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Tumor Necrosis Factor in Experimental Cancer Regression with Alphatocopherol, Beta-Carotene, Canthaxanthin and Algae Extract

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Abstract—*Regression of established hamster buccal pouch carcinoma has recently been demonstrated in asssociation with an induction of tumor necrosis factor alpha in macrophages. Regression of hamster buccal pouch tumors has also been demonstrated following the local injection of alphatocopherol, canthaxanthin and an extract of* Spirulina–Dunaliella *algae. The current study demonstrates that cancer regression is also accompanied by a significant induction of tumor necrosis factor in macrophages in the tumor area, suggesting a possible mechanism of tumor destruction.*

One hundred and forty young, male adult hamsters were divided into seven equalgroups of 20 *animals. Epidermoid carcinomas were induced in right buccal pouches by* 14 *weeks of painting, three times per week, of a* 0.5% *solution of* 7,12-*dimethylbenz(a)anthracene. Groups* 1 *and* 2 *were untreated and sham injected controls. Groups* 3–7 *had injected twice weekly into the right buccal pouches* 0.1 ml (1.9 mg/ml *of* 13-cis-*retinoic acid, canthaxanthin, algae extract, beta-carotene and alphatocopherol. After* 4 *weeks the tumors in groups* 3–7 *demonstrated varying degrees of regression and the animals were sacrificed and the right buccal pouches excised. Tumor necrosis factor alpha (TNF-*α*) was demonstrated by immunohistochemical techniques. A very significant increase in TNF-*α *positive macrophages was found in the tumor-bearing pouches of animals in groups* 5–7. *Smaller numbers of TNF-*α*-positive macrophages were found in group* 4 *pouches and a very slight increase in group* 3 *pouches.*

INTRODUCTION

REGRESSION of established epidermoid carcinomas of hamster buccal pouch was shown to be possible, following local injection or topical application of beta-carotene [1]. The tumor regression was associated with an induction of tumor necrosis factor alpha in macrophages at the tumor site [1]. Other micronutrients have now been demonstrated to be capable of regressing carcinomas of the hamster buccal pouch, following local injection into the tumor-bearing tissue. These include alphatocolpherol [2], canthaxanthin [3] and an extract of *Spirulina* and *Dunaliella* algae [4]. Since one of the mechanisms of tumor regression by beta-carotene may be the stimulation of an enhanced immune response, with selective destruction of cancer cells by tumor necrosis factor alpha, we wondered whether tumor regression by alphatocopherol, canthaxanthin and algae extract may be mediated through a similar immunoenhancement. An experiment was designed to study regression of chemically induced epidermoid carcinomas of the hamster buccal pouch. The production of tumor necrosis factor alpha was to be demonstrated with immunohistochemical technique, as in previous experiments dealing with tumor regression. Since beta carotene is considered to be metabolically converted to retinoid [5], the effect of a retinoid, 13-*cis*-retinoic acid, was also considered for the study. Canthaxanthin is a carotenoid which does not convert to retinoid [6] and its action could differ from that of beta-carotene in tumor regression.

The hamster buccal pouch tumor model is an ideal experimental model for the demonstration of cancer regression and the various possible mechanisms related to the cancer regression. A specific oncogene (C-erbB) is expressed during hamster buccal pouch carcinogenesis [7] and this is the same oncogene expressed in many oral epidermoid carcinomas in human subjects [8]. Furthermore, the hamster buccal pouch carcinomas and human

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oral carcinomas demonstrate a number of similar biochemical markers such as gamma glutamyl transpeptidase (GGT) [9, 10].

Immune mediation of hamster buccal pouch carcinogenesis has also been adequately demonstrated. Immunosuppression by methotrexate [11], cortisone [12] or specific antilymphocyte serum [13] enhances carcinogenesis, while immunoenhancement by BCG [14] or levamisole [15, 16] has been shown to retard carcinogenesis.

Retinoids, beta-carotene, canthaxanthin and alphatocopherol have all been shown to enhance immune activity in various animal models [17–21].

MATERIALS AND METHODS

One hundred and forty young adult male Syrian hamsters (*Mesocricetus auratus*) had their right buccal pouches painted three times weekly with a 0.5% solution of 7,12-dimethylbenz(a)anthracene (DMBA) in heavy mineral oil USP, using a number 3 sable brush. Each painting placed approximately 0.6 mg of DMBA in oil upon the epithelial surface of the pouch. The hamsters were randomly bred (Lakeview strain LVG, Charles River Breeding Laboratories) and were fed standard Purina laboratory pellets and water *ad libitum*. The animals were housed five to a cage and maintained in a controlled environment under standardized conditions of temperature and humidity with an alternating 12 h light–dark cycle. The animals were 60–90 days of age and weighed 95–125 g at the beginning of the experiment.

After 14 weeks all animals had obvious gross tumors of the right buccal pouches of variable size and number. At this point the animals were divided into seven equal groups for the study of tumor regression following the local injections into the right pouches of 13-*cis*-retinoic acid, canthaxanthin, algae extract, beta-carotene and alphatocopherol. Both untreated and sham injected controls were used.

Group 1	Untreated control
Group 2	Sham-injected control (MEM)
Group 3	Injected with 13-*cis*-retinoic acid
Group 4	Injected with canthaxanthin
Group 5	Injected with an extract of *Spirulina* and *Dunaliella* algae
Group 6	Injected with beta-carotene
Group 7	Injected with alphatocopherol

Injections into the pouches were carried out twice weekly for 4 weeks. The injected agents were each injected in a solution–suspension of 1.9 mg/ml and each injection consisted of 0.1 ml of the agents in minimal essential medium (MEM). None of the agents used were completely soluble in MEM and the solution–suspension was shaken prior to each injection.

At the termination of the 4 weeks experimental period, the animals were killed in a carbon dioxide chamber. The right buccal pouches were photographed and tumors were counted and measured. The pouches were then excised, fixed in 10% formalin and sectioned in paraffin. Serial sections were prepared for standard hematoxylin–eosin staining as well as for the immunohistochemical demonstration of tumor necrosis factor alpha.

IMMUNOHISTOCHEMICAL DEMONSTRATION OF TUMOR NECROSIS FACTOR ALPHA

Antibody to tumor necrosis factor

A polyclonal highly specific rabbit antiserum directed against murine cachectin/tumor necrosis factor was obtained through the courtesy of Dr. Bruce Beutler. A dilution of 1:20 was utilized for the primary antibody.

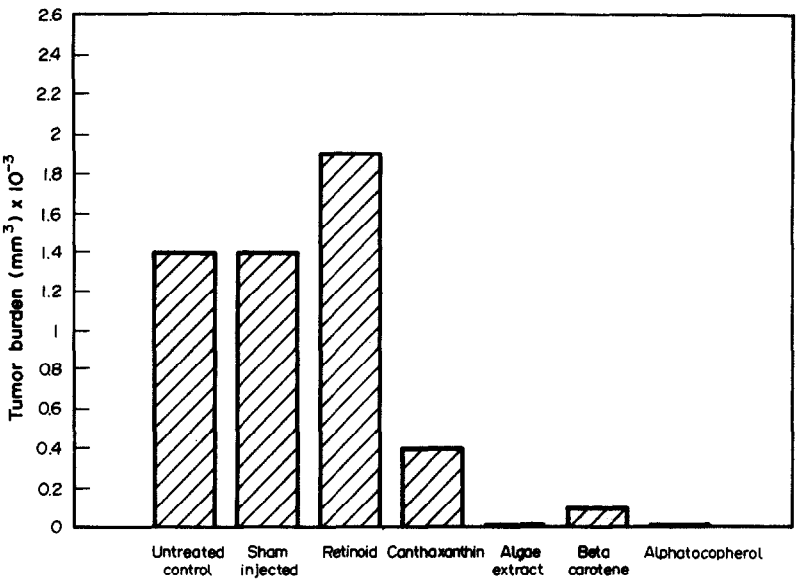
Peroxidase anti-peroxidase staining (PAP)

Paraffin-embedded sections were placed in an oven at 50°C for one half hour, then quickly placed into xylene for 1–2 min, and then 80% alcohol for 2 min. The PAP staining method was identical to the instructions in PAP, DAKO Kit (Boenisch, T., ed., Reference Guide Series 1, PAP/Immunoperoxidase, Santa Barbara, DAKO Corporation). Controls for PAP reaction: in the experimental procedure swine serum was utilized. Control sera included mouse and hamster. Antibody control included the substitution of a different primary antibody or a different secondary antibody.

Determination of histochemical reaction

The observation of cytoplasmic granules or intense red-brown stain was judged to be positive. The determination resulted from the counting of the number of positive cells per cellular dense field at 400 × magnification in a light microscope. At least two fields per slide were obtained from each animal. The square area was determined using a calibrated micrometer. The values obtained were the result of the blind assessment of histochemical appearance by at least two pathologists. The code for the group relationships was disclosed following these determinations. The determination of the relative percentage of positive cells was derived from the procedures mentioned above, and was calculated as follows:

Number of positive cells per mm^2 (400 ×)/ Total number of cells present per mm^2.



Graph I

RESULTS

Gross observations

At the end of the 4 week experimental period the animals in groups 1 and 2 (control groups) demonstrated numerous, moderately sized papillary tumors of the right buccal pouches. The animals in groups 3–7 demonstrated fewer and smaller tumors. The most notable tumor regression was seen in groups 4–7. Less pronounced tumor regression was seen in group 3 (Graph I, Fig. 1).

Microscopic observations

The tumors were epidermoid carcinomas. Large tumors were apparent in groups 1 and 2 control animals. Smaller tumors were seen in groups 3–7 animals. Several animals in groups 5, 6 and 7 had right buccal pouches free of tumor. Where tumors were present in these three groups, areas of degeneration could be seen and the connective tissue beneath the tumors was densely infiltrated with macrophages and lymphocytes.

Immunohistochemical observations

Tumor necrosis factor alpha (TNF-α) was minimally seen in the right buccal pouches of control groups 1 and 2, with many histologic fields showing no evidence of TNF-α-positive cells. Group 3 (retinoid) pouches showed a slight increase of TNF-α-positive cells when compared with the control groups 1 and 2. The right pouches in group 4 (canthaxanthin) animals showed a significant rise in TNF-α-positive cells and some positive cells could be seen in most histologic fields. In groups 5, 6 and 7 (algae, beta-carotene, alphatocopherol), the percentage of TNF-α-positive cells was notably elevated, with over half the cells in each field showing positivity for TNF-α. The positive cells were primarily macrophages and were seen in great numbers adjacent to the regressing epidemoid carcinomas and within the tumors where degeneration of tumor cells was apparent (Graph II, Table 1, Figs. 5–7).

CONTROLS FOR IMMUNOHISTOCHEMICAL REACTION

A number of *in vivo* and *in vitro* controls were carried out to reassure us of the validity of the observations.

In vivo

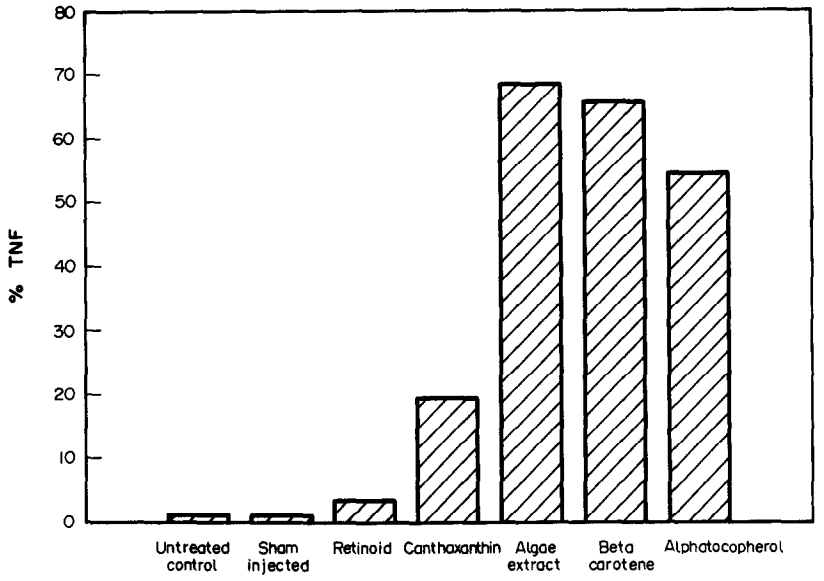
Serial sections of the hamster buccal pouch mucosa were stained with the peroxidase anti-peroxidase technique. In control sections, the primary antibody was replaced with (1) rabbit serum, (2) mouse serum, (3) hamster serum, (4) substitution of a different antibody with the same isotype. The control sections stained negative for tumor necrosis factor alpha (Figs. 7, 8).

In vitro

Peritoneal derived macrophages from Syrian hamsters were placed on to round coverslips (00, American Scientific Prod).

Adherent macrophages were obtained following 3 h of incubation and washing of cell populations with DMEM + 10% FCS + 100 units Pen/Strept/ml. These cells have been assessed histochemically and functionally in a previous study [22].

Macrophages or macrophages incubated with lipopolysaccaride (LPS) (Sigma Chemical, St. Louis, MO) 5 μg/ml, beta-carotene, canthaxanthin or carotenoids from an algae extract (350 μg/ml) were observed for production of tumor necrosis factor alpha (TNF-α) (24 h incubation). The pri-



Graph II

Table 1. *The relative percentage of tumor necrosis factor alpha following the administration of various agents to hamsters with oral squamous cell tumor*

Groups		Percentage (per 100 cells in a 40× field, three fields obs.)
I.	Untreated + DMBA-induced tumor	1.10 ± 0.35
II.	Sham-injected + DMBA-induced tumor	1.25 ± 0.42
III.	13-*Cis*-retinoic acid-injected + DMBA-induced tumor	3.60 ± 3.3
IV.	Canthaxanthin-injected + DMBA-induced tumor	19.6 ± 2.2
V.	*Spirulina*-injected + DMBA-induced tumor	68.8 ± 2.6
VI.	Beta-carotene-injected + DMBA-induced tumor	65.7 ± 3.5
VII.	Alphatocopherol + DMBA-induced tumor	54.5 ± 2.4

mary antibody was purchased from Endogen (Waltham, MA) (1:50) and then stained using PAP.

Controls included the substitution of the primary antibody with control serum (rabbit).

Positive control of PAP staining included the above incubation, with or without the coincubations with HCPC-1 tumor cells ($\times 10^4$ cells/ml) (Figs. 9, 10).

In addition a cytotoxicity assay with Na(^{51}Cr) radiolabelled HCPC-1 tumor target cells was performed.

Macrophages (5×10^5 cells) were incubated for 18 h with HCPC-1 tumor cells radio-labelled with sodium chromate-51 (10^4 cells). Specific lysis of tumor cells was assessed as follows

$$\% \text{ Specific lysis} = \frac{100\ (R_E - R_S)}{100 - R_S}$$

where: R_E = experimental release; R_S = spontaneous release.

The macrophages were found to be actively cytoxic to the tumor target cells; in this case an established carcinoma cell line derived from a chemically induced epidermoid carcinoma of hamster buccal pouch [23] (Fig. 11).

DISCUSSION

Those injected agents that significantly induced tumor regression, also stimulated large numbers of tumor necrosis factor alpha-positive macrophages to locate at the site of the degenerating, regressing tumors. Initial studies had shown this relationship with beta-carotene. This study confirms the earlier observation and also shows that alphatocopherol and an algae extract can stimulate tumor necrosis factor alpha. They are also capable of regressing established epidermoid carcinomas of hamster buccal pouch, following local injection into the tumor site.

One might hypothesize that the stimulation of TNF-α in macrophages at the tumor site may be a mechanism for the tumor degeneration and regression. Thus, beta-carotene, alphatocopherol and an algae extract rich in beta-carotene, other carotenes and alphatocopherol may induce tumor

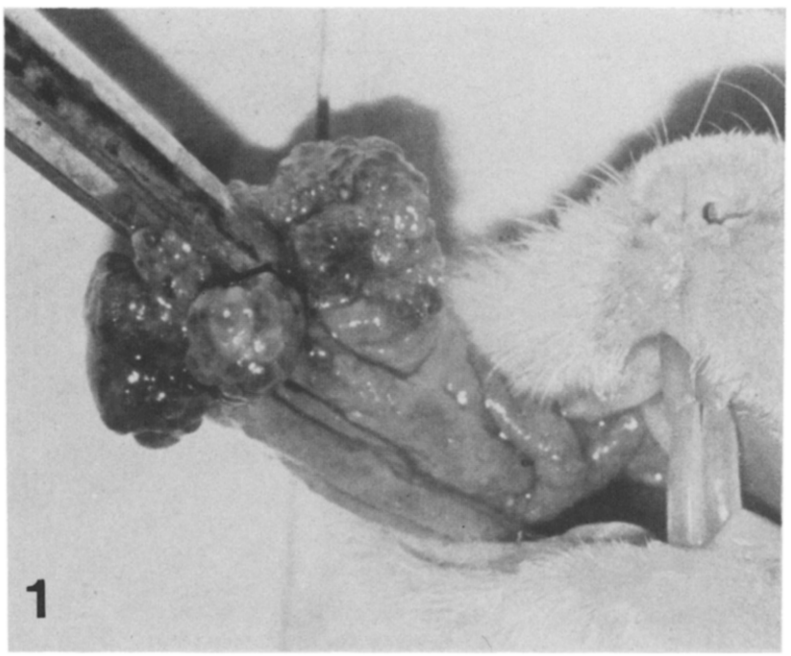


Fig. 1. *Large epidermoid carcinoma of right buccal pouch of hamster.*

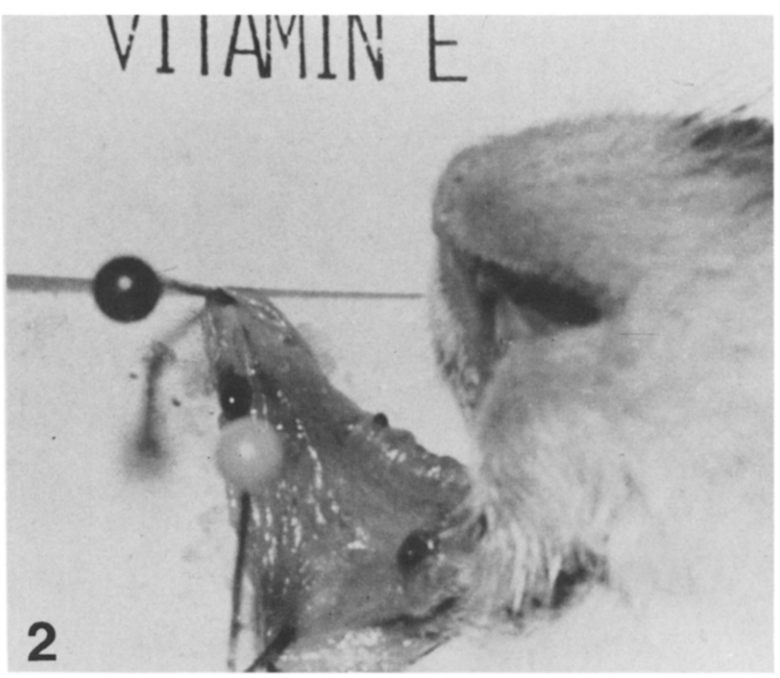


Fig. 2. *Regression of tumor in Fig.* 1 *after* 4 *weeks of injection with algae extract.*

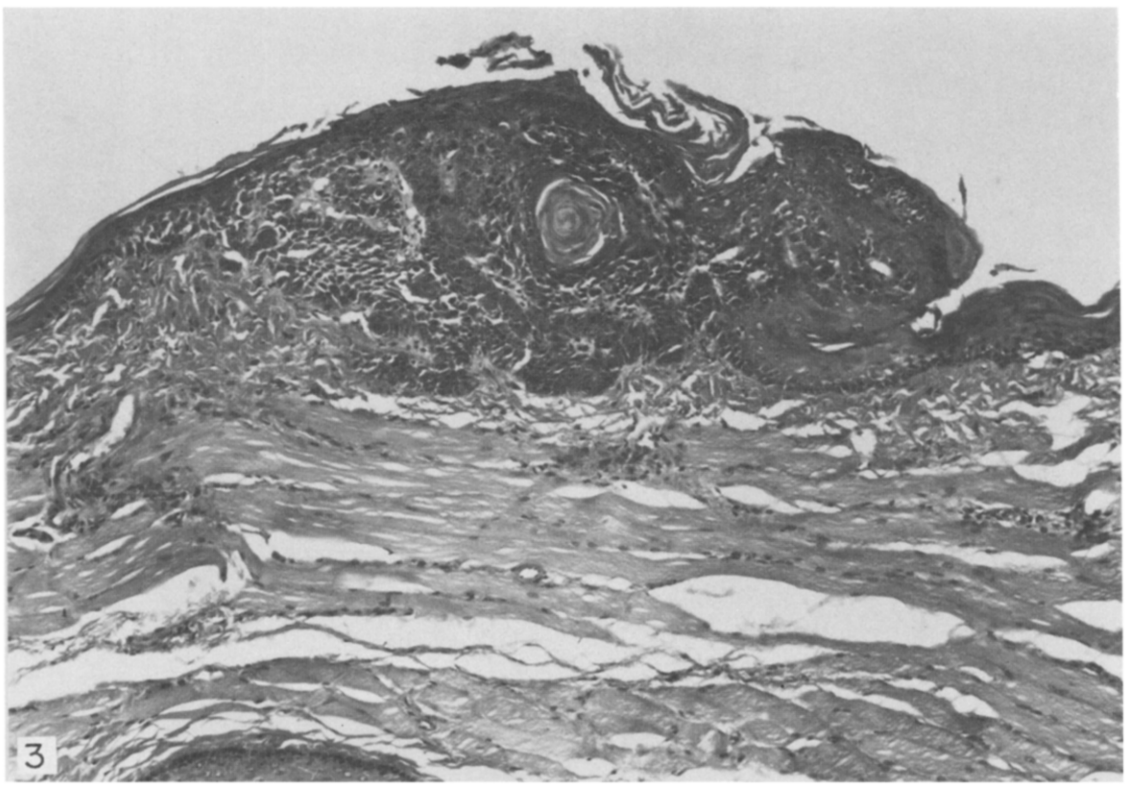


Fig. 3. Microscopic appearance of tumor undergoing regression in animal receiving algae extract (hematoxylin–eosin stain × 72).

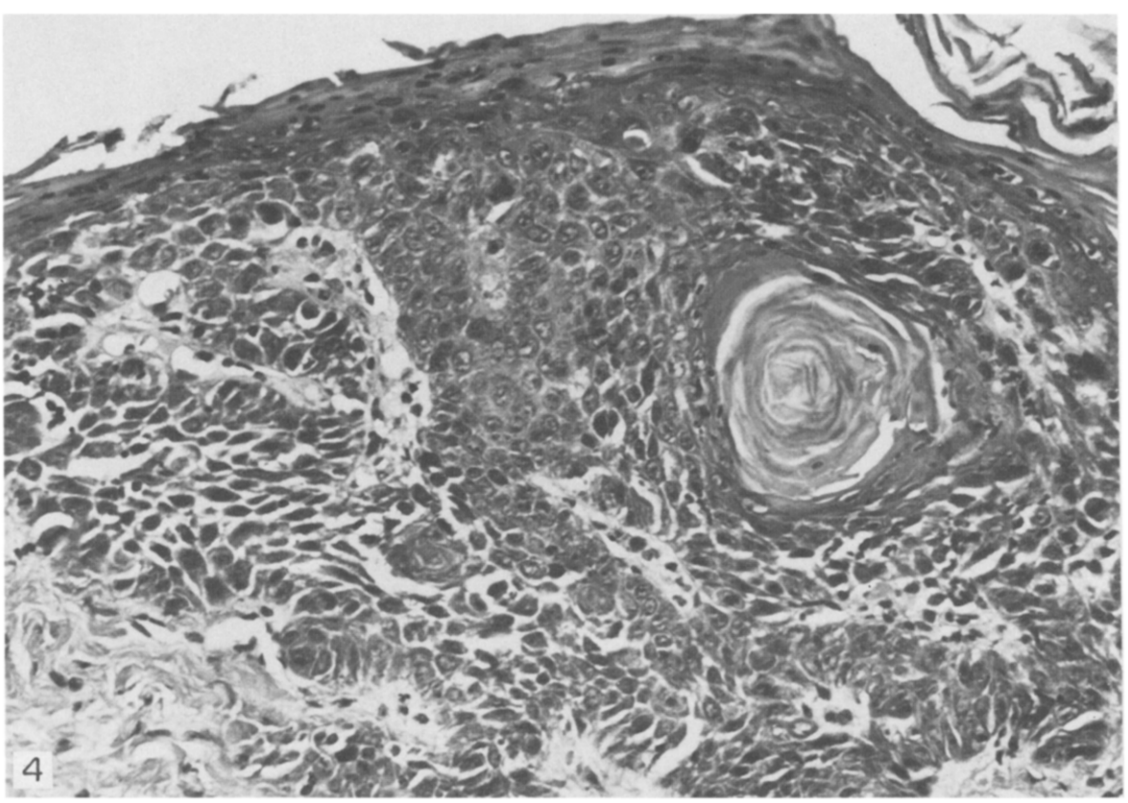


Fig. 4. High power view of Fig. 3 showing degenerating cancer cells and an infiltrate of mononuclear cells (hematoxylin–eosin stain × 180).

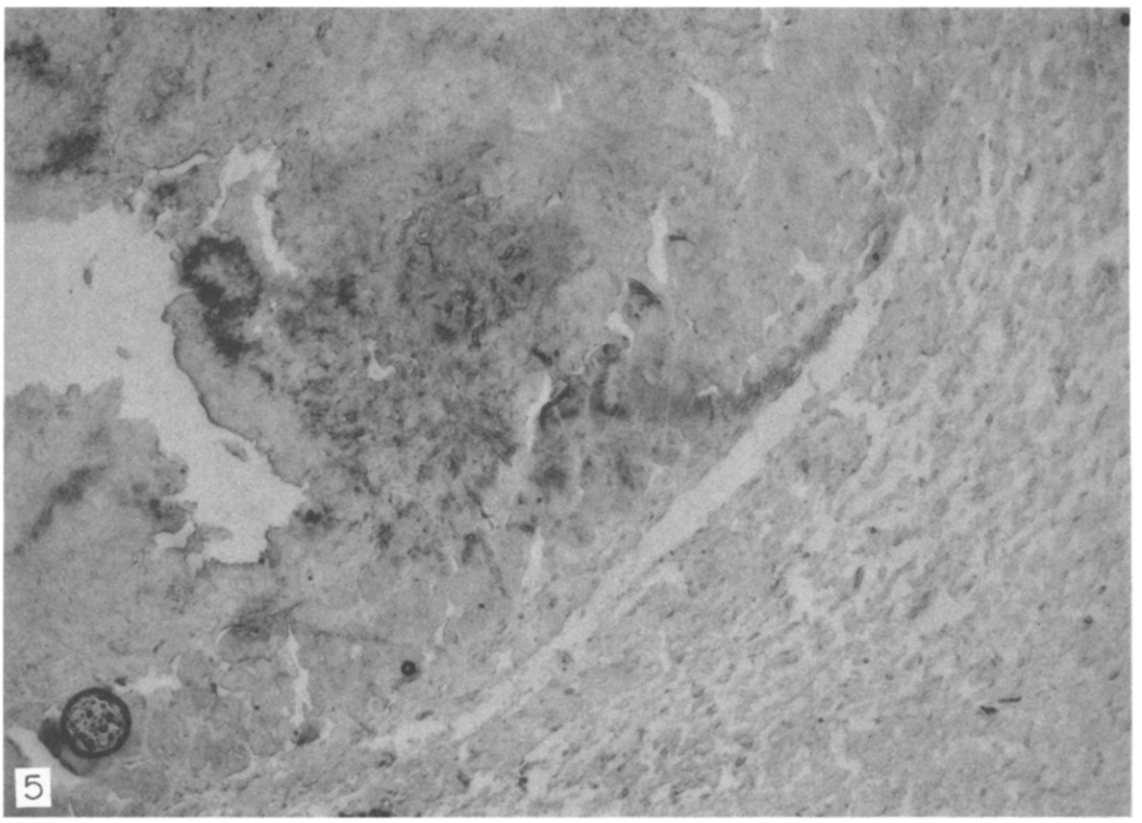


Fig. 5. Low power photomicrograph of epidermoid carcinoma of hamster buccal pouch showing an intense reaction for TNF-α (PAP technique; magnification × 90).

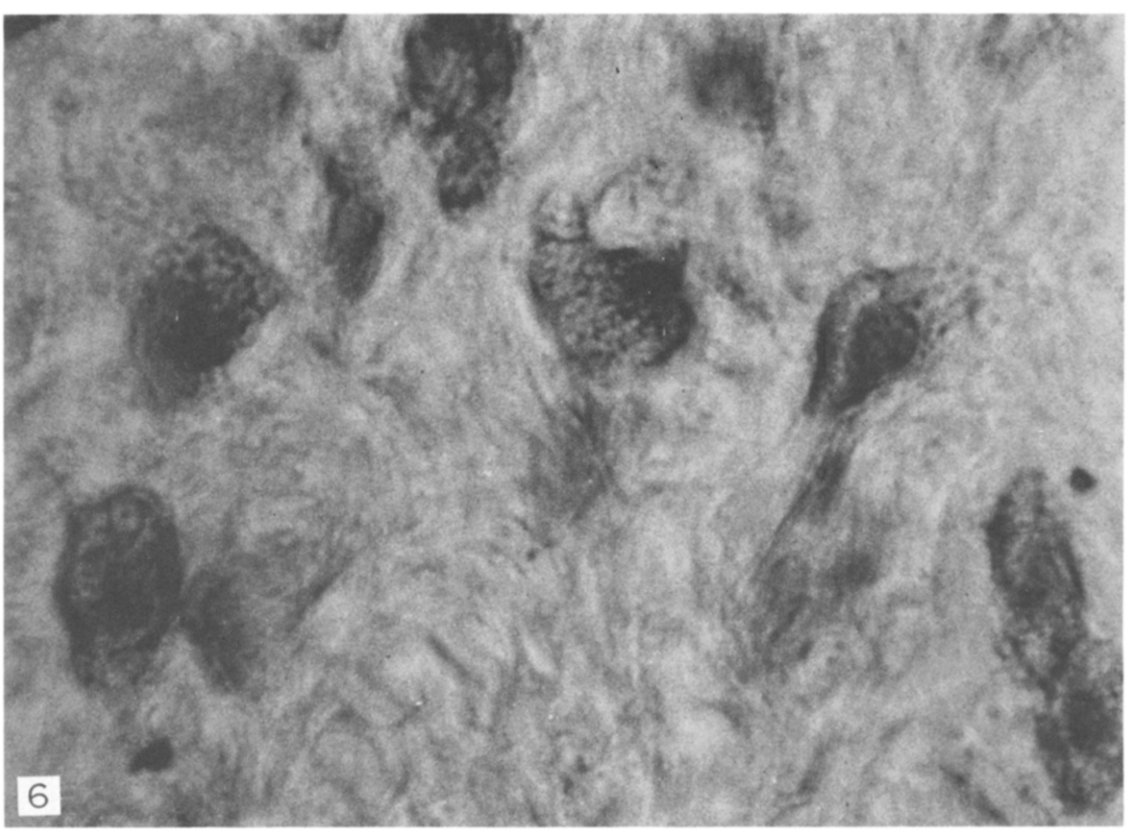


Fig. 6. High power view of Fig. 5 showing macrophages at tumor site containing brown granules of TNF-α (PAP technique; magnification × 720).

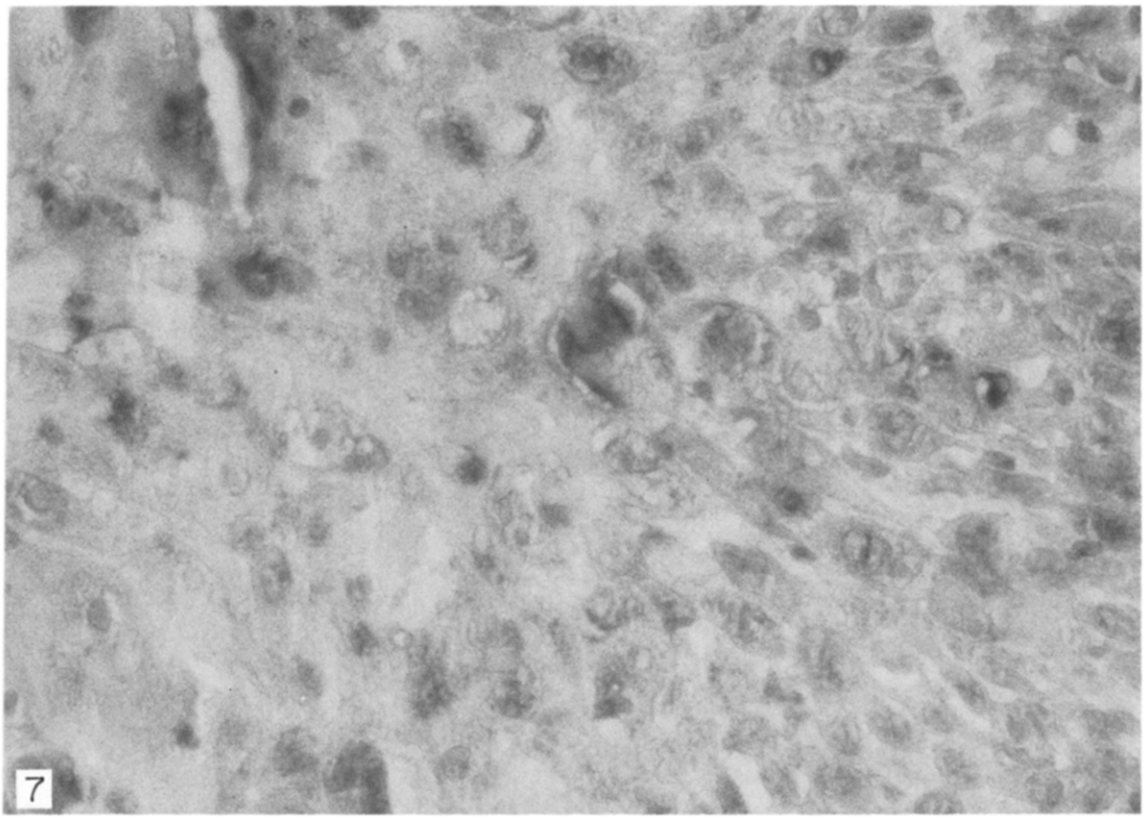


Fig. 7. Epidermoid carcinoma with numerous TNF-α positive macrophages (PAP technique; magnification × 315).

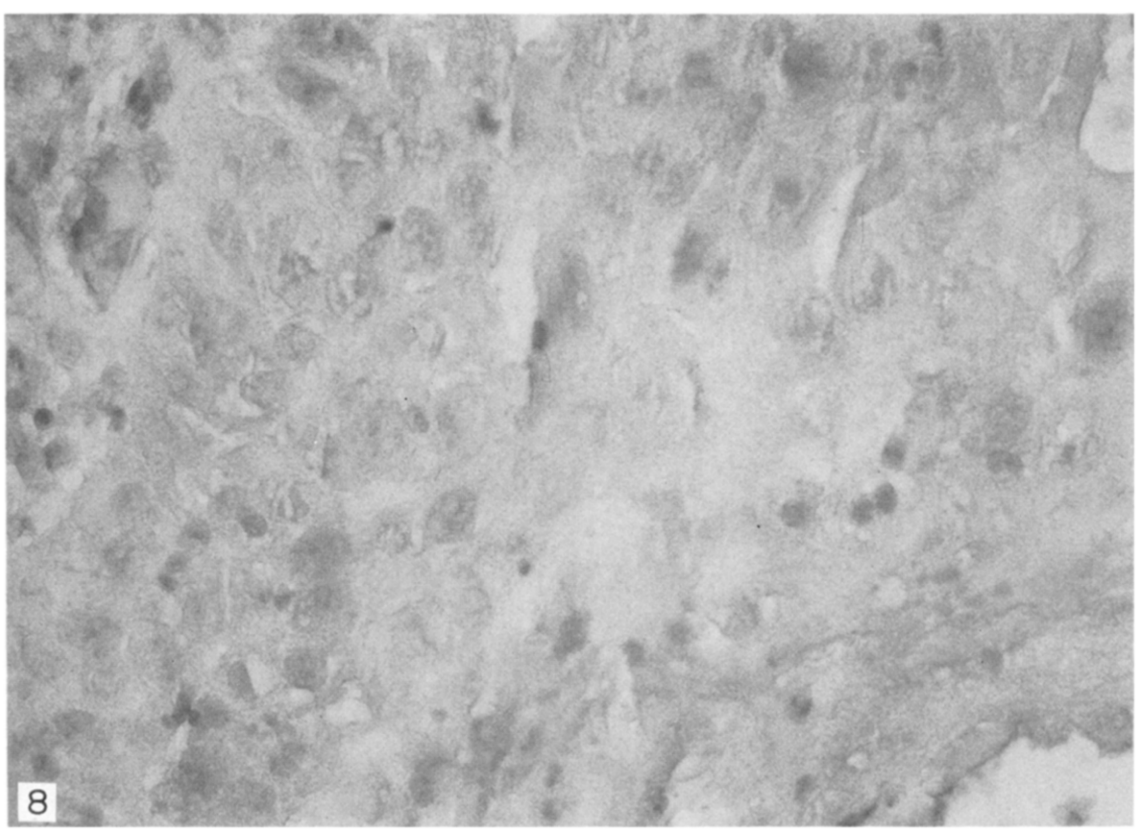


Fig. 8. Control section for Fig. 7 with TNF-α antibody replaced by hamster serum. No stain for TNF-α is seen.

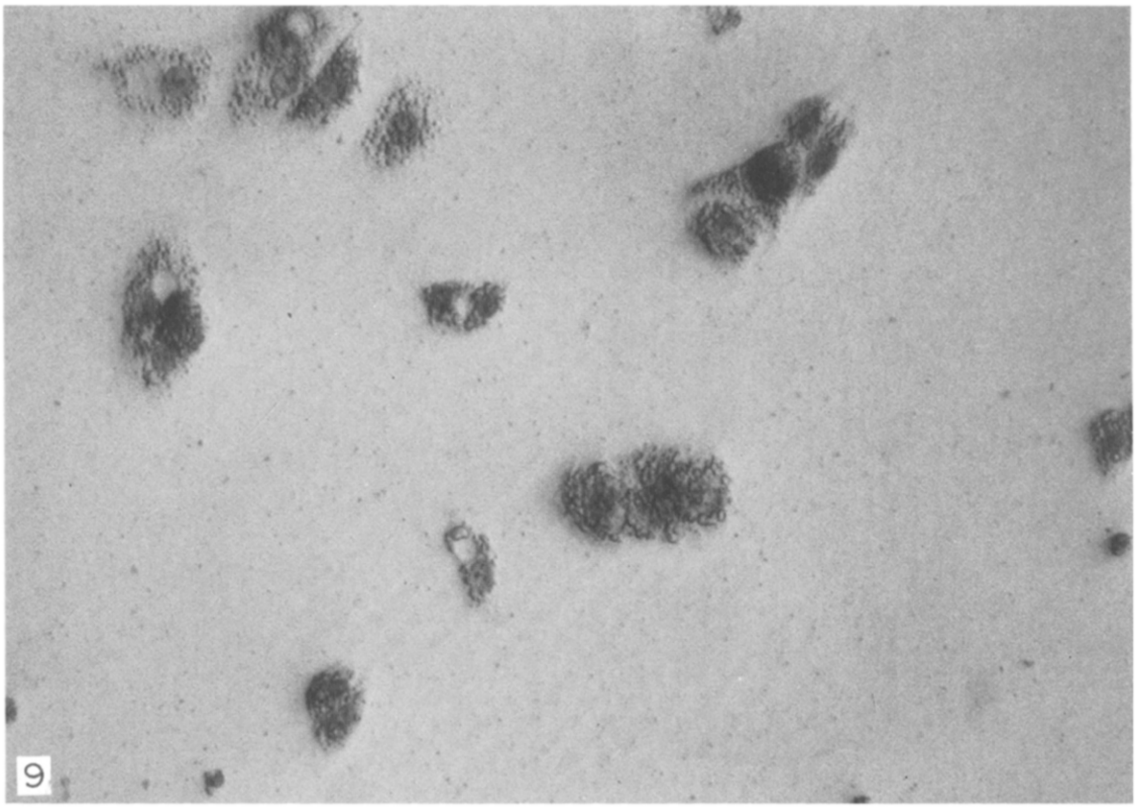


Fig. 9. *Macrophages* in vitro *stimulated by LPS to produce TNF-α (PAP technique; magnification* × 720).

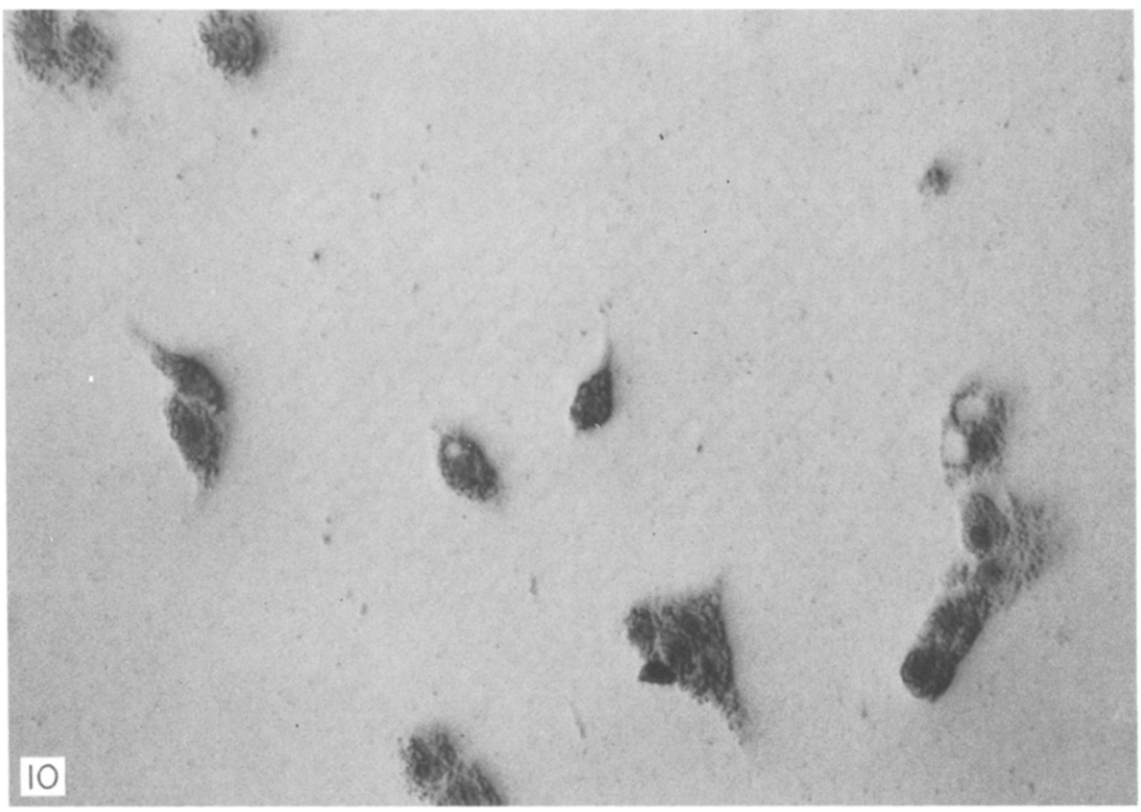


Fig. 10. *Macrophages* in vitro *stimulated by beta-carotene to produce TNF-α (PAP technique; magnification* × 720).

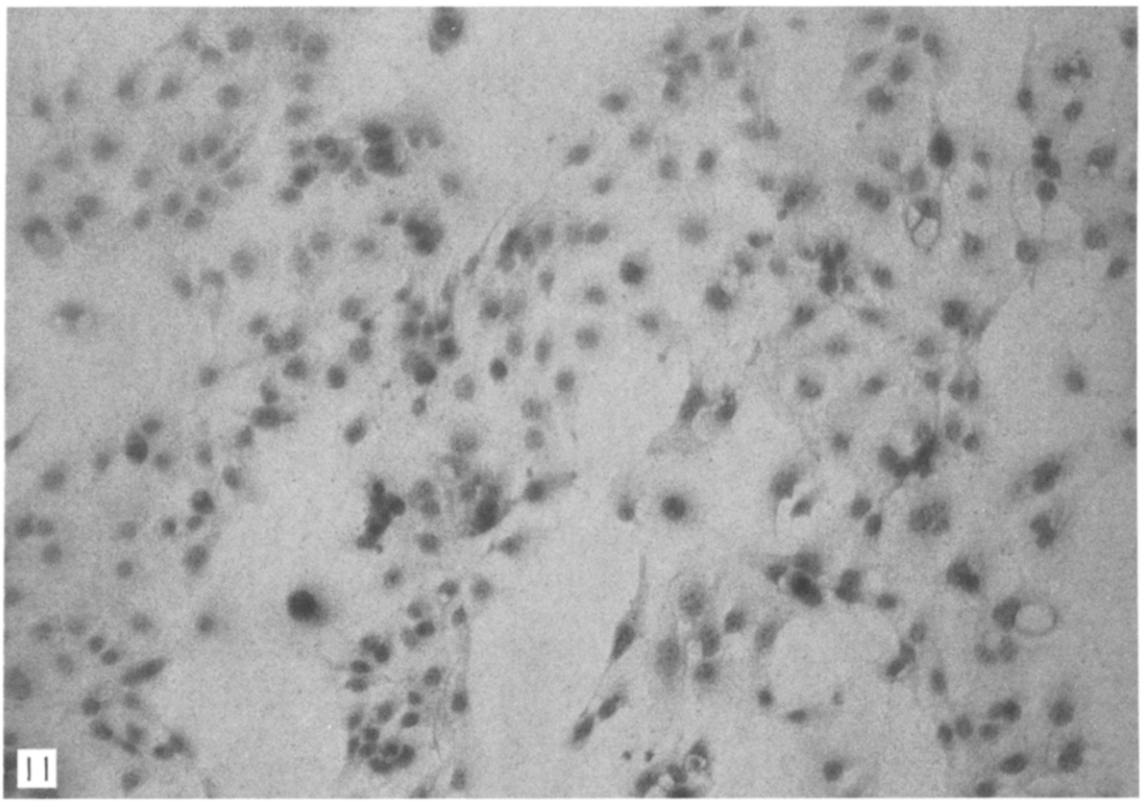


Fig. 11. *Macrophages positive for TNF-α lying upon a culture of HCPC* 1 *carcinoma cell line (PAP technique; magnification* × 225).

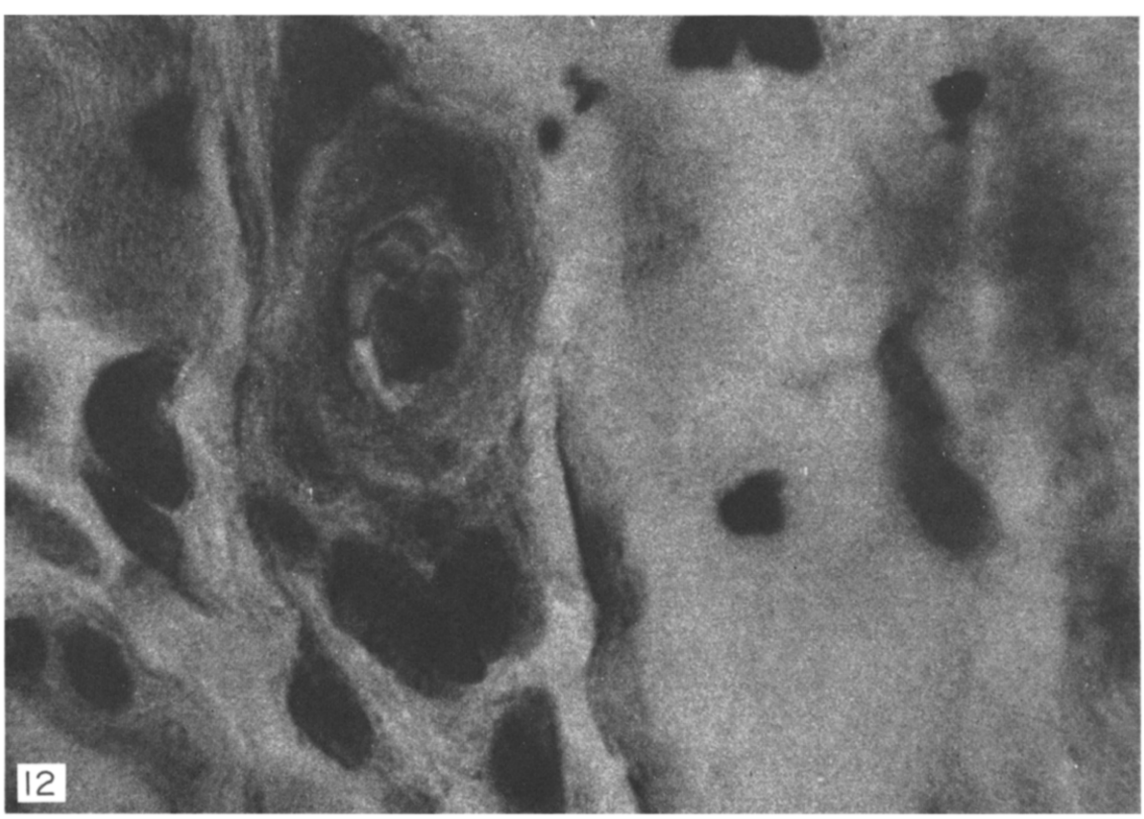


Fig. 12. *Macrophages positive for TNF-α oriented around a small arteriole within a hamster buccal pouch carcinoma (PAP technique; magnification* × 720).

regression by immunoenhancement. Since 13-*cis*-retinoic acid was only slightly effective, it could be assumed that beta-carotene, when injected locally into tissues, did not convert metabolically to retinoid. Furthermore, the carotenoid, canthaxanthin, which does not convert to retinoid, was significantly more effective than 13-*cis*-retinoic acid—both in stimulating TNF and in causing tumor regression.

Tumor necrosis factor or cachectin, as it has been referred to in its early history [24], is induced by bacterial endotoxin [25] or lipopolysaccharide [26]. It was found to be a product of mononuclear phagocytes in many different species [27, 28]. Its role as a potential antitumor agent was mentioned by Mannel *et al.* in 1980 [29], but Coley, as early as 1906, had found that malignant tumors could be treated by repeated inoculation of erysipelas toxin [30]. Necrosis of tumors was also reported by an endotoxin-induced serum factor [31] and by bacterial polysaccharide [32].

The results presented in this study demonstrate that macrophages can be activated *in vivo* to become cytotoxic to tumor targets. Previous studies have shown that elicited macrophage populations can be primed by gamma interferon (IFN-γ), and subsequently triggered by endotoxin, to express full cellular cytotoxicity [33, 34].

It seems likely the production of TNF-α by macrophages is enhanced with the secretion of INF-γ by activated T lymphocytes [35]. It is our assumption that the carotenoid or alphatocopherol acts in conjunction with activated T lymphocytes (producers of INF-γ) which prime the macrophages which then become tumoricidal upon triggering by the carotenoid or alphatocopherol and release TNF-α at the tumor site *in vivo*. This assumption is based upon preliminary studies *in vitro* (manuscript in preparation) in which cytotoxicity activity was observed following incubation of peritoneal-derived macrophages (>97% purified) with T cell conditioned media (Splenic T cell activation with concanavalin A 5 μg/ml 10 h), beta-carotene, canthaxanthin, and with or without LPS (5 μg/ml). The results indicated that while LPS incubation (5 μg/ml 20 h) can induce cytotoxicity by macrophages, the incubation with the carotenoids (350 μg/ml) was sufficient to induce a greater effect after 20 h of incubation.

In addition, it is important to note that in this study there was no obvious evidence of necrosis of normal tissue, perhaps indicating a selective targeting of tumor cells. These observations agree with the recent studies of Fletcher *et al.*, who show that gap junctional communication is associated with resistance to the cytotoxic action of lymphotoxin or TNF-α [36]. Therefore the triggering of TNF-α production by macrophages at the tumor site would most likely be directed to those tumor cells in the process of mitosis or spread.

The induction of tumoricidal activity (TNF-α positivity) by macrophages appears to be an early association event following carotenoid injection, for the observation has been made of TNF positive monocytes juxtaposed with numerous arterioles adjacent to the developing tumor site (Fig. 12).

Taken together, these results indicate that perhaps carotenoids or alphatocopherol which are relatively non toxic may be used to trigger immune responsiveness to growing tumours *in vivo* in a therapeutic manner.

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