Delayed neuropathology after carbon monoxide poisoning is immune-mediated

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The neuropathological sequelae of carbon monoxide (CO) poisoning cannot be explained by hypoxic stress alone. CO poisoning also causes adduct formation between myelin basic protein (MBP) and malonylaldehyde, a reactive product of lipid peroxidation, resulting in an immunological cascade. MBP loses its normal cationic characteristics, and antibody recognition of MBP is altered. Immunohistochemical evidence of degraded MBP occurs in brain over days, along with influx of macrophages and CD-4 lymphocytes. Lymphocytes from CO-poisoned rats subsequently exhibit an autoreactive proliferative response to MBP, and there is a significant increase in the number of activated microglia in brain. Rats rendered immunologically tolerant to MBP before CO poisoning exhibit acute biochemical changes in MBP but no lymphocyte proliferative response or brain microglial activation. CO poisoning causes a decrement in learning that is not observed in immunologically tolerant rats. These results demonstrate that delayed CO-mediated neuropathology is linked to an adaptive immunological response to chemically modified MBP.

myelin basic protein | malonylaldehyde | lymphocyte activation | CD-40 | microglia

Carbon monoxide (CO) is the leading agent of injury and death by poisoning worldwide (1). Annually in the United States, ≈40,000 individuals are treated for CO poisoning (2). Over half of those with serious poisoning develop an encephalopathy between 3 days and 4 weeks afterward (3). Because the affinity of CO for hemoglobin (to form carboxyhemoglobin) is >200-fold greater than that of O_2, CO-mediated hypoxic stress is a recognized insult. However, because carboxyhemoglobin values correlate poorly with clinical outcomes, additional pathophysiological mechanisms for delayed sequelae are thought to exist (2–4). Damage to brain related to excitatory amino acids and oxidative stress has been demonstrated in several animal models (5–10). Experimental CO poisoning causes activation of *N*-methyl-D-aspartate neurons, and subsequent overactivity of neuronal nitric oxide synthase gives rise to perivascular changes that cause neutrophil sequestration/activation (10–14). Reactive O_2 species produced by activated neutrophils, mitochondria, and xanthine oxidase cause brain lipid peroxidation, but how these events trigger delayed neurological injuries is not understood (8–14).

Aldehydic lipid peroxidation products, such as malonylaldehyde (MDA), react with cell proteins and may alter protein ionic charge and/or tertiary structure, and/or render proteins immunogenic (15, 16). Inflammatory responses play an important role in the pathogenesis of many brain disorders, including excitotoxicity (17, 18). Therefore, we evaluated immunological responses in brain after CO poisoning. We focused our attention on myelin basic protein (MBP), because it is the major myelin protein of central nervous system (≈30%). Others have shown that chemical modifications of MBP can alter three-dimensional structure, enhancing protease attack, and influence antibody recognition (19–21). A highly encephalitogenic agent, MBP causes an autoimmune disorder when injected into animals, and it may play a role in CNS inflammation after transient focal ischemia (22, 23). Anionic isoforms of MBP occur in a number of neurodegenerative diseases (24–26). We hypothesized that acute CO-mediated oxidative stress causes alterations in MBP and that immune responses to the modified protein precipitate delayed neurological dysfunction.

Methods

Animals and Reagents. Wistar male rats (Charles River Laboratories) weighing 200–290 g were fed a standard diet and water ad libitum for most studies. All animals' care was in accordance with institutional guidelines. Unless otherwise stated, chemicals were purchased from Sigma–Aldrich. Rabbit antibody against MDA-protein adducts used in the ELISA was a kind gift from Dennis Petersen (University of Colorado, Denver). Commercial antibodies used were as follows: monoclonal anti-ED-1 (Serotec); monoclonal anti-MHC II (Accurate Chemicals); polyclonal anti-CD40 (Santa Cruz Biotechnology); monoclonal anti-GLUT3 (Calbiochem); monoclonal anti-glial fibrillary acidic protein (Pharmingen); monoclonal anti-actin (Sigma); polyclonal anti-MDA antibody conjugated to FITC (Academy Biomedical Company, Houston); and monoclonal anti-CD4 and all antibodies against MBP, including monoclonals to amino acid segments 67–74, 82–87, 129–138, a polyclonal anti-MBP, and anti-degraded MBP (Chemicon). The anti-degraded-MBP antibody will only recognize protein epitopes on MBP that is undergoing degeneration by activated microglia and macrophages (27).

Animal Manipulations. CO poisoning was performed according to the published protocol in a 7-liter Plexiglas chamber (9). Rats breathed 1,000 ppm CO for 40 min then 3,000 ppm for up to 20 min, until they lost consciousness, and then they were removed to breathe room air and regain consciousness. Bovine MBP was administered by repetitive gavage with 1 mg of protein (or ovalbumin as control) in 0.5 ml of PBS every 2–3 days for 2 weeks for a total of five feedings according to published methods (22), and CO poisoning occurred 2 days after the final gavage.

Tissue Preparation for Chemical Assays. Rats were anesthetized, and brain tissue was processed for Western blotting as described in ref. 10. In brief, after decapitation and isolation, brains were frozen in liquid nitrogen, homogenized in 5 ml of chloroform:methanol (2:1 vol/vol), and then diluted with chloroform:methanol to obtain a ratio of 1 g of brain to 19 ml of ice-cold chloroform:methanol. This volume of solvent was defined as "one volume" for subsequent steps. After centrifugation (2,000 × *g* for 5 min) and washing twice in one volume of fresh chloroform:methanol, the residue was washed once in ice-cold acetone. After centrifugation (2,000 × *g* for 5 min) the

Abbreviations: CM, carboxymethylcellulose; MBP, myelin basic protein; MDA, malonylaldehyde.

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pellet was washed three times with 10 ml of water and then suspended in 10 vol of 0.03 M HCl. The suspension was incubated for 1 h at 4°C while stirring and centrifuged at 44,000 × *g*, and material in the clear aqueous-acid extract was obtained by overnight vacuum evaporation. Acid-soluble brain material was chromatographed after two different published procedures (28, 29). In one, material was dissolved in 500 μl of 80 mM sodium glycinate buffer (pH 9.5) containing 6 M urea and applied to a carboxymethylcellulose (CM) column equilibrated with in 80 mM sodium glycinate buffer (pH 10.5) containing 6 M urea. Protein was eluted by using a linear gradient of sodium chloride (0–0.75 M) in an 80 mM sodium glycinate buffer (pH 10.5) containing 6 M urea and collecting as 300-μl fractions (28). In the second method, the lyophilized sample was dissolved in 500 μl of 0.1 M acetic acid and applied to a CM column equilibrated with 0.1 M acetic acid (29). Protein was eluted by using a linear gradient of sodium chloride (0–0.75 M) in 0.1 M acetic acid and collecting as 300-μl fractions. The charge pattern observed for the protein eluates was identical with both methods, so the second method was chosen as the standard procedure and used in all studies reported in this article. Protein concentration in the fractions was determined by absorbance at 280 nm. Fractions of pooled protein were desalted on a Sephadex G-25 column prepared with 7% formic acid. The pooled material was lyophilized and stored at −80°C until being resuspended in water for additional analysis.

Assessment of MDA-Reacted Substances in Brain Fractions with ELISA. Resuspended fractions from CM cation-exchange chromatography were applied to Immunolon 4 96-well microtiter immunoassay plates (Dynatech) at a concentration of 0.05 to 1 μg/50 μl of water per well and incubated at 4°C overnight. The antigen solution was then removed, wells were washed three times with PBS plus 0.05% Tween-20 (PBS-T), and the remaining sites were blocked by incubating with 300 μl of PBS-T containing 10% BSA for 1 h. The solution was removed, wells were rinsed twice with PBS-T, and samples were probed by incubating for 3 h at room temperature with a 1:2,000 dilution of anti-malonaldehyde antibody obtained from Dennis Peterson. Standards were used to quantify binding to plates for each experiment by plating known concentrations of BSA that had been reacted with MDA so that data are expressed as equivalents of MDA-BSA (in micrograms). After incubation with the rabbit anti-MDA antibodies, wells were washed three times with PBS-T. Secondary goat-anti rabbit antibody conjugated to alkaline phosphatase was added to the wells and incubated for 3 h at 37°C. Plates were again rinsed three times with PBS-T, and 200 μl of a 1 mg/ml solution of *p*-nitrophenyl phosphate in 0.05 M sodium carbonate buffer (pH 9.8, containing 1 mM $MgCl_2$) was slowly added until a yellow color developed (≈10 min). The reaction was stopped by addition of 50 μl of 2 M NaOH, and the absorbance of each well was measured at 405 nm.

Analysis of MBP. MS and protein sequencing were done by the Proteomics Core Facility of the Genomics Institute and the Abramson Cancer Center of the University of Pennsylvania. Protein bands were cut from nitrocellulose paper and eluted with a solution of 0.1% formic acid and 50% acetonitrile for matrix-assisted laser desorption/ionization time-of-flight analysis (MALDI-TOF). These analyses were carried out with a Voyager DE Pro (Applied Biosystems) mass spectrometer, and the data were analyzed by using MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) software. For microsequencing, peptide samples were digested with either trypsin or Lys C (both approaches gave the same results), dissolved in 10 μl of 0.1% formic acid, injected (1 $\mu l \cdot min^{-1}$) into the mass spectrometer, and eluted by linearly increasing the mobile phase composition from 0.1% formic acid to 100% acetonitrile. To identify the eluting peptides, samples were subjected to tandem ion-trap MS analysis on a LCQ Deca XP mass spectrometer (ThermoFinnigan, San Jose, CA) coupled online with a mini C18 reversed-phase capillary HPLC column by using electrospray ionization interphase. The precursor MS was scanned, and up to four abundant ions were dynamically selected for subsequent collision induced dissociation (CID) with the energy set to 35%, generating the tandem MS spectrum of the peptide. The CID ion-trap spectra were analyzed by using SEQUEST (http://fields.scripps.edu/sequest).

Lymphocyte Proliferation. The neck and axillary fur of anesthetized rats was soaked in ethanol and opened, and the lymph nodes were removed. Nodes were pulverized with a sterile glass syringe plunger and wire mesh screen to release individual cells. Cells were aspirated into a syringe through a 25-gauge needle to release clumps, washed in RPMI medium plus 10% heat-inactivated FBS plus 50 μg/ml gentamicin, and counted. Individual wells of a 96-well microtiter plate were inoculated with 300,000 cells, stimulatory agents were added to each well, and then plates were incubated at 37°C in a CO_2 incubator. After 48 h, 0.5 μCi (1 Ci = 37 GBq) [^{3}H]thymidine was added to each well, and plates were incubated for an additional 21 h. Cells were then removed and washed, and radioactivity was assessed in a scintillation counter. Cultures were run in triplicate, and the mean was taken as the proliferative response for cells from each rat. Cells were incubated with PBS (control), 5 μg of 18.5-kDa MBP, chromatography fraction 5 obtained from control rats and rats killed 90 min after CO poisoning, or 5 μg of Con A.

Rat MBP Isolation for Lymphocyte Activation Assays. Procedures followed were exactly as described in ref. 30. Homogenates from control rats and those killed 90 min after CO poisoning were subjected to SDS/PAGE and transferred to nitrocellulose paper. Bands were visualized by using Aurodye reagent (Amersham Biosciences), and the 18.5-kDa band corresponding to MBP was cut out and dissolved in DMSO. These samples were precipitated by adding 50 mM carbonate/bicarbonate buffer (pH 9.6), centrifuged at 10,000 × *g* for 10 min, resuspended in RPMI medium 1640, divided into aliquots, and frozen at −20°C until use.

Histochemistry. Immunohistochemistry was performed on paraffin-embedded sections after perfusion/fixation of anesthetized rats according to published methods (11). Deparaffinized slides were stained overnight with primary antibody dilutions. This procedure was followed by counterstaining with the appropriate anti-IgG conjugated to Cy3 or FITC for 2 h. Staining with FITC-conjugated isolectin B_4 (1:500) was performed for 2 h. Microscope examinations were carried out with a Bio-Rad Radiance 2000 attached to a Nikon TE 300 inverted-stage confocal microscope that was operated with a red diode laser at 638 nm and krypton lasers at 488 nm and 543 nm. The number of activated microglia, positive for both CD-40 and isolectin B_4, were taken as the average count from two brain sections separated by at least 60 μm to avoid double-counting of cells and visualized with a Nikon Diaphot-TND epifluorescence inverted microscope at ×200 magnification.

Maze Studies. Cognitive function was examined by using an eight-arm radial maze according to methods we described in ref. 10. In brief, familiarization and testing procedures were performed every other day, 3 days per week. CO poisoning occurred after the third familiarization session on Friday afternoon, and rats were left undisturbed for 2 days. Rats underwent 12 test sessions over 4 weeks by someone blinded to which rats were poisoned. Data recorded were the number of arm entries until a repeat occurred (choice accuracy), and the time to complete

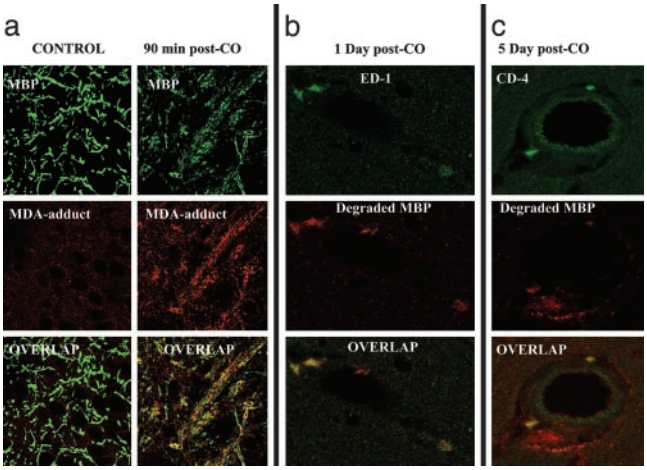

Fig. 1. Immunohistochemical evaluations. (*a*) Control brain section showing low-level staining by antibody to MDA-protein adducts but no colocalization with native MBP, and extensive staining for MDA-adducts with colocalization to MBP (shown in yellow in ''overlap'' images) in brains of rats killed 90 min after CO poisoning. (*b*) Colocalization among the microglia/macrophage marker, ED-1, and ''degraded MBP'' at 1 day after CO poisoning. (*c*) Infiltration of CD-4$^+$ lymphocytes observed in close proximity to an accumulation of ''degraded MBP'' at 5 days after CO poisoning.

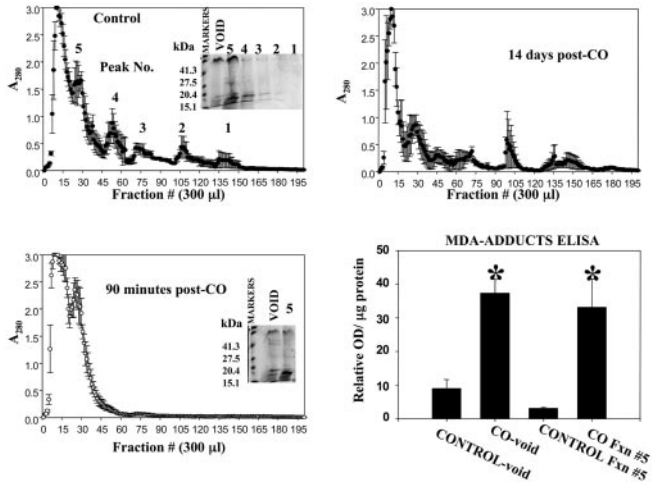

Fig. 2. Column chromatography and analysis of acid-soluble material from brain. (*Upper Left*) Pattern for control rats. (*Lower Left*) Pattern for rats killed 90 min after CO poisoning. (*Upper Right*) Pattern for rats killed 14 days after CO poisoning. Identical protein amounts were loaded onto CM columns, and the results are mean ± SE for four rats in each group. (*Lower Right*) Data from an ELISA demonstrating the presence of MDA adducts in void and fraction 5 material obtained from control rats and rats killed 90 min after CO poisoning (mean ± SE, $n = 4$). *, $P < 0.05$, ANOVA. (*Insets*) SDS/PAGE patterns for the various fractions.

the session (in seconds) divided by the total number of arm entries (latency or response duration).

Statistics. Statistical analysis of maze scores was determined by repeated-measures ANOVA followed by the Tukey test (SIGMASTAT, Systat, Point Richmond, CA). To compare the efficacy of different treatments, the ''entry-to-repeat'' scores for the final 3 days of testing in week 4 were examined. Dunn's method of statistical comparison was used, because these groups had unequal numbers of rats. The level of significance was taken as $P < 0.05$. Results are expressed as mean ± SE.

Results

MDA-MBP Adducts and MBP Charge Isomers. Brain sections from CO-poisoned rats exhibited staining for MDA-protein adducts, and colocalization was observed with an antibody to rat MBP (Fig. 1*a*). Staining was most prominent 90 min after CO poisoning, the time when lipid peroxidation products are at their highest concentration (9). No MDA-modified protein staining was observed in control brains. To more closely investigate changes in MBP, acid-soluble material from brain homogenates was fractionated by carboxymethylcellulose (CM) cation-exchange column chromatography (Fig. 2). Whereas five peaks were resolved in control samples, at 90 min after CO poisoning only one peak was observed, and the majority of the protein was eluted in the void volume. SDS/PAGE indicated that a large portion of the proteins in the fractions had molecular weights consistent with isoforms of rodent MBP: 14, 17, 18.5, and 21.5 kDa (Fig. 2 *Insets*). The CM chromatography pattern reverted to one similar to control in the days subsequent to CO poisoning. By 14 days there were once again five peaks, although the pattern was not exactly the same as in the control (Fig. 2). The CM-chromatography void volume and fraction 5 material from control and CO-exposed animals were analyzed by ELISA with an antibody against MDA adducts (Fig. 2). As shown, there was a significantly higher recognition of MDA-adducts in material isolated from CO-poisoned brains.

Western blots of brain homogenates exhibited prominent staining for the 18.5-kDa MBP isoform (Fig. 3*a*, see control). These proteins were subjected to MALDI-TOF, and microsequences were assessed by tandem MS analysis. We identified peptides that cover residues 6–117, 91% of the 128 aa in rat MBP. No differences were detected. Despite these findings,

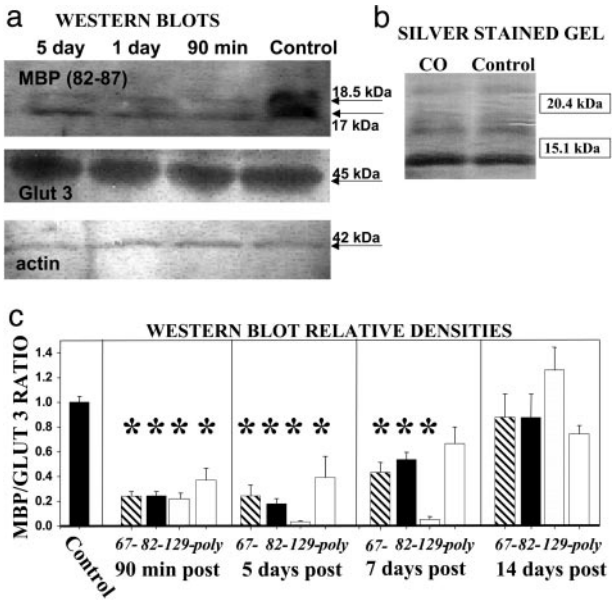

Fig. 3. Western blots of MBP after CO poisoning. (*a*) Representative Western blot probed with a monoclonal antibody that recognizes the MBP epitope between amino acids 82 and 87, GLUT 3, and actin-using brain homogenates from rats killed at different times after CO exposure. (*b*) Silver stain of SDS/PAGE gel from a control rat and one killed 90 min after CO poisoning. (*c*) Quantification of MBP staining expressed as the ratio of band densities for MBP versus GLUT 3 with four different antibodies to MBP (monoclonal antibodies to amino acid segments 67–74, 82–87, and 129–138, and a polyclonal anti-MBP). Blots were generated for rats killed at different times after poisoning. Data are expressed relative to a control ratio of 1.0. Homogenates from five different rats were used for all blots probed. Values are mean ± SE. *, $P < 0.05$.

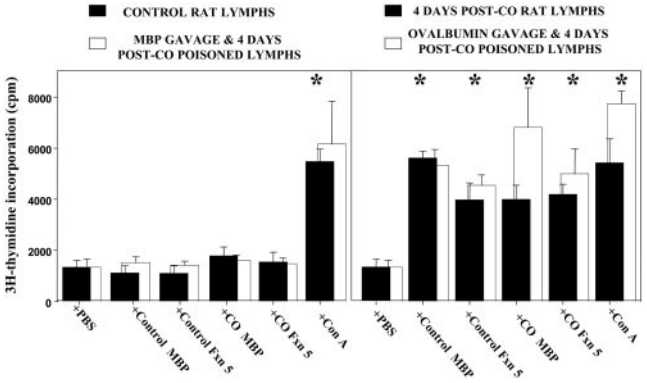

Fig. 4. Lymphocyte proliferative responses to rat MBP and CM chromatography fraction 5 material. Results are expressed as the mean from triplicate samples (five rats for each group). Groups shown are from control rats, rats killed 4 days after CO poisoning, and rats subjected to gavage with MBP or ovalbumin, poisoned with CO, and killed 4 days later. Cells were exposed to PBS, 5 μg of 18.5-kDa MBP or CM chromatography fraction 5 eluates from control rat brain, 5 μg of 18.5-kDa MBP or CM chromatography fraction 5 eluates from rats killed 90 min after CO poisoning, or 5 μg of Con A. Values are mean $\pm$ SE. *, $P < 0.05$ vs. PBS control count.

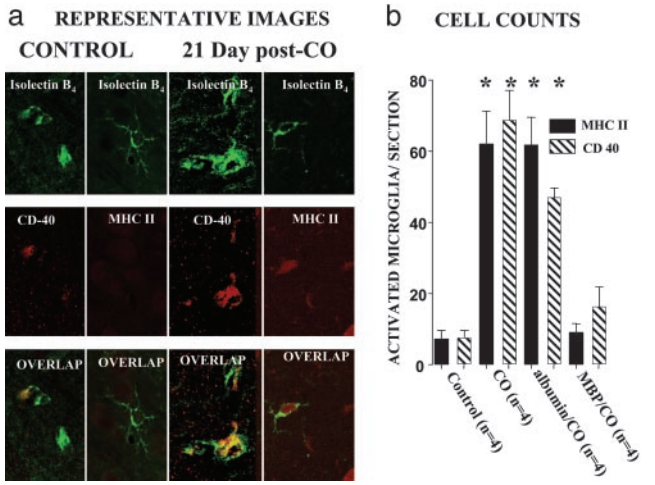

Fig. 5. Microglia are activated after CO poisoning. (*a*) Cells exhibiting dual staining for FITC-conjugated isolectin B_4 (green) and MHC II or CD-40 counterstained with Cy3 (red) were observed frequently in brains from rats 21 days after CO poisoning but not in control. (*b*) Activated microglia were counted on brain sections as cells expressing isolectin B_4 and MHC II, or isolectin B_4 and CD-40. Data are mean $\pm$ SE (n = number of rats used in each group). *, $P < 0.05$. CO, brains from rats killed 21 days after CO poisoning. Where indicated, rats were subjected to gavage with ovalbumin (albumin gavage) or gavage with bovine MBP gavage (MBP).

when Western blots of brain homogenates were probed by using a polyclonal antibody and three monoclonal antibodies that recognize different MBP epitopes, recognition was markedly reduced in samples taken as early as 90 min after poisoning (Fig. 3*a*). However, no significant loss of protein was detected when polyacrylamide gels were analyzed by silver stain (Fig. 3*b*), indicating that proteolysis had not occurred. In Western blot analyses, the band density of glucose transporter 3 (GLUT 3) was chosen as a standard for comparison against MBP. GLUT 3 is the major glucose transporter for neuronal and glial cells (14). Analyses were similar when MBP band densities were compared by using less specific proteins such as actin (data not shown). A reduction in the ratio of MBP to GLUT 3 band density was found in samples obtained from 90 min through 7 days after CO poisoning (Fig. 3*c*). The ratio was insignificantly different from control at 2 weeks. No alterations in band density ratios versus GLUT 3 were found when blots were probed for glial fibrillary acidic protein, the major protein of glial filaments. The glial fibrillary acidic protein/GLUT 3 band density ratio for control brains was 4.4 $\pm$ 0.9 ($n = 4$) and 4.7 $\pm$ 1.0 ($n = 4$, NS vs. control) for brains taken 90 min after CO poisoning.

Lymphocyte Activation. Evidence for an immunological response to MBP was sought in CO-poisoned animals. Lymphocytes harvested from cervical and axillary nodes of control rats and rats killed 4 days after CO poisoning were screened for *in vitro* proliferative responses to MBP. Rat 18.5-kDa MBP from control rats and rats killed 90 min after CO poisoning was removed from Western blot nitrocellulose strips according to published procedures (30), and fraction 5 protein was collected from CM chromatography elutions (as shown in Fig. 2). A proliferative response to materials obtained from both CO-poisoned and control rats occurred with lymphocytes from the former rats but not the latter (Fig. 4). If rats were first subjected to gavage with bovine MBP according to methods shown to induce immunological tolerance (22), lymphocytes did not exhibit a proliferative response (Fig. 4). Proliferation was observed with lymphocytes from rats subjected to ovalbumin gavage as a control (Fig. 4). As was expected, however, MBP gavage did not change the CO-induced abnormalities detected by CM chromatography or antibody recognition on Western blots. In three rats killed 90 min after CO poisoning, the chromatography elution pattern was identical to untreated CO-poisoned rats (data not shown), and the MBP/GLUT3 band density ratio was 0.22 $\pm$ 0.04 (SE), virtually the same as for CO-poisoned rats receiving no treatment. Therefore, although MBP still underwent chemical modification, lymphocytes did not respond to it once rats were rendered tolerant.

MBP Degradation and Microglial Activation. Paraffin brain sections were stained with antibody that only recognizes MBP undergoing degradation by activated microglia and macrophages (27). Antibody did not bind in control brains or brains from rats killed 90 min after CO poisoning, but staining was prominent 1–5 days later (Fig. 1 *b* and *c*). Colocalization was often found between the degraded MBP and cells positive for ED-1, an antigenic determinant on activated macrophages and microglia (31) (Fig. 1*b*). We also found that $CD\text{-}4^+$ lymphocytes were recruited to brain in the days after poisoning and were usually in close proximity to accumulations of degraded MBP (Fig. 1*c*).

Brain microglia were identified on brain sections by isolectin B_4 staining, and cell activation was assessed by colocalization with either MHC II antigens or CD-40, the glycoprotein ligand for the T cell receptor (Fig. 5*a*) (32–34). In control brain, microglia exhibited only faint staining for MHC II and CD-40 in comparison with observations in CO-poisoned brains. When activated microglia were counted across entire brain sections, there were significantly higher numbers after CO poisoning (Fig. 5*b*). Rats subjected to gavage with bovine MBP exhibited fewer activated microglia after CO poisoning than rats subjected to ovalbumin gavage and CO poisoning (Fig. 5*b*).

CO-Mediated Cognitive Deficits. Cognitive function of rats was assessed as the ability to learn to maneuver in an eight-arm radial maze, according to our published methods (10). Control rats exhibited progressive improvement in "entry-to-repeat" scores (Fig. 6), reflected by comparing the scores on individual days and by the slope of the linear regression line. Scores obtained in the final week, days 24–28, were significantly greater than the first three scores. There were no significant differences in the "entry-to-repeat" scores of CO-poisoned rats, and the regression line showed no significant trend (Fig. 6). No motor deficit occurred

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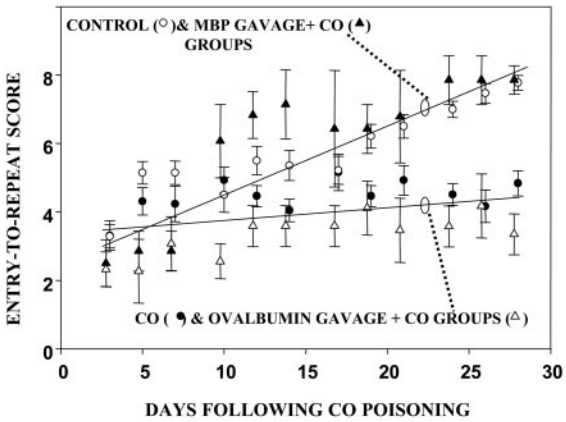

Fig. 6. Maze maneuvering scores. Entry-to-repeat scores for control rats (○, $n = 6$) and CO-poisoned rats first subjected to gavage with MBP (▲, $n = 4$) increased over the test period. Entry-to-repeat scores for last 3 days of testing were all significantly greater than the starting values (repeated-measures ANOVA). Values for CO-poisoned rats (●, $n = 6$) and CO-poisoned rats first subjected to gavage with ovalbumin (△, $n = 4$) were not significantly different over the 4-week testing period.

because of CO poisoning, however, because CO-poisoned rats explored the maze with the same speed as control animals (data not shown). Expressed as the response duration parameter or latency, this is the same finding we reported in ref. 10. Rats subjected to bovine MBP gavage before CO poisoning exhibited significant improvement in "entry-to-repeat" scores (Fig. 6). Latency scores for the treated rats exhibited the same pattern of improvement as was observed for both the control and untreated CO-poisoned rats.

Discussion

These findings provide insight into the pathophysiology of brain injury due to CO poisoning. Biochemical and immunological studies indicate that MBP undergoes charge and antigenic alterations. A causal relationship between lipid peroxidation and MBP modifications is supported by colocalization of MDA-adducts with MBP on tissue sections and results with the ELISA. MALDI-TOF and tandem MS analyses confirm that the antibodies detected MBP, although the CO-mediated chemical alterations were not identified. They may have reversed during proteolytic digestion, or lipid adducts may have been formed in the 9% of MBP we were unable to analyze. There is strong precedence that aldehydic lipid peroxidation products can react with amino acid residues to neutralize positive charges and alter protein conformation (15, 16).

There are a number of lines of evidence indicating that CO poisoning elicits an adaptive immunological response. Rat MBP causes a proliferative response in lymphocytes from CO-poisoned rats but not in lymphocytes from control rats. Interestingly, the responsive lymphocytes exhibit crossreactivity to materials from CO-poisoned and control rats. Colocalization between degraded MBP and ED-1-positive cells indicates that myelin is an early target for inflammatory cells entering brain after CO poisoning. We also found increased expression of MHC II and CD-40 markers on microglia in CO-poisoned rats. Studies with chimeric mice have shown that CD-40 expression by endogenous microglia controls the migration and retention of MBP-reactive T cells in the CNS (35). Others have shown that if interactions between microglia and T cells are inhibited, CD-40 expression and chemokine secretion by microglia are diminished (34–36). Similarly, we found fewer activated microglia in rats rendered tolerant to MBP by gavage. MBP gavage did not prevent structural changes in MBP, however, as reflected by CM chromatography and antibody recognition. These findings, along with histochemical evidence for CD-4$^+$ T cell infiltration, point to a complex interplay between systemic immune cells and resident cells in the brain as a consequence of CO poisoning.

Finally, CO-poisoned rats exhibited impaired learning in maze studies that did not occur in rats rendered immunologically tolerant to MBP. This result supports a causal relationship between CO-mediated oxidative stress, structural MBP changes, immunological responses, and learning dysfunction. Activated microglia can mediate cognitive dysfunction by impairing neurogenesis and by causing neuronal necrosis or apoptosis (37, 38). Necrosis and apoptosis have been reported after CO poisoning (5–7). It is notable that motor deficits and gross demyelination were not observed in the CO-poisoned rats. Clearly, the immunological responses after CO poisoning differ from experimental allergic encephalomyelitis, which arises after injection of adjuvant and MBP chemical extracts (23).

These results provide a cohesive mechanism that links acute CO-mediated insults with delayed brain dysfunction and may explain why the outcome from clinical CO poisoning does not follow a simple dose–response relationship with CO "dose" measured as the carboxyhemoglobin level (2–4). Identification of immunological responses after CO poisoning offers hope that delayed neurological sequelae might be treatable with anti-inflammatory agents.

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1. Raub, J. A., Mathieu-Nolf, M., Hampson, N. B. & Thom, S. R. (2000) *Toxicology* **145,** 1–14.
2. Hampson, N. B. (1998) *J. Emerg. Med.* **16,** 695–698.
3. Weaver, L. K., Hopkins, R. O., Chan, K. J., Churchill, S., Elliott, C. G., Clemmer, T. P., Orme, J. F., Thomas, F. O. & Morris, A. H. (2002) *N. Engl. J. Med.* **347,** 1057–1067.
4. Thom, S. R., Taber, R. L., Mendiguren, I. I., Clark, J. M., Hardy, K. R. & Fisher, A. B. (1995) *Ann. Emerg. Med.* **25,** 474–480.
5. Nabeshima, T., Katoh, A., Ishimaru, H., Yoneda, Y., Ogita, K., Murase, K., Ohtsuka, H., Inari, K., Fukuta, T. & Kameyama, T. (1991) *J. Pharmacol. Exp. Ther.* **256,** 378–384.
6. Ishimaru, H. A. (1992) *J. Pharmacol. Exp. Ther.* **261,** 349–352.
7. Piantadosi, C. A., Zhang, J., Levin, E. D., Folz, R. J. & Schmechel, D. E. (1997) *Exp. Neurol.* **147,** 103–114.
8. Zhang, J. & Piantadosi, C. A. (1992) *J. Clin. Invest.* **90,** 1193–1199.
9. Thom, S. R. (1990) *J. Appl. Physiol.* **68,** 997–1003.
10. Thom, S. R., Fisher, D., Zhang, J., Bhopale, V. M., Cameron, B. & Buerk, D. G. (2004) *Toxicol. Appl. Pharmacol.* **68,** 280–295.
11. Thom, S. R. (1992) *J. Appl. Physiol.* **73,** 1584–1589.
12. Ischiropoulos, H., Beers, M. F., Ohnishi, S. T., Fisher, D., Garner, S. E. & Thom, S. R. (1996) *J. Clin. Invest.* **97,** 2260–2267.
13. Thom, S. R. (1993) *Toxicol. Appl. Pharmacol.* **123,** 234–247.
14. Thom, S. R., Fisher, D. & Manevich, Y. (2001) *Am. J. Physiol.* **281,** H923–H930.
15. Steinbrecher, U. P. (1987) *J. Biol. Chem.* **262,** 3603–3608.
16. Thiele, G. M., Tuma, D. J., Willis, M. S., Miller, J. A., McDonald, T. L., Sorrell, M. F. & Klassen, L. W. (1998) *Alcohol. Clin. Exp. Res.* **22,** 1731–1739.
17. Barone, F. C. & Feuerstein, G. Z. (1999) *J. Cereb. Blood Flow Metab.* **19,** 819–834.
18. Tikka, T., Fiebich, B. L., Goldsteins, G., Keinanen, R. & Koistinaho, J. (2001) *J. Neurosci.* **21,** 2580–2588.
19. Cao, L., Goodin, R., Wood, D., Moscarello, M. A. & Whitaker, J. N. (1999) *Biochemistry* **38,** 6157–6163.

20. Pritzker, L. B., Shashikant, J., Gowan, J. J., Harauz, G. & Moscarello, M. A. (2000) *Biochemistry* **39,** 5374–5381.
21. Zhou, S. R., Moscarello, M. A. & Whitaker, J. N. (1995) *J. Neuroimmunol.* **62,** 147–152.
22. Becker, K. J., McCarron, R. M., Ruetzler, C., Laban, O., Sternberg, E., Flanders, K. C. & Hallenbeck, J. M. (2000) *Proc. Natl. Acad. Sci. USA* **94,** 10873–10878.
23. McFarlin, D. E., Blank, S. E. & Kibler, R. F. (1973) *Science* **179,** 478–480.
24. Beniac, D. R., Wood, D. D., Palaniyar, N., Ottensmeyer, F. P., Moscarello, M. A. & Harauz, G. (2000) *Neurochem. Res.* **5,** 319–336.
25. Boggs, J. M., Rangaraj, G., Koshy, K. M., Ackerley, C., Wood, D. D. & Moscarello, M. A. (1999) *J. Neurosci. Res.* **57,** 529–535.
26. Tranquill, L. R., Cao, L., Ling, N. C., Kalbacher, H., Martin, R. M. & Whitaker, J. N. (2000) *Mult. Scler.* **6,** 220–225.
27. Matsuo, A., Lee, G. C., Terai, K., Takami, K., Hickey, W. F., McGeer, E. G., McGeer, P. L. (1997) *Am. J. Pathol.* **150,** 1253–1266.
28. Palma, A. E., Owh, P., Fredic, C., Readhead, C. & Moscarello, M. A. (1997) *J. Neurochem.* **69,** 1753–1762.
29. Martenson, R. E. & Gaitonde, M. K. (1969) *J. Neurochem.* **16,** 333–347.
30. Abou-Zeid, C., Stele, J. & Rook, G. A. W. (1987) *J. Immunol. Methods* **98,** 5–9.
31. Bauer, J., Sminia, T., Wouterlood, F. G. & Dijkstra, C. D. (1994) *J. Neurosci. Res.* **38,** 365–375.
32. Streit, W. J. & Kreutzberg, G. W. (1987) *J. Neurocytol.* **16,** 249–260.
33. Sedgwick, J. D., Schwender, S., Imrich, H., Dorries, R., Butcher, G. W. & Meulen, V. T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7438–7442.
34. Becher, B., Durell, B. G., Miga, A. V., Hickey, W. F. & Noelle, R. J. (2001) *J. Exp. Med.* **193,** 967–974.
35. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. & Weiner, H. L. (1994) *Science* **265,** 1237–1240.
36. D'Aversa, T. G., Weidenheim, K. M. & Berman, J. W. (2002) *Am. J. Pathol.* **160,** 559–567.
37. Ekdahl, C. T., Claasen, J.-H., Bonde, S., Kokaia, Z. & Lindvall, O. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 13632–13637.
38. Wu, D. C., Jackson-Lewis, V., Vila, M., Teismann, P., Vadseth, C., Choi, D.-K., Ischiropoulos, H. & Przedborski, S. (2002) *J. Neurosci.* **22,** 1763–1771.