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Urine antibody against human cancer antigen NY-ESO-1

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Abstract

NY-ESO-1 is one of the most immunogenic tumor antigens known to date. Spontaneous humoral and cellular immune responses against NY-ESO-1 are detected in a substantial proportion of patients with NY-ESO-1 positive cancers. NY-ESO-1 serum antibody is dependent on the presence of NY-ESO-1+ cancer cells, and antibody titers correlate with the clinical development of the disease. NY-ESO-1 serum antibody is associated with detectable NY-ESO-1-specific CD8+ T cell reactivity. High titers of NY-ESO-1 serum antibodies are found in patients with advanced NY-ESO-1+ malignancies. Urine samples of seropositive patients with normal kidney function were tested for NY-ESO-1 antibody by Western blotting and enzyme-linked immunosorbent assay (ELISA). Antibodies to NY-ESO-1 were found in the urine of patients whose NY-ESO-1 serum antibody titers were 1:10,000 or higher by Western blotting. In patients with weak (positive at 1:250, negative at 1:1,000) or no reactivity, urine antibody was not detectable. No urine NY-ESO-1 antibody was found in patients without detectable NY-ESO-1 serum antibody. Our results show that urine analysis for NY-ESO-1 antibody identifies patients with strong NY-ESO-1 immunity. Urine antibody detection may also be of value in the monitoring of spontaneous and vaccine-induced immunity against other defined tumor antigens.

Introduction

Major progress has been made in the analysis of immune responses against cancer through the molecular characterization of human cancer antigens. Based on the specific recognition by humoral or cellular effectors of the immune system, a large number of tumor-associated antigens has been identified (1, 2, 3). They can be categorized according to their expression patterns, function or origin into 6 groups: (i) Cancer-Testis (CT) antigens, i.e. MAGE, NY-ESO-1, LAGE1, SSX, which are expressed in a range of different tumor types and normal germ cells (4, 5, 6, 7, 8); (ii) differentiation antigens, i.e. Melan A/MART-1, tyrosinase, gp100, NY-BR-1,

which are expressed in cancer cells and the corresponding normal tissue (9, 10, 11, 12, 13); (iii) mutated normal genes, i.e. p53, MUM-1, CDK-4 (14, 15, 16); (iv) overexpressed `self´ antigens, i.e. HER2/neu, p53 (16, 17); (v) viral antigens, i.e. EBV, HPV (18, 19) and (vi) splice variants of normal genes, i.e. ING1, NY-CO-37, NY-CO-38 (20, 21). Most of these antigens were initially defined by either cellular or humoral immune responses. Some of these were later shown to elicit integrated immune responses involving both humoral and cellular effectors (22, 23, 24).

The CT antigen NY-ESO-1, initially identified by serological expression cloning of a recombinant cDNA library obtained from a squamous cell carcinoma of the esophagus, elicits both humoral and cellular immune responses in patients with NY-ESO-1+ cancers (8, 23, 25, 26). The presence of NY-ESO-1 serum antibody was found to be predictive of CD8+ T cell responses against NY-ESO-1-derived peptide epitopes presented by different MHC class I alleles (23, 27). CD4+ T cell responses to MHC class II-presented peptides were also found in patients with serum antibody to NY-ESO-1 (28, 29). Spontaneous changes in NY-ESO-1 antibody titers analyzed over extended periods of time were directly correlated with the clinical development of NY-ESO-1+ disease (25). Because specific IgG antibody in urine has been reported in various infectious diseases (30, 31, 32, 33), we investigated whether NY-ESO-1 antibody could be detected in urine. Our results have implications for the development of urine-based tests for the monitoring of antibody responses against NY-ESO-1 and other cancer antigens.

Results

NY-ESO-1 serum antibody

Western blot assays and ELISAs have previously been standardized to detect NY-ESO-1 serum antibody. Both methods yield consistent results for large series of serum samples (25, 26). In this study, NY-ESO-1 serum antibody reactivity was assessed primarily by Western blot analysis. Of 47 patients tested, 21 had a strong NY-ESO-1 serum antibody reactivity detectable at a serum dilution of 1:10,000 (Table 1). Seven patients had weak serum antibody reactivity (faint bands at serum dilutions of 1:250, negative at 1:1,000), and 19 patients had no detectable NY-ESO-1 serum antibody. ELISA and Western blotting was performed in parallel on a group of sera. Sera with strong reactivity in Western blotting were positive in ELISA, while sera with weak reactivity in Western blotting were negative in ELISA (data not shown).

NY-ESO-1 urine antibody

Urine samples of all 47 patients were tested for NY-ESO-1 antibody by Western blot. The results presented in Table 1 show that strong NY-ESO-1 serum antibody reactivity (at a dilution of 1:10,000) was associated with detectable NY-ESO-1 urine antibody in all 21 patients at urine dilutions of 1:1 and 1:100. Western blot analysis of serum and urine samples for patients with strong, weak and no serum reactivity is shown in Figure 1. No NY-ESO-1 urine antibody was found when serum reactivity was weak or negative. Since changes in NY-ESO-1 serum antibody titers over time were observed for some patients, the serum and urine samples analyzed in this study were collected on the same day or within 4 weeks. ELISA results were uninterpretable for most urine samples due to high background reactivity, though a low specific reaction with urine was seen for a few patients with antibody-positive serum. Patients with weak or negative serum reactivity in Western blots had negative ELISA results for both serum and urine samples.

Table 1. NY-ESO-1 serum and urine antibody status in patients with advanced NY-ESO-1+ cancers[a].

Patient	Primary Cancer	Metastases	Serum Ab	Urine Ab
NW29	melanoma	lymph nodes	++	+
NW923	urothelial	liver	++	+
NW1073	soft tissue sarcoma	liver	++	+
NW1106	melanoma	lung, lymph nodes	++	+
NW1123	breast	lung, liver	++	+
NW1231	thyroid	pleura	++	+
NW1288	stomach	liver	++	+
NW1310	breast	brain, bone	++	+
NW1418	breast	skin, lung	++	+
NW1453	melanoma	lymph nodes	++	+
NW1474	leiomyosarcoma	peritoneum	++	+
NW1496	multiple myeloma	bone	++	+
NW1539	melanoma	brain, lymph node	++	+
NW1558	ovary	peritoneum	++	+
NW1567	ovary	peritoneum	++	+
NW1620	soft tissue sarcoma	peritoneum	++	+
NW1622	NSCLC	liver	++	+
NW1624	stomach	lymph nodes	++	+
NW1714	prostate	bone	++	+
NW1715	NSCLC	lung, lymph nodes	++	+
NW1716	ovary	peritoneum	++	+
NW1241	NEC	liver	+	-
NW1312	NHL	lymph nodes	+	-
NW1366	ovary	peritoneum	+	-
NW1374	SCLC	lung	+	-
NW1621	NSCLC	lung	+	-
NW1717	prostate	bone	+	-
NW1718	NSCLC	pleura, lung	+	-
NW475	melanoma	lymph nodes	-	-
NW555	melanoma	liver, lung	-	-
NW597	melanoma	bone	-	-
NW846	soft tissue sarcoma	lymph nodes, bone	-	-
NW886	melanoma	lung	-	-
NW1045	melanoma	lung	-	-
NW1071	NSCLC	lung	-	-
NW1189	breast	lymph nodes	-	-
NW1409	CAC	liver	-	-
NW1431	prostate	bone	-	-
NW1439	prostate	bone	-	-
NW1451	NSCLC	lung	-	-
NW1497	melanoma	lung, lymph nodes	-	-
NW1719	prostate	bone	-	-
NW1720	soft tissue sarcoma	peritoneum	-	-
NW1721	melanoma	lung	-	-
NW1722	NSCLC	lung	-	-
NW1723	NSCLC	lung	-	-
NW1724	melanoma	brain, bone	-	-

[a]Sera were analyzed for the presence of NY-ESO-1 antibody by Western blotting at dilutions ranging from 1:250 to 1:10,000. Antibody reactivity was considered strong (++) when detectable at a serum dilution of 1:10,000, weak (+) when detectable at a serum dilution 1:250, and negative (-) when no reactivity was detected. Urine samples were assessed at dilutions ranging from 1:1 to 1:1,000 for the presence of NY-ESO-1 antibody. Antibody reactivity was considered positive (+) when detectable at a urine dilution of 1:100 and negative (-) when no reactivity was detected in the undiluted sample. Abbreviations: NSCLC, non-small cell lung cancer; NEC, neuro-endocrine carcinoma; NHL, non-Hodgkin's lymphoma; SCLC, small cell lung cancer; CAC, cholangiocellular carcinoma.

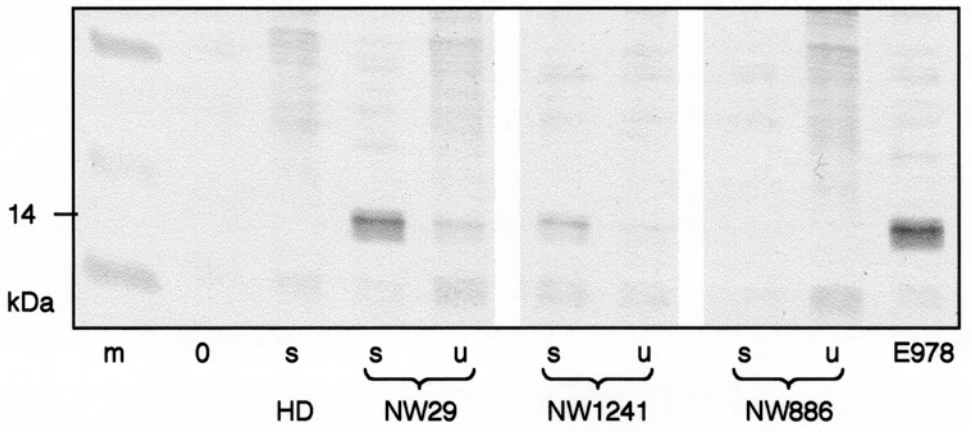

Figure 1. Western blot analysis of serum and urine samples. These are shown for a patient with strong (NW29), weak (NW1241), and negative (NW886) NY-ESO-1 serum antibody reactivity. Blots with 1 µg/lane recombinant NY-ESO-1 `short´ protein (14 kDa) were probed with serum (s; 1:1,000 dilution), urine (u; 1:100 dilution) and the E978 mouse monoclonal antibody as positive control (1:250 dilution). As negative controls, no serum (0) and serum obtained from a healthy donor (HD) were used at a 1:250 dilution.

To exclude non-specific reactivity with the recombinant NY-ESO-1 protein, sera and urine samples were also tested against naturally produced NY-ESO-1 protein in cell lysates. Figure 2 shows a Western blot using lysates of COS-7 cells transfected with NY-ESO-1 and different urine dilutions. A Western blot using lysates of the NY-ESO-1-expressing melanoma cell line NW-MEL-38 and serum and urine samples from the same patient is shown in Figure 3.

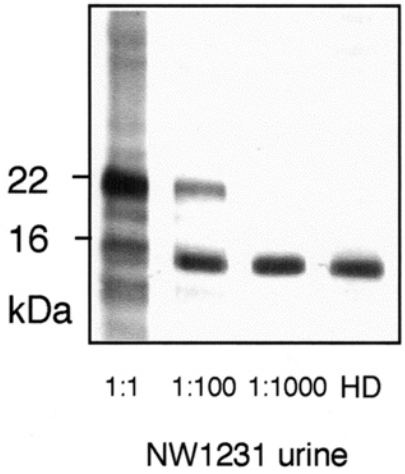

Figure 2. Western blot analysis of urine from a patient with NY-ESO-1+ thyroid cancer (NW1231). The urine, diluted 1:1, 1:100, and 1:1,000, was used to probe a blot with the lysate of $5x10^6$ COS-7 cells transfected with NY-ESO-1 and thus containing naturally produced NY-ESO-1 protein (22 kDa). NY-ESO-1 urine antibody was detected at urine dilutions of 1:1 and 1:100. Urine from a healthy donor (HD) at a dilution of 1:1 served as a negative control. All samples tested show non-specific reactivity with a COS-7 cell component (16 kDa).

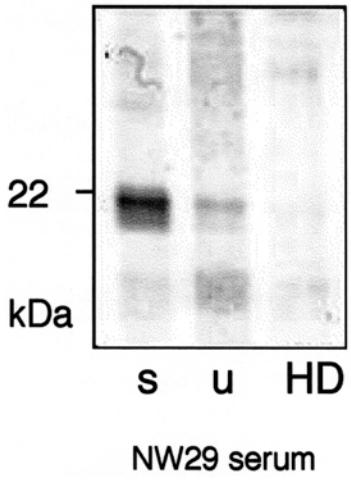

Figure 3. Western blot analysis of samples from melanoma patient NW29. A lysate of $5x10^6$ NW-MEL-38 melanoma cells, which naturally express NY-ESO-1 (22 kDa), was used for the Western blot. Serum (s; dilution 1:250) and urine (u; dilution 1:1) samples from melanoma patient NW29 were tested for the presence of NY-ESO-1 antibody. Serum from a healthy donor (HD) at a dilution of 1:250 served as a negative control.

Discussion

Humoral and cellular immune responses against human tumor antigens have been observed in patients with different types of cancer (34). The CT antigen NY-ESO-1 is one of the most immunogenic tumor antigens, eliciting spontaneous immune responses in a substantial proportion of patients with NY-ESO-1+ cancers. NY-ESO-1 serum antibody is a reliable indicator for NY-ESO-1-specific CD8+ T cell responses, and antibody titers correlate with the clinical course of NY-ESO-1+ malignancies. Therefore, the assessment of NY-ESO-1 serum antibody has become an important tool to monitor spontaneous and vaccine-induced immunity to NY-ESO-1.

NY-ESO-1 urine antibody was detected in cancer patients who had strong serum reactivity. Because of the high non-specific background reactivity often observed in ELISA with urine samples, Western blotting was the more reliable method for the assessment of NY-ESO-1 urine antibody. Nephropathy was excluded as a possible reason for increased renal protein loss in all patients. In accordance with previous studies (35), strong NY-ESO-1 antibody reactivity correlated with NY-ESO-1-specific CD8+ T cell reactivity. All eight HLA-A2+ NY-ESO-1 serum and urine antibody-positive patients tested showed a strong NY-ESO-1-specific CD8+ T cell reactivity (data not shown). In contrast, none of the patients with weak (3 patients tested) or no NY-ESO-1 serum and urine antibody reactivity (5 patients tested) had detectable CD8+ T cell reactivity. There was no correlation between clinical features (metastatic sites, treatment) and NY-ESO-1 serum and urine antibody status for the patients with NY-ESO-1+ malignancies analyzed in this study. However, detectable serum antibody titers were found to correlate with the clinical evolution of NY-ESO-1 positive disease in individual patients over time (25). As an easily accessible source of material, urine can now be included as a monitoring element in assessing changes in NY-ESO-1 antibody status.

High-titered antibody responses against several infectious diseases, such as HIV, infection with *Helicobacter pylori*, schistosomiasis, have been found to be associated with detectable levels of urine antibody in patients with normal renal function (30, 31, 32, 33). Based on the high concordance of serum and urine antibody determinations, urine-based tests are increasingly being considered for antibody screening because they are non-invasive, painless, and easy to administer. Screening tests have been developed which facilitate the rapid detection of urine antibodies with defined specificity for the monitoring of patients at risk for infection.

Based on the high concordance of NY-ESO-1 serum and urine antibody in strongly seropositive patients, we are extending the search for urine antibody reactivity to other defined human cancer antigens that frequently induce humoral immune responses (i.e. p53). If urine excretion of antibodies against cancer antigens is not restricted to NY-ESO-1, urine-based test systems may be of value for the monitoring or follow-up of spontaneous and vaccine-induced humoral immune responses against the different cancer antigens expressed by individual tumors. Furthermore, the search for new cancer antigens may be improved by screening tumor-derived cDNA expression libraries with autologous or allogeneic urine samples, which may represent a source of high-titered antibody responses in individual patients.

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Materials and methods

Patients

All patients in this study were treated in the outpatient department of II. Medizinische Klinik at Krankenhaus Nordwest in Frankfurt. Forty-seven patients with advanced NY-ESO-1+ malignancies were selected for the assessment of NY-ESO-1 serum and urine antibody. The distribution of patients was as follows: melanoma (12), prostate cancer (5), breast cancer (4), stomach cancer (2), non-small cell lung cancer (8), ovarian cancer (4), soft tissue sarcoma (4), leiomyosarcoma (1), cholangiocarcinoma (1), non-Hodgkin's lymphoma (1), small cell carcinoma (1), multiple myeloma (1), bladder cancer (1), thyroid cancer (1), and neuroendocrine carcinoma (1). Multiple serum and urine samples were collected from the patients. Serum and urine samples tested in this analysis were taken on the same day or within 4 weeks.

Cell culture

The tumor cell line NW-MEL-38 was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10 mM HEPES buffer, L-arginine (84 mg/l), L-glutamine (584 mg/l), penicillin (10 IU/ml), streptomycin (100 µg/ml), and 10% FCS (23).

Eukaryotic expression of NY-ESO-1

The full-length coding sequence of NY-ESO-1 was cloned into the pcDNA3.1(-) expression vector (Invitrogen), and this construct was used for the transfection of COS-7 cells as described (23).

Immunoblot Analysis

Serum and urine antibody responses against the recombinant NY-ESO-1 protein were tested by standard Western blot analysis (36). Briefly, 1 µg of purified recombinant NY-ESO-1 protein was diluted in SDS and

electrophoresed on a 15% SDS polyacrylamide gel. In all assays, the recombinant NY-ESO-1 `short´ protein (amino acids 10-121; molecular weight 14 kDa) was used, as we have previously shown that the full length and recombinant NY-ESO-1 `short´ proteins are recognized equally well by serum IgG antibodies (26). After overnight blotting on a nitrocellulose filter (0.45 µm, Sartorius, Göttingen) and blocking with 3% BSA in TBS, blots were incubated with either patients' sera at 1:250, 1:1,000, and 1:10,000 dilution, patients' urine at 1:1, 1:100, 1:250, and 1:1,000 dilution, or the E978 mouse monoclonal antibody (1:250 dilution) against NY-ESO-1 as a positive control (26). To show that recognition of the recombinant NY-ESO-1 `short´ protein (14 kDa) by serum and urine IgG antibodies corresponds to recognition of the naturally expressed NY-ESO-1 protein (22 kDa), 5 µl lysate of the NY-ESO-1-expressing melanoma cell line NW-MEL-38 or of COS-7 cells transfected with NY-ESO-1 were used. Serum and urine antibodies binding to NY-ESO-1 were detected by incubation with goat anti-human IgG (Fc-spec.; Sigma Chemical Co., St. Louis, MO) diluted 1:10,000, or in the case of E978 with goat anti-mouse IgG (Bio-Rad, Hercules, CA) diluted 1:3,000, and visualized with NBT/X-phosphate (Sigma). Serum and urine samples were considered positive for NY-ESO-1 antibody when reactivity with the 14 kDa recombinant NY-ESO-1 `short´ protein and/or the naturally expressed 22 kDa NY-ESO-1 protein was detectable.

ELISA

Recombinant NY-ESO-1 `short´ protein at a concentration of 1 µg/ml 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 6 x 10 (Nunc, Roskilde, Denmark) overnight at 4°C at 10 µl/well. Plates were washed with PBS and blocked overnight at 4°C with 10 µl/well 2% BSA in PBS. After washing, serum and urine dilutions in 2% BSA (10 µl/well) were added and the plates incubated for 2 h at room temperature. The plates were then washed, 10 µl/well diluted secondary antibody in 2% BSA added (goat anti-human IgG-AP; Southern Biotechnology, Birmingham, AL), and the plates incubated for 1 h at room temperature. The plates were then washed, incubated with 10 µl/well substrate solution (AttoPhos® substrate; JBL Scientific, San Louis Obispo, CA) for 25 min at room temperature and the fluorescence immediately determined (Cytofluor 2350; Millipore, Bedford, MA). Four-fold dilutions of sera, ranging from 1:100 to 1:100,000, and of urine samples, ranging from 1:1 to 1:250, were tested as described previously (26).

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