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Obligatory role of Ca^{2+} in the cytotoxic activity of dengue virus-induced cytotoxin

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Summary. The role of calcium ions (Ca^{2+}) in the cytotoxic activity of the dengue type 2 virus (DV)-induced macrophage (Mϕ) cytotoxin (CF_2) was investigated in the present study. The findings show that CF_2 prepared in Ca^{2+}-free medium had no cytotoxic activity on normal mouse spleen cells suspended in Ca^{2+}-free medium but killed the cells suspended in the medium with Ca^{2+}. Substitution with calcium chloride restored the cytotoxic activity of CF_2 the optimal dose being 10^{-4} M concentration. CF_2 induced an influx of Ca^{2+}, as assayed by uptake of radiolabelled calcium chloride (^{45}Ca), in the susceptible target cells, viz. Mϕ and T lymphocytes. The cytotoxic activity of CF_2 as well as the CF_2-induced influx of ^{45}Ca was inhibited by treatment of the target cell with the calcium channel blocking drugs verapamil and nifedipine. Thus, the presence of Ca^{2+} is obligatory for the cytotoxic activity of CF_2 and cell death is associated with increased intracellular Ca^{2+}.

Keywords: dengue virus, cytotoxin, Ca^{2+}, calcium channel blockers, macrophages, T cells

Progressive damage of spleen occurs during dengue type 2 virus (DV) infection of mice which is characterized by hypocellularity, disorganization and atrophy of follicles, and necrosis of cells. This damage has been attributed to the cytotoxic pathway induced in DV-infected mice which involves generation of a cytotoxic factor (CF) by T lymphocytes. CF induces I-A positive macrophages to produce a cytotoxin (CF_2). CF_2 is a biologically active protein which kills various types of cell and produce various immunopathological effects including increased capillary permeability (Dhawan *et al*, 1990; Khanna *et al*, 1989, 1990; reviewed by Chaturvedi 1986, 1989).

The types of cell which are adversely affected by CF_2 are known but not the precise mechanism of cell injury. Some of the mechanisms which have been implicated in cell cytotoxicity depend on calcium ions (Ca^{2+}), while others do not. Disruption of cytoskeleton, DNA fragmentation and extensive damage to other subcellular components leading to cell death have been associated with a sustained increase in cytosolic Ca^{2+} (reviewed by Orrenius *et al*, 1989). The present study was undertaken to investigate

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the role of Ca^{2+} in cytotoxic activity of CF_2 and demonstrates that the presence of Ca^{2+} is obligatory for the cytotoxic activity of CF_2 and that cell death is associated with an influx of Ca^{2+} into the susceptible target cells, namely T lymphocytes and macrophages.

Materials and methods

Animals

The study was carried out on inbred Swiss albino mice aged 2–4 months obtained from the colony maintained in this Department.

Virus

The brain of adult mouse, infected with dengue type 2 virus (DV), strain P23085, was used as a source of the virus (Chaturvedi *et al*, 1977).

Reagents

Verapamil and nifedipine were purchased from Sigma Chemicals Co., St Louis. The stock solutions of the drugs were prepared and used on the same day. Radiolabelled calcium chloride (^{45}Ca) was obtained from Bhabha Atomic Research Centre, Bombay and had a specific activity of 77.4 mCi/g. It was appropriately diluted and used in doses of 1–3 μCi/ml of the cell suspension.

Preparation of CF_2

CF_2 was prepared from the mouse peritoneal macrophage monolayers in 5-cm glass Petri dishes by the technique of Gulati *et al.* (1983a). Normal mouse peritoneal macrophage culture supernatants were similarly prepared and used in control groups.

Preparation of target cells

A single-cell suspension prepared from normal mouse spleen was treated with Tris ammonium chloride solution to haemolyse the red blood cells. The cells were washed, suspended in minimum essential medium (MEM) containing 10% bovine serum and the viable nucleated cells were counted using trypan blue dye (Chaturvedi *et al*, 1978). T lymphocyte subpopulations were enriched by filtration through glass-wool and nylon-wool columns (Julius *et al*, 1973; Trizio & Cudkowicz 1974). The purity of the cell population was screened as described (Tandon *et al*, 1979).

Mouse peritoneal cells were used to obtain a Mϕ-enriched subpopulation. The peritoneal cavity was lavaged with 5 ml heparinized MEM and the cells obtained were layered on 5-cm glass Petri dishes and incubated at 37°C in the presence of 5% CO_2 for 90 min (Chaturvedi *et al*, 1982). Cells non-adherent to glass were removed by repeated washing and the glass-adherent cells were scrapped off with the help of a rubber-tipped policeman rod and suspended in MEM after washing thrice. About 98% of these cells phagocytosed latex particles and were therefore considered as a Mϕ enriched subpopulation (Chaturvedi *et al*, 1982).

Assay of cytotoxic activity of CF_2

Normal mouse spleen cells were used as targets to assay the cytotoxic activity of the preparations. In some experiments a Mϕ or T cell-enriched subpopulation was used. The assay of cytotoxic activity is described elsewhere (Chaturvedi *et al*, 1980). Briefly, the target cells (2×10^6/ml) and the test solution were mixed in equal volumes in a micro-titre U-well Perspex plate and incubated at 4°C for 1 h. Viable cells were counted using trypan blue dye and the percentage of non-viable cells was calculated. The data has been presented after deducting background non-viable cells.

Assay of ^{45}Ca uptake

The Ca^{2+} influx into the target cells was assayed by measuring the uptake of radiolabelled calcium chloride (^{45}Ca) by the tech-

nique of Freedman and Khan (1979) and Birx *et al* (1984). The cells (1×10^7 cells/ml) were incubated with 1–3 μCi of ^{45}Ca/ml at 37°C for 30 min. The cells were transferred to an ice-bath and washed with chilled 0.15 M PBS containing 1.8 mM $CaCl_2$ to remove extracellular calcium. The cells were transferred to a tenplace filtration assembly (Millipore Co., Bedford, MA01730) and the cells harvested onto a fibreglass filter were washed repeatedly with chilled 0.15 M PBS containing 1.8 mM $CaCl_2$. The filters were transferred to glass vials containing 10 ml scintillant fluid and the radioactivity was determined with a LKB (Wallac) beta-counter. The counts per min (c.p.m.) in control and experimental groups were expressed after deducting background c.p.m. The background c.p.m. was obtained from tubes containing medium, CF_2, drugs and ^{45}Ca, without cells. The control group data consisted of c.p.m. in cells and ^{45}Ca without CF_2 or drugs. The experiments were set up in triplicate and were repeated at least three times. Mean values ± standard error of the mean have been presented. The data was analysed statistically using Student's *t*-test for *P* value. A *P* value of less than 0.05 was considered significant.

Results

The presence of calcium is essential for the cytotoxic activity of CF_2

This experiment was made to investigate if the presence of Ca^{2+} is essential for the mediation of cytotoxic activity of CF_2 on the target cells. CF_2 prepared in Ca^{2+} -free medium was assayed for cytotoxic activity on target spleen cells suspended in Ca^{2+} -free medium. The control groups included were

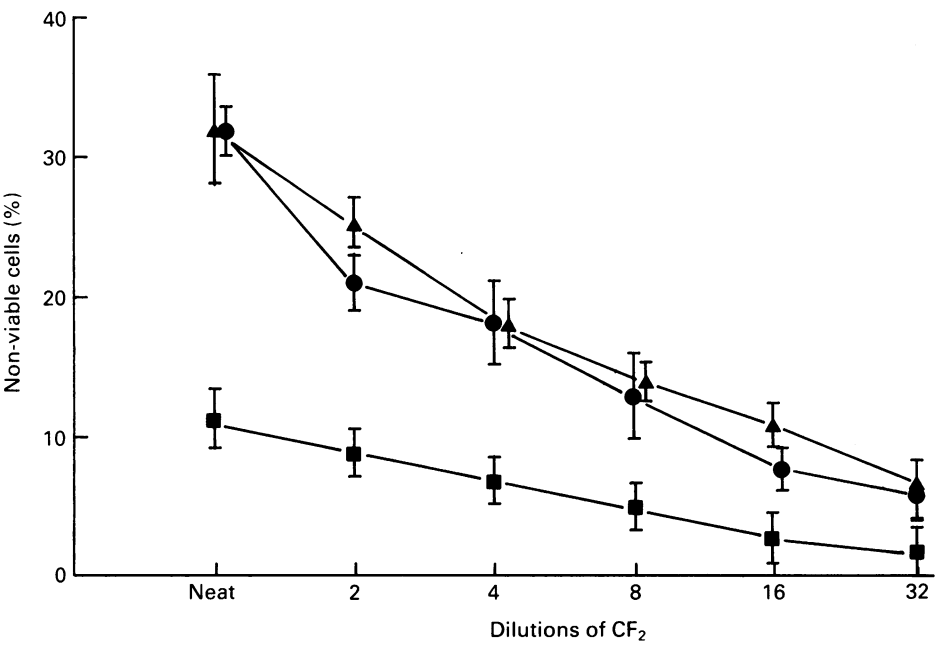

Fig. 1. Effect of absence of Ca^{2+} from the milieu on the cytotoxic activity of CF_2. The activity of CF_2 was assayed using ■, CF_2 prepared in Ca^{2+}-free medium and the target cells suspended in Ca^{2+}-free medium; ▲, CF_2 prepared in Ca^{2+}-free medium and the target cells suspended in the medium containing Ca^{2+}; and ●, CF_2 and the target cells both prepared in medium containing Ca^{2+}.

(i) CF_2 assayed using target cells suspended in MEM (containing Ca^{2+}) and (ii) CF_2 prepared in the usual way (in medium containing Ca^{2+}) assayed on target cells suspended in MEM. The data presented in Fig. 1 demonstrate that the cytotoxicity of CF_2 was significantly reduced when Ca^{2+} was absent from both the CF_2 preparation and the test system. The activity of CF_2 prepared in Ca^{2+}-free medium but tested on target cells suspended in the presence of Ca^{2+} was similar to that in which Ca^{2+} was present in both preparations. Thus the findings show that calcium is essential for mediation of cytotoxic activity by CF_2.

Substitution of calcium restores the cytotoxic activity of CF_2

The following experiment was performed to investigate the effect of substitution of Ca^{2+} in the test system where CF_2 and the target cells were prepared in Ca^{2+}-free medium. Target cells suspended in Ca^{2+}-free medium were treated with graded molar concentrations of $CaCl_2$ for 1 h at 37°C. To these cells CF_2 (undiluted) prepared in Ca^{2+}-free medium was added and the mixture was further incubated at 4°C for 1 h; the non-viable cells were then counted. It was observed that in the presence of 10^{-6}–10^{-10} M $CaCl_2$, the cytotoxic activity of CF_2 was negligible. Cytotoxic activity appeared with 10^{-5} M $CaCl_2$ reaching levels similar to that of controls with 10^{-4} M concentrations (Fig. 2). Higher concentrations of $CaCl_2$ (10^{-1} or 10^{-3} M) killed large numbers of the target cells without addition of CF_2 so a higher percentage of non-viable cells was seen.

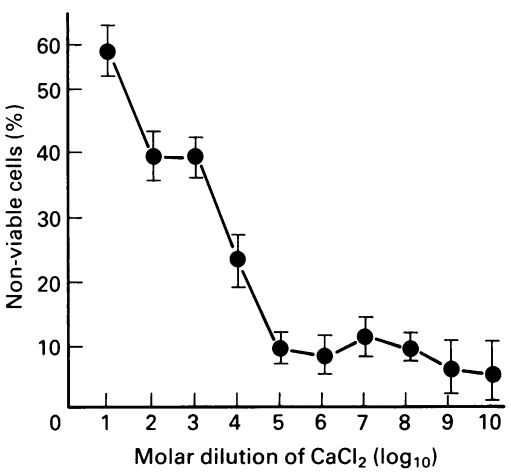

Fig. 2. Effect of substitution of calcium on the cytotoxic activity of CF_2. The target cells prepared in Ca^{2+}-free medium were treated with various concentrations of $CaCl_2$ and then used to assay the cytotoxic activity of CF_2 prepared in Ca^{2+}-free medium.

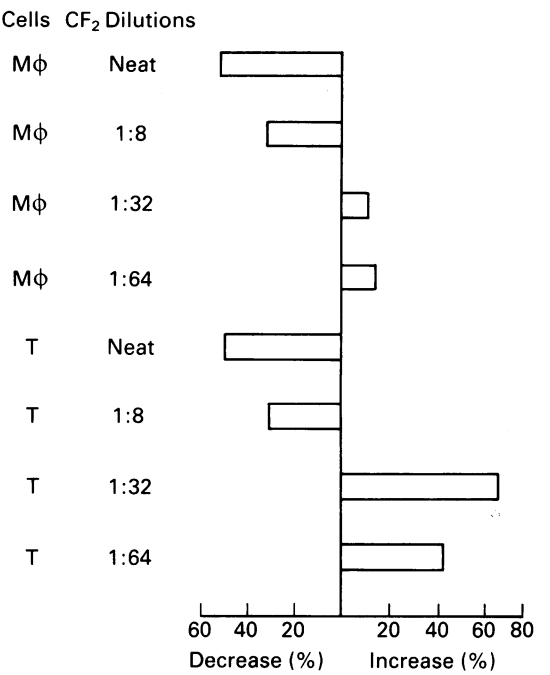

Fig. 3. Effect of CF_2 on Ca^{2+} influx into the target cells. Enriched subpopulations of macrophages (Mϕ) and T lymphocytes (T) were treated with various doses of CF_2 and the intracellular calcium was measured by estimation of radiolabelled calcium chloride (^{45}Ca). The percentage increase/decrease of ^{45}Ca was calculated as follows:

$$\%\ \text{increase/decrease} = 100 \times \frac{\text{C.p.m. of cells treated with } CF_2 - \text{background c.p.m.}}{\text{c.p.m. of untreated cells} - \text{background c.p.m.}} - 100$$

Effect of CF_2 on calcium influx into target cells

The above experiments showed that the presence of Ca^{2+} in the milieu is essential for the activity of CF_2. The present set of experiments were carried out to investigate if target cell killing was due to enhanced Ca^{2+}influx into the target cells. Enriched subpopulations of Mϕ and T lymphocytes were used and intracellular calcium was measured by the estimation of radiolabelled ^{45}Ca.

Four doses of CF_2 were used starting from undiluted CF_2, which killed most of the susceptible target cells, to a 1:64 dilution which killed a negligible number of the cells. CF_2 was added to 2×10^6 target cells and incubated at 37°C for 15 min followed by the addition of 100 μl of ^{45}Ca and a further incubation at 37°C for 30 min. The cells were then washed with 1.8 mM cold calcium chloride and were processed for intracellular ^{45}Ca estimation. The findings, summarized in Fig. 3, show that with a 1:32 dilution of CF_2 the ^{45}Ca uptake by Mϕ was increased by 10% while with 1:64 dilution the increase was 13%. Undiluted CF_2 quickly killed all the susceptible target cells so intracellular ^{45}Ca was reduced.

In another similar experiment T cell enriched subpopulations were used as targets. The findings presented in Fig. 3 show that treatment with 1:32 diluted CF_2 increased the intracellular ^{45}Ca by 65%. The increase was 42% with 1:64 dilution of CF_2. In contrast, treatment with undiluted CF_2 or CF_2 diluted 1:8 resulted in a decrease of intracellular Ca^{2+} by 30–49% (Fig. 3).

Effect of calcium channel blocking drugs on cytotoxicity of CF_2

Calcium ion influx into the cells can be inhibited by certain drugs through blocking passive slow channels (Braunwald 1982). In the present experiment, therefore, the effect of various calcium channel blocking drugs on the cytotoxic activity of CF_2 was investigated. The drugs used were verapamil and nifedipine.

Spleen cells were treated with various doses of different calcium channel blocking drugs at 37°C for 30 min. The cells were centrifuged at 2000 *g* for 10 min, resuspended in MEM and used as targets to assay the cytotoxic activity of CF_2. For the controls, drug-treated cells or untreated spleen cells treated with CF_2 were included. The data presented in Fig. 4 show that CF_2 killed 33±5% untreated target spleen cells. The cytotoxic activity of CF_2 was inhibited to a similar extent in a dose-dependent manner by treatment of the target cells with both the drugs. The inhibition was 67% with 10^{-3} M concentration of verapamil and 30% with 10^{-7} M. Treatment of target cells with nifedipine produced similar results (statistically insignificant). The drugs by themselves did not kill the target cells in the concentrations used in the test (data not presented here).

It has been observed that CF_2 kills T cells and Mϕ but has no effect on B cells (Gulati *et al*, 1983b); therefore, in the next experiment enriched T cells and Mϕ subpopulations were used in place of total spleen cells as

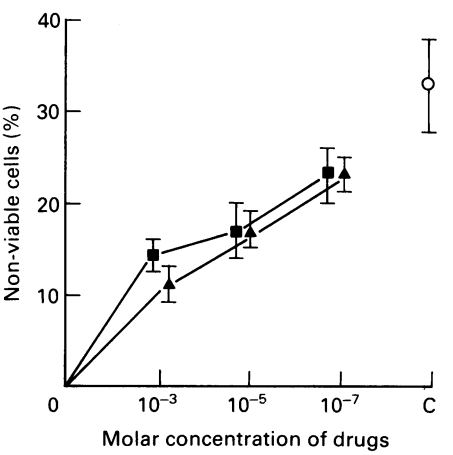

Fig. 4. Effect of calcium channel blocking drugs on the cytotoxic activity of CF_2. Normal mouse spleen cells were treated with various doses of ▲, verapamil ■, nifedipine, or C, O, remained untreated for control. The cytotoxic activity of CF_2 was assayed using these cells as targets.

targets to study the effect of calcium channel blocking drugs on the cytotoxic activity of CF_2. The data presented in Fig. 5a show that CF_2 killed $24 \pm 2\%$ of cells in an untreated T-enriched subpopulation. The cytotoxicity of CF_2 was inhibited in a dose-dependent manner by treatment of Mϕ with verapamil or nifedipine. The proportion of cells killed was $9 \pm 2\%$ by treatment with 10^{-3} M and $17 \pm 3\%$ with 10^{-7} M verapamil. The cytotoxicity of CF_2 on Mϕ-enriched subpopulation was similarly inhibited in a dose-dependent manner by treatment of the cells with verapamil or nifedipine (Fig. 5b).

Effect of calcium channel blocking drugs on CF_2-induced influx of ^{45}Ca in target cells

The data from previous experiments show that CF_2 induced an increase of intracellular

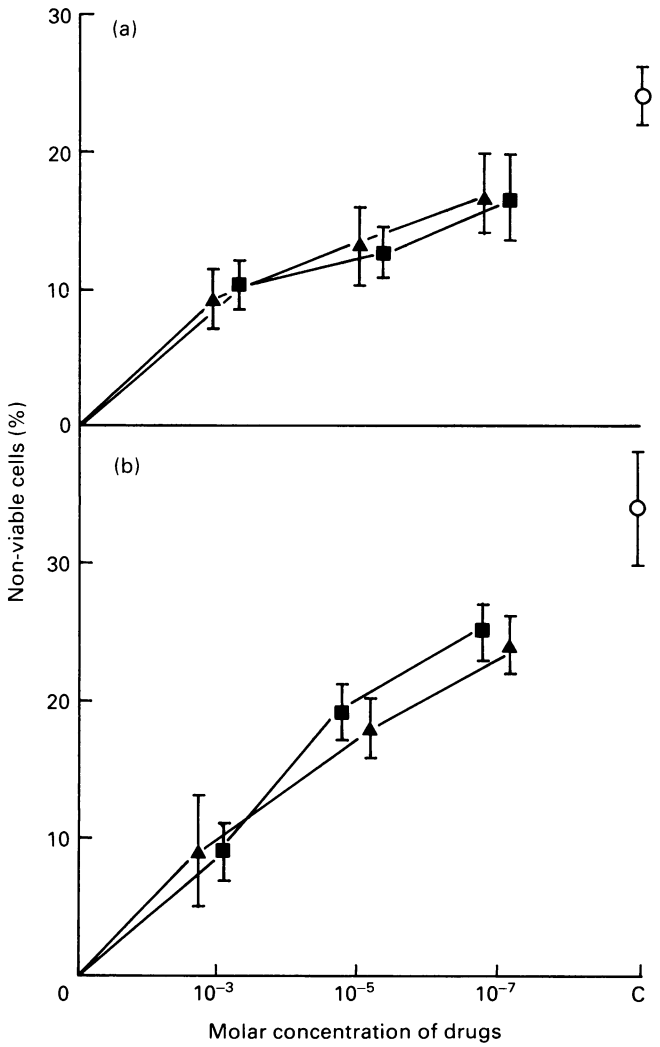

Fig. 5. Effect of calcium channel blocking drugs on the cytotoxic activity of CF_2 on enriched subpopulations of a, T lymphocytes and b, macrophages. The remaining description is as in Fig. 4.

calcium in Mϕ and T cells. Therefore, the effect of calcium channel blocking drugs on the CF_2-induced Ca^{2+} influx in an enriched subpopulation of Mϕ and T lymphocytes was investigated using verapamil and nifedipine. The target cells (2×10^6) were treated with various doses of verapamil or nifedipine for 30 min at 37°C followed by treatment with CF_2 (1 : 32 dilution) and a further incubation for 15 min at 37°C. ^{45}Ca was added to the cell mixture which was then incubated at 37°C for 30 min. The cells were washed with 1.8 mM of cold calcium chloride and prepared for the assay of intracellular ^{45}Ca.

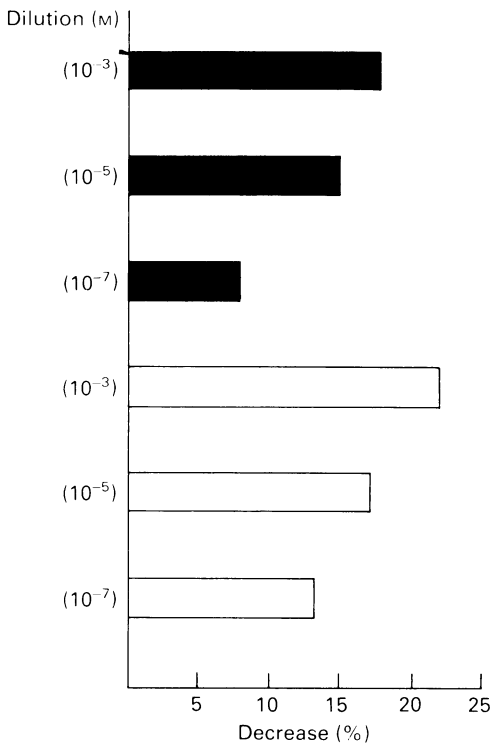

Fig. 6. Effect of calcium channel blocking drugs ■, verapamil; □, nifedipine on CF_2-induced influx of ^{45}Ca into enriched subpopulations of macrophages. The percentage decrease of intracellular ^{45}Ca was calculated as follows:

$$\% \text{ decrease} = 100 - \frac{\text{C.p.m. of cells treated with drug and } CF_2 - \text{c.p.m. of cells treated with drug}}{\text{C.p.m. of cells treated with } CF_2 - \text{c.p.m. of untreated cells.}} \times 100$$

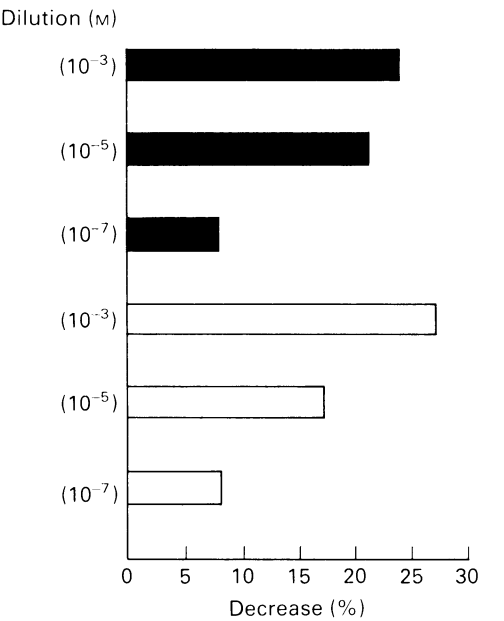

Fig. 7. Effect of calcium channel blocking drugs ■, verapamil; □, nifedipine, on CF_2-induced influx of ^{45}Ca into enriched subpopulations of the T cells. For details see legend to Fig. 6.

The findings summarized in Fig. 6 show that pretreatment of Mϕ with 10^{-3} M of verapamil inhibited influx of CF_2-induced calcium by 18% whereas with 10^{-5} or 10^{-7} M of verapamil, the inhibition was 15 or 8%. Similarly, with 10^{-3} M nifedipine the decrease in calcium uptake was 22%, while with 10^{-5} or 10^{-7} M of the drug the decrease in calcium was 17 or 13%.

When the above experiment was repeated using a T-cell-enriched subpopulation as target, similar findings were obtained (Fig. 7). A dose-dependent decrease in calcium influx was observed by treatment with the two drugs. Thus with 10^{-3} M verapamil the decrease was 29% while with 10^{-7} M it was 8%. A similar effect was observed by pretreatment of the cells with nifedipine (Fig. 7).

Discussion

The main findings of the present study are that the cytotoxic activity of CF_2 is inhibited

by the absence of calcium from the milieu and by pretreatment of target cells with calcium channel blocking drugs such as verapamil or nifedipine. On the other hand, substitution of Ca^{2+} restored the cytotoxic activity of CF_2, the optimum dose of calcium chloride being 10^{-4} M. Therefore, killing of susceptible target cells by CF_2 appeared to be a calcium-dependent phenomenon.

Cytotoxic effector mechanisms may be Ca^{2+}-dependent or Ca^{2+}-independent; for example, the cytotoxic activity of lymphotoxins, tumour necrosis factor, serine esterases and perforins is Ca^{2+}-independent. Some clones of cytotoxic T lymphocytes are Ca^{2+}-dependent for their activity, others are not (reviewed by Clark 1988). CF_2 differs from these cytotoxic molecules in its obligatory requirement of the presence of Ca^{2+} for its activity.

A sustained increase of Ca^{2+} in the cytosol of a cell may activate cytotoxic mechanisms which disrupt the cytoskeleton, fragment DNA and damage other subcellular organelles causing cell death. The Ca^{2+}-dependent processes which lead to cell death are the activation of proteases, phospholipases and endonucleases, ATP depletion, and thiol modification (reviewed by Orrenius *et al*, 1989). It was observed in the present study that CF_2 induced an influx of Ca^{2+} in the Mϕ and T lymphocytes in a dose-dependent manner, the maximum increase in intracellular ^{45}Ca in T cells being 65% compared to untreated control cells. We have recently shown an increase in capillary permeability by inoculation of CF_2 in mice through release of histamine (Dhawan *et al*, 1990). It appears that CF_2-induced increase in Ca^{2+} in cells (which are a source of vasoactive mediators), as described in the present study, results in their degranulation and release of histamine etc. increasing the capillary permeability.

Verapamil and nifedipine inhibit Ca^{2+} influx into cells through blockade of passive slow channels (New & Trautwein 1972; Beeler & Reuter 1980; Braunwald 1982). The data presented here show inhibition of the cytotoxic activity of CF_2 in a dose-dependent manner by pretreatment of the target cells with the calcium channel blockers. The effect of each of the drugs used, viz. verapamil and nifedipine, was similar. The effect of these drugs on the CF_2-induced Ca^{2+} influx in Mϕ and T cells was investigated. It was observed that both drugs inhibited ^{45}Ca influx to the extent of 22–29% in both types of cells.

CF_2 selectively kills Mϕ and DV-induced helper T cells (Th) but has no effect on other cells (Gulati *et al*, 1983b; Khanna *et al*, 1989). The determinant of this selectivity of CF_2 are not known. It has been shown that $CD4^+$ and $CD8^+$ cells have a different type of K^+ channels, and K^+ and Ca^{2+} influx may be coordinately regulated in lymphocytes (reviewed by Klaus 1988). It is therefore likely that Ca^{2+} channels which could be activated by CF_2 are present only on Th and Mϕ, thus determining its selectivity of action.

The findings of the present study thus demonstrate that the presence of Ca^{2+} is obligatory for the mediation of the cytotoxic activity of CF_2, and cell death is associated with increased intracellular Ca^{2+}.

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