Effects of Melanin-Induced Free Radicals on the Isolated Rat Peritoneal Mast Cells

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Pheomelanin from human red hair (RHM) produces considerably more cellular damage in Ehrlich ascites carcinoma cells when subjected to radiations of wavelength 320–700 nm than eumelanin from black hair (BHM). Irradiation of RHM generated large amounts of superoxide while BHM did not produce detectable amounts of superoxide. The present investigations describe the effects of irradiation of mast cells in the presence of various natural and synthetic melanins. Irradiation of mast cells in the presence of RHM and red hair melanoprotein released large amounts of histamine while BHM and synthetic melanins prepared from dopa, cysteinyldopa, or a mixture of dopa and cysteinyldopa did not release histamine. The release of histamine at lower concentrations of RHM was not accompanied by the release of ^{51}Cr from chromium-loaded cells, suggesting that this release was of noncytotoxic nature. On the other hand, the release of histamine at higher concentrations of RHM was due to cell lysis since both histamine and cytoplasmic marker ^{51}Cr were released to the same extent. The release evoked by large concentration RHM was not inhibited by superoxide dismutase or catalase. This suggests that the cell lysis under these conditions was not due to H_2O_2 or O_2^-. The finding that mast cells release histamine when irradiated in the presence of RHM suggests that the immediate and late-phase reactions seen in sunburn may in part be due to the release of mediators from these cells.
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The characteristic manifestations of sunburn reactions such as erythema, edema, dermal infiltrate, hyperpigmentation, and peeling off or scaling of the skin are more common in persons with fair skin than in those with darker skin [1]. It is generally believed that this is due to the lesser protection afforded by the melanin in the skin of the former, and that this is probably due to the lower concentrations of melanin in their skin [2]. Clinical evidence indicates that in addition to sunburn reactions, many cutaneous cancers are more prevalent and severe among people with red hair and "Celtic type" skin [3]. The generation of O_2^- during the irradiation of melanin has been reported earlier [4,5]. The comparison of O_2^- generation by eumelanin and pheomelanin during irradiation revealed that the pheomelanin produces larger amounts of O_2^- than does eumelanin [6]. This is interesting since recent studies show that pheomelanin, a pigment in red hair, when irradiated produces free radicals which are capable of inflicting cell injury [7–9]. On the other hand, eumelanin, a constituent pigment of black hair produces considerably less cellular damage [7]. These observations suggest that pheomelanin in the skin may be positively contributing to the inflammatory response in the skin induced by light.

It is known that the shorter-wavelength UV radiations are more erythemogenic than the longer-wavelength light. However, the high UVA irradiance present in sunlight may contribute significantly to the sunburn reactions [10]. The solar erythemogenesis and subsequent delayed reactions seem to be due to mediators released by direct injury to the target cells as well as the cascade of events leading to inflammation [11–13]. The initial reaction may be due to the interaction between cutaneous mast cells and reactive species and/or oxygen-based free radicals generated from melanin during irradiation. The release of histamine from mast cells by H_2O_2 [14] and by myeloperoxidase(MPO)-H_2O_2-halide system [15] has been shown to occur in the isolated cell system. Late-phase reactions, a prolonged inflammatory response subsequent to immediate allergic reaction, has been shown to be propagated by mast cell degranulation [16].

The formation of melanin-free radicals in human skin as a result of exposure to light has been reported by Pathak and Stratton [17]. Although melanin is mainly present in melanocytes located in the epidermal region while mast cells are found in the dermal and subepidermal region, the relatively long-lived reactive intermediates such as H_2O_2 and lipid hydroperoxides formed by the action of free radicals can diffuse to the site of target cells. It should be noted that melanin is also present in a diffuse state as "melanin dust" [18].

In the present paper, the effects of reactive species generated from melanins by exposure to light on isolated rat peritoneal mast cells have been reported.

MATERIALS AND METHODS

Melanins Red hair melanin (RHM), black hair melanin (BHM), and red hair melanoprotein (RHMP) were prepared as described by Menon et al [19]; dopa melanin, cysteinyldopa melanin, and dopa-cysteinyl-dopa melanin were synthesized according to Sealy et al [20].

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Abbreviations:
BHM: black hair melanin
EAC: Ehrlich ascites carcinoma
ESR: electron spin resonance
MPO: myeloperoxidase
RHM: red hair melanin
RHMP: red hair melanoprotein
SOD: superoxide dismutase

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Chemicals Superoxide dismutase (SOD) (bovine liver, sp act 2700 units/mg protein), catalase (bovine liver, sp act 3500 units/mg protein), compound 48/80, and histamine (free base) were purchased from Sigma Chemical Co., St. Louis, Missouri; chromium-51 (Na_2CrO_4) was obtained from Amersham Corporation, Oakville, Ontario, and Ficol 400 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Mast Cells Peritoneal mast cells were isolated from peritoneal washings of male Wistar rats weighing 350–400 g as described by Ranadive and Ruben [21]. Mast cell preparations were about 90% pure and contained less than 3% nonviable cells as determined by trypan blue dye test. The final mast cell suspensions were prepared in Tyrode's solution containing 0.1% gelatin.

Light Source Westinghouse (400 W) mercury vapor lamp (H33C) was used for irradiation. The specifications have been described earlier [22].

Chromium Labeling of Cells Labeling of mast cells with ^{51}Cr was done as described previously [23]. The chromium released was determined by γ counting in LKB Compu-gamma counter. The amount of chromium released is expressed as the percentage of the total released by lysing the cells by freezing and thawing.

Histamine Assay Histamine in the supernatants after removal of the cells was determined using Shore's fluorometric method as described in the *Manual of Clinical Immunology* [24] and is expressed as percentage of the total histamine in the cells. Total histamine in a given number of cells was obtained by placing the cell suspensions in a boiling waterbath for 2 min.

Irradiation of Mast Cells Mast cells (2×10^5) were irradiated with various melanins at various concentrations in a final volume of 1 ml Tyrode's solution. Cell suspensions were taken in 10-ml beakers and were placed in a metabolic shaker bath at 21°C. The samples were kept centrally under the lamp at a distance of 14 cm for the outer envelope of the lamp. For each set of irradiated samples, a similar set was kept in the dark as a control. All the experiments were done with triplicate samples. The solutions of SOD, catalase, and compound 48/80 were prepared in saline and used in 0.1-ml volume to give the final desired concentration in 1 ml.

RESULTS

Rat peritoneal mast cells irradiated in the presence of RHM released histamine. This histamine release was dependent on the concentration of the melanin added to the cell suspension (Fig 1). When the cells were incubated in the dark in the presence of low concentrations of RHM, there was no release of histamine. At high concentration of RHM (100 μg/ml) a slight increase in spontaneous histamine release was observed. Mast cells either incubated in the dark or irradiated in the presence of BHM did not release appreciable amounts of histamine.

In Fig 2 is shown the relationship between the period of irradiation and release of histamine. The release of histamine was found to be related to the dose of radiation and the maximum release by 100 μg/ml of RHM occurred in 60 min.

Red hair melanin is known to contain largely pheomelanin derived from cysteinyldopa, while eumelanin is derived from dopa. An attempt was made to see whether the synthetic melanins prepared from dopa, cysteinyldopa, or a mixture of dopa and cysteinyldopa would induce the release of histamine from mast cells on irradiation. The data in Table I show that none of these synthetic melanins induced release of histamine from the mast cells even at concentrations as high as 200 μg/ml. Since melanins in the natural state are associated with proteins, the effects of red hair melanin-protein complex (RHMP) were investigated. It was found that the release of histamine by irradiation in the presence of RHMP was comparable to that seen with RHM.

We have shown earlier that Ehrlich ascites carcinoma (EAC) cells, when irradiated in the presence of RHM, undergo cell lysis

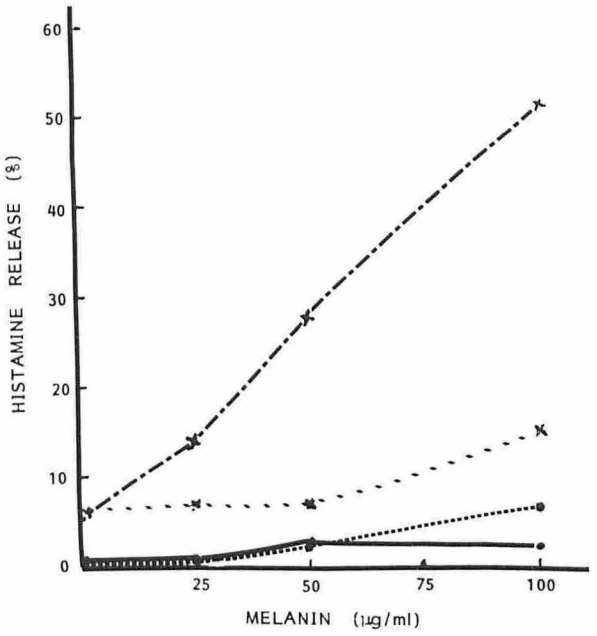

Figure 1. Release of histamine from mast cells irradiated in the presence of various concentrations of melanins for 60 min at room temperature. ●------●, RHM in dark; ×——×, RHM in light; ●——●, BHM in dark; ×- - - -×, BHM in light.

and this lysis is probably due to the free radicals generated during irradiation [7–9]. It is also shown that O_2^-, H_2O_2, and H_2O_2-MPO-halide system induce release of histamine from mast cells both thorugh cytotropic and cytotoxic reactions [14,15]. Experiments were therefore designed to study the mode of histamine release from mast cells during irradiation in the presence of RHM. Mast cells labeled with ^{51}Cr were irradiated in the presence of various concentrations of RHM, and release of ^{51}Cr as well as histamine was determined. It will be seen from Fig 3 that at lower concentrations of RHM, the release of histamine was not accompanied by the release of ^{51}Cr; however at higher concentrations of RHM, histamine release was comparable to the ^{51}Cr release. The bars in Fig 3 show the release of histamine and ^{51}Cr from the same mast cells by 1.0 μg/ml of compound 48/80, an agent known to induce secretory release of histamine.

The data in Table II show that the release of histamine induced by RHM and irradiation was not inhibited by the addition of

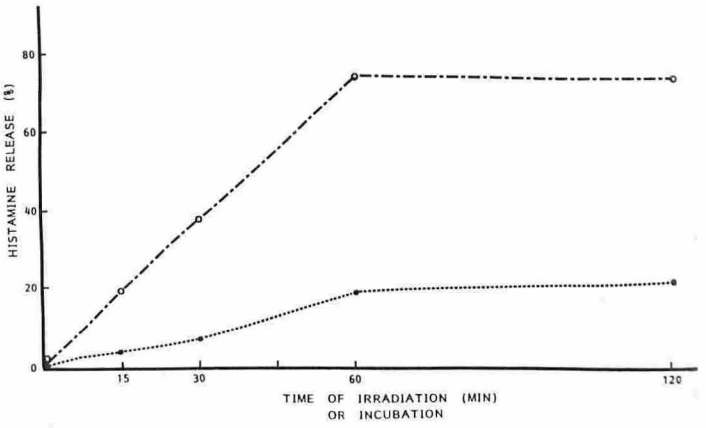

Figure 2. Release of histamine from mast cells by 100 μg/ml of RHM as a function of period of irradiation at room temperature. ●-----●, Cells in dark; ○——○, cells in light.

Table I. Release of Histamine from Mast Cells by Synthetic Melanins, RHM, and RHMP

Melanin Added (μg/ml)[a]	Histamine Release (%) In Dark	In Light
None	7	8
Cysteinyldopa (25)	7	8
Cysteinyldopa (50)	8	8
Cysteinyldopa (100)	11	9
Cysteinyldopa (200)	14	10
Dopa-cysteinyldopa (25)	12	13
Dopa-cysteinyldopa (50)	13	13
Dopa-cysteinyldopa (100)	14	14
Dopa-cysteinyldopa (200)	18	12
Dopa (200)	10	10
RHM (50)	20	62
RHMP (50)	18	50

[a]Concentration of melanins added to 2×10^5 mast cells.

SOD. In fact SOD slightly enhanced the release. The presence of catalase during irradiation either with or without SOD had no effect on histamine release.

DISCUSSION

Mast cells are known to participate in various cutaneous phototoxic reactions [25–27]. It is also known that melanin, an important pigment in the skin, can act as a biologic electron exchange polymer [28,29]. A number of studies have shown that melanins can generate free radicals on exposure to UV or visible radiations

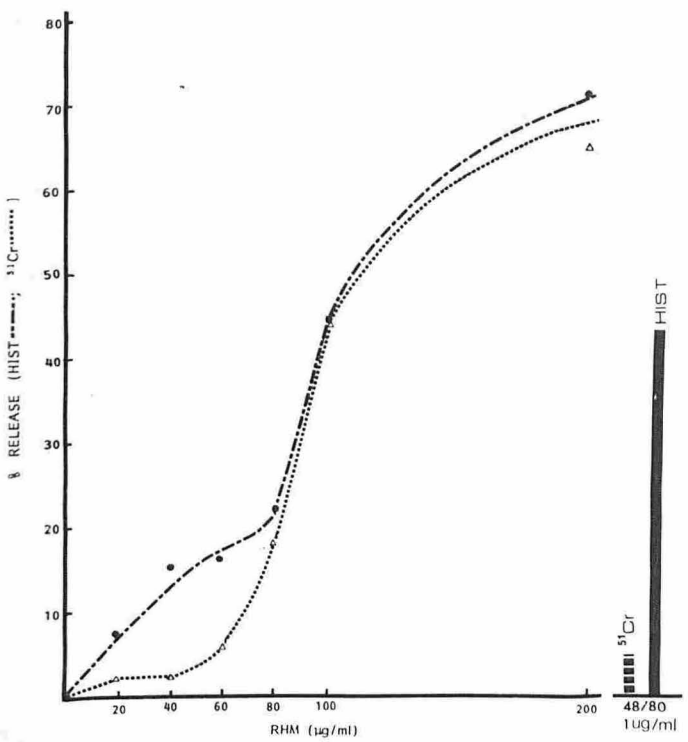

Figure 3. Comparison of histamine release and ^{51}Cr release from chromium-loaded mast cells by various concentrations of RHM in the presence of light for 60 min at room temperature. △------△, ^{51}Cr release; ●——●, histamine release. *Bars on right* show ^{51}Cr release and histamine release by 1 μg/ml compound 48/80 in 10 min at room temperature from the same cell preparation.

Table II. Effect of SOD and Catalase on the Release of Histamine and ^{51}Cr from Chromium-Loaded Mast Cells

	^{51}Cr Released (%) In Dark	In Light	Histamine Release (%) In Dark	In Light
Mast cells (2×10^5)	6	8	8	10
Mast cells + RHM[a]	12	43	19	43
Mast cells + RHM + SOD[b]	12	50	20	46
Mast cells + RHM + Catalase[c]	13	42	17	36
Mast cells + RHM + SOD + Catalase	12	44	18	40

[a]RHM (100 μg/ml).
[b]SOD (165 units/ml).
[c]Catalase (3500 units/ml).

[30,31]. Recent electron spin resonance (ESR) spectroscopy studies by Sealy et al [20,32] on BHM and RHM suggest that RHM contains a specific kind of free radical(s) which is not present in BHM. The generation of melanin-free radicals, O_2^-, and subsequent formation of other reactive oxygen species and their cytotoxic properties have been reported from our laboratories [7–9]. Mast cells are shown to be activated by H_2O_2 and H_2O_2-MPO-halide system [14,15] as well as by irradiation of the cells in the presence of protoporphyrin [33,34]. In the present studies it was found that irradiation of mast cells alone or in the presence of BHM had no significant effect on the release of histamine. On the other hand the cells that were irradiated in the presence of RHM released histamine which was related to the period of irradiation as well as the concentration of RHM. Red hair melanoprotein also exhibited the ability to release histamine from mast cells on irradiation. However, synthetic dopa melanin, cysteinyldopa melanin, and dopa-cysteinyldopa melanin did not activate mast cells for histamine release. These results are interesting in view of the ESR studies on melanins which show that synthetic pheomelanin (cysteinyldopa-melanin) does exhibit the ESR signal similar to that seen with RHM [20,32]. It is possible that the active entity related to the release of histamine from mast cells may be different from the one detected by the ESR studies or, alternatively, the photobiologic properties of the free radicals responsible for cell lysis might be modified by the physical state, such as the water of hydration, aggregation, etc. In fact it has been observed that free-radical properties of melanin are altered by the drying treatment, indicating that the hydrated layer of melanin granules influences its free-radical properties [31,35].

The comparison of the release of histamine from mast cells with the release of ^{51}Cr from chromium-loaded cells suggests that at lower concentrations of RHM the release is of a noncytotoxic nature while at higher concentrations cell lysis occurs. The mechanism by which the noncytotoxic release of histamine is triggered is still to be investigated.

Since the release of histamine was not inhibited by SOD, catalase, or SOD plus catalase, it may be suggested that although O_2^- and H_2O_2 are generated during the irradiation of RHM, the release seen under the present experimental conditions was due to the reactive species other than O_2^- or H_2O_2. The reaction sequence involved in formation of the reactive species during irradiation of melanin has been discussed elsewhere [9].

A few implications of these in vitro studies in cutaneous phototoxicity are worth noting. First, most of our in vitro studies presented here were carried out using protein-free melanin. Previously it has been reported that the oxidation of NADH by synthetic dopa melanin was inhibited by the addition of bovine serum albumin [36]. This oxidative reaction could not be detected when melanoprotein isolated from B16 melanoma was used. When proteins were removed from this preparation, the resulting melanin showed NADH oxidizing activity similar to that of synthetic melanin. Therefore it was important to investigate whether the

results using protein-free melanin with isolated cell systems would be applicable to the in vivo conditions. The present studies showed that melanoprotein produced histamine release very similar to that brought about by the protein-free melanin. Second, in the skin, melanin is mostly present within the melanocytes and keratinocytes, while in in vitro experiments melanin was added to the cell suspension. We have not yet examined whether the melanin penetrates into the mast cells. However electron microscopic studies have shown that under similar conditions melanin penetrated EAC [37]. Morphologic studies have shown occasional presence of melanin granules in the mast cells, although it is disputable whether this represents the phagocytosis of melanin granules by these cells or a histogenetic relationship between mast cells and melanocytes [38]. It should be noted that although melanin is present in the skin mostly within the melanosomes, it is also present in a more diffused state as melanin dust [18]. The question of the location of melanin within the melanosomes or as melanin dust with respect to the target cell(s) involved in the in vivo effects of UV radiation is difficult to answer directly. However our in vitro studies lend themselves to some relevant considerations. Irradiation of melanin, especially in the presence of naturally occurring sulfhydryl compounds such as cysteine, produces H_2O_2 which is relatively stable and could diffuse across a number of cell layers [9]. The irradiation of melanin in the presence of free fatty acid has been shown to produce lipid peroxidation [39,40]. The peroxidation of cellular lipid components by irradiation of cells in the presence of melanin has also been reported [40,41]. Some of the intermediates in lipid peroxidation such as hydroperoxides are fairly stable [42]. Thus, although the reactive free radicals in melanin produced by irradiation are short-lived, it is conceivable that these free radicals could react with several biomolecules producing reactive compounds which are relatively long-lived and could permeate to other sites in the skin.

The studies reported here clearly show that the RHM on exposure to UV visible radiation produces reactive species which can induce release of histamine by both cytotoxic and cytotropic mechanisms, depending on the concentration of melanin. The mast cells in cutaneous tissue represent an effector cell capable of elaborating the essential mediators of inflammation [43]. The fact that the exposure of RHM to UV visible radiation generates active compounds suggests the possibility that sunburn reactions more frequently observed in Celtic skin may be related to the type of melanin present in the skin.

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