Commentary

New Paths in Human Cancer Serology

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Two questions have dominated 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. However, as in so many other areas of research where technical advances provide the means to advance fields, this has also been the case in cancer immunology. With regard to T cell–defined tumor antigens, the discovery of IL-2 permitted the isolation of stable lines of cytotoxic T cells with specificity for autologous melanoma cells (1), and this, in turn, led to the identification of T cell–recognized epitopes on human tumor cells (2, 3). With regard to antibody-defined tumor antigens, hybridoma technology for generating mouse and human monoclonal antibodies and advances in cloning and biochemical characterization of tumor antigens have given rise to an increasingly detailed picture of the surface antigenic structure of cancer cells. Nevertheless, these serological efforts did not bring answers to the issue of tumor-specific recognition by the humoral immune system of the tumor-bearing host. For this purpose, a new approach introduced by Pfreundschuh and his colleagues Sahin and Türeci at the University of Saarland (Homburg, Germany; references 4–6) has inaugurated a new phase in cancer serology, bringing with it the prospect of providing a comprehensive view of the immune recognition of human cancer. They call their approach SEREX, for serological analysis of recombinant cDNA expression libraries of human tumors with autologous serum. In their initial application of the method, tumor antigens such as MAGE and tyrosinase that had originally been defined as T cell–recognized epitopes were detected by autologous antibody. SEREX analysis has now identified a series of provocative cancer antigens that have relevance to the etiology, diagnosis, and therapy of cancer. What is so encouraging about SEREX is that it provides a way to analyze the humoral immune response to intracellular cancer antigens, a generally impenetrable forest for cancer serologists in the past.

Two papers related to antigens defined by SEREX have appeared in *The Journal of Experimental Medicine* (7, 8). As background for these studies, this commentary is intended to give a historical perspective to the development of SEREX and to review the current status of SEREX analysis of human cancer.

A Brief History of Cancer Serology. The search for antibodies that distinguish cancer cells from normal cells is one of the longest uninterrupted inquiries in cancer research (9). The history of this pursuit can be divided into four phases. The first, dominated by immunologists such as Witebsky and Hirszfeld, dealt mainly with the analysis of heteroimmune sera obtained from rabbits and other animals immunized with human cancer (10). The challenge, generally unmet, was to remove antibodies reactive with normal tissue antigens, and a variety of absorption techniques were devised to accomplish this. Complement fixation and later agar gel immunoprecipitation provided the primary assay systems to analyze the heteroimmune sera. Although little of enduring value came from this vast effort, two useful antigens were identified: alpha fetoprotein, a serum marker for hepatoma and germ cell tumors (11), and carcinoembryonic antigen (CEA), a serum marker for colon and other epithelial cancers (12). The second phase in this odyssey was initiated by the work of Gorer, a scientist best known for his discovery of the mouse major histocompatibility locus. Gorer also had an intense interest in tumor antigens and he introduced the approaches and test systems involving cytotoxic alloantibodies prepared in inbred mice that led to the serological dissection of normal and malignant lymphoid cells and the discovery of cell surface "differentiation antigens" such as thymus leukemia (TL), Ly1, Lyt2 (CD8), Thy-1, and PCA (13), and endogenous retroviral-coded antigens such as GCSA, G_{IX}, and ML (14). The emergence of hybridoma technology transformed the field of serology and opened the floodgates for identifying new cell surface antigens in mice and humans and for analyzing the antigenic phenotype of human cancer. Although there was great hope that monoclonal antibodies would uncover tumor-specific antigens in humans, this has not proven to be the case. Rather, experience has shown that even the most restricted tumor antigen generally turns out to be a restricted normal differentiation antigen (15). In addition to the use of polyclonal and monoclonal antibodies of heterologous and allogeneic origin in the search for tumor-specific antigens, there has been a sustained effort to determine whether the autologous host recognizes cancer cells. To establish as rigorous and unambiguous a serological test system as possible for this purpose, an approach called autologous typing was developed (16), initiating what can now be seen as the third phase in human cancer serology. The intention of autologous typing was to restrict the analysis to autologous reagents (tumor cells, serum, normal cells such as fibroblasts, and lymphocytes from the same patient) to eliminate the contribution of alloantigens in the reactions

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observed and to establish tumor specificity by absorption with autologous normal cells. With the exception of leukemia and lymphoma cells, cultured tumor cell lines were required for autologous typing, and this limited analysis to tumor types that could be adapted to growth in vitro with some regularity, i.e., melanoma (17, 18), renal cancer (19), and brain cancer (20). The conclusion coming from the autologous typing of a large series of patients is that a small fraction of patients develop demonstrable autologous antibody with specificity for cell surface antigens of the tumor. However, with few exceptions (21, 22), molecular characterization of the antigen was generally beyond reach, primarily because the antibodies were not of sufficient titer to monitor biochemical purification or cloning. With the development of SEREX, ushering in the fourth phase of cancer serology, autologous typing can now be carried to a new level of specificity analysis and comprehensiveness that could only be dreamed about in the past.

SEREX Methodology. Although the concept behind SEREX is straightforward, there were a number of technical challenges that needed to be resolved. One of the most crucial involved eliminating antibodies in human sera that react with bacterial or phage components. This is not a trivial exercise and is absolutely essential because such contaminating antibodies completely obscure the detection of other classes of antibodies. The presence of variable numbers of B cells in tumors gives rise to IgG mRNA, which is expressed and detected in SEREX, and a strategy to eliminate these clones also needed to be developed. There was an initial suspicion that the majority of antibodies detected in SEREX would be autoantibodies with little or no relevance to cancer. Experience has shown that antibodies such as those related to known autoimmune states have not been overrepresented in SEREX studies to date, and this may have to do with Pfreundschuh and his colleagues' decision to exclude IgM from the analysis and to focus on high titered IgG antibodies.

In their original analysis, Sahin et al. (4) established the basic strategy for the initial study of SEREX-defined clones: (*a*) DNA sequencing to establish identity, similarity, or uniqueness with regard to genes in the existing data banks and the search for possible structural (e.g., mutational) abnormalities, (*b*) analysis of the mRNA expression pattern in normal tissues and in tumors, and (*c*) immunogenicity as measured by the frequency of antibodies in a limited panel of sera from normal individuals and patients with the same tumor type. Subsequent analysis of interesting clones showing cancer relatedness in terms of sequence abnormalities, expression patterns, or seroreactivity includes chromosomal mapping, generating monoclonal antibodies for biochemical and immunohistochemical studies, and serological surveys of antibody reactivity in patients with various types of human cancer.

Categories of SEREX-defined Antigens. During the past two years, SEREX has been applied to a range of tumor types, including melanoma, renal cancer, astrocytoma, Hodgkin's disease (4), esophageal cancer (23), lung cancer (24, 25), colon cancer (26), gastric cancer, breast cancer, and prostate cancer. (A SEREX collaborative group has been established by the Ludwig Institute for Cancer Research involving investigators at the University of Saarland [Homburg, Germany]; Ludwig Institute Branches in New York, San Diego, CA, Melbourne [Australia], and London [UK, University College]; Aichi Cancer Center [Nagoya, Japan]; Krankenhaus Nordwest [Frankfurt, Germany]; Mie University School of Medicine [Mie, Japan] Moscow State University [Moscow, Russia], and the Institute of Molecular Biology and Genetics [Kyiv, Ukraine].) This survey has identified a large number of tumor antigens, >400, only a small fraction of which has been analyzed beyond the initial sequencing stage. Approximately one third of the SEREX-defined genes are novel.

Table 1 lists the categories of tumor antigens that have been identified to date. The prime example of a mutational antigen is p53 isolated from a case of colon cancer, showing the potential of SEREX to identify etiologically relevant gene products in cancer. Three SEREX-defined antigens, which we have called 3p antigens, are encoded for by the p21 region on chromosome 3 (25 and Gordan, J.D., and M.J. Scanlan, unpublished data), a region known to be a hot spot of genetic abnormalities in many cancer types. Although mutation may likely be the underlying mechanism for the immunogenicity of these antigens, no mutations have been detected as yet in the coding sequences of the 3p antigens. The classic differentiation antigen tyrosinase has been detected in SEREX, and other examples, including a gastrointestinal tract–related differentiation antigen galectin 4, have been identified (26). Several antigens coded for by amplified or overexpressed genes have also been identified, e.g., a new isoform of carbonic anhydrase in renal cancer (4), aldolase A (25) and eIF-4γ (24) in lung cancer, and galectin 9 in Hodgkin's disease (27), indicating that antigen overexpression can lead to immunogenicity, as it does in the case of HER2/neu (28).

A fascinating category of tumor antigens, first discovered in the analysis of T cell–recognized epitopes, has been referred to as cancer-testis (CT) antigens (Table 2). CT antigens are expressed by a variable proportion, ~10–40%, of a wide range of different human tumor types. In normal tissues, expression is highly restricted, with testis being the sole or predominant site of CT expression. The CT^+ cell type in the testis appears to be spermatogonia (33). Three antigens in this category, MAGE (3), BAGE (34), and GAGE (35), were initially identified as targets for cytotoxic T cells. HOM-MEL-40/SSX2, NY-ESO-1, SCP1, and CT7 were uncovered by SEREX analysis. In total, there are now seven genes or gene families belonging to the CT category, and four of them have been shown to be coded for by the X chromosome (MAGE, GAGE, SSX, and NY-ESO-1). Since no evidence has been obtained for mutation or other gene rearrangements involving CT-coding genes (the single exception to this is the characteristic t[X;18] translocation of synovial sarcoma that involves SSX genes (36), but this translocation is not found in other SSX ex-

Table 1. *Categories of SEREX-identified Human Tumor Antigens*

Antigen category	Examples	Tumor source	References
Mutational	p53	Colon cancer	26
3p	NY-LU-12	Lung cancer	25
Differentiation	Tyrosinase	Melanoma	4
	Galectin 4	Colon cancer	26
Amplified/overexpressed	Carbonic anhydrase	Renal cancer	4
	Galectin 9	Hodgkin's disease	27
	Aldolase A	Lung cancer	25
	eIF-4γ	Lung cancer	24
Retroviral	HERV-K10	Renal cancer	5
Splice variant	Restin	Hodgkin's disease	4
	NY-CO-38	Colon cancer	26
CT	(see Table 2)		

pressing cancers.), the likely explanation for expression of CT antigens in cancer cells is gene activation or derepression, and the well-known precedent for this in animal systems is the anomalous expression of TL antigens in the leukemias of TL^- mice (37). De Smet et al. (38) have correlated MAGE expression with the state of global hypomethylation generally associated with cancer and spermatogenesis. However, this cannot be the whole explanation because CT antigens are not coordinately expressed, and certain tumor types, such as colon cancer, rarely express known CT antigens. SCP1, the only CT antigen with a known function, is a synaptonemal complex protein involved in chromosome reduction during meiosis, and it is intriguing to speculate what role aberrant expression of a meiotic protein in a somatic cell plays in the origins and progression of cancer. CT7 is the most recent CT antigen defined by SEREX. (Because the function of only one CT antigen is known, a standardized nomenclature for these antigens has not been established. We have suggested that new CT antigens be numbered in the order of their discovery, e.g., CT7 for the seventh CT antigen or antigen family to be identified. In the case of CT antigens belonging to a multigene family, each member would be distinguished by a number following the CT designation, e.g., CT7.1, CT7.2, etc.) The CT7 gene encodes a protein with >1,000 amino acid residues, with the COOH terminus highly homologous to the MAGE-10, and other MAGE genes over a ~200 amino acid stretch. Sequences N-terminal to this segment, however, show no homology to the MAGE family. To find new members of the CT family, SEREX analysis is being extended to screening expression libraries derived from normal testis (30, 32) and tumor cell lines expressing one or more of the known CT antigens.

Future Directions. The rise in interest in T cell, particularly CTL, recognition of cancer over the past two decades has been associated with a corresponding diminution in attention paid to the humoral immune response to cancer. As the cellular and humoral immune systems work in concert, it would be surprising if cancer antigens induced only a cellular response and no antibodies. In fact, SEREX has taught us that antigens, such as MAGE and NY-ESO-1, elicit both cellular and humoral immune responses, and we

Table 2. *SEREX-identified CT Antigens*

Antigen	Chromosomal locus	No. of genes in family	References
MAGE-1	Xq28	13	3, 4, 29
MAGE-4a	Xq28		5, 29, and our unpublished data
SSX2	Xp11.2	5	4, 30
NY-ESO-1	Xq28	2	23, 31, and our unpublished data
SCP1	1p13	Unknown*	32
CT7	Unknown	1–2‡	Our unpublished data

*Southern blot analysis with a SCP1 probe suggests a multigene family.
‡Based on Southern blot and preliminary cloning results.

can expect that this will be the case for the broad generality of cancer antigens. In addition, as the development of high titered IgG requires CD4 T cell help, SEREX provides a direct route to the analysis of the CD4 T cell repertoire against tumor antigens. Although antibodies against intracellular proteins, in contrast to CTL against peptides derived from them, cannot generally be expected to have antitumor activity, antibodies have enormous advantages over CTL as probes for structural cloning and monitoring immune responses. The methodology for defining T cell–recognized tumor antigens has depended on stable T cell lines and established tumor cell lines, conditions that are frequently difficult to meet and virtually impossible in the case of certain tumor types. SEREX bypasses both these requirements. Accumulating knowledge about HLA-binding motifs provides a guide to identifying T cell–recognized peptides associated with SEREX-defined antigens, and Jäger et al. (7) have recently used this approach to define NY-ESO-1 peptides recognized by CTL from a patient with high NY-ESO-1 antibody titers. SEREX-defined antigens also provide targets to carry out broad scale serological surveys of antibody responses in normal individuals and patients with cancer. Stockert et al. (8) have conducted such a survey with a panel of tumor antigens, including several CT antigens, and found that NY-ESO-1 appears to be particularly immunogenic, with ~40–50% of patients with NY-ESO-1–expressing tumors developing an antibody response at some stage in the disease. With the rapid increase in the number of SEREX-defined antigens, the challenge is to choose those with the most promise for further analysis. Cancer-restricted immunogenicity, as indicated by a high frequency of antibody response in cancer patients, is one of the criteria we believe to be of greatest significance in this selection process. In this context, SEREX has awakened once again the old hope of finding antibody-based screening tests for general use in the diagnosis of cancer. Finally, SEREX-defined antigens are prime targets for cancer vaccine development. One of the obstacles confronting the development of maximally immunogenic vaccine, whether involving peptide, protein, DNA, or RNA, is monitoring the immune response to the vaccine. Although most current emphasis is on measuring CTL responses, serological assays may provide simple and reliable end points for monitoring the immunogenicity of cancer vaccines.

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