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IMMUNOMODULATING ACTIVITIES OF BRAZILIN AND HEMATOXYLIN IN NORMAL YOUNG MICE

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ABSTRACT: *Experiments were undertaken to evaluate the effects of brazilin and hematoxylin on immune functions in normal young mice. Brazilin and hematoxylin decreased the circulating leucocyte counts. Both compounds did not produce a significant change of immunoorgan weights in comparison with those in the control group. Brazilin decreased significantly IgM plaque forming cells. Brazilin and hematoxylin decreased significantly Arthus reaction at 3 hours-post challenge with respect to aggregated bovine serum albumin. Brazilin augmented delayed-hypersensitivity at 24 hours-post challenge while no effect was observed in the case of hematoxylin. Phagocytic activity was increased slightly by treating brazilin. These results suggest that brazilin and hematoxylin may affect various types of immune function.*

Keywords: *Brazilin, Hematoxylin, IgM plaque forming cell, Delayed-hypersensitivity, Arthus reaction, Carbon clearance, Phagocytic activity.*

INTRODUCTION

Brazilin and hematoxylin, an active principle of *Caesalpinia sappan* and *Hematoxylin campenchianum*, respectively, have been previously reported to possess widespread biological activities including antimicrobial (Sanches-Maroquin *et al.*, 1958; Goncalves de Lima *et al.*, 1961), antiinflammatory (Hikino *et al.*, 1977), anticarcinogenic (Aizenman *et al.*, 1961; Rogers, 1970), epinephrine sparing (Gabor *et al.*, 1952b), and hypoglycemic (Moon and Lee, unpublished) properties. They have also been shown to inhibit several enzyme activities such as histidine decarboxylase (Gabor *et al.*, 1952d), lens aldose reductase (Moon *et al.*, 1985), and cyclic nucleotide phosphodiesterases (Nikaido *et al.*, 1981). In addition, specific effects of brazilin and hematoxylin on blood cells have been demonstrated, *e.g.* they inhibit collagen- and ADP-induced platelet aggregation *in vivo* as well as *in vitro* (Moon and Hwang, unpublished) and improve the erythrocyte deformability (Moon *et al.*, 1988).

Both substances have been also demonstrated to have antioxidant properties; they inhibited lipid peroxidation in various tissues of animals (Moon *et al.*, 1984; Moon *et al.*, 1987) and protect hepatotoxicity induced by CCl_4 (Moon and Ahn, unpublished). Another reports suggested that antioxidants may influence a variety of immune responses, in part, by modulating reactions of cyclooxygenase and lipoxygenase pathways (Ball *et al.*, 1986). In view of the lack of information about the effects on immune functions, it was considered of interest to study whether the immune functions might be modulated by brazilin and hematoxylin. Therefore, this study attempted to examine the immunomodulating effects of brazilin and hematoxylin in normal young mice.

MATERIALS AND METHODS

Animals

Male Balb/c, C57BL/6 inbred and ICR outbred mice supplied from Laboratory Animal Center of Seoul National University, 6-8 weeks of age, weighing 18-22 gm, were used. Animals were mechanically maintained on a diurnal cycle of 12 hours-interval. The room temperature was at 20-24°C and relative humidity at 50-60%. Laboratory rodent chow (Sam Yang Co, Korea) and tap water were provided *ad libitum*.

Chemicals

Brazilin monohydrate was purchased from Fluka (Swiss) and hematoxylin from Sigma (St. Louis, MO, USA). Brazilin and hematoxylin were administered intraperitoneally for ten consecutive days at the doses of 10 mg/kg and 100 mg/kg. All treatments and samplings were made at 9:00-11:00 a.m., taking into consideration of the chronobiological aspects of immunological status (Fernandes *et al.*, 1977).

Immnopathology

Blood samples were collected via retroorbital plexus with heparinized capillary and the number of leucocytes were determined on hemacytometer. Body, spleen, thymus and liver weights were measured. Plasma corticosterone was determined by the method of Silber *et al.* (1954).

IgM Plaque Forming Cell (PFC) Assay

Splenic IgM PFCs SRBC were quantified at 4th day followings a single intraperitoneal injection with 8×10^7 SRBC by the method of Cunningham and Szenberg (1968). Reaction mixture was composed of spleen cell suspension (50 μl), 12.5% SRBC (100 μl), 1/5 diluted complement (100 μl) and balanced salt solution (250 μl), and pipetted into Cunningham chamber.

Delayed-Hypersensitivity and Arthus Reaction

Mice were sensitized with 100 μg bovine serum albumin (BSA; Sigma) emulsified in complete Freund's adjuvant (Sigma) subcutaneously at the base of tail. Seven days later delayed hypersensitivity and arthus reaction were elicited by challenging mice in the footpad with 30 μl of 2% heat aggregated BSA in saline, as described by Titus and Chiller (1981). At 3 and 24 hours-post challenge, footpad swelling thickness was measured with micrometer (Mitsutoyo, Japan) and the extent of swelling was calculated by

substracting the thickness of the negative footpad from that of the antigen injected footpad.

In Vivo Phagocytosis

Intravascular phagocytosis by hepatic kupffer cell and splenic macrophage were determined by the method of Biozzi *et al.* (1954). Colloidal carbon (Pelikan drawing ink, D-3000. Hannover 1, 17 Black, Germany) suspended in 1% gelatin-saline was injected intravenously at a dose of 16 mg/100 gm of body weights.

Statistical Analysis

Student's *t*-test was employed to assess the statistical significance. Values which differ from control over $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

In order to evaluate the effects of brazilin and hematoxylin on immune functions, numbers of the circulating leukocytes and weights of immunoorgans were investigated first. As shown in Table 1, circulating leucocyte count in control group was about 11,000 and this result was consistent with the previous reports (Mitruka and Rawnsley, 1981). Leucocyte counts were significantly decreased by treatments of brazilin (100 mg/kg) and hematoxylin (100 mg/kg). Reduction in leucocyte counts was much pronounced in brazilin trated group compared with hematoxylin treated group. When treated with low dose of brazilin, there were no significant differences in leucocyte counts compared with the treatment of high dose of brazilin. The precise nature of these leucocytopenias is not yet clear. But, brazilin and hematoxylin were reported to have inhibitory effects on the growth of rapidly dividing cells such as bacteria, viruses and tumors (Aizenman *et al.*, 1961; Sanches-Maroquin *et al.*, 1958; Goncalves de Lima *et al.*, 1961; Rogers, 1970; Au and Hsu, 1982) and according to our preliminary study, brazilin strongly inhibited ^{3}H-thymidine incorporation in bone marrow cells (EC_{50}; about 10^{-5} M). Most chemotherapeutic agents that are cytotoxic, alter the bone marrow cell functions and subsequently bring about leucopenia (Perry and Yar-

Table 1. Effects of brazilin and hematoxylin on the circulating leucocytes and immunoorgans (spleen, thymus and liver) in normal young male C57BL/6 inbred mice.

Group	N	Circulating leucocyte/μl	Spleen/ body(10^{-3})	Thymus/ body (10^{-3})	Liver/ body (10^{-2})
Control	7	11,850 ± 375	4.19 ± 0.22	1.77 ± 0.38	4.39 ± 0.10
Brazilin (10mg/kg)	7	8,625 ± 263[a]	3.80 ± 0.17	1.85 ± 0.22	4.24 ± 0.12
Brazilin (100mg/kg)	7	8,025 ± 255[b]	4.10 ± 0.34	1.99 ± 0.17	4.61 ± 0.15
Hematoxylin (100mg/kg)	7	10,650 ± 315[c]	5.00 ± 0.30	1.68 ± 0.26	4.26 ± 0.25

Brazilin and hematoxylin were administered intraperitoneally for ten consecutive days at doses of 10 mg/kg and 100 mg/kg.

Each value represents mean ± SE; $p < 0.01$; control vs a,b and d vs a,b; $p < 0.05$; control vs c

bro, 1984). From this reason, it could be suggested that cytotoxic activities of brazilin and hematoxylin might contribute to leucocytopenia. In this point, it should be noted that lymphocytes have still high percentage of circulating leucocytes in rodents compared with those in other species (above 70%; Mitruka and Rawnsley, 1981). Therefore, it could be inferred that brazilin-induced leucocytopenia might result from lymphocytopenia and in turn, this would affect the specific immune responses. Immunoorgan weights were not altered by treatments of brazilin and hematoxylin.

The numbers of IgM plaque forming cells (PFC) were significantly reduced by treatment of brazilin but this result was not observed in the case of hematoxylin. Regardless of treatment, splenic cellularities were statistically equal among groups. Although what caused the inhibition of IgM PFCs is not yet elucidated, direct cytotoxicity seems not to be responsible, since splenic celluralities were not changed. Another explanation could be due to the intraperitoneal co-administration of brazilin and sheep red blood cells (SRBC). Intraperitoneal injection of 10 mg/ml and 1 mg/ml of brazilin produces about 32 mM and 3.2 mM in the peritonium of mouse, respectively. It can not be excluded that these concentrations of brazilin might induce hemolysis of SRBC, alter immunogenecity of SRBC, or inhibit activation of peritoneal macrophages.

As shown in Table 3, brazilin significantly lowered arthus reaction indices by more than 90%. Hematoxylin also significantly decreased the reaction by 40%. Brazilin and hematoxylin are of high potent nonsteroidal antiinflammatory activities, and brazilin is still more potent than hematoxylin (Gabor, 1952a; Gabor, 1952c; Hikino *et al.*, 1977). The decrease in arthus reaction which is true immunologically-mediated inflammation, might result from the inhibitory effects of brazilin and hematoxylin on acute inflammation, and differences in reduction of Arthus reaction between both substances might be explained by the fact that brazilin has more potent antiproliferative action than hematoxylin. (Seng *et al.*, 1987; Hikino *et al.*, 1977).

Delayed hypersensitivity (DH) reaction indices were greatly increased by the treatment of brazilin. High dose of brazilin (100 mg/kg) showed larger increment in DH reaction than the low dose (10 mg/kg). Hematoxylin did not altered DH-index.

Considering previous results, it suggested that helper T cell was not affected by treatment of brazilin. Suppression of IgM PFC and potentiation of DH reaction might result from the inhibition of B cell and/or suppressor T cell in the early antigen specific stages of the immune response, since brazilin was given 8 days before the injection of

Table 2. Effects of brazilin and hematoxylin on the IgM PFC in normal young male Balb/C inbred mice.

Group	N	Lymphocyte (10^7) m*l* spleen suspension	PFC (IgM) 10^6 spleen cells	PFC (IgM)/ spleen
Control	5	2.08 ± 0.18	706 ± 80	117,438 ± 13,791
Brazilin (10 mg/kg)	5	2.20 ± 0.13	412 ± 24[a]	72,513 ± 4,161
Brazilin (100 mg/kg)	5	2.23 ± 0.11	328 ± 57[c]	58,149 ± 9,285
Hematoxylin (100mg/kg)	5	2.08 ± 0.38	672 ± 95	101,660 ± 18,454

Brazilin and hematoxylin were administered intraperitoneally for ten consecutive days at doses of 10 mg/kg and 100 mg/kg.
Each value represents mean ± SE; $p < 0.01$; control vs a,b; $p < 0.05$; a vs b

Table 3. Effects of brazilin and hematoxylin on the Arthus reaction (AH) and delayed-type hypersensitivity (DH) in normal young male ICR mice

Group	N	Footpad thickness (10^{-1}mm) AH (3 hr)	DH (24 hr)
Negative Control	5	10.58 ± 0.23	3.06 ± 0.20
Control	10	16.63 ± 0.71	6.95 ± 0.30
Brazilin (10 mg/kg)	10	10.78 ± 0.27[a]	9.67 ± 0.43[d]
Brazilin (100 mg/kg)	10	11.16 ± 0.26[b]	12.12 ± 0.88[e]
Hematoxylin (100 mg/kg)	10	14.16 ± 0.35[c]	7.40 ± 0.78

Brazilin and hematoxylin were administered intraperitoneally for ten consecutive days at doses of 10 mg/kg and 100 mg/kg.
Each value represents mean ± SE; $p<0.01$; control vs a,b,d,e; $p<0.05$; control vs c and d vs e

SRBC.

The effects of brazilin on nonspecific phagocytic function of splenic macrophages and kupffer cells were presented in Table 4. Brazilin increased the phagocytic index as well as corrected phagocytic index.

Plasma corticosterone was assayed in order to evaluate the microenvironmental influences on the immunological data (Riley, 1981). Plasma corticosterone level was 14.21 ± 0.48 ug/d*l*, which indicated that mice were not stressed under our experimental conditions.

In the light of these results, special attention should be given to the fact that brazilin shows the considerable immunomodulating activity, while hematoxylin possesses a little. A similar tendency was already reported by Hikino *et al.* (1977); that is, brazilin was highly potent in the treatment of chronic inflammation but hematoxylin was not in the case. Seng *et al.* (1987) reported that inhibitory effect of indomethacin on lymphocyte proliferation occurred at doses that were more closely associated with the inhibitory effect on chronic inflammation. As described above, circulating leucocyte counts were more profoundly decreased by treatment of brazilin than by that of hematoxylin, and brazilin has more potent antiproliferative activity than hematoxylin (Aizenman *et al.*, 1961; Rogers, 1970; Au and Hsu, 1979). Based on these facts, the an-

Table 4. Effects of brazilin on colloidal carbon clearance as macrophage function in normal male young ICR mice.

Group	N	Phagocytic Index (10^{-3})[a]	Corrected Phagocytic[a] Index
Control	6	14.93 ± 0.54	3.59 ± 0.05
Brazilin (10 mg/kg)	6	17.64 ± 0.75[b]	3.88 ± 0.10[d]
Brazilin (100 mg/kg)	5	16.91 ± 0.64[c]	3.80 ± 0.10[e]

Brazilin was administered intraperitoneally for ten consecutive days at doses 10 mg mg/kg and 100 mg/kg.
Each value represents mean ± SE; $p<0.05$; control vs b,c and control vs d,e

tiproliferative action of brazilin might contribute to its immunomodulating effects as well as to chronic antiinflammatory effects.

It is well known that antioxidants influence the various immune function (Fidelus, 1988; Ball *et al.*, 1986) and prostaglandins have been regarded as one of the endogenous immunoregulator (Grinwich and Plescia, 1977; Jordan and Simmons, 1987; Ceuppens and Goodwin, 1984). It is, in particular, noteworthy that brazilin and hematoxylin are potent antioxidnats (Lea, 1944; Yuichiro and Fumiko, 1979; Moon *et al.*, 1984; Moon *et al.*, 1987) and inhibitors of prostaglandin synthesis (Hikino *et al.*, 1977; Moon, unpublished). Therefore, it is easily deducible that, these agents directly and/or indirectly affected the immune status in normal young mice, and they may have still more profound effects in the experimental conditions where lymphocytes are highly sensitive to prostaglandins and antioxidants, such as tumor, autoimmune and aging state (Fenichel and Chirigos, 1984; Ceuppens and Goodwin, 1984; Goodwin and Messner, 1979; Grinwich and Plescia, 1977). From these points of view, further studies on the immunomodulating activities under various conditions such as aged, immunosuppressed, tumor bearing and diabetic mice are worth while.

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