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Respiratory Burst by Dengue-Virus-Induced Cytotoxic Factor

Key Words
Dengue virus
Cytotoxic factor
Respiratory burst
Cytotoxicity
Pathogenesis

Abstract

Objective: This study investigates the induction and release of the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by mouse spleen cells on stimulation with dengue type 2 virus (DV) and a DV-induced cytokine, the cytotoxic factor (mCF). **Methods:** Normal mice or their spleen cell cultures were inoculated with DV or mCF. At different time periods, the spleen cell supernatants were assayed for the production of O_2^- and H_2O_2. **Results:** Inoculation of DV in spleen cell cultures resulted in peak production of O_2^- and H_2O_2 at 48 and 72 h, respectively, while in DV-infected mouse spleen, the maximum production was on days 7 and 8, which correlated with the appearance of mCF in the milieu. Maximum O_2^- and H_2O_2 production occurred at 45 min and 1 h after inoculation of 5 μg of mCF. Pretreatment of mCF with anti-mCF-antiserum inhibited O_2^- and H_2O_2 release indicating the specificity of the induction by mCF. The enriched subpopulations of macrophages and T cells produced O_2^- and H_2O_2 and not B cells. Treatment of the cells with superoxide dismutase increased H_2O_2 release but inhibited O_2^- release and the cytotoxicity in a dose-dependent manner. **Conclusion:** This showed that O_2^- is responsible for the cytotoxic activity of mCF and not H_2O_2. In conjunction with our earlier findings that pretreatment with N^G-monomethyl-*L*-arginine inhibited mCF-induced production of NO and the cytotoxicity, it is concluded that the presence of both O_2^- and NO is required for the cytotoxic activity of mCF, thereby indicating a possible role of peroxynitrite.

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Introduction

Professional phagocytic cells, upon appropriate stimulation undergo a respiratory burst leading to production of reactive oxygen intermediates (ROI) which include superoxide anions (O_2^-) hydroxyl radicals, hydrogen peroxide (H_2O_2), singlet oxygen and HOCl [1]. These cells also produce reactive nitrogen intermediates (RNI), which include nitrite (NO_2^-) and related highly reactive nitric oxide (NO) and nitrogen dioxide. Simultaneous production of NO and O_2^- may form peroxynitrite. Both ROI and RNI form an important line of host defense against infectious agents and parasites [1–3]. It has been shown that the tissue injury by ROI is of necrotic type while that with RNI and peroxynitrite is of apoptotic type [4]. Various agents, including cytokines, stimulate phagocytic cells directly or by triggering stimuli to generate RNI and ROI [2, 4].

Dengue type 2 virus (DV)-infected mice produce a cytokine, the cytotoxic factor (mCF), which kills normal mouse spleen cells in vitro. Inoculation of mCF depresses T and B cell responses of mice to sheep erythrocyte antigens, and the number and functions of macrophages are also depressed [5–7]. mCF is a highly potent pathogenesis-related protein, capable of reproducing in mice all the pathological lesions that are seen in patients with dengue hemorrhagic fever, including an increase in capillary permeability and damage to the blood-brain barrier [8–11]. Recently, the presence of an mCF-like protein has been shown in sera of cases of dengue hemorrhagic fever and cultures of human peripheral blood mononuclear cells (PBMC) inoculated with DV [12, 13]. mCF kills H-2A-negative macrophages, T helper cells and mast cells in 1 h by inducing influx of Ca^{2+} in the target cells, but has no effect on B cells [14–17]. Production of nitrite by mouse splenic macrophages and T cells occurs on stimulation with mCF [18, 19]. The present study was undertaken to investigate these aspects and show that mCF induces production of ROI in the form of O_2^- and H_2O_2.

Materials and Methods

Animals

This study was carried out on inbred Swiss albino mice aged 6–8 weeks, obtained from the mouse colony maintained by the Department.

Virus

DV strain P23085 was used in the form of infected adult mouse brain suspension [20]. The virus was used in doses of 1,000 LD_{50}. Normal mouse brain (NMB) was used in the controls.

Preparation of Cytotoxic Factor (mCF)

mCF was prepared from the spleen cells of DV-infected mice and purified as described by Khanna and Chaturvedi [11], and its protein contents were estimated [21]. A similar preparation (NF) obtained from the normal mouse spleen was used in the controls. Antiserum against purified mCF (mCF-As) was prepared in mice [11].

Preparation of Spleen Cell Cultures

The spleen was teased out with the help of forceps in chilled minimum essential medium containing 10% fetal calf serum. A single-cell suspension was prepared and viable cells were counted using trypan blue dye exclusion [14]. The cells were cultured at 37 °C in the presence of 5% CO_2 in air of 4 ml volume (4×10^6 cells/ml) in 5-cm glass petri dishes.

Preparation of Enriched Cell Population

The enriched subpopulations of splenic T and B cells were obtained by filtration through glass wool and nylon wool columns as described earlier [22, 23]. Macrophage-enriched cells were prepared from the peritoneal lavage cells of normal mice [6].

Assay of Cytotoxic Activity

The cytotoxic activity of mCF was assayed using normal mouse spleen cells as target. Equal volumes of the test solution and the target cells (2×10^6 cells/well) were mixed in a microtitre U-well Perspex plate and incubated at 4 °C for 1 h. Nonviable cells were counted

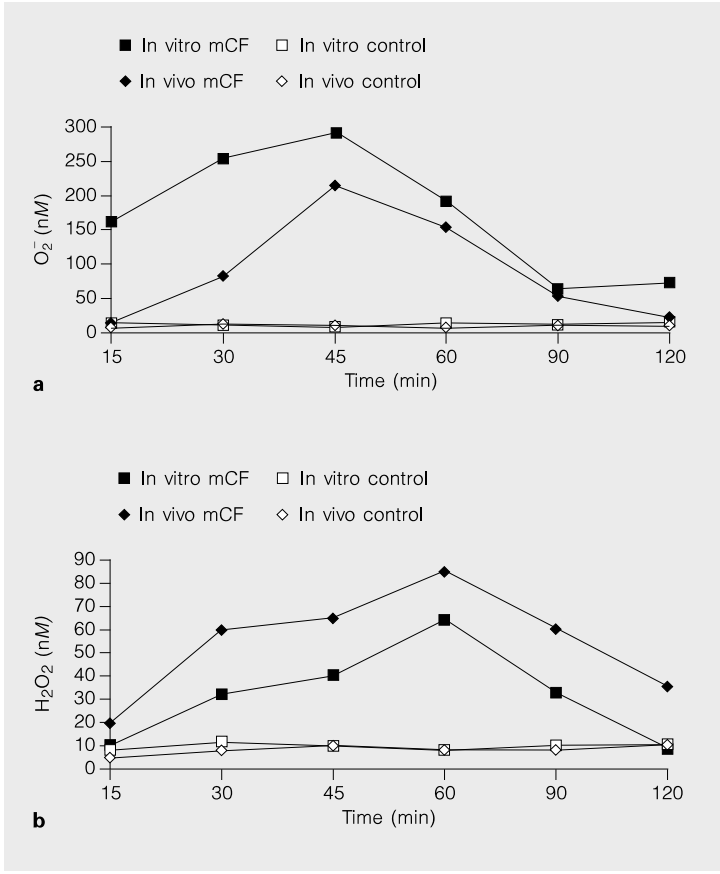

Fig. 1. mCF-induced production of O_2^- (**a**) and H_2O_2 (**b**). The results are presented after deduction of background values as mean values of 10 cultures (in vitro) or 8 mice (in vivo).

using trypan blue dye, and the percentage of the nonviable cells was calculated [14].

Assay of O_2^- Release

The O_2^- release was measured by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C [24]. Briefly, spleen cells (4×10^6 cells/ml) were incubated with mCF or DV in the presence of 80 μ*M* ferricytochrome C in phenol-red-free minimum essential medium at 37°C in the presence of 5% CO_2 in air. Parallel experiments were done in the presence of varying concentrations (1–70 μg) of SOD. The control cells were inoculated with NF/NMB in the place of mCF/DV, respectively. For background values, the cells contained ferricytochrome C but no stimulants. Following incubation, the experiments were terminated by placing the tubes in an ice bath, followed by centrifugation at 2,000 *g* for 10 min at 4°C. The OD of the supernatants was measured immediately at 540 nm in a microplate reader (Biotek Instruments Inc., Burlington, Ontario, Canada). The amount of O_2^- produced was calculated after deducting the background value by the method indicated by Pick and Mizel [25], and was expressed as O_2^- released by 4×10^6 cells.

Assay of H_2O_2 Release

The release of H_2O_2 by mouse spleen cells in response to mCF or DV was assayed as described earlier [26–28].

The chemicals and reagents used in the study were purchased from the Sigma Chemical Co. St. Louis, Mo., USA). All the experiments were set up in triplicate and repeated. The data were analyzed using Student's t test. A p value less than 0.05 was considered significant.

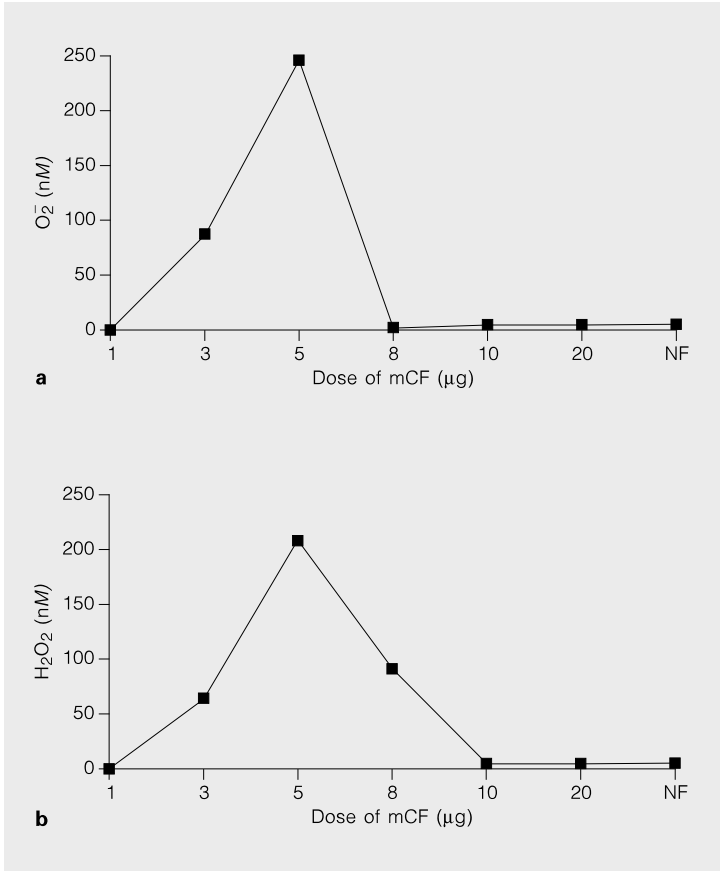

Fig. 2. Dose-dependent production of O_2^- (**a**) and H_2O_2 (**b**) by mCF. The results are presented after deduction of background values as mean values of 8 cultures.

Results

mCF-Induced Production of O_2^- and H_2O_2

The effect of mCF on O_2^- and H_2O_2 induction was studied in vitro and in vivo. For in vitro studies, normal mouse spleen cell cultures (4×10^6 cells/ml) were treated with 5 μg mCF at 37°C. Control cultures were treated with NF. The culture supernatants were studied at different times. For in vivo studies, groups of mice were inoculated with 5 μg mCF intravenously, and the control mice were inoculated with NF. The spleens were removed at different times, and a single-cell suspension was prepared and cultured (4×10^6 cells/ml) at 37°C in the presence of 5% CO_2. The culture supernatants were collected after 24 h and studied. Time course studies revealed that maximal release of O_2^- occurred at 45 min (fig. 1a) and that of H_2O_2 occurred at 60 min (fig. 1b) after inoculation of mCF in vitro and in vivo. To study the dose response, spleen cell cultures (4×10^6 cells/ml) were injected with different doses of mCF. The data presented in figure 2 show a dose-dependent production of mCF-induced O_2^- and H_2O_2. Another experiment conducted on enriched subpopulations of spleen cells showed that macrophages and T cells released O_2^- (fig. 3a) and H_2O_2 (fig. 3b), while B cells did not produce them.

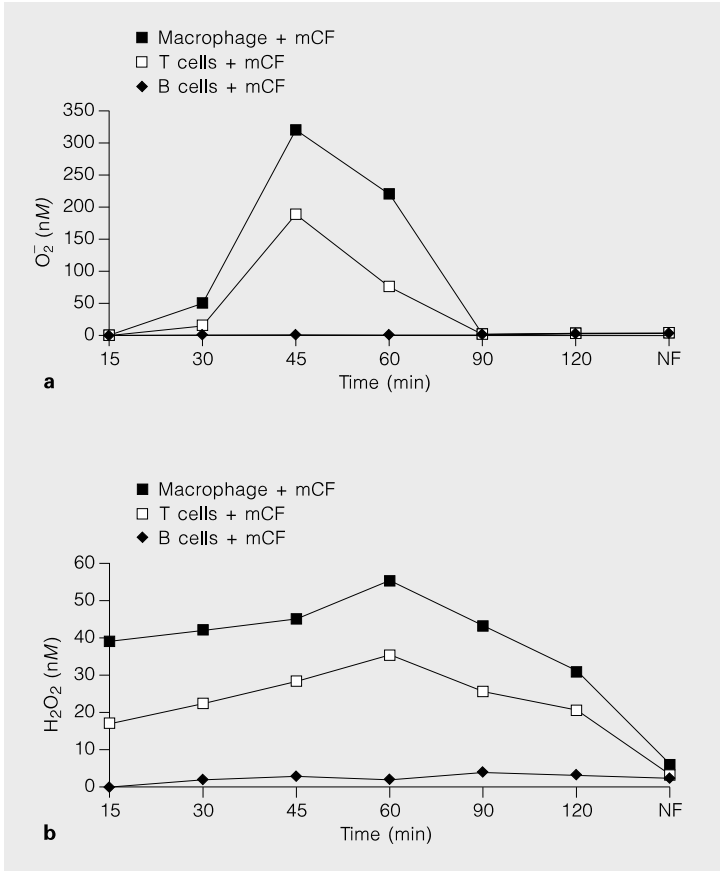

Fig. 3. mCF-induced production of O_2^- (**a**) and H_2O_2 (**b**) by the enriched cell subpopulations of the spleen. The results are presented after deduction of background values as mean values of 10 cultures (**a**) and 6 cultures (**b**). For the sake of clarity, the mean values of all the cultures inoculated with NF has been presented together, as there was no difference.

Effect of Anti-mCF-Antiserum on mCF-Induced Production of O_2^- and H_2O_2

mCF was mixed with different dilutions of mCF-As, incubated for 1 h at 37°C followed by addition of 4×10^6 cells/ml normal mouse spleen cells and further incubated at 37°C for 45 min or 1 h. The control group was treated with normal mouse serum in place of mCF-As. The viability of the cells was studied, and the supernatants were assayed for the production of O_2^- or H_2O_2. The data presented show that the production of O_2^- (fig. 4a) and H_2O_2 (fig. 4b) and the cytotoxicity of mCF were inhibited by pretreatment with mCF-As in a dose-dependent manner.

DV-Induced Production of O_2^- and H_2O_2

Normal mouse spleen cell (4×10^6 cells/ml) cultures were inoculated with 1,000 LD_{50} DV or with NMB for controls. The cultures were incubated at 37°C in the presence of 5% CO_2, and at different times the culture supernatants were harvested and assayed for the production of O_2^-, H_2O_2 and the cytotoxic activity. The data presented in figure 5a show that O_2^- production was maximal at 48 h after DV inoculation, while H_2O_2 (fig. 5b) production was maximal at 72 h. The cytotoxic activity appeared at 48 h and persisted untill 96 h, when the experiments were terminated.

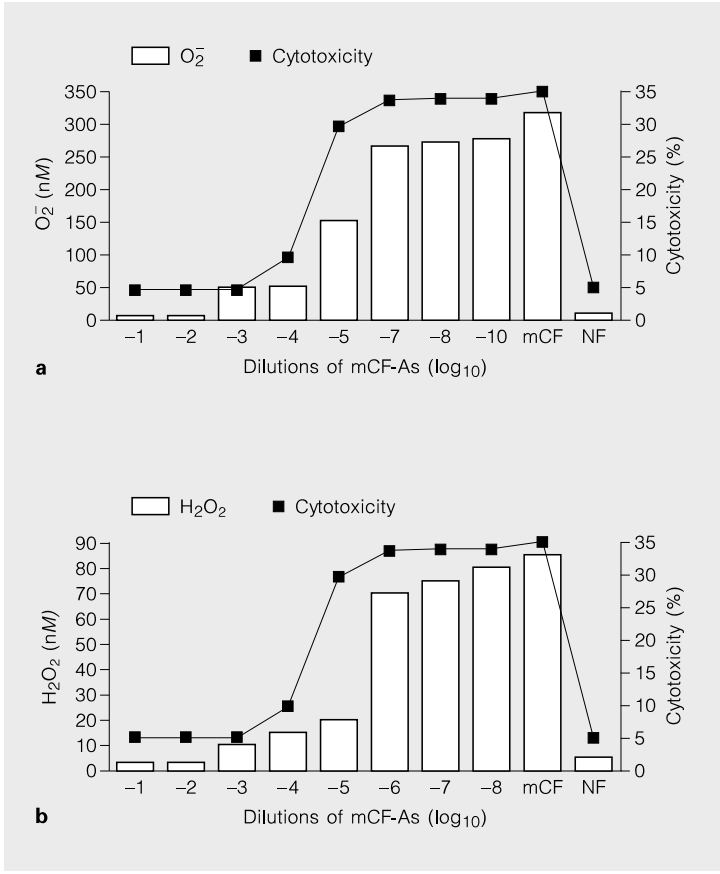

Fig. 4. Effect of pretreatment of mCF with mCF-As on its cytotoxic activity and the mCF-induced production of O_2^- (**a**) and H_2O_2 (**b**). The results are presented as mean values of 6 cultures after deduction of background values.

For in vivo studies, groups of mice were inoculated with a dose of 1,000 LD_{50} of DV, and the control group was inoculated with NMB. At different times, the spleens were harvested and cultured (4×10^6 cells/ml) for 24 h at 37°C in the presence of 5% CO_2, and the culture supernatants were studied. It was observed that the production of O_2^- and H_2O_2 was maximal on days 7 and 8, respectively, while the peak cytotoxic activity was seen on days 8, 9 and 10 after infection (fig. 6). For the sake of clarity, the mean values of all the groups inoculated with NF or NMB have been presented together, in figures 1–6, as there was no difference.

Role of SOD in the Release of O_2^- and H_2O_2

The major pathways for the generation of H_2O_2 are spontaneous dismutation of O_2^- or generation by SOD. Normal mouse spleen cells (4×10^6 cells/ml) were cultured with different doses of SOD followed by injection of 5 µg of mCF or NF in the controls. After incubation for 1 h at 37°C, the cultures were harvested and studied. The findings presented in figure 7 show that SOD pretreatment inhibited the production of O_2^-, but had no effect on the mCF-induced production of H_2O_2.

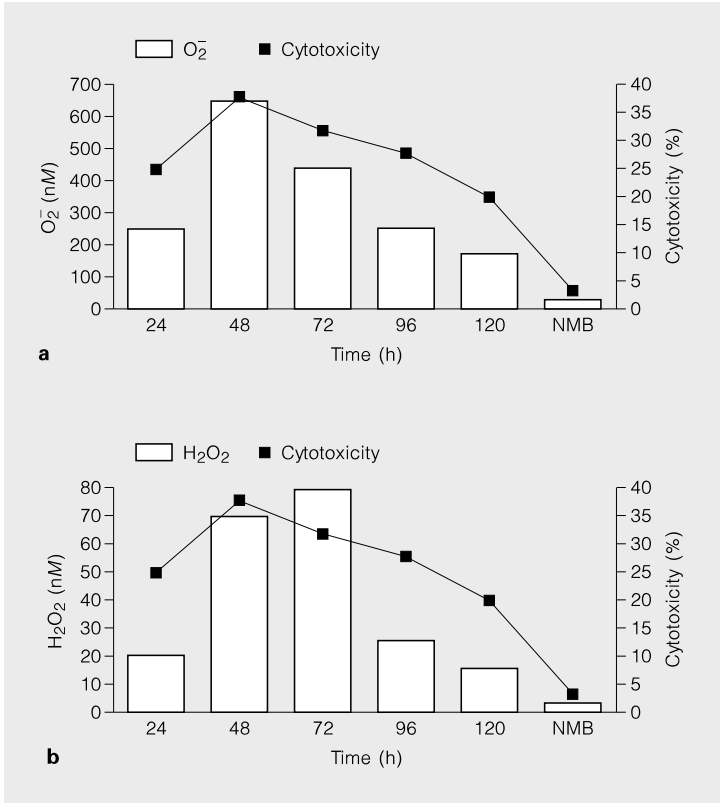

Fig. 5. Production of O_2^- and mCF (**a**) and H_2O_2 and mCF (**b**) by DV-stimulated spleen cell cultures. The results are presented after deduction of background values as mean values of 8 cultures.

Discussion

The findings of the present study demonstrate the production of O_2^- and H_2O_2 by the spleen cells during DV infection and also following the treatment with the DV-induced cytokine mCF. Abrogation of the production of O_2^- and H_2O_2 and the cytotoxic activity by pretreatment of mCF with the specific antiserum confirmed that the cytokine was responsible for these effects. Normal mouse spleen cell cultures inoculated with DV produce mCF at 2–4 days, while in mice inoculated with DV, peak production of mCF occurs at 8–10 days after infection. Similar observations have been made with DV-infected human PBMC cultures and the PBMC obtained from the cases of dengue hemorrhagic fever [13, 22, 29, 30]. The findings of the present study showed that the peak production of O_2^- and H_2O_2 correlated with the peak production of mCF, in vitro and in vivo. This further supported the role of mCF in the generation of O_2^- and H_2O_2. However, at the earlier periods, the amount of O_2^- and H_2O_2 produced did not correlate with the cytotoxic activity (fig. 5, 6). It has been shown that the minimum amount of mCF that can be detected by the cytotoxicity assay is 500 ng/ml, and that with ELISA is 7 ng/ml [31]. It appears that the amount of mCF required to induce production of O_2^- and H_2O_2 is very small. mCF is cytotoxic to a selected group of cells: namely, H-2A-negative macrophages, T helper and mast cells, but

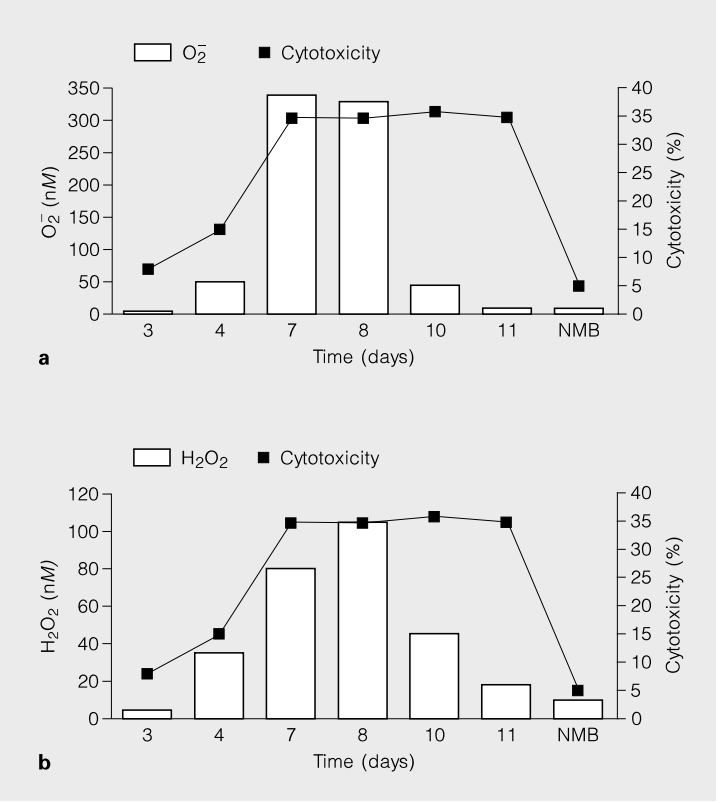

Fig. 6. Production of O_2^- and mCF (**a**) and H_2O_2 and mCF (**b**) by the spleen cells of DV-infected mice. The results are presented after deduction of background values as mean values of 9 mice.

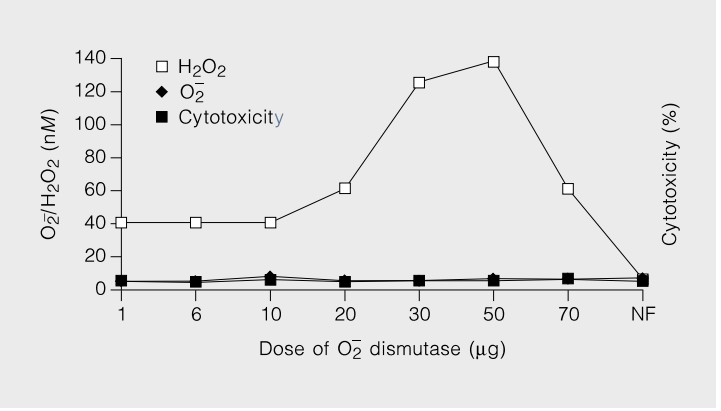

Fig. 7. Effect of pretreatment of spleen cell cultures with O_2^- dismutase on the mCF-induced production of O_2^- and H_2O_2. The results are presented after deduction of background values as mean values of 8 cultures.

has no effect on various cell lines [14–16]. The present study showed that mCF induced macrophages and T cells to produce O_2^- and H_2O_2, but not B cells. Pretreatment of the spleen cells with SOD inhibited the production of O_2^- and the cytotoxic effects of mCF, while the production of H_2O_2 was enhanced. These findings suggest that the killing of the target cells by mCF was mediated via O_2^- and not via H_2O_2.

We have observed production of NO_2^- by the spleen cells of mice following stimulation with DV or mCF [18, 19]. The production of ROI, as described here and elsewhere [31], and the production of RNI reported earlier [18, 19] have many similarities: (1) the peak response occurs in both in 45 min with 5 μg of mCF; (2) both are produced by macrophages and T cells, but not by B cells; (3) the amount produced in response to DV correlates in both with the peak production of mCF, and (4) both are specifically inhibited by anti-mCF antibodies and a calcium-channel-blocking drug. Ding et al. [2] screened 12 cytokines out of which only IFN-γ induces production of both NO_2^- and H_2O_2 by independent pathways. It has been proposed that a ligand binding to the cell surface receptor may stimulate the nitric oxide synthase and the NADPH oxidase pathways, resulting in production of NO and O_2^- [3].

It has been shown that O_2^- on dismutation produces H_2O_2 or reacts with NO to produce highly toxic peroxynitrite. These products, singly or in combination, results in target cell death by apoptosis [3, 32]. We have shown that macrophages and lymphocytes treated with mCF have fragmented DNA and electron microscopic appearance of apoptotic cells [10; Misra et al., unpubl. data]. The cascade of the events during the production and the mechanism of action of mCF have been presented elsewhere [33]. The cytotoxic activity of mCF is abrogated by treatment with: (1) N^G-monomethyl-*L*-arginine, an inhibitor of NO_2^-/NO_3^- [18, 19], and (2) SOD which dismutates O_2^- as shown here. This showed that production of both NO_2^- and O_2^- is required for the cytotoxic activity of mCF. Since both the pathways run concomitantly, it is proposed that the mechanism of target cell killing by mCF works via the formation of peroxynitrite. But we still do not know why and how mCF kills a very selected group of cells.

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