SUMMARY: Production of a factor with a biological activity to inhibit the in vitro tumor-cell migration (TCM) from peripheral blood E rosette-forming cells (ERFC), CD4+ and CD8+ T cells in patients with gastric and breast carcinoma was investigated. The cells were stimulated for 2 or 24 hr with allogeneic gastric or breast cancer extracts in samples of cell suspensions. A microculture system at an initial cell concentration from 2,500 cells to 1 cell per well was used. Feeder cells, PHA, IL-2-containing supernatant and cancer extract were added to each well. Ehrlich ascites tumor cells were employed in the migration-inhibition assay. ERFC and CD4+ T cells produced in the culture supernatants a factor inhibiting TCM, when these cells were stimulated with cancer extracts, but not with extracts of benign tissue. Stimulated CD8+ T cells did not produce such a factor. The production of the factor inhibiting TCM in the microculture system was also significantly correlated with the type of cells in the wells, particularly with ERFC and CD4+ T cells, but not with CD8+ T cells (r=0.94, p<0.001). It could be suggested that this factor probably took part in in vivo blockading the migration of tumor cells in small cancer foci.

INTRODUCTION

A factor inhibiting the migration of tumor cells has been obtained from different human lymphoid cell lines and murine lymphocytes stimulated with concanavalin A (1,2). In these investigations, target cells from various experimental tumors, including Ehrlich ascites and solid tumor, were used. We have reported that the migration of transplanted sarcoma cells and Ehrlich ascites tumor cells out of capillary tubes may be inhibited by the factor found in the culture supernatants.
tants, when the spleen cells of BALB/c mice transplanted with sarcoma cells or immunized with killed sarcoma cells were stimulated in vitro with a 3 M KCl extract of the sarcoma (3). In our previous study, it was shown that the regional lymph node lymphocytes from patients with cancer of the stomach, after a stimulation with gastric cancer extracts, produced a factor which inhibited the migration of gastric cancer cells and Ehrlich cells (4,5). In these lymph nodes, without cancer metastases or with small metastatic foci, immunoreactive changes were found. No essential difference in migration inhibition was observed with the following target cells: gastric cancer cells, transplanted sarcoma cells (in experiments) and Ehrlich cells.

The aim of this study was to determine whether the peripheral blood E rosette-forming cells (ERFC), CD4+ and CD8+ T cells would produce a factor inhibiting in vitro tumor-cell migration (TCM) by stimulation with cancer extracts. The results of the study together with the data mentioned above could contribute to the interpretation of the factor, whose biological activity is expressed in inhibition of TCM, to be an in vivo protective phenomenon in tumor pathology.

MATERIALS AND METHODS

Patients: Peripheral blood mononuclear cells (PBMC) were obtained from 44 patients with gastric cancer (23 males and 21 females), 66 women with breast cancer and controls. The patients whose cancer referred to the stages I and II in TNM system had undergone radical subtotal gastric resection, respectively total mastectomy and axillary dissection at the University Hospital of the Medical University, Pleven. Patients with chronic ulcer of the stomach and fibrocystic disease of the breast as well as healthy persons were taken as controls.

Cell preparations: PBMC were isolated from heparinized venous blood of cancer patients, one or two days before the operation, and from controls by centrifugation at 800 ×g for 15 min over a cushion of lymphocyte separation medium (Polysep, Pharmachim, Bulgaria). PBMC were partially depleted of adherent cells by incubation in plastic flasks at 37 C for 35 min. ERFC were separated by rosetting with sheep erythrocytes. The rosettes were resuspended, layered on Polysep, and centrifuged at 500 ×g for 15 min. ERFC were freed of attached erythrocytes by treatment with Tris-buffered 0.83% ammonium chloride (pH 7.2).

Complement (C)-dependent lysis of lymphocytes with monoclonal antibodies (6,7) was employed to obtain preparations of CD4+ and CD8+ T cells. Briefly, ERFC were suspended in PBS (pH 7.2) containing 2% fetal calf serum (FCS) in a
concentration of $1 \times 10^7$ cells/ml, and 50-$\mu$l samples of the suspension ($5 \times 10^5$ cells) were mixed with 20-$\mu$l portions (0.20 $\mu$g) of anti-CD4 or anti-CD8 monoclonal antibody. The mixtures were incubated for 30 min on ice and then centrifuged at 400 $\times g$ for 5 min. The supernatant was discarded, and 0.2 ml of rabbit C was added. After incubation at 37 C for 45 min, the cells were centrifuged, and their viability was determined by the trypan blue dye exclusion test. As a control, the cells were mixed with C alone. CD2$^+$ cells, CD3$^+$, CD4$^+$, CD8$^+$ T cells and NK cells in ERFC as well as CD4$^+$ and CD8$^+$ T cells obtained by C-dependent lysis of lymphocytes were determined with the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8 and anti-CD57 FITC conjugated (National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria), OKT11/FITC (Ortho, Raritan, NY), anti-Leu-11b (CD16) phycoerythrin (PE) conjugated and anti-Leu-19 (CD56) PE (Becton Dickinson). Cytofluorographic analysis was performed with an FACStar (Becton Dickinson). Suspensions of ERFC, CD4$^+$ and CD8$^+$ T cells were prepared in RPMI 1640, supplemented with 10% heat-inactivated pooled human serum, penicillin (100 IU/ml) and streptomycin (100 $\mu$g/ml).

Preparation of a cancer extract and stimulation of the lymphocytes with it: To obtain preparations of cancer extract, an extraction procedure with hypertonic potassium chloride was used (8,9). Ten milliliters of cold 3 M KCl in PBS (pH 7.2) was added to every 1 g of tumor tissue of solid tumor (gastric and breast cancers from various patients), which was finely minced and then passed through a steel sieve. The preparation contained approximately $3 \times 10^8$ tumor cells. The mixture was shaken at 4 C for 16 hr and then centrifuged at 160,000 $\times g$ for 1 hr. Further centrifugation of the mixture was done at 40,000 $\times g$ and 18,000 $\times g$. The supernatants were dialyzed against PBS for 24 hr, and the protein content was adjusted to 0.35-0.55 mg/ml.

Samples of 0.1 ml of suspension of ERFC, CD4$^+$ and CD8$^+$ T cells at a concentration of $20 \times 10^6$ cells/ml ($2 \times 10^6$ cells) were each mixed with 0.05 ml of allogeneic cancer extracts corresponding to the kind of the carcinoma in the patients. The mixtures were incubated for 2 hr at 37 C in a 5% CO$_2$ atmosphere. On the other hand, samples of 0.2 ml of cell suspensions containing $1 \times 10^6$ cells/ml ($2 \times 10^5$ cells) were mixed with 40 $\mu$l of allogeneic, corresponding and non-corresponding cancer extract and incubated for 24 hr. The mixtures were centrifuged at 18,000 $\times g$ at 4 C for 20 min. The supernatants were dialyzed against PBS for 24 hr, and the protein concentration was determined was between 0.18 to 0.40 mg per ml. Then, the supernatants were irradiated with ultraviolet light and were stored at $-25$ C. ERFC from cancer patients and controls were stimulated for 2 and 24 hr with either the allogeneic cancer extracts or extracts of benign tissue (chronic ulcer of the stomach and fibrocystic disease of the breast), both obtained from surgical specimens of the patients who had undergone an operative treatment. The non-cancer extracts were prepared as were the cancer extracts. For each assay, two or three cancer and non-cancer extracts were used. The supernatants were obtained as described above.
**Microcultures of ERFC, CD4+ and CD8+ T cells:** The method of limiting-dilution microcultures was used (10,11) in 22 cases, in which the previously examined supernatants of ERFC stimulated with cancer extract for 24 hr showed an inhibition effect (IE) on Ehrlich-cell migration. Peripheral blood ERFC, CD4+ and CD8+ T cells were seeded in 96-well round-bottom plates (Linbro, Flow Laboratories, McLean, VA) in a range from 2,500 to 1 cell per well in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum and antibiotics. Irradiated (5,000 rads) allogeneic PBMC (5×10^4) from healthy donors were added to each well as feeders in a final volume of 200-μl culture medium as well as PHA (1% v/v). After 48 hr of culture, the supernatant containing IL-2 (50% v/v) was added. This supernatant was obtained from PBMC of healthy donors (1×10^6 cells/ml) stimulated with PHA (2 μg/ml) for 48 hr (12) and then depleted of PHA with chicken erythrocytes (13). The supernatant of cultures of PHA-stimulated (1% PHA v/v) allogeneic human spleen cells (5×10^6 cells/ml) was also a source of IL-2 (14). The activity of IL-2-containing supernatants was assessed by its ability to promote DNA synthesis in blast cells obtained by PHA stimulation of PBMC from healthy donors (12,15). The supernatants which showed an IL-2 activity approximately 90% of that of the IL-2 standard were used. The 1st International Human IL-2 Standard was kindly provided by Dr. A. Meager of the National Institute for Biological Standards and Control, London, UK.

The microcultures of ERFC, CD4+ and CD8+ T cells were incubated at 37 C in a humid atmosphere of 5% CO2. Twelve days later, cancer extract of 20 μl was added to wells with growing microcultures for 24 hr. The culture supernatants were decanted and assayed for migration inhibition. Then, the seeded cells in each well were removed with an automatic pipette and counted in a hemocytometer. This procedure defined the proliferation of the cells in microcultures at various initial cell concentrations (high and small). As a control, feeder cells were stimulated with PHA and an IL-2-containing supernatant. Control microcultures of ERFC and CD4+ T cells from patients with gastric cancer were stimulated with extract of breast cancer. On the contrary, ERFC and CD4+ T cells from patients with breast cancer were stimulated with extract of gastric cancer, or were not stimulated with a cancer extract.

**Target cells:** Ehrlich ascites tumor maintained by serial ip transplantation (1×10^6 cells) every 10 days in BALB/c mice was used as the source of target cells. The suspension of Ehrlich cells was easily prepared, and the migration areas out of capillary tubes were exactly measured. Peritoneal exudates were induced by oil in normal guinea pigs and peritoneal exudate cells (macrophages) were obtained (16).

**Assessment of migration inhibition:** Samples of 0.2 ml of target cells at a concentration of 2×10^7 cells per ml of RPMI 1640 supplemented with 5% fetal calf serum and antibiotics were mixed with 0.1 ml of the supernatants of ERFC, CD4+ and CD8+ T cells. The mixtures were incubated for 2 hr at 37 C in an atmosphere of 5% CO2. Capillary tubes sealed at one end were filled with Ehrlich
cells or peritoneal macrophages and centrifuged at 250 × g. The portions with the sedimented cells were cut off, placed in chambers filled with medium and incubated at 37 C for 24 hr. The areas of target-cell migration were projected and measured by planimetry. The migration index (MI) was calculated by the following formula:

\[
MI = \frac{\text{Average area of migration of target cells incubated with the supernatant}}{\text{Average area of migration of target cells incubated without the supernatant}}
\]

Fraction of responding microcultures (FrRMs) for each initial cell concentration was calculated as number of responding microcultures per total number of microcultures. Microcultures were designated as responding when the supernatants obtained from them showed an IE against Ehrlich-cell migration (MI < 0.80).

The frequency (F) of the inhibitory factor-producing cell precursors was calculated by analysis of the Poison distribution relationship between the number of cells per well (in a range from 1 to 50 cells) and the negative logarithm of the fraction of non-responding microcultures (17). Fraction of non-responding (negative) microcultures (FrNRMs) was calculated as number of non-responding microcultures in which the supernatants obtained did not show an IE per total number of microcultures. The weighted mean (WM) estimator was used for determination of F (18).

**Fractionation on Sephadex G-100 column:** Active supernatants of ERFC, from patients with gastric or breast cancer, stimulated with cancer extracts for 24 hr and possessed a migration-inhibition effect were fractioned by gel chromatography on a 10×700 mm column containing Sephadex G-100 up to 640 mm. For the eluant solution, 0.15 M NaCl was used. The following molecular-weight markers were used: human-plasma albumin with mol.wt. of 66,500, peroxidase (E. Merck, Darmstadt, Germany) with mol.wt. of 40,000, lysozyme (Sigma, St. Louis, MO) with mol.wt. of 14,300 daltons, and Komb-Insulin S (Hoechst AG, Frankfurt am Main, Germany) with mol.wt. of 5,800. The extinctions of the eluting material were recorded at 280 nm on a VSU 2 spectrophotometer (Carl Zeiss, Jena, Germany).

**Statistics:** The methods for variational and correlational analysis were applied. The chi-square method and the Student's t test were used to assess the values obtained.
RESULTS

Cell Populations and Active and Inactive Supernatants

ERFC from the patients with gastric and breast cancer were 42.05 ± 5.32% of PBMC. The following proportions of CD2+ cells and T cell subsets in ERFC were determined: 94.80 ± 2.42% for CD2+ cells, 80.23 ± 2.13% for CD3+ T cells, 54.95 ± 3.39% for CD4+ T cells, and 40.24 ± 7.32 for CD8+ T cells. CD3-, CD57+ NK cells comprised <10% (7.90-9.10%) of ERFC, while CD3-, CD16+, CD56+ NK cells possessing the most cytotoxic efficiency (19) were less than 5%.

Inhibition effects (IE) against the migration of Ehrlich ascites tumor cells for the supernatants of ERFC, CD4+ and CD8+ T cells stimulated with allogenic cancer extracts in terms of MI are shown in Table I. In addition to Table I, MI for the inactive supernatants of ERFC (MI > 0.80) in patients with gastric cancer for 2 hr of stimulation was 1.00 ± 0.09, and for 24 hr, it was 0.91 ± 0.06. In patients with breast cancer, MIs were 1.05 ± 0.05 and 0.99 ± 0.07, respectively. Supernatants of cancer-extract-stimulated CD4+ T cells for 2 hr in patients with cancer of the stomach and the breast showed an IE (active supernatants, MI < 0.80) in 94.44% and in 88.24% of the tests performed. For the stimulation for 24 hr, active supernatants were found in 100% of the tests. On the contrary, the supernatants of cancer-extract stimulated CD8+ T cells did not show an IE (inactive supernatants) as follows: for the stimulation for 2 hr in 100% of the tests and for 24 hr in 92.1% and in 100%. In cases with stimulation of ERFC by non-corresponding cancer extract, i.e. in patients with gastric cancer by extract of breast cancer and vice versa, the supernatants obtained did not inhibit Ehrlich-cell migration. MIs were 1.16 ± 0.07 and 1.13 ± 0.08, respectively. The active supernatants did not inhibit macrophage migration (MI - 0.96 ± 0.10).

MIs for the culture supernatants of ERFC from cancer patients and controls stimulated with either the allogeneic cancer extracts or extracts of benign tissue are shown in Table II. In this table, only cancer patients with active supernatants obtained from cancer-extract-stimulated ERFC are included. In addition to the present study, supernatants of ERFC from patients with gastric and breast cancer stimulated with autologous cancer extracts for 24 hr showed an IE against Ehrlich-cell migration. MIs were 0.60 ± 0.06 and 0.66 ± 0.03, respectively. In some cases of gastric and breast cancer patients, ERFC were stimulated with an extract of non-malignant adjacent autologous tissue for 24 hr, and the supernatants obtained did not inhibit Ehrlich-cell migration. MIs ranged from 0.90 to
Table I. Inhibition of Ehrlich-cell migration by culture supernatants of peripheral blood ERFC, CD4+ and CD8+ T cells from patients with gastric and breast cancer

<table>
<thead>
<tr>
<th>MI of culture supernatants of cells from patients with</th>
<th>Cancer of the stomach</th>
<th>Cancer of the breast</th>
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<tbody>
<tr>
<td>Stimulation by allogeneic gastric cancer extract on</td>
<td>ERFC</td>
<td>CD4+ T cells</td>
</tr>
<tr>
<td>Stimulation for (hr)</td>
<td>2</td>
<td>0.56±0.05</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.58±0.06</td>
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<tr>
<td></td>
<td></td>
<td>1.06±0.05</td>
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<td></td>
<td></td>
<td>0.65±0.06</td>
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<td></td>
<td></td>
<td>0.62±0.05</td>
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<td></td>
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<td>1.02±0.08</td>
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</table>

The supernatants of cancer-extract stimulation of ERFC for 2 hr showed in the patients with gastric cancer an IE against Ehrlich-cell migration (active supernatants, MI<0.80) in 45.46% of the tests performed, and after stimulation of ERFC for 24 hr in 75% of the tests. Active supernatants in patients with breast cancer were detected in 34.09%, and in 71.42% of the tests.
Table II. Inhibition of Ehrlich-cell migration by culture supernatants of peripheral blood ERFC from cancer and control patients

<table>
<thead>
<tr>
<th>Stimulation of ERFC cells by allogeneic extracts of</th>
<th>MI of culture supernatants of cells from patients with</th>
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<tr>
<td>Gastric cancer</td>
<td>Breast cancer</td>
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<td>Stimula-</td>
<td>Gastric</td>
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<td>tion for (hr)</td>
<td>cancer</td>
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<tr>
<td>2</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>24</td>
<td>0.63 ± 0.04</td>
</tr>
</tbody>
</table>

ERFC from the cancer and control patients were stimulated for 2 and 24 hr with either the allogeneic extracts, obtained from cancers, non-cancer gastric mucosa and breast tissue. The supernatants received were assayed for their IE. In patients with chronic gastric ulcer, the supernatant of ERFC-stimulation with gastric cancer extracts for 24 hr showed an IE in 20% of the tests performed.
1.27. The supernatants of CD4+ and CD8+ T cells from gastric and breast cancer patients stimulated with allogeneic extracts of gastric mucosa or breast tissue for 24 hr did not show IE. The values of MI were: 0.92±0.09 and 0.94±0.07 in gastric cancer patients, and 0.85±0.06 and 0.95±0.10 in breast cancer patients. In healthy persons, the supernatants of ERFC did not show IE, when the cells were stimulated for 2 hr with the extracts of either gastric cancer or gastric mucosa (MIs - 0.85±0.09 and 1.04±0.10), and the extract of breast cancer or breast tissue (MIs - 1.16±0.08 and 1.05±0.05). The inactive supernatants were also obtained after 24 hr of stimulation. MIs were: 0.98±0.11, 1.06±0.09 and 0.90±0.07, 1.04±0.09.

**Inhibition Effect of the Culture Supernatants Obtained from the Microcultures**

The culture supernatants of all the series of microcultures with ERFC and CD4+ T cells in patients with gastric and breast cancer at all initial cell concentrations possessed IEs on Ehrlich-cell migration (mean MI<0.80). The mean values of MI showing the level of the supernatants obtained did not essentially differ among various series of microcultures. As an example, MIs for the series of 2,500 and 1,000 ERFC and CD4+ T cells per well (initial cell concentration) were: 0.66±0.03, 0.57±0.05 (ERFC), and 0.55±0.08, 0.66±0.08 (CD4+ T cells). For the series at initial cell concentrations of 50, 25 and 5 cells/well, MIs were: 0.61±0.10, 0.52±0.08, 0.63±0.10 (ERFC), and 0.68±0.11, 0.58±0.06, 0.60±0.08 (CD4+ T cells). On the contrary, the culture supernatants from all series of microcultures with CD8+ T cells did not show IE (mean MI>0.80). Individual cases showed for the various series of microcultures MIs from 0.75 to 0.44 for the supernatant of ERFC and CD4+ T cells, and 0.82 to 1.28 for CD8+ T cells. A significant difference was observed in the level of IE (values of MI) between the supernatants of ERFC and CD4+ T cells and those of CD8+ T cells (p<0.001).

The number of the cells removed from the well with growing microcultures at the end of culture was found to have increased. For example, from 2,500-2,000 cells/well of the initial cell concentration to 25,000-150,000, from 1,500-1,000 cells/well to 14,000-120,000, from 150-100 cells/well to 10,000-75,000, from 25-10 cells/well to 5,000-27,500, from 5-3 cells/well to 5,600-40,000 and from 1 cell per well to 4,600-25,000; only in two wells to 100,000 and 110,000 cells. In 12 of 22 patients, a relatively regular decreasing number of proliferative cells in microcultures was found, which corresponded to the initial cell number in the wells (2,500-
1), but in 10 patients such was not observed. The minimal number of cells necessary to obtain an inhibitory factor after incubation with a cancer extract for 24 hr was determined at 2,500-3,000 cells per sample.

FrRMs > 0.5 indicated that the culture supernatants obtained from more than half of the microcultures at certain initial cell concentrations, in patients with gastric and breast cancer, showed an IE on Ehrlich-cell migration. FrRMs higher than 0.5 was observed for the supernatants of all series (100%) of microcultures with ERFC and CD4+ T cells and the mean values for the various series of microcultures varies from 0.63 to 1. This was observed also in the individual cases with an exception of one patient with breast cancer (M.T.) in which the FrRMs for a serie of 5 ERFC/well was 0.39. On the contrary, an FrRMs lower than 0.5 (0.07 and 0.12) was found only in one patient with gastric cancer for the supernatants obtained from two series of microcultures with CD8+ T cells (25 and 150 cells/well). A significant correlation was found between the production of the factor inhibiting Ehrlich-cell migration according to the data of the FrRMs and the type of cells in microcultures, particularly with ERFC and CD4+ T cells, but not with CD8+ T cells (r = 0.94, p < 0.001). FrRMs = 1 demonstrated that the supernatants received from all microcultures at a certain initial cell concentration possessed an IE on Ehrlich-cell migration. In the patients with breast cancer, the FrRMs for the supernatants of CD4+ T lymphocytes in all the 13 series of microcultures was higher than FrRMs of ERFC. Moreover, five of the series with CD4+ T lymphocytes showed FrRMs = 1.

Only in six cases, a regression of FrNRMs in relation to the initial number of cells per microculture (1-50 cells) was observed, but in most of the cases (16 of 22) such was not found. For example, FrNRMs in one patient with breast cancer (M.P.) at an initial number of 3 CD4+ T cell well was 0.14 and for 25 cells/well, it was 0.33.

The frequencies (Fwm) of inhibitory factor-producing cell precursors could be determined in the six patients mentioned above, i.e. two with gastric cancer, two with breast cancer in ERFC and CD4+ T cells, and two with breast cancer separately in ERFC and CD4+ T cells. In the patients with gastric cancer, the frequencies of cell precursors in ERFC were 0.22 (one cell in 4.5) and 0.07 (one cell in 14.2), and in CD4+ T cells they were 0.13 (one cell in 7.6) and 0.11 (one cell in 9). The frequencies of cell precursors in the patients with breast cancer in ERFC were 0.20 (one cell in 5), 0.15 (one cell in 6.6) and 0.08 (one cell in 12.5). In CD4+ T cells they were: 0.31 (one cell in 3.2), 0.55 (one cell in 1.8) and 0.51 (one cell in
Fig. 1. Frequencies of inhibitory factor-producing cell precursors in ERFC (•) and CD4+ T cells (▲) in patient M.T. with breast cancer. The cells were seeded in limiting numbers in wells in the presence of feeder cells. PHA, IL-2-containing supernatant, and 12 days later cancer extract were added for 24 hr. The culture supernatants were assayed for migration inhibition. The fractions of non-responding microcultures (not-producing inhibitory factor) are plotted on a log scale. The frequencies (F) were calculated by the weighted mean (WM) estimator.

The frequencies of inhibitory factor-producing cell precursors in ERFC and CD4+ T cells from one patient with breast cancer (M.T.) are shown in Fig. 1. FrRMs in representative growing microcultures give information for the cell clones producers (11) of inhibitory factor. For example, FrRMs of growing microcultures with ERFC in four patients with gastric cancer (25 in each case) were 0.84, 0.80, 0.76 and 0.68, and 21/25 (84%), 20/25 (80%), 19/25 (76%), and 17/25 (68%) of clones were producers. The inhibition activity was demonstrated in 77% of clones in the four patients.

When a non-corresponding extract obtained from breast cancer instead of gastric cancer, and vice versa, was added to the wells with ERFC and CD4+ T
Fig. 2. Fractionation of active crude supernatant of cancer-extract-stimulated ERFC in patient N. B. with gastric cancer by gel chromatography on a 10×700 mm column containing Sephadex G-100. Six fractions were isolated. The fractions 1, 2 and 3 were inactive (MI - 0.95, 1.15 and 0.86). Inhibition effect against Ehrlich-cell migration was found with the fractions 4, 5 and 6 (MI - 0.70, 0.58 and 0.65). The positions of the calibrating molecular-weight markers are indicated with arrows.

cells, or no cancer extract was added, the culture supernatants obtained did not show IE against Ehrlich cell migration.

*Inhibition Effects of the Fractions of Crude Supernatants*

Active crude supernatants were chromatographed on a Sephadex G-100 column, and six fractions with low protein contents (0.06-0.11 mg/ml) were isolated (Fig. 2). IE against migration of Ehrlich cells was found with the fractions 4, 5 and 6; the factor was distributed in the range of molecular weights between 14,300 and 5,800 for the fraction 4 and lower than 5,800 for the fractions 5 and 6. The fraction 6 did not appear in all cases.
DISCUSSION

Our results showed that the factor inhibiting the in vitro migration of Ehrlich ascites tumor cells was produced from peripheral blood ERFC and CD4+ T cells in patients with gastric and breast cancer, when the cells were stimulated for 2 hr and mostly for 24 hr with 3 M KCl allogeneic extracts corresponding to the kind of carcinoma in patients and containing tumor-associated antigens. Moreover, this factor could be produced by ERFC from some patients with chronic gastric ulcer after stimulation of the cells with a cancer extracts for 24 hr, but not with extracts of gastric mucosa. Our data from the stimulation of ERFC in cancer and control patients by allogeneic extracts of gastric mucosa and breast tissue showed that the organ-specific antigens did not stimulate the production of the inhibitory factor. In the assay for migration inhibition with Ehrlich cells, the non-specific effect of the factor inhibiting tumor cell migration (TCM) against different target cells (1-3,5) was used. The factor inhibiting TCM was produced in the limiting dilution microculture system of ERFC and CD4+ T cells at an initial concentration from 2,500 cells to 1 cell per well. This was supported by the findings of MI < 0.80 and FrRMs > 0.5 for the culture supernatants obtained from microcultures. For estimation of the production of the inhibitory factor, FrRMs showing the proportion of microcultures by which this factor was produced was more informative than MI.

We admitted that in microcultures with initial large number of ERFC and CD4+ T cells, numerous cells could proliferate and produced an inhibitory factor in a higher concentration, estimated by the values of MIs and mainly by the FrRMs than in microcultures with a small number of cells. But no relationship between the initial and terminal numbers of cells in wells and the production of the inhibitory factor in most of the cases was found, although the various numbers of proliferated cells in microcultures in 12 patients corresponded to the number of cells/well at the onset of culture.

The high frequency of the inhibitory factor-producing cell precursors was found in six cases in which the line of regression of the values of FrNRM could be fitted (from 1/14.2 to 1/4.5 in ERFC and from 1/9 to 1/1.8 in CD4+ T cells). In patients with breast cancer, the frequencies of cell precursors in CD4+ T cells was higher than those in ERFC. The cell precursors did not originate from CD8+ T cell subset, as the inhibitory factor was not produced from cancer-extract stimulated CD8+ T cells.
At the limiting dilution procedure, cell precursors dispersed in most of the wells of the series of microcultures including those with a low initial cell concentration. Then, the cells proliferated and multiple clones produced an inhibitory factor as the cell number in wells with growing microcultures at the termination of culture exceeded the relatively low number of cells necessary for production of this factor. This explained the lack of significant difference in production of the factor from various initial concentrations of cells in wells. The data for the production of the factor from cancer extract stimulated CD4⁺ T cells in microcultures were confirmed in patients with breast cancer. As it was shown, in all series of microcultures FrRMs for the supernatants of CD4⁺ T cells, including FrRMs = 1, was higher than that of ERFC. The production of the factor inhibiting TCM in microcultures of ERFC was determined by the content of CD4⁺ T cells in ERFC and their activity. Previously, we have indicated that in patients with cancer of the stomach, when active crude supernatants of regional lymph node lymphocytes stimulated with gastric cancer extracts were chromatographed on a Sephadex G-100 column, four active fractions were isolated (5). Inhibition effect against migration of gastric cancer cells was associated with molecular weights ranging between 14,000 and 5,800 for fractions 4 and 5 and lower than 5,800 for fractions 6 and 7. Similar active fractions from crude supernatants were isolated now, although less in number as well as from supernatants of stimulated spleen cells of sarcoma-bearing mice (3). For the reason of these findings the factor inhibiting TCM could be determined as cytokines probably of protein and mainly of polypeptide nature with molecular heterogeneity. In the present article the term “factor” was used, because cytokines of uncharacterized nature were quite reasonably referred to as “factors” of some defined biological activities (20).

As the result of our investigations, it has been suggested that the production of the factor with IE against tumor-cell migration, which is one of the elements of the metastatic cascade (21), could be accepted as a protective phenomenon in in vivo tumor pathology probably expressed in blockading the migration of tumor cells in small cancer foci. It could be argued whether this factor is related to Interleukin 10 (IL-10), designated also as cytokine synthesis-inhibitory factor (22), or supresses the effect of some chemoattractants, particularly autocrine motility-stimulating factors (21,23). Further investigations will be required in this field.
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