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Circulating melanoma-associated antigen detected by monoclonal antibody NKI/C-3

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Summary. NKI/C-3 and NKI/black-13 are monoclonal antibodies recognizing different epitopes on a melanoma-associated antigen that is preserved after fixation in formalin and embedding in paraffin in virtually all melanoma tissues. The antigen, a predominantly cytoplasmic vesicle membrane-bound heterogeneous glycoprotein of $25-110\times10^3$ daltons, was shown to be a single 25×10^3 dalton polypeptide when incorporation of N-linked carbohydrates was inhibited by tunicamycin. The antigen was measured in a double determinant enzyme immunoassay (DDEIA) using NKI/C-3 as catcher antibody. Results from in vitro experiments indicated that the antigen is actively shed from living cells. In sera from melanoma patients with a small tumor burden, the antigen concentrations were in the range of those of controls (0–22 U/ml). Significantly increased values (33–600 U/ml) were found in sera from patients with a moderate or large tumor burden. The antigen concentrations in sera from patients with multiple metastases of other tumors were within the range of controls. Several sera from patients with multiple metastases of colon, pancreatic, and stomach carcinoma, however, contained increased antigen concentrations (45–80 U/ml). These results correspond with the reactions of NKI/C-3 in tissue sections of some malignancies other than melanoma.

During the follow-up of melanoma patients the concentrations of circulating antigen correlated with tumor progression. The predictive value of the NKI/C-3 assay was no better than determination of serum lactate dehydrogenase, alkaline phosphatase or gamma glutamyl transferase activity.

Introduction

Recently we have described a monoclonal antibody (NKI/C-3) that recognizes a melanoma-associated antigen in formalin-fixed paraffin-embedded tissue sections [28]. This antibody reacted in the immunoperoxidase test with more than 90% of primary and metastatic melanomas [13, 20, 23, 27, 28]. Usually, a strong staining of the melanoma cells is observed, although heterogeneity of staining has also been described [13, 27]. The antigen is a heterogeneous $25-110\times10^3$ dalton glycoprotein which is stable to relatively extreme conditions like dilute acid and dilute alkali as well as heating at 100 °C [28]. In the present study we have examined whether the antigen detected by NKI/C-3 could serve as a reliable melanoma-associated serum marker. A double determinant enzyme immunoassay (DDEIA) was used with a second monoclonal antibody (NKI/black-13) directed against a different epitope on the antigen recognized by NKI/C-3. We also investigated the cause of the heterogeneity of the antigen by analyzing the antigenic peptide as it is synthesized in melanoma cells before the incorporation of N-linked carbohydrates.

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Material and methods

Preparation of monoclonal antibody NKI/black-13. A smooth membrane fraction of a fresh lymph node metastasis of melanoma was prepared as described elsewhere [28], and used to immunize a BALB/c mouse with 0.1 mg protein emulsified in complete Freund's adjuvant by an i.p. and a s.c. injection on day 0. Booster injections of 0.1 mg protein were given on day 7 i.p. (emulsified in incomplete Freund's adjuvant), day 14 s.c., and days 74, 75, and 76 i.v. Then 2 days later the spleen cells of the mouse were fused with the mouse myeloma cell line Sp 2/0 [7]. The supernatants were initially screened in an enzyme immunoassay (EIA [28]) for reactivity with Nonidet P-40 (NP-40) lysates of membranes of melanoma tissue, as well as for absence of reactivity with NP-40 lysates of membranes of buffy coat cells (prepared from a pool of 20 donors) and NP-40 lysates of ghosts of AB-positive erythrocytes (prepared according to Harris [14]). The hybridoma producing NKI/black-13 was one of the cultures that were selected in a second screening on the basis of production of monoclonal antibodies reacting with melanoma cells in both cryostat and formalin-fixed paraffin-embedded tissue sections, but not with lymphocytes, erythrocytes, or non-malignant tissue in the melanoma sections. After two additional cloning procedures (1 cell/well) a stable production of monoclonal antibody was achieved. The antibody was of the IgG2a isotype as determined in an Ouchterlony immunodiffusion test with antisera against different mouse immunoglobulin isotypes (IgM, IgG1, IgG2a, IgG2b and IgG3; Littton Bionetics, Kensington, Md., USA).

Purification and biotinylation of monoclonal antibodies. Serum or ascites containing high concentrations of monoclonal antibody were obtained between 10 and 14 days after inoculation of pristane-primed BALB/c mice with 4×10^6

viable hybridoma cells producing either NKI/C-3 [28] or NKI/black-13. IgG was precipitated repeatedly with ammonium sulfate and dissolved in phosphate buffered saline (PBS) [15]. The final precipitate was free of non-IgG protein contamination as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

The purified antibodies were labeled with biotin according to the procedure described by Bayer et al. [2], except that 25 moles biotinyl-N-hydroxysuccinimide ester per mol IgG were used [4].

Binding assays. Recognition sites for NKI/C-3 and NKI/black-13 were tested with an additive EIA according to Friguet et al. [8] using the test system described earlier [28]. In addition, an EIA was used where binding of biotinylated monoclonal antibody to solid phase bound antigen was inhibited by unlabeled antibody, the binding of biotinylated antibody being tested with avidin and biotinylated peroxidase as described below for the DDEIA. The affinity constants of NKI/C-3 and NKI/black-13 were determined as described by Friguet et al. [9], assuming a mean molecular weight of 47.5×10^3 daltons for the antigen (see Fig. 1).

For the DDEIA the conditions were as follows. Incubations were in EIA plates (3590, Costar, Cambridge, Mass., USA). Catcher antibody was bound by incubating the wells overnight at 4 °C with 50 µl purified monoclonal antibody, 10 µg/ml, in 0.02 M sodium tetraborate buffer, pH 8.2, containing 0.02% sodium azide. After five washings in 0.15 M NaCl containing 0.01 M Tris-HCl, pH 7.2, the residual protein binding sites were blocked by incubation for 1 h at room temperature with 0.3 ml 2% BSA buffer (2% bovine serum albumin in 0.15 M NaCl, 0.01 Tris-HCl, pH 7.2, and 0.08% sodium azide). The plates were used either immediately or after storage at 4 °C. The solution was aspirated and the wells were filled with 50 µl biotinylated monoclonal antibody (0.15 µg/ml in 2% BSA buffer, unless noted otherwise), which served as tracer antibody, and 50 µl of either reference antigen or sample to be measured for antigen content (dilutions in 2% BSA buffer). After incubation overnight at 4 °C the test plates were washed and the wells incubated for 1 h at room temperature with 50 µl of a mixture containing avidin and biotinylated peroxidase (Vecta stain, ABC Standard Kit, Vector Laboratories, Burlingame, Calif., USA; dilution 1:500 in a 0.5% BSA containing solution of 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.2). The plates were washed and the wells incubated for 1 h at room temperature with 0.1 ml substrate solution consisting of 0.01% 3,3',5,5'-tetramethylbenzidine in 0.1 M sodium acetate-citric acid buffer, pH 4.0, and 1.8 mM H_2O_2 [3, 10]. The reaction was terminated by the addition of 0.1 ml 0.8 M H_2SO_4 and the absorbance was read in a Titertek multiscan spectrophotometer at 450 nm.

Calculation of antigen concentration. Samples were tested in the DDEIA in duplicate. Twofold serial dilutions were used, starting from a 1:2 dilution. Duplicates of at least three different concentrations of 150-fold immunoaffinity chromatography purified antigen [28] within the linear part of the calibration curve (see Fig. 3) were included in each test plate as reference. The antigen concentrations in the samples were calculated by interpolation using the linear regression method. Twice the value of the blank obtained in the control without antigen was taken as the lower threshold for calculation of antigen concentration. One unit (U) of antigen per ml was defined as the absorbance obtained with the reference antigen at a protein concentration of 10 ng/ml.

Radioimmunoprecipitation of the antigen. Cells of the melanoma cell line MeWo were cultured to 3/4 confluent monolayers and labeled by incubation for 18 h at 37 °C with 0.1 mCi/ml ^{3}H-threonine (sp. act. 20 Ci/mmol, Amersham International, Amersham, England) in threonine-deficient culture medium (RPMI-Selectamine kit, Gibco, Paisley, Scotland) containing 10% fetal calf serum dialyzed against PBS. Labeling in the presence of tunicamycin (10 µg/ml; Calbiochem, La Jolla, Calif., USA) was performed similarly, except that the tunicamycin was added to the cells 5 h prior to the addition of threonine [17]. After labeling the cells were washed and lysed as described previously [28]. The lysate was centrifuged at 10,000 *g* and subsequently at 100,000 *g*. The supernatant was precleared with protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) that had been incubated successively with rabbit anti-mouse Ig and normal mouse Ig. The antigen was precipitated with protein A-Sepharose CL-4B beads to which NKI/C-3 had been bound by rabbit anti-mouse Ig (incubations 16 h at 4 °C). After washing the beads were heated for 5 min at 100 °C in sample buffer containing 3% SDS. Electrophoresis was performed in 5% to 15% polyacrylamide slab gels containing the SDS-buffer system described by Laemmli [18]. The gel was processed with Enhance (New England Nuclear, Dreieich, FRG) according to the recommendation of the manufacturer. Autoradiography was performed at −70 °C with Kodak XAR-50 films and Dupont Cronex intensifying screens. Reference markers were myosin (220×10^3 daltons), β-galactosidase (116×10^3 daltons), phosphorlylase b (93×10^3 daltons), bovine serum albumin (66×10^3 daltons), ovalbumin (45×10^3 daltons), carbonic anhydrase (29×10^3 daltons), soy bean trypsin inhibitor (21×10^3 daltons) and lysozyme (14×10^3 daltons; Bio Rad Laboratories, Richmond, Calif. USA).

Sera, tissue culture supernatants, and cell lysates. Sera from healthy donors and patients were stored between −20° and −35 °C. Artificial sera containing elevated levels of tumor markers (NMS T-markers) and other components (NMS) were obtained from Pharmachemie (Haarlem, The Netherlands). The artificial sera were reconstituted and stored as recommended by the manufacturer. Tissue culture supernatants were centrifuged at $100 \times g$. Cells from $100 \times g$ tissue culture supernatants and monolayers were combined and lysed as described previously [28]. Both the tissue culture supernatants and the cell lysates were filtered through 0.22 µm Millipore filters and stored at −20 °C.

Results

The antigen detected by NKI/C-3

In a previous study the antigen recognized by monoclonal antibody NKI/C-3 appeared to be a heterogeneous $25-110 \times 10^3$ dalton glycoprotein [28]. In immunoprecipitates of ^{3}H-threonine labeled melanoma cells a heterogeneous distribution of the antigen was found in the region of 30 to 65×10^3 dalton proteins (Fig. 1). After labeling of the

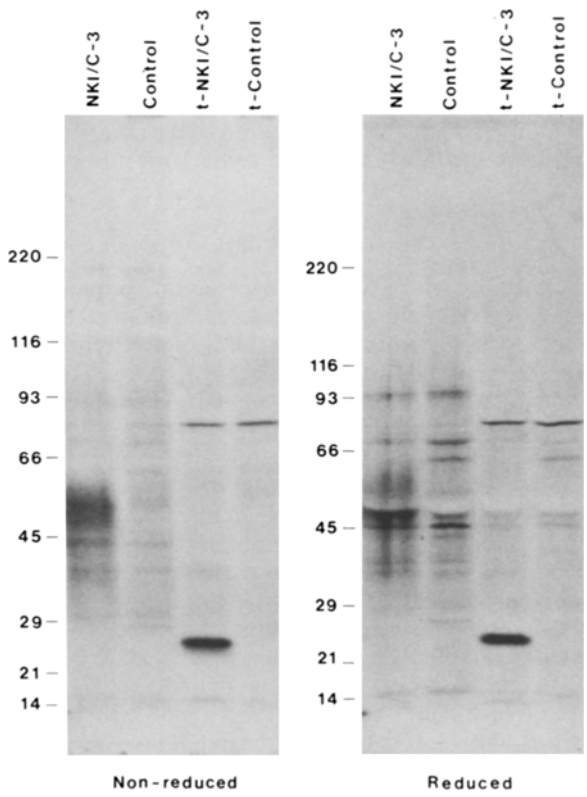

Fig. 1. Immunoprecipitation of the antigen detected by monoclonal antibody NKI/C-3 and control antibody (Sp 2/0 ascites). The melanoma cells were labeled with ^{3}H-threonine. Immunoprecipitates of cells labeled in the presence of tunicamycin are indicated by the prefix t. Note that in the immunoprecipitates with NKI/C-3 of cells labeled in the absence of tunicamycin only the faint staining between 30 and 65×10^3 daltons, which is lacking in the control, represents the antigen

cells in the presence of tunicamycin, however, discrete protein bands were specifically precipitated by NKI/C-3 of 25 (majority) and 24 (trace) $\times 10^3$ daltons in the non-reduced, and of 24×10^3 daltons in the reduced state of the antigen. Since it is known that tunicamycin inhibits the incorporation of N-linked carbohydrates into glycoproteins [19], the results indicate that the heterogeneity of the antigen arises after N-linked sugar components have been attached to the peptide chain.

Monoclonal antibodies NKI/C-3 and NKI/black-13 recognize different epitopes on the same antigen

The following findings indicated that monoclonal antibody NKI/black-13 recognizes the same antigen as NKI/C-3. In the immunoperoxidase test on formalin-fixed and paraffin-embedded tissue sections a similar distribution of antigen was found. In a Western blot the antigen showed the same heterogeneous molecular weight. Antigen purified by affinity chromatography with NKI/C-3 was recognized equally well with NKI/black-13 as with NKI/C-3. NKI/C-3 and NKI/black-13 recognized different but partially overlapping epitopes on the same antigen because of the following findings. In the EIA with solid phase bound antigen the results of simultaneous incubations of NKI/C-3 and NKI/black-13 were additive (additive index AI = 56%, [8]). Using the avidin and biotinylated peroxidase system, binding of biotinylated NKI/C-3 was inhibited only partially (33%) by a 2000-fold excess of unlabeled NKI/black-13, while binding of biotinylated NKI/black-13 was inhibited only partially (36%) by a 2000-fold excess of unlabeled NKI/C-3. In both cases binding of the biotinylated monoclonal antibody was inhibited by more than 85% by the same unlabeled antibody. Therefore, monoclonal antibodies NKI/C-3 and NKI/black-13 can be used in a double determinant assay.

Using antigen purified by immunoaffinity chromatography an affinity constant K of 6×10^8 M^{-1} was found for NKI/C-3 and $7 \times 10^7 M^{-1}$ for NKI/black-13.

DDEIA with monoclonal antibodies NKI/C-3 and NKI/black-13

The results in the DDEIA with the monoclonal antibodies NKI/C-3 and NKI/black-13 are shown in Fig. 2. In subsequent tests we have used NKI/C-3 as catcher antibody and biotinylated NKI/black-13 as tracer antibody. Up to 10 U/ml a linear relationship was found between the antigen concentration and the absorbance at 450 nm (Fig. 3). Taking twice the value of the blank without antigen as the

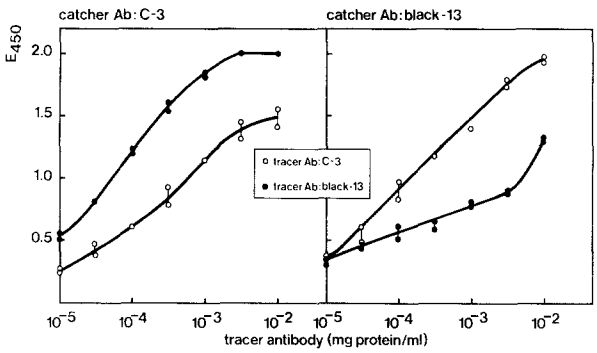

Fig. 2. Detection of melanoma-associated antigen in the DDEIA. NP-40 lysate of membranes of a diffuse liver metastasis of melanoma was used as source of antigen. C-3: monoclonal antibody NKI/C-3; black-13: monoclonal antibody NKI/black-13. The tracer antibodies were labeled simultaneously under identical conditions

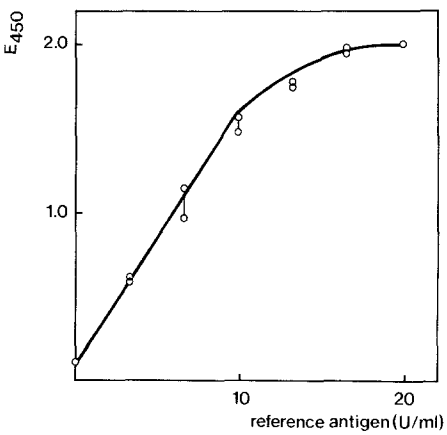

Fig. 3. Relation between the concentration of reference antigen and the absorbance at 450 nm in the DDEIA. The reference antigen was diluted in 2% BSA buffer. Identical results were obtained when the reference antigen was diluted in normal human serum

lower threshold for antigen detection in a 1 :2 diluted sample, antigen concentrations could be determined from 2 U/ml.

Detection of melanoma-associated antigen in tissue culture supernatants and in pleural effusions from melanoma patients

Soluble antigen was detected in tissue culture supernatants of melanoma cells as well as in pleural effusions from melanoma patients. In pleural effusions from six melanoma patients antigen concentrations of 10, 10, 25, 32, 59, and 111 U/ml were found. Culture medium in which MeWo melanoma cells had been grown to confluency contained 4% (97 U) of the total antigen as measured in both the culture supernatant and the NP-40 lysate of the cells (2379 U). Then 6 days later, when the cells had become detached completely and 58% of the cells were still alive as measured by trypan blue exclusion, the antigen content in the culture supernatant had increased to 13% (632 U) of the total antigen content of the culture (4910 U). When the cells had been incubated in the presence of 10 mM sodium azide, which is an inhibitor of cytochrome c oxidase in mitochondria of mammalian cells [5, 29] during these 6 days, no increase in antigen content of the culture supernatant nor of the cell lysate occurred, although the cells started to detach and die 3 days earlier than in the untreated culture. These observations do not support the hypothesis that the antigen in the culture supernatant originates from dead or dying cells, but they indicate that an active, energy-dependent mechanism is responsible for shedding of the melanoma-associated antigen.

Elimination of false positive results in the DDEIA with sera

Initially the sera were tested in the absence of irrelevant mouse immunoglobulins. In 2/405 individuals, however, false positive results were found due to the presence in the sera of antibodies reacting with mouse immunoglobulins. One of these persons, a healthy donor, was occupationally working with mice. The other person, however, a melanoma patient with no evidence of disease (NED), had never been in contact with mice. The false positive results were easily avoided by incubating the sera in the presence of purified mouse monoclonal antibody not reacting with the antigen to be measured in the DDEIA (Fig. 4). Addition of irrelevant mouse IgG1 had no effect on a positive and a negative control serum. Therefore, in subsequent tests irrelevant mouse IgG1 at a concentration of 0.05 mg/ml was present during the incubation with serum and tracer antibody.

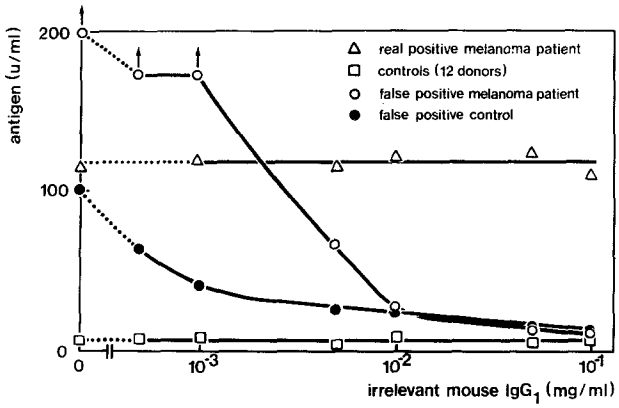

Fig. 4. False positive reactions in the DDEIA as a result of anti-mouse immunoglobulins. The false positive reactions were not found when a sufficient amount of mouse IgG1 not reacting with the antigen recognized by NKI/C-3 was present during the incubation with tracer antibody

Absence of reactivity in the DDEIA of other serum markers

To exclude the possibility that known serum components not related to melanoma were detected, we tested an artificial serum (NMST) containing the following components: adrenocorticotrophic hormone (60 pg/ml), alpha fetoprotein (60 ng/ml), aldosterone (1300 pg/ml), carcinoembryonic antigen (40 ng/ml), calcitonin (30 pg/ml), creatinine kinase (9 ng/ml), beta-human chorionic gonadotropin (70 mU/ml), ferritin (300 ng/ml), gastrin (4500 pg/ml), placental alkaline phosphatase (25 ng/ml), prolactin (30 ng/ml), pituitary thyroid hormone (20 ng/ml), and thyroglobulin (90 ng/ml). No measurable antigen activity was found in the DDEIA. Likewise, an artificial serum (NMS) containing follicle stimulating hormone (5 mU/ml), immunoglobulin E (20 U/ml), insulin (30 µU/ml), luteinizing hormone (50 mU/ml), myoglobin (80 ng/ml), thyronine (600 ng/dl), thyroxine (15 µg/dl), thyroxine binding globulin (3 µg/dl), thyroid stimulating hormone (30 mU/l) and many other compounds, was negative in the DDEIA.

Identity of the antigen in serum with the antigen detected in melanoma tissue

In the DDEA a number of sera from melanoma patients contained elevated antigen levels compared with sera from healthy donors. Dilution curves of these sera were parallel with the dilution curve of the reference antigen, which was purified from a liver metastasis of melanoma (Fig. 5). This result indicates that in the DDEIA identical antigens are detected in melanoma tissue and in sera from melanoma patients.

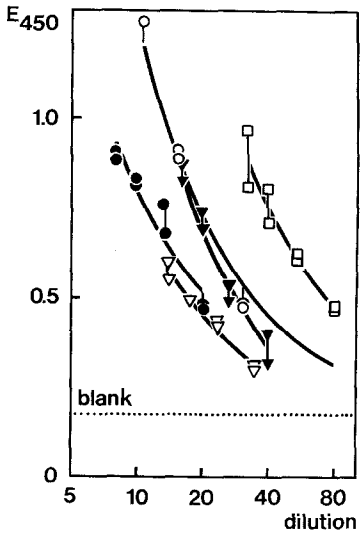

Fig. 5. Results in the DDEIA with different dilutions of reference antigen (○ :100 U/ml) and four sera from melanoma patients (▽ : 51 U/ml; ● : 58 U/ml; ▼ : 94 U/ml and □ : 215 U/ml)

Table 1. Reproducibility of the DDEIA with monoclonal antibodies NKI/C-3 and NKI/black-13[a]

Serum source	No.	U/ml			Coefficient of variation (%)
		Range	Mean	SD	
Melanoma patients	1	221–399	332	70	21
	2	216–301	283	46	16
	3	215–289	256	37	14
	4	86–141	113	19	17
	5	52– 78	68	10	15
	6	52– 80	66	10	15
	7	28– 58	46	14	30
	8	29– 55	39	10	26
	9	22– 49	35	12	34
	10	20– 61	33	13	40
Control	11	3– 15	7	3	42

[a] Each serum was tested on 9 different days

Reproducibility of the DDEIA

The reproducibility of the DDEIA was examined in nine repeated tests with 10 sera from melanoma patients as well as with a control serum obtained by pooling 12 sera from healthy donors (Table 1). From antigen levels of 75 U/ml and higher a mean coefficient of variation of 17% was found. At antigen levels lower than 75 U/ml the coefficient of variation increased proportionally with decreasing antigen levels up to a maximum of 47% at 2 U/ml.

Within a test the coefficient of variation was considerably smaller. In six simultaneous tests with a serum from a melanoma patient (mean 118 U/ml, SD 5) the coefficient of variation was 4%, whereas with a control of 12 pooled sera (mean 7 U/ml, SD 1) it amounted to 10%.

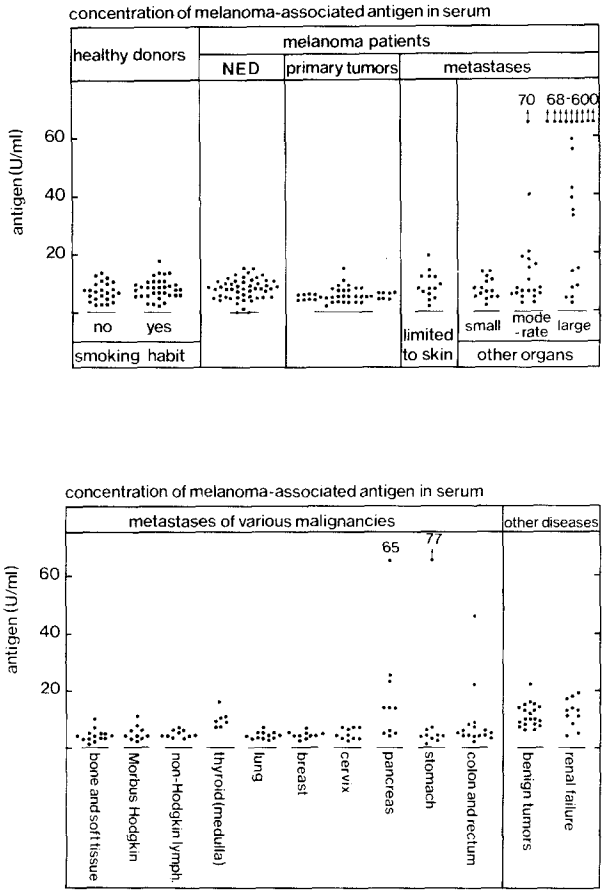

Fig. 6. Concentrations of melanoma-associated antigen in serum as measured in the DDEIA. The patients with non-melanoma malignancies had widespread metastases, except for the patients with medullary carcinoma of the thyroid, where 4 patients had a large, 1 patient had a moderately large, and 2 patients had a small tumor mass

Comparison of antigen levels in sera from melanoma patients and controls

The serum antigen levels varied in controls, i.e., in healthy donors ($n=76$, age 20–62 years) from less than 2 to 18 U/ml (mean 8 U/ml, SD 4), in patients with renal failure ($n=11$) from 4 to 19 U/ml (mean 12 U/ml, SD 5), and in patients with benign tumors ($n=20$) from 6 to 22 U/ml (mean 11 U/ml, SD 4; see Fig. 6). No difference was found between healthy donors with or without a smoking habit. For controls an upper limit of antigen level in serum of 22 U/ml was used, which corresponds to the mean value of the group of patients with renal failure plus $2 \times$ SD. Using this limit, only 1/107 control sera was positive.

In sera from patients with extended metastases of malignancies other than melanoma the antigen levels were comparable with those of the healthy donors, except for 3/10 patients with pancreatic, 1/10 patients with stomach, and 1/10 patients with colon carcinoma, who had significantly elevated antigen levels. No elevated antigen levels were found in patients with a large tumor burden of medullary carcinoma of the thyroid, whereas this type of tumor has been strongly positive with NKI/C-3 in the immunoperoxidase test [27, 28].

In sera from melanoma patients with NED after treatment of the tumor, and in sera from melanoma patients with a primary tumor or only skin metastases the antigen levels were within the range of healthy donors (8 ± 4, 6 ± 2, and 9 ± 5 U/ml respectively). In 2/20 sera from melanoma patients with a moderately large tumor load with metastases (also) present in organs other than skin and in 15/22 sera from melanoma patients with a large tumor load, significantly elevated antigen levels of more than 30 U/ml were found. These patients had metastases in several organs such as liver, lung, adrenal, brain, bone, spleen, and lymph nodes. In 7 patients, however, the antigen levels were below 22 U/ml, in spite of a large tumor load; 2 of these patients had their main tumor mass in the skin, and 1 patient had a large bone metastasis (7.5×6 cm as measured on a CT scan) and pleural effusion. Another patient had the main tumor mass in a lymph node metastasis (3.5 cm diameter), and 1 patient was negative with liver, brain, and lung metastases. Later on, however, this patient had a pleural effusion which contained a high antigen level (more than 160 U/ml). Other seronegative patients had brain and lymph node metastases, and lung and lymph node metastases, respectively.

Most of the patients who had clinical progression of the disease showed increasing antigen levels in time (Fig. 7). In 2 melanoma patients (nos. 2 and 6), however, decrease of antigen level was found with no clinical regression of the tumor.

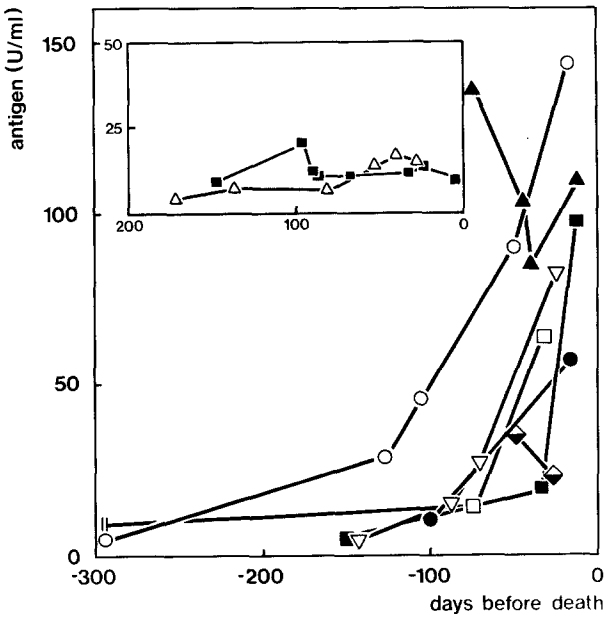

Fig. 7. Antigen levels in serum of melanoma patients developing a moderate or large tumor mass during the disease. ○: Patient no. 1 with one small lymph node metastasis on day −295, developed multiple skin and multiple lung metastases (day −170), an adrenal metastasis (day-145), a brain and new skin metastases (day −123), growth of the adrenal and lung metastases (day −78) and pleural effusion (day −18). ▲: Patient no. 2 with a large tumor mass because of skin, lung and liver metastases as well as pleural effusion on day −75, showed decreased antigen level during DNCB treatment from day −75 to day −45, and a rise in antigen level from day −45. Clinically only progression of the disease was noticed. ▽: Patient no. 3 with three small skin metastases on day −143 which were removed, suddenly developed a large tumor mass in liver and lungs (day −73) and a large visceral tumor mass (day −24). □: Patient no. 4 with a few very small skin metastases on day −504, developed some other skin metastases (day −103), suddenly entered a progressive phase (day −33) with increasing skin metastases, a lung hilus metastasis, metastases in the mediastinum and liver, and a small brain metastasis. ■: Patient no. 5 with multiple small lymph node metastases (day −54), liver metastases (day −47), and lung metastases (day −12). ◈: Patient no. 6 with a large tumor mass because of multiple skin, lung, bone, and perhaps liver metastases (day −49), developed new metastases and ascites (day −28). On day −49 the patient received the first treatment with combination chemotherapy (DTIC, vindesine, and cisplatinum). Clinically no tumor regression was observed. ●: Patient no. 7, who had a primary ocular melanoma, with a large liver metastasis which increased between day −101 and −17.

Inset: melanoma patients whose antigen levels did not rise significantly above the levels of the control group during progression of the disease. ■: Patient no. 8 with many skin metastases on day −148, developed multiple lung metastases (day −95), a brain metastasis (day −92), and pleural effusion (day −5). The patient received combination chemotherapy (DTIC, vindesine, cisplatinum) from day −195 to day −68. In the period between day −89 and −68 partial regression of the lung and skin metastases was noticed. The patient died with a moderate tumor burden. △: Patient no. 9 with many large skin metastases on day −172, developed more skin and small esophagal metastases (day −137), and metastases in the mediastinum (day −27). The patient died with a large tumor burden, mainly because of the skin metastases

Correlation between antigen level and lactate dehydrogenase, alkaline phosphatase, and gamma glutamyl transpeptidase activity

In 8/10 patients who were followed during progression of the disease three other serum markers, alkaline phosphatase (AP, which is released by liver and bone), lactate dehydrogenase (LDH, which is released after cell damage) and gamma glutamyl transpeptidase (γGTP, which is widely distributed in mammalian tissues and elevated in sera from patients with some malignant diseases [26]) were

Table 2. Correlation between antigen level and LDH, AP, and γGTP, respectively

Patient no.	Correlation with antigen level						Presence of liver or bone metastases	
	LDH activity		AP activity		γGTP activity			
	No. of observations	Correlation coefficient (r)	No. of observations	Correlation coefficient (r)	No. of observations	Correlation coefficient (r)	Liver	Bone
1[a]	3	0.95	3	0.84[b]	3	0.35	No	No
2	4	−0.08	4	−0.54	4	0.41	Yes	No
3	4	0.97	4	0.90	3	0.96	Yes	No
4	3	0.77[c]	3	0.89	3	1.00	Yes	No
5	2	−1	3	1.00	3	1.00	Suspected	No
6	1	–	2	−1	NT[d]	–	Suspected	Yes
7	2	1	2	1	2	−1	Yes	No
8	4	−0.15[e]	5	0.44[e]	5	0.82[e]	No	No
9	3	−0.77[e]	6	0.71[e]	6	0.85[e]	No	No
10	3	−0.74	4	0.84	3	0.92	Yes	Yes
All	32	0.33	22	0.83[f]	32	0.54	–	–
observations	19	0.29[f]	–	–	18	0.50[f]	–	–

[a] The numbers refer to the same patients as in Fig. 7, except for patient no. 10 who was not shown in that Fig.
[b] All values for AP activity were less than 110 U/ml
[c] All values for LDH activity were less than 200 U/ml
[d] NT = not tested
[e] All antigen levels were less than 22 U/ml
[f] Only the data of patients with (suspected) liver metastases were used

routinely tested. Comparison of the levels of these enzymes with the antigen can be found in Table 2.

Elevated AP activity was found in all 7 patients with (suspected) liver metastases. In 4/7 patients with liver metastases (nos. 3, 5, 7, and 10) elevated AP activity was found in a serum sample just preceeding the serum sample with elevated antigen level. In the group of patients with (suspected) liver metastases a correlation was found between antigen level and AP activity.

Increased γGTP activities were found in all 10 patients followed until death, irrespective of the presence of liver metastases, however, the correlation with γ-GTP activity was considerably less than with AP activity. In 4/7 patients elevated γGTP activity was found in one serum sample (nos. 5, 7, and 10) or two serum samples (no. 3) preceeding the serum samples in which elevated antigen level was measured. In 1 patient (no. 1) elevated antigen level preceeded elevated γGTP activity in one serum sample.

The correlation between antigen level and LDH activity was least consistent. Of 4/7 patients an elevated antigen level was found in one (nos. 5, 7, and 10) or two (no. 3) serum samples after raised LDH activity was measured. No correlation between antigen level and LDH activity was found in the group of melanoma patients as a whole.

Discussion

In a previous study we observed that the antigen recognized by NKI/C-3 is a highly glycosylated protein of heterogeneous molecular weight distribution [28]. From inhibition studies of the EIA with antigen that had been subjected to either proteolysis or periodate oxidation it had already been concluded that the antigenic site had to be located on the peptide backbone of the antigen. We have also found that the antigenic determinant for NKI/C-3 is disulfide configuration-dependent [28]. We now show that the antigen originates from a single precursor peptide of 25×10^3 daltons. This indicates that the heterogeneity of the antigen is caused by heterogeneous glycosylation of the antigenic peptide.

The antigen appears to be very similar to the antigen recognized by the monoclonal antibodies ME 491, ME 492, and ME 493 described by Atkinson et al. [1], on account of both its antigen distribution and its molecular nature. In our hands, the molecular weight of the antigenic protein core was somewhat higher than has been reported for the antigen recognized by ME 491 [1, 25]. We found that in the EIA with solid phase bound antigen binding of biotinylated NKI/C-3 was inhibited by unlabeled ME 491. Also, but to a lesser extent, binding of biotinylated NKI/black-13 to antigen was inhibited by unlabeled ME 491 (unpublished results). Inhibition by unlabeled ME 491 was also found in the DDEIA. This result indicates that monoclonal antibody ME 491 recognizes the same antigen as NKI/C-3, and that the epitope for ME 491 is adjacent to the epitope to, if not identical with, the epitope for NKI/C-3. Monoclonal antibody MAb8-1H, raised against ocular melanoma, seems to recognize the same antigen as ME 491, although it binds a different epitope [6]. It remains to be determined whether the epitope recognized by MAb8-1H is identical or different from the epitope for NKI/black-13. Another monoclonal antibody, HMSA-2, also reacts with melanoma in formalin-fixed and paraffin-embedded tissues [21]. This antibody, however, recognizes a different antigen, namely 47×10^3 and 59×10^3 dalton polypeptide chains. In our hands binding of neither biotinylated NKI/C-3, nor of biotinylated NKI/black-13 to solid phase antigen was inhibited by unlabeled HMSA-2 (unpublished result).

The significantly elevated antigen levels found with the DDEIA in some of the sera from colon and pancreatic carcinoma patients are not surprising, since in the immunoperoxidase test NKI/C-3 has occasionally shown a weak reaction with tissue from colon carcinoma [28] and the monoclonal antibody is also positive with pancreatic tissue (Ph. Hageman, personal communication). The melanoma-associated antigen was shown to be elevated in sera of only 15/22 melanoma patients with a large tumor burden and metastases outside skin. Such a tumor mass usually consists of multiple metastases in different organs. Most of the patients who were positive in the DDEIA died within 3 months; 2 melanoma patients with a large tumor burden and antigen levels of 332 U/ml and 283 U/ml, respectively, died after 8.5 and 7.5 months (not shown). The negative reactions with sera from melanoma patients with a large tumor burden in our study may be attributed to the heterogeneity of some melanomas for NKI/C-3 [13, 27]. Also, the degree of vascularization of the metastases may influence the appearance of the antigen in the circulation. A rather strong correlation was found between the serum antigen level and the serum AP activity. This suggests that high antigen levels may be caused mainly by liver and/or bone metastases. Tumor progression as measured by elevated antigen level in the DDEIA was usually not detected at an earlier stage of the disease compared with the rise in serum LDH, AP, and γGTP-activity. With antibodies against other melanoma-associated antigens elevated serum levels have also been detected only in patients with progressive disease [11, 12, 16, 22, 24]. However, in spite of the lack of sensitivity, serum testing with monoclonal antibodies against melanoma-associated antigens could be helpful in cases where more knowledge of tumor-specificity is required, for example in patients with a history of ocular melanoma suspected of tumor recurrence in the liver.

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