Therapy of Chemically Induced Skin Tumors of Mice with Vitamin A Palmitate and Vitamin A Acid

Animal experiments carried out by several authors have shown that the induction of benign as well as of malignant epithelial tumors could be retarded or even prevented by systemically applied retinol[1] or retinylpalmitate[2] (CHU and MALMGREN[3], DAVIES[4], MCMICHAEL[5], ROWE and GORLIN[6], SAFFIOTTI et al.[7], SCHMÄHL[8]). Whereas these investigators were able to demonstrate a prophylaxis of epithelial tumors, a therapy of established tumors was only rarely attained (BOLLAG[9]). This paper deals with the therapeutic influence of systemically applied retinylpalmitate and retinoic acid[10] on established skin papillomas of mice, induced by dimethylbenzanthracene and croton oil.

Materials and methods. Female Swiss albino mice of the random bred Füllinsdorf strain were fed the mouse diet Nafag 199, containing 2500 IU of vitamin A per kg food. Mice were housed in individual cages. At the beginning of the experiment the mice weighed 20–22 g.

Induction of skin papillomas. 7,12-Dimethylbenz(a)-anthracene (DMBA) was applied twice – on day 1 and on day 15 – on the shaved skin of the back of mice. 150 γ DMBA dissolved in 0.2 ml acetone were painted on the skin of each mouse on an area of approximately 5 cm^2. After an interval of 3 weeks 0.5 mg of croton oil dissolved in 0.2 ml acetone were applied on the skin twice a week for 3 to 4 months. When the papillomas, which appeared mostly as multiple ones, varying in most cases between 5 and 10, had reached mean diameters of at least 4 mm, the therapeutic test was started.

Characterisation of papillomas. Various specimens of the papillomas were histologically verified. In the control groups the great majority of papillomas continued to grow. Less than 10% of the papillomas showed a tendency to regress spontaneously. Carcinomas arising either primarily or out of the papillomas appeared about 5 to 8 months after the first application of DMBA.

Therapy of skin papillomas. Groups of 3–6 mice, each mouse bearing an average of 7 tumors, received retinylpalmitate or retinoic acid i.p. by injection or orally by stomach tube either daily on 5 days of the week or once a week for 2 and 6 weeks respectively. Retinylpalmitate was prepared as a 2% water miscible solution by means of a non ionic emulsifier. The vehicle contained 8% Cremophor® und 10% propylene glycol. Retinoic acid was prepared as a suspension in arachis oil or as a 1% water miscible solution in a similar way as retinylpalmitate. The water miscible solutions were diluted with water to obtain lower concentrations. The volumes given varied between 0.2 ml and 0.8 ml per 20 g body weight. Controls received arachis oil or the vehicle with the emulsifier.

Evaluation of numerical results. The volumes of the papillomas were calculated with the formula $4/3\ r^3\pi$, r being the measured mean radius. The sum of papilloma volumes was determined for each mouse and the mean of these values was calculated. This was done before the beginning of treatment (day 0), as well as 2 weeks (day 14) and 6 weeks (day 42) respectively after the first drug application. The change of the mean volumes was expressed in percent of the values of day 0. Statistical analysis was done by testing the significance of the differences between mean papilloma volumes of day 0, day 14 and day 42 respectively by means of the (one-sided) t-test. The calculated error probabilities α varied between 0.05 and 2.5% except in some controls or lowest dosage groups. In addition the distribution free Wilcoxon test for matched samples was applicated. The latter led only to neglectible differences in the levels of significance compared with those of the t-test.

Results. Within the period of observation, the control animals showed a progression of the mean papilloma growth. There was an increase in the mean volume of their papillomas varying in the first 2 weeks' period between 22.3 and 56.5%. This signifies that at this stage of the papillomas the progressive growth exceeded by far the tendency to spontaneous regression. In contrast to the controls, the treated animals showed a regression of their papillomas. With retinylpalmitate as well as with retinoic acid, the papillomas decreased in size. From Table I it can be seen that retinoic acid applied i.p. in the form of oily suspensions has a marked effect on the papillomas. 100 mg/kg once a week led within a period of 2 weeks to a reduction of the mean papilloma volume of 60.9%, whereas 400 mg/kg even reduced the papillomas by 86.0%. The shrinkage of the papillomas can first be observed 4–5 days after an injection of retinoic acid. The papillomas become pale and dry, they decrease in size and sometimes disappear completely. Tables II and III demonstrate that water miscible solutions of retinylpalmitate as well as of retinoic acid given by the oral route elicit the same effect on papillomas. The extent of regression is dose-dependent. There is no significant difference in the effects between retinylpalmitate and retinoic acid when taking into account the different molecular weights

[1] Retinol = all-*trans*-β-retinol = vitamin A alcohol.
[2] Retinylpalmitate = palmitate of all-*trans*-β-retinol = vitamin A palmitate.
[3] E. W. CHU and R. A. MALMGREN, Cancer Res. *25*, 884 (1965).
[4] R. E. DAVIES, Cancer Res. *27*, 237 (1967).
[5] H. MCMICHAEL, Cancer Res. *25*, 947 (1965).
[6] N. H. ROWE and R. J. GORLIN, J. dent. Res. *38*, 72 (1959).
[7] U. SAFFIOTTI, R. MONTESANO, A. R. SELLAKUMAR and S. A. BORG, Cancer *20*, 857 (1967).
[8] D. SCHMÄHL, personal communication.
[9] W. BOLLAG, Int. J. Vit. Res. *40*, 299 (1970).
[10] Retinoic acid = all-*trans*-β-retinoic acid = vitamin A acid = Retinsäure = Tretinoin.

Table I. Results of treatment of skin papillomas during 2 weeks with oily suspensions of retinoic acid

Retinoic acid dose	Mean papilloma volume per animal in mm^3 day 0	day 14	Change in mean papilloma volume per animal (%)	Error probabilities α (%)
Controls	1272.9	1841.4	+44.7	$\alpha < 0.1$
100 mg/kg once a week i.p.	910.3	356.3	−60.9	$\alpha < 2.5$
200 mg/kg once a week i.p.	1705.4	442.1	−74.1	$\alpha < 0.1$
400 mg/kg once a week i.p.	1132.8	159.1	−86.0	$\alpha < 0.25$

Table II. Results of treatment of skin papillomas during 2 weeks with water miscible solutions of retinylpalmitate and retinoic acid

Compound dose	Mean papilloma volume per animal in mm³ day 0	day 14	Change in mean papilloma volume per animal (%)	Error probabilities α (%)
Controls (vehicle daily p.o.)	456.8	558.7	+ 22.3	$\alpha > 5$
Controls (vehicle once a week p.o.)	188.5	295.0	+ 56.5	$\alpha < 2.5$
Retinoic acid				
20 mg/kg daily p.o.	381.2	151.8	—60.2	$\alpha < 0.5$
40 mg/kg daily p.o.	773.5	240.5	—68.9	$\alpha < 0.05$
100 mg/kg once a week p.o.	203.9	146.1	—28.3	$\alpha > 5$
200 mg/kg once a week p.o.	961.6	389.5	—59.4	$\alpha < 0.05$
400 mg/kg once a week p.o.	897.8	193.1	—78.4	$\alpha < 0.05$
Retinylpalmitate				
40 mg/kg daily p.o.	286.9	154.8	—46.0	$\alpha < 0.5$
80 mg/kg daily p.o.	394.3	60.4	—84.7	$\alpha < 0.05$
200 mg/kg once a week p.o.	323.6	435.5	+ 34.6	$\alpha > 5$
400 mg/kg once a week p.o.	247.0	70.7	—71.4	$\alpha < 2.5$
800 mg/kg once a week p.o.	460.8	52.5	—88.6	$\alpha < 0.05$

Table III. Results of treatment of skin papillomas during 6 weeks with water miscible solutions of retinylpalmitate and retinoic acid

Compound dose	Mean papilloma volume per animal in mm³ day 0	day 42	Change in mean papilloma volume per animal (%)	Error probabilities α (%)
Controls (vehicle daily p.o.)	456.8	723.4	+ 58.4	$\alpha > 10$
Retinoic acid				
20 mg/kg daily p.o.	381.2	124.6	—67.3	$\alpha < 5$
40 mg/kg daily p.o.	773.5	63.5	—91.8	$\alpha < 0.05$
Retinylpalmitate				
40 mg/kg daily p.o.	286.9	41.2	—85.6	$\alpha < 0.05$
80 mg/kg daily p.o.	394.3	50.3	—87.3	$\alpha < 0.05$

of retinylpalmitate (524.8) and of retinoic acid (300.4). The daily administration induces a somewhat higher extent of regression than the weekly application if the total doses are compared. After cessation of treatment, papillomas slowly tended to increase again in size. The toxic effects of the highest applied doses were moderate. They consisted in a temporary weight loss in the case of weekly administration, which did not exceed 10% of the body weight. Some alopecia and skin desquamation were observed.

Discussion. The above mentioned results show clearly that the treatment with retinylpalmitate as well as with retinoic acid lead to the regression of skin papillomas previously induced in mice by painting with dimethylbenzanthracene and croton oil. Notable regressions take place very rapidly. The fact that cancer chemotherapeutic substances e.g. cyclophosphamide, fluorouracil and procarbazine have almost no effect on these skin papillomas (BOLLAG[11]), confers particular interest to the anti-papilloma effect of retinylpalmitate and retinoic acid. This phenomenon could be interpreted either as a direct effect on the papillomas in the proper sense of a chemotherapeutic agent or as an indirect effect whereby a defence mechanism may be stimulated. Lacking definite proof we can only speculate about the mode of action of retinylpalmitate and retinoic acid.

Vitamin A is known as a regulator of growth and differentiation of epithelial tissue. Therefore retinylpalmitate and retinoic acid respectively may be able to induce a reconversion of neoplastic epithelial tissue to normal epithelial tissue, in the same way as it was shown in organ culture that a carcinogen-induced epithelial metaplasia which may be considered as a prerequisite for a neoplastic transformation was reconverted to normal epithelial tissue by excess of retinol (LASNITZKI[12]).

Vitamin A in high doses has a lysosome labilizing effect (DINGLE[13], LUCY and DINGLE[14], DINGLE and LUCY[15], BRANDES and ANTON[16], ANTON and BRANDES[17]) whereby hydrolases are released from the lysosomes. Proteases and nucleases may be responsible for the destruction of tumor cells, which are perhaps more sensitive to the activity of these enzymes than non neoplastic cells.

Retinylpalmitate as well as retinoic acid in high doses induce a marked proliferation in the epidermis (STUDER[18,19], STUDER and FREY[20], LÄUPPI[21]). The mitotic rate of the cells of the basal layer is increased. If this phenomenon is elicited in the papilloma epithelium, which shows already a high mitotic activity, this tissue may be

[11] W. BOLLAG, unpublished.
[12] I. LASNITZKI, Natn. Cancer Inst. Monograph *12*, 381 (1963).
[13] J. T. DINGLE, in *Lysosomes*, CIBA Foundation Symposium (Little, Brown and Co., Boston 1963), p. 384.
[14] J. A. LUCY and J. T. DINGLE, Nature, Lond. *204*, 156 (1964).
[15] J. T. DINGLE and J. A. LUCY, Biol. Rev. *40*, 422 (1965).
[16] D. BRANDES and E. ANTON, Lab. Invest. *15*, 987 (1966).
[17] E. ANTON and D. BRANDES, Expl molec. Path. *7*, 156 (1967).
[18] A. STUDER, Schweiz. Z. allg. Path. *13*, 799 (1950).
[19] A. STUDER, Z. ges. exp. Med. *121*, 287 (1953).
[20] A. STUDER and J. R. FREY, Dermatologica *104*, 578 (1952).
[21] E. LÄUPPI, unpublished.

driven to a 'lethal proliferation'. The growth becomes unbalanced to such an extent that the cells do not survive.

A stimulation of the defence mechanism may also play a role in the regression of skin papillomas. Spontaneous regression of papillomas occur. It was shown that X-rays and thymectomy delayed, whereas a methanol extract of bacille Calmette-Guérin (BCG) accelerated the regression of papillomas (LAPPÉ and PREHN[22]). It was assumed that an immunologic surveillance is operative with regard to premalignant skin papillomas. The balance can be disturbed in both ways. Whereas immunosuppressive measures like irradiation and thymectomy may decrease the regression rate by diminishing immune reactions, immunostimulating agents, like extracts of BCG acting as adjuvants may enhance the regression rate. Vitamin A has been shown to have an adjuvant effect. Under the influence of retinol it was possible to obtain a certain titer of humoral antibodies towards an otherwise non immunogenic protein (DRESSER[23]).

The above mentioned results show that it is possible to induce regressions of a benign epithelial tumor by compounds which do not belong to the well known classes of cytotoxic or antimitotic agents. Vitamin A compounds do not suppress mitotic activity but even enhance it. Thus the mode of action must be very different from that of the compounds used until now in the chemotherapy of tumors. After these first positive results in the treatment of a benign epithelial tumor we tried in animal as well as in clinical experiments to influence malignant epithelial tumors. We succeeded to induce partial and in a few cases even total regressions of chemically induced skin carcinomas of mice arising from papillomas (BOLLAG[11]). Furthermore, in clinical trials locally applied retinoic acid besides reducing or eliminating actinic or senile keratoses, considered as precancerous epithelial lesions also caused partial or complete regressions of basal cell carcinomas of the skin (BOLLAG und OTT[24]). Thus vitamin A and particularly vitamin A acid may be considered as therapeutic agents affecting special types of tumors, but possessing a mode of action completely different from that of the known cancer chemotherapeutic agents.

Zusammenfassung. Mittels Dimethylbenzanthracen und Krotonöl wurden bei Mäusen Hautpapillome erzeugt. Diese Tumoren wurden durch systemische Anwendung von Retinylpalmitat und Retinsäure therapeutisch beeinflusst. Es kam zu einer deutlichen Regression der Tumoren, die dosisabhängig war. Sowohl orale wie parenterale tägliche oder wöchentliche Applikation der geprüften Vitamin-A-Verbindungen waren wirksam. Der Wirkungsmechanismus wird diskutiert. Er unterscheidet sich grundsätzlich von demjenigen anderer tumorhemmender Substanzen.

W. BOLLAG

Department of Experimental Medicine,
F. Hoffmann-La Roche & Co. Ltd.,
CH-4002 Basel (Switzerland), 26 June 1970.

[22] M. A. LAPPÉ and R. T. PREHN, Cancer Res. *29*, 2374 (1969).
[23] D. W. DRESSER, Nature, Lond. *217*, 527 (1968).
[24] W. BOLLAG and F. OTT, Agents and Actions *1*, 172 (1970).

Interaction of Lead with Erythrocytes

Blood lead is found mainly in association with the erythrocytes and only 5% or less is in the plasma. Studies with radioactive lead (Pb-203) in vitro have shown that plasma is rapidly cleared of lead by erythrocytes and that equilibrium is reached after approximately 15 min (unpublished data). The kinetics of the reaction in vivo using Pb-210 have been reported by other authors[1] with essentially similar findings. The nature of this interaction has been the subject of speculation. Most workers have assumed that the principal site involved was the erythrocyte membrane. AUB et al.[2] postulated that lead was precipitated at the surface of the membrane as the phosphate, but later workers suggested that it was as the diphosphoglycerate[3]. Support for this view was given by the demonstration that membrane permeability was altered by low concentrations of lead[4,5]. The interference with ATP synthesis within the cell has also been attributed to the interaction of lead with a ligand at the cell membrane rather than with an intracellular ligand[6]. Conversely, lead is known to traverse the plasma membrane of nucleated cells without difficulty[7,8] and also that it traverses the erythroblast membrane since mitochondrial abnormalities result in that cell series in experimental lead poisoning[9]. Evidence also exists for a lead binding compound within the erythrocyte[10,11].

We have therefore re-examined the lead-binding properties of human erythrocytes and attempted to identify the cell fractions in which binding occurs. 2 techniques have been employed to separate erythrocytes into fractions of varying molecular weight, namely, Sephadex gel filtration and ultracentrifugation. Fresh, washed erythrocytes were haemolyzed by freezing and thawing, and diluted 10-fold with *tris*-maleic acid buffer pH 7.0. 5 ml of haemolysate was passed upwards through a 100 × 2.5 cm Sephadex 200 column at 20 ml/h which had been calibrated with cytochrome C (Sigma Chemical Co., St. Louis); transferrin (Sigma Chemical Co., St. Louis) and γ-globulin (A. B. Kabi, Stockholm). The elution volumes were cytochrome C 395 ml, transferrin 325 ml, γ-globulin 290 ml. The effluent was monitored with a double-beam, flow-through UV-analyser at 254 nm (ISCO, model UA) prior to collection in 10 ml aliquots with a drop counting

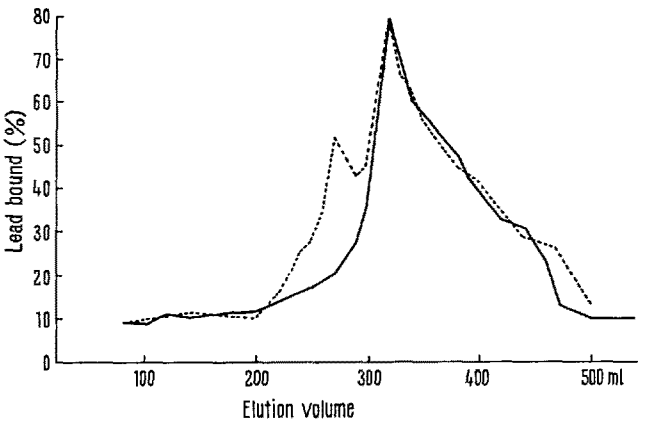

Fig. 1. Gel filtration of human erythrocyte haemolysates on a column of Sephadex G-200. Elution with *tris*-maleic acid buffer pH 7.0. Lead binding of fractions determined at equilibrium by ultra-filtration ....., before; ——, after preliminary ultra-centrifugation at 60,000*g* for 4 h (3 × 23 ml M.S.E. 65 swing-out rotor).