ± 5.4	4/5	1

Exp. B: Effect of 2 ml Cl_2MDP-containing PCMAN-liposomes (Cl_2MDP MANL) injected intravenously at different timepoints post-immunization (days six and eight, seven and nine, and eight and ten) and Cl_2MDP-containing PC-liposomes (Cl_2MDP L) (days eight and ten p.i.), on the expression of clinical signs of EAE. Averages are given ± SD. Difference with control animals is statistically significant: $^*p < 0.05$ (χ_y^2 Test).

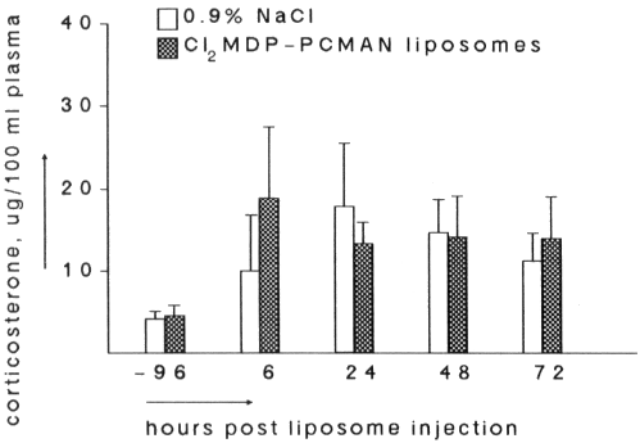

Figure 6. Effect of 2 ml, i.v. injected, 0.9% NaCl and Cl_2MDP-containing PCMAN-liposomes on plasma corticosterone level at 6 h and 1, 2, and 3 d post-injection. No difference between saline and liposome effects were observed (Wilcoxon Rank sum Test). Values given are means ± SD, five animals per group.

intravenous injections of mannosylated Cl_2MDP PC-liposomes shortly before onset of clinical signs. No significant effect was found on weight loss normally accompanying expression of EAE. Radioactive labeled mannosylated PC-liposomes can be demonstrated in the CNS after intraperitoneal injection (28). This is in contrast with radiolabeled non-mannosylated PC-liposomes, which can not be detected in the CNS after intravenous injection (30). Both kinds of liposomes, when containing Cl_2MDP, eliminated Mφ in spleen liver when injected intravenously (27; this study). Cl_2MDP PC-liposomes without mannose incorporated had no effect on the expression of EAE. Elimination of Mφ in spleen and liver was apparently not sufficient to suppress clinical signs of EAE. The suppressive effect of the Cl_2MDP PCMAN-liposomes is therefore likely to be due to elimination of Mφ infiltrating the CNS.

In contrast to silica (23–25), Cl_2MDP PC-liposomes selectively kill phagocytizing cells (29) and do not affect proliferation and functions of T and B cell clones in vitro (39). Suppression of EAE by Cl_2MDP-containing liposomes is thus due to direct or indirect effects of Mφ elimination.

Histological evaluation of CNS tissue revealed a considerable reduction of the number of infiltrated Mφ in Cl_2MDP PCMAN-liposome–treated animals. This finding supports the fact that the mannosylated PC-liposomes reach the CNS and exert their effect there. On light microscopal level no changes in glial cell populations could be observed.

Recent intriguing data on involvement of corticosteroids in immune-regulatory events show the need of elevated plasma corticosterone levels for spontaneous recovery of rats from EAE (40, 41). To rule out the possibility that the Cl_2MDP PCMAN-liposomes evoke a stress response in the CNS by stimulating the hypothalamic-pituitary-adrenal axis and subsequently suppress expression of EAE by elevation of corticosterone levels, we studied the effect of intravenous injections of Cl_2MDP PCMAN-liposomes on plasma corticosterone levels. No difference in effect was observed on corticosterone levels between saline and Cl_2MDP PCMAN-liposome injections. Slight elevations, probably due to the stress induced by the injections and repetition of blood sampling under ether anesthesia were observed, but did not reach corticosteroid levels of more then 30–40 μg/100 ml, which are described to be necessary for EAE recovery (40).

Timing of the injections appeared to be very important. Suppression of EAE after Cl_2MDP PCMAN-liposomes injected 6 and 8 d p.i. was not as strong as after Cl_2MDP PCMAN-liposome injections 7 and 9 and 8 and 10 d p.i. Injections of Cl_2MDP PCMAN-liposomes on two successive days post-immunization showed an almost constant time interval of 3 d between the day of the second injection and the day of onset of clinical signs (Table 1). In those 3 d liposomes may partially have been cleared from the blood, and new monocytes may have been recruited from the bone marrow as a source of new activated Mφ. These Mφ can then be responsible for retarded and possibly less severe clinical signs. This indicates a very quick succession of immunological events, such as attraction of immunocompetent cells into the CNS, initiation of effector mechanisms resulting in tissue damage, and subsequent generation of suppressor mechanisms during acute EAE.

Our results indicate that Mφ are essential for the pathogenesis of EAE. Sedgwick et al. (42) reported, however, that $CD4^+$ lymphocytes alone are responsible for the development of clinical signs of EAE in irradiated rats after sensitization with MBP-reactive $CD4^+$ cells. These recipient rats had only a very few infiltrating cells in the CNS. Hardly any Mφ were present in the CNS, as was demonstrated by the mAbs W3/25 (CD4) (43, 44) and MRC OX-42 (35). It must be noted, however, that neither W3/25 nor OX-42 are specific Mφ markers, nor do they recognize all Mφ as does mAb ED1, for example (32, 35, 43, 44). Therefore, it is possible that the Mφ in the lesions of the CNS of the irradiated rats were not recognized by the used mAbs. Another explanation for the discrepancy between the results of Sedgwick et al. (42) and our results is that the mechanism that causes paralysis in the irradiated rats differs from that in unirradiated rats. The pathology in the CNS of irradiated rats includes, in contrast to the usual EAE models, hemorrhages that also can cause paralysis. A third possibility is that the effect of Mφ elimination in our study is not caused directly by the lack of Mφ but by the lack of their stimulatory activity for $CD4^+$ cells. Our immunohistochemical findings reveal no difference in the number and localization of T cells in the CNS between animals treated with Cl_2MDP PCMAN-liposomes and controls.

The difference between the effects of mannosylated and non-mannosylated Cl_2MDP PC-liposomes was striking. Mannosylated liposomes are found to pass the BBB (28). It is not clear how the liposomes pass the BBB, and Umezawa and Eto (28) mention mannose as being a "recognition marker" for an unknown molecule or mechanism. With respect to the present study, it should be kept in mind that a mannosyl receptor can be expressed on activated monocytes (45, 46).

This phenomenon leads to a more efficient in vitro binding of mannosylated liposomes to macrophages (47). However, both kinds of liposomes, with and without mannose incorporated, eliminate Mφ in spleen and liver equally well after intravenous injections. In addition, i.v. injections of 2 ml of Cl_2MDP PCMAN- as well as Cl_2MDP PC-liposomes eliminate all monocytes in the blood (Huitinga, I., J.G.M.C. Damoiseaux, N. Van Rooijen, and C.D. Dijkstra, manuscript submitted for publication). Elimination of Mφ in spleen and liver and monocytes in the blood, was apparently not sufficient to suppress EAE. This raises the question why silica dust suppresses EAN (20–22) and EAE (19). Possible explanations are the following: (*a*) silica exerts additional effects on other immunocompetent cells (23–25), (*b*) silica is capable of entering the CNS, and (*c*) silica is more efficient in eliminating Mφ than are the Cl_2MDP PC-liposomes.

In conclusion, the results of our study show that Mφ play an important role in the pathogenesis of neurologic disorders in the CNS of EAE animals. As long as the cause of demyelinating disease like MS is uncertain, modulation of effector mechanisms as seen in animal models for demyelinating diseases such as EAE, is of interest. The Mφ seem to be an interesting target, and apparently can be reached and influenced within the CNS by using the appropriate liposomes. Studies are underway to determine which specific functions of activated Mφ are involved in the pathogenesis of EAE.

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