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### *Paramecium* as a bioassay system for elucidation of cytotoxicity and biocompatibility of nanoparticles: effects of carbon nanofibers on proliferation and survival

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#### SUMMARY

Carbon nanofibers (CNF), composed of carbon nanotubes, are a recent technological advance with wide applications in nano-engineering fields including biotechnology and biomedicine. However, little is known about the environmental effects of CNF, or their potential danger to human health. To elucidate the safety of CNF, we examined the cytotoxicity of CNF in *Paramecium*. In this study we considered the cytotoxicity effect of CNF in two categories of cellular properties, cell survival and cell proliferation. We show that CNF are ingested and concentrated as efficiently as nutritive bacteria by paramecia, revealing a means by which CNF could be introduced into the food webs of aquatic ecosystems. Clear cytotoxicity of CNF was detected in survival tests by extracellular application

at extremely high concentration (up to 50 mg/ml) in culture medium containing nutritive bacteria. Contrary to this effect, no cytotoxicity was detected in survival tests using the modified Dryl's solution (K-DS) that is used as a buffered saline of culture medium. The cytotoxicity of CNF suggests an interaction between CNF and the components in culture medium or their metabolic products produced by digestion of components in culture medium. Another cytotoxicity effect was detected in proliferative activity test at lower concentrations of CNF (up to 500 µg/ml). The cytotoxicity on proliferative activity was reversible and recovery occurred within 24 hours after removal of CNF. Our results suggest that *Paramecium* is useful for a bioassay of nanoparticle cytotoxicity. For the elucidation of safety of CNF we have to examine both the optimum concentration of CNF and co-existing biomaterials in a test solution. In conclusion, CNF have a high potential for cytological and biomedical application under precise control of concentration.

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## INTRODUCTION

The safety of materials to living organisms usually depends upon particle size: substances that are safe in macroscopic quantities can become dangerous when reduced to microscopic particles (Brown et al, 2001). As the behavior of nanoparticles in the environment and in cells is not well understood, it is important to establish an experimental system in which to assess the effects of nanoparticles on the environment and on living organisms.

*Paramecium*, a ciliated eukaryotic unicellular organism that lives in fresh water, has several features that make it a potentially valuable model to assay the cytotoxicity of environmental agents (Rajini et al, 1989, Smith-Sonneborn et al, 1983, 1986): they can ingest both soluble molecules and many different types of particle, including polyethylene particles, India ink, iron filings and sand, by phagocytosis, and they show very stable cellular functions such as phagocytosis, cell division and swimming behavior. In this study, we examine the use of *Paramecium* as a bioassay for evaluating the cytotoxicity of nanoparticles and have established a standard experimental method that enables micro liter-sized assays.

The CNF used in this experiment have a structure of hollow graphitic tubules of nanometer dimensions, and are comprised of coaxial tubes of helically arranged carbon-hexagon sheets. Typically, their diameters ranged from 2 to 20 nm, and they were several micrometers in length (Iijima, 1991).

## MATERIALS AND METHODS

### Cells and culture medium

*Paramecium caudatum*, syngen 3 was used in this study. They were grown at 25°C in a culture medium containing 1.25% (w/v) fresh lettuce juice

diluted with K-DS (Dryl's solution (Dryl, 1959) modified by substituting  $\text{KH}_2\text{PO}_4$  for  $\text{NaH}_2\text{PO}_4$ , pH 7.0 (Yanagi, 1987)) and inoculated with *Klebsiella pneumoniae* one day before use (Hiwatashi, 1968).

### CNF production

CNF were provided by Prof. K. Tohji (Tohoku University, Japan). They were produced in a conventional flow reactor system (Rodriguez, 1993) in which a hydrocarbon/carrier-gas mixture ( $\text{C}_2\text{H}_4/\text{H}_2$  (4:2)) was pyrolysed at 873 K for 4 hours in the presence of powdered Ni catalyst. The catalyst was positioned in an  $\text{Al}_2\text{O}_3$  boat and set in a quartz tube assembled in a horizontal tube furnace; it had been reduced in advance in a 10%  $\text{H}_2/\text{He}$  stream for 2 hours at 873 K.

As the CNF inevitably contain an appreciable amount of metallic elements, such as Ni, a purification process is vital to both fundamental studies and potential applications of the material (Ebbesen et al, 1994; Tohji, 1996). The CNF used in this study were purified by hydrochloric acid treatment (Tohji et al, 1996).

### CNF ingestion

CNF were washed twice with K-DS with centrifugation at 10,000 rpm for 5 min. The washed CNF were suspended at a concentration of 100 mg/ml in K-DS. Paramecia were washed twice with K-DS by a hand centrifuge and suspended in K-DS at the cell density of about 4,000 cells/ml. Then, 40  $\mu\text{l}$  of the CNF suspension was mixed with an equal volume of the cell suspension and the mixture was incubated at 25°C. The paramecia were periodically isolated from the mixture with a hand-made glass micropipette and put into a drop of 2.5%(w/v) methyl cellulose dissolved in K-DS on a glass slide. Photographs were taken under a light microscope (ZEISS AX10) by a digital camera (Nikon, DS-5Mc).

### Cytotoxicity test of CNF

A CNF suspension was prepared in an autoclaved glass depression slide with an autoclaved microchip by mixing CNF stock suspension with either K-DS or fresh culture medium. Then, a single paramecium was added into 100  $\mu$ l of the CNF mixture with a hand-made glass micropipette under a binocular microscope. The CNF suspension with the paramecium was incubated at 25°C.

### Capillary culture method

We developed a small-sized bioassay using a glass capillary (0.6 mm inside diameter and 35 mm length) by modification of the method (Haga and Hiwatashi, 1981). Twenty  $\mu$ l of a CNF suspension containing paramecia was put on a glass depression slide and then the suspension was drawn into a capillary by capillary action. The capillary was placed in a laboratory dish with a sheet of wet paper. This method allows a one-week incubation at 25°C without any serious problem of evaporation of the medium.

### Statistic analysis

Statistical analysis of the data from the dose-effect experiments was performed using the Dunnett test (Graph pad Prism 4).

## RESULTS

### Ingestion of CNF and food vacuole formation

Paramecia ingest nutritive bacteria via the buccal cavity and concentrate the bacteria, together with a small amount of fluid, in food vacuoles. CNF-containing food vacuoles are shown in Figure 1a and 1b. Immediately after initial contact with CNF Paramecia began to ingest CNF by forming a food vacuole. CNF particles were concentrated in each food vacuole. No CNF particle that escaped from a food vacuole into cytoplasm was detected by a light microscope observation.

### Effect of CNF on non-proliferating cell survival

Before testing the cytotoxicity of CNF, we examined experimental conditions to prepare homogeneous dispersion of CNF suspension in K-DS. By varying the concentrations of CNF and the time of sonication, we confirmed that a homogeneous suspension of CNF could be obtained in up to a concentration of 50  $\mu$ g/ml in K-DS for 20 min sonication.

Paramecia do not undergo proliferation when incubated in a bacteria-free solution. The survival of non-proliferating paramecia was examined by using a capillary culture with 20  $\mu$ l of CNF suspension in K-DS. In the presence of 50 mg/ml of CNF, all of the tested cells remained alive through-



Fig. 1. A photograph of a living *Paramecium* ingesting CNF. a: At 1 min after the initiation of incubation in a CNF suspension (50 mg/ml), a cell was isolated in 2.5% (w/v) methyl cellulose dissolved in K-DS and mounted on a glass slide. Then, about 5 min later, a photograph was taken with a digital camera (Nikon, DS-5Mc) attached to a light microscope (ZEISS AX10). A white arrow shows aggregating CNF particles in a food vacuole. b: A photograph was taken at 20 min after the initiation of incubation in the CNF suspension. Black particles, one of which is shown by a white arrow, indicate CNF-containing food vacuoles. Bar is 30  $\mu$ m.

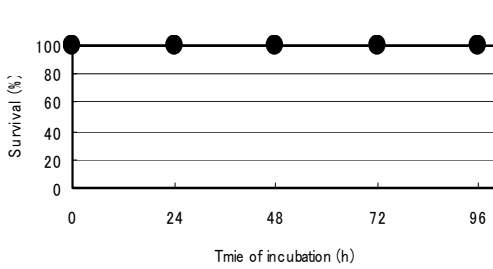


Fig. 2a. Survival rates of non-proliferating paramecia in a 50 mg/ml CNF suspension. The abscissa shows length of time of incubation in the CNF suspension, and the ordinate shows the mean percentage of cells surviving up to 96 h after the initiation of incubation (n=6).

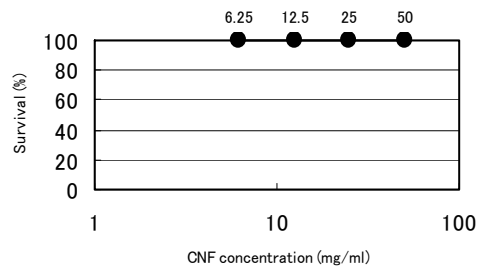


Fig. 2b. Effect of different doses of CNF on survival of non-proliferating paramecia. The abscissa shows the amount of CNF suspended in K-DS. Scale of abscissa was shown by logarithm. The ordinate shows the mean percentage of cells surviving up to 96 h after the initiation of incu-

out the period of 96 hours incubation, without any noticeable detrimental response to CNF exposure (Fig. 2a). No cytotoxicity was detected over a range of CNF concentrations up to 50 mg/ml in non-proliferating paramecia (Fig. 2b).

**Effect of CNF on proliferation**

The effect of CNF on the proliferation of paramecia was tested in fresh culture medium containing nutritive bacteria in a glass depression slide

culture. A single cell was suspended in 200 µl of the CNF suspension (50 mg/ml) and then was kept at 25°C. Under these conditions, during the first 24 hours the paramecia underwent cell division once, but their daughter cells gradually lost the ability to swim and, after 48 hours of incubation, more than 95% died. On the other hand, cells in the control culture continued cell divisions during 48 hours of incubation (Fig. 3a).

We examined cytotoxicity effect of CNF at

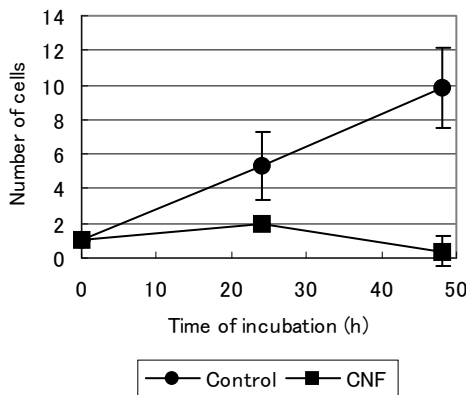


Fig. 3a. Survival of proliferating paramecia cultured in a CNF suspension. The abscissa shows time after the initiation of incubation and the ordinate shows the mean number of surviving cells in fresh culture medium containing nutritive bacteria and 50 mg/ml CNF (n=6). Control means the culture containing 0 mg/ml of CNF (n=6).

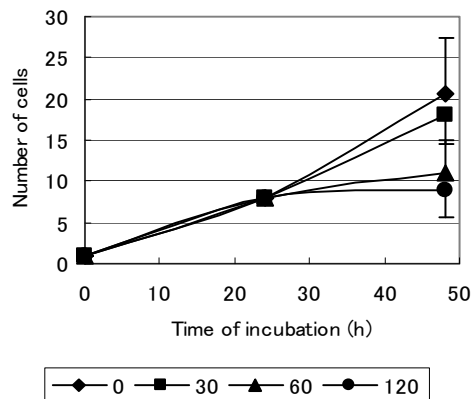


Fig. 3b. The time course of proliferation in different concentrations of CNF. The mean numbers of cells in fresh culture medium containing nutritive bacteria are shown (n=6). Diamond, 0; square, 30; triangle, 60; and circle, 120 µg/ml CNF. The abscissa shows time after the initiation of incubation and the ordinate the mean number of living

lower amount of CNF suspension. A single cell was suspended in 200  $\mu$ l of culture medium containing bacteria in a glass depression slide culture. The time course of the CNF effect is shown in Fig. 3b. During the first 24 hours in bacteria-containing culture medium, CNF did not inhibit proliferation at any concentration. However, during the second 24 hours incubation, 60 and 120  $\mu$ g/ml of CNF inhibited cell division.

The dose effect of CNF on proliferation at 48 hours after incubation is shown in Fig. 4. The mean number of cells at 30  $\mu$ g/ml of CNF was about 80% of that of the control culture (Dunnett,  $P < 0.05$ ). At 60  $\mu$ g/ml of CNF, the mean number of cells decreased to about 40% of the control (Dunnett,  $P < 0.01$ ), and remained at this level as the concentration of CNF increased. Thus, no dose effect was observed above 60  $\mu$ g/ml of CNF.

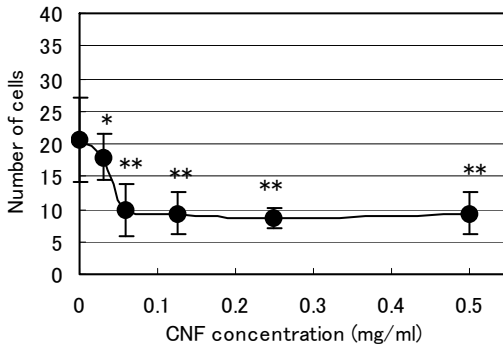


Fig. 4. Dose effect of CNF on proliferation. The abscissa shows the concentration of CNF and the ordinate the mean number of surviving cells at 48 h after the initiation of incubation. Bars show the standard deviation and \* indicates a significant statistical difference at  $P < 0.05$  (Dunnett test) and \*\* at  $P < 0.01$  (Dunnett test) ( $n = 6$ ).

**Reversibility of the cytotoxicity effect of CNF**

The reversibility of cytotoxicity of CNF was examined by transferring paramecia cultured for

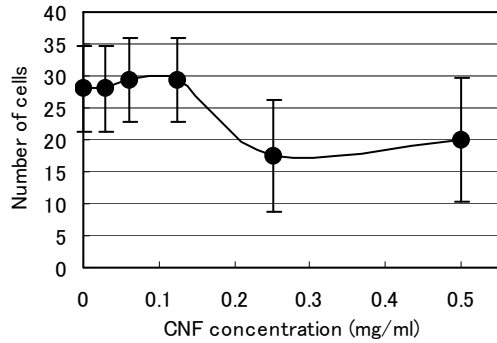


Fig. 5. Recovery of proliferative activity after removal of CNF. The abscissa shows the concentration of CNF and the ordinate the mean number of surviving cells at 48 h after the initiation of incubation. Bars indicate standard deviations ( $n = 6$ ).

48 hours in various CNF concentrations to CNF-free fresh culture medium in a glass depression slide. As shown in Fig. 5, at 30, 60 and 125  $\mu$ g/ml of CNF, the mean number of cells at 48 hours incubation recovered to control levels (CNF: 0  $\mu$ g/ml). At 250 and 500  $\mu$ g/ml, the mean number of cells did not recover fully, although statistical analysis showed no significant differences among the CNF concentrations (Dunnett,  $P < 0.09$  at 250  $\mu$ g/ml).

**DISCUSSION**

The health risks to humans and other organisms from exposure to nanoparticles in the environment are one of the most important concerns regarding the use of these potentially valuable materials. The *Paramecium* bioassay system described here provides a useful means by which to determine the cytotoxicity of environmental and ingested CNF.

Paramecia play an important role in food webs in aquatic ecosystems. They ingest diverse bacteria growing in water (Taylor, 1979) and, in turn, are

eaten by other protozoa such as amoebas, didinia and other metazoan animals or their larvae (Porter et al, 1979; Taylor, 1980). Thus, CNF could move into food webs of aquatic ecosystems following ingestion by paramecia. The speed of a CNF-containing food vacuole formation and the maximum number in a cell were similar to those observed after ingestion of nutritive bacteria (data not shown)

Two types of nuclear division occur in *Paramecium*: mitotic nuclear division performed by the micronucleus, and amitotic division by the macronucleus. For the purposes of this article, the cells incubated in K-DS are referred to as "non-proliferating" cells.

At the beginning of this study, we set two categories of cellular properties for an assessment of the cytotoxicity effect of CNF. One is the property of cell survival and the other cell division. For cell survival tests we used the buffered saline, K-DS, and examined survival ratio and swimming behavior. In non-proliferating paramecia, CNF had no cytotoxicity effect on both cellular properties in all experiments performed in this study.

However, we have found strong cytotoxic effect of CNF in the cell division tests. In this test we used culture medium containing lettuce juice and nutritive bacteria as described in "Materials and Methods". The cytotoxic effect of CNF was observed in both properties of cell survival and cell division. At 50 mg/ml of CNF, cytotoxic effect was detected in the property of cell division during the initial 24-hour incubation and no effect on the property of cell survival. In the following 24-hour incubation, however, cytotoxic effect was detected in the property of cell survival, too.

We considered the possibility of causal relation of CNF effect on the property of cell survival. The simplest case is the inhibition of energy source uptake by CNF. But, it is hard to assume this case because, under our experimental conditions, paramecia can survive more than 1 week without any

nutrient biomaterials indicating that the energy pool of cytoplasm is enough for surviving in the period of experiments performed in this study. However, an energy source uptake under the presence of CNF is remained to estimate experimentally.

An alternative possibility is the production of hazardous chemical substances by interaction between the metabolic products of culture medium and CNF. At this moment we have no information about chemical compositions of the culture medium. However, one candidate of the hazardous chemical substances is suggested by the following observation. One of the remarkable characteristics observed regarding cytotoxicity of CNF was the cessation of ciliary movement on the cell surface of proliferating paramecia. Ciliary movement in paramecia is essentially controlled by membrane potential (Dunlap, 1977; Kung and Saimi, 1982). Because the control of membrane potential is dependent on several kinds of ion channels (Kung and Saimi, 1982), chemical substances that affect ion channel functions will be one of target molecules to understand cytotoxicity of CNF.

Alternative experimental approach to the molecular mechanisms of CNF cytotoxicity is the use of complete synthetic culture medium in a cell division test. In this experiment we could systematically change chemical compositions in culture medium, so that the cytotoxicity of CNF control.

As shown in Fig. 4, the dose effect of CNF cytotoxicity was biphasic: at lower concentrations (from 30 to 60  $\mu\text{g/ml}$  of CNF), a dose dependency was observed, while the effect appeared independent of dose at higher concentrations (from 60 to 500  $\mu\text{g/ml}$  of CNF). The number of cells in culture medium depends on both the number of cell divisions and the number of cell death during cultivation. To obtain precise information about the contribution of cell division and cell death we have to perform a single cell isolation line culture under the presence of CNF after every cell division in

future experiments.

Our findings indicate that the effect of CNF on living organisms might not be as simple as previously predicted. We anticipate the presence of some complexity involving the molecular interactions between CNF and intracellular chemicals, which, in turn, may have harmful effects on cells.

In summary, the *Paramecium* bioassay system reveals that it is important to distinguish the property of cell division and cell survival in the elucidation of CNF cytotoxicity. As our studies have indicated, developing the appropriate regulatory guidelines to ensure the safety of nanotechnology will require biochemical examination and identification of chemical substances that interact with and modulate CNF cytotoxicity.

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