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IMMUNOSUPPRESSIVE EFFECTS OF SAFROLE IN BALB/c MICE

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Abstract: *The immunosuppressive effects of safrole were studied in female BALB/c mouse. Mice were given 100, 200 and 400 mg safrole/kg daily for 14 days and evaluated on day 15. The day 4 immunoglobulin-M antibody response to T-dependent antigen, sheep red blood cells (SRBC) was inhibited dose-dependently in all doses studied. In vitro antibody response to polyclonal antigen, lipopolysaccharide (LPS) by spleen cell suspensions from safrole-treated mice were also significantly inhibited. When safrole was treated for 14 days to mice, and mitogen-induced proliferation of splenocytes were assayed on day 15, there were significant suppression of responses to B-cell mitogen, LPS and T-cell mitogen concanavalin A (Con A) at a dose of 400 mg safrole/kg. Direct addition of safrole on the splenocyte culture also produced a dose dependent suppression of in vitro antibody response to LPS, and mitogen-induced lymphoproliferation at doses of 100, 200, 400 and 800 μM safrole. The role of metabolic activation in safrole-induced suppression of in vitro antibody response was studied using splenocyte-hepatocyte coculture system. The suppression of in vitro antibody respose to LPS by safrole was not altered when safrole were incubated in the splenocyte-hepatocyte coculture system for 4 hr as compared with direct addition of safrole in splenocytes culture. Neither the addition of salicylamide, sulfotransferase inhibitor, nor the addition of inorganic sulfate, sulfation cofactor to the splenocyte-hepatocyte coculture, altered the suppression of antibody response by safrole. These results suggest*

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that the immunosuppression by safrole may not be produced by the reactive metabolites which are mediated in carcinogenesis of safrole.

Key words: *Immunosuppression, Safrole, Antibody respose, Mitogen-induced proliferation, Reactive intermediate.*

INTRODUCTION

Safrole (1-allyl-3,4-methylenedioxybenzene) is a naturally occuring plant oil which is mutagenic and carcinogenic (Long *et al.,* 1963; Borchert *et al.,* 1973). It is contained in plant oils as a major component of sassafras and minor component of several other essential oils, herbs, and spicess such as anise, basil, nutmeg, mace and pepper (Hickey, 1948; Guenther and Althausen, 1949; Jacobs, 1958). Although safrole is no longer used as a food additive, it is ingested in small amounts by humans since it is contaminated in certain spices and plant derivatives.

Safrole need metabolic activation to exert their mutagenic and carcinogenic activity. Safrole is metabolized by drug metabolizing enzymes cytochrome P-450 to 1-hydroxy-safrole (Epstein *et al.,* 1970; Swanson *et al.,* 1975). And then 1'-hydroxysafrole could be conjugated with sulfate by a cytoplasmic sulfotransferase to 1'-sulfooxysafrole, the ultimate form of metabolite which binds covalently to DNA, and causes hepatocarcinogenesis (Boberg *et al.,* 1983; 1986). These are supported by the observations that the tumor incidence caused by safrole parallels sulfotransferase activity in the experimental animals (Bock and Schirmer, 1987).

Recently, the effects of chemical carcinogens on immune system has brought an increasing attention. It is because many human chemical carcinogens are known to be immunosuppressive (Dean *et al.,* 1982: Luster *et al.,* 1987) and immunosuppression is closely related to incidence of tumor formation by carcinogens (Kalland and Forsbery, 1981). However, the effects of safrole on immune system have not been reported yet. This study was, therefore, undertaken to examine the effects of safrole on the immune system. The immunosuppressive effects of safrole were studied *in vivo* and *in vitro*. Antibody responses to sheep red blood cells (SRBC) and lipopolysaccharide (LPS) along with mitogen-induced proliferative responses to LPS and concanavalin A (Con A) were used as the immunological parameters.

And to characterize the role of metabolism in safrole-induced immunosuppression, hepatocyte-splenocyte coculture system was used. In our previous studies, we demonstrated that this coculture system can be used succesfully to characterize the immunosuppressive effects of procarcinogens, such as cyclophosphamide, aflatoxin B1, dimethylnitrosamine, and 2-acetylaminofluorene (Yang *et al.,* 1986; Kim *et al.,* 1989a, 1989b).

This study reports that safrole produce immunosuppression in BALB/c mice. Safrole produce suppression of antibody responses and mitogen-induced lymphoproliferation *in vivo*. It also produce immunosuppression in *in vitro* spleen cell cultures. This study suggests that safrole-induced immunosuppression might not be produced by reactive metabolites mediated in carcinogenesis.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 6-8 wk of age were used for immunological studies *in vivo* and *in vitro*. Mice of 12-16 wk age (25-30 g) were used for isolation of mouse hepatocytes. All animals were housed in plastic cages and maintained on Purina Lab Chow (Purina Korea) and tabwater *ad libitum*. The animal room was maintained at 21-24℃ and 40-60% relative humidity. A 12 hr light/12 hr dark cycle was used.

Safrole Exposure and Spleen Cell Culture

Safrole was purchased from Sigma chemical Co. (St. Louis, MO) and prepared in corn oil at the desired concentrations. Safrole was administered daily by oral intubation for a period of 14 days with a dose volume of 10 μl/g body weight. 24 hr after the final injection of safrole (Day 15), mice were immunized with SRBC or sacrificed for the assay of mitogen-induced proliferation of splenocytes. To study direct effect of safrole on splenocyte cultures, safrole was also diluted in acetone and added directly to spleen cell cultures.

In Vivo Antibody Responses

Mice were exposed to safrole for 14 days. On day 15, mice were immunized with 5×10^8 SRBC i.p. (0.2 m*l*). SRBC were purchased from Korea Med Co. (Seoul, Korea), stored at 50% concentration in Alsever's solution. SRBC were washed in balanced salt solution (BSS), counted and resuspended for immunization. Mice were sacrificed on day 19, the spleens dissociated into single cell suspensions and immunoglobulin M-secreting antibody forming cells (AFC) were enumerated using the modified Jerne-Nordin plaque assay (Bullock and Moller, 1972) as described previously (Holsapple *et al.*, 1984). The results were calculated as AFC/million recovered cells. Viabilities were measured in all experiments by the pronase method as previously described (Holsapple *et al.*, 1984).

In Vitro Antibody Responses

Mice were treated with safrole or vehicle for 14 days. On day 15, the animals were sacrificed and the spleens removed aseptically. Single cell suspensions of splenic lymphocytes were obtained by pressing with a sterile syringe plunger in a 35 mm petri dish (Falcon) and suspending the cells in RPMI 1640 (GIBCO). The cells were collected into a centrifuge tube, centrifuged, and resuspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/m*l* of penicillin, 100 μg/m*l* of streptomycin, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 5×10^{-5} M of 2-mercaptoethanol. The cell numbers were counted and adjusted to 5×10^6 cells/m*l*. The cultures were set up in quadriplicate (1.0 m*l*) in individual wells of 24-well culture plate (Falcon) and lipopolysaccharide (LPS: 100 μg/m*l*: from *Salmonella typhosa* 0901, Difco) was added. The cultures were incubated at 37℃, 5% CO_2 incubator. The polyclonal antibody response to LPS was measured on Day 2. The AFC were enumerated using the modified Jerne-Nordin plaque assay. To enumerate AFC to LPS, the

indicator SRBC was densely coupled with trinitrophenyl following the method of Rittenberg and Pratt (1969).

The direct effect of safrole on the *in vitro* antibody response to LPS was assessed by adding concentrations of safrole from 100 to 800 μM to splenic suspensions from untreated BALB/c mice. Acetone served as a vehicle control. The culture conditions and assay procedures were as described above.

Mitogen-Induced Proliferative Responses

Mice were sacrificd 24 hr after final administration of safrole (on Day 15) and spleens were removed. The mitogenic response of splenocytes to B-cell mitogen, LPS and T-cell mitogen, Con A were assayed as described by Yang *et al.* (1986) with the following modifications. Single cell suspension of spleen cells were obtained in RPMI 1640 culture medium supplemented with 5% heat-inactivated human AB serum (Flow Lab) and were assayed in quadriplicate cultures (0.1 m*l*, 2×10^5 cells/culture well) for each treatment group in 96-well microtiter plates for their responses to LPS (100 μg/m*l*). Cultures were harvested on Day 3 after an 18 hr pulse with 1 μCi of ^{3}H-thymidine (Amersham, Buckinghamshire, England) onto glass fiber filters with an automated multiple sample harvester (Flow Lab). The filters were then dried, placed in scintillation vials with scintillation fluid, and counted in a liquid scintillation counter.

Direct Effect of Safrole on the Mitogen-Induced Proliferative Responses.

The direct effect of safrole on the mitogen-induced proliferative responses was assessed by adding concentrations of safrole from 100 to 800 μM to splenic suspensions from untreated BALB/c mice. Acetone served as a vehicle control. The culture conditions and assay procedures were as described in "Mitogen-Induced Proliferative Responses".

Coculture of Primary Mouse Hepatocytes and Splenocytes.

The cocultures of hepatocytes and spleen cells from BALB/c mice was established as described previously (Kim *et al.* 1987). Briefly, hepatocytes were isolated by collagenase perfusion and a monolayer culture was established in the wells of 6-well culture plate (Costar) during 20-hr incubation. After the 20 hr incubation, the medium was aspirated off and the cells washed twice with RPMI 1640. Spleen cells (1 m*l* of 3×10^7 cell/m*l*) from untreated mice were added to the hepatocyte wells along with various concentrations of safrole for 4 hr at 37℃ and 5% CO_2. After the incubation, splenocytes were then gently removed, washed and cultured with LPS as described before in "In vitro antibody responses".

Statistical Analysis

Results were expressed as mean± S.E. of replicates. As for the results of all studies, a Dunnet's T-test (Dunnet, 1955) was performed if an one-way analysis of variance of the means showed treatment effects.

RESULTS

Table 1 shows the effects of safrole on the body, spleen, thymus, liver and kidney

weights of mice. Animals up to 400 mg/kg safrole treatment, there was no significant changes in body, thymus and kidney weights. The spleen and liver werghts of 400 mg/kg treatment groups were increased by 54 and 107% respectively as compared to naive groups.

Figure 1 shows the dose-response effects of safrole on the antibody respose to SRBC, a T-dependent antigen. Animals exposed to 50, 100, 200 and 400 mg/kg of safrole for 14 days demonstrated a dose-related, significant suppression

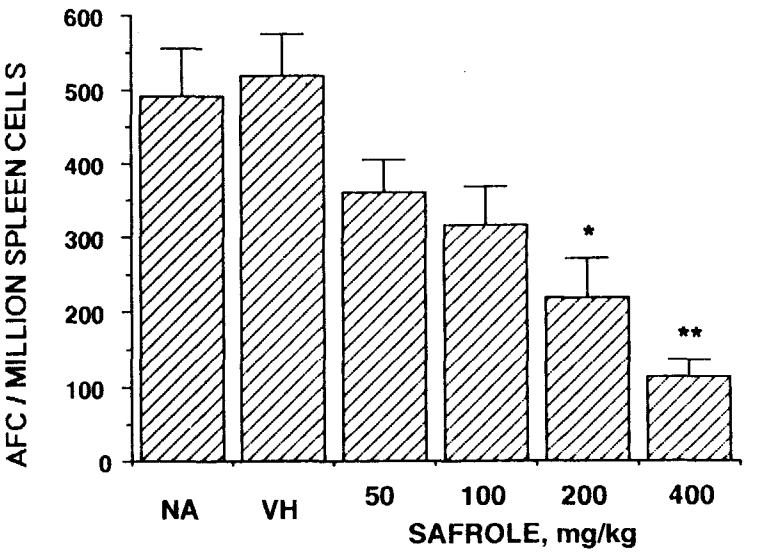

Figure 1. Suppression of *in vivo* antibody responses to SRBC in mice treated with safrole. Adult female BALB/c mice were exposed (oral intubation) to nothing (NA), corn oil (VH) or 50, 100, 200, or 400 mg/kg of safrole daily for 14 days. Twenty-four hours after the final exposure, mice were immunized with SRBC. The antibody response to SRBC were measured on Day 4 by enumerting the number of AFC per million splenocytes. The suppression of AFC by safrole was not associated with an alteration in spleen cell recovery or percentage of viability of recovered cells in any dose group when compared to naive controls ($2.1 \pm 0.22 \times 10^8$ cells/spleen and $92 \pm 3\%$ viability). Each value represents the mean± S.E. for five mice/treatment group. Values significantly different from NA as determined by Dunnett's T-test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

Table 1. Effects of Safrole on Body and Organ Weights in BALB/c Mice

Exposure Group	Body wt. (g)	Spleen wt. (mg)	Thymus wt. (mg)	Liver wt. (g)	Kidney wt. (mg)
Naive	28.0± 1.2	119± 13	67± 11.6	1.5± 0.6	268± 16
Vehicle Safrole(mg/kg)	27.7± 1.2	112± 19	65± 8.5	1.6± 0.4	260± 22
50	28.5± 1.2	99± 12	59± 6.5	1.4± 0.2	222± 19
100	27.5± 1.1	129± 12	52± 5.2	1.6± 0.3	242± 15
200	30.1± 1.2	136± 14	54± 3.4	1.8± 0.3	265± 12
400	29.1± 1.4	185± 22*	53± 5.6	2.8± 0.3*	278± 15

Groups of BALB/c female mice were garvaged with nothing (Naive), corn oil (Vehicle), or safrole in corn oil daily for 14 days. On day 15, the body and organ weights of mice were measured. Values represent mean± S.E. of 5 mice. The asterisks (*) indicates $P<0.05$ when comared to Naive values.

of the AFC response to SRBC when immunized on Day 15 and assayed on Day 19. The suppression of humoral immunity is not due to an alteration in spleen cell viability and is not associated with an alteration of spleen cell numbers (Figure 1 Legend).

Proliferative responses of spleen cells to B-cell mitogen, LPS (10 and 100 μg/ml) and T-cell mitogen, Con A (0.5, 1.0, 5.0 and 10.0 μg/ml) were examined. As the trend of effects were similar for both mitogen concentrations, only the results for the optimum concentration are shown. As shown in Figure 2, exposure to 100, 200 μg/kg safrole for 14 days did not exhibit inhibition in both mitogen assays. However, responses to LPS and Con A was significantly inhibited at 400 mg/kg safrole. These effects on the mitogenic responses occured in the absence of any changes in spleen cell number.

Figure 3 shows the suppression of *in vitro* antibody response by safrole. Spleno-

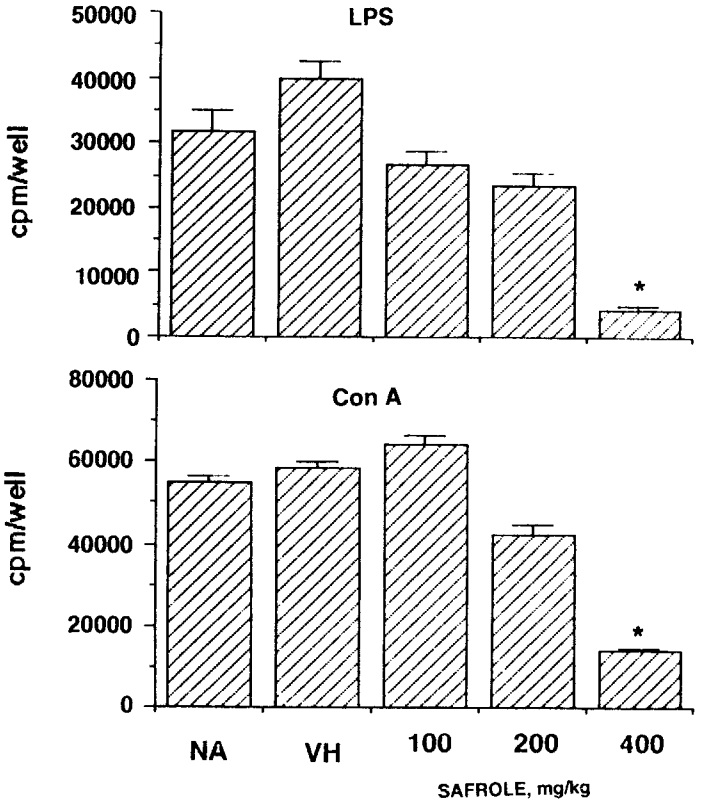

Figure 2. Suppression of the mitogen-induced proliferation of spleen cells by safrole. Female BALB/c mice were exposed (oral intubation) to nothing (NA), corn oil (VH) or 100, 200 or 400 mg/kg of safrole daily for 14 days. Twenty-four hours after th final exposure, mice were sacrificed and spleen cell suspensions were aceptically prepared. Aliquots were incubated with LPS (100 μg/ml) or Con A (0.1 μg/ml). Quadriplicate cultures were set up for each treatment group. The proliferation of splenocytes were measured by culturing for 3 days after an 18 hr pulse with ^{3}H-thymidine. Each value represents the mean cpm± S.E. for four replicate cultures. Values significantly different from NA as determined by Dunnett's T-test at $P<0.01$ are indicated by *.

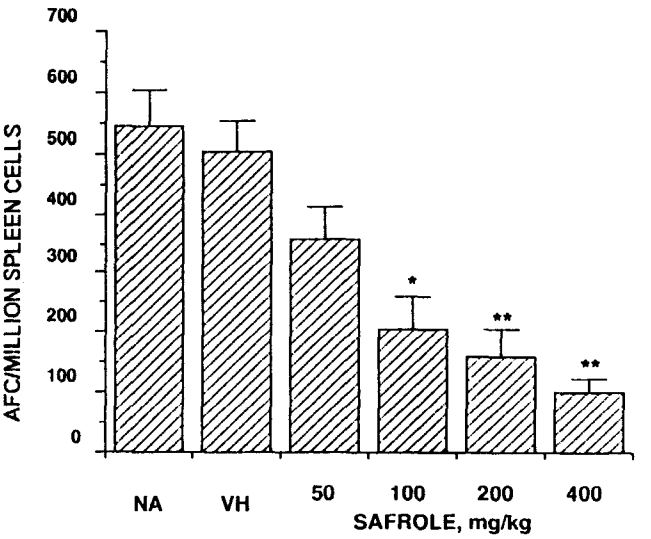

Figure 3. Suppression of *in vitro* antibody responses to SRBC in mice treated with safrole. Female BALB/c mice were exposed (oral intubation) to nothing (NA), corn oil (VH) or 10, 100, 200 or 400 mg/kg of safrole daily for 14 days. Twenty four hours after the final exposure, mice were sacrificed and spleen cell suspensions were prepared. The suspensions from each mouse was incubated with 100 μg/ml LPS. The polyclonal antibody responses by LPS were measured on day 3. Each value represents the mean ± S.E. for five mice/treatment group. Values significantly different from NA as determined by Dunnett's T-test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

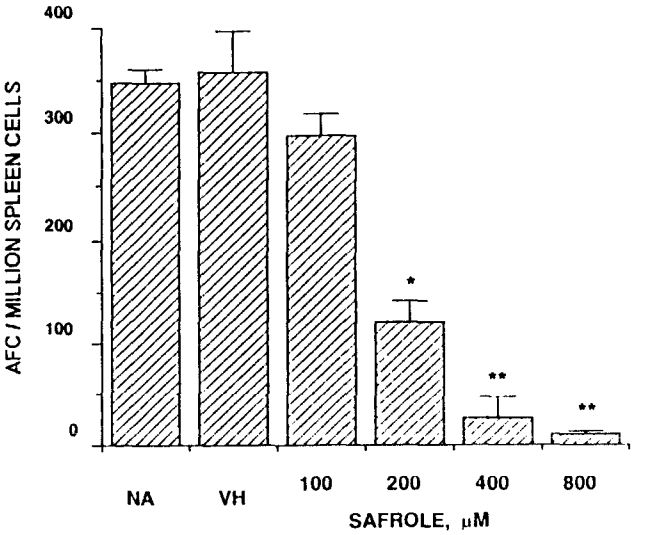

Figure 4. Suppression of *in vitro* polyclonal antibody response of splenocytes by safrole. Splenocytes, isolated from untreated mice were cultured with nothing (NA), 0.5% acetone (VH), or 100, 200, 400 or 800 μM of safrole. LPS (100 μg/ml) was added and AFC was enumerated on Day 2. Results were expressed as the mean± S.E. of quadriplicate cultures. Values significantly different from NA as determined by Dunnett's T-test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

cytes isolated from 100, 200, 400 mg/kg treated mice produced dose-dependent suppression of antibody response to LPS significantly as compared with naive group (51, 22, 20% of Naive at 100, 200, 400 mg/kg safrole treatment groups respectively).

To study the effects of safrole addition to splenocyte cultures, splenocytes from untreated mice were cultured along with safrole and antibody response and mitogen-induced splenocyte proliferation assay were performed. Figure 4 shows the dose response effects of safrole on the *in vitro* antibody response to polyclonal antigen, LPS. At 100 μM, safrole did not produce inhibition in *in vitro* antibody response to LPS. Safrole, however, produced a dose-related inhibition in responses to LPS at 200, 400 and 800 μM. The percent viability of the recovered cell after 2-day antibody response culture period in the naive (NA) and vehicle (VH) control groups were 68.3± 3.0 and 73.6± 4.1 respectively. And at 100, 200 400 and 800 μM of safrole treated group, the percent viability were 73.4± 2.5, 74.6± 4.2, 72.5 ± 4.4 and 71.6± 3.7 respectively. This results shows that the suppression of the AFC response to LPS is not due to an alteration of spleen cell viabilities.

The effects of safrole on the mitogen-induced proliferative responses in *in vitro* cell culture were studied. As shown in Figure 5, the responses to LPS and Con

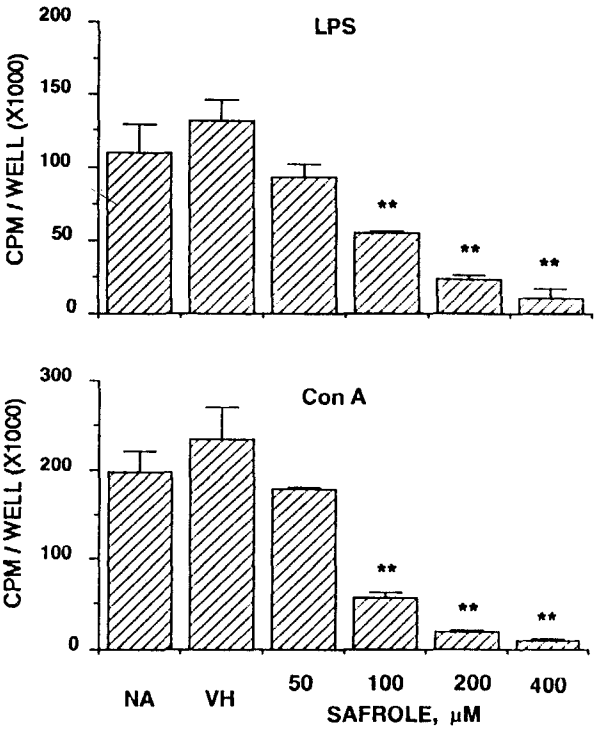

Figure 5. Suppression of mitogen-induced proliferative responses by safrole in *in vitro* splenocyte cultures. Splenocytes, isolated from untreated mice were cultured with nothing (NA), 0.5% acetone (VH), or 100, 200, 400 or 800 μM of safrole. LPS (100 μg/ml) or Con A (1 μg/ml) was added and cultures were harvested on Day 3 after an 18 hr pulse with ^{3}H-thymidine. Results were expressed as the mean± S.E. of quadriplicate cultures. Values significantly differnt from NA as determined by Dunnett's T test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

A were significantly inhibited at 100, 200, 400 and 800 μM of safrole. These effects on the mitogenic responses occured in the absence of any changes in spleen cell number.

To study the role of metabolic activation on the immunosuppression induced by safrole, primary cultures of mouse hepatocytes were used as an *in vitro* metabolic activation system and spleen cells were cocultured with hepatocytes along with safrole. Cyclophophamide (CP, 1 mM) was used as a positive control. After coculturing for 4 hr, polyclonal antibody response to LPS was determined. As shown in Figure 6, CP produced inhibition in response to LPS only if CP was activated in the hepatocytes-splenocytes coculture. Meanwhile, the suppresion of *in vitro* antibody response by safrole was not altered when safrole was incubated in the splenocyte-hepatocyte coculture for 4 hr as compared with direct addition of safrole in splenocyte cultures.

Drug metabolizing enzymes, cytochrome P-450 metabolizes safrole to 1′-hydroxysafrole and cytoplasmic sulfotransferase catalyzes the sulfation of 1′-hydroxysafrole to 1′-sulfooxysafrole, which is a ultimate carcinogenic metabolite of safrole (Boberg *et al.*, 1983). To study the relationship between immunosuppressive effects and sulfate conjugation of safrole, the effects of sulfation cofactor and sulfotransferase inhibitor on the coculture were studied. As shown in Figure 7, the addition of sulfation cofactor, sulfate (2 mM) and sulfotransferse inhibitor, salicylamide (SAM; 1 mM) along with safrole to hepatocytes-splenocytes coculture produced no effect on the suppression of antibody response induced by safrole.

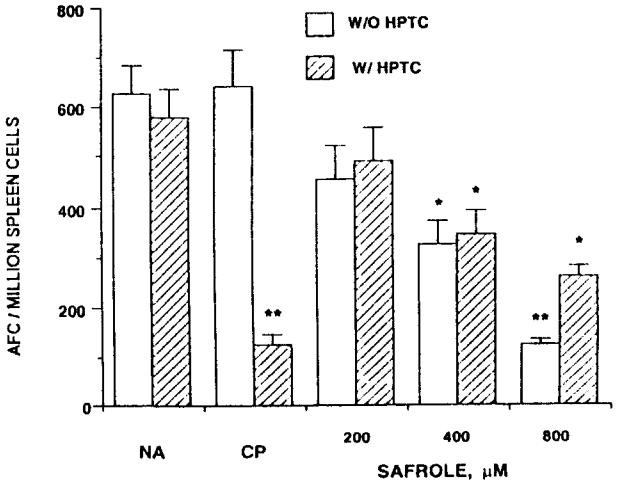

Figure 6. Effects of hepatocytes coculture on the suppression of polyclonal antibody response by safrole. Spleen cells were cultured alone (open bar) or cocultured with mouse hepatocytes (hatched bar) for 4 hr along with 200, 400 or 800 μM of safrole. Additional groups include 0.5% of acetone as a vehicle (VH) and 1 mM cyclophosphamide (CP) as a positive control. At the end of the coculture, the splenocytes were harvested from hepatocytes and washed twice with RPMI 1640. The cell numbers were adjusted and LPS was added. The polyclonal antibody response was measured on Day 2 by enumerating AFC. Results were expressed as the mean± S.E. of quadriplicate cultures. Values significantly different from VH as determined by Dunnett's T test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

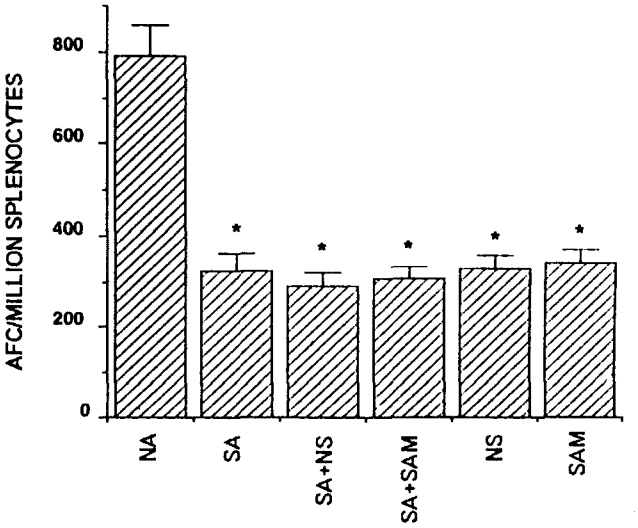

Figure 7. Effects of salicylamide or sulfate on the safrole-induced suppression of *in vitro* antibody responses. Spleen cells were cocultured with nothing (NA), 200 μM of safrole (SA), 200 μM of safrole+10 mM of sodium sulfate (SA+NS), 200 μM of safrole+1 mM of salicylamide (SA+SAM), 10 mM of sodium sulfate (NS), or 1 mM of salicylamide (SAM). At the end of the coculture, the splenocytes were harvested from hepatocytes and washed twice with RPMI 1640. The cell numbers were adjusted to 5×10^6 cells/m*l* and LPS was added. The polyclonal antibody response was measured on Day 2 by enumerating AFC. Results were expressed as the mean± S.E of quadriplicate cultures. Values significantly different from NA as determined by Dunnett' T test at $P<0.05$ and $P<0.01$ are indicated by * and **, respectively.

DISCUSSION

There are considerable evidences that an increased incidence of tumors may be induced by chemical carcinogens. Incidences of tumors induced by UV and 3-methylcholanthrene in mice were enhanced by co-treatment of immunosuppressive agents (Outzen, 1980). And many human carcinogens, including polycyclic aromatic hydrocarbons, dimethynitrosamine, benzidine, cyclophosphamide, and heavy metals suppress immune responses of experimental animals *in vivo* (Luster *et al.*, 1987). This relationship between carcinogenicity and suppression of the host immune response provoked studies on the effects of chemical carcinogens on the immune responses. In this paper, we studied the immunosuppressive effects of safrole, one of the environmental chemical carcinogens.

Our findings that *in vivo* and *in vitro* exposure of safrole could suppress the antibody responses to SRBC and LPS, and proliferative responses of spleen cell to B-cell mitogen LPS and T-cell mitogen Con A is the first report of suppression of the immune responses by safrole. T-and B-cells seem to be evenly damaged by safrole since the magnitude of the inhibition in proliferative responses of spleen cell culture was similar.

The spleen has limited amounts of drug metabolizing enzymes including enzymes to metabolize safrole while liver is the most active tissue for metabolism and

is the target organ of safrole (King *et al.*, 1981; Reddy and Randeraty, 1990). To characterize the role of metabolic activation in safrole-induced immunosuppression, the antibody response to LPS was evaluated in splenocytes cocultures with mouse hepatocytes. If metabolites mediate the suppression of antibody response by safrole via activation in splenocytes, then one would expect a marked shift in the dose-response curve in the presence of hepatocytes. On the contrary, the suppression of antibody response by safrole was not altered even if safrole was added to the splenocytes cocultured with hepatocytes.

It has been known that the ultimate carcinogenic metabolite of safrole is 1′-sulfooxysafrole (Boberg, 1986). 1′-Sulfooxysafrole could be produced by cytopalsmic sulfotransferase which catalyze the reaction between 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and 1′-hydroxysafrole. The synthesis of PAPS requires ATP and inorganic sulfate. RPMI 1640 culture medium contains 0.41 mM of inorganic sulfate concentration while the sulfotransferase activity in hepatocytes is maximum at 10 mM of inorganic sulfate (Moldeus *et al.*, 1979). To characterize the role of sulfate conjugation in immunosuppressive effects of safrole, we studied the effects of inorganic sulfate and salicylamide, the sulfotransferase inhibitor (Bennet, 1975). Neither the addition of inorganic sulfate (10 mM), nor of salicylamide (2 mM) to hepatocyte-splenocyte coculture along with safrole produced suppression of antibody response of splenocytes. These results suggested that the immunosuppressive effects of safrole may not be mediated by the reactive metabolites which produce carcinogenicity. In our previous studies, we also suggested that the immunospuression induced by 2-aminofluorene, 2-acetylaminofluorene and dimethylnitrosamine might not be mediated by reactive intermediates which produce carcinogenicity (Kim *et al.*, 1989a, 1989b; Haggerty *et al.*, 1990).

The mechanism by which safrole produce the suppression on the immune responses is not known. It might be possible that safrole is endogeneously metabolized in splenocytes, not related to the cytochrome P-450 associated monooxygenase system, to produce immunosuppressive effects. Experiments are currently undreway to identify the metabolism of safrole in spleen cells and the immunosuppressive metabolite(s) of safrole.

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