



In vivo hydroquinone exposure impairs allergic lung inflammation in rats

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Abstract

Hydroquinone (HQ) is naturally found in the diet, drugs, as an environmental contaminant and endogenously generated after benzene exposure. Considering that HQ alters the immune system and its several source of exposures in the environment, we hypothesized that prolonged exposure of HQ could affect the course of an immune-mediated inflammatory response. For this purpose, male Wistar rats were intraperitoneally exposed to vehicle or HQ once a day, for 22 days with a 2-day interval every 5 days. On day 10 after exposure with vehicle or HQ, animals were ovalbumin (OA)-sensitized and OA-aerosolized challenged on day 23. HQ exposure did not alter the number of circulating leukocytes but impaired allergic inflammation, evidenced by lower number of leukocytes in the bronchoalveolar lavage fluid 24 h after OA-challenge. Reduced force contraction of *ex vivo* tracheal segments upon OA-challenge and impaired mesentery mast cell degranulation after *in situ* OA-challenge were also detected in tissues from HQ exposed animals. The OA-specificity on the decreased responses was corroborated by normal trachea contraction and mast cell degranulation in response to compound 48/80. In fact, lower levels of circulating OA-anaphylactic antibodies were found in HQ exposed rats, as assessed by passive cutaneous anaphylaxis assay. The reduced level of OA-anaphylactic antibody was not dependent on lower number or proliferation of lymphocytes. Nevertheless, lower expression of the co-stimulatory molecules CD6 and CD45R on OA-activated lymphocytes from HQ exposed rats indicate the interference of HQ exposure with signaling of the humoral response during allergic inflammation. Together, these data indicate specific effects of HQ exposure manifested during an immune host defense.

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Keywords: Hydroquinone; Allergic inflammation; Ovalbumin; Anaphylactic antibodies; CD45R; CD6; Mast cell degranulation

1. Introduction

Hydroquinone (HQ) is a naturally occurring agent in plants or plant derived products (Deisinger et al., 1996), synthetically produced to be used in chemical industries, black and white photographic developers, and in cos-

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metic products as a skin lightening agent (Stanfield et al., 2006). Moreover, the main portion of environmental contamination occurs through cigarette smoke or benzene exposure in petroleum refining, petrochemical and chemical industries (Dimitrova et al., 2005) or by traffic exhaust fumes (Darrall et al., 1998; Snyder, 2002, 2004). Experimental assays have proposed that the immunosuppressive effects of benzene intoxication are associated with its phenolic bio-products, of which HQ is a pivotal compound (Li et al., 1996, 1997; McCue et al., 2000; Snyder, 2002, 2004).

Accordingly, after benzene exposure, HQ is endogenously produced via oxidation reactions catalyzed by cytochrome P450 enzymes in the liver (Snyder, 2002, 2004). Once circulating, HQ passively reaches the bone marrow where it is metabolized and might account for the myelotoxicity in benzene intoxication (Henderson, 1996; Snyder, 2002, 2004). Taking this into consideration, HQ is potentially a toxic agent capable of affecting immune cell responses. In fact, *in vitro* assays have demonstrated that HQ blocks the production of lymphocytes and impairs their proliferation (Li et al., 1996, 1997; Poirier et al., 2002; McCue et al., 2000, 2003). The mechanisms involved are not completely understood and are related to inhibition of DNA synthesis or blastogenesis, alteration of cell cycle entry and progression through the G(1) phase (McCue et al., 2000, 2003), in addition to inhibition of cytokine production. Conversely, it was shown that HQ sensitizes mice by enhancing B cells in the popliteal lymph node (Ewens et al., 1999) and significantly increasing *in vitro* and *in vivo* IL-4 production by stimulated CD4⁺ T cells, accompanied by an increment in circulating immunoglobulin (Ig) E levels (Lee et al., 2002). Also, Kim et al. (2005) showed that HQ may enhance allergic immune responses by inhibiting *in vitro* IL-12 production by LPS-activated macrophage, in which the mechanism proposed is the suppression of NF κ B activity by inhibiting the degradation of the I κ B protein.

The allergic response is triggered by mast cell-derived mediators, released after the antigen cross-links the IgE on the mast cells surface. In the sensitization phase, antigen is presented by antigen-presenting cells (APC, dendritic cells, macrophages and Langerhans cells) to CD4 lymphocytes, which signal to the B lymphocytes to produce immunoglobulins (Murphy and Reiner, 2002; Careau et al., 2002; Ansel et al., 2003). Upon antigen challenge, the sensitized mast cell activates multiple signaling pathways causing its degranulation with a consequent release of a wide range of mediators accounting for leukocyte infiltration, plasma extravasation, airway smooth muscle contraction and mucus secretion (Galli

et al., 2005; Okayama and Kawakami, 2006). In this context, it is known that accessory surface receptors and adhesion molecules in APC, T and B cells are involved in mediating the highly regulated phosphorylation and dephosphorylation of tyrosine residues on target proteins, regulating antigen presentation, lymphocyte proliferation, cytotoxicity, humoral activities and apoptosis (Iezzi et al., 1998; Burastero and Rossi, 1999; Kiefer et al., 2002). Therefore, the successful immune response is dependent on a coordinated cascade of events mediated by complex membrane and intracellular events (Burastero and Rossi, 1999; Kiefer et al., 2002).

As HQ is an environmental contaminant and displays toxicity to immune system, here we investigated the role of prolonged HQ exposure on allergic lung inflammation triggered by unrelated antigen to HQ. Data obtained show that HQ exposure impairs the expression of CD45R and CD6 co-stimulatory molecules on activated spleen lymphocytes and diminishes the levels of OA-anaphylactic antibodies and the subsequent mast cell degranulation in sensitized rats challenged with OA. These effects might be important mechanisms to account for the impaired allergic inflammation, here characterized by decreased airway reactivity and leukocyte infiltration.

2. Materials and methods

2.1. Chemicals

Pentobarbital sodium (Cristália, Brazil); FITC-labeled anti-rat CD86, CD40, CD6 and PE-labeled anti-rat CD45R and CD80 were purchased from BD Biosciences (USA); Heparin (Liquemine®, Roche, Brazil); Ovalbumin (chicken egg albumin crude, grade II), Concanavalin A from *Canavalia ensiformis* (type IV), RPMI 1640 medium, θ -dianisidine, hexadecyltrimethyl ammonium bromide, compound 48/80, EDTA and HQ were purchased from Sigma (St. Louis, MO, USA); Chloral hydrate (Quims, Brazil) ammonium chloride, aluminum hydroxide, blue toluidine, Evans blue, May-Grünwald and Giemsa dyes, H₂O₂ and ethanol were purchased from Merck (USA); [³H] thymidine (Amersham Pharmacia Biotech UK Limited, UK); Fetal calf serum (Gibco-BRL, MD, USA).

2.2. Animals

Adult male Wistar rats weighing 180–220 g were used. Animals were kept under a light/dark cycle (12 h on, 12 h off), allowed a standard pellet diet and water *ad libitum*. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

2.3. Protocol of *in vivo* HQ exposure

HQ (50 mg/kg in a final volume of 1 mL) was intraperitoneally injected, once a day. Animals used as controls received the same volume of vehicle by the same route. HQ was dissolved in absolute ethanol (5% of the final solution) and the volume was completed with sterile saline solution. The animals received 16 daily doses of treatment, with 2-day intervals every 5 doses. Assays were carried out 24 h after the last dose of HQ or vehicle exposures.

2.4. Number of circulating leukocytes

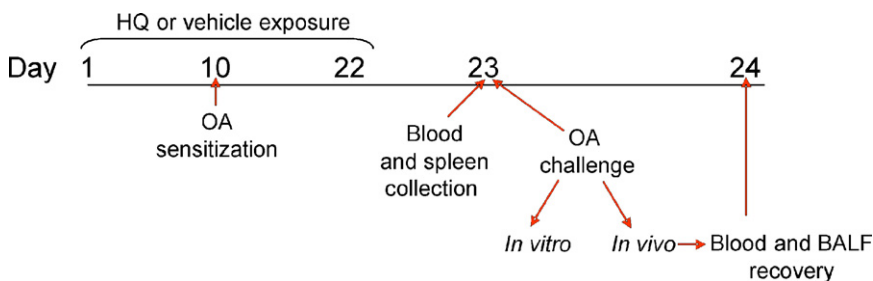
Blood was collected from orbital plexus from animals before and after HQ or vehicle exposures. The total number of leukocytes was assessed using a Neubauer chamber. Differential counts were performed on the basis of 500 cells per slide in cytocentrifuge smears stained with May-Grünwald and Giemsa dyes.

2.5. Antigen sensitization

Rats were sensitized by a single intraperitoneal injection of ovalbumin (OA, 10 µg) mixed with aluminum hydroxide (10 mg) as adjuvant on the 10th day of HQ or vehicle exposure. Experiments were performed 13 days later. The time frame was chosen based on Coleman et al. (1983), who demonstrated that the levels of circulating IgE antibodies in rats increases rapidly 7–14 days after intraperitoneal OA injection.

2.6. *In vivo* antigen challenge

On day 13 after OA-sensitization and 24 h after the last injection of HQ or vehicle, rats were subjected to a single 15 min-exposure of aerosolized OA (1% in phosphate buffered saline, PBS) using an ultrasonic nebulizer device (Icel®, SP, Brazil) coupled to a plastic inhalation chamber (18.5 cm × 18.5 cm × 13.5 cm).



2.7. Number of leukocytes in bronchoalveolar lavage fluid (BALF)

Twenty-four hours after OA challenge, animals were anesthetized with pentobarbital sodium (65 mg/kg, intraperitoneal injection) and exsanguinated by abdominal aorta. In brief, a plastic cannula coupled to syringe was inserted into the

trachea and 20 mL (2 × 10 mL) PBS was injected. Immediately following PBS injection, BALF was collected and the fluid was centrifuged at 500 × g for 10 min at 4 °C. The cell pellet was re-suspended in 1 mL of PBS. The number of cells was determined using a Neubauer chamber and stained cell suspensions (0.5% crystal violet dissolved in 30% acetic acid). Differential cell counts were performed from cytopspin preparations and then stained with May-Grünwald.

2.8. *In vitro* anaphylactic reaction

Twenty-four hours after the last injection of HQ or vehicle, rats were killed under deep chloral hydrate anesthesia (>400 mg/kg, i.p.) and exsanguinated via the abdominal aorta. The trachea was removed and dissected free of connective tissue. Tracheal rings corresponding to the last 3–5 cartilaginous rings closest to the carine, designated the distal trachea, were set up for the measurement of isometric contractions by suspending them with two steel hooks in 8 mL organ baths containing Krebs-Henseleit solution with the following composition (mM): 115.0 NaCl, 4.6 KCl, 2.5 CaCl₂·2H₂O, 1.2 KH₂PO₄, 2.5 MgSO₄·7H₂O, 25.0 NaHCO₃ and 11.0 glucose. The solution was gassed with 95% O₂ and 5% CO₂ and maintained at 36 °C. The tissue was allowed to equilibrate for 60 min under an initial tension of 500 mg. During this period, the tension was adjusted to 1.0 g, and the contractile activity of the tissue was then assessed by the addition of iso-osmolar Krebs-bicarbonate solution containing 60 mM KCl. The tissue was subsequently challenged by the addition of OA at a final concentration of 100 µg/mL (Schultz, 1910; Dale, 1913; de Lima and da Silva, 1998). The force contraction elicited by the tracheal segment due to the OA addition in the organ bath system was recorded using an isometric transducer coupled to the PowerLab 4sp system; the data were analyzed using the Chart 3.4® software (Ad Instruments, Australia).

2.9. *In situ* mast cell degranulation

Twenty-four hours after the last injection of HQ or vehicle, rats were anesthetized intraperitoneally with pentobarbital sodium (65 mg/kg) and the mesentery was exteriorized and analyzed by intravital microscopy. After surgery, the ani-

mals were kept on a special board thermostatically controlled at 37 °C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with a warmed Ringer-Locke solution (pH 7.2–7.4; 154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂·2H₂O, 6 mM NaHCO₃ and 5 mM glucose) containing 1% gelatine. The rate of outflow of the solution onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of the liquid. Transilluminated images were obtained by optical microscopy (Axioplan II, Carl-Zeiss equipped with 5.0/0.30× plan-neofluar or 10.0/0.25× Achroplan longitudinal distance objectives/numeric aperture and 1.0×, 1.25× or 1.60× optovar). The images were captured by a video camera (ZVS, 3C75DE, Carl-Zeiss) and were transmitted simultaneously to a TV monitor and to a computer. Images obtained on the TV monitor were recorded on video-tape. Digitized images on the computer monitor were subsequently analyzed by image-analyzing software (KS 300, Kontron).

The degranulation of mast cells under topical application of 10 µL of OA 1% saline solution or 10 µg of compound 48/80 was determined in the tissue adjacent to postcapillary venules of the mesentery. The images were recorded 5 min after stimulus application and degranulated mast cells were dyed by topical application of blue toluidine dye. Ten areas were evaluated in each animal.

2.10. Passive cutaneous anaphylaxis (PCA) reaction

PCA reaction is a typical assay to indirectly measure anaphylactic antibodies levels (Mota and Wong, 1969; Shin et al., 2000; Hong et al., 2003). An IgE-dependent cutaneous reaction was generated by sensitizing the skin of non-manipulated rats with an intradermal injection of diluted sera from vehicle- or HQ-exposed and OA-sensitized rats. Twenty-four hours after the intradermal injections, animals received an intravenous injection of 1 mL of solution containing 500 µg of OA and 2.5 mg of Evans blue. After 30 min, the rats were sacrificed and the dorsal skin was removed for measurement of the pigmented area. Data are expressed by PCA titer, which represents the serum dilution capable of inducing a dyed area greater than 5 mm in diameter as described by Mota and Wong (1969).

2.11. Cell proliferation assay

Twenty-four hours after the last injection of HQ or vehicle, the spleen was harvested and 2×10^5 cells per well were cultured in 96-well flat bottom tissue culture plates with 25–125 µg/mL of OA in complete RPMI with 5% fetal calf serum (FCS) or with concanavalin A (ConA; 7 µg/mL). During the last 18 h of the 4-day culture period, [³H]thymidine was added (1 µCi/well). At the end of this period of incubation, cells were collected with an automated cell harvester, and incorporated radioactivity was measured by liquid scintillation spectrometry. Data are expressed as mean ± standard error of mean counts per minute of [³H]thymidine incorporation.

2.12. Blood flow cytometry

Thirteen days after OA-sensitization or 24 h after OA-challenge, leukocytes were isolated from spleen to quantify the expression of CD45R, CD40, CD80, CD86 and CD6. Briefly, erythrocyte lysis was performed using ammonium chloride solution (0.13 M) and leukocytes were recovered after washing with Hanks's Balanced Salt Solution (HBSS). Leukocytes were incubated for 30 min at 4 °C in the dark with 10 µL of monoclonal antibodies. Immediately after incubation, cells were analyzed on a FACScalibur flow cytometer (Becton & Dickinson – San Jose, CA, USA). Data from 10,000 events were obtained and only the morphologically viable cells were considered for analysis. Median of the fluorescence intensity and percentage of labeled lymphocytes was obtained and results express the mean of 4 assays, performed in duplicate.

2.13. Statistical analysis

Means and standard errors of means (S.E.M.) of all data are presented and were compared by Student's *t*-test or ANOVA with a significance probability of less than 0.05. Individual comparisons were subsequently performed with Bonferroni's test for unpaired values and Tukey test.

3. Results

3.1. Effects of HQ exposure on leukocyte migration into the lungs of rats upon antigen challenge

There were significantly fewer leukocytes present in the BALF 24 h after OA challenge in HQ exposed rats when compared to number of leukocytes found in BALF of allergic rats exposed to vehicle. Differential analysis showed that HQ exposure reduced the influx of both polymorphonuclear (PMN) cells and mononuclear (MN) cells into the bronchoalveolar cavity (Fig. 1). The numbers of cells collected in BALF of naïve rats consisted of 5.04×10^5 MN and 0.17×10^5 PMN cells.

HQ exposure did not change the number of circulating leukocytes, as number of cells at the peripheral compartment was equivalent before and after HQ exposure and similar to those found in vehicle exposed rats (before HQ exposure = 7867 ± 322 cells/mm³; after HQ exposure = 8134 ± 497 cells/mm³; before vehicle exposure = 7833 ± 450 cells/mm³; after vehicle exposure = 8317 ± 473 cells/mm³; *n* = 6 for each group).

3.2. Influence of HQ exposure on the ex vivo OA-induced rat tracheal contraction

As shown in Fig. 2, the force of contraction after the ex vivo antigen challenge (OA) of tracheal segments

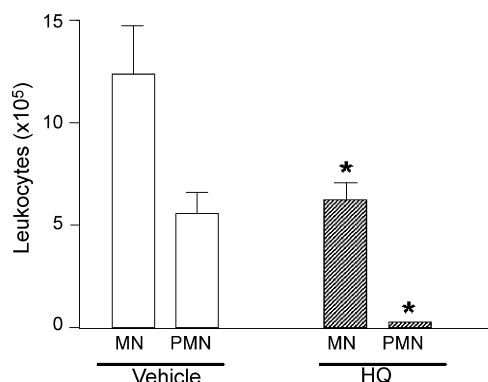


Fig. 1. Number of leukocytes in the bronchoalveolar lavage fluid (BALF) from rats exposed to vehicle or HQ (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses). Animals were OA-sensitized (10 μ g of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposure and OA-challenged (1% solution, 15 min, i.n.) on day 23. BALF was obtained 24 h after OA challenge. MN=mononuclear; PMN=polymorphonuclear. Results are expressed as mean $\pm$ S.E.M. of samples collected from 6 animals in each group. * $p < 0.001$ values obtained for vehicle-exposed rats.

from HQ exposed rats was significantly lower than that observed in tracheal segments of vehicle exposed rats. In contrast, HQ exposure failed to modify the force of contraction caused by compound 48/80. Trachea of naïve rats did not respond to *ex vivo* OA addition (data not shown).

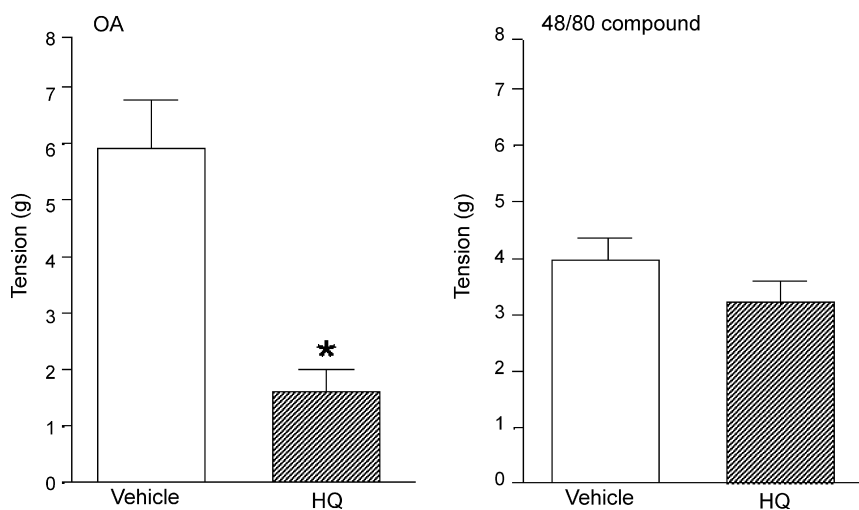


Fig. 2. *In vitro* anaphylactic response in isolated distal tracheal segments from rats exposed to vehicle or HQ (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses). Animals were OA-sensitized (10 μ g of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposure and tracheas removed 13 days later. The contractile activity of the tissue was assessed by the addition of iso-osmolar Krebs-bicarbonate solution containing 60 mM KCl and subsequently challenged by addition of OA at a final concentration of 100 μ g/mL or 48/80 compound (10 μ g). Results are expressed as mean $\pm$ S.E.M. of tracheas collected from 4 animals in each group. Assays were performed in duplicate. * $p < 0.001$ values obtained in tracheas from vehicle-exposed rats.

3.3. Effects of HQ exposure on mesenteric mast cell degranulation

Mesentery tissue from HQ or vehicle and OA sensitized rats was exteriorized and the degranulation of mast cells adjacent to the microvascular network was observed using intravital microscopy. As demonstrated in Fig. 3A and B and represented in Fig. 3E, OA induced-degranulation of mesenteric mast cells was significantly reduced in HQ exposed rats as compared to that observed in mesenteric tissue of allergic rats upon vehicle exposure. In addition, the level of mast cells degranulation after topical application of compound 48/80 was similar in both HQ and vehicle exposed rats (Fig. 3C–E).

3.4. Effect of HQ exposure on the circulating levels of anaphylactic antibodies

Data presented in Fig. 4 show the ability of sera obtained from rats exposed to HQ or vehicle to induce increased cutaneous microvascular permeability in naïve rats. The amount of serum from HQ exposed rats required to induce the reaction was higher than the amount of serum from vehicle exposed rats, indicating lower levels of IgE and IgG OA-anaphylactic antibodies in HQ exposed rats.

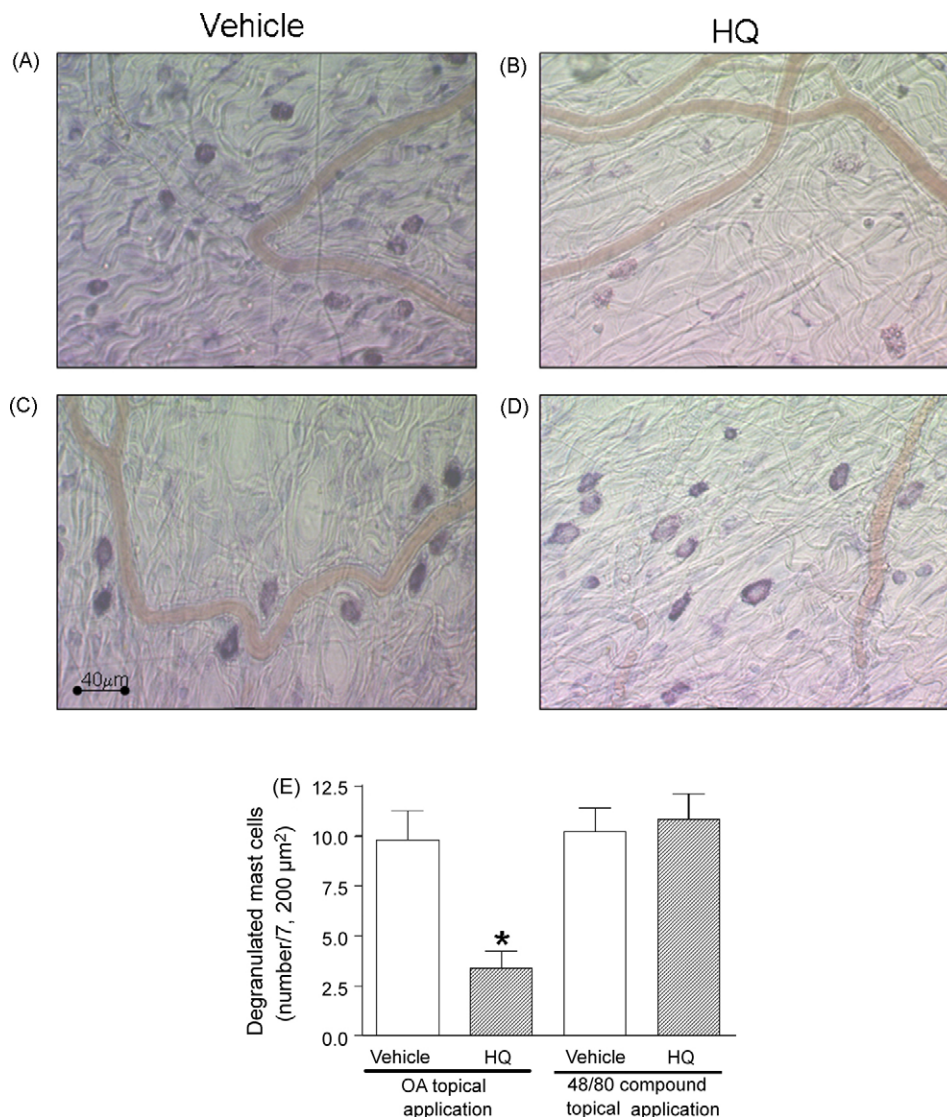


Fig. 3. Mesentery mast cell degranulation from rats exposed to vehicle or HQ (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses) and sensitized with OA (10 μg of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposures. Degranulation was induced 13 days after OA sensitization by topical application of OA or 48/80 compound. Images were obtained by intravital microscopy 5 min after topical application of stimuli. Degranulated mast cells were dyed by blue toluidine dye. (A) and (C) represent mast cells from vehicle exposed rats stimulated by OA or compound 48/80, respectively; (B) and (D) represent mast cells from HQ exposed rats and stimulated with OA or compound 48/80, respectively. (E) Number of degranulated mast cells in perivascular tissue. Four animals were employed per group. * $p < 0.001$ values obtained for vehicle exposed rats.

3.5. Influence of HQ exposure on the lymphocyte proliferation

In vitro lymphocyte proliferation in response to OA or ConA challenge was not altered by HQ exposure, as demonstrated in Fig. 5. Lymphocyte proliferation was equivalent in spleen cells collected from HQ or vehicle exposed rats. HQ exposure also did not modify the total number of spleen leuko-

cytes (HQ = $18.6 \pm 1.7 \times 10^6$ cell/mL; vehicle = $18.6 \pm 1.1 \times 10^6$ cell/mL; $n = 4$ for each group).

3.6. Effect of HQ exposure on CD45R and CD6 expression on spleen leukocytes

Results depicted in Fig. 6 show that HQ exposure does not alter the expression of CD80, CD86 or CD40, but reduces the expressions of CD45R and CD6 co-

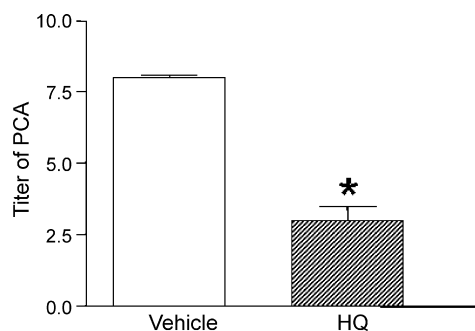


Fig. 4. Passive cutaneous anaphylaxis (PCA) reaction on the skin of naïve animals sensitized with an intradermal injection of diluted serum obtained from rats exposed to vehicle or HQ (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses) and sensitized with OA (10 µg of OA plus 10 mg of aluminum hydroxide, i.p., day 10 of exposure). Twenty-four hours after the i.d. injections, animals were intravenously challenged with OA solution. Evans blue intravenous injection was used as a dye. Thirty minutes later, the dorsal skin was removed for measurement of the pigment area. The PCA titer was expressed as the mean of the highest dilution resulting in a PCA reaction of 5 mm in diameter or greater. Results are expressed as mean ± S.E.M. of samples collected from 4 animals in each group. * $p < 0.001$ values obtained for vehicle exposed rats.

stimulatory molecule on surface of spleen leukocytes after *in vivo* OA-challenge. Interestingly, the reduced expression of CD6 or CD45R detected in cells collected from HQ-exposed rats is not dependent on the number of lymphocytes in the spleen as presented in Table 1. The percentage of cells expressing the CD45R or CD6 is equivalent in HQ or vehicle exposed animals in both the basal state and after *in vivo* OA-challenge (Table 1).

4. Discussion

Benzene concentration in the environment, especially in industrial centers, has reached levels that raise public health concerns (Fustinoni et al., 2005). Determination of the effects of benzene exposure and its mechanisms of action on different tissues may improve risk assessment. Here we show that *in vivo* exposure of rats to HQ, an endogenous metabolite of benzene, results in changes of immune response mirrored by impairing allergic lung response to ovalbumin, an antigen unrelated to HQ.

It is of interest to note that the limits for benzene and HQ exposures are dependent on dose and duration of exposure, route of administration, and animal species (IPCS, 1994). A controlled study in humans volunteers using high doses and prolonged exposure of HQ (300–500 mg HQ daily for 3–5 months, oral route), did not show any observed pathological changes in blood or urine. On the other hand, intraperitoneal administration of lower doses of HQ to mice was cytotoxic as evidenced

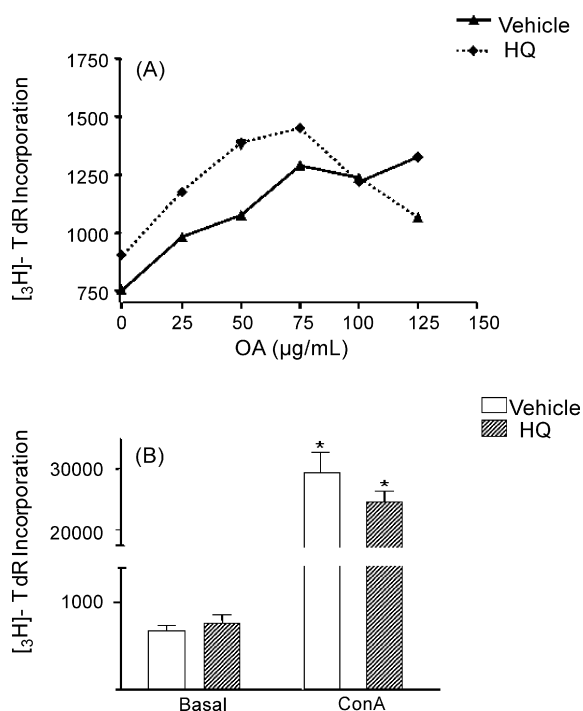


Fig. 5. *In vitro* spleen lymphocyte proliferation from vehicle or HQ exposed rats (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses) sensitized with OA (10 µg of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposures. Cells were collected 13 days after sensitization and 2×10^5 cells were cultured per well with 25–125 µg/mL of OA or with Concanavalin A (ConA; 7 µg/mL). During the last 18 h of the 4-day culture period, [^{3} H]thymidine was added (1 µCi/well). At the end of this period of incubation, cells were collected with an automated cell harvester, and incorporated radioactivity was measured by liquid scintillation spectrometry. Data are expressed as mean ± S.E.M. counts per minute of [^{3} H]thymidine incorporation in cells obtained from 3 animals in each group. Assays were performed in triplicate. * $p < 0.001$ for basal values.

by bone marrow reduction and splenic hypocellularity (IPCS, 1994; Hazel et al., 1996; Ewens et al., 1999). Accordingly, this study employed a dosing schedule for HQ that did not cause any apparent toxicity, as reflected by unaltered animal body weights, the normal blood and spleen cellularity, and normal morphology and function of both liver and kidney (data not shown).

Allergic lung inflammation is characterized by onset airway constriction, increased vascular permeability, mucous secretion and recruitment of inflammatory cells (Galli et al., 2005), contributing to pulmonary dysfunction. In this context, we observed that HQ exposure reduced airway reactivity and pulmonary inflammation, as evidenced by decreased *ex vivo* tracheal force contraction and diminished inflammatory cells recovered in BALF after antigen challenge. Although eosinophils are linked to genesis of allergic inflammatory disease,

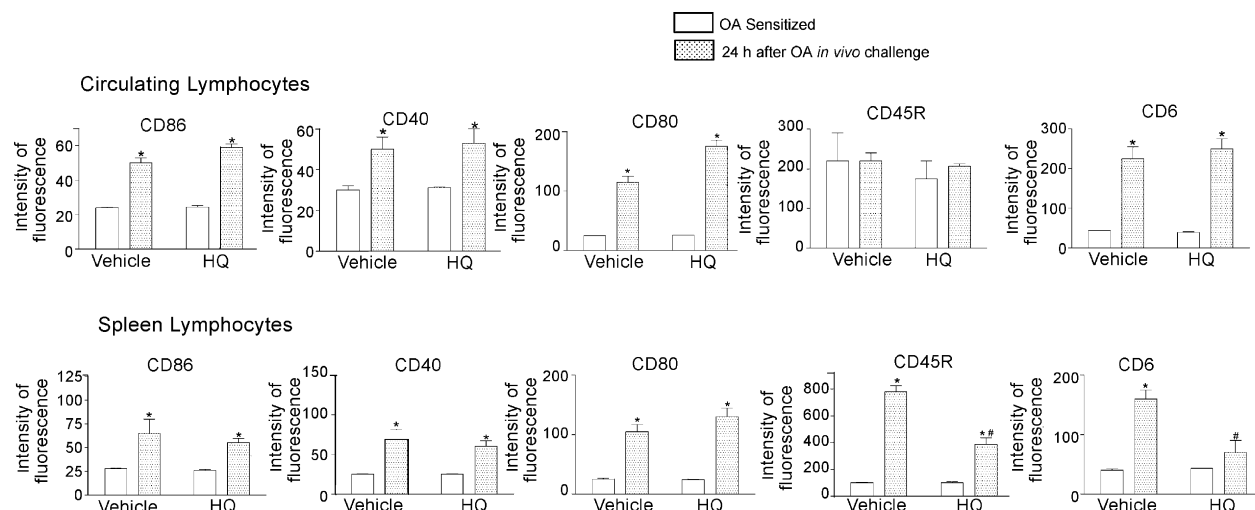


Fig. 6. Expression of co-stimulatory molecules on spleen leukocytes before and 24 h after *in vivo* OA challenge. Spleen cells were obtained from vehicle- and HQ-exposed rats (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses) and sensitized with OA (10 µg of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposures. OA-challenge (1% solution, 15 min, i.n.) was performed on day 23. Leukocytes were obtained from blood samples from the orbital plexus and co-stimulatory molecule expression was quantified by blood flow cytometry. Results are expressed as mean ± S.E.M. of samples collected from 4 animals in each group. Assays were performed in duplicate. **p* < 0.001 respective values obtained before OA-challenge and #*p* < 0.05 values obtained from vehicle exposed rats after OA-challenge.

in our model of acute allergic inflammation neutrophils are predominantly cells in the BALF, as they are one of the first inflammatory cells to be recruited into the airways after either allergen exposure or injury (Foley and Hamid, 2007). Our data, therefore, clearly revealed that HQ exposure modified the acute and late allergic lung response. Because, cellular recruitment during inflammatory process involves cellular traffic from bloodstream to the injured site, we decide to investigate if the HQ exposure could be associated with changes in leukocyte traffic. Interestingly, HQ exposure did not alter the profile of circulating leukocytes, a fact that ruled out the involvement of hematotoxicity of HQ (Ross et

al., 1996; Smith et al., 2000; Kalf et al., 1996; King et al., 1987, 1989; Snyder, 2002; Macedo et al., 2006). Moreover, HQ exposure did not change the expression of adhesion molecules L-selectin and β₂ integrin on the membrane of peripheral leukocytes (data not shown). Overall, the impaired lung leukocyte migration of allergic rats exposed to HQ appears not to depend on changes of peripheral mobilization of leukocytes from the bone marrow or their activation state in the blood compartment.

Therefore, we proposed a hypothesis that HQ exposure could modify the immunological activation of mast cells. Sensitized mast cells release a number of inflam-

Table 1
Percentage of spleen leukocytes expressing co-stimulatory molecules collected from HQ or vehicle exposed rats

	Vehicle		HQ	
	Before	After OA	Before	After OA
CD86	27.01 ± 3.24	73.21 ± 12.10*	27.28 ± 5.02	81.61 ± 2.66*
CD80	41.09 ± 2.55	78.93 ± 5.47**	30.63 ± 8.10	92.75 ± 1.64**
CD40	29.03 ± 3.91	77.74 ± 9.35**	21.42 ± 1.21	86.19 ± 8.10**
CD45R	60.11 ± 5.97	94.29 ± 2.10**	64.49 ± 3.60	97.44 ± 1.18**
CD6	52.95 ± 4.47	89.64 ± 1.79**	48.49 ± 3.89	90.34 ± 5.45**

Spleen cells were obtained from vehicle or HQ exposed rats (50 mg/kg; i.p. route; once a day; 16 doses with a 2-day interval after every 5 doses) and sensitized with OA (10 µg of OA plus 10 mg of aluminum hydroxide, i.p.) on day 10 of exposures. OA-challenge (1% solution, 15 min, i.n.) was performed on day 23. Cells were collected before and 24 h after OA-challenge. Percentage of leukocyte expressing each co-stimulatory molecule was determined by flow cytometer. Data represent mean ± S.E.M. of 4 animals in each group. **p* < 0.01 and ***p* < 0.001 in comparison to respective values before OA-challenge.

matory mediators within a few minutes after antigen challenge, including histamine, serotonin, cytokines, proteases, eicosanoids and interleukins (Galli et al., 2005). As consequence, these mediators induce an initial contraction of airway smooth muscles, stimulate nerve endings and cause mucus secretion. Additionally, they contribute to the development of the allergic response by activating resident and vascular cells to secrete additional mediators and to induce leukocyte recruitment to the inflammatory focus (de Lima and da Silva, 1998; Nemmar et al., 1999; Damaso et al., 2001; Wyss et al., 2005; Lukacs et al., 2005; Lino dos Santos Franco et al., 2006). In this study we observed that HQ exposure reduced the OA-induced tracheal contraction and the degranulation of mesentery perivascular mast cells. Overall, the relevance of the immunological response was supported by similar tracheal contraction and mesenteric mast cell degranulation in HQ or vehicle exposed rats after topical administration of compound 48/80, a mast cell secretagogue agent that evokes activation of phospholipase D and G protein-independent-receptors (Palomaki and Laitinen, 2006).

Reduced PCA titers were found in serum of HQ exposed allergic rats, reinforcing the suggestion that HQ might to interfere with the immune mechanisms associated with sensitization phase. Being so, we hypothesized that reduced levels of IgE and IgG anaphylactic antibodies, well established as mediators of allergic inflammation, might impaired the allergic response observed in rats subjected to HQ exposure, likely explained by the decreased mast cells degranulation in response to antigen challenge. Our data did not reveal the mechanisms triggered by HQ in order to cause impairment on levels of circulating OA-immunoglobulins. However, a putative mechanism proposed to the immunotoxicity of HQ is the inhibition of lymphocyte differentiation and proliferation, resulting in a reduced humoral activity. Despite *in vitro* assays showing the ability of high doses of HQ to inhibit T and B-lymphocyte proliferation (Li et al., 1996; Doepker et al., 2000; McCue et al., 2000; Poirier et al., 2002), there is no conclusive evidence about *in vivo* exposure of HQ. Conversely, it was shown that HQ sensitizes mice by enhancing B cells in popliteal lymph nodes (Ewens et al., 1999) and enhances the levels of IL-4 produced by CD4⁺ T cells and circulating levels of IgE in keyhole limpet haemocyanin-primed mice (Lee et al., 2002). Results herein do not corroborate these previous studies, since the schedule of HQ exposure employed did not alter the number of lymphocytes in circulation, in the spleen or their *in vitro* proliferation induced by

antigenic stimulus or by ConA. These divergent results may be accounted by different protocols of exposure. Indeed, in our model, the rats were subjected to extended treatment with phenolic compound prior to antigen sensitization whereas Lee et al. (2002) injected HQ in rats daily during a week after initial contact with the antigen.

Interaction of the T cell receptor (TCR) complex with the antigen peptide presented by the MHC on the APC is a pivotal event in the T cell activation. However, sustained T cell activation requires signal amplification by accessory molecules (Gimferrer et al., 2004). The amplification and modifications of the TCR signal by the co-stimulatory signals enable the antigen-specific pre-activated T lymphocyte to proliferate, secrete cytokines, and express cell-surface molecules for further cell–cell interactions involved in the development of the immune response (Mueller et al., 1989; Kallinich et al., 2005). Thus, we proposed to investigate the role of HQ exposure on expression of co-stimulatory molecules involved in T cell proliferation and B cell activation. We verified that *in vivo* HQ exposure impairs the expression of the CD45 receptor and CD6 molecules in OA activated spleen lymphocytes. CD6 acts as a co-stimulatory molecule synergizing with the TCR or CD28 to enhance T cell proliferation (Gimferrer et al., 2004). As lymphocyte proliferation is not altered by HQ exposure, it is possible to suggest that other molecules, such as CD28 may have a main role in the process. This hypothesis may be reinforced by normal expressions of CD28 ligands, CD80 and CD86. However, the importance of CD28 in our experimental model of HQ exposure will be further investigated. CD45 is an important surface protein on nucleated hematopoietic cells, comprising about 10% of T and B surface area involved in proliferation, differentiation and functions of lymphocytes (Thomas, 1989). Anti-CD45 antibodies alter signaling in B and T cells (Morikawa et al., 1991; Hasegawa et al., 1990; Faris et al., 1994; Mittler et al., 1994) and the CD45R inhibitor negatively regulates IgE-dependent anaphylaxis and contact hypersensitivity reactions *in vivo* and *in vitro* (Hamaguchi et al., 2001). These results provide a basis to support the hypothesis that HQ exposure may alter the interaction between T and B cells, with the subsequent impairment of the humoral response detected here.

Taken together the data show that the impairment of allergic lung inflammation after prolonged HQ exposure is associated with a deficiency in T and B lymphocyte co-stimulatory molecules expression, which perhaps reduces antibody production leading to failure of mast cell activation.

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