

## Release of reactive oxygen intermediates by dengue virus-induced macrophage cytotoxin

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**Summary.** Dengue type 2 virus (DV) induces a subpopulation of T lymphocytes of mice to produce a cytokine, cytotoxic factor (mCF), which induces H-2A positive macrophages to produce macrophage cytotoxin (CF2). The present study was undertaken to investigate the mechanism of cytotoxicity of CF2. It was observed that CF2 induced production of superoxide anion ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) by the spleen cells of mice *in vitro* and *in vivo*. The maximum production of  $O_2^-$  ( $260 \pm 10 \text{ nmol}/4 \times 10^6$  cells) was at 45 minutes while that of  $H_2O_2$  was at 90 minutes after inoculation of CF2. Pretreatment of mice or spleen cells with anti-CF2-antisera inhibited  $O_2^-$  and  $H_2O_2$  production in a dose-dependent manner. Superoxide dismutase (SOD) inhibited  $O_2^-$  production and cytotoxicity while  $H_2O_2$  production was increased by increasing SOD concentration in the culture. This indicated that  $O_2^-$  production is necessary for the cytotoxic activity of CF2. Pretreatment of the cells with  $Ca^{2+}$  channel blocking drugs, nifedipine or verapamil, inhibited CF2-induced  $O_2^-$  and  $H_2O_2$  production in a dose-dependent manner. We have shown earlier that the cytotoxic activity of CF2 is known to be  $Ca^{2+}$  dependent and CF2-induced production of nitrite and the cytotoxicity is inhibited by  $N^G$ -monomethyl-L-arginine. Thus, it is suggested that  $O_2^-$  and nitrite are necessary for cell killing by CF2 in a  $Ca^{2+}$ -dependent manner and the killing may possibly be by generation of peroxynitrite.

**Keywords:** dengue virus, macrophage cytotoxin, oxygen intermediates, cytotoxicity

The adverse effects of dengue type 2 virus (DV) on the immune system appear to be mediated by a DV-induced cytotoxic pathway which involves the production of a unique cytokine, cytotoxic factor (mCF), by the T-lymphocytes. It kills H-2A negative macrophages, T-helper cells, mast cells, etc. in 1 hour by inducing an influx of  $Ca^{2+}$  into the target cells

(reviewed by Chaturvedi 1986; Khanna *et al.* 1988; Dhawan *et al.* 1990; 1995) but recruits the H-2A positive macrophages to induce them to produce macrophage cytotoxin (CF2) which amplifies the effects of mCF (Gulati *et al.* 1983a).

CF2 is a heat and pH labile, biologically active protein. It has a molecular weight 10–12 kDa on SDS-PAGE. CF2 kills H-2A negative and H-2A positive macrophages and T-helper cells in 1 hour by inducing  $Ca^{2+}$  influx and adversely effecting leucocyte functions. Cytotoxicity of CF2 is dependent on the  $Ca^{2+}$  and production of nitrite

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(Gulati *et al.* 1983b; Dhawan *et al.* 1991; Mukerjee *et al.* 1996).

CF2 produces various other immunopathological effects including increased capillary permeability and damage to the blood–brain barrier by release of histamine (Khanna *et al.* 1988; Dhawan *et al.* 1991; 1994). Cytokines are among the various agents which stimulate phagocytic cells alone or in the presence of triggering stimuli to generate reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). Some cytokines stimulate ROI and RNI pathways; some of them, e.g. interleukin-1 (IL-1), interferon- $\gamma$  (IFN- $\gamma$ ), stimulate both (Ding *et al.* 1988; Blanco *et al.* 1995). Important lines of host defence against infectious agents, parasites and perhaps tumour cells are ROI and RNI (reviewed by Ding *et al.* 1988; Cao *et al.* 1995; Dugas *et al.* 1995). Appropriate stimulation of phagocytic cell surface receptors or intracellular signal transduction undergo respiratory bursts leading to the production of ROI which include superoxide anions, hydroxyl radicals, hydrogen peroxide, singlet oxygen and HOCl (reviewed by Cao *et al.* 1995). It has been reported that tissue injury by ROI is necrotic while that by RNI and peroxynitrite is apoptotic type (reviewed by Blanco *et al.* 1995). Spleen macrophages and T-lymphocytes produce nitrite following stimulation with CF or CF2 (Misra *et al.* 1996; Mukerjee *et al.* 1996). The present study was therefore undertaken to investigate the production of superoxide and H<sub>2</sub>O<sub>2</sub> and their role in the cytotoxic activity of CF2.

## Materials and methods

### Animals

Inbred Swiss albino mice aged 3–4 months obtained from the mouse colony maintained in this Department were used.

### Virus

The dengue type-2 virus (DV), strain P23085 obtained from the National Institute of Virology, Pune, was used in the form of infected adult mouse brain suspension (Chaturvedi *et al.* 1977).

### Preparation of macrophage cytotoxin (CF2)

Spleen cells of DV-infected mice were used as a source of mouse cytotoxic factor (mCF) and purified by the technique described (Khanna & Chaturvedi 1992). CF2 was prepared by the technique of Gulati *et al.* (1983a, b). Briefly, normal mouse peritoneal lavage cells were

adhered to 10-cm glass Petri dishes for 2 hours at 37°C in presence of 5% CO<sub>2</sub>. The non-adherent cells were washed off and the glass-adherent cell sheet was exposed to 5  $\mu$ g of mCF for 1 hour at 4°C. The cell sheet was washed three times and further incubated at 37°C for 24 hours with normal saline. The supernatant fluid and the cells were collected, sonicated and centrifuged at 2000 *g* for 10 minutes at 4°C and the supernatant was assayed for cytotoxic activity. The crude CF2 thus prepared was purified by high performance liquid chromatography (HPLC System, Pharmacia, Sweden) using a reverse-phase C18 column. Purified CF2 was freeze dried in Speed Vac (Savant Instruments Inc., USA). The amount of protein was estimated by the method of Lowry *et al.* (1951). The supernatant obtained from the glass-adherent cell sheet treated with normal spleen homogenate was similarly prepared (NF) and used in controls in place of CF2.

### Preparation of antisera against CF2 (CF2-As)

Antisera against CF2 was prepared in mice by giving 5  $\mu$ g CF2 protein emulsified with Freund's complete adjuvant (FCA; Sigma Chemical Co., St Louis, USA) intraperitoneally (i.p.). Fifteen days later a booster dose of 5  $\mu$ g CF2 mixed with Freund's incomplete adjuvant (FIA) was given. At the 30th day after the first dose, mice were bled. The sera were tested for neutralization of the cytotoxic activity of CF2.

### Preparation of spleen cell culture

Normal mouse spleens were harvested and teased out with the help of forceps in chilled Eagle's minimum essential medium (MEM) containing 10% fetal calf serum. A single-cell suspension was prepared and viable nucleated cells were counted using the trypan blue exclusion test. The cell count was adjusted to  $4 \times 10^6$  cells/ml and the cells were cultured by layering 4 ml of the cell suspension in 5-cm glass Petri dishes. The cultures were incubated at 37°C in presence of 5% CO<sub>2</sub>.

Groups of mice were inoculated with 20  $\mu$ g CF2 intravenously. At different time periods the spleens were removed, a single-cell suspension was prepared from individual mouse spleens and  $4 \times 10^6$  cells/ml were cultured for 24 hours at 37°C in presence of 5% CO<sub>2</sub>.

### Assay of cytotoxic activity

The cytotoxic activity of CF2 was assayed using normal mouse spleen cells as a target. Equal volumes of the test

solution and the target cells ( $2 \times 10^6$ /well) were mixed in a microtitre U-well Perspex plate and incubated at  $4^\circ\text{C}$  for 1 hour. Non-viable cells were counted using trypan blue dye and the percentage of non-viable cells was calculated after deduction of background non-viable cells.

#### Superoxide release assay

Superoxide was detected by its ability to reduce ferricytochrome C (Horse heart, Type III Sigma Chemical Co., St Louis, MO) (Babior *et al.* 1973; Cao *et al.* 1995). Briefly, spleen cells ( $4 \times 10^6$ /ml) were incubated in duplicate samples in the presence of  $80 \mu\text{M}$  ferricytochrome C in phenol red-free MEM at  $37^\circ\text{C}$  in the presence of 95% air and 5%  $\text{CO}_2$ . Simultaneously, experiments were conducted in the presence of varying concentrations (1–70  $\mu\text{g}$ ) of exogenous superoxide dismutase (Sigma, St Louis, MO). A control group of cells were inoculated with NF. For background values, cells were cultured identically in all respects except for the omission of the protein (CF2) under study. After completion of the incubations, reactions were terminated by placing the tubes in an ice-bath followed by centrifugation at 2000 *g* for 10 minutes at  $4^\circ\text{C}$  to remove spleen cells. The optical density of the supernatants was measured immediately at 540 nm in a Titertek Multiskan MC-plate reader (Biotek Instrument Inc., Burlington Ontario).

The amount of superoxide anion produced was calculated by subtracting the background values respectively and using the following formula: Nanomoles per well = (absorbance at 540 nm  $\times$  100)/6.3.

This takes into account the difference of extinction coefficients between oxidized and reduced cytochrome C, as well as the length of the light-path under the experimental conditions described (Pick & Mizel 1981). Finally, the values were multiplied to give the nanomoles of  $\text{O}_2^-$  produced per  $4 \times 10^6$  cells.

#### Assay for $\text{H}_2\text{O}_2$ release

Estimation of  $\text{H}_2\text{O}_2$  production was based on the  $\text{H}_2\text{O}_2$  mediated and horse-radish peroxidase-dependent oxidation of phenol red (Pick & Keisari 1981; von Asmuth & Buurman 1995). Briefly,  $4 \times 10^6$ /ml spleen cells were suspended in Earle's balanced salt solution (BSS) containing 0.5% phenol red and 8.5 U/ml horse-radish peroxidase (Type II, 170 purpurogallin units per mg solid, Sigma, St Louis, MO). CF2 was added to the cells and incubated for different time periods at  $37^\circ\text{C}$  in presence of 5%  $\text{CO}_2$  air. Cell cultures inoculated with NF were used as controls. For background values the tubes contained cells suspended in BSS. At the completion of

incubation, tubes were centrifuged for 10 minutes at 2000 *g*. The cell-free supernatants were made alkaline by the addition of 100  $\mu\text{l}$  of 1M NaOH and the absorbance was measured at 620 nm and compared with the standard curve generated with dilutions of a reference solution of  $\text{H}_2\text{O}_2$ . The experiments were repeated 3–5 times and the data have been presented, after deduction of the background values, as mean value  $\pm$  s.d. from 9 to 15 assays from individual mouse spleen cell cultures.

#### Statistical analysis

Data were analysed using Student's *t*-test. A *P*-value less than 0.05 was considered significant.

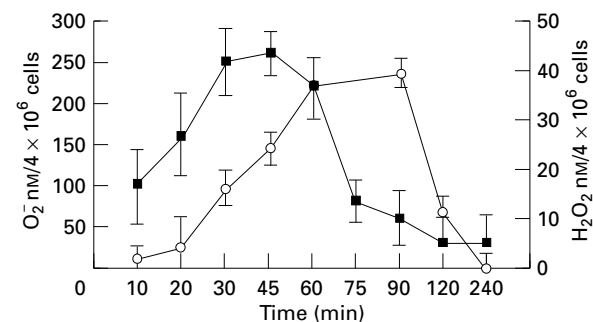
## Results

#### CF2-induced $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ release

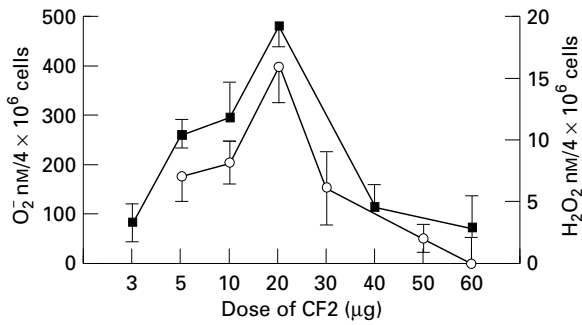
The effect of CF2 on  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  production was studied *in vitro* and *in vivo*. Time course studies have revealed that maximum release of  $\text{O}_2^-$  occurred at 45 minutes after deduction of background value ( $7 \pm 2.5 \text{ nm}$ ) and that of  $\text{H}_2\text{O}_2$  at 90 minutes (background value  $1 \pm 1.4 \text{ nm}$ ) after CF2 inoculation *in vitro* ( $P < 0.05$ ) (Figure 1) and *in vivo* (data not shown). The data presented in Figure 2 show a dose-dependent production of CF2-induced  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ . Maximum response was observed with 20  $\mu\text{g}$  CF2.

#### Effect of CF2-AS on $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ release

The effect of pretreatment of CF2 with CF2-AS on its capacity to induce  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  production was



**Figure 1.** CF2-induced production of  $\blacksquare$ ,  $\text{O}_2^-$  and  $\circ$ ,  $\text{H}_2\text{O}_2$  by mouse spleen cells *in vitro*. Normal mouse spleen cell cultures ( $4 \times 10^6$ /ml) were treated with 20  $\mu\text{g}$  CF2 at  $37^\circ\text{C}$ . At different time periods the culture supernatants were collected and assayed as described in Materials and methods. Results are presented after deduction of background values as mean  $\pm$  s.d. of 10 cultures.

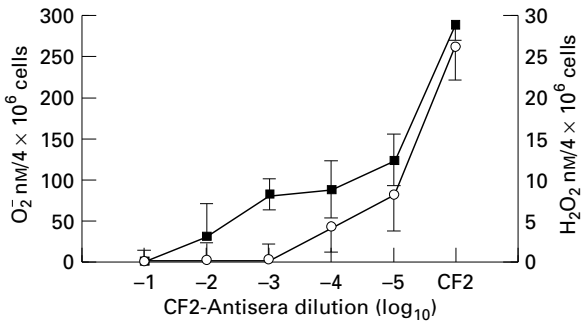


**Figure 2.** Dose-dependent  $O_2^-$ / $H_2O_2$  production by CF2. Normal mouse spleen cells ( $4 \times 10^6$  cells/ml) were inoculated with various doses of CF2 and cultured at  $37^\circ C$  in presence of 5%. The production of  $\blacksquare$ ,  $O_2^-$  and  $\circ$ ,  $H_2O_2$  was assayed at 45 and 90 minutes respectively in the supernatants as described in Materials and methods. Results are presented after subtraction of background values ( $4 \pm 1.2$  nm for  $O_2^-$ ,  $1 \pm 0.5$  nm for  $H_2O_2$ ) as mean values  $\pm$  s.d. from 8 cultures.

evaluated. CF2 was mixed with CF2-AS dilutions ( $10^{-1}$ – $10^{-6}$ ) for 1 hour at  $37^\circ C$  followed by addition of spleen cells. Data presented in Figure 3 show that production of  $O_2^-$ / $H_2O_2$  was inhibited by CF2-AS in a dose-dependent manner ( $P < 0.01$ ). In *in vivo* studies, the mice were inoculated with different doses of CF2-AS i.v. followed 24 hours later with CF2 inoculation i.v. A similar pattern of inhibition was seen as in *in vitro* treatment (data not shown).

**Role of SOD in  $O_2^-$  and  $H_2O_2$  release**

The dismutation of  $O_2^-$  by enzyme SOD results in generation of  $H_2O_2$  so the effect of exogenous SOD on



**Figure 3.** Effect of pretreatment of CF2 with anti-CF2-antisera (CF2-AS) on CF2-induced production of  $O_2^-$  and  $H_2O_2$ . CF2 was mixed with different dilutions of CF2-AS and incubated for 1 hour at  $37^\circ C$  followed by addition of normal mouse spleen cells ( $4 \times 10^6$ /ml) and further incubated. Culture supernatant was assayed for  $\blacksquare$ ,  $O_2^-$  and  $\circ$ ,  $H_2O_2$  production at  $37^\circ C$  for 45 and 90 minutes respectively as described in Materials and methods. Results are presented after deduction of background values as mean  $\pm$  s.d. from 6 cultures.

the generation of  $O_2^-$  and  $H_2O_2$  by spleen cells was investigated. The findings presented in Figure 4 show that SOD treatment inhibited the production of  $O_2^-$  but had no effect on  $H_2O_2$  production ( $P < 0.05$ ) in a dose-dependent manner after subtraction of background values ( $5 \pm 1.3$  nm for  $O_2^-$ ,  $2.5 \pm 1$  nm for  $H_2O_2$ ).

**Effect of SOD on cytotoxicity**

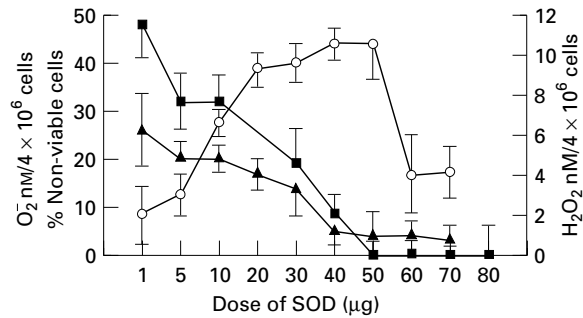
Cytotoxic activity of CF2 in the cell cultures was assayed by counting non-viable cells. Addition of increasing amounts of SOD to CF2-stimulated spleen cells resulted in the abrogation of cytotoxic activity in a dose-dependent manner. The data are presented in Figure 4 after deduction of background values ( $4 \pm 1$ ).

**Effect of  $Ca^{2+}$  channel blocking drugs on  $O_2^-$  and  $H_2O_2$  release**

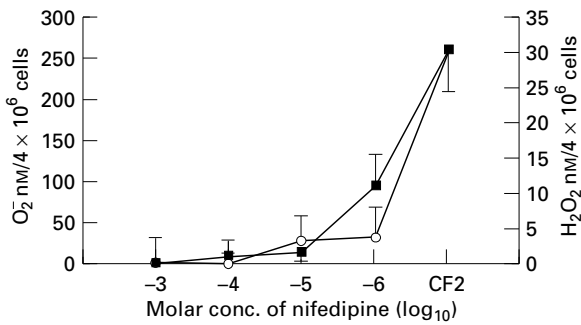
The role of calcium in  $O_2^-$  and  $H_2O_2$  release was investigated by using  $Ca^{2+}$  channel blockers, nifedipine and verapamil. Normal mouse spleen cells pretreated with nifedipine or verapamil for 30 minutes at  $37^\circ C$  were inoculated with CF2. The data presented here showed that  $O_2^-$ / $H_2O_2$  release was inhibited by these drugs. Maximum inhibition occurred with  $10^{-3}$  M concentration of both verapamil and nifedipine ( $P < 0.025$ ) (Figures 5 and 6).

**Discussion**

The findings of the present study show production of  $O_2^-$

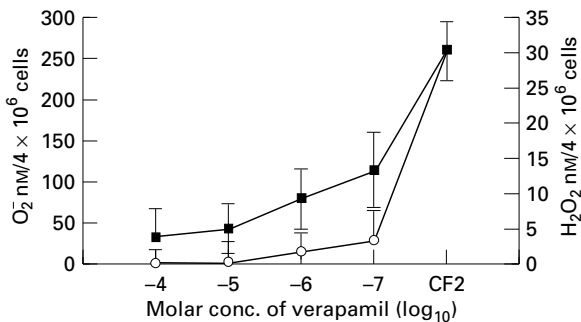


**Figure 4.** Effect of exogenous superoxide dismutase (SOD) on the production of  $\blacksquare$ ,  $O_2^-$  and  $\circ$ ,  $H_2O_2$ . Normal mouse spleen cells ( $4 \times 10^6$ /ml) were cultured with different doses of SOD followed by inoculation of  $20 \mu g$  CF2. After incubation for 45 and 90 minutes at  $37^\circ C$  the culture supernatants were assayed for the production of  $O_2^-$ / $H_2O_2$  and  $\blacktriangle$ , cytotoxicity respectively as described in Materials and methods. Results are presented after deduction of background values as mean  $\pm$  s.d. from 8 cultures.



**Figure 5.** Inhibition in O<sub>2</sub><sup>-</sup>/H<sub>2</sub>O<sub>2</sub> production by treatment with nifedipine. Normal mouse spleen cells (4 × 10<sup>6</sup>/ml) were pretreated for 30 minutes with different molar concentrations of nifedipine. After 30 minutes CF2 was added to the cells and further incubated for 45 and 90 minutes at 37°C in presence of 5% air. ■, O<sub>2</sub><sup>-</sup> and ○, H<sub>2</sub>O<sub>2</sub> production were assayed as described in Materials and methods. The results are presented after deduction of background values (6 ± 2 nM for O<sub>2</sub><sup>-</sup>, 1.5 ± 0.3 nM for H<sub>2</sub>O<sub>2</sub>) as mean ± s.d. from 8 cultures.

and H<sub>2</sub>O<sub>2</sub> by the mice spleen cells following treatment with DV-induced cytokine, CF2. Inhibition of the cytotoxic activity and the production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> by pretreatment of CF2 with anti-CF2-antisera confirmed that the cytokine was responsible for these effects. CF2 is cytotoxic to a selected group of cells, namely macrophages, T-helper cells, mast cells, etc. but has no effect on various other cell lines (Chaturvedi *et al.* 1987; Khanna *et al.* 1988). The findings presented here show that treatment of the cells with SOD inhibited the production of O<sub>2</sub><sup>-</sup> and the cytotoxic activity of CF2 while production of H<sub>2</sub>O<sub>2</sub> was enhanced. These findings suggest that killing



**Figure 6.** Inhibition in O<sub>2</sub><sup>-</sup>/H<sub>2</sub>O<sub>2</sub> production by treatment with verapamil. Normal mouse spleen cells (4 × 10<sup>6</sup>/ml) were pretreated for 30 minutes with different molar concentrations of nifedipine. After 30 minutes CF2 was added to the cells and further incubated for 45 and 90 minutes at 37°C in presence of 5% air. ■, O<sub>2</sub><sup>-</sup> and ○, H<sub>2</sub>O<sub>2</sub> production were assayed as described in Materials and methods. The results are presented after deduction of background values (4 ± 1.3 nM for O<sub>2</sub><sup>-</sup>, 2 ± 1.2 nM for H<sub>2</sub>O<sub>2</sub>) as mean ± s.d. from 8 cultures.

of the target cells by CF2 was mediated via O<sub>2</sub><sup>-</sup> and not via the H<sub>2</sub>O<sub>2</sub>.

The roles of various components, which include protein kinase C, phospholipase C, etc., in the respiratory burst have been described. Ca<sup>2+</sup> also plays an important role in the respiratory burst (Morel *et al.* 1991). The production, secretion and cytotoxic activity of CF2 is Ca<sup>2+</sup> dependent. The Ca<sup>2+</sup> channel blocking drugs, nifedipine and verapamil, inhibit Ca<sup>2+</sup> ion influx by blocking passive slow channels (Braunwald 1982; New & Trautwein 1972). It has been shown that an influx of Ca<sup>2+</sup> (measured by uptake of <sup>45</sup>Ca) induced by treatment of the spleen cells with 5 μg CF is inhibited to the extent of 85% with 10<sup>-3</sup> M concentrations of either verapamil or nifedipine. At higher doses both drugs are toxic to the cells (Dhawan *et al.* 1990). The O<sub>2</sub><sup>-</sup> production in the present model was Ca<sup>2+</sup>-dependent as shown by inhibition of its production by pretreatment with the calcium channel blocking drugs, nifedipine or verapamil.

We have observed production of NO<sub>2</sub><sup>-</sup> by the spleen cells of mice following inoculation of dengue virus or mCF/CF2 (Misra *et al.* 1996; Mukerjee *et al.* 1996). The production of O<sub>2</sub><sup>-</sup> as described here, and the production of NO reported earlier (Misra *et al.* 1996), have many similarities. It has been reported that IFN-γ is the only one among the 12 cytokines to induce production of both NO<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> by independent pathways (Ding *et al.* 1988). Recently, Dugas *et al.* (1995) have proposed that a ligand binding to the cell surface receptor may stimulate nitric oxide synthase and NADPH oxidase pathways resulting in the production of NO and O<sub>2</sub><sup>-</sup>.

We have observed that macrophages and lymphocytes treated with mCF/CF2 show fragmentation of DNA and electron microscopic appearance of apoptosis (Nath *et al.* 1983; A. Misra *et al.*, unpublished data). The cascade of events during the production, and the mechanisms of action, of mCF/CF2 have been presented elsewhere (Mukerjee & Chaturvedi 1995). On dismutation O<sub>2</sub><sup>-</sup> produces H<sub>2</sub>O<sub>2</sub> or reacts with NO to produce highly toxic peroxynitrite (ONOO<sup>-</sup>). The NO<sub>2</sub><sup>-</sup> production and cytotoxicity of mCF/CF2 is inhibited by treatment with (i) N<sup>G</sup>-monomethyl-L-arginine (NMMA), an inhibitor of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> (Misra *et al.* 1996; Mukerjee *et al.* 1996) while treatment with SOD reduces cytotoxicity but has no effect on NO<sub>2</sub><sup>-</sup> production (data not shown) and (ii) SOD (present study), which dismutates O<sub>2</sub><sup>-</sup>, inhibited O<sub>2</sub><sup>-</sup> release and cytotoxicity but did not affect the H<sub>2</sub>O<sub>2</sub> or NO<sub>2</sub> release. This indicated that production of both NO<sub>2</sub><sup>-</sup> and O<sub>2</sub><sup>-</sup> is required for the cytotoxic activity of CF2. Both pathways run concomitantly; therefore, it is proposed that the mechanism of target cell killing by CF2 may be via formation of peroxynitrite. But we still do not

know why or how CF2 kills only a very selected group of cells.

## References

- BABIOR B.M., KIPNES R.S. & CURNUTTE J.T. (1973) Production by leukocytes of superoxide. A potential bactericidal agent. *J. Clin. Invest.* **52**, 741–744.
- BLANCO F.J., OCHS R.L., SCHWARZ H. & LOTZ M. (1995) Chondrocyte apoptosis induced by nitric oxide. *Am. J. Path.* **146**, 75–85.
- BRAUNWALD E. (1982) Mechanism of action at calcium channel blocking agents. *N. Engl. J. Med.* **307**, 1618–1627.
- CAO D., MIZUKAMI I.F., GARNI-WAGNER B.A., KINDZELSKII A.L., TODD III R.F., BOXER L.A. & PETTY H.R. (1995) Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. *J. Immunol.* **154**, 1817–1829.
- CHATURVEDI U.C. (1986) Virus-induced cytotoxic factor in AIDS and dengue. *Immunol. Today* **7**, 159.
- CHATURVEDI U.C., NAGAR R., GULATI L. & MATHUR A. (1987) Variable effects of dengue virus-induced cytotoxic factors on different subpopulations of macrophages. *Immunology* **61**, 297–303.
- CHATURVEDI U.C., TANDON P. & MATHUR A. (1977) Effect of immunosuppression on dengue infection in mice. *J. Gen. Virol.* **36**, 449–458.
- DHAWAN R., CHATURVEDI U.C., KHANNA M., MATHUR A., TEKWANI B.L., PANDEY V.C. & RAI R.N. (1991) Obligatory role of Ca<sup>2+</sup> in the cytotoxic activity of dengue virus-induced cytotoxin. *Int. J. Exp. Path.* **72**, 31–39.
- DHAWAN R., KHANNA M., CHATURVEDI U.C. & MATHUR A. (1990) Effect of dengue virus-induced cytotoxin on capillary permeability. *J. Exp. Path.* **71**, 83–88.
- DHAWAN R., KHANNA M., CHATURVEDI U.C. & MATHUR A. (1994) Dengue virus-induced cytokine damages the blood–brain barrier. *Proc. Ind. Natl Sci. Acad.* **B60**, 45–52.
- DHAWAN R., MUKERJEE R., CHATURVEDI U.C. & KHARE S.D. (1995) Flow cytometric analysis of T4:T8 splenic cells during dengue virus infection of mice. *Ind. J. Exp. Biol.* **33**, 816–823.
- DING A.H., NATHAN C.F. & STUEHR D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol.* **141**, 2407–2412.
- DUGAS B., MOSSALAYI M.D., DAMAIS C. & KOLB J.P. (1995) Nitric oxide production by human monocytes: evidence for a role of CD23. *Immunol. Today* **16**, 574–580.
- GULATI L., CHATURVEDI U.C. & MATHUR A. (1983a) Dengue virus-induced cytotoxic factor induces macrophages to produce a cytotoxin. *Immunology* **49**, 121–130.
- GULATI L., CHATURVEDI U.C. & MATHUR A. (1983b) Characterization of the cytotoxin produced by macrophages in response to dengue virus-induced cytotoxic factor. *Br. J. Exp. Path.* **64**, 185–190.
- KHANNA M. & CHATURVEDI U.C. (1992) Purification and aminoterminal sequence of the dengue virus-induced cytotoxic factor. *Int. J. Exp. Path.* **73**, 43–49.
- KHANNA M., CHATURVEDI U.C. & MATHUR A. (1988) Abrogation of helper T cells by dengue virus-induced cytotoxic factor. *Curr. Sci.* **57**, 411–414.
- LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J. (1951) Protein measurement with follin's phenol reagents. *J. Biochem.* **193**, 265–275.
- MISRA A., MUKERJEE R. & CHATURVEDI U.C. (1996) Production of nitrite by dengue virus-induced cytotoxic factor. *Clin. Exp. Immunol.* **104**, 406–411.
- MOREL F., DOUSSIERE J. & VIGNAIS V. (1991) The superoxide-generating oxidase of phagocytic cells. *Eur. J. Biochem.* **201**, 523–546.
- MUKERJEE R. & CHATURVEDI U.C. (1995) Cytokine antagonism by active vaccination. *Curr. Sci.* **69**, 900–902.
- MUKERJEE R., MISRA A. & CHATURVEDI U.C. (1996) Dengue virus-induced cytotoxin releases nitrite by spleen cells. *Int. J. Exp. Path.* **77**, 45–51.
- NATH P., TANDON P., GULATI L. & CHATURVEDI U.C. (1983) Histological and ultrastructural study of spleen during dengue virus infection of mice. *Ind. J. Med. Res.* **78**, 83–90.
- NEW W. & TRAUTWEIN W. (1972) The ionic nature at slow inward current and its relation to contraction. *Pflüger's Arch.* **334**, 24–28.
- PICK E. & KEISARI Y. (1981) Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages – induction by multiple nonphagocytic stimuli. *Cell. Immunology* **59**, 301–318.
- PICK E. & MIZEL D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immuno. Methods* **46**, 211–215.
- VON ASMUTH E.J.U. & BUURMAN W.A. (1995) Endothelial cell associated platelet – activating factor (PAF), a costimulatory intermediate in TNF- $\alpha$ -induced H<sub>2</sub>O<sub>2</sub> release by adherent neutrophil leukocytes. *J. Immunol.* **154**, 1383–1390.