

Isolation and partial characterisation of a new antiproliferative substance from human leucocytes inhibiting growth of *Candida albicans*

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Aim: To purify and partially characterise a fraction from human leucocytes containing a substance cytotoxic to *Candida albicans*.

Methods: Leucocytes were isolated from the buffy coats of healthy blood donors. The cytotoxic factor (CF) was isolated from the soluble fraction of the cells. A cell lysate was passed through a filter with a cut off value of 3 kDa, and the filtrate was processed by anionic exchange chromatography and gel filtration. The purified CF was analysed for its chemical and biological properties. The cytotoxicity of CF was tested on *C albicans* grown on agar plates.

Results: Mass spectrometry showed a molecular mass of 2.148 kDa. CF was found in polymorphonuclear neutrophilic cells only. No amino acids were detected, and a low ultraviolet absorbance at 260 nm and resistance to nuclease indicate the absence of nucleic acids. An anthrone test was positive for carbohydrate. The substance was soluble in water. CF showed a dose related cytotoxicity in the range of 0.1–1 mg/ml. The cytotoxic effect was abrogated by zinc ions. Preliminary testing indicated that CF also had cytotoxic effects against some bacteria.

Conclusions: This report describes a factor from isolated human leucocytes that is cytotoxic to *C albicans*. The substance contains a carbohydrate moiety, whereas no amino acids were detected. The cytotoxicity can be abrogated by zinc ions in vitro. This substance is probably part of the repertoire by which leucocytes prevent infections.

This report deals with the partial characterisation of a new cytotoxic factor (CF) isolated from human neutrophilic granulocytes. Leucocytes are reported to produce several antimicrobial substances that can destroy invading microbes. Most of the identified substances are peptides and proteins—for example, defensins, serpicidins, cathelicidins, calprotectin, and other proteins with cytotoxic effects.¹ However, there are several other small endogenous substances, redox active molecules such as superoxide anion, hydrogen peroxide, and substances containing halogens, which are all very reactive.² To increase selectivity and efficiency phagocytic leucocytes ingest and sequester microbes in intracellular vacuoles.³

“We suggest that this substance is an integral part of the granulocyte defence system, being cytotoxic against both eukaryotic and prokaryotic cells”

We have observed a small molecular mass substance, which could be purified by DEAE anion exchange chromatography. The purpose of our present study was to isolate and characterise this molecule, which has cytotoxic properties. We have demonstrated cytotoxicity and the presence of carbohydrate, in addition to the absence of amino acids and nucleic acids.

We suggest that this substance is an integral part of the granulocyte defence system, being cytotoxic against both eukaryotic and prokaryotic cells, and have named it cytotoxic factor (CF).

Preliminary results (not shown) have also indicated that CF has some cytotoxic effects on various fungi and bacterial strains.

MATERIALS AND METHODS

Preparation of the crude leucocyte extract

All steps in the isolation procedure were performed at 4°C. The buffy coats from standard 500 ml blood units were

prepared from healthy blood donors on the day of collection. The volumes of the buffy coats were 60 ml and they contained approximately 3×10^9 leucocytes and 7.5×10^9 thrombocytes. Leucocytes were isolated from the buffy coats after lysis of the red blood cells with isotonic ammonium chloride (0.155M) and sodium hydrogencarbonate (0.01M) for 10 minutes. The leucocytes were spun down at 160 $\times g$ for 10 minutes. The supernatant was discarded and the leucocytes were washed three times with isotonic saline. The cell pellet was frozen in Falcon polypropylene tubes (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) at -50°C , and subsequently thawed to rupture the leucocytes. The cell pellet was then suspended in distilled water.

This suspension was centrifuged, the supernatant was collected, the cell pellet was washed with two volumes of distilled water, and the supernatants containing mainly the cytosol were combined. This was designated crude leucocyte extract.

Preparation of CF

Crude leucocyte extracts from four buffy coats were filtered through a Sterivex-HV 0.45 μm filter (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate was transferred to an Amicon stirred filter cell model 8050 with a Diaflo YM-3 ultrafiltration membrane, with a nominal 3 kDa molecule mass cut off (Amicon Inc, Danvers, Massachusetts, USA). The low molecular filtrate was mixed with 1/3 volume 0.625M EDTA₂ buffer, adjusted to pH 7.5 with NaOH, and applied to a DEAE Sepharose fast flow ion exchange column (1 \times 7 cm, Pharmacia Biotech, Uppsala, Sweden). The DEAE ion exchange column was equilibrated with the EDTA₂ buffer.

Abbreviations: CF, cytotoxic factor; ESI-MS, electrospray ionisation mass spectrometry; UV, ultraviolet

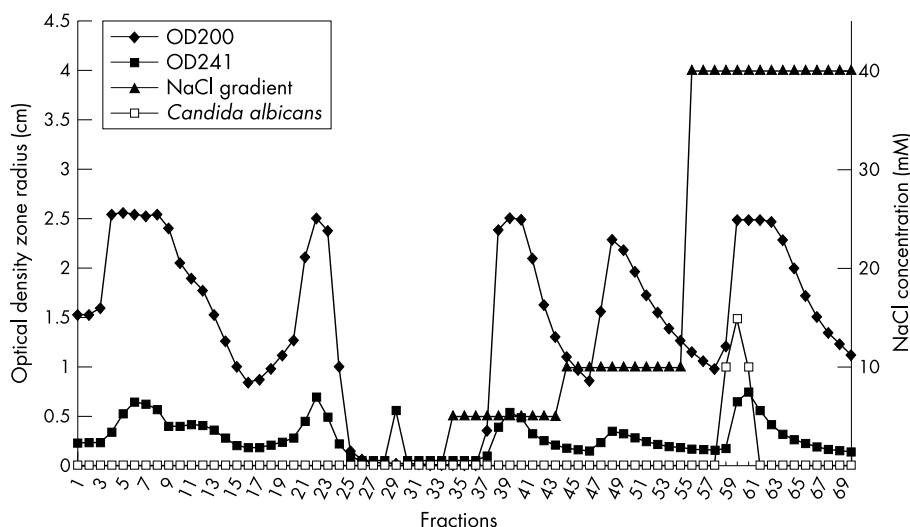


Figure 1 Elution profile of the ultrafiltrate from the crude leucocyte extract from the DEAE Sepharose fast flow anion exchange column (1×7 cm). The column was equilibrated with EDTA buffer, pH 7.5, and eluted stepwise with NaCl. The cytotoxic factor appeared when eluted with 40mM NaCl (in fractions 57–61). The cytotoxic factor was determined by measuring inhibition zones in *Candida albicans* plates. OD, optical density.

The column was eluted with 20 ml EDTAK₂ buffer, 30 ml distilled water, and stepwise 20 ml of 5mM, 20 ml of 10mM, and 40 ml of 40mM NaCl; the fraction size was 2.0 ml (fig 1).

The fractions containing cytotoxic substance were combined and lyophilised, dissolved in 1.8 ml distilled water, and applied on to a P-2 extra fine gel filtration column (1.5×162 cm) (Bio-Rad Laboratories, Hercules, California, USA). The void volume (V_0) was 74.8 ml, as determined with haemoglobin. Fractions of 2.5 ml were collected, tested for cytotoxicity, and their ultrasound (UV) absorbance was measured at 200 and 241 nm (fig 2).

Testing for cytotoxicity

The agar diffusion method described by Ericsson and Sherris⁴ was used with some modifications. We added 35 μ l of the test solutions to each of 39 wells made with a gel puncher on YNB agar plates seeded with *C albicans*. The plates were incubated at 37°C for 24 hours, and the inhibition zones were measured.

The cell density of the inoculum was adjusted to produce nearly confluent colony growth. This was achieved by making up a saline solution with the initial density of cells corresponding to 0.5 McFarland units. Five drops of this solution were added to 10 ml of saline. This inoculum was evenly

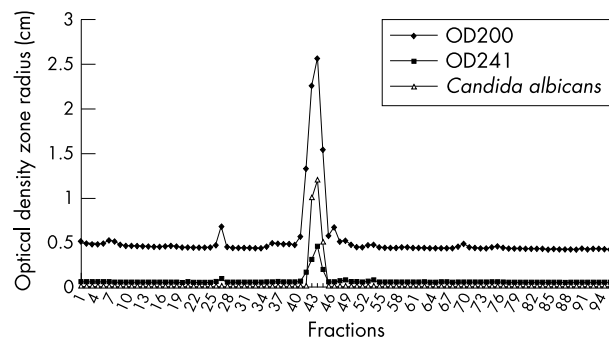


Figure 2 Elution profile when concentrated fractions 57–61 from DEAE chromatography were run on the P-2 extra fine gel filtration column (1.5×162 cm). The column was equilibrated and eluted with 20mM NaCl. The fractions were 2.5 ml. The cytotoxic factor was assayed by measuring the inhibition zones on YNB plates incubated with *Candida albicans*. The elution volume (V_0) for the cytotoxic factor was 105 ml (fractions 40–45), corresponding to a molecular mass of 2.4 kDa. OD, optical density.

distributed by gentle rotation. The plates were tipped to drain and the surplus inoculum was then removed with a pipette.

Subcultures of *C albicans* were grown on Sabouraud plates.

Determination of the molecular mass

Electrospray ionisation mass spectrometry (ESI-MS) was used to obtain the molecular mass of CF. The ESI-MS system used was a Waters 2690 (Massachusetts, USA) mobile phase pump coupled to a Quatro LC-MS (liquid chromatography-mass spectrometry/MS triple quadrupole mass spectrometer (Micromass Ltd, Altrincham, UK), equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing were performed using a Maslynx NT 3.1 computer system. To obtain the molecular mass, MaxEnt algorithms were used, based on the method of maximum entropy. The analyte was introduced into the mass spectrometer directly via a 10 μ l LC injection loop. The LC conditions were as follows: mobile phase: 50 vol% methanol/water, flow rate 0.2 ml/minute. ESI-MS conditions were as follows: nitrogen was used as both drying gas and nebulising gas at flow rates of approximately 1000 litre/hour and 80 litre/hour, respectively. The cone voltage was set to 50 V and the electrospray capillary was at 4 kV. The source was operating at a temperature of 100°C and the desolvation temperature at 180°C. The pressure in the analyser was 6.7×10^{-6} mbar and 3.7×10^{-5} mbar in the gas cell. Both positive and negative ionisation modes were used. The mass scan range was 50–2500 amu.

The molecular mass of CF was also determined by gel filtration using the P-2 extra fine gel filtration column (1.5×162 cm). Vitamin B₁₂, sucrose, and glucose were used as standards.

The elution volumes were measured and the molecular mass of CF was determined by plotting log molecular mass against K_{av} values. The exponential regression formula was found to be $y = -0.18 \ln(X) + 1.5$. Calculation using the regression formula gave the molecular mass of the molecule.

Distribution of CF in blood

Blood samples were collected from blood donors using Vacutainer tubes with SST gel and clot activator to obtain serum. To collect plasma, both EDTA tubes and ACD tubes were used (Becton Dickinson, UK). To isolate the different cell types from 5 ml EDTA blood, PMN separation medium (Nycomed Pharma, Oslo, Norway) was used, according to the

manufacturer's instructions. The bands containing the different cells were harvested using a pasteur pipette, suspended in 3 ml 0.9% saline each, and frozen separately at -50°C .

After thawing, each of the cell types was centrifuged and washed with two volumes of distilled water. The supernatants from each of the cell types were combined and treated as crude leucocyte extract.

Estimation of the CF content in each cell

Leucocytes from eight buffy coats were processed in two batches, each from four buffy coats, according to our procedure for the preparation of CF. After gel filtration, the fractions containing CF were combined and lyophilised. The residue was weighed, dissolved in distilled water, lyophilised again, and weighed for control of constant weight. The content of CF in each cell was calculated, assuming no loss during this procedure.

Estimation of heat stability

Samples of CF in 100 μl distilled water were heated in a boiling water bath for five minutes, tested for cytotoxicity against *C. albicans*, and compared with untreated samples.

Effects of different enzymatic treatments

To test the susceptibility of CF to a protein degrading enzyme, we added 10 μl of proteinase K suspension (EC 3.4.21.64; Sigma Chemical Co, Missouri, USA) to a 100 μl sample of CF (in 18.75mM Diemal buffer, pH 7.5, containing 10mM CaCl_2), and incubated it at 37°C for 18 hours. After incubation, the mixture was heated to 95°C for 10 minutes to denature the enzyme. A control with only the proteinase K was included to test for enzyme toxicity.

Peptidase (Sigma) has general proteolytic and aminopeptidase activity. We added 300 μl of CF in Diemal buffer and 300 μl Diemal buffer to 0.6 U peptidase and incubated and tested the solution as above.

To test for the presence of nucleic acids we used nuclease S1 (EC 3.1.30.1, Molecular Biology Boehringer Mannheim, Mannheim, Germany), which has endonuclease and exonuclease activities. A 500 μl sample of CF and 500 μl of water were each added to 500 μl of incubation buffer and 20 μl (80 U) of nuclease S1. The tubes were incubated for one hour at 37°C and then heated in a boiling water bath for five minutes to inactivate the enzyme. The mixtures were tested for toxicity.

Thin layer chromatography to detect amino acids

Glass ampoules with CF dissolved in 1 ml 6M HCl were flushed with nitrogen, evacuated, and sealed. The ampoules were heated at 110°C for 26.5 hours. The hydrochloric acid was evaporated over NaOH in an evacuated desiccator. The hydrolysate was dissolved in 50 μl water to 2.5 mg/ml.

Thin layer chromatography on silica plates (Merck, Darmstadt, Germany) was carried out on samples of CF before and after hydrolysis. The thin layer plates were developed with n-butanol/acetic acid/water (2/1/7), sprayed with ninhydrin reagent, and heated at 110°C as described by Fahmy *et al.*⁵

Test for total carbohydrate

To test for the presence of carbohydrates, the anthronetest was performed, as described by Pons *et al.*,⁶ with glucose as a standard.

The phenol sulfuric test for carbohydrates was performed as a qualitative test according to the procedure described by Dubois *et al.*⁷

Effect of metal ions on the cytotoxicity of CF

To test the effects of metal ions on the cytotoxicity of CF against *C. albicans*, we added different concentrations of ZnSO_4 , FeSO_4 , MnCl_2 , and $\text{Cu}(\text{Ac})_2$. The toxicity of these metal ions alone on *C. albicans* was also examined.

To test whether metal ions with approximately the same ionic radius and same charge could interfere with the effect of zinc, various concentrations of iron, copper, and manganese were added to a CF solution where the toxic effect had been abrogated by zinc.

Dose-response of CF toxicity

To determine within assay and between assay variations, agar diffusion tests were performed on 10 plates using the same concentrations of CF. On the first day, five YNB plates were inoculated and tested with twofold dilutions of CF, starting with 1 mg/ml. On each of the following five days, one YNB plate was inoculated and tested with the same concentrations of CF. The zones of inhibition for different concentrations of CF were measured. Mean values and SDs were calculated. The figures from these 10 plates were included in a dose-response study.

Chemicals

All buffers and salt solutions were made from filtered, deionised water (Milli-RO 4; Millipore Corporation, Bedford, Massachusetts, USA) or bottled sterile water (Baxter Healthcare Corporation, Deerfield, Illinois, USA).

All chemicals were of reagent grade. EDTA_{K₂} was from Fluka Chemie AG, Switzerland. Other salts and reagents were from Merck. Special care was taken not to introduce cytotoxic or enzyme inhibiting agents to the buffer, reagents, or equipment used.

RESULTS

Molecular mass

ESI-MS experiments of CF were performed in both positive and negative mode. The mass spectrum of the CF, obtained in negative mode, shows molecular ions at m/z 2148; that is, a molecular mass of 2.148 kDa.

The molecular mass as determined by gel filtration was approximately 2.4 kDa. Calculation of the molecular mass using $K_{av} = 0.117$ in the regression formula gives the value 2.364 kDa.

Distribution of CF in blood

We followed the isolation procedure for CF on plasma, serum, and leucocytes. CF was detected in preparations from polymorphonuclear neutrophilic cells only. However, its presence in monocytes cannot be excluded because of the low numbers of cells available for extraction.

Estimation of CF in each cell

The estimated number of granulocytes in eight buffy coats is 12×10^9 and 3.3 mg of CF was recovered from eight buffy coats. Therefore, the calculated amount of CF in each cell was 0.275 pg/cell (0.128 fmol/cell).

Temperature stability

Samples of CF that had been heated in a boiling water bath for five minutes retained their cytotoxic effect on *C. albicans*.

Enzymatic treatment

Proteinase K and peptidase, which degrade proteins and peptides, had no effect on the cytotoxicity of CF, and neither did nuclease S1, which degrades nucleic acids.

Search for amino acids

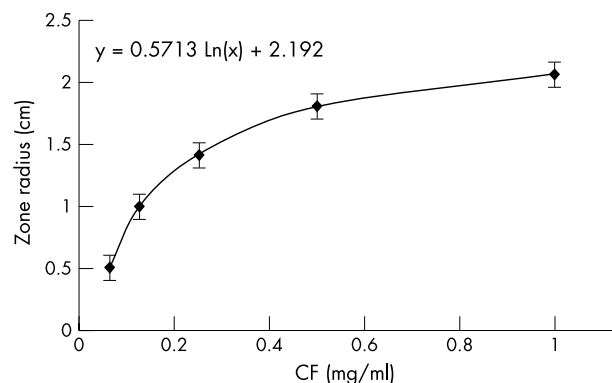
No amino acids were detected by thin layer chromatography of untreated CF or acid hydrolysed CF followed by ninhydrin spray.

Total carbohydrate

The results from the anthronetest indicated that the concentration of carbohydrate measured as glucose was 41 $\mu\text{g}/\text{ml}$. The ratio of carbohydrate, measured as glucose, to

Table 1 Effect of different metal salts on the cytotoxic effect of cytotoxic factor

Salt	Concentration				
	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
ZnSO ₄	-	-	-	+	+
FeSO ₄	-	-	-	-	-
MnCl ₂	-	-	-	-	-
Cu(Ac) ₂	-	-	-	-	-

**Figure 3** Dose-response curve showing the relation between the concentration of cytotoxic factor (mg/ml) and the inhibition radius (in cm) on *Candida albicans* plates. The formula for the regression line is shown.

total CF was 1 : 1 (41 µg of carbohydrate/400 µg of CF). The phenol sulfuric acid test confirmed the presence of carbohydrate.

Effect of metal salts on the cytotoxic effect

Table 1 shows the effect of different metal salts on the cytotoxic effect of CF. The addition of up to 10⁻⁵M ZnSO₄ did not alter the toxic effect, whereas at higher concentrations of zinc the growth inhibition was abrogated. In contrast, concentrations of 10⁻⁶M to 10⁻³M FeSO₄, MnCl₂, and Cu(Ac)₂ did not affect the growth inhibitory effect of CF on *C. albicans*.

No toxic effects on *C. albicans* were seen for the metal ions in the concentrations used—that is, up to 10⁻³M.

Dose-response

The dose-response curve (fig 3) shows the relation between the concentration of CF and the growth inhibitory zone for *C. albicans*. The inserted logarithmic regression equation describes the curve. The curve was made from the mean of the measured values of 10 inhibition zones at each concentration of CF. Tables 2 and 3 show the results and the calculated means and SDs of the values obtained.

DISCUSSION

In our study, we have presented a procedure for the isolation and purification of a small (2.148 kDa) anionic molecule from neutrophil granulocytes with a cytotoxic effect on *C. albicans*. This cytotoxic factor was isolated from the cytosol fraction of leucocytes from healthy blood donor buffy coats. We have shown that polymorphonuclear granulocytes are the main source of CF in blood. The cytotoxicity was abrogated by the addition of zinc ions.

Mass spectrometry using the MaxEnt algorithms gave the molecular mass of 2.148 kDa, which fits well with the data from the gel filtration experiments. We have also demonstrated the presence of carbohydrate in this molecule, but its structure remains to be studied.

To our knowledge, this cytotoxic substance has not previously been isolated or described.

We did not analyse tissues, but it is known that other cytotoxic substances can be found elsewhere, in particular in epithelial cells.⁸ The recovery of CF for each granulocyte was about 0.275 pg, which is 0.6% of total cell weight. We have not been able to test the recovery rate of the extraction and purification processes.

CF was isolated under very mild conditions: no detergents, high salt concentrations, or extreme pH values were used. Furthermore, the first step after thawing was ultrafiltration, which removes all molecules with a molecular mass higher than 3 kDa. Therefore, we do not believe that CF is a fragment of a cytotoxic molecule of higher molecular mass.

To test for carbohydrates, we used the anthrone method (Pons and colleagues⁶), which measures total carbohydrates. The authors stated that all carbohydrates react in this test, but

Table 2 Radius (in cm) of inhibition zones on *Candida albicans* plates: between assay variations

Concentration (mg/ml)	Well	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean	SD	CV	Mean SD
1.0	1	2.2	2.1	2.0	2.1	2.2	2.1	0.1	3.9	0.1
0.5	2	1.9	1.9	1.8	1.8	1.8	1.8	0.1	3.0	
0.25	3	1.5	1.6	1.5	1.4	1.3	1.5	0.1	7.8	
0.13	4	1.1	1.2	0.9	1.0	1.0	1.0	0.1	11.0	
0.06	5	0.5	0.5	0.5	0.6	0.6	0.5	0.1	10.1	

CV, coefficient of variation.

Table 3 Radius (in cm) of inhibition zones on *Candida albicans* plates: within assay variations

Concentration (mg/ml)	Well	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV	Mean SD
1.0	1	2.1	2.0	2.1	2.0	1.9	2.0	0.1	4.1	0.1
0.5	2	1.8	1.7	1.8	1.9	1.7	1.8	0.1	4.7	
0.25	3	1.4	1.3	1.4	1.6	1.2	1.4	0.1	10.7	
0.13	4	1.0	1.0	0.9	1.1	0.8	1.0	0.1	11.9	
0.06	5	0.5	0.5	0.5	0.6	0.3	0.5	0.1	22.8	

CV, coefficient of variation.

Take home messages

- We have isolated and characterised a factor from human polymorphonuclear neutrophils that is cytotoxic to *Candida albicans*, in addition to various other fungi and some bacteria
- The substance is approximately 2.148 kDa, contains a carbohydrate moiety, but no amino acids
- The cytotoxicity of this molecule can be abrogated by zinc ions *in vitro*
- This substance is probably part of the repertoire by which leucocytes prevent infections

there is a difference in colour yield between different carbohydrates. Because the carbohydrate in this CF is unknown, our quantitative results must be interpreted with caution. We used these tests as estimates relative to standard curves made from glucose. The phenol sulfuric acid method of Dubois and colleagues⁷ confirmed the presence of carbohydrate.

Acid hydrolysis of peptides may lead to the loss of amino acids, in particular tryptophan and tyrosine, but also serine and threonine. If carbohydrates and amino acids are present, darkening of the hydrolysis product can be seen as a result of the formation of humins.⁹ We saw no darkening after hydrolysis, and UV scanning of solutions of native CF did not show a peak in the 260–280 nm region, which indicates that aromatic substances were not present.

“Preliminary tests on some Gram positive and on Gram negative bacteria have shown that cytotoxic factor affects the growth and the morphology of several bacterial cells”

The CF molecule has an anionic character, and this may be because of the presence of sulfate, phosphate, or carboxylic groups.

We tried to use a cation exchanger for purification purposes, but did not succeed (not shown), which suggests that this substance is devoid of cationic groups.

The toxicity of CF is comparable to that of common antimycotic drugs, because 8.75 µg of CF gave a growth inhibition diameter of 3 cm. Antibiotics are usually tested *in vitro*, with tablets or discs containing 1–50 µg of active substance. The difference in test method makes a direct comparison difficult, but our well method indicates the same dose–response level.

The mechanism by which zinc abrogates the antiproliferative effects of CF is not known. Zinc is an essential element in most organisms, being a required cofactor for many enzymes. There are several different theories that could explain its

inhibitory effect. Zinc may be sequestered by the CF via a liganding or chelating mechanism, thus making the zinc ion unavailable to the organism. However, we tested this theory by adding solutions of EDTA to the wells, but no effect was seen on growth during 24 hours (data not shown). Zinc may alter the conformation of the molecule so that it takes on a non-toxic form, or zinc may take the place of another metal ion that makes the substance toxic. We have not yet carried out binding studies to establish whether CF can bind zinc or other metal ions.

It has been shown that the cytotoxic effect may be regulated by the zinc concentration. However, the physiological consequences of such a regulation are not known.

Preliminary tests on some Gram positive and on Gram negative bacteria have shown that CF affects the growth and the morphology of several bacterial cells. We regard this as an important observation that will be the subject of further studies on eukaryotic and prokaryotic organisms.

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