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CD40–CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis

(autoimmunity/T-helper cell/gp39/therapy)

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ABSTRACT **We investigated the role of CD40–CD40 ligand (CD40L) interactions in multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE). Activated helper T cells expressing CD40L (gp39) surface protein were found in MS patient brain sections, but not in brain tissue sections of normal controls or patients with other neurological diseases. CD40L-positive cells were co-localized with CD40-bearing cells in active lesions (perivascular infiltrates). Most of these CD40 bearing cells proved to be of the monocytic lineage (macrophages or microglial cells), and relatively few were B cells. To functionally evaluate CD40–CD40L interactions, EAE was elicited in mice by means of proteolipid-peptide immunization. Treatment with anti-CD40L monoclonal antibody completely prevented the development of disease. Furthermore, administration of anti-CD40L monoclonal antibody, even after disease onset, shortly before maximum disability score was reached led to dramatic disease reduction. The presence of helper T cells expressing CD40L in brain tissue of MS patients and EAE animals, together with the functional evidence provided by successful experimental prevention and therapy in an animal model, indicates that blockade of CD40–CD40L-mediated cellular interactions may be a method for interference in active MS.**

Interactions between CD40, constitutively expressed on B cells, and CD40 ligand [CD40L; gp39; TBAM (T-B cell activation molecules); TRAP (tumor necrosis factor-related activation protein)], transiently expressed on activated $CD4^+$ T cells, are essential for B-cell responses against thymus-dependent antigens. CD40L provides a number of signals to the B cell, some of which require additional cytokine stimuli, such as upregulation of cell surface markers, homotypic adhesion, proliferation, and isotype switching (reviewed in ref. 1). *In vivo* treatment of mice with anti-CD40L monoclonal antibody (mAb) prevents thymus-dependent antibody responses (2, 3), and generation of B-cell memory (4).

Although less well described, CD40–CD40L interactions are now thought to play a role in activation of cells of the monocytic lineage as well. Treatment of human monocytes with granulocyte/macrophage colony-stimulating factor, interleukin 3 (IL-3) or interferon γ resulted in the induction of CD40 mRNA and enhancement of cell surface CD40 protein expression. CD40 was found to mediate monocyte adhesion to cells transformed to express CD40L. The CD40L-transfected cells provided a costimulatory signal for monocytes to produce tumor necrosis factor α and IL-6 in the presence of granulocyte/macrophage colony-stimulating factor or IL-3 (5).

Adoptive transfer studies have established the crucial role of $CD4^+$ T-cells in experimental allergic encephalomyelitis (EAE), but both B cells and monocytes are considered to be involved in the pathogenesis of multiple sclerosis (MS) as well. Active lesions in the central nervous system (CNS) of MS patients are characterized by mononuclear cell infiltrates. The infiltrating cells comprise T cells, B cells, and macrophages (6). About 50% of the mononuclear cells in the perivascular lesions in the CNS of EAE animals, an animal model considered to represent the effector phase of MS, are blood-borne monocytes/macrophages (7) and microglial cells (8). In Lewis rats, in which EAE was induced either by adoptive spleen cell transfer or by active immunization with CNS white-matter homogenate, the elimination of macrophages significantly suppressed the development of EAE neurological signs (7). This suggests an important functional role for macrophages in EAE.

Recently, we have detected autoantigen specific B cells in human cerebellum and cerebrum *in situ* (9). Anti-myelin basic protein (MBP)-specific B cells were found in CNS tissue sections of only MS patients, not in patients with other neurological diseases nor in CNS tissue sections of normal controls. It is not clear whether these autoantigen-specific B cells were activated by $CD40L^+$ helper T (Th) cells locally in the demyelinated areas of the CNS or in peripheral lymphoid tissues.

In this study, we investigated whether interactions between CD40L on activated $CD4^+$ T cells and CD40 on B cells and/or cells of the monocytic lineage are involved in development and progression of EAE and MS. Using immunohistochemistry, we evaluated expression of CD40 and CD40L in CNS tissue sections of MS autopsy material. Furthermore, functional experiments blocking CD40–CD40L interactions in a mouse EAE model were performed. Collectively, our data support the hypothesis that the CD40–CD40L system is indeed involved in EAE and MS, providing a target for therapeutic intervention.

MATERIALS AND METHODS

Histochemistry. Human autopsy CNS tissues from patients with MS (n = 12), normal controls, and controls with other neurological disease were obtained from the Multiple Sclerosis/Control Brain Bank (supported by the Netherlands Foundation for the Support of MS Research; Amsterdam). All histochemical analyses of both human and mouse tissues were performed on cryosections (8 μm) from snap-frozen material. In a pilot study, selected CNS tissues of MS patients (n = 4) were used in which anti-MBP-specific B cells (putatively

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Abbreviations: CNS, central nervous system; DAS, disability scale; EAE, experimental allergic encephalomyelitis; mAb, monoclonal antibody; MBP, myelin basic protein; MS, multiple sclerosis; CD40L, CD40 ligand; PLP, proteolipid protein; Th, helper T; IL-n, interleukin n.

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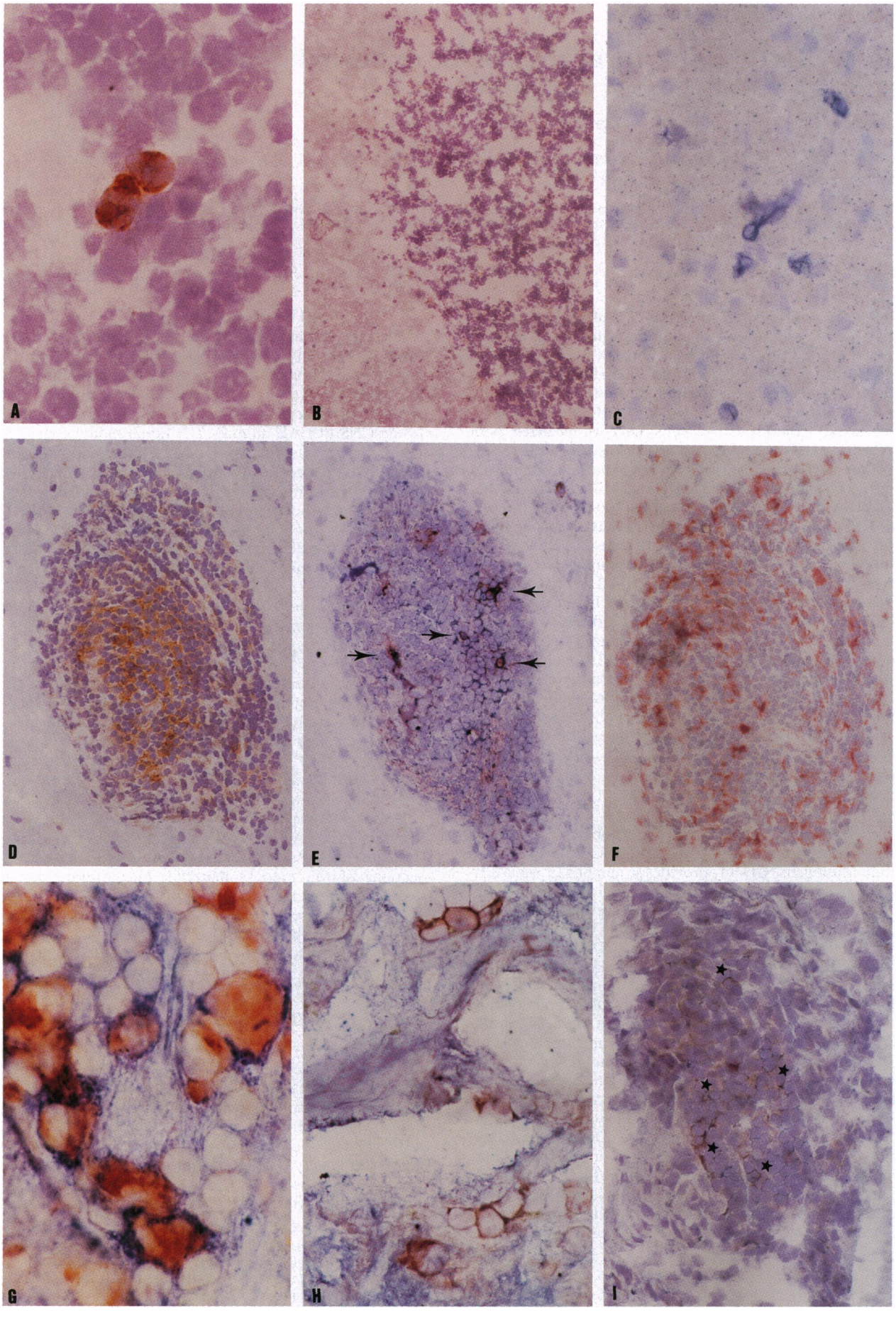

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

bearing CD40) were detected on a previous occasion (9). $CD40L^+$ cells were detected immunohistochemically in human tissue by using a CD40–Ig fusion protein, as described (3), and in the mouse with the mAb MR1 (3, 10) directly labeled with alkaline phosphatase. Alkaline phosphatase was revealed by using napthol-AS-MX-phosphate and fast blue BB base resulting in a blue precipitate, as described (11). CD40 was revealed with a mouse anti-human CD40 antibody (5D12; a kind gift from M. de Boer, PanGenetics BV, Heemstede, The Netherlands; ref. 12), followed by rabbit-anti-mouse IgG conjugated with horseradish peroxidase (RAMPO, Dako, Denmark), and immunochemistry with 3-amino-9-ethylcarbazole (AEC, Sigma), resulting in a red precipitate, as described (11). CD40 was revealed in a blue color after incubation with mAb 5D12, followed by horse-anti-mouse-IgG coupled with biotin (Vector Laboratories), and subsequent application of streptavidin-alkaline phosphatase (GIBCO/BRL). Cells of the monocytic lineage (11–13) were detected by acid-phosphatase staining demonstrating endogenous enzyme activity in lysosomes by using naphthol AS-BI phosphate (Sigma), as described (11). CD11b (complement receptor 3; ref. 13) was revealed in red after incubation with Leu-15 (Becton Dickinson) followed by RAMPO and AEC. B cells were demonstrated in red as described (9) with mouse anti-human IgG/IgM directly labeled with horseradish peroxidase and revealed with AEC.

EAE Induction and Anti-CD40L mAb Administration. EAE was induced according to a standard protocol in three groups (n = 18) of female SJL/J mice (12–15 weeks old) by two subcutaneous injections of 75 μg, 150 μg, or 300 μg of proteolipid protein (PLP) peptide in the abdominal flanks. The PLP peptide contained amino acids 139–151 of rat PLP (14). Peptide synthesis was performed by using 9-fluorenylmethoxcarbonyl (Fmoc) amino acids with the 9500 Milligen synthesizer according to standard protocols. The standard EAE-induction procedure using this peptide results in the development of acute EAE, clinically and pathologically identical to EAE induced by using whole CNS myelin or MBP (15). The emulsion contained 25 μg of *Mycobacterium tuberculosis* organisms (H37RA; Difco) in 50 μl of Freund's complete adjuvant and 37.5 μg, 75 μg, or 150 μg of peptide in 50 μl of phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.2/0.9% NaCl). On days 0 and 2, each mouse was injected intravenously with 200 μl of *Bordetella pertussis* suspension (10^{10} organisms per animal). Mice were injected intraperitoneally with 125 μg of hamster anti-CD40L mAb (2, 10) in 200 μl of PBS on days 0, 2, and 4; 4, 6, and 8; or 7, 9, and 11. Mice of the control groups received 125 μg of normal hamster antibodies (Serva) in 200 μl of PBS. The severity of EAE clinical signs was evaluated each day and graded according to discrete criteria (15): Disability scale (DAS) units; grade 0 = no clinical signs, grade 1 = tail weakness, grade 2 = mild paraparesis and ataxia of the hind legs, grade 3 = severe paraparesis or ataxia of the hind legs, grade 4 = moribund, grade 5 = death due to EAE. Blood samples were obtained on days 4, 9, 14, 21, 31, and 40 by tail-vein puncture. Sera were screened in a standard direct ELISA (16) for the presence of anti-PLP peptide antibodies by using the PLP peptide at a concentration of 10 μg/ml in PBS for coating of microtiter plates.

RESULTS

Detection of $CD40L^+$ Th Cells. As shown in Fig. 1*A*, $CD40L^+$ cells were detected in CNS tissue sections from MS patients (n = 12). Cells were shown to be of the Th phenotype by double staining for CD4 (data not shown). In control CNS tissue sections from normal individuals (n = 5) or from Alzheimer patients (n = 5), no cells stained with CD40–Ig fusion protein were observed (Fig. 1*B*). Similarly, CD40L-bearing cells could be demonstrated in cryosections of EAE mice with either CD40–Ig fusion protein (overlap of CD40–Ig and MR1 immunochemical staining of mouse tissue was shown before; ref. 3) or the anti-CD40L antibody MR1 (Fig. 1*C*; day 12 after induction of disease). CD40L-bearing cells were found in white matter lesions and not outside these lesions nor in control animals.

Detection of CD40-Bearing Cells. In Fig. 1 *D–I* serial sections of a representative perivascular infiltrate in a cryosection of MS brain are shown. From Fig. 1*D*, it is clear that a majority of the infiltrating cells bear CD40. From our previous study on anti-MBP antibody production in MS brain (9), we would have expected these cells to be CD40-bearing B cells; however, only a few cells (10–20%) were B cells, on the basis of double staining for IgM/IgG and CD40 (Fig. 1*E*). Staining for either acid phosphatase (Fig. 1 *F* and *G*) or CD11b (Fig. 1*H*) showed that most CD40-bearing cells belonged to the monocytic lineage. Only a very few of these cells were detected in control cerebrum tissue sections. Cells expressing CD40 and cells expressing CD40L were found in close juxtaposition after double staining (Fig. 1*I*), suggesting ongoing cellular interactions.

Prevention of EAE by Anti-CD40L mAb. Administration of PLP peptide resulted in EAE of dose-dependent severity. After EAE induction with 75 μg or 300 μg of PLP peptide (data of 150 μg not shown), control mice or those receiving irrelevant hamster antibodies showed a significant reduction in body weight (25–30%) from day 11 until day 17 (Figs. 2 *Top* and 3 *Top*; shaded bars), the first clinical signs of EAE becoming apparent on day 11. Severe disease (most animals moribund or dead) was induced with all peptide doses. The highest average DAS score of the control groups in animals in which EAE was induced with 300 μg of PLP peptide was 3.6, observed on days 16–23 (Fig. 3 *Middle*), and a score of 2.3 was observed on days 15–22 (Fig. 2 *Middle*) in animals in which EAE was induced with 75 μg of PLP peptide. Strikingly, no body-weight reduction was observed in animals which were treated on days 0, 2, and 4 with 125 μg of anti-CD40L mAb, irrespective of the peptide dose used for disease induction

FIG. 1 (*on opposite page*). *In vivo* evidence for involvement of CD40–CD40L interactions in EAE and MS. (*A*) Red, CD40L. CD40L-positive cells in a perivascular infiltrate in human MS brain. (×325.) (*B*) No specifically stained cells. No CD40L-positive cells were found in human brain tissues from "normal" controls or from patients with other neurological disease. Shown here is a representative section of Alzheimer brain. (×40.) (*C*) Blue, CD40L. CD40L-positive cells in a perivascular infiltrate of mouse brain during EAE. (×200.) Note: *D–I* are serial sections from the same plaque in human MS brain. (*D*) Red, CD40. Numerous CD40-positive cells are present. (×65.) (*E*) Red, IgG/IgM; blue, CD40; violet, double staining, both IgG/IgM and CD40. Only a few cells positive for CD40 also contain IgG or IgM (arrows). This indicates that only a minority of CD40-expressing cells in the infiltrates belongs to the B-cell subset (10–20%). (×65.) (*F*) Red, acid phosphatase. Numerous cells having acid phosphatase activity in lysosomal compartments are present. This indicates that these cells have phagocytic properties and are therefore monocytes/macrophages or microglia (monocytic lineage). (×65.) (*G*) Red, acid phosphatase; blue, CD40. The large majority of cells bearing CD40 on their membrane also have acid phosphatase activity in the cytoplasm. This indicates that CD40-positive cells in infiltrates are presumably monocytes and/or microglia. (×650). (*H*) Red, CD11b (CR3); blue, CD40; violet, double staining, both CD11b and CD40. The large majority of CD40-positive cells also express complement receptor 3. Taken together with the acid phosphatase activity, this indicates that CD40-positive cells in infiltrates are monocytes or microglia. (×325.) (*I*) Red, CD40; blue, CD40L. Cells expressing CD40 and cells expressing CD40L are juxtaposed (stars). (×130.) This suggests that CD40–CD40L interactions are ongoing in perivascular infiltrates in human MS brain. For technical details of immunohistochemical staining, see *Materials and Methods.*

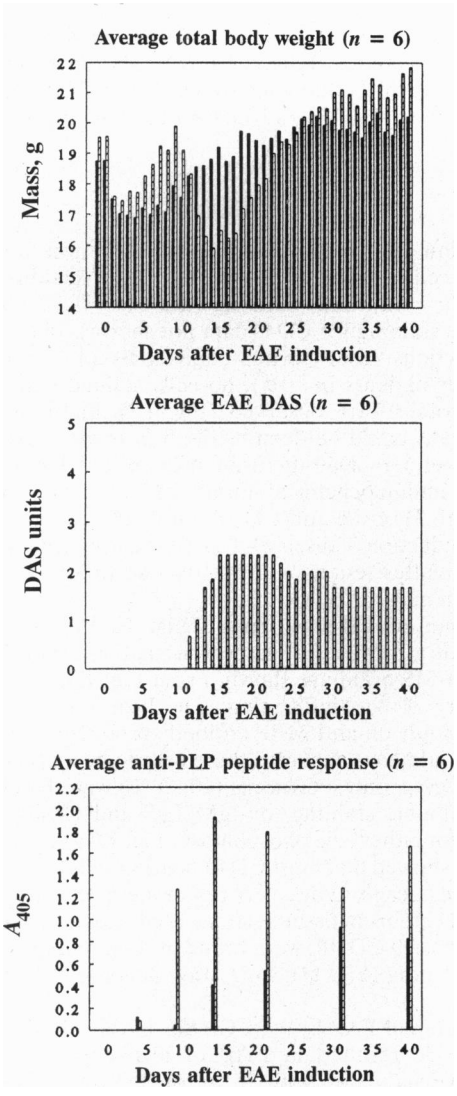

FIG. 2. Prevention of EAE induced with low-dose peptide by using anti-CD40L mAb. EAE was induced with 75 μg of PLP peptide in female SJL/J mice ($n = 6$) according to a standard procedure. Animals were treated with anti-CD40L mAb on days 0, 2, and 4 (black bars). Control mice received normal hamster antibodies on the same days (hatched bars). The effect of anti-CD40L mAb treatment was monitored by determination of the body weight (*Top*), by evaluation of clinical signs (*Middle*; note: no black bars/no disease), and by determination of serum anti-PLP peptide antibody responses by standard direct ELISA (*Bottom*).

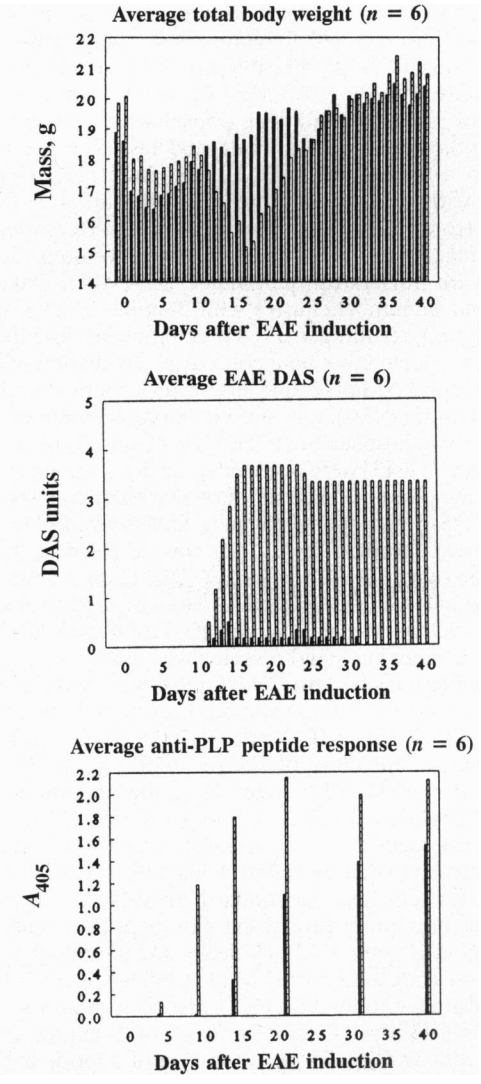

FIG. 3. Prevention of EAE induced with high-dose peptide by using anti-CD40L mAb. EAE was induced with 300 μg of PLP peptide in female SJL/J mice ($n = 6$) according to a standard procedure. Animals were treated with anti-CD40L mAb on days 0, 2, and 4 (black bars). Control mice received normal hamster antibodies on the same days (hatched bars). The effect of anti-CD40L mAb treatment was monitored by determination of the body weight (*Top*) by evaluation of clinical signs (*Middle*; note: only very low black bars/minimal disease), and by determination of serum anti-PLP peptide antibody responses by standard direct ELISA (*Bottom*).

(Figs. 2 *Top* and 3 *Top*; black bars). Even more dramatic, MR1-treated animals showed only minimal or no clinical signs at both doses of PLP peptide used for EAE induction (Figs. 2 *Middle* and 3 *Middle*; note: no or only very low black bars). Clinical signs in the anti-CD40L-treated mice were only found when EAE was induced with the highest dose (300 μg) of PLP peptide and disappeared on day 31, whereas clinical signs of the control group remained severe (Fig. 3 *Middle*). In control animals, anti-PLP peptide serum antibody responses were observed from day 9 until day 40, with maximal responses on day 14 (titer = 1433) and 21 (titer = 2710) after EAE induction with 75 μg or 300 μg of PLP peptide, respectively. In contrast, anti-PLP peptide antibody responses in animals treated with CD40L mAb on days 0, 2, and 4 were severely delayed and decreased, with highest levels on day 31 (titer = 571) and 40 (titer = 1034) after EAE induction with 75 μg and 300 μg of PLP peptide, respectively (Figs. 2 *Bottom* and 3 *Bottom*).

Effect of Anti-CD40L Treatment During Disease. Treatment with anti-CD40L mAb around day 6 and day 9 after EAE induction with 150 μg of PLP peptide still resulted in blockade of disease by 80% and 67%, respectively, as compared with the complete inhibition (100%) in animals treated with anti-CD40L mAb around day 2 (Fig. 4). Of the animals treated with anti-CD40L, none died due to EAE. In control animals, the first EAE clinical signs (and death) were found on day 11.

DISCUSSION

This study provides evidence that CD40–CD40L interactions are involved in development of EAE in mice and MS in man. Functionally, treatment of mice with antibodies against CD40L both prevented development of disease (prophylaxis) and dramatically suppressed clinical signs when treatment was started after onset of disease (therapy). Histologically, cells expressing CD40 and CD40L were found in the perivascular infiltrates in the CNS of both EAE mice and MS patients but not in control tissues. Double-staining procedures revealed

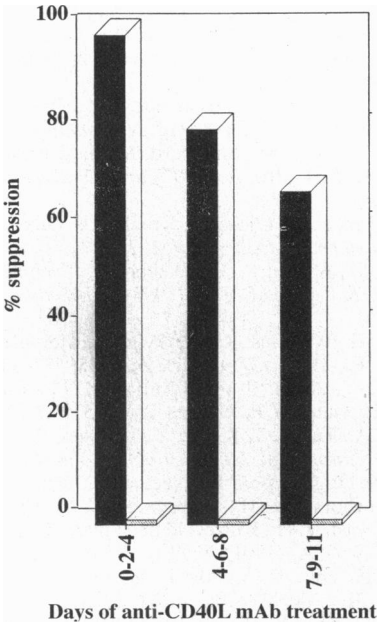

FIG. 4. Effect of delayed anti-CD40L treatment on EAE. EAE was induced with 150 μg of PLP peptide in female SJL/J mice (n = 6) according to a standard procedure. Animals were treated with anti-CD40L mAb on days 0, 2, and 4; 4, 6, and 8; or 7, 9, and 11 (black bars). Control mice received normal hamster antibodies on the same days (hatched bars). The effect of anti-CD40L mAb treatment was monitored by evaluation of clinical signs. The cumulative DAS scores from day 12 until day 28 of mice treated with anti-CD40L mAb on days 0, 2, and 4 was set at 100% EAE suppression. Cumulative DAS scores from day 12 until day 28 of the other groups were related to this percentage.

that the majority of CD40-expressing cells in human MS brain were cells of the monocytic lineage, while a minority belonged to the B-cell subset. Finally, juxtaposition of CD40- and CD40L-expressing cells *in situ* was demonstrated, indicative of ongoing cellular interactions.

The interaction between CD40L on activated $CD4^+$ T cells and CD40 on B cells has been shown to be indispensable for antibody responses against thymus-dependent antigens (1–4). However, recent data indicate that the CD40–CD40L axis is involved not only in humoral immunity but in development of some autoimmune diseases as well, such as collagen-induced arthritis (17) and lupus nephritis (18).

Here, we show that cells expressing CD40L could be found in perivascular infiltrates of CNS tissue of both EAE mice and MS patients but not in control tissues. Frequencies of CD40L-expressing cells in these infiltrates were modest. This is in accordance with frequencies of $CD40L^+$ cells induced in the spleen by immunization of mice with thymus-dependent antigen (TNP-KLH)(3), where ≈1 $CD40L^+$ cell was found per 12 KLH-specific B cells. Cells expressing CD40 were abundantly present in perivascular infiltrates of MS brain. By using acid phosphatase and CD11b (CR3) as markers for monocytes/macrophages and microglia (8, 13), it was shown that CD40 expression was predominantly restricted to cells of the monocytic lineage, while B cells formed a minority of the $CD40^+$ population.

To functionally assess the role of CD40 and its ligand, we blocked this cognate interaction *in vivo* by administration of anti-CD40L antibody in a mouse EAE model. Treatment with anti-CD40L mAb during disease induction (days 0–4) completely prevented development of disease, indicating that CD40–CD40L interactions play an important role in the induction phase of EAE. Importantly, delaying treatment with anti-CD40L mAb by initiating it shortly before maximal clinical disease score was reached, resulted in near-total suppression of disease (Fig. 4). These results indicate that CD40–CD40L interactions are not only important in the induction phase but also during the clinical phase of EAE, implying that the development of this inflammatory disease of the CNS is dependent on continuous or repeated CD40–CD40L interactions, as was shown for humoral responses against thymus-dependent antigens (1–3).

What effector mechanisms are induced by CD40–CD40L interactions in the CNS during EAE and MS? The immunocytochemical data discussed above clearly showed that the majority of CD40-bearing cells were macrophages or microglia. In addition, a small subpopulation of $CD40^+$ cells belonged to the B-cell lineage. Consequently, CD40L-induced functions of both macrophages/microglia and B cells should be considered. With respect to B cells, antibodies specific for or crossreactive with myelin components may be involved in the process of demyelination in EAE (see, for example, refs. 19 and 20). However, several findings argue against a central role for B cells in development of EAE and MS. It has been convincingly demonstrated that EAE can be adoptively transferred by T cells but not by B cells (21). Furthermore, we have found only limited numbers of MBP-specific plasma cells in CNS tissue of MS patients and EAE rhesus monkeys (9). The current study demonstrates that the number of immunoglobulin-containing $CD40^+$ B cells in lymphoid infiltrates of MS brain is also limited in relation to the total number of infiltrating cells and the number of $CD40^+$ cells of the monocytic lineage. Finally, the anti-PLP peptide antibodies found at later time points after anti-CD40L treatment indicate that, despite intact B-cell function, no clinical signs develop. Apparently, either B-cell responses are not crucial to disease induction or B cells/antibodies become unable to induce disease after a critical susceptible period.

This leaves macrophages/microglia ($CD11b^+$, acid phosphatase-containing cells: see Fig. 1 *D–H*) in perivascular infiltrates as the CD40-bearing population crucial to disease development. Consistent with this possibility, Huitinga *et al.* (7) have elegantly demonstrated that macrophages are required for development of EAE in rats. What macrophage effector mechanisms contributing to inflammation and/or demyelination in the CNS may be activated through CD40 triggering? Alderson *et al.* (5) have shown that CD40 triggering of human monocytes induced tumoricidal activity, and in the presence of appropriate cytokines, tumor necrosis factor α, IL-6, and IL-8 was produced. In addition, CD40 triggering induces IL-1 and IL-12 (22, 23) and can enhance nitric oxide production (24). Interestingly, antibodies against IL-12 prevent development of EAE in mice (25). It remains to be determined which of these compounds are actually produced *in vivo* in response to CD40–CD40L interactions and what their relative contributions to disease development are.

How does treatment with anti-CD40L antibody prevent disease development and suppress established disease? As we discussed before (4), anti-CD40L mAb does not induce unresponsiveness by a direct cytotoxic effect on T cells, and it does not affect the frequencies of IL-2, IL-4, and interferon γ-producing cells *in situ* (3). Collectively, these observations and the current study suggest that amelioration of EAE by anti-CD40L treatment may result from direct blocking of interaction of CD40L on activated T cells with CD40 on monocytes/macrophages and microglial cells.

Alternatively, anti-CD40L antibody may induce Th-cell unresponsiveness in EAE by interference with the CD40-related expression of additional costimulatory molecules on antigen-presenting cells, resulting in impaired antigen presentation. Other studies support this hypothesis in view of the fact that the blocking of interactions between B7 family members and their ligands *in vivo* induce a state of allo-specific T-cell unresponsiveness (26). Therefore, receptors like CD40, the triggering of which was shown to regulate the expression of

B7.1 and B7.2 (27), might play an important role in controlling tolerance and immunity.

Although further details of the mechanism(s) of action are clearly needed, the co-localization of CD40-bearing macrophages/microglia and CD40L-bearing cells in affected CNS tissue of patients suggests that CD40–CD40L interactions may play an important role in the immunopathology of MS. By analogy with the results obtained in EAE, blockade of CD40–CD40L interactions should be considered as a method to interfere in active episodes of MS as well. Preventing CD40–CD40L interactions is potentially useful in limiting duration, intensity, and neurological damage of disease exacerbations. A significant advantage of CD40L as a target for intervention, in comparison with the constitutively expressed CD40, is its transient expression restricted to activated $CD4^+$ T cells. This feature allows targeting of only those T cells actively participating in the response without affecting the population of T cells at large.

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