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Role of Ca^{2+} in induction and secretion of dengue virus-induced cytokines

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Abstract. The present study was undertaken to investigate the role of calcium ions (Ca^{2+}) in the induction and secretion of the dengue type 2 virus induced cytotoxic factor and the cytotoxin. This was done by using calcium channel blocking drugs such as verapamil, nifedipine or diltiazem hydrochloride. The production of cytotoxic factor was significantly reduced by treatment of dengue type 2 virus infected mice with verapamil. Similarly, a dose-dependent inhibition of the secretion of cytotoxic factor was observed, when spleen cells of the virus-primed mice were treated *in vitro* with the 3 calcium channel blockers. The production of cytotoxin by macrophages was abrogated by pretreatment with calcium channel blockers but had little effect on its secretion as shown by treatment of macrophages with verapamil at 1 h after the induction to later periods up to 18 h. The findings thus show that in the induction of both the cytokines Ca^{2+} plays a critical role; on the other hand it is required for the secretion of the cytotoxic factor but not for that of the cytotoxin.

Keywords. Ca^{2+}; cytokines; dengue virus; cytotoxic factor; calcium channel blockers.

Introduction

Calcium plays an important role in the regulation of a large number of vital cellular activities and mediates cellular responses to a wide range of stimuli. When calcium ion (Ca^{2+}) is bound, to calmodulin, it modulates the activities of a number of intracellular enzymes, including those involved in protein phosphorylation, secretory functions and calcium flux etc. Ca^{2+} also plays a critical role in a number of pathological and toxicological processes (Orrenius *et al.,* 1989). Activation of T lymphocytes (Lichtman *et al.,* 1983), natural killer cells (Hiserodt *et al.,* 1982) and macrophages (Wright *et al.,* 1985) have been shown to be Ca^{2+}-dependent. Further, target cell killing by various activated cells may be mediated by cytotoxic molecules, secretion of which may be Ca^{2+}-dependent or independent (Kelly, 1985; reviewed by Young and Liu, 1988).

Dengue type 2 virus (DV) induces a subpopulation of T lymphocytes of mice to produce a cytotoxic protein, the cytotoxic factor (CF). CF kills I-A negative macrophages and helper T cells (Chaturvedi *et al.,* 1987; Khanna *et al.,* 1988), but induces I-A positive macrophages to produce another cytotoxin (CF_2). Both CF and CF_2 are biologically active protein molecules which kill different types of cells and produce various immunopathological effects including increased capillary

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Abbreviations used: Ca^{2+}, Calcium ion; DV, dengue type 2 virus; CF, cytotoxic factor; CF_2, cytotoxin; PBS, phosphate buffered saline; MEM, minimum essential medium; M∅, macrophage.

permeability (Dhawan *et al.,* 1990; Khanna *et al.,* 1989, 1990; reviewed by Chaturvedi, 1986, 1989). The present study demonstrates the importance of Ca^{2+} in the production and secretion of CF and CF_2 by the primed cells.

Materials and methods

Animals

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

Virus

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

Reagents

Verapamil and nifedipine were purchased from the Sigma Chemical Co., St. Louis, Missouri, USA and were dissolved in 30% ethyl alcohol as stock solution. Diltiazem hydrochloride was from the Anglofrench Drug Co. Eastern Ltd., Bangalore and was dissolved in the medium used. The stock solutions of the drugs were prepared and used on the same day. The drugs had no cytotoxicity in the concentrations used in the tests.

Preparation of CF

CF was prepared from the spleen cells of DV-infected mice by the technique described elsewhere (Chaturvedi *et al.,* 1980a, b; Khanna *et al.,* 1989). Briefly, mice were given DV intracerebrally in doses of 10^3LD_{50} and the spleens were harvested aseptically from the sick moribund mice on 9–11 postinfection day. The spleens were teased out in chilled phosphate-buffered saline (PBS), pH 7 with the help of forceps, and a single cell suspension was obtained. The cells were cultured for 24 h at 37°C in the presence of 5% CO_2 and then centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was assayed for cytotoxic activity and stored at – 70°C for further use as CF.

Preparation of CF_2

CF2 was prepared by the technique described elsewhere. (Gulati *et al.,* 1983a, b). Briefly, 5 ml of heparinized minimum essential medium (MEM) was inoculated intraperitoneally into the mouse and the peritoneal lavage was aspirated under aseptic conditions. It was layered onto 5 cm glass Petri dishes and incubated at 37°C for 2 h in the presence of 5% CO_2. Glass-non-adherent cells were removed by washing 3 times with MEM. These cells were considered macrophages (M∅) as described elsewhere (Chaturvedi *et al.,* 1982). One ml of CF (1:30) was layered on

glass-adherent cell sheet and incubated for 1 h at 4°C. The cell sheet was then washed thoroughly with MEM and cultured in normal saline for 24 h. The supernatant fluid was collected and the cells were scraped off with a rubber policeman and clear supernatant obtained after sonication. Both the preparations were cleared by centrifugation at 2000 *g* for 10 min and were stored at – 70°C and used as CF_2 .

Assay of cytotoxic activity

Cytotoxic activity of the preparations was assayed using a single cell suspension of normal mouse spleen cells as target. The technique for the assay of cytotoxic activity of CF and CF_2 has been described elsewhere (Chaturvedi *et al.,* 1980a, 1983; Gulati *et al*., 1983a; Khanna *et al.,* 1989). Briefly, equal volumes (100 μl) of the test solution and the target cells (2×10^6/ml) were mixed in microtitre U-well persplex plate and incubated at 4°C for 1 h. Viable target cells were counted using trypan blue dye exclusion method and the percentage of non-viable cells was calculated after counting 200–300 cells. The tests were set up in triplicate and were repeated at least thrice. The results have been presented after substracting the background non-viable cells. The data have been analysed using Students *t* test. A P value of more than 0·05 was considered insignificant.

Results

Effect on secretion of CF

Mouse spleen cells obtained 9–11 days after intracerebral infection with DV, secrete CF in culture fluid (Chaturvedi *et al*., 1980a; Khanna *et al*., 1989). Effects of calcium channel blockers on the secretion of CF by such DV-primed spleen cells was investigated. The drugs used were verapamil, nifedipine or diltiazem hydrochloride at concentrations of 10^{-3}, 10^{-5} or 10^{-7} M. A single cell suspension of DV-infected mice spleen cells (2×10^7 cells) was mixed with various calcium channel blocking drugs and the mixture was cultured for 24 h at 37°C in the presence of 5% CO_2. After centrifugation at 2000 *g* for 10 min at 4°C the supernatant was collected and assayed for cytotoxic activity. Untreated cells mixed with the diluent or the drug alone were used as controls.

Effect of various drugs on secretion of CF by spleen cells is shown in figure 1. It was observed that the culture fluid obtained from untreated cells killed 36 ± 2% target cells. In contrast, the culture fluid from cells treated with 10^{-3}, 10^{-5} or 10^{-7} M concentration of verapamil killed 7 ± 2, 19 ± 4, 31 ± 1% target cells respectively. The effect of the drug was thus, found to be dose dependent. Similar results were obtained by treating target cells with nifedipine or diltiazem (figure 1). The drugs as such had no adverse effect on the target cells with the concentrations used in the test (data not presented).

Effect on production of CF

The findings of the above experiment showed that secretion of CF is blocked by

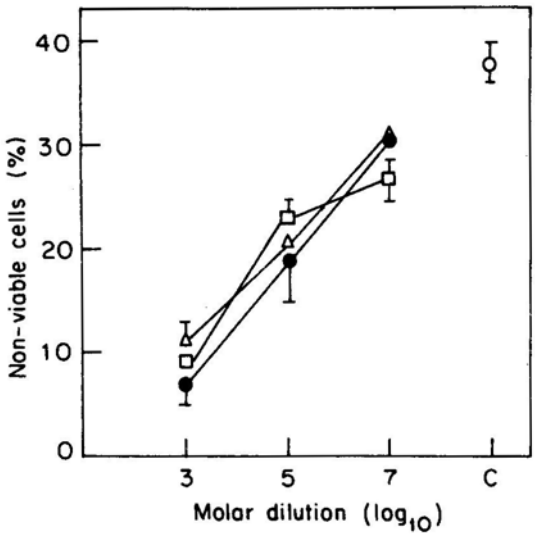

Figure 1. Effect of calcium channel blocking drugs on the secretion of CF by the spleen cells obtained from DV-primed mice. (●), Verapamil; (Δ),nifedipine; (□), diltiazem. (C) Untreated control.

treatment of DV-primed cells with various calcium channel blocking drugs. In another series of experiments, an effort was made to investigate the effect of such drugs on the production of CF. Mice were inoculated at 0 h with 0·1 ml of various dilutions of verapamil intraperitoneally and DV intracerebrally. Mice were sacrificed on the 10th day and the spleen cells were either sonicated or cultured for 24 h. The sonicate and the culture fluid were cleared at 2000 *g* and assayed for the cytotoxic activity. It was observed that the cytotoxic activity of the culture supernatants from mice not treated with verapamil was 33±4%. The supernatant from mice treated with 10^{-3} M verapamil had an activity of 18±3% ($P < 0{\cdot}001$) while that obtained from mice treated with 10^{-7} M dilution of the drug was 27 ± 2%. This showed that the drug significantly inhibited the production of CF in a dose dependent manner (figure 2). Among the sonicates, the cytotoxic activity of

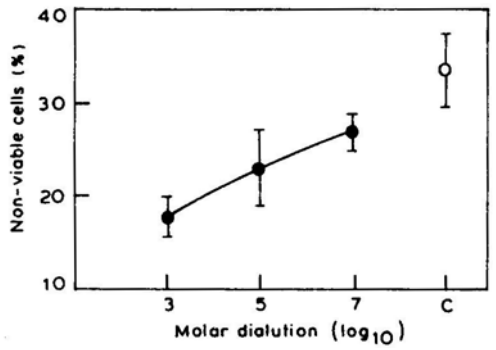

Figure 2. Effect of pretreatment of DV-primed mice with verapamil on the production of CF.

control, drug untreated mice was 40 ± 6 %. The sonicates obtained from mice treated with verapamil had a cytotoxic activity of 18 ± 3, 26 ± 2 and 33 ± 3 % respectively at 10^{-3}, 10^{-5} and 10^{-7} M dilutions of the drug. In another set of experiments the mice were given two doses of the various dilutions of the drug, one at 0 h and the second on the 4th day. It was observed that with the dose of 10^{-3} M the cytotoxic activity was 13 ± 3 %. A similar effect was observed by giving two doses of 10^{-5} or 10^{-7} M dilution of the drug.

Effect on secretion of CF_2

CF induces mouse macrophages to produce CF_2 which is secreted out of the cells (Gulati *et al.,* 1983a, b; Chaturvedi *et al.,* 1987). The effect of calcium channel blocking drugs on the secretion of CF_2 by MØ was investigated in the present experiment. Mouse peritoneal MØ monolayers were prepared. The MØ monolayers were pretreated with calcium channel blockers, at 37°C for 1 h (verapamil, nifedipine or diltiazem) at 10^{-3}, 10^{-5} or 10^{-7} M concentration. The fluid was then decanted and the MØ monolayers were inoculated with CF (1:30 dilution). After a further incubation at 4°C for 1 h the MØ monolayers were washed thrice and cultured for 24 h at 37°C in the presence of 5% CO_2. The culture fluid was then collected and clear supernatant obtained after centrifugation at 2000 *g* for 10 min was assayed for cytotoxic activity. MØ-monolayers were similarly inoculated with CF but were not treated with the drugs, and were used as controls. In another control the cells were treated with the drugs only.

The data presented in figure 3 show that the culture fluid obtained from untreated MØ-monolayers killed 39 ± 3% target cells, thus showing secretion of CF2 in the fluid. On the other hand culture fluid from cells treated with 10^{-3}, 10^{-5} or 10^{-7} M verapamil killed 10±2, 16 ± 3 and 20 ± 2 % target cells respectively. Thus

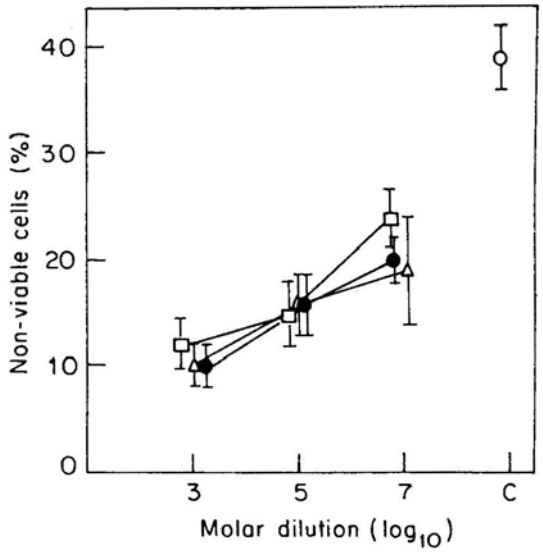

Figure 3. Effect of calcium channel blocking drugs on the induction of macrophages to produce CF_2. (n), Verapamil; (Δ), nifedipine; (c), diltiazem. (C) Untreated control.

a dose dependent inhibition of the secretion of CF_2 was observed. A similar significant reduction in the secretion of CF_2 was observed with nifedipine and diltiazem. The 3 drugs bind to 3 distinct sites on the calcium channel receptors of the cells and may synergise the effect when used in combination. In the next set of experiments, therefore, the 3 drugs were used in doses of 10^{-5} M in various combinations as shown in table 1, using above protocol. No summation of the effect was seen by using different combinations of the 2 drugs but by using all the 3 drugs simultaneously a higher reduction in cytotoxic activity was noted (table 1).

Table 1. Effect on the production of CF_2 by treatment of M∅ with various combinations of the calcium channel blocking drugs.

Groups	Drug treatment (10^{-5} M)	Cytotoxicity (%)	Reduction in cytotoxicity (%)
1	Ver + Nif*	13 ± 3	63
2	Ver + Dil	16 ± 2	54
3	Nif + Dil	13 ± 2	63
4	Ver + Nif + Dil	9 ± 5	74
5	None (control)	35 ± 4	0

*Ver, Verapamil; Nif, nifedipine; Dil, diltiazem hydrochloride.

From the above experiments it could not be concluded whether the reduced cytotoxic activity in the culture fluid was due to blockade of secretion or due to blockade of production of CF_2. Therefore, in another series of experiments M∅ sheets were treated with CF for 1 h at 4°C and then after thorough washing were cultured in normal saline. After 1, 3, 5 or 18 h the cultures were washed thoroughly and then recultured with normal saline containing 10^{-3} M verapamil. The fluid of the control cultures to which the drug was not added were similarly processed. At 24 h the culture fluid was collected and assayed for cytotoxicity. Further, the M∅ cells were scraped off, sonicated and centrifuged at 2000 *g* for 10 min. The clear supernatants thus obtained were also assayed for cytotoxicity. The findings presented in figure 4 show that the cytotoxic activity of culture supernatants of the|M∅ treated with verapamil at 1–18 h was 19 ± 2 – 27 ± 4 %. The cytotoxic activity in the control cultures, not treated with the drugs, was similar in the various groups and the mean value obtained was 29 ± 2%. Similar findings were observed when the cell sonicates were tested. Thus verapamil appears to block production of CF_2.

Discussion

The role of Ca^{2+} in production/secretion of two DV-induced cytokines, the CF and CF_2, has been demonstrated in the present study. This has been shown by the inhibition of their production/secretion by treatment with various calcium channel blocking drugs, namely verapamil, nifedipine or diltiazem. It was observed that the production of CF was significantly reduced by treatment of DV-infected mice with verapamil. Similarly, when spleen cells obtained from DV-primed mice were treated *in vitro* with either of the above drugs a dose dependent inhibition of the secretion of CF was observed. Thus Ca^{2+} appears to play an important role both in production as well as secretion of CF. The production of CF_2 was inhibited by

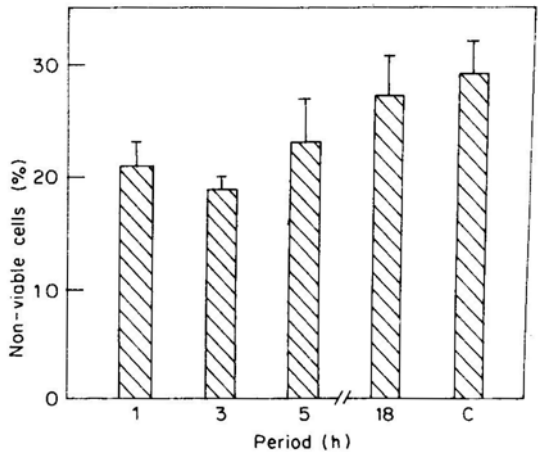

Figure 4. Effect of pretreatment of primed macrophages with verapamil on the secretion of CF_2.

pretreatment of M∅ by any of the 3 drugs. A higher degree of inhibition was seen when all the 3 drugs were used simultaneously but no summation was noted by using combination of any of the 2 drugs. On the other hand, the calcium channel blockers had almost no effect on its secretion. It has been shown earlier that following induction with CF the M∅ start producing CF_2 which continues to be secreted up to 24 h (Chaturvedi *et al.,* 1983a). The findings of the present study show that treatment of such M∅ with verapamil after 1 h of induction or at later periods up to 18 h had no effect on the secretion of CF_2.

In several studies the calcium channel blocking drugs have been used to elucidate the role of Ca^{2+}. For example suppression of mitogen-induced activation of lymphocytes by such drugs suggests a pivotal role of calcium in initial activation of T cells (Birx *et al*., 1984). Activation of macrophages to a tumoricidal state is inhibited by the pharmacological agents that inhibit calcium influx or inhibit calmodulin function (Wright *et al*., 1985). We have recently demonstrated that the presence of Ca^{2+} is obligatory for the cytotoxic activity of CF and CF_2 and the target cell killing is associated with an influx of Ca^{2+} in the susceptible target cells (Khanna *et al*., 1990; Dhawan *et al.,* 1991).

Secretion of the cytotoxic molecules can be *via* calcium-dependent or independent pathways. When such molecules are in the form of secretory granules, their exocytosis requires Ca^{2+} as a second messenger, while in certain systems the release is constitutive which does not require calcium (Kelly, 1985). The release of tumour necrosis factor is Ca^{2+} independent (Liu *et al.,* 1987); CF_2 resembles it in this aspect. On the other hand, perforin/cytolysin secreted by cytotoxic T lymphocyte are absolutely dependent on Ca^{2+} for assembly, secretion and function (Henkart et *al*., 1984; Podack *et al*., 1984, 1985). Similarly secretion of serine esterase is Ca^{2+} dependent (Ostergaard *et al*., 1987). Ca^{2+} is an important mediator in M∅ activation also as shown by enhanced secretion of cytolytic factor from M∅ by calcium ionophore A23187 (Drysdale *et al*., 1983). The data presented here, besides demonstrating the role of Ca^{2+} in secretion of CF also shows that Ca^{2+} plays an

important role in the transmission of the signal in T lymphocytes to produce CF and in M∅ to produce CF_2.

The findings of the present study thus show that besides playing an important role in mediation of the cytotoxic activity of CF/CF_2, as described elsewhere, Ca^{2+} is also necessary for their production/secretion from the primed cells.

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