Eur J Cancer Clin Oncol, Vol. 24, No. 2, pp. 151–159, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00

The Effectiveness of Intravenously Administered Immune Lymphoid Cells Against Intraperitoneally Growing Tumor Correlates with Their Homing in the Recipients' Lymphoid Organs

RADISLAV VUKIĆ,* RUDOLF TOMEK,† FILIP ČULO and MATKO MARUŠIĆ
Department of Physiology, Zagreb University School of Medicine, Zagreb, Yugoslavia

Abstract—*Intraperitoneally growing Ehrlich ascites tumor (EAT) was eradicated by both i.p. and i.v. injection of serum, and by i.p. injection of spleen cells from mice immune to EAT. However, the i.v. injected immune spleen cells were completely ineffective unless the recipients had been pretreated with cyclophosphamide. The analysis of immune response in mice cured by the combination of cyclophosphamide and cell transfer revealed that they developed a humoral-type immunity to EAT and that the transferred spleen cells did not penetrate into their abdominal cavity. The effect of cyclophosphamide correlated with the extent of seeding of the donor-type cells in the recipients' lymphoid organs. Inasmuch as homing in cyclophosphamide-pretreated mice surpassed that in normal mice three to four times, it appeared that the beneficial effect of cyclophosphamide was primarily founded on its enhancement of seeding of the transferred lymphoid cells, implying that homing of these cells is a prerequisite for their anti-tumor activity.*

INTRODUCTION

PASSIVE tumor immunotherapy has been extensively studied [1], obviously in relation to its potential clinical application. Generally, the transferred immune lymphoid cells are most effective when admixed with tumor cells prior to inoculation [1]. Unfortunately, such experimental protocols bear little or no resemblance to clinical reality; more relevant models are those in which immune cells are injected i.v. to animals with clinically ascertained tumors. However, in such 'distant immunotherapy' models, the transferred lymphoid cells have proven to be ineffective [1–3]. Their effectiveness could be improved by various pretreatments of tumor-bearing recipients, primarily by sublethal irradiation [4], cyclophosphamide injection [3, 5] or T-cell depletion [2, 6]. Such enhancement of anti-tumor effectiveness of the transferred immune lymphoid cells was ascribed to eradication of the recipients' suppressor lymphocytes that allegedly thwart the action of the transferred lymphoid cells [2, 3]. The idea is based on the observation of the suppressor cells being selectively depleted due to their greater sensitivity to such treatments [2–4, 7].

Still respecting this concept, in the present report we describe another factor that may prove significant in regard to the effectiveness of passive tumor immunotherapy, i.e. the extent of homing of the transferred lymphoid cells in the recipients' lymphoid organs.

In our previous studies [8], the i.v. injected spleen cells from donors immune to Ehrlich ascites tumor (EAT) were found to be ineffective against an i.p. growing tumor. In the present study, this ineffectiveness was found to be completely reversed by cyclophosphamide, the EAT cells being destroyed by the humoral-type of immune response. A cytologic analysis of the transferred immune lymphoid cell homing has revealed, however, that these cells never penetrate into the peritoneal cavity of EAT-bearing animals. Their effectiveness correlated not only with cyclophosphamide pretreatment but also with the extent of their homing in the recipients' lymphoid organs. It is thus possible that the improvement of seeding of the transferred cells underlies the beneficial effects of cyclophosphamide in the passive tumor immunotherapy protocols.

Accepted 4 August 1987.
Correspondence to: Professor Matko Marušić, Department of Physiology, Zagreb University School of Medicine, Šalata 3, 41000 Zagreb, Yugoslavia.
*Present address: Department of Gynecology, University of Banja Luka, Banja Luka, Yugoslavia.
†Present address: Institute of Tumors and Allied Diseases, Zagreb, Yugoslavia.

MATERIALS AND METHODS

Mice

Inbred mice from our animal colony (CBA/Zgr and CBAT6T6/Zgm) were used in all experiments. They were derived from original strains obtained 20 years ago from the Medical Research Council, Harwell, U.K. Mice of both sexes, aged 3–5 months, were used. The CBAT6T6/Zgm strain is syngeneic with the CBA/Zgr strain, differing in the shape of the sixth pair of chromosomes only. This difference is a basis of the cytologic analysis that allowed us to determine the origin of mitotic cells in cured mice.

Tumor

Ehrlich ascites tumor (EAT) is a highly transplantable tumor that grows in all mouse strains. Its origin is unclear; it is believed to represent a spontaneous mammary carcinoma able to grow in an ascitic form [9]. After an s.c. injection, it grows in a solid form. Our line is an aneuploid one, maintained by i.p. passages in CBA/Zgr mice.

In the present study, EAT cells were injected i.p., 5–10 × 10^5/recipient. This dose killed all the recipients within a month.

Immunization to EAT was achieved by two consecutive (administered in a 2-week interval) injections of 2 × 10^7 100 Gy-irradiated EAT cells, and a challenge with 5 × 10^5 live cells. The mice which survived tumor-free for 2 months were used as donors of immune serum and spleen cells.

Cyclophosphamide

Cyclophosphamide (Endoxan produced by Bosnalijek, Sarajevo, Yugoslavia, under license from Asta-Werke, Brackwede, F.R.G.) was disssolved in sterile isotonic saline and appropriate concentrations were injected i.v. within 1–2 h, 0.01 ml/kg body wt.

Cell suspension and transfer

Spleen and EAT cells were always harvested under aseptic conditions and injected in 0.5 ml phosphate-buffered saline (PBS).

Scoring of chimerism

To determine the incidence of donor cells among mitotic lymphoid cells, the cells were analyzed for the presence of autosomal T6T6 markers in the recipient CBA/Zgr mice. The procedure used for scoring mitotic cells was that described by Ford [10].

Assessment of the distribution of radioactively labelled spleen cells

5 × 10^8 spleen cells were incubated for 45 min at 37°C with 200 μCi of ^{51}Cr (B. Kidrič Institute, Vinča, Yugoslavia). After three washings, the recipients were injected with an appropriate number of viable cells. The radioactivity of the spleen, liver and peritoneal eluate was determined and expressed as fraction of total body radioactivity determined for each individual mouse.

Immunofluorescence

The EAT cells, harvested from treated or untreated animals, were washed three times in PBS containing 0.01% azide and 2% fetal calf serum. Thereafter, they were exposed to fluorescein-labelled rabbit anti-mouse IgG serum (Hyland, Travenol Laboratories, California, U.S.A.) at 4°C for 30 min (10^6 cells in 0.1 ml 1/12 diluted antiserum). After three washings, the cells were resuspended in PBS and azide and analyzed immediately under a u.v.-microscope. At least 100 cells were scored in each sample.

Elution of immunoglobulins from the surface of EAT cells

The procedure of Witz *et al.* [1] was used. In short, a sample of 400 mg of packed tumor cells was suspended in 2.5 ml cold glycine–HCl buffer, 30 min at pH 2.5. After centrifugation, the supernatant pH was changed to 7.5 and the specimen centrifuged at 4°C for 30 min at 8000 ***g***. The concentration of proteins was determined in the new supernatant and adjusted by ultrafiltration to 5 and 30 mg/ml, respectively.

Testing of sensitivity of EAT cells to complement in vitro

The EAT cells were harvested from treated mice, washed three times in minimal essential medium (MEM) and admixed to the 1 : 10 diluted complement (lyophilized guinea pig complement, Institute of Immunology, Zagreb, Yugoslavia), at a concentration of 2 × 10^6 cells/0.2 ml. In the negative control groups, the EAT cells were mixed with 0.2 ml MEM. The cells were then incubated for 45 min at 37°C in water bath, washed and checked for viability by the dye-exclusion method (0.05% Trypan blue in PBS). The percentage of stained (dead) cells was determined in each individual sample for at least 200 cells.

Tumor growth-inhibition tests

Equal volumes of EAT and spleen cell suspensions (or serum of the desired dilution) were mixed together and injected immediately afterwards. Mice received 10^6 EAT cells i.p. or 10^5 EAT cells s.c.

Cell-mediated cytotoxicity

10^5 live ^{51}Cr-labelled EAT cells in RPMI-1640 (Gibco, U.S.A.) were admixed in triplicate samples with 25 × 10^5, 50 × 10^5 or 100 × 10^5 spleen cells from EAT-immune donors. Culture supernatants were checked for ^{51}Cr-release after a 4 h incubation

Table 1. Passive immunotherapy of i.p. growing Ehrlich ascites tumor with various doses of spleen cells and serum from EAT-immune donors†*

Spleen cells ($\times 10^6$)	Serum (ml)	Dead/total mice injected i.p.	i.v.
200		0/10	10/10
100		0/10	10/10
50		0/10	10/10
20		5/10	10/10
10		10/10	ND
	0.2	0/10	0/10
	0.1	0/10	0/10
	0.05	4/10	5/10
None		10/10	

*10^6 cells injected i.p. Immunotherapy was performed on the same day.

†Donor mice were immunized twice with 2×10^7 100 Gy-irradiated EAT cells and challenged with 10^5 EAT cells/mouse. Two months later, tumor-free mice were sacrificed to obtain immune spleen cells and anti-EAT serum.

at 37°C in a humidified atmosphere containing 5% CO_2.

Statistics

The survival times in mice were evaluated by the simplified Mann–Whitney *U*-test [12], at a 95% significance level. The incidence of survivors in particular experimental groups was compared by the chi-square test.

RESULTS

Failure of intravenously injected immune spleen cells to constrain intraperitoneal EAT growth

Mice were injected with 10^6 EAT cells/mouse i.p. and simultaneously i.p. or i.v. with various doses of syngeneic immune spleen cells, ranging from 2×10^8 to 10^6 cells/mouse (Table 1). Some mice were injected i.p. or i.v. with 0.2, 0.1 or 0.05 ml/mouse of serum from EAT-immune donors (Table 1). Mortality was followed up for 3 months. Intraperitoneally injected immune spleen cells constrained EAT growth completely in doses of 5×10^7 cells/mouse and higher, partially when given in a dose of 2×10^7, and were completely ineffective in a dose of 10^7 cells/recipient. Intravenously injected immune spleen cells were completely ineffective; up to 2×10^8 cells neither cured any mouse nor influenced the median survival times (not shown). On the contrary, anti-EAT serum from immune donors inhibited tumor growth when administered either i.p. or i.v.; 0.1 ml was effective in all recipients, whereas the dose of 0.05 ml protected approximately half of the tested population (Table 1).

The ability of spleen cells from EAT-immune donors to kill EAT cells in a short-term *in vitro* culture was tested using the 25:1, 50:1 and 100:1 spleen cell to EAT cell ratios. The experiment was performed with a pool of donor spleen cells that had proved inhibitory for the EAT growth in the tumor growth-inhibition tests (Table 1), but no significant cytotoxicity was detected (data not shown).

The next experiment was designed to test whether the inability of the i.v. injected immune spleen cells to influence i.p. EAT growth was due to their failure to contact the antigen (most probably restricted to the peritoneal cavity) and become adequately stimulated. Therefore, in addition to 10^6 live EAT cells i.p. and 10^8 immune spleen cells i.v., the groups of recipient mice were also injected i.v. with 4×10^7 irradiated EAT cells. Irradiated cells were supposed to provide the lacking antigenic stimulus; they were injected 2 days before, simultaneously or 2 days after immune spleen cells. However, in any schedule they were unable to reverse the inability of i.v. injected immune spleen cells to constrain i.p. EAT growth (data not shown).

Restoration of the activity of i.v. administered immune spleen cells against i.p. growing EAT by a cyclophosphamide pretreatment

Recipient mice were injected with 10^6 EAT cells i.p. and 5 days later with 200 mg/kg cyclophosphamide and 9×10^7 immune spleen cells i.v. The injection of spleen cells was delayed by at least 6 h after cyclophosphamide in order to avoid adverse effects of the drug on the transferred lymphocytes [13]. Mortality was followed up for 3 months (Table 2). As expected, the immune spleen cells alone had no influence on the EAT growth.

Cyclophosphamide alone was found to moderately increase the survival time, but had no curative effect (Table 2). On the other hand, all mice treated with both cyclophosphamide and immune spleen cells rejectd EAT and survived tumor-free throughout the observation period.

To investigate the characteristics of the immune reaction against EAT in mice cured by the combination of cyclophosphamide and spleen cells, in the following experiment the presence and activity of anti-EAT antibodies in these animals were analyzed. An immunofluorescence study of the i.p. growing EAT cells revealed that the majority of tumor cells (69% on day 2 and 45% on day 5 following the drug and cell injection) was coated with anti-EAT antibodies (Table 3). When exposed to complement *in vitro*, these EAT cells appeared to be sensitive to complement-mediated lysis (Table 3, right columns). In contrast, the EAT cells from mice treated with cyclophosphamide or immune spleen cells only were antibody-free and resistant to complement lysis *in vitro*.

Further evidence of the antibody-mediated mechanism of inhibition of i.p. EAT growth in mice

*Table 2. Restoration of anti-EAT activity of i.v. injected immune spleen cells against i.p. growing EAT by the cyclophosphamide pretreatment**

Injection (i.v.)	Dead/total mice	Median survival time (days) (range)	$< P$
—	10/10	17 (15–21)	
Immune spleen cells	10/10	18 (16–23)	NS
Cyclophosphamide	10/10	26 (23–31)	0.05
Immune spleen cells + cyclophosphamide	0/10	> 90	0.001

*10^6 EAT cells were injected i.p. and 5 days later 200 mg/kg cyclophosphamide was injected i.v. Immune spleen cells (9×10^7/mouse) were injected at least 6 h after cyclophosphamide.

*Table 3. Humoral immunity to EAT cells in mice protected by passive immunotherapy**. In vivo *antibody binding*† *to tumor cells and* in vitro *sensitivity of tumor cells to complement-mediated lysis*‡

Treatment of mice bearing i.p. EAT	Percentage of fluorescent EAT cells (range) on the day after EAT inoculation		Percentage of dead EAT cells after exposure to complement,§ on the day after EAT inoculation	
	2	5	2	5
None	ND‖	ND	2	5
Immune spleen cells	3.5 (2–5)	4.5 (3–6)	3	5
Cyclophosphamide	ND	ND	7	6
Cyclophosphamide + immune spleen cells	69 (61–76)	46 (41–53)	15	42
Positive control¶	96	99	ND	ND

*Mice were injected with 10^6 EAT cells/mouse i.p., 5 days later with 200 mg/kg cyclophosphamide i.v. and 6 h after the drug with 9×10^7 immune spleen cells/mouse i.v. EAT cells were harvested and tested after 2 and 5 days, respectively.

†Tumor cells were washed and exposed to fluorescein-labelled rabbit anti-mouse IgG antiserum. Three mice per group.

‡Tumor cells were pooled from three mice per group, exposed to complement and checked for their ability to exclude trypan-blue.

§Numbers represent the net difference as compared to the samples with no complement added.

‖Not done.

¶Tumor cells pretreated with mouse anti-EAT serum and then exposed to the rabbit anti-mouse IgG.

Table 4. Neutralization of s.c. EAT growth with sera of survived mice and eluate from EAT cells from EAT-bearing mice treated with cyclophosphamide and immune spleen cells

Treatment of EAT-bearing serum and eluate donors*	Tested	Tumor incidence	Tumor diameter (mm; $\bar{X} \pm$ S.E.)†
None	Serum	6/6	11.7 ± 0.93
None	Eluate	6/6	10.8 ± 1.05
Immune spleen cells + cyclophosphamide	Serum	3/6	5.2 ± 1.2‡
	Eluate	6/6	9.5 ± 0.99
	Eluate (concentrated 5 times)	6/6	6.0 ± 1.15‡

*Mice were injected with 10^6 EAT cells i.p.; 5 days later with cyclophosphamide (200 mg/kg) i.v. and 6 h after the drug with immune spleen cells (9×10^7/mouse). Their EAT cells and sera were harvested 2 and 5 days later. Tumor cell eluates and sera (0.1 ml/recipient) were tested in the admixture-inhibition assay of growth of 10^5 s.c. injected EAT cells.

†Determined 35 days after s.c. EAT injection.

‡Significant difference ($P < 0.01$) as compared to the group given serum or eluate from normal (untreated) mice.

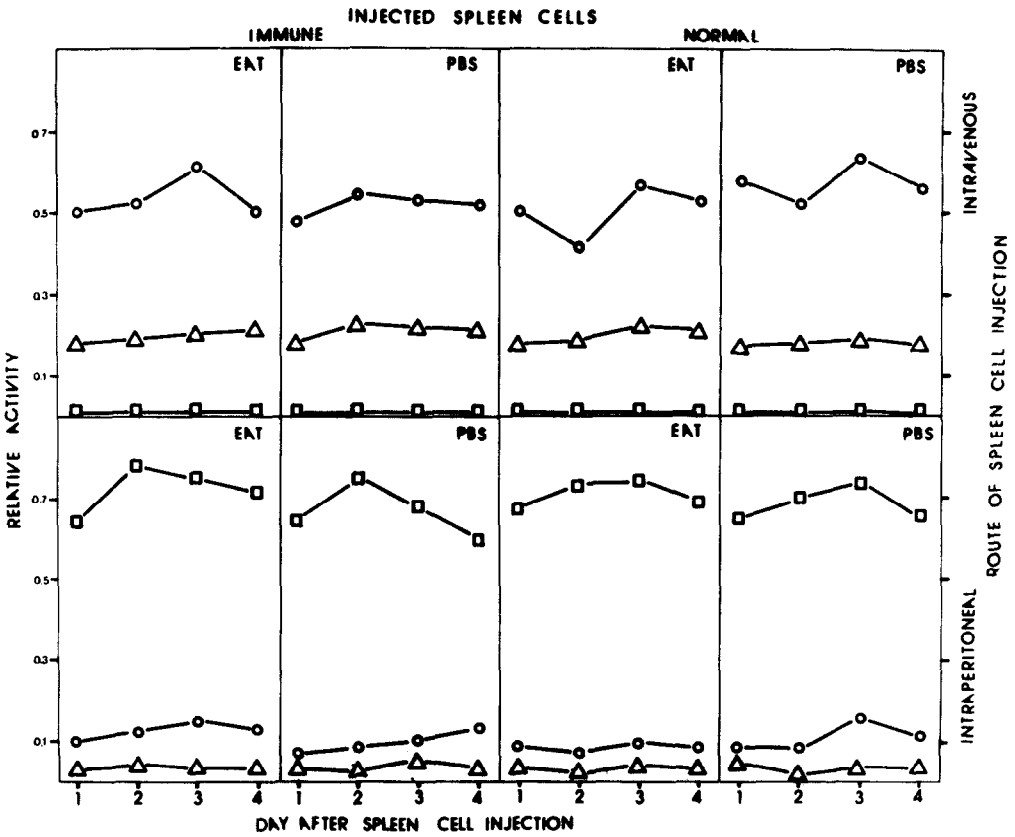

Fig. 1. Distribution of i.v. and i.p. injected[51] cr-labelled spleen cells (5 × 10[7]/recipient) from mice immune to EAT and normal mice in syngeneic recipients which simultaneously received i.p. EAT cells (5 × 10[5]/mouse) or PBS. Radioactivity relative to total body radioactivity is shown in the liver (○), spleen (△) and peritoneal cavity (□). Three mice per point.

treated with the combination of the drug and cells is given in Table 4. Serum of cured animals as well as the eluate from EAT cells were both able to inhibit the growth of EAT cells injected s.c. to normal recipients.

Homing of intravenously administered spleen cells

Homing of the normal and immune spleen cells injected i.v. or i.p. to EAT-bearing or EAT-free mice were tested first. The ^{51}Cr-labelled spleen cells were injected in a dose of 5 × 10^7/recipient. EAT cells were injected i.p. on the same day in a dose of 5 × 10^5 cells/mouse. During the next 4 days, the radioactivity was daily determined in the liver, spleen and peritoneal cavity, and compared to the total body radioactivity. Each experimental point contained data obtained from three mice. The data in Fig. 1 clearly show that i.v. injected spleen cells migrated mainly to the liver and the spleen but did not penetrate into the abdominal cavity. In contrast, the majority of i.p. injected labelled cells was retained in the abdominal cavity, migrating to a rather small extent to the liver and only minimally to the spleen. This general pattern was not altered, regardless of whether immune or normal spleen cells were used, or whether or not the recipient mice were injected with EAT (Fig. 1).

In the following experiment, normal CBA mice were injected with syngeneic CBAT6T6 spleen cells in order to evaluate the extent of their homing in the recipients' lymphoid organs by cytologic analysis. Homing of the transferred immune spleen cells in mice treated with cyclophosphamide was also analyzed, since only in these mice the growth of tumor was inhibited (see Table 2). The cytologic data in Table 5 show that neither in cyclophosphamide-pretreated mice nor in normal EAT-bearing mice did the transferred immune spleen cells penetrate significantly into the peritoneal cavity. Furthermore, a detailed analysis of homing of transferred immune spleen cells in lymphoid organs of cyclophosphamide-pretreated recipients (Figs. 2 and 3) revealed that the i.v. injected immune spleen cells seeded rather poorly in spleens and lymph nodes of either normal (Fig. 2A) or EAT-bearing (Fig. 2B) recipients, but that in cyclophosphamide-pretreated recipients they constituted more than 50% of the total mitotic cell population (Fig. 2C). The highest chimerism was found when the immune spleen cells were injected early after cyclophosphamide (Fig. 2C) and when lymphoid organs were analyzed up to 8 days after the immune spleen cell

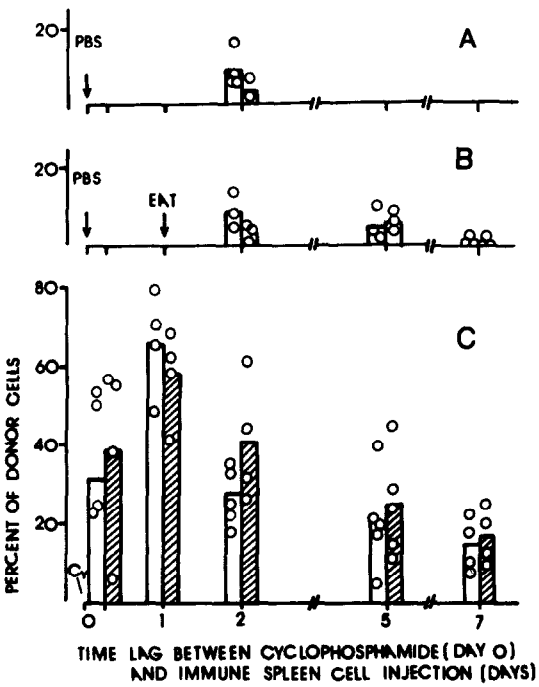

Fig. 2. Percentage of donor-type cells in spleens (white columns) and lymph nodes (shaded columns) of mice injected i.v. with 5 × 10^7 spleen cells from donors immune to EAT. The interval is indicated at which the immune spleen cells were injected after PBS (A,B) or cyclophosphamide (C). The chimerism was always determined 7 days after the injection of spleen cells. (A) Mice pretreated with PBS on day 0. (B) Mice pretreated with PBS on day 0 and one day later injected i.p. with 5 × 10^5 EAT cells. (C) Mice injected with 180 mg/kg cyclophosphamide on day 0. Each point represents one mouse.

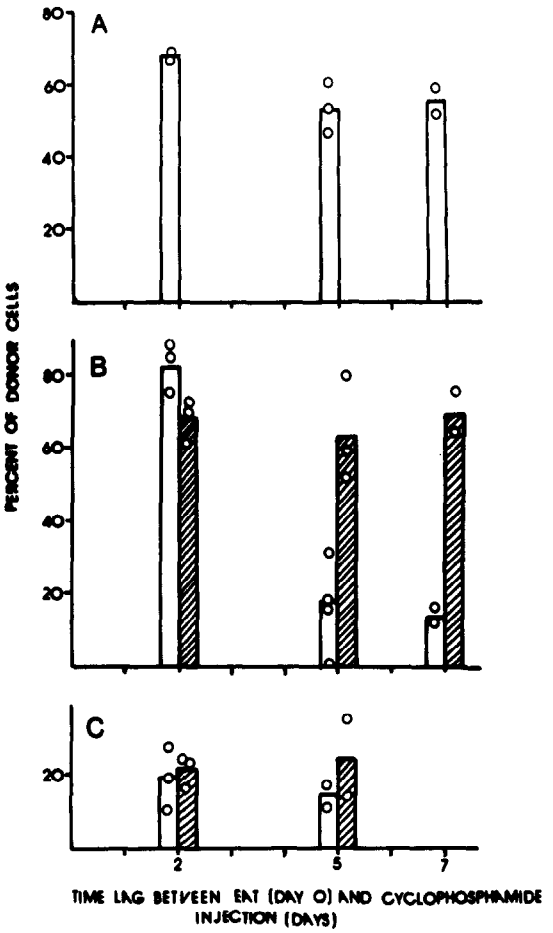

Fig. 3. Percentage of donor-type cells in spleens (white columns) and lymph nodes (shaded columns) of mice injected i.p. with 5 × 10^5 EAT cells/mouse and 2, 5 or 7 days later i.v. with 180 mg/kg cyclophosphamide and 5 × 10^7 spleen cells from EAT-immune donors. The chimerism was determined 3 (A), 8 (B) and 115 (C) days after the injection of spleen cells. Each point represents one mouse.

injection (Fig. 3). However, up to 20% of the donor-type cells could be detected in the recipients' spleens and lymph nodes even 4 months after the spleen cell injection into cyclophosphamide-pretreated mice (Fig. 3C).

DISCUSSION

In the previous study of immune mechanisms in EAT growth in mice [8], anti-EAT immune serum was observed to inhibit i.p. EAT growth when injected either i.p. or i.v., whereas spleen cells from immune donors were effective when injected i.p. only. In the present report, these findings are confirmed and documented in a dose–response manner (Table 1). Even very high doses of immune spleen cells (e.g. 2 × 10^8/mouse) were completely ineffective when injected i.v. (Table 1). This phenomenon

*Table 5. The proportion of donor-type cells among the cells infiltrating i.p. growing EAT in a model of adoptive immunotherapy of EAT in mice**

Treatment of mice bearing i.p. EAT	No. of donor cells/total no. of mitotic non-tumor cells on the day after injection of immune spleen cells	
	2	5
Immune spleen cells	2/78	0/60
Immune spleen cells +cyclophosphamide	2/92	1/38

*CBA mice were injected with 10^6 EAT cells/mouse i.p.; after 5 days with cyclophosphamide (200 mg/kg) i.v., and 6 h after the drug with 9 × 10^7 CBAT6T6 EAT-immune spleen cells i.v. The chimerism of peritoneal cell population was determined after 2 and 5 days, respectively. Two to three mice per group.

deserves full attention inasmuch as it may turn out to be rather relevant to the clinical application of passive tumor immunotherapy. Namely, the cells to be transferred to a cancer patient would mostly be administered systematically in an attempt to locally reduce the growing tumor mass (see [1]). In addition to the well known problems of tumor antigenicity and extracorporeal stimulation of immunocytes, such apparently banal obstacles may crucially determine the final outcome of the immunologic intervention. Hence we used a strongly immunogenic tumor and adequately stimulated cells, but the degree of anti-EAT protection was still found to completely depend on the route of injecting the transferred lymphoid cells (Table 1). The present experimental study was devoted to an analysis of this finding.

Failure of i.v. injected immune spleen cells to constrain i.p. EAT growth was not due to the protocol-related reasons that could prevent immune cells contacting tumor antigens and becoming activated. They were also ineffective when combined with irradiated tumor cells that could provide the antigenic stimulus. The data on hematologous dissemination of i.p. growing EAT cells [14] also speak against that possibility, because such disseminated EAT cells could potentially provide the necessary stimulation.

Pretreatment of EAT-bearing mice with cyclophosphamide allowed the i.v. injected immune spleen cells to completely constrain i.p. tumor growth (Table 2). Comparable findings were often reported by other researchers [3, 5, 15, 16] and generally ascribed to a cyclophosphamide-induced depletion of suppressor cells [3, 6, 16]. However, we were aware of Celada's finding [17] that an adoptive transfer of immune cells to normal adult mice often failed to confer immunity upon the recipients, although the same immune cells were capable of doing so when transferred to newborn or irradiated recipients. We felt that the effectivenss of the transfer might, among other possible factors, greatly depend on the extent of *homing* of transferred cells in the new environment. The effectiveness of transferred cells in irradiated recipients was ascribed to a release from suppression normally exerted by host suppressor lymphocytes [4, 18]. However, a much simpler alternative has never been considered: it may be that the effectiveness of transferred cells firstly depends on the effectiveness of their seeding in the recipients' lymphoid organs. The question of homing of transferred cells as a prerequisite for their effectiveness of any kind, especially in a syngeneic combination, has never been adequately assessed although the data showing a relevant correlation are quite old [19, 20]. Although it appears reasonable to assume that suppressor cells are scarcer in the T-cell deficient [2, 6], irradiated [4, 7, 21] or cyclophosphamide-treated [7, 16] mice, it is equally reasonable to suspect that these mice provide more inevitable 'room' for transferred cells to home and function. Indeed, the data showing cyclophosphamide to enhance the action of transferred syngeneic cells even when given before tumor [20] strongly suggest that the effect was due to better colonization and 'long persistence' of donor cells in the lymphoid tissues depleted of cyclophosphamide.

Our data indirectly support a view that the helper T-cells are responsible for an adoptive transfer to immunity to EAT. The immune spleen cells, although effective against EAT upon the adoptive transfer to normal recipients, did not exert any cytotoxicity for EAT in a short-term ^{51}Cr-release assay (data not shown). However, the activity of such cells could be entirely abolished by the pretreatment of spleen cells with anti-Thy-1 antibodies [8]. The transferred helper T-cells ($L3T4^+$) can mediate their antitumor activity in at least three ways [22]: (a) they can function as helper cells to recruit and augment the production of host cytotoxic lymphocytes; (b) by secreting lymphokines, initiate a delayed-type hypersensitivity reaction, which is mediated by host cells; and (c) provide help for the production of antibodies.

The third mechanism, i.e. the induction of antitumor antibodies, was the most probable mechanism for the rejection of EAT in our model. We have clearly shown that cytotoxic antibodies are present at the tumor site (Table 3) and in the serum of cured animals (Table 4), and that the EAT cells can be destroyed by local or systemic administration of antitumor antibodies (Tables 1 and 4). Thus, EAT is similar to some virus-induced tumors, in which such a role of antibodies has been ascertained [20, 23]. Accepting this, one has to answer two questions. First, whether the immunity to EAT is tumor-specific or is directed to some virus-related structure, which might be secondarily incorporated in EAT cells due to its long transplantation history. Although we cannot exclude that immune cells or antibodies recognize some virus-related structure, such a reaction is not crucial for the rejection mechanism, since immunity to EAT was shown to be tumor-specific, i.e. mice immune to EAT did not reject other mouse tumors [24, 25].

The second question concerns the origin of B cells producing the antitumor antibodies. In the view of the findings showing B cells to be extremely sensitive to cyclophosphamide metabolites *in vivo* [26], one has to assume that antibody response is mediated exclusively by donor cells. However, we have previously shown [27, 28] that immune reaction was not sensitive to the suppressive action of cyclophosphamide if the drug was applied 5 or more days after priming with dead tumor cells. Thus, it is

possible that the primed host B cells might escape the toxic action of cyclophosphamide and, therefore, be able to cooperate with donor T cells in producing antitumor antibodies. Experiments with the transfer of purified T cells would provide an unequivocal direct answer to this question.

Since anti-EAT antibodies inhibited i.p. EAT growth when injected not only by the i.p. route but also when given i.v. (i.e. after penetration from circulation, Table 1), we have to speculate why spleen cells do not function when injected i.v. One of the possible reasons would be that spleen cells must penetrate into the abdominal cavity in order to suppress tumor growth, since the i.p. injected cells did elicit their action (Table 1). Indeed, there are well documented findings showing that the transferred donor cells infiltrate tumor (mostly T cells), and that local immune reaction initiated by donor cells leads to regression of tumors in cyclophosphamide-pretreated mice [29]. In our experiments, however, neither in normal or EAT-bearing mice nor in cured, cyclophosphamide-pretreated mice did the transferred spleen cells penetrate into the abdominal cavity. This was shown by the analysis of the distribution of cells with T6T6 marker (Table 5) and ^{51}Cr-labelled cells (Fig. 1). Although the data obtained with T6T6 marker might not be conclusive, since they are based on scoring of cells in mitosis (which need not necessarily be of an immunological origin), their consistency with data obtained for the distribution of ^{51}Cr-labelled cells supports the conclusion that the transferred cells did not penetrate into the abdominal cavity. Obviously, as discussed before, if spleen cells act by producing antibodies, and antigen circulates in the blood [13], there is no need for immune cells to penetrate at the tumor site.

The second possible reason for the failure of i.v. injected spleen cells from EAT-immune donors to inhibit i.p. EAT growth would be that something outside the peritoneal cavity normally prevents the transferred cells acting. This factor is neutralized by cyclophosphamide (Table 2), so that transferred immune spleen cells become effective and destroy EAT cells from a distance, by circulating serum antibodies. These antibodies penetrate into the abdominal cavity and bind to tumor cells (Tables 3 and 4).

We focused on the idea of a homing prerequisite and found a clear-cut correlation between the effectiveness and homing of transferred immune spleen cells (Figs. 2 and 3). Naturally, this does not prove the causative relation between the two events, since the donor cells proliferating in CY-pretreated host lymphoid tissues might equally be of hematopoietic origin. Therefore, our data still do not challenge the possibility that the cyclophosphamide-related depletion of suppressor cells was important. However, it should be noted that in tumor bearing mice treated with cyclophosphamide only, no *in vitro* sensitivity of EAT cells to complement-mediated lysis could be detected (Table 3), suggesting that the drug did not release the otherwise suppressed anti-EAT immune response. It is certainly true that cyclophosphamide-induced enhancement of anti-EAT activity of transferred immune spleen cells is partly due to its direct anti-tumor effects; however, the tumoricidal cyclophosphamide action could not account for the complete effect, because when immune spleen cells were not added, cyclophosphamide was only marginally effective (Table 2). Another possibility is that cyclophosphamide renders tumor cells more sensitive to the effects of immune mechanisms, which we actually have already shown [30].

Leaving all these possibilities open, our present data still clearly show that cyclophosphamide enabled the transferred lymphoid cells to seed to the hosts' lymphoid organs to a much higher extent than they did in untreated mice (Figs. 2 and 3). Since the effect correlated with the appearance of anti-tumor activity of the transferred cells, the importance of homing of the cells used for passive tumor immunotherapy should be seriously taken into account in the relevant research. Our current studies have been aimed at separating the mentioned effects of cyclophosphamide and determining their individual relative importance.

REFERENCES

1. Rosenberg SA, Terry WD. Passive immunotherapy of cancer in animal and man. *Adv Cancer Res* 1977, **25**, 323–388.
2. North RJ. The murine antitumor immune response and its therapeutic manipulation. *Adv Immunol* 1984, **35**, 89–155.
3. Evans R. Combination therapy by using cyclophosphamide and tumor-sensitized lymphocytes: a possible mechanism of action. *J Immunol* 1983, **130**, 2511–2513.
4. North RJ. γ-Irradiation facilitates the expression of adoptive immunity against established tumors by eliminating suppressor T cells. *Cancer Immunol Immunother* 1984, **16**, 175–181.
5. Greenberg PD, Cheever MA, Fefer A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1$^+$2$^-$ lymphocytes. *J Exp Med* 1981, **154**, 952–963.
6. North RJ. Models of adoptive T-cell-mediated regression of established tumors. *Contemp Topics Immunobiol* 1984, **13**, 243–257.
7. Greene MI, Perry LL, Benacerraf B. Regulation of the immune response to tumor antigen.

V. Modulation of suppressor T-cell activity *in vivo*. *Am J Pathol* 1979, **95**, 159–179.
8. Marušić M. Immune mechanisms in Ehrlich ascites tumor growth in mice. *Eur J Cancer* 1979, **15**, 39–45.
9. Baillif RN, Jones EL. Delayed tumors of mice immunized against Ehrlich ascites tumor. *Cancer* 1968, **21**, 178–183.
10. Ford CE. Appendix. In: Micklem HS, Loutit JF, eds. *Tissue Grafting and Radiation*. New York, Academic Press, 1966, 217–238.
11. Witz I, Yagi Y, Pressman D. IgG associated with microsomes from autochthonous hepatomas and normal liver of rats. *Cancer Res* 1967, **27**, 2295–2299.
12. Campbell R. The Mann–Whitney procedure. In: Campbell R, ed. *Statistics for Biologists*. London, Oxford University Press, 1974, 49–52.
13. Kiseleva Y, Magamadov M. Hematologous dissemination of tumor cells and metastases formation in Ehrlich ascites tumor. *Neoplasma* 1972, **19**, 257–275.
14. Kline I, Gang M, Tyrer D, Mantel N, Veniditti J, Goldin A. Duration of drug levels in mice as indicated by residual anti-leukemic efficacy. *Chemotherapy (Tokyo)* 1968, **13**, 28–41.
15. Fefer A. Adoptive tumor immunotherapy in mice as an adjunct to whole-body irradiation and chemotherapy. A review. *Isr J Med Sci* 1973, **9**, 350–375.
16. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med* 1982, **155**, 1063–1074.
17. Celada F. Quantitative studies of the adoptive immunological memory in mice. I. An age-dependent barrier to syngeneic transplantation. *J Exp Med* 1966, **124**, 1–14.
18. Bell EB, Shand FL. Changes in lymphocyte recirculation and liberation of the adoptive memory response from cellular regulation in irradiated recipients. *Eur J Immunol* 1975, **5**, 1–7.
19. Lance EM, Taub RM. Segregation of lymphocyte populations through differential migration. *Nature* 1969, **221**, 841–843.
20. Fefer A. Immunotherapy of primary Moloney sarcoma-virus-induced tumors. *Int J Cancer* 1970, **5**, 327–337.
21. Hellstrom I, Hellstrom KE. Antitumor effect of whole-body X-irradiation: possible role of an X-ray-sensitive T suppressor cell population. *Transpl Proc* 1979, **9**, 1073–1081.
22. Fujiwara H, Fukuzawa M, Yoshioka T, Nakajima H, Hamaoku T. The role of tumor-specific Lyt-1^+2^- T cells in eradicating tumor cells *in vivo*. I. Lyt-1^+2^- T cells do not necessarily require recruitments of host's cytotoxic T cells precursors for implementation of *in vivo* immunity. *J Immunol* 1984, **133**, 1671–1676.
23. Collins JJ, Snyderman R. Absence of macrophage involvement in the passive serum therapy of Friend leukemia virus-induced disease. *Cancer Res* 1980 **40**, 557–562.
24. Shohat B, Gitter S, Lavie D. Effect of withaferin A on Ehrlich ascites tumor cells—cytological observations. *Int J Cancer* 1970, **5**, 244–252.
25. Čulo F, Marušić M, Allegretti N. Immunization of mice to Ehrlich ascites tumour with heat-killed tumor cells in complete Freud adjuvant. *Period Biol* 1975, **77**, 107–112.
26. Poulter LW, Turk JL. Proportional increase in the θ carrying lymphocytes in peripheral lymphoid tissue following treatment with cyclophosphamide. *Nature New Biol* 1972, **238**, 17–18.
27. Čulo F, Allegretti N, Marušić M. Lymphotoxic effect of cyclophosphamide in therapy of Ehrlich ascites carcinoma in mice. *J Natl Cancer Inst* 1977, **58**, 1759–1764.
28. Vidović D, Čulo F, Marušić M. Interference of anti-tumor and immunosuppressive effects of cyclophosphamide in tumor-bearing rats. Analysis of factors determining resistance or susceptibility to a subsequent tumor challenge. *Cancer Immunol Immunother* 1982, **14**, 36–40.
29. Evans R, Duffy TM. Distribution of tumor-sensitized cells during the induction of permanent tumor regression by chemoimmunotherapy: the use of glucose phosphate isomerase as a marker. *Int J Cancer* 1984, **34**, 575–580.
30. Marić M, Čulo F, Juretić A, Allegretti N. Transplantability of tumor cells pretreated with cyclophosphamide. *Period Bol* 1983, **85** (*Suppl* 3), 325–326.