A Cytotoxic Substance (CTS-51) Produced by Human Buffy Coat Cultures Stimulated by Staphylococcal Enterotoxin B: Further Characterizations and Combined Action with Interferon

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*The First Department of Internal Medicine, School of Medicine, Kanazawa University, Kanazawa 920, †Wadley Institutes of Molecular Medicine, Dallas, Texas 75235, USA, and ‡Immuno Modulator Laboratories, Stafford, Texas 77036, USA

HIRAI, N., HATTORI, N., GEORGIADES, J., OSTER, K. and BERG, K. A Cytotoxic Substance (CTS-51) Produced by Human Buffy Coat Cultures Stimulated by Staphylococcal Enterotoxin B: Further Characterization and Combined Action with Interferon. Tohoku J. exp. Med., 1986, 148 (1), 87-97 — A recently recognized unique cytotoxic substance, CTS-51, was tested for the heat or acid stability, trypsin digestion and dialysis. Moreover, influences of elevated incubation temperatures or serum concentrations of medium on the cytotoxic activity of CTS-51, and the combination effects of CTS-51 and human leucocyte interferon (HuIFN-α (Le)) were investigated. The cytotoxic activity of CTS-51, which is promoted by a small molecule easily passable the dialysis membrane, was found to be very stable to heat (even at 100°C for 30 min) or acid (pH 2.0 for 24 hr at 4°C) treatments. The treatment with 0.75% trypsin for 1 hr did not diminish the CTS-51 activity. The susceptibility of Daudi lymphoma cells to the antiproliferative action of HuIFN-α (Le) was further potentiated by treating the cells with CTS-51 for 16 hr. On the other hand, the CTS-51 activity which was revealed to be prescribed by its concentration in the medium, was not potentiated at 39°C when compared to that at 37°C in contrast to HuIFN-α (Le) action, and was reduced according to the increase of the fetal calf serum concentration in the medium.

— cytokine; interferon; cytotoxicity

It is generally known that lymphocytes or monocytes, when activated by the antigen- or mitogen-stimulation, release a mixture of several biologically active soluble factors, with various molecular sizes, which are usually called lymphokines or monokines, generally called “cytokines” as a whole (Evans 1982). It has been argued, from several quarters (Evans 1982; Ransom and Evans 1982;
Dimolfo and Chadlia 1983), that a very complex interplay should take place among these factors and immune system and as a result of this, tumor cells or virus infected cells are often damaged severely or even killed. Some of these factors, such as, for example, interferons and interleukin-2, are revealed to have a property to potentiate the cytotoxic activities of several cellular immunological systems, such as, for example, natural killer cell system or MLC derived cell mediated cytotoxicity system (Heron et al. 1976, 1979; Marx 1983). On the other hand, several other cytokines have been reported to have a property directly cytotoxic to the target cells in vitro, which include lymphotoxins (Evans 1982), tumor necrosis factors (Williamson et al. 1983), natural killer cytotoxic factors (Bonavida et al. 1982) and immune interferons (Rubin and Gupta 1980).

Recently, we have recognized that unique tumor cytotoxic substance (CTS-51), which is probably one of the cytokines, was produced in human buffy coat cultures during the mass production of immune interferon and interleukin-2 (Hirai et al. 1985). In this description, CTS-51 was further investigated in some biological or physicochemical characteristics, the mode of action, and the influences of fetal calf serum (FCS) concentrations in the test medium or the virus induced human leucocyte interferon. As a result of this, it was found that CTS-51 has properties distinct from any ever reported cytotoxic cytokine, and that CTS-51 may potentiate the interferon anticellular action whereas the cytotoxic activity of this substance may be antagonized by some components in the FCS preparation.

**Materials and Methods**

**Cells.** Daudi cells, a Burkitt lymphoma derived cell line, and G-361 cells, a human malignant melanoma origined cell line, were used to test the cytotoxic activity of CTS-51. Both cell lines were obtained from American Type Culture Collection (ATCC), and maintained in RPMI-1640 medium (GIBCO) supplemented with 10% heat inactivated fetal calf serum (FCS, GIBCO), 100 U/ml of penicillin and 100 µg/ml of streptomycin (GIBCO).

**CTS-51.** CTS-51 was isolated during the process of interferon-γ and interleukin-2 production from human buffy coat cultures as described previously (Hirai et al. 1985). CTS-51 was used in a partially purified form. The content of lytic units (LU) of the material used in this study (Lot No. A041C) was found to be 818±32 LU (mean±s.d. from 9 assays on Daudi cells). LU was defined as the reciprocal of the dilution which suppressed the cell growth (or killed the cells) in 50% of the controls as calculated from the cell growth inhibition curves using the 14C-thymidine incorporation method.

**Interferon.** Native human leucocyte interferon (HuIFN-α (Le)) was produced from donor buffy coats induced by Sendai virus and partially purified following the method described by Cantell et al. (1981). Interferon titer was assayed by the plaque reduction assay using NIH standard IFN-α (Lot No. G023-901-527) as a reference, and the specific activity of the HuIFN-α(Le) used in this study was 1.0 × 10⁶ IU/mg protein.

**Assay of the anticellular actions of CTS-51 and interferon.** 1.5 × 10⁴/well of Daudi cells or 3 × 10⁴/well of G-361 cells were cultured in 0.2 ml medium (10% FCS in the standard assay) per well containing several serial dilutions of CTS-51 or interferon in 96-well flat bottom microplates (Nunc, Denmark), for three days. Cell propagation was then measured by 14C-thymidine (14C-TdR) incorporation technique as Hilfenhaus et al. (1976) previously
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described, and the cell growth inhibition rates were calculated.

Heat stability of CTS-51. CTS-51 was treated at 56°C for 30 min or 1 hr, and at 100°C for 1 or 30 min in a water bath, respectively. After these treatments, the cytotoxic activity of each sample was tested on Daudi cells.

Acid stability of CTS-51. The pH of CTS-51 preparation was lowered down to 2.0 by adding an adequate volume of 1N-HCl solution and it was kept at 4°C for 24 hr. Then the pH was elevated up to 7.2 by adding 1N-NaOH solution. The parallel control material of CTS-51 was added the same volumes of medium as HCl and NaOH added to the test material, and was also kept at 4°C for 24 hr. Both preparations were assayed for the cytotoxic activity on Daudi cells.

Trypsin digestion of CTS-51. CTS-51 preparation was treated with 0.75% trypsin (GIBCO) at 37°C for 1 hr. Then the medium containing 10% FCS, that was enough to inactivate the trypsin activity completely, was added. The cytotoxic activity of the sample was assayed on Daudi cells.

Dialysis of CTS-51. Two ml of CTS-51 preparation was dialyzed by using a dialysis membrane (Cellulose tubing, Union Carbide) for 24 hr at 4°C against more than 200 ml of Dulbecco’s PBS (without Ca or Mg). Then, the volume of the dialyzed material was adjusted back to the original, and the cytotoxic activity was assayed on the Daudi cell cultures.

Mode of CTS-51 action. To investigate what parameter would prescribe the degree of cytotoxic action of CTS-51, it was tested in two different modes against Daudi lymphoma cells; using different volumes of CTS-51 preparations but in a constant concentration, or different volumes but in a constant LU amounts of CTS-51.

Effects of combination of CTS-51 and interferon. After 1.5 X 10⁶ cells/ml of Daudi cells were pretreated with human leucocyte interferon or CTS-51 for several length periods and washed, 1.5 X 10⁶ cells/0.2 ml of them were cultured with the other agent for three days. Cell propagation was measured as mentioned above.

Effects of elevated incubation temperature on the CTS-51 and interferon activity. To test the effect of an elevated temperature, Daudi and G-361 target cells were cultured in 0.2 ml medium containing various dilutions of CTS-51 and interferon-α (Le) at 37°C or 39°C for three days. Temperature effect was evaluated by comparing the cell growth inhibition rates among both temperatures.

Influence of serum concentration on the CTS-51 activity. Influences of difference of FCS concentration in the assay medium on the cytotoxic activity of CTS-51 were tested in the several assays on Daudi and G-361 cells. The cell growth inhibition rate in each FCS concentration group was estimated against the control cell growth cultured in the same FCS concentration, respectively.

RESULTS

Fig. 1 shows the results from the experiments where the heat or acid stability, the effects of trypsin digestion or dialysis of CTS-51 were examined. As indicated in Fig. 1A, the cytotoxic activity of this substance was not inactivated at all after the treatments at 56°C for 30 min or 1 hr, nor even at 100°C for 1 or 30 min. As for the effect of the trypsin digestion, the cytotoxic activity of CTS-51 was not destroyed by the treatment with 0.75% trypsin for 1 hr (Fig. 1A), by which treatment the interferon activity was completely destroyed at all (data not shown). As indicated in Fig. 1B, the treatment of CTS-51 in a low pH circumstance at 2.0 for 24 hr neither could inactivate the cytotoxic activity of this substance. Only the dialysis of this partially purified CTS-51 preparation
against Dulbecco's PBS diminished the cytotoxic activity, indicating that this property should be carried by some small molecules in size. Also, it was noted that the CTS-51 activity was stable to low temperature treatment, either (cf. parallel control material in Fig. 1B).

To explore which parameter, the concentration or the total amounts of
CTS-51, would prescribe the outcomes of the cytotoxic efficacy of this substance, several experiments were performed. Fig. 2 indicates the results of these experiments where a constant amount of Daudi cells was incubated in different volumes of medium containing the same concentration or the same LU amount of CTS-51. When the concentrations of CTS-51 was constant, the differences in the volumes made only minimal differences in the CTS-51 efficacy (Fig. 2A). On the other hand, when the total LU was constant, the moderate differences in the CTS-51 efficacy were noted relating to the concentrations of CTS-51 (Fig. 2B); the higher concentration appears to be more efficient compared to the lower, suggesting the concentrations of this substance seem to prescribe the cytotoxic efficacy.

Fig. 3. Potentiation of CTS-51 and interferon activities by increased temperature. $3 \times 10^3$ of G-361 melanoma cells (A), and $1.5 \times 10^4$ of Daudi lymphoma cells (B) were cultured in 0.2 ml medium containing various dilutions of CTS-51 or human leucocyte interferon at $37^\circ C$ ($\circ$) or at $39^\circ C$ ($\bullet$) for three days. Cell propagation was evaluated by the $^{14}$C-TdR incorporation method. Experiments were performed in triplicate and mean values were plotted.
The combination effects of CTS-51 and interferon were investigated by treating Daudi cells with one agent followed by the other one. Fig. 3A shows the results of the experiments where Daudi cells were pretreated with HuIFN-α (Le) for 16 hr and cultured with CTS-51 for further three days. It appears that pretreatment of cells with interferon does not change the efficacy of CTS-51. Similar results were obtained when preincubating the cells for 1 or 5 hr with

Fig. 4. Effects of combinations of CTS-51 and human leucocyte interferon. A: Potentiation of CTS-51 activity by interferon pretreatment. 1.5×10⁶ cells/ml of Daudi cells were pretreated with no (○), 1 (■), 5 (△), 10 (▲), and 100 (▲) IU/ml of human leucocyte interferon in 4 ml medium for 16 hr. Cells were washed and 1.5×10⁴ cells/well were cultured in 0.2 ml medium containing several dilutions of CTS-51 for three days. B and C: Potentiation of interferon activity by CTS-51 pretreatment. 1.5×10⁶ cells/ml of Daudi cells were pretreated with no CTS-51 (○), or 1:10 (▲), 1:100 (■), and 1:1000 (●) dilutions of CTS-51 in 4 ml medium. After 5 (B) or 16 (C) hr pretreatments, cells were washed and 1.5×10⁴ cells/well were cultured in 0.2 ml medium containing various concentrations of human leucocyte interferon for three days. Cell propagation was measured by ¹⁴C-TdR incorporation methods. Experiments were done in triplicate and mean values were plotted.
The reverse sequence was also tried (Fig. 3B & 3C). As can be seen from Fig. 3C, the antiproliferative activity of human leucocyte interferon at each tested concentration was remarkably potentiated when the cells were pretreated with 1:10 and 1:100 dilutions of CTS-51, respectively, for 16 hr. Pretreatment with CTS-51 at a dilution of 1:1000 did not yield any effect on the antiproliferative activity of any of the interferon concentrations employed. Furthermore, this potentiation at 1:10 dilution was more prominent compared to that at 1:100 dilution, indicating a dosedependent relationship. A similar experiment employing only 5hr incubation with CTS-51 was performed (Fig. 3B), but no potentiation was noted.

The possibility that the CTS-51 action can be potentiated by elevated incubation temperature as the virus induced interferon has been reported to be (Heron and Berg 1978; Hirai et al. 1984) was also tested. Cultures of G-361 cells (Fig. 4A) or Daudi cells (Fig. 4B) were incubated at different dilutions of CTS-51 and HuIFN-α (Le), respectively, and cultured at 37°C and 39°C for three days. As indicated in Fig. 4, no potentiation at the elevated temperature occured with respect to CTS-51. In contrast, the antiproliferative action of interferon was clearly potentiated at 39°C compared to 37°C.

Fig. 5 shows the influences of FCS concentrations in the assay medium on the cytotoxic property of CTS-51 on Daudi and G-361 cell cultures. As shown in Fig. 5A, the cytotoxic activity of CTS-51 on the Daudi cell line was observed to be more prominent when the FCS concentration was lowered. The same tendency was also noted in the experiments performed on the G-361 cell line as indicated in Fig. 5B. The means of the control cell growth rates (¹⁴C-TdR incorporation rates)}
in these experiments were 48.2% at 50%-FCS, 83.9% at 20%-FCS, 106.1% at 5%-FCS and 32.7% at 1%-FCS, respectively, when compared to that at 10%-FCS (standard procedure) in the Daudi cell experiments. Those in G-361 cell experiments were 80.8% at 5%-FCS and 57.4% at 1%-FCS, respectively, when compared to that at 10%-FCS (standard procedure). Both cell lines, Daudi and G-361, could hardly grow (less than 2% compared to the growth at 10%-FCS) in the serum free medium.

**DISCUSSION**

Lymphokine preparations are generally known to contain mixtures of several biological active molecules, each of which may have individual immunological activities in vivo (Evans 1982). The CTS-51 preparation used in this study was isolated from the culture supernatant of the human buffy coat cultures stimulated by staphlococcal enterotoxin B (SEB) during the mass production process of the immune interferon and interleukin-2, as previously described (Hirai et al. 1985).

In the previous investigation, we have found that this substance has a cytotoxic property against several human malignant cell lines in vitro at the concentrations where the normal cells or non-malignant cells are not killed, and also that the cytotoxic property may not be so active on the murine tumor cells. The present investigations have revealed that this substance is very stable to heat and low pH treatments; CTS-51 was not inactivated even at 100°C for 30 min nor at pH 2.0 for 24 hr. These treatments are known to inactivate a number of lymphokines or monokines. Furthermore, this substance was not destroyed by the usual trypsin digestion procedure. These properties may evoke a question whether CTS-51 is a protein or not. However, trypsin is known to act on the specific amino acid sequence which is not probably included in the small biologically active molecules of CTS-51, which can easily pass the cellulose dialysis membrane. Another cytotoxic lymphokine, rabbit tumor necrosis factor, was also reported to be resistant to trypsin digestion (Matthews et al. 1980).

There have been four groups of cytokines reported to be directly cytotoxic to malignant cell cultures in vitro; namely, lymphotoxins (LT), tumor necrosis factors (TNF), natural killer cytotoxic factors (NKCF) and immune interferons (IFN-\(\gamma\)), as mentioned above. CTS-51 was isolated in the process of the immune interferon production. However it does not show any antiviral activity at all, and the cytotoxic activity of CTS-51 is preserved even after the acid treatment at pH 2.0 for 24 hr, where IFN-\(\gamma\) is known to be inactivated (Rubin and Gupta 1980). These observations seem to deny the possibilities that CTS-51 is one of the immune interferons nor that the cytotoxic activity of IFN-\(\gamma\) preparations may be exerted by this cytotoxic substance contaminated in them. Also, in respects that this substance has very high heat or acid stability and much smaller molecular weights, CTS-51 does not seem to be one of the other three cytotoxic factors, LT (Granger et al. 1979), TNF (Williamson et al. 1983) and NKCF (Bonavida et al.
As for the mode of CTS-51 action, we have investigated the influence of the change in the medium volume with a constant concentration or amount of CTS-51. From these experiments, it was suggested that the concentration of CTS-51 prescribes the cytotoxic efficacy even if this substance is introduced in a variety of volumes against target cells. Similar modes of action are also noted in other cytokines including interferons.

The question of whether interferon and CTS-51 would interfere with each other was examined using Daudi cells that are known to be highly sensitive to both agents (Hirai et al. 1984, 1985). Our results indicated that the anti-cellular effects of HuIFN-α (Le) were prominently and dosedependently potentiated by the pretreatment with CTS-51 (at 1:10 and 1:100 dilutions) for 16 hr compared to control, whereas in the reverse sequence of treatments no potentiation was noted. Lately, synergistic combination effects of human TNF and interferons are reported (Williamson et al. 1983). To our knowledge, however, there have been no reports in the literature describing this kind of potentiation in a delayed killing process produced by a partially purified cytotoxic substance from white blood cell culture which acts on tumor cells in a rather specific manner.

The influence of the elevated incubation temperature at 39°C on CTS-51 activity was tested against Daudi and G-361 target cells comparing to that of virus induced human leucocyte interferon. The temperature dependent potentiations of IFN-α (Le) actions are well established by the authors previously (Heron and Berg 1978; Hirai et al. 1984). In contrast to this IFN property, CTS-51 action was not potentiated at all by the elevated temperature (Fig. 4). These phenomena seem to imply that the development mechanism of the anticellular action of CTS-51 inside the target cells should be independent from that of the anticellular effect of IFN-α (Le), which is well-known to be promoted by some intracellular proteins inside the target cells (Zilberstein et al. 1978).

The influence of the FCS concentrations in the assay medium on the cytotoxic activity of CTS-51 was further explored in vitro to investigate whether this substance is still active even in the higher serum concentration circumstances. Unfortunately, the cytotoxic activity of CTS-51 was found to be reduced when the FCS concentration was elevated, and increased when it was lowered (Fig. 3). Recently, Oleszak and Ingolet (1980) have suggested that the antiproliferative activity of interferon is antagonized by the platelet derived growth factor (PDGF) that is known to be contained in the serum preparations and help the growth of the in vitro cultured cells. Our results seem to suggest the possibility that there may be present the same kind of antagonism also between the cytotoxic activity of CTS-51 and some components in the FCS preparations such as PDGF. Although further investigations are needed in this point, this property of CTS-51 may offer some minority in the in vivo trials of this substance.

In conclusion, we have described the biological or physicochemical properties
of a unique tumor specific cytotoxic substance, CTS-51, produced together with
immune interferon and interleukin-2 in the human buffy coat cultures stimulated
by staphylococcal enterotoxin B (SEB), which have been found to be distinct
from those of any ever known cytotoxic lymphokines. Namely, CTS-51, whose
activity seems to be promoted by small active molecules has a cytotoxic activity
very stable after the treatments of heat even at 100°C for 30 min, low pH at 2.0
for 24 hr and the trypsin digestion with 0.75% trypsin for 1 hr. The cytotoxic
activity of CTS-51 seems to be prescribed by the concentration of this substance.
This substance can potentiate the antacellular activity of HuIFN-α (Le), whereas
the cytotoxic activity of CTS-51 may be antagonized by some components in FCS
preparations, in vitro.

Further investigations and the complete characterization are needed before
elucidating if this unique cytotoxic cytokine, CTS-51, will be of any clinical
usefulness.

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