

IMMUNOLOGICAL PHENOMENA IN HAIRLESS MICE DURING EXPERIMENTAL CARCINOGENESIS INDUCED WITH LONG-TERM TOPICAL APPLICATION OF 7, 12-DIMETHYLBENZANTHRACENE (DMBA)

Cellular Immunity in DMBA-carcinogenesis

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Abstract. To estimate the influence of topical treatment with DMBA and induced tumors on delayed hypersensitivity, the response of spleen lymphocytes to PHA in vitro and macrophage migration inhibition with PHA were studied in DMBA-treated hairless mice. DNA synthesis and blastic transformation of cultured lymphocytes decreased after 6-12 weeks of DMBA application. Lymphocyte response to PHA gradually diminished during the experiment, as compared with control animals. Since the malignant transformation of skin tumors was not observed before 16 weeks of DMBA carcinogenesis, it seems that derangements in cellular immunity preceded the malignant proliferation. The increase in spleen weight and the absence of PHA-induced inhibition of macrophage migration in hairless mice with malignant tumors may also be related to the influence of the tumor itself on the lymphatic system of experimental animals.

Key words: DMBA-carcinogenesis; Tumour immunity; Hairless mice; Lymphocyte transformation

Polycyclic aromatic hydrocarbons are well-known, strongly active carcinogens which induce, depending on the manner of their application and the animal species, malignant tumors of varied histologic structure. In mice and rats, DMBA (7, 12-dimethylbenzanthracene), one of the strongest carcinogenic chemicals, applied externally, can induce during long-term treatment acanthosis of the epidermis, papillomas, lesions of the keratoacanthoma type, or squamous-cell carcinoma.

Besides the cancerogenic activity, polycyclic aromatic hydrocarbons may have an immunosuppressive effect on experimental animals. The production of antibodies decreases, the graft rejection time increases, and the graft-versus-host reaction is inhibited in the animals receiving DMBA or methylcholanthrene (2, 5, 11, 14, 15, 16).

The relationship between the carcinogenic and the immunosuppressive activities of polycyclic aromatic hydrocarbons has not yet been established. Szakal et al. (17) have found that in hamsters treated topically with DMBA, the immunosuppressive action of carcinogen is primary, and it seems possible that the development of neoplasm may depend on the elimination of the host defense system.

There are numerous reports (1, 3, 4, 6, 7, 18) on depression of cell-mediated immune response in animals with advanced malignant tumors, but it remains to be established whether inhibition of cell-mediated immunity during experimental carcinogenesis is related to the immunological influence of the neoplasm itself or rather to a primary immunosuppressive action of the carcinogen.

The purpose of the present work was to investigate the cellular immunological response at various stages of experimental carcinogenesis in hairless mice and to determine the relationship between the immune response of the animals and the development of malignant tumors.

MATERIAL

The investigations were carried out in 3 groups of hairless, but not athymic mice:

1. 40 hairless mice (20 males and 20 females), aged about 3 months, were treated with 0.5% DMBA solution in acetone, once weekly, in the dorsal region.

The control group consisted of:

2. 10 hairless mice treated with acetone in the dorsal area once weekly, and
3. 20 hairless mice of the same age as the animals in groups 1 and 2, not subjected to any procedures.

Table I. PHA-stimulated spleen lymphocyte transformation in hairless mice bearing DMBA-induced skin tumors

Experimental group	Length of treatment	Lymphocyte transformation							
		0		48 h		72 h		96 h	
		ARG	BLAST	ARG	BLAST	ARG	BLAST	ARG	BLAST
1	0.5% DMBA externally for 4 months	0.6	1	11.8	23	4.8	25	6.7	18
2	Acetone externally for 12 months	4	6	31	45	14.5	67	21.5	50
3	Without treatment	3	7	29	32	12	68	20	51

ARG = the percentage of ^3H -thymidine incorporating cells; BLAST = the percentage of blast cells.

METHODS

In experimental animals at various stages of carcinogenesis and tumor growth (after 3, 6, 9, 12, 15, 18, 20 and 25 weeks of DMBA application) and prior to treatment, the following investigations were made:

(a) lymphocyte transformation test using PHA as a non-specific mitogen: A suspension of spleen lymphocytes in Parker's medium (TC 199) with addition of 15% calf serum inactivated at 56°C, containing 2×10^6 cells in 1 ml, was incubated at 37°C with or without PHA (0.1 ml PHA to 4 ml culture). On the 2nd, 3rd and 4th day of culture, smears were prepared after a one-hour incubation with ^3H -thymidine. The results of transformation were evaluated by the autoradiographic method and morphologically, counting the percentage of cells synthesizing DNA or blast cells (labelled spleen lymphocytes/spleen lymphocytes $\times 100$ or blast cells/spleen lymphocytes $\times 100$) in 2 000 consecutive cells.

(b) macrophage-migration inhibition test with PHA: Fragments of spleen about 1 mm in diameter, obtained after removal of the splenic capsule, were placed in culture medium (containing Parker's fluid 80% plus 20% of calf serum inactivated at 56°C) in separate chambers. PHA (0.1 ml PHA per 1 ml of medium) was added to half of the fragments. After 24 hours the zone of macrophage migration was planimetrically measured and the percentage of migration inhibition was calculated by the formula:

$$\frac{\text{migration radius with PHA}}{\text{migration radius without PHA}} \times 100\%$$

(c) histological examination of skin sections obtained from the areas treated with DMBA and from skin tumors.

(d) measurement of tumor diameters and spleen weights.

RESULTS

In hairless mice treated with 0.5% DMBA solution, thickening of the epidermis was observed after 2 weeks and, commencing from the 8th–9th week of

the experiment, the papillomatous proliferation of the epidermis and tumor growth occurred.

Histological examination of tumors at various intervals showed the structure of papilloma, keratoacanthoma or squamous cell carcinoma. Squamous cell carcinoma did not develop in all animals and malignant proliferation only appeared after at least 16 weeks of DMBA application.

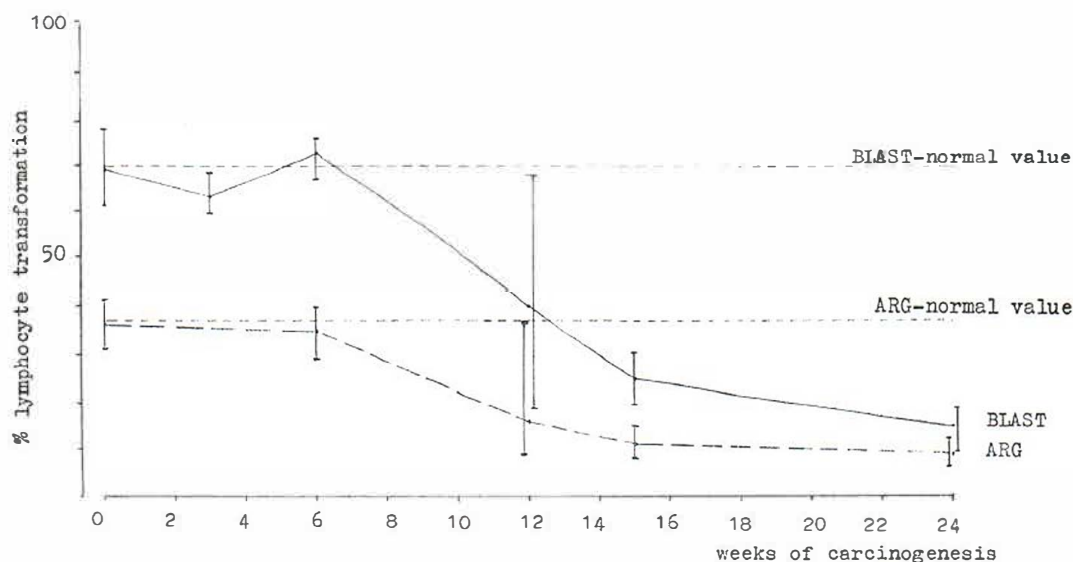
Regardless of the benign or malignant character of skin tumors, the mice showed gradually progressive cachexia and died within 4–7 months. Between the 15th and 25th week of the experiment the spleens of the animals enlarged gradually, particularly in mice with squamous cell carcinoma. A correlation was observed between the increase in spleen weight and the size of the growing tumor (Fig. 2).

The hypertrophy of the epidermis at the site of acetone application was also observed in hairless mice treated with acetone alone. In some animals small tumors had been noticed as well as erosions and ulcerations at the site of the skin treated with acetone. Histological examination of these lesions showed inflammatory infiltrations and benign proliferation of the epidermis.

The control group was followed up for 12 months.

Table I shows the results of the lymphocyte transformation test in hairless mice with active tumors treated for 4 months with 0.5% DMBA solution, and in control hairless mice after 12 months of acetone treatment or in normal non-treated animals.

The threefold decrease of cells incorporating ^3H -thymidine during 48 hours of culture with PHA



Notes: ARG - the percentage of ^3H -labelled cells
BLAST - the percentage of blast cells

Fig. 1. PHA-stimulated spleen lymphocyte transformation in hairless mice in different stages of DMBA carcinogenesis.

ARG = the percentage of ^3H -labelled cells. BLAST = the percentage of blast cells.

(11.8%) was noted in DMBA-smear mice, which was statistically significant as compared with control groups. Similarly, in mice treated with carcinogen the percentage of blast cells after PHA stimulation was significantly lower (25%) after 72 hours' culture than in control cultures (68%).

Although in mice receiving acetone applications the epidermis was thickened and acanthosis as well as ulcerations were present, PHA-induced lymphocyte transformation was normal.

Disturbances in lymphocyte response to PHA in hairless mice treated with DMBA were noted not earlier than after 6 weeks of the experiment. The lymphocyte transformation inhibition of varying degree after 12 weeks of DMBA application was observed in most animals. We have found 19-49% (mean 36%) of blast cells and 9-26% (mean 16.5%) of DNA-synthesizing cells in PHA-stimulated cultures. After 15 weeks of experiment, immunological deficiencies were present in all animals. The degree of lymphocyte transformation decreased gradually in the further course of DMBA carcinogenesis (Fig. 1).

The macrophage migration inhibition test with PHA was carried out at various stages of carcinogenesis in mice treated with DMBA, starting on the 12th week of the experiment when disturbances

in spleen lymphocyte response to PHA became evident (Table II).

The inhibition index of macrophage migration after 12 weeks of DMBA treatment, by the direct method using spleen fragments, was similar in animals with evident neoplasms (33.4%) and in both control groups (36%). No PHA-induced migration inhibition after 20 and 24 weeks of carcinogenesis was observed; the inhibition index was 81% and 93.5%, respectively. In all experimental models, macrophage migration without presence of antigen (PHA) was completely normal.

Fig. 2 demonstrates a comparison of immunological and pathological phenomena during carcinogenesis induced with DMBA.

Table II. Inhibition of macrophage migration with PHA in different stages of DMBA carcinogenesis (%)

Experimental group	Length of treatment			
	12 weeks	20 weeks	24 weeks	12 months
1. DMBA	33.4	81	93.5	×
2. Acetone	36	×	34.5	30.5
3. Normal mice	37.7	×	×	35

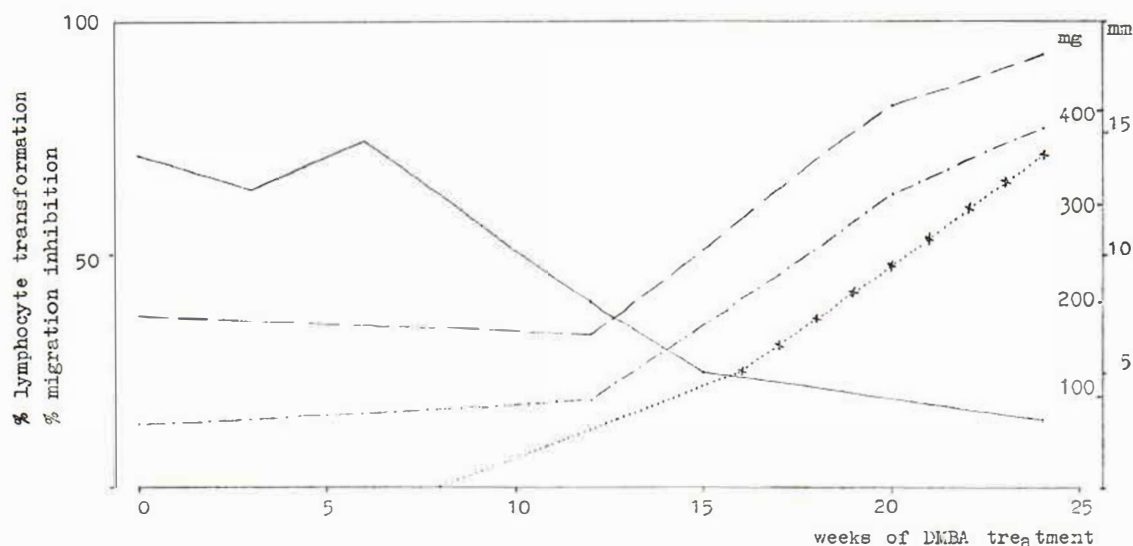


Fig. 2. Immunological and histopathological findings during DMBA carcinogenesis in hairless mice.—, PHA-induced lymphocyte transformation (% blast cells); ---, Macrophage migration test (inhibition index with PHA); ····,

Spleen weight (mg); ----, Diameter of tumor (mm) histologically papilloma; ··· + ···, diameter of tumor, histologically carcinoma spinocellulare.

DISCUSSION

The degree of blastic transformation of lymphocytes induced with PHA is known as an index of the immunological status of the organism (8, 9, 10, 12, 13). The use of spleen lymphocytes in the lymphocyte transformation test with PHA confirms the significance of this test for studying the cell-mediated immune response in hairless mice during experimental carcinogenesis. In the control groups (including the hairless mice not subjected to any treatment or treated with acetone) the percentage of blast cells ranged from 58 to 80% and the percentage of cells incorporating radioactive thymidine ranged from 35 to 45%. Lymphocyte response to PHA during the first 6 weeks of DMBA application in experimental animals did not differ from that in controls. The number of blast cells or cells incorporating radioactive thymidine after PHA stimulation was decreased and this preceded by about 8–10 weeks the appearance of malignant tumors. However, in some animals treated with carcinogen, despite the derangement of lymphocyte transformation, only a slight hypertrophy of the epidermis was noted.

The present results confirm the investigations of authors who observed a primary immunosuppressive action of DMBA. The inhibition of blastic transformation, however, became much more evident

when the malignant tumors appeared. These findings seem to be dependent on the influence of the neoplasia on the immunological phenomena in the host. It may be that the carcinogenic effect of DMBA on the epidermis of hairless mice and its immunosuppressive action occurred synergistically in the process of neoplasia. The hypothesis of Szakal et al. (17) that the development of tumor depends exclusively on the immunosuppressive action of DMBA seems unlikely.

In mice with malignant neoplasms the PHA-induced macrophage migration inhibition was absent. Since the agglutinating properties of PHA may cause a non-specific inhibition of migration without participation of specific lymphokines (MIF), these findings are indecisive. However, they were reproducible in all animals with malignant tumors and seem to be dependent on the action of the neoplasm. This supposition is more likely in view of a great increase in spleen weight in these animals, although histological examination of the spleen failed to demonstrate any significant abnormalities except hypertrophy and congestion. The enlargement of the spleen was not related to the secondary infection and inflammation in the skin, since it was never observed in mice painted with acetone, which induced inflammatory changes and ulcerations. The test for macrophage migration showed a significant

inhibition of migration induced with PHA in hairless mice treated with acetone.

CONCLUSIONS

1. Lymphocyte transformation of spleen lymphocytes under the influence of PHA as a non-specific stimulator is a useful method for evaluation of the immunological state of hairless mice during experimental carcinogenesis.

2. Inhibition of lymphocyte transformation in animals treated externally with 0.5% acetone solution of DMBA occurs between the 6th and 12th week of the experiment and it precedes the development of malignant neoplasms. These data suggest an immunosuppressive effect of DMBA.

3. After development of malignant tumor, the lymphocyte transformation shows further gradual decrease, which may indicate an effect of the neoplasm on the immunological response.

4. The absence of PHA-induced migration inhibition of macrophages derived from the spleen and the increase of spleen weight following the growth of malignant skin neoplasm seemed to confirm the depressive effect of the neoplasm on the lymphatic system of hairless mice.

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