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The role of antioxidants in skin immune reactions: The use of flow cytometry to determine alterations in Ia-positive epidermal cells in allergic contact dermatitis

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Synopsis

Earlier experiments from our laboratory revealed that common parasubstituted phenolic compounds such as monobenzyl ether of hydroquinone (MBEH), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA), ubiquitous compounds found in medications, cosmetics, foods, and various industrial products, can alter Ia+ Langerhans cells (epidermal macrophages) and epidermal immune responsiveness (i.e., exacerbate allergic contact dermatitis) when topically applied to the epidermis of various strains of mice. The current experiments demonstrated that a precise and sensitive instrumental approach, fluorescent activated cell sorting (FACS), could be used to quantify changes more precisely in the expression of the membrane-bound Ia antigen found on epidermal antigen-presenting-Langerhans cells during contact hypersensitivity reactions. Further, this technique can be used to screen potential vehicle and/or medicament allergens as likely sources for allergic contact dermatitis at home and in the work place.

INTRODUCTION

Earlier studies from our laboratory have documented that topical applications of common antioxidants such as monobenzyl ether of hydroquinone (MBEH), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), ubiquitous compounds found in medications, cosmetics, food, and industrial rubber products, can alter the contact hypersensitivity responsiveness (CHS) (e.g., allergic contact dermatitis, ACD) in various mice strains when epicutaneously applied (1). These compounds, when topically applied for a short period of time (i.e., daily for five days) to various epidermal sites, can alter the density (cells/mm^2) of Ia+ (immune associated antigen)/ATPase+ Langerhans cells (LC) (epidermal immune macrophages). Further, this increase or decrease in identifiable Langerhans cells could be correlated with alterations in CHS responsiveness following sensitization and challenge with common allergens/haptens (i.e., dinitrofluorobenzene, oxazalone) (1). Other studies suggest that the mechanism responsible for the alteration of Ia+ Langerhans cell density and functional CHS reactivity following topical antioxidant treatment may be mediated via arachidonic acid (AA) and/or its metabolites (i.e., prostaglandins, thromboxanes, leukotrienes) (1,2).

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The current study was designed to examine, using more precise methodology (flow cytometry), the density of the Class II major histocompatability (MHC) Ia antigen on epidermal immune cells that had been previously treated in intact epidermis with various antioxidants or a biologically relevant molecule, arachidonic acid. It has been previously documented that the density of the Ia antigen membrane marker correlates with the intensity of epidermal immune reactivity (e.g., allergic contact dermatitis (1,3,5). We suggest that the described methodology may be useful as a sensitive screening technique for ascertaining whether common and/or new antioxidants used in medications and/or cosmetics may exacerbate common immune-mediated dermatoses such as allergic contact dermatitis.

MATERIALS AND METHODS

Four-to-six-week-old male DBA/2^d and C57BL/6^b mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed in University facilities, four to six per cage with free access to water and Purina Mouse Chow®. The animals were exposed to an ambient 12-hour light:dark photoperiod. The experiments were performed at least twice and produced similar results.

IN VIVO EXPOSURE TO MBEH AND AA

DBA/2 mice were treated topically with either 50 μl of 20% MBEH in 95% ETOH or 0.05% and 1.0% AA in DMSO:H_2O (1:1) applied to the dorsal pinnal epidermis daily for five days. Control animals were topically treated with 95% ETOH diluent or the DMSO:H_2O (1:1) diluent. On day 6, treated and diluent control ears were surgically excised and epidermal suspensions were prepared and subjected to fluorescent-activated cell sorting analysis as described by others (6,8,9) with modifications.

IN VITRO EXPOSURE TO AA, BHT, BHA

4×10^5 viable unenriched epidermal cell suspensions were prepared and plated in 24-well Linbro polystyrene tissue culture plates. To the unenriched epidermal cells, various concentrations of AA, BHT, and BHA were added in complete RPMI 1640 cell culture media supplemented with 1.5 μg/ml of indomethacin. A series of pilot experiments was performed at various concentrations to determine the *in vitro* effects of the specific phenols BHT, BHA, and AA. The maximum biological effects were produced by the following solutions: BHT, 2.5 μg/ml; BHA, 5 μg/ml; and AA, 66 ng/ml and 666 ng/ml; therefore, these concentrations were used throughout the studies. Following 48 hours of incubation at 37°C in 5% CO_2, the cells were harvested, washed in complete 1640 RPMI medium, and incubated with appropriate primary and secondary fluorochrome-conjugated antibodies at 4°C (8,9). Treated and control epidermal cell preparations were then subjected to FACS analysis and the density of Ia expression quantified. To ensure that the cells were Langerhans cells and not keratinocytes which can also express the Ia membrane antigen in allergic contact dermatitis, unenriched epidermal cell suspensions were double stained with the Ia and Fc receptor antigens. The antigens were then conjugated with appropriate secondary antibodies, specifically fluorescein isothiocyanate and streptavidin phycoerythrin red.

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RESULTS

TOPICAL ADMINISTRATION OF MBEH: *IN VIVO* ANALYSIS

Figures 1a and 1b depict the results of experiments in which mice were treated for five days with 20% MBEH or 95% ethanol (diluent) to dorsal pinnal epidermal surfaces. On day 6, the ears were surgically excised and the cells were subjected to fluorescent-activated cell sorting using a FACS 440 (Becton Dickinson, Mountain View, CA). 30,000 events (e.g., cell parameters, of fluorescein intensity and size) were examined. 1.4% of the cells proved to be Ia+ Langerhans cells in the control (diluent) versus 3.3% in the MBEH treatment group (see Figures 1a and 1b). Normal Langerhans cell density, cells/mm^2, makes up approximately 2% of the total epidermal cell density in this particular strain of mice. In addition, the mean fluorescence intensity increased from 69.51% (control) to 72.99% (treatment) (Figures 1a, 1b). These results corroborate our earlier *in vivo* light microscopy experiments in that the numbers of Langerhans cells, i.e., density of cells/mm^2 (1), and the expression of the Ia antigen marker are increased following topical treatment with the common antioxidant MBEH.

IN VITRO FACS ANALYSIS OF BHT, BHA AND AA

Table I shows the results of experiments in which unenriched epidermal cell suspensions were exposed to various concentrations of arachidonic acid alone or with BHA, BHT, and the control diluents. Following 48 hours of incubation, the cell preparations were harvested and subjected to FACS analysis to quantify changes in Ia+ antigenic cell membrane expression. The results demonstrate that there is an apparent biphasic effect following exposure to low or high doses of AA, with or without the antioxidants BHA and BHT. There was a significant decrease ($p \leq 0.05$) (Student t-test) in the quantity of Ia+ expression from unenriched epidermal cell suspensions following exposure to a large dose of arachidonic acid. Further, the addition of indomethacin (IND) to the cell culture wells containing high doses of AA could restore Ia antigenic expression to control values (data not shown). These data corroborate our earlier *in vivo* studies in which a large 1% AA concentration decreased the number of Ia+ Langerhans cells as well as CHS responsiveness, while a more dilute concentration of 0.05% AA could increase both LC density (cells/mm^2) and enhance the CHS or allergic contact dermatitis response in several mice strains (1).

DISCUSSION

It has been reported earlier that the mechanism by which MBEH, BHT, and BHA may alter the Ia antigenic marker expression in epidermal cells is via the arachidonic acid/prostaglandin biochemical pathway (1,10). The quenching of the radical oxygen molecule in this pathway by these common antioxidants irreversibly inhibits the cyclooxygenase enzyme, thereby affecting arachidonic acid metabolism directly and/or indirectly via the oxidation of AA into its products, namely the prostaglandins and leukotrienes. Other investigators have documented that common antioxidants can affect systemic immune reactivity (11,14). However, there has been a paucity of studies regarding the role of topically applied antioxidants on the skin immune system. Earlier studies from our laboratory have demonstrated that short-term (i.e., 5-day daily) topical applications

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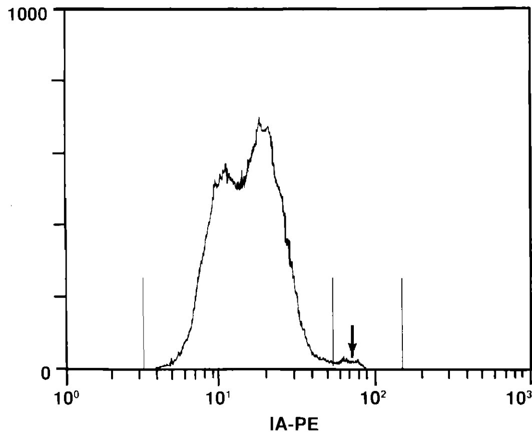
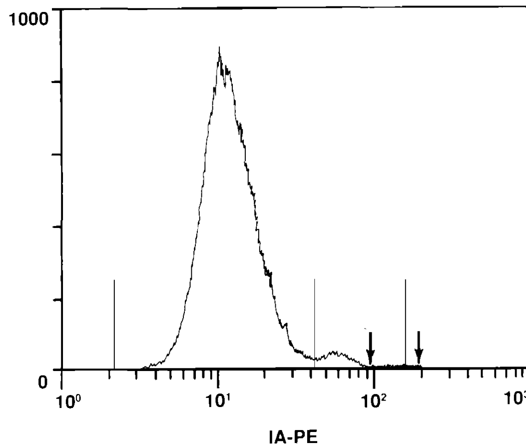


Single Histogram Statistics

Parameter: FL2 *Gated events: 30000* *Total events: 30000*

	Left	Right	Events	% Gated	% Tot	Mean	Mode	Peak	CV
	3.21	52.20	29503	98.3	98.3	16.86	18.15	702	45.1
a	53.63	150.13	427	1.4	1.4	69.51	63.10	31	19.0



Single Histogram Statistics

Parameter: FL2 *Gated events: 30000* *Total events: 30000*

	Left	Right	Events	% Gated	% Tot	Mean	Mode	Peak	CV
	2.19	40.90	28938	96.5	96.5	12.96	10.27	897	43.5
b	42.03	973.27	990	3.3	3.3	72.99	53.63	52	62.0

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Table I
Effect of AA, BHA, and BHT on Mean Phenotypic Ia+ Epidermal Cell Expression: Effects of Low- and High-Dose AA With Antioxidant on Mean Ia+ Expression

Control	% Ia+ Expression	% Ia+ Expression
Diluent (DMSO)* 3.81	Arachidonic acid 66 ng AA 4.72 (+24%)	Arachidonic acid 666 AA ng 1.42 (−63%)
BHA BHA diluent (DMSO) + BHA 5 μg/ml 2.88	AA + BHA 66 ng AA + 5 μg/ml BHA 3.62 (+25%)	AA + BHA 666 ng AA + 5 μg/ml BHA 2.11 (−27%)
BHT BHT diluent (DMSO) + BHA 2.5 μg/ml 3.99	BHT + AA 66 ng AA + 2.5 μg/ml BHT 4.44 (+11%)	BHT + AA 666 ng AA + 2.5 μg/ml BHT 3.00 (−25%)

* DMSO: 100 μl was used as delivery for BHT, BHA, and AA concentrations.
4×10^5 unenriched epidermal cells were plated into 24-well polystyrene plates containing antioxidant-supplemented RPMI-1640 followed by a pulse of either low- or high-dose AA. Following a 48-hour incubation at 37°C, 5% CO_2, the cells were harvested and stained with appropriate primary and secondary fluorochrome-conjugated antibodies and then subjected to FACS analysis. There is an apparent biphasic dose effect on the mean expression of the Ia^+ antigen, suggesting that the dose/concentration of the antioxidant can contribute significantly to altering immune phenotypic marker expression.

of common parasubstituted phenolic antioxidants, namely MBEH, BHT, and BHA, appear to alter the density of identifiable Ia+ Langerhans cells in various strains of mice. Further, these alterations in Ia+ Langerhans cells were dose-related and correlated with either enhancement or suppression of the contact hypersensitivity response in these mice strains (1). In addition, this effect could be totally reproduced by using a biologically relevant molecule, namely arachidonic acid. However, these earlier *in vivo* experiments performed in our laboratory were "gross," using the imprecise mouse ear swelling assay, which lacks the precision and sensitivity needed to validate our earlier functional CHS observations.

The current studies were carried out using a more precise methodology, that of fluorescent-activated cell sorting, to determine whether it could serve as a screening technique to determine which of these ubiquitous antioxidants/preservatives may contribute to

Figure 1. Changes in Langerhans cell density and Ia expression following topical treatment with MBEH: Increase in density (number) and class II MHC product (Ia antigen) of unenriched murine Langerhans cells following five days of *in vivo* topical treatment with diluent (a) or MBEH (b). To identify Ia+ Langerhans cells, dual labeling immunofluorescence was performed using fluorescein isothiocyanate (FITC)-labeled anti-Fc receptor and monoclonal anti-class II MHC streptavidin phycoerythrin red (PE) antibodies. In other words, two cell membrane markers were used to identify Langerhans cells from keratinocytes (i.e., the Ia marker and the Fc marker are found only on Langerhans cells in normal epidermis). The percentage of identifiable Ia+ Langerhans cells increased significantly from 1.4% to 3.3% in fluorescent intensity as shown in the histogram, Figures 1a and 1b, respectively. Arrows indicate positive signal gated regions (i.e., FACS is able to detect fluorochrome marker expression). The mean fluorescent intensity (i.e., measurement/density of Ia marker) also increased from 69.51% (diluent) to 72.99% (treated). X-axis denotes fluorescent intensity; y-axis denotes the number of events.

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exacerbated allergic contact dermatitis via the alteration of Ia+ epidermal immune cells.

In the present study we were able to demonstrate that a short-term application of MBEH could significantly increase the density of Langerhans cells (1.4% to 3.3%) as well as the mean phenotypic expression of the Ia antigenic marker. Several investigators have reported that the increase in Ia+ antigen or analogue in man, HLA-DR, can be directly correlated with an intensified allergic contact dermatitis reaction. Further, our current studies examine more ubiquitous antioxidants, namely BHT and BHA, using an *in vitro* cell culture system. Results from these experiments strengthen our earlier findings that antioxidants can play a role in altering epidermal immune reactivity. Further, this reponse could be reproduced using a biologically relevant molecule, arachidonic acid.

In summary, we have presented the results of experiments which demonstrate the use of FACS as a sensitive and precise tool to measure small yet significant identifiable changes in epidermal immune molecules residing in the epidermis. This technique, along with the use of human epidermal cell suspensions obtained from various sources (i.e., surgical specimens), theoretically provides one with a technique to determine which cosmetics and/or pharmaceutical formulations may alter the epidermal immune homeostasis, thereby potentially leading to exacerbated allergic contact dermatitis both in the home and in the workplace.

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