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The Reverse Proteomics for Identification of Tumor Antigens

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Abstract The identification of tumor antigens is essential for the development of anticancer therapeutic vaccines and clinical diagnosis of cancer. SEREX (serological analysis of recombinant cDNA expression libraries) has been used to identify such tumor antigens by screening sera of patients with cDNA expression libraries. SEREX-defined antigens provide markers for the diagnosis of cancers. Potential diagnostic values of these SEREX-defined antigens have been evaluated. SEREX is also a powerful method for the development of anticancer therapeutics. The development of anticancer vaccines requires that tumor antigens can elicit antigen-specific antibodies or T lymphocytes. More than 2,000 antigens have been discovered by SEREX. Peptides derived from some of these antigens have been evaluated in clinical trials. This review provides information on the application of SEREX for identification of tumor-associated antigens (TAA) for the development of cancer diagnostics and anticancer therapeutics.

Keywords: DC-based anticancer vaccine, diagnostics, reverse proteomics, serological analysis of recombinant cDNA expression libraries, tumor-associated antigens

There are two questions dominant in the field of human cancer immunology throughout its history. Do cancer-specific antigens exist and, if so, are they recognized by the autologous host? Until recently, attempts to provide definitive answers to these questions have not been rewarded with much success.

Sahin and his colleagues [50] have introduced a method for the identification of tumor antigens recognized by autologous serum IgG of cancer patients. This method, termed SEREX (serological analysis of recombinant cDNA expression libraries), has led to the identification of a series of provocative cancer antigens that have relevance to the etiology, diagnosis, and therapy of cancer. As annotated in the SEREX database (Cancer Immunome database: http://www2.licr.org/CancerImmunomeDB), more than 2,000 different antigens have been defined by SEREX analysis of more than 15 different tumor types. It is generally accepted that immune recognition involves both cell-mediated and humoral immunity. The recent development of a new approach to dissect the humoral immune response to cancer has opened the way to establish a comprehensive picture of the immune repertoire against human cancer antigens. SEREX allows systematic and unbiased search for cancer-specific antigens, and immunogenic proteins based on their reactivity with autologous patient serum.

Serology plays central roles in two phases of SEREX analysis. The first phase is antigen identification. A second phase involves screening panels of serum from normal individuals and cancer patients with the aim of defining antigen panels that demonstrate cancer-restricted immune recognition [53–55]. SEREX-defined antigens showing cancer-restricted seroreactivity offer a range of opportunities for cancer diagnosis and disease monitoring, and perhaps immunotherapy. As opposed to methods used to assay serum antigen concentrations, such as PSA or CA125 assays, assays for cancer-restricted seroreactivity measure serum titers of tumor-specific antibodies. SEREX analysis of renal cancer [54], colon cancer [55], and breast cancer [53] have led to the identification of 13, 32, and 40 different antigens, respectively, which react exclusively with sera from cancer patients, but not with sera from healthy controls.

In addition to the serological definition of human tumor antigens, this past decade has seen a revolution in the structural identification of human tumor antigens recognized by the cellular immune system as well. Pioneering work of van der Bruggen *et al.* [69] has opened the way to identify peptide/protein antigens recognized by autologous cytotoxic T cells (CTL), and the list of new antigens identified in this way has grown rapidly, particularly in the case of malignant melanoma [70]. This methodology, however, can only be applied to defining antigens on target cells that can be adapted to growth in culture, which express appropriate

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MHC molecules, and are recognized by antigen-specific CTL lines. Alternatively, peptide elution from MHC molecules of tumor cells and the use of these purified fractions to stimulate CTL responses *in vitro* has also been used to define antigenic structures recognized by T lymphocytes [12]. This method employs costly and highly specialized mass spectrometric techniques for peptide sequencing, a process not readily adaptable to even the most highly sophisticated research laboratories. In the case of $CD4^+$ T lymphocytes, progress in the identification of antigenic structures recognized by this arm of the cellular immune system has been hindered by the complexity of the MHC class II presentation pathway, *i.e.*, a requirement for specialized accessory molecules and processing vesicles, and the variable length of MHC class II binding peptides. In spite of these limitations, several peptide epitopes recognized by $CD4^+$ T lymphocytes of cancer patients have been identified [75, 76]. In relation to the identification of antigens recognized by the T-cell compartments, antibodies detected by SEREX are high-titered IgG, the generation of which requires $CD4^+$ T lymphocyte help, implying $CD4^+$ cell recognition. Thus, SEREX analysis can also be seen as an analysis of the CD4 repertoire against tumor antigens. The demonstration of cytotoxic T lymphocytes (CTL) reactive with SEREX-defined antigens [28, 50], and the SEREX identification of antigens that were originally defined as CTL-recognized peptides [28, 50, 53], indicate that SEREX detects tumor antigens eliciting CTL immunity as well. Thus, in comparison with the other methods, SEREX is less technically demanding and can yield similar information regarding host immune responses to cancer.

SEREX Method

The SEREX approach offers the following features : (i) the use of fresh tumor specimens restricts the analysis to genes that are expressed by the tumor cells *in vivo*; (ii) the use of patients' sera allows for the identification of multiple antigens, (iii) the screening is restricted to antigens against which patients raised high-titer antibody responses.

SEREX (Fig. 1) employs a bacteriophage recombinant cDNA expression library prepared from tumor tissues, tumor cell lines, and testis tissues. The use of tumor cell lines for SEREX analysis has benefits. The main advantage would be that cell lines circumvent the need for tumor/normal tissue, which can be difficult to obtain. The cDNA expression library is used to transduce *E. coli*. The recombinant protein library is induced and transferred to nitrocellulose membranes. These membranes are then incubated with diluted (1:100–1:1,000) extensively preabsorbed pooled sera from the autologous patient. Clones reactive with high-titer antibodies are identified using an enzyme (alkaline phosphatase-conjugated secondary antibody specific for human IgG). Positive clones are then subjected to DNA sequencing. Sequence information of the DNA insert can be used to determine expression profile of the transcript and to evaluate the incidence of antibody responses to the respective antigens. Many antigens identified by SEREX have also been identified by many other investigators working on different cancers. This led us to believe that there may be a defined number of antigens recognized by the immune systems of patients with cancers, collectively referred to as cancer "immunome" by Dr. Lloyd Old (Ludwig Institute for Cancer Research).

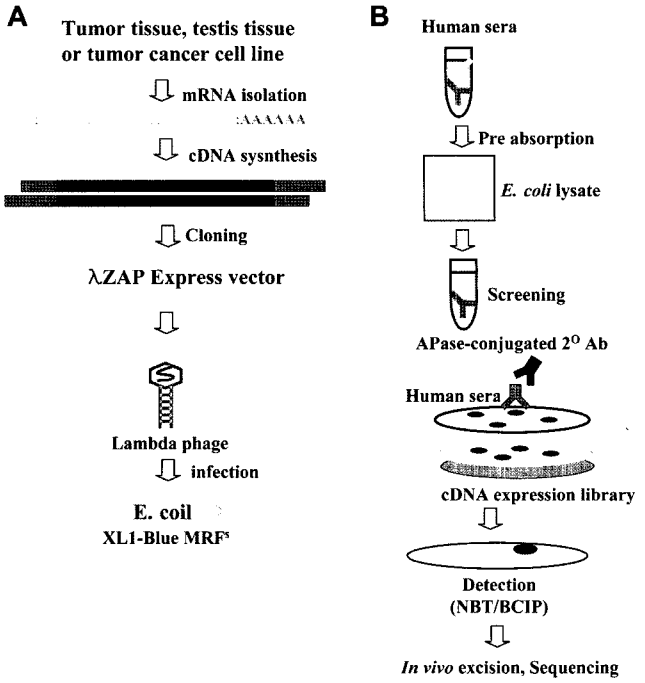

Fig. 1. Identification of SEREX-defined antigens and its application in clinical diagnosis.
A. cDNA expression libraries are made from tumor tissues, tumor cell lines, or testis tissues. For this, 5 μg of poly(A)$^+$ RNA is converted into cDNA by reverse transcriptase. Thus obtained cDNA library is cloned into λ ZAP expression vector. Each library usually consists of 2×10^6 primary recombinants on average and 5×10^5 of them are used for immunoscreening. Each of these recombinant cDNA libraries is transformed into *E. coli.* to yield the recombinant cDNA expression library. **B.** Immunoscreening of the recombinant cDNA expression library. cDNA expression libraries are screened with pooled sera of patients with cancers. Immune reactive clones are selected by reacting a nitrocellulose membrane containing recombinant clones with pooled sera of patients with cancers, followed by incubation with alkaline phosphatase-conjugated secondary antibody. Thus selected clones are subjected to *in vivo* excision and sequencing to determine the identity of each immune reactive clone. Sensitivity and specificity of each clone are determined by incubating each clone with the individual serum of cancer patients or healthy controls.

Practical Problems Associated with SEREX

SEREX combines serological analysis with antigen cloning techniques to identify human tumor antigens eliciting high-titer immunoglobulin G (IgG) antibodies. Although the concept behind SEREX is straightforward, there are several challenges that need resolution: (i) It involves eliminating

antibodies in human sera that react with bacterial or phage components. This step is done by repeated absorption of the diluted pooled sera of patients with bacterial and phage lysates. This is absolutely required because such contaminating antibodies would completely obscure the detection of other classes of antibodies; (ii) B cells in tumors often give rise to IgG, which is detected in SEREX; (iii) It is quite possible that many of those autoantibodies identified by SEREX may have no relevance to cancer. This is understandable in that SEREX focuses on high-titered IgG. Therefore, it is required that the function of each of these genes should be thoroughly investigated.

Classification of the SEREX-defined Antigens

SEREX provides broad targets for large-scale analysis of humoral response in cancer patients and healthy individuals. Cancer-restricted recognition suggests common origins of immunogenicity, such as gene mutation or aberrant expression, indicating that serological methods of gene discovery can be used to identify molecules of etiologic relevance to cancer. Indeed, several antigens isolated by SEREX analysis [4] are directly involved in tumor development.

A number of SEREX-defined antigens with these cancer-related characteristics have been identified and can be classified into one of the following categories; differentiation antigens, mutational antigens, overexpressed antigens, and cancer/testis antigens.

Mutational Antigens

Several mutational antigens have been isolated by SEREX. Tumor suppressor gene p53 has been identified by SEREX for ovarian cancer [53]. In the case of colon cancer, a single base substitution of p53 (A to G) was identified, confirming this mutation as the basis for the observed immunogenicity. The CDX2 mutation is in a microsatellite sequence within the coding region, and is believed to result from microsatellite instability in patients. A different type of mutation, namely translocation, was found in the E-cadherin gene, detected in the SEREX analysis of gastric cancer. Two of the three genes, NY-REN-9 and NY-REN-10, were derived from renal carcinoma and corresponded to LUCA-15 and gene 21, respectively [54].

Differentiation Antigens

Differentiation antigens are expressed in tumors in a lineage-specific pattern, but also in normal cells of the same origin. The classic example of a differentiation antigen recognized by SEREX is the melanocyte-specific protein tyrosinase [50]. Other examples include NY-BR-1 in breast cancer [26], and glial fibrillary acidic protein (GFAP) in glioma [57]. Normal tissue expression of galectin-4 is restricted to the normal colon and small intestine. Because galectin-4 is localized to the leading edge of lamellipodia, it is thought to have a role in cell adhesion [51].

Amplified or Overexpressed Antigens

Many SEREX-defined genes are overexpressed in cancer, based on Northern blot analysis and real-time RT-PCR. Amplified or overexpressed antigens identified include carbonic anhydrase XII in breast [77], and eIF-4 gamma [22] in lung cancers. Several mechanisms can account for amplified expression of gene products in cancer, including gene amplification (*e.g.*, eIF-4 gamma), increased steady-state mRNA (*e.g.*, KOC3), and increased protein stability (*e.g.*, p53) [44].

Cancer/Testis Antigens (CT)

Certain CT gene families contain multiple members (*e.g.*, MAGEA, GAGE1), as well as splice variants (*e.g.*, XAGE1a, XAGE1b), and a total of 89 distinct transcripts are currently known to be encoded by CT genes. CT antigens share the following characteristics: (i) predominant mRNA expression in testis, but generally not in other normal somatic tissues; (ii) gene activation and mRNA expression in a wide range of human tumor types; (iii) existence of multigene families; and (iv) with rare exception, localization of coding genes to chromosome X. Table 1 shows a partial list of CT antigens identified by various methods, including SEREX [10, 50, 66].

Many testis-specific transcripts and proteins are under such tight regulatory controls that they are almost never expressed in cancers other than germ-cell tumors.

The frequent expression of CT antigens in various types of tumors is an exception to this general rule. It suggests that the CT antigens, most of them with unknown function at present, are a distinct group of proteins in terms of their regulation and possibly their biological function.

Immunogenicity of SEREX-defined Antigens

Immunogenicity of SEREX-defined antigens can be ascribed to several mechanisms: gene activation, mutation, amplification, overexpression, or expression of abnormal splice variants. The antibody responses to cancer/testis antigens are related to the abnormal expression of these

Table 1. Examples of cancer/testis antigens.

C/T antigen family	# Genes	Chromosome	Detection
MAGE	16	Xq28	$CD8^+$, Ab
BAGE	2	Unknown	$CD8^+$
GAGE	9	Xp11	$CD8^+$
SSX	>5	Xp11	Ab
NY-ESO-1	2	Xq28	Ab, $CD8^+$, RDA
SCP-1	3	P12-p13	Ab
MAGE-C1	1	Xq26	Ab, RDA
CTP11	1	Unknown	Ab
SAGE	1	1p	Ab
cTAGE-1	1	Xq27	Ab, RDA
CAGE	1	Xp22	Ab

antigens in cancer that are usually expressed only in germ cells. Abnormal antigen expression is also found in paraneoplastic syndromes affecting the central nervous system. These syndromes result from autoimmune recognition of neural antigens aberrantly expressed by nonneural cancers, and specific autoantibodies are often found to be associated with specific tumor types [42].

Mutation forms the basis for the immunogenicity of SEREX-defined antigens. In the case of mutation, it is quite possible that the resulting antibodies recognize the wild-type. Therefore, sequencing of the independent clones from the same library is required. Many examples of autoantibodies to overexpressed proteins in cancers have been identified by SEREX. This indicates that the immune system responds well to quantitative as well as qualitative changes in antigen expression.

Clinical Diagnosis Employing SEREX-defined antigens

The identification of biomarkers for diagnosis, prognosis, and therapy of human cancer has been a long-standing challenge in cancer research. With regard to serum markers for cancer, a limited number of clinically beneficial antigenic markers have been defined, such as carcinoembryonic antigen in gastrointestinal cancers, α-fetoprotein in hepatoma and germ-cell tumors, CA125 in ovarian cancer, and prostate-specific antigen in prostate cancer [64]. Growing evidence indicates that the humoral immune system of cancer patients recognizes tumor-associated antigens [50]. In contrast to the detection of serum antigens, the detection of serum antibody responses to tumor antigens could represent a novel form of serum marker for cancer diagnosis [56]. SEREX analysis has defined a subset of tumor antigens that react exclusively with serum antibodies derived from multiple cancer patients but do not react with sera from normal individuals. SEREX analysis of renal cancer has led to the identification of 12 antigens associated with a cancer-related serological response in which 72% of serum samples from renal cancer patients had serum antibodies to at least one of these antigens, whereas sera from normal individuals did not [54]. Serum antibodies detecting this subset of tumor antigens represent potentially valuable serum markers for cancer. Determination of antibody responses to SEREX-defined antigens is critical for evaluating the clinical relevance of SEREX-defined antigens. For this, a small panel of sera from normal individuals and patients with cancer are screened against the SEREX-defined bacteriophage clone, a process called petit serology. The SEREX-defined clones showing a cancer-restricted reactivity are then tested in a large-scale analysis using purified recombinant proteins in ELISA. Extensions of the petit serology have been devised by Scanlan *et al.* [55], and given the name SADA (serial analysis of defined antigen). This involves arranging the phage clones on filters in a "dot blot" fashion, thus allowing simultaneous testing of a large panel of antigens (Fig. 2). This method works very well for clones that show strong seroreactivity. Scanlan *et al.* [5] evaluated, by employing SADA, the serological response of 74 colon cancer patients and 75 normal individuals to a panel of 77 SEREX-defined antigens. In this, they identified a subset of cancer antigens that are recognized in a cancer-specific manner. This subset of cancer antigens includes tumor-related antigens, transcriptional regulators, and antigens with unknown functions. The diagnostic value of these results is supported by the fact that 34 of 74 serum samples (46%) from colon cancer patients detect 1 or more of the 13 antigens associated with a colon cancer-related serological response, whereas serum samples from 75 normal blood donors are not reactive with this subset of antigens.

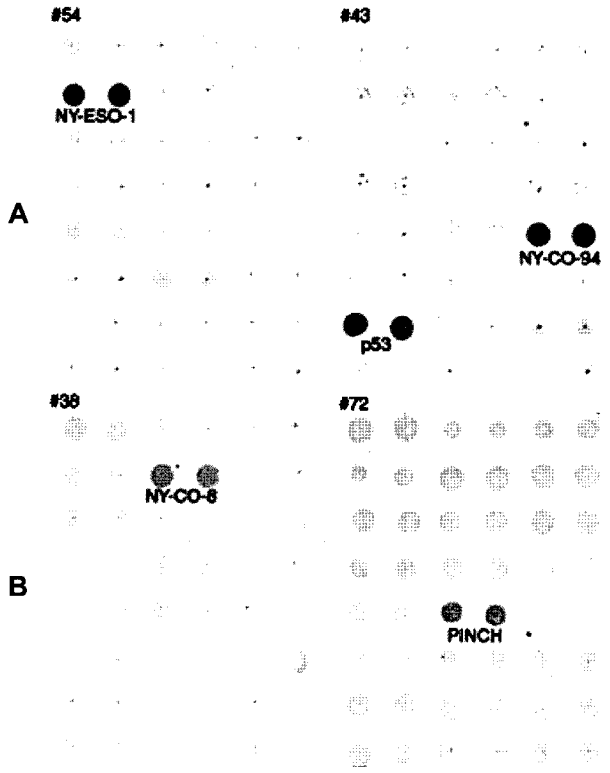

Fig. 2. SADA (serial analysis of defined antigens) analysis of 24 SEREX-defined antigens in duplicate against serum samples from 4 colon cancer patients (38, 43, 54, and 72).
* Scanlan *et al.* [55].

Ever since SEREX was first applied to melanoma, renal cancer, and Hodgkin disease, this technique had been employed for the identification of the new tumor antigens in various tumors, including gastric cancer. Gastric cancer is one of the popular malignancies in the world. The majority of gastric cancer patients are diagnosed at an advanced stage. The key in diagnosis and treatment of gastric cancer is to identify reasonable target antigens that not only provide early diagnosis of the disease but also can boost antitumor immune responses in cancer patients.

Table 2. SEREX-defined antigens in human gastric cancer.

Designation	Accession No.	Gene	SEREX DB	No. clones
St-1	XM010732	ADPRT	Yes	15
St-2	BC000672	G prot.	No	5
St-4	NM006940	SOX5	No	1
St-8	XM003095	ZNF288	No	7
St-9	AF309034	SOX6	No	1
St-15	XM007263	KSN2	No	3
St-17	XM008359	HDAC5	Yes	1
St-19	XM008972	DDXL	No	2
St-21 (CAGE)	AY039237	Novel	No	1
St-28	NM002752	JNK2	No	8
St-30	XM018280	Poly(A) BP	No	35
St-31	NM015874	RBPJK	Yes	11

Gastric cancer is one of those cancers resistant to radiation and chemotherapy. Therefore, an alternative way of treatment is needed for the treatment of gastric cancer. So far, only a few genes whose alteration leads to gastric cancer have been identified. We performed SEREX to identify gastric cancer-associated antigens by using sera of patients with gastric cancers.

In order to identify antigens associated with gastric cancers, we screened recombinant cDNA expression libraries of human gastric cancer cell lines MKN74 and SNU 601 and testis tissues. We identified 39 clones that react with pooled sera of five gastric cancer patients. Table 2 shows a partial list of genes identified by SEREX. Among these clones, we were interested in a novel gene (st-21, later named as CAGE) According to the expression profile analysis, CAGE showed restricted expression in testis among normal tissues, whereas it showed a wide expression in various tumor tissues. It showed localization in the X chromosome. Expression of CAGE was shown to be under epigenetic regulation [11]. In other words, hypermethylation leads to silencing of CAGE expression in normal tissues, except the testis. Hypomethylation of CAGE was seen in premalignant lesions, suggesting that CAGE can be employed for early detection of gastric cancer (Table 3).

Iwata *et al.* [25] later cloned CAGE by screening cDNA expression libraries made from testis or endometrial cancer cell lines using sera from patients with endometrial cancer or melanoma. Anti-CAGE IgG antibody was detected in sera from 5 of 45 endometrial cancer, 2 of 24 melanoma, and 2 of 33 colon cancer patients, but not in sera from healthy individuals. By ELISA analysis, anti-CAGE antibody was detected in 12 of 45 endometrial cancer, 2 of 20 melanoma, and 4 of 33 colon cancer patients. Intriguingly, anti-CAGE antibody was highly positive in 7 of the 13 (53.8%) microsatellite instability (MSI)-H patients with endometrial cancer, but negative in 20 non-MSI-H patients (P=0.001). Taken together, these data suggest that CAGE would be valuable for the diagnosis and prognosis of cancer patients.

Table 3. Summary of the hypomethylation frequency of CAGE.

Samples	Frequency
Archival samples	
Breast cancer (n=24)	(20/24,83%)
Lung cancer (n=25)	(18/25,72%)
Uterine cervix cancer (n=22)	(2/22, 9%)
Larynx cancer (n=19)	(4/19, 21%)
Prostate cancer (n=23)	(8/23, 34%)
Hepatic cancer (n=31)	(19/31, 61%)
Colorectal cancer (n=16)	(4/16, 25%)
Normal prostate (n=14)	(0/14, 0%)
Liver cirrhosis (n=22)	(13/22, 59%)
Chronic gastritis (n=55)	(19/55, 35%)
Chronic hepatitis (n=11)	(0/11, 0%)
Normal colon (n=14)	(0/14, 0%)
Fresh-frozen tissues	
Hepatic cancer (n=6)	(5/6, 83%)
Colorectal cancer (n=9)	(8/9, 88%)
Gastric cancer (n=64)	(50/64, 78%)

Evaluation of antibody responses to the SEREX-defined antigens using allogeneic sera from cancer patients would offer valuable cancer diagnostics employing antibody-based screening.

Recognition of SEREX-defined antigens

Growing evidence suggests that the immune system interacts with tumor cells during the course of the disease. Much has been learned in the past 20 years concerning cancer antigens. General properties of cancer antigens have been described (Fig. 3). These include the following principles: (1) cancer antigens contain epitopes binding to various

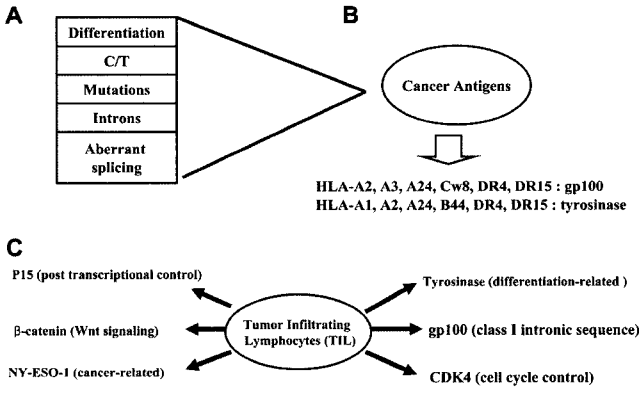

Fig. 3. Properties of SEREX-defined antigens (TAAs).
A. Caner antigens can arise from differentiation antigens, cancer/testis antigens, mutant antigens, intronic sequences, and splicing variants. **B.** A single cancer antigen contains various epitopes that can be presented on many different alleles of HLA molecules. **C.** A single cancer patient usually develops immune response to multiple antigens.

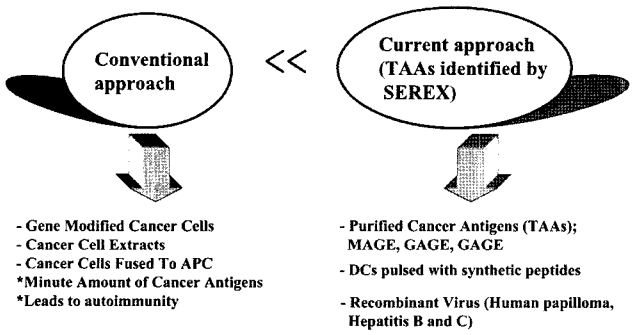

Fig. 4. Vaccine approaches to the treatment of cancer patients.

HLA alleles; and (2) tumor-infiltrating T lymophocytes contain various cancer antigens. A large number of TILs from tumors greatly increased T-cell populations capable of recognizing cancer antigens [39]. The presence of tumor-infiltrating T cells (TILs) is associated with better prognosis in individual patients, indicating that TILs recognize specific antigens expressed by the tumor (Fig. 3). TILs, when adoptively transferred along with IL-2, led to a significant tumor regression rate. This rate was twice that seen with IL-2 alone [47]. Before the identification of TAA by SEREX, conventional immunotherapy focused mainly on immunization with either autologous or allogeneic cancer cells or cancer cell extracts (Fig. 4). This approach is not effective because of minute amounts of cancer antigens present in the intact cells. It also causes autoimmunity since many of the antigens overexpressed in tumors are also expressed in normal cells. Various approaches had been tried to increase the immunogenicity of tumor cells, including injection of tumor cells along with adjuvants, or transducing cells with genes encoding cytokines such as TNF, IFN-γ, or GM-CSF. However, these approaches have not been successful in generating T cells that recognize intact tumor cells. Cancer antigens identified by SEREX have offered new approaches to the development of anticancer therapeutic vaccines (Fig. 4). Tumor cells express antigens that can be recognized by the host's immune system. These tumor-associated antigens (TAAs) can be injected into cancer patients in an attempt to induce a systemic immune response that may result in the destruction of the cancer growing in different body tissues.

Studies on experimental animals showed that cellular immunity rather than a humoral response was responsible for rejection of transplanted tumors or tissues. Therefore, identification of antigens recognized by human lymphocytes is critical for the development of anticancer therapeutics. Identification of antigens recognized by $CD8^+$ CTL cells has been achieved by transfecting cDNA libraries from tumor cells into the target cells with appropriate MHC molecules, and then using antitumor cells to identify such transfectants [6]. Once the gene is identified, the region encoding the antigenic peptide can be narrowed down by transfecting gene fragments. The synthetic peptides were then made and tested for recognition by the original tumor-specific CTL clone.

Alternatively, peptides eluted from the surface of tumor cells are pulsed onto antigen-presenting cells, such as dendritic cells, and tested for reactivity with specific antitumor lymphocytes [9, 23].

SEREX has been used to identify cancer antigens that are recognized by $CD8^+$ T cells (CTL). Both $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper cells recognize antigens presented as small peptides in the MHC. $CD8^+$ CTL cells recognize peptides of 8–10 amino acids presented on the class I MHC. Peptides recognized by CTL are digested in proteasomes and presented *via* the endoplasmic reticulum. Experimental evidence in mice suggests that the effectiveness of $CD8^+$ T cells is dependent on factors from $CD4^+$ T-helper cells. Peptides recognized by $CD4^+$ T-helper cells are derived from extracellular proteins presented on class II MHC. The peptide-MHC complex is recognized by the T-cell receptor (TCR) on the surface of T lymphocytes. In addition to directly contacting the MHC molecule, other peptide residues are available to establish a direct contact with the TCR [4]. Thus, whether a TAA peptide elicits a T-cell response is governed both by the ability of the peptide to bind the presenting MHC allele and by the resulting affinity of the peptide-MHC complex for the TCR.

T-cell defined TAAs include (a) differentiation antigens, which are expressed in a lineage-related manner and are detected in the normal counterpart of neoplastic tissue; (b) tumor-restricted antigens, which are expressed only on neoplastic cells; and (c) mutated antigens. Tumor-restricted antigens encompass both shared antigens of different origin and unique antigens. For unique TAAs [58], the immunogenic peptide includes a mutated amino acid sequence that confers immunogenicity through the exposure of an altered nonself epitope.

Some tumor-restricted TAA epitopes are encoded by intron sequences [31]. These TAAs appear to be highly immunogenic, because a high frequency of specific CTLs directed against them has been detected in cancer patients with a favorable prognosis after therapy [2].

It was not until 1991 that the first report describing the cloning of a gene encoding a human TAA, the melanoma antigen-1 (MAGE-1), was published. The identification of its nonamer peptide, which is recognized by human leukocyte antigen (HLA)-A1-restricted cytotoxic T lymphocytes (CTLs), was published the following year [67]. This T-cell epitope cloning technique was further employed to identify tumor antigens, such as the BAGE and GAGE gene family [5, 69]. These antigens are typical of cancer/testis antigens in that their expression in normal tissues is restricted to the testis and ovary, whereas these antigens are widely expressed in various tumor tissues. With regard to gastric cancer,

peptides from SEREX-defined antigens were able to induce MHC-specific CTL responses, indicating their potential uses as cancer vaccines in certain patients with gastric cancer [31]. These cancer/testis antigens are likely to be oncogenes, in that they are expressed only in the testis among normal tissues, an immune-privileged site, and widely expressed in various tumor tissues. Therefore, these cancer/testis antigens are ideal targets for the development of anticancer therapeutic vaccines.

The differentiation antigens comprise a group of antigens that are recognized by CTL. These include tyrosinase [36], MEL-1 [73], and gp100 [34].

Epitopes from differentiation antigens often exhibit low affinities for MHC molecules. Identification of class II MHC-restricted cancer antigens was made possible by fusing cDNA libraries to an invariant chain sequence to guide the transfected proteins into the class II presentation pathways [75].

Mutated antigens are another group of TAAs. The p53 gene is the most commonly mutated gene in human tumors. According to several studies, peptides derived from p53 induced CTL response *in vitro* [24].

Examples of the antigens [1, 3, 7, 8, 15, 20, 21, 30, 34–36, 41, 43, 52, 59, 62, 63, 71–74] recognized by $CD8^+$ CTL cells and presented on class I MHC molecules are shown in Table 4. Examples of the antigens [27, 32, 60, 61, 75, 76, 78] recognized by $CD4^+$ T-helper cells are also shown in Table 4.

Identification of TAA peptides expressed by different human tumors provides the basis for vaccination or active immunotherapy. Immunization studies using these TAA peptides have been relatively successful in generating high levels of T cells against cancer antigens [29, 49].

The advantages of peptide-based vaccines include (1) the simplicity of peptide administration in a clinical setting; (2) the possibility of treating only those patients whose tumors express the cognate epitopes, thus avoiding the useless immunization of patients whose tumors are TAA-negative; and (3) the availability of *in vitro* or *ex vivo* assays that can assess patients' immune responses to vaccine epitopes.

Although multitudes of T-cell-defined TAA epitopes are now available for potential clinical application as vaccines, they are still of limited clinical use for the majority of cancers. Problems include that (1) most of the available TAAs are expressed by melanoma, whereas relatively few TAA epitopes have been characterized in other tumors; (2) most of the already known TAA epitopes are recognized by only a few HLA alleles that are widely represented in the Caucasian population, leaving few epitopes available for recognition by T cells of subjects with less frequent HLA alleles; and (3) the majority of TAA epitopes are derived from normal proteins for which immune tolerance may prevent immunogenicity. Most T-cell responses require repeated *in vitro* stimulation with TAA epitopes [46] and show limited immunogenicity when used as vaccines for cancer patients [37]. Therefore, most of the TAA peptides have elicited insufficient immune response to control cancer growth.

The immunogenicity of TAA peptides can be increased by altering amino acid residues at positons that anchor the peptide to the appropriate HLA molecules [45]. This

Table 4. Examples of SEREX-defined antigens recognized by human T cells.

Antigen	Reference	Antigen	Reference
I. Class I-restricted antigens recognized by CD8 + T cells			
Differentiation antigens		**Cancer-testes antigens**	
MEL-1	74	MAGE-1	41
Gp100(pmel-17)	34	MAGE-2	63
Tyrosinase	36	MAGE-3	35
Tyrosinase-related protein 1	75	MAGE-12	15
Tyrosinase-related protein 2	43	NY-ESO-1	20
Melanocyte-stimulating hormone receptor	52		
Mutated antigens		**Mutated antigens**	
CDK-4	60	α-Fetoprotein	8
Caspase-8	1	G-250	72
KIAA0205	21	Telomerase catalytic protein	73
HLA-A2-R	7	CEA	30
		P53	64
II. Class II-restricted antigens recognized by $CD4^+$ lymphocytes			
Epitopes from nonmutated proteins		**Epitopes from mutated proteins**	
gp100	32	Triosephosphate isomerase	62
MAGE-3	79	CDC-27	76
Tyrosinase	61	LDLR-FUT	77
NY-ESO-1	27		

modification induces a qualitatively and quantitatively improved T-cell response without changing the HLA binding affinity or stability. This variant presumably improves immunogenicity through a more efficient interaction with the TCR.

The strategy of modifying TAA peptides to enhance antitumor T-cell responses represents a new way for the development of anticancer therapeutic vaccines.

Often, peptide vaccine alone does not give rise to sufficient immune responses to control cancer growth. Modified peptide from the gp100 (gp100:209–217), when used along with IL-2, led to a significantly higher regression rate than when peptide alone was administered [48]. The chimeric form of IL-2 with TNF was shown to display antitumor activity [59].

This approach may result in a decrease in circulating antitumor cells, possibly due to traffic of specific lymphocytes to the tumor site, resulting in decrease of these cells in circulation.

Immunotherapy Employing Dendritc Cells Presenting TAAs

The goal of active immunotherapy concerning tumors is to use tumor antigens to prime specific antitumor immunity by generating effector cells such as CTL to lyse tumor cells. Dendritic cells (DCs) are antigen-presenting cells that have the function of presenting antigens, including TAAs, to naive T cells in lymph nodes. DCs have specialized characteristics that make them efficient at capturing presenting antigens and activating T cells. The generation of tumor-specific T cells against TAA-derived peptides requires a phase of "antigen presentation" by cells expressing MHC class I molecules (*i.e.*, the antigen-presenting cells), of which the most efficient appear to be DCs [18]. They express high levels of MHC class I and II antigens in addition to various immunomodulatory molecules that are essential for cancer immunotherapy. For immunotherapy, DCs are pulsed with TAA-derived peptides, tumor cell lysates, or naked DNA. DCs can be fused to tumor cells to induce antitumor immune responses [17]. The efficacy of DC-based vaccination against tumor growth can be substantially improved by combining the injection of TAA-loaded DCs with the administration of cytokines such as IL-12 [19].

Immunotherapy employing DC has been successfully used in the treatment of various cancers, including gastric cancer, melanoma, colon cancer, and non-Hodgkin lymphoma. DC pulsed with peptides derived from Her-2/neu and MAGE-3 to immunize cancer patients yielded encouraging results in clinical trials [13].

Tumor cell lysates provide DCs with multiple TAA peptides, some of which have not yet been characterized. Such an approach has been used in vaccinating patients with melanoma [16]. More information is needed to define the repertoire of TAA peptides included in tumor lysates and their immunogenicity *in vivo*. In addition, lysates include normal proteins that should be ignored and tolerated by the host, but that, under certain conditions, may cause autoimmunity.

In the past two decades, the number of SEREX-defined antigens has been greatly increasing. Identification of these antigens makes it possible for early detection and the development of anticancer vaccines. However, SEREX has its own problems that need resolution. For example, using *E. coli* expression system may result in identification of antigens with affected functions and immunogenicity. This problem can be solved by employing eukaryotic expression vector systems such as the yeast [38].

Only some of those SEREX-defined antigens are recognized by multiple patients. SEREX shows bias towards selecting antigens that are highly expressed in the tumors, as recombinant cDNA expression libraries are made from tumor tissues or tumor cell lines. Antigen expression levels are not necessarily related to tumorigenesis. The relevance of these antigens in clinical diagnosis should be addressed by extensive functional studies. SEREX does not preferentially select for antigens that are oncogenic. Often, the identified antigens are patient-specific, but not tumor-specific. It would therefore be necessary for using sera selected from cancer patients with known pathologic diagnosis and outcome. Autoantibodies are part of the normal immune response, making it difficult to distinguish tumor-associated antibodies from those irrelevant antibodies. SEREX has identified a number of potential biomarkers that may have relevance to the diagnosis and therapy of cancer. However, identification of serologic markers with definitive predicting ability has not been successful with SEREX. Thus, modifications of SEREX have been tried to minimize the identification of irrelevant antibodies and increase the number of cancer-related antibodies [14]. These include (1) selection of sera from cancer patients with known pathologic diagnosis; (2) omission of absorption steps with non-transfected host cells; (3) use of a T7 phage display library made from non-autologous cells. This approach decreases bias towards antigens overexpressed in only autologous cancer cells; and (4) employment of immunoreactivity rather than random selection, to select positive clones. By modification of SEREX, it is now possible to demonstrate tumor relevance early in the screening process and identify cellular proteins related to tumerogenesis [65].

Identifying the complete repertoire of immunogenic gene products in human cancer is now an achievable goal for tumor immunology. Since the establishment of the SEREX database in 1997, 2,593 sequences derived from 2,169 clones have been deposited (as of February 2004). Many of the genes have been isolated repeatedly by SEREX, from the same and/or from different tumor types, indicating that these gene products are highly immunogenic in the human host. On the other hand, even in a very recent study

of sarcoma and lung cancer, only about one-third of the isolated genes were already in the database, suggesting that the pool of immunogenic cancer antigens, although apparently finite in size, is still far from being completely defined.

Immunotherapy employing DCs pulsed with HLA-restricted peptides derived from various TAAs has been tried with some success. There are several points that should be addressed before DC-based immunotherapy becomes a viable option. Tumor cells often escape immune recognition through the loss of antigen expression, secretion of local immunosuppressive factors, inhibition of antigen processing and presentation, and the inability of tumor cells to activate antitumor precursors. There are several ways to avoid an immune escape mechanism. These include (1) vaccination to suppress immune tolerance or immunosuppression caused by factors released by tumor cells; (2) use of cytokine adjuvants, such as GM-CSF or IL-2, to improve antigen presentation; and (3) use of multiepitope vaccines to bypass the heterogeneity in TAA expression. Immunotherapeutic approaches, including DCs pulsed with TAA peptides, have resulted in objective CTL response and tumor regression, in some cases. However, the efficient therapeutic effect is still limited. Identification of more target antigens by proteomics approaches, including SEREX, would be necessary for increasing the number of patients that can be treated with cancer vaccines. Using a vaccine against multiple TAA epitopes could be more effective than a vaccine against a single epitope. Clinical trials involving many of the TAA peptides are currently under way. Patient monitoring systems should be enhanced and standardized in clinical immunotherapy.

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