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Note

Liquid chromatographic determination of serum lipid peroxides as diphenylthiobarbituric acid-reactive substance to collagen disease patients – a preliminary study

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Toxic oxygen species, such as hydroxyl and superoxyl radicals and hydrogen peroxides, appear to be closely linked to certain pathologic disorders, such as cancer [1], diabetes [2], pancreatic disease [3], Down's syndrome [4], etc. The levels of lipid peroxide (LPO) have served as an index of the oxidative status of tissues or biofluids [5, 6]. In view of this, the serum LPO levels in patients suffering from serious metabolic disorders should also be examined. However, not very much information is available on the correlation between serum LPO levels and autoimmune disorders. The generalized methods for LPO determination in the assay of biofluids (thiobarbituric acid, TBA, methods according to Yagi [7]) are subject to various limitations [8] that hamper the examination of such a correlation.

We report here an examination of the serum LPO levels of 30 patients suffering from collagen diseases and related autoimmune disorders by the colorimetric determination of the malondialdehyde (MDA)–TBA complex by high-performance liquid chromatography (HPLC). This makes possible the selective determination of serum TBA-reactive substances without interference from bilirubin, sugars or sialic acid. The effects of high-dose steroid infusion on the serum LPO levels of these patients were also examined.

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EXPERIMENTAL

LPO measurement

An aliquot (25 μl) of serum was added to 0.5 ml of a solution of 0.01 *M* 1,3-diphenyl-2-thiobarbituric acid (DPTBA) in phosphate (pH 3.0). The solution was well mixed and incubated at 95°C for 40 min, then cooled for 5 min under tap water, before 0.25 ml of acetonitrile–pyridine (4:1, v/v, 0.25 ml) were added. After 1 min of vortex-mixing, the mixture was centrifuged at 1600 *g*, and an aliquot (20 μl) of the supernatant was subjected to HPLC [8]. The HPLC conditions were as follows: column, JASCO Fine Pack C_{18} (10 μm particle size, 25×0.46 cm I.D.); mobile phase, acetonitrile–0.1 *M* sodium chloride (52:48, v/v); detection, 537 nm. The peak height of the MDA condensate with DPTBA was determined. LPO concentrations were calculated as nmol MDA per ml serum from a calibration curve made at various concentrations (0–0.5 nmol) of tetraethoxypropane as the standards.

Patients and controls

Serum samples were obtained from thirty patients suffering from systemic lupus erythematosus (SLE; eight patients), chronic rheumatoid arthritis (RA; twelve patients), idiopathic thrombocytopenic purpura (ITP; three patients) and pernicious anemia (PA; seven patients). Control sera were obtained from fourteen healthy persons (mean age, 44.4 ± 19.0 years). The blood was centrifuged at 1600 *g* and the separated serum used for LPO determinations.

Materials

DPTBA was a kind gift of Dr. K. Nakashima, Nagasaki University. Other reagents were of the best available grade, from Wako.

RESULTS

Fig. 1A shows the chromatogram of the products of the reaction of a patient's serum with DPTBA, monitored at 537 nm. Fig. 1B shows the chromatogram of the products of the reaction of MDA with DPTBA, as the standard. A linear relationship (r=0.99999) was obtained between peak height of the MDA–DPTBA complex and the standard MDA added to the test solution in the concentration range 0–6.0 nmol/ml.

The effects of glucose, bilirubin and sialic acid on the assay were examined. The peak height of MDA–DPTBA complex was not influenced by the presence of 0.1–1.0 mg/ml glucose and decreased to 96–89% of that of the control in the presence of higher concentrations (5–100 mg/ml) of glucose (Fig. 2A). The peak height was not influenced by 10 μg/ml bilirubin (Fig. 2B), but 25 or 50 μg/ml bilirubin decreased the peak height to 98.2 or 95.3% of that of the control, respectively. The assay was unaffected by 2.5–5000 μg/ml sialic acid (Fig. 2C).

The serum LPO concentrations of the thirty patients and fourteen healthy subjects, as determined by the present method, are displayed in Fig. 3, which shows the mean LPO level of the patients with RA, SLE, ITP and PA to be

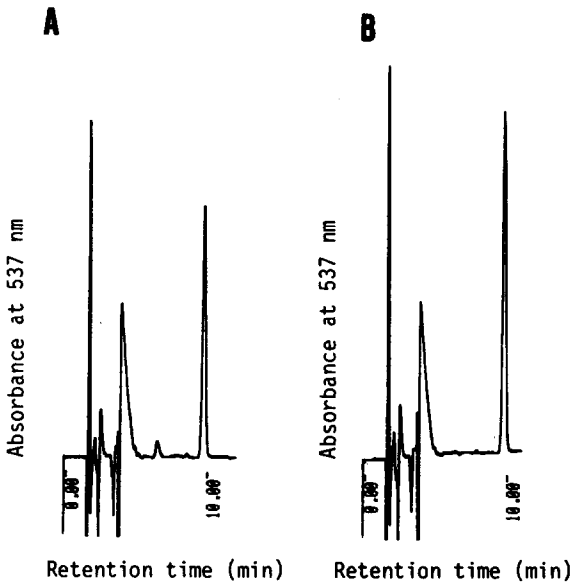

Fig. 1. Chromatogram of reaction products of DPTBA with (A) patient's serum (25 μl) and (B) MDA (0.3 nmol) as a standard.

1.87 ± 1.47, 2.81 ± 1.79, 1.11 ± 0.37 and 1.48 ± 0.52 nmol/ml, respectively, whereas that of the healthy subjects was 1.74 ± 0.69 nmol/ml. The difference between each group of patients and the healthy subjects is not significant. Three patients, two with SLE and one with RA, were gravely ill and required high-dose steroid pulse therapy (1.0 g of methylprednisolone sodium hemisuccinate, intravenous drip). It is significant that the LPO levels of these patients were 2.30–3.87 times those of the healthy subjects (Fig. 3a–c). The serum LPO concentrations of the former were measured one to seven days after infusion of methylprednisolone and were found to drop to nearly the normal range within one to three days of this treatment accompanied by pulse therapy (Fig. 4).

DISCUSSION

Increased serum LPO levels have been noted in acute hepatitis, fulminant hepatitis, active chronic hepatitis [5], diabetes mellitus [2], Down's syndrome [4], myocardial infarction [6] and numerous other diseases, including autoimmune disorders. However, the correlation between LPO levels and autoimmune diseases remains unknown. For instance, no significant difference was observed between the serum LPO levels of osteoarthritis and rheumatoid arthritis patients, although the LPO levels in the synovial fluids of the latter significantly exceeded those of the former [9]. The following reasons may explain the discrepancy: (i) serum LPO concentrations may directly reflect the LPO levels of local lesions, such as synovial and cerebrospinal fluids, tissues or skin, since antioxidant levels in the blood differ from those in other biofluids or tissues; (ii) LPO levels may change, depending on the particular point to which the disease has progressed i.e.

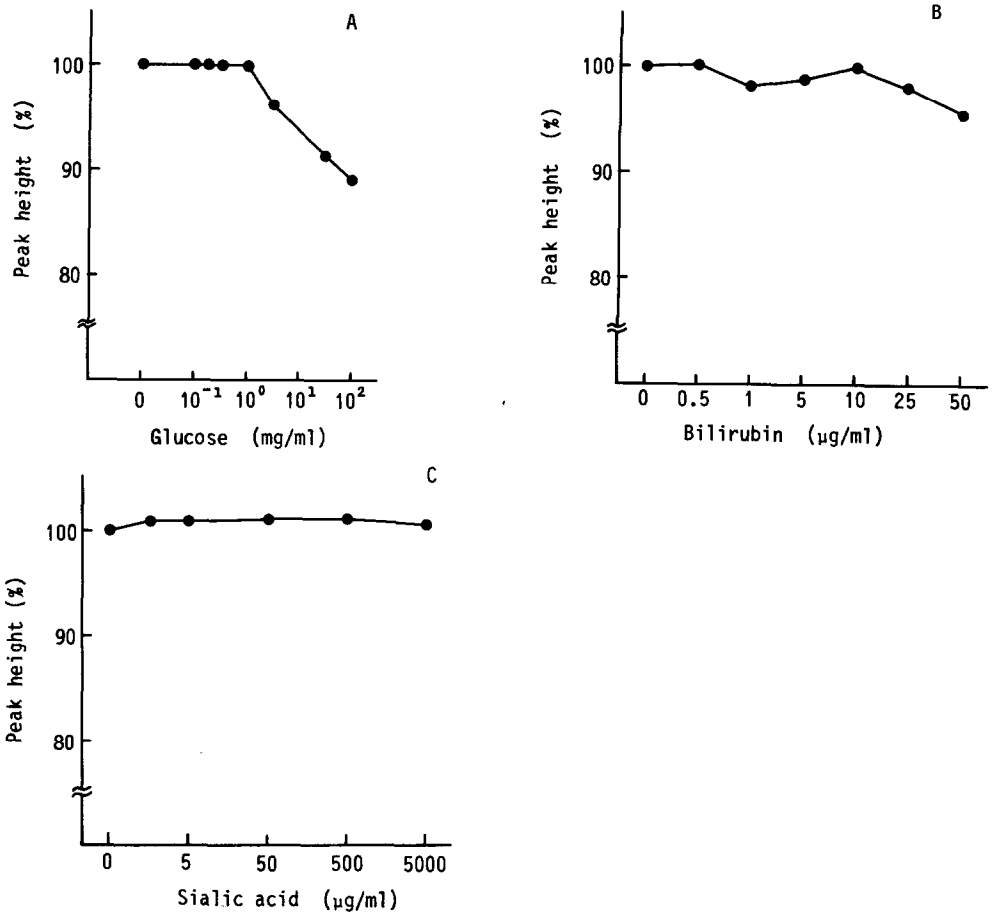

Fig. 2. Effects of (A) glucose, (B) bilirubin and (C) sialic acid on the peak height of MDA-DPTBA complex (100% corresponds to the peak height obtained in the absence of each agent).

they may increase at the time the disease becomes active and vice versa; (iii) generalized methods for the determination of LPO (TBA methods according to Yagi [7]) are hampered by various limitations, such as the influence of bilirubin, sugars, aldehydes and sialic acid. Such factors often result in an overestimate of LPO in human biofluids and tissues. This was avoided in the present study by separation of the MDA-DPTBA complex by HPLC. Indeed, the serum LPO levels of healthy subjects reported here are only half those reported by Suematsu et al. [5], who used the conventional TBA method.

In the present study, significant differences were not observed in the serum LPO levels between collagen disease patients and healthy subjects. However, it should be noted that extraordinarily high values were obtained in two patients with SLE and one patient with RA, both gravely ill and requiring high-dose glucocorticoid therapy. The possibility that a diminished clearance rate may increase serum LPO levels is avoided by the fact that the serum LPO levels of these patients

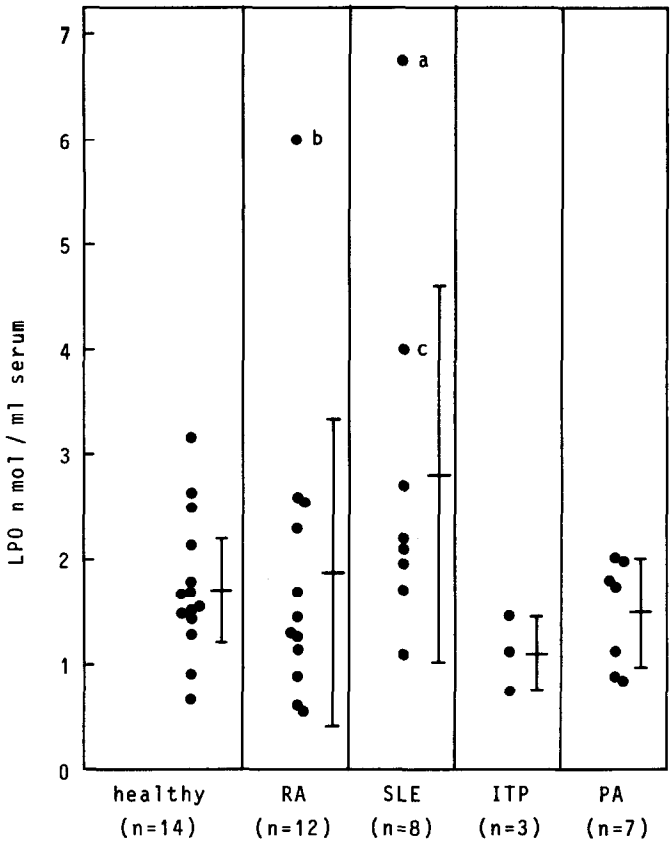

Fig. 3. LPO levels in the serum of fourteen healthy subjects and thirty patients with collagen diseases and related autoimmune disorders. Patients a and c with SLE and patient b with RA were subsequently treated with high-dose glucocorticoid therapy. Horizontal bars represent the mean ±S.D. values for each group.

increased while serum creatinine levels were unchanged (data not shown). The authors consider the serum LPO levels in these patients to have been normal at the time the disease was dormant, but to have exceeded more than twice the normal range with the onset of the disease. High-dose glucocorticoid pulse treatment to such patients has been carried out empirically, although the mechanism of the action involved remains unclear. The results from recent experiments on animals suggest that high-dose methylprednisolone is directly capable of scavenging toxic oxygen in a model of brain ischemia or trauma [10]. The serum LPO levels of our patients dramatically decreased to nearly the normal range within one to three days following pulse therapy, indicating the possible antioxidant effects of high-dose glucocorticoids.

The assay used in this study is simple, rapid and sensitive, and needs only a small amount of serum (25 μl). Moreover, under the conditions of the assay, glucose, bilirubin and sialic acid do not interfere. Thus, this assay should be applicable to many other metabolic disorders, such as hepatic diseases, diabetes mellitus, cancer, etc., in place of the conventional TBA method.

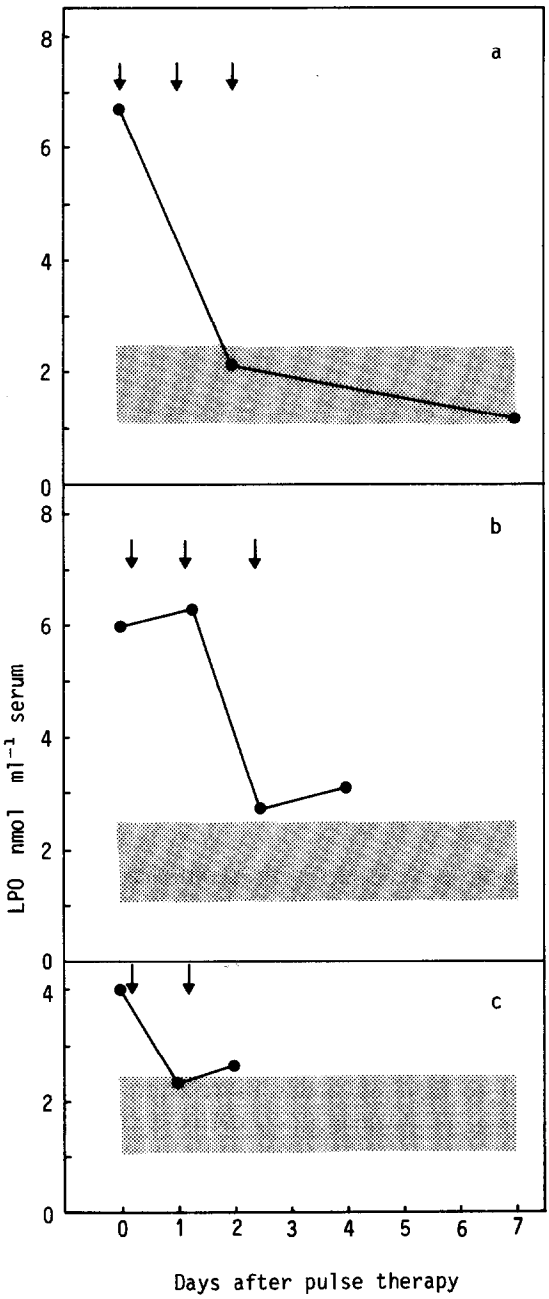

Fig. 4. Effects of high-dose glucocorticoid on serum LPO levels in patients a–c from Fig. 3. Arrows indicate methylprednisolone pulse therapy (1.0 g/day, intravenous drip). Shaded areas indicate normal range.

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