Brief Definitive Report

A Survey of the Humoral Immune Response of Cancer Patients to a Panel of Human Tumor Antigens

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Summary

Evidence is growing for both humoral and cellular immune recognition of human tumor antigens. Antibodies with specificity for antigens initially recognized by cytotoxic T lymphocytes (CTLs), e.g., MAGE and tyrosinase, have been detected in melanoma patient sera, and CTLs with specificity for NY-ESO-1, a cancer-testis (CT) antigen initially identified by autologous antibody, have recently been identified. To establish a screening system for the humoral response to autoimmunogenic tumor antigens, an enzyme-linked immunosorbent assay (ELISA) was developed using recombinant NY-ESO-1, MAGE-1, MAGE-3, SSX2, Melan-A, and tyrosinase proteins. A survey of sera from 234 cancer patients showed antibodies to NY-ESO-1 in 19 patients, to MAGE-1 in 3, to MAGE-3 in 2, and to SSX2 in 1 patient. No reactivity to these antigens was found in sera from 70 normal individuals. The frequency of NY-ESO-1 antibody was 9.4% in melanoma patients and 12.5% in ovarian cancer patients. Comparison of tumor NY-ESO-1 phenotype and NY-ESO-1 antibody response in 62 stage IV melanoma patients showed that all patients with NY-ESO-1^+ antibody had NY-ESO-1^+ tumors, and no patients with NY-ESO-1^- tumors had NY-ESO-1 antibody. As the proportion of melanomas expressing NY-ESO-1 is 20–40% and only patients with NY-ESO-1^+ tumors have antibody, this would suggest that a high percentage of patients with NY-ESO-1^+ tumors develop an antibody response to NY-ESO-1.

Analysis of the human immune response to cancer has had a long and complex history (1). Although serological methods dominated initial efforts to find evidence for immune recognition of cancer, advances in analyzing cell-mediated immunity have now permitted exploration of T cell recognition of human cancer (2). Interpreting the specificity of an observed humoral or cellular immune response to cancer cells has always been the critical issue in human tumor immunology. Test systems restricting the analysis to autologous systems, i.e., antibody or T cells from the same patient, eliminated the contribution of alloantigens and provided provocative evidence for humoral (3) and cellular (4) immunity to human cancer cells. However, the molecular cloning of tumor antigens recognized by CTLs (2) and antibodies (5) has opened a new era in tumor immunology, and the list of defined immunogenic human tumor antigens is growing rapidly. These antigens fall into one of the following categories: (*a*) cancer-testis (CT) antigens, e.g., MAGE (2), SSX2 (HOM-MEL-40; reference 5), and NY-ESO-1 (6), which are expressed in a variable proportion of a wide range of human tumors, but show a highly restricted expression pattern in normal tissues, namely testis; (*b*) antigens coded for by mutated genes, e.g., p53 (7) and CDK4 (8); (*c*) differentiation antigens, e.g., tyrosinase (9, 10) and Melan-A (11); (*d*) amplified gene products, e.g., HER2/neu (12) and carbonic anhydrase (5); and (*e*) viral antigens, e.g., retrovirus (13), HPV (14), and EBV (15). Although some of these antigens, e.g., MAGE and tyrosinase, were initially detected by CTLs and cloned via their CTL-recognized epitopes, they also elicit humoral immunity and can be identified and cloned by using antibodies from cancer patients (5, 16). This study initiates a survey of the human humoral immune response to a panel of those recently defined autoimmunogenic human tumor antigens.

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Materials and Methods

Tissues and Sera. Tumor tissues were obtained during routine surgical procedures, frozen in liquid nitrogen, and stored at −80°C until use. Human sera were obtained from patients with various tumor types and from normal blood donors and were stored at −80°C (IRB No. 87-20; Memorial Sloan-Kettering Cancer Center, New York; and IRB No. 0596-336, Cornell University Medical College, New York). The patients with melanoma, breast, and ovarian cancer had metastatic disease, whereas the majority of patients with lung and colon cancer had primary operable disease.

ELISA. 10 μl/well of a 1 μg/ml recombinant protein in coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6, with 0.02% NaN_3) was adsorbed to TC microwell plates 60 × 10 (Nunc, Roskilde, Denmark) overnight at 4°C. Plates were washed with PBS and blocked overnight at 4°C with 10 μl/well of 2% BSA/PBS. After washing, 10 μl/well of serum dilutions in 2% BSA was added and incubated for 2 h at room temperature. Plates were washed and 10 μl/well diluted secondary antibody/2% BSA was added (Goat anti–human IgG-AP; Southern Biotechnology, Birmingham, AL) and incubated for 1 h at room temperature. Plates were washed, incubated with 10 μl/well of substrate solution (Attophose substrate; JBL Scientific, San Louis Obispo, CA) for 25 min at room temperature, and immediately read (Cyto-Fluor 2350; Millipore, Bedford, MA). For the serological survey of human sera, sera were tested over a range of serial fourfold dilutions from 1:100 to 1:100,000. A positive reaction is defined as an OD value of a 1:400 diluted serum that exceeds the mean OD value of sera from normal donors ($n = 70$) by three standard deviations. Fig. 2 shows characteristic titration curves of negative and positive sera.

Reverse Transcription PCR. Messenger RNA (mRNA) expression of NY-ESO-1 in normal and malignant tissues was evaluated by RT-PCR assays as previously described (6, 17).

Expression of Recombinant Tumor Antigens in Escherichia coli. The tumor antigens listed in Table 1 were expressed in *E. coli* using histidine-tag–containing vector pQE9 (Qiagen, Chatsworth, CA). Various cDNA amplification primers were designed to encompass entire or partial coding sequences of these genes, corresponding to amino acid positions shown in Table 1. The induction of recombinant protein synthesis and subsequent purification by Ni^{+2} column were performed as described (17).

Immunoblotting Analysis. Serum antibody responses against the purified recombinant protein were tested by standard Western blot analysis (22) using 1 μg of the purified protein and human serum at 1:1,000, 1:10,000, and 1:100,000 dilutions, or with 1:50 diluted mouse mAb supernatants (see below). Goat anti–human IgG (Fc specific; Sigma Chemical Co., St. Louis, MO) diluted 1:10,000 and goat anti–mouse IgG (Bio-Rad, Hercules, CA) diluted 1:3,000 were used as secondary reagents.

Monoclonal Antibodies. A series of mAbs were generated against NY-ESO-1, MAGE-3, and SSX2 (HOM-MEL-40) using methods previously described (18). Three representative mAbs were used in this study, E978 for NY-ESO-1, M3-6 for MAGE-3, and HM498 for SSX2.

Expression of NY-ESO-1 in Baculovirus. The NY-ESO-1 cDNA insert from the pQE9 recombinant clone was released and subcloned in pBlue BacHis2A vector (Invitrogen, Carlsbad, CA) and positive clones were isolated. Transfection of Sf9 cells with pBlueBacHis2A/NY-ESO-1 and isolation of recombinant viruses were accomplished following the protocol from Invitrogen. Infection of insect cells was performed in IPL-41 medium with 10% fetal calf serum at a multiplicity of infection (MOI) of 20. Expression of recombinant NY-ESO-1/His-tag protein was confirmed by Western blot analysis with NY-ESO-1 E978 mAb, and purification was by Ni^{2+} affinity chromatography.

Results

Establishment of ELISA Systems for Tumor Antigen Typing. Table 1 lists the seven protein antigens included in the typing panel. NY-ESO-1 (full length), Melan-A, and SSX2 represent full-length products, whereas NY-ESO-1 (long and short), MAGE-1, MAGE-3, tyrosinase, and car-

Table 1. *Characteristics of Recombinant Tumor Antigens Used for Serological Analysis*

Recombinant protein	Amino acid No.		Amino acid position	Apparent mol wt*	References
	Full length	Included			
NY-ESO-1					
Full length	180	180	1–180	~22 kD	6
Long	180	171	10–180	~20 kD	6
Short	180	112	10–121	~14 kD	6
Baculovirus	180	171	10–180	~20 kD	6
MAGE-1	309	163	57–219	~20 kD	17
MAGE-3	309	163	57–219	~20 kD	2 and our unpublished data
Melan-A	118	118	1–118	~23 kD	18
Tyrosinase	529	452	23–474	~52 kD	19, 20
SSX2	188	188	1–188	~30 kD	21 and our unpublished data
Carbonic anhydrase	354	157	198–354	~15 kD	5 and our unpublished data

*By SDS–polyacrylamide gel electrophoresis under reducing conditions.

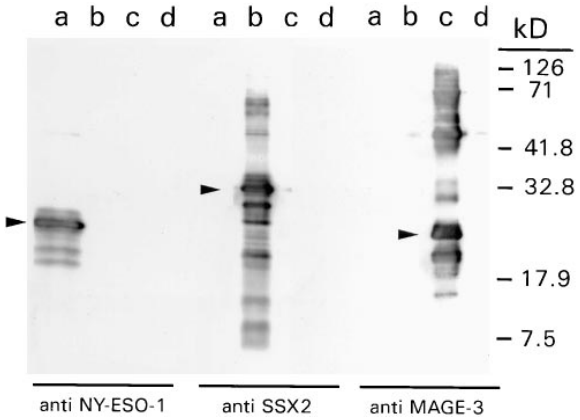

Figure 1. Western blot analysis of mouse mAbs against recombinant tumor antigens. NY-ESO-1 (full length), SSX2, MAGE-3, and carbonic anhydrase (lanes *a*, *b*, *c*, and *d*) were purified and reacted with mAbs against NY-ESO-1 (mAb E978), SSX2 (mAb HM498), and MAGE-3 (mAb M3-6), respectively. *Arrowheads*, the main reactive protein species in each lane, migrating at the expected mol wt of these proteins (see Table 1).

bonic anhydrase represent truncated products involving the internal region. These expressed proteins were used to generate mAbs, and prototype mAbs for each antigen were selected by specific reactivity in ELISA and Western blots. mAbs against MAGE-1, tyrosinase, and Melan-A have been previously reported (17–19), and detailed characterization of NY-ESO-1, MAGE-3, SSX2, and carbonic anhydrase mAbs will be published elsewhere. Each of the mAbs showed specificity for the immunizing antigen and did not cross-react with the other antigens in the panel. Fig. 1 shows the reactivity pattern of the NY-ESO-1, MAGE-3, and SSX2 mAbs used as typing reagents for standardizing and optimizing conditions for ELISA, e.g., protein concentration, conditions for antigen adsorption, test antigen stability, blocking, and washing conditions.

Survey of Human Sera for Antibodies to the Panel of Human Tumor Antigens. Table 2 summarizes the results with 234 sera from patients with cancer and 70 from normal individuals and Fig. 2 illustrates titrations of sera from selected individuals. Positive sera were tested at least three times on the seven antigens, and most negative sera were tested twice. Absorbing reactive sera with lysates of *E. coli* and bacteriophages did not reduce serum titers, nor did it affect the background reactivity of unreactive sera. A small fraction of sera in this series (one colon cancer, one ovarian cancer, four melanomas, and two normal blood donors) showed a nonspecific reactivity pattern with the entire antigen panel and were easily distinguished and eliminated. These non-specifically reactive sera also bound strongly to the assay plates in the absence of adsorbed protein. Our survey showed that 9.4% (12/127) of melanoma patients, 12.5% (4/32) of ovarian cancer patients, 4.2% (1/24) of patients with lung cancer, and 7.7% (2/26) of patients with breast cancer have antibody against NY-ESO-1. No specific antibody reactivity to NY-ESO-1 was detected in sera of 25 patients with colon cancer and in 70 normal human sera. MAGE-1 antibodies were found in three patients in this study, one with melanoma, one with ovarian cancer, and one with lung cancer, MAGE-3 antibody was found in two patients with melanoma, and SSX2 antibody was found in one patient with melanoma. No antibody against Melan-A, tyrosinase, or carbonic anhydrase was found.

Reactivity of Human NY-ESO-1 Antibodies with Recombinant NY-ESO-1 Protein Produced in Baculovirus. NY-ESO-1 produced by baculovirus was as reactive as NY-ESO-1 of bacterial origin in tests with human sera. Reactivity with mouse mAb against NY-ESO-1 also showed that the bacterial and baculovirus NY-ESO-1 products were equally recognized.

Correlation of NY-ESO-1 Expression and Presence of NY-ESO-1 Antibodies. Fresh-frozen tumor specimens and serum samples were available from 62 patients with melanoma. All patients had a history of metastatic melanoma for >2 yr before serum and tumor samples were collected, and had undergone different chemo- and immunotherapeutic regimens. Tumors were typed for NY-ESO-1 expression by RT-PCR and sera were assayed for NY-ESO-1 antibody by ELISA and by Western blotting (Table 3 and Fig. 3). In this series of 62 patients, 15 had NY-ESO-1 positive tumors and 8 of these patients had NY-ESO-1 antibodies.

Table 2. *Survey of Sera from 70 Normal Blood Donors and 234 Cancer Patients: ELISA Reactivity with Recombinant Tumor Antigens*

		Recombinant tumor antigens						
	Number of sera tested	NY-ESO-1	MAGE-1	MAGE-3	SSX2	Melan-A	Tyrosinase	Carbonic anhydrase
Blood donors	70	0	0	0	0	0	0	0
Cancer patients								
Melanoma	127	12	1	2	1	0	0	0
Ovarian	32	4	1	0	0	0	0	0
Lung	24	1	1	0	0	0	0	0
Breast	26	2	0	0	0	0	0	0
Colon	25	0	0	0	0	0	0	0

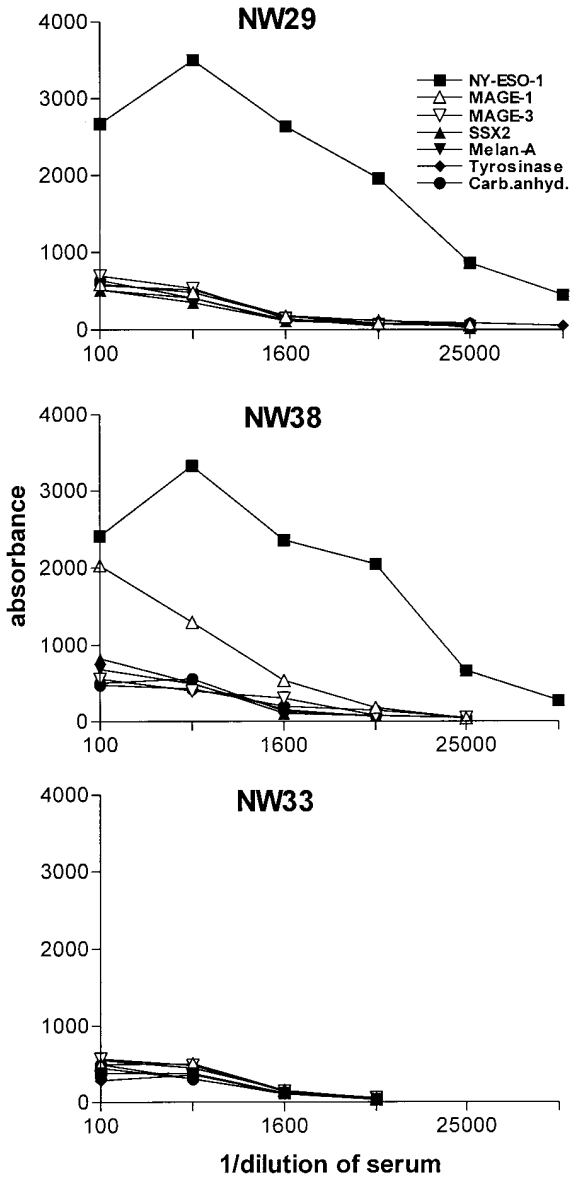

Figure 2. Representative results of ELISA reactivity with sera from melanoma patients NW29, NW38, and NW33, against a panel of seven recombinant tumor antigens.

The two NY-ESO-1 detection systems, ELISA and Western blotting, gave identical results. No NY-ESO-1 antibody was detected in patients with NY-ESO-1 negative tumors. Seven patients had NY-ESO-1 positive tumors and no detectable NY-ESO-1 antibody. Although the RT-PCR analysis was not designed to be a quantitative assay, the PCR signals in this group of tumors appeared to be lower than the signal in NY-ESO-1 positive tumors from NY-ESO-1 antibody positive patients.

Discussion

Based on the recognition by humoral or cellular immune responses in the autologous human host, a number of human antigens have been identified (2, 5, 6, 16, 23). These antigens provide attractive new targets for vaccine-based therapies, and a range of different strategies including peptide, protein, RNA, DNA, and viral vector vaccines are being pursued. The availability of these cloned tumor antigens also permits the development of serological or cell-based assays for screening human populations for specific antibody or T cell responses. Because a number of these antigens appear to be recognized by humoral and/or cellular immune reactions, we have chosen serological assays to initiate this survey because of their greater simplicity and speed. The ELISA screening system established in this study has been standardized using mouse mAbs with specificity for each of the antigens. In tests of human sera, the use of different antigens prepared in the same bacterial expression system provides a critical internal specificity control to eliminate reactions directed against contaminating bacteria and phage proteins. To control for the influence of protein folding and glycosylation on antibody detection, the reactivity of tumor antigens purified from mammalian sources will be compared with the corresponding antigens produced in bacterial systems.

Of the seven antigens tested in this study, antibodies to NY-ESO-1 were observed most frequently. Reactivity to NY-ESO-1was found in ~10% of patients with melanoma and ovarian cancer and in lung and breast cancer with a lower frequency. No reactivity was found in the sera of patients with colon cancer. MAGE-1 reactivity was found in one patient each with melanoma, ovarian, or lung cancer, MAGE-3 reactivity was found in two patients with melanoma, and SSX2 was found in one patient with melanoma. In the study of Sahin et al., SSX2 antibodies were found in 2 of 11 patients with melanoma using a plaque assay for antibody detection (5), and we are currently comparing the sensitivity of ELISA and plaque assays. The sera from 70 healthy blood donors were negative with all seven antigens. Hoon et al. (24) reported that MAGE-1 antibodies were frequently found in normal individuals as well as in melanoma patients, and that the titer of MAGE-1 increases after vaccination with MAGE-1^+ tumor cells. This discrepancy between our results and Hoon et al. (24) may reflect differences in the assay system or the MAGE-1 antigen constructs.

Table 3. *Correlation between NY-ESO-1 mRNA Expression and NY-ESO-1 Antibody Response in 62 Melanoma Patients**

RT-PCR typing	Antibody detection system		Number of cases
	ELISA	Western blot	
+	+	+	8
+	−	−	7
−	−	−	47
−	+	+	0

*These correlations are statistically highly significant (Fischer's exact test, $P < 0.001$).

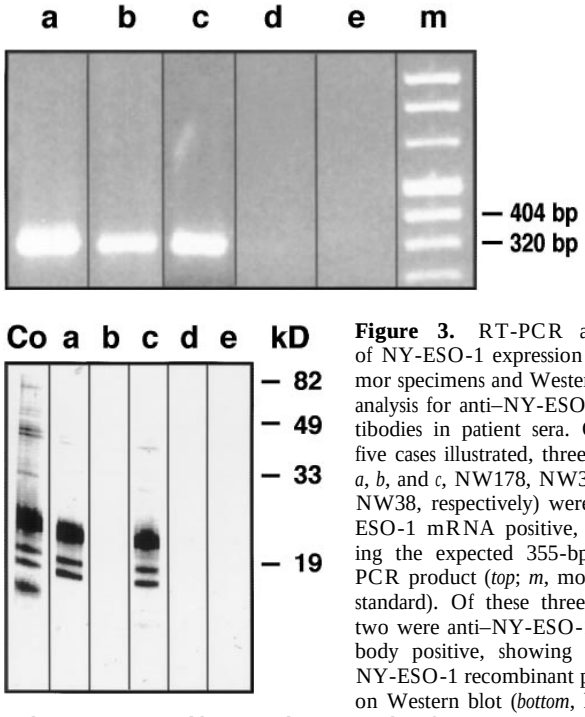

Figure 3. RT-PCR analysis of NY-ESO-1 expression in tumor specimens and Western blot analysis for anti–NY-ESO-1 antibodies in patient sera. Of the five cases illustrated, three (lanes *a*, *b*, and *c*, NW178, NW33, and NW38, respectively) were NY-ESO-1 mRNA positive, showing the expected 355-bp RT-PCR product (*top*; *m*, molecular standard). Of these three cases, two were anti–NY-ESO-1 antibody positive, showing 22-kD NY-ESO-1 recombinant protein on Western blot (*bottom*, lanes *a* and *c*, 1:1000 serum dilution), whereas one (lane *b*) was negative. Positive control on Western blot was provided by using anti–NY-ESO-1 mouse mAb (lane *Co*). Two cases (lanes *d* and *e*, NW309 and NW145) were negative for both NY-ESO-1 mRNA and anti–NY-ESO-1 antibody. ELISA and Western blotting gave identical results.

In our initial study, we found NY-ESO-1 mRNA in 20–40% of melanomas (6). As 10% of unselected melanoma patients have NY-ESO-1 antibody (Table 2), this would suggest that ~50% of patients with NY-ESO-1 positive melanomas develop NY-ESO-1 antibodies at some time during the course of their disease. To make a direct test of this relationship, we studied a series of 62 patients with stage IV melanomas, where tumor and serum specimens from the same patient were available and compared the NY-ESO-1 antibody status with NY-ESO-1 mRNA expression in the autologous tumor. The results showed that NY-ESO-1 antibodies were found in patients with NY-ESO-1 positive tumors, and that no patients with NY-ESO-1 negative tumors had NY-ESO-1 antibody. However, there were seven patients with NY-ESO-1 positive tumors with no detectable antibody, suggesting that these patients did not form antibody, that they made antibody not detectable by ELISA or immunoblotting, or that they developed cellular but not humoral immunity against NY-ESO-1. Analyses of NY-ESO-1 antibody status and patient characteristics (sex, stage, state, and extent of disease and previous therapies) has not revealed any correlation with serological status, but this will need more extensive study, particularly with sera from patients with less advanced disease.

To explore the relationship between humoral and cellular immune recognition of NY-ESO-1, CD4 and CD8 T cell responses to NY-ESO-1 also need to be evaluated. We have recently described a patient with high NY-ESO-1 antibody titers and strong CTL reactivity against autologous melanoma cells (25). Transfection experiments showed that NY-ESO-1 coded for the CTL-recognized antigen, and using motif analysis, NY-ESO-1-related peptides were synthesized that were efficiently recognized by the CTLs. This patient showed that strong humoral and cellular immunity to a tumor antigen can coexist in the same patient.

One of the major challenges confronting the clinical testing of vaccines is the availability of reliable assays that can monitor specific immune responses to the vaccine as a way to guide the development of maximally immunogenic vaccines. With regard to vaccines aimed at eliciting cytotoxic antibodies to cell surface antigens, such as GM2, sensitive and specific assays have been developed (26). However, monitoring the immune response to vaccines aimed at eliciting cellular immunity, particularly CTL generation, has been problematic and difficult to interpret. No CTL responses were seen in vaccine trials with MAGE peptides (27), and even though CTL responses can be elicited in patients immunized with peptides derived from melanoma-associated differentiation antigens such as Melan-A or tyrosinase, nonimmunized normal individuals can also generate CTL responses to these antigens (28, 29). The presence of high titered NY-ESO-1 antibodies in patients with melanoma, ovarian, lung, and breast cancer indicates CD4 recognition of NY-ESO-1, and serological tests therefore provide a way to monitor the CD4 repertoire to tumor antigens. Should other patients with NY-ESO-1 antibody also have a specific CTL response, as we have already shown in one patient (25), serological tests may be useful as a way to identify patients with a CTL response to NY-ESO-1.

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