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LIPID PEROXIDATION PRODUCTS AND ANTIOXIDANT PROTEINS IN PLASMA AND CEREBROSPINAL FLUID FROM MULTIPLE SCLEROSIS PATIENTS

M. I. S. HUNTER[1], B. C. NLEMADIM[1], AND D. L. W. DAVIDSON[2]

[1]*Department of Biochemistry and Microbiology, University of St. Andrews St Andrews, Fife*

and

[2]*Department of Neurology Dundee Royal Infirmary Dundee, Angus*

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Lipid peroxidation (LPx) products were measured as thiobarbituric acid-reactive substances (TS) and lipid-soluble fluorescent pigments (FP) in both plasma and CSF from MS patients and controls. Although no significant changes were found in MS plasma, we report here for the first time increases in both TS and FP in MS CSF ($p < 0.05$ and $p < 0.01$, respectively, compared with patients with other neurological diseases), indicating that increased LPx in CNS may be a feature of MS. Levels of transferrin were normal but caeruloplasmin (CP), a major antioxidant plasma protein, was significantly raised in MS patients ($p < 0.01$) and this may represent an adaptive response to increased oxidative challenge. Neither of these proteins was detectable in CSF using radial immunodiffusion. There was no significant correlation between the severity or duration of the disease nor the period since the last relapse and either LPx products or CP suggesting that the changes observed in this work are not simply the direct result of demyelination and tissue damage.

INTRODUCTION

Several lines of evidence, summarised in (1), support the possibility that lipid peroxidation (LPx) may be a factor in membrane damage in MS.

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These include: (i) increased osmotic and mechanical fragility of MS erythrocytes (2–4); (ii) abnormal glutathione peroxidase (GPx) activity in MS erythrocytes and leukocytes (5–10); (iii) increased erythrocyte superoxide dismutase activity and peroxidisability (11); (iv) elevated plasma malondialdehyde (MDA) levels (12); (v) decreased proportions of phospholipid classes which have the highest polyunsaturated fatty acid (PUFA) content in MS myelin (13–15); (vi) decreased PUFA (especially linoleic acid) in several MS tissues (16–21); (vii) increased incidence of MS in some populations consuming high proportions of animal fats (deficient in vitamin E) (22–25); (viii) increased incidence of MS and also nutritional muscular dystrophy in cattle, associated with deficiency of vitamin E and Se (an essential cofactor for GPx), in certain areas of Finland (26).

There has been one report that malondialdehyde is significantly raised in MS plasma and the present work was undertaken to repeat this test for plasma, but also to examine CSF and to assay another reliable, sensitive index of LPx, lipid-soluble fluorescent pigments (27) in both plasma and CSF. Increased levels of these materials, particularly in CSF, would be strong evidence for the occurrence of LPx in CNS in MS.

In addition, ceruloplasmin, the major circulating antioxidant (28) along with the functionally associated, iron-binding protein transferrin were assayed in both plasma and CSF.

EXPERIMENTAL PROCEDURE

MS Patients and Controls. MS patients were predominantly in-patients, although a minority were attending out-patient clinics, at the Department of Neurology, Dundee Royal Infirmary. Most patients were diagnosed as having definite MS by the McAlpine criteria although a small number were designated possible or probable. The numbers in these three categories are indicated at appropriate points in the Results section. Patients were also rated for severity of the disease using the Kurtzke scale and the time elapsed since the most recent relapse was also ascertained.

Normal controls for plasma studies were healthy volunteers from the staff and students of the authors[1] Departments. Control CSF samples were selected from samples undergoing protein analysis for routine neurological investigation in the Department of Biochemical Medicine, Ninewells Hospital, Dundee, and which were subsequently confirmed to have come from subjects suffering from neurological disorders other than MS (e.g. Parkinson's disease, Friedrich's ataxia, Guillaine Barre syndrome, cerebral tumour). Control samples of plasma were also obtained from patients, attending the Department of Neurology, Dundee Royal Infirmary, who had neurological diseases other than MS (OND).

Assay of Lipid Peroxidation Products

(i) Thiobarbituric Acid (TBA)-Reactive Substances. The method used was essentially that of Satoh (29) modified to estimate total, rather than only trichloroacetic acid-precipitable, TBA-reactive materials. This method therefore measures any free malondialdehyde in plasma as well as lipoperoxides associated with serum lipoproteins.

To 0.5 ml plasma or CSF was added 0.5 ml 35% TCA. After vortex mixing, 0.5 ml Tris/HCl buffer (50 mM; pH 7.4) was added followed by further mixing and incubation at room temperature for 10 min. 1.0 ml 0.75% TBA in 2 M Na_2SO_4 was added and the mixture heated at 100° for 45 mins. After cooling 1 ml 70% TCA was added, the mixture vortexed and then centrifuged at 950 g for 10 mins. The absorption spectrum of the supernatant was scanned between 500 and 600 nm to ensure the absence of any interfering absorption peaks and the absorbance at 530 nm recorded. Total TBA-reactive materials were expressed as MDA, using a molar extinction coefficient for MDA of 1.52×10^5 (30).

(ii) Fluorescent Pigments. Fluorescent pigments (FP) were extracted from plasma and CSF by a minor modification of the method of Dillard et al. (31).

0.5 ml plasma or CSF was extracted using 3 ml chloroform-methanol, 2:1 (v/v) at room temperature by vortex mixing for 1 min. The extracts were centrifuged for 2 min after the addition of 3 ml distilled water and mixing. 1.0 ml of the lower chloroform layer was then removed, 0.1 ml methanol added and the fluorescence spectrum determined. The fluorescence intensity at the emission maximum (450–470 nm) was measured and expressed as arbitrary fluorescence units per unit volume of fluid.

Immunoassay of Caeruloplasmin and Transferrin. Radial immunodiffusion assay of these two plasma proteins was carried out using antisera obtained from the Scottish Antibody Production Unit, Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire (transferrin) and Hoescht U.K. Ltd., Parmaceuticals Division, Hounslow, Middlesex (caeruloplasmin).

Antibody titres were 1.1 g/L (TF) and 0.4 g/L (CP) and the final antibody concentration used was 1.7%. Plasma samples (10 μl:diluted 1:2 for TF) were applied to the plates and the diameters of the immunoprecipitate rings measured after 48 h. incubation at room temperature. The system was calibrated using standard plasma (Hoescht).

Statistical Analysis. Data for MS patients and controls were compared using Student's *t* test. Possible correlation between levels of lipid peroxidation products and clinical parameters was tested using linear regression analysis. Both these tests were carried out using a statistical package for the BBC B microcomputer (32).

RESULTS

Although neither lipid peroxidation (LPx) product was significantly elevated in plasma, both MDA and FP were elevated in CSF of MS patients compared with normal controls ($P < 0.05$ and $P < 0.01$ respectively), as shown in Table I and II and Figure 1.

Whilst plasma transferrin was found not to be statistically significantly different from normal, plasma caeruloplasmin was significantly increased in MS patients compared with both healthy controls ($P < 0.01$) and OND controls ($P < 0.02$). Neither of these proteins was detectable in CSF using the immunodiffusion method described here.

There was no significant correlation between any of the parameters investigated and either age of subject, duration or severity of MS, or period since last relapse.

TABLE I

LIPID PEROXIDATION PRODUCTS AND ANTIOXIDANT PROTEINS IN PLASMA FROM MS PATIENTS AND CONTROLS

	Normal Controls Mean (SD) ⟨Range⟩	MS Patients Mean (SD) ⟨Range⟩	OND Controls Mean (SD) ⟨Range⟩
Malondialdehyde	8.0 (1.5)	9.0 (1.5)	N.D.[2]
$nmol.ml^{-1}$	⟨5.5–11.0⟩	⟨6.4–11.0⟩	
n	10	14[3]	
Fluorescent Pigments	30.5 (4.3)	29.4 (3.9)	N.D.[2]
$RFU^{1}.ml^{-1}$	⟨23.0–35.0⟩	⟨24.5–36.0⟩	
n	10	8[4]	
Caeruloplasmin	25.3 (4.9)	30.4** (5.8)	25.1 (5.2)
$mg.dl^{-1}$	⟨16.0–33.0⟩	⟨17.5–42.0⟩	⟨16.0–32.5⟩
n	16	24[3]	10
Transferrin	2.7 (0.3)	2.6 (0.5)	N.D.[2]
$mg.dl^{-1}$	⟨2.1–3.3⟩	⟨1.5–3.7⟩	
n	10	16[3]	

[1] Relative fluorescence units.
[2] Not determined.
[3] Includes 2 possible + 1 probable.
[4] Includes 1 possible + 2 probable.
** $P < 0.01$ vs. normal controls; $P < 0.02$ vs. OND controls.

TABLE II

LIPID PEROXIDATION PRODUCTS IN CEREBROSPINAL FLUID FROM MS PATIENTS AND CONTROLS WITH OTHER NEUROLOGICAL DISORDERS (OND)

	OND Controls Mean (SD) ⟨Range⟩	MS Patients Mean (SD) ⟨Range⟩
Malondialdehyde	1.1 (1.9)	5.3* (4.2)
$nmol.ml^{-1}$	⟨0.0–4.8⟩	⟨0.0–11.0⟩
n	6	7[2]
Fluorescent Pigments	12.6 (2.1)	17.4** (2.5)
$RFU^{1}.ml^{-1}$	⟨10.0–15.0⟩	⟨13.5–20.0⟩
n	5	7[3]

[1] Relative fluorescence units.
[2] Includes 2 probable + 1 possible.
[3] Includes 1 probable + 2 possible.
* $p < 0.05$
** $p < 0.01$

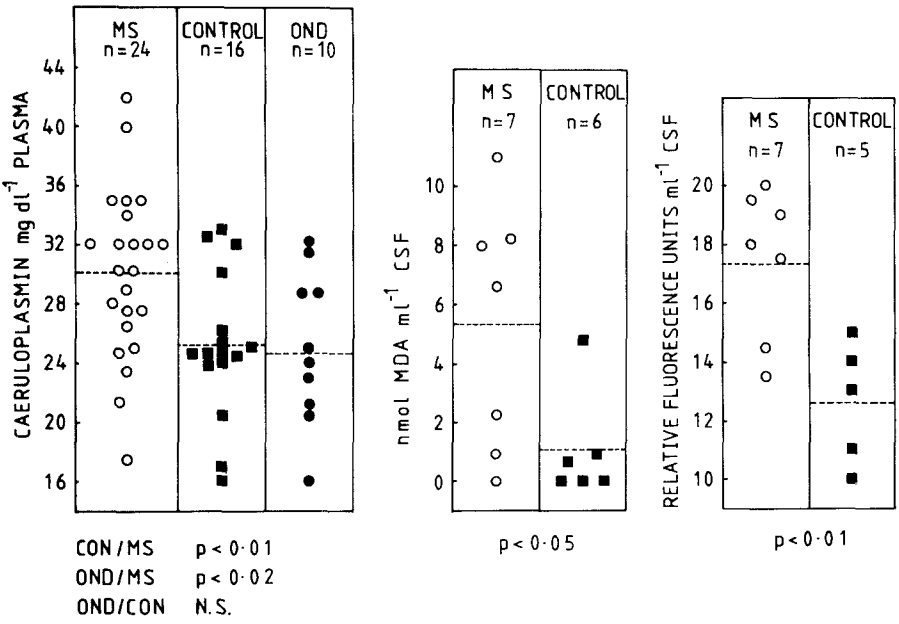

FIG. 1. Plasma caeruloplasmin concentrations in MS patients, patients with other neurological diseases (OND) and healthy controls. Thiobarbituric acid-reactive materials and fluorescent pigments in CSF from MS patients and OND controls.

DISCUSSION

The current evidence supporting a role for lipid peroxidation in the pathogenesis of MS is, at best, tentative. It includes cellular and biochemical abnormalities which would certainly be consistent with autoxidative degradation of membrane phospholipids (2–26) but which might also have other bases. In erythrocytes, the finding of both decreased (5–9) and increased (10, 11) activities of antioxidant enzymes has been used as an argument in favour of increased lipid peroxidation (LPx) in MS. However, careful studies in our laboratory have been unable to demonstrate any significant abnormality in these and other enzymes in MS erythrocytes (1). Only one other group has attempted to search directly for LPx products in body fluids from MS patients and found a 1.5–2 fold increase in plasma malondialdehyde (12). We have been unable to confirm this finding in plasma but were able to detect both thiobarbituric acid-reactive substances (TS) and fluorescent pigments (FP) in significantly elevated amounts in CSF from MS patients compared with normal controls ($P <$

0.05 and 0.01, respectively). This constitutes important and direct evidence in favour of LPx occurring in the CNS, associated with the disease.

The precise origin of these LPx products is unclear. Tissue insult of various kinds is known to lead to increased LPx (33) but our data suggest that, in MS, the increased TS and FP are not the direct result of demyelination and associated cellular damage since there was no correlation with either severity, duration or time elapsed since last relapse. However, this interpretation is only preliminary in view of the small numbers of subjects studied. Another possible source of TS and FP might be cells of the immune system, e.g. macrophages, since local extracellular and intracellular production of free radicals is known to accompany phagocytosis of material by such cells (34) and increased immune cell activity has been implicated in the pathogenesis of MS.

If the findings presented here could be confirmed with a larger number of subjects and, in particular, the difference between MS patients and those suffering from other neurological diseases were substantiated, the use of LPx products as diagnostic indices for MS might well be indicated, perhaps in conjunction with the other CSF parameters already measured to this end, oligoclonal IgG bands and IgG/albumin ratio. A fuller characterisation of the components responsible for the fluorescence and TBA-reacting properties of MS CSF might also allow a particular component (or components), preferentially raised in MS, to be identified and thereby improve the discrimination of such a diagnostic test.

The significant elevation of plasma caeruloplasmin (CP) found in MS patients has also never been previously described. CP is an acute phase protein whose plasma concentration is known to rise in response to tissue damage or inflammation (35). We therefore expected that CP levels might be related to either severity, duration or time since last relapse, but no such correlation was found. The main extracellular antioxidant role of CP is as a ferroxidase (36) thereby converting ferrous ions (a potent catalyst for LPx) into ferric ions. It is tempting to speculate that increased LPx in CNS, and possible escape of LPx products and/or ferrous ions into the circulation, is the trigger for the adaptive increase in CP. An important role for free iron in the pathogenesis of experimental allergic encephalomyelitis has recently been demonstrated since the course of this experimental model for autoimmune demyelinating disease can be arrested *in vivo* by the iron chelator, desferrioxamine (37).

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REFERENCES

1. HUNTER, M. I. S., LAO, M. S., BURTLES, S. S., and DAVIDSON, D. L. W. 1984. Erythrocyte antioxidant enzymes in multiple sclerosis and the effect of hyperbaric oxygen. Neurochem. Res. 9:507–516.
2. KURANTSIN-MILLS, J., SAMJI, N., MOSCARELLO, M. A., and BOGGS, J. M. 1982. Comparison of membrane structure, osmotic fraglity and morphology of multiple sclerosis and normal erythrocytes. Neurochem. Res. 7:1523–1540.
3. CASPARY, E. A., SEWELL, F., and FIELD, E. J. 1967. Red blood cell fragility in multiple sclerosis. Brit. Med. J. 2:610–611.
4. SCHAUF, C. L., FRISCHER, H., and DAVIS, F. A. 1980. Mechanical fragility of erythrocytes in multiple sclerosis. Neurology 30:323–325.
5. SHUKLA, V. K. S., JENSEN, G. E., and CLAUSEN, J. 1977. Erythrocyte glutathione peroxidase deficiency in multiple sclerosis. Acta Neurol. Scand. 56:542–550.
6. SZEINBERG, A., GOLAN, R., EZZER, J. B., SAROVA-PINHAS, I., and KINDLER, D. 1981. Decreased erythrocyte glutathione peroxidase activity in multiple sclerosis. Acta Neurol. Scand. 60:265–271.
7. SZEINBERG, A., GOLAN, R., BEN-EZZER, J., SAROVA-PINHAS, I., and KINDLER, D. 1981. Glutathione peroxidase activity in various types of blood cell in multiple sclerosis. Acta Neurol. Scand. 63:67–75.
8. JENSEN, G. E., GISSEL-NEILSEN, G., and CLAUSEN, J. 1980. Leukocyte glutathione peroxidase E.C.1.11.1.9 activity and selenium level in multiple sclerosis. J. Neurol. Sci. 48:61–68.
9. JENSEN, G. E., and CLAUSEN, J. 1984. Glutathione peroxidase and reductase, glucose-6-phosphate dehydrogenase and catalase activities in multiple sclerosis. J. Neurol. Sci. 63:45–53.
10. ZACHARA, B., GROMADZINSKA, J., CZERNICKI, J., MACIEJEK, Z., and CHMIELEWSKI, H. 1984. Red blood cell glutathione peroxidase activity in multiple sclerosis. Klin. Wochenschr. 62:179–182.
11. POLIDORO, G., DI ILIO, C., ARDUINI, A., LA ROVERE, G., and FEDERICI, G. 1984. Superoxide dismutase, reduced glutathione and thiobarbituric acid-reactive products in erythrocytes of patients with multiple sclerosis. Int. J. Biochem. 16:505–509.
12. ROGOVINA, N. I., and KOKLOV, A. P. 1980. Metabolism of lipid peroxidation products in multiple sclerosis patients. Zh. Nevropatol. Paikhiatr. Im. S. S. Korsakova 80:696–700.
13. YU, R. K., UENO, K., GLASER, G. H., and TOURTELLOTTE, W. W. 1982. Lipid and protein alterations of spinal cord and cord myelin of multiple sclerosis. J. Neurochem. 39:464–477.
14. WOELK, H., and BORRI, P. 1973. Lipid and fatty acid composition of myelin purified from normal and MS brains. Eur. Neurol. 10:250–260.
15. GOPFERT, E., PYTLIK, S., and DEBUCH, H. 1980. 2′,3′-Cyclic nucelotide 3′-phosphohydrolase and lipids of myelin from multiple sclerosis and normal brains. J. Neurochem. 34:732–739.
16. THOMPSON, R. H. S. 1975. Unsaturated fatty acids in multiple sclerosis. Pages 184–191. *In* DAVISON, A. N., HUMPHREY, J. H., LIVERSEDGE, L. A., MCDONALD, W. I., and PORTERFIELD, J. S. (eds). Multiple sclerosis research. HMSO:London.
17. BAKER, R. W. R., THOMPSON, R. H. S., and ZILKHA, K. J. 1964. Serum fatty acids and multiple sclerosis. J. Neurol. Neurosurg. Psychiat. 27:408–414.
18. SANDERS, H., THOMPSON, R. H. S., WRIGHT, H. P., and ZILKHA, K. J. 1968. Further studies on platelet adhesion and serum cholesteryl linoleate levels in multiple sclerosis. J. Neurol. Neurosurg. Psychiat. 31:321–325.

19. NEU, I. S. 1983. Essential fatty acids in the serum and cerebrospinal fluid of multiple sclerosis patients. Acta Neurol. Scand. 67:151–163.
20. GUL, S., SMITH, A. D., THOMPSON, R. H. S., WRIGHT, H. P., and ZILKHA, K. J. 1970. Fatty acid composition of phospholipids from platelets and erythrocytes in multiple sclerosis. J. Neurol. Neurosurg. Psychiat. 33:506–510.
21. CHERAYIL, G. D. 1984. Sialic acid and fatty acid concentrations in lymphocytes, red blood cells and plasma from patients with multiple sclerosis. J. Neurol. Sci. 63:1–10.
22. BERNSOHN, J., and STEPHANIDES, L. M. 1967. Aetiology of multiple sclerosis. Nature 215:821–823.
23. DICK, G. 1976. The aetiology of multiple sclerosis. Proc. Roy. Soc. Med. 69:611–615.
24. JONES, R., PREECE, A. W., LUCKMAN, N. P., and FORRESTER, J. A. 1983. The analysis of the red blood cell unsaturated fatty acid test for multiple sclerosis using laser cytopherometry. Phys. Med. Biol. 28:1145–1151.
25. MERTIN, J., and MEADE, C. J. 1977. Relevance of fatty acids in multiple sclerosis. Brit. Med. Bull. 33:67–71.
26. WICKSTROM, J., WESTERMARK, T., and PALO, J. 1976. Selenium, vitamin E and copper in multiple sclerosis. Acta Neurol. Scand. 54:287–290.
27. FLETCHER, B. L., DILLARD, C. J., and TAPPEL, A. L. 1973. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. Anal. Biochem. 52:1–9.
28. AL-TIMIMI, D. J., and DORMANDY, T. L. 1977. The inhibition of lipid autoxidation by human caeruloplasmin. Biochem. J. 168:283–288.
29. SATOH, K. 1978. Serum lipid peroxide in cerebrovascular disorders detected by a new colorimetric method. Clin. Chim. Acta 90:37–43.
30. STOCKS, J., and DORMANDY, T. L. 1971. The autoxidation of human red cell lipids induced by hydrogen peroxide. Brit. J. Haematol. 20:95–111.
31. DILLARD, C. J., and TAPPEL, A. L. 1971. Fluorescent products of lipid peroxidation of mitochondria and microsomes. Lipids 6:715–721.
32. BEYNON, R. J. 1983. In: Contributed Programs Vol. 1., Biochemistry Microcomputer Group, University of Liverpool.
33. HALLIWELL, B., and GUTTERIDGE, J. M. C. 1984. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. Lancet (i):1396–1397.
34. SLATER, T. F. 1984. Free-radical mechanisms in tissue injury. Biochem. J. 222:1–15.
35. GUTTERIDGE, J. M. C., and STOCKS, J. 1981. Caeruloplasmin: physiological and pathological perspectives. CRC Crit. Rev. Clin. Lab. Sci. 14:257–329.
36. GUTTERIDGE, J. M. C., RICHMOND, R., and HALLIWELL, B. 1980. Oxygen free radicals and lipid peroxidation. Inhibition by the protein caeruloplasmin. FEBS Lett. 112:269–272.
37. BOWERN, N., RAMSHAW, I. A., CLARK, I. A., and DOHERTY, P. C. 1984. Inhibition of autoimmune neuropathological process by treatment with an iron-chelating agent. J. Exp. Med. 160:1532–1543.