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Bracken Fern Carcinogenesis: Multiple Intravenous Doses of Activated Ptaquiloside Induce DNA Adducts, Monocytosis, Increased TNFα Levels, and Mammary Gland Carcinoma in Rats

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Aims: **(1) establish a rat model for investigating ptaquiloside (PT) carcinogenesis via intravenous dosing; (2) determine the role of activated PT (APT) in this model; and (3) monitor changes at molecular (DNA adducts, TNFα levels) and cellular (histopathology) levels.** ***Methods:*** **Sprague-Dawley rats were dosed with PT or APT intravenously for 10 consecutive weeks. One group of animals was sacrificed immediately for TNFα and DNA adduct analyses. A second group of animals was kept alive for 30 more weeks to allow for tumour formation. Tissues were collected at the end of the experiment for histopathological studies.** ***Results:*** **Rats dosed with PT or APT showed marked increase in monocyte and TNFα levels. These levels remained high even 30 weeks after the last dosing. Analysis of DNA showed the presence of DNA adducts in APT-treated animals in target organs. In addition, 40% of APT-treated rats developed mammary gland carcinomas.** ***Conclusion:*** **This is the first study to demonstrate the potential of activated PT as a carcinogen *in vivo.* In addition, our findings suggest that PT exposure can be monitored using monocyte and TNFα levels.**

Key Words: **Ptaquiloside, monocytosis, DNA adduct, TNFα, adenocarcinoma.**

It is known that bracken fern (*Pteridium* spp.) produces a number of toxic effects in grazing animals including carcinomas of the upper alimentary tract and urinary bladder (1-4). The toxic syndromes induced by ingestion of large quantities of bracken are different in different species (5-7) and include anorexia, staggers and incoordination in horses, bright blindness due to the neuroepithelium degeneration in sheep and depression of bone marrow associated with leucopaenia, thrombocytopenia and hematuria in cattle. Rats fed on diets containing bracken developed multiple ileal, urinary bladder and mammary gland adenocarcinomas (1, 8 and 9).

The principle carcinogen in bracken is PT (Figure 1), whose reactivity is pH dependent. Under acidic conditions it undergoes aromatisation to give pterosine B (Figure 1). Under weakly alkaline conditions, PT converts into an unstable dienone, APT (10a). APT is known to alkylate adenines and guanines in DNA in a sequence selective fashion (11). However, the role of APT itself in the initiation of carcinogenesis is yet to be demonstrated. Recently we showed that bracken-fed calves harbored PT-DNA adducts which resulted in H-*ras* activation via mutation in codon 61 (12).

Rodents have also been shown to be sensitive to oral administration of PT. Depending on dose and time of exposure, multiple ileal, oesophageal and pharyngeal neoplasia, bladder and mammary gland neoplasms have been observed in rats (10b, 10c).

The present study was carried out using PT and APT to establish a tumour model in rats using intravenous dosing. The potential advantages of this mode of PT and/or APT dosing over dosing by other routes are: 1) the amount of toxin required is an order of magnitude lower, 2) the toxin reaches target organs (ileum, mammary glands and urinary bladder) with minimal loss of activity and 3) much of the toxin reaches target organs via direct blood circulation rather than first having to pass through the tissues of the gastrointestinal wall. The results presented here show that female Sprague-Dawley rats exposed to PT or APT via an intravenous tail vein led to monocytosis and a corre-

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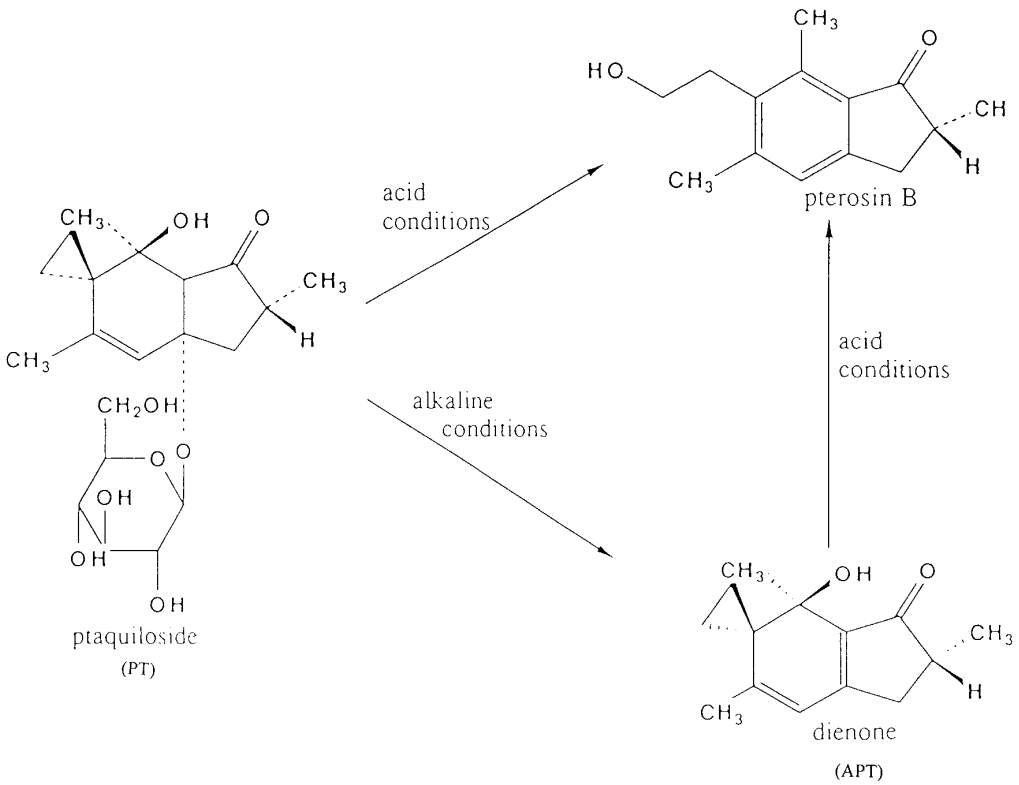

FIG. 1. Chemical structure of PT and formation of DNA adducts via its reactive intermediates.

sponding increase in TNFα levels. In addition, this is the first study to demonstrate the potential of activated PT as a carcinogen *in vivo*, as 40% of the APT-treated animals developed mammary gland adenocarcinomas within 40 weeks.

MATERIALS AND METHODS

The bracken fronds were collected from southeast Queensland (Maleny) and PT was isolated and purified using a published method (13). *Trans-anti* (±) benzo(a)pyrenediolepoxide (BPDE), which was used in the positive control, was obtained from Midwest Research Institute, USA. The enzymes, proteinase K, micrococcal nuclease, spleen phosphodiesterase, nuclease P1 and TLC plates were purchased from Sigma. Polynucleotide kinase (PNK) was obtained from Boehringer-Mannheim. The radiolabel γ-^{32}P-APT was obtained from Bresatec, Australia.

Animal Studies

Tumour studies. Two groups of 10 female Sprague Dawley rats (mean age, 6 weeks; mean body weight, 145 grams) were dosed over 10 weeks with 3 mg/week of PT or APT via an intravenous tail vein using saline buffer as a vehicle. Activation of PT to APT was carried out by incubation in 10 mM NaOH for an hour at 37°C. The solutions for dosing were then neutralized with HCl prior to dilution in the saline buffer. A control group receiving only saline buffer was also included in the study. The animals were kept alive for a further 30 weeks after the last dose (total 40 weeks) to allow for tumor formation. Animals were weighed weekly and were cared for in accordance with the Australian National Health & Medical Research Council ethical requirements. At the end of the experiment (40 weeks) all the animals were put down following carbon dioxide anesthesia.

DNA damage studies. One further group of 10 animals was dosed with 3 mg APT every week for 10 weeks. It is known from other studies that the repair of chemically induced DNA lesions in tissues occurs in a short time after the completion of last dosing (14). Therefore, in order to maximize the possibility of detection of such DNA lesions in the present study the animals were killed as early as 24 hours after the last dose. A control group of 10 rats dosed with vehicle only was also included in the study.

Peripheral blood was collected into EDTA fortnightly from a tail vein in order to measure total white blood cell (TWBC), differential leukocyte and platelet counts. TWBC count was performed using 0.5 volume of blood and 9.5 volumes of dilution buffer (1:20) using a Neubauer haemocytometer. The dilution buffer was made of 2% acetic acid to lyse the red blood cells. Blood smears were stained using a Commercial Diff Quick staining kit (Lab Aids, Australia).

Plasma samples were collected from individual rats at the end of the experiment and stored at −80°C for TNFα analysis (15). Gross pathology was recorded for liver, kidney, ileum, urinary bladder, lung, lymph nodes and mammary gland. Tissue samples were preserved in 10% buffered neutral formalin and were subsequently embedded in paraffin. Sections for pathological examination were prepared and stained with haematoxylin and eosin (H&E). DNA was extracted from ileum as described elsewhere (16).

DNA Adduct Analysis

DNA (20 μg) isolated from the ileum of each rat was used in the ^{32}P-postlabelling assay carried out as described elsewhere (12). Adducts were visualized using autoradiography. Each assay was performed in duplicate. A positive control in which calf thymus DNA

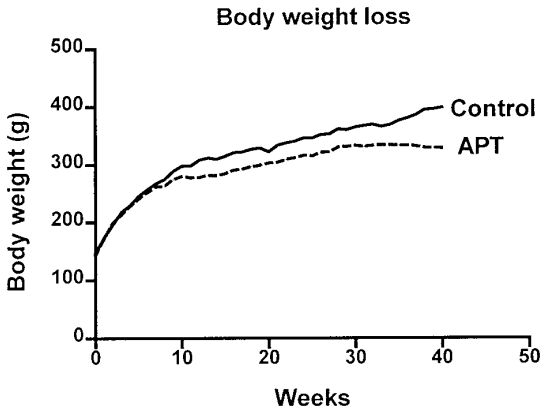

FIG. 2. Body weight loss by APT.

was adducted with BPDE (data not shown) and a negative (no DNA) control were routinely included in each assay.

RESULTS

Figure 2 shows the progressive body weight change in animals dosed with APT compared with controls. The PT - treated animals showed a similarly reduced weight gain at the end of the experiment (data not shown).

Monocytosis was evident in both PT and APT groups (Figures 3A and 3B). Monocytes increased after the first treatment and remained elevated even at the end of the experiment. Although the dosing was done on a weekly basis, blood for hematological examination was taken only fortnightly in order to minimize stress to the animals. Total leucocyte, polymorphonuclear leucocyte, lymphocyte and platelet counts showed no significant changes.

The levels of TNFα measured at end of the experiment were significantly higher in both PT- and APT-treated rats compared with controls (Figure 4) but the difference in the TNFα levels between the two treated groups were not statistically significant ($P > 0.05$).

Gross pathological examination showed that four out of 10 (40%) animals that received APT had enlarged mammary glands up to 1 to 2 cm, compared to unaffected glands which were less than 0.5 cm in diameter. These animals had macroscopically visible tumors and the tumour location was different in each animal (two in the inguinal area and one each in abdominal and thoracic areas). On histopathological examination they were found to be either mammary gland adenocarcinomas or papillary carcinomas (Figures 5A-C). The proliferating neoplastic cells replaced the normal epithelium of the acini, ductal and alveolar buds. Further they occupied the acinar spaces in which the lumens were not apparent (Figure 5A). In addition, macrophages and mononuclear cells infiltrated into the acinar epithelium and surrounded the area by connective fibrous

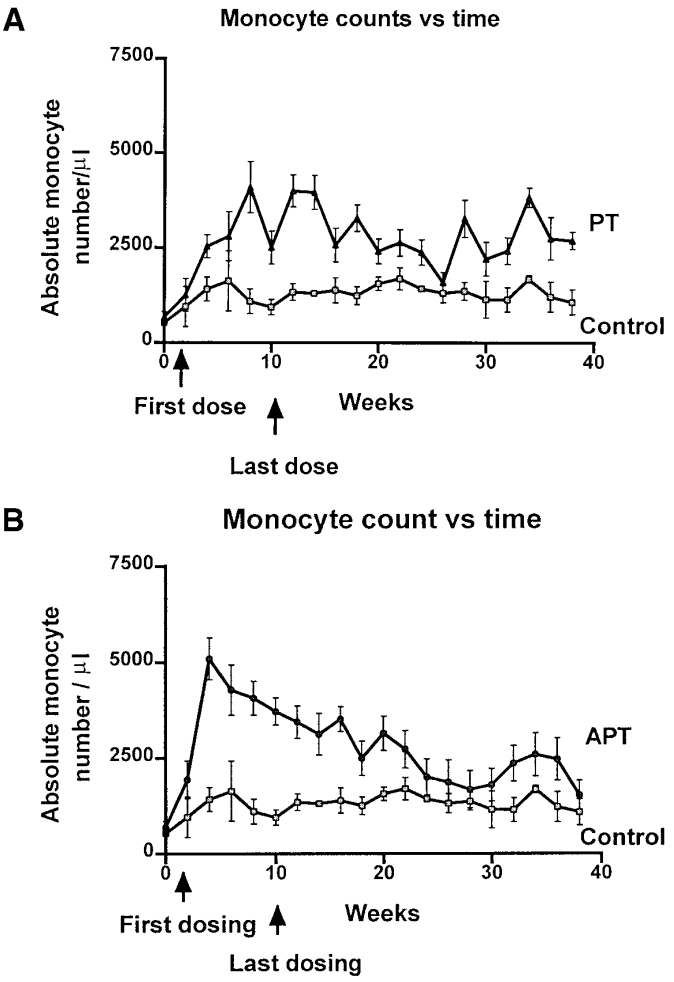

FIG. 3. Progressive monocyte counts for the duration of the experiment: (**A**) PT treated rats; (**B**) APT treated rats.

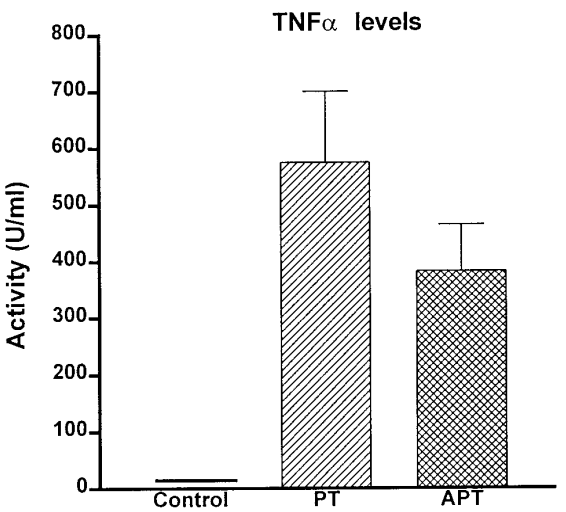

FIG. 4. TNFα levels in PT and APT treated rats.

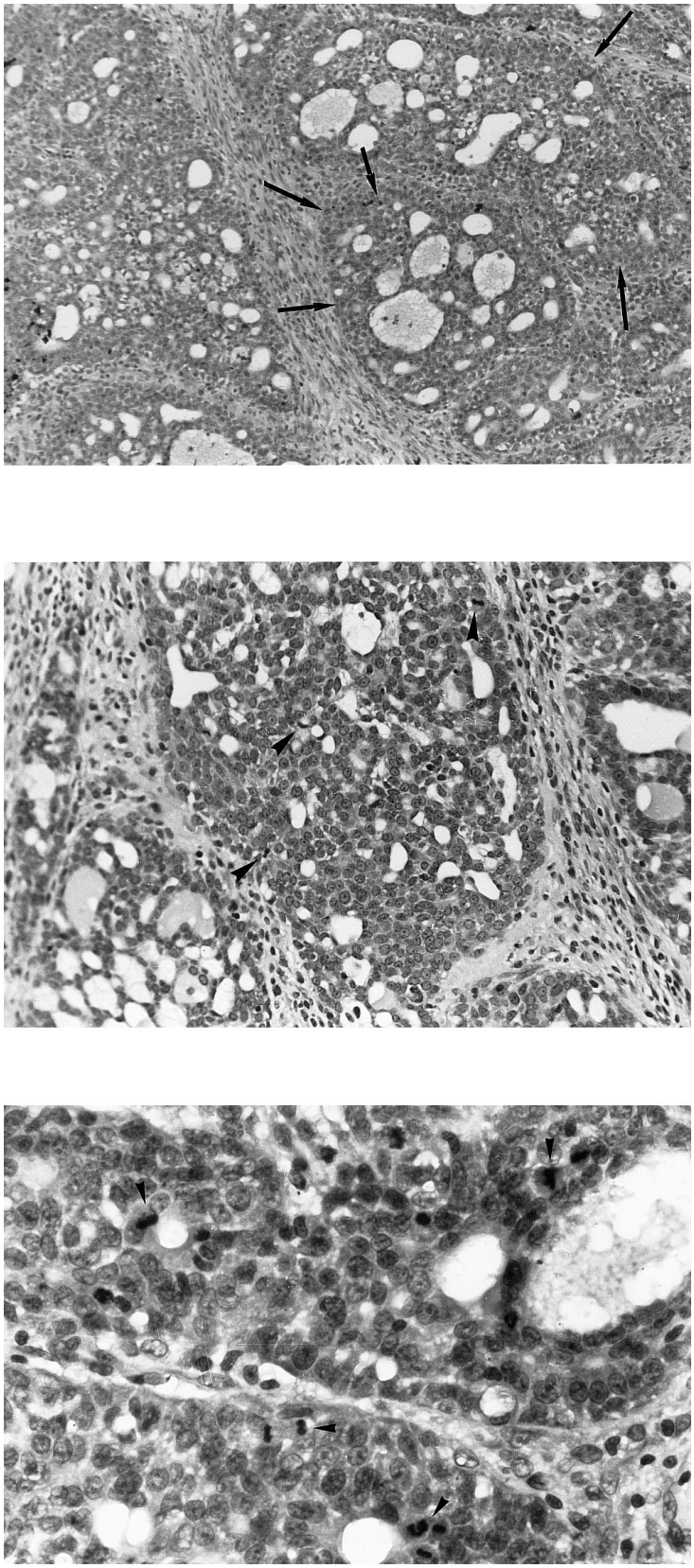

FIG. 5. Mammary gland adenocarcinoma. (**A**) Neoplastic cells occupy the mammary gland acini. The lumen is not visible. H&E × 95. (**B**) The acini, of irregular size and shape, are lined by well-differentiated columnar epithelial cells in which mitotic figures are common (see arrowheads). Also, acinar epithelium is heavily infiltrated by mononuclear cells. H&E × 190. (**C**) Higher magnification of the epithelium lining in the acini. Hyperchromatic tumour cells and mitoses are evident. (see arrowheads). H&E × 380.

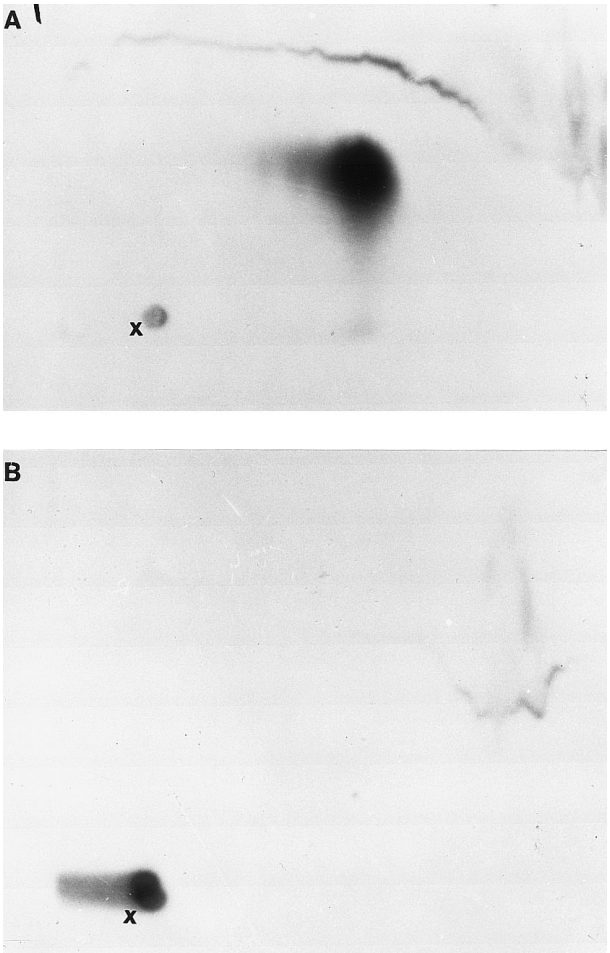

FIG. 6. ^{32}P-Postlabeling assay. Formation DNA adducts in the ileum of (**A**) APT-treated rat. The cross (**x**) indicates the point of sample application (origin). (**B**) Control rat.

tissues (Figure 5B) and the epithilium lining in the acini contained hyperchromatic tumor cells and mitotic figures (Figure 5C). The acini shown in these figures are in a mammary gland that is not lactating. In contrast normal mammary gland in control animals had acini containing a layer of cuboidal epithelium surrounded by mesenchymal layers and myeloepithelial cells (data not shown). In addition to mammary gland carcinoma, one of the APT treated animals also had an ileal adenocarcinoma. In contrast none of the PT-dosed rats and the control rats developed tumors.

^{32}P-postlabeling showed the presence of DNA adduct formation in the ileum of all the APT-treated animals which were sacrificed at the end of the dosing period (Figure 6A). The position of the spot on the TLC is chromatographically similar to that of the PT-DNA adduct formed *in vitro* as reported in earlier studies (10, 11,17,18). In comparison, control animals showed no spots (Figure 6B). Mammary gland and urinary bladder tissue samples could not be tested for DNA adducts because of insufficient quantities of tissue.

DISCUSSION

The role of APT itself in the initiation of carcinogenesis is yet to be demonstrated. We carried out the present study to: a) establish a rat model for PT carcinogenesis using the iv route and b) to directly evaluate the potential of APT to induce tumor in animals.

Our findings show that the intravenous route of administration does induce tumors in rats and hence is a satisfactory model for studying PT carcinogenesis. This is the first study to demonstrate the carcinogenic potential of the activated form of PT thus supporting its probable role in the mechanism of PT carcinogenesis.

It is believed that DNA adduct formation is an early event in the initiation of chemically induced carcinogenesis. Most of the adducts disappear within a few days after exposure to DNA damaging agents due to efficient DNA repair mechanisms present within cells (14). For that reason a separate experiment was set up in which a group of animals was sacrificed 24 hours after the last dose. The postlabeling assay showed that the ileum of all ten APT-treated rats contained DNA-adducts which chromatographically corresponded to the PT- DNA adducts observed *in vitro* (10, 11), providing further confirmation of the role of APT as the eventual metabolite involved in the DNA damage and subsequent initiation of PT carcinogenesis.

In contrast to the earlier studies with PT administered by the oral route over 218 days (total 9.4g) in Charles River S-D rats (12a) tumors were not observed with this compound in the present study. This may be due to the fact that the dose rate (total 30 mg) used in this study was 313 times less than in the earlier study. The dose rate was nevertheless sufficient to cause the development of mammary gland tumors in 40% of the animals when APT was used. However, only one in ten rats developed ileal tumors even with APT in contrast to a 100% incidence rate with PT in Hirono's study (12). The particular susceptibility of the mammary glands as seen in the present study may be due to the fact that terminal end buds (TEB) in the young virgin rat mammary gland have high mitotic and DNA synthetic activities (20). Carcinogens are believed to damage primarily the epithelium of the TEBs while they are developing into alveolar buds and terminal ducts (20).

An unexpected finding of this study is that both PT and APT treated animals exhibited high monocyte and TNFα levels during dosing, which persisted long after the last dose. Activated monocytes and macrophages release soluble cytotoxic molecules including TNFα, capable of lysing cells *in vitro* (21, 22). TNFα causes necrosis and regression of some animal tumors (23, 24). Peripheral blood monocyte and macrophages have been shown to exhibit tumoricidal activity both *in vitro* and *in vivo* (25, 26). The increase in labile circulatory serum TNFα found in this study is likely to be due to either a host response to the toxin or as part of the overall

process of tumorigenesis. Further studies in our laboratory are being directed towards developing the monocyte response and TNFα level as biomarkers of carcinogen exposure in both animals and humans.

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