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Pharmacological and biochemical studies on the role of free radicals during stress-induced immunomodulation in rats

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ABSTRACT

The present study was designed to evaluate the role of free radicals in restraint stress (RS)-induced modulation of immune responses in rats. RS significantly suppressed both humoral and cell-mediated immune responses as evidenced by reduced (a) anti-SRBC antibody titre (b) splenic Plaque Forming Cell counts, (c) footpad thickness response, and (d) IFN-γ and IL-4 levels. Assay for oxidative stress markers in blood showed that there was significant enhancement in plasma corticosterone and products of lipid peroxidation, viz. malondialdehyde and lowered reduced glutathione levels on exposure to RS. Further, this was associated with decreased antioxidant enzyme activity, viz. superoxide dismutase and catalase. These RS-induced changes in immunological and oxidative stress markers were markedly attenuated by pretreatment with the antioxidants, L-ascorbic acid (100 and 200 mg/kg) and α-tocopherol (30 and 60 mg/kg), by differential degrees. The combination of L-ascorbic acid and α-tocopherol was shown to have synergistic effects on reversal of these RS-induced effects. The results suggest that reactive oxygen species may be involved in stress-induced immunomodulation.

1. Introduction

A variety of psychological and environmental factors can act as stressful stimuli and disrupt the physiological homeostasis of the organism. Such stressors (external or internal) can alter the dynamic equilibrium of the biological system and the inability to cope with such aversive inputs can precipitate disease states [1,2]. Since Hans Selye's introduction of stress in biology and medicine, the concept of stress has evolved into that of a "stress system" wherein complex interactions between central nervous system, neuroendocrinal system, gastrointestinal and immune system have been proposed, and such interactions play a significant role in the outcome of the stress response [3–7]. Complex neural networks are also known to regulate the activity of stress axis and drugs modulating neurotransmitter/neuromodulators exert differential effects on the various organs and systems [8–12]. Stress, depending on its nature and intensity, can influence the functioning of the immune system and complex interactions between CNS and immune system have been proposed to explain stress-induced immunomodulation. Physical or psychological stressors have also been linked with an increased susceptibility to a number of immune related disorders such as infections and neoplastic diseases. For example, depressed patients have been found to have an increased rate of cancer morbidity and mortality. The involvements of catecholamines and glucocorticoids have been reported to modulate stress-induced immune regulation [13–18].

The role of free radicals in health and diseases is well documented and both reactive oxygen (ROS) and reactive nitrogen (RNS) species, which are extremely reactive molecules, are generated as byproducts of the body's normal metabolic pathways. Free radicals are so destructive that they are now regarded as primary agents of degeneration and death in all living organisms, and are responsible for the initiation and progress of various diseases including cancer, inflammatory and neurodegenerative disorders [19–21]. The endogenous antioxidant defense system neutralizes the toxic effects of these free radicals by donating electrons to these toxic species, thereby reducing their damaging abilities and an imbalance between prooxidant and antioxidants forces are crucial determinants of health and disease. Some recent experimental studies have indicated the role of ROS in the regulation of stress-induced neurobehavioral changes [22–24]. Though stress is known to disturb the oxidant/antioxidant balance in these situations, the role of antioxidant defense mechanisms in stress-induced immunological changes is not clearly defined. Thus, the present study was designed to evaluate the possible involvement of free radicals and antioxidant defense systems during stress-induced immunomodulation in rats.

2. Materials and methods

2.1. Animals

Inbred male Wistar rats of (150–200 g) were used. All animals were housed in standard laboratory conditions with food and water

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available *ad libitum*. The care of animals was done as per guideline in Care and Use of Animals in Scientific Research prepared by Indian National Science Academy (INSA) New Delhi and the study protocol was approved by the Institutional Animal Ethical Committee (IEAC).

2.2. Stress procedure

Restraint stress (RS), which is a widely used and most accepted experimental model to induce emotional stress in rodents [22–24], was used as the experimental stressor. In this method, rats were immobilized in Plexiglas restrainers (INCO, Ambala) for 1 h at room temperature. Separate groups of rats were pretreated with different drug doses ($n=7$, per group), followed by exposure to RS (1 h)/day, for 5 consecutive days. After the completion of the stress procedure, blood samples were collected for the various immunological and biochemical assays. This particular stress paradigm was chosen on the basis of our earlier pilot studies which had shown that a single RS exposure did not influence any of the immune parameters tested, and the most consistent and reproducible effects were seen after 5 sessions of RS. The 5 days treatment schedule with antioxidants was selected in order to have consistent effects on all aspects of adaptive immune response development.

2.3. Drugs

L-Ascorbic acid was procured from the Sigma Aldrich Chemical Co. USA and α-tocopherol obtained from Cipla Ltd India. L-Ascorbic acid dissolved in distilled water and α-tocopherol was emulsified in a drop of tween 20 in distilled water. Both the drugs were administered intraperitoneally (i.p.) in a volume of 2 ml/kg.

2.4. Immunological assays

2.4.1. Tests for humoral immunity

2.4.1.1. Haemagglutination assay: [8,25]. Fresh blood from healthy sheep was collected by venepuncture and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and RBCs were washed three times with 0.9% normal saline. The RBCs were diluted to 0.5×10^9 cells/ml based on the cells counted on the Neubaur's chamber under the microscope. All the animal groups were immunized with i.p. injection of 0.5×10^9 SRBC on day 0 and were treated with different doses of drugs for 5 consecutive days, whereas, the control group received only vehicle. The stress (RS) exposure was done for 1 h daily from the day of immunization (day 0) for 5 consecutive days. On day 6, they were bled from the supraorbital plexus by microcapillary technique under light ether anesthesia. The serum of each rat was collected in the Eppendorf tubes. The serum samples were kept at −80 °C until analyzed for anti-SRBC antibody titre by hemagglutination technique. The serum samples were assayed for anti-SRBC antibody titre using 96 well titre plates (Tarson). The samples were diluted 2 folds with phosphate buffer saline (150 Mm/l, pH 7.4) and to each well 0.025 ml of 1% v/v SRBC (fresh) in phosphate buffer was added. The plates were incubated at 37 °C for 1 h and then observed for hemagglutination. The highest dilution showing visible hemagglutination was taken as the antibody titre. The antibody titre was expressed in a graded manner, the minimum dilution (1/2) being ranked as one (1), and the mean ranks for different groups were compared for statistical significance.

2.4.1.2. Splenic plaque forming cell (PFC) assay [26,27]. This assay is an in vitro method for identifying antibody forming cells by the action of the freshly synthesized antibodies against SRBC around antibody forming cells. The spleen was dissected out and rinsed in 2 ml of RPMI-1614 (pH 7.2) and dissociated with the help of sterile forceps on the small wire-mesh under aseptic conditions to recover lymphoid cells. 1 ml of histopaque (1.077 g/ml density) and 2 ml cell suspension was taken and centrifuged at 1000 rpm for 20 min at 4 °C. The whitish interface of the lymphocytes between button red pellet and uppermost layer was taken and adjusted to a volume of 1 million lymphocytes per millions/ml, i.e. 1×10^6 cells/ml. SRBC were washed with PBS (150 mM/l, pH 7.4) and the cell count was adjusted to 800×10^6 cells/ml. Guinea pig serum was taken as complement and diluted 5 times in PBS. For assay, 1 ml of SRBC suspension, 1 ml of lymphocytes and 0.5 ml of guinea pig serum were taken in the micro tube. From this mixture, a known volume was loaded into a Cunningham's chambers, sealed with vacuum grease, and incubated for 1 h at 37 °C. The plaques were counted under light microscope and results were expressed as plaques/10^6 spleen cells.

2.4.2. Tests for cell-mediated immunity

2.4.2.1. Delayed type hypersensitivity (DTH) reaction: [28]. After the immunization with SRBC on day 0, all rats were exposed to the different treatment schedules and RS (1 h) for 5 consecutive days. After this, all groups were challenged with 0.2 ml of 2% v/v fresh SRBCs in 0.9% normal saline into the right hind paw, while left paw received normal saline. After 24 h the footpad edema was measured and difference in the footpad volume (Right paw − Left paw) was measured by a dial calliper (Mututio, Japan) and expressed as percentage difference in DTH response.

2.4.2.2. Cytokine assays. The cytokines assays were carried out by the commercially available cytokine assays kits (Diclone, France). Briefly, the Th_1 (IFN-γ) and Th_2 (IL-4) cytokines assays were performed by using solid phase sandwich enzyme linked immunosorbent assay (ELISA). A monoclonal antibody specific for IL-4 or IFN-γ was coated on to the wells of the microtitre strips provided. Antigen and biotinylated polyclonal antibody specific for rIL-4 and IFN-γ were simultaneously incubated at 37 °C for 1 h. The revelation steps included streptavidin horse-radish peroxidase and TMB as chromogen. The readings were taken on Microscan-5405A ELISA reader (ECIL) and results were expressed in pg/ml.

2.5. Estimation of oxidative stress markers

2.5.1. Lipid peroxidation (TBARS) assay

The serum samples were analyzed for lipid peroxidation using the method of Satok [29]. Briefly, 0.5 ml of the serum samples and 2.5 ml TCA (20%) were taken in test tubes and centrifuged at 3500 rpm for 10 min. The supernatant was decanted and precipitate was washed once with 2.0 ml of 0.05 M H_2SO_4. This washed precipitate was dissolved in 2.0 ml of the H_2SO_4, and 3.0 ml of thiobarbituric acid (0.68% TBA in Na_2SO_4) was added and vortexed. The tubes were then incubated in boiling water bath for 30 min, taken out, and kept under tap water. To this chromogen 4.0 ml of n-butanol was added with vigorous shaking and centrifugation was done at 3000 rpm for 10 min. The upper organic layer was separated and absorbance was taken at 530 nm on spectrophotometer (UV 5740 SS, ECIL) for malondialdehyde (MDA). The calculation was carried out using extinction coefficient and results were expressed in nM/ml.

2.5.2. Superoxide dismutase (SOD) assay

A stock RBC hemolysate was prepared from the blood sample collected, by addition of four parts of ice cold triple distilled water to one part of washed red cells and frozen at −20 °C. This was used for estimation of the SOD by using the method of Nandi and Chatterjee [30]. Briefly 0.5 ml of RBC hemolysate mixed with 3.0 ml of ice cold H_2O, 1.0 ml of ethanol and 0.6 ml of $CHCl_3$, mixed properly after each addition, and finally shaken for 10 min and centrifuged at 3000 rpm for 30 min and the supernatant was used for SOD estimation. 100 μl of pyrogallol was added in cuvette containing 2.7 ml of tris HCl buffer