

Endothelial cell cytotoxicity in inflammatory vascular diseases—the possible role of oxidised lipoproteins

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SUMMARY One of the proposed mechanisms of vascular damage in connective tissue disease is the direct action of a cytotoxic serum factor inducing endothelial cell damage. The nature of this serum factor is unclear, but has been suggested to be a lipoprotein. Sera from patients with (1) systemic necrotising arteritis (polyarteritis nodosa, Wegener's granulomatosis, and necrotising arteritis associated with rheumatoid synovitis), (2) systemic or joint restricted rheumatoid disease, and (3) large vessel/giant cell arteritis have been examined for cytotoxicity to human cultured endothelial cells and azide-resistant ferroxidase-like activity (indicative of the oxidised lipoprotein content). *Stored* sera from patients with necrotising arteritis showed a significantly enhanced tendency to develop oxidised lipoprotein, which correlated closely with human endothelial cell cytotoxicity. *Fresh sera* also contained this factor, but to a lesser extent. It is argued that the cytotoxic factor detected in previous clinical studies is in part an in-vitro artefact, although its accelerated development in certain patient groups might suggest an excess of pro-oxidants that have developed in vivo.

Stored and frozen sera from patients with scleroderma and other connective tissue disorders including rheumatoid arthritis are reported to be occasionally cytotoxic to cultured human endothelial cells.¹⁻⁴ It has therefore been suggested that a factor in serum has a pathogenic role in the vascular and microvascular complications of these diseases. The observation that patients with giant cell arteritis also have a serum cytotoxic factor which falls with steroid therapy would support this view.³

The nature of the serum factor has not been defined, nor is it established whether it is also present in fresh sera. Kahaleh and LeRoy⁵ have recently published evidence that endothelial cell cytotoxicity is mediated in vitro via a protease mechanism associated with a functional deficiency of protease inhibitors in scleroderma sera that develops in vivo. However, Cohen *et al.*⁴ demonstrated that the factor, which was contained within the protein fraction and was heat-stable, did not have esterolytic activity and suggested that it may be a lipoprotein. As low density lipoprotein (LDL) is known to be cytotoxic to human endothelial cells,^{6,7} this hypothesis is attractive.

LDL contains a large proportion of unsaturated lipid and is unique among the serum lipoproteins in its susceptibility to autoxidation with storage, to give peroxides.^{8,9} Human endothelial cell and fibroblast cytotoxicity can be inhibited by preparing LDL in the presence of antioxidants and promoted by increasing their content of lipid peroxides.⁹⁻¹² The direct measurement of peroxidised LDL is difficult. However, it shares with the copper-containing protein caeruloplasmin the ability to oxidise ferrous iron. This, however, is a stoichiometric rather than enzymatic reaction.¹³⁻¹⁵ Such activity can be measured directly within the serum and distinguished from that of caeruloplasmin by its resistance to the enzyme inhibitor azide.¹⁶

In this paper we report an association between azide resistant ferroxidase-like activity (ARFLA) and human endothelial cell cytotoxicity in stored sera. We also demonstrate that the progressive accumulation of ARFLA that occurs in stored sera is greater in patients with necrotising arteritis than in those with rheumatoid synovitis with or without extra-articular disease, or polymyalgia rheumatica/giant cell arteritis. We suggest that the ARFLA measured in these sera is due to the presence of oxidised lipoprotein and speculate that differences between patient groups reflect the concentrations of

circulating pro-oxidants in vivo. The observed functional defect in protease inhibitors⁵ may thus be explained by oxidative damage in vivo and by lipid peroxide damage in vitro.

Materials and methods

PATIENTS

The sera from 43 patients were examined for azide-inhibitable and azide-resistant ferroxidase activity (see below). Blood samples were collected into plastic containers to avoid contamination with iron from glass (promotes lipid peroxidation) and without exposure to anticoagulants. Sera, when aliquoted into plastic containers, were frozen at -20°C immediately and stored under identical storage conditions until use. All samples were stored for between 24–30 months. Patient sera were divided into three groups. Group A consisted of 12 patients with systemic necrotising arteritis (polyarteritis nodosa (six), Wegener's granulomatosis (three), and necrotising arteritis associated with rheumatoid synovitis (three)). Group B included 25 patients with definite rheumatoid arthritis (American Rheumatism Association criteria). These patients had either uncomplicated synovitis or rheumatoid disease with active extra-articular disease, but not necrotising arteritis. Group C consisted of six patients with large vessel/giant cell arteritis (Takayasu's arteritis (three), giant cell arteritis/polymyalgia rheumatica (three)).

FERROXIDASE ASSAY

Ferroxidase activity was determined by the method of Johnson *et al.*¹⁷ This method quantitates the catalysed oxidation of a ferrous salt to the ferric state. The resulting ferric salt binds to apotransferrin, resulting in a complex with an absorbance at 460 nm. Changes in absorbance were measured by a Pye Unicam SP8000 UV recording spectrophotometer coupled to a Unicam AR25 linear pen recorder.

Apotransferrin (human, substantially iron-free) and ferrous ammonium sulphate were obtained from Sigma Chemical Co., Poole, UK. All solutions were made up in Chelex-100 treated distilled water to remove contaminating metal ions, while glassware was washed in diluted nitric acid followed by Chelex-treated water. Solutions of ferrous salt were kept anaerobic by bubbling with nitrogen before use and by then keeping the vessel on ice and firmly stoppered.

FERROXIDASE-LIKE ACTIVITY OF PEROXIDISED LDL

This non-enzymic activity can be distinguished from the ferroxidase activity of caeruloplasmin by its

resistance to 1mM azide.¹⁶ For the purpose of comparison the units of this second ferroxidase-like activity have also been expressed as mg/l of caeruloplasmin. To confirm that LDL has iron (II) oxidising capacity, immunoelectrophoresis and staining was performed on sera possessing ARFLA, according to the method of Rabinovitz *et al.*¹⁸ against monospecific sheep antihuman LDL antiserum (gift from Professor K. W. Walton, Department of Investigative Pathology, University of Birmingham). After immunoelectrophoresis the slide was dried and immersed in a solution of ferrous sulphate for 15–30 minutes. After washing and drying, Perls's reagent was used to detect the presence of the ferric salt. To confirm that the ARFLA of oxidised LDL was non-enzymic, phospholipase C treatment was performed on sera possessing ARFLA, according to the method of Sung and Topham.¹⁹ Reaction mixtures were incubated for 2 hours at 37°C . Each contained 0.25 ml of serum and 0.75 ml of 0.1 M potassium buffer, pH 7.4, (containing 4 mM CaCl_2) and 25 $\mu\text{g/ml}$ phospholipase C (type 1; Sigma), omitting phospholipase C in controls. The ARFLA of 25 μl of each mixture was determined.

CELL CULTURE

Human endothelial cells (EC) were cultured essentially by the method of Jaffe *et al.*²⁰ Briefly, human umbilical cords were washed with 0.1 M phosphate buffered saline (PBS), pH 7.2, and the umbilical vein was cannulated and washed through with PBS. Collagenase (2 mg/ml in PBS, Sigma) was passed in and the cord incubated at 37°C for 15 minutes. Endothelial cells were collected by flushing through with PBS and pelleted. They were then resuspended in 5 ml medium 199 tissue culture medium (Flow, Irvin, UK), containing 10% fetal calf serum (Gibco, Uxbridge, UK,) or pooled human AB serum, penicillin, streptomycin, and 25 mM HEPES buffer per cord, and cultured in a 25 cm^3 flask. The flask was incubated in an atmosphere of 5% CO_2 /95% air at 37°C . The next day the cells were washed once with PBS, and fresh medium replaced. EC were confirmed by morphological appearance and factor VIII staining by indirect immunofluorescence (>90% positive).

CYTOTOXICITY ASSAY

Serum cytotoxicity measurements were performed by a modification of the method described by Hawker and Hawker.²¹ EC were harvested by digestion with 5 mg/l trypsin or dispase (Gibco) for five minutes, followed by collection and pelleting. Excess tryptic activity was suppressed by addition of 2 ml of fetal calf serum. The pellet was resuspended in 1 ml minimum essential medium (MEM) (Flow),

50–100 μ l of 111 indium (50–100 μ Ci) (Amersham, UK) added and incubated for two minutes. This was followed by two washes of 10 ml MEM. Cells were resuspended at 1×10^6 /ml in MEM and 100 μ l dispensed to each well of a Nunc 96 well microtitre plate (Gibco). A further 100 μ l of serum (test) or medium (control) was added, and the plate incubated for three hours at 37°C. After incubation 100 μ l Triton X-100 (BDH, Poole, UK) was added to selected wells to establish maximal release, and the plate was centrifuged for five minutes at 100 g. A 100 μ l aliquot was taken for counting on a single-well type gamma counter for 10 seconds. The cytotoxicity index (CI) was calculated according to the following method:

$$CI = \frac{\text{Serum (test) release} - \text{control (medium) release}}{\text{Maximal release (Triton X-100)}} \times 100\%$$

The cytotoxicity index was defined from the mean of 2–3 separate estimations, dependent on the amount of available serum.

Results

Immunoelectrophoresis of sera containing significant amounts of azide-resistant ferroxidase-like activity (ARFLA) against monospecific antihuman LDL antiserum gave a precipitin band which stained strongly with Perl's reagent after pretreatment with a ferrous salt solution. These sera also showed no loss of activity on incubation with phospholipase C, indicating a non-enzymic ferrous iron oxidising activity of LDL.¹⁹

In fresh sera, from both the clinical groups and normal controls, ARFLA accounted for 0 to 10% of the total ferroxidase activity in the serum. There were no significant differences in this activity between the groups. In terms of caeruloplasmin 'equivalents' (see 'Patients and methods') six normal fresh sera contained 10 ± 3 (ISD) mg/l. In normal sera (six samples stored at -20°C for 2–4 years) the mean ARFLA had increased to 280 ± 68 mg/l, or roughly 30 times; 6–9 months' storage produced no significant change, though the mean level of ARFLA in this group was raised (Fig. 1). The CI of normal sera also increased with time of storage at -20°C : 2–4 years' storage of normal sera resulted in a mean increase in cytotoxicity which was $2\frac{1}{2}$ times that of fresh normal sera. However, even after this period of storage it was still significantly lower ($p < 0.001$) than in the group A sera stored for the shorter period (24–30 months).

ARFLA was found in most of the clinical samples that had been stored at -20°C for similar periods of time (see 'Patients and methods'), and values are

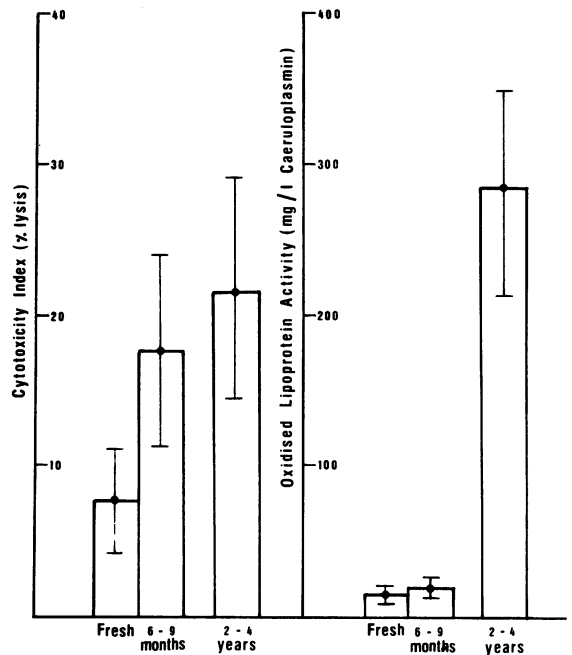


Fig. 1 Comparisons between oxidised lipoprotein activity (ARFLA) and cytotoxicity index for fresh normal sera and normal sera stored for 6–9 months and 2–4 years. The results are represented as the mean of each group ($n=6$) \pm 1 SD.

depicted according to clinical groups (A–C) in Fig. 2. Group A, necrotising arteritis patients, had significantly increased ARFLA (\pm 1 SEM) compared with patients with rheumatoid arthritis – Group B (641 ± 52 mg/l; 230 ± 32 mg/l; $p < 0.001$) despite identical storage conditions.

The data from patients with large vessel vasculitis in group C are clearly not normally distributed, but with the exception of one value, are represented among the lowest recorded ARFLA values for the whole group. The patient with the atypical (high) value had no other obvious distinguishing clinical or laboratory features.

To 'mimic' the effect of prolonged storage fresh serum from one normal control was incubated at 37°C along with fresh sera from one patient with a necrotising arteritis and one with rheumatoid synovitis. ARFLA was measured after four days' incubation. The normal control value increased by $> 100\%$ (12.6 mg/l– 30.0 mg/l). The rheumatoid sample showed no significant difference from control (14.4 – 20.8 mg/l). The necrotising arteritis serum had

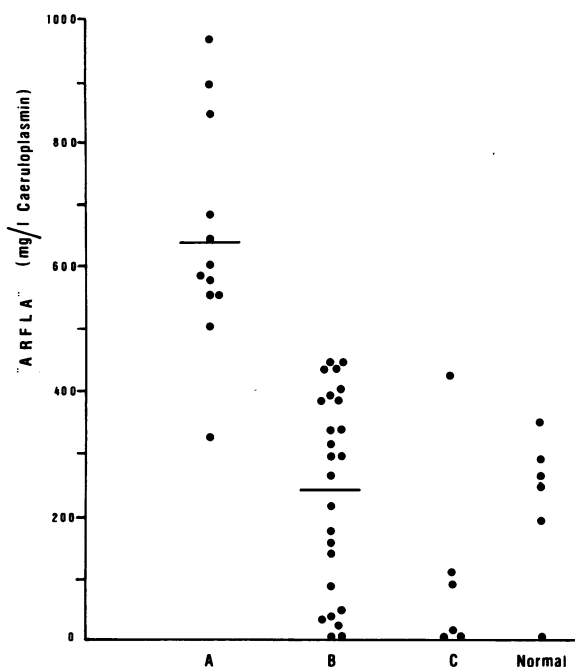


Fig. 2 Differences in azide-resistant ferroxidase-like activity (ARFLA) between disease groups in sera stored at -20°C for similar periods of time. Group A: systemic necrotising arteritis (polyarteritis nodosa, Wegener's granulomatosis). Group B: rheumatoid arthritis (either uncomplicated synovitis or with active extra-articular disease, but not necrotising arteritis). Group C: large vessel/giant cell arteritis (Takayasu's arteritis, giant cell arteritis/polymyalgia rheumatica). ARFLA is expressed as the concentration of caeruloplasmin giving equivalent ferroxidase activity. Sera from groups A to C were stored for 24–30 months. Values for ARFLA measured in normal sera stored for 2–4 years are shown for comparison. Values are the mean of duplicate or triplicate analyses. Horizontal bars indicate mean values for each group.

increased ARFLA initially (28.6 mg/l), which increased 500% on incubation to 180 mg/l. No patient or control was receiving drug therapy in any form.

Sufficient stored sera for repeated cytotoxicity measurements were available for 15 of the clinical samples and six normal controls. Control samples had been processed in an identical fashion to test sera (see 'Methods'). A direct correlation between the cytotoxicity index and ARFLA was found and is displayed in Fig. 3. The value of Spearman's rank correlation coefficient, r_s , is 0.8, though the relationship is logarithmic and is displayed as the log 10 of ARFLA against cytotoxicity. The correlation

without ranking, r , for the points in the logarithmic plot represented is 0.84 ($p < 0.001$). The data are displayed in terms of the patients' diagnostic groups. Patients from group A with necrotising arteritis have the highest values for both ARFLA and cytotoxicity, though distinctions are not absolute. Age of sample (24–30 months) had no relation to eventual cytotoxicity.

Discussion

We have observed that stored sera from patients with connective tissue disease have a variable cytotoxic effect on human endothelial cells in culture. This serum-induced cytotoxicity is enhanced in patients with necrotising arteritis when compared with sera from patients with either rheumatoid disease or polymyalgia/giant cell arteritis. This phenomenon does not relate to the intensity of the acute phase response to inflammation, which is most pronounced within the polymyalgia subgroup, nor is it found in fresh sera with these relatively insensitive techniques.

We would argue that the cytotoxic factor that develops in sera only after storage is related to increased predisposition to oxidation that occurs in vivo. Lipid conjugated dienes have been described in fresh rheumatoid sera,²² and Cawood *et al.*²³ have suggested that this lipid is a 'preperoxide.' This slight molecular rearrangement is derived from the initial autoxidation of polyunsaturated fatty acids, the reaction step prior to the addition of molecular oxygen.²⁴ Polyunsaturated fatty acids are extremely sensitive to free radical attack, because they contain an allylic hydrogen which is susceptible to abstraction from the molecule by radical species, such as the hydroxyl radical. This reaction also generates a radical product, setting up a chain reaction in the presence of O_2 . Under conditions of insufficient O_2 tension molecular O_2 will not react with the first radical product and a preperoxide will be formed by a radical termination reaction.²⁵ Such preperoxides are considerably more likely to peroxidise in vitro than the original polyunsaturated fatty acid, and in the presence of O_2 in vitro will generate a hydroperoxide.²⁵ Frieden *et al.*^{13, 14} have suggested that lipoprotein hydroperoxides are generated on storage and have ferroxidase-like activity. We have demonstrated that such ferroxidase-like activity is associated with serum-induced cell cytotoxicity. The rate of formation of ARFLA on storage is characteristic of an autoxidative reaction. However, although ARFLA levels were correlated with a corresponding increase in cytotoxicity, there was a contrastingly disproportionate increase of cytotoxicity with time of storage. This may relate to the insensitivity of the

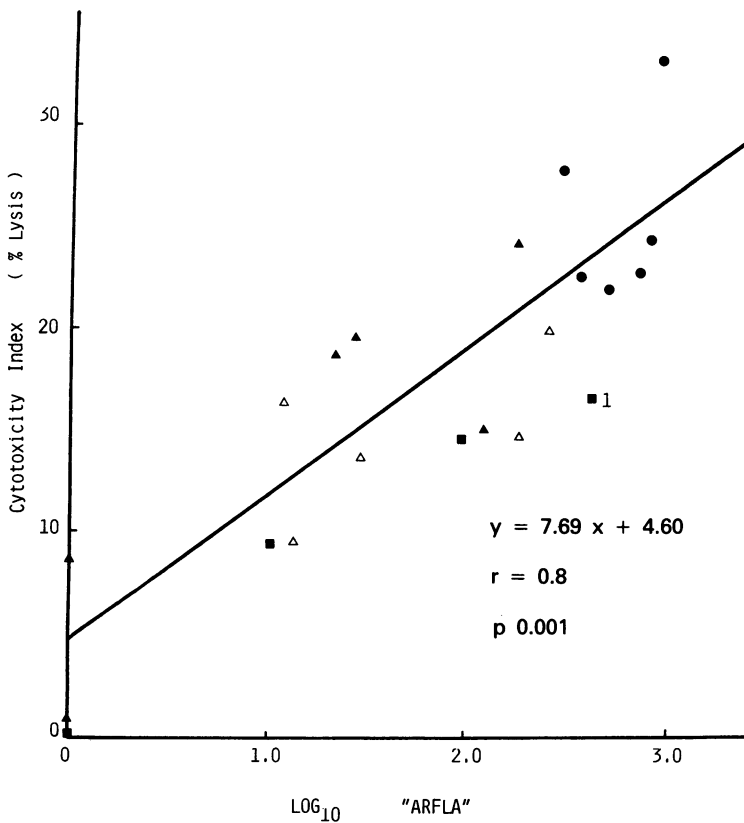


Fig. 3 Correlation between cytotoxicity index (CI) and log₁₀ ARFLA (azide-resistant ferroxidase-like activity) measured in 15 patients and six normal sera stored at -20°C. CI and ARFLA were measured in duplicate or triplicate for each point. ARFLA was expressed as mg/l caeruloplasmin (see 'Patients and methods') before logging. Group A. ●. Group B, ▲. Group C, ■. Normal. △. 1: This point corresponds to the group C patient mentioned in the text (see 'Results') with an unusually high serum ARFLA compared with the rest of the group.

cytotoxicity assay, though it is reasonable to conclude that free radicals and lipid peroxides are responsible for endothelial cell damage indirectly.

It has recently been suggested that endothelial cell cytotoxicity is mediated via a proteolytic mechanism involving a depression of antiprotease activities in scleroderma sera.⁵ Such a concept is compatible with our data, as in scleroderma the defect in the antiprotease system is not a depression of antigenic levels of α_2 -macroglobulin and α_1 -antitrypsin, but a functional deficiency of trypsin inhibitory capacity. One of the mechanisms for functional defects in serum protease inhibitors is by direct damage from reactive oxygen species generated by phagocytosing neutrophils.²⁶ Active oxygen products from normal stimulated neutrophils can also damage vascular endothelium directly *in vitro*.²⁷ This cytotoxic effect appears to be predominantly mediated via hydrogen peroxide.²⁸ Since hydrogen peroxide in the presence of traces of metal ions can also cause the oxidation of lipids, whether they are attached to lipoproteins or not, endothelial cells can be damaged either

directly by inorganic oxygen radicals or indirectly through lipid peroxidation, both as a result of neutrophil activation. In addition it has recently been reported²⁹ that free radicals generated by human macrophages or neutrophils can oxidise LDL *in vitro* and render it toxic to fibroblasts. Human umbilical vein endothelial cells and bovine aortic smooth muscle cells but not human fibroblasts or bovine aortic endothelial cells also oxidised LDL and rendered it toxic to fibroblasts.²⁹ Neutrophils may also be stimulated to produce further free radical products directly by oxidised LDL (J. Lunec, unpublished observations), providing the potential for a continuous feed-back system.

Our data suggest that patients with necrotising arteritis develop this vasculitic complication in a fashion that is distinct from patients with large vessel/giant cell arteritis. A neutrophil-mediated free radical reaction would appear to be a likely basis for the underlying pathology, uniting the mechanistic concepts that have been reported to date^{5 29 30} (Fig. 4). This concept is further supported

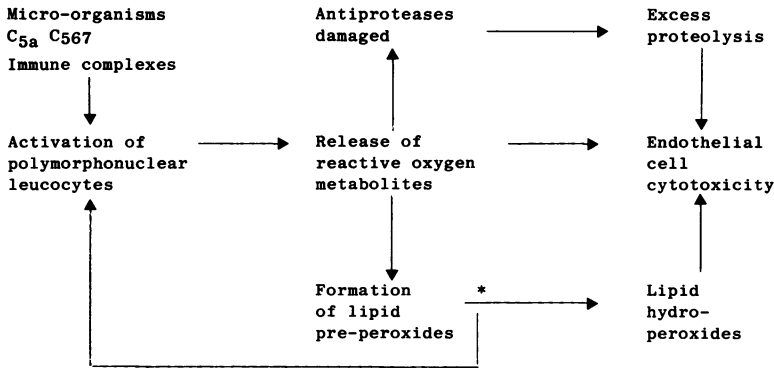


Fig. 4 Possible mechanisms of endothelial cell damage by reactive oxygen metabolites. *Enhanced by storage.

by simple histological examination, which shows that polymorphonuclear leucocytes are the dominant cells infiltrating blood vessel walls in patients with necrotising arteritis but not in patients with large vessel/giant cell arteritis.³¹

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