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Infiltrating CD8$^+$ T Lymphocytes, Natural Killer Cells, and Expression of IL-10 and TGF-β1 in Chemically Induced Neoplasms in Male Wistar Rats

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ABSTRACT

The present study aimed to estimate the number of CD8$^+$ T and natural killer (NK) infiltrating cells and the expression of interleukin-10 (IL-10) and transforming growth factor beta 1 (TGF-β1) in chemically induced neoplasms in an initiation-promotion bioassay for carcinogenesis. Male Wistar rats were treated with *N*-nitrosodiethylamine, *N*-methyl-*N*-nitrosourea, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, dihydroxy-di-*N*-propylnitrosamine, and 1,2-dimethylhydrazine for 4 weeks. Two groups were subsequently exposed through diet to phenobarbital (0.05%) or 2-acetylaminofluorene (0.01%) for 25 weeks. An untreated group was used as a control. Immune cells and cytokines were immunohistochemically evaluated in neoplasms and in surrounding normal tissues at the liver, kidneys, lung, and small and large intestines. When compared to the respective normal tissues, an increased number of NK cells was verified infiltrating the colon, lung, and kidney neoplasms, while the number of CD8$^+$ T cells decreased in the intestine and lung neoplasms. Expression of IL-10 was found mainly in kidney tumors. TGF-β1 was expressed mainly in the liver and kidneys tumors. The results indicate that the differential occurrence of immune cells between neoplastic and normal tissues could be dependent upon tumor microenvironment.

Keywords. Initiation-promotion; neoplasia; cytokines; tumor-infiltrating lymphocytes; natural killer cells.

INTRODUCTION

Tumor-infiltrating immune cells have been reported in several neoplasms, such as those of the breast, colon, lung, and ovary, with lymphocytes being the major component of mononuclear cell infiltrates in solid tumors (Merogi et al., 1997; Ropponen et al., 1997; Asselin-Paturel et al., 1998; Wong et al., 1998). Many authors have demonstrated that infiltration by CD8$^+$ T and natural killer (NK) cells in human colorectal cancer with cytotoxic phenotype is associated with favorable outcome and can be used as a prognostic factor (Coca et al., 1997; Ropponen et al., 1997; Naito et al., 1998).

Increasing evidence has suggested that soluble mediators like cytokines play a role in neoplastic development, modulating tumor growth and immune response (Chouaib et al., 1997; Seung et al., 1999). However, the biological activity of cytokines depends on the local microenvironment where they are produced. The aberrant expression of cytokines such as the transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) either by tumor cells or by tumor-infiltrating lymphocytes (TILs) may confer a selective growth advantage to neoplasia by suppressing the activity of the infiltrating immune cells (O'Hara et al., 1998; Seung et al., 1999; Pasche, 2001).

TGF-β is a multifunctional peptide expressed by endothelial, hematopoietic, and connective tissue cells that regulates a variety of cellular processes, including proliferation, differentiation, migration, and adhesion (Massague, 1998). In advanced stages of cancer TGF-β does not act as a bystander; it is actively secreted by tumor cells and contributes to cell growth, invasion, metastasis, and inhibits the host-tumor immune responses (Pasche, 2001).

IL-10 is a cytokine produced by B lymphocytes, macrophages, and type-2 helper T cells (Pretolani et al., 1999). It can inhibit T cell and macrophage activation in vivo, crippling two potential mediators of the anti-tumor immune response (Mossman, 1994). In addition, IL-10 inhibits the production of IL-2 and IFN-γ by T cells (de Wall Malefyt et al., 1993; Taga et al., 1993). The production of IL-10 by human tumors like renal cell carcinoma, ovarian tumors, and non-Hodgkin's lymphoma suggests an in vivo potential immunosuppressive microenvironment within these tumors (Pisa et al., 1992; Blay et al., 1993; Filgueira et al., 1993; Nakagomi et al., 1995; Wang et al., 1995).

The initiation-promotion bioassay protocols, in contrast to the transplantable tumor models, are convenient tools for the in vivo evaluation of the role of the immune system on the neoplastic development (Spinardi et al., 1999; Spinardi-Barbisan et al., 2000, 2004). The initiation-promotion protocol for chemical carcinogens adopted in this study has been proposed as an alternative or complement to the conventional long-term bioassay for detection of chemical carcinogens (Ito et al., 1992, 1998; Takahashi et al., 1992; IARC, 1999). In this context, the present study aimed to investigate the infiltration of T lymphocytes and NK cells and to evaluate the expression of IL-10 and TGF-β1 in chemically induced neoplasms of male Wistar rats submitted to a multiorgan carcinogenesis bioassay. The analysis of the tumor-infiltrating immune cells and of the soluble factors released at the tumor site can contribute to the knowledge of the biological behavior of neoplasms induced chemically.

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METHODS

Animals

Male Wistar rats (4 weeks old) were purchased from CEMIB (Centro Multidisciplinar de Investigação Biológica, UNICAMP, Campinas, SP, Brazil) and housed in polypropylene cages (4 animals/cage) in an environment-controlled room maintained at 22 ± 2°C, 55 ± 10% relative humidity, and with a 12/12-hour light/dark cycle. Animals were supplied with filtered water and fed NUVILAB-CR1 (NUVITAL, Curitiba, PR, Brazil) ad libitum. After a 4-week acclimation period, animals weighing 225–250 g were used in this study.

Chemical Carcinogens

N-nitrosodiethylamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), 1,2-dimethylhydrazine (DMH), and 2-acetylaminofluorene (2-AAF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) and phenobarbital (PB) were purchased from Tokyo Kasei Industries Co. (Tokyo, Japan); and dihydroxy-di-*N*-propylnitrosamine (DHPN) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

Experimental Design

The experimental protocol is shown in Figure 1. Animals were allocated to 4 groups of 15–20 rats each. An untreated group was used as a control and maintained on a basal diet. Three groups (DMBDD, DMBDD/PB, and DMBDD/2-AAF) were treated with 5 chemical initiators of carcinogenesis: DEN (100 mg/kg body weight, ip, single dose at the commencement), MNU (20 mg/kg body weight, ip, 4 times, 2 doses/week), and BBN (0.05% in drinking water for 2 weeks) administered during weeks 1 and 2; DHPN (0.1% in drinking water for 2 weeks) and DMH (40 mg/kg body weight, sc, 4 times, 2 doses/week) administered during weeks 3 and 4. After initiation the DMBDD/PB and the DMBDD/2-AAF groups were supplied with PB (0.05%) or 2-AAF (0.01%) in the diet for 25 weeks, respectively.

Body weight and water and food consumption were evaluated during the experiment (data not shown). The animals were sacrificed by exsanguinations under pentobarbital anesthesia (45 mg/kg body weight) at the 30th week. The protocols used were in accordance with the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Local Ethics Committee for Animal Research (protocol no. 116).

Tissue Processing and Histological Analysis

Liver, kidneys, lung, small and large intestines, spleen, thymus, mesenteric lymph nodes, and bone marrow from all animals were fixed in JB Fix buffer for 24 hours (Beckstead, 1994). All organs removed were embedded in paraffin and stained with hematoxylin and eosin (H&E) for histological analysis (Figure 2). Sections were also prepared on glass slides precoated with poly-D-lysin for immunostaining procedures. Histological diagnosis was performed according to the Monographs on Pathology of Tumors in Laboratory Animals (IARC, 1990).

Immunohistochemistry

Detection of $CD8^+$ T and NK cells (Figures 3 and 4) and expression of IL-10 and TGF-β1 (Figure 5) were performed

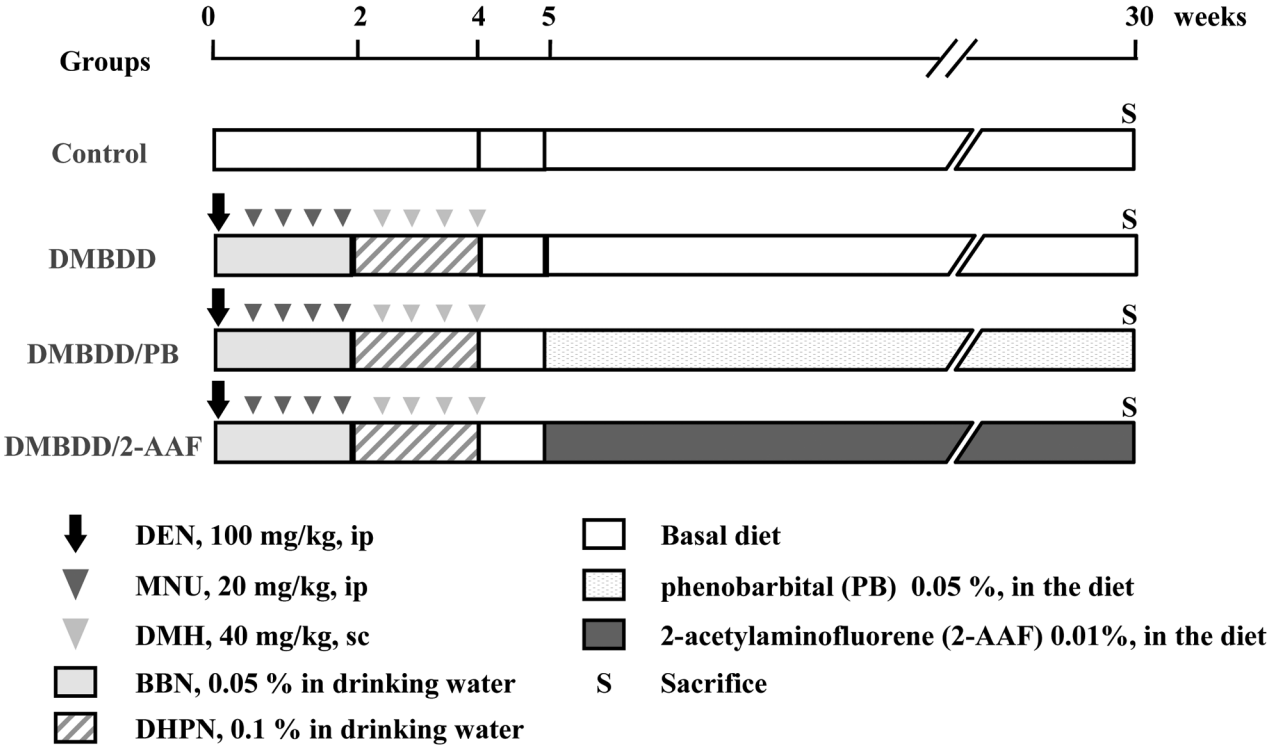

FIGURE 1.—Experimental design (for details, see Methods).

TABLE 1.—Primary antibodies used in immunohistochemistry.

Antibody	Clone	Specificity	Dilution	Catalog number
CD8 (monoclonal)	OX-8	Mature T lymphocyte subsets	1:800	22071D/Pharmingen
NKR-P1A (monoclonal)	10/78	Natural killer cells	1:50	22641D/Pharmingen
IL-10 (policlonal)	M-18	Rat and mice IL-10	1:50	sc-1783/Santa Cruz
TGF-β1 (policlonal)	V	Rat, mice, and human TGF-β	1:200	sc-146/Santa Cruz

immunohistochemically using the avidin-biotin-complex method in serial sections (Hsu et al., 1981). Positive and negative control sections were used in all reactions. Samples from thymus and spleen were used as positive controls for T lymphocyte and lymph node samples for NK cells (Figures 4b and 4d). Histological sections of the small intestine with Peyer's patches and liver were used as positive controls for IL-10 and TGF-β1, respectively. Negative controls (Figures 4a, 4c, 4e, and 4g; Figures 5b, 5d, 5e, 5g, and 5i) were performed by incubating the sections with phosphate buffered saline (PBS) instead of primary antibodies. After deparaffinization and hydration in xylol and alcohol, all sections were submitted to endogenous peroxidase blocking treatment (H_2O_2 3%) in PBS for 5 min followed by nonspecific binding sites blocked with skimmed milk (1% in PBS) for 1 hour. Slides were submitted to overnight incubation in a humid chamber at 4°C with the respective primary antibody (Table 1), then rinsed in PBS solution and incubated with appropriated biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA), 1:200 dilution, in bovine serum albumin (BSA) 1% (Sigma, St Louis, MO, USA) for 1 hour. The slides were rinsed again in PBS and incubated with the avidin-biotin-peroxidase solution (ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 45 minutes. Antigen-antibody complexes were detected with a solution of 0.038% 2,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.025% hydrogen peroxide in 0.1 M Tris-HCl, pH 7.4 for 5 minutes, and the slides were counterstained with Harris hematoxylin. The slides stained for TGF-β1 were previously immersed in citrate buffer pH 6.0 and submitted to microwave treatment for antigen retrieval (two 7-minute cycles) before peroxidase blocking (Park et al., 2001).

Analysis of Immune Cells and Cytokines

The numbers of $CD8^+$ T and NK cells were estimated using a Nikon photomicroscope (Microphot-FXA) connected to an image analysis KS-300 system (Kontron Electronic, Berlin, Germany). Immune cells were quantified in 10 random microscopic fields at 40× magnification and data were calculated and expressed as cell number per normal or neoplastic tissue area (cells/mm^2). The overlapping between the markers for $CD8^+$ T and NK cells was considered in the final counts. Cytokine positive reactions were qualitatively evaluated as weak, moderate, or intense based on the intensity of staining for both normal and neoplastic tissues. Immune cells and cytokines were evaluated in all tumor areas, including the center and the periphery of the tumors.

Statistical Analysis

Statistical analysis was performed with SigmaStat Statistical Analysis System for Windows Version 2.0 (Jandel Scientific, Erkrath, Germany). The comparisons of the numbers of $CD8^+$ and NK cells/mm^2 between normal and neoplastic tissues were done by the Student t-test. The incidence of benign and or malignant neoplasms per group was analyzed by Fisher's Exact Test or the Cui-square. Differences were considered significant when $p < 0.05$.

RESULTS

Histological Analysis

At the 30th week, benign and malignant tumors were found in the liver, kidneys, lung, and small and large intestines of the initiated and promoted groups (Table 2). The lymphohematopoietic organs did not present any tumors in any of the groups. The incidences of hepatic adenomas and of cholangiocarcinomas were significantly higher in the DMBDD/PB ($p < 0.04$) and DMBDD/2-AAF ($p < 0.03$) groups, respectively, when compared to the DMBDD group (Table 3). Tumor multiplicity was higher in the DMBDD/PB and DMBDD/2-AAF groups than in the DMBDD group (Table 2). Figure 2 demonstrates the morphology of some tumors observed in this study.

Immunolabeling and Quantification of Immune Cells

In the normal liver, labeled cells were distributed in the portal area, with a predominance of $CD8^+$ T cells. In the kidneys, both $CD8^+$ T and NK cells were observed in the interstitial tissue around the glomeruli and between renal tubules. In

TABLE 2.—Incidence and frequency of neoplasms.

	Noninitiated	Initiated		
	Control	DMBDD[a]	DMBDD/PB[b]	DMBDD/2-AAF[c]
Number of animals	15	20	20	17
Liver	15[d]	20	20	17
Adenoma	0	1 (05)[1]	7 (35)*	5 (29)
Cholangioma	0	2 (10)	1 (05)	7 (41)
Hepatocellular carcinoma	0	0	1 (05)	3 (18)
Cholangiocarcinoma	0	1 (05)	1 (05)	6 (35)*
Kidneys	15	20	20	17
Tubular adenoma	0	3 (15)	6 (30)	3 (18)
Tubular carcinoma	0	2 (10)	1 (05)	2 (12)
MRT[e]	0	6 (30)	7 (35)	4 (24)
Nephroblastoma	0	0	0	1 (06)
Lung	15	20	20	17
Adenoma	0	7 (35)	8 (40)	2 (12)
Adenocarcinoma	0	0	2 (10)	0
Small intestine	15	20	20	17
Adenoma	0	0	0	0
Adenocarcinoma	0	4 (20)	3 (15)	5 (29)
Large intestine	15	20	20	17
Adenoma	0	0	0	0
Adenocarcinoma	0	4 (20)	8 (40)	6 (35)

[a] Animals initiated with DEN, MNU, BBN, DHPN, and DMH.
[b] Animals initiated and treated with Phenobarbital.
[c] Animals initiated and treated with 2-acetylaminofluorene.
[d] Number of analyzed organs.
[e] Mesenchymal renal tumor.
[1] Percentage of animals with lesion.
* Significantly different from DMBDD group ($p < 0.05$).

TABLE 3.—Incidence of animals with neoplasms and tumor multiplicity.

Groups	No. of animals with tumor	Benign neoplasia		Malignant neoplasia		Tumor multiplicity[a]
		No. of animals	No. of tumors	No. of animals	No. of tumors	
Noninitiated						
Control	0 (15)[b]	0	0	0	0	0
Initiated						
DMBDD[c]	14 (20)	10 (50%)	19	11 (55%)	21	2.86
DMBDD/PB[d]	18 (20)	15 (75%)	28	12 (60%)	32	3.33
DMBDD/2-AAF[e]	16 (17)	13 (76%)	25	14 (82%)	38	3.94

[a] Number of tumors/tumor-bearing animals.
[b] Effective number of animals.
[c] Animals initiated with the carcinogens DEN, MNU, BBN, DHPN, and DMH.
[d] Animals initiated and treated with Phenobarbital.
[e] Animals initiated and treated with 2-acetylaminofluorene.

the lung, $CD8^+$ T cells and rare NK cells were encountered in the bronchi, bronchioles, alveoli, and bronchi-associated lymphoid tissue (BALT). In the normal small intestinal mucosa, $CD8^+$ T cells were detected predominantly at the intraepithelial location, mainly along the top of the villi, and NK cells were found mostly in the lamina propria. In the large intestine, both $CD8^+$ T and NK cells were detected in the lamina propria.

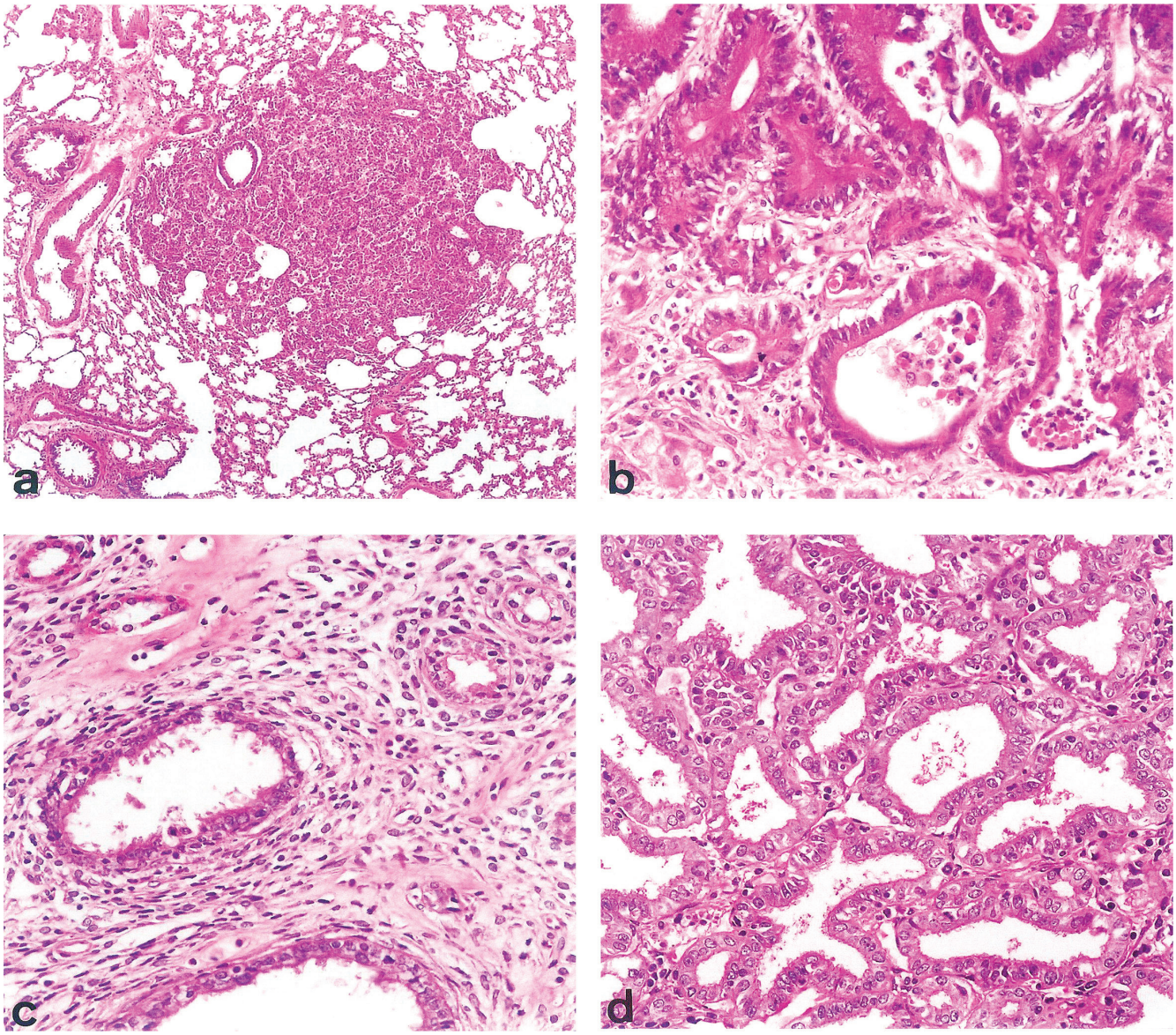

FIGURE 2.—Neoplasms of male Wistar rats chemically induced in an initiation-promotion model for carcinogenesis, stained with H&E. (a) Lung: adenoma; (b) large intestine: adenocarcinoma; (c) kidney: mesenchymal renal tumor; (d) liver: cholangiocarcinoma.

In tumors from the liver $CD8^+$ T and NK cells were seen mainly in the stroma of lesions. The distribution of $CD8^+$ T (Figure 4f) and NK cells in the tumors from the kidneys was similar to that in the respective normal tissue. In the lung tumors, $CD8^+$ T and NK cells were observed permeating tumor mass. In the small and large intestinal tumors, $CD8^+$ T and NK cells (Figure 4h) were found mainly in the stroma, which was abundant in these neoplasms.

The numbers of $CD8^+$ T and NK cells in the different organs in the control group were similar to those found in normal tissues surrounding the tumors in the groups treated with the carcinogens (data not shown).

The number of infiltrating $CD8^+$ T cells was significantly lower in tumors from the lung ($p < 0.001$), small intestine ($p < 0.001$), and large intestine ($p < 0.02$) when compared to the respective normal tissues (Figure 3). In contrast, the number of infiltrating NK cells was significantly higher in neoplasias of kidneys ($p < 0.001$), lung ($p < 0.02$), and large intestine ($p < 0.008$) (Figure 3). In tumors from the liver, the number of infiltrating $CD8^+$ T cells in the DMBDD and DMBDD/2-AAF groups was significantly higher in the lesions (43.10 ± 17.07 vs. 15.52 ± 7.31; 48.85 ± 60.66 vs. 9.05 ± 4.10) than in the respective normal tissue ($p < 0.001$). The number of NK cells was very low in liver tumors (data not shown). Table 4 summarizes the distribution of tumor-infiltrating immune cells in the different tumors.

Immunolabeling and Expression of Cytokines

In normal tissues, IL-10 expression was observed in the epithelial cells of renal tubules, predominantly at the cortical zone (Figure 5a). Weak labeling for IL-10 was seen in the intestines in intraepithelial lymphocytes and in follicular center cells of lymphoid aggregates. Expression of TGF-β1 was detected mainly in hepatocytes (Figure 5c) and in the renal epithelial cells of proximal and cortical collector tubules.

Tumors from the kidneys, lung, and intestines presented positive labeling for IL-10. In renal cell carcinomas there was moderate to intense labeling for IL-10 in epithelial cells (Figure 5f). No expression for IL-10 was observed in mesenchymal renal tumors (Figure 5h). Weak labeling for IL-10 was seen in tumors from the lung and the small and large intestines, mainly in lymphocytes and macrophages.

Expression of TGF-β1 was observed in tumors from the liver, kidneys, lung, and small and large intestines. In the liver, there was moderate to intense labeling for TGF-β1 in the stroma in both cholangiomas and cholangiocarcinomas (Figure 5j). In the kidneys, a weak to moderate labeling for TGF-β1 was detected in epithelial cells from renal cell carcinomas. In mesenchymal renal tumors intense labeling for TGF-β1 was observed in stromal lymphocytes and some normal tubules imprisoned into the tumors. Weak labeling for TGF-β1 was seen in tumors from the lung and the small and large intestines, mainly in stromal lymphocytes. The overview of the expression of cytokines by tumors is presented in Table 4.

DISCUSSION

The occurrence of $CD8^+$ T and NK cells and the expression of IL-10 and TGF-β1 were investigated in order to estimate the migration of immune cells towards chemically induced tumors and to determine whether chemical mediators produced by tumor-infiltrating lymphocytes (TILs) or by neoplastic cells are related to the tumor infiltration by immune cells.

Neoplasms were induced in all groups of male Wistar rats chemically initiated for carcinogenesis and sacrificed 26 weeks later. The distribution of tumors in several different organs demonstrates the amplitude of the initiating treatment used, which established DNA damage in different target organs (IARC, 1999). This alteration probably led to mutations of critical genes involved in the processes of cellular proliferation, differentiation, and cell death (Afshari and Barrett, 1993) and also influenced the expression of different antigens on the cellular surface favoring the tumor infiltration by T lymphocytes and or NK cells (Houbiers et al., 1993). Other factors that interfere with tumor-infiltrating cells

TABLE 4.—Infiltration of immune cells and immunohistochemical expression of cytokines in chemically induced neoplasms.

	Immune cells		Cytokine expression	
Target organs	$CD8^+$ T	NK	IL-10	TGF-β1
Liver				
Adenoma	⇧[a]	Rare cells[b]	—	—
Adenocarcinoma	⇧	Rare cells	—	—
Cholangioma	⇧	Rare cells	—	+ Moderate/intense[c]
Cholangiocarcinoma	⇧	Rare cells	—	+ Moderate/intense
Kidneys				
Adenoma	⇧	⇧	—	—
Carcinoma	⇧	⇧	+	+ Weak/moderate
MRT*	⇧	⇧	Moderate/intense	
			—	+ Intense
Lung				
Adenoma	⇩	⇧	+ Weak	+ Weak
Adenocarcinoma	⇩	⇧	+ Weak	+ Weak
Small intestine				
Adenocarcinoma	⇩	⇩	+ Weak	+ Weak
Large intestine				
Adenocarcinoma	⇩	⇧	+ Weak	+ Weak

[a] Alteration related to respective normal tissue.
[b] Absence or few cells.
[c] Intensity of staining.
*MRT = mesenchymal renal tumor.

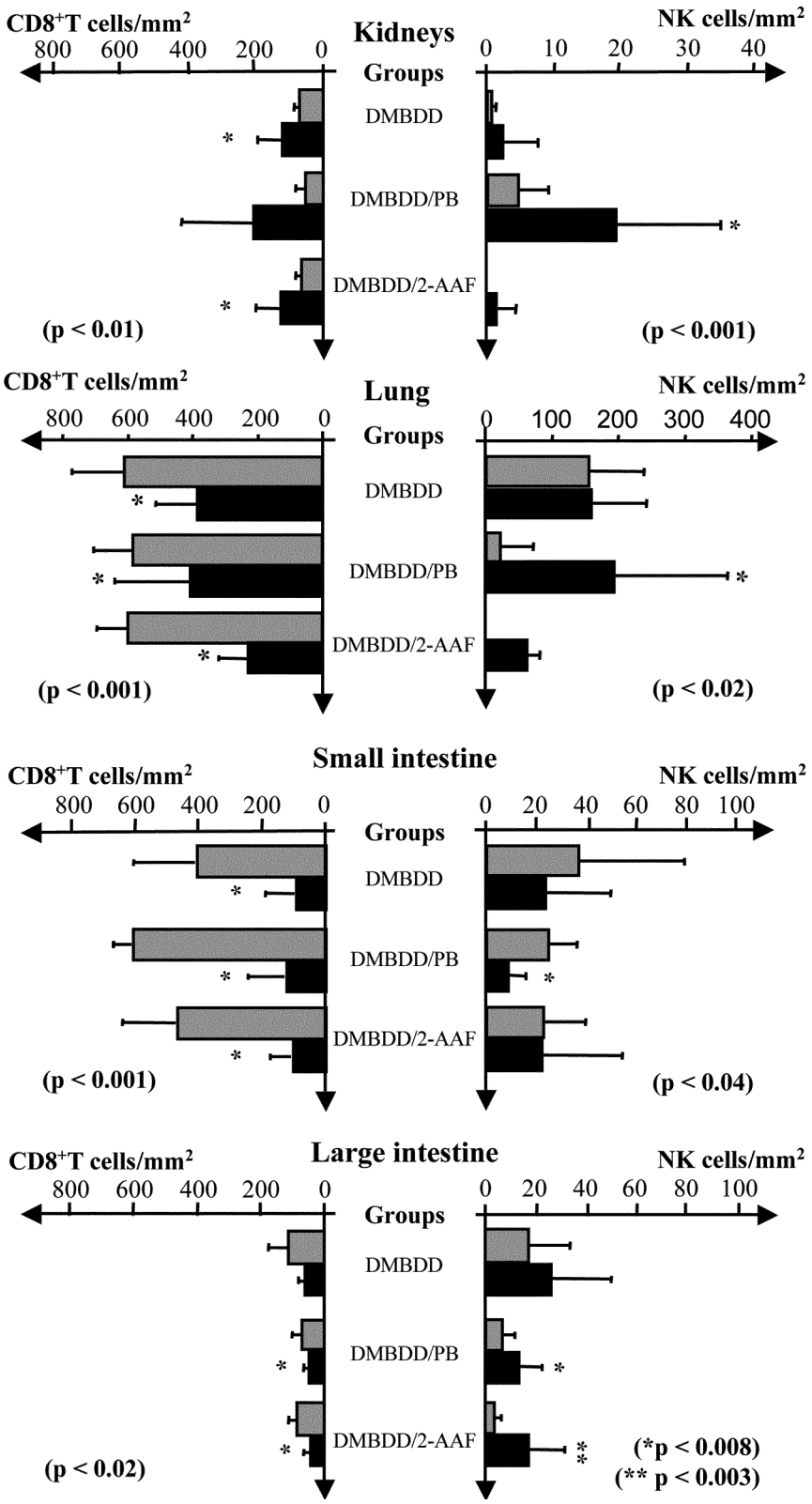

FIGURE 3.—Number of CD8$^+$ T and NK cells in normal □ and neoplastic tissues ■ from kidneys, lung, and small and large intestines from animals initiated and treated with Phenobarbital (PB) or 2-acetylaminofluorene (2-AAF). *, ** Significantly different from normal tissue.

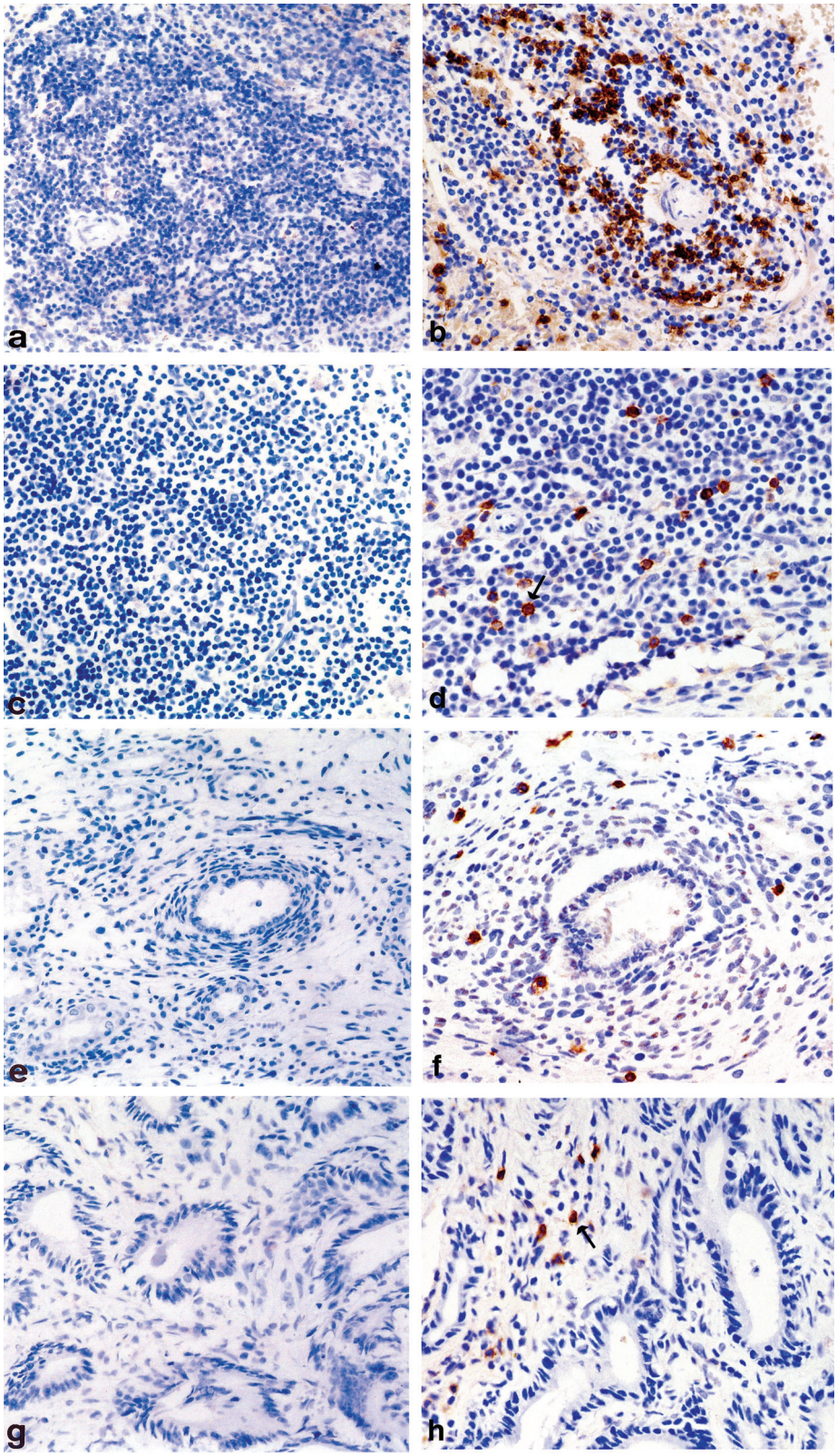

FIGURE 4.—Immunolabeling for $CD8^+$ T and NK cells. Spleen: (a) negative and (b) positive labeling for $CD8^+$ T cells in lymph nodule; lymph node: (c) negative and (d) positive labeling for NK cells; kidney: (e) negative and (f) positive labeling for $CD8^+$ T cells in mesenchymal renal tumor; large intestine: (g) negative and (h) positive labeling for NK cells in colon adenocarcinoma.

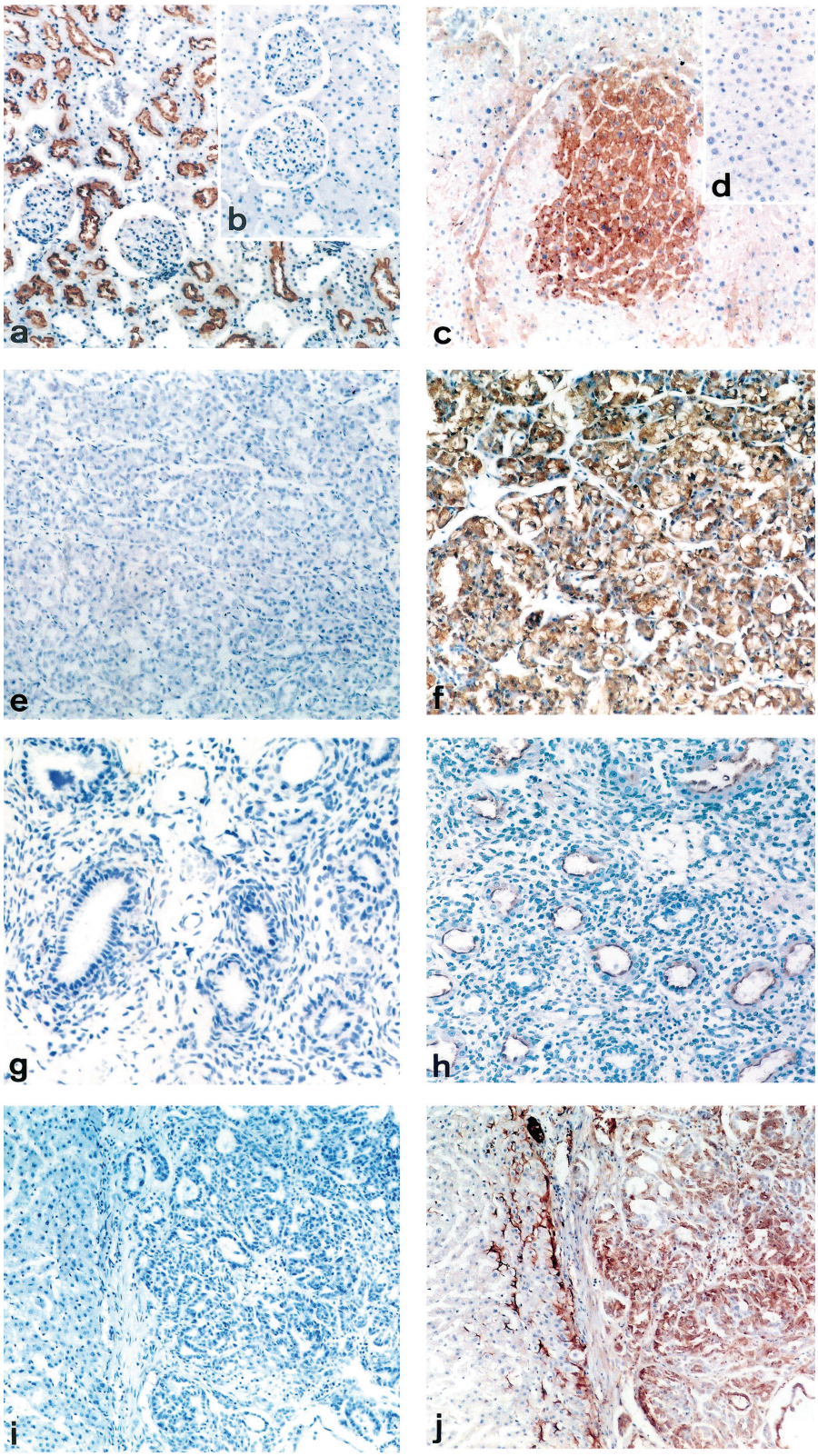

FIGURE 5.—Immunolabeling for IL-10 and TGF-β1. Kidney: (a) positive and (b) negative labeling for IL-10 in cortical zone; liver: (c) positive and (d) negative labeling for TGF-β1; kidney: (e) negative and and (f) positive labeling for IL-10 in renal cell carcinoma; (g) negative and (h) positive labeling for IL-10 in mesenchymal renal tumor; liver: (i) negative and (j) positive labeling for TGF-β1 in cholangiocarcinoma.

include downregulation of the expression of the major histocompatibility complex (MHC) class I molecules, alteration of stromal components such as adhesion molecules, and release of immunosuppressive cytokines by tumor cells (Anderson et al., 1996; Tlsty, 2001). In this study, an increased number of NK cells was observed infiltrating neoplasms of the lung and colon. It is possible that the chemical agents used in the present models or peculiar features of the neoplasms in these organs could have contributed to the loss of MHC class I molecules, favoring the infiltration of NK cells instead of the $CD8^+$ T cells. This phenomenon could inhibit the infiltration of $CD8^+$ T lymphocytes favoring the infiltration of NK cells into these neoplasms (Kaklamanis et al., 1994).

The low frequency of $CD8^+$ T cells observed in tumors of the small and large intestines could also be related to the intrinsic characteristics of their stroma. Ohno et al. (2002) demonstrated in human gastric carcinoma that stromal collagen works as a barrier for $CD8^+$ T cell infiltration, and this might be one of the mechanisms of tumor escape from the host immune attack. Many alterations in tumor microenvironment, including the release of cytokines by stromal cells (e.g., growth factors and extracellular matrix compounds) could facilitate the escape of malignant cells from the host defenses (Tlsty, 2001). In this context, expression of TGF-β1 by tumor cells can contribute to immunosuppression by affecting the differentiation or activation of lymphocytes and the inhibition of perforines liberation by $CD8^+$ T lymphocytes, resulting in reduction of the immune cytotoxic activity (Smyth et al., 1991). TGF-β1 is also responsible for inhibition of chemotactic factors involved in migration of inflammatory cells. In this study, cholangiomas and cholangiocarcinomas presented moderate to intense labeling of TGF-β1 in ductal cells and in the stroma cells within the neoplasia. Interestingly, these tumors presented increased infiltration of $CD8^+$ T lymphocytes when compared to the respective normal tissue. It is possible that TGF-β1 did not interfere in lymphocyte infiltration, but inhibited the cytotoxic activity of these cells.

Moderate to intense expression of IL-10 was observed in renal cell carcinoma, but this cytokine was not expressed in mesenchymal renal tumors. This observation could explain the low infiltration of $CD8^+$ T and NK cells in these epithelial neoplasms. IL-10 may affect the migratory abilities of immune effector cells and/or the metastatic abilities of tumor cells (Marrogi et al., 1997). These results are in agreement with those by Wang et al. (1995) and Nakagomi et al. (1995), who have demonstrated the expression of IL-10 mRNA or protein in renal cell carcinomas, suggesting an in vivo local immunosuppressive effect of this cytokine. Other studies have demonstrated that cells of human renal carcinomas induce the production of IL-10 by monocytes, which downregulate the expression of cell–surface molecules involved in antigen presentation (Ménétrier-Caux et al., 1999).

These results show that the neoplastic development occurs even in the presence of immune cells, but it is possible that these cells present reduced function when facing neoplastic growth. In previous studies we have demonstrated that the chemicals used in the present protocol did not change the ratio $CD4^+/CD8^+$ T cells as determined by flow cytometry (Spinardi-Barbisan et al., 2000). However, the lymphoproliferative response and the NK activity by spleen cells were decreased (Spinardi et al., 1999; Spinardi-Barbisan et al., 2000). We have also demonstrated that the in vitro cytokine production such as IL-2, IL-12, IL-10, TGF-β, and IFN-γ in supernatants of spleen cells of rats submitted to the same chemical carcinogenesis protocol was not altered (Spinardi-Barbisan et al., 2004). Therefore, the biologic activity of cytokines might be related to factors released locally by the tumors. In this context, the differential occurrence of immune cells within chemically induced neoplasms could be dependent on the tumor microenvironment, which is modulated by the neoplastic growing cells.

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