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The Molecular Heterogeneity of Nonspecific Cross-reacting Antigen Synthesized by Tumor Cells and Granulocytes

Motomu KUROKI,[*1] Masahide KUROKI,[*1] George E. MOORE,[*2] Sadanori ICHIKI[*1] and Yuji MATSUOKA[*1, *3]

[*1] *First Department of Biochemistry, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01, Japan and* [*2] *Department of Surgical Oncology, Denver General Hospital, 645 Bannock, Denver, CO 80204, USA*

The molecular heterogeneity of nonspecific cross-reacting antigen (NCA) was examined. Metabolically-labeled glycoproteins were precipitated from cell lysates of human tumor cell lines and of normal peripheral granulocytes with antibodies specific for NCA, and analyzed by SDS-PAGE. NCA components synthesized by three tumor cell lines, QGP-1 (pancreas), HLC-1 (lung) and CAOV-2 (ovary) showed slightly different migration patterns on SDS-PAGE, but the molecular weights of their unglycosylated peptides synthesized in the presence of tunicamycin were all found to be 35K. On the other hand, two molecular species of NCA were identified in normal granulocytes: an 80K mature form derived from a 69K precursor peptide and a 58K mature form from a 41K precursor peptide. Upon SDS-PAGE, the migration pattern of the unglycosylated NCA peptides from tumor cells was affected by the presence of 2-mercaptoethanol, while that of the peptides of granulocytes was not. All the NCAs identified in this study possessed antigenic determinants common to carcinoembryonic antigen as well as those unique to NCA. These results suggest that the molecular heterogeneity of NCA observed thus far resulted from diverse glycosylation of the three fundamental molecular forms of unglycosylated peptides: one with a molecular weight of 35K produced by tumor cells and two with molecular weights of 69K and 41K produced by granulocytes.

Key words: Nonspecific cross-reacting antigen — Unglycosylated peptide — Human carcinoma cell lines — Human granulocyte

Nonspecific cross-reacting antigen (NCA)[*4, *5] is a glycoprotein that partially shares antigenic determinants with CEA.[1] NCA has been found in various tissues or cells, such as normal lung,[1–3] spleen,[1, 4] colon mucosa,[5] granulocytes,[6, 7] monocytes,[6, 7] colon tumor[8–11] and established tumor cell lines.[12, 13] A variety of molecular weights (50K–130K) have been reported for the NCAs isolated from different tissues as summarized by Krop-Watorek *et al.*,[14] but the molecular relationships among these NCAs remain to be elucidated. Recently, Buchegger *et al.*[15] have identified two molecular forms of NCA: NCA-55 (55K) existing in both granulocytes and epithelial cells, and NCA-95 (95K) present only in granulocytes. Grunert *et al.*[16] have isolated these two antigens from both colon tumor and normal lung and also a 75K antigen from colon tumor. Neumaier *et al.*[17] and Burtin *et al.*[18] also found in normal serum and in normal lung, respectively, three NCAs with molecular weights ranging from 48K to 130K. To analyze the apparent molecular heterogeneity of NCA, two different aspects should be taken into consideration: the difference in the cell types producing NCA and the difference in the glycosylation of NCA.

[*3] To whom correspondence should be addressed.

[*4] Abbreviations used are: CEA, carcinoembryonic antigen; 2-ME, 2-mercaptoethanol; NCA, nonspecific cross-reacting antigen; R-a-CEA, rabbit antibody against CEA unabsorbed; R-a-NCA, rabbit antibody against NCA unabsorbed; R-a-NCA-abs, rabbit antibody against NCA absorbed with CEA; SaCI, heat-killed *Staphylococcus aureus* Cowan I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TM, tunicamycin.

[*5] Several similar antigens reported with different names (NGP,[2] CCEA-2,[8] CCA-III[9] or TEX[10]) are collectively called NCA in this paper.

In the present study, we compared NCAs synthesized by established tumor cell lines with those by normal peripheral granulocytes. The cells in the tumor cell lines are much more homogeneous than those in tumor tissues, if not completely monoclonal. The effect of glycosylation on the heterogeneity of NCA was analyzed by comparing metabolically-labeled whole NCA molecules with their unglycosylated peptides synthesized in the presence of TM, a inhibitor of asparagine-linked glycosylation.[19] We found three species of unglycosylated NCA peptides, one in tumor cell lines and two in normal granulocytes, which seem to be the fundamental molecular forms of the heterogeneous NCAs thus far reported.

MATERIALS AND METHODS

Cell Lines The human cell lines used in this study are: a pancreatic carcinoma cell line, QGP-1,[20] obtained from Dr. M. Kaku of Kyushu Cancer Center (Fukuoka), a lung carcinoma cell line, HLC-1,[21] from Dr. T. Hamaoka of Osaka University (Osaka) and an ovarian carcinoma cell line, CAOV-2, from Dr. J. Fogh of Memorial Sloan-Kettering Cancer Center (New York). The cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum.

Preparation of Granulocytes Granulocytes were obtained from heparinized human venous blood by the method of Ferrante and Thong.[22] Briefly, 5 ml of blood was centrifuged at 500*g* for 20 min on 3 ml of Ficoll-Hypaque medium (d=1.114). The granulocyte fraction was separated and washed by suspending it in phosphate-buffered saline. More than 95% of the cells were found to be granulocytes by Giemsa's staining.

Reference Preparations of NCA and CEA NCA was purified from the perchloric acid extracts of pooled normal human lungs by a combination of affinity chromatography on an immunoadsorbent and gel filtration as described previously.[23] CEA was prepared from metastatic liver tumors from colon cancer using a purification procedure described earlier.[24,25] The NCA and CEA preparations were practically pure as demonstrated by immunoelectrophoresis and SDS-PAGE,[23] and were used as reference preparations and for immunization.

Antibodies A rabbit anti-NCA antiserum was raised by immunization with the NCA preparation. The antiserum was specifically purified by adsorption on NCA-coupled Sepharose 4B (Pharmacia, Uppsala, Sweden) and elution with 0.175*M* glycine-HCl, pH 2.8. The purified antibody (R-a-NCA) was then absorbed thoroughly with CEA-Sepharose. This absorbed antibody (R-a-NCA-abs) was used mainly in this study. R-a-CEA was prepared as described before.[12,25] A monoclonal anti-CEA antibody, F4-82, which shows no cross-reactivity with NCA, has been described in detail.[26] The specificities of the polyclonal antibodies are shown in Fig. 1.

Radiolabeling of Glycoproteins About 1×10^6 cells were cultured with 50 μCi of [^{3}H]leucine, 20 μCi of [^{14}C]glucosamine or 50 μCi of [^{35}S]methionine (Amersham, Bucks, UK) in 1 ml of leucine or methionine-deficient medium for 16 hr. For pulse-chase experiments, cells were labeled with [^{35}S]-methionine for 30 min, then washed and suspended

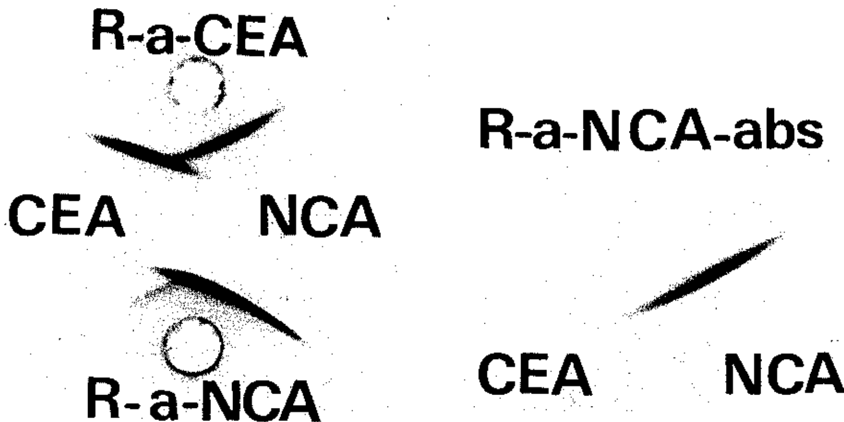

Fig. 1. Double immunodiffusion in agarose gels of the polyclonal antibodies against purified NCA and CEA.

in fresh medium for a different chase time. In several experiments, 10 μg/ml of TM (Sigma, St. Louis, MO) was added to the culture to inhibit glycosylation 3 hr before addition of the radioactive precursors. As reported previously,[27] this concentration of TM completely inhibited the incorporation of [^{14}C]glucosamine into CEA and NCA.

Immunoprecipitation and Gel Electrophoretic Analyses After radiolabeling, cells were washed with phosphate-buffered saline and solubilized in 1% Nonidet P-40 in 50m*M* Tris-buffered saline, pH 7.6, containing 5m*M* EDTA, 2 mg/ml bovine serum albumin, 2m*M* phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, pepstatin A and chymostatin (Protein Research Foundation, Osaka) for 1 hr at 4°. The cell lysate was centrifuged at 39,000*g* for 30 min to remove cell debris. The supernatant (1 ml) was precleared with 100 μl of 10% (w/v) SaCI (Wako Junyaku, Tokyo), then 10 μg of antibody was added, and the mixture was allowed to stand overnight at 4°. The antigen-antibody complex was precipitated by incubation with 50 μl of SaCI for 30 min at 4°. Immunoprecipitation with F4-82 was performed according to the method of Lampson[28] using SaCI precoated with rabbit anti-mouse IgG. The immunoprecipitates thus obtained were washed five times with 50m*M* Tris-HCl buffer, pH 8.0, supplemented with 0.5% Nonidet P-40, 5m*M* EDTA, 0.5*M* NaCl and 0.1% SDS.

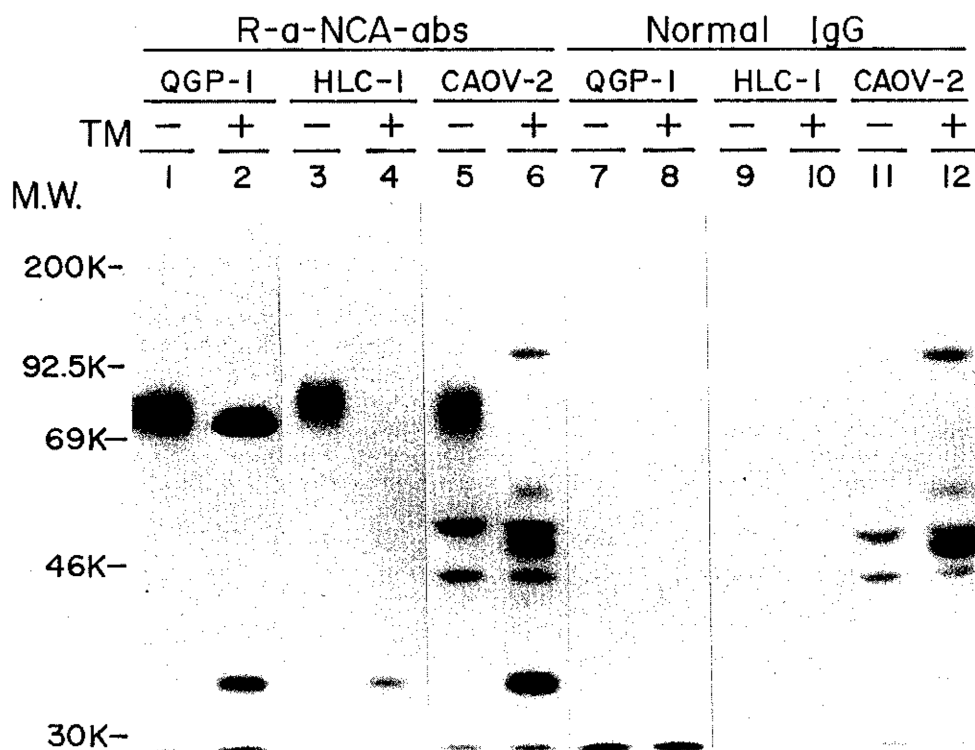

Fig. 2. SDS-PAGE profiles of NCAs synthesized by tumor cell lines. QGP-1 (lanes 1, 2, 7 and 8), HLC-1 (lanes 3, 4, 9 and 10) and CAOV-2 (lanes 5, 6, 11 and 12) were metabolically labeled with [^{3}H]-leucine in the absence (lanes 1, 3, 5, 7, 9 and 11) or presence (lanes 2, 4, 6, 8, 10 and 12) of TM for 16 hr before solubilization. Immunoprecipitates made from the cell lysates with R-a-NCA-abs (lanes 1–6) or normal rabbit IgG (lanes 7–12) were subjected to SDS-PAGE (7.5% gel) under reducing conditions. A prominent band of about 75K in lane 2 represents unglycosylated CEA (see Fig. 3). Since several bands of 40K–110K were nonspecifically precipitated with normal IgG, as can be seen in lanes 11 and 12, the corresponding bands in lanes 5 and 6 were identified as nonspecific precipitates. Molecular weight standards were as follows: myosin (200K), phosphorylase b (92.5K) bovine serum albumin (69K) ovalbumin (46K) carbonic anhydrase (30K).

SDS-PAGE was carried out according to the method of Laemmli.[29] The immunoprecipitates were boiled in SDS-sample buffer and the eluates were applied to 7.5% gels. Unless otherwise mentioned, electrophoresis was carried out under reducing conditions.

Two-dimensional gel electrophoresis (nonequilibrium pH gradient electrophoresis and SDS-PAGE) was performed as described by O'Farrell *et al.*[30]

After electrophoresis, gels were fixed with methanol (46%)-acetic acid (8%) solution and then immersed in Amplify (Amersham). The gels were dried and exposed to X-OMAT-S film (Eastman-Kodak, Rochester, NY) at −70° for 2–14 days. The ^{14}C-labeled molecular markers were obtained from Amersham.

Results

NCAs Synthesized by Tumor Cells NCAs synthesized by three different cancer cell lines, QGP-1, HLC-1, and CAOV-2 were identified by SDS-PAGE after immunoprecipitation with R-a-NCA-abs (Fig. 2). In each cell line, a broad band of NCA labeled with [^{3}H]-leucine was observed within the molecular weight range of 70K–90K (lanes 1, 3 and 5). By precise examinations, slight differences in molecular weights were observed among the NCAs synthesized by the three cell lines. When the asparagine-linked glycosylation[3] was inhibited by addition of TM to the culture, the molecular weights of the NCA components synthesized by the three cell lines were all found to be 35K (lanes 2, 4 and 6). These unglycosylated NCAs were labeled with [^{35}S]methionine but not with [^{14}C]-glucosamine and precipitated with R-a-CEA (not shown).

In addition to the 35K NCA, a component of about 75K was precipitated with R-a-NCA-abs from TM-treated QGP-1 cells (Fig. 2, lane 2). This 75K band was identified as the unglycosylated CEA peptide of QGP-1 on the basis of the following results. 1) The 75K component was bound by the monoclonal anti-CEA antibody F4-82 (Fig. 3, lane 2) which reacted with 180K native CEA but not with the native NCA (lane 1). 2) By preabsorption of the cell lysate with F4-82, the 75K component which was precipitated with R-a-NCA-abs (Fig. 3, lane 3) was markedly diminished but the 35K band remained unchanged (lane 4). 3) The 75K band was hardly detectable in the cell lysates of HLC-1 and CAOV-2 (Fig. 2, lanes 4 and 6) which produce NCA actively but CEA hardly at all.

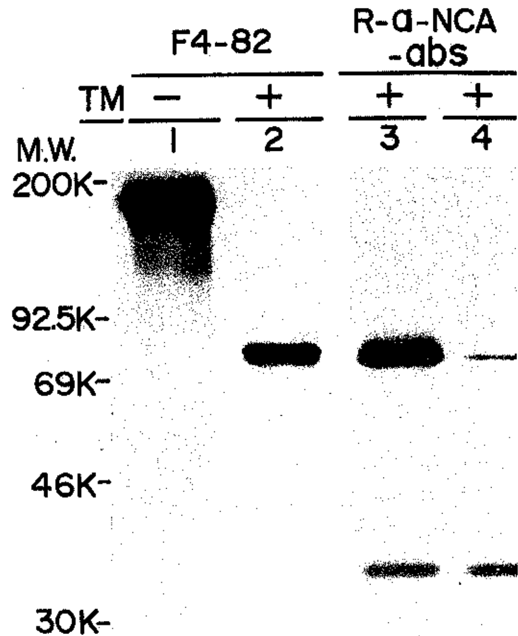

Fig. 3. Immunoprecipitation of unglycosylated CEA with R-a-NCA-abs. QGP-1 cells were labeled with [^{3}H]leucine in the absence (lane 1) or presence (lanes 2–4) of TM. The cell lysates obtained were precipitated with F4-82 (lanes 1 and 2) or with R-a-NCA-abs (lanes 3 and 4). The cell lysate for lane 4 was preabsorbed with F4-82 before immunoprecipitation with R-a-NCA-abs. The immunoprecipitates were subjected to SDS-PAGE.

NCAs Synthesized by Granulocytes From the cell lysate of normal peripheral granulocytes labeled with [^{35}S]methionine, four NCA components of 80K, 75K, 53K and 48K were precipitated with R-a-NCA-abs (Fig. 4A, lane 1). In the presence of TM, two components of 69K and 41K were detected (lane 2). The 35K peptide detected in the tumor cells (Fig. 4B, lane 2) was not found in granulocytes. All these NCA components identified with R-a-NCA-abs in granulocytes were also labeled with [^{3}H]leucine and were reactive with R-a-CEA but not with F4-82 (not shown).

The relationships among these NCA components in granulocytes were analyzed by a pulse chase experiment with [^{35}S]methionine

(Fig. 5). Two bands of 75K and 48K were detected in the cell lysate obtained just after pulsing (lane 1). During subsequent chase, the radioactivities of both components decreased with a concomitant increase of the 80K and 53K bands by 90 min (lanes 2 and 3). In culture medium, the radioactivities of the two components of 80K and 58K increased gradually with the chase time (lanes 7–10). After 4 hr of chase, the radioactive NCA components in the cell lysate decreased (lane 4) and those in the culture medium became dominant (lane 10). The 58K NCA was the major component released into the medium and was larger than the corresponding precursor component (53K) in the cell lysate. These results suggested that the 69K and 41K components identified in the presence of TM were unglycosylated precursor peptides for the mature 80K and 58K NCAs, respectively, and that the other three components of 75K, 53K and 48K detected in the cell lysates were intermediate products. Individual variations of these NCA components in granulocytes obtained from other subjects were not observed (lanes 5, 6, 11 and 12).

Effects of Reducing Conditions on the Mobility of the NCA Peptides on SDS-PAGE The native and unglycosylated NCAs of granulocytes and QGP-1 were subjected to SDS-PAGE in the presence or absence of 5% 2-ME. Figure 4A shows that the migrations of both the native and unglycosylated NCAs synthesized by granulocytes were not affected at all by the presence of 2-ME. On the other hand, the 35K unglycosylated NCA of QGP-1 (and also the 75K unglycosylated CEA) migrated faster in the absence of 2-ME than

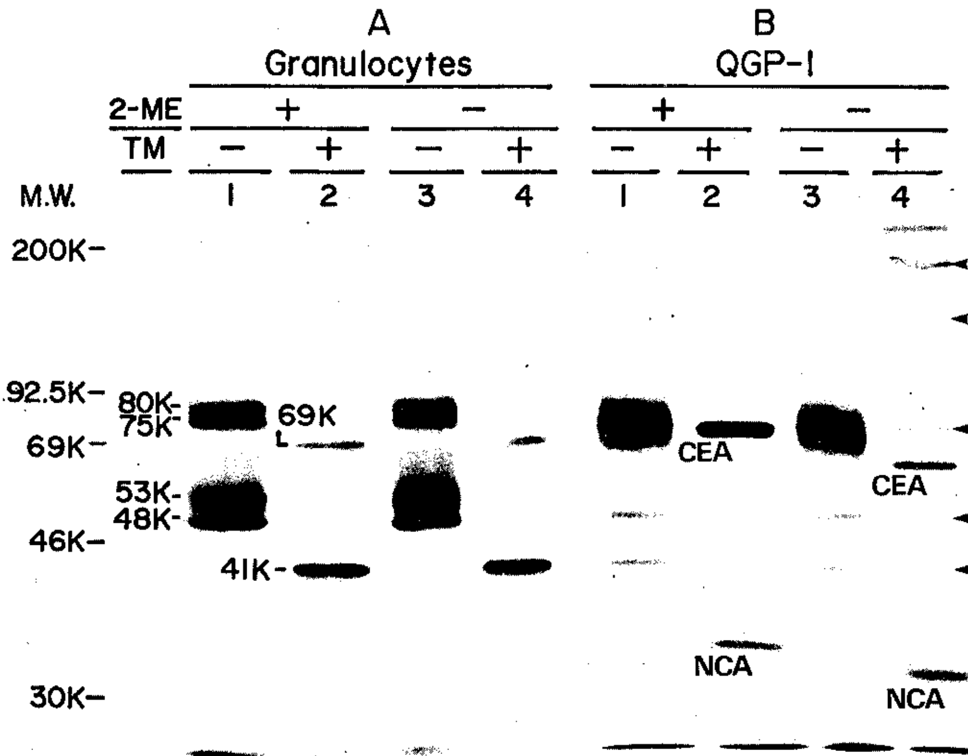

Fig. 4. SDS-PAGE profiles of native and unglycosylated NCAs synthesized by granulocytes and QGP-1 under reducing and nonreducing conditions. Granulocytes (panel A) and QGP-1 (panel B) were labeled with [^{35}S]methionine and [^{3}H]leucine, respectively, in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of TM. Immunoprecipitates with R-a-NCA-abs from cell lysates were subjected to SDS-PAGE under reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. Arrows in panel B indicate nonspecific bands.

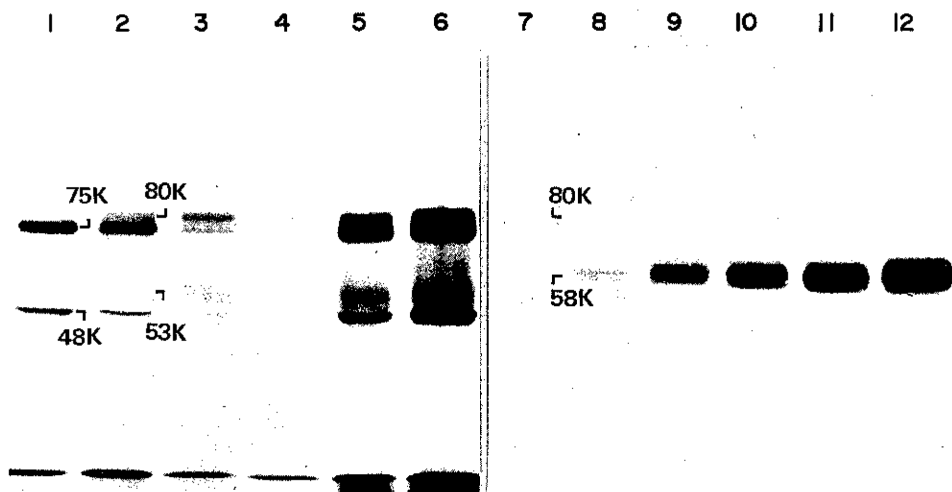

Fig. 5. Alteration in the molecular weights of NCAs synthesized by granulocytes during processing and secretion. Granulocytes were pulse-labeled with [^{35}S]methionine for 30 min, then chased. Immunoprecipitates with R-a-NCA-abs from cell lysates (lanes 1–6) and spent medium (lanes 7–12) were subjected to SDS-PAGE. Samples were obtained 0 min (lanes 1 and 7), 30 min (lanes 2 and 8), 90 min (lanes 3 and 9) and 4 hr (lanes 4 and 10) after pulsing. Samples for lanes 5, 6, 11 and 12 were obtained from granulocytes of different individuals labeled for 16 hr.

under reducing conditions (Fig. 4B, lanes 2 and 4), although the mobility of the native glycosylated NCA synthesized by QGP-1 cells was little affected by the presence of 2-ME (Fig. 4B, lanes 1 and 3), suggesting unfolding of the naked peptides of QGP-1 by 2-ME.

Analysis by Two-dimensional Gel Electrophoresis The isoelectric points of the 35K NCA peptide of QGP-1 (Fig. 6A) and the 69K and 41K NCA peptides of granulocytes (Fig. 6B) were found to be different from one another by two-dimensional gel electrophoresis.

Discussion

The heterogeneity observed in the molecular form of NCA has been poorly explained. In the present study, we analyzed the effect of glycosylation on the molecular heterogeneity of NCA and compared unglycosylated NCAs synthesized by established tumor cell lines with those synthesized by normal granulocytes.

It is evident from the results presented here that the slight differences in the molecular size of NCA observed among the tumor cell lines are mainly attributable to heterogeneous glycosylation of peptides with the same molecular weight of 35K. Tumor cells originated from different organs seem to produce very similar, if not identical, NCA peptides. This 35K peptide identified in the tumor cell lines might correspond to that of the tumor-extracted antigen (TEX) reported by Kessler *et al.*[10] In a previous study,[13] we detected two broad bands of NCA in tumor cell lysates by Western blotting. It seems probable that these two components resulted from diverse glycosylation of the 35K peptide identified in this study.

On the other hand, in normal peripheral granulocytes, seven components of NCA with different molecular weights were observed in all. The mature forms were found to be the 80K and 58K components secreted into culture medium, which are probably equivalent to the NCA-95 and NCA-55, respectively, identified in granulocytes by Buchegger *et al.*[15] The 75K, 53K and 48K components in cell lysate were identified as intermediate products, and the two components of 69K and 41K as unglycosylated precursor peptides synthesized in the presence of TM. These unglycosylated NCA peptides of granulocytes were quite different from the NCA peptide of the tumor

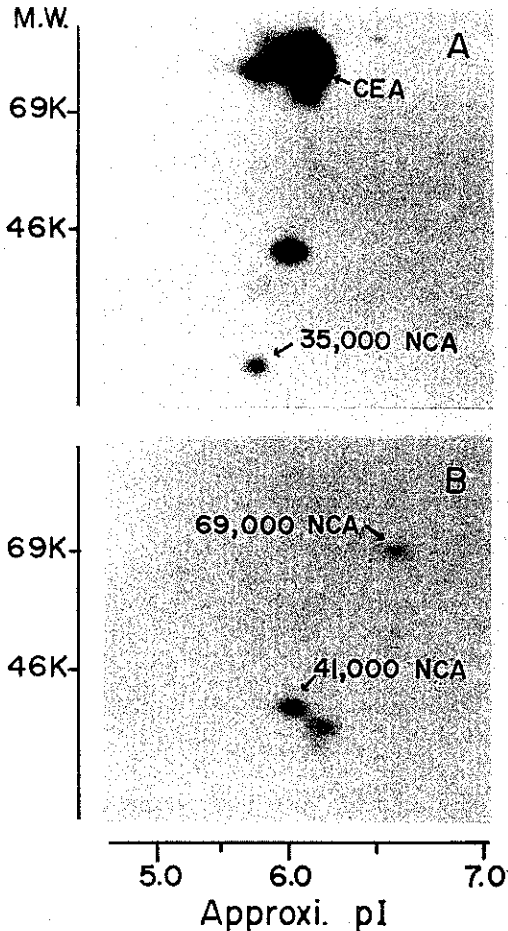

Fig. 6. Two-dimensional gel electrophoresis patterns of unglycosylated NCAs synthesized by QGP-1 (panel A) and granulocytes (panel B). QGP-1 and granulocytes were labeled with [^{3}H]-leucine in the presence of TM. Immunoprecipitates with R-a-NCA from cell lysates were subjected to nonequilibrium pH gel electrophoresis followed by SDS-PAGE. In panel A, a spot of 42K and pI 6.0 is a component nonspecifically precipitated.

cells in molecular size, in sensitivity to 2-ME on SDS-PAGE, and in isoelectric point, though no antigenic difference was detected between them so far as tested. Thus, although the apparent molecular forms of NCA are quite heterogeneous, the fundamental forms of NCA may consist of three peptides: one of 35K produced by tumor cells and two of 69K and 41K produced by granulocytes.

The existence of methionine in NCA is one of the features that distinguish it from CEA.[3,10] In the present study, all the NCA components synthesized by the tumor cells and granulocytes were labeled with both [^{3}H]-leucine and [^{35}S]methionine. This confirms the presence of methionine in NCA, as also reported recently by Paxton *et al.*,[31] although AbuHarfeil *et al.*[32] have claimed the absence of methionine in NCA.

R-a-NCA-abs showed no binding with the reference CEA (Fig. 1) and the glycosylated CEA molecules synthesized by QGP-1 (Fig. 2), but it reacted with the unglycosylated 75K CEA peptide of QGP-1(Figs. 2 and 3). This suggests that some of the antigenic determinants on the CEA peptide, shared by NCA, are completely buried by sugar chains of the CEA molecule. The recent sequence analysis[31] has shown that a great homology exists between the peptides of CEA and NCA. To elucidate further the molecular difference among NCAs produced by different cells and that between NCAs and CEA, complete sequence analyses of the NCA peptides identified in this study are necessary.

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