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Initiation-Promotion Skin Carcinogenesis and Immunological Competence[1, 2, 3] (38974)

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The immunological status of an animal has been reported to play an important role in the induction and progression of carcinogenesis (1–5). Animals with a lowered immune competence have been shown to develop tumors more rapidly than animals with normal immunological reactivity (6, 7). Conversely, tumor growth is inhibited by pretreatment of animals with adjuvants which increase immunological reactivity (8). In several studies, various chemical carcinogens have been shown to be immunosuppressive (2, 9) and DMBA, in particular, has been shown to depress both humoral and cell-mediated immune responses (10, 11).

The immune competence of mice subjected to initiation-promotion carcinogenesis was determined by the length of time required to reject allografts of tail skin and by the incorporation of tritiated thymidine ([^{3}H]thymidine) by lymphocytes stimulated with the mitogens PHA and PWM. In addition, during initiation-promotion carcinogenesis, mice were treated with chlorphenesin, a compound reported to increase immunological reactivity particularly cellular immunity (12).

Materials and Methods. Skin grafting. The host animals were female Strain A mice, an inbred strain maintained at the Eppley Cancer Institute. They were fed pelleted food and water *ad libitum*. There were five groups of animals in the skin grafting experiment. Animals were treated with one application of 100 μg of DMBA in acetone followed after 10 days with 20 μl of 2.5% croton oil in acetone applied to the interscapular area of the skin twice a week for 30 wk (13). At the end of the treatment, the mice were separated into two groups, those developing tumors and those not developing tumors. To assess the effect of the initiating and promoting agents, one 100 μg application of DMBA was applied to the interscapular area of the third group of mice 10 days before grafting. The fourth group was treated on the interscapular area with 20 μl of 2.5% croton oil 10 days before grafting. The fifth group of animals received no treatment. Skin grafting was performed by removing tail skin from female C3H mice and grafting this skin onto the dorsum of the Strain A mice and care was taken not to place the graft on the area which had been treated with carcinogen or promoter. The procedure is described in detail elsewhere (14).

Lymphocyte cultures. There were five groups of mice in the lymphocyte experiment. Group I was treated with 100 μg of DMBA applied to the interscapular area of the skin and Group 2 received 20 μl of 2.5% croton oil also on the skin. Group 3 received 5 mg. (two 2.5 mg doses given on the same day) of chlorphenesin intraperitoneally (ip). Group 4 mice received 20 μl of 2.5% croton oil on the skin and were injected with 5 mg (two 2.5 mg doses) of chlorphenesin ip, and Group 5 received no treatment. These treatments were initiated 10 days before the mice were bled. The mitogenic response of lymphocytes to PHA and PWM was determined on whole blood lymphocyte cultures by the method of Heiniger *et al.* (15). Blood was pooled from three mice for each group. The blood was collected in 50 USP units of heparin/ml. The media for the cultures contained 500 μl of RPMI-1640 (Grand Island

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[2] Abbreviations used in this paper: 7,12-dimethylbenzanthracene (DMBA), 3-*p*-chlorphenoxy-1,2-propanediol (chlorphenesin), Adenosine 3′,5′-cyclic monophosphate (cyclic AMP), phytohemoagglutinin (PHA), pokeweed mitogen (PWM).

[3] Chlorphenesin was a generous gift from H. B. Zimmerman of Wallace Laboratories, Canberg, New Jersey.

Biological Company) to which 50 μg/ml Gentamicin (Schering Diagnostics) had been added. To the cultures were added either 200 μg of PHA in 20 μl (M. form), 200 μg PWM in 20 μl (Grand Island Biological Company), or 20 μl of RPMI-1640 in the control cultures. 50 μl of blood was added to triplicate cultures and incubated for 36 hr at 37° in a humidified incubator with 5% CO_2 in air. 2 μCi of [^{3}H]thymidine (sp act = 20 Ci/mmole) (New England Nuclear) was added and incubation continued for 16 hr. The cultures were removed from the incubator, placed on ice and 2 ml of cold distilled water was added to each culture to lyse the erythrocytes. The samples were collected on Shatman GF/A glass filters using a Millipore sampling manifold (3025), washed three times with 0.85% saline, then 5% trichloroacetic acid was added followed by methanol. All reagents were at 5°. Each filter was transferred to a scintillation vial and allowed to dry. 10 ml of Omnifluor in toluene (New England Nuclear) was added to each vial and allowed to stand for 1 hr at room temperature after which time they were counted in a Beckman LS-230 liquid scintillation counter. The data for the stimulated and unstimulated cultures for each group are given in cpm/10^6 cultured lymphocytes and are also expressed as a mitogenic index, which is defined as the ratio of radioactivity in PHA or PWM stimulated cultures over nonstimulated cultures.

Tumor initiation-promotion. Two groups each of 30 Swiss mice received 100 μg of DMBA applied to the interscapular area of the skin followed 3 wk later by 20 μl of 2.5% croton oil applied to the skin twice a week for 20 wk. Group 2, in addition to receiving DMBA and croton oil, also received 5 mg (two 2.5 mg injections given on the same day) of chlorphenesin ip twice a week at the same time that croton oil was applied. The animals were examined weekly and the number of tumors recorded. The experiment was terminated at 20 wk at which time the animals were autopsied and examined for internal pathology. If an animal had several tumors, not all of the tumors were examined microscopically.

Results. In the skin grafting experiment (Table I) the allografts on the DMBA plus croton oil treated tumor bearing mice were retained significantly longer ($P < 0.02$) than were the grafts on either the control mice or the DMBA plus croton oil treated mice which had not developed tumors. The mice which received one application of DMBA 10 days before grafting were also inhibited ($P < 0.02$) from rejecting their skin grafts.

TABLE I. SKIN GRAFT SURVIVAL OF CARCINOGEN AND CROTON OIL TREATED MICE.

Group	No. grafts	Mean day of rejection ± SD
DMBA, croton oil with tumors	10	[a]12.7 ± 1.88
DMBA, croton oil no tumors	10	10.9 ± 1.87
DMBA	9	[a]12.8 ± 1.32
Croton Oil	8	9.9 ± 0.99
Control	12	10.9 ± 0.95

Animals treated with 100 μg of DMBA plus repeated application of 20 μl croton oil twice a week for 30 wk were separated into tumor-bearing and nontumor-bearing groups prior to skin grafting. The DMBA group received 100 μg of DMBA and the croton oil group received 20 μl of croton oil in acetone 10 days before grafting.

Statistical analysis of results was done by the Mann-Whitney *U* test, with each of the groups compared to the control group. [a] Groups which are significantly different from the control group $P < 0.02$.

In the *in vitro* lymphocyte experiments seen in Table II, DMBA was found to inhibit both PHA and PWM stimulation of [^{3}H]thymidine incorporation into DNA ($P < 0.01$). The promotor croton oil significantly enhanced the PWM stimulation of lymphocytes of mice 10 days after their treatment with this agent ($P < 0.01$). However, croton oil did not cause a corresponding enhancement of the PHA response. Chlorphenesin caused an enhancement of PHA ($P < 0.01$) but not PWM response. Treatment of mice with both croton oil and chlorphenesin abolished the enhancement of PWM stimulation seen with croton oil alone.

As seen in Fig. 1, initiation with DMBA followed by croton oil induced 30 tumors (57% of the animals with tumors) in the Group 1 animals and 16 tumors (33% of the animals with tumors $P < 0.05$) in the

TABLE II. EFFECT OF DMBA, CROTON OIL, AND CHLORPHENESIN ON PWM AND PHA RESPONSE OF LYMPHOCYTES.

Treatment	Control	PWM (mitogenic index)	PHA (mitogenic index)
Normal	3031 ± 713	13469 ± 4332 (3.9)	12181 ± 3545 (3.6)
DMBA	3449 ± 342	[a]2685 ± 587 (0.9)	[a]5181 ± 1066 (1.8)
Croton oil	2388 ± 952	[a]31253 ± 8379 (13.1)	4229 ± 325 (1.8)
Chlorophenesin	832 ± 188	4326 ± 1995 (5.2)	[a]5398 ± 1288 (6.5)
Chlorophenesin + croton oil	1625 ± 222	3665 ± 627 (2.3)	4988 ± 1265 (3.1)

The PWM and PHA response of lymphocytes from mice treated 10 days before bleeding with 100 μg of DMBA, 20 μl of 2.5% croton oil, 5 mg of chlorphenesin, or both 20 μl of 2.5% croton oil and 5 mg of chlorphenesin. The data is expressed in cpm/10^6 lymphocytes and as a mitogenic index which is the ratio of radioactivity in the PHA and PWM stimulated cultures over the corresponding unstimulated controls. Statistical analysis of results was done by the Fisher's Analysis of variance. Values are triplicate samples from two experiments. [a] Cultures are significantly different from the normal untreated cultures $P < 0.01$.

Group 2 animals which also received chlorphenesin. The histological verified skin tumors in Group 2 consisted of 11 papillomas and 5 uncharacterized tumors. In Group 1, there were 16 papillomas, 2 keratoacanthomas, and 1 squamous cell carcinoma and 11 uncharacterized tumors.

Discussion. Initiation-promotion carcinogenesis is divided into several separate phases. There is first the treatment with a carcinogen (DMBA) at a subtumorigenic dose; this is followed by treatment two times a week with a promoting agent (croton oil) which produces intense inflammation and finally tumors appear in the treated area. Our purpose was to examine the immune response of the host during each of these phases by determining the rate of graft rejection and lymphocyte response to mitogens.

In the experiments reported here, Strain A mice treated with DMBA were found to retain their skin allografts significantly longer than did the control mice. This suggests that DMBA, as reported by other investigators, is immunosuppressive. DMBA also reduced the stimulation of lymphocytes by PWM and PHA. This also supports the reports of others (10, 11).

Mice treated with one application of croton oil 10 days prior to receiving a skin allograft were not immunosuppressed. These animals appeared to reject their skin grafts more rapidly than did the control mice although the difference was not statistically significant. Croton oil was found to enhance the PWM but not the PHA response of lymphocytes. This may indicate stimulation of only B cells since PHA stimulates T-lymphocytes and PWM stimulates T and B lymphocytes (16). One of the tumor-promoting esters from croton oil is PMA (phorbol myristate acetate) and it has been shown to stimulate proliferation of T-lymphocytes (17). TPA (tetradecanoyl-phorbol-acetate), another promoting ester of croton oil, stimulates mouse spleen cells (18). Chlorphenesin, when injected, inhibits the stimu-

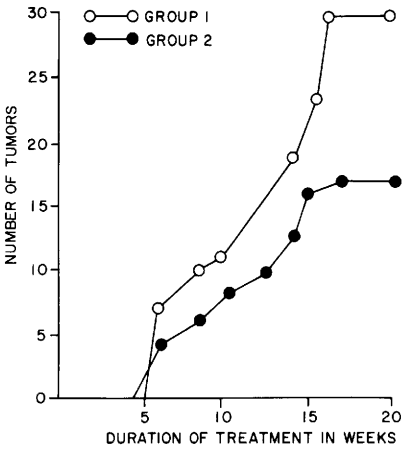

FIG. 1. Effect of chlorphenesin on DMBA and croton oil induction of tumor formation. Group I received 100 μg of DMBA (on day 0) applied to the skin followed by 20 μl of 2.5% croton oil 2 times/wk for 20 wk. Group 2 received, in addition to DMBA and croton oil, two 2.5 mg intraperitoneal injections of chlorphenesin at the same time that croton oil was applied. Statistical analysis of results was done by the Chi squared test with the chlorphenesin treated group differing significantly from the control group at $P < 0.01$.

lation of PWM mitogenesis observed in lymphocytes from croton oil treated mice. This compound stimulates cell-mediated immunity and inhibits virus-induced murine leukemias and transplanted tumors (12).

Following the development of tumors from DMBA and promotion, the Strain A mice were separated into two groups: those that developed tumors and those which did not develop tumors. These mice were then grafted with tail skin allografts. The mice which had tumors retained their skin graft significantly longer than did either the non-treated control mice or the DMBA-croton oil treated mice which did not develop tumors. The finding that skin tumors affect skin allograft survival supports the suggestion that animals and patients who develop neoplasia have a reduced cell-mediated immunity (19–22).

Chlorphenesin when injected intraperitoneally throughout promotion reduced tumor formation by 47%. Chlorphenesin has been reported to increase intracellular levels of cyclic AMP (23) and cyclic AMP has been shown to modify immunological responses (24–26). In addition, cyclic AMP has recently been reported to inhibit chemical carcinogenesis by one dose of DMBA followed by repeated application of croton oil (initiation-promotion) (27) but to stimulate tumor formation when administered to mice receiving repeated doses of DMBA without croton oil (28). Chlorphenesin may inhibit initiation-promotion carcinogenesis by raising intracellular levels of cyclic AMP and altering the immune competence of the host.

Summary. The immune competence of mice during initiation-promotion skin carcinogenesis was determined by skin allograft rejection and lymphocyte mitogenesis. The carcinogen 7, 12-dimethylbenzanthracene inhibited the cellular immune competence of mice while lymphocytes from croton oil treated mice had enhanced PWM response. Chlorphenesin, a stimulator of cellular immunity, was found to inhibit tumorigenesis in initiation-promotion skin carcinogenesis when injected during promotion.

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