Early tumor effect on splenic Th lymphocytes in mice

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Received 7 July 1997

Abstract Tumors are characterized by their ability to avoid the host immune system. Ehrlich ascites tumor cells were used to investigate the early alterations of the host immune system after tumor inoculation. The results show that frequencies of splenic Th lymphocytes were drastically reduced during tumor growth, reaching a minimum only two days after tumor inoculation. The frequency of splenic CD4$^+$ lymphocytes expressing IFN-γ was significantly increased, although the total number was unchanged, suggesting that there was no net induction of Th1-type response. Splenic macrophages were increased, in both frequency and cell number, after four days of tumor growth. The same pattern was observed when mice were inoculated with cell free ascitic fluid. TGF-β precursors were detected in tumor cells as well as in ascitic fluid. The data suggest that tumor actively interacts with host immune system by means of tumor cell secreted factors.

Key words: Ehrlich ascites tumor cell; Spleen; CD4$^+$ lymphocyte; TGF-β

1. Introduction

Host immune response to tumors is still not well understood. A remarkable consequence of tumor growth is a decline in the host immune function, partially explained by a diminished responsiveness of T lymphocytes and NK cells [1,2]. Other studies in mice inoculated with plasma cell and hepatoma tumors report that Th, but not cytotoxic lymphocytes are susceptible to tumor derived inhibitory factors, manifested by a reduced proliferation of Th cells when they are stimulated with different mitogens [3,4]. Some mechanisms have been described that account for such immune suppression in tumor bearing hosts, including down-regulation of growth factors [5], production of immunosuppressive cytokines [6,7], and the induction of suppressive macrophages [8–10,5] and T cells [11,2]. In addition, tumors are very poor in initiating effective immune responses; one reason for that is the lack of costimulation necessary to effectively activate T cells [12]. It has been proposed that the tumor induces a Th2-type response, which does not negatively affect the tumor, and depresses a Th1-type response which would promote tumor regression [13–15].

Mice bearing Ehrlich ascitic tumor, a rapidly growing experimental model, show several of the above indicated features: reduced responsiveness of T cells to mitogens [16,17] and a progressive loss of splenic NK activity [9]. Macrophages are in part responsible for this suppressive effect [18,9]. In other tumor models, macrophages have been shown to secrete a panel of immunosuppressive cytokines [7,19]; they also present low levels of MHC class II molecules [20] and induce oxidative stress that causes the disappearance of the CD3 ζ chain of the T cell receptor complex [21].

Since host immune responses to tumor antigens are weak and not immediate [12], the early contacts between host and tumor are critical in determining the future directions of the immune response elicited by tumor secreted factors. Most tumor-host interaction studies are carried out when the tumor is well developed, actually reflecting the new steady state induced by the tumor. The present model enables us to investigate, at earlier stages, the effects of the implantation of Ehrlich ascites tumor cells on the immune system of mice, as well as during the exponential tumor growth phase, revealing some alterations caused by tumor secreted products. Previous studies of our group have demonstrated that tumors induce early effects on host nitrogen metabolism [22,23].

2. Materials and methods

2.1. Animals and tumor cells

A hyperdiploid Lettré strain of Ehrlich ascites tumor cells was maintained in female albino Swiss CD1 mice (24–25 g), purchased from Criffa (Barcelona, Spain), as described elsewhere [24]. The life span of animals after inoculation with 5×10^6 tumor cells was 16 ± 1 days.

2.2. Antibodies

Antibodies used were: Interferon-γ (IFN-γ) (rat IgG1, XMG 1.2) from Pharmingen (San Diego, California); Transforming growth factor-β (TGF-β) (polyclonal) from R&D Systems (Minnealopis, MN); CD4 (RM4-5) and CD18 (C71/16) from Sigma (St. Louis, MO).

2.3. In vitro stimulation of spleen cells

Mice were sacrificed by cervical dislocation. Spleens were homogenated and cells incubated in RPMI medium containing 0.3 mg/ml glutamine, 10% FCS (Whittaker), 100 units/ml Penicillin, 0.1 mg/ml Streptomycin and 1.25 units/ml Amphotericin B (Gibco BRL), in 100 mm diameter petri dishes at 2×10^6 cells/ml. Cells were stimulated for 5 h with 5 ng/ml PMA and 1 μM Ionomycin. Brefeldin A (Sigma) was added at 10 μg/ml, using a stock of 1 mg/ml in ethanol, 2 h before the cells were harvested to prevent secretion of cytokines into the medium [25]. Afterwards, the cells were washed and resuspended in PBS at 2×10^6 cells/ml. Cells were fixed for 20 min at room temperature by addition of 1 vol of 4% formaldehyde in PBS. Fixed cells were washed twice in PBS and resuspended in PBS containing 0.5% BSA and 0.02% NaN_3 (buffer A) at $1–2\times10^6$ cells/ml. Cells were stored until processing at 4°C, in the dark.

2.4. Cytokine stainings

Spleen cells were analysed for production of IFN-γ by intracellular staining as described by Assenmacher et al. [26]. Briefly, cells were incubated with the FITC-conjugated anti IFN-γ antibody for 15 min at $1–2\times10^6$ cells/ml (room temperature) in buffer A containing 0.5% saponin, and washed once with the same buffer. For subsequent surface staining, intracellularly stained cells were resuspended in buffer A. EAT cells were fixed and stained intracellularly with a pan-specific polyclonal antibody against TGF-β, detected with a biotinilated goat anti rabbit IgG and phycoerithrin conjugated streptavidin. Control

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Abbreviations: EAT, Ehrlich ascites tumor; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TBM, tumor bearing mice; FCS, fetal calf serum

PII S0014-5793(97)00943-5

stainings were made using purified rabbit IgG from non immunized rabbits.

All antibody incubations were supplemented with 10% normal mouse serum (Sigma) to prevent inspecific binding. Samples were analysed by flow cytometry in a FACSort apparatus with CellQuest software (Beckton Dickinson).

2.5. Western blotting

Ascitic fluid was analysed by analytical SDS-PAGE as described by Laemmli [27], using 5–15% gradient polyacrilamide gels. Samples were transferred to nitrocellulose membranes as described elsewhere [28] and protein stained with Ponceau red to control the plotting efficiency. The membranes were then blocked for 2 h with a solution of 3% BSA in PBS containing 0.1% Tween-20 (TPBS). Incubations with the primary antibody at 1 μg/ml were performed overnight at 4°C, followed by 3 washes with TPBS and incubated with the second antibody. Membranes were washed and the immune complexes were detected with a biotin-avidin peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) following the manufacturers directions. Finally the membranes were washed three times with PBS and color development was performed using 3–3' diaminobenzidine tetrahydrochloride and 0.02% (v/v) H_2O_2 in 20 mM phosphate buffer pH 6. All antibodies were diluted with TPBS supplemented with 1% BSA.

3. Results and discussion

3.1. Tumor growth kinetics

Ehrlich ascites tumor (EAT) cells were implanted in the peritoneal cavity of mice as described in Section 2. Exponential growth was observed until day 10 after inoculation, starting a reduction in the number of cells at day 13 [29]. Mice were euthanized 10 days after tumor development.

3.2. Effects of tumor growth on splenic Th and macrophage populations

The aggressive behavior shown by the EAT cells causes dramatic changes in the energy and nitrogen metabolism of the host; our previous results have shown that as early as 24 h after tumor implantation, the plasma [24] and liver [23] concentrations of some amino acids change, and glutamine metabolism is modified in order to satisfy the tumor needs [30]; questions arise whether or not the tumor also exerts early systemic effects on the host immune system.

The designed experiment involved transplantation of mice with 5×10^6 EAT cells at the exponential phase of growth, 7 days after tumor inoculation. Mice were sacrificed after 1, 2, 4 and 7 days of tumor growth and spleen cells were fixed in formaldehyde, stained and analysed by flow cytometry. Control mice were inoculated with the same volume of sterile 9‰ saline solution. The results shown in Fig. 1A demonstrated that the frequency of $CD4^+$ lymphocytes rapidly diminished by 50% of controls in the spleen of mice after 2 days of tumor inoculation. Since changes in other splenic populations could alter the percentage of $CD4^+$ cells, total splenic $CD4^+$ lymphocytes were calculated. Total cell numbers in the spleen showed a progressive reduction until day 4 after tumor inoculation, but they were recovered at day 7 (results not shown). On the contrary, total $CD4^+$ cells (Fig. 1B) showed a more pronounced decrease and did not recover the original number, confirming the loss of $CD4^+$ cells from the spleen.

$CD18^+$ macrophage frequencies remained unchanged at days 1 and 2 after tumor inoculation (Fig. 1), but later on,

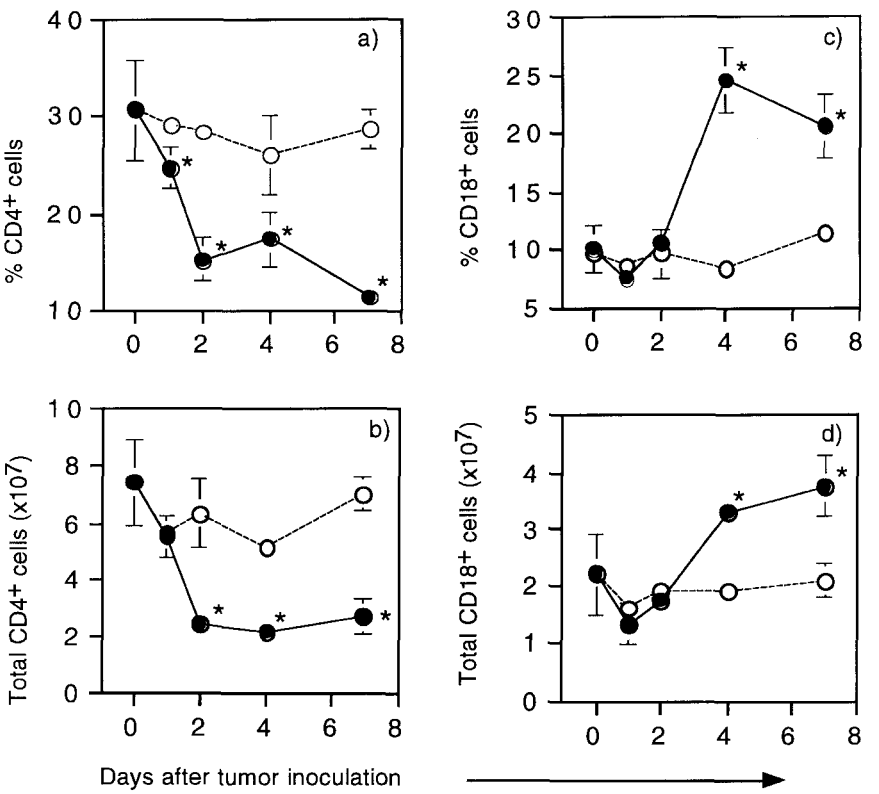

Fig. 1. Frequencies and number of $CD4^+$ and $CD18^+$ cells in the spleen of tumor bearing mice. Mice were inoculated i.p. with 5×10^5 EAT cells (●); sterile saline were given to control mice (○). The spleen cells were fixed, stained with phycoerythrin-conjugated CD4 or CD18 antibodies and analysed by flow cytometry. Data are represented as mean ± S.D. of at least 3 mice. Values significantly different from controls (using the U-Mann Whitney test) are marked with an asterisk * ($p < 0.01$).

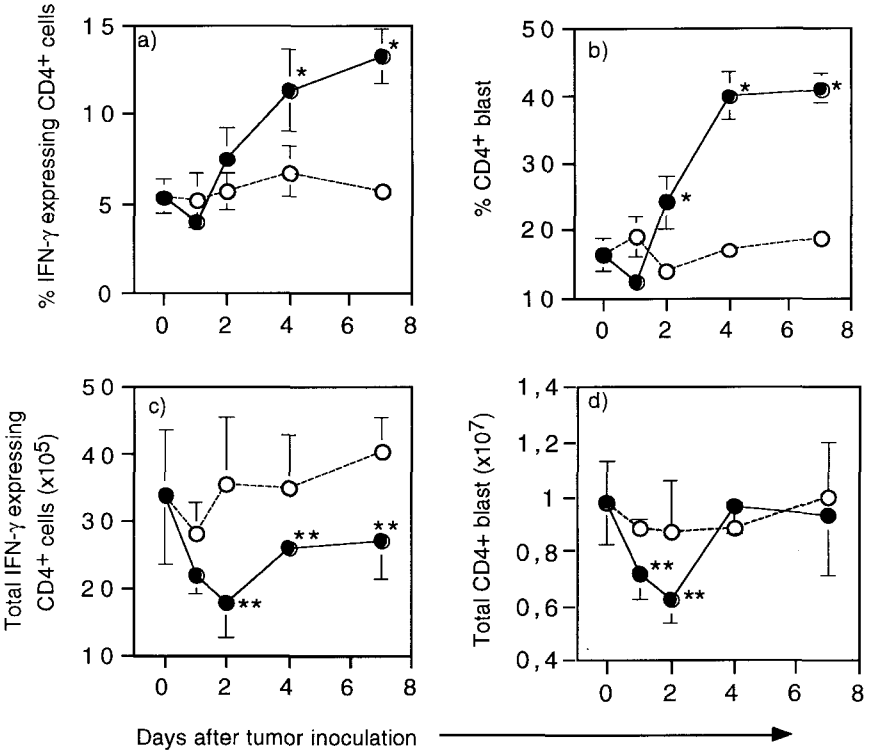

Fig. 2. Frequencies and number of splenic $CD4^+$ IFN-γ expressing and blast lymphocytes in the spleen of tumor bearing mice. Spleen cells were stimulated with PMA and Ionomycin as described in Section 2. Tumor bearing mice are represented with full circles (●), and control mice with open circles (○). $CD4^+$ blast frequencies were obtained by analysing forward and side scatter plots of $CD4^+$ gated cells. Data are represented as mean ± S.D. of at least 3 mice. Values significantly different from the controls (using the U-Mann Whitney test) are marked with * ($p < 0.01$) or ** ($p < 0.05$).

at day 4, there was a dramatic increase in frequencies and total numbers in the spleens of tumor bearing mice.

These results clearly show that the tumor induces early effects on the host immune system, reducing Th lymphocytes and increasing macrophage numbers and frequencies in the spleen of tumor bearing mice. Both affected cell populations, the Th lymphocytes and macrophages, are key targets in order to depress an effective immune response against the tumor invasion. In fact, a population of suppressive macrophages have been shown to be responsible for a diminished responsiveness of splenic lymphocytes to mitogens, and a suppression of the NK activity in mice inoculated with EAT cells [9,17] and other tumors [5,6,21]. Thus, Th cells seem to be a special target for the tumor signals. Ruzek et al. [3] observed a diminished proliferating response to mitogens of $CD4^+$ lymphocytes, and not $CD8^+$, in the spleen of mice bearing plasma cell tumors. Other authors have observed similar imbalances in lymphocytic and monocytic lineages. In the bone marrow of mice inoculated with EAT cells, lymphocytes of the null, Nk1.1 and $Thy1^{lo}$ lineages are increased during the early growth of the tumor [31], in accordance with an enhanced hematopoietic activity [16]. In the spleen of mice implanted with mammary adenocarcinoma and Morris hepatoma there is an increased number of macrophages, exerting a suppressive activity [4,6], and in patients with gastric cancer, splenic NK and Th cells are present in lower frequencies compared to normal controls [2].

The reduced numbers of splenic Th lymphocytes in the tumor bearing mice is not explained by a reduction in the spleen size. In order to determine if those remaining lymphocytes had suppressed their capability for an effective cytokine-mediated anti-tumor response, we evaluated, by intracellular staining, the frequency of IFN-γ expressing Th lymphocytes in the spleen of tumor bearing mice after mitogenic stimulation. As can be seen in Fig. 2a, the frequency of IFN-γ expressing Th lymphocytes slightly decayed at day 1 after tumor inoculation, but it rapidly increased until day 7. This apparent activation was parallel to an increase in the frequency of $CD4^+$ blast cells during tumor growth (Fig. 2b); and should reflect an activation process. Nevertheless, due to the progressive loss of splenic $CD4^+$ lymphocytes, the total number of IFN-γ expressing $CD4^+$ cells did not increase, but rather decreased until day 2 and did not reach the control numbers (Fig. 2c), indicating that there was not a net induction of IFN-γ expression, but a selective loss of $CD4^+$ cells not primed to express IFN-γ in the spleen of tumor bearing mice. A similar pattern was shown by total $CD4^+$ blast cells (Fig. 2d). If we interpret these results as a consequence of the immune response against the tumor, they would be in accordance with the data suggesting that the tumor induces a Th 2-like response while depressing or leaving the Th 1-like response unaffected [3,13–15]. Moreover, the early effects of the tumor inoculation may suggest that a factor secreted by the tumor is giving rise to a loss of $CD4^+$ cells in the spleen of tumor bearing mice.

3.3. *Effects of ascitic fluid inoculation on splenic Th and macrophage populations*

To test the possibility that the tumor secretes a humoral factor that may induce the observed effects on the host im-

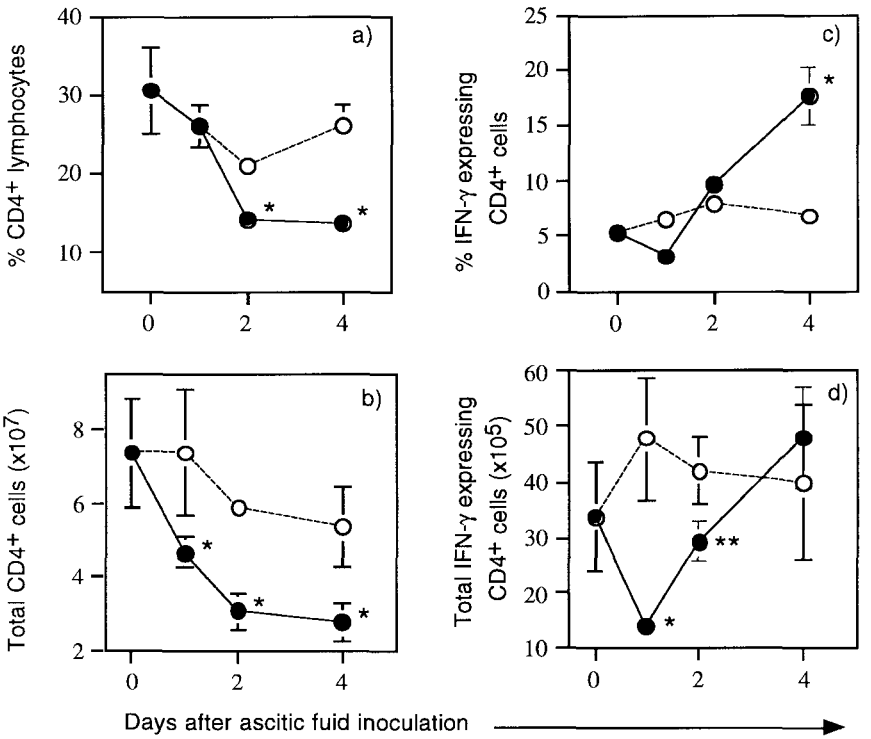

Fig. 3. Frequencies and number of splenic CD4+ and IFN-γ expressing Th lymphocytes in the spleen of mice inoculated with ascitic fluid. 5 mg of cell-free ascitic fluid of protein were daily inoculated to a panel of mice (●). Control mice were inoculated with sterile saline (○). Spleen cells from mice inoculated with ascitic protein were directly fixed for CD18 staining, or stimulated with PMA and Ionomycin and fixed for IFN-γ staining. Data are represented as mean ± S.D. of at least 3 mice. Values significantly different from the controls (using the U-Mann Whitney test) are marked with * ($p < 0.01$) or ** ($p < 0.05$).

mune system, about 100 μl of cell free ascitic fluid (5 mg of total protein from 7-day tumor bearing mice) were daily inoculated to another group of healthy mice, and their spleens were obtained at days 1, 2 and 4 after beginning the experiment. Healthy mice inoculated daily with equal volumes of sterile saline solution were used as controls. The results are shown in Fig. 3. Frequencies and total number of CD4+ lymphocytes were significantly decreased in animals inoculated with ascitic proteins (Fig. 3a and b), showing a similar pattern as that previously found in tumor bearing mice. Similarly, the frequencies of IFN-γ expressing CD4+ lymphocytes were increased in the spleen, although the number of cells reached control levels at day 4, after a pronounced reduction that was observed at day 1 (Fig. 3c and d). The frequency and number of CD18+ macrophages were also similar to those in tumor bearing mice (results not shown).

The reduced number of CD4+ lymphocytes in the spleen together with the higher frequencies of IFN-γ expressing CD4+ cells, suggest that previously activated lymphocytes would be selectively retained in the spleen of tumor bearing mice. It has been demonstrated that EAT cells produce a factor that inhibits lymphocyte migration [32]; in the same way, CD4+ T cells from patients with breast carcinoma inhibit T cell migration [33]. One possible explanation for a preferential retention of IFN-γ expressing CD4+ cells in the spleen of tumor bearing mice, could be the inhibition of migration in preactivated lymphocytes.

Suppressive macrophages are another source of inhibitory function; we have shown that both, the presence of the tumor and the cell-free ascitic fluid, induce an increase in both number and frequencies of splenic macrophages. In the EAT bearing mice, macrophages actively inhibit T cell activation [9]; in the same way, a five fold increase in Mac-1+ splenic macrophages was described in mice with mammary adenocarcinoma [6].

The results shown suggest that some humoral factor(s) produced by the tumor may induce marked systemic effects on the host immune system.

3.4. *Presence of tumor secreted TGF-β precursors in ascitic fluid of tumor bearing mice*

Ascitic fluid from mice bearing EAT cells has been previously shown to exert immunoregulatory activity, depressing splenic T cell polyclonal activation [32]. Many tumors produce soluble factors with pleiotropic activity that interact with the host immune system [33,34]. One of such a factor is the cytokine TGF-β, that possesses angiogenic activity but it is also characterised for its ability to suppress immune response [35]. Since EAT cells have been described to have TGF activity [36], we have made a first attempt to detect TGF-β in ascitic fluid of TBM as well as in EAT cells. Fig. 4 shows an immunoblotting of a gradient SDS gel containing ascitic fluid of 7 day TBM, revealed with a polyclonal pan-specific anti TGF-β antibody. Under non reducing conditions (Fig. 4a, lane 1), a single band of ca. 221 kDa can be observed, which agrees with the molecular weight of the latent high molecular weight complex of TGF-β_1 [37]. When reducing conditions were used (Fig. 4a, lane 2), a major band of ca. 72 kDa was detected, which corresponded with the molecular weight of the TGF-β_1 precursor after reduction of all disulphide

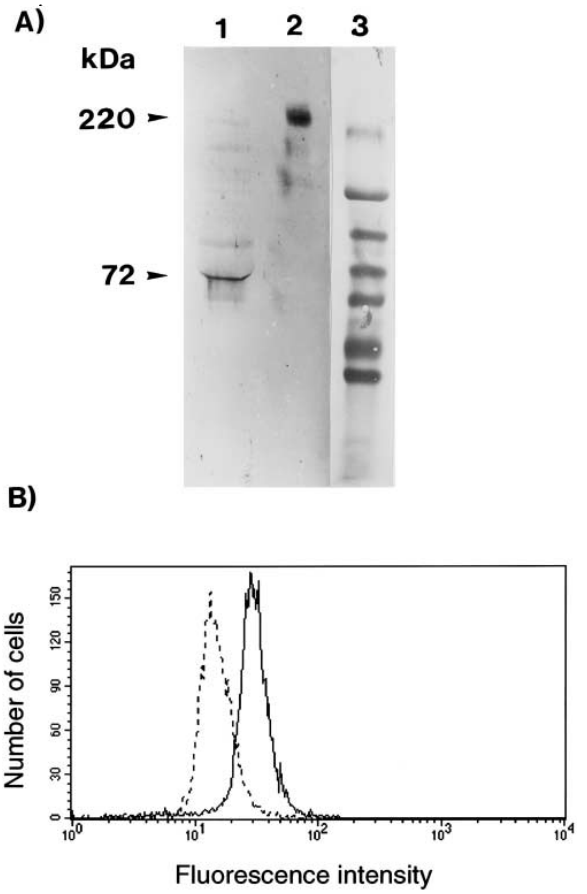

Fig. 4. Detection of TGF-β in cell free ascitic fluid and Ehrlich cells. (a) Immunoblots analysis of ascitic fluid of 7 days TBM. Samples revealed with a polyclonal anti-TGF-β antibody. Lane 1, sample treated with 1 M β-mercaptoethanol (reducing conditions). Lane 2, sample with β-mercaptoethanol omitted (non reducing conditions). Line 3: molecular weight markers: α_2-macroglobulin (193 kDa), β-galactosidase (112 kDa), fructose-6-phosphate kinase (70 kDa), fumarase (57 kDa), lactate dehydrogenase (39.5 kDa), triose-phosphate-isomerase (36 kDa). (b) Intracellular stainings of EAT cells with anti TGF-β antibody (solid line). Control stainings were made using unrelated rabbit IgG as primary antibody (dotted line).

bonds [37]. Furthermore, TGF-β was detected in EAT cells by intracellular staining (Fig. 4b) with the same antibody used for immunoblots.

Active forms of TGF-β_1 could not be detected in our assays in the ascitic fluid of TBM, which makes sense taking into account the potent anti-proliferative activity of TGF-β. The activity of this cytokine is mainly regulated by its realising from the latent TGF-β complex and not by its secretion [38]. Physiologically, this is achieved by the presence of plasmine and urokinase; activated macrophages being one of the cell types that activates endogenous latent TGF-β [38]. In this way, tumor would ensure that TGF-β would exert its activity in the places where its needs are satisfied, i.e. in the host spleen.

All the tumor effects on the host immune system are orchestrated to enhance its tumorigenicity. We have shown in mice with a highly malignant tumor (EAT cells), that early systemic effects could be induced by means of soluble factors secreted by the tumor. This rapid silencing of the host immune response may be of critical importance in order to ensure tumor progression. It has been demonstrated that mice develop a strong and memory immune response against irradiated EAT cells [39,40], which reinforces the view that factors secreted by the tumor are crucial during the establishment of the tumor in the host. The characterization of such factors is important in order to prevent tumor growth and might allow an effective immune response against the tumor.

Acknowledgements: We are indebted to Professor I. Núñez de Castro for helpful suggestions during the course of this work. We thank Professor Dr. Juan Giménez (Departamento de Genética) for use of his FACSort apparatus, and Mrs. Norma I. McVeigh McIvor for the English grammar corrections. Juan A. Segura is recipient of a Training Through Research fellowship from the European Union. This work was supported by a research contract with the European Union. (ERBFMBICT950040) and by grant PB 94-1471 from the Spanish DGICYT.

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