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THE INFLUENCE OF C_{60} POWDERS ON CULTURED HUMAN LEUKOCYTES

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Abstract. In order to check its possible acute toxicity, C_{60} was incorporated into living human phagocytes. It was observed that C_{60} has no influence on the survival of human leukocytes.

Introduction.

C_{60} has recently been described as a new kind of carbon molecule and at present is being extensively studied for its physical and chemical properties [1, 2]. However, although it has been postulated that a water soluble derivative of C_{60} can inhibit the human immunodeficiency virus (HIV) [3] and it has been shown

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that a C_{60} carboxylic acid can kill cells in the presence of light [4], the biological properties of C_{60} itself are still to be determined ; in particular its high redox potential [5] and its ability to photosensitise the conversion of triplet to singlet oxygen [6] suggest that footballene will have an acute toxicity.

We therefore tried to incorporate C_{60}, in living cells for informations on its possible acute toxicity. To transfer C_{60} into biological systems, different studies were carried out in aqueous solutions [7-10] but the concentration of solubized C_{60} was very low. To study its possible acute toxicity, we choose to use free C_{60} directly suspended in culture media at sufficient level (actually, 5 to 10 grains per cell at minimum). In order to by-pass this insolubility, we used living cells which are able to phagocyte solid particles (human polymorphonuclear neutrophils) [11-12]. After the C_{60} incorporation had been demonstrated, we measured parameters that usually reveal cytotoxic activity [13].

Experimental.

C_{60} was prepared and purified as previously described [5, 14]. Its purity was tested by HPLC, IR, UV and mass spectrometry. No impurity could be observed. It was used as such and also after further sublimation. No differences could be observed in the results obtained with both kinds of product.

Human leucocytes were obtained from healthy donor's peripherical veinous blood collected in heparinized tubes. Leucocytes fractionation and incubation were performed under aseptic atmosphere to prevent activation.

A 24/10 (v/v) mixture (DRS) of a 9% Dextran T500 (Pharmacia) and a 38% Radio-Selectan (Schering, N.Y.) solutions were first

prepared for human leucocytes frationation [15]. 40 ml blood were carefully set above 40 ml cold (+4°C) DRS solution. After 1 hour sedimentation, the leucocyte containing supernatant was collected, washed twice with a 0.1 M Phosphate Buffer Saline (PBS) (pH = 7.4), and centrifugated at 600 g for 10 minutes. The resulting pellet was resuspended at a concentration of 10^6 cells per ml in RPMI 1640 cell medium (Eurobio) supplemented with 1% l-glutamine (Gibco), 10% FCS (Eurobio), 1% streptomycin (Eurobio) and 1% penicillin (Eurobio).

Three series of 10^6 leucocytes per ml of culture media were prepared in triplicate and maintained at 37°C. One of them was used as a negative control. The two others were supplemented with either graphite (which was used for C_{60} production) particles used as usual positive phagocytosis control [11,12] (at the level of 1 mg/ml) or cubic C_{60} powder suspended at the same level. The size of C_{60} and graphite grains typically ranged from 1 to ~ 100 mm [20 % <1 mm, 60 % 1 to 20 mm and 20 % > 20mm].

Leucocytes were counted at t = 0, 1, 5, and 24 hours: all leucocytes, by means of a Coultronics T660 counter, and surviving ones, by using a Malassez cell after they were coloured thanks to a 0.4 % Trypan blue dye solution.

Three other series prepared in the same conditions as previously described were used for determining the ratio of lysis according to the LDH activities [13]. Extracellular (EC LDH) and intracellular LDH (IC LDH) were determined in culture media by the usual method [16]. To sum it up, after incubation, supernatant and residual cells were separated by centrifugation (600 g, 10 mn). LDH activities were determined in the supernatant (EC LDH) as well as in the residual cells (IC LDH) [after lysing

residual cells with 40 ml water diluted Triton X100 (9/1, v/v) and adding 1 ml of culture media]. The ratio of cellular lysis (LDH index) was calculated using extracellular versus total LDH activity ratio [(EC LDH/EC LDH + IC LDH) x 100].

Samples for Transmission Electron Microscopy (TEM) examination were fixed in 1% glutaraldehyde, 4% paraformaldehyde in 0.1 M PBS (pH=7.4), post-fixed in 0.1 M OsO_4, dehydrated in graded ethanol and embedded in Epon (glycidyl ether 100) [17]. Ultrathin sections cut by means of a Reichert Ultracut E were contrasted with uranyl acetate and lead citrate and examined by a JEOL 1010 electron microscope. For Scanning Electron Microscopy (SEM), samples were fixed in the same fixatives, dehydrated in graded ethanol, dried using carbon dioxide at its critical point, and coated by a thin sputtered platinum layer. They were examined at 25 kV with a JEOL 35 C SEM device.

Results and discussion

SEM photograph (Figure 1) shows an activated normal human neutrophil (A) incubated with C_{60} grains (B). This photograph reveals that contact between cells and C_{60} really occurs. This is confirmed by TEM photographs (Figures 2, 3) and by electron diffraction of the incorporated crystal chunks (Figure 3, insert). Figure 2 shows a human neutrophil trying to engulf crystalline C_{60} particles. Furthermore, the photograph of Figure 3 proves that phagocytosis is achieved whenever crystalline C_{60} particles are small enough. Therefore, the ensuing toxicity experiments will be significant.

Figure 4 exhibits the variation of number of surviving cells as a function of time for the three series of samples described above. It appears that these counts are similar for C_{60} grains, negative control and positive phagocytosis control. Indeed, the

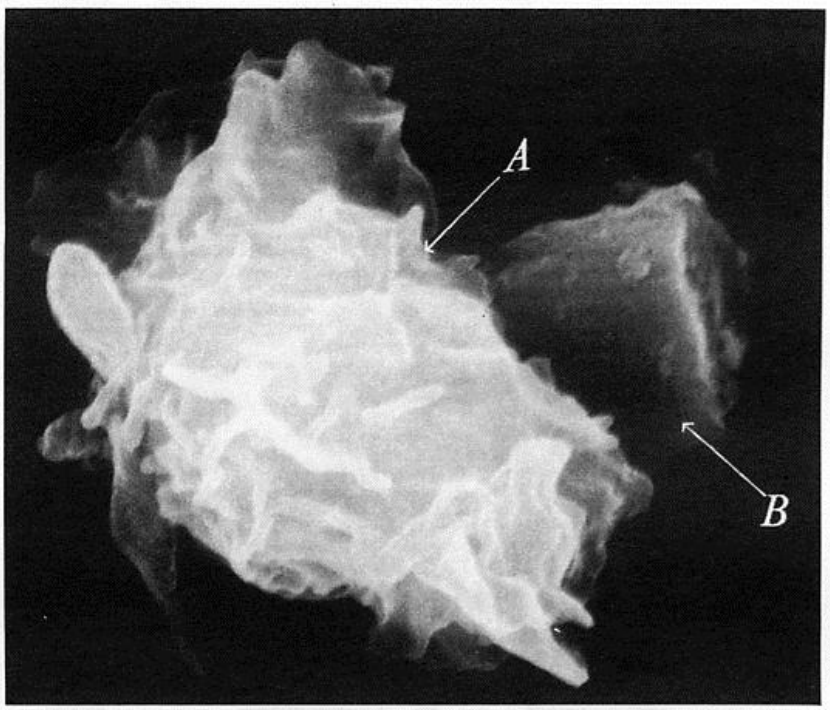

FIG 1 : SEM photograph of an activated human leukocyte (A) in contact with a C_{60} particle (B) (the length of the scale bar is 1 µm).

discrepancies observed are below the CV % of the method (CV % = 16, n = 5). Thus, C_{60} has no effect on the survival of human leukocytes in these conditions.

Figure 5 shows the variation of the LDH activity index as a function of time for the three series of samples. These results exhibit no difference in the percentage of lysed cells between C_{60} and positive phagocytosis control, according to the accuracy of the method (CV % = 6, n = 5). In these conditions, free C_{60} does not entail cytolytic effects on cultured human leukocytes. These results confirm the ones obtained by leukocytes count determination. As to the LDH index results, the discrepancy

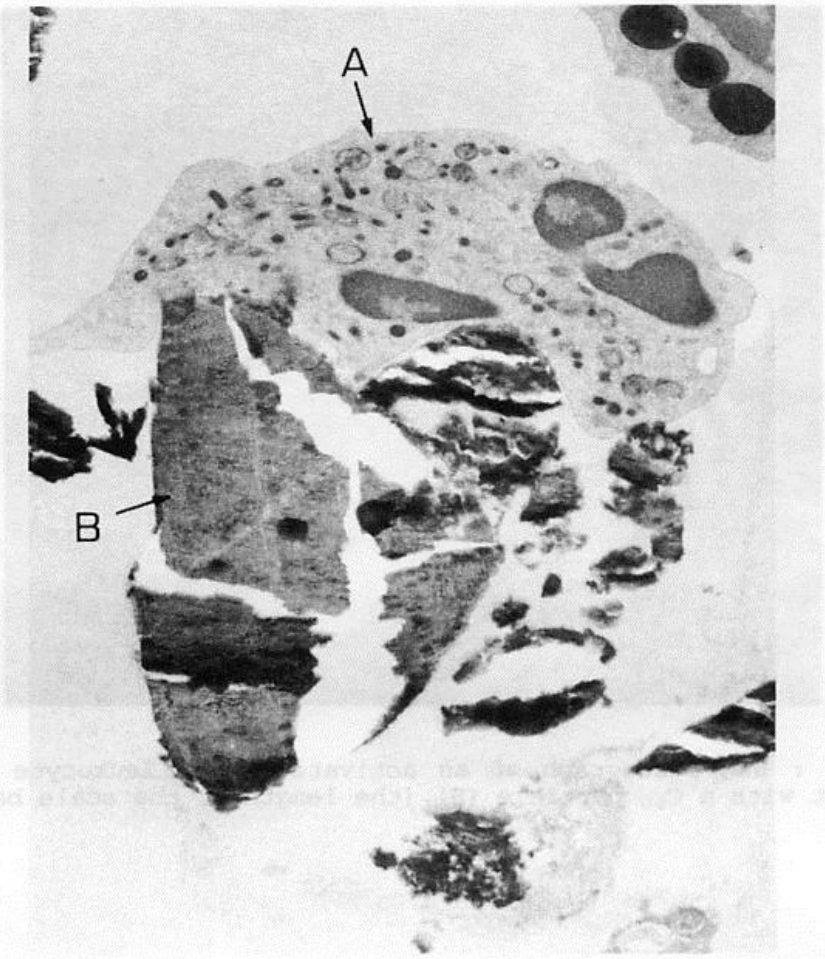

FIG 2 : TEM photograph of an activated human leukocyte (A) phagocyting a C_{60} particle (B) (15 µm long).

between the negative control and the positive phagocytosis control is ascribable to activation of leukocytes during phagocytosis process [11,12]. Because it is less accurate, trypan blue dye test does not allow to reveal the difference between these controls.

These results agree with the previous data of Scrivens et al. [10]. Using ^{14}C labelled C_{60} in the form of fine aqueous suspension these researchers showed that C_{60} is rapidly taken up

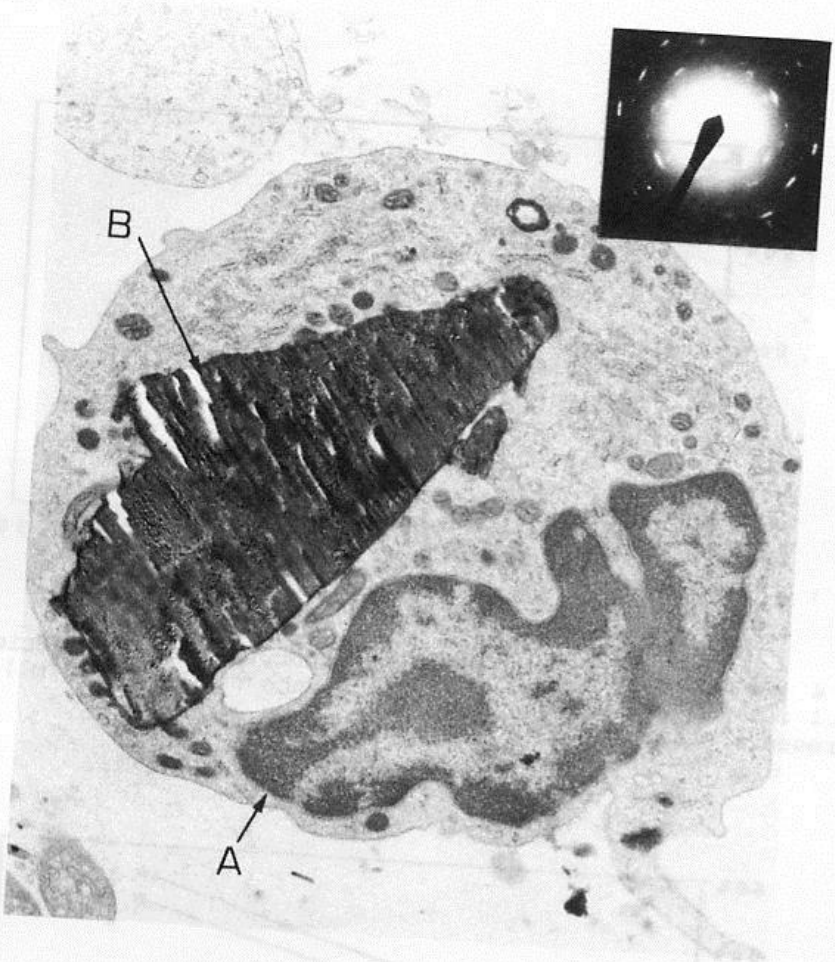

FIG 3 : TEM photograph of a 5 µm long C_{60} particle (B) inside a human leukocyte (A) ; insert : elctron diffraction spectrum of (B).

by human skin cells in culture and apparently does not show any acute toxic effects. However, in their experiments, the C_{60} particles could only be adsorbed to the outside of the cell. In order to visualize C_{60} inside the cell, if this phenomena occurs, we decided to put large size grains (1 to 20 µm) in contact with cultured phagocytes. Although this method is not rigourously quantitative as to the number of grains per cell,

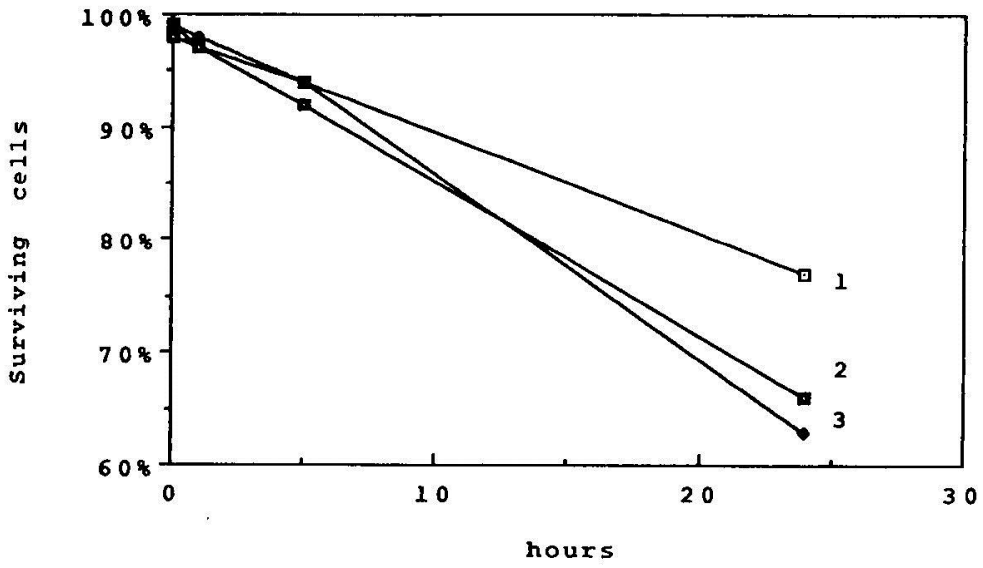

FIG 4 : Percentage of surviving leukocytes as a function of time.1 : negative control, 2 : in presence of C_{60} (1 mg/ml), 3 : in presence of graphite (1 mg/ml).

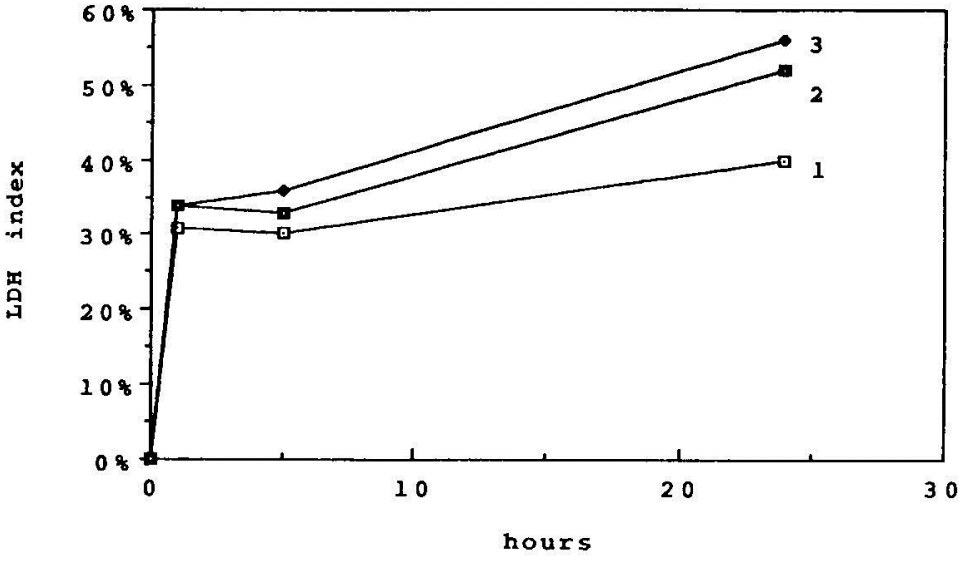

FIG 5 : LDH index [(EC LDH/EC LDH + IC LDH) x 100] as a function of time. 1, 2, 3 : see FIG 4.

our results show that : i- the contact between C_{60} and leukocytes realy occurs, ii- even large excess has no acute toxicity in the case of cultured human leukocytes.

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