The Journal of Immunology

Fullerene Nanomaterials Inhibit the Allergic Response

John J. Ryan, Henry R. Bateman, Alex Stover, Greg Gomez, Sarah K. Norton, Wei Zhao, Lawrence B. Schwartz, Robert Lenk and Christopher L. Kepley

J Immunol 2007; 179:665-672; ;
doi: 10.4049/jimmunol.179.1.665
http://www.jimmunol.org/content/179/1/665

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Fullerene Nanomaterials Inhibit the Allergic Response[1]

John J. Ryan,* Henry R. Bateman,‡ Alex Stover,‡ Greg Gomez,‡ Sarah K. Norton,* Wei Zhao,† Lawrence B. Schwartz,‡ Robert Lenk,§ and Christopher L. Kepley[2‡]

Fullerenes are a class of novel carbon allotropes that may have practical applications in biotechnology and medicine. Human mast cells (MC) and peripheral blood basophils are critical cells involved in the initiation and propagation of several inflammatory conditions, mainly type I hypersensitivity. We report an unanticipated role of fullerenes as a negative regulator of allergic mediator release that suppresses Ag-driven type I hypersensitivity. Human MC and peripheral blood basophils exhibited a significant inhibition of IgE dependent mediator release when preincubated with C_{60} fullerenes. Protein microarray demonstrated that inhibition of mediator release involves profound reductions in the activation of signaling molecules involved in mediator release and oxidative stress. Follow-up studies demonstrated that the tyrosine phosphorylation of Syk was dramatically inhibited in Ag-challenged cells first incubated with fullerenes. In addition, fullerene preincubation significantly inhibited IgE-induced elevation in cytoplasmic reactive oxygen species levels. Furthermore, fullerenes prevented the in vivo release of histamine and drop in core body temperature in vivo using a MC-dependent model of anaphylaxis. These findings identify a new biological function for fullerenes and may represent a novel way to control MC-dependent diseases including asthma, inflammatory arthritis, heart disease, and multiple sclerosis. *The Journal of Immunology,* **2007, 179: 665–672.**

Nanotechnology, the use of nanomaterials at the molecular level, is a multidisciplinary scientific field undergoing exponential growth and has broad applications among all divisions of science (1–3). One form of nanomaterials, fullerenes, are soccer ball-shaped, carbon cages (C_{60}) that can be functionalized and derivatized with a wide array of molecules (3, 4). Given their unique structure, inertness, and characteristic stability, fullerenes are being investigated as a novel way to improve upon modalities used to diagnose, monitor, and treat certain conditions.

Type I hypersensitivity is the result of B cell-produced, specific IgE Ab to common, normally innocuous Ags. The allergen-specific IgE sensitizes mast cells (MC)[3] and peripheral blood basophils (PBB). Re-exposure to the allergen triggers an allergic response through the release of inflammatory mediators from MC and PBB. Indeed, many allergy medications are aimed at neutralizing (antihistamines, H_1-receptor blockers) or preventing (anti-IgE; Omalizumab) this response.

No studies have examined the effects that C_{60} fullerenes have on the MC and PBB allergic responses. Previous studies in other cellular systems demonstrated that these molecules can inhibit various cellular pathways. We used human skin and lung MC, PBB, and an in vivo model for MC-dependent anaphylaxis to investigate the effects these molecules have on the allergic response.

Materials and Methods

Reagents

Water-soluble fullerene derivatives (polyhydroxy C_{60}, *N*-ethyl-polyamino C_{60}) referred to as "poly" or "*N*-ethyl" were obtained from BuckyUSA. Stem cell factor was acquired from BD Biosciences. The human IgE anti-NP Ab (used for in vitro studies) was purified from the JW8 cell line. Mouse anti-DNP IgE (used for in vivo studies) was obtained from Dan Conrad (Virginia Commonwealth University Health Systems, Richmond, VA). Anti-fullerene Abs (5) were a gift from Bernard F. Erlanger (Columbia University, New York, NY). All Western blotting Abs were purchased from Cell Signalling.

Skin MC and PBB

Human skin/lung MC and PBB were purified and cultured as described (6–8). The majority of the experiments were performed using human MC. Skin MC cultures were maintained for up to 2 mo and were ~100% skin MC. Lung MC were used within 1 wk after purification from tissue. PBB obtained from buffy coats were purified to >85%. All studies were approved by the Human Studies Committee at Virginia Commonwealth University Health Systems. For some experiments, bone marrow-derived MC from mice were obtained as described (9).

MC and PBB viability

PBS was added to water-soluble fullerenes to achieve a final concentration of 10 mg/ml. Skin MC and PBBs were incubated with varying concentrations up to 72 h. Cells were washed, collected, and stained with trypan blue (0.04%) for assessing viability. Cell counting was accomplished with an improved Neubauer hemocytometer.

Activation of skin MC and PBB

Human skin MC and PBB were sensitized with anti-NP IgE Abs (50 ng/ml for at least 24 h). All experiments were performed in duplicate or triplicate. Varying concentrations (0.001–1000 ng/ml) of fullerenes were added to the cells in minimal medium. After 24 h, the cells were centrifuged and 100 μl of medium was removed for β-hexosaminidase and histamine measurement. The cells were washed and exposed to 100 ng/ml NP-BSA (Biosearch Technologies) with 50 μg/ml soybean tissue inhibitor in X-Vivo medium. In some experiments, the anti-FcεRI Ab (3B4) was used to activate non-IgE-sensitized cells. After incubation at 37°C for 30 min, the cells were centrifuged and 100 μl of medium was removed for degranulation and PGD_2 assessment using a kit from Cayman Chemical. The cells

*Department of Biology, [s]†Department of Pediatrics, and [s]‡Department of Internal Medicine, Virginia Commonwealth University Health Systems, Richmond, VA 23294; and §Luna Innovations, Danville, VA 24541

Received for publication January 26, 2007. Accepted for publication April 15, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by National Institutes of Health Grants R01-AI27517 (to L.B.S.) and K08-AI057357 (to W.Z.) and by the Food Allergy and Anaphylaxis Network (to C.L.K.).

[2] Address correspondence and reprint requests to Dr. Chris Kepley, PhD, MBA Department of Internal Medicine, Division of Rheumatology, Allergy and Immunology, P.O. Box 980263 MCV Station, Richmond, VA 23298. E-mail address: clkepley@vcu.edu

[3] Abbreviations used in this paper: MC, mast cell; PBB, peripheral blood basophils; ROS, reactive oxygen species; DCF, dichlorofluorescein.

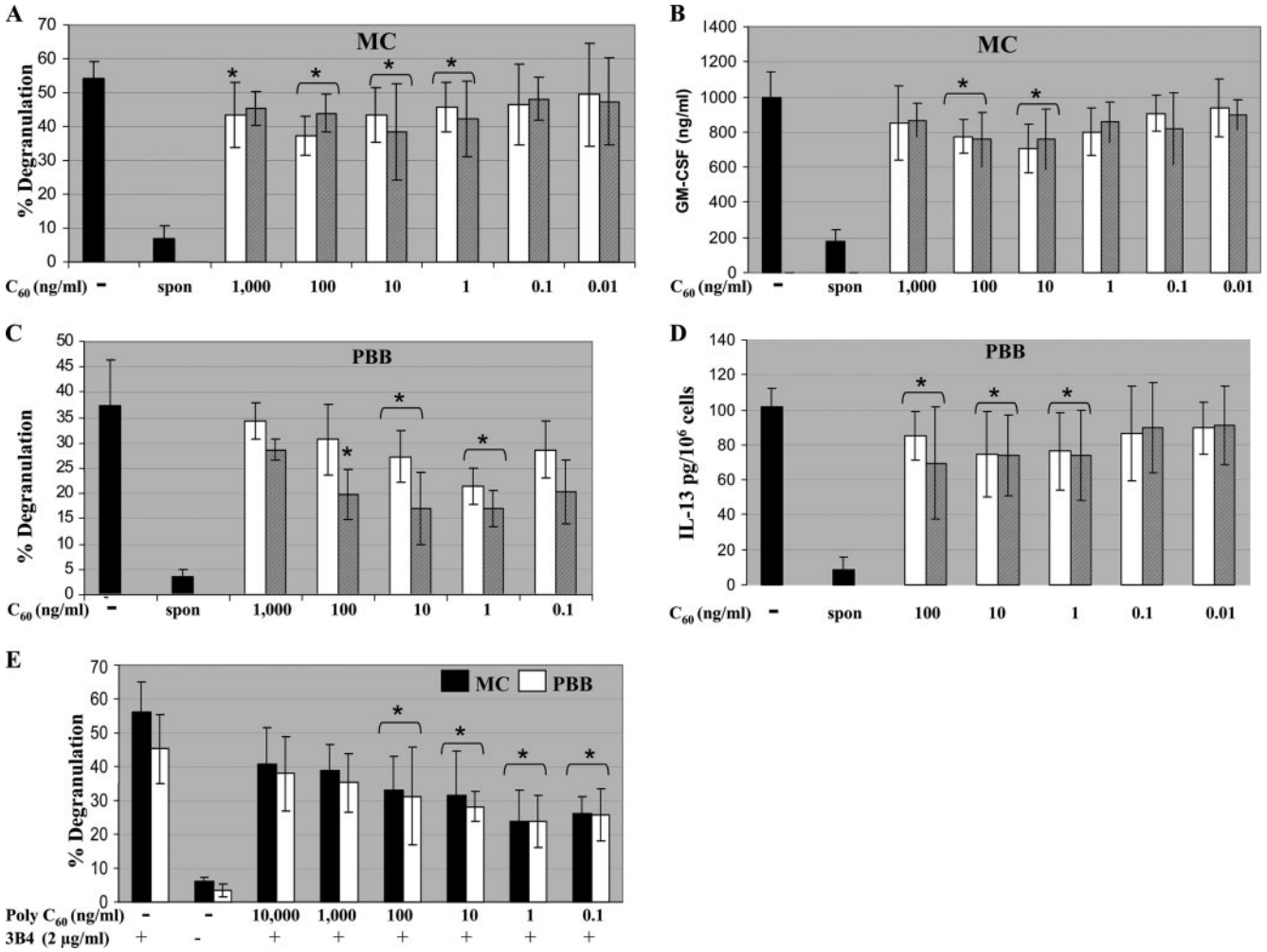

FIGURE 1. The effects of fullerenes on skin MC and PBB. Skin MC (*A* and *B*) or PBB (*C* and *D*) were sensitized with NP-IgE. Fullerenes (white, poly C_{60}; striped, *N*-ethyl-C_{60}) were added for 24 h at the indicated concentrations. For comparison, equal number of cells did not receive C_{60} (indicated by the (−)). The cells were washed and activated with or without (spontaneous; spon) optimal concentrations of NP-BSA (100 ng/ml) for 0.5 (degranulation) or 30 h (cytokine production), centrifuged and the supernatants examined for mediator release. In *F*, MC or PBB were incubated with or without the indicated concentrations of fullerenes. The next day cells were washed and activated with optimal concentrations of 3B4 and the supernatants assayed for degranulation. The results represent the average release with each condition and the SD. ∗, statistically significant changes in mediator levels comparing the treated cells with the untreated cells (−) using the Student *t* test ($p < 0.05$). All experiments were performed at least four times with separate donors.

were suspended in the remaining medium and placed back in an incubator. After 24 h, the cells were centrifuged and 50 μl of medium was removed for cytokine (GM-CSF, TNF-α, IL-13) measurement as described (7).

Kinex Ab array

The Kinex Ab microarray service tracks the phosphorylation (with phospho-site-specific antibodies) of ~600 proteins in dye-labeled cell lysates. For these experiments, cells were activated with or without prior incubation with 10 ng/ml poly C_{60} or *N*-ethyl C_{60} under the same conditions where maximal inhibition of mediator release was observed. Cells treated under the four conditions were lysed and cell lysates prepared according to the company protocol. Complete quantitation information for all detected spots was analyzed and data presented as the percentage of change from control, which measures of the change in normalized signal intensity averages between the treated sample and the control sample. Data was filtered such that only those signals that were inhibited greater than 50- and 100-fold are presented. All data are normalized to correct for systematic errors such as the difference in total protein amount between the control and experimental samples.

Measurement of reactive oxygen species (ROS) by FACS

ROS production was measured using dichlorofluorescein (DCF), a cell-permeant indicator that becomes fluorescent upon oxidation by H_2O_2. Skin MC or PBB were sensitized, exposed to fullerenes, and activated as above. After washing, cells were resuspended in X-Vivo medium containing 5 μM DCF and incubated at 37°C for 45 min. Cells were washed twice with skin MC medium, suspended in PBS and the mean fluorescence measured by FACS analysis. The data are presented as the mean intensity fluorescence in which the negative control (DMSO only; see below) was adjusted to approximately the same value (125–150 mean intensity fluorescence) for each experiment. All experiments were performed in duplicate.

Immunohistochemical staining of fullerenes

Skin MC were incubated with different concentrations of fullerenes at various time intervals (4, 8, and 24 h). Cytospins were made and each slide fixed in methanol/acetone/formalin (19:19:4) for 5 min and methanol/hydrogen peroxide (40:1) for 30 min. After washing in Tris-buffered saline with 0.2% Tween (TTBS) three times, the cells were placed in blocking buffer (TTBS with 1% BSA, human serum, and goat serum) for 1 h. The slides were washed three times in TTBS, and 200 μl of an Ab specific for fullerenes (5) was added to each slide. After washing the slides as before, peroxidase conjugated goat anti-mouse Abs was added. After washing, the substrate 3-amino-9-ethyl-carbazole was added and the reaction visualized and photographed with a Zeiss microscope with an Olympus digital camera.

Mouse model of anaphylaxis

Mice (C57BL/6, aged 16–20 wk) were injected i.p. with 50 μg of anti-DNP IgE or PBS. Mice then received 50 ng of poly C_{60} fullerene in 100 μl

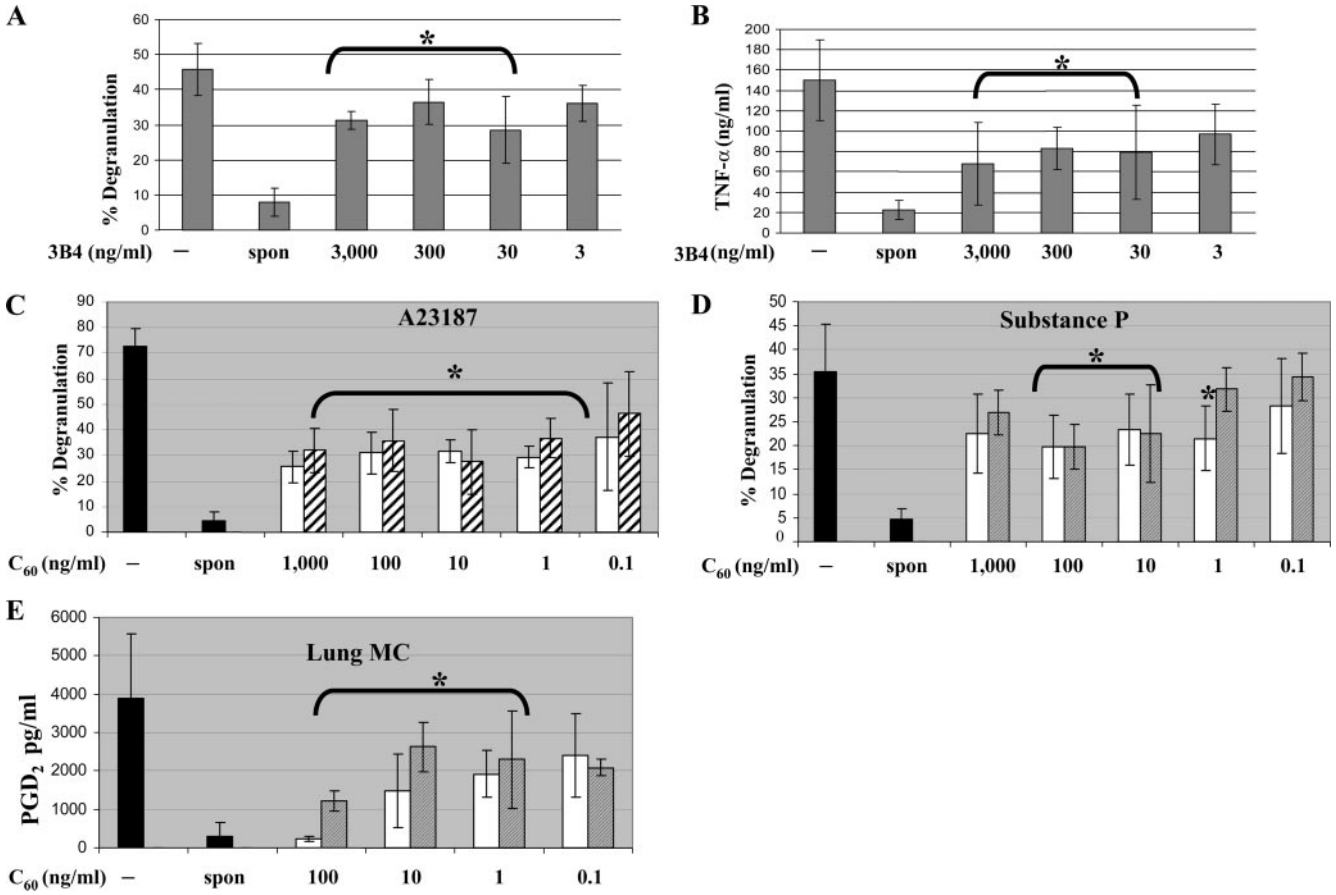

FIGURE 2. *A* and *B*, The effects of fullerenes on lung MC. Lung MC (73–86% purity) were cultured with fullerenes (10 ng/ml) overnight. The next day the cells were washed and activated with or without (spontaneous; spon) the indicated concentrations of the anti-FcεRI-α Ab 3B4 for 0.5 (degranulation; *A*) or 30 h (cytokine production; *B*), centrifuged and the supernatants examined for mediator release. $*$, Significant changes ($p < 0.05$). *C* and *D,* The effects of fullerenes on non-IgE-mediated degranulation Cells were cultured with (white, poly C_{60}; striped, *N*-ethyl C_{60}) or without (black) the fullerenes at the indicated concentrations. Cells were washed and activated with or without (spontaneous; spon) A23187 (1000 ng/ml; *C*) or substance P (100 μM/ml; *D*) for 30 min and the supernatants examined for mediator release. $*$, Statistically significant changes ($p < 0.05$). *E*, The effects of fullerenes on PGD_2 release. DNP-IgE-sensitized lung MCs were cultured with (white, poly C_{60}; striped, *N*-ethyl C_{60}) or without (black) fullerenes at the indicated concentrations. Cells were washed and activated with or without (spontaneous; spon) anti-FcεRI-α Ab 3B4 (3 μg/ml) for 30 min, centrifuged and the supernatants examined for PGD_2. $*$, Statistically significant changes in mediator levels ($p < 0.05$). All experiments above were performed at least three times with separate donors.

of PBS. Twenty four hours later, mice were challenged with an i.p. injection of 100 μg of DNP-albumin (Sigma-Aldrich) in 100 μl of PBS. Body temperature measurements were recorded with a digital rectal thermometer every 10 min for 30 min. Peripheral blood was harvested by cardiac puncture 30 min after Ag injection, and serum histamine measurements were determined by ELISA.

Results

No studies have examined the effects fullerenes have on human MC and basophils. Thus, we first tested the effects fullerene derivatives have on cell growth and viability of skin MC and PBB. Cells incubated with up to 1000 ng/ml either polyhydroxy C_{60} or *N*-ethyl C_{60} for up to 72 h did not demonstrate any significant changes in cell numbers or viability (data not shown). These results suggest fullerenes are not toxic to human MC and PBB.

We next investigated the effects of fullerenes on MC and PBB Ag-induced mediator release. NP-IgE-sensitized MC and PBB were incubated with varying concentrations of fullerenes before challenge with NP-BSA. Fullerenes alone did not induce MC or PBB mediator release (data shown not shown). However, when cells were incubated with water-soluble fullerenes before challenge with optimal concentrations of Ag (100 ng/ml) there was a significant inhibition of both degranulation (Fig. 1, *A* and *C*) and cytokine production (Fig. 1, *B* and *D*) compared with skin MC and PBB not incubated with the fullerenes. Similar inhibition was observed when cells were challenged with anti-IgE receptor Abs (Fig. 1*E*).

MC found in the skin are phenotypically different from those found in the lung. For this reason we investigated the effects fullerenes had on freshly isolated human lung MC mediator release. As seen in Fig. 2, *A* and *B*, lung MC also exhibited inhibited IgE responses when cells were first exposed to fullerenes compared with cells not treated with fullerenes. The inhibitory effect was also observed from non-IgE/FcεRI-mediated stimulation using calcium ionophore and substance P, a potent neuropeptide (Fig. 2, *C* and *D*) and when PGD_2 release was measured (Fig. 2*E*). Fullerene-treated skin MC also had inhibited PGD_2 release compared with nontreated and IgE-activated cells (data not shown).

We next investigated the effect fullerenes had on IgE binding to MC and levels of FcεRI expression on MC because the inhibitory effect observed above could be due to steric hindrance of IgE binding and/or reduced FcεRI expression. As seen in Fig. 3*A*, MC incubated with or without fullerenes demonstrated similar IgE binding. The expression levels of FcεRI were not changed when

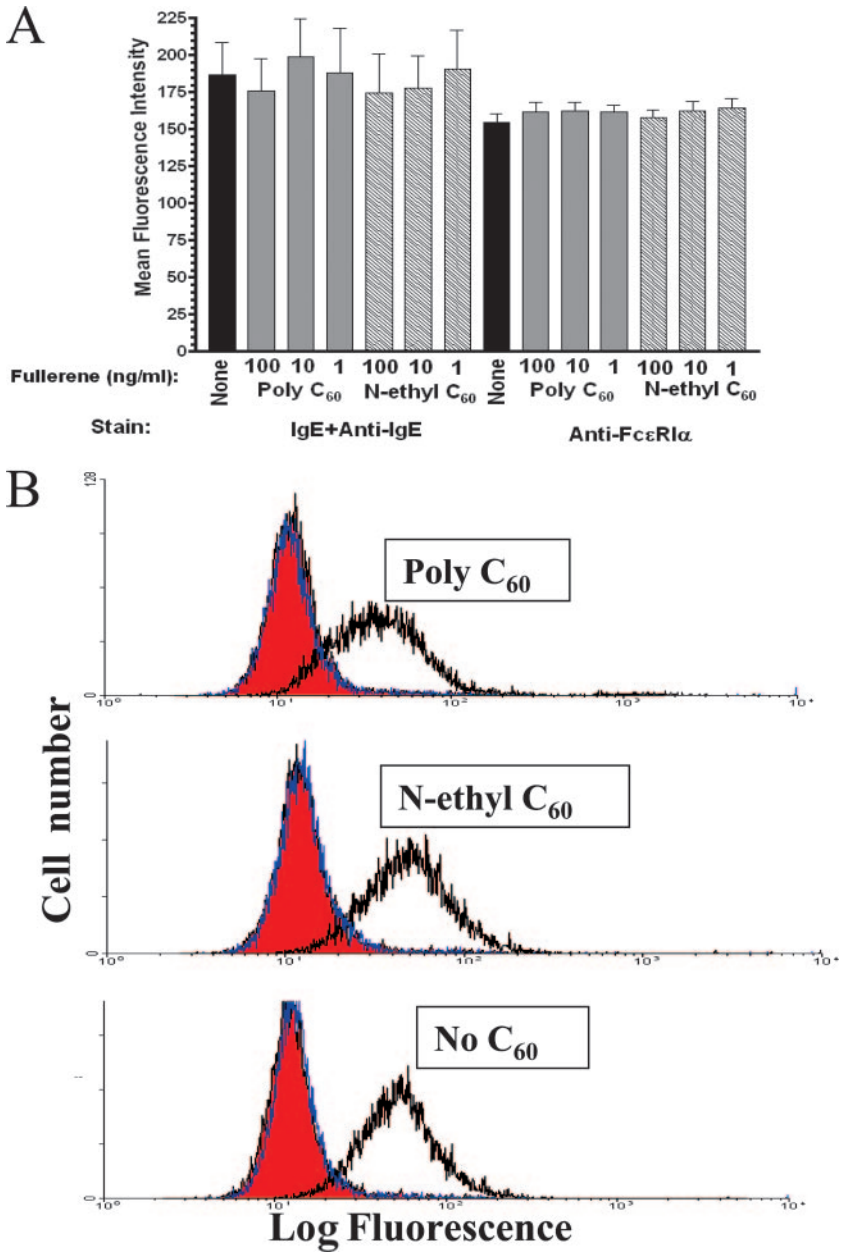

FIGURE 3. *A*, Fullerenes do not inhibit IgE binding to MC or reduce FcεRI levels. *Left*, Mouse MCs were incubated with IgE for 24 h, after which fullerenes were added at the indicated concentrations (ng/ml) for 24 h. Cells were then stained with IgE plus anti-IgE, and mean fluorescence intensity was measured by flow cytometry. *Right*, A second group of MCs received fullerenes alone for 24 h and was then stained for surface IgE receptors using anti-FcεRIa. Data shown are mean ± SEM of six samples. *B*, Fullerenes are not detected on the cell surface under conditions that inhibit mediator release. SMC were incubated with 10 ng/ml poly (*top*), *N*-ethyl (*middle*), or no (*bottom*) fullerenes overnight. The next day, cells were washed and incubated with anti-fullerene Abs (blue), anti-FcεRI Abs (black), or nonspecific mouse IgG isotype-matched control Abs (red) followed by fluorescent-labeled anti-mouse $F(ab')_2$ Abs. After washing, cells were subjected to FACS analysis.

cells were incubated with fullerenes. In addition, fullerenes were not detected on the cell surface when cells were analyzed by FACS analysis after fullerene incubation and staining with fullerene-specific Abs (Fig. 3*B*). These results, along with those in Figs. 1*F* and 2, *A* and *B*, strongly suggest that the inhibitory effect seen with fullerene preincubation is not simply due to steric hindrance of IgE binding to Ag, IgE binding to FcεRI, nor to reduced FcεRI levels. Thus, fullerene derivatives inhibit the signaling pathways leading to human MC and PBB mediator release through mechanisms that do not involve Ag-Ab or Ab-anti-FcεRI receptor inhibition.

To assess the cellular localization of fullerenes within human MC and PBB, we incubated these cells with water-soluble fullerenes and detected fullerenes with imunohistochemistry using an anti-fullerene Ab. As seen in Fig. 4, both skin MC and PBB incubated with the fullerenes for at least 4 h reacted with the fullerene Abs. The staining was mostly within the cytoplasm of the cells further indicating that the fullerenes exert their inhibitory affect inside the cell.

To determine the mechanisms of fullerene inhibition, we used protein microarray to provide detection and quantitation of ~600 known phospho-activated signaling molecules. As seen in Table I, ~35 Ag-induced signaling intermediates were inhibited by greater than 50-fold when cells were preincubated with the fullerenes and Ag activated compared with those nontreated and Ag activated. Of these, ~12 signaling intermediates were affected greater than 100-fold compared with Ag-only affected cells (Table I, underlined). For example, the tyrosine hydroxylase enzyme was up-regulated greater than 100-fold following IgE receptor stimulation (and was the signaling molecule up-regulated the highest by Ag stimulation). However, in cells first treated with fullerenes, this molecule was not up-regulated following Ag stimulation as detected using the Kinexus assay system. This analysis also revealed several signaling intermediates previously shown in other cellular systems to be associated with the generation of reactive oxygen species (ROS; Table I, italics).

Syk levels correlate with MC/PBB secretion and is a critical kinase in the FcεRI signaling cascade (10). The protein microarray data suggested that the tyrosine kinase Syk was inhibited greater than 50-fold following Ag stimulation when cells were first

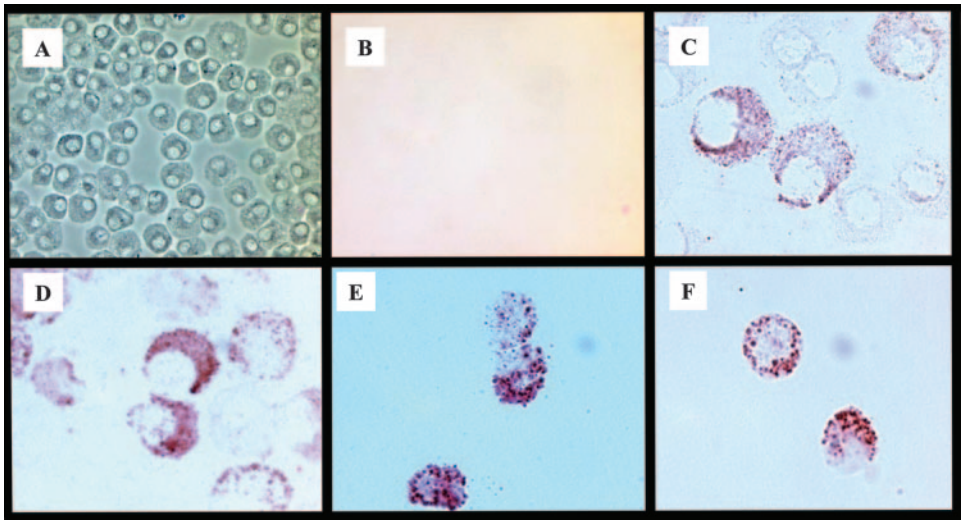

FIGURE 4. Carbon fullerenes localize within the cytoplasm of MC and PBB. Skin MC (*A–D*) or PBB (*E* and *F*) were incubated with poly C_{60} (10 ng/ml; *A*, *B*, *C* and *F*) or *N*-ethyl C_{60} (10 ng/ml; *D* and *F*) for 4 h, washed and cytocentrifuged onto slides. The cytocentrifuge preparations were incubated with anti-C_{60} fullerene Abs (*C–F*) or with an isotype-matched control (*A* and *B*) overnight. The cytospins were washed and incubated with a 1/50 dilution of peroxidase-conjugated, anti-mouse Abs and developed with 3-amino-9-ethyl-carbazole as described. Photographs A and B are the same field taken under phase-contrast (*A*) or light (*B*) at a magnification of ×400. *C–F*, ×1000.

incubated with fullerenes. We performed Western blotting of Syk following Ag stimulation with or without fullerene preincubation. As seen in Fig. 5*A*, Syk phosphorylation was dramatically inhibited when cells were preincubated with fullerenes before activation. Similar observations were observed with Lyn and total cellular tyrosine phosphorylation (not shown) further suggesting that fullerenes inhibit MC/PBB mediator release through the reduction in FcεRI-associated kinase activation.

The protein microarray data also suggested that the fullerenes inhibited several molecules involved in the regulation of cellular ROS levels. We measured ROS levels in Ag-challenged cells with or without preincubation with fullerenes. As seen in Fig. 5*B*, preincubation of skin MC and PBB with fullerenes significantly reduced the Ag-induced increase in ROS levels within the cells. This effect was comparable to that observed with *N*-acetylcysteine; a powerful antioxidant. These data suggest that the inhibition of MC and PBB mediator release observed with fullerene preincubation may be due to reductions in ROS levels after Ag challenge.

Systemic anaphylaxis is predominately driven by the activation of MC and PBB through IgE-FcεRI cross-linking (11). We showed these cell types were inhibited by preincubation with fullerene derivatives. Thus, we hypothesized that fullerenes could inhibit MC- and PBB-induced anaphylaxis in vivo using IgE-sensitized mice. As shown in Fig. 6, animals injected i.p. with fullerenes 24 h before systemic Ag challenge had a significant decrease in the MC/PBB-derived histamine response when the animals were challenged with Ag. In addition, fullerenes injected 24 h before systemic Ag challenge significantly reduced the anaphylactic-induced drop in core body temperature. Of note, the fullerene injection did not noticeably affect the behavior in any of the mice tested ($n = 35$).

Table I. *The effects of fullerenes on SMC signaling intermediates; protein array of signaling molecules affected by fullerenes in SMC*[a]

14-3-3 protein ζ: adaptor protein of intracellular signal pathways	Tumor suppressor protein p53 (antigenNY-CO-13)
B23 (nucleophosmin, numatrin, nucleolar protein NO38)	*Phosphoinositide-3-kinase (PI3K),*
ErbB2 (Neu, HER2) receptor-tyrosine kinase	Platelet-derived growth factor receptor kinase αβ
Extracellular regulated protein-serine kinase 1/2 (p44/p42 MAP kinases)	Protein-serine kinase C αβ2, δ, γ, η, θ
Heat shock 47 kDa protein (collagen-binding protein 1, colligin 1)	Protein kinase C-related protein-serine kinase 1/2
Focal adhesion protein-tyrosine kinase (FAK)	Progesterone receptor
Intestinal cell protein-serine kinase (ICK)	*Pro-caspase 9*
Inhibitor of NF-κ-B protein-serine kinase (IKK) α and β	Rad17 homolog
Integrin-linked protein-serine kinase 1 (ILK)	Raf1 proto-oncogene-encoded protein-serine kinase
Jun protooncogene-encoded AP1 transcription factor	Ribosomal S6 protein-serine kinase 1/2
Yes-related protein-tyrosine kinase (Lyn)	Retinoblastoma-associated protein 1
MAPK/ERK protein-serine kinase 1 (MKK1)	*Second mitochondria-derived activator of caspase*
Hepatocyte growth factor (HGF) receptor-tyrosine kinase	Ret receptor-tyrosine kinase
Jun N-terminus protein-serine kinases (JNK)	*Sma- and Mad-related protein 2 (SMAD2)*
Mitogen & stress-activated protein-serine kinase 1	Silencer of death domains (Bcl2-associated athanogene 4)
Mitogen-activated protein-serine kinase p38 α	Synapsin 1 isoform Ia
	Spleen tyrosine kinase (Syk)
	Tyrosine hydroxylase isoform a
	Wee1 protein-tyrosine kinase

[a] Each signaling intermediate was inhibited greater than 50-fold (or 100-fold shown as underlined) when cells were first incubated with poly-C_{60} (overnight) before being washed and challenged with Ag (100 ng/ml) for 10 min. For example, in cells exposed to fullerenes before Ag activation, Lyn was inhibited greater than 100-fold compared with non-fullerene-treated, Ag-activated cells. The intermediates in *italics* represent those molecules previously shown to be involved in ROS signaling pathways.

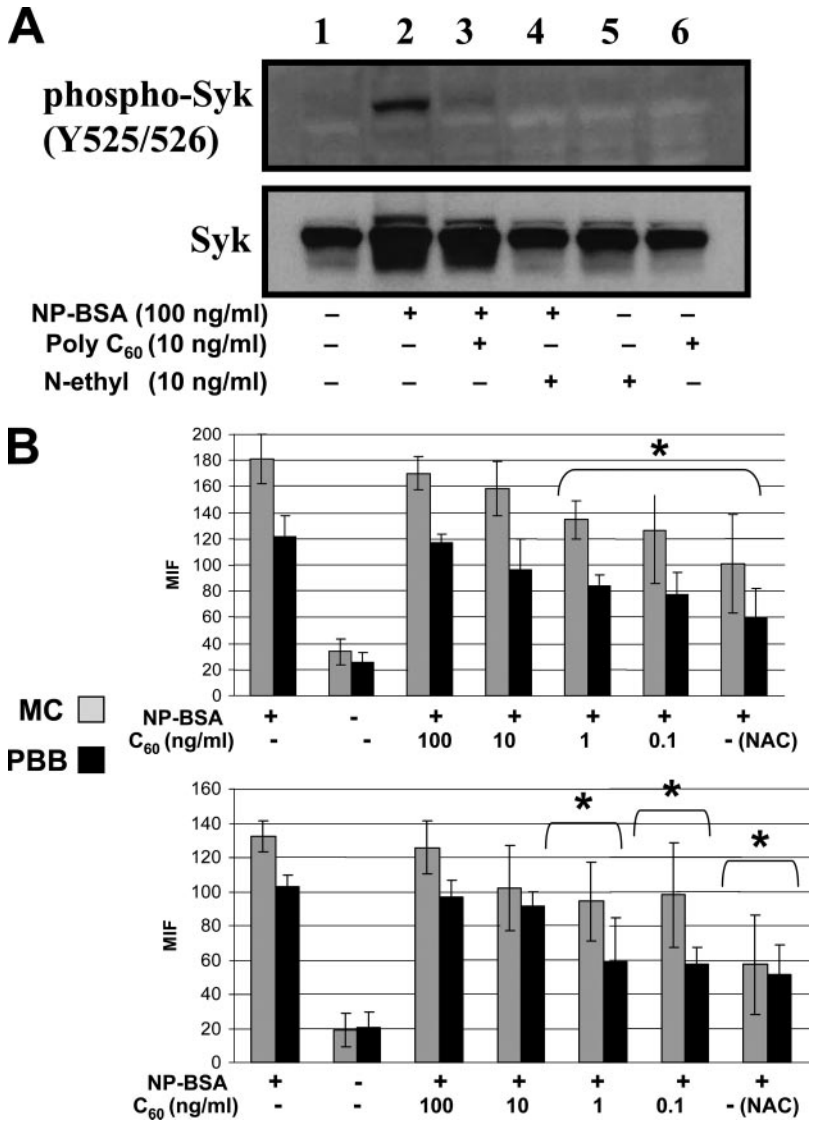

FIGURE 5. *A*, Fullerenes inhibit tyrosine phosphorylation of Syk. NP-IgE-sensitized MC were incubated overnight with or without polyhydroxy-C_{60} or *N*-ethyl-polyamino-C_{60} (10 ng/ml). Cells were washed and activated for 10 min with or without NP-BSA (100 ng/ml). Western blotting was performed as described previously (60). *B*, Fullerenes inhibit ROS production in skin MC and PBB. Skin MC (■) or PBB (■) were sensitized overnight with NP-IgE. Cells were washed and activated for 30 min with NP-BSA (100 ng/ml) with or without preincubation with polyhydroxy-C_{60} (*top panel*) or *N*-ethyl-polyamino-C_{60} (*bottom panel*) at the indicated concentrations or NAC (10 mmol). Supernatants were assayed for degranulation and cellular ROS levels were detected by incubating the cells in DCF and detecting the fluorescence by FACS analysis. The results are representative of three separate MC or PBB cultures. ∗, Statistically significant changes in ROS levels for MC and PBB, respectively ($n = 3$; $p < 0.05$).

Discussion

Fullerenes or "Bucky-balls" represent the third allotrope of carbon. In this form, 60 or 70 carbon molecules are arranged in a cage structure and are water insoluble unless derivatized with various compounds. The small size of fullerenes, their inertness, and their ability to be manipulated with a host of drugs, polymers, and ligands make them attractive for the use as new therapeutics and improving the efficacy of drugs already developed. Our studies have clear implications for their use as a new way to "turn off" MC and PBB and, in turn, those disease in which these cells have a critical role.

The fullerene core can react with free radical species given its capacity to absorb electrons and disperse them through the 20 benzene rings distributed over its surface. In fact it is one of the most potent free radical scavengers known with the potential for being "sponges" in diseases involving ROS (4). This property makes them attractive therapeutic options in acute and chronic neurodegenerative diseases such as Parkinson, Alzheimer, and Lou Gehrig, which involve ROS, probably due to the over-excitation of glutamic acid receptors (12–14). To this end fullerenes derivatized with OH species have been shown to prevent ischemia (poisoning due to lack of oxygen) which is initiated and propagated through sudden increases in ROS as tissues react to energy depletion (15–17). Derivatized fullerenes also reduce ROS-induced neuronal apoptosis and have been proposed as a potential therapeutic for neurodegenerative disorders (14). Other derivatives of fullerenes include hexosulfobutyl and C3, the Tris malonate derivative, and polyethylene glycol.

The mechanism of inhibition by the fullerenes in MC and PBB appears to be, in part, through the inhibition of cellular ROS levels. As seen in Table I, fullerenes inhibited several signaling pathways previously been shown to be involved in ROS generation (18–26). For example inhibiting procaspase 9 prevents mitochondrial uncoupling and ROS production (27). Procaspase 9 was inhibited over 50-fold in MC incubated with fullerenes before Ag activation and could help explain the reduced ROS levels within the cells treated with fullerenes. Thus, fullerenes appear to act as an antioxidant that can absorb the free radical electrons of ROS. Our results are similar to previous studies demonstrating that fullerenes are powerful antioxidants in a variety of cell types (4).

The role of ROS in human MC and PBB-induced responses has not been investigated thoroughly. Work in rat basophil leukemic cells (RBL) has shown that stimulation through the high-affinity IgE receptor induces the production of ROS which regulate various MC responses, including degranulation, leukotriene secretion, and cytokine production (28). Conversely, antioxidants that quench intracellular ROS in rodent MC inhibit degranulation (29).

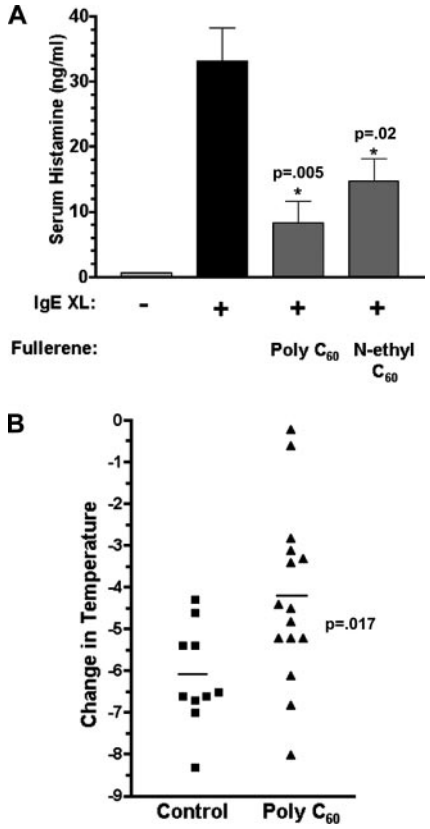

FIGURE 6. Fullerenes inhibit IgE-induced anaphylaxis in vivo. Mice were sensitized with or without (black) DNP-IgE overnight. The mice were then given vehicle alone (■) or fullerenes (▲) i.v. (50 ng/ml) overnight. The next morning the animals were challenged with DNP-BSA (100 μg/ml) and the core body temperature measured for up to 30 min. Serum histamine levels were measured by ELISA (*A*, 3–5 per group; $n = 3$ separate experiments, $p < 0.05$, *B*. $n = 10$ control, 13-poly-treated).

Several secretogogues induced intracellular increases of ROS levels in rodent MC (30–33), PBB (33), and human blood-derived MC (34). We demonstrated ROS generation in human PBB that had released significantly more mediators when challenged with diesel exhaust particles compared with nonchallenged cells (35). Some evidence indicates that allergic and inflammatory skin diseases like atopic dermatitis, urticaria and psoriasis are mediated by MC-initiated oxidative stress (36) while recent studies suggest antioxidants can reduce asthma symptoms in mice (37, 38). Previous studies have demonstrated that ROS, possibly from MC, play a significant role in the pathogenesis of a number of disorders such as inflammation, rheumatoid arthritis, asthma, psoriasis, urticaria, and contact dermatitis (39). Our studies further suggest a critical role for ROS in human MC mediator release and inhibiting ROS levels by fullerenes leads to a blunted Ag-induced response.

We found that both Lyn and Syk were inhibited by fullerene preincubation. These 2 kinases have been shown to be linked to FcεRI-regulated mediator release in MC and PBB (40, 41). These are the first studies linking the inhibition of tyrosine phosphorylation of these kinases to the activity of fullerenes. However, in B cells, Syk has a crucial role in intracellular signal transduction induced by oxidative stress as well as Ag receptor engagement (42). These studies found that that oxidative stress-induced Syk activation triggers the activation of several pathways, such as pro-apoptotic and survival pathways, and the balance among these various pathways is a key factor in determining the fate of a cell exposed to oxidative stress. Although a direct link between oxidative stress and up-regulation of Syk has not been demonstrated in MC our studies are similar to that reported in the B cell where Ag activation induces oxidative stress as well as Syk activation. The area of ROS/oxidative stress signal transduction mechanisms (secretion of ROS, ROS dependent mediator release, etc) in human MC/PBB has simply not been investigated thoroughly and represents an area of research that remains to be explored.

Given the relative infancy of the field, the studies examining the toxicity of fullerenes on biological systems are still emerging. Toxicological studies using a wide range of nanomaterials have been conflicting and inconclusive (1, 2, 43–48). Water solubility, dose, exposure time, and similar parameters all appear to influence the cytotoxicity of the fullerenes. For example, unfunctionalized C_{60} appears to be cytotoxic in certain systems given its highly charged core structure but as functional side chains are added to the carbon skeleton the level of cytotoxicity appears to diminish (45, 49). Previous studies have demonstrated that fullerenes have no cytotoxic effects on keratinocytes or fibroblasts and can protect blood mononuclear cells and macrophages from oxidative stress (50–52). The level of cytotoxicity also depends on the concentration of fullerenes exposed to cells. Yamawaki showed the cytotoxic effects of hydroxyl C_{60} fullerenes on endothelial cells using 100 μg/ml while Chen demonstrated that polyalkylsulfonated caused renal damage with an LD50 of 600 mg/kg in rats (53, 54). In addition, high concentrations of fullerene-based amino acid nanoparticles were cytotoxic to epidermal keratinocytes, while low concentrations displayed no cytotoxic effects (55).

We show that an injection of 50 ng into mice weighing ~25 g (0.002 mg/kg) significantly inhibits anaphylaxis and does not affect the mice adversely. We have also injected five-fold higher (250 ng) with no toxicity (data not shown). Hence, the concentrations of fullerenes that are capable of inhibiting anaphylaxis and MC/PBB mediator release are 400–300,000-fold lower than the concentrations observed to have in vivo toxicity. More studies are needed to determine their effects on cell and cellular systems before they can be used as a therapeutic intervention.

In conclusion, fullerenes are capable of inhibiting MC and PBB allergic responses in vitro and anaphylaxis in vivo and suggests these molecules have previously unrecognized antiallergic properties. Fullerenes may represent a new way to control diseases largely influenced by these cells including type I hypersensitivity (anaphylaxis, hay fever, etc) (11), asthma (56), arthritis (57), multiple sclerosis (58), and other autoimmune diseases (59). For future applications, fullerenes could be modified with functional groups to specifically target their uptake to MC and PBB. For example we are investigating the effects fullerene-coated stem cell factor and IgE Fc peptide, molecules that will specifically target MC and PBB, have on mediator release.

Disclosures

The authors have no financial conflict of interest.

References

1. Emerich, D. F., and C. G. Thanos. 2003. Nanotechnology and medicine. *Exp. Opin. Biol. Ther.* 3: 655–663.
2. Moghimi, S. M., A. C. Hunter, and J. C. Murray. 2005. Nanomedicine: current status and future prospects. *FASEB J.* 19: 311–330.
3. Satoh, M., and I. Takayanagi. 2006. Pharmacological studies on fullerene (C_{60}), a novel carbon allotrope, and its derivatives. *J. Pharmacol. Sci.* 100: 513–518.
4. Bosi, S., T. Da Ros, G. Spalluto, and M. Prato. 2003. Fullerene derivatives: an attractive tool for biological applications. *Eur. J. Med. Chem.* 38: 913–923.
5. Chen, B. X., S. R. Wilson, M. Das, D. J. Coughlin, and B. F. Erlanger. 1998. Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics. *Proc. Natl. Acad. Sci. USA* 95: 10809–10813.
6. Kepley, C. L., and N. Cohen. 2003. Evidence for human mast cell nonreleaser phenotype. *J. Allergy Clin. Immunol.* 112: 457–459.

7. Kepley, C. L. 2005. Antigen-induced reduction in mast cell and basophil functional responses due to reduced Syk protein levels. *Int. Arch. Allergy Immunol.* 138: 29–39.
8. Kepley, C. L., L. Youssef, R. P. Andrews, B. S. Wilson, and J. M. Oliver. 2000. Multiple defects in FcεRI signaling in Syk-deficient nonreleaser basophils and IL-3-induced recovery of Syk expression and secretion. *J. Immunol.* 165: 5913–5920.
9. Gillespie, S. R., R. R. DeMartino, J. Zhu, H. J. Chong, C. Ramirez, C. P. Shelburne, L. A. Bouton, D. P. Bailey, A. Gharse, P. Mirmonsef, et al. 2004. IL-10 inhibits FcεRI expression in mouse mast cells. *J. Immunol.* 172: 3181–3188.
10. Siraganian, R. P., J. Zhang, K. Suzuki, and K. Sada. 2002. Protein tyrosine kinase Syk in mast cell signaling. *Mol. Immunol.* 38: 1229–1233.
11. Schwartz, L. B. 2004. Effector cells of anaphylaxis: mast cells and basophils. *Novartis Found. Symp.* 257: 65–74; discussion 74–79, 98–100, 276–285.
12. Dugan, L. L., J. K. Gabrielsen, S. P. Yu, T. S. Lin, and D. W. Choi. 1996. Buckminsterfullerenol free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons. *Neurobiol. Dis.* 3: 129–135.
13. Dugan, L. L., D. M. Turetsky, C. Du, D. Lobner, M. Wheeler, C. R. Almli, C. K. Shen, T. Y. Luh, D. W. Choi, and T. S. Lin. 1997. Carboxyfullerenes as neuroprotective agents. *Proc. Natl. Acad. Sci. USA* 19: 94: 9434–9439.
14. Dugan, L. L., E. G. Lovett, K. L. Quick, J. Lotharius, T. T. Lin, and K. L. O'Malley. 2001. Fullerene-based antioxidants and neurodegenerative disorders. *Parkinsonism Relat. Disord.* 7: 243–246.
15. Chen, Y. W., K. C. Hwang, C. C. Yen, and Y. L. Lai. 2004. Fullerene derivatives protect against oxidative stress in RAW 264.7 cells and ischemia-reperfused lungs. *Am. J Physiol.* 287: R21–R26.
16. Taylor, A. E. 2004. Fullerene derivatives protect against oxidative stress in murine macrophage line cells and ischemia-reperfused lungs. *Am. J. Physiol.* 287: R1–R2.
17. Lai, Y. L., P. Murugan, and K. C. Hwang. 2003. Fullerene derivative attenuates ischemia-reperfusion-induced lung injury. *Life Sci.* 72: 1271–1278.
18. Kamata, H., and H. Hirata. 1999. Redox regulation of cellular signalling. *Cell. Signal.* 11: 1–14.
19. Sonoda, Y., N. Aiba, R. Utsubo, E. Koguchi, M. Hasegawa, and T. Kasahara. 2004. Induction of antioxidant enzymes by FAK in a human leukemic cell line, HL-60. *Biochim. Biophys. Acta* 1683: 22–32.
20. Shibanuma, M., K. Mori, J. R. Kim-Kaneyama, and K. Nose. 2005. Involvement of FAK and PTP-PEST in the regulation of redox-sensitive nuclear-cytoplasmic shuttling of a LIM protein, Hic-5. *Antioxid. Redox Signal* 7: 335–347.
21. Chiarugi, P., G. Pani, E. Giannoni, L. Taddei, R. Colavitti, G. Raugei, M. Symons, S. Borrello, T. Galeotti, and G. Ramponi. 2003. Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J. Cell Biol.* 161: 933–944.
22. Natarajan, V., W. M. Scribner, M. al Hassani, and S. Vepa. 1998. Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells. *Environ. Health Perspect.* 106(Suppl. 5): 1205–1212.
23. Song, Y. S., Y. S. Lee, and P. H. Chan. 2005. Oxidative stress transiently decreases the IKK complex (IKKα, β, and γ), an upstream component of NF-κB signaling, after transient focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* 25: 1301–1311.
24. Bubici, C., S. Papa, K. Dean, and G. Franzoso. 2006. Mutual cross-talk between reactive oxygen species and nuclear factor-κB: molecular basis and biological significance. *Oncogene* 25: 6731–6748.
25. Ozaki, M., S. Haga, H. Q. Zhang, K. Irani, and S. Suzuki. 2003. Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling: role of PI3-K and Akt kinase upon rac1. *Cell Death Differ.* 10: 508–515.
26. Li, W. Q., H. Y. Qureshi, A. Liacini, F. Dehnade, and M. Zafarullah. 2004. Transforming growth factor β1 induction of tissue inhibitor of metalloproteinases 3 in articular chondrocytes is mediated by reactive oxygen species. *Free Radical Biol. Med.* 37: 196–207.
27. Cepero, E., A. M. King, L. M. Coffey, R. G. Perez, and L. H. Boise. 2005. Caspase-9 and effector caspases have sequential and distinct effects on mitochondria. *Oncogene* 24: 6354–6366.
28. Chen, S., J. Gong, F. Liu, and U. Mohammed. 2000. Naturally occurring polyphenolic antioxidants modulate IgE-mediated mast cell activation. *Immunology* 100: 471–480.
29. Yoshimaru, T., Y. Suzuki, T. Matsui, K. Yamashita, T. Ochiai, M. Yamaki, and K. Shimizu. 2002. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* 32: 612–618.
30. Matsui, T., Y. Suzuki, K. Yamashita, T. Yoshimaru, M. Suzuki-Karasaki, S. Hayakawa, M. Yamaki, and K. Shimizu. 2000. Diphenyleneiodonium prevents reactive oxygen species generation, tyrosine phosphorylation, and histamine release in RBL-2H3 mast cells. *Biochem. Biophys. Res. Commun.* 276: 742–748.
31. Swindle, E. J., J. A. Hunt, and J. W. Coleman. 2002. A comparison of reactive oxygen species generation by rat peritoneal macrophages and mast cells using the highly sensitive real-time chemiluminescent probe pholasin: inhibition of antigen-induced mast cell degranulation by macrophage-derived hydrogen peroxide. *J. Immunol.* 169: 5866–5873.
32. Wolfreys, K., and D. B. Oliveira. 1997. Alterations in intracellular reactive oxygen species generation and redox potential modulate mast cell function. *Eur. J. Immunol.* 27: 297–306.
33. Yoshimaru, T., Y. Suzuki, T. Matsui, K. Yamashita, T. Ochiai, M. Yamaki, and K. Shimizu. 2002. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* 32: 612–618.
34. Swindle, E. J., D. D. Metcalfe, and J. W. Coleman. 2004. Rodent and human mast cells produce functionally significant intracellular reactive oxygen species but not nitric oxide. *J. Biol. Chem.* 19: 279: 48751–48759.
35. Kepley, C. L., F. T. Lauer, J. M. Oliver, and S. W. Burchiel. 2003. Environmental polycyclic aromatic hydrocarbons, benzo(a) pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. *Clin. Immunol.* 107: 10–19.
36. Okayama, Y. 2005. Oxidative stress in allergic and inflammatory skin diseases. *Curr. Drug Targets Inflamm. Allergy* 4: 517–519.
37. Lee, K. S., H. S. Park, S. J. Park, S. R. Kim, K. H. Min, S. M. Jin, L. Li, and Y. C. Lee. 2006. An antioxidant modulates expression of receptor activator of NF-κB in asthma. *Exp. Mol. Med.* 38: 217–229.
38. Lee, K. S., S. R. Kim, S. J. Park, K. H. Min, K. Y. Lee, S. M. Jin, W. H. Yoo, and Y. C. Lee. 2006. Antioxidant down-regulates IL-18 expression in asthma. *Mol. Pharmacol.* 70: 1184–1193.
39. Bowler, R. P., and J. D. Crapo. 2002. Oxidative stress in allergic respiratory diseases. *J. Allergy Clin. Immunol.* 110: 349–356.
40. Tkaczyk, C., and A. M. Gilfillan. 2001. FcεRI-dependent signaling pathways in human mast cells. *Clin. Immunol.* 99: 198–210.
41. Nadler, M. J., S. A. Matthews, H. Turner, and J. P. Kinet. 2000. Signal transduction by the high-affinity immunoglobulin E receptor FcεRI: coupling form to function. *Adv. Immunol.* 76: 325–355.
42. Takano, T., K. Sada, and H. Yamamura. 2002. Role of protein-tyrosine kinase syk in oxidative stress signaling in B cells. *Antioxid. Redox Signal* 4: 533–541.
43. Fenoglio, I., M. Tomatis, D. Lison, J. Muller, A. Fonseca, J. B. Nagy, and B. Fubini. 2006. Reactivity of carbon nanotubes: free radical generation or scavenging activity? *Free Radical Biol. Med.* 40: 1227–1233.
44. Fiorito, S., A. Serafino, F. Andreola, A. Togna, and G. Togna. 2006. Toxicity and biocompatibility of carbon nanoparticles. *J. Nanosci. Nanotechnol.* 6: 591–599.
45. Sayes, C. M., F. Liang, J. L. Hudson, J. Mendez, W. Guo, J. M. Beach, V. C. Moore, C. D. Doyle, J. L. West, W. E. Billups, et al. 2006. Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. *Toxicol. Lett.* 161: 135–142.
46. Mori, T., H. Takada, S. Ito, K. Matsubayashi, N. Miwa, and T. Sawaguchi. 2006. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. *Toxicology* 225: 48–54.
47. Worle-Knirsch, J. M., K. Pulskamp, and H. F. Krug. 2006. Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano. Lett.* 6: 1261–1268.
48. Nakamura, E., and H. Isobe. 2003. Functionalized fullerenes in water: the first 10 years of their chemistry, biology, and nanoscience. *Acc. Chem. Res.* 36: 807–815.
49. Sayes, C. M., A. M. Gobin, K. D. Ausman, J. Mendez, J. L. West, and V. L. Colvin. 2005. Nano-C60 cytotoxicity is due to lipid peroxidation. *Biomaterials* 26: 7587–7595.
50. Fumelli, C., A. Marconi, S. Salvioli, E. Straface, W. Malorni, A. M. Offidani, R. Pellicciari, G. Schettini, A. Giannetti, D. Monti, et al. 2000. Carboxyfullerenes protect human keratinocytes from ultraviolet-B-induced apoptosis. *J. Invest. Dermatol.* 115: 835–841.
51. Monti, D., L. Moretti, S. Salvioli, E. Straface, W. Malorni, R. Pellicciari, G. Schettini, M. Bisaglia, C. Pincelli, C. Fumelli, et al. 2000. C60 carboxyfullerene exerts a protective activity against oxidative stress-induced apoptosis in human peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun.* 277: 711–717.
52. Porter, A. E., K. Muller, J. Skepper, P. Midgley, and M. Welland. 2006. Uptake of C60 by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography. *Acta Biomater.* 2: 409–419.
53. Castells, M. C., A. M. Irani, and L. B. Schwartz. 1987. Evaluation of human peripheral blood leukocytes for mast cell tryptase. *J. Immunol.* 138: 2184–2189.
54. Chen, H. H., C. Yu, T. H. Ueng, S. Chen, B. J. Chen, K. J. Huang, and L. Y. Chiang. 1998. Acute and subacute toxicity study of water-soluble polyalkylsulfonated C60 in rats. *Toxicol. Pathol.* 26: 143–151.
55. Rouse, J. G., J. Yang, A. R. Barron, and N. A. Monteiro-Riviere. 2006. Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytes. *Toxicol. In Vitro* 20: 1313–1320.
56. Bradding, P., A. F. Walls, and S. T. Holgate. 2006. The role of the mast cell in the pathophysiology of asthma. *J. Allergy Clin. Immunol.* 117: 1277–1284.
57. Lee, D. M., D. S. Friend, M. F. Gurish, C. Benoist, D. Mathis, and M. B. Brenner. 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297: 1689–1692.
58. Secor, V. H., W. E. Secor, C. A. Gutekunst, and M. A. Brown. 2000. Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J. Exp. Med.* 191: 813–822.
59. Gregory, G. D., A. Bickford, M. Robbie-Ryan, M. Tanzola, and M. A. Brown. 2005. MASTering the immune response: mast cells in autoimmunity. *Novartis Found. Symp.* 271: 215–225; discussion 225-31.
60. Kepley, C. L., B. W. Wilson, and J. M. Oliver. 1998. Identification of the FcεRI-activated tyrosine kinases Lyn, Syk, and Zap-70 in human basophils. *J. Allergy Clin. Immunol.* 102: 304–315.