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Editors

Karl M. Kadish
Department of Chemistry
University of Houston
Houston, Texas 77204-5641

Rodney S. Ruoff
Department of Physics
Washington University
St. Louis, Missouri 63130-4899

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C_{60} FULLERENE TOXICITY : PRELIMINARY ACCOUNT OF AN *IN VIVO* STUDY

Fathi MOUSSA[1*], Monique PRESSAC[2], Michelle HADCHOUEL[3], Brigitte ARBEILLE[4], Pascale CHRÉTIEN[5], François TRIVIN[1], René CÉOLIN[1] and Henri SZWARC[6].

[1] Jeune Equipe 1990 et [4] Service de Microscopie Electronique, Facultés de Pharmacie et de Médecine, 31, Avenue Monge, F-37200 Tours; [2] Laboratoire d'Endocrinologie, Faculté de Pharmacie, 4, Avenue de l'Observatoire, F-75270 Paris Cedex 06; [3] Génétique et Mécanismes des Maladies du Foie de l'Enfant, INSERM U 347, 80, Rue du Général Leclerc, Hôpital de Bicêtre, F-94276 Le Kremlin-Bicêtre; [5] Laboratoire d'Immunologie et d'Hématologie, Centre Hospitalier Intercommunal, F-94000 Créteil; [6] Laboratoire de Chimie Physique des Matériaux Amorphes, URA D 1104 du CNRS, Bâtiment 490, Université Paris XI, F-91405 Orsay Cedex.

ABSTRACT

In order to seek *in vivo* a possible subacute toxicity of C_{60} fullerene and to investigate the proliferation kinetics of C_{60}-activated fat-storing cells, we have injected intraperitoneally micronized C_{60} into Swiss mice. Despite high amounts injected (2.5 g/kg of body weight), all mice were still alive, without any behavioural trouble, at the end of the study (eight weeks).

In early stages, C_{60} induced hypertrophy and hyperplasia of fat-storing cells where it mainly accumulates. At the later stages, the architecture of the liver remained normal and there was no fibrosis either perisinusoidal or portal. At D_{49} and D_{56}, C_{60} is still present in Kupffer cells and fat-storing cells but the latter ones decrease in number, without any transformation into myofibroblast-like cells. C_{60} was also observed in the cytoplasm of a few hepatocytes without any liver cell injury.

INTRODUCTION

Water-soluble derivatives of C_{60} exhibit interesting properties in various biological systems. However, the biological properties of C_{60} itself, have not been extensively studied (1). After showing that cultured human leukocytes are able to phagocyte C_{60} without any acute toxic effects (2), we have injected intraperitoneally micronized C_{60} into Swiss mice, in order to check *in vivo* its possible acute toxicity (3). We observed that C_{60} have neither lethal effect nor acute toxicity related to this animal species. Nevertheless, C_{60} induces hypertrophy and hyperplasia of fat-storing cells in which it mainly accumulates (3). This phenomenon usually occurs under different conditions leading to fibrosis (4). In order to investigate the possible emergence of a subacute toxicity and to study the kinetics of C_{60}-activated fat storing cells, we carried out again the same experiments for a period of eight weeks.

EXPERIMENTAL

Reagents

Micronized C_{60} (C_{60m}) (Type M, purity 99.9 %) was purchased from Technocarbo (Plan de Grasse, France). An aqueous suspension of C_{60m} (smC_{60} = 100 mg/ml) was prepared as described previously (3).

Animal preparation, C_{60} administration and samples collection

Mice were housed at 20 ± 1 °C (hygrometry = 60 % relative humidity) during 48 hours before injection. Food and tap water were provided *ad libitum* throughout the study. A group of 16 specific pathogen free male Swiss mice ($C_{60}G$) weighing (20 ± 2) g received intraperitoneally injection (BC needles adjusted to 1.0 ml single use syringes) of a single dose of smC_{60} (0.5 ml/20 g) ; another group used as a control group (CG) received 0.5 ml/20 g of suspending solution. After treatment, animals were maintained in a filter paper-covered flat bottom vessel for 30 min to facilitate diuresis and defecation and then filled with wood wool. After 3 hours of examination, animals were caged by groups of eight.

At D_1, D_7, D_{14}, D_{21}, D_{28}, D_{35}, D_{42}, D_{49} and D_{56} (day n = D_n), four animals (one animal of each subgroup) were weighed and sacrificed, after anesthesia with diethylether, for pathological examination and blood and tissue collection.

After abdomen incision, the following organs were collected : liver, spleen, lung, heart, kidney and brain. They were then weighed and observed by optical (OM) and transmission electronic (TEM) microscopies as described previously (3). Blood specimens of approximately 0.8 ml were collected by intracardiac puncture. Blood

aliquots (200 µl) were transferred into EDTA-containing tubes for blood counts and blood formulae; the remaining samples were centrifuged (10 min at 3 000 g) and the sera collected and stored at - 20 °C until analysis.

Methods

Complete blood counts were performed using a STKS counter (Coulter, Miami, Florida, USA). Blood formulae were determined by light microscopy after May-Grunwald-Giemsa staining.

Serine albumin levels, used as markers for hepatic protein synthesis, were determined with an automated bromocresol green staining method (Hitachi 911 Automatic analyser, Bohringer, Mannheim, Germany).

RESULTS AND DISCUSSION

Despite high amounts injected (2.5 g/kg of body weight) all mice were still alive, without any behavioural trouble, at the end of the study (eight weeks). Under these conditions C_{60} does not alter the mice growth. The average weights of the C_{60}G mice, measured weekly and expressed in relative percentages with respect to the starting body average weights, exhibit no difference with those obtained for CG mice (Fig. 1).

Pathological examination of C_{60}G mice shows hypertrophy of livers and spleens. They exhibit the same colour as the injected product ; then the colours of these organs fade slightly away. At D_7, a little part of the injected product was not absorbed and remained in the vicinity of the injection area.The average weights of the livers (C_{60}LW) and the spleens (C_{60}SW) of C_{60}G mice, measured weekly and expressed in relative percentage with respect to the whole body average weights, increased as compared to those of CG mice ones (CSW and CLW for spleens and livers, respectively). The average of C_{60}LW to LCW ratios increased by 122 per cent at D_7 and then decreased to reach 102 to 112 per cent from D_{14} to D_{56}, while the average of C_{60}SW to CSW ratios increased by 250 per cent at D_7 and then decreased to reach 150 to 180 per cent between D_{14} and D_{56}.

The complete blood counts determined weekly remained normal until the end of the study. Furthermore no difference was observed between blood formulae of the C_{60}G mice and those of the CG ones. However, at D_7, grains of C_{60}, appearing as yellow-brown granules, were observed into 36 to 46 per cent of hypertrophic circulating monocytes (5 to 15 granules per monocyte). The percentage of monocytes containing the C_{60} granules as well as the number of granules per monocyte decreased progressively to reach 8 to 14 per cent monocyte containing granules and 2 to 3 granules per monocyte at D_{56}.

These results show that C_{60} has no subacute toxic effect under these conditions. The persistence of spleen hyperplasy and the presence of circulating monocytes

containing C_{60} granules, at the end of the study, can be ascribed to the high amounts of C_{60} injected which exceed the absorption capacity of the mice. Nevertheless, the injected C_{60} is well distributed as shown by its localisation in different organs, mainly in spleen and liver, as demonstrated by light and electron microscopies (3). Inside the cells, C_{60}-containing accumulations appear as yellow-brown granules brilliantly birefringent when viewed through polarized light under an optical microscope. In spleens, lungs, hearts, kidneys, deposits were present in reticuloendothelial cells. No deposit was found in the brain. C_{60} accumulations within cells are confirmed by TEM and by electron diffraction of the incorporated crystal chunks as described previously (3).

In the livers, C_{60} is detected at D_1 in macrophages and Kuppfer cells. At D_7 and D_{14}, it is detected in hepatocytes as well as in Kupffer cells and in endothelial cells. As we reported previously (3), C_{60} induces at this stage hypertrophy and hyperplasia of fat-storing cells where it mainly accumulates. The identity of fat-storing cells is confirmed by their typical location in the space of Disse between the sinusoidal endothelium and hepatocytes (4).

To our knowledge, the activation of fat-storing cells leads incurably to their further differentiation into myofibroblast-like cells associated with progressive liver injury. This phenomenon is observed in different pathological conditions of hepatocellular damage, inflammation, fibrosis, reflected in acute or chronic CCl_4 intoxication, drug-induced hepatitis, vitamin A intoxication and alcoholism (4). Surprisingly, C_{60} accumulation creates neither differentiation of fat-storing cells into myofibroblast-like cells nor liver cell injury. C_{60} seems to generate a different pattern. At the later stages of this study, the architecture of the liver remained normal and there was no fibrosis either perisinusoidal or portal. There was no liver cell necrosis. At D_{49} and D_{56}, C_{60} is still present in Kupffer cells and fat-storing cells, but the latter ones decreases in number, without any transformation into myofibroblast like-cells. C_{60} was also observed in the cytoplasm of a few hepatocytes without inducing any liver cell injury. This is confirmed by measuring weekly serine albumin levels, wich remained normal until the end of the study (results not shown).

If C_{60} happens to inhibit differentiation of activated fat-storing cells into myofibroblast-like cells, it would be the first compound exhibiting such a property to our knowledge.

It now remains to investigate whether the penetration of C_{60} into hepatocytes is followed by some biotransformation of this compound and to look for the possible emergence of a chronic toxicity.

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FIGURE

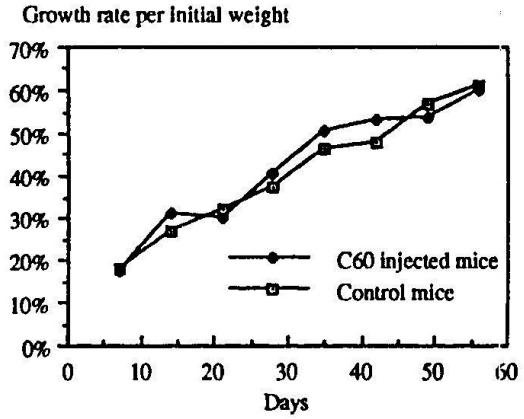

Figure 1 : Comparative growth of C_{60} injected mice and control mice (growth is expressed as rate of initial body weight. Each point represents the median of two animals).