Brief Definitive Report

Elevated Secretion of Reactive Nitrogen and Oxygen Intermediates by Inflammatory Leukocytes in Hyperacute Experimental Autoimmune Encephalomyelitis: Enhancement by the Soluble Products of Encephalitogenic T Cells

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Summary

Perivascular lesions within the central nervous system (CNS) of rats with hyperacute experimental autoimmune encephalomyelitis (HEAE) contained large numbers of peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN), cells enzymatically capable of producing reactive nitrogen and oxygen intermediates (RNI and ROI), which, in excess, are mediators of tissue damage. PBMC and PMN isolated from the CNS and periphery of HEAE-affected rats secreted significantly ($p < 0.01$–0.0001) elevated levels of ROI and RNI compared with that of similar cell populations from pertussis- and saline-treated control animals. Coincubation of systemically derived PBMC and PMN with antigen-stimulated myelin basic protein–specific T cell lines led to further increases in ROI and RNI output of between 15.3 and 83.1%, an effect that could be largely attributed to heat-labile, soluble products released by these T cell lines. Our studies suggest a putative neuropathological role for ROI and RNI in HEAE, which may be mediated via cytokines emanating from autoreactive T lymphocytes.

Inflammatory cells, notably T cells and macrophages, are found within lesions in the central nervous systems (CNS) of animals with experimental autoimmune encephalomyelitis (EAE) (1). In hyperacute EAE (HEAE), a disease variant used as a laboratory model for rapid onset multiple sclerosis and acute necrotising hemorrhagic leukoencephalopathy, significant numbers of polymorphonuclear leukocytes (PMN) are also present (2). Upon activation, both macrophages (3) and PMN (4) are major cellular sources of reactive oxygen intermediates (ROI). It is now established that these cells also secrete an additional class of effector molecules collectively termed the reactive nitrogen intermediates (RNI), which include the highly unstable species nitric oxide (NO·), as well as its derivatives, nitrite (NO_2^-) and nitrate (NO_3^-) (5, 6). The strong oxidant properties of ROI and RNI provide potent antimicrobial (3, 7), parasiticidal (8), and tumoricidal (9) defense mechanisms. They are, however, also injurious to normal host tissue components (10), suggesting a possible pathogenic role in HEAE.

We report here an elevated secretion of RNI and ROI by both systemic and CNS-derived PBMC and PMN from rats during HEAE. In addition, the effects of encephalitogenic T cells or their soluble products on ROI and RNI secretion by these effector cells were examined, since it has been proposed recently that neurospecific T lymphocytes, through the action of cytokines such as TNF-α and -β, and IFN-γ (11, 12), recruit and activate inflammatory cells during EAE. The priming effects of these cytokines for inducible ROI (4) and RNI (13) release are well documented.

Materials and Methods

Induction of HEAE. Under ether anesthesia, 8–10-wk-old Lewis × DA(F_1) hybrid rats (180–220 g) were injected intradermally with 125 μl of guinea pig spinal cord homogenate (400 mg/ml in 0.9% NaCl) into each hind footpad along with 0.1 ml of *Bordetella pertussis* vaccine (10^{10} heat-killed organisms/ml) (Commonwealth Serum Labs, Melbourne, Australia) administered dorsally. Control groups received pertussis vaccine or saline alone. Clinical HEAE, which manifested 6–8 d postinoculum (p.i.), was graded according to the following criteria: 0, asymptomatic; 1, loss of distal half of tail tonicity; 2, loss of entire tail tonicity; 3, hindlimb paresis; 4, hindlimb paralysis; 5, tetraplegia; 6, death.

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Cell Isolation. On day 8 p.i., PBMC and PMN from the peripheral blood of HEAE-affected rats, pertussis-immunized, and saline-treated controls were separated by Ficoll-Paque centrifugation (density 1.089 g/ml; Pharmacia Fine Chemicals AB, Uppsala, Sweden) and dextran sedimentation (T-500; Pharmacia Fine Chemicals AB) subsequent to hypertonic lysis to remove erythrocytes (14). Differential staining (Diff Quik stain; Lab Stains, Narabeen, Australia) showed that PMN fractions were 96.5 ± 1.2% pure, while PBMC fractions exhibited the following leukocyte profile: 38.7 ± 2.8% monocytes, 57.4 ± 2.4% lymphocytes, and 1.5 ± 0.8% PMN. Cell viability, as assessed by trypan blue exclusion, was consistently >95%. CNS-derived PBMC and PMN were obtained from animals that were thoroughly perfused with saline in order to remove nonadherent blood cells. Spinal cords were removed aseptically and dissected in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 50 mg/ml type I collagenase (Sigma Chemical Co., St. Louis, MO), gently agitated (15 min, 4°C), and screened before separating by Ficoll-Paque centrifugation. Cell yields ranged from 2 to 3.5 × 10^4 PBMC and ~5 × 10^3 PMN per 100 mg tissue wet weight. The negligible hemoglobin content of these tissue preparations showed that the perfusion technique employed was effective.

Myelin Basic Protein–reactive T Cell Lines. Cell lines reactive to myelin basic protein (MBP-C) were generated essentially as described by Ben-Nun et al. (15). At the time of use these cells had been in continuous culture for ~5 mo and had $CD4^+$, $CD8^-$, $OX22^-$ phenotypes.

Cell Activation and Coculture Conditions. MBP-C (1.5 × 10^6/ml) were activated by coculturing them with irradiated (2,000 rad) syngeneic thymocytes (IT) (3.5 × 10^7/ml) for 3 d with 20 μg/ml MBP in 50 ml of DME containing 1% autologous serum, 5 × 10^{-5} M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, plus penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2. Nonactivated cells were isolated from the culture by Ficoll-Paque centrifugation (14) and cultured for a further 3 d or more in DME with 10% FCS and 15% Con A–activated spleen cell supernatant as a source of IL-2. After the culture period, activated or nonactivated MBP-C were centrifuged (500 *g*, 5 min, 4°C) and washed twice with PBS. Subsequently, 1.5 × 10^6 activated or nonactivated MBP-C were added to either 10^6 PBMC for 8 h or 10^5 PMN for 2 h, respectively, in 5.0 ml fresh DME. After coincubation, cell suspensions were centrifuged (750 *g*, 10 min, 4°C), and the pellet was prepared for chemiluminescence (CL), while supernatants were set aside for assay of total RNI. Sterile filtered (0.22 μm) supernatants from MBP-C, HEAE-primed PBMC (8 h), or PMN ((2 h) were used to investigate the contribution by soluble factors to ROI or RNI production, and contained <20 pg/ml endotoxin as assessed by limulus amebocyte assay (M. A. Bioproducts, Walkersville, MD).

Unstimulated Chemiluminescence. 10^6 PBMC or 10^5 PMN in 900 μl HBSS containing 5 mM glucose were added to dark-adapted disposable glass tubes in the presence of either 20 μl luminol (LUM) (10 mM) (Boehringer Mannheim Biochemicals, Indianapolis, IN) or 50 μl lucigenin (LUC) (5 mM) (Sigma Chemical Co.) for detection of H_2O_2/HOCl and O_2^-, respectively (16). Cell-free suspensions served as background controls. CL was recorded over 30 min at 37°C in an unstirred system using a multichannel Lumicon luminometer (Hamilton Bonaduz AG, Bonaduz, Switzerland). Cubic spline interpolated chemiluminescent traces (ANUGraph; ANU Tech, Canberra, Australia) were integrated using an Apple Macintosh IIcx minicomputer to obtain the total counts/30 min.

No_2^- and NO_3^- Determination. A microplate assay method (essentially as described by Rockett et al. [8]), using the Griess reagent (1% sulfanilamide plus 0.1% *N*-[1-naphthyl]ethylenediamine dihydrochloride [Sigma Chemical Co.] in 2.5% H_3PO_4), and a Zn/Cd/Cu catalyst to discriminate between NO_2^- and NO_3^-, was used to determine total secreted RNI.

Cytokine Assays. TNF-α, IFN-γ, and IL-6 in cell culture supernatants were measured in triplicate using the following methods: (*a*) TNF-α by double sandwich ELISA (17); (*b*) IFN-γ by WEHI-279.1 proliferation assay with rRat IFN-γ (Holland Biotechnology bv, CA Leiden, The Netherlands) as the standard (18); and (*c*) IL-6 via a [^{3}H]thymidine incorporation assay using the B cell hybridoma, B9 (19).

Results and Discussion

Heightened RNI and ROI Secretion from Systemic and CNS-derived PBMC and PMN during HEAE. Independent biosynthetic pathways for RNI and superoxide (O_2^-) production have been proposed for murine monocytes/macrophages (5, 13) and human PMN (6), although this does not preclude their concomitant secretion when elicited by suitable physiological stimuli. Indeed, circulating and CNS-derived monocytes from rats clinically ill with HEAE secreted significantly more ($p < 0.0001$) RNI and ROI compared with pertussis-immunized and saline-treated controls (Fig. 1, *a*, *b*, and *c*). Levels of NO_2^- + NO_3^- in 8-h supernatants of PBMC taken from rats with neurological symptoms were some 10-fold higher (systemically derived) and 10–15-fold higher (CNS-derived) than those from pertussis or saline-treated rats (Fig. 1 *a*). ROI release, like RNI output, was substantially elevated in PBMC from sick animals compared with either control group as shown by unstimulated chemiluminescence. Here, increases in H_2O_2/HOCl output of ~8.0 (systemically derived) and between 9.4 and 10.2 times (CNS-derived) that of either control group were found (Fig. 1 *b*). Corresponding differences for O_2^- production (Fig. 1 *c*) were less pronounced, with HEAE-affected rats secreting ~4 (systemically derived) and between 3.5 and 3.8 (CNS-derived) times more than controls.

PMN isolated from HEAE-affected animals exhibited elevations in ROI output compared with PMN from control rats (H_2O_2/HOCl, >30 and >35 times, and O_2^- >40 and >37 times that of control animals for systemic and CNS-derived PMN, respectively; $p < 0.0001$ in each case) (Fig. 1, *e* and *f*). Cumulative RNI production by PMN (Fig. 1 *d*) was also greater in sick rats than controls.

Together these findings suggest that elevations in ROI and RNI output are specifically associated with the disease process, a contention supported by the following evidence. First, quantitative similarities in all three parameters for cells taken from both control groups rule out the possibility that the adjuvant, *B. pertussis* vaccine, alone caused increased ROI or RNI secretion. Second, to assess more accurately their state of activation in vivo, PBMC and PMN were isolated without elicitation and their CL measured without stimulation in vitro. Concurrent experiments using serum-opsonized zymosan were also undertaken, and significant differences between HEAE and control groups were found (data not shown).

In addition, the finding that the levels of RNI and ROI production by cells obtained from the peripheral circulation

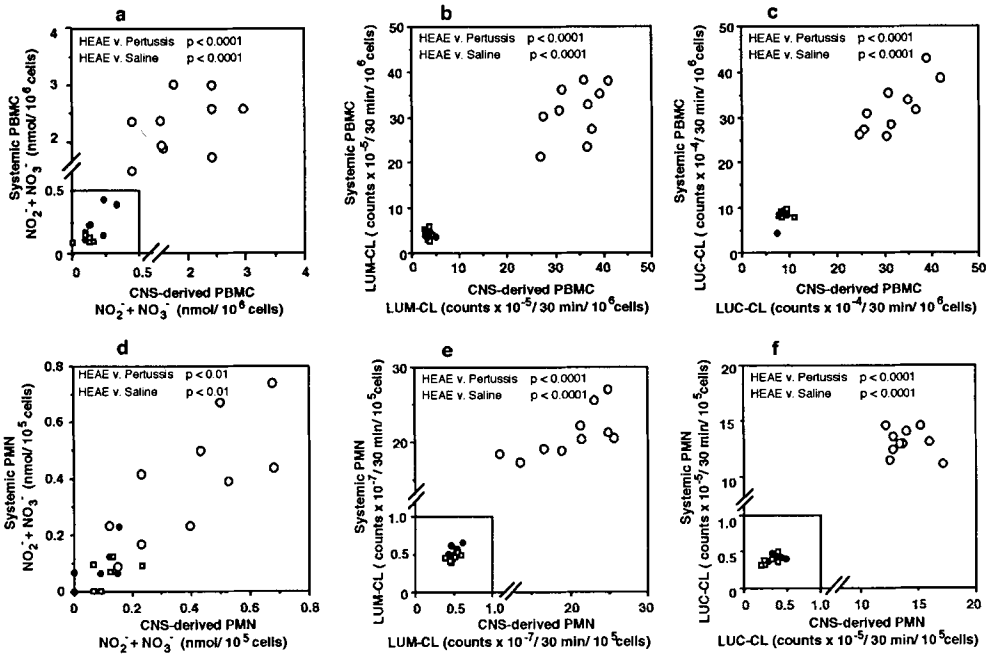

Figure 1. Individual comparison of NO_2^- + NO_3^-, LUM-CL, and LUC-CL production by systemic and CNS-derived PBMC (*a–c*) and PMN (*d–f*) of HEAE (○) (n = 10) versus pertussis-immunized (●) (n = 6) and saline-treated (□) (n = 6) control rats. Cells were harvested day 8 p.i., coinciding with peak clinical severity (score: 4.71 ± 1.03). All measurements were performed in duplicate; statistical differences were measured via student's t test.

versus the central nervous system of the same animal were quantitatively similar (Fig. 1) suggests that those cells entering the CNS probably do so already activated, contrary to the parenchymal activation proposed by others (20). The recent finding that NO· selectively contributes to the LUM-CL of PMA-stimulated macrophages (21) may under some circumstances be a limitation to the use of CL as a measure of ROI (e.g., after pretreatment with PMA [21]).

Encephalitogenic T Cells and Their Soluble Products Augment RNI and ROI Release from HEAE-primed PBMC and PMN. Autoreactive $CD4^+$ T lymphocytes are not only responsible for disease induction in EAE, but are also thought to help localize and orchestrate the cellular events leading directly to CNS damage (22). The increased secretion of ROI and RNI by HEAE-primed PBMC and PMN prompted us to consider whether such secretory functions may be influenced by MBP-C. CL and RNI responses of either PBMC (10^6 cells) or PMN (10^5 cells) incubated for 8 and 2 h, respectively, in the presence of activated MBP-C (1.5×10^4 cells) plus IT (10^5 cells) showed that encephalitogenic T cell lines have the ability to enhance these effector functions in vitro (Fig. 2). MBP-C were only effective if first activated with antigen (Fig. 2; activated MBP-C vs. nonactivated MBP-C), and their contribution to the levels of ROI in these coincubations, like that of IT, was negligible (<6.7×10^4 counts/30 min/10^6 cells for LUM-CL, and <4.4×10^4 counts/30 min/10^6 cells for LUC-CL) (data not shown). Similarly, neither the MBP-C nor IT secreted significant amounts of RNI, typically <0.07 nmol/10^6 cells (data not shown).

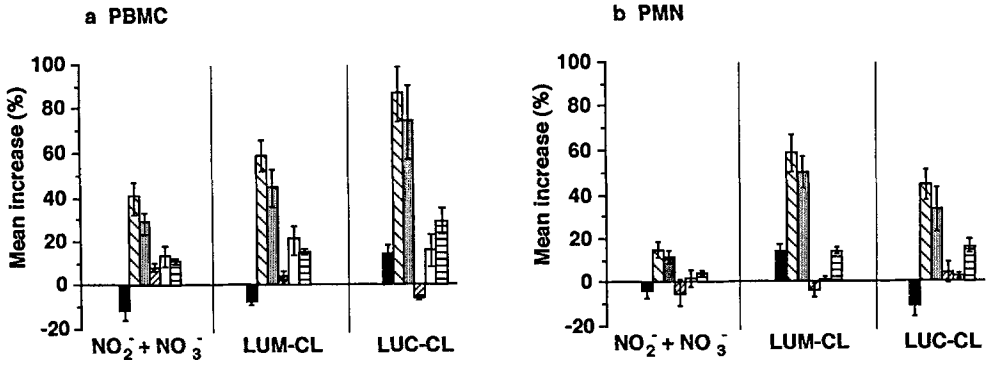

Figure 2. Effect on NO_2^- + NO_3^- or CL of systemically derived PBMC or PMN coincubated with the following: (■) nonactivated MBP-C, (▨) activated MBP-C, (▩) activated MBP-C supernatants, (▨) heat-inactivated MBP-C supernatants, or (□) autologous supernatants. PBMC or PMN from normal donors (▤) cocultured with activated MBP-C. Mean percent increases were obtained by subtracting the sum of concurrently run separate incubations, e.g. PBMC + MBP-C, from the corresponding coincubation values (PBMC + MBP-C). Cells for these experiments were taken from eight different HEAE-affected rats or four different normal rats, and measurements were performed in duplicate. Supernatants of activated MBP-C were heat inactivated at 80°C for 30 min. NO_2^- + NO_3^- enhancement was corrected for the original amount of RNI in all supernatants before coincubation with PBMC or PMN.

Table 1. *Cytokine Profiles in Supernatants of MBP-C or PBMC (8 h) and PMN (2 h) Isolated from Animals with HEAE*

Supernatant	TNF-α*	IFN-γ‡	IL-6‡
		U/ml	
Activated MBP-C	7.0 ± 0.24	26.76 ± 0.5	5.56 ± 0.47
HIA§ MBP-C	0.32 ± 0.11	<0.4	<0.5
PBMC	1.32 ± 0.24	3.72 ± 0.83	59.68 ± 6.73
PMN	0.72 ± 0.19	<0.4	2.28 ± 0.54

Data are means ± sem for at least three supernatants.
* Measured by ELISA.
‡ Measured by bioassay.
§ Heat inactivated.

In PBMC activated by MBP-C, O_2^- production was increased 83.1% above the added values for separate MBP-C and PBMC incubations. Comparable large increases for H_2O_2/HOCl (57.5%) and NO_2^- + NO_3^- (42.3%) were measured (Fig. 2 *a*). A role for sensitized T cells in regulating PBMC in EAE has previously been demonstrated (23), with LUM-CL of EAE donors being reduced by as much as 32% after anti-CD4 antibody treatment. Coincubation of PMN with activated MBP-C also enhanced the release of O_2^- by 45.1%, H_2O_2/HOCl by 59.4%, and NO_2^- + NO_3^- by 15.3% (Fig. 2 *b*) compared with the added values for MBP-C and PBMC alone.

When activated MBP-C were coincubated with PBMC or PMN from normal donors, enhancement of ROI and RNI release was much less than that seen in cells from HEAE-affected animals (Fig. 2). This may merely reflect a greater degree of priming of cells taken from sick animals, and equate, for example, with an increased level of receptor expression for cytokines, as has been shown previously in SJL and B10.PL mice with EAE (24).

The disparate effects of antigen-activated and nonactivated MBP-C on RNI and ROI production by phagocytes led us to examine whether enhancement by activated MBP-C might be due to the release of soluble mediators, since autoantigenic encounter is known to cause MBP-reactive T cell clones to produce TNF-α/β, IFN-γ, and IL-2 (11). Activated MBP-C supernatants were, on average, 75% as effective as activated MBP-C at inducing the release of ROI and RNI from either PBMC (Fig. 2 *a*) or PMN (Fig. 2 *b*). This observation may be accounted for by the presence of cytokines in MBP-C supernatants, since this effect was abolished by heating at 80°C for 30 min (Fig. 2, *a* and *b*). Autologous PBMC and PMN supernatants did not induce notable increases in RNI and ROI secretion (Fig. 2 *b*). The cytokine profiles of these supernatants are shown in Table 1. TNF-α, and especially IFN-γ, levels were highest in the supernatants that exhibited the greatest enhancing effect (i.e., those from activated MBP-C). Low levels of these cytokines were also detected in PBMC supernatants, which had a correspondingly smaller influence on PBMC effector functions. In the remaining supernatants, TNF-α and IFN-γ were near or below the levels of assay sensitivity, and these supernatants did not alter RNI or ROI output (Fig. 2, *a* and *b*). IL-6 is reportedly elevated within the CNS during EAE (25) and capable of priming the oxidative burst in PMN and PBMC (26). This cytokine, however, was not effective at increasing the oxidative response of phagocytic cells from animals with HEAE, since its level was >10 times higher in PBMC supernatants (59.7 U/ml; <20% enhancement) than in supernatants from activated MBP-C (5.6 U/ml; 15–75% enhancement) (Table 1).

In summary, our studies indicate a potential role for RNI and ROI in the pathogenesis of HEAE. A role for the secretory products of encephalitogenic T cells in the induction of ROI and RNI release has also been identified. Whether this relationship translates too a disease-specific mechanism is unclear, since nonencephalitogenic MBP-specific cell lines were unavailable for this study. Nevertheless, the involvement of ROI and RNI in HEAE pathology is, we believe, certainly plausible given that the CNS is not particularly well protected against oxidative stress (27). Moreover, being both a potent vasodilator (28) and neurotoxic (29), NO· may contribute to the edema and motor dysfunction characteristic of HEAE.

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