

Piroxicam, indomethacin and aspirin action on a murine fibrosarcoma. Effects on tumour-associated and peritoneal macrophages

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SUMMARY

Growth of a methylcholanthrene-induced fibrosarcoma in BALB/c mice was accompanied by an increase in the activation state of tumour-associated macrophages (TAM), as measured by their FcIgG receptor expression, phagocytic index and β -glucuronidase levels. All of these parameters were markedly higher in TAM than in peritoneal macrophages (PM) derived from the same animal. On the other hand, PM from tumour-bearing mice showed lower activation parameters than PM from normal animals. We also studied the effect on tumour development of three inhibitors of prostaglandin synthesis: indomethacin, piroxicam and aspirin. Intraperitoneal administration of these drugs during 8 d was followed by the regression of palpable tumours. Indomethacin (90 mg/d) induced 45% regression, while with piroxicam (two 400 mg/d doses and six 200 mg/d doses) and aspirin (1 mg/d) 32% and 30% regressions, respectively, were observed. The growth rate of non-regressing tumours, which had reached different volumes by the end of the treatment, was delayed to a similar extent by the three anti-inflammatory non-steroidal drugs (NSAID). With respect to TAM, the treatment did not induce any significant change in their activation state, though both piroxicam and indomethacin increased slightly the TAM number. In contrast, NSAID administration was followed by a remarkable increase in the activation parameters of PM when compared with PM from tumour-bearing mice receiving no treatment. Indeed, these parameters were in some cases higher than those of PM from normal mice. The leukocytosis (60 000/ μ l) with neutrophilia (80%) induced by tumour growth on peripheral blood leukocytes (PBL) was reversed by the treatment to values close to normal, in parallel with the reduction of tumour size. A drop in haematocrit was also noted which was most probably a consequence of tumour growth rather than of the treatment. This study reveals that the three NSAID tested have a remarkable antitumour activity, which correlates with the restoration of PM activity and PBL values.

Keywords anti-inflammatory drugs macrophages tumour growth

INTRODUCTION

A large body of evidence indicates that metabolites of arachidonate, especially prostaglandins of the E series, are directly involved in the initiation, promotion and progression of tumoural processes (Fischer, 1985; Trosko *et al.*, 1985; Fletcher, 1989) or indirectly by their negative feed-back effect on the immune response and mielopoiesis. Through these activities these metabolites would influence tumour invasion, metastasis, angiogenesis, osteolysis, etc. With respect to the immune response, prostaglandins, especially E_2 , would have a pan-suppressor effect, hindering blastogenesis and the antitumour cytotoxicity of T cells (Young & Hoover, 1986; Myers, Hanafin

& Schook, 1989), NK, lymphokine activated killer and macrophages (Imir, Sibbit & Bankhurst, 1987; Parhar & Peeyush, 1988). This effect would be exerted through the inhibition of the IL-1 synthesis by macrophages (Kunkel, Chensue & Phan, 1986; Bernheim, 1986; Otterness *et al.*, 1988), inhibition of the antitumour activity of these cells by interferon- γ (IFN- γ) (Schultz, 1983), inhibition of IL-2 synthesis and IL-2 receptors from T cells (Rappaport & Rodgi, 1982), inhibition of macrophage/granulocyte colony-stimulating factor (MG-CSF) and CSF-1 (Kurland *et al.*, 1978; Vore *et al.*, 1989) and generation of T suppressor cells (Elmasry, Fox & Rich, 1987).

Prostaglandins could be produced by neoplastic cells at levels much higher than normal cells (Trosko *et al.*, 1985; Fischer, 1985; Lau *et al.*, 1987) and also by cells from the macrophage-monocyte series (Hardy & Balducci, 1986; Elmasry *et al.*, 1987; Myers *et al.*, 1989). The activity of the latter, as well as that of other cells of the immune system is

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regulated by prostaglandins (Parhar & Peeyush, 1988). Since high concentrations of these substances disrupt the immune system homeostasis they might well be a contributing factor in tumour development. In this connection, there is convincing evidence for the antitumour effects of cyclooxygenase inhibitors. Thus, experimental models have shown that chronic oral administration of indomethacin impedes mammary tumour development (Fulton, 1984). Piroxicam inhibits colon carcinogenesis (Reddy, Masuyama & Kelloff, 1987) and indomethacin prevents tumour metastases from mammary carcinomas (Lalal, Parhar & Singh, 1986). When employed in humans, piroxicam induces either tumour regression or a slowing of the growth rate of pulmonary metastases from a variety of tumours (Breau, Morere & Israel, 1989). Indomethacin inhibits the growth of the Desmoid tumour (Klein *et al.*, 1987) and of a hepatocellular carcinoma with hypercalcaemia (Ikeda *et al.*, 1988), to list just two of the cases studied.

We report here a comparative study on the effects of three classical inhibitors of cyclooxygenase, indomethacin, aspirin and piroxicam, on the progression of a transplantable fibrosarcoma induced by methylcholanthrene (MCA) in mice. Since macrophages are the immune and inflammatory cells that predominate within neoplasias, either experimental or in humans (McBride, 1986; Wei *et al.*, 1986), we have chosen to evaluate the activation state of both tumour-associated (TAM) and peritoneal macrophages (PM) in mice, either treated or untreated with these drugs. To this end, we measured the β -glucuronidase content, phagocytic index and FcIgG receptor expression in these cells. Additionally, quantitative alterations of peripheral blood leukocytes (PBL) induced by both tumour growth and treatment with non-steroidal anti-inflammatory drugs (NSAID) were examined.

MATERIALS AND METHODS

Mice

Inbred BALB/C mice aged between 5 and 10 weeks were used. Each experimental group consisted of 40–50 mice.

Tumour

The tumour used was a fibrosarcoma induced by s.c. implantation of MCA crystals. It was maintained by serial passages *in vivo*. Transplants were made by s.c. inoculation of 5×10^5 viable tumour cells into the lateral left flank. Experiments were performed from the sixth transplant onwards.

Measurement of tumour size

Tumour growth was evaluated by caliper measurement of tumour length and width. Tumour volume was determined using the formula proposed by Attia & Weiss (1966), $V = 0.4 \cdot d^2 \cdot D$, where V = tumour volume in cm^3 , D and d = longest and shortest diameters, respectively. The tumour reached a volume of 0.05 cm^3 after approximately 10 d, being at this stage perfectly palpable. By days 20–25 it was around 5 cm^3 in diameter. Mice died between 30 and 40 d following inoculation of tumour cells.

Treatment with cyclooxygenase inhibitors

Once tumours had reached a volume of 0.05 cm^3 the animals were divided into four groups. One group was inoculated with $90 \mu\text{g/d}$ indomethacin (IM 75; Montpellier Laboratory, Argen-

tina). Another group received 1 mg/d aspirin (Egalgic, Armstrong Laboratory, Argentina) for 10 d, except on days 6 and 7. A third group was inoculated with piroxicam (Solocalm, Microsules and Bernabo Laboratory, Argentina) according to the following schedule, taking into account that doses of $400 \mu\text{g/d}$ may have detrimental effects on the general health of mice: day 1, $400 \mu\text{g}$; days 2, 3 and 4, $200 \mu\text{g}$; day 5, $400 \mu\text{g}$; days 8, 9 and 10, $200 \mu\text{g}$. All inoculations were i.p. in 0.1 ml volumes.

A fourth group received no treatment and served as control. Mice were sacrificed at 5 d after the end of treatment, when the volume of control tumours was large ($> 5 \text{ cm}^3$). Evaluation of tumour growth was made over a 12-d period from the onset of the treatment. Tumour size was recorded every 4 d and they were classified into the following five categories according to their volume: regressing, $< 0.05 \text{ cm}^3$; initial, $= 0.05 \text{ cm}^3$; small, $0.05 - > 0.5 \text{ cm}^3$; medium, $0.5 - > 1.35 \text{ cm}^3$; large, $> 1.35 \text{ cm}^3$.

Preparation of cell suspensions

Tumour cell suspensions were obtained by enzymatic digestion of tumours with HBSS containing 0.05% protease type XXV, 0.05% collagenase type IV and 0.001% deoxyribonuclease type I (all enzymes were from Sigma).

Peritoneal cell suspensions were prepared by vigorous lavage of the peritoneal cavity with 5 ml HBSS containing 10 U/ml Heparine (Abbott Laboratories, Wiesbaden, Germany).

Quantification of TAM

The percentage of macrophages in tumour cell suspensions was determined by erythrocyte-antierythrocyte (EA) rosette formation. This is the method of choice for the identification of TAM, since the FcIgG receptor appears in an early stage of their maturation and displays in these cells a higher activity than in other cells (McBride *et al.*, 1982; McBride, 1986).

Preparation of macrophage monolayers

Peritoneal or tumour cell suspensions at concentrations of 10^6 and 10^7 cells/ml, respectively, were prepared in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma) and $50 \mu\text{g}$ gentamicin/ml. Macrophages from 1 ml of either suspension were isolated by allowing them to adhere to a glass surface. In the case of tumour cell suspensions, 20% FBS and 0.05% protease were added to prevent adherence of tumour cells (McBride *et al.*, 1982).

Phagocytosis test

FITC-labelled *Candida albicans* (5×10^6) opsonized with mouse autologous serum were resuspended in 1 ml of RPMI-1640 medium containing 10% FBS and $50 \mu\text{g}$ gentamicin/ml and added to a monolayer of macrophages. Phagocytosis was allowed for 60 min at 37°C . Monolayers were rinsed with HBSS and the phagocytic index was determined with a Leitz fluorescence microscope.

Cytochemical assay of enzymes

β -glucuronidase activity was evidenced in smears of cell suspensions and in macrophages isolated from them with kit 180-C (Sigma).

Peripheral blood leukocytes

Blood samples from tumour-bearing mice, either inoculated with NSAID or non-inoculated, and from normal mice were

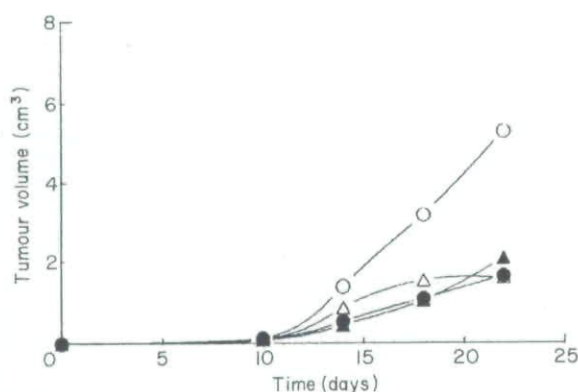


Fig. 1. Growth curve of the tumour in mice, untreated (control) (○) and treated with indomethacin (●), piroxicam (△) and aspirin (▲) as indicated in Materials and Methods. Values are the means of tumour volumes for 20–30 animals in each group.

collected by tail incision at days 4, 10, 17 and 27 after tumour passage. Samples were diluted in Turk solution for total leukocyte counting and smears stained with Giemsa solution to differentiate between granulocyte, lymphocyte and monocyte populations.

Statistical analysis

Statistical analysis of data was performed using the Student's *t*-test.

RESULTS

Effect of the treatment on tumour growth

The percentages of mice showing tumour regression at the end of treatment with cyclooxygenase inhibitors were as follows: indomethacin, 45%; aspirin, 32% and piroxicam, 30%. For the remaining animals, in which tumours did not regress, average volumes as a function of time are shown in Fig. 1, which shows the remarkable delay in tumour growth rate in the treated groups and also that the effect of the different drugs was similar.

Percentage of EA rosettes in tumour cells (TAM) and peritoneal cells

The percentage of TAM in tumour cell suspensions increased significantly only in piroxicam-treated mice (Fig. 2a). Peritoneal cells from mice treated with the three NSAID displayed a significant increase in the percentage of rosettes, when compared with untreated mice, while no differences existed between treated and normal animals (Fig. 2b).

Phagocytic capacity of TAM and PM from mice treated and untreated with NSAID

No significant difference was noted between the phagocytic indexes of TAM from treated and control mice (Fig. 3a). With regard to PM, only the group treated with piroxicam showed an important increase in their phagocytic index. For all groups, with the exception of that treated with piroxicam, the phagocytic index of TAM was higher than that of PM (Fig. 3b).

Study of the cell β -glucuronidase content

Treatment with the three cyclooxygenase inhibitors was not followed by changes in β -glucuronidase content, either in tumour cell suspensions or in TAM isolated from them (Fig. 4a).

In contrast, treatment with any of the drugs increased the enzymatic levels in total peritoneal cells and PM derived from them (Fig. 4b).

For the treated groups, the β -glucuronidase content of macrophages (TAM and PM) was similar. In the control group, the enzymatic level of PM was lower than in TAM and PM from the normal group.

Effect of tumour growth on PBL

The influence of tumour growth on PBL was explored, starting at day 4 after tumour cell implantation. Leukocytes were augmented ($12000 \pm 300/\mu\text{l}$) with a concomitant neutrophilia ($31 \pm 7\%$) and a relative decrease of lymphocytes ($60 \pm 17\%$). Normal values were as follows: leukocytes, $5700 \pm 640/\mu\text{l}$; neutrophils, $20.5 \pm 5.5\%$; and lymphocytes, $74 \pm 4.8\%$. By day 27 after tumour implantation, when it had reached the stage of a large tumour, the leukocyte number was $63500 \pm 14200/\mu\text{l}$, with $80 \pm 4\%$ neutrophils and $17 \pm 3.4\%$ lymphocytes.

Summarizing, tumour growth induced an increase mainly in the absolute number of neutrophils (see Fig. 5).

Effect of treatment with NSAID on PBL

Five days after the end of treatment with each of the cyclooxygenase inhibitors, animals bearing regressing tumours showed a significant decrease in leukocytosis, with a percentage drop of granulocytes and a relative increase of lymphocytes. In the rest of the tumours (small, medium and large), the leukocytosis and percentage of granulocytes rose proportionally to the tumour volume (Fig. 6).

Effect of tumour growth and treatment on the hematocrit

Tumour growth reduced the hematocrit with regard to normal mice ($58 \pm 4.3\%$). In fact, values dropped to $50 \pm 4\%$ by the middle of tumour growth and finally to $34 \pm 4\%$ when the tumour was fully developed. Regarding the effect of treatment on hematocrit, average values observed on day 5 after treatment was completed were as follows: R = $40 \pm 10\%$; S = $39 \pm 5\%$; M = $45 \pm 7\%$; L = $35 \pm 6\%$.

DISCUSSION

We have previously demonstrated that PM from fibrosarcoma-bearing mice display a lower activation state than TAM, as indicated by their β -glucuronidase content, phagocytic index, FcIgG-receptor expression and cytotoxic activity (Valdéz *et al.*, 1990). In the present report, we have compared both macrophage populations with PM from normal mice. These latter showed a basal activation level which was higher than the activation state of PM from tumour-bearing mice and lower than that of TAM. This correlates well with other studies also performed on a MCA-induced fibrosarcoma in BALB/c mice, in which tumour growth was shown to reduce the markers 2⁺, 3⁺ and Ia⁺ in PM with respect to PM from normal mice. This was interpreted as an influx into the peritoneum of immature macrophages (Yurocho, Pyle & Elgert, 1989). It was noted that cancers, both human and experimental, reduce monocyte-macrophage activity (Snyderman *et al.*, 1976; Obrist, 1987; Fletcher, 1989).

Tumours produce high levels of prostaglandins (Trosko *et al.*, 1985; Fischer, 1985), which down-regulate IL-1 synthesis, class II antigens of the major histocompatibility complex

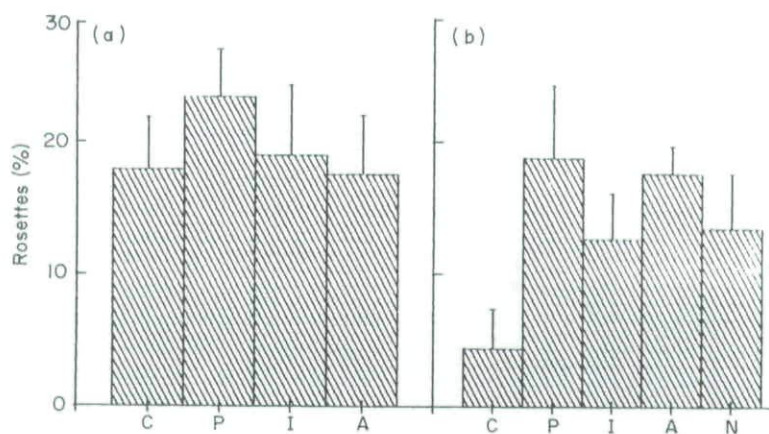


Fig. 2. Erythrocyte-antierythrocyte rosette formation in (a) tumour cell suspension and (b) peritoneal cell suspension from: untreated (control) mice (C), normal mice (N) and mice treated with piroxicam (P), indomethacin (I), and aspirin (A). Values represent the mean of the percentage of rosettes \pm s.d. from five experiments. Each experiment was performed with cells pooled from four mice.

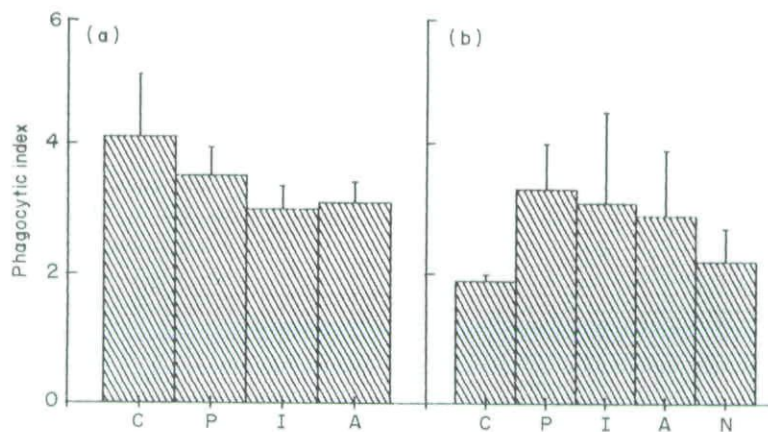


Fig. 3. Phagocytic index of (a) tumour-associated macrophages and (b) peritoneal macrophages from: untreated (control) mice (C), normal mice (N) and mice treated with piroxicam (P), indomethacin (I), and aspirin (A). Values are the mean of the phagocytic index \pm s.d. from five experiments. Each experiment was performed with cells pooled from four mice.

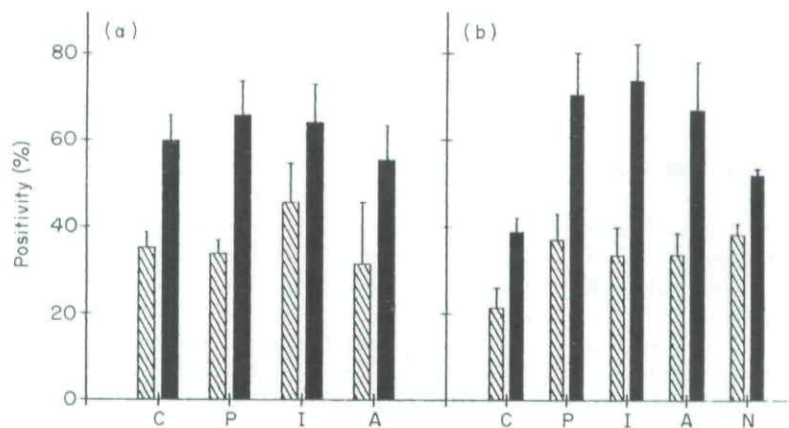


Fig. 4. β -glucuronidase assay. (a) Total tumour cells (▨) and tumour-associated macrophages (■); (b) total peritoneal cells (▨) and peritoneal macrophages (■) from: untreated (control) mice (C), normal mice (N) and mice treated with piroxicam (P), indomethacin (I), and aspirin (A). Values are the mean \pm s.d. from five experiments. Each experiment was performed with cells pooled from four mice.

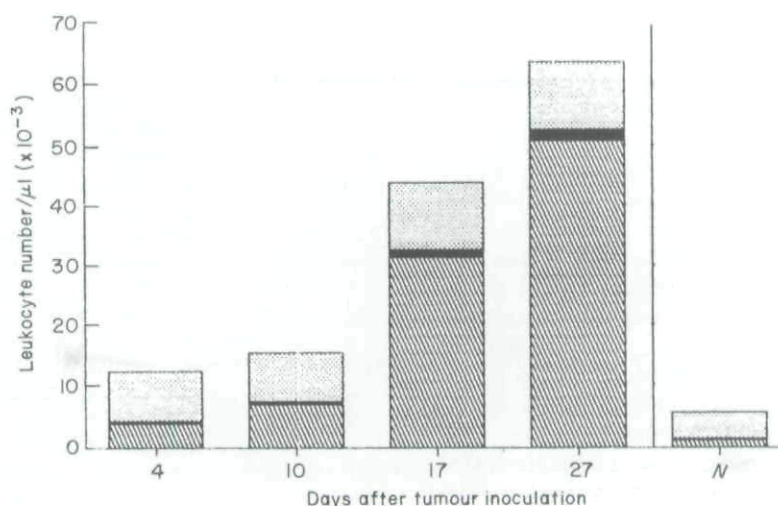


Fig. 5. Changes in leukocyte count and in the percentage of granulocytes (▨), monocytes (■) and lymphocytes (□) with tumour growth. N, values corresponding to normal mice. Each bar is a mean of five experiments.

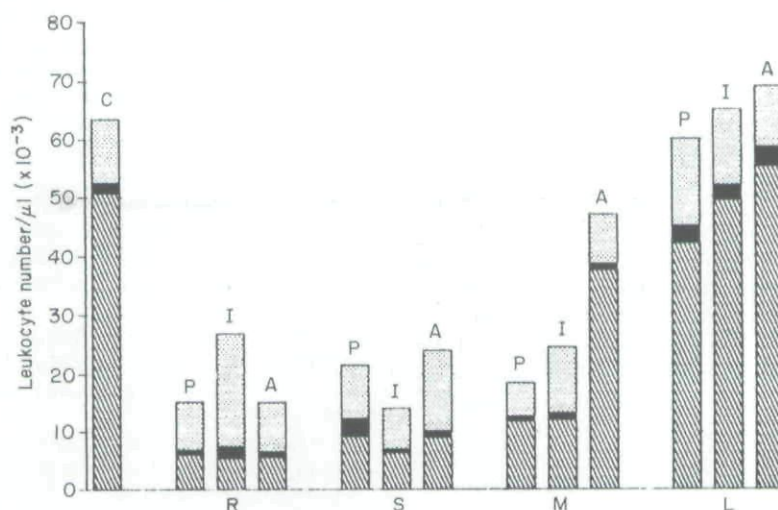


Fig. 6. Leukocytosis and percentages of granulocytes (▨), monocytes (■) and lymphocytes (□) in control (untreated) mice (C) and in mice treated with piroxicam (P), indomethacin (I) and aspirin (A). Experiments were performed for each tumour volume on day 5 after the end of treatment. R, regressing; S, small; M, medium and L, large. Values represent the mean of five experiments.

(Snyder, Beller & Unanue, 1982; Goldyne, 1986) and exert a suppressor effect on immune cells with antitumour activity (Parhar & Peeyush, 1988).

Several studies have demonstrated the beneficial effect of cyclooxygenase inhibitors, especially indomethacin and piroxicam, in the therapy of a variety of primary tumours and metastases not only in experimental models (Fulton, 1984; Lala *et al.*, 1986; Young & Hoover, 1986; Reddy *et al.*, 1987) but also in some human tumours (Klein *et al.*, 1987; Ikeda *et al.*, 1988; Breau *et al.*, 1989).

We assumed that the tumour used in the present work produced prostaglandins, originated either in the tumour cells and/or in activated TAM. One of their effects on PM would be a decreasing of their activation level. Myers *et al.* (1989) demonstrated that i.p. inoculation of mice with methylnitrosamine induced the production of high levels of prostaglandin E₂ in PM, which correlated directly with the activation state of these cells. This caused a generalized depression of cell-mediated immunity.

In the treatments with indomethacin and aspirin we have employed doses (mg/kg body weight) that are usually used in human therapy, whereas piroxicam doses were 10-fold higher, since lower ones were not as effective as for the other drugs. However, no marked side-effects were observed, because of the low toxicity of piroxicam (Reddy *et al.*, 1987). Our results show that the treatment with indomethacin has a discrete advantage over treatments with aspirin and piroxicam, taking into account the percentage of tumour regressions, despite the fact that the effect in delaying the growth of tumours that did not regress was similar for all three inhibitors.

Only the treatment with piroxicam was followed by a greater macrophage infiltration of the tumour. This is a remarkable finding, in view of the fact that the TAM percentage is fairly stable in these experimental models, and does not vary after inoculation of immunopotentiators (McBride *et al.*, 1982; Valdéz *et al.*, 1990).

Based mainly on the increased expression of FcIgG-receptor

and β -glucuronidase levels, we believe that the ultimate effect of NSAID on PM from tumour-bearing mice is the recovery of their activation level, which would improve extratumoural macrophage response in the immunoinflammatory reaction against tumour.

Tumour growth selectively induced a large increase of neutrophils in peripheral blood, as noted before by others (Wei *et al.*, 1986; Bartholeyns, Frendenberg & Galanos, 1987). Monocyte and lymphocyte counts increased only slightly, while erythrocytes were diminished. Since treatment with NSAID restored cell counts to values close to normal, prostaglandins would probably be involved. However, further studies are required to explain this effect of NSAID, because of the complex interrelationship between growth factors, such as colony-stimulating factors, prostaglandins (produced by tumour cells and macrophages) and mielopoiesis (Boardman & Hart, 1967; Kurland *et al.*, 1978; Hardy & Balducci, 1986; Vore *et al.*, 1989).

Summarizing, the above-mentioned effect of prostaglandin inhibitors on PBL and macrophages would allow a better response of lymphocytes, monocytes and macrophages to lymphokines, IL-1, IL-2, IFN- γ , CSF-1, etc., thereby facilitating their antitumour activity. This would probably account for the regression and/or slowing of tumour growth rate.

We are presently performing histopathological studies which reveal that tumours from treated animals are well encapsulated, with less mitotic figures and aggressivity. We cannot rule out the possibility that the restoration of both hematic values and PM activity are the consequence and not the cause of the reduced aggressivity and encapsulation that follow NSAID administration. Although there is evidence indicating that such drugs, especially indomethacin, have no direct antitumour activity (Fulton, 1984; Lala *et al.*, 1986), the effectiveness of NSAID permits them to be considered as substances of potential use as adjuvants in the treatment of tumours, even though the tumour employed in the present work, which is non-metastatic, is somewhat different from human tumours.

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