A trial of Bacillus Calmette Guerin (BCG) to cure the Skin Cancer

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Objectives: In pursuit of cancer cure, the present studies, using mice as a model, evaluated the BCG. to prevent and revert the structural and biochemical changes that were induced in skin by carcinogens DMBA¤ and TPA¤¤.

Methods: Eighty mice distributed into eight groups of ten mice each were utilized. Two separate control groups were maintained. In the first experimental group, DMBA alone and in the second experimental group, DMBA and TPA were applied on the skin of mice. In the third and the fourth experimental groups, these carcinogens were applied alongwith the administration of BCG. In the fifth and sixth experimental groups, BCG, was administered after applications of these carcinogens. The development / fate of lesions were evaluated.

Results: It was observed that the repeated application of DMBA alone produced more malignant while the repeated application of TPA produced more benign varieties of skin cancer; the repeated application of these carcinogens with the administration of BCG did not result into epidermal tumor, but resulted into only dermal cancer; administration of BCG. also arrested the lesions pre-produced by these carcinogens . The statistical analysis of DNA, RNA and proteins concentrations estimates were found significant.

Conclusion: Results of this study point out that repeated application of DMBA, or TPA cause both epithelial and mesenchymal tumorigenesis in the skin. However, exposures to these carcinogens with the administration of BCG do not produce epidermal tumors. Moreover, administration of BCG arrests the epidermal lesions already produced by these carcinogens.

Abbreviations:

- ¤ **DMBA** (Dimethyl Benz anthracene) is derived from anthracene i.e. polyaromatic hydrocarbon found in commonly used coal tar.
- ¤¤ **TPA** (tetradecanyl phorbol acetate) is derived from acetate that is salt or ester of ethanoic acid manufactured by oxidation of ethanol and commonly used for production of vinegar.

Introduction

Tumor is produced due to irreversible changes in the genes that regulate growth and differentiation of normal developing tissue thus results in un-controlled abnormal production of cells[1]. A number of initiating inherited or exogenous (carcinogenic) factors for this genetic abnormality are identified[2]. Tumorigenesis by carcinogens usually occurs in multi-steps. The first two steps are known as initiation and promotion. A final step is progression during which the transformed cell develops into a malignant tumor[3]. Hamster cheek pouch (HCP) serves as an excellent model system for the studies on initiation and promotion of carcinogenesis[4]. Some other Researchers have reported "the establishment of cancer in a host involving at least two major events: the escape of tumor cells from normal growth control and their escape from immunological recognition[5]"; "malignancy is due to depressed immunity both cellular and humoral[6]"; "immunotherapy arrests growth of cutaneous malignant melanoma[7]"; BCG Vaccine assists in arresting tumor by acting as non-specific stimulant to enhance the host's immune system against melanoma and renal cell carcinoma[8] and BCG is the most active agent for therapy of the superficial transitional epithelial cell carcinoma of urinary bladder.[9]

No researcher has reported the role of BCG for skin cancer induced by carcinogens. Therefore, in the present studies effect on developing and developed tumour induced by the application of certain carcinogens of BCG were evaluated through a complete follow up studies of histopathological and biochemical changes.

Material and Methods

Eighty albino mice (*Mus musculus*) of both genders, 5-6 week old and weighing between 22 to 25 gm were distributed in eight groups "1," "2," "3", "4", "5", "6", "7" and "8", ten mice in each group. (Table 1). They were kept in separate iron cages under controlled conditions of a 12-h light / 12-h dark cycle at the animal house of Zoology Department Government College University Lahore.. Air conditioning was used to keep temperature $22 \pm 2\ c^{a}$ Cages and animals were labeled with waterproof marker. The animals quarantined / acclimatized for a week before start of the experiment and their backs were shaved with electric clippers 3 days prior to the first skin treatment and thereafter

shaved when needed. Commercial national mice food and tap water regularly provided to mice were changed with fresh one each day. The particulars of each animal such as the name of the group, age at start of the experiment, period for keeping the animal alive, gender, weight in grams at start and at the end of the experimentation, the period of application / administration of the carcinogen, diluent and vaccine for each animal were recorded throughout the length of experiment for the objective of correlation. Gross tumor appearing features were counted and measured by Vernier Caliper weekly. The lesions and the surrounding skin were also examined macroscopically with magnified lens. The required skin tissues of all animals were taken by needle biopsy at twentieth and thirtieth week of the experiment while all animals of each group at end of forty weeks were sacrificed by decapitation after resection of the required tissue under anesthesia.

Carcinogens and Vaccine

Both carcinogens DMBA and TPA were obtained from Sigma Chemical Company (St. LOUIS, MO, USA). 100 mg of DMBA (available in ampoule of dried powder) was dissolved in one liter of acetone to make concentration of 100 µg per ml of acetone while 1mg dry powder of TPA was dissolved in 100 ml of acetone to get concentration 10µg per ml of acetone. The working solutions of both DMBA and TPA were prepared just before the use. Stock and working solutions of both carcinogens were kept at 20c°. Automatic pipettes were used for topical application. The vaccine BCG was obtained from Aventis Pasteur Pharmaceutical, which provided Freeze-dried live vaccine that contains attenuated strain 1077 of Mycobacterium bovis (Merieux seed) and Excipients: Dextran- Glusoe - Titon WR 1339- Human Albumin and Diluent (Water). These contents were mixed to reconstitute the working vaccine just before

Table 1: *Purpose and Dose Schedule of Different Groups in the Present Forty Week Studies.*

		Dose schedule			
Group	**Purpose**	**Twice a week topical application of 20*u*g DMB per 0.2ml of acetone**	**Twice a week topical application of 2*u*g of TPA per 0.2 ml of acetone**	**Intradermally $4 \times 10^5 - 16 \times 10^5$ units of BCG once after two weeks**	**Twice a week topical application of 0.2ml of acetone**
1	Control	–	–	–	–
2	Placebo Control	–	–	–	First 20 weeks of the study
3	To test DMBA as skin cancer initiator as well as promoter agent.	First 20 weeks of the study.	–	–	–
4	To test DMBA and TPA as skin cancer initiator and promoter agents respectively.	Initial single application.	First 20 of weeks of the study except Ist. one .	–	–
5	To test the effect of BCG on the developing carcinogenic effect of DMBA.	First 20 weeks of the study.	–	First 20 weeks of the study.	–
6	To test the effect of BCG on the Pre-produced carcinogenic effect of DMBA.	First 20 weeks of the study.	–	Last 20 weeks of the study.	–
7	To test the effect of BCG on the developing carcinogenic effect repeated application of TPA after single application of the cancer initiator.	Initial single application.	First twenty weeks.	First 20 weeks of the study.	–
8	To test the effect of BCG on the Pre-produced carcinogenic effect of by repeated application of TPA after single application of the cancer initiator.	Initial single application.	First twenty weeks.	Last 20 weeks of the study.	–

administration and it's 0.1ml contains 800,000 and 3200 000 units of the live bacillus.

Light Microscopy and spectrophotometry

The tissues obtained by biopsy / resection were given 2-3 washes with 0.85% saline solution. each specimen of the tissues was then divided into two parts, one part stored in the frozen nitrogen while the other piece kept in Neutral buffered formaline solution for twenty-four hours. Subsequently the frozen tissues were subjected to standard DNA, RNA and proteins spectrophotometric procedures. The estimates for concentrations of the DNA, RNA and proteins molecules were measured through the comparative optical density change of the absorbance for DNA, RNA and Proteins molecules of different samples with that of the control. Then they were analyzed statistically using "t" test. The paraffin sections 1-5 *u* of formaline fixed tissues were prepared by standard histological techniques and stained with Haemotoxylin & Eosin (H& E).

Results

Effects of Carcinogens

The repeated topical application of DMBA alone produced skin cancer of the following types and frequencies: papilloma 10% (Fig. 1), dysplasia, 10%, keratoacanthoma 10%, squamous cell carcinoma *in situ* 10%, extensive squamous cell carcinoma, 40%, and fibrosarcoma (Fig.2), 20%, in addition to some areas of chronic inflammation, loss of hair,ulceration punched, inverted or everted margins of the lesion and pleomorphism etc.

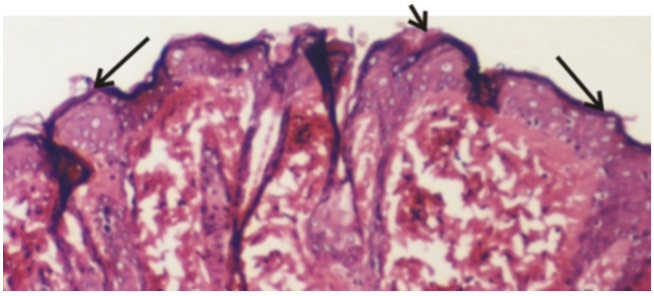

Fig. 1: *Microphotograph showing squamous cell papilloma in the skin of Mice exposed to DMBA (H & E, 100 x).* ***Black Arrows*** *pointing to papilloma.*

The repeated application of TPA for 20 weeks after single application of DMBA in the present studies also produced skin cancer but varieties included were 30% papilloma, 30% dysphasia (Fig. 3), 20% Osteoma, 20%% squamous cell carcinoma in situ, in addition to some areas of acute inflammation and of precancerous changes like hyperplasia, pleomorphism etc. The loss of hair, ulceration and outgrowth in skin observed here displayed the sequence and progression resembling that observed with repeated application of DMBA alone.

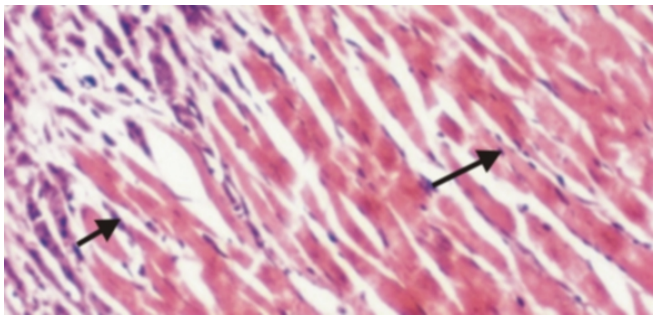

Fig. 2: *Microphotograph showing fibrosarcoma. Infiltrating muscular tissue in the skin of mice exposed to DMBA (H & E, 400 x).* ***Black Arrows*** *pointing to malignant spindly cells invading muscle fibers.*

Effects of Carcinogens under the Umbrella of BCG

Under such situation, DMBA or DMBA and TPA did not result into the neoplastic changes in the epidermis of the skin. Howere it resulted in only dermal cancer.

Effects of BCG on the lesions pre-produced by Carcinogens

BCG arrested further progression in the lesions produced already by DMBA or DMBA and TPA.

Effects on body weight

Topical application of these carcinogens with & without BCG administration had no effect on the body weight of the treated mice.

StatisticalAnalysis

The estimates of the concentrations of DNA, RNA and Proteins of various groups were found statistically significant for various levels of significance ($p<0.05$, $p<0.01$, $p<0.001$: Table 2).

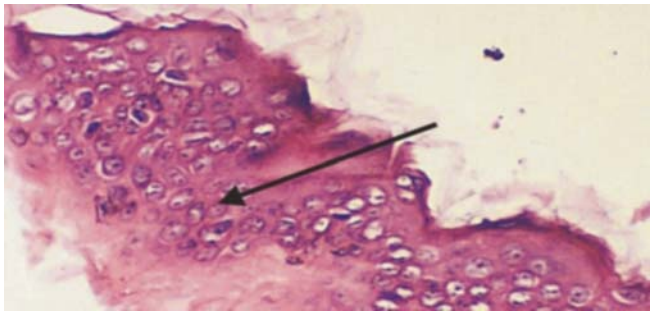

Fig. 3: *Photomicrograph showing squamous cell dysplasia in the skin of mice exposed to DMBA & TPA. (H & E, 400 x).* ***Black Arrow*** *pointing to disorganized cells.*

Discussion

It is evident from the present studies that repeated topical application of DMBA alone produced both benign and

malignant epidermal (epithelial) tumors, which indicates

Fig. 4: *Photograph showing skin outgrowths in mice treated with DMBA.* ***White arrow*** *pointing to hemorrhagic and necrotic outgrowth.*

that DMBA alone initiates as well as promotes epidermal cancer. These findings are in agreement with those of Gijare *et al*[4]. Another inference of the present investigations is that all epidermal tumors remain benign during the early period of the experiment (up to 30 weeks), but most become malignant after 40 weeks showing that chemical carcinogenesis occurs as a multistage process in epithelial tissue leading to malignant tumor This observation is consistent with reports of Lakshma-Reddy *et al* [10], Hennings *et al* [11], Slaga and **Skarin**[12]. The present observation that epidermal tumors developed in 80 % of the cases and dermal in 20% indicates that epidermal cells are more likely get affected by DMBA, probably because they are more in ready contact and therefore more prone to the action of DMBA than the underlying dermal cells. However early development of malignancy in dermal cells indicates that if the dermal cells happen to be exposed to DMBA, the cancerous changes develop more rapidly in the dermal cells unlike to the multistage process (first benign and then malignant) of epithelial carcinogenesis. Deep ulceration is seen in our study indicates one way of penetration of DMBA to dermis. In the present investigations, DMBA was also found to be a skin irritant as it caused inflammation of the skin. This finding is not in line with **Lewis** *et al*[13] who reported that cancer promoter and not the cancer initiator causes skin inflammation in mice. This study has also shown that repeated application of TPA produced benign skin tumors and very few papilloma progressed into squamous cell carcinoma *in situ* in late phase of the experiment exhibiting that malignant conversion is slower with the repeated application of the cancer promoter (TPA). This is consistent with the findings of Hennings *et al*[11] who reported that the malignant conversion of the skin tumors is enhanced by repeated application of tumor initiator and not by repeated application of tumor promoter. Inflammation of the skin with repeated TPA application noted in the present study agrees with observations of **Lewis** *et al*[13] indicating the promoter's irritating ability of causing inflammation. The biochemical estimates of concentrations of DNA, RNA and Proteins in the skin exposed to DMBA and TPA in the present studies and those of Manjanatha *et al*[14] are significantly higher indicate that repeated application of carcinogens causes stimulation of more DNA synthesis in the exposed cells leading uncontrolled multiplication of cells representing the mechanism of carcinogenisis by these carcinogens. The finding "no weight loss in DMBA/ DMBA & TPA treated mice" of the present research studies is consistent with finding of jiska *et al*[15]. Because of this feature, DMBA more commonly is used as cancer initiator than other cancer initiators. The observation of present study that DMBA or DMBA & TPA topical application under the parallel administration of BCG vaccine failed to produce neoplastic growth in the epidermis strongly indicates that BCG is protective for epidermis against these carcinogens by acting as non-specific stimulant to enhance the host's immune system enabling it's recognition to capture and kill

Table 2: *Statistical Analysis for Estimates of DNA and RNA Proteins Concentrations for a 20-Week Application of DMBA (20 MG / 0.2 ML Acetone) on the Skin of Mice (group 3) Compared with that of Control (Group 1).*

Weeks	DNA			RNA			PROTEINS		
	Group 1	Group 3	"t" test	Group 1	Group 3	"t" test	Group 1	Group 3	"t" test
20	2.79± 0.02	2.86*± 0.04	$p_{< 0.05}$	12.7 ± 0.04	12. 9*± 0.05	$p_{< 0.05}$	11.01± 0.007	12.812*± 0.009	$p_{< 0.05}$
30	2.79± 0.02	2.93**± 0.08	$p_{< 0.01}$	12.7 ± 0.04	13.36**± 0.030.04	$p_{< 0.01}$	11.01± 0.007	12.813**± 0.009	$p_{< 0.01}$
40	2.79 ±	3.65***±	$p_{< 0.001}$	12.65 ±	16.481***	$p_{< 0.001}$	11.01±0.0	14.717***±	$p_{< 0.001}$

	0.02	0.09		0.04	± 0.004		07	0.009	

the cancerous cells. These reports are also in agreement with the findings of Leong *et al*[8]; Bassi *et al*[16]; and Kamat *et al* [17]. One finding of present study is that BCG did not permit further progression of the skin tumour produced by carcinogens is in agreement with DeLa *et al*[7] and Ishihara *et al*[18]. On the basis of this study, it is suggested: "BCG should be administered in skin cancer suffering patients to prolong their survival and as protective for the people work in the environment contaminated with these carcinogens.

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