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Detection of Blood-borne Cells in Colorectal Cancer Patients by Nested Reverse Transcription-Polymerase Chain Reaction for Carcinoembryonic Antigen Messenger RNA: Longitudinal Analyses and Demonstration of Its Potential Importance as an Adjunct to Multiple Serum Markers[1]

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ABSTRACT

The use of reverse transcription-PCR (RT-PCR) to analyze cells in the blood of cancer patients for the detection of mRNA expressed in tumor cells has implications for both the prognosis and the monitoring of cancer patients for the efficacy of established or experimental therapies. Carcinoembryonic antigen (CEA) is expressed on ~95% of colorectal, gastric, and pancreatic tumors, and on the majority of breast, non-small cell lung, and head and neck carcinomas. CEA shed in serum is useful as a marker in only ~50% of colorectal cancer patients and rarely is shed by some other carcinoma types. RT-PCR has been used previously to detect CEA mRNA in cells in the blood and lymph nodes of cancer patients. Under the assay conditions validated in the studies reported here, 34 of 51 (67%) patients with different stages of colorectal cancer had blood cells that were positive by RT-PCR for CEA mRNA, whereas none of 18 patients with colonic polyps were positive; 2 of 60 apparently healthy individuals (who were age and sex matched with the carcinoma patients and were part of a colon cancer screening program as controls) were marginally positive. The results of CEA PCR in the blood of the carcinoma patients and the other groups showed strong statistical correlation with the disease ($P2 < 0.0001$). Analyses were carried out to detect both serum CEA protein levels and CEA mRNA in blood cells of colorectal carcinoma patients by RT-PCR. For all stages of disease, 18 of 51 patients (35%) were positive for serum CEA, whereas 35 of 51 (69%) were positive by RT-PCR. More importantly, only 5 of 23 (20%) of stage B and C colorectal cancer patients were positive for serum CEA, whereas 16 of 23 (70%) were positive by RT-PCR. The use of two other serum markers (CA19.9 and CA72-4) for colorectal cancer in combination with serum CEA scored two additional patients as positive; both were positive by RT-PCR for CEA mRNA. Pilot long-term longitudinal studies conducted before and after surgery identified some patients with CEA mRNA in blood cells that were negative for all serum markers, who eventually developed clinical metastatic disease. The studies reported here are the first to correlate RT-PCR results for CEA mRNA in blood cells with one or more serum markers for patients with different stages of colorectal cancer, and are the first long-term longitudinal studies to use RT-PCR to detect CEA mRNA in blood cells of cancer patients. Larger cohorts will be required in future studies to define the impact, if any, of this technology on prognosis and/or disease monitoring.

INTRODUCTION

One of the newer areas being explored in the management of cancer is the use of RT-PCR[3] to analyze the blood of cancer patients for the detection of mRNA expressed in tumor cells (1–7). This technology may aid in three major areas in the management of cancer: (*a*) predicting which patients will have a favorable outcome following removal/treatment of the primary lesion; (*b*) more efficiently analyzing ways to follow the efficacy of known therapies; and (*c*) defining ways to more rapidly determine the efficacy of a new experimental therapy.

The gene for CEA is one of the most widely expressed genes in cancer cells. It is expressed in ~95% of colorectal, gastric, and pancreatic cancers; on the majority of non-small cell lung cancers and other carcinoma types, such as squamous cell cancer of the head and neck; and in ~50% of breast cancers (8). Serum CEA protein currently is used to follow the course of therapy in the management of colorectal carcinoma (9). Although CEA protein is expressed in tumors of other types, it rarely is shed into the serum in levels that can be detected. Furthermore, even in the case of colorectal cancer, it has been shown that only approximately half of colorectal cancers shed CEA in levels sufficient for their detection in monitoring therapy (10). Moreover, it has been shown by statistical analysis that there is no correlation between the level of CEA expression in tumor biopsies and the presence of CEA protein in the serum (9). Thus, for various aspects in the management of colorectal cancer and perhaps for other carcinoma types, detection in the blood of tumor cells that express CEA could conceivably help in the prognosis of patients, following the clinical course of patients, and analysis of standard therapies as well as new experimental therapies.

Numerous studies have reported the use of RT-PCR technology to detect CEA message in tumor cells from peripheral blood, lymph nodes, or peritoneal washes of cancer patients (2, 11–17). These methods have varied in terms of (*a*) primers used, (*b*) amount of RNA used, (*c*) amount of blood analyzed, (*d*) assay conditions, and (*e*) methodology to amplify the CEA message. As a result, the literature contains reports with conflicting findings as to the detection of CEA-containing cells in peripheral blood of "apparently healthy" donors as well as cancer patients. Some of the studies in which healthy donors were reported to have CEA-containing cells in their blood used primers that were not unique to CEA and indeed contained sequences shared between CEA and CEA-related proteins, such as NCA, which is expressed in skin and in granulocytes. There are several reports, however, in which primers specific for CEA were used and, to

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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen; NCA, normal cross-reacting antigen; MAb, monoclonal antibody; AI, antigen index.

enhance the sensitivity, nested RT-PCR was performed (14, 18). With these methodologies, CEA-positive cells were not detected in the blood of healthy donors, but were found in approximately one-half to two-thirds of colon cancer patients. These studies analyzed peripheral blood samples by RT-PCR prior to surgery and ~24 h after surgery (19). In the studies reported here, we have essentially confirmed these previous findings, using minor modifications of previously published methods. Additionally, we report here, for the first time, long-term longitudinal analyses of blood cells from patients of different stages of colorectal cancer by nested RT-PCR for CEA mRNA. We have also compared, for the first time, these results with three known serum markers used in the management of colorectal cancer patients. The results demonstrate that the nested RT-PCR technology for CEA mRNA can, in some cases, detect CEA-expressing blood-borne cells in patients negative for the serum markers and can, in some cases, precede clinical detection of disease. We also show, in patients with early-stage disease, a lack of association between the detection of CEA mRNA in blood-borne cells and the detection of serum CEA, although there is some evidence of a relationship between these two measures in patients with more advanced disease. These studies thus form the basis for larger scale prospective studies toward the goal of using nested RT-PCR to detect CEA mRNA in blood-borne cells in the management of colorectal cancer and, perhaps, other CEA-expressing tumors.

MATERIALS AND METHODS

Patients and Samples

Patients' Characteristics. We evaluated 51 patients with histologically diagnosed primary colorectal adenocarcinoma (27 males, 24 females; mean, 60.1 years; range, 44–80 years). All patients with malignant disease underwent surgery for their primary tumor at the Regina Elena Cancer Institute or the University of Rome "Tor Vergata," Rome, Italy. Malignant colorectal disease was pathologically staged according to the Astler-Coller classification: stage A ($n = 6$), stage B1 ($n = 7$), stage B2 ($n = 8$), stage C1 ($n = 3$), stage C2 ($n = 5$), stage D ($n = 17$), and locoregional recurrences ($n = 5$). Tissue biopsies of histologically confirmed neoplastic tissues were obtained at surgery, immediately fixed in formalin, embedded in paraffin, and subsequently evaluated for CEA expression by immunohistochemistry. Carcinoma specimens were evaluated by histopathology and were shown to be free of necrotic areas and to consist of at least 50% malignant cells.

Forty apparently healthy donors and 18 patients with benign colorectal disease were also included in the study. Healthy donors were subjects enrolled in an institutional program of screening and surveillance for gastrointestinal cancer. Blood samples from patients with benign lesions were obtained just prior to digestive endoscopy. Diagnosis was confirmed by histological evaluation.

Blood Samples. A 7-ml sample of venous blood was collected at the time of diagnosis by standard transcutaneous needle venipuncture into 7-ml Vacutainers containing lithium heparin from colorectal patients with either benign ($n = 18$) or malignant ($n = 51$) disease. Similarly, blood samples were obtained from apparently healthy donors ($n = 40$). Moreover, in 10 of the healthy donors, multiple Vacutainers (up to five), each containing 7 ml of peripheral blood, were obtained by the same needle transcutaneous venipuncture and numbered in sequence from 1 to 5, with 1 representing the first blood sample collected. Furthermore, in 13 of the 51 colorectal carcinoma patients, two to three Vacutainers containing 7 ml of peripheral blood (numbered in sequence from 1 to 3) were obtained on 2 different days no more than 1 week before surgery. Blood cells were isolated as peripheral blood mononuclear cells by Histopaque (Sigma Chemical Co., St. Louis, MO) and subjected to density gradient centrifugation at 2000 rpm for 30 min at room temperature. Cells were recovered from the plasma-Histopaque interface and washed twice with sterile phosphate buffer solution (GIBCO BRL, Life Technologies, Gaithersburg, MD). Cell pellets were snap frozen and stored at −80°C until RNA extraction.

Immunohistochemical Analyses

Immunohistochemical analyses were performed on paraffin-embedded sections that were cut at 5 μm using a modification of the avidin-biotin-peroxidase complex immunohistochemical method (20). To evaluate the expression of CEA in tissue-tumor biopsies, the anti-CEA mouse MAb COL-1 was used at a concentration of 20 μg/ml (20). The negative control antibody MOPC-21 (Sigma) was used at the same concentration.

All tumors were independently evaluated by two pathologists. For each slide, three to five different fields were scored for the presence of tumor cells and the cell-associated diaminobenzidene precipitate staining pattern. Staining intensity was scored as weak (+) for pale brown reactivity, moderate (++) for intermediate brown intensity, and strong (+++) for intensive, dark brown precipitate. The percentage of MAb-positive cells for each slide represented an analysis of the three to five fields for each tissue specimen. In an attempt to unify the two parameters (*i.e.*, percentage of MAb-positive cells and staining intensity) used to define CEA expression, an arbitrary AI was used as reported by Roselli *et al.* (21). Briefly, the AI is the product of the percentage of MAb-positive tumor cells and the staining intensity (+ = 1, ++ = 2, +++ = 3). For example, a sample in which 15% of the tumor cells were MAb positive with a relative staining intensity of ++ would have an AI of 30.

Determination of CEA, CA19.9, and CA72-4 in Serum Samples

Serum samples from 51 colorectal cancer patients were drawn no more than 1 week before surgery. Patients were monitored during the perioperative period and postoperatively (*i.e.*, every 3 months at the time of scheduled clinical follow-up). All samples were coded and stored at −20°C until the assays were performed. Serum CEA levels were determined by an enzyme immunoassay test kit (AMDL, Inc., Tustin, CA), using a value of 5 ng/ml as positive (7). CA19.9, an indicator of mucin TAG-72, was determined using the CA19.9 kit (Fujirebio, Malvern, PA), with the suggested value 37 units/ml as positive (22). Serum CA72-4 antigen levels, another measure of mucin TAG-72, were determined using the CA72-4 kit (Fujirebio), with the suggested value 6 units/ml as positive (23).

Statistical Analyses

The association between RT-PCR classification (positive, borderline, or negative) and type of subject (healthy donor, patient with benign colorectal disease, and patient with colorectal cancer) was determined by the Jonckheere-Terpstra test for trend (24), the Cochran-Armitage trend test (25), and Fisher's exact test, as appropriate.

The difference in actual CEA, CA19.9, CA72-4, or AI values between RT-PCR dichotomized as positive (+) *versus* negative (−) was tested using the Wilcoxon rank-sum test. The same parameters [whether actual values or dichotomized as (−) or (+)] were compared between four levels of RT-PCR (−, +, ++, and +++) using the Jonckheere-Terpstra test for trend. Any results from analyses of 2 × 2 tables were obtained using either the χ^2 test or Fisher's exact test, as appropriate. All *P*s are two-tailed and denoted *P*2.

Cell Lines

The murine colon adenocarcinoma MC38 and MC38(CEA2) cell lines have been described and characterized (26). The MC38(CEA2) cell line, developed by transduction of the MC38 cell line with a retroviral construct containing cDNA encoding the human CEA gene, is designated as MC38(CEA) here (26). The MC38(NCA) line is the result of transduction of MC38 with the human NCA gene and has been characterized (26). These three cell lines were used as positive and negative controls to establish the CEA-specific RT-PCR methodology. All cell lines were maintained in DMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (Life Technologies).

RNA Extraction

Total RNA from cell lines and blood cells was extracted using TRIZOL (Life Technologies) according to the manufacturer's protocol. The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. The integrity and quality of the patients' RNA were assessed by electrophoresis in Tris-acetate-EDTA buffer on 1% agarose gel (FMC Bio-

products, Rockland, ME), with the DNA bands visualized by ethidium bromide staining.

Primers

CEA and β-actin primers were designed according to published sequence information (18, 27), synthesized, and cartridge-purified by ANNOVIS (Aston, PA). These primers were as follows:

β-actin forward primer: 5′-GCTCACCATGGATGATGATATCGC-3′
β-actin reverse primer: 5′-GGAGGAGCAATGATCTTGATCTTC-3′
CEA A primer: 5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′
CEA B primer: 5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′
CEA C primer: 5′-GGGCCACTGCTGGCATCATGATTG-3′

cDNA Synthesis

cDNA was synthesized using the GIBCO BRL SuperScript Preamplification System (Life Technologies) with 1–5 μg of total RNA, 20 pM of random hexamers, and Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions.

The integrity of patients' RNA samples and the fidelity of cDNA synthesis were verified using 2 μl of cDNA to amplify β-actin in a standard RT-PCR reaction. Each 50-μl reaction contained 20 mM Tris (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 2 mM deoxynucleotide triphosphate; 1 unit of Hot Tub (Amersham Pharmacia Biotech, Piscataway, NJ), and diethyl pyrocarbonate-treated water to 48 μl, and 200 ng each of β-actin forward and reverse primers. β-Actin was amplified at 94°C for 2 min, and for 25 cycles at 94°C (30 s), 55°C (30 s), and 72°C (30 s), with a final extension step at 72°C (7 min), using the Perkin-Elmer GeneAmp PCR System 9600 (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). The β-actin PCR product, a DNA fragment of 1 kb, was resolved for each patient's sample by electrophoresis on 1% agarose in Tris-acetate-EDTA buffer and visualized by ethidium bromide staining of the gel.

CEA Nested RT-PCR

The nested RT-PCR was performed as described previously (18), with some modifications. For the first round of PCR, 80 μl of PCR Buffer (Applied Biosystems Division, Perkin-Elmer), containing 1.5 mM $MgCl_2$, 0.8 μM CEA primers A and B, 200 μM deoxynucleotide triphosphate, 2 μl of cDNA, and 2 units of AmpliTaq Gold (Applied Biosystems Division, Perkin-Elmer) were added to the tubes. CEA amplification was performed by heating at 95°C (10 min), followed by 20 rounds of denaturation at 95°C (1 min), annealing and polymerase extension at 72°C (2 min), and a final extension step at 72°C for 10 min. Amplified product (5 μl) was transferred to a second tube with the above buffer containing 0.4 μM primers C and B and 2 units of AmpliTaq Gold; the tubes were heated to 95°C for 10 min to activate the Taq polymerase, followed by 30 cycles of denaturation at 95°C (1 min), annealing at 69°C (1 min), polymerase extension at 72°C (1 min), and a final extension step for 10 min at 72°C. The 131-bp CEA PCR product was identified by electrophoresis of 10 μl through 4% NuSieve 3:1 plus agarose (FMC Bioproducts) in Tris-borate-EDTA buffer and ethidium bromide staining.

Each RT-PCR run included a positive control synthesized from the transduced MC38(CEA) cell line (CEA positive), the negative cell line MC38, and cells from a negative blood donor. Each PCR run also included a sample containing PCR buffer but no cDNA. The RT-PCR method was standardized by loading equal volumes of PCR product on agarose gels to ensure that the intensity of the β-actin bands reflected an equivalent amount of ethidium bromide-detectable products.

The 131-bp products from blood cells and the MC38(CEA) line were sequenced and found to be identical to the CEA sequence published by Schrewe *et al.* (27).

RESULTS

Studies were first conducted to determine the optimal amount of RNA necessary for the detection of CEA-positive cells in blood samples by nested RT-PCR using the primers described in "Materials and Methods." As seen in Table 1 (*series I*), total RNA was extracted from peripheral blood mononuclear cell preparations of 20 apparently healthy donors (age- and sex-matched with colorectal cancer patients as part of a cancer surveillance study) and 20 patients with metastatic colorectal cancer; 1, 2, and 5 μg of total RNA were used to synthesize cDNA. cDNA was then analyzed by nested RT-PCR using the CEA-specific primers. With these methods, none of the 20 healthy donors were positive for CEA expression at any of the three RNA levels. The percentage of positive colorectal cancer patients with advanced disease clearly went up with increasing amounts of RNA to a detection level of 75% when 5 μg of RNA were used. This is exemplified in Fig. 1*A*, where total RNA extracted from three different patients with advanced colorectal cancer was analyzed using 5 and 1 μg of RNA. As seen in Fig. 1*A*, the 1 μg samples (*Lanes 4–6*) were negative, whereas the 5 μg samples (*Lanes 1–3*) gave detectable signals by ethidium bromide staining.

Table 1 *Summary of CEA RT-PCR results in blood samples from apparently healthy volunteers and patients with benign or malignant colorectal disease*

	No. of cases	No. of positive cases[a]	No. of borderline positive (+/−) cases[a]	No. of negative cases
Series I				
Donors				
1 μg of RNA	20	0	0	20
2 μg of RNA	20	0	0	20
5 μg of RNA	20	0	0	20
Patients				
1 μg of RNA	20	3 (15)	0	17
2 μg of RNA	20	7 (35)	0	13
5 μg of RNA	20	15 (75)	0	5
Series II[b]				
Donors[c]	40	0	2 (5.0)	38 (95.0)
Patients				
Benign	18	0	0	18 (100.0)
Malignant	51	34 (66.7)	1 (2.0)	16 (31.4)

[a] Numbers in parentheses represent percentages.
[b] 5 μg of RNA were used for all series II samples.
[c] Donors are apparently healthy individuals, as described in "Materials and Methods."

The specificity of the CEA-nested RT-PCR used in this study was then determined. To accomplish this, the murine carcinoma cell line MC38 was used. MC38 has previously been shown to lack the human CEA gene (26). The MC38 cell line was transduced with the human CEA gene [designated MC38(CEA)] or the human NCA gene [designated MC38(NCA)], which shares ~80% homology with CEA (26). As seen in Fig. 1*C*, CEA DNA product could easily be detected in MC38(CEA) (*Lane 1*) but not in MC38(NCA) (*Lane 2*) or control MC38 cells (*Lane 3*). Dilution experiments demonstrated that with the methodology and primers used in this study, we could detect one MC38 tumor cell transduced with the CEA gene in 10^6 mononuclear cells (data not shown). Figs. 1*C* (*Lanes 4–11*) and 1*D* (*Lanes 1–11*) demonstrate results from RNA extracted from 19 different, apparently healthy donors; no CEA DNA bands were detected. As an additional control to rule out that NCA and/or CEA were not detected from skin as a result of needle puncture, multiple samples of blood were obtained from the same needle transcutaneous venipuncture from 10 apparently healthy volunteers; all samples were negative. To further rule out variations in different samples from the same patient, blood samples were drawn prior to surgery at two different times within 1 week from 13 different patients with various stages of colorectal carcinoma. Ten samples were positive at both time intervals, and 3 samples remained negative at both time intervals. This is exemplified in Fig. 1*B*, where RNA was obtained from blood-borne cells 7 days prior to surgery (*Lanes 3* and *4*) and 1 day prior to surgery (*Lanes 5* and *6*) from either 7 or 15 ml of blood. Consistency of results with these samples was demonstrated.

RNA was then extracted from blood cells from an additional 40 apparently healthy donors who were enrolled in an institutional gas-

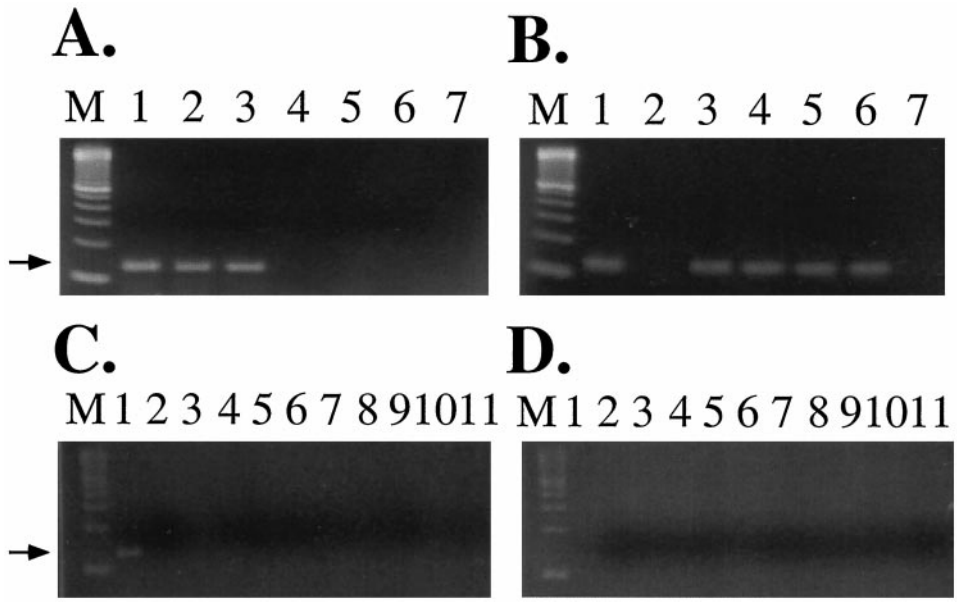

Fig. 1. Optimization of the sensitivity and specificity of the RT-PCR assay for CEA mRNA. *A*, improvement in RT-PCR sensitivity using different amounts of total RNA. Agarose gel electrophoresis of CEA RT-PCR amplification products from peripheral blood cells of three different metastatic colorectal carcinoma patients when cDNA was synthesized using either 5 μg (*Lanes 1–3*) or 1 μg (*Lanes 4–6*) of total RNA. *Lane 7*, cDNA negative control. *Arrow*, 131-bp CEA band. *M*, 100-bp DNA ladder (also seen in *B–D*). *B*, evaluation of two different blood samples obtained at days 7 and 1 prior to surgery, using two different blood volumes from the same colorectal cancer patient. *Lane 1*, MC38(CEA) cell line. *Lane 2*, MC38 cell line. *Lanes 3* and *4*, 15 and 7 ml of blood, respectively, from a colorectal cancer patient 7 days prior to surgery. *Lanes 5* and *6*, 15 and 7 ml of blood, respectively, from the same patient 1 day prior to surgery. *Lane 7*, cDNA negative control. *C*, evaluation of the specificity of the RT-PCR reaction. *Lane 1*, MC38(CEA) cells expressing CEA. *Lanes 2* and *3*, MC38 and MC38(NCA), respectively. *Lanes 4–11* (and *Lanes 1–11* in *D*), blood samples from 19 different, apparently healthy donors.

trointestinal cancer screening program (Table 1, *series II*). These subjects were age and sex matched with the colorectal cancer patients analyzed in this study. RNA was also extracted from blood cells obtained prior to colonoscopy from 18 patients in whom adenomatous polyps had been detected via colonoscopy. RNA was also extracted from blood cells of 51 patients with various stages of colorectal carcinoma, as will be detailed below. As can be seen in Table 1 (*series II*), 95% of the donors in the surveillance program were negative by CEA RT-PCR, whereas 2 of the 40 demonstrated a marginal positivity. It should be pointed out that the 20 donors initially tested in the RNA titration experiments (Table 1, *series I*) were again negative when retested using 5 μg of total RNA. It should also be pointed out that the RNA from these 20 patient samples has subsequently been tested by RT-PCR five additional times, always with negative results. Thus, a total of 60 apparently healthy individuals were tested at the 5 μg of RNA level; of these individuals, 2 (3.3%) were marginally positive (Table 1, *series I* and *II*). All 18 patients with polyps tested in an identical manner were negative (Table 1); 34 of 51 (67%) patients with different stages of colorectal cancer were positive, and 1 additional patient was marginally positive (Table 1). When we compared these three groups and included the marginally positive result as a separate, intermediate category, there was a strong statistically significant association between the RT-PCR result and type of subject evaluated ($P2 < 0.0001$). When donors and patients with benign colorectal disease were pooled, the difference remained strongly significant ($P2 < 0.0001$). Finally, when we used a dichotomized RT-PCR result (−) *versus* (+, ±) and compared the combination of donors and patients with benign disease *versus* those with malignant disease, we found an extremely strong relationship between RT-PCR result and the presence or absence of malignant disease ($P2 < 7.5 \times 10^{-17}$). Thus, regardless of the categorization, there is a strong association between the RT-PCR result and type of specimen evaluated.

CEA mRNA from blood cells of patients with different stages of colorectal carcinoma was then analyzed by RT-PCR prior to surgery (Table 2). Surprisingly, two of six patients with stage A colorectal cancer were positive by RT-PCR. All six of these patients were negative for serum CEA protein (Table 2). Follow-up studies of the two positive patients are detailed below. Four of seven patients with stage B1 colorectal cancer were also positive by RT-PCR prior to surgery, whereas only one of these seven patients was positive for serum CEA. Five of eight stage B2 patients were positive by RT-PCR, whereas only one of these patients was positive for serum CEA (Table 2). Analysis of blood cells by RT-PCR revealed that seven of eight patients with stage C1 and C2 colorectal carcinoma were positive by RT-PCR prior to surgery (Table 2). Three of these patients were positive for serum CEA, and one of these patients was also negative by RT-PCR. This patient (patient 25) will be discussed below. As seen in Table 2, 13 of 17 patients with stage D colorectal cancer with metastases at various sites were positive by RT-PCR prior to surgery; moreover, four of five patients with locoregional recurrence were positive by RT-PCR prior to surgery. Many of these patients with stage D and locoregional recurrence were also positive for serum CEA (Table 2).

Using the data from all 51 patients presented in Table 2, we classified serum CEA values as positive (+) if CEA was ≥5.0 ng/ml, and negative (−) if CEA was <5.0 ng/ml, and dichotomized RT-PCR results as (+) *versus* (−). Only 2 of 16 patients classified as negative by RT-PCR had a positive serum CEA, compared with 16 of 35 classified positive by RT-PCR ($P2 = 0.021$). The same degree of difference was present when we compared the actual serum CEA values between the dichotomized RT-PCR categories [median = 1.5 (−) *versus* 4.2 (+); $P2 = 0.021$]. This association was largely related to stage of disease, however. For example, among patients with stage B and C disease, 1 of 7 who were negative by RT-PCR were positive by serum CEA, and 4 of 16 who were positive by RT-PCR were positive by serum CEA levels ($P2 = 1.0$), whereas in patients with stage D or recurrent disease, 1 of 5 who were negative by RT-PCR were positive by serum CEA compared with 12 of 17 who were positive by RT-PCR and positive by CEA ($P2 = 0.12$). Although neither set of comparisons demonstrated a statistically significant difference, it is noteworthy that of 17 stage D and recurrent patients who were RT-PCR positive, 12 were also positive for serum CEA compared with only 4 of 16 patients with stage B and C disease. When we used the actual serum CEA values, a similar result was obtained (for stage B and C, $P2 = 0.37$; for stage D and recurrent, $P2 = 0.10$). Classifying RT-PCR into four categories (−, +, ++, and +++) also demonstrated an overall tendency toward a positive trend in the association with serum CEA in all patients, whether considered dichotomously ($P2 = 0.12$) or using the actual values of serum CEA

Table 2 *RT-PCR results and serum tumor marker levels in nonmetastatic colorectal cancer patients*

Serum CEA levels were determined by an enzyme immunoassay test kit, using a value of 5 ng/ml as positive. CA19.9 was determined using the CA19.9 kit with the suggested value 37 units/ml as positive. Serum CA72-4 antigen levels were determined using the CA72-4 kit with the suggested value 6 units/ml as positive.

Patient	Site of primary	Stage	Site of metastasis	Serum levels			CEA RT-PCR
				CEA (ng/ml)	CA19.9 (units/ml)	CA72-4 (units/ml)	
1	Colon	A		2.9 (−)	8.0 (−)	1.7 (−)	−
2	Rectum	A		1.4 (−)	39.9 (+)	10.0 (+)	+++
3	Rectum	A		0.8 (−)	3.7 (−)	0.1 (−)	−
4	Colon	A		0.6 (−)	25.6 (−)	1.9 (−)	++
5	Colon	A		0.6 (−)	12.2 (−)	2.1 (−)	−
6	Colon	A		0.5 (−)	15.3 (−)	1.4 (−)	−
7	Rectum	B1		3.0 (−)	6.2 (−)	1.8 (−)	++
8	Rectum	B1		2.8 (−)	30.9 (−)	0.7 (−)	++
9	Rectum	B1		2.7 (−)	4.8 (−)	2.0 (−)	−
10	Rectum	B1		2.1 (−)	7.0 (−)	1.9 (−)	−
11	Sigma	B1		11.5 (+)	8.3 (−)	0.9 (−)	+
12	Rectum	B1		0.9 (−)	15.8 (−)	2.2 (−)	−
13	Colon/Rectum	B1[a]		1.7 (−)	8.7 (−)	2.4 (−)	++
14	Colon	B2		77.3 (+)	40.0 (+)	22.9 (+)	++
15	Sigma	B2		3.1 (−)	9.3 (−)	4.5 (−)	+++
16	Rectum	B2		1.8 (−)	7.4 (−)	12.5 (+)	+
17	Rectum	B2		1.7 (−)	7.3 (−)	2.1 (−)	+
18	Rectum	B2		1.4 (−)	4.0 (−)	0.8 (−)	−
19	Rectum	B2		1.1 (−)	8.1 (−)	1.8 (−)	+
20	Colon	B2		0.4 (−)	18.3 (−)	3.4 (−)	−
21	Colon	B2		0.2 (−)	2.2 (−)	3.0 (−)	−
22	Rectum	C1		6.2 (+)	12.7 (−)	1.8 (−)	+
23	Rectum	C1		1.0 (−)	12.1 (−)	2.3 (−)	+
24	Colon	C1		0.5 (−)	8.9 (−)	2.0 (−)	+
25	Sigma	C2		16.1 (+)	0.1 (−)	1.6 (−)	−
26	Colon	C2		5.6 (+)	0.4 (−)	0.4 (−)	++
27	Colon	C2		1.2 (−)	87.1 (+)	18.5 (+)	++
28	Rectum	C2		0.8 (−)	6.2 (−)	2.3 (−)	+
29	Rectum	C2		0.6 (−)	11.8 (−)	2.8 (−)	+++
30	Colon	D	Liver	433.5 (+)	1364.2 (+)	18.9 (+)	+
31	Colon	D	Liver	311.6 (+)	6490.1 (+)	31.3 (+)	+++
32	Colon	D	Liver	257.4 (+)	44.7 (+)	65.3 (+)	+
33	Sigma	D	Liver	139.0 (+)	963.0 (+)	151.9 (+)	−
34	Colon	D	Multiple	126.2 (+)	53.4 (+)	2.6 (−)	+
35	Cecum	D	Liver	20.5 (+)	75.8 (+)	0.7 (−)	+
36	Rectum	D	Peritoneum	20.0 (+)	238.6 (+)	15.5 (+)	+
37	Colon	D	Liver	13.5 (+)	84.6 (+)	22.5 (+)	+++
38	Rectum	D	Liver	10.2 (+)	2.0 (−)	0.1 (−)	+++
39	Colon	D	Peritoneum	8.7 (+)	1.7 (−)	3.8 (−)	+
40	Rectum	D	Lung-Liver	5.0 (+)	43.4 (+)	2.0 (−)	+
41	Rectum	D	Liver	4.7 (−)	25.7 (−)	1.6 (−)	+
42	Colon	D	Liver	4.2 (−)	34.9 (−)	0.1 (−)	+
43	Rectum	D	Liver	2.1 (−)	8.3 (−)	2.8 (−)	−
44	Colon	D	Multiple	1.5 (−)	99.5 (+)	22.7 (+)	−
45	Colon	D	Liver	1.5 (−)	148.3 (+)	10.2 (+)	−
46	Sigma	D	Liver	1.0 (−)	0.4 (−)	0.3 (−)	++
47	Sigma	REC[b]	Locoreg.	278.1 (+)	25.5 (−)	1.9 (−)	+
48	Rectum	REC	Locoreg.	47.4 (+)	124.2 (+)	3.9 (−)	+
49	Rectum	REC	Locoreg.	3.9 (−)	9.8 (−)	2.0 (−)	+
50	Rectum	REC	Locoreg.	3.1 (−)	13.4 (−)	1.9 (−)	−
51	Colon	REC	Locoreg.	0.9 (−)	127.3 (+)	104.4 (+)	+

[a] Synchronous adenocarcinomas of the right colon and rectum.
[b] REC, recurrent; Locoreg, locoregional.

($P2 = 0.11$; see Fig. 2*A*). This trend tended to be more pronounced among patients with stage D or recurrent disease ($P2 = 0.06$ for dichotomous CEA; $P2 = 0.10$ for continuous CEA). Therefore, there appears to be modest evidence of a trend toward an association between RT-PCR and serum CEA, which is true in the entire set of patients examined but appears to be concentrated in patients with stage D and recurrent disease much more so than in patients with earlier stage disease.

Studies were then conducted to compare the detection of CEA mRNA in blood cells by RT-PCR to that of three different colorectal cancer markers, CEA, CA19.9, and CA72-4 (which detects the mucin TAG-72), in sera of patients with various stages of colorectal cancer prior to surgery. The standardized cutoffs for a positive value are 5 ng/ml for CEA, 37 units/ml for CA19.9, and 6 units/ml for CA72-4. As seen in Table 2, of the two patients with stage A colorectal cancer, one (patient 2), who was positive by RT-PCR and negative for serum CEA, was indeed positive for both CA19.9 and CA72-4. On the other hand, the other stage A patient positive for RT-PCR (patient 4) was negative for all three serum tumor markers. Analysis of 15 patients with stage B colorectal cancer (Table 2) revealed that 6 of these patients who were positive by RT-PCR were negative for all three serum markers (Table 3). Analysis of patients with stage C colorectal cancer (Table 2) revealed one patient (patient 27) who was positive by RT-PCR and negative for serum CEA but positive for both CA19.9 and CA72-4. On the other hand, four of the eight patients with stage C colorectal cancer were positive by RT-PCR and negative for all three serum markers (Table 3). Analysis of results of stage D colorectal cancer patients prior to surgery revealed that whereas most patients were positive by RT-PCR and positive for one or more serum markers, three patients (patients 41, 42, and 46) were positive by RT-PCR and negative for all three serum markers. This was also the case for one patient (Table 2, patient 49) of the five with locoregional recurrence. Evaluation of the association between CA19.9 and RT-PCR results indicated that the two measures were, in general, unre-

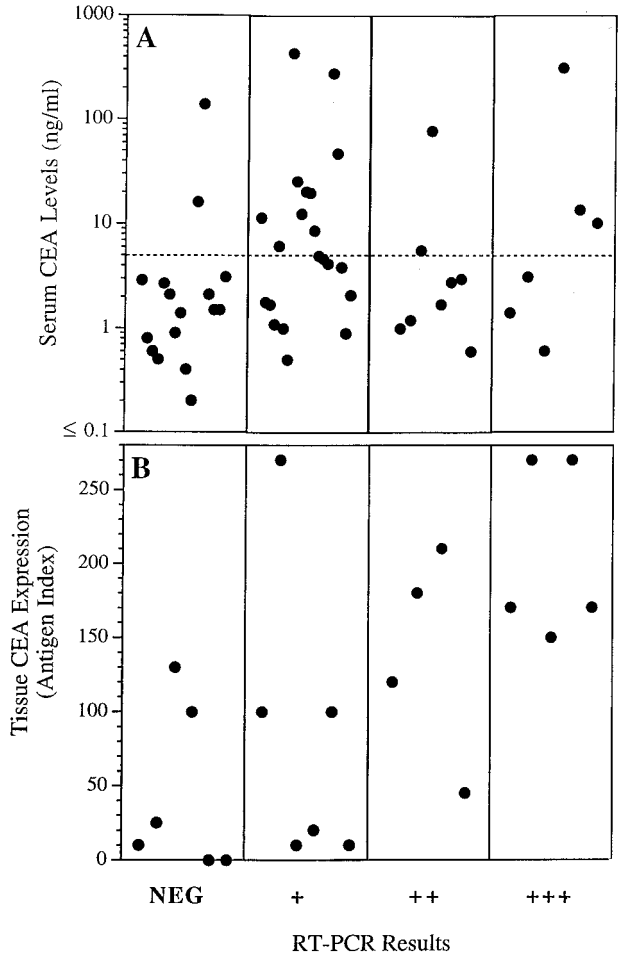

Fig. 2. Relationship between CEA RT-PCR results and CEA serum levels and CEA expression in tumor tissue. *A*, modest overall association between serum CEA levels and RT-PCR analysis for CEA in the blood of colorectal cancer patients. [$P2 = 0.11$, comparing four groups as shown; $P2 = 0.02$ comparing (+) *versus* (−)]. *B*, strong relationship between CEA expression in tumor tissue by immunohistochemistry and RT-PCR analysis for CEA in the blood of colorectal cancer patients ($P2 = 0.0041$). *NEG*, negative.

lated statistically, either overall or by stage. For example, among the 16 patients who were RT-PCR (−), three were CA19.9 (+), whereas 13 of 35 who were RT-PCR (+) were CA19.9 (+) ($P2 = 0.19$). Among patients with stage B or C disease, and separately among patients with stage D or recurrent disease, there was no evidence of association ($P2 = 1.00$ for both stage groupings). Among patients with stage B or C disease, there was, however, a weak trend toward higher CA 19.9 values in patients with a positive RT-PCR result compared with a negative result (median, 8.8 *versus* 4.8; $P2 = 0.13$). With respect to the association between CA72-4 and RT-PCR, there was no evidence of any clear relationship regardless of the type of evaluation used. Results presented in Table 3 show that in stage A, B, and C patients, RT-PCR can detect the probable presence of disease not detected by any of the three serum markers. The use of RT-PCR for the detection of CEA mRNA in conjunction with the three serum markers revealed the probable presence of disease in more patients (39 of 51; 76.5%) than use of the serum markers alone (24 of 51; 47.1%; Table 3). More importantly, among these 15 additional patients in whom use of RT-PCR alone was able to detect disease, 10 were in stages B and C. In these stages, the serum markers alone were able to detect only 7 of 23 cases; thus, in these stages, RT-PCR appeared to be a potentially more useful indicator. In addition, among 27 patients negative for all three serum markers, 15 were positive by RT-PCR. In contrast, of 24 patients positive for at least one marker, only 4 were negative by RT-PCR. Thus, although there was agreement for 32 of 51 cases, when there was disagreement, RT-PCR was a better indicator of disease (in 15 of 19 cases) than the combination of the three markers ($P2 = 0.02$, McNemar's test for paired categorical data).

Studies were then undertaken to determine whether there was a correlation between RT-PCR results detecting CEA-positive blood-borne cells prior to surgery and CEA expression in tumor biopsies from the same patient, using immunohistochemistry. As seen in Table 4, 12 patients with stage A, B, or C colorectal cancer were evaluated by RT-PCR of blood-borne cells and histochemistry of tissue biopsies. Of the four cases that were negative by RT-PCR, two (patients 9 and 12) demonstrated a low percentage of cells positive for CEA in tumor biopsy and a weak staining intensity; two others showed a moderate percentage of CEA-positive cells and staining intensity in the biopsy. More strikingly, however, were the results obtained for stage D colorectal cancer patients and one patient with a locoregional recurrence (Table 4). Two of these nine patients (patients 44 and 45) were negative by RT-PCR for CEA RNA in blood cells. In both cases, the tumors were also negative for CEA expression by immunohistochemistry. Four additional patients were positive but showed low levels of CEA RNA expression in blood cells by RT-PCR analysis. Of these patients, three (patients 30, 32, and 49) showed very weak staining of CEA in tumor biopsies. Overall, in patients with earlier stage disease (A, B, or C), there was a trend toward an increase in the percentage of CEA-positive cells among subjects who were positive compared with those who were negative by CEA RT-PCR (median, 77.5 *versus* 37.5, respectively; $P2 = 0.07$); a similar result was obtained for patients with stage D or recurrent disease (median, 20.0 *versus* 0.0; $P2 = 0.055$). A trend of correlation between the intensity of the band detected by ethidium bromide staining of the CEA RT-PCR product from the blood-borne cells and the expression of CEA protein in tumor biopsy as determined by AI (21) based on immunohistochemical staining for CEA is illustrated in Fig. 2*B* ($P2 = 0.0041$ for the

Table 3 *Detection of CEA-specific mRNA by nested RT-PCR in peripheral blood cells vs. serum marker levels of colorectal cancer patients*

Stage	No. of patients	No. of patients positive for[a]					
		CEA RT-PCR	Serum CEA	Any of the three serum markers[b]	Any of the three serum markers and RT-PCR	CEA RT-PCR and negative by all three serum markers	Any of the three serum markers and/or RT-PCR
A	6	2 (33.3)	0 (0.0)	1 (16.7)	1 (16.7)	1 (16.7)	2 (33.3)
B	15	9 (60.0)	2 (13.3)	3 (20.0)	3 (20.0)	6 (40.0)	9 (60.0)
C	8	7 (87.5)	3 (37.5)	4 (50.0)	3 (37.5)	4 (50.0)	8 (100.0)
D	17	13 (76.5)	11 (64.7)	13 (76.5)	10 (58.8)	3 (17.6)	16 (94.1)
Locoregional recurrence	5	4 (80.0)	2 (40.0)	3 (60.0)	3 (60.0)	1 (20.0)	4 (80.0)
Total	51	35 (68.3)	18 (35.3)	24 (47.1)	20 (39.2)	15 (29.4)	39 (76.5)

[a] Values in parentheses are percentages.
[b] CEA, CA19.9, CA72-4.

Table 4 *Correlation between RT-PCR results and tissue CEA expression in colorectal cancer patients*

Patients diagnosed with	Patient	Site of primary tumor	Stage	Site of metastasis	Serum CEA (ng/ml)	CEA RT-PCR	Tissue CEA expression[a] (%)
Primary nonmetastatic cancer	2	Rectum	A		1.4 (−)	+++	85 (++)
	7	Rectum	B1		3.0 (−)	++	40 (+++)
	8	Rectum	B1		2.8 (−)	++	>90 (++)
	9	Rectum	B1		2.7 (−)	−	10 (+)
	12	Rectum	B1		0.9 (−)	−	25 (+)
	13	Colon & rectum	B1[b]		1.7 (−)	++	70 (+++)
	14	Sigma	B2		2.3 (−)	+++	>90 (+++)
	17	Rectum	B2		1.7 (−)	+	50 (++)
	19	Rectum	B2		1.1 (−)	+	>90 (+++)
	20	Colon	B2		0.4 (−)	−	65 (++)
	21	Colon	B2		0.2 (−)	−	50 (++)
	29	Rectum	C2		0.6 (−)	+++	50 (+++)
Metastatic disease	30	Colon	D	Liver	433.5 (+)	+	10 (+/−)
	31	Colon	D	Liver	311.6 (+)	+++	>90 (+++)
	32	Colon	D	Liver	257.4 (+)	+	20 (+)
	38	Rectum	D	Liver	10.2 (+)	+++	85 (++)
	39	Sigma	D	Peritoneum	8.7 (+)	+	50 (++)
	44	Colon	D	Multiple	1.5 (−)	−	Neg[c]
	45	Colon	D	Liver	1.5 (−)	−	Neg
	46	Sigma	D	Liver	1.0 (−)	++	15 (+++)
	49	Rectum	REC[d]	Locoregional	3.9 (−)	+	10 (+/−)

[a] CEA tissue expression was evaluated by immunohistochemical analysis as described in "Material and Methods."
[b] Synchronous adenocarcinomas of the right colon and the rectum.
[c] Neg, CEA was evaluated only on biopsies obtained from liver metastases (taken for diagnostic purposes).
[d] REC, recurrent.

statistical significance of the association between RT-PCR result and the AI value).

Patients with various stages of colorectal cancer were analyzed, both prior to surgery and at various time intervals after surgery. Longitudinal studies were then conducted by RT-PCR for CEA expression in blood-borne cells and by three different serum markers. Initial studies were conducted with short-term follow-up (*i.e.*, within 39 days postsurgery). Overall, the conclusion from these studies (data not shown) was that spurious results may be obtained from RT-PCR analysis of cells from blood obtained less than 40 days after surgery. For example, three patients who were positive by RT-PCR prior to surgery were also positive postsurgery. Four patients who were negative prior to surgery remained negative by RT-PCR postsurgery. Three patients who were positive prior to surgery became negative by RT-PCR for at least two more time points postsurgery. Although these results were not unexpected, results with spurious points were obtained from five other patients. For three patients, blood-borne cells that were positive for CEA by RT-PCR analysis prior to surgery became negative 24 h postsurgery and then became positive again, according to at least one blood sample. Most disturbing, however, were results obtained for two patients who were negative at surgery and 1 day postsurgery. These patients tested positive once before they became negative at a subsequent time point. Taken together, these results indicate that analysis of blood samples by RT-PCR within 40 days of surgery can lead to spurious and inconclusive results. These findings also underscore the point that conclusions based on one time point should not be considered reliable before they are validated by similar findings by subsequent assays. The same is true when serum protein markers are used for clinical evaluation of cancer, *i.e.*, more than one time point is used. Possible reasons for the spurious results obtained from cells from blood collected early following surgery will be discussed below. Therefore, experiments with blood samples obtained from longer term follow-up were then undertaken.

Four patients with stage A colorectal carcinoma were followed by RT-PCR and serum markers for 122–675 days postsurgery (Table 5). Prior to surgery, patient 2 was positive for CEA by RT-PCR, positive for serum markers CA19.9 and CA72-4, but negative for serum CEA. As shown in Table 5, five longitudinal samples postsurgery were negative for CEA by RT-PCR as well as for all three serum markers. Patient 4, on the other hand, was positive by RT-PCR analysis prior to surgery, as well as at days 47 and 122 postsurgery, but remained negative for all three serum markers throughout the 122-day observation period. Approximately 1 month after the day 122 RT-PCR analysis, this patient was diagnosed with peritoneal recurrence of colorectal carcinoma. The two other stage A patients with colorectal carcinoma were negative for CEA by RT-PCR and for all three serum markers, both prior to surgery and at the several time intervals analyzed postsurgery (Table 5).

Five stage B2 patients with colorectal cancer who did not receive chemotherapy or any other therapy postsurgery were also analyzed longitudinally (Table 5). One patient (patient 19) was positive for CEA by RT-PCR both prior to surgery and 5 days postsurgery, but became negative at days 39 and 126 postsurgery; serum markers were negative pre- and postsurgery. Another patient (patient 17) was also positive prior to surgery and at day 47 postsurgery by RT-PCR, and then became negative at subsequent evaluations. Similarly, patient 15 was positive for CEA by RT-PCR before surgery and at days 41 and 62 postsurgery, whereas four subsequent evaluations between days 90 and 309 postsurgery were negative. These results support the findings outlined above, namely, that analysis of blood cells by RT-PCR at short time intervals postsurgery does not adequately reflect long-term presence of CEA-positive cells in the blood. Patient 20 was negative for CEA by RT-PCR prior to surgery and at all time points postsurgery. Whereas serum CEA values were negative prior to surgery and for 236 days postsurgery, CA19.9 and CA72-4 values were negative prior to surgery and became positive at all time points from 90 days postsurgery. This patient was diagnosed with metastatic peritoneal disease at ~230 days postsurgery. This is clearly an example in which CEA RT-PCR results are negative, whereas serum markers are positive. In contrast are the results obtained with another stage B2 patient (patient 21; Table 5). CEA RT-PCR and all three serum markers were negative prior to surgery. At 108 days postsurgery, all three serum markers remained negative, whereas the CEA RT-PCR analysis of blood cells became strongly positive. At 677 days postsurgery, CEA RT-PCR of blood remained strongly positive, serum CEA and serum CA19.9 values remained negative, and serum CA72-4 became positive. At ~737 days postsurgery, this patient was diagnosed with recurrent pelvic metastases, and CA72-4 values rose to 56.3 units/ml.

Table 5 *Summary of the CEA RT-PCR results on blood samples from stage A and B2 colorectal cancer patients during postsurgical follow-up*

Patient	Stage	Days post surgery	CEA RT-PCR	Serum levels[a] CEA	CA19.9	CA72-4
2	A	−2	+++	1.4 (−)	39.9 (+)	10.0 (+)
		30	−	1.3 (−)	27.3 (−)	2.1 (−)
		206	−	1.6 (−)	28.1 (−)	1.8 (−)
		367	−	1.2 (−)	29.5 (−)	2.2 (−)
		444	−	1.8 (−)	22.7 (−)	2.5 (−)
		675	−	2.2 (−)	28.5 (−)	1.7 (−)
4	A	−37	++	0.6 (−)	25.6 (−)	1.9 (−)
		0	++	2.8 (−)	29.8 (−)	0.7 (−)
		3	+	2.1 (−)	25.6 (−)	1.2 (−)
		47	+	2.2 (−)	26.4 (−)	1.3 (−)
		122	+	2.2 (−)	24.9 (−)	1.3 (−)
5	A	−3	−	0.6 (−)	12.2 (−)	2.1 (−)
		2	−	0.8 (−)	11.3 (−)	1.8 (−)
		4	−	1.2 (−)	6.9 (−)	1.7 (−)
		8	−	0.9 (−)	12.4 (−)	2.3 (−)
		120	−	1.4 (−)	7.9 (−)	2.1 (−)
		240	−	1.1 (−)	9.8 (−)	1.9 (−)
1	A	0	−	2.9 (−)	8.0 (−)	1.7 (−)
		3	−	2.3 (−)	8.1 (−)	1.5 (−)
		31	−	1.8 (−)	9.5 (−)	1.8 (−)
		90	−	1.9 (−)	6.8 (−)	1.6 (−)
		202	−	2.0 (−)	8.1 (−)	1.3 (−)
19	B2	−2	+	1.1 (−)	8.1 (−)	1.8 (−)
		5	+	2.1 (−)	9.9 (−)	2.3 (−)
		39	−	2.4 (−)	6.2 (−)	3.7 (−)
		126	−	3.1 (−)	4.9 (−)	4.2 (−)
17	B2	−1	+	1.7 (−)	7.3 (−)	2.1 (−)
		47	+	0.6 (−)	5.5 (−)	1.6 (−)
		75	−	2.7 (−)	3.5 (−)	1.7 (−)
		103	−	1.0 (−)	5.5 (−)	2.0 (−)
15	B2	−1	+++	3.1 (−)	9.3 (−)	4.5 (−)
		41	+++	2.3 (−)	4.7 (−)	1.9 (−)
		62	+	1.8 (−)	6.7 (−)	2.0 (−)
		90	−	2.6 (−)	8.9 (−)	1.5 (−)
		118	−	1.9 (−)	9.7 (−)	1.7 (−)
		146	−	2.5 (−)	13.6 (−)	2.0 (−)
		309	−	2.0 (−)	12.0 (−)	1.9 (−)
20	B2	0	−	0.4 (−)	18.3 (−)	3.4 (−)
		30	−	1.4 (−)	25.3 (−)	5.2 (−)
		90	−	1.8 (−)	225.4 (+)	7.4 (+)
		120	−	3.0 (−)	525.3 (+)	13.1 (+)
		236	−	2.8 (−)	1368.4 (+)	21.0 (+)
		280	−	10.3 (+)	2466.6 (+)	57.4 (+)
		292	−	15.1 (+)	4273.5 (+)	60.6 (+)
21	B2	−2	−	0.2 (−)	2.2 (−)	3.0 (−)
		108	+++	2.6 (−)	0.1 (−)	1.5 (−)
		677	+++	2.5 (−)	4.1 (−)	7.8 (+)

[a] Serum CEA levels were determined by an enzyme immunoassay test kit, using a value of 5 ng/ml as positive. CA19.9 was determined using the CA19.9 kit with the suggested value 37 units/ml as positive. Serum CA72-4 antigen levels were determined using the CA72-4 kit with the suggested value 6 units/ml as positive.

Four stage D patients with metastatic colorectal carcinoma and one stage C2 patient who received chemotherapy immediately after surgery were also analyzed by RT-PCR for CEA-positive cells in the blood and by three serum markers (Table 6). Two of these patients (patients 26 and 38) were positive prior to surgery and when analyzed postsurgery by both CEA RT-PCR and serum CEA. The stage C2 patient (patient 26) underwent a course of adjuvant chemotherapy after removal of the primary tumor; during the 6 months of treatment, CEA RT-PCR remained positive. Subsequently, the patient developed lung metastases, which were diagnosed on day 420. Two additional patients (patients 31 and 37) were positive by RT-PCR prior to surgery, became negative during chemotherapy, and subsequently became positive. Both of these patients were initially characterized as "responsive" to chemotherapy but were diagnosed with progressive disease. Patient 46, on the other hand, was negative for all three serum makers prior to surgery and at all subsequent samples to day 219 postsurgery. This patient, however, was positive for CEA by RT-PCR prior to surgery and remained positive through the 219-day observation period (Table 6). At this time, however, this patient is defined clinically as a "complete response."

DISCUSSION

The data presented here demonstrate the potential use of RT-PCR to detect CEA-positive cells in the blood of cancer patients using the appropriate primers, assay conditions, and amount of RNA. To our knowledge, this is the first report of longitudinal analyses of patients using CEA RT-PCR, and the first comprehensive analysis comparing RT-PCR values for CEA with those of serum CEA levels or other markers used to monitor colorectal cancer patients at various stages of disease. It should be noted that the term "detection of tumor cells in the blood" was not used in this report. To make such a statement, one would have to isolate the cells scoring positive by RT-PCR for CEA mRNA and demonstrate that those same cells are phenotyped as neoplastic by cytological and/or biological criteria. In two series of analyses, blood from 2 of 60 (3.3%) apparently healthy individuals scored marginally positive for CEA mRNA by RT-PCR. These patients were age- and sex-matched controls for the cancer patients and were part of a cancer screening program. In light of the fact that 18 additional patients with benign gastrointestinal disease (polyps) also scored negative by RT-PCR, 2 of 78 (2.6%) blood samples from "noncancer" patients were considered as positive. Because of the incidence of colorectal cancer and other CEA-positive tumors such as breast cancer (50% are CEA positive) and non-small cell lung cancer (70% are CEA positive), we cannot rule out the possibility that the two marginally positive results from blood cells of apparently healthy individuals are attributable to artifacts in the assay (*i.e.,* false positives) or to the presence of occult tumor cells. Subsequent long-term follow-up studies with large cohorts of patients will be required to answer this question. As shown in Tables 2 and 3, 51 colorectal cancer patients were analyzed by RT-PCR and for serum CEA. Sixty-eight percent of these patients were positive for blood-borne cells expressing CEA mRNA by RT-PCR. Sixteen of these patients were positive by both RT-PCR and serum CEA assay, and 14 were negative by both tests. Whereas two patients were positive for serum CEA and negative by RT-PCR, 19 patients negative for serum CEA were positive for CEA by RT-PCR. This phenomenon was particularly striking, moreover, when the analysis was restricted to patients with stage A, B, or C colorectal cancer only. Of 29 patients studied, 10 were negative by both tests and 4 were positive by both tests. Although only 1 patient in this group was positive for serum CEA and negative by RT-PCR, 14 (48.3%) of these patients were positive for CEA by RT-PCR and negative for serum CEA. The findings may have important implications in the management of patients with no known metastatic disease and who are negative for serum CEA.

These comparisons were extended to include three serum markers (*i.e.,* CEA, CA19.9, and CA72-4) that are used to follow colorectal cancer disease progression (Table 2). Of the 51 patients analyzed, 20 were positive for at least one of three serum markers and by RT-PCR, and 12 were negative for all serum markers and negative by RT-PCR. Four of the 51 patients were negative by RT-PCR and positive for at least one serum marker. However, 15 of the 51 patients (29.4%) were negative for all three serum markers and positive for CEA mRNA in blood-borne cells by RT-PCR. These studies *in toto* indicate that the detection of CEA-expressing blood-borne cells by RT-PCR is potentially different from all three serum markers and, at times, can be advantageous when used in combination with serum markers. The lack of general correlation between the detection of blood-borne cells expressing CEA by RT-PCR and CEA protein in the blood, although varying to some degree by stage of disease, is not surprising in light of the fact that previous studies (9) demonstrated a lack of correlation between serum CEA levels and CEA expression in tumor biopsies from the same patient. Indeed, two phenomena are likely occurring here: the CEA RT-PCR analysis is most likely detecting the presence

Table 6 *Summary of the CEA RT-PCR results on blood samples from colorectal cancer patients undergoing chemotherapy*

Patient	Stage	Site of metastasis	Days post surgery	CEA RT-PCR	Serum levels[a]			Postsurgical treatment
					CEA	CA19.9	CA72-4	
26	C2		−5	++	5.6 (+)	0.4 (−)	0.4 (−)	CHT-RT[b]
			90	+	9.0 (+)	18.4 (−)	1.2 (−)	
			210	+	12.5 (+)	29.3 (−)	1.5 (−)	
		Lung	420	+++	793.8 (+)	2319.0 (+)	0.6 (−)	Lung metastasis
		Lung	483	+++	894.0 (+)	1090.0 (+)	1.8 (−)	
38	D	Multiple	−2	+++	10.2 (+)	2.0 (−)	0.1 (−)	CHT
			36	++	9.4 (+)	0.1 (−)	0.1 (−)	Stable disease
			53	++	16.2 (+)	0.1 (−)	1.8 (−)	Stable disease
			235	++	51.3 (+)	0.1 (−)	2.6 (−)	Stable disease
			319	+++	82.0 (+)	0.8 (−)	2.4 (−)	PD
			530	+++	573.1 (+)	5.3 (−)	3.3 (−)	PD
31	D	Liver	−1	+++	311.6 (+)	6490.1 (+)	1.3 (−)	CHT
			33	−	224.0 (+)	182.9 (+)	2.4 (−)	
			51	−	60.5 (+)	462.0 (+)	2.6 (−)	Responsive
			182	++	226.5 (+)	487.1 (+)	42.6 (+)	PD
37	D	Liver	−1	+++	13.5 (+)	84.6 (+)	22.5 (+)	CHT
			33	−	4.3 (−)	45.3 (+)	8.4 (+)	Responsive
			51	−	4.1 (−)	39.5 (+)	6.9 (+)	Responsive
			182	++	9.5 (+)	76.4 (+)	18.9 (+)	PD
46	D	Liver	−11	+++	1.0 (−)	0.4 (−)	0.3 (−)	CHT
			41	++	1.4 (−)	0.1 (−)	1.4 (−)	Responsive
			56	++	2.2 (−)	0.1 (−)	0.4 (−)	CR
			219	+	1.1 (−)	0.8 (−)	2.1 (−)	CR

[a] Serum CEA levels were determined by an enzyme immunoassay test kit, using a value of 5 ng/ml as positive. CA19.9 was determined using the CA19.9 kit with the suggested value 37 units/ml as positive. Serum CA72-4 antigen levels were determined using the CA72-4 kit with the suggested value 6 units/ml as positive.
[b] CHT-RT, chemotherapy + radiotherapy; PD, progressive disease; CR, complete remission.

of tumor cells in peripheral blood, whereas levels of serum CEA have been shown to generally correlate with tumor mass. The studies reported here confirm and extend the observations of Mori *et al.* (1), who demonstrated a correlation between the stage of disease and the detection of CEA-positive cells by RT-PCR in the blood.

The studies reported here also demonstrate a positive relationship between the detection of CEA-positive blood-borne cells by RT-PCR and CEA expression in tumor biopsy from the same patient by immunohistochemistry (Table 4). It is of interest to note that two of nine patients with metastatic disease were negative by RT-PCR. Analysis of tumor biopsies of both of these patients demonstrated that they were indeed also negative for CEA expression in the tumor. These studies thus indicate that an optimal analysis of blood for the detection of cells may ultimately involve the use of primers for two or more tumor-associated genes. Other studies have used the detection of mRNA of cytokeratin 19 or 20 (28, 29), or MUC-1 (6) toward this goal. Future studies for the detection of these and other genes, in combination with the use of probes for the detection of CEA, will perhaps bear out this point.

The studies reported here also demonstrate that minor changes in methodology can manifest substantial alterations in results. As seen in Table 1, the use of 5 μg of RNA was better than using the smaller amounts that were used in several previous reports (1, 15–18). These findings, as well as a review of the literature, underscore the need for eventual standardization of methodologies and reagents to detect antigen-expressing cells in blood by RT-PCR.

The studies reported here are the first to analyze blood samples longitudinally by RT-PCR for colorectal patients at various stages of disease. Our results indicate that analysis of blood samples within ~1 month of surgery is not reliable in adequately reflecting the long-term prospect for detection or lack of detection of CEA-expressing cells by RT-PCR. This may be attributable to the fact that after surgery, patients receive numerous transfusions that can dilute and mask the detection of CEA-positive cells in the blood. Other factors not understood at this time may also be responsible for this phenomenon. Of interest, however, were the results obtained here from longer term follow-up of patients at different stages of colorectal cancer by both CEA RT-PCR and three serum markers. One of four stage A colorectal cancer patients remained positive for CEA-expressing cells in the blood by RT-PCR up to 122 days postsurgery, but remained negative for all three serum markers (Table 5). This patient was subsequently diagnosed with peritoneal metastases. Also of interest is the analysis of one of five patients with stage B2 colon carcinoma (Table 5, patient 21). Prior to surgery, this patient was negative for CEA by RT-PCR and all three serum markers. At 108 days postsurgery, the patient remained negative for all of the serum markers but was strongly positive for RT-PCR. At day 677 postsurgery, the patient remained strongly positive for CEA-expressing cells in the blood by RT-PCR and negative for serum CEA, but became slightly positive for the CA72-4 serum marker. This patient was also subsequently diagnosed with recurrent pelvic carcinoma; here, the presence of CEA-positive cells in the blood was detected 629 days prior to recurrence of disease. Large randomized prospective studies will be required to define the role of CEA RT-PCR in the management of colorectal cancer. The studies reported here do demonstrate, however, the possibility that longitudinal analyses of blood-borne cells expressing CEA by RT-PCR may be useful for the management of colorectal cancer and other CEA-expressing tumors in terms of prognosis, in the analysis of the response to existing therapies, and perhaps in the analysis of new therapies.

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Detection of Blood-borne Cells in Colorectal Cancer Patients by Nested Reverse Transcription-Polymerase Chain Reaction for Carcinoembryonic Antigen Messenger RNA: Longitudinal Analyses and Demonstration of Its Potential Importance as an Adjunct to Multiple Serum Markers

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