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 (Cl2MDP). Cl2MDP-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 Cl2MDP-containing mannosylated 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. Cl2MDP-containing liposomes without mannose incorporated had no effect. Cl2MDP-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) 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-
development 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 composed of phosphatidylcholine and cholesterol containing dichloromethylene diphosphonate (Cl2MDP) (Cl2MDP PC-liposomes). Cl2MDP PC-liposomes injected intravenously eliminate almost all MΦ in spleen and liver (26, 27). Free Cl2MDP 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 Cl2MDP PC-liposomes (Cl2MDP PCMAN-liposomes). Mannosylated 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 Cl2MDP PCMAN-liposomes injected on days 8 and 10 post-immunization (p.i.) completely suppressed or markedly attenuated the expression of EAE. Non-mannosylated Cl2MDP 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 foodpad. 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 Cl2MDP into PC-liposomes, 1.89 g Cl2MDP (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 Cl2MDP, was added. Liposomes were sonicated and centrifuged as described above.

Treatment. Two experiments were 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 at 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 Cl2MDP 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 mannoside 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 Cl2MDP 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 Cl2MDP PCMAN-liposomes on days 6 and 8, 7 and 9, and 8 and 10 p.i., or two intravenous injections of 2 ml Cl2MDP 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, paralyse 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 mg/kg bodyweight) and killed by an intracardiac 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 Cl2MDP-liposomes in spleen and liver. The following mAbs were used: mouse anti-panC cell (OX-19), mouse anti-IA (OX-4), both from Serotec, Oxford, UK; mouse anti-iat-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 rat MΦ as well as glial cells (33, 34). ED8 which recognizes rat MΦ as well as glial cells (33, 34). ED8 shows a staining pattern comparable with OX-42, which recognizes rat MΦ as well as glial cells (33, 34).
Chemical Co.) in 0.05 M Tris buffer, pH 7.6, containing 0.03% H2O2. 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 C12MDP 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. I. iews rats were injected intravenously with either 2 ml 0.9% NaCl or 2 ml C12MDP 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 C12MDP 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 C12MDP PCMAN-liposomes showed a striking absence of neurological disorders. Weight loss was apparent in all three groups, though animals in the C12MDP 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,
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 Cl3MDP 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 Cl3MDP 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 Cl3MDP PC- and Cl3MDP 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](image-url)

**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 Cl3MDP 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 Cl3MDP-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 C12MDP 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 C12MDP 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 C12MDP PCMAN-liposome–treated EAE group.

EAE–Experiment B

Clinical Data. The effect of C12MDP PCMAN-liposomes injected at three different time points (days 6 and 8, 7 and 9, and 8 and 10 p.i.) and of C12MDP 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 C12MDP PC-liposome–treated group, subsequently followed by the control animals (10.2 ± 0.4 d), and the C12MDP 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 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 C12MDP 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 C12MDP 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 C12MDP 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 C12MDP PCMAN-liposome–treated groups (Fig. 5). In contrast, animals that were treated with C12MDP 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 detection. ED1+ and ED3+ cells were present as normal. Recovery within 2 mo of MΦ in the spleen after depletion by C12MDP-containing liposomes has been described (27).

Effect of C12MDP PCMAN-Liposomes on Plasma Corticosterone Levels

No difference was observed between the effect of injection of C12MDP 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
days post immunization

![Graph showing clinical signs of EAE](image)

Figure 5. Exp. B: Effect of 2 ml Cl2MDP-containing PCMAN-liposomes injected intravenously at different timepoints post-immunization (days 6 and 8, 7 and 9, and 8 and 10) and Cl2MDP-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 |
|--------------------|------------------------------------|-----------|----------------------------------------|-------------------------------------------------------------|----------|
| No treatment (n = 6) | 10.2 ± 0.4 | 100 | 6.2 ± 1.4 | 6/6 | 0 |
| days 6 and 8 p.i. | Cl2MDP MANL (n = 5) | 10.8 ± 1.0 | 80 | 4.3 ± 2.5 | 2/5 | 2 |
| days 7 and 9 p.i. | Cl2MDP MANL (n = 5) | 12.7 ± 0.6 | 60 | 3.0 ± 0.0 | 1/5* | 1 |
| days 8 and 10 p.i. | Cl2MDP MANL (n = 5) | 13.0 ± 0.0 | 60 | 2.3 ± 1.2 | 1/5* | 0 |
| days 8 and 10 p.i. | Cl2MDP L (n = 5) | 9.4 ± 1.3 | 100 | 9.0 ± 5.4 | 4/5 | 1 |

Exp. B: Effect of 2 ml Cl2MDP-containing PCMAN-liposomes (Cl2MDP MANL) injected intravenously at different timepoints post-immunization (days six and eight, seven and nine, and eight and ten) and Cl2MDP-containing PC-liposomes (Cl2MDP 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 (X2 Test).
intravenous injections of mannosylated Cl2MDP 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 cannot be detected in the CNS after intravenous injection (30). Both kinds of liposomes, when containing Cl2MDP, eliminated Mcp in spleen liver when injected intravenously (27; this study). Cl2MDP PC-liposomes without mannose incorporated had no effect on the expression of EAE. Elimination of Mcp in spleen and liver was apparently not sufficient to suppress clinical signs of EAE. The suppressive effect of the Cl2MDP PCMAN-liposomes is therefore likely to be due to elimination of Mcp infiltrating the CNS.

In contrast to silica (23–25), Cl2MDP 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 Cl2MDP-containing liposomes is thus due to direct or indirect effects of Mcp elimination.

Histological evaluation of CNS tissue revealed a considerable reduction of the number of infiltrated Mcp in Cl2MDP PCMAN-liposome–treated animals. This finding supports the fact that the mannosylated PC-liposomes reach the CNS and exert their effect there. On light microscopic 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 Cl2MDP 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 Cl2MDP PCMAN-liposomes on plasma corticosterone levels. No difference in effect was observed on corticosterone levels between saline and Cl2MDP 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 corticosterone levels of more than 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 Cl2MDP PCMAN-liposomes injected 6 and 8 d p.i. was not as strong as after Cl2MDP PCMAN-liposome injections 7 and 9 and 8 and 10 d p.i. Injections of Cl2MDP 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 Mcp. These Mcp 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 Mcp 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 Mcp 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 Mcp markers, nor do they recognize all Mcp as does mAb ED1, for example (32, 35, 43, 44). Therefore, it is possible that the Mcp 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 Mcp elimination in our study is not caused directly by the lack of Mcp 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 Cl2MDP PCMAN-liposomes and controls.

The difference between the effects of mannosylated and non-mannosylated Cl2MDP 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 mannos 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 mannosate incorporated, eliminate Mφ in spleen and liver equally well after intravenous injections. In addition, i.v. injections of 2 ml of C12MDP-PCMAN - as well as C12MDP-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 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 C12MDP 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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References

1. Raine, C.S. 1985. Experimental allergic encephalomyelitis and experimental allergic neuritis. In Handbook of Clinical Neurology, J.C. Koetsier, editor, Elsevier Science Publishers B.V., Amsterdam 47:429.
2. Hickey, W.F., N.K., Gonatas, H. Kimura, and D.B. Wilson. 1983. Identification and quantitation of T-lymphocyte subsets found in the spinal cord of the Lewis rat during acute experimental allergic encephalomyelitis. J. Immunol. 131:2805.
3. Sobel, R.A., B.W. Blanchette, A.K. Bahn, and R.B. Colvin. 1984. The immunopathology of experimental allergic encephalomyelitis, I. Quantitative analysis of inflammatory cells in situ. J. Immunol. 132:2293.
4. Traugott, U., C.S. Raine, and D.E. McFarlin. 1985. Acute experimental encephalomyelitis in the mouse: immunopathology of the developing lesion. Cell. Immunol. 91:240.
5. Antoniou, A.V., D. Parker, J.L. Turk, B.T.G. Tan, and R.J. Schepel. 1986. Immunocytochemical identification and quantitation of mononuclear cells in the meninges during the development of chronic relapsing experimental allergic encephalomyelitis (CREAB) in the guinea pig. Cell. Immunol. 97:386.
6. Polman, C.H., C.D. Dijkstra, T. Sminia, and J.C. Koetsier. 1986. Immunohistological analysis of macrophages in the central nervous system of Lewis rats with experimental allergic encephalomyelitis. J. Neurol. Immunol. 11:215.
7. Lampert, P.W. 1965. Demyelination and remyelination in experimental allergic encephalomyelitis — further electron microscopic observations. J. Neuropathol. Exp. Neurol. 24:371.
8. Epstein, L.G., J.W. Prineas, and C.S. Raine. 1983. Attachment of myelin to coated pits on macrophages in experimental allergic encephalomyelitis. J. Neurol. Sci. 61:341.
9. Hartung, H.-P., and K. Heiniger. 1989. Non-specific mechanisms of inflammation and tissue damage in MS. Res. Immunol. 140:226.
10. Cammer, W., B.R. Bloom, W.T. Norton, and S. Gordon. 1978. Degradation of basic protein in myelin by neutral proteases secreted by stimulated macrophages: a possible mechanism of inflammatory demyelination. Proc. Natl. Acad. Sci. USA. 75:1534.
11. Konat, G.W., and R.C. Wiggins. 1985. Effect of reactive oxygen species on myelin proteins. J. Neurochem. 45:1113.
12. Selmaj, K.W., and C.S. Raine. 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann. Neurol. 23:339.
13. Hamann, K.P., and H.C. Hopf. 1988. The significance of the inflammatory reactions for the development of clinical signs in multiple sclerosis and acute experimental autoimmune encephalomyelitis as assessed by means of the spontaneous chemiluminescence activity of peripheral blood monocytes. J. Neuroimmunol. 20:239.
14. Cazner, M., R.O. Barnard, B.J.L. MacGregor, N.J. Borshell, and A.N. Davison. 1976. Myelin composition in acute and chronic multiple sclerosis in relation to cerebral lysosomal activity. J. Neurol. Sci. 29:323.
15. Marks, N., A. Grynaus, and S. Levine. 1977. Proteolytic enzymes in ordinary, hyperacute, monocytic and passive transfer forms of experimental allergic encephalomyelitis. Brain Res. 123:147.
16. Brosnan, C.F., W. Cammer, W.T. Norton, and B.R. Bloom. 1980. Proteinase inhibitors suppress the development of experimental allergic encephalomyelitis. Nature (Lond.). 285:235.
17. Osanai, T., and Y. Nagai. 1984. Suppression of experimental
allergic encephalomyelitis (EAE) with liposome-encapsulated protease inhibitor: therapy through the blood-brain-barrier. *Neurochem. Res.* 9:1407.

18. Hartung, H.-P., B. Schäfer, K. Heining, and K.V. Toyka. 1988a. Suppression experimental autoimmune neuritis by the oxygen radical scavengers superoxide dismutase and catalase. *Ann. Neurol.* 23:466.

19. Brosnan, C.F., M.B. Bornstein, and B.R. Bloom. 1981. The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. *J. Immunol.* 126:614.

20. Tansey, F.A., and C.F. Brosnan. 1982. Protection against experimental allergic neuritis with silica quartz dust. *J. Neuroimmunol.* 3:169.

21. Heining, K., B. Schäfer, H.-P. Hartung, W. Fierz, C. Linnigton, and K.V. Toyka. 1988. The role of macrophages in experimental autoimmune neuritis induced by a P2-specific T-cell line. *Ann. Neurol.* 23:326.

22. Hartung, H.-P., B. Schäfer, K. Heining, G. Stoll, and K.V. Toyka. 1988b. The role of macrophages and eicosanoids in the pathogenesis of experimental allergic neuritis: serial clinical, electrophysiological, biochemical and morphological observations. *Brain.* 111:1039.

23. Levy, M.H., and E.F. Wheelock. 1975. Effects of intravenous silica on immune and non-immune functions of the murine host. *J. Immunol.* 115:41.

24. Wirth, J.W., W.P. Carney, and E.F. Wheelock. 1980. The effect of particle size on the immunodepressive properties of silica. *J. Immunol. Methods.* 32:357.

25. Zimmerman, B.T., B.P. Canono, and P.A. Campbell. 1986. Silica decrease phagocytosis and bacterial activity of both macrophages and neutrophils in *vitro*. *Immunology.* 59:521.

26. Van Rooijen, N., and R. van Nieuwmegen. 1984. Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. *Cell Tissue Res.* 238:355.

27. Van Rooijen, N., N. Kors, M.v.d. Ende, and C.D. Dijkstra. 1990. Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate. *Cell Tissue Res.* 260:215.

28. Umezawa, F., and Y. Eto. 1988. Liposome targeting to mouse brain: mannose as a recognition marker. *Biochim Biophys. Res. Commun.* 153:1038.

29. Van Rooijen, N. 1989. The liposome-mediated macrophage 'suicide' technique. *J. Immunol. Methods.* 124:1.

30. Claassen, E., and N. van Rooijen. 1984. The effect of elimination of macrophages on the tissue distribution of liposomes containing [3H]methotrexate. *Biochim. Biophys. Acta.* 802:428.

31. Matthaei, I., C.H. Polman, C.J.A. de Groot, C.D. Dijkstra, J.C. Koetsier, and T. Sminia. 1989. Observer agreement in the assessment of clinical signs in experimental allergic encephalomyelitis. *J. Neuroimmunol.* 23:25.

32. Dijkstra, C.D., E.A. Döpp, P. Joling, and G. Kraal. 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology.* 54:589.

33. Danoiseaux, J.G.M.C., E.A. Döpp, J.J. Neefjes, R.H.J. Beelen, and C.D. Dijkstra. 1989. Heterogeneity of macrophages in the rat evidenced by variability in determinants: two new anti-rat macrophage antibodies against a heterodimer of 160 and 95 kd (CD11/CD18). *J. Leukocyte Biol.* 46:556.

34. De Groot, C.J.A., C.D. Dijkstra, and T. Sminia. 1988. Discrimination between different types of neuroglial cells in rat central nervous system using combined immuno- and enzymehistochemical methods. *Immunobiology.* 178:177.

35. Robinson, A.P., T.M. White, and D.W. Mason. 1986. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and MRC OX-42, the latter recognizing complement receptor type 3. *Immunology.* 57:239.

36. Vermes, I., F. Berkenbosch, F.J.H. Tilders, and P.G. Smelik. 1981. Hypothalamic deafferentation in the rat appears to discriminate between anterior lobe and intermediate lobe response to stress. *Neurosci. Lett.* 27:89.

37. Berkenbosch, F., I. Vermes, and F.J.H. Tilders. 1984. The β-adrenoceptor-blocking drug propranolol prevents secretion of immunoreactive β-endorphin and α-melanocyte-stimulating hormone in response to certain stress stimuli. *Endocrinology.* 115:1051.

38. Glick, D., D. Von Redlich, and S. Levine. 1964. Fluorometric determination of corticosterone and cortisol in 0.02-0.05 milliliters of plasma or submilligram samples of adrenal tissue. *Endocrinology.* 74:653.

39. Claassen, Y., N. Van Rooijen, and E. Claassen. 1990. A new method for removal of mononuclear phagocytes from heterogenous cell populations in vitro, using liposome encapsulated dichloromethylene diphosphonate. *J. Immunol. Methods.* In press.

40. MacPhee, I.A.M., F.A. Antoni, and D.W. Mason. 1989. Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J. Exp. Med.* 169:431.

41. Mason, D., I.A.M. MacPhee, and F.A. Antoni. 1990. The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. *Immunology.* 70:1.

42. Sedgwick, J., S. Brostoff, and D. Mason. 1987. Experimental allergic encephalomyelitis in the absence of a classical delayed-type of hypersensitivity reaction. *J. Exp. Med.* 165:1058.

43. Williams, A.F., G. Gallré, and C. Milstein. 1977. Analysis of cell surfaces by xenogenic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell.* 12:663.

44. Barclay, A.N. 1981. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues. *Immunology.* 42:593.

45. Stahl, P.D., J.S. Rodman, M.J. Miller, and P.H. Schlesinger. 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. USA.* 75:1399.

46. Ezekowitz, R.A.B., and P.D. Stahl. 1988. The structure and function of vertebrate mannose lectin-like proteins. *Cell Sci. Suppl.* 9:121.

47. Garçon, N., G. Gregoriadis, M. Taylor, and J. Summerfield. 1988. Mannose-mediated targeted immunoadjuvant action of liposomes. *Immunology.* 64:743.