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Production of nitrite by dengue virus-induced cytotoxic factor

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SUMMARY

Dengue type 2 virus (DV) infection induces production of a cytokine, the cytotoxic factor (CF) in the spleen of mice. The present study was undertaken to investigate the production of nitrite (NO_2^-) by the spleen cells of mice *in vitro* and *in vivo* following inoculation of DV or CF. Maximum NO_2^- production occurred at 45 min after inoculation of 5 μg CF, both *in vitro* and *in vivo*. The NO_2^- was produced by macrophages and T cells and not by B cells. Pretreatment of CF produced by macrophages and T cells and not by the B cells. Pretreatment of CF with anti-CF antisera inhibited production of NO_2^-. DV-stimulated spleen cell culture supernatants showed peak production of CF and NO_2^- at 72 h. In DV-infected mouse spleen, maximum NO_2^- production occurred at 8–11 days post-infection, which correlated with peak cytotoxic activity in the spleen. Pretreatment of spleen cells with N^G monomethyl L-arginine (NMMA) inhibited NO_2^- production. NO_2^- production was abrogated in a dose-dependent manner by treatment of spleen cells with Ca^{2+} channel blocking drug, Nifedipine. The findings demonstrate that DV-induced CF induces production of NO_2^- in spleen cells, probably in a Ca^{2+}-dependent manner, and may be a mechanism of target cell killing.

Keywords dengue virus cytotoxic factor nitrite cytotoxicity

INTRODUCTION

Nitric oxide (NO) is an important paracrine and autocrine signal used by different cell types, and is synthesized by a variety of cells and tissues including vascular endothelium, macrophages, neutrophils, hepatic Kupffer cells, adrenal tissue and cerebellar tissue (reviewed in [1]). NO mediates a number of effects, for example vaso dilation, inhibition of platelet aggregation, cytotoxic action of macrophages and neutrophils, and neurotransmission (reviewed in [2]). NO is synthesized from L-arginine by the enzyme NO synthase in presence of NADPH [3,4], and its synthesis is inhibited by the NO_2^-/NO_3^- synthesis inhibitor N^G-monomethyl-L-arginine (NMMA) [5]. The synthesis of NO appears to be tightly regulated in the cell, as it has protective or regulatory functions in the cell at low concentration and toxic effects at higher concentration. Ding *et al.* [6] screened the effect of 12 cytokines on NO_2^- production, and found that the most effective of them was interferon-gamma (IFN-γ).

A unique cytokine, the cytotoxic factor (CF) is produced by T lymphocytes of dengue type 2 virus (DV)-infected mouse spleen [7–13]. It kills H2-A-negative macrophages, T helper (Th) cells and mast cells, etc., in 1 h by inducing influx of Ca^{2+} in the target cells. It has no effect on other cells and cell lines. It has recently been shown that CF is a pathogenesis-related protein, capable of reproducing all the pathological lesions in mice that are seen in cases of human dengue [14–16]. CF is a highly potent molecule of 22–25 kD on SDS–PAGE with an isoelectric point of pH 6·5. The N-terminal sequence of 19 amino acids of CF does not match with any other known cytokines or DV-specific proteins [17]. Oligonucleotide probes derived from this sequence show the presence of mRNA for CF in the spleen cells of DV-infected mice and cultures of human peripheral blood mononuclear cells (PBMC) [18] by Northern hybridization. The cDNA library for CF has been constructed and recombinant CF is expressed in *Escherichia coli* (U. C. Chaturvedi *et al.*, unpublished data). Recently, the presence of CF has been shown in the sera of several patients suffering from dengue haemorrhagic fever (DHF), collected at Shahjahanpur epidemic during 1993 [19]. This finding was validated at US Army Medical Component (AFRIMS, Bangkok Thailand) by screening 340 sera from cases of dengue disease. We have succeeded in purifying CF proteins from the sera of human cases of DHF by chromatography (R. Mukerjee *et al.*, unpublished data). The present study was planned to investigate if CF induces production on NO_2^- in spleen cells of mice to mediate its cytotoxic effect on target cells.

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MATERIALS AND METHODS

Animals

The study was carried out on inbred Swiss albino mice aged 6–8 weeks, obtained from the mouse colony maintained in this department.

Virus

The dengue type-2 virus (DV) P23085 was used in the form of infected adult mouse brain suspension [20]. The virus titre was estimated by intracerebral (i.c.) inoculation of the preparation as described earlier [15,20]. Briefly, serial 10-fold dilutions of the suspension were inoculated intracerebrally in groups of adult mice, and mortality was recorded. The virus titre was calculated by the method of Reed & Muench [21] expressed as $\log_{10}$ LD_{50} for 30 μl of the suspension. Normal mouse brain suspension (NMB) was used in controls.

Preparation of cytotoxic factor

CF was prepared from the spleen cells of DV-infected mice and purified as described earlier [7,17]. A similar preparation obtained from normal mouse spleen (NF) was used in the controls.

Preparation of antisera against CF

Antisera against purified CF (CF-As) was prepared in mice. Briefly, mice were inoculated with 5 μg CF emulsified with Freund's complete adjuvant (FCA; Sigma Chemical Co., St Louis, MO) intraperitoneally. Fifteen days later a booster dose of 5 μg CF mixed with Freund's incomplete adjuvant (FIA) was given intraperitoneally. At day 30 after the first dose, mice were bled and sera collected. The capacity of the sera to neutralize the cytotoxic activity of CF was assayed.

Preparation of spleen cell culture

The spleen was teased out with the help of forceps in chilled minimum essential medium (MEM) containing 10% fetal calf serum (FCS; Armour Pharmaceutical Co., UK). A single-cell suspension was prepared and viable nucleated cells were counted using the trypan blue exclusion test [22]. Cells (10×10^6/ml) were suspended in MEM containing 10% FCS. The cultures were set up by layering 4 ml of the cell suspension in 5-cm glass Petri dishes and were incubated at 37°C in presence of 5% CO_2 in air [23].

Preparation of enriched cell populations

The enriched subpopulations of splenic T and B lymphocytes were obtained by filtration through glass wool and nylon wool columns and the purity of the cells was ascertained [18]. Macrophage-enriched glass-adherent monolayers were prepared from the peritoneal lavage cells of normal mice as described elsewhere [24]. In different preparations 92–95% of the cells were phagocytic [24].

Assay of cytotoxic activity

Cytotoxic activity was assayed using normal mouse spleen cells as a 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. Non-viable cells were counted using trypan blue dye and the percentage of non-viable cells was calculated [22].

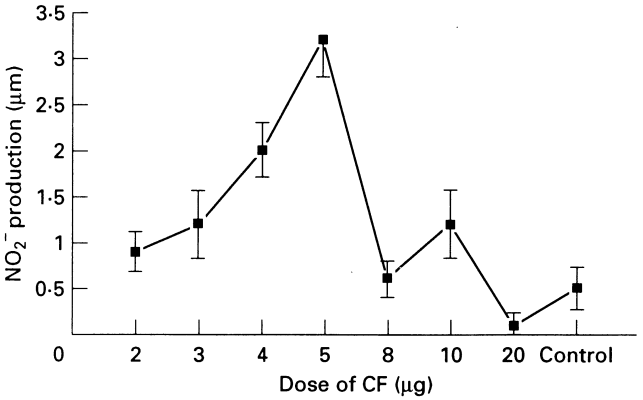

Fig. 1. Production of NO_2^- by the mouse spleen cell culture treated with cytotoxic factor (CF). Groups of spleen cell cultures (10×10^6 cells/ml) treated with various doses of CF were inoculated at 37°C in the presence of 5% CO_2 in air. For control, spleen cells were inoculated with normal mouse spleen (NF). After 45 min the cultures were harvested and production was assayed in the cell-free supernatants as described in Materials and Methods. Results are presented after deduction of background values as mean ± s.d. of 10 cultures.

Nitrite measurement

Nitrite measurement in the culture supernatant was determined by a microplate assay method [6]. The culture was incubated in the presence of phenol red free medium (Earle's solution containing Earle-1X, dextrose, $NaHCO_3$ 5%, 5% FCS and antibiotics). Briefly, 100 μl test samples were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0·1% naphthylethylene diamine dihydrochloride, 2·5% H_3PO_4) (LOBA Chemie Ltd., Bombay, India) and incubated at room temperature for 10 min in a flat-bottomed microtitre plate. Absorbance at 540 nm was measured in a microplate reader (Multiskan MC-Plate Reader; Biotek Instrument Inc., Burlington, Ontario). The NO_2^- concentration was determined using $NaNO_2$ as standard.

Plan of study

The production of NO_2^- by mouse spleen cells was investigated *in vitro* in spleen cell cultures and *in vivo* in mouse spleen cells. The experimental groups consisted of a test group inoculated with DV or CF; a control group inoculated with NF (in place of CF) or NMB (in place of DV); and a blank group inoculated with the diluents. Each group consisted of 10–12 cultures/mice and the data were obtained from experiments repeated at least three times. Data have been presented, after deduction of the background value, as mean value ± s.d.

RESULTS

Induction of NO_2^- production by CF

Induction of NO_2^- in normal mouse spleen cells was investigated both *in vitro* and *in vivo*. Maximum production of NO_2^- was observed after 45 min of CF treatment. The experiment repeated using different doses of CF showed that maximum production of NO_2^- occurred with 5 μg of CF (Fig. 1). Among the enriched cell populations, macrophages and T cells produced NO_2^- on treatment with CF *in vitro*, while there was no such production from B cells (Fig. 2). In *in vivo* studies, maximum NO_2^- production occurred in the spleen harvested after 45 min of CF inoculation (Fig. 3).

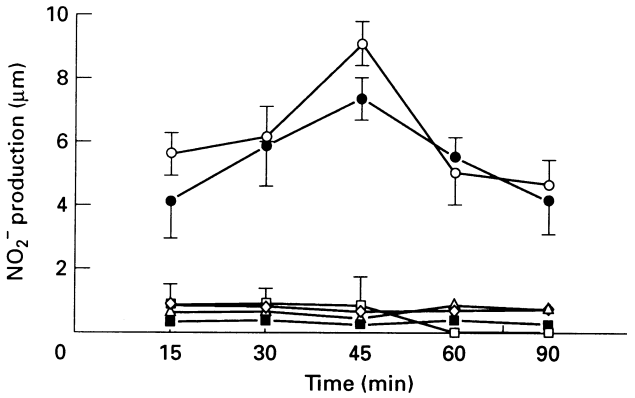

Fig. 2. Production of NO_2^- by the enriched subpopulations of macrophage (○), T cell (●) and B cell (□) cultures treated with 5 μg cytotoxic factor (CF) for different time periods at 37°C in the presence of 5% CO_2 in air. Control groups of macrophage (■), T cell (▽) and B cell (△) cultures were inoculated with normal mouse spleen (NF). Cultures were harvested and NO_2^- production was assayed in the cell-free supernatants as described in Materials and Methods. Results are presented after deduction of background values as mean ± s.d. of six cultures each.

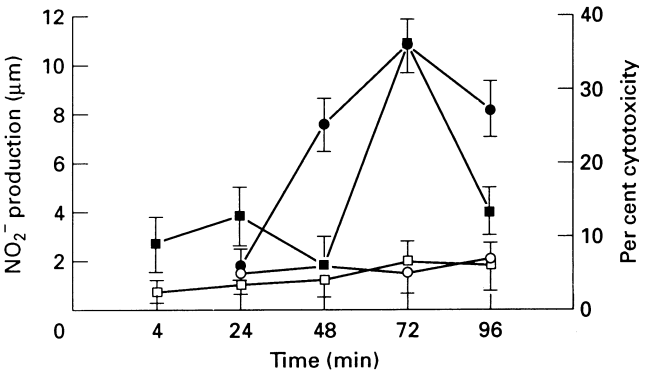

Fig. 4. Normal mouse spleen cell (10×10^6/ml) cultures were inoculated with 1000 LD_{50} dengue virus (DV) and incubated at 37°C in the presence of 5% CO_2 in air. At different time periods NO_2^- production (■) and cytotoxic activity (●) were assayed in the culture supernatants as described in Materials and Methods. For control groups, normal mouse spleen cells were inoculated with normal mouse brain suspension (NMB) in place of DV, and NO_2^- production (□) and cytotoxic activity (○) were assayed as described above. Each point represents mean value ± s.d. from 10 cultures.

Effect of anti-CF antisera on NO_2^- production

CF-As neutralizes the cytotoxic effect of CF in a dose-dependent manner [14], and the effect of CF-As on NO_2^- production by CF-treated cells was therefore investigated to find if it is specifically induced by CF. The antiserum dilution was mixed with equal volume of 5 μg CF, incubated at 37°C for 1 h, and then the mixture was added to the normal mouse spleen cells (10×10^6/ml) followed by further incubation at 37°C in the presence of 5% CO_2 for 45 min. Control groups of cells were treated with normal mouse sera in place of CF-As or with CF only. The culture supernatant was assayed for NO_2^- production. It was observed that the amount of NO_2^- in control group of cells given CF alone was $7{\cdot}3 \pm 0{\cdot}14$ μM, while a dose-dependent decrease in CF-induced NO_2^- production ($0{\cdot}08 \pm 0{\cdot}003$ μM to $3{\cdot}87 \pm 0{\cdot}41$ μM) was observed in spleen cells treated with various dilution of CF-As.

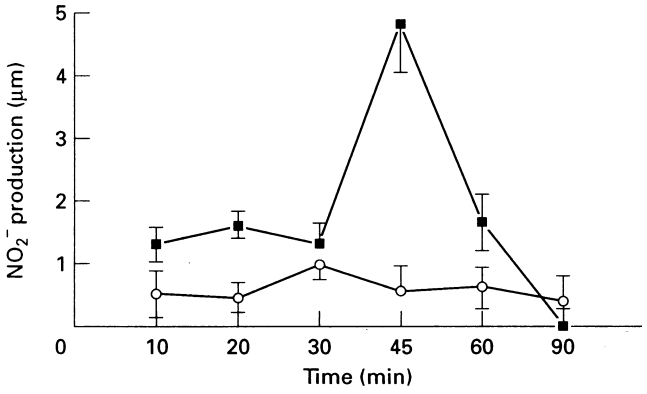

Fig. 3. Production of NO_2^- by the spleen cells of cytotoxic factor (CF)-inoculated mice. Groups of eight mice each were inoculated with CF (5 μg/mouse) intravenously (■) or with normal mouse spleen (NF) for controls (○). At different time periods the spleens were harvested and the cells (10×10^6/ml) cultured for 24 h at 37°C in the presence of 5% CO_2 in air. NO_2^- production in the cell-free culture supernatants was assayed as described in Materials and Methods. Data are presented after subtraction of background values as mean values ± s.d. from eight mice in each group.

Induction of NO_2^- by DV infection

Induction of NO_2^- production by CV infection was investigated. The findings summarized in Fig. 4 show a maximum production of NO_2^- at 72 h after DV inoculation *in vitro*, which was $10{\cdot}8 \pm 0{\cdot}85$ μM. Cytotoxic activity in the culture supernatants appeared at 48 h, being maximum at 72 h and remaining up to 96 h, when the experiment was terminated (Fig. 4). The data presented in Fig. 5 show that maximum production of NO_2^- occurred *in vivo* at day 11 of DV inoculation. Assay for the cytotoxic activity showed that the activity gradually increased, reaching a peak value of $36 \pm 4{\cdot}5$% on day 11 after DV inoculation (Fig. 5).

Effect of NMMA on CF-induced production of NO_2^-

The data summarized in Fig. 6a show that maximum inhibition of CF-induced NO_2^- production in the presence of NMMA was 85 ± 3%. To determine the optimum dose of NMMA, spleen cell cultures were pretreated with different doses of NMMA (5–150 μM) followed by inoculation of CF and assay of NO_2^- production as described above. Maximum inhibition of CF-induced NO_2^- production was observed with 100 μM NMMA. The effect of NMMA on cytotoxic activity of CF in the cells of the cultures was assayed. The data presented in Fig. 6b show that the cytotoxic activity of CF was inhibited by NMMA, and maximum inhibition was obtained with 100 μM NMMA.

Role of Ca^{2+} on production of NO_2^-

The role of Ca^{2+} in CF-induced production of NO_2^- by spleen cells was investigated. The data presented in Fig. 7 show inhibition of NO_2^- production by the calcium channel blocking drug, Nifedipine, in a dose-dependent manner.

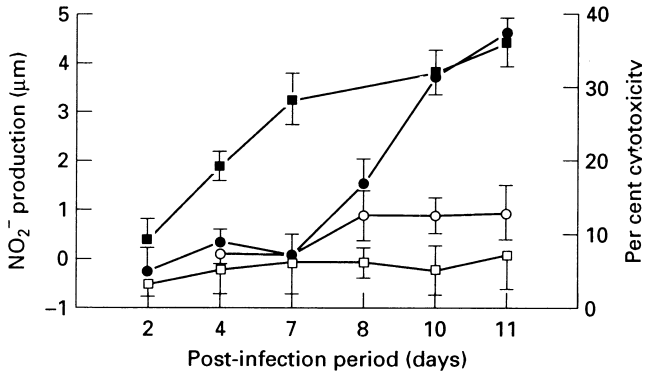

Fig. 5. Production of NO_2^- by spleen cells of dengue virus (DV)-infected mice. Groups of 10 mice each were inoculated with DV intracerebrally. Spleens were harvested at different time periods post-infection, a single-cell suspension was prepared (10×10^6 cells/ml) and cultured for 24 h at 37°C in the presence of 5% CO_2 in air. NO_2^- production (●) and cytotoxic activity (■) were assayed in the culture supernatants as described in Materials and Methods. For control, mice were inoculated intracerebrally with normal mouse brain suspension (NMB) in place of DV, and NO_2^- production (○) and cytotoxic activity (□) were assayed as described above. Each point represents mean values ± s.d. from 10 mice.

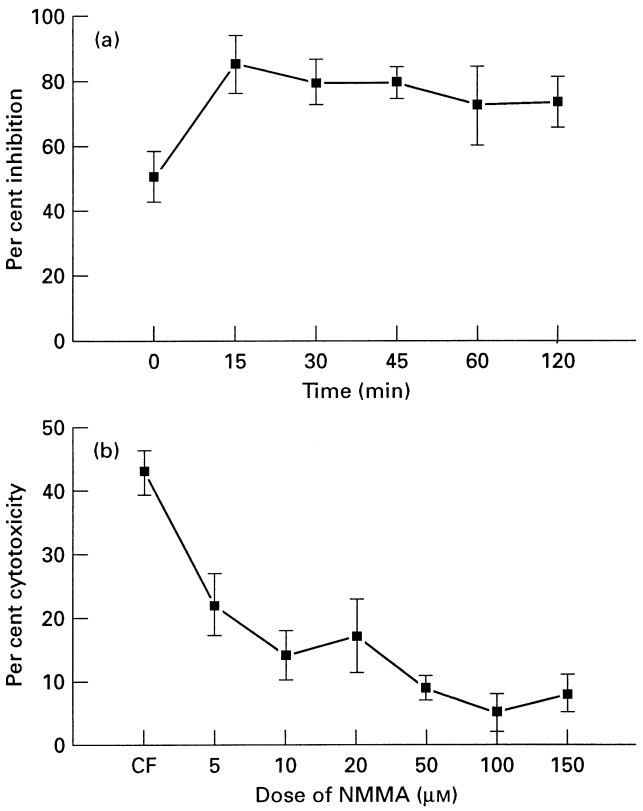

Fig. 6. (a) Inhibition of NO_2^- production by treatment with N^G monomethyl L-arginine (NMMA). Normal mouse spleen cell cultures (10×10^6/ml) were pretreated with 100 μM NMMA followed by inoculation of cytotoxic factor (CF; 5 μg) at different periods after NMMA treatment. Control group of cells were treated with CF only. Cells treated with 100 μM NMMA for different time periods were used for background values. After further incubation for 45 min at 37°C, NO_2^- production was assayed as described in Materials and Methods. Results are presented after deduction of background values as mean ± s.d. from eight cultures. (b) Effect of different doses of NMMA on cytotoxic activity. Normal mouse spleen cell cultures (10×10^6/ml) pretreated with different doses of NMMA for 15 min followed by inoculation of CF and incubated for 45 min at 37°C in the presence of 5% CO_2 in air. Control groups of cells were treated with CF only. Cells treated with different doses of NMMA only for 15 min were used for background values. The percentage of non-viable cells in each well was counted as described in Materials and Methods. Results are presented after deduction of background values as mean ± s.d. from eight cultures.

DISCUSSION

The findings of the present study demonstrate production of NO_2^- by the spleen cells of DV-infected mice, and also after treatment with DV-induced cytokine CF. Abrogation of NO_2^- production by pretreatment of CF with the specific antisera confirmed that the cytokine was responsible for the induction of NO_2^- production. Normal mouse spleen cell cultures inoculated with DV produced CF at 48–96 h, while in mice inoculated with DV intracerebrally the production of CF was maximum at days 10 to 11 post-inoculation. These findings support our earlier observations on production of CF in DV-infected mice and human PBMC cultures [7,18,24]. The data presented here show that peak NO_2^- production correlated with the peak of CF produced, both *in vitro* and *in vivo*, further confirming the role of CF in NO_2^- production. However, at earlier periods the amount of NO_2^- and cytotoxic activity did not correlate (Figs 4 and 5). It has been observed that the minimum amount of CF that can be detected by cytotoxicity is 500 ng/ml, and that with ELISA is 7 ng/ml [19]. The amount of CF required to produce NO_2^- was at least four times more than that required to produce cytotoxicity (Fig. 1), which explains the apparent discrepancy. NO can produce protective or regulatory functions in the cell at low concentration, while the main action of large quantities of NO production by macrophages is cytotoxic on different types of cells [4,25]. The mechanism of cytotoxicity appears to be NO-mediated nitrosation of key iron-containing enzymes or iron–sulphur proteins in the target cells [25,26]. CF is cytotoxic to a selected group of cells, namely H2-A-negative macrophages, Th cells and mast cells, etc., but has no effect on various cell lines [8–10]. The findings presented here show that CF induced macrophages and T cells to produce NO_2^-, but not B cells (Fig. 2). Pretreatment of the cells with NMMA inhibited the cytotoxic activity of CF. These findings suggest that killing of target cells by CF via NO_2^- production may be one of the mechanisms. At present, it is not clear whether NMMA inhibits at the same step where CF stimulates.

The action of NO synthase is controlled by various mechanisms, e.g. (i) calmodulin and Ca^{2+}-dependent type, as seen in endothelium [27] and cerebellum [28]; (ii) Ca^{2+}-independent type, as shown in macrophages [29]; and (iii) dependent on Ca^{2+} but not on calmodulin type, as shown in polymorphonuclear neutrophils [30]. The expression of the Ca^{2+}-independent cytokine-inducible NO synthase by IFN-γ) and tumour necrosis factor-alpha (TNF-α) has been described in various cell types, while IL-8 blocks both the release of NO and NO synthase induction by lipopolysaccharide (LPS) at the transcriptional level. A similar antagonistic response is observed between transforming growth factor-beta (TGF-β) and IFN-γ in the NO release in macrophages [31]. NO_2^- production in the present model is Ca^{2+}-dependent, as shown by abrogation of NO_2^- production by pretreatment with the calcium channel blocking drug, Nifedipine. It has been shown earlier that production and

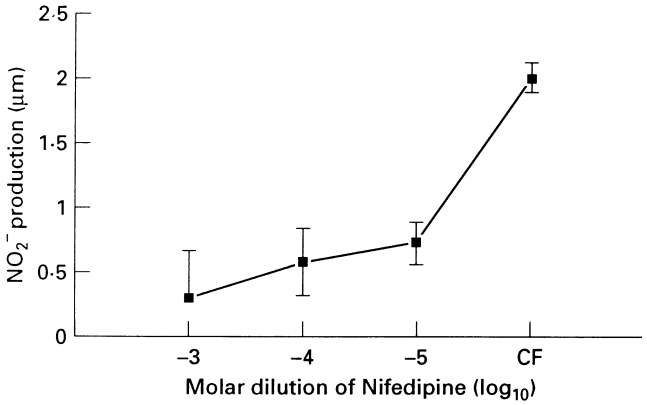

Fig. 7. Reduction in NO_2^- production by treatment with Ca^{2+} channel blocking drug, Nifedipine. Normal mouse spleen cells (10×10^6/ml) were pretreated for 30 min with different molar dilutions of Nifedipine. After 30 min, cytotoxic factor 5 μg (CF) were added in the pretreated cells and incubated for 45 min at 37°C. The control group of cells (10×10^6/ml) were treated with CF alone. NO_2^- production was assayed as described in Materials and Methods. Results are presented after deduction of background values as mean ± s.d. from nine cultures in each group.

secretion of CF by T cells and its cytotoxic activity is Ca^{2+}-dependent [11,12], therefore the findings of the present study are in conformity with the above reports. It has been shown that NO-mediated cell killing of murine peritoneal macrophages is of apoptosis type [32]. We have observed that macrophages and lymphocytes treated with CF show fragmentation of DNA and the electron microscopic appearance of apoptotic cells [33]. The cascade of events during production and the mechanism of action of CF has been presented elsewhere [34]. With the findings of the present study it is suggested that NO_2^- may be a mechanism of cytotoxicity of CF on target cells.

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