A unique cytotoxic substance (CTS-51), which is rather specific to malignant cell cultures in vitro, was found to be produced together with human immune interferon and interleukin-2 in Buffy coat cultures stimulated with staphylococcal enterotoxin B. CTS-51, which is stable at 100°C and has a molecular weight of 8,000-10,000, was found to have biological properties distinct from those of known cytotoxic cytokines. CTS-51 was able to kill four human malignant cell lines at concentrations that did not affect normal or non-malignant cell lines. The mode of CTS-51 action was found to be cytotoxic rather than cytostatic. Several kinetic studies showed that the cell killing activity seemed to be dose-dependent, and the appearance of the activity required about 24 hr. The minimum effective exposure time of the target cells to CTS-51 also seemed to be dose-dependent.

Key words: Lymphokine — Monokine — Cytotoxicity
supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), 100 U/ml of penicillin and 100 μg/ml of streptomycin (GIBCO). The other cells were cultured in Eagle's MEM (GIBCO) supplemented with 10% FCS, 100% non-essential amino acids (NEAA, GIBCO), 100 U/ml of penicillin and 100 μg/ml of streptomycin.

CTS-51 CTS-51 was isolated during the process of human IFN-γ and interleukin-2 production as a by-product. A full description of the purification and characterization will be published elsewhere (Georgiades et al., manuscript in preparation).

Briefly, humanuffy coats were isolated from red blood cells and washed with saline. After contaminating red blood cells had been lysed by ammonium chloride, the white blood cells were suspended in RPMI 1640 medium supplemented with 1% human a-gamma serum and 2.5% albumin at a cell concentration of 1.2 x 10^6 cells/ml. Then 100 ng/ml of staphylococcal enterotoxin B (SEB) was added and the cells were kept at 37°C for 2-24 hr. The supernatant was clarified by centrifugation and after several steps it was passed through a Millipore filter with a molecular weight cut-off of 10,000 daltons. The filtrate, containing all molecules of less than 10,000 daltons, was concentrated by means of another Millipore filter with a 1,000 dalton cut-off and the pH was adjusted to 7.0. The material was sterilized by filtration, and stored in ampules at 4°C.

Interferon Human leucocyte interferon (IFN-α(Le)) was produced from donor buffy coats induced with Sendai virus and partially purified according to the method described by Cantell et al. The interferon titer was estimated by means of the plaque reduction assay on U cells challenged with vesicular stomatitis virus (VSV). All units are expressed as international units (IU) referring to the NIH standard IFN-α (Lot No. G023-901-527). The specific activity of the partially purified leucocyte IFN (PIF) used in this study was 1.0 x 10^6 IU/mg protein.

Assays of Anticellular Effects of CTS-51 and Interferon Cells were cultured in 200 μl of medium/well containing serial 3-fold dilutions of CTS-51 or various concentrations of interferon in 96-well flat-bottomed microplates (Nunc, Denmark) for three days at 37°C in a 5% CO₂ atmosphere. Cell propagation was then evaluated by counting cell numbers, measuring [³H]-thymidine (³H-TdR) incorporation, or measuring neutral red dye incorporation, and the % cell growth inhibition (relative to controls) was calculated. Cell counting was performed by counting total and viable cells in a hemocytometer chamber (Fisher Sci. Co.) using 2.5% Trypan Blue solution (GIBCO). ³H-TdR incorporation studies were done as previously described. Briefly, 30 nCi of [³H]-TdR (ICN Chemical & Radioisotope) in 15 μl was added to each well. After being incubated at 37°C for 24 hr, the cells were harvested by a Mash II apparatus on filter paper (Whatman Ltd.), and washed with distilled water. The radioactivity on the dried filters was counted in a liquid scintillation counter. Dye incorporation was measured as described by Kull and Cuatrecasas. Cells were incubated with 150 μl/well of medium containing 0.01% neutral red dye (Difco) for 2 hr at 37°C, washed twice gently with 0.9% NaCl solution and lysed with adding 100 μl of solution consisting of equal parts of ethanol and Sorenson's citrate buffer, pH 4.2. The A₅₄₀ of each well was measured with a Titertek Multiskan (Flow Laboratories).

In experiments involving normal WBC cultures, the effect of CTS-51 was evaluated in terms of the % decrease in viability using Trypan Blue solution.

**RESULTS**

In order to evaluate the extent to which CTS-51 can suppress the growth of various types of cells, a number of experiments were performed. Fig. 1 shows the results of four experiments where the suppressive effects of various concentrations of CTS-51 on the six different target cells were tested by means of the dye uptake method. As indicated in Fig. 1, the growth of each cell type tested was suppressed by this substance dose-dependently. The sensitivity of a normal cell (primary fibroblast) or a non-malignant cell line (U amnion) to CTS-51 was rather lower than that of cell lines derived from malignancies (G-361 melanoma, PLC/PRF/5 hepatoma, CCRF-CEM lymphoblastoid and Daudi lymphoma cells). At the dilution of 1:54, the growth of malignant cell lines was completely (100%) suppressed, whereas that of normal or non-malignant cell types was not inhibited at all. Even at the dilution of 1:162, the growth of three malignant cell lines out of four was suppressed by more than 75%.

This difference in sensitivity between malignant and non-malignant cells was also tested using normal peripheral white blood cells (WBC) and two lymphoblastoid cell lines (CCRF-CEM and Daudi cells) by means of a viability test, because normal WBC do not grow without any stimulation in vitro. As can be seen from Fig. 2, normal WBC were impaired only slightly in viability...
at a dilution of 1:18. In contrast, both malignant lymphoblastoid cell lines had considerably lower viabilities (below 50% even at 1:54 dilution).

The possibility, based on the viability study (Fig. 2), that CTS-51 suppresses cell growth by killing the target cells (cytotoxic effect), was further investigated by testing the parallelism between the cell growth suppression curve and the viability curve using CCRF-CEM and Daudi target cells. In these experiments, the cell propagation and viability were evaluated by counting the total and viable cell numbers using 2.5% Trypan Blue solution, and the mode of action of this material was compared with that of virus-induced interferon (IFN-α(Le)). As shown in Fig. 3A and 3B, the antiproliferative efficacies of IFN-α(Le) against these two cell lines were different; namely, Daudi cells were highly sensitive but CCRF-CEM cells were insensitive to IFN-α(Le). Similarly, the sensitivity to CTS-51 of Daudi cells was considerably higher than that of CCRF-CEM cells (also indicated in Figs. 1 and 2). As for the mode of action, CTS-51 clearly showed cytotoxic action (indicated by the parallel reductions of the growth and the viability of both target cells, independently of their sensitivities to this material). On the other hand, the action of IFN-α(Le) was not cytotoxic but cyto-

Fig. 1. Cell growth inhibition by CTS-51. Normal human fibroblasts (○), U-amnion (□), or PLC/PRF/5 (▲) at 1.0 × 10^4 cells/well, Daudi (●), or CCRF-CEM (◆) at 1.5 × 10^4 cells/well, or G-361 cells at 3.0 × 10^4 cells/well were cultured with various dilutions of CTS-51 for three days at 37°C in 96-well microculture plates. Cell propagation was measured by the neutral red dye incorporation method. Cell growth inhibition was assessed by comparison with controls without CTS-51. Results are the mean values of four experiments.

Fig. 2. Viability of cells treated with CTS-51. WBC (○) at 1.0 × 10^4 cells/well, Daudi (●) at 1.0 × 10^4 cells/well or CCRF-CEM (▲) cells at 1.5 × 10^4 cells/well were cultured with various dilutions of CTS-51 for three days at 37°C in 96-well microculture plates. Cell propagation and viability were measured by direct cell counting using 2.5% Trypan Blue solution. The % reduction in viability was calculated relative to the control. Cell counts from control cultures were (9.2 ± 0.6) × 10^4 cells/well (n=6) for WBC, (7.2 ± 0.4) × 10^4 cells/well (n=5) for Daudi, and (2.1 ± 0.2) × 10^5 cells/well (n=7) for CCRF-CEM cells. Viabilities of the control cell cultures were 96.68 ± 1.44% (n=6) for WBC, 92.72 ± 3.73% (n=5) for Daudi, and 97.12 ± 1.19% (n=7) for CCRF-CEM cells. These experiments were done at least in triplicate and mean values are plotted.
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Several kinetics studies were performed with Daudi cells as target cells in order to investigate the appearance of the cytotoxic effect of CTS-51. Fig. 4 shows the course of the cell viability and the concentration of viable cells up to 48 hr after the start of incubation of Daudi cells with three different dilutions of CTS-51. At the highest concentration (the lowest dilution; 1:10) of CTS-51, the cytotoxic effect appeared between 16 and 24 hr after the start of incubation. At the dilution of 1:100, however, it was observed between 24 and 48 hr, indicating that the appearance of the CTS-51 action (early cytotoxic action) seems to be dose-dependent. At 1:1000 dilution, this material did not show any cytotoxic action at all within 48 hr.

In order to investigate how long an exposure of the target cells to CTS-51 is needed to produce cell damage or lysis (late cytotoxic action), the following experiment was performed. After Daudi cells had been incubated with various concentrations of CTS-51 for different lengths of time (see Fig. 5; 1, 5, 16, 24 and 48 hr), the target cells were washed, the volume was adjusted back to the original, and the cells were further incubated in CTS-51-free medium for three days. Then the cell propagation was determined by means of the 14C-TdR incorporation method and the % growth inhibition was calculated.

As indicated in Fig. 5, contact with the highest concentration of CTS-51 (1:10 dilution) for 16 hr resulted in more than 80% reduction of thymidine incorporation of cells after three days of culture in CTS-51-free medium. At 1:1000 dilution, there was a slight reduction of thymidine incorporation after 24 or 48 hr exposure to CTS-51. Thus, the minimum exposure time required to pro-

Fig. 3. Comparison of actions of CTS-51 with IFN-α(Le). Daudi (A) and CCRF-CEM (B) cells at 1.5 x 10^4 cells/well were cultured with various dilutions of CTS-51 (○, ■) or human leucocyte interferon (□, ▲) for three days. Cell propagation and viability of the cells were measured as described in the legend to Fig. 2. Cell counts of control cultures were (7.6 ± 1.7) x 10^4 cells/well (n = 4) for Daudi, and (2.1 ± 0.2) x 10^5 cells/well (n = 7) for CCRF-CEM cells. Viability levels of the control cell cultures were 96.1 ± 1.4% (n = 4) for Daudi and 97.1 ± 1.2% (n = 7) for CCRF-CEM cells. Experiments were done in at least triplicate and mean values are plotted.
duce the late cytotoxic action of CTS-51 also seems to be dose-dependent. It should be noted that a 16 hr incubation at the high concentration (1:10 dilution) of CTS-51 showed no early cytotoxic effect (Fig. 4) but produced a delayed cytotoxicity of about 80% (Fig. 5). In the case of 1:100 dilution of CTS-51, similar results were obtained at an incubation time of 24 hr (Figs. 4 and 5).

**DISCUSSION**

Lymphokine preparations are generally known to contain mixtures of several biologically active substances, such as lymphotoxins, tumor necrosis factors, interferons, interleukin-2, etc. Although numerous reports have appeared on the diverse activities of these substances, no mechanisms have yet been proposed which would encompass all the different actions ascribed to these molecules.

In the present study, we have investigated the cell growth-inhibitory or cytotoxic properties of a unique substance produced by human buffy coat cultures during the mass production of immune interferon and interleukin-2. This substance, CTS-51, was subjected to several purification steps including membrane filtration and ion exchange chromatography, after which no interferon activity at all was detected by the CPE inhibition assay on GM cells (data not shown). Our results have revealed that monolayer cultures of normal human fibroblast cells and the U cell line, a non-malignant amnion cell line, were not affected to the same degree as four human malignant cell lines (Fig. 1). On the basis of these experiments, it is possible to define a lytic unit (LU) of the CTS-51 preparation used in this study with respect to an individual cell type by employing the reciprocal of the dilution which will yield 50% reduction of cell propagation relative to the control. Our preparation showed activities of 55 LU on fibroblast cells, 40 LU on U cells, 130 LU on CCRF-CEM, 213 LU on G-361, 281 LU on PLC/PRF/5 and 678 LU on
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Daudi cell line. Thus, it appears that CTS-51 is three to thirteen times more active on the latter four tumor cell lines than on the normal fibroblast cells and U cells. Similar assay results were obtained when \(^{14}\)C-TdR uptake and direct cell-counting methods were employed to assess the cell propagation in other experiments (data not shown).

The putative tumor specificity of this material was further substantiated by the viability study (Fig. 2). Normal WBC were only damaged moderately at high concentrations of CTS-51 (at 1:6 and 1:18 dilutions), whereas the viability of Daudi cells was severely impaired even at 1:162 dilution and moderately at 1:4,374. With regard to CCRF-CEM cells, the viability was reduced by more than 50% at 1:54 dilution of CTS-51.

When the CTS-51 activity was tested on tumor cell lines of both human and mouse origin using the tumor stem cell assay technique, a similar trend was noted with regard to the human cell lines in the same dose range as used in the above study. However, this material was less active on the murine cell lines (data not shown). This indicates that CTS-51 is not likely to be one of the lymphotoxins (LT) or so-called tumor necrosis factors (TNF). These two cytotoxic cytokines are well established to be active on murine target cells.19, 21

The results shown in Fig. 3 clearly revealed that the mode of CTS-51 action is cytotoxic, and not cytostatic as that of virus-induced interferon is. This mode of action was also substantiated by the results in Fig. 5; after a sufficiently long exposure to CTS-51 the target cells subsequently died even in the CTS-51-free medium.

The kinetics of the killing process were examined (Fig. 4), and the minimum exposure time to this substance that results in the cell lysis was explored (Fig. 5). Interestingly, the incubation periods required for the appearance of the early and late cytotoxic actions were dose-dependent. Moreover, there was a considerable time delay in the appearance of the early cytotoxic effect as compared with the late one; namely, at 1:10 dilution, the early cytotoxic effect appeared after 16–24 hr of incubation but the minimum exposure time required to produce the late cytotoxic effect was less than 16 hr. A similar relationship was noted at 1:100 dilution. At present, however, it is not known whether the process of killing requires \emph{de novo} protein synthesis in the target cells, as is the case with interferon actions.11, 13, 22

Currently, there appear to be five groups of cytotoxic cytokines; namely, lymphotoxins (LT), tumor necrosis factors (TNF), natural killer cytotoxic factors (NKCF), thymodin and immune interferons (IFN-\(\gamma\)).4, 21 Since this material, CTS-51, does not possess any interferon activity (antiviral activity tested on GM cells), and the production of CTS-51 does not require thymus cells, the latter two groups can be excluded but the former three deserve further discussion.

The assay system of LT usually includes a protein synthesis inhibitor.19 It is generally known that LT is inactivated above 80 degrees C and has a molecular weight in the range above 10,000. Human TNF, which is known to have some tumor specificity,21 was reported to have a molecular weight of approximately 70,000, to have no interferon activity, to be acid-labile, and to be destroyed by heating at 70 degrees C for 1 hr.21 NKCF was reported to have a molecular weight of more than 12,000, to be labile at 100 degrees C and to be inactivated by trypsin treatment.1 In addition, all three cytokines are known to be active in murine cell systems, for instance on mouse L-929 cells.1, 5, 19, 21 TNF requires the presence of divalent metal ions.21

We used CTS-51 in a partially purified form. Recently, however, CTS-51 has been purified further (not published), giving only three protein bands on SDS-PAGE; two of the bands showed the cytotoxic activity and had molecular weights between 8,000 and 10,000. Recent studies in CTS-51 have also revealed that this material is not inactivated by heating at 100 degrees C, by treatment at pH 2.0, or by trypsin digestion (manuscript in preparation). The responses of this substance to other proteolytic enzymes and to glycosidases are now under investigation.

Based upon these preliminary observations, this material is not considered to be one of the known cytotoxic lymphokines or monokines. CTS-51 seems to show some similarity to TNF in its tumor specificity, but also seems to have some species specificity, as men-
tioned above, based on the preliminary results of tumor stem cell assay. Moreover, CTS-51 has much lower molecular weight than any previously reported cytotoxic cytokine, and seems to be very stable to heat or acid. Thus, this substance appears to have characteristics distinct from those of any reported lymphokine or monokine.

In conclusion, we have demonstrated that a cytotoxic substance, probably a lymphokine or monokine with properties distinct from those of any presently known cytokine, can be produced in large amounts from human buffy coat cultures stimulated by SEB to produce human immune interferon and interleukin-2. It is tempting to suggest that the activity of CTS-51 is directed mainly against tumor cells. CTS-51 does not require the presence of any cytotoxic effector cells in order to exert its cytotoxic actions, nor does it require any metabolic inhibitor. Thus, CTS-51 appears to be an interesting cytokine with a rather unusual tumor-specific cytotoxicity. Further experiments are needed before it will be possible to establish whether this substance is likely to be of any clinical value.

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