

A Panel of Candidate Tumor Antigens in Colorectal Cancer Revealed by the Serological Selection of a Phage Displayed cDNA Expression Library¹

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In the last few years it has been shown that the humoral immune response in cancer patients is a rich source of putative cancer vaccine candidates. To fully explore the complex information present within the Ab repertoire of cancer patients, we have applied a method, serological Ag selection, to molecularly define tumor Ags recognized by the humoral immune response in colorectal cancer (CRC). First, we built a cDNA display library by cloning a cDNA library from CRC cell line HT-29 for expression as a fusion protein with a filamentous phage minor coat protein, pVI. This cDNA display library was then enriched on pooled sera from CRC patients who had undergone active specific immunization with autologous tumor. We identified a panel of 19 clones reactive with the serum pool. Seventeen of 19 (89%) clones showed reactivity with one or more of the eight Ag-reactive sera, conversely six of eight (75%) sera were reactive with at least one of the 19 clones. Sequencing revealed that these 19 clones represented 13 different Ags. A detailed serological analysis of the 13 different Ags showed preferential reactivity to sera of cancer patients for six different Ags. Four of these Ags displayed increased serum reactivity after the active specific immunization procedure. Furthermore, one of the six Ags, a novel Ag homologous to HSPC218, showed restricted expression in normal testis, suggesting that it belongs to the cancer-testis Ag family. Some of the Ags we have identified may be candidates for tumor vaccination, for sero-diagnosis of cancer, as prognostic markers, or as probes for monitoring tumor cell-based vaccination trials. *The Journal of Immunology*, 2002, 169: 2772–2780.

The isolation of Ags that distinguish normal cells from cancer cells is an important challenge in the field of tumor immunology and immunotherapy. In the 1970s autologous typing was introduced as a stringent approach to the serological identification of human tumor Ags (1, 2). Although the molecular characterization of these Ags was generally beyond reach, this approach provided the first real evidence for the existence of tumor-specific structures with an associated specific Ab response in cancer patients. More recent attempts at isolating new tumor Ags have relied on the isolation of tumor Ag-specific T cell clones through the production of tumor cell lines (3). This dependence on the generation of tumor cell lines has meant most Ags isolated to date are from easy to culture tumor types such as melanoma, e.g., MAGE-1, BAGE, GAGE, MART-1/melan A, gp100, and tyrosinase (3). For many tumors other than melanoma, the identification of unique tumor Ags has been less successful, particularly for solid tumor types of epithelial cell origin such as colo-

rectal cancer (CRC).⁴ The advent of the serological identification of Ags by recombinant cDNA expression cloning (SEREX) procedure (4) has allowed the direct molecular definition of immunogenic tumor proteins in many tumor types (4, 5), including CRC (6), without the need to establish tumor cell lines or cloned tumor cell-reactive T cells. In the original SEREX procedure, a primary tumor cDNA expression library is screened for Ab-reactive clones using autologous serum in a filter screening assay. An interesting finding of SEREX has been that Ags relevant to known autoimmune states have not dominated the procedure. This may be because SEREX is heavily biased toward the identification of Ags that elicit a high titer IgG response and as such immune responses that require T cell help (7). This has led to applications of SEREX in an allogeneic setting using cDNA libraries derived from tumor cell lines (8, 9). These cell line-based cDNA expression libraries have the added advantage that they are not contaminated with IgG transcripts, which can be a serious problem when screening cDNA repertoires prepared from primary tumor material infiltrated with B lymphocytes. Although the SEREX approach has led to the assembly of a database of >1500 Ags (<http://www-ludwig.unil.ch/SEREX/>), it is a labor-intensive procedure. Furthermore, the non-quantitative format of the secondary screening assay on individual sera is prohibitive when performing analysis of large panels of candidate Ags against large panels of individual sera. In searching for an alternative method, we have recently described a novel procedure dubbed serological Ag selection (SAS) (10, 11). This procedure uses repeated cycles of selection and amplification of phage

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⁴Abbreviations used in this paper: CRC, colorectal cancer; ASI, active specific immunization; CT, cancer-testis; pIII, bacteriophage coat protein 3; pVI, bacteriophage coat protein 6; SAS, serological Ag selection; SEREX, serological identification of Ags by recombinant cDNA expression cloning; SLE, systemic lupus erythematosus.

cDNA libraries on patient serum to enrich immunoreactive cDNA products displayed on the surface of phage. Furthermore, display on the surface of phage allows for the potential development of sensitive high throughput binding assays. We sought to explore this method to identify a broad profile of Ags in CRC, which is one of the most difficult cancers to immunologically characterize.

Phage display has developed as a powerful technique to select ligands to essentially any chosen target from diverse repertoires of peptides, proteins, or Abs displayed on the surface of a phage particle (12). Previous applications have mainly focused on Ab libraries (13, 14) and constrained or linear peptide libraries (15). This technology has also been used to identify immunogenic targets or epitopes recognized by Abs; for example, selection of peptide libraries on mAbs and complex sera of patients with disease has led to the isolation of immunoreactive peptide epitopes (16–19). Although mapping sera with peptide phage libraries has had some success with small viral genomes such as hepatitis B (16), the selection of peptide repertoires appears not to be a suitable approach when profiling the complexity of the immune response in the context of the human genome. One of the drawbacks is that often predominantly Ag mimotopes are recovered, for which further complicated analysis is required to recover the original Ag that elicited the immune response (16, 19). A more successful approach is to display cDNA expression libraries on filamentous phage (18). In contrast to the use of peptide display repertoires, there have been significantly fewer reports on cDNA display due to technical challenges in their construction and expression. For example, due to stop codons inherent to cDNA, cDNA display libraries cannot be fused to the N terminus of the popular phage anchor protein pIII. As such, anchoring of the cDNA product on the phage coat has to be performed via indirect linkage to pIII (20, 21) or by direct fusion to the C terminus of another phage coat protein, pVI (10, 22). Early reports on the selection of phage-displayed cDNA libraries with patient sera were based on either high titer IgE allergic response to the fungus *Asperigillus fumigata* in affected individuals (20, 21) or high titer IgG response in patients afflicted with the chronic autoimmune disease, Sjogren's syndrome (23). In the case of low titer IgG responses that predominate in immunosuppressed conditions such as cancer, our early studies using autologous selection of phage-displayed primary tumor cDNA repertoires were unsuccessful, although selections with both mAbs and polyclonal rabbit sera did recover specific ligands (10, 11). Selections performed with immobilized autologous cancer patient IgG using an anti-human IgG capture Ab resulted in the preferential recovery of only IgG transcripts that were present due to tumor-infiltrating B cells. Here we report the use of an optimized SAS procedure, in which we have successfully established methods for enrichment of particular cDNA phage clones from phage cDNA libraries displayed on minor coat protein pVI.

We have applied SAS to CRC, as this tumor type remains a very difficult cancer to immunologically characterize from a molecular perspective and as such was predicted to benefit from the very sensitive and powerful enrichment possible with phage cDNA selection. We have also used CRC sera from patients who have undergone active specific immunization (ASI). ASI involves the removal of autologous tumors, irradiation of the tumor cells, and readministration to patients. This approach has been shown to give a significant longer recurrence-free period and 61% risk reduction for recurrences in stage II CRC patients (24). Our motivation for using sera from immunized patients was that these patients may be expected to have a higher titer of tumor-specific IgGs, which may allow for more powerful selection of tumor Ags. We report here the isolation of a panel of tumor Ags in CRC and the detailed serological characterization of these Ags using a quantitative

phage ELISA. To investigate whether the immunogenicity of these tumor Ags increased during ASI, we compared serum reactivity in the same patient before and after vaccination.

Materials and Methods

Cloning of a HT-29 tumor cell line cDNA expression library into pVI phage display vectors

A custom λ HT-29 adenocarcinoma cell line library (2.0×10^6 primary recombinants, unidirectionally cloned into the UniZAP XR vector with *EcoRI* and *XhoI* cloning sites; Stratagene, La Jolla, CA) was mass excised according to the manufacturer's instructions, resulting in a library size of 3×10^8 CFU. Plasmid DNA was prepared from the excised library and further digested with *BamHI* and *KpnI*. DNA fragments were gel-purified (Qiagen, Valencia, CA) and ligated into the three vectors, pSP6A, pSP6B, and pSP6C (10), digested with *BglIII* and *KpnI*. The ligation mixture was used to transform *Escherichia coli* TG1 cells by electroporation to obtain libraries pSP6A-HT-29, pSP6B-HT-29, and pSP6C-HT-29, respectively. cDNA inserts were amplified with the oligonucleotide primers 12 (5'-AGC GGA TAA CAA TTT CAC ACA GG-3') and 195 (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'), with insert sizes ranging from 500 bp to 2.5 kb.

Serum samples

Sera were obtained from 60 CRC patients (31 men and 29 women; age, 26–86 years) after surgical resection. This patient group includes serum samples from 30 CRC patients (16 men and 14 women; age, 26–80 years) who had undergone active specific immunization (ASI) with autologous tumor cell preparations (24). In addition, a gender/age-matched control group of 60 healthy individuals was collected, sera from 30 patients with autoimmune diseases, of which 10 sera were from patients with inflammatory bowel diseases (five men and five women; age, 23–72 years) and 20 sera were from patients with systemic lupus erythematosus (SLE; one man and 19 women; age, 19–79 years). Serum samples were stored at -20°C after collection.

Sera used for the selection procedure (1/10 diluted in $1 \times$ TBS (50 mM Tris-HCl (pH 7.9) and 150 mM NaCl)) were adsorbed by repeated passage through columns of Sepharose 6 MB (Pharmacia, Uppsala, Sweden) coupled to lysates of *E. coli* Y1090 and bacteriophage-infected *E. coli* XL1-Blue to remove Abs reactive with Ags related to the phage-host infection. Following adsorption steps, final serum dilutions (1/10) were prepared in 0.2% (w/v) skimmed milk powder in TBS (MPBS) and stored at -20°C .

Serological Ag selection of phage pVI-displayed cDNA repertoires

Serum samples from 10 CRC patients who had undergone one cycle of ASI (four men and six women; age, 26–76 years; five Dukes stage II and five Dukes stage III) were pooled and used for affinity selections as described previously (10, 11) with slight adaptations. In brief, an immunotube (Nunc, Roskilde, Denmark) was coated with rabbit anti-human IgG (Dako, Glostrup, Denmark) at a concentration of $10 \mu\text{g/ml}$ in coating buffer (0.1 M sodium hydrogen carbonate, pH 9.6) for 2 h at 37°C . After washing the immunotube twice with PBS/Tween 20 (PBST: 50 mM Tris, 150 mM NaCl (pH 7.5), and 0.1% Tween 20 (w/v)) and twice with PBS, the tube was blocked for 2 h at room temperature with 2% MPBS. Phage were prepared from libraries pSP6A-HT-29, pSP6B-HT-29, and pSP6C-HT-29 as described previously (25). Approximately equal numbers of each phage library ($\sim 3 \times 10^{12}$ CFU in $500 \mu\text{l}$ of PBS) were added to $500 \mu\text{l}$ of 4% MPBS and 1 ml of pooled serum (1/100 diluted in 4% MPBS). This mixture was incubated in a glass tube to avoid binding of phage to the tube for 1.5 h at room temperature on a rotating platform. The coated immunotube was washed twice with PBST and twice with PBS, and the preincubated serum plus phage mix was added and incubated for 30 min on a rotating platform, followed by 120 min of standing at room temperature. The tube was then washed 20 times with PBST and 20 times with PBS. Phage were eluted with 1 ml 100 mM triethylamine for 10 min on an end-over-end rotating device and were further neutralized with 0.5 ml 1 M Tris-HCl (pH 7.4) by mixing with inversion. Input and output phage were titrated on $2 \times$ TY agar plates containing ampicillin and glucose (16 g/L bacto-tryptone, 10 g/L yeast extract, 5 g/L NaCl, 15 g/L bacto-agar, 100 $\mu\text{g/ml}$ ampicillin, and 2% (w/v) glucose) at each round of selection to monitor enrichment of specific clones. The ratio of output/input phage was determined. After five rounds of selection, individual clones were selected, and the insert size was determined with primers 306 (5'-CTC TCT GTA AAG GCT GC-3') and 195, which annealed at either end of the cDNA insert.

Plasmid DNA was isolated from clones of fifth round of selection and sequenced by Greenomics (Wageningen, The Netherlands) using an ABI automated DNA sequencer (PerkinElmer, Norwalk, CT). Sequences were submitted to GenBank for BLAST homology search.

ELISA of cDNA displaying phage

Ninety-six-well, flat-bottom microtiter plates (Falcon, Franklin Lakes, NJ) were coated overnight at 4°C with 200 μ l rabbit anti-human IgG (Dako; 10 μ g/ml) in coating buffer and blocked with 200 μ l 2% MPBS for 1 h at room temperature. For the primary ELISA screening, the pool of 10 human CRC sera was diluted 1/100 in 4% MPBS (each individual serum was diluted 1/1000), and 50 μ l diluted serum was used for preincubation with 100 μ l crude phage supernatant in a 96-well, round-bottom plate (Costar, Corning, NY). Other serum dilutions were also tested, resulting in lower signals (data not shown). Phage particles were incubated with patient serum for 1 h at 37°C, followed by 30 min of shaking at room temperature. After washing three times with PBST and three times with PBS, the preincubated serum plus phage mixture was transferred to the plate coated with rabbit anti-human IgG for 1 h at 37°C, followed by shaking at room temperature for 30 min. After washing, 150 μ l of a peroxidase-conjugated anti-phage (anti-M13) mAb (Amersham/Pharmacia/Biotech, Uppsala, Sweden), diluted 1/5000 in 2% MPBS was incubated for 1 h shaking at room temperature. After washing the plates three times with PBST and three times with PBS, 130 μ l 3,3',5,5'-tetramethyl-benzidine dihydrochloride chromogen solution (10 mg/ml) was added, and color development was stopped with 65 μ l/well 2 N H₂SO₄. The plates were read at 450 nm in a Novapath microplate reader (Bio-Rad Laboratories, Hercules, CA).

Secondary ELISA on individual patient sera was performed using polyethylene glycol-precipitated phage particles (10¹⁰ phage/well), prepared as previously described (25), preincubated with a 1/100 dilution of individual patient serum. Both *E. coli* extract-adsorbed and nonadsorbed patient sera were tested in ELISA experiments. However, adsorption of sera against bacterial and phage-related proteins was not necessary, as no differences in background signals were seen between adsorbed and nonadsorbed sera.

Northern blot analysis

Northern blot analysis was performed as described previously (26).

Results

Selection of a phage-displayed tumor cell line cDNA expression library with patient serum

A cDNA library from a CRC cell line, HT-29, was cloned into the pVI phage display vectors pSP6A, -B, and -C (10) in three reading frames to give a total library size of 1.0×10^8 . These vectors had previously been validated by the display on the filamentous phage minor coat protein pVI of primary tumor cDNA repertoires and successful selection with both homogeneous ligands, such as a mAb to β_2 -microglobulin, and also heterogeneous ligands, such as polyclonal anti-human IgG (10). The insert sizes in our phage-displayed tumor cell line cDNA repertoire ranged from 650 to 1800 bp.

The SAS procedure is shown in Fig. 1. Equal volumes of phage from each of the three libraries were used for selections with equal volumes of each of 10 pooled sera from randomly selected patients

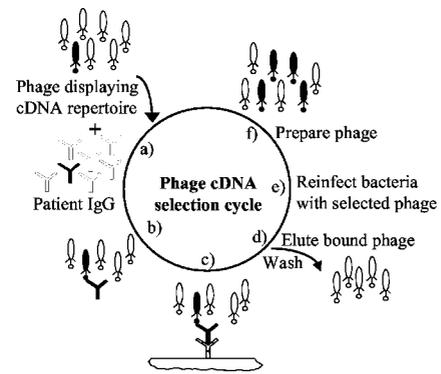


FIGURE 1. SAS procedure. *a*, A phage-displayed tumor cDNA repertoire is preincubated with patient serum Abs. *b*, Tumor-specific Ags displayed on phage (black) bind to tumor Ag-specific patient IgG (black). *c*, Phage Ag-IgG complexes (black) are captured on a surface coated with polyclonal anti-human IgG (checked). *d*, Nonrelevant phage are washed away, and serum IgG-specific phage are eluted. *e*, Selected phage are used for reinfection of bacteria. *f*, Selected phage are amplified and used for further rounds of selection.

admitted for one cycle of ASI (24). We chose to pool sera for the selection process, because previous SEREX analysis had shown that the frequency of serum reactivity to any given tumor Ag is low, ranging from 10–30% (5, 6). After five rounds of selection, the ratio of the output phage titer to the input phage titer increased substantially, reflecting the enrichment of specific phage clones (Table I). Fingerprint analysis showed that several clones had been selected multiple times, which was again indicative of selective enrichment of serum reactive clones (data not shown). The range of insert sizes did not change significantly during selection (insert sizes from the fifth round of selection ranged from 600–1600 bp), suggesting that there was no apparent negative selection on the basis of size constraints of the cDNAs.

ELISA screening of Ags selected on CRC patient sera pool with sera used for selection

The efficiency of selection was monitored during the selection as an increase in the percentage of phage clones reactive in ELISA with the serum pool used for the selection (Table I). Initial efforts to develop an ELISA screening procedure were based on either phage binding to a precaptured serum IgG surface or by direct coating of phage onto a solid surface, followed by probing with patient serum (10, 11); in both cases the ELISA format was unsatisfactory. We subsequently optimized an ELISA procedure involving the postbinding capture of phage-serum IgG complexes onto a surface coated with rabbit anti-human IgG. This phage

Table I. Selection of pVI phage-displayed CRC cDNA library on CRC patient sera

Round	Input ^a	Output ^a	Ratio (O/I)	Primary ELISA ^b	Secondary ELISA ^c
1	7.3×10^{13}	2.6×10^6	3.6×10^{-8}	0/19 (0)	ND
2	1.0×10^{13}	9.8×10^6	9.8×10^{-7}	0/19 (0)	ND
3	1.4×10^{13}	2.0×10^7	1.4×10^{-6}	2/19 (11)	ND
4	7.2×10^{12}	4.0×10^7	5.6×10^{-6}	4/19 (21)	ND
5	4.0×10^{12}	7.4×10^6	1.9×10^{-6}	8/19 (42)	19/19 (100)

^a The titration of input (I) and output (O) phage is presented as CFU per milliliter.

^b The binding of pooled CRC patient sera to selected clones was detected in a primary phage ELISA using crude phage supernatant. The number of positive clones of the total number of clones tested is shown, and the percentage of positive clones is indicated in parentheses.

^c The binding of individual CRC patient sera to selected clones in round 5 was detected in a secondary phage ELISA using purified phage. The number of positive clones of the total number of clones tested is shown, and the percentage of positive clones is indicated in parentheses. ND, not determined.

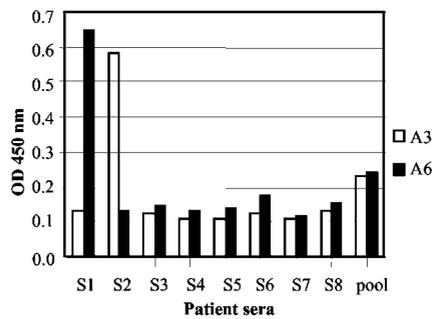


FIGURE 2. Reactivity of two selected Ags on individual and pooled CRC patient sera. The ODs of eight individual patient sera used for selection and serum pool to A3 and A6 are indicated. Average values (OD 450 nm) from two independent experiments are shown. Both clones A3 and A6 were negative on 10 normal sera (data not shown).

ELISA format was suitable for the detection of phage in crude culture supernatant, and it was used as the primary screen for serum-reactive phage clones. The use of purified phage resulted in a more sensitive assay giving a higher percentage of positive clones (Table I), and as such we used purified phage for all secondary screening.

The percentage of reactive clones in ELISA was shown to increase during five rounds of selection on CRC sera (Table I). Positive clones could be detected after the third round of selection, which increased to 42% (8 of 19) by primary ELISA and 100% (19 of 19) by secondary ELISA in the fifth round. Screening of the 10 individual CRC sera for specificity to the selected phage clones showed two sera with Ag-independent reactivity. These were termed phage-reactive sera and were excluded from further analysis. Fig. 2 represents a secondary ELISA screen of the immunoreactivity of the eight remaining individual immunized sera and the serum pool tested to phage clones A3 and A6. Reactivity to both clones A3 and A6 was found in one of 8 (12.5%) individual sera tested, while both clones were negative on 10 normal (non-age-matched) sera tested (data not shown). The signal on individ-

ual sera was much higher than that on pooled sera, which was consistent with the dilution of the Ag-specific Igs in the pooled sera.

There was an excellent association between the selected Ags and reactivity to the individual sera of the serum pool used for selection. Of the phage clones retrieved after five rounds of selection 17 of 19 (89%) showed reactivity to one or more of the sera used in the selection. Conversely, six of eight (75%) of reactive sera were positive with at least one of the 19 phage clones (Table II). We could not detect any reactivity for clones B4 and B5 in the remaining eight CRC patient sera after exclusion of the two phage-reactive patient sera.

Sequence analysis of Ags selected on CRC sera

Sequence analysis revealed that the 19 selected phage clones represented 13 different Ags, which we annotated with the name AMCOL-number, which is short for Amsterdam-Maastricht-Colon, following a similar nomenclature used for clones identified with SEREX. In Table III are listed the five sequences that are homologous to known gene products. Table III shows selected clones that have as yet no homology to any functionally annotated sequences in the public databases. The sizes of the putative serum reactive epitopes ranged from 6–175 aa. All five clones representing annotated gene products were in the correct orientation and reading frame for display on phage. The serum reactivity was located to a gene fragment of the parental protein (Fig. 3).

Detailed serological analysis of the CRC Ag panel

To determine whether the cDNA clones from selection on pooled CRC sera showed cancer-related immunoreactivity, sera from 30 CRC patients admitted to the ASI program (24) (stage II, *n* = 19; stage III, *n* = 11) and from 30 CRC patients (stage I, *n* = 5; stage II, *n* = 13; stage III, *n* = 11; stage IV, *n* = 1) were tested against the antigenic panel with a phage secondary screening ELISA. An age/gender-matched control group of 60 healthy individuals plus a group of sera from autoimmune patients composed of 10 patients with inflammatory bowel diseases and 20 patients with SLE were

Table II. Reactivity of panel of 19 phage clones on individual CRC sera used for the selection campaign^a

Phage cDNA Clones	Serum								Number of Reactive Sera
	S1	S2	S3	S4	S5	S6	S7	S8	
A3	-	+	-	-	-	-	-	-	1/8
A4	-	+	-	-	-	-	-	-	1/8
A5	-	+	-	-	-	-	-	-	1/8
A6	+	-	-	-	-	-	-	-	1/8
A7	-	-	-	+	-	-	-	-	1/8
A8	-	-	-	+	+	-	-	-	2/8
A9	-	+	-	-	-	-	-	-	1/8
A10	-	-	+	+	-	-	-	-	2/8
A11	-	-	-	+	-	-	-	-	1/8
B1	-	-	-	+	-	-	-	-	1/8
B2	-	-	-	+	-	-	-	-	1/8
B3	-	-	+	+	-	-	-	-	2/8
B4	-	-	-	-	-	-	-	-	0/8
B5	-	-	-	-	-	-	-	-	0/8
B6	-	-	+	+	-	-	-	-	2/8
B7	-	-	-	+	+	-	-	-	2/8
B8	-	-	+	+	-	-	-	-	2/8
B10	-	-	-	-	-	+	-	-	1/8
B11	-	-	-	-	-	+	-	-	1/8
Number of positive phage cDNA clones	1/19	4/19	4/19	10/19	2/19	2/19	0/19	0/19	

^a +, positive ELISA signal at OD₄₅₀ (>2× background); -, negative ELISA signal at OD₄₅₀ (<2× background).

Table III. Sequence analysis of Ags retrieved by the selection of phage displayed CRC cDNA expression library on patient sera

Name	Clone No.	No. of Clones ^a	GenBank No.	Identity	Vector pSP ^b	Correct Reading Frame	Fusion Junction	Size
Ags with a functional annotation								
AM-COL-1	A3/A4	2	X99209.1	Arginine methyltransferase	A	Y	SKPKYNHILK	148
AM-COL-2	A6	1	J00277.1	c-Ha-ras 1 protooncogene	B	Y	EDIHQYREQI	99
AM-COL-3	A7/B2	2	AF151052.1	HSPC218 mRNA	B	Y	EEHEWFIPAR	59
AM-COL-4	A8/B7	2	X69150.1	Ribosomal protein S18	A	Y	DGRRKIAFAI	132
AM-COL-5	B11	1	M84711	v-Fos transformation effector protein	C	Y	DVQGKNCLTN	175
Ags with no functional annotation								
AM-COL-6	A5	1	AC012064.2		B		ICLQLHLSME	18
AM-COL-7	A9	1	AC012586.6		C		RESITTF*	7
AM-COL-8	A10/B3/B6/B8	4	AL133444.2		C		SVNIWASPKT	115
AM-COL-9	A11	1	AC068738.2		A		GLCEKTKSTS	15
AM-COL-10	B1	1	AC020894		C		DTSPGKMVI	11
AM-COL-11	B4	1	BF002749.1		A		VYFKTHTQCQ	23
AM-COL-12	B5	1	AI379862.1		C		RKLLNL*	6
AM-COL-13	B10	1	AL162074.1		C		RGIEGREGITQ	12

^a Number of times clone recovered.

^b Reading frame of vector (10).

^c Size of protein product in amino acids fused to pVI coat protein. *, Stop codon.

used. For all Ags that were retrieved multiple times (A3/A4, A7/B2, A8/B7, and A10/B3/B6/B8), we obtained identical serological profiles (Table II). This clearly demonstrates the consistency and robustness of the phage ELISA screening test.

Of the 13 different Ags screened, 6 Ags reacted either exclusively or preferentially with sera from CRC patients (Table IV). AM-COL-4 (ribosomal protein S18) gave a significantly higher frequency of reactivity with serum from CRC patients ($n = 4/58$) compared with the frequency of reactivity with sera from normal patients ($n = 1/60$), and as such this clone was defined as having a cancer-related serological profile (Table IV). AM-COL3 (HSPC218) and AM-COL8 (A10) reacted exclusively with CRC sera, and AM-COL-5 (v-Fos transformation effector protein) reacted exclusively with CRC and SLE sera. The two unknown se-

quences (AM-COL-9 and AM-COL-10) reacted only with the patient sera used in the selection. Although in some cases we did find reactivity in SLE patients, this was not taken as an exclusion criterion for further analysis, as there is a growing list of normal self proteins that are targets for CTL reactivity in cancer patients (27, 28). The remaining seven Ags reacted with a subset of sera from both healthy individuals and CRC patients and showed no clear tumor-related immunogenicity (Table V).

In total, 8 of 58 (14%) CRC patients had serum IgG Abs reactive with at least one of the panel of 6 Ags with a tumor-related immunogenicity (Table IV). Of these patients five had classified stage II disease, and three had classified stage III disease. Furthermore, 2 of 58 (3%) of these patients, with classified stage II disease, had Abs to 2 or more of this Ag panel. The highest frequency of Ab responses (4 of 58) in CRC sera was found to AM-COL-4 (ribosomal protein S18), which is a protein known to be overexpressed in cancer (29).

To evaluate the possible relatedness of serum reactivity to the ASI vaccination event in some of the patients, a comparative secondary ELISA screen was performed using matched individual pre- and postimmunization sera. We observed higher titer Ag reactivity for four different Ags in four postimmunization sera compared with matched preimmunization sera (Fig. 4). Quantitative differences did not appear to be due to variations in total serum IgG, because all sera tested showed equivalent total serum IgG levels pre- and postvaccination.

Expression pattern of novel tumor Ags

Northern blot analysis of the novel candidate tumor Ags with a cancer-related serological profile on a variety of normal human tissues was performed. AM-COL-4 (ribosomal protein S18) was found to be universally expressed in the tissues tested and gave a transcript of the expected size (Fig. 5). AM-COL-10 gave a transcript of 0.4 kb and showed a restricted expression pattern, with some expression in spleen and, to a lesser extent, in testis. AM-COL-3 (HSPC218) was only expressed in testis and ovarian tissue and gave a dominant transcript of 1.4 kb and a smaller product of 1.09 kb. A transcript of the expected size of 0.8 kb for AM-COL-5 (v-Fte-1) was preferentially expressed in ovarian tissue, with some

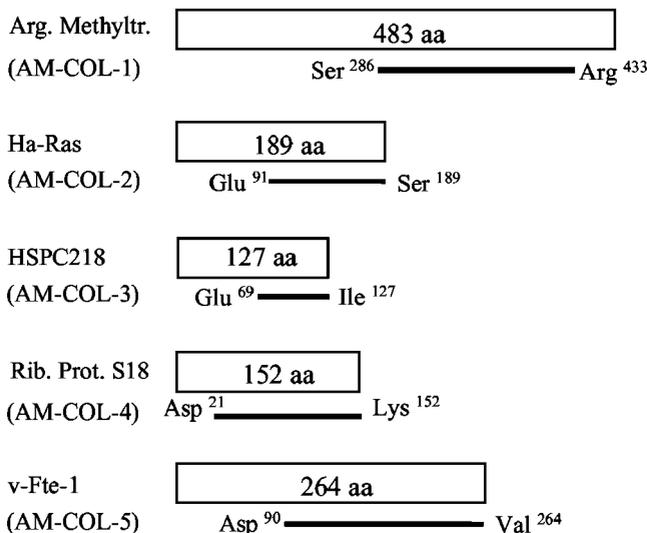


FIGURE 3. Localization of serum reactivity for Ags homologous to known proteins. Rectangles indicate full coding sequences of selected protein products, and the length is given in amino acids. The identity of the protein is given on the left, with the Ag name in parentheses. The selected protein fragments are indicated by a line. The residue numbers defining the phage-displayed protein product are indicated.

Table IV. *Ags with a CRC-related serological profile*

Name	Homology	Sera					
		Normal	Immunized selection ^a	Immunized nonselection ^b	Nonimmunized CRC ^c	IBD ^d	SLE ^e
AM-COL-3	HSPC218	0/60	1/8	0/20	1/30	0/10	0/18
AM-COL-4	Ribosomal protein S18	1/60	2/8	2/20	0/30	0/10	0/18
AM-COL-5	v-Fte-1	0/60	1/8	0/20	1/30	0/10	1/18
AM-COL-8	Est	0/60	2/8	0/20	1/30	0/10	0/18
AM-COL-9	Est	0/60	1/8	0/20	0/30	0/10	0/18
AM-COL-10	Est	0/60	1/8	0/20	0/30	0/10	0/18

^a Individual Ag-reactive sera from ASI patients used in the selection campaign.

^b Individual sera from ASI patients not used in the selection campaign.

^c Individual sera from nonimmunized CRC patients.

^d Sera from patients with inflammatory bowel diseases.

^e Sera from patients with SLE.

expression in spleen. No transcript could be detected for Ags AM-COL-8 and AM-COL-9.

Discussion

The present study demonstrates that SAS is an efficient technique for molecular Ag profiling of the humoral immune response in CRC. We report the identification of a panel of 13 different Ags, of which five showed homology to known gene products (none of which is currently present in the SEREX database) and eight of which represent novel gene products. Four functionally defined gene products have a known or suspected etiological association with cancer, i.e., arginine methyltransferase (AM-COL-1) (30), c-Ha-Ras-1 (AM-COL-2) (31), ribosomal protein S18 (AM-COL-4) (29), and v-Fos transformation effector protein (AM-COL-5), also known as S3a ribosomal protein or TNF- α -induced TU-11 gene (32, 33). The isolation of a high proportion of cancer-associated genes by SAS is in contrast to SEREX analysis, in which the majority of targets have no known or suspected association with cancer (6). This finding may be explained by the powerful repeated cycles of selective enrichment possible with phage cDNA selection compared with the single step screening possible with SEREX. In SAS the repeated cycles of selection and amplification may bias for Ags with the highest titer IgG responses, which, in turn, could be induced by aberrant gene expression, a phenomenon closely associated with the cancer phenotype.

Four of the five selected Ags with defined open reading frame (c-Ha-Ras-1, arginine methyltransferase, ribosomal protein S18, and v-Fte-1) are all predicted to be intracellular products. This finding is in agreement with reports using the SEREX procedure,

where the majority of Ags are also intracellular, and their probable release by necrosis or cell lysis at the tumor site is an initiating factor in eliciting an immune response (34). Nevertheless, the panel of selected cDNAs recovered by either SEREX or SAS may be subject to different constraints or biases peculiar to each of the protein expression/display methods.

With the rapid increase in the number of serologically defined Ags, the challenge is to choose the most promising candidates for further analysis, and cancer-related or restricted serum reactivity is the most important criteria. Our initial ELISA screening assays (10, 11), using the methods described for IgG responses to hepatitis B (16) and for allergic IgE responses (35) to *Asperigillus fumigatus*, were not sufficiently sensitive for evaluating low titer IgG responses in CRC patients. As such we have developed robust phage ELISA screening assays using both crude phage preparations (which may be amenable to automation) and also a secondary phage ELISA screen using purified phage as a more sensitive serological test. The use of a high throughput quantitative serological screening test was proven to be crucial for analyzing the cancer-associated immunogenicity of the selected Ags.

To determine which of our panel of 13 Ags showed a tumor-related immunogenicity, we analyzed the frequency of the Ab responses in a large panel of sera from diseased and healthy individuals. Six of 13 Ags did show a tumor-related immunogenicity and for 5 Ags no reactivity was seen with 60 age-matched normal sera. The finding that 8 of 58 (14%) sera from CRC patients showed reactivity to at least one of these 6 Ags indicates the low diagnostic potential of the Ag panel. Overall, this frequency of serum reactivity is lower than that found by SEREX analysis of

Table V. *Ags with a non-cancer-related serological profile*

Name	Homology	Sera					
		Normal	Immunized selection ^a	Immunized nonselection ^b	Immunized nonimmunized CRC ^c	IBD ^d	SLE ^e
AM-COL-1	Arginine methyltransferase	3/60	1/8	0/20	1/30	1/10	0/18
AM-COL-2	c-Ha-ras 1	2/60	1/8	0/20	1/30	0/10	0/18
AM-COL-6	Est	9/60	1/8	1/20	6/30	2/10	0/18
AM-COL-7	Est	2/60	1/8	1/20	0/30	0/10	1/18
AM-COL-11	Est	3/60	0/8	1/20	0/30	0/10	0/18
AM-COL-12	Est	2/60	0/8	1/20	1/30	1/10	0/18
AM-COL-13	Est	2/60	1/8	0/20	1/30	0/10	0/18

^a Individual Ag-reactive sera from ASI patients used in the selection campaign.

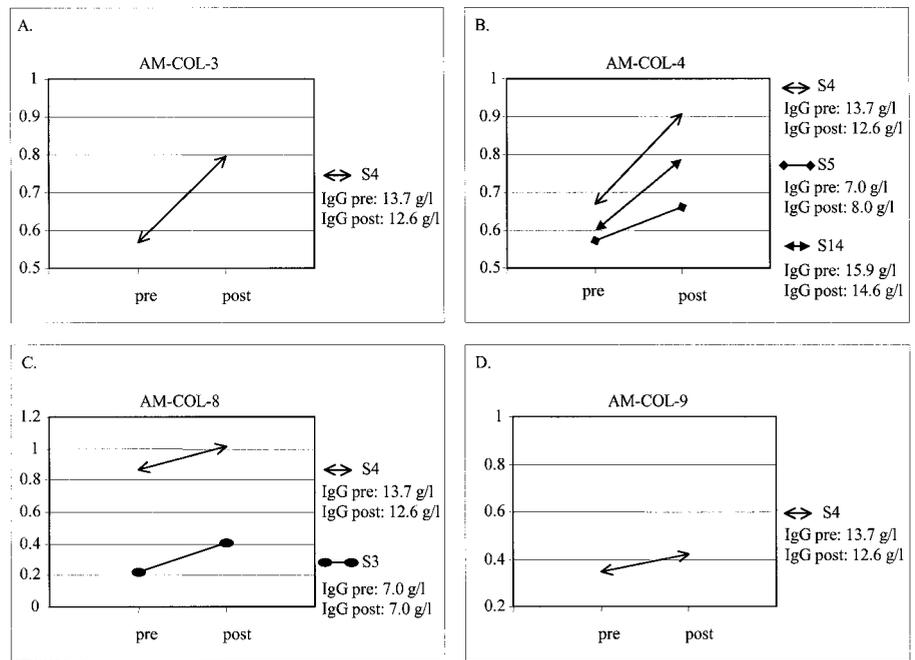
^b Individual sera from ASI patients not used in the selection campaign.

^c Individual sera from nonimmunized CRC patients.

^d Sera from patients with inflammatory bowel diseases.

^e Sera from patients with SLE.

FIGURE 4. Reactivity of pre- and postimmunization tumor sera to selected Ags. Sera were obtained from ASI patients before (pre) and after (post) immunization. The serum reactivity is shown for AM-COL-3 (a), AM-COL-4 (b), AM-COL-8 (c), and AM-COL-9 (d). Average values (OD₄₅₀) from two independent experiments are shown. The threshold value is set when OD values exceed twice the background values from controls. The serum IgG concentration (pre and post) for each serum is shown on the right of each panel.



CRC (6) and renal cell carcinoma (5), which show serum reactivities of 14–27 and 5–25%, respectively. The remaining seven Ags were recognized by IgG Abs in both sera from CRC patients and healthy individuals. This is presumptive, but not conclusive, evidence that the immune response to these Ags is not related to cancer. Indeed, c-Ha-Ras-1, which is a member of the Ras family and is frequently mutated in human cancer and to which tumor-related CD4⁺, CD8⁺, and B cell immune responses have been reported (36–38), was placed in this group.

There are several perceived mechanisms by which tumor Ags can elicit an immune response in the autologous host. These include gene activation, gene overexpression, viral gene expression, gene mutation, abnormal post-translational modifications, or incorrect processing and presentation to the immune system. However, in many cases it is not easy to predict the mechanism of immunogenicity without further study. Gene activation is a possible mechanism of immunogenicity for the novel Ag HSPC218 (AM-COL-3). The preferential expression of the AM-COL-3 in testis and not in normal tissues, including colon, suggests that it is a member of the cancer-testis (CT) family of Ags (8). Other Ags

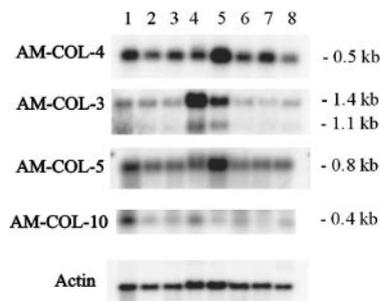


FIGURE 5. Expression profile of Ags with a tumor related serological profile in normal tissues. Expression patterns are shown for AM-COL-4 (ribosomal protein S18), AM-COL-3 (HSPC18), AM-COL-5 (v-Fte-1), and AM-COL-10 (Est). The lower panel shows a control hybridization experiment with an actin probe. Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, peripheral blood lymphocytes.

that belong to this family are MAGE (39), BAGE (40), and GAGE (41), which were initially defined as targets for T cells. Later studies using SEREX, which in addition to rediscovering the Ags listed above has also identified HOM-MEL-40/SSX2, NY-ESO-1, SCP1, and CT7 as new CT Ags (42). A possible explanation for the expression of CT Ags is through gene activation by cellular DNA demethylation, which occurs constitutively in testis, but also in tumors (8). Previous studies have correlated MAGE-1 expression with the genomic demethylation (43). As the testis is an immune-privileged site, the immune system will not have previously been challenged with CT Ags, and as such, following expression in the tumor, will be seen as foreign by the immune system. As ribosomal protein S18 has previously been reported to be overexpressed in cancer (29), this is likely to be the mechanism of immunogenicity by way of overriding the thresholds required for maintaining peripheral immune tolerance (44). This is also likely to be the mechanism of immunogenicity for v-Fte-1, which is reported to be overexpressed in transformed and tumor cells (32, 33). We could not detect any gene mutations in the panel of antigenic cDNAs isolated in our study, which is largely in agreement with SEREX analysis in that only p53 has previously been isolated as a mutated gene product with associated immunoreactivity (6). In this case although immunoreactivity was probably raised against the mutated p53 product, it was probably not specific for the mutation, as previous studies of the anti-p53 response reported cross-reactivity of the immune response between mutant and wild-type p53 (45). Here we have found a similar example in the selection of a cDNA clone encoding a C-terminal protein fragment of c-Ha-Ras 1. Serum reactivity in cancer patients to p21-Ras has been reported to be directed to the nonmutated C-terminal region rather than the codon 12 mutational hotspot (46). For this reason we believe that, as for mutant p53 (45), an immune response was initially raised against mutant p21-Ras. However, serum reactivity is unlikely to be mutation specific and therefore has resulted in the selection of the wild-type C-terminal fragment of c-Ha-Ras-1. As such, it is difficult to unambiguously eliminate gene mutation as a mechanism of immunogenicity for many tumor Ags, as serum reactivity initially raised against the mutant product may cross-react with the wild-type product.

In most cases the cDNAs that we have identified contain a large portion of the open reading frame of the parental protein; however, for some selected expressed sequence tag sequences, the reading frame is limited to a short peptidic sequence (Table III). A number of these clones may represent mimotopes of an immunogen rather than the immunogen itself, and their selection may be due to the cross-reactivity of the patient's serum Abs specific for the initial immunogen with the mimotope sequence. This is analogous to previous studies that have selected randomized peptide libraries on patient serum from hepatitis B patients (19). These Ag mimotopes may then be used to isolate the original target. The Ag mimotopes that we have selected, although of little value as cancer vaccine candidates, could still be of use to identify the original immunogen or could be used as serological diagnostic markers in their own right. Further, mimotopes selected in allergic conditions or auto-immune conditions may be of therapeutic value in inhibiting pathological immune responses (47).

A clear understanding of the correlation of Ag expression to the associated immune response and subsequently to clinical parameters such as patient age, sex, extent of disease, clinical outcome, and previous therapeutic history is of critical importance for the development of cancer vaccines. Such complex analysis may only be possible using a high throughput automated procedure combining selection of phage-displayed cDNA repertoires with an automated screening ELISA or, possibly, with DNA microarray analysis. Indeed, we have noted that the Ags isolated using SAS all show reactivity to the serum pool used for selection (Table I). With this high degree of association, the serological screening of Ags may in the future be replaced by postselection analysis using the selected cDNAs as probes for DNA microarrays. We envisage that by performing multiple parallel selections on individual sera and such microarray-based screening, it will be possible to quickly define both generic tumor Ags and Ags directly correlated with clinical parameters. The greater simplicity and speed of phage cDNA selection and the rapid screening of candidate Ags with large serological databanks make SAS a valuable alternative to SEREX, and it may be better placed to profile the humoral immune response on a genomics scale.

In our selections we used serum from patients who had undergone vaccination with autologous tumors in the ASI trial (24) with the purpose of identifying tumor Ags relevant to the vaccination strategy. Despite not taking any special precautions in the selection procedure (e.g., depletion on sera of patients pre-ASI), we found an increase in tumor Ag-specific IgG responses when using pre- and postvaccination serum for some patients (Fig. 4). Further analysis is required to evaluate whether any of the Ags have a vaccination-enhanced immune response and whether this indeed relates to clinical parameters. This is nevertheless a surprising finding because of the intrinsic complexity of the immunogen used in ASI. Our data hint of applications of SAS in the identification of Ag probes for guiding the development of maximally immunogenic vaccines. Indeed, a major challenge in the clinical evaluation of cancer vaccines is the availability of reliable assays for monitoring a patient's vaccine-induced immune response. Monitoring vaccine-induced cellular immunity has been problematic, and previous vaccination trials with defined tumor Ags such as MAGE-3 have reported no CTL responses, (48), although CTL responses have clearly been reported in patients immunized with melanoma-associated Ags (49). Eventually, some of the Ags identified in this study may be suitable probes for serological monitoring of the ASI vaccination trial. Furthermore, as Ags have been isolated by virtue of a high titer IgG response that requires T cell help, such serological assays could also provide an indication of the extent of the vaccination-induced CD4⁺ T cell response.

In conclusion, we have isolated a panel of six Ags with a tumor-related serological profile, including two Ags, ribosomal protein S18 (AM-COL-4), and v-Fte-1 (AM-COL-5), which have a suspected etiological association with cancer. Of those six, one is a novel Ag HSPC218 (AM-COL-3) that may be a new member of the important CT Ag family (50). Given progress in developing other CT Ags as cancer vaccines, e.g., MAGE-3 (51), we believe that HSPC218 is a promising candidate for further study. The technology presented in this study holds much promise for application in the cancer field and also to certain diseases that have to date been serologically undefined, e.g., diseases of the CNS, certain bacterial infections, and diseases of unknown etiology. Furthermore, we predict that the amenability of SAS to automation will allow in the future the generation of complex serological Ag profiles on a genomics scale that will help to unravel the intricacies of the host-vs-disease immune response.

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