Increased serum concentrations of conjugated diens and malondialdehyde in patients with pulmonary tuberculosis

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During pulmonary inflammation increased amounts of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are produced as a consequence of phagocyte respiratory burst. One of the manifestations of these free radical-mediated processes is lipid peroxidation (LP).

The aim of our study was to assess the concentration of lipid peroxidation products (LPPs), conjugated diens (CD) and malondialdehyde (MDA), in patients with active TB.

Forty-two patients were enrolled into the study. Half (group I) had advanced TB and were sputum smear-positive. The remainder (group II) had only small radiographical changes and were sputum smear-negative. Serum concentrations of CD and MDA were measured at days 0, 7, 14 and 28 in group I and day 0 in group II. We found that in all patients with active TB CD (1.0 ± 0.05 A₉₅₀) and MDA (2.01 ± 0.16 nmol dl⁻¹) were significantly elevated compared to healthy controls (0.67 ± 0.03 A₉₅₀ and 1.36 ± 0.08 nmol dl⁻¹, respectively) (P<0.001). The highest levels of LPPs were in patients with advanced TB. These concentrations were stable during the first month of anti-tuberculous therapy.

Our data indicated that, as in bacterial pneumonia, LPPs were enhanced in active TB. The levels of LPPs depended on the form of the disease as they were higher in subjects with advanced disease than in those with only small radiographical changes. Further studies are needed to assess the role of antioxidants as adjuvant therapy in patients with pulmonary TB.

Introduction

About one-third of world's population is thought to be infected with Mycobacterium tuberculosis (1). Mycobacteria are intracellular pathogens which grow and replicate in the host macrophages. After phagocytosis survival of mycobacteria depends on their ability to avoid destruction by macrophages. It is well known that phagocytes, e.g. neutrophils, macrophages and monocytes, undergo respiratory burst after contact with microorganism. These cells possess the capacity to generate huge amounts of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), which are essential for the destruction of ingested microorganisms but also contribute to inflammatory injury of host tissue.

Increased amounts of ROS and RNI are produced as a consequence of phagocyte respiratory burst and serve as markers of the free-radical-mediated processes. These reactive oxygen and nitrogen species induce lipid peroxidation (LP), a chain process which affects unsaturated fatty acids mainly localized in cell membranes, in which such products as conjugated diens (CD) and malondialdehyde (MDA) are generated (2). Lipid peroxidation products (LPPs) diffuse from the site of inflammation and can be measured in the blood.

It was well established in an animal model as well as in patients that serum concentrations of LPPs were increased in pulmonary inflammation (3-5). In a previous study we have demonstrated that in subjects with pneumonia concentrations of lipid peroxides and malondialdehyde were significantly enhanced and they correlated with other markers of inflammation like erythrocyte sedimentation rate (ESR) and white blood cell count (WBC) (3). In mycobacterial infection the interactions between macrophages and lymphocytes mediated by the cytokines seem to be the most important in host defence (6,7). Chan et al. (8) have shown in a murine model that inhibition of Mycobacterium tuberculosis growth is independent of the generation of ROS. On the other hand, MacMicking et al. (9) have recently demonstrated that among mice with genetic inability to produce inducible nitric oxide synthase (iNOS) replication of Mycobacterium tuberculosis is faster than in wild-type animals. Taking these data into consideration it can not be excluded that enhanced generation of ROS may
 occur in subjects with active TB, but this process may have a limited, if any, role in the defence against Mycobacterium tuberculosis. Therefore in our study we decided to investigate whether: (1) LP would enhance during active pulmonary TB; (2) LP would depend on the form of TB: advanced versus small radiographical changes; and (3) if 1 month of anti-tuberculous treatment would change the intensity of lipid peroxidation.

Methods

PATIENTS

Forty-two patients with pulmonary tuberculosis treated in Department of Pneumology and Allergology in Łódź were enrolled into the study. Group I consisted of 21 patients (14 men and seven women; mean age 50 ± 2 years; 15 smokers). All of them had: (1) clinical manifestations of TB (temperature, cough, sweat, weight loss, fatigue); (2) advanced radiographical changes (at least three zones on X-ray); (3) positive sputum smear. Group II consisted of 21 patients (eight men and 13 women, mean age 43.3 ± 3 years; 12 smokers) with: (1) small radiographic changes (one zone on X-ray); (2) usually no symptoms; (3) negative sputum smear. All patients from the group II revealed radiographical regression during anti-tuberculous treatment, but only nine of them had bacteriological confirmation either by culture on Lowenstein–Jensen medium, radiometric system of Bactec 460 or Ligase Chain Reaction (LCx-Abbott) diagnostic methods. The patients had been treated with standard chemotheraphy: isoniazid (INH 5 mg kg⁻¹ day⁻¹), pyrazinamide (PZA 25 mg kg⁻¹ day⁻¹), rifampin (RFP 10 mg kg⁻¹ day⁻¹, no more than 0.6 g day⁻¹) daily for 2 months and then INH (15 mg kg⁻¹) plus RFP (0.6 g) twice a week for the next 4 months. Patients from the group I also received streptomycin (SM 15 mg kg⁻¹ day⁻¹, no more than 1 g day⁻¹) for the first 2 months of treatment. During the study patients did not receive any known antioxidants such as N-acetylcysteine, ambroxol, vitamin C or E (10–13). In group I, on days 0 (pre-treatment), 7, 14 and 28 blood samples were taken for the assessment of CD and MDA. In the group II blood samples were taken only on day 0, because of low pre-treatment values and lack of changes in LPPs concentration in preliminary studies performed in this group.

On days 0 and 28, ESR and WBC were also performed. Body temperature was measured at 06:00 h and 16:00 h each day and expressed as a mean value. An X-ray was taken before introducing anti-tuberculous drugs, after 1 month of treatment and at the end of therapy. Each X-ray was analysed independently by two physicians.

As a control we used 10 healthy volunteers (six men and four women; mean age 41 ± 2 years; five smokers) without any changes on X-ray. They were not currently taking medication. It was shown that LPP concentrations were stable in healthy people over the few weeks of observation (3).

All patients gave informed consent and the study was approved by the Local Ethics Committee.

LIPID PEROXIDATION PRODUCTS IN TB

Determination of CD and MDA

Conjugated dienes were measured according to the method described previously (14). Briefly, 0.5 ml of serum were mixed with 7 ml of a chloroform (Ubichem Ltd, Eastleigh, U.K.)/methanol (POCh, Gliwice, Poland) mixture (1:2 v/v), shaken for 2 min and centrifuged (1500 g for 5 min). A 5 ml aliquot of the lower layer was mixed with 2 ml of distilled water acidified with HCl to pH 2.5. The solution was shaken again for 2 min and centrifuged as above. The chloroform layer was aspirated and dried under a flow of nitrogen. The residues were diluted with 2 ml of heptane and its absorbance was read against a heptane blank at 233 nm using an Ultraspec III (Pharmacia LKB, Pharmacia Biotech Ltd, Cambridge, U.K.). Results were expressed in absorbance units.

Malondialdehyde concentration was estimated according to the method of Yagi with some modifications (3,15). Briefly, 0.25 ml of serum were mixed with 2.5 ml of a 0.069 m solution of phosphotungstic acid (pH 1.3–1.5) (Merck, Darmstadt, Germany). The sample was then centrifuged (4°C, 3000 g) for 15 min and 0.5 ml of a 0.055 m solution of TBA (thiobarbituric acid, Sigma Chemical Co, St. Louis, U.S.A.) and 1 ml of distilled water were added to the residue. After vigorous shaking the sample was boiled for 60 min and again centrifuged at 6000 g for 10 min. TBA-reactive substances were extracted with 2 ml of N-butanol and measured spectrofluorometrically (excitation 515 nm, emission 555 nm) on a Perkin Elmer (Beaconsfield, U.K.) Luminescence Spectrometer (LS-50B). Tetramethoxypropane (0.1–10 nmol dl⁻¹) (Sigma, St. Louis, MO, U.S.A.) was used as an external standard and the concentrations of TBA-reactive substances were expressed in nmol dl⁻¹ (16).

Statistical analysis

Results were expressed as mean ± SEM. For non-paired samples the Student’s t-test and Kolmogorov–Smirnov test were used depending on sample distribution. Correlations were estimated by Pearson’s and Spearman’s tests, respectively.

Results

LPPs in the whole group of patients (with advanced and small radiographical changes) were much higher than in healthy controls. C_CD was 1.0 ± 0.05 A_233 as compared to 0.67 ± 0.03 A_233 (P < 0.001) and C_MDA was 2.01 ± 0.16 nmol dl⁻¹ in TB patients and 1.36 ± 0.08 nmol dl⁻¹ in the control group (P < 0.001) (Fig. 1).

Patients from group I had the highest levels of LPPs. The concentration of CD before treatment reached 1.14 ± 0.09 A_233 and was significantly higher than in patients in group II (P < 0.01) and the control group (P < 0.05) (Fig. 2). The levels of CD did not change significantly during the first month of anti-tuberculous therapy: day 0, 1.14 ± 0.09 A_233; day 7, 1.22 ± 0.07 A_233; day 14, 1.2 ± 0.07 A_233; day 28, 1.15 ± 0.07 A_233 (Fig. 3).
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**2.01f 0.16 1**

**J 1.0 f0.05 n 0.67 f 0.03**

**A B C D**

**FIG. 1.** Concentration of CD and MDA in all patients with TB and healthy controls. A, CD in healthy controls; B, CD in patients with TB; C, MDA in healthy controls; D, MDA in patients with TB.

*P<0.001 vs. healthy controls.

**2.00**

**$1.00**

**0.00**

**FIG. 2.** The serum concentration of CD in healthy controls, subjects with small radiographic changes (group II) and patients with advanced TB (group I).

*P<0.05 vs. small radiographical changes; **P<0.01 vs. advanced TB; ***P<0.05 vs. healthy controls.

**FIG. 3.** No significant changes of serum concentration of CD (●) and MDA (■) in patients with advanced TB during the first month of anti-tuberculous therapy.

**FIG. 4.** The serum concentration of MDA in healthy controls, patients with small radiographic changes (group II) and patients with advanced TB (group I).

*N.S. vs. small radiographical changes; **P<0.001 vs. advanced TB; ***P<0.05 vs. healthy controls.

Apart from day 0 (r=0.37; Spearman's test) there was a correlation between C\textsubscript{CD} and C\textsubscript{MDA} in patients from group I during the first month of therapy: r=0.52 (P<0.05; Pearson's test) on day 7; r=0.45 (P<0.05; Spearman's test) on day 14; and r=0.52 (P<0.05; Pearson's test) on day 28.

The positive correlation between C\textsubscript{CD} and body temperature (r=0.43; P<0.05; Spearman's test) was only found in patients with advanced TB before treatment. After 4 weeks of anti-tuberculous therapy all patients from group I were still smear-positive and only seven of them (33.3%) revealed small radiographical regression. There was no correlation between LPPs and body temperature, WBC or ESR after 1 month of treatment.

Mean ESR, WBC and temperature in patients from groups I and II are shown in Table 1.

Patients in group II exhibited lower concentrations of LPPs. The level of CD was \(0.85 \pm 0.05\) A\textsubscript{233} and was significantly higher than in healthy controls C\textsubscript{CD}=0.67 \pm 0.03 A\textsubscript{233} (P<0.05), but C\textsubscript{MDA} was \(1.40 \pm 0.16\) nmol dl\(^{-1}\) and did not differ from the control group C\textsubscript{MDA}=1.36 \pm 0.08 nmol dl\(^{-1}\) (P>0.05). After 1 month of treatment their X-ray pictures were stable except in four cases with slight regression.

There was no correlation between LPPs and ESR, WBC or body temperature in patients from the group II before treatment and after 1 month of therapy (P>0.05).

At the end of the treatment (after 6 months) patients from the group I had marked fibrotic changes on their X-ray (narrowed affected zones, shifted mediastinum, elevation of diaphragm and hilum, many linear shadows). Patients from the group II had only few linear shadows on their X-ray.

**Discussion**

In our study we have demonstrated that LPPs were elevated in patients with active pulmonary TB, which provides the evidence for enhanced free-radical-mediated processes. Moreover, the high concentrations of CD and MDA corresponded with more advanced disease. Among patients...
with marked clinical manifestations of TB, large radiographical changes and positive sputum smears, concentrations of CD and MDA were significantly higher than in patients with small changes on X-ray and negative sputum smears. Jack et al. (17), studying 17 selected patients with TB, found out that CD concentrations were significantly higher only in cases with active process, while thiobarbituric acid reactive substances (TBARS) were elevated both in patients with active and inactive disease. It corresponds with our present results showing no difference in MDA concentration between group II and healthy controls. This may be a consequence of the fact that concentration of MDA measured as TBARS is a relatively non-specific method assessing the end products of LP as well as oxidation products of amino acids, nucleic acids, carbohydrates and prostanoid metabolism (2).

There is much evidence that LP during oxidative stress is common in inflammatory processes (18-21). In our previous study we have demonstrated that LPPs were enhanced in serum of patients with pneumonia and gradually declined during recovery (3). Recently Hull et al. (21) found that increased serum LPPs in children with cystic fibrosis were associated with the presence of pulmonary inflammation. This indicates that CD and other LPPs could be used as indicators of oxidative tissue injury independent of the species of pathogenic bacteria. Unlike bacterial pneumonia, in patients with TB elevated levels of MDA and CD did not change significantly during the first month of anti-tuberculous therapy. This is not surprising as regression of TB is a long-lasting process, even up to 18 months after diagnosis (22).

Only in the group of smear-positive patients with advanced TB was there a correlation between the concentration of CD and body temperature ($r=0.43$, $P<0.05$). These results are in agreement with Ward et al.’s study (23) pointing out that CD is a more sensitive indicator of free-radical-mediated processes related to inflammation than MDA. Other investigators found a correlation between $C_{CD}$ and non-specific markers of inflammation such as ESR and the serum level of C-reactive protein (3,21).

It has been shown that LP increased synthesis of TGF-$\beta$ (transforming growth factor $\beta$) (24), which plays a key role in tissue repair and fibrogenesis (25). On one hand it stimulates synthesis of procollagen type I and fibronectin (26), while on the other it downregulates the gene expression of collagenase (27). We suppose that high concentrations of CD and MDA in serum of patients with advanced TB, not only at the onset of the disease but also during treatment, may contribute to fibrosis of the lungs. Indeed, all patients from group I revealed marked fibrotic changes on X-ray at the end of therapy, while patients from the group II exhibited only small changes and the concentrations of CD and MDA in this group were lower than in patients from group I. Such a relationship has been shown in interstitial pulmonary fibrosis caused by bleomycin in an animal model (28). However, our hypothesis on the role of LPPs in fibrogenesis in tuberculosis should be verified in quantitative studies.

During the last few years, new therapeutic possibilities have been explored to control intracellular infections. It was shown that selective iron depletion (desferrioxamine) could prevent the damage associated with ROS and inhibited the intracellular infection of Plasmodium falciparum (29). It might be of particular importance in elderly, in whom oxidative stress during anti-tuberculous therapy is higher than in younger patients and who are at a greater risk of lung damage because of poor antioxidant mechanisms (30). Up to now there have been conflicting data about the role of ROS and RNI in host defense against mycobacteria (31,32). In future investigations, if the beneficial effect of the scavengers of toxic oxygen or nitrogen species on lung injury outweighs their influence on the inhibition of the growth of mycobacteria, antioxidants may be very useful in anti-tuberculous treatment.

In summary, we found that, as in bacterial pneumonia (3), lung inflammation due to Mycobacterium tuberculosis is accompanied by enhanced lipid peroxidation. The serum levels of LPPs were higher in subjects with advanced TB than in those with only small radiographical changes. This suggests the presence of systemic oxidative stress in these patients in response to M. tuberculosis infection.

References


<table>
<thead>
<tr>
<th>Patients</th>
<th>Temperature (°C)</th>
<th>WBC x $10^9$ cells l$^{-1}$</th>
<th>ESR mm h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After 1 month</td>
<td>Before treatment</td>
</tr>
<tr>
<td>Advanced TB</td>
<td>37.7 ± 0.2</td>
<td>36.9 ± 0.1*</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>TB with small radiographical changes</td>
<td>37.1 ± 0.1</td>
<td>36.7 ± 0.1*</td>
<td>7.4 ± 0.4</td>
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Mean body temperature of two measurements at 06:00 h and 16:00 h each day.

WBC: white blood cell count; ESR: erythrocyte sedimentation rate.

* $P<0.1\%$ vs. value before treatment; ** $P<5\%$ vs. value before treatment.


