Pergamon

European Journal of Cancer Vol. 33, No. 4, pp. 676–682, 1997
© 1997 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(96)00506-0

Original Paper

Hydrogen Peroxide is Involved in Lymphocyte Activation Mechanisms to Induce Angiogenesis

M. Monte, L.E. Davel and E. Sacerdote de Lustig

Dpto. Immunobiologia, Area Investigación, Instituto de Oncología "Angel H. Roffo", Universidad de Buenos Aires, Av. San Martin 5481, 1417-Buenos Aires, Argentina

T-lymphocytes from tumour-bearing mice are able to trigger the angiogenic cascade. Since it is known that tumour growth produces reactive oxygen species (ROS), the aim of this study was to evaluate the role of hydrogen peroxide (H_2O_2) on the activation of lymphocytes and their induction of this vascular response. Studies on lymphocytes, stimulated *in vitro* by ROS to induce angiogenesis, showed that only the enzyme catalase (CAT) could block the activation. The incubation of normal lymphocytes with H_2O_2 stimulated these cells to induce angiogenesis. The administration of H_2O_2 or an oxidative stress-producing drug (doxorubicin) to normal mice activated *in vivo* angiogenesis. In tumour-bearing mice, high levels of lipid peroxidation products were observed in the spleen, but not in the liver or kidney. Moreover, when the ROS scavenger enzyme activities (superoxide dismutase (SDM) and CAT) were determined, we observed low CAT activity in normal spleens, reflected in a high SDM/CAT ratio, when compared to liver or kidney values. We also showed an increasing value of the SDM/CAT ratio with tumour growth. These results strongly suggest that H_2O_2 could be involved in the mechanisms of lymphocyte activation and their induction of angiogenesis during tumour growth. **© 1997 Elsevier Science Ltd. All rights reserved.**

Key words: angiogenesis, lymphocytes, tumour, oxygen free radicals, hydrogen peroxide, superoxide dismutase, catalase, TBARS

Eur J Cancer, Vol. 33, No. 4, pp. 676–682, 1997

INTRODUCTION

SOLID TUMOURS induce an angiogenic response by the host blood vessels to form a new vascular network for the supply of nutrients and oxygen [1]. This neovascular response is partly responsible for tumour growth and metastatic spread [2, 3].

Davel and associates [4, 5] have previously observed that, in addition to tumour cells, T-lymphocytes from tumour-bearing mice induced angiogenesis in syngeneic animals. This response, called syngeneic lymphocyte-induced angiogenesis (SLIA), was detected early after tumour implant, showing a fast spleen-cell recognition of tumour presence.

During tumour development, reactive oxygen species (ROS) are produced [6, 7]. Although high concentrations of ROS are known to cause serious injury to tissues and cell death [8], they are also involved in normal cell mechanisms. There is now a large body of evidence that different cell types are able to enhance ROS production following extracellular stimulation with phorbol 12-myristate-13-acetate (PMA) and physiological concentrations of cytokines, such as tumour necrosis factor (TNF) or interleukin-1 (IL-1) [9, 10]. Moreover, it has been demonstrated that oxidative phenomena are implicated in T-cell stimulation [11, 12] and that IL-2 production could be regulated by hydrogen peroxide (H_2O_2) [13], suggesting that ROS can be used for intracellular signalling.

We have recently demonstrated that lymphocyte-induced angiogenesis is triggered by ROS stimulation, and that this response can be blocked by the administration of a free radical scavenger to tumour bearing mice. Furthermore, we have also stimulated normal lymphocytes to induce angiogenesis with oxygen free radicals *in vitro* [14].

In the present study we investigated the role of different ROS scavengers on the stimulation of lymphocytes and their

Correspondence to M. Monte.
Received 14 May 1996; revised 27 Sep. 1996; accepted 10 Oct. 1996.

induction of angiogenesis, and the effect of *in vivo* administration of oxidative stress-related compounds (doxorubicin and H_2O_2). Finally, we determined lipid peroxidation and antioxidant enzyme activities in the spleen of normal and S13 tumour-bearing mice in order to observe whether oxidative stress takes place in this organ.

MATERIALS AND METHODS

Animals

All the assays were performed with 3 to 4 month-old BALB/c male mice obtained from our animal breeding centre.

Tumour

A syngeneic adenomammary carcinoma, S13, which originally appeared spontaneously in BALB/c mice was used. It was maintained by serial subcutaneous (s.c.) transplantations into the right flank of normal mice. Mice died approximately 30 days after tumour inoculation, when the tumour reached 30% of the body weight. At time of death, 80% of animals had developed lung metastasis [15, 16].

Animal treatments

Treatments were performed as follows. Three groups consisted of four mice each, and each animal was injected i.v. via the caudal vein with doxorubicin (DOX)(Farmitalia, Milan, Italy), 10 mg/kg body weight DOX 20 mg/kg b.wt., or lactose (vehicle) in 0.2 ml. DOX was injected in a single dose 48 h before the SLIA assay. Mice in another three groups were injected intraperitoneally (i.p.) with 0.5 ml of H_2O_2 (Sigma Chemical Co.) 100 μM, H_2O_2 500 μM or saline (vehicle) once a day for 2 days.

Spleen cell suspensions

Spleen cells were obtained from tumour-bearing mice 25 days after tumour transplantation or from normal mice. Spleen cell suspensions were performed by teasing and abrading spleen tissue against a stainless steel screen immersed in TC 199 (Gibco, Grand Island, New York, U.S.A.). Further disruption of clumps was achieved by passing the cell suspension in and out of a syringe. These suspensions were used in the SLIA assay.

Lymphocyte exposure to oxidant injury

Normal spleen cells were exposed to oxidant injury by incubation with the xanthine–xanthine oxidase system (X/XO) or H_2O_2. Ten milliliter suspensions with 2×10^7 lymphocytes were incubated at 37°C for 90 min in a humidified 95% air 5% CO_2 atmosphere. Incubation took place in the presence of the following media: TC 199 supplemented with 2 μM xanthine (Sigma Chemical Co., St Louis, Missouri, U.S.A.) and 0.0075 U/ml xanthine oxidase (Sigma, Grade 1, 0.64 U/mg protein), or TC 199 supplemented with H_2O_2 (Sigma Chemical Co.). Anti-oxidant enzymes or compounds such as CU–Zn superoxide dismutase (SDM from bovine erythrocyte, 790 U/mg protein) (Biosidus, Buenos Aires, Argentina), catalase (CAT from bovine liver, 2000 U/mg protein) (Sigma Chemical Co.) or dimethyl sulphoxide (DMSO) (Sigma Chemical Co.) were used in different incubations.

Angiogenesis assay

Lymphocytes obtained from different experimental groups were tested in the SLIA assay. A drop of diluted trypan blue was added to the final suspension to facilitate subsequent location of the injection site. Spleen cells (4×10^6 in 0.1 ml of TC 199) were injected intradermally (i.d.) into normal syngeneic mice. Each mouse received two injections midlaterally (one on each site) in the thoracolumbar region of the trunk. Five days later mice were killed with ether. The skin was carefully separated from underlying tissues and examined under a dissecting microscope (WILD) at 6.4× magnification [17, 18].

Quantification of vascularisation

The quantification of vascularisation was determined by measuring the vessel density as follows: photographs were taken of mouse skin, placing the site of inoculation in the middle of the slide. The slides were projected on to a reticular screen, each square corresponding to 1 mm^2 of skin. The total area measured was 216 mm^2 in all cases. To count the number of vessels in each square (double blind), the criteria of the original Auerbach's methods was applied [19]. The density (d) of the vessels around each inoculation site was determined as:

$$d = \frac{\sum \text{number of blood vessels per square}}{\text{number of total counted squares}}$$

Tissue sample preparation

Liver, kidney and spleen from normal or tumour-bearing mice were washed in cold saline solution several times and then homogenised in cold potassium phosphate buffer 50 mM and pH 7.0 with a tissue homogeniser Ultraturrax Antrieb T-25 (Janke & Kunkel IKA Laboratory) at a setting of 20 500 rpm and centrifuged at 3000*g* for 10 min. The cold homogenate was aliquoted for the lipid peroxidation assay (LPO) and for superoxide dismutase (SDM) and catalase (CAT) determination. Butylated hydroxytoluene (BHT) was added to the LPO sample, and then assayed, while SDM and CAT samples were frozen (−40°C) until enzyme determination. The SDM sample was sonicated in ice by a Vibra-Cell (High Intensity Ultrasonic Processor, Sonics & Materials, Inc., Danbury, Connecticut, U.S.A.) before the assay.

Superoxide dismutase assay

Total SDM activity was determined by the adrenalin to adenochromo autoxidation assay [20]. The reaction mixture contained 50 mM glycine buffer at pH 10.2 and 60 mM adrenalin pH 2. The reaction was carried out at 30°C. The rate of increased absorbance at 480 nm was recorded with a Metrolab 325 digital spectrophotometer equipped with a thermostatised cell compartment. One SDM unit was defined as 50% inhibition of the autoxidation of adrenalin under assay conditions. Enzyme activity was expressed as units/mg of proteins measured according to Lowry and associates [21].

Catalase assay

Catalase activity was determined in tissue samples (1% Triton X-100) following the decomposition of H_2O_2 at 240

nm [22]. The reaction mixture contained 10 mM H_2O_2 in 3 ml of 50 mM potassium phosphate buffer at pH 7.0. The reaction was carried out at 25°C. One catalase unit was defined as the amount of enzyme that decomposes 1 μmol H_2O_2/min/ml. Enzyme activity was expressed as units/mg of protein.

Lipid peroxidation assay

LPO was assayed by measuring the production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) [23]. Proteins from homogenate samples supplemented with 0.2% BHT were precipitated with one volume of 20% trichloroacetic acid (TCA). The reaction of 2-thiobarbituric acid (0.67% w/v) with the samples was carried out at 100°C for 30 min and the absorbance was measured at 532 nm. The TBARS content was calculated by using a calibration curve prepared from malonaldehyde bis (diethyl acetal) (Aldrich Chemical Co., Milwaukee, U.S.A.) and expressed as TBARS/mg protein.

Statistical analysis

The significance of the differences between groups was found either by Student's *t*-test or ANOVA test.

RESULTS

Effect of reactive oxygen species on in vitro *lymphocyte stimulation*

In order to find out the role of different ROS on lymphocyte stimulation and induction of angiogenesis, we exposed normal lymphocytes to the superoxide anion generator system, xanthine–xanthine oxidase. Spleen lymphocytes obtained from normal BALB/c mice were incubated at 37°C for 90 min in TC 199 supplemented with xanthine (X) and xanthine oxidase (XO) and separated into groups according to the type of free radical scavenger added. As shown in Figure 1, lymphocytes incubated with xanthine–xanthine oxidase evoked a strong vascular response, which was inhibited by the addition of both SDM and CAT prior to the X–XO reaction. Unexpectedly, the addition of SDM alone (a specific superoxide anion scavenger) did not result in inhibition of angiogenesis, while the addition of CAT (H_2O_2 scavenger) dramatically blocked this response. It is noteworthy that the addition of SDM increased the vascular density induced by lymphocytes. We also tested the effect of DMSO (HO scavenger) on this incubation. As shown in Figure 1, neither 250 μM DMSO nor 500 μM DMSO inhibited lymphocyte activation. In all cases, lymphocyte viability was higher than 80%, with no significant cell death produced by ROS incubation.

Hydrogen peroxide-induced lymphocyte angiogenesis

In view of our results, we investigated whether H_2O_2 could be an oxygen derivative molecule able to trigger the angiogenic response induced by lymphocytes. Lymphocytes obtained from normal mice were incubated in TC 199 supplemented with a graded concentration of H_2O_2 for 90 min as was done with X–XO. Then, cells were injected into normal mice to test the angiogenic response. As shown in Figure 2, while 0.6 and 2.5 μM H_2O_2 could not elicit a neovascular response, 10 and 40 μM H_2O_2 strongly stimulate normal lymphocytes to induce angiogenesis. The addition of CAT to the culture medium completely suppressed lymphocyte stimulation. With the doses of H_2O_2 used, no significant cell death was observed compared with controls (less than 15%).

In vivo *stimulation of lymphocyte angiogenesis*

We performed experiments to find out whether lymphocytes could be stimulated *in vivo* by the administration of the anthracycline, DOX or H_2O_2. As shown Figure 3, lymphocytes obtained from DOX-treated mice (Figure 3a) or H_2O_2-treated mice (Figure 3b) strongly induce the angiogenic response.

TBARS determination during tumour growth

In order to study whether oxidative stress occurred during S13 tumour growth, we measured thiobarbituric acid reac-

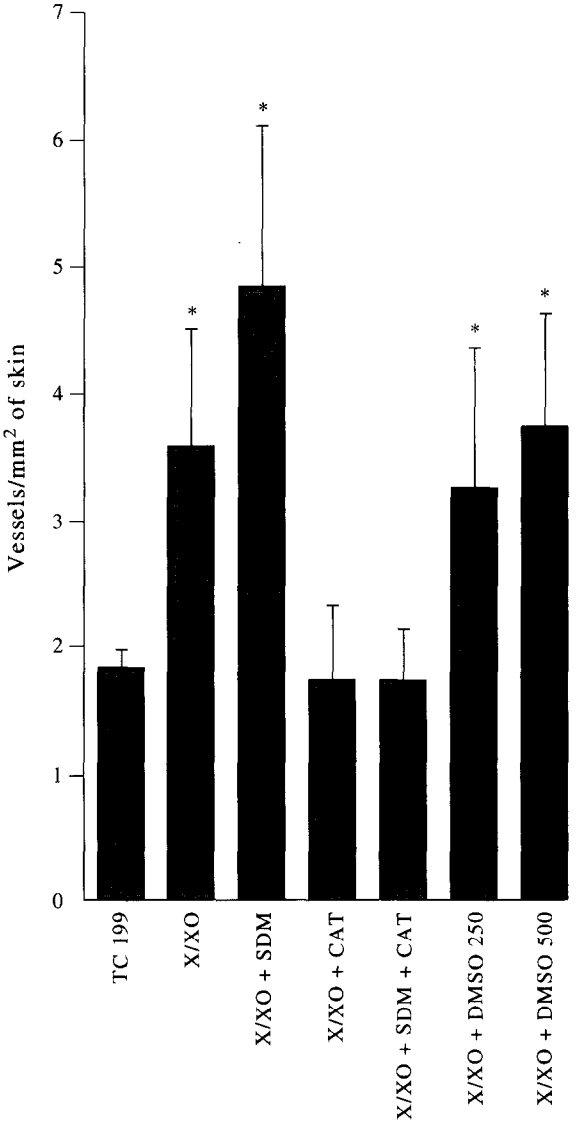

Figure 1. Effect of different free radical scavengers on angiogenesis induced by lymphocytes stimulated with the xanthine–xanthine oxidase (X–XO) system. Normal lymphocytes were incubated with: TC199 X–XO; X–XO plus 50 U/ml SDM; X–XO plus 60 U/ml CAT; X–XO plus 50 U/ml SDM and 60 U/ml CAT and X–XO plus 250 or 500 uM dimethyl sulphoxide (DMSO). The vascular density of 10 inoculated sites were determined in each experiment. *$P < 0.05$, ANOVA—compared with TC 199 levels.

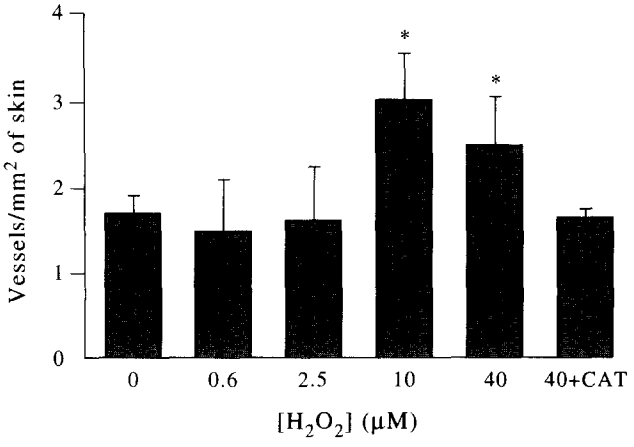

Figure 2. Dose–response study on the ability of H_2O_2 to induce lymphocyte angiogenesis. Normal lymphocytes were incubated with graded concentrations of H_2O_2. The addition of 100 U/ml CAT and H_2O_2 is also shown. Each mean density value is the average of 10 inoculated sites. *$P < 0.05$, ANOVA.

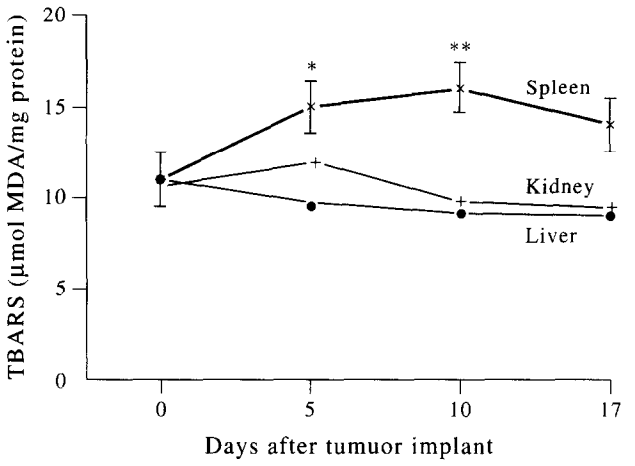

Figure 4. TBARS measurement during tumour growth. MDA concentration was determined in liver, kidney and spleen, 5, 10 and 17 days after S13 tumour implantation. Each point represents the mean of six independent measurements. Differences between each measurement and day 0 was done using Student's *t*-test *$P < 0.02$, **$P < 0.001$.

tive substances (TBARS) in the spleen, kidney and liver of tumour-bearing mice. As shown in Figure 4, an increase in the values of TBARS was observed in the spleen as early as 5 days after tumour implant, when s.c. transplants usually remain in a lag period. Liver and kidney did not show significant modifications during the first 17 days following tumour implant.

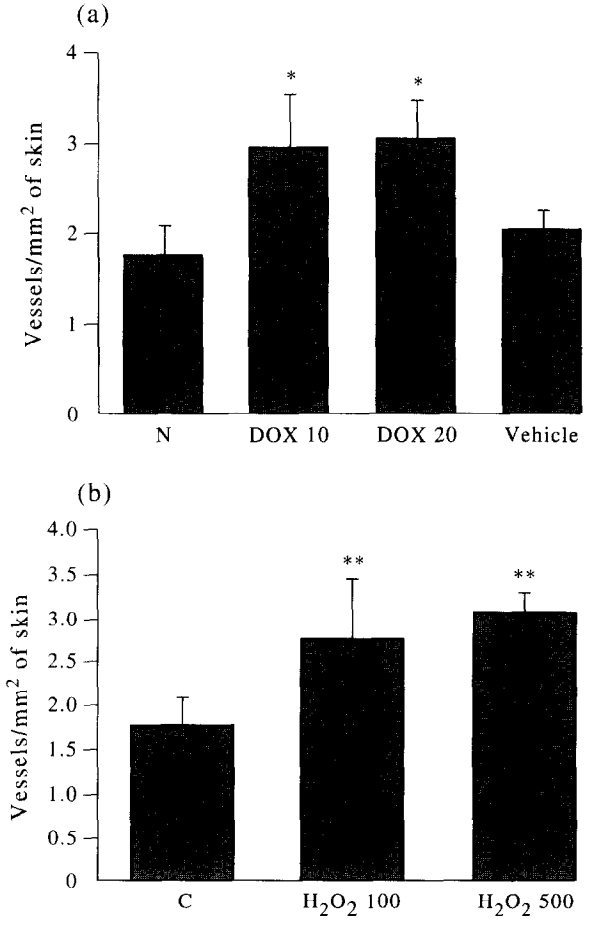

Figure 3. Effect of the *in vivo* administration of doxorubicin (DOX) and H_2O_2 on SLIA. (a) Groups of mice were injected i.v. with, 10 (DOX 10) or 20 mg DOX/kg b.wt. (DOX 20) or lactose (vehicle). *N* is the value corresponding to normal lymphocytes (no treatment). (b) Groups of mice were injected i.p. with 0.5 ml of saline (C); 100 (H_2O_2 100) or 500 uM H_2O_2 (H_2O_2 500). Spleen lymphocytes were isolated and tested in the SLIA assay. *$P < 0.03$, **$P < 0.001$, Student's *t*-test.

Spleen antioxidant enzymes in normal and tumour bearing mice tissues

In order to determine the capability of different organs to eliminate ROS, we first studied the activities of the main antioxidant enzymes SDM and CAT in normal mice. As shown in Figure 5, the spleen contained lower CAT activity than kidney and liver. Since, during oxygen metabolism, CAT eliminates H_2O_2, mainly produced by SDM activity, we compared SDM and CAT activities in each organ. While liver and kidney had a SDM/CAT ratio of approxi-

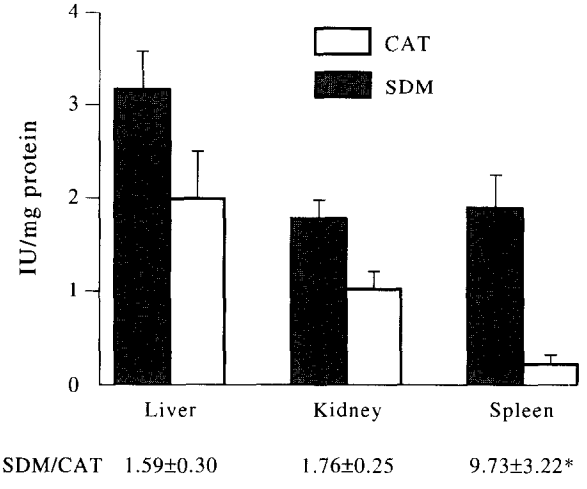

Figure 5. Anti-oxidant enzyme determination on normal mice tissues. Superoxide dismutase and catalase activities were determined on liver, kidney and spleen. SDM/CAT ratio is shown in the insert below the figure. Each mean value is the average of at least six independent determinations. SDM/CAT values were compared using ANOVA test. *$P < 0.05$.

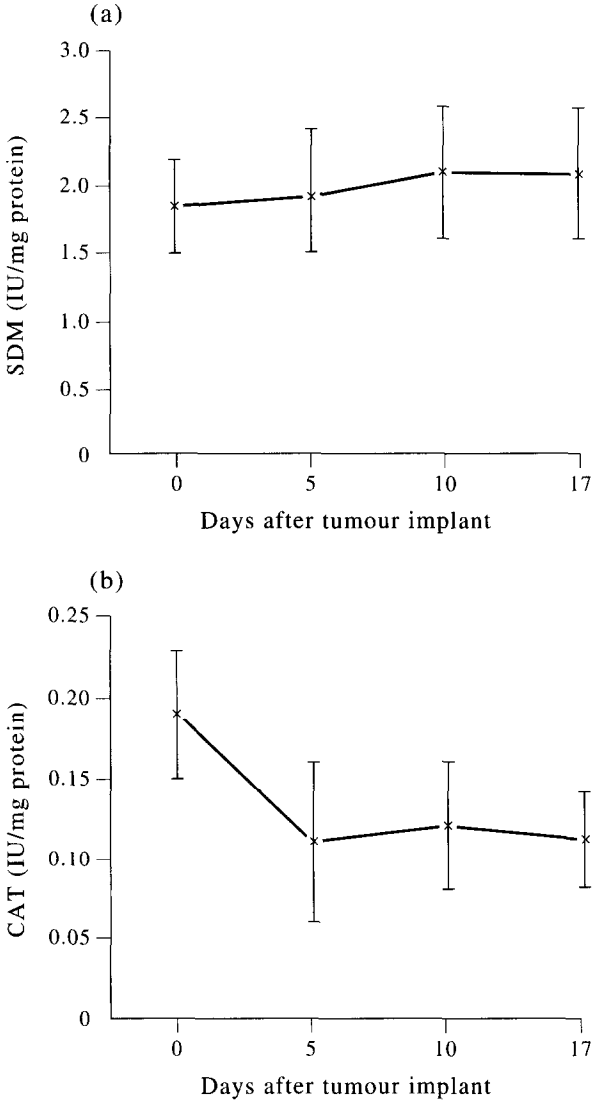

Figure 6. Spleen SDM and CAT activities during tumour growth. (a) SDM and (b) CAT activities were determined in spleen tissues during tumour growth 5, 10 and 17 days after s.c. S13 tumour transplant. Each point is the mean of six determinations. No significant differences were observed in the enzyme activity during tumour growth.

mately 1.59 ± 0.30 and 1.76 ± 0.25, respectively, this value was 9.73 ± 3.22 in the spleen. When enzyme activities were determined in tumour-bearing mice, greater differences between SDM and CAT were observed (Figure 6). Moreover, as early as 5 days after tumour implant, the SDM/CAT ratio reached a value of 17.45 ± 4.31 (not shown in the figure), mainly due to a decrease of CAT, pointing that during tumour growth this ratio became higher than in the normal spleen.

DISCUSSION

Angiogenesis is a very complex process that mainly involves the release of angiogenic factor/s, resulting in tissue neovascularisation. This is a key process for tumour development and metastasic spread. Angiogenic factors are not only released by tumour cells. Watt and Auerbach [24] have reported the production of a mitogenic factor for endothelial cells obtained from activated mouse lymphocytes.

Early experiments in our laboratory indicated that T-lymphocytes from tumour-bearing mice could induce angiogenesis, with a direct correlation between the density of vessels and the metastatic capabilities of different tumours [4, 18]. Moreover, we showed that different antigenic non-tumoral stimuli, with the capacity to activate the immune system, were unable to stimulate spleen cells to evoke an angiogenic response. Therefore, tumour cells seem to be the only antigenic stimuli able to activate spleen lymphocytes to induce SLIA [25].

We carried out several experiments to elucidate the SLIA mechanisms, which included polyamine synthesis inhibition [26, 27] and treatment with certain biological modulators (indomethacin, copper, TNF, peptides of extracellular matrix) that showed anti-angiogenic properties because they could block the angiogenic response of lymphocytes from tumour-bearing mice [17, 28, 29]. Soluble factors released by tumour cells growing within diffusion chambers placed i.p. could also activate lymphocytes to induce angiogenesis [30].

We have recently demonstrated that active oxygen-derived species can play a role in angiogenesis induction. The anti-angiogenic effect, using a ROS scavenger (EGb-761), correlated with a decrease in the incidence and number of lung metastasis of S13 tumour [14]. The exposure of normal lymphocytes to ROS generator medium, as we have already demonstrated, induced angiogenesis (Figure 1).

In order to elucidate which ROS was involved in angiogenesis, we used specific ROS scavengers, SDM, CAT or DMSO. Although the X–XO system specifically generates superoxide anion and uric acid, dismutation of the superoxide anion to H_2O_2 spontaneously occurs. The presence of superoxide anion and H_2O_2 facilitates hydroxyl radical production, since its generation could arise from the Habber–Weiss reaction or the iron-catalysing Fenton reaction [31, 32]. When SDM was added, angiogenesis increased over that produced with X–XO stimulation alone. This might be due to an overproduction of H_2O_2 caused by SDM activity. Treatment with both SDM and CAT entirely blocked the angiogenic response. CAT alone also abolished SLIA, indicating that H_2O_2 may be a key molecule in this process. With DMSO, no modification over background vascularisation was observed. In addition, concentrations of H_2O_2 over 10 μM triggered SLIA (Figure 2).

Thus, this is the first study to show that H_2O_2 is a key intermediate for stimulating lymphocytes to induce angiogenesis. The same effect was obtained *in vivo* with H_2O_2 (Figure 3a). The administration of DOX known to induce oxidative stress *in vivo* [33, 34] also induced a strong neovascular response (Figure 3b). Tesoriere and associates [35] showed evidence of oxidative stress in rats 48 h after the administration of DOX 10 mg/kg body weight, supporting our results.

The fact that TBARS values in the spleen, but not in the liver or kidney, were increased in tumour-bearing mice, indicates that only spleen cells have increased oxidative stress when tumour cells are present. Moreover, during tumour growth, the spleen size of S13-tumour-bearing mice increases up to 4-fold, showing the spleen host response to the tumour (data not shown). The oxidative stress in the spleen could be the result of an active cell metabolism com-

bined with low anti-oxidant defences. Normal spleens exhibited low CAT activity compared to kidney and liver, and also low CAT activity compared to spleen SDM activity (Figure 5). During tumour growth, CAT activity tends to decrease in the spleen, increasing the difference with SDM activity (Figure 6). This feature of the spleen could be indicating the importance of H_2O_2 in the lymphocyte activiting process.

Our findings suggest that, during tumour growth, H_2O_2 plays an important role in lymphocyte activation. Although, in the S13 tumour, it has not yet been determined, several authors have reported superoxide anion and H_2O_2 production by tumour cells [36, 37]. Another possibility of H_2O_2 increase is that cells such as neutrophils and/or macrophages, activated by the tumour, release H_2O_2 and superoxide anion during the oxidative burst [38], stimulating lymphocytes to induce angiogenesis. Sherck and associates [39] have reported that the transcription factor NF kappa-B is activated by the concentrations of H_2O_2 used in this study. This fact, in addition to the effect of H_2O_2 on T-cell growth factor production reported by Roth and associates, support our findings.

In conclusion, oxygen derivatives could act as important biological messengers or response modifiers and the control of free radical overproduction may be a potential anti-angiogenic tumour therapy, particularly in tumours infiltrated by lymphocytes.

1. Folkman J. Tumor angiogenesis. *Adv Cancer Res* 1985, **43**, 175–208.
2. Liotta LA. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991, **64**, 327–336.
3. Blood C, Zetter B. Tumor interaction with the vasculature: angiogenesis and tumor metastasis. *Biochim Biophys Acta* 1990, **1032**, 89–118.
4. Davel LE, Miguez MM, Sacerdote de Lustig E. Angiogenesis induction by lymphocytes from tumor-bearing mice in a syngeneic combination. *Transplantation* 1984, **37**, 327–328.
5. Davel LE, Miguez MM, Kohan S, *et al.* Angiogenic activity from tumor bearing mice: nature of the spleen cells population capable of inducing neovascular response. *J Exp Clin Cancer Res* 1988, **7**, 111–117.
6. Boveris A, Liesuy SF, Fraga GC. Increased liver chemiluminiscence in tumor bearing mice. *Free Radic Biol Med* 1985, **1**, 131–138.
7. Perez Soler R, Lopez Berestein G, Cabanillas F. Superoxide anion production by peripheral blood monocytes in Hodgkin's disease and malignant lymphoma. *J Clin Oncol* 1985, **3**, 641–645.
8. Sies H, de Groot H. Role of reactive oxygen species in cell toxicity. *Toxicol Lett* 1992, **64/65**, 547–551.
9. Meier B, Radeke HH, Selle S, *et al.* Human fibroblasts release reactive oxygen species in response to interleukin-1 and tumor necrosis factor alpha. *Biochem J* 1989, **263**, 539–545.
10. Meier B, Radeke HH, Selle S, Habermehl GG, Resch K, Sies H. Human fibroblasts release low amounts of reactive oxygen intermediates in response to the potent phagocyte stimulants serum-treated symozan, *N*-formyl-methionyl-leucyl-phenylalanine, leukotriene B4 or 12-*O*-tetradecanoylphorbol 13-acetate. *Biological Chem Hoppe–Seyler* 1990, **371**, 1021–1025.
11. Sekkat C, Dormand J, Gerber M. Oxidative phenomena are implicated in human T-cell stimulation. *Immunology* 1988, **63**, 431–437.
12. Chaudhri G, Clark IA, Hunt NH, Cowden WB, Ceredig R. Effect of antioxidants on primary alloantigen-induced T cell activation and proliferation. *J Immunol* 1986, **137**, 2646–2652.
13. Roth S, Droge W. Regulation of T-cell activation and T-cell growth factor (TCGF) production by hydrogen peroxide. *Cell Immunol* 1987, **108**, 417–424.
14. Monte M, Davel L, Sacerdote de Lustig E. Inhibition of lymphocyte inducted angiogenesis by free radical scavengers. *Free Radic Biol Med* 1994, **17**, 259–266.
15. Jasnis M, Klein S, Bonaparte Y, Oisgold-Dagá S, Pirosky R. Delayed hypersensitivity in tumor-bearing mice. In vitro activation of "eclipsed" spleen cells. *Int J Cancer* 1977, **20**, 394–399.
16. Fuchs A, Sacerdote de Lustig E. Copper histochemistry of 5 murine tumors and their respective metastasis. *Tumor Biol* 1989, **10**, 3847–3852.
17. Davel LE, Miguez MM, Sacerdote de Lustig E. Evidence that indomethacin inhibits lymphocytes induced angiogenesis. *Transplantation* 1985, **39**, 564–565.
18. Miguez MM, Davel LE, Sacerdote de Lustig E. Lymphocyte-induced angiogenesis: correlation with the metastatic incidence of two murine mammary adenocarcinomas. *Invasion & Metast* 1986, **6**, 313–320.
19. Sidky, Auerbach R. Lymphocyte-induced angiogenesis: a quantitative and sensitive assay of graft-vs-host reaction. *J Exp Med* 1975, **141**, 1084–1100.
20. Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972, **247**, 3170–3175.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
22. Aebi H. Catalase *in vitro*. *Methods Enzymol* 1984, **105**, 121–126.
23. Estrebauer H, Cheeseman KH. Determination of lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* 1990, **186**, 407–412.
24. Watt SL, Auerbach R. A mitogenic factor for endothelial cells obtained from mouse secondary mixed leukocyte cultures. *J Immunol* 1986, **136**, 197–202.
25. Davel LE, Jasnis MA. Lymphocyte-induced angiogenesis: effect of different antigenic stimuli. *Onology* 1995, **52**, 226–229.
26. Monte M, Klein S, Jasnis MA, Davel LE, Algranati ID, Sacerdote de Lustig E. Inhibition of lymphocyte and tumor-induced angiogenesis by the administration of difluoromethylornithine. *Cancer J* 1993, **6**, 147–150.
27. Jasnis MA, Klein S, Monte M, Davel L, Sacerdote de Lustig E, Algranati ID. Polyamines prevent DFMO-mediated inhibition of angiogenesis. *Cancer Lett* 1994, **79**, 39–43.
28. Davel LE, Fuchs AG, Miguez MM, Schiavi G, Jasnis MA, Sacerdote de Lustig E. Inhibition of lymphocyte-induced angiogenesis by copper and TNF. *J Exp Clin Cancer Res* 1993, **12**, 111–114.
29. Eijan A, Davel L, Oisgold-Dagá S, Sacerdote de Lustig E. Modulation of tumor-induced angiogenesis by proteins of extracellular matrix. *Mol Biother* 1991, **3**, 38–40.
30. Klein S, Jasnis MA, Daiment M, Davel LE, Aguirre J, Bonaparte Y. Immunomodulation by soluble factor from tumor cells culture in vivo in diffusion chambers. *Tumor Biol* 1994, **15**, 160–166.
31. Aruoma OL, Halliwell B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radical from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl radical generation? *Biochem J* 1987, **241**, 273 278.
32. Haber F, Weiss JJ. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc R Soc Lond* 1934, **147**, 332–351.
33. Doroshow JH, Davies KJA. Redox cycling of anthracyclines by cardiac mitochondria. II. Formation of superoxide anion, hydrogen peroxide and hydroxyl radical. *J Biol Chem* 1986, **261**, 3061–3067.
34. Doroshow JH, Locker GY, Myers CE. Enzymatic defenses of the mouse heart against reactive oxygen metabolites. Alteration produced by doxorubicin. *J Clin Invest* 1978, **65**, 955–961.
35. Tesoriere L, Ciaccio M, Valenza M, *et al.* Effect of vitamin A administration on the resistance of rat heart against doxorubicin-induced cardiotoxicity and lethality. *J Pharmacol Exp Ther* 1994, **269**, 430–436.

36. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med* 1995, **18**, 775–794.
37. Szabrowski T, Nathan C. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 1991, **51**, 794–798.
38. Babior BM. Oxygen-dependent microbicidal killing by phagocytes. *N Engl J Med* 1978, **298**, 659–665.
39. Sherck R, Albermann K, Baeuerle PA. Nuclear factor κB: oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* 1992, **17**, 221–237.

Acknowledgements—We are grateful to Ileana Lotersztain for her excellent and valuable assistance in this work. We are also grateful to Dr Laura Borodinsky and Dr Maria Adela Jasnis for their collaboration. This work was supported by grants from the National Research Council (CONICET) and the University of Buenos Aires.