

Detection of Circulating Tumor Cells Is Improved by Drug-induced Antigen Up-regulation: Preclinical and Clinical Studies

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Abstract. ⁵¹Cr-prelabelled colon cancer cells (simulating 'circulating tumor cells', CTCs) were added to human peripheral blood and exposed to staurosporine (ST) to increase carcinoembryonic antigen (CEA) expression. CTCs were captured with immunomagnetic beads coated with Ber-EP4 monoclonal antibody, recognizing the common epithelial antigen present in the majority of cancer cells of epithelial origin, with capture efficiency of more than 80%. Moreover, ST treatment increased CEA expression without compromising Ber-EP4 capture efficiency. In a pilot clinical study on 37 patients, CTCs were captured using Ber-EP4 beads, and recognized by RT-PCR set for CEA or cytokeratin-19 (CK) mRNA detection. The results showed that: (a) the percentage of CEA-positive CTCs (CTC_{CEA}, 54.1%) was lower than that of CK-positive CTCs (CTC_{CK}, 70.3%); (b) *in vitro* ST treatment converted a significant number of CTC_{CEA}-negative into CTC_{CEA}-positive cases. Therefore, immunomagnetic capture combined with exposure

to ST provides a feasible and sensitive technique for the detection of functionally-active CTCs responsive to ST-mediated CEA up-regulation.

Recent advances in the detection of circulating tumour cells (CTCs) have opened up new horizons in the management of cancer patients by monitoring early tumour spread, recurrence, metastasis and response to therapy (1-4). In addition, the molecular characterization of CTCs might provide important diagnostic information, including assessment of the tumour of origin (5, 6).

A number of different techniques have been adopted to evaluate CTCs, including immunomagnetic capture of CTCs followed by microscopic recognition (7, 8), or by molecular recognition of tumour-associated mRNA using RT-PCR detection (9).

Morphological recognition of cells of epithelial origin showing a malignant pattern and inappropriately circulating in peripheral blood, does not determine that the host carries metabolically viable CTCs, possibly endowed with metastatic potential. A further step in defining the biological properties of these cells, is provided by molecular recognition of transcripts of genes actively expressed in CTCs, (e.g. cytokeratins (10), or more selective tumour markers, such as carcinoembryonic antigen [CEA] (9)).

For several years, our attention has been focused on drug-induced antigen remodelling, as shown by the finding that a certain number of drugs can modify substantially the antigenic pattern of malignant cells (11, 12). This effect

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could be exploited to amplify antitumor host responses (13, 14) and possibly to increase target cell recognition, as described in our preliminary report (15).

In the first part of the present study, a preclinical simulation model was used to analyze the efficiency parameters of the technique designed to detect viable and metabolically active CTCs. The model also permitted the exploration of whether 5-fluorouracil (5-FU), a classic antitumour agent (16) or staurosporine (ST), a protein kinase C inhibitor able to amplify CEA expression (17), could interfere with target cell capture from whole blood.

In the clinical section of the present report, a small-size study on CTC detection in cancer patients using a 3-phase sequential procedure: CTC binding by immunomagnetic beads; selective magnetic capture by external magnetic field and molecular recognition of captured cells by CEA and cytokeratin-19 (CK) mRNA detection using the RT-PCR technique, is described.

Materials and Methods

Chemicals. 5-FU (Roche, Milan, Italy; 250 mg vials in alkaline solution) was stored until use at room temperature and brought to the desired final concentrations after having adjusted pH to 7 with 0.1 N HCl. ST was purchased from Alexis, (Laufelfingen, Switzerland) and dissolved in dimethyl sulfoxide at a concentration of 1 mM and stored at -20°C .

Immunomagnetic beads. The capture of cancer cells was performed with two types of immunomagnetic beads, namely Ber-EP4 beads and Pan Mouse beads. The term Ber-EP4 beads was used to indicate beads coated with Ber-EP4 monoclonal antibody (mAb) against human epithelial antigen (Dynabeads Epithelial Enrich, Dynal, Oslo, Norway). The term Pan Mouse beads was used to indicate beads coated with Pan Mouse mAb that recognizes all mouse IgG subclasses (CELLlection Pan Mouse IgG, Dynal). The immunomagnetic beads were washed with PBS containing 0.1% bovine serum albumin (BSA) and resuspended in the same washing buffer before use.

Antibodies. CEA antigen recognition was attained by using a mouse IgG2a mAb termed COL-1 that was prepared, purified and characterized in our laboratory at the NCI, as previously described (18). Fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ rabbit antimouse IgG was obtained from Dako (Dakopatts, Copenhagen, Denmark). Alkaline phosphatase-coupled secondary rabbit antimouse antibody, used for Western blot analysis, was purchased from Promega Biotec (Madison, WI, USA).

Cell cultures and separation of mononuclear cells from blood. Two human colon cancer subclones derived from HT-29 cells, *i.e.* C22.20 and C6.6 cells, expressing low or high levels of CEA antigen, respectively, were generated in our laboratory (19). The cells were routinely grown in DMEM, supplemented with 2 mM glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin and 10% heat-inactivated FCS, hereafter referred to as complete medium (CM). Tumour cells growing as plastic adherent cells were removed using trypsin-ethylenediaminetetraacetic acid (EDTA) solution, (0.05% trypsin and 0.02% EDTA in PBS without

calcium and magnesium). All the reagents for the cell cultures were obtained from HyClone, Laboratories, Inc. (Logan, UT, USA). Peripheral blood mononuclear cells (MNC) were separated from the blood samples by centrifugation on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient.

Tumour cell labelling and evaluation of radiolabelled cell recovery for preclinical studies. Colon cancer cells (1×10^6 cells) were washed with CM without FCS, centrifuged and the pellet was suspended in 5 µl of FCS + 100 µCi of Na₂⁵¹CrO₄ (NEN Life Science Products Inc., Boston, MA, USA) and incubated at 37°C for 1 h. Thereafter, the cells were washed and resuspended in CM. At the end of the cell capture process (see below), the extent of cell recovery was evaluated on the basis of sample radioactivity that was measured in a γ-scintillation counter (Cobra II Monodetector, Mod. 5003, Packard Instrument Co., Meriden, CT, USA) with 4-6 replicates per point.

Preclinical simulation experiments: drug treatment and cell capture. Exponentially growing CEA-deficient C22.20 cancer cells (2.5×10^6 in 12 ml of CM) were left untreated or exposed to ST (5 nM) on day 3 after seeding, and collected on day 6, unless otherwise stated. Untreated CEA-proficient C6.6 tumour cells were kept in the same culture conditions without ST and collected on the same day. In some cases, the cells were treated with a clinically relevant concentration of 2.5 µM 5-FU (20) for 48 h. The malignant cells were then labelled with ⁵¹Cr and admixed with 5 ml of CM or of 0.5-5 ml heparinised peripheral blood from healthy donors. Blood without tumor cells was used as a negative control. In other experiments, non-pretreated unlabelled or radiolabelled cells were added to normal blood and exposed to 5 nM ST in sterile tubes (Eppendorf, Hamburg, Germany or Falcon, Lincoln Pak, NJ, USA) with gentle rotation at 37°C for 24 h. At the end of treatment, the tumour cells were captured using two alternative methods, *i.e.* capture with Ber-EP4 beads, or capture with Pan Mouse beads after the addition of COL-1 mAb to blood. Cell capture with Ber-EP4 beads was performed by incubating the test samples with 2.5×10^6 immunomagnetic beads at 4°C with gentle rotation in a Dynal mixer for 60 min. When Pan Mouse beads were used for target cell capture, 6 µg of COL-1 mAb were added to 0.5 ml of blood containing the desired number of tumour cells and incubated at 4°C for 30 min. Thereafter, tumour cells were captured with 2.5×10^6 Pan Mouse beads using the same method described for Ber-EP4 beads. At the end of the procedure, the tumour cells bound to the beads were retrieved with a magnetic particle concentrator (MPC, Dynal) and washed twice with PBS.

FACS analysis. One million cancer cells were incubated with an excess of the anti-CEA primary mAb for 30 min, washed and incubated with 1:10 dilution of FITC-conjugated F(ab)₂ rabbit anti-mouse IgG (second antibody) in an ice bath for 30 min, washed again and analyzed using a FACScan (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed using CellQuest software (Becton-Dickinson).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Polyadenylated [PolyA(+)] RNA was isolated using a Dynabeads mRNA direct micro-kit (Dynal). The mRNA was eluted in 10 mM Tris-HCl (20 µl) after incubation of the dynabeads-mRNA complex at 65°C for 2 min. cDNA was synthesized using 10 µl of eluted mRNA and a sensiscript RT kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR was performed by

adding cDNA samples (¼ of the RT reaction volume) to a solution (total volume 50 µl) containing 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin) and 200 µM (each) dCTP, dATP, dGTP and dTTP. Ten picomoles each of two synthetic CEA-specific oligonucleotide primers [5'-GATCCTA TACGTGCCAAG-3' (exon 4) and 5'-CGGTAATAGGTGTATG AGGG-3' (exon 6)] (21) or CK-specific primers [5'-GTGGA GGTGGATTCCGCTCC-3' (exon 4) and 5'-TGGCAATCTCCTGCT CCAGC-3' (exon 6)] (22) were added to the mixture, and amplification was performed using Taq DNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN, USA) for 30 cycles in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 53°C for 90 s and extension at 72°C for 120 s. Five µl of each sample were re-amplified under the same conditions with nested primers for 20 cycles, using CEA- [5'-GATCACAGTCTATGC AGAGC-3' (exon 4/exon 5 junction) and 5'-GGCCATAGAGG ACATT CAGG-3' (exon 5/exon 6 junction)] or CK- [5'-ATGGCCGAGCAGAA CCGGAA-3' (exon 4) and 5'-CCATGAGCCGCT GGTACTCC-3' (exon 6)] specific primers (21, 22). Twenty µl of the nested PCR reaction were electrophoresed through a 2% agarose gel containing ethidium bromide. The PCR negative control contained the reagents of the RT-PCR reaction, but lacked the RT enzyme.

Cell extraction electrophoresis and immunoblotting. Captured cells were suspended in 100 µl extraction buffer (50 mM Tris-HCl, pH 7.5; 1 mM phenylmethylsulfonyl fluoride; 2 mM ethylene glycol-bis[betamino-ethyl ether]; *N,N,N',N'*-tetra-acetic acid; 400 mg/ml soybean trypsin inhibitor; 10 mM dithiothreitol; 5 µg/ml aprotinin; 200 µg/ml leupeptin; 1% Triton X-100) kept on ice for 10 min, sonicated for 5 sec and centrifuged for 5 min at 15,000 × *g* at 4°C in an Eppendorf microcentrifuge. The protein concentrations were determined with BioRad protein reagent (BioRad, Hercules, CA, USA). The proteins were heated in a boiling water bath for 2 min and separated in 10% SDS (w/v) polyacrylamide gels using a Bio Rad Mini-protein electrophoresis apparatus. All the reagents, except the BioRad protein reagent, were obtained from Sigma (St. Louis, MO, USA).

Protein electrotransfer to nitrocellulose filters was performed with BioRad Mini *trans-blot* apparatus at 25 V overnight. Thereafter, the membranes were incubated with 3% non-fat dry milk (BioRad) in Tris buffered saline (TBS) at pH 7.5, for 30 min and then incubated at room temperature with COL-1 mAb diluted (14 µg/ml) in TBS containing 0.05% Tween 20 (TBST) for 30 min, washed twice with TBST and incubated with alkaline phosphatase-coupled secondary antibody diluted 1:7,500 in TBST for 30 min. The bands were visualized using the Protoblot (Promega Biotec) color development system, as described by the manufacturer.

Clinical studies. A small size study was performed on cancer patients at different stages of malignant disease of epithelial origin. The patient characteristics (22 females and 15 males, ranging between 45 and 81 years of age) and site of primary tumour and metastases are listed in Table I. Ten ml of venous blood were collected in heparinised tubes by standard transcutaneous needle venipuncture. Samples were maintained at 4°C, and processed within 4 h from collection. Informed consent was obtained in all cases and approval was furnished by the Ethic Committee of the 'Istituto Dermatologico dell'Immacolata'-IRCCS (Rome, Italy) in compliance with the Helsinki Declaration. Each sample was divided into two aliquots of 5 ml, which were incubated with or without ST

(5 nM), at 37°C for 24 h. Thereafter, all the samples were subjected to the same procedure of immunomagnetic capture using a high number (*i.e.* 20×10⁶) of Ber-EP4 beads with the intent to assure the maximum efficiency of target cell capture, followed by RT-PCR analysis, as described above.

The CTC recognition assay, based on the drug-mediated tumour marker amplification strategy, was performed in 15 patients with colorectal cancer (12 with metastatic disease), 10 patients with breast cancer (7 with metastatic disease), and 12 patients with other tumours, including two with non-small cell lung cancer (11 with metastatic disease), as illustrated in Table I. In some cases, 2-5 blood sample collections were taken from the same patient at different times within 3 months, except for patient no. 6 in which blood was collected up to 8 months. However, in the majority of cases, only one blood sample was obtained from each donor. In all instances, the CTCs were captured with Ber-EP4 beads. The recognition phase of captured cells was performed using RT-PCR-based semi-quantitative evaluation. The patients were considered to be positive for CTCs bearing the tumour-selective CEA (CTC_{CEA}) or the non-selective CK (CTC_{CK}) marker when at least one collected blood sample, incubated with medium alone or with ST, was found to be positive for CTC_{CEA} or CTC_{CK}, respectively.

Statistical analysis. Means, standard errors (SE) and Student's *t*-test analysis were performed to process normally distributed data. When results of different experiments were expressed in terms of percentage inhibition, the mean % and SE were calculated after angular transformation of each percentage inhibition in order to obtain statistical parameters based on normally distributed data. In this case, mean % + SE and mean% – SE were not symmetrical.

The statistical analysis of the percentages of CTC-positive patients was performed according to Chi-square analysis with Yates correction.

Results

Preclinical studies

Capture of prelabelled cells from medium or from whole blood. The percentage recovery of the labelled cells from the blood was assessed on the basis of radioactivity measurement. As illustrated in Table II, cell capture with Ber-EP4 beads was by far more efficient than capture with the CEA-selective Col-1 mAb + Pan Mouse beads. Non-specific capture with Pan Mouse beads alone was found to be marginal (data not shown). Additional experiments were performed with untreated or ST-treated C22.20 or untreated C6.6 cells pre-incubated with anti-CEA COL-1 mAbs *in vitro* prior to ⁵¹Cr labelling. In this case, radiolabelled cancer cells pre-coated with mouse COL-1 mAbs were added to blood and captured with Pan Mouse beads. However, even in these experimental conditions, capture efficiency with Pan Mouse beads was not substantially higher than that illustrated in Table II (data not shown).

The capture with Ber-EP4 beads from blood was highly efficient and close to that obtained when the cells were admixed with medium alone (Figure 1a). The results in Table II highlight that *in vitro* treatment with ST considerably

Table I. Patient characteristics and presence of CTCs.

Patient number	Age (years)	Gender	Site of primary tumour	Site of distant metastasis ^a	No. of blood samples	Presence of ^b	
						CTCC _{EA}	CTC _{CK}
1	71	M	Colon	Liver	1	–	+
2	62	F	Colon	Liver	4	+	+
3	64	M	Colon	Liver	1	–	–
4	59	F	Colon	None (Dukes C2)	1	–	+
5	68	F	Colon	Liver	1	+	–
6	68	F	Colon	Liver	5	+	+
7	67	F	Anus	Liver	3	+	+
8	71	M	Colon	None (Dukes C2)	1	+	+
9	72	F	Rectum	None (Dukes C2)	1	+	+
10	72	M	Colon	Lung, liver	2	+	+
11	60	F	Anus	Bone	1	–	+
12	71	F	Colon	Lung	1	–	–
13	73	F	Colon	Liver	1	–	–
14	68	M	Rectum	Liver	4	+	+
15	68	M	Colon	Multiple	2	–	+
16	69	F	Breast	Lung	1	–	–
17	64	F	Breast	Bone	2	+	+
18	81	F	Breast	None (T2N2M0)	1	–	+
19	60	F	Breast	Contralateral axillary nodes	1	+	+
20	59	F	Breast	Bone	2	+	+
21	54	F	Breast	None (T2N2M0)	1	+	+
22	78	F	Breast	Liver	2	–	+
23	72	F	Breast	Lung	1	–	+
24	49	F	Breast	None (T2N2M0)	1	+	–
25	62	F	Breast	Bone	2	+	+
26	64	M	Lung	Liver	3	+	+
27	63	M	Kidney	Lung, liver	1	–	+
28	67	F	Gastric	None (T1N1M0)	1	–	–
29	72	M	Penis	Pelvic nodes	3	+	+
30	67	M	Pancreas	Liver	1	–	+
31	73	M	Bladder	Lung	1	+	+
32	72	F	Ovary	Peritoneum	1	–	+
33	62	M	Penis	Pelvic nodes	1	–	–
34	57	F	Ovary	Liver	1	–	–
35	58	M	Oesophagus	Brain	1	+	–
36	45	M	Prostate	Liver, bone, Pelvic nodes	1	+	–
37	50	M	Lung	Bone	1	+	+
Total ^c					59	20 (54.1%)	26 (70.3%)

^aIn non-metastatic cases, stage at diagnosis is indicated. ^bThe presence of CTCs expressing CEA or CK considered '+' when at least one blood sample, treated or not with ST, was found to be positive for the indicated marker. ^cNumber of patients in which CTCs positive for CEA or CK were detected.

increased the CEA expression on the C22.20 cells and, as expected, ameliorated CEA-selective capture efficiency, although to a limited extent. In addition, the CEA-selective capture was higher when the CEA-proficient C6.6 target cells were used, compared with that obtained with untreated CEA-deficient C22.20 cells. It must be stressed that ST treatment did not significantly modify the highly efficient recovery of the C22.20 prelabelled cells achieved by the Ber-EP4 beads. Moreover, no significant difference of capture

efficiency by the same beads was found between the C22.20 and C6.6 cells treated or not treated with ST (Table II and data not shown).

Capture of non-treated prelabelled cells from medium or from whole blood. A series of assays was performed to establish the influence of different experimental conditions including Ber-EP4 bead number, tumour cell number or modality of capture (*i.e.* directly from peripheral blood, or from MNC) on the

Table II. Capture from blood of ^{51}Cr -labelled cells untreated or treated with ST.

Tumor cells	ST	% Growth inhibition	CEA ^b	Percentage capture of labelled cells Mean (SE)		P-Value ^c	P-Value ^d
				COL-1+ Pan mouse beads	Ber-EP4 beads		
C22.20	-	-	2.1	0.66 (0.17)	89.2 (3.4)	<0.01	-
C22.20	5 nM	20.6 (13.8-28.4) ^a	25.4	9.45 (1.15)	79.7 (3.6)	<0.01	NS
C6.6	-	-	35.0	19.1 (0.3)	93.1(0.47)	<0.01	-

5000 Labelled cells incubated with 0.5 ml whole blood. ^aMean and SE (viable cell count) of 8 distinct experiments. Data were processed after angular transformation (see methods). ^bPercentage of CEA-positive cells according to FACS analysis. ^c*p*-value COL-1+Pan Mouse beads vs. Ber-EP4 beads. ^d*p*-Value Ber-EP4 beads capture of ST treated cells vs. untreated C22.20 cells. NS, Not significant.

Table III. Influence of 48 h pretreatment with 5-FU on ^{51}Cr -labelled cell capture by Ber-EP4 beads from blood.

Tumour cells	5-FU	Mean % capture (SE) ^a	% Reduction	P-Value ^b
C22.20	-	82.4 (2.4)	-	<0.05
	2.5 μM	75.3 (0.4)	8.6	
C6.6	-	78.3 (6.7)	-	NS
	2.5 μM	73.3 (2.6)	6.4	

5000 ^{51}Cr -labelled cells (pretreated or not pretreated with 5-FU) added to 1 ml whole blood, captured with 2.5×10^6 Ber-EP4 beads. ^aMean of 4 replicas of 1 representative experiment, showing results similar to those of 2 additional experiments. No angular transformation of replica percentages applied. ^bMean % capture of untreated cells vs. 5-FU-treated cells. NS, Not significant.

Table IV. Cumulative CTC_{CEA} detection in cancer patients.

Tumour	Number of patients tested	Number of CTC _{CEA} -positive patients			
		Basal CTC _{CEA} ^a	ST-induced ^b	ST-suppressed ^c	Overall ^d
Colorectal	15	5	3	1	8
Breast	10	4	2	3	6
Other	12	3	3	3	6
Total ^e	37	12 (32.4%)	8	7	20 (54.1%) ^f

^aNumber of patients with CTC_{CEA} in absence of *in vitro* treatment with ST. ^bNumber of patients negative for CTC_{CEA} without ST treatment and converted into CTC_{CEA}-positive by ST treatment. ^cNumber of patients positive for CTC_{CEA} without ST treatment, were converted into CTC_{CEA}-negative after ST treatment. ^dCumulative data including all patients positive for CTC_{CEA} with or without exposure to ST, in at least one of the assays. ^eNumbers and percentages of the total number of patients tested. ^f*p*<0.05 % total basal CTC_{CEA} vs. overall CTC_{CEA}.

percentage recovery of prelabelled cells. The main findings consistently showed that a high cell recovery (*i.e.* more than 80%) was obtained when prelabelled cells suspended in 5 ml of culture medium as well as in 5 ml of peripheral blood, were captured by 5×10^6 to 20×10^6 beads (Figure 1a). Moreover, MNC separation on ficoll-hypaque was not required for efficient immunomagnetic tumour cell capture from whole blood. On the contrary, direct capture of malignant cells without gradient separation on ficoll-hypaque was preferable (Figure 1b).

Capture of 5-FU-treated cells from whole blood. Since CTC are frequently tested in patients with advanced carcinoma treated with antineoplastic agents, additional simulation experiments were performed with C22.20 and C6.6 cells treated *in vitro* with 5-FU for 48 h. Table III shows that 5-FU treatment of the cancer cells only slightly reduced, although significantly in the case of C22.20 cells, the extent of prelabelled cell recovery. Moreover, exposure of the C22.20 and C6.6 sublines to 5-FU resulted in a modest cell growth

inhibition, not exceeding 10% (data not shown), indicating that these cells were relatively resistant to the fluoropyrimidine compound in these experimental conditions.

ST-mediated up-regulation of CEA expression in C22.20 cells added to normal peripheral blood. The expression of CEA protein and mRNA was evaluated by Western blot analysis or nested PCR, respectively. The results of the immunoblots with anti-CEA COL-1 mAb (Figure 2a) indicated that the ST treatment of whole blood containing C22.20 cells was followed by a substantial increase of CEA expression of the cancer cells. In addition, a high molecular weight band corresponding to the Ber-EP4 mAb, present on the Ber-EP4 beads, was detectable in all the samples containing beads. In contrast, when the nitrocellulose filter was incubated with the anti-mouse secondary antibody only, the CEA band was no longer detected, whereas the band corresponding to the Ber-EP4 mAb was still present.

RT-PCR analysis of the CEA transcript (Figure 2b) from the C22.20 captured cells showed that CEA mRNA was detected only in the ST-treated whole blood. In this experiment RNA corresponding to 125 cells was used. This selected condition did not allow the detection of CEA transcript in the untreated control. In contrast, similar levels of CK transcript were found in the untreated or ST-treated blood samples.

Clinical studies. Table I illustrates the CTC detection relative to each single case. More than 50% of all the patients showed the presence of CTC_{CEA}, whereas a higher percentage of patients (*i.e.* approximately 70%) revealed the presence of CTC_{CK}. As expected, in the majority of cases (*i.e.* 16 cases), the patients carrying CTC_{CEA} were also positive for CTC_{CK}, only four patients (*i.e.* patient 5, 24, 35, 36) were positive for CTC_{CEA} and negative for CTC_{CK}. In contrast, 10 patients positive for CTC_{CK} were negative for CTC_{CEA}. When detection of CTC_{CEA} and CTC_{CK} were considered together, in the absence or presence of ST, 30 out of 37 (81.1%) patients were found to be positive for CTCs showing at least one of the two markers.

In the 30 patients with metastatic disease, CTC_{CEA} were detected in 17 cases (56.7%) and CTC_{CK} in 21 cases (70.0 %). In this instance also, if detection of CTC_{CEA} and CTC_{CK} were considered together, 24 out of 30 (80%) patients with metastasis were positive for CTCs showing at least one of the two markers. Moreover, of note is the observation that in the 7 non-metastatic patients, 4 were positive for CTC_{CEA} and 5 for CTC_{CK}. In this situation, if detection of CTC_{CEA} and CTC_{CK} were considered together, 6 out of 7 non-metastatic patients were found to be positive for CTCs, showing at least one of the two markers. However, a higher number of non-metastatic patients should be analyzed for a sound statistical analysis of frequency of CTC_{CEA} and CTC_{CK} detection. In no case was CTC_{CEA} or CTC_{CK} detected in the blood of 12 control healthy donors (data not shown).

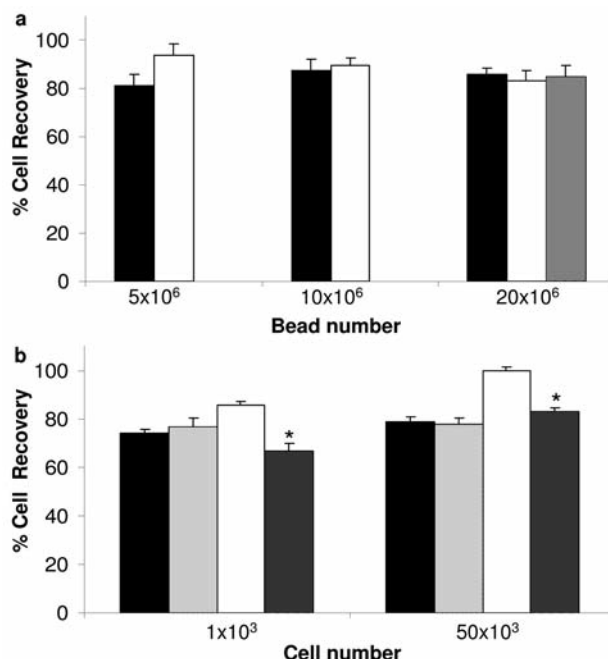


Figure 1. Immunomagnetic capture of ⁵¹Cr-labelled C22.20 cells by Ber-EP4 beads. Data from one experiment representative of 3 (a) or 2 (b) assays that gave comparable results. Each value represents the mean of four observations. Bars indicate standard error of the mean. (a) ■ 500 or □ 5000 ⁵¹Cr-labelled cells added to 5 ml complete medium; ■ 500 ⁵¹Cr-labelled cells added to 5 ml of whole blood. (b) ⁵¹Cr-labelled cells admixed with 5 ml of whole blood. Direct capture from blood using ■ 5x10⁶ or □ 40x10⁶ Ber-EP4 beads or by capture from blood-derived MNC population, after separation on ficoll-hypaque gradient, using ■ 5x10⁶ or ■ 40x10⁶ Ber-EP4 beads. **p*<0.05 direct capture from blood vs. capture from MNC.

Further detailed analysis of the results of CTC_{CEA} detection in the blood of the 37 patients examined is illustrated in Table IV. *In vitro* ST treatment of the blood samples induced the appearance of CTC_{CEA} in 8 cases that were CTC_{CEA}-negative in the absence of ST, and suppressed CTC_{CEA} recognition in 7 cases that were found to be CTC_{CEA}-positive without exposure to ST. However, when the results of CTC_{CEA} detection in the presence or absence of ST were pooled (see overall column, total row, Table IV), a net and statistically significant gain in CTC detection was attained. It must be noted that the number of 'ST-suppressed' cases was irrelevant. All the cases that became negative after *in vitro* exposure to ST derived from CTC_{CEA}-positive patients that were included in the overall list (Table IV).

A similar analysis performed on CTC_{CK}, revealed that *in vitro* treatment of the blood samples with ST did not produce a statistically significant increase of the extent of CTC_{CK} detection, *i.e.* bringing the number of CTC_{CK}-positive

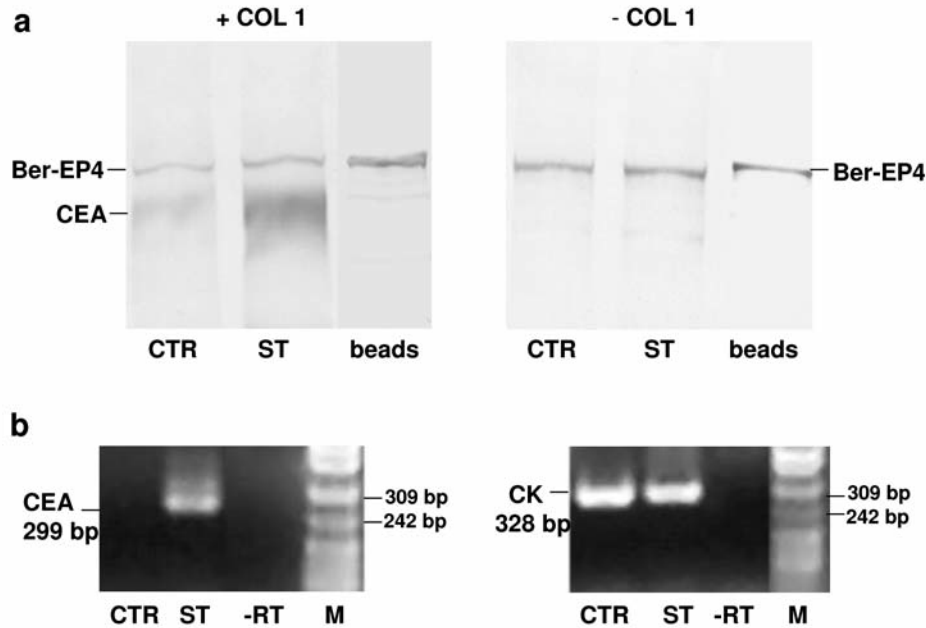


Figure 2. CEA expression of C22.20 cells admixed with normal peripheral blood in the presence or in the absence of ST. Cells (3×10^6 cells for Western blot analysis or 2×10^3 cells for RT-PCR analysis) admixed with whole blood (1ml), exposed to 5nM ST for 24 h, recovered by Ber-EP4 beads. a: Western blot of cells bound to beads, or of beads alone, performed with the anti-CEA COL-1 mAb followed by secondary anti-mouse antibody (+COL-1) or with the secondary antibody alone (-COL-1). b: RT followed by nested PCR of CEA or CK. M, pBR322 Plasmid digested with *Msp*I (Gibco-BRL); -RT, PCR-negative control containing the reagents of the PCR reaction but lacking RT enzyme.

patients from 22 to 26 (data not shown). On the contrary, following *in vitro* exposure to ST 8 out of 22 CTC_{CK}-positive patients became negative (data not shown).

Discussion

The preclinical simulation studies, illustrated in the present report, confirmed previous findings described in the literature (23, 24) showing that immunomagnetic capture of tumour cells of epithelial origin ("capture phase"), followed by target cell identification through a molecular marker ("recognition phase") could be used for CTC detection in cancer patients. The use of ^{51}Cr -prelabelled cells provided the opportunity to adopt a quantitative and extremely precise method for determining the extent of target cell recovery and to ascertain the best experimental conditions to be applied in the clinic.

Not less importantly, drug-mediated enhancement of a tumour marker was shown to provide an attractive strategy for increasing the sensitivity of cancer cell detection, even in those cases in which the marker, under investigation, was minimally expressed (CEA in C22.20 cells). In the present investigation, while our previous results on ST-induced increase of CEA transcript, CEA protein expression and CEA density on the cell membrane (17, 25), were confirmed (Table II, Figure 2), the diagnostic application of this finding in the area of CTC capture and recognition was emphasized.

In particular, the results demonstrated that CEA-targeted tumour cell capture from whole blood was increased by ST treatment. Obviously, the efficiency of the capture process was proportional to the extent of CEA density on the tumour cell membrane, as shown by the degree of target cell capture of CEA-proficient C6.6 tumour or of ST-treated C22.20 cells, as opposed to the absence of tumour capture in the case of CEA-deficient untreated C22.20 targets. However, the results with the Ber-EP4 beads clearly indicated that non-selective capture targeting an ubiquitous epithelial antigen present on malignant cells was by far more efficient (Table II). Moreover, the Ber-EP4 capture phase could be easily performed directly from whole blood without previous separation of the mononuclear cells (Figure 1b). This capture strategy combined with the ST-dependent increase of CEA expression for optimizing the recognition phase, appeared to provide a feasible and most advantageous technique for CTC detection. This was supported by the RT-PCR analysis which revealed the presence of a band corresponding to the CEA transcript in captured CEA-deficient C22.20 cells exposed to ST, but not in those not treated with the CEA enhancing agent. Moreover, it must be considered that ST treatment did not play a negative role in the capture process, as shown by the simulation experiments.

The evaluation of CTC in operable epithelial cancer has been shown to provide promising information on prognosis

(26). However, the determination of CTC in patients with advanced disease has attracted the most attention, especially regarding prognosis (26-28) and response to therapy (29, 30).

In the present preliminary clinical study on patients, mostly with advanced disease, the detection technique was based on the preclinical investigation, suggesting a possible strategy for optimal CTC evaluation. Positivity for circulating epithelial cells provides evidence for the presence of CTCs (31, 32), although the presence of epithelial cells of non-malignant origin in the peripheral blood cannot be ruled out (32). A further step in interpreting the neoplastic origin of a circulating cell is the presence of CK, as indicated by different studies reported in the literature (33, 34). However, the most compelling evidence of malignant origin applies to a circulating cell captured on the basis of an epithelial antigen on its membrane expressing a tumour marker (*e.g.* CEA), either in basal conditions (35, 36) or under the influence of *in vitro* treatment with a drug, such as ST (15).

Interestingly, treatment of the target tumour cells with a clinically relevant concentration of the classic antineoplastic agent 5-FU, largely used in colorectal cancer (16), did not interfere substantially with the Ber-EP4 based capture phase of the assay. Nevertheless, the possible influence of other antineoplastic agents on tumour cell capture efficiency by Ber-EP4 beads should be considered and properly tested in future studies concerning CTC detection in cancer patients undergoing chemotherapy.

In the present report, mainly on patients with colorectal and breast cancer, the percentage of patients positive for CTC_{CEA} or CTC_{CK} was of the order of 50 and 70% respectively. Most of the tested patients had metastatic disease and higher percentages of CTC-positive cases would have been expected. The molecular approach, adopted for the recognition phase of the assay, could have missed cells that morphologically appear to be neoplastic using the 'cell search' methodology (7, 8). However, the latter technique might overestimate the number of effective CTCs. Actually, the cell search methodology could detect cells that lack a molecular signature of malignancy, or a functional marker of viability such as that manifested by mRNA expression. The observation that CTCs were not always detected in CTC-positive patients when sequential blood samples were collected from the same donor, could have been due to variability in the release rate of CTCs from overt metastases (37), or to epithelial-to-mesenchymal transition of CTCs with loss of epithelial marker proteins (38).

Based on our previous preclinical studies (17, 25) and on the present experiments, it was reasonable to predict that *in vitro* exposure of the blood samples to ST could have improved the sensitivity of the recognition phase of the present CTC assay in patients. In particular, the expected gain in the CEA-related recognition phase stemmed from the

observation that ST treatment of the blood-tumour mixture was able to augment substantially the CEA mRNA and protein levels (Figure 2) of the low-expression C22.20 sub-line. Actually, the present pilot clinical study showed that *in vitro* treatment of the blood samples with ST converted eight CTC_{CEA}-negative patients into CTC_{CEA}-positive cases. However, seven CTC_{CEA}-positive patients were converted into CTC_{CEA}-negative subjects by the *in vitro* exposure of their blood to ST (Table IV). It is reasonable to hypothesize that the cytotoxic effect of ST (39, 40) could have masked, at least in part, its potential role as a tumour marker amplifier. This interpretation was further supported by the finding that eight patients at first CTC_{CK}-positive became CTC_{CK}-negative following *in vitro* exposure of their blood samples to ST (data not shown). In any case, in spite of a presumed *in vitro* cytotoxic effect of ST, a substantial and statistically significant increase in the number of CTC_{CEA}-positive patients was detected when the blood samples either not treated or exposed to ST were collectively evaluated.

In conclusion, the present results open up the possibility that appropriate pharmacological manipulation, able to up-regulate tumour marker expression, could be employed to increase CTC detection efficiency in various clinical conditions, including non-metastatic malignancies. This approach which detects both viability (supported by mRNA transcription) and malignant origin (supported by tumour marker transcription) is particularly attractive.

Prostate specific antigen that can be up-regulated by androgens in prostate cancer cells (41, 42) appears to be a good candidate for future development of the present CTC detection strategy in patients with prostate neoplasias. Therefore, studies are in progress to explore whether a number of chemically or biologically inducible tumour markers could be employed for CTC recognition to extend this approach to a large variety of malignant diseases.

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