OXIDATIVE STRESS IN ADULT DENGUE PATIENTS

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Abstract. An association between viral diseases and increased oxidative stress has been suggested. The time course of serum levels of total antioxidant status (TAS), peroxidation potential (PP), glutathione (GSH), lipid peroxidation measured as hydroperoxides, and malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA), as well as antioxidant enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx), were measured in 22 serologically confirmed dengue patients. Most of the patients had dengue fever and three of them had dengue hemorrhagic fever. The redox parameters were compared with those of age- and sex-matched controls. No significant difference was observed for levels of GSH and TAS between patients and controls. Levels of PP, MDA + 4-HDA, and SOD were significantly higher. Levels of GPx and total hydroperoxides were significantly lower in patients in comparison with controls. These findings suggest that the alteration in redox status could result of increased oxidative stress and it may play a role in the pathogenesis of the disease.

INTRODUCTION

The pathogenesis of dengue hemorrhagic fever (DHF) is not well understood. Several epidemiologic studies demonstrate the importance of secondary infection by a different dengue serotype as the main risk factor for this severe disease. It is hypothesized that non-neutralizing cross-reactive antibodies bind to the new virus serotype; however, in conditions where neutralization does not occur, virus-antibody complexes are taken up more readily than uncoated virus particles by cells expressing Fc receptors, and consequently a higher viremia is observed. Although widely accepted, it is not clear how higher viremia levels cause the pathology and symptoms of DHF. A high level of different cytokines produced both by dengue virus-infected monocytes and activated specific T lymphocytes could explain the main manifestation of DHF, plasma leakage. A shift from a Th1-dominant response to a Th2-biased response has been proposed by some as a mechanism of DHF. Recently, Mongkolsapaya and others proposed that profound T cell activation and death by apoptosis may contribute to the systemic disturbances leading to DHF, and that “original antigenic sin” of T cells occurs, suppressing or delaying virus elimination. Despite these new findings, the basic molecular mechanisms leading to DHF are not defined.

During immune activation by viral entities, neutrophils and others cells produce reactive oxygen species (ROS) as a mechanism of signal amplification for protection. Endothelial cell growth, death, and functions are important determinants of vascular homeostasis. Although the role of in promoting endothelial dysfunction and death has been well studied, the role of endogenously generated ROS in endothelial cell survival is relatively unknown. Research on the relationship between ROS and vascular dysfunction originated in a report in 1981 by Granger and others. Additional data were later provided by Irani.

Oxidative stress initiates and regulates the transcription and activation of a large series of other mediators in cells, which culminate in common mechanism of damage: apoptosis, necrosis, inflammation, immune response, ischemia, vascularitis, altered gene expression, and regeneration. The prevalence and the persistence of one or more of these aspects may influence the occurrence of different types of diseases. Oxygen-centered free radicals and others chemical species, often referred to as ROS, are well known as oxidants. The ROS have been recognized as widespread mediators both of cell injury and either intercellular or intracellular signaling processes. They are formed by several physiologic processes, but are currently thought to be involved in the pathogenesis of several disorders, sometimes as causes, sometimes as effects.

Oxidative stress arises when the balance between oxidants and antioxidants is tipped in favor of the former. This phenomenon may be influenced by exogenous agents but also by endogenous ones such as viruses. There is now much evidence that oxidants play a complex role in viral diseases, starting from influences on host cell metabolism and viral replication and extending to desirable inactivating effects on viruses and less desired toxic effects on host tissue. Recently, several approaches to study antioxidant consumption and markers of free radical-induced damage have been described. Oxidative damage may affect all biochemical compounds including lipids, proteins, nucleic acids, carbohydrates, and macromolecules of connective tissue. This process might causes the loss of fluidity, leads to the destruction of cell membrane because of structural deformity and the production of lipo- peroxides and their products, such as malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). Inactivation and removal of these ROS depend on relations involving a wide spectrum of antioxidative defense mechanisms.

The capacity of defense is determined by a dynamic interaction between individual components, which comprises vitamins and metabolites such as glutathione (GSH) and antioxidant enzymes. Among these enzymes, the most important are superoxide dismutase (SOD) and glutathione peroxidase (GPx). Malondialdehyde is the most abundant aldehyde generated by the attack of free radicals on polyunsaturated fatty acids of cell membranes. It should be noted that total hydroperoxides (THs) are not only passive markers of oxidizing stress, but are also cytotoxic products that could modify DNA and proteins.

While alterations of redox status have been observed in
several viral diseases such as acquired immunodeficiency syndrome, hepatitis C, bronchitis, pneumonia (influenza virus), and lymphoma (human T lymphotropic virus type I), they have not been reported during a dengue infection. The aim of this investigation was to study the status of some oxidative stress markers in serologically confirmed adult dengue patients comparing with those observed in healthy individuals.

METHODS

Study sample. The study sample consisted of 22 adults (age range = 21−58 years, 11 females) diagnosed as having a dengue infection during the dengue 3 Cuban epidemic in 2001. Fifteen were clinically classified as having dengue fever (DF), four as having DF with hemorrhagic manifestations (DFHM), and three as DHF (two with grade II and one with grade III) according to the Pan American Health Organization/World Health Organization Guidelines for Control and Prevention of Dengue and Dengue Hemorrhagic Fever in the Americas. Patients were bled at days 3, 5, and 7 after the onset of fever. Dengue infection was confirmed by IgM detection and viral isolation.

Informed consent was obtained from patients after they were given a verbal and written explanation of the objectives and risks of the study. Procedures were previously reviewed and approved by the Committee for Research on Human Subjects of the Pedro Kourí Tropical Medicine Institute. Controls included 22 sex- and age-matched healthy individuals. Oxidative stress indices in controls were used to compare those in dengue patients. Serum samples of both patients and controls were stored at −70°C until analysis.

Total antioxidant status (TAS). For TAS quantitation, a commercial kit (catalog no. NX2332; Randox, Ltd., Crumlin, United Kingdom) was used. Briefly, ABTS (2,2’-azino-di-(3-ethylbenzthiazoline sulfonate) was incubated with metmyoglobin and hydrogen peroxide to produce the radical cation ABTS+•. This has a relatively stable blue-green color that can be measured at 600 nm. Based on their concentration, antioxidants will cause a suppression of the color production.

Peroxidation potential (PP). For the determination of the susceptibility to lipid peroxidation, serum samples were incubated with a solution of cupric sulfate (final concentration = 2 mM) at 37°C for 24 hours. The PP was calculated by subtracting the MDA concentration at time 0 from the one obtained at 24 hours.

Glutathione. Serum reduced GSH was analyzed with the method described by Sedlak and Lindsay. Glutathione (Sigma, St. Louis, MO) was used to generate the standard curves.

Glutathione peroxidase. Evaluation of GPx activity was determined using a commercial kit (catalog no. RS505; Randox, Ltd.). Briefly, GPx catalyzes the oxidation of GSH by cumene hydroperoxide. In the presence of GSH reductase and NADPH, the oxidized GSH is immediately converted to the reduced form with a concomitant oxidation of NADPH to NAPD+*. The decrease in absorbance at 340 nm is measured.

Superoxide dismutase. Evaluation of SOD activity was determined using a commercial kit (catalog no. SD125; Randox, Ltd.). Briefly, this method uses xanthine and xanthine oxidase to generate superoxide radicals, which reacts with 2-(4-iiodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the inhibition of this reaction.

Total hydroperoxides (THs). The THs were measured with a commercial assay (Bioxytech H2O2-560 kit catalog no. 21024; Oxis International, Inc., Portland, OR). The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. Ferric ions bind with the indicator dye xylene orange (3,3’-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresolsulfone-phatein, sodium salt) to form a stable colored complex that can be measured at 560 nm.

Malondialdehyde and 4-hydroxyalkenal concentration. The MDA + 4-HDA concentrations were analyzed with a commercial kit (LPO-586; Calbiochem, La Jolla, CA). In this assay, stable chromophore production after incubation for 40 minutes at 45°C is measured at 586 nm using a Pharmacia (Piscataway, NJ) spectrophotometer. Lipid peroxidation was expressed as the normalized content of MDA + 4-HDA. Values were expressed in nanomoles.

RESULTS

The PP and TAS measure serum antioxidant capacity. The former is a marker of serum susceptibility to lipid peroxidation. The TAS levels at days 3 and 5 (Figure 1A) were lower in patients than in controls and were significantly higher at day 7 (P < 0.05). Conversely, higher levels of PP (Figure 1B) were detected in patients at the three days tested (P < 0.05). Serum GSH levels were not significantly different between patients and controls (Figure 1C). The activities of the antioxidant enzymes GPx and SOD are shown in Figure 1D and E. The GPx activity was significantly (P < 0.05) decreased in dengue patients, while the SOD activity was significantly (P < 0.05) increased. Serum levels of TH were significantly (P < 0.05) decreased in dengue patients (Figure 1F). Lipid peroxidation determined by MDA and 4-HDA serum concentrations was significantly (P < 0.05) higher in the dengue group (Figure 1G).

Figure 2 shows the kinetics of redox markers according to the clinical classification. Sample sizes were not large enough to detect differences between groups (DF, DFHM, and DHF); however, the results were analyzed in descriptive form. The highest level of TAS was detected in DF patients (Figure 2A). This group showed the lowest PP levels (Figure 2B), suggesting that total antioxidant depletion is higher in DHF patients. The GPx activity was also lower in DHF cases (Figure 2C). The SOD activity in serum was higher in DHF and DFHM patients (Figure 2D), suggesting that the generation of the superoxide species is greater. This enzyme produces the oxidant species known as hydroperoxide, which could damage nearby biomolecules. Serum levels of TH and MDA + 4-HDA were lower in DF patients, suggesting that...
lipid peroxidation was higher in DHF cases (Figure 2E and 2F).

**DISCUSSION**

Redox equilibrium is an important factor for the normal function of several cell species, and is involved in different functions such as activation, maturation, and cell signaling and death. Disturbance of this equilibrium contributes to the pathogenesis of a wide array of diseases, including viral diseases. Virus-induced oxidative stress could be mediated by an early phase of liberation of pro-inflammatory cytokines. In some situations, the host inflammatory response appears to be an important contributor to the pathogenesis of the disease.

In this investigation, we demonstrated a redox status alteration in the sera of dengue patients. To our knowledge, this is the first study documenting an increase in lipid peroxidation with a pro-oxidant state, a reduced antioxidant activity of GPx, an increase in SOD activity, and a low antioxidant capacity evaluated as PP during an infection with dengue virus. This finding is consistent with another report of increased oxidant and lowered antioxidative serum capacity associated with viral illness.

The levels of hydrophilic and lipophilic antioxidants were measured in serum samples collected at three different times during the acute phase of the disease. The PP was significantly high in dengue patients compared with controls. In contrast, TAS was similar in both groups except at day 7 after the onset of fever. The higher PP values found in our patients suggest a
decrease in lipid scavenger species or an increase in ROS generation. The dramatic increase of the byproduct of lipid peroxidation (MDA + 4-HDA) is consistent with a previous observation that showed the disruption of the redox balance.  

Antioxidant enzyme levels are sensitive to oxidative stress. Both increased and decreased levels have been reported in different diseases in which an enhancement of ROS is a cause or a consequence of the illness.  

In this condition, the detoxification capacity evaluated as PP is decreased and is probably related to the high levels of lipid peroxidation.

The THs were significantly lower in the patients than in the control group. This chemical species is a stable intermediate that could generate peroxyl and other ROS by interaction with organic metal. Generally, in oxidative stress conditions, this index shows higher values because of accumulation of TH. In this case, the lower values could indicate enhanced peroxidation due to an increase in metal concentration that may produce a lipid byproduct such as MDA and 4-HDA. This result needs further corroboration. The hydrophilic antioxidant GSH and the hydrophilic TAS did not show significant changes in the patients.

Two interesting observations were made. The first was the alteration of the redox markers, which probably started before day 3, suggesting an early compromise of the oxidant/antioxidant balance. The second was the increase or decrease in the levels of the studied markers on day 5. This observation was preceded by defervescence at day four in most of the patients. Conversely, thrombocytopenia and hemoconcentration was observed on days 4 and 5 in DHF and DFHM cases, respectively. Unfortunately, we could not collect a convalescent serum sample to evaluate the redox at recovery. How-
ever, a tendency to normality was observed in some of the indices. The present results also suggest a higher severity of oxidative stress in DHF patients, but due to the small size of the samples, statistical significance could not be determined.

Few redox diagnostic schedules have been followed during acute infections. As a result, comparisons of altered redox equilibrium associated with acute viral or bactericidal infections are not available. However, a recent investigation compared heme oxygenase-1 (HO-1) production by monocytes in vivo in various acute inflammatory illnesses and in healthy controls. Heme oxygenase-1, an inducible heme-degrading enzyme, exerts a potent anti-inflammatory effect through the production of carbon monoxide and bilirubin. Significantly elevated HO-1 mRNA levels seen in acute inflammatory illnesses suggest that monocyte production of HO-1 serves as a potent anti-inflammatory agent in controlling excessive cell or tissue injury in the presence of oxidative stress and cytokine-mia.

Different cells, such as leukocytes and endothelial cells, contribute to ROS production. Specifically, ROS generation has been detected when endothelial cells are stimulated by cytokines, a process reported to occur during dengue infection. The oxidative stress processes resulting from cytoxic lipid products may modify proteins and cell membranes. The ROS can attack polyunsaturated fatty acids and initiate lipid peroxidation, a process that can ultimately lead to a loss of membrane function and in some situations alterations in the integrity of the membrane. It has been suggested that the increased vascular permeability observed in DHF is caused by a malfunction rather than a structural destruction of the endothelial cells. It is possible that also endothelial cells contribute to ROS production during a dengue infection.

The present study suggests the relationship of in vivo oxidative stress, as indicated by high levels of sensitive markers of lipid peroxidation, with the pathogenesis of dengue virus infection. Lipid peroxidation levels and endothelial cell dysfunction could be related and would acutely enhance local or systemic vascular leakage. However, it is necessary to study the kinetic of these markers in a more representative number of DHF serum samples.

Our results will contribute to an integral overview of dengue infection and open a new window for the study of this disease. Further investigations are needed to elucidate the role of redox status in the context of virus replication, T cell activation, and apoptosis.

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