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Production of dengue virus-induced macrophage cytotoxin *in vivo*

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Summary. We have observed that dengue virus-induced cytotoxic factor (CF) induces peritoneal and splenic macrophages *in vitro* to produce a cytotoxin (CF_2). This study demonstrates also production of CF_2 *in vivo* in DV-infected mice and following inoculation with CF. The cell-type responsible for CF_2 production *in vivo* is the macrophage (Mϕ) as Mϕ-depleted mice failed to produce CF_2. CF_2 activity could not be observed in the serum or peritoneal fluid though it is produced in peritoneal Mϕ. Once stimulated, CF_2 is present for 4 h in Mϕ. Mϕ can be restimulated to produce CF_2 only after a refractory period of 48 h.

Keywords: cytotoxic factor, macrophage, cytotoxin, dengue virus

Antigen-non-specific immunosuppression observed in dengue type 2 virus (DV)-infected mice is mediated by a cytotoxic factor (CF) produced by the T-lymphocytes of spleen (Chaturvedi *et al.* 1980*a*; 1980*b*; Shukla *et al.* 1982). CF kills lymphoid cells in several species of animals and adversely affects the functions of those cells which escape death (Chaturvedi *et al.* 1981; 1982; 1983*a*; Nagar *et al.* 1984). CF induces normal macrophages of the mouse spleen and peritoneal cavity to produce a cytotoxin (CF_2) which is similar in function to CF and which amplifies the effect of CF (Chaturvedi *et al.* 1983*b*; Gulati *et al.* 1983*a*). The production of CF_2 has been demonstrated previously *in vitro*. This study was undertaken to examine whether or not *in vivo* production of CF_2 occurs and, further, to attempt to delineate the biological requirements for production of CF_2 during immunosuppression.

Materials and Methods

The details of the animals used, dengue type 2 virus (DV), the DV-mouse model and preparation of the cytotoxic factor from DV-infected mouse spleen have been reported previously (Chaturvedi *et al.* 1977; 1978; 1980*a*; 1980*b*).

Depletion of macrophages in mice. Silica, prepared according to the method of O'Rourke *et al.* (1978), was used to deplete the Mϕ in mice as described previously (Shukla & Chaturvedi 1982). Briefly, each mouse was inoculated with protein-coated silica particles in doses of 50 mg i.p. and 3 mg i.v., followed 24 h later by inoculation of CF i.v. Efficacy of the silica treatment was confirmed using latex particles as described previously (Shukla & Chaturvedi 1982).

Preparation of Mϕ cultures. Resident peritoneal cells were aspirated with 5 ml hepari-

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nized medium, layered onto a glass petri dish and incubated for 2 h at 37°C. The glass-adherent Mϕ cell sheet was washed and then cultured after layering with 3 ml of MEM-HEPES at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of macrophage cell-homogenates. Mϕ monolayers were prepared by layering splenic or peritoneal lavage cells into glass petri dishes and incubating for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The glass-adherent cell sheets were washed thoroughly to remove glass-non-adherent cells. The glass-adherent cells were scraped off with a rubber tipped policeman rod and homogenized in 2 ml chilled phosphate buffered saline (PBS) pH 7.0. Cell debris was removed by centrifugation at 2000 *g* for 10 min and the supernatants collected.

Collection of serum. Blood was collected after 3 h and 6 h from the hearts of mice inoculated with CF i.v. and after 4 and 6 days from DV i.c.-inoculated mice. Sera were separated and assayed for cytotoxicity.

Preparation of cell-free peritoneal fluid. Peritoneal lavage cells were collected at 3 and 6 h after inoculation of CF i.v. and at 4 and 6 days after DV i.c. The peritoneal cavity was lavaged with 5 ml heparinized MEM containing 10% fetal calf serum. The collected washings were centrifuged at 2000 *g* for 10 min in the cold, and cell-free supernatant was collected and assayed for cytotoxicity.

DEAE cellulose chromatography. The technique of DEAE cellulose chromatography has been described in detail elsewhere (Gulati *et al.* 1983*a*; Dalakoti *et al.* 1983). The sample is layered on top of the column and elution performed immediately with 0 to 0.4 M NaCl at a flow rate of 0.5 ml/min. Fractions (4 ml) were collected and monitored for absorbance of ultraviolet light at 280 nm wavelength and also assayed for cytotoxic activity.

Agarose gel electrophoresis. The details of agarose gel electrophoresis have been described elsewhere (Chaturvedi *et al.* 1980*b*). The sample was electrophoresed for 4 h at 4°C using a constant voltage of 2 mA/cm of gel. Slices of 1 cm were cut and homogenized in a pestle and mortar with 1 ml of PBS to elute the proteins. The fractions were centrifuged at 3000 *g* for 15 min and clear supernatants were tested for cytotoxicity and screened for absorbance at 280 nm wavelength.

Cytotoxicity assay. Cytotoxicity was assayed as described previously (Chaturvedi *et al.* 1980*a*; Gulati *et al.* 1983*a*). Briefly a 0.1 ml aliquot was incubated with 0.1 ml of normal mouse spleen cell suspension (2×10^6 cells) at 4°C for 1 h. The non-viable cells were counted using the trypan blue dye exclusion method.

The mean values and standard deviations were calculated for 3 or 4 duplicate experiments. Data were analysed using Student's *t*-test for the *P* value; *P* values less than 0.05 were considered significant.

Protocol. In vivo production of CF_2 was screened in CF-inoculated and in DV-infected mice. Mice given CF in doses of 0.25 ml (1:30) i.v. were killed in groups at different times from 1 to 24 h. Single cell suspensions of the spleens were prepared. Mϕ monolayers, prepared in glass petri dishes from the peritoneal and splenic cells, were homogenized in 2.0 ml of chilled PBS and the homogenates screened for cytotoxicity.

Other groups of adult mice were inoculated with 10^3 LD_{50} of DV by different routes, *viz*: i.c., i.p., i.v. or s.c. Mice were killed in batches from day 1 to 10. Macrophage monolayers were prepared as described above and the cytotoxic activity of their homogenates screened.

Results

Production of CF_2 in CF inoculated mice

With CF-inoculated mice, cytotoxic activity

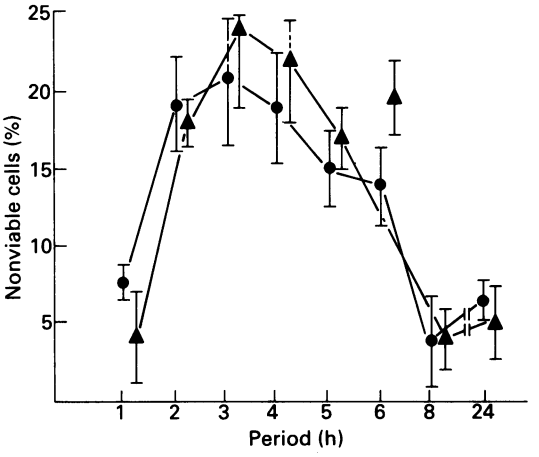

Fig. 1. Mice were dosed i.v. with 0.25 ml CF. Their peritoneal and splenic Mϕ were collected at times from 1–24 h. Mϕ homogenates were prepared and screened for CF_2 activity. (●), splenic Mϕ homogenate; (▲), peritoneal Mϕ-homogenate.

was present in cell homogenates 2 h after CF inoculation, reaching peak levels by 3 h and then gradually declining to reach insignificant levels by 8 h; the findings were similar in both peritoneal and spleen cells (Fig. 1).

The association of cytotoxic activity of cell homogenates with CF_2

To determine whether the cytotoxic activity of cell homogenates was attributable to CF_2, DEAE cellulose chromatography of splenic cell homogenates obtained 3 h after CF inoculation was performed. A single peak with cytotoxic activity was eluted with 0.32 M NaCl (Fig. 2) which corresponded to that for CF_2 described previously (Gulati *et al.* 1983*a*).

Titration of CF_2 activity in cell homogenates

The cytotoxicity titre of splenic cell-homogenates obtained at different periods after CF i.v. was estimated. An inverted bell-shaped curve for cytotoxic titre was obtained (Fig. 3).

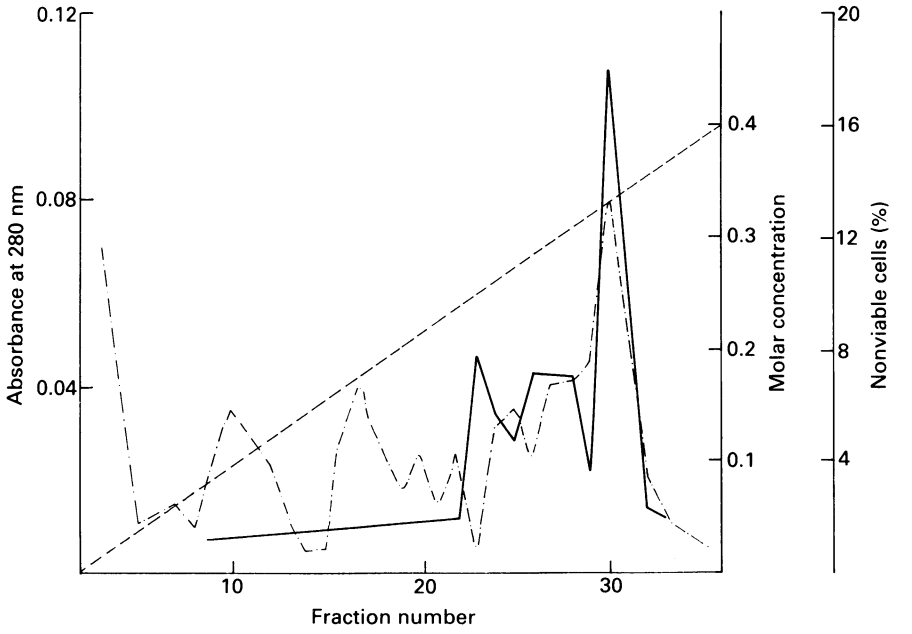

Fig. 2. DEAE-cellulose chromatography of splenic Mϕ-homogenate obtained 3 h after i.v. CF. elution with 0–0.4 M NaCl. Eluted fractions were screened for protein by their UV absorption at 280 nm (–·–), and for cytotoxic activity (——).

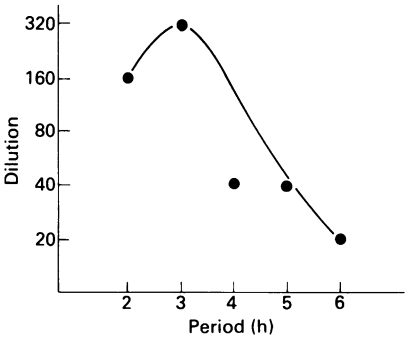

Fig. 3. Cytotoxic titre of splenic Mϕ-homogenates obtained at different times after i.v. CF.

CF_2 production by macrophages

This experiment was designed to determine whether CF_2 is produced only by macrophages. Mice, depleted of Mϕ by silica treatment, 24 h later were given CF i.v. The cytotoxic activities of the splenic and peritoneal cell-homogenates were assayed at 3 h. Cytotoxic activity was negligible in cell homogenates of splenic and peritoneal cells obtained from silica-treated mice (Table 1).

Screening of CF_2 activity in sera and peritoneum

Peritoneal fluid and serum collected 3 h and 6 h after inoculation of CF i.v. were screened for cytotoxic activity as also were the cell-homogenates of peritoneal cells and splenic glass-adherent cells of the same mice. CF_2 activity was absent from the sera as well as from the peritoneal fluid at both times though it was present in homogenates of peritoneal and splenic cells (Table 2).

Table 1. Cytotoxic activity of Mϕ-homogenates from CF-injected mice (i.v.)

	Non-viable cells (%)	
Sample	Peritoneal Mϕ-homogenate	Splenic Mϕ-homogenate
Silica treated	5.5±1.8	4.4±4.1
Untreated	24±6	21±4.6

Mϕ were depleted in mice by inoculating protein coated silica particles in doses of 50 mg i.p. and 3 mg i.v. After 24 h, animals were dosed i.v. with 0.25 ml CF, and their peritoneal and splenic Mϕ-homogenates screened for CF_2 activity.

Table 2. Cytotoxic activity of CF_2 in sera and peritoneal fluid of CF-inoculated mice, 3 and 6 h after inoculation

	Non-viable cells (%)	
Sample	CF i.v. 3 h	CF i.v. 6 h
Sera	7±5	4±2
Peritoneal fluid	3±3	4±2
Peritoneal cell-homogenate	19±3	16±2
Splenic cell-homogenate	23±2	20±3

Production of CF_2 in DV-infected mice

Cytotoxic activity was present in cell homogenates from mice given DV by any route (Fig. 4). Cytotoxicity was observed from day 1 to 6, peaking on day 4.

Comparison of cytotoxicity in DV-infected and CF-inoculated mice

To determine whether the cytotoxic activity of the cell homogenates from both DV-infected and CF-inoculated mice is attributable to the same product, agarose gel electrophoresis of peritoneal cell homogenates prepared from both experimental groups was performed. Peak cytotoxic activity was associated with fraction 7 of both samples, indicating that the cytotoxic activity is likely to be attributable to the same product (Fig. 5).

CF_2 activity in sera and peritoneal fluid

Mice inoculated with DV i.c. were killed after

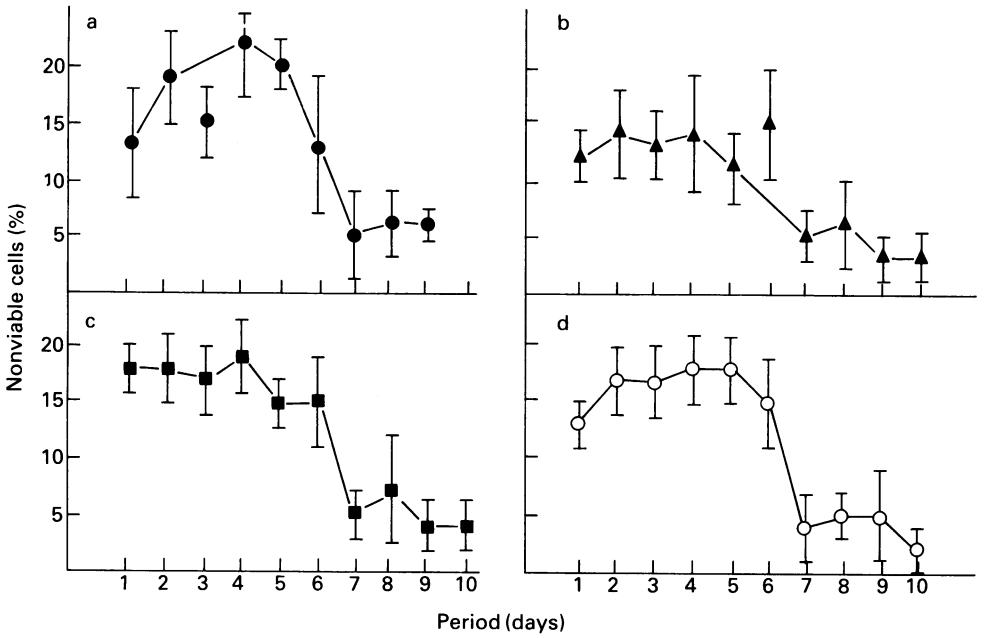

Fig. 4. Mice were inoculated with 10^3 LD_{50} of DV by different routes, *viz*: i.c., i.p., i.v. or s.c., and CF_2 activity was screened in peritoneal Mϕ-homogenates from days 1 to 10. *a*, DV i.c.; *b*, DV i.p.; *c*, DV i.v.; d DV s.c.

4 and 6 days and their sera and peritoneal fluids assayed for cytotoxic activity. CF_2 activity was absent from both sera and peritoneal fluid on both days even though it is present within the cells suspended in these fluids, for cell homogenates were cytotoxic on both the 4th and 6th days (Table 3).

Duration of CF_2 activity in in vivo-induced Mϕ

Peritoneal Mϕ were prepared 3 h after inoculation of CF i.v. and then cultured for various periods from 2 to 24 hr in 2 ml MEM-HEPES. Cultures were harvested at different times and their supernatants and cell homogenates screened for cytotoxicity. CF_2 was present in Mϕ only up to 4 h (Table 4). The supernatants and Mϕ homogenates prepared at later times had no significant activity.

Reinduction of Mϕ by CF inoculation

The above experiment demonstrates that CF_2 activity was absent from Mϕ homoge-

Table 3. Cytotoxic activity of CF_2 in sera and peritoneal fluid of DV i.c. inoculated mice after 4 & 6 days

	Non-viable cells (%)	
Sample	DV i.c. 4th day	DV i.c. 6th day
Sera	6±2	4±2
Cell free peritoneal fluid	6±4	4±3
Peritoneal cell-homogenate	17±4	10±3

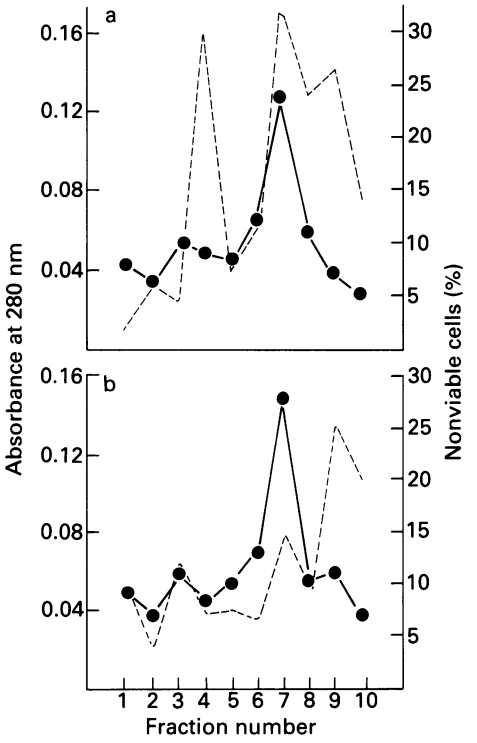

Fig. 5. Comparison of cytotoxic activity in peritoneal Mϕ-homogenates of DV-infected and CF-inoculated mice by prolonged agarose gel electrophoresis. Fractions were tested for protein at 280 nm (– – – –) and for cytotoxicity (●); *a*, peritoneal Mϕ-homogenate after CF i.v.; b, peritoneal Mϕ-homogenate after DV i.c.

nates at 6 h Mϕ were reinduced with CF at 6, 8, 24 and 48 h. The cell monolayers were washed thoroughly and cell homogenates prepared to screen for CF_2 activity. The Mϕ remained refractory for 24 h during which time they could not be induced to produce CF_2 (Table 5). After 48 h they could be reinduced and produced significant amounts of CF_2.

Discussion

This *in vivo* study confirms and extends the previous *in vitro* observation that CF induces Mϕ to produce CF_2 within 45 min (Gulati *et al.* 1983*a*; Chaturvedi *et al.* 1983*b*). CF_2 is

Table 4. Duration of CF_2 activity in *in vivo* induced Mϕ

	Non-viable cells (%)	
Period (h)	Culture supernatant	Mϕ homogenate
2 h	23±1.8	22±3
4 h	22±2	22±7
6 h	3±2	3±2
8 h	4±2	3±2
24 h	7±5	6±2

Mice inoculated with 0.25 ml of CF i.v. were killed after 3 h and their peritoneal Mϕ were cultured. Cultures were harvested between 2 h and 24 h and their culture supernatants and Mϕ-homogenates screened for CF_2 activity.

Table 5. Restimulation by CF of *in vivo* induced Mϕ to produce CF_2

Reinduced with CF at	Non-viable cells (%)
6 h	5±3
8 h	3±2
24 h	3±2
48 h	18±5

Mice were given CF i.v. and the peritoneal Mϕ were collected after 3 h and cultured. The Mϕ were stimulated *in vitro* at 6 h, 8 h, 24 h and 48 h by layering them with 1 ml of CF (1:30) for 1 h at 4°C. CF_2 activity was screened in these Mϕ after homogenization.

detectable in mouse splenic and peritoneal cells within 2 h, with peak activity 3 h after i.v. inoculation of CF. The cell responsible for production of CF_2 is the Mϕ and CF is not produced in Mϕ-depleted mice; this is in accord with *in vitro* observations (Gulati *et al.* 1983). Elution of cytotoxic activity with 0.32 M NaCl in DEAE cellulose chromatography and its presence in fraction number 7

on agarose gel electrophoresis, indicates that the cytotoxicity was attributable to CF_2 and not to CF.

It is interesting that CF_2 is produced in mice following inoculation of DV. Can DV directly induce the production of CF_2 or does it have to be *via* CF production? DV inoculated parenterally induces CF *in vivo* (Chaturvedi *et al.* 1980*a*; Dalakoti *et al.* 1983), as does inoculation of cell cultures *in vitro* with CV (unpublished observations). Further CF production is not related to DV replication (Chaturvedi *et al.* 1980*a*; Dalakoti *et al.* 1983) and thus it appears that the virus per se is capable of stimulating T cells to produce DF. This, in turn, induces CF_2 production (Chaturvedi *et al.* 1983*b*). The presence of CF_2 in cells from the first day after DV inoculation thus is not surprising. CF_2 activity persists only until the day 6 and attempts to detect it thereafter have been unsuccessful (Fig. 4). This may reflect both a marked reduction in Mϕ number and function in DV-infected mice from day 5 (Gulati *et al.* 1982; Chaturvedi *et al.* 1983*a*; unpublished data) and also the fact that Mϕ become refractory to CF for 3 days after first exposure during which time they do not produce CF_2 (Chaturvedi *et al.* 1983*b*).

Lymphokines, including lymphotoxins, are found in sera and body fluids (Neta & Salvin 1981) and may be involved in antibody-dependent cell-mediated cytotoxicity (Kondo *et al*, 1981). CF_2 was not found in sera and peritoneal fluid though it was produced by the peritoneal and splenic Mϕ. The capacity to release lymphokines in the spleen may be independent of the capacity to release them in other lymphoid organs or into the circulation, perhaps because suppressor cells are sequestered in the spleen (La Grange & Mackaness 1978). Suppressor cells can be generated in the spleen of DV-infected mice (Tandon *et al.* 1979; Shukla & Chaturvedi 1981, 1984) and they may contribute to the reduction of CF_2 activity in sera and peritoneal fluid, for DV-induced suppressor factor is known to inhibit the production of CF in mice and to abrogate the DV- or CF-induced alterations in macrophage function (Nagar *et al.* 1985*a*).

Several workers have reported the production of Mϕ cytotoxins (Reed & Lucas 1975; Walker *et al.* 1976; Aksamit & Kim 1979). Beaman *et al.* (1983) noted that Ly 1^+2^- lymphocytes elaborate a lymphokine which activates Mϕ across H-2 barriers to produce a cytotoxin; this is similar to the CF-induced production of CF_2 by Mϕ reported here and previously (Chaturvedi *et al.* 1980*a*; Shukla *et al.* 1982; Gulati *et al.* 1983*a*). Männel *et al.* (1980) observed the production of a lymphokine-induced Mϕ-cytotoxin within 2 h, which is stable at 56°C for 30 min and has a mol. wt. of from 55 000 to 60 000; by comparison, CF_2 is heat-labile and has a low molecular weight (Gulati *et al.* 1983*b*).

The interactions of ligands with specific receptors on Mϕ have multiple consequence which can include either suppressed or enhanced secretion of neutral proteases (Edelson 1980). Activated murine Mϕ secrete a potent cytolytic neutral protease (Adams *et al*, 1980). Its production which occurs in 30 min, is not inhibited by treatment with cycloheximide or mitomycin C (Johnson *et al.* 1981). Similarly, CF_2 is produced in 45 min and this is not prevented by pretreating the Mϕ with cycloheximide or mitomycin C (Gulati *et al.* 1983*a*; Chaturvedi *et al.* 1983*b*).

Engagement of the Fc-receptors on Mϕ results in the production of oxygen metabolites (Johnson *et al.* 1978) including the strongly cytotoxic hydrogen peroxide (Nathan *et al.* 1980). Halstead (1981) suggested that pharmacologically active substances, including proteases secreted by Mϕ during DV infection, may be involved in production of the shock and haemorrhage syndrome. Fc-mediated attachment and phagocytosis of opsonized sheep erythrocytes by Mϕ of DV-infected mice, is modulated by CF (Chaturvedi *et al.* 1983*a*; Nagar *et al.* 1984, 1985*a*, 1985*b*). It will be of interest to determine the chemical nature of CF_2.

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