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ORIGINAL PAPERS

Evaluation of the antitumor activity of interleukin-12 in an experimental murine model of colorectal cancer induced by 1,2 dimethylhydrazine (DMH)

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ABSTRACT

Objective: interlukin 12 (IL-12) is a cytokine that may enhance the proliferation and cytotoxic activity of T lymphocytes and natural killer (NK) cells. A relationship between extensive intratumoral infiltration of NK cells and longer survival rates in colorectal cancer (CRC) patients was previously noted. Preliminary evidence suggests that the combined administration of IL-12 and IL-2 may produce additive immunomodulatory activity. The purpose of this study was to determine whether the systemic administration of IL-12 (+/- IL-2) may induce an immune response against CRC as induced by 1,2-dimethylhydrazine (DMH).

Methods: sixty-five 6-week-old Wistar rats were treated with weekly subcutaneous injections of DMH for 26 weeks at a dose of 20 mg/kg of body weight. Once tumoral induction was over, the animals were randomly allocated to one of three groups: I, control; II, intraperitoneal injections of IL-12; III, intraperitoneal injections of IL-12 combined with IL-2. At 30 weeks, all surviving animals were sacrificed. We studied the following parameters in each rat – number of tumors, size of tumors, and total tumoral volume.Tumor samples were studied using the monoclonal antibody CD 57 for the detection of NK cells. The extent of NK infiltration was classified as small, less than 50 NK cells/50 high-power field (HPF); moderate, 50 to 150 NK cells/50 HPF, and extensive, more than 150 NK cells/50 HPF.

Results: thirty-five rats died before completion of the carcinogen exposure, and 30 rats were randomized (10 each group). In group II, 2 animals died during treatment. All rats in groups I and III developed tumors, while in group II two rats (25%) were tumor-free. Moreover, only one rat in group II developed multiple neoplasms, in contrast with group I and group III, where six rats (60%) and seven rats (70%), respectively, had more than one tumor. We found statistically significant differences in the mean number of tumors found in group II when compared to group I (p=0.028) and group III (p = 0.019). Other parameters measured, such as biggest tumor size and total tumoral volume were found to be lower in group II, although no statistical differences were found between groups. Only 10% of rats in group I showed moderated/extensive NK cell infiltration, vs. 60% of rats in group II (p = 0.077) and 70% in group III (p = 0.02).

Conclusion: The administration of DMH to rodents provides a reliable and consistent means of inducing CRC that may be suitable for the evaluation of anti-cancer therapies. Our findings suggest that IL-12 is effective against the development of experimental CRC. Its antineoplastic effect could be attributed to the stimulus of this cytokine on the intratumoral infiltration of NK cells.

Key words: NK cells. Experimental colorectal cancer. Interleukin-12. Interleukin-2.

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INTRODUCTION

Colorectal cancer (CRC) is still an important health problem. It is the second neoplastic cause of death in developed countries (10% of cancer deaths in males and 11% in females) (1). In spite of advances in early diagnose and treatment, mortality by CRC has only slightly decreased during the last five years (2).

The average rate of survival in patients with disseminated CRC in the absence of cytostatic therapy is 6 to 8 months. With chemotherapy, survival ranges from 12 to 18 months. Current treatments elicit a response in 20-40% of patients. Combined chemotherapy-radiotherapy has not shown benefits in patients with stage-III CRC (3). Until now, results obtained with the systemic treatment of

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CRC have been unsuccessful. For this reason it is necessary to investigate new alternative therapies. In this way immunotherapy is the fourth type of treatment for malignant neoplasms, after surgery, chemotherapy, and radiotherapy. This kind of treatment could be a promising type of therapy, and the beginning of a new strategy for the treatment of cancer.

Natural killer cells (NK) are a subset of lymphocytes that express different membrane antigens (CD56, CD 57, CD 16 and FcR). They are especially involved in immune antitumoral response, make up for 1-3% of blood mononuclear cells, have natural antitumoral capabilities, and previous immunization is not required to act against neoplastic cells. In other words, they play an important role in antitumoral defense, since they have a spontaneous ability to recognize and lyse both neoplastic and virus-infected cells (4,5).

The antitumoral action of NK cells is modulated by cytokines, especially by inteleukine 2 (IL-2). Also, inteleukine 12 (IL-12) has shown a powerful antitumoral effect on some tumoral models in animals (6-11), with less toxic effects and more effectiveness than IL-2 (6,7,9,12,13). Even more, a combination of both IL-12 and IL-2 showed better results than either cytokine alone for experimental kidney cancer (14). These results suggest that CD4 and CD8 T cells may be stimulated to act against tumoral growth after treatment with IL-12. However, it seems that the antitumoral effect of IL-12 is not only mediated by T cells but also by NK cells (8,15,16). In this way, previous studies carried out by our group have found a relationship between the intratumoral infiltration of NK cells and survival in patients with colon cancer (17).

Due to the known capacity of IL12 to stimulate the antitumoral effect of NK cells, we think that the systemic administration of IL12 may increase the number of these cells in tumors, and this effect may be improved by the combined administration of IL12-IL2. As a result, a partial or total destruction of tumors may be expected.

The purpose of this study was to determine whether the systemic administration of IL-12 (± IL-2) may induce an immune response against CRC in an experimental model induced by 1,2-dimethylhydrazine (DMH) (18). For that reason, we tried to reach two objectives –one was the development of a valid experimental model of CRC, and the other was the evaluation of intratumoral infiltration with NK cells and the outcome of experimental tumors after treatment with IL-12 (± IL-2).

MATERIAL AND METHODS

Sixty-five 6-week-old Wistar rats were treated with weekly subcutaneous injections of DMH for 26 weeks at a dose of 20 mg/kg of body weight. The carcinogen was freshly prepared every week prior to the time of injection. DMH was prepared by diluting 400 mg of DMH in 100 ml of destilled water containing 37 mg of EDTA (0.001 M), with a pH of 6.5 being adjusted using sodium hydroxide (1% NaOH). All rats were fed on a fat –and carbohydrate-rich, calcium– and fiber-poor diet. After tumor induction, the animals were randomly allocated to one of three groups –group I: control; group II: intraperitoneal injections of IL-12, 1 mg/day, 5 days a week, for weeks 28-29; group III: intraperitoneal injections of IL-12, 0,5 mg/day, 5 days a week, for weeks 28-29, combined with IL-2, 300,000 IU twice daily, one day per week, for weeks 27-28-29. None of the animals in group II or III received any treatment before week 28. At week 30, all surviving animals were sacrificed. At the autopsy, the thoracic and abdominal cavities were examined. The entire gastrointestinal tract was removed, longitudinally opened, and cleaned of residue with water.

We examined all abdominal viscera and gastrointestinal tract, and studied the following parameters in each rat –number of tumors, size of tumors, and tumor load (tumor total volume: $\sum V_T = \sum[^3/_4 \pi R^3]$). Tissue specimens were fixed in 10% formalin and embedded in paraffin. Six-micrometer paraffin sections were stained with hematoxylin and eosin. Tissue sections were analyzed by a pathologist, and all diagnoses of tumor tissue were confirmed and studied by at least two pathologists. Tumor slices were studied using monoclonal antibody CD 57 for the detection of NK cells. Tissue sections were analyzed by two independent observers. The extent of NK-cell infiltration was classified as small, when we found fewer than 50 NK cells/50 high-power intratumoral fields (HPF); moderate, for 50 to 150 NK cells/50 HPF, and extensive, for more than 150 NK cells/50 HPF. When no interobserver agreement was reached, tissue specimens were studied by another observer.

Statistical analyses were performed using the SPSS for Windows 11.0 program. Descriptive statistics included relative frequencies for categorical variables, and mean and standard deviation for quantitative variables. For group comparisons, a Mann-Whitney non-parametric U test was used. The minimum level of statistical signification used was $p < 0.05$.

RESULTS

Survival

During the 26 weeks of tumor induction 35 rats died before completion of carcinogen exposure (rate of mortality, 53%). But mortality did not follow a lineal course, because only 15% of deaths occurred during the first 16 weeks. After that, 30 rats were randomized into three groups (10 in each group). In group II, two animals died during treatment.

Experimental tumors (Table I)

In group I (control) all rats developed tumors (100%). We found one tumor in four rats, two to four tumors in

Table I

		Treatment			Total
		I (Control)	II (IL-12)	III (IL-2 + IL-12)	
Tumors	No		2 (25%)		2 (7.1%)
	Yes	10 (100%)	6 (75%)	10 (100%)	26 (92.9)
Histological type	Gastric adenocarcinoma		1 (12.5%)		1 (1.7%)
	Small-bowel adenocarcinoma	3 (10.3%)	4 (50.0%)	9 (39.1%)	16 (26.7%)
	Proximal colon adenocarcinoma	1 (3.4%)		5 (21.7%)	6 (10%)
	Distal colon and rectal adenocarcinoma	25 (86.2%)	2 (25%)	8 (34.8%)	35 (58.3%)
	Pleural mesotheliomal		1 (12.5%)		1 (1.7%)
	Squamous-cell carcinoma			1 (4.3%)	1 (1.7%)
Total		29 (100%)	8 (100%)	23 (100%)	60 (100%)
Degree of differentiation	Well differentiated	12 (41.4%)	3 (42.9%)	12 (54.5%)	27 (46.6%)
	Moderately differentiated	4 (13.8%)	1 (14.3%)	5 (22.7%)	10 (17.2%)
	Poorly differentiated	13 (44.8%)	3 (42.9%)	5 (22.7%)	21 (36.2%)
Tumor extension	T1	3 (10.3%)	1 (14.3%)	4 (18.2%)	8 (13.8%)
	T2	19 (65.5%)		6 (27.3%)	25 (43.1%)
	T3	3 (10.3%)	4 (57.1%)	11 (50%)	18 (31%)
	T4	4 (13.8%)	2 (28.6%)	1 (4.5%)	7 (12.1%)
Lymph node/peritoneal infiltration	No	10 (100%)	7 (87.5%)	8 (80%)	25 (89.3%)
	Yes		1 (12.5%)	2 (20%)	3 (10.7%)
Liver metastases	No	9 (90%)	7 (87.5%)	10 (100%)	26 (92.9%)
	Yes	1 (10%)	1 (12.5%)		2 (7.1%)

five rats, and eleven microcarcinomas in one rat, with a total of 29 tumors. Tumor sites included: 25 in the distal colon or rectum (86%) (Figs. 1, a, b), three in the small bowel (10%), one in the proximal colon, and one liver metastasis. Tumor extension was: 10% T1, 65% T2, 10% T3, and 13% T4. In all, 41% adenocarcinomas were well differentiated, 13% were moderately differentiated, and 44% were poorly differentiated.

In group II (IL-12), eight rats were still alive at the end of treatment, two rats (25%) were tumor-free, and only one rat developed three tumors. Of the eight tumors found in this group, two (25%) were adenocarcinomas of the distal colon or rectum, four (50%) were adenocarcinomas of the small bowel, one (12%) was a gastric adenocarcinoma, and one (12%) was a pleural mesothelioma. One of the animals developed liver metastases, and another animal developed mediastinal lymph-node metastases and peritoneal implants. Of seven gastrointestinal tumors, four were in stage T3 (57%), one in T1 (14%), and two in T4 (43%). Three tumors (43%) were well-differentiated adenocarcinomas, whereas one (14%) was moderately differentiated, and three (43%) were poorly differentiated.

In group III (IL-2+ IL-12), all rats developed tumors (100%). In this group we found a total of 23 tumors, and seven animals developed multiple neoplasms. Eight tumors (35%) were adenocarcinomas of the distal colon or rectum, nine (39%) were adenocarcinomas of the small bowel, five (22%) were adenocarcinomas of the proximal colon, and one (4%) was a squamous-cell carcinoma of the external auditory canal. Two animals (20%) developed regional lymph-node metastases. Tumor extension was: 18% T1, 27% T2, 50% T3, and 4% T4. In all, 54% of adenocarcinomas were well differentiated, 23% were moderately differentiated, and 23% were poorly differentiated.

Rats in group II (Table II) showed the lesser number of tumors, with a rate of 1 tumor/animal (SD = 0.9) *vs.* rats in group I, with a rate of 2.9 tumors/animal (SD = 3) ($p = 0.028$), and *vs.* rats in group III, with a rate of 2.3 tumors/animal (SD = 1.3) ($p = 0.019$). Other parameters measured included: a) average biggest tumor size, which was also lower in group II (0.8 cm, SD = 0.6) *vs.* group I (0.9 cm, SD = 0.7) and group III (1 cm, SD = 0.8); and b) total tumor volume, which showed a lower average in group II, which was 0.5 cm^3 (SD = 0.7), *vs.* 0.8 cm^3 (SD = 1.2) for group I and 1.2 cm^3 (SD = 2) for group III. However, we did not find significant statistical differences between groups regarding these two parameters.

Intratumor infiltration of NK cells (Table III)

Only 10% of rats in group I showed moderate/extensive NK-cell infiltration, *vs.* 60% (40% moderate and 20% extensive) of rats in group II ($p = 0.077$) and 70% in group III (50% moderate and 20% extensive) ($p = 0.02$). Between groups II and III no statistical differences were found ($p = 1$) (Figs. 1, c-f).

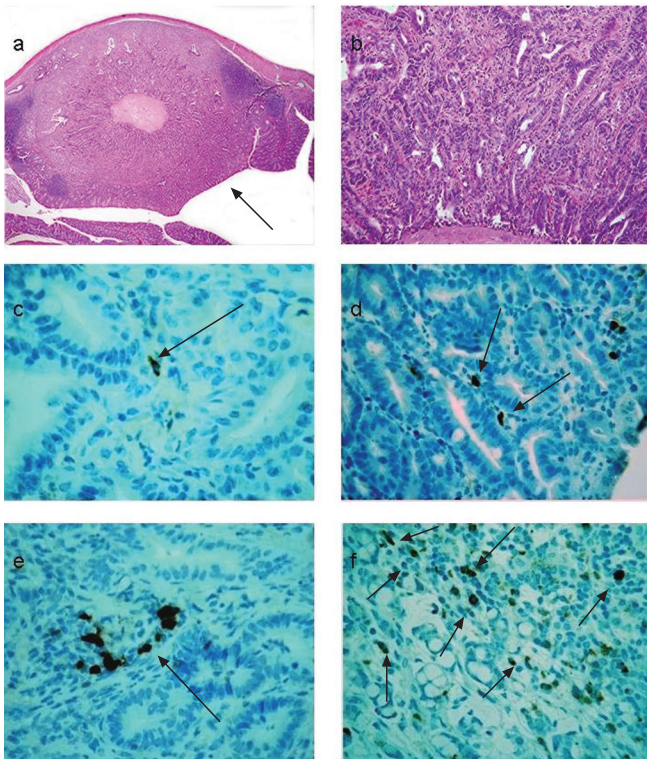

Fig. 1.– a: Intestinal tumor induced by DHM (arrow). b: Well-differentiated adenocarcinoma. c: Small intratumor NK-cell infiltration (arrow). d: Moderate intratumor NK-cell infiltration (arrows). e: Interaction of NK cells with tumor cells (arrow). f: Extensive intratumor NK-cell infiltration (arrows).

a: Tumor intestinal inducido mediante DHM (flecha). b: Tumor inducido de tipo adenocarcinoma bien diferenciado. c: Infiltración tumoral de células NK (flecha) escasa. d: Infiltración intratumoral moderada de células NK (flechas). e: Interacción de células NK con células tumorales (flecha). f: Infiltración tumoral abundante de células NK (flechas).

Table II

		Treatment		Total
		I (Control)	II (IL-12)	III (IL-2 + IL-12)
*Number of tumors**	Mean (SD)	2.90 (3.07)	1 (0.92)	2.30 (1.33)
Biggest tumor size (cm)	Mean (SD)	0.89 (0.72)	0.8 (0.62)	1.05 (0.79)
Tumor load (cm^3)	Mean (SD)	0.78 (1.16)	0.5 (0.74)	1.15 (2.16)

SD: standard deviation.
*Statistical differences: (Control) *vs.* (IL12) p = 0.028
*Statistical differences: (IL12) *vs.* (IL12 + IL2) p = 0.019

Table III. Intratumor infiltration of NK cells

	Group I (control)	Group II (IL-12)	Group III (IL-2 + IL1-2)
Small	9 (90%)	2 (40%)	3 (30%)
Moderate	1 (10%)	2 (40%)	5 (50%)
Extensive		1 (20%)	2 (20%)

DISCUSSION

The model of induction using subcutaneous DMH is a very well known and experimented model (19,20) used to obtain tumors resembling human colorectal cancer, both microscopically and in clinical behavior (18). Most authors consider that only one dose of 20 mg per kilogram of weight, injected weekly for 26 weeks, may induce a very high incidence of colorectal adenocarcinomas (18,19,21-24). However, even in these models a high incidence of tumors may be found, which rarely reaches 100% (25). Several modifications of these models were proposed in order to facilitate carcinogenesis, such as the addition of 5FU (26) or complex surgical modifications (25). In our model, we decided to use a 20-mg dose in subcutaneous injection plus the addition of a diet that was low in fiber but very high in fat and carbohydrates; based on recent studies over the anticarcinogenic action of calcium-rich diets (27), we used a diet poor in calcium. In a previous step, we began with DMH tumor induction in 30 experimental animals, and obtained tumors in all rats that were alive at 26 weeks after induction (28); nevertheless, we noted a very high mortality rate. This mortality rate was similar to that described by other authors (22), and higher than that noted by other authors (23,27). With these controversial results we began our study with 65 rats, 35 (53%) of which died before induction completion. This high mortality rate, in our opinion, could be due to the high toxicity of DMH. In the group of animals that completed the induction process, we obtained, as expected, 100% of tumors in the control group, results that confirm the high safety level in this model. Nevertheless, the high rate of mortality in this model should be kept in mind for futures studies.

In the group of rats treated with IL-12 we observed a lower tumor incidence *versus* the control group, with a significant statistical difference. Also a lower tumor size and tumor load were found in rats in group II *vs.* rats in group I (control), but with no statistical difference. These findings were associated with a higher presence of intratumor infiltration of NK cells in rats in group II *vs.* rats in group I, with significant statistical differences. The association found between a higher presence of NK cells in inflammatory tumoral infiltrates and a lower incidence of experimental tumors in animals treated with IL-12 is in accordance with the favorable prognosis attributed to intratumor infiltration of NK cells, as has been described by our group in human colorectal cancer (17), and by other authors in gastric cancer (29) or in lung cancer (30, 31). We believe that this is due to the stimulating effect of IL-12 on NK cells at the dose used.

Nevertheless, the results found when we used IL-12 plus IL-2 in group-III rats were apparently contradictory

as they were unexpected. We did not find statistically significant differences in number of tumors, tumor size or tumor mass between this group and group I, in spite of a general increase of NK-cell infiltrates in group-III rats. In our opinion these results may be due to the IL-12 dose used, since in an attempt to avoid potential higher toxicity with the addition of IL-2, we reduced the IL-12 dose to a half for animals in group III. Another possibility is that the effectiveness of combined IL-12 + IL-2 as seen in other tumors (14) may not be that high in CRC. This hypothesis has yet to be confirmed in future experimental studies searching for an optimal dose of cytokines, as the potential activity of IL-12 together with IL-2 continues to be an attractive option. In this way, recent studies (32,33) are going to open up new options in the selection of patients for immune treatments, where not only the stimulation of a number of intratumor infiltrates of NK cells, but also the antineoplastic activity of this kind of cells *in vitro* should be considered (34,35).

Nevertheless, we think that the tumor model used is a useful model for antitumor treatment trials, as was the case with our study of intestinal cancer. We strongly believe that IL-12 may have an antineoplastic effect against tumor development, and this effect may be linked, partly at least, to the stimulation produced by this cytokine on intratumor infiltrates with NK cells. However, new studies are needed to analyze not only the quantification of NK-cell infiltrates, but also the antitumor activity of these cells, and to find out the correct dose of IL-12/IL-2 in order to obtain the best antitumor response.

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