PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 535–542 (1983)

A 38,000-Dalton Antigen Found in Namalwa Cells Induced by Newcastle Disease Virus[1] (41599)

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Abstract. An antigen has been isolated from Namalwa cells, a Burkitt lymphoma line, that was induced by Newcastle disease virus (NDV) for interferon production. The antigen was extracted by 3 *M* NaCl from ribonucleoprotein particles (RNP), obtained from the nuclear 0.01 *M* Tris extract, and was purified by hydroxylapatite chromatography, phosphocellulose chromatography, and preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Its molecular weight was 38 kilodalton (kDa) as determined by SDS–PAGE. The tryptic peptide map of ^{125}I-labeled antigen contained seven major peptides. The antigen was not found in HeLa cells, normal human liver or in Namalwa cells that had not been induced by the virus. This result suggests that this antigen was produced in Namalwa cells as a result of induction by the NDV virus.

The presence of common nucleolar antigens has been demonstrated in a broad range of human malignant tumor cells but not in nontumor tissues by indirect immunofluorescence (1–4). To identify the molecular species responsible for antigenicity, efforts were made to isolate the individual antigen(s) from HeLa and Namalwa cells (5). Using antisera to HeLa nucleolar proteins and the DE-0.15 *M* eluate of Namalwa nuclear Tris extract (5), two antigens of molecular weight 54 kd and pI 6.3 were identified from nuclear Tris extracts of HeLa and Namalwa cells (5).

It was found that the pellet fraction obtained by centrifugation of Namalwa nuclear 0.01 *M* Tris extract at 100,000*g* was a rich source of nucleolar antigens. In this communication, we report the presence of a 38 kDa antigen recognized by anti-DE-0.15 *M* antibodies in the RNP fraction of Namalwa cells that were induced by the NDV virus used for interferon production.[3] The antigen was absent from the uninduced cells, HeLa cells or normal human liver.

Materials and Methods. *Chemicals.* Acrylamide and *N,N'*-methylene-bis-acrylamide and hydroxylapatite (Bio Gel HTP) were purchased from Bio-Rad Laboratories, Richmond, California. Peroxidase conjugated IgG of goat antirabbit IgG was obtained from Miles Laboratories, Elkhart, Indiana; the protease inhibitors phenylmethylsulfonylfluoride (PMSF), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), ethylene glycol-bis-(β-amino ethyl ether) *N,N*-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co., St. Louis, Missouri; leupeptin was purchased from the Peptide Institute, Osaka, Japan; low-molecular-weight protein markers and Protein A were from Pharmacia Fine Chemicals, Piscatawa, New Jersey; ^{125}I (NaI in 0.02 *N* NaOH) was from Iso-Tex, Inc., Friendswood, TX; Bolton-Hunter reagent was purchased from Amersham, Arlington Heights, Illinois; nitrocellulose sheets were from Schleicher and Schuell, Inc., Keene, New Hampshire; precoated TLC plastic sheets of cellulose were obtained from E. Merck, Darmstadt, West Germany. All other chemicals were standard reagent grade chemicals.

Cells. The Namalwa cells (a Burkitt lym-

[1] These studies were supported by the Cancer Research Grant CA-10893, P-1, the Human Tumor Nucleolar Antigen Grant, CA-27534, the Michael E. DeBakey Medical Foundation, the Davidson Fund, and the Pauline Sterne Wolff Memorial Foundation.

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[3] Abbreviations used: HAP, hydroxylapatite; RNP, ribonucleoprotein; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; phosphate-buffered saline, PBS; Newcastle disease virus, NDV; ^{125}I-protein A, IPA; Ethylene diamine tetracetic acid, EDTA; *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, TLCK; phenylmethyl sulfonyl fluoride, PMSF; Ethylene glycol-bis-(β-aminoethyl ether), *N,N*-tetracetic acid, EGTA; 2,2'-azino-di-[3-ethyl benzthiazolin sulfonate (6)], ABTS.

0037-9727/83 $1.50
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phoma line containing Epstein–Barr viral genome currently used at the Fredrick Cancer Laboratories for the synthesis of interferon) were the generous gift of Mr. Fred Klein, Dr. John Douros, Dr. Richard F. Geoghegan, and Dr. Vincent DeVita of the Frederick Cancer Research Laboratories and the USPHS, Bethesda, Maryland. The cells were induced for interferon production by NDV in the form of infectious allantoic fluid (6).

Antiserum. Rabbit antiserum to the DE-0.15 *M* fraction of Namalwa nuclear Tris extract was prepared as described previously (5, 14). The nuclear extract was obtained from Namalwa cells that had been induced by NDV for interferon production. IgG was prepared from the sera by ammonium sulfate precipitation and DEAE-cellulose chromatography (7). It was absorbed with placental Tris nuclear extract, fetal calf serum, and normal human serum coupled to Sepharose CL-4B.

Three molar NaCl and 9 M urea extract of human liver nuclei. Normal human liver nuclei prepared by the method of Davis *et al.* (4) were homogenized with 3 *M* NaCl, 10 m*M* Tris–HCl, 1 m*M* PMSF, 0.1 m*M* each of TLCK and leupeptin (pH 8) in a Teflon–glass type homogenizer, centrifuged at 100,000*g* for 16 hr and the supernatant was collected. The pellet was homogenized with 9 *M* urea, 10 m*M* Tris–HCl (pH 8), 1 m*M* PMSF, 0.1 m*M* each of TLCK and leupeptin and centrifuged at 27,000*g* for 20 min. The supernatant was used for further studies.

Isolation of antigen from Namalwa ribonucleoprotein (RNP) particles. The Tris extract of Namalwa nuclei was prepared by the method of Chan *et al.* (5). A mixture of protease inhibitors consisting of 1 m*M* PMSF, 5 m*M* EGTA, 0.1 m*M* each of TLCK and leupeptin were added to the buffers at all steps following the rupture of the cells.

The Tris extract obtained after centrifugation at 27,000*g* for 10 min was further centrifuged at 100,000*g* for 16 hr. The pellet (RNP) was then homogenized with 3 *M* NaCl, 10 m*M* potassium phosphate, 1 m*M* PMSF, 0.1 m*M* each of TLCK and leupeptin (pH 7) in a tight (Teflon–glass) homogenizer by 15 up-and-down strokes and centrifuged at 30,000*g* for 20 min. The supernatant was used for the purification step.

Hydroxylapatite chromatography. Hydroxylapatite (Bio Gel HTP) was packed into a column (1.5 × 8 cm) after removal of the fines and equilibrated with 10 m*M* potassium phosphate, 1 m*M* PMSF, 0.1 m*M* TLCK, and 0.1 m*M* leupeptin (pH 7). The RNP extract was loaded on the column and after washing with the buffer, the column was eluted with a linear gradient of 0.01–0.8 *M* potassium phosphate (pH 7) containing the protease inhibitors. Most of the antigenically active proteins were eluted between 0.35–0.45 *M* potassium phosphate. These fractions were used for purification of the antigen (active HAP fraction).

Phosphocellulose chromatography. Phosphocellulose (Whatman P11) was sequentially treated with 0.5 *N* NaOH, washed free of alkali, followed by treatment with 0.5 *N* HCl, washed free of acid and then equilibrated with 0.2 *M* potassium phosphate (pH 7), 10% glycerol, 0.1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 1 m*M* PMSF, 0.1 m*M* leupeptin, and 0.1 m*M* TLCK until the wash had the same pH as the starting buffer. The active HAP fraction, after dilution to a phosphate concentration of 0.2 *M* was applied to the column (1 × 7 cm) and eluted with a linear gradient of phosphate between 0.2–0.8 *M* (pH 7). Most of the antigenic activity was eluted between 0.7–0.8 *M* phosphate but most of the protein was eluted at a higher phosphate concentration.

SDS–polyacrylamide gel electrophoresis and immunochemical localization of antigen with ^{125}I-protein A (IPA). The protein samples were dissolved in the sample buffer of Laemmli (8) and electrophoresis was carried out by the method of Laemmli using a 4% stacking gel and a 10% separating gel. The electrophoretically separated proteins were transferred to nitrocellulose sheets by the method described by Burnette (9). The transfer was done for 8 hr at 25 V in cold. After the transfer, the sheets were either stained with amido black or blocked with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 3% bovine serum albumin (essentially globulin free). Washing was done with PBS–0.05% Tween-20. The antibody and IPA were taken up in PBS–0.05% Tween-20 containing BSA (fraction V, Sigma).

Preparative SDS–PAGE. The method was performed according to that described by Knecht and Busch (10) as modified by Catino *et al.* (11) and Goldknopf *et al.* (12).

Radioiodination of proteins. Protein A was radiolabeled with Bolton–Hunter reagent as described (13). All other protein samples were labeled with ^{125}I by the method of Fraker and Speck (14).

Tryptic peptide mapping of purified ^{125}I-labeled antigen. The method was the same as that described by Chan *et al.* (15).

Enzyme immunoassay. The method used was that described by Kelsey *et al.* (16) with the modification that the peroxidase-conjugated goat anti-rabbit IgG (1:300 dilution in PBS–0.5% Tween-20) was used instead of alkaline phosphatase-conjugated goat antirabbit IgG. Color development was done with the substrate 2,2′-azino-di-[3-ethyl-benzthiazolin sulfonat (6)] (ABTS) (15 mg/ml diluted 1:100 with 0.1 *M* citrate, pH 4, containing 0.5 μl H_2O_2/ml).

ELISA competition assay. The method was the same as that described above except that the anti-DE-0.15 *M* antibody was preincubated with various extracts of competing antigen for 1 hr before being applied to microtiter well.

Results. *Fractionation of the nuclear antigens.* The 3 *M* NaCl extract of Namalwa RNP particles was subjected to hydroxylapatite column chromatography using a linear gradient of potassium phosphate (0.01–0.8 *M*). ELISA analysis was done on the various fractions with the IgG fraction of anti-DE-0.15 *M* antibody. The antigen(s) which reacted with anti-DE-0.15 *M* antibody appeared mainly in fractions 60–70 (Fig. 1). After dilution to 0.2 *M* phosphate, the HAP fraction (fraction 60–70) was subjected to phosphocellulose chromatography (Fig. 2). ELISA analysis with anti-DE-0.15 *M* antibody showed that much of the antigen was eluted by 0.7–0.8 *M* phosphate in fractions 50–60 (Fig. 2). The HAP (Fraction 60–70) and phosphocellulose (Fraction 50–60) were subjected to SDS–PAGE (Fig. 3). The phosphocellulose fraction contained a major 38-kDa protein band besides three other protein bands and they were well separated from each other in the gel. Accordingly, it was possible to excise the 38-kDa band from the gel and obtain it in highly purified form by electrophoretic elution.

Identification of the antigen(s) on nitrocellulose sheets. The antigenically active Namalwa RNP HAP fraction (60–70) and phosphocellulose fraction (50–60) were separated

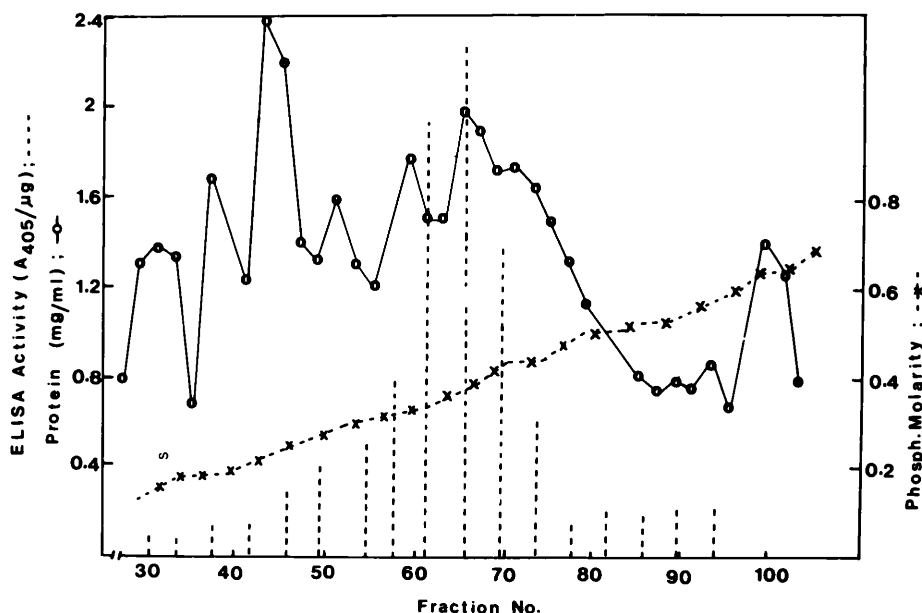

FIG. 1. Hydroxylapatite chromatography of RNP extract of Namalwa cells. Tumor antigenic activity was measured by enzyme immunoassay (ELISA) using anti-DE-0.15 *M* antibody (– – –). The protein concentration was measured by Bio-Rad protein assay method (O). The conductance of the effluent was also measured (-X-X-) and was found to be fairly linear. The antigenic peak (fractions 60–70) were pooled (active HAP fraction).

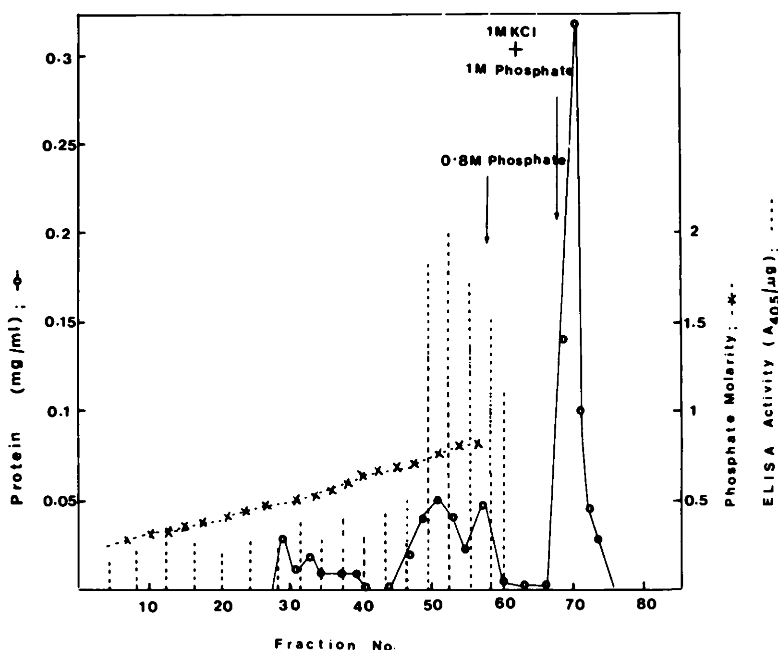

FIG. 2. Phosphocellulose chromatography of active HAP fraction. Fractions were tested for antigenic activities against anti-DE-0.15 *M* antibody (- - -) and protein was measured by Bio-Rad method (O). The conductance of the effluent was measured for the gradient (0.2–0.8 *M*). The antigen peak (fractions 50–60) was pooled.

by SDS–PAGE and analyzed by the "Western Blotting" technique (see Materials and Methods).

The protein transferred to nitrocellulose sheets was incubated with anti-DE-0.15 *M* antibody preabsorbed with fetal calf serum, normal human serum and placental Tris extract. Figure 3B shows that both these fractions contained one major 38-kDa antigen recognized by this antibody; its molecular weight was approximately 38 kDa. Similar experiments with phosphocellulose fractions incubated with preimmune rabbit antibody did not develop a dense band (Fig. 4).

To determine whether this antigen was present in normal human liver nuclei, extracts of liver nuclei were made with 0.01 *M* Tris–HCl (pH 8)/3 *M* NaCl or 0.01 *M* Tris–HCl (pH 8)/9 *M* urea containing 1 m*M* PMSF and 0.1 m*M* each of leupeptin and TLCK. The proteins from these extracts and from HAP fraction of Namalwa RNP were electrophoretically separated, transferred to nitrocellulose sheets, and subsequently immunolocalized by incubation with anti-DE-0.15 *M* antibody. Figure 5 shows that the 38-kDa protein from Namalwa cells was immunoreactive, no corresponding band was identified in the normal liver extracts.

The immunoreactivity of the 38 kDa protein was also analyzed by ELISA competition assays (Fig. 6). Both the phosphocellulose fraction and Namalwa nuclear Tris extract competed with the 38-kDa antigen but the Namalwa nuclear Tris extract competed at a level 100-fold less than that of the phosphocellulose fraction. The liver nuclear extract did not compete with the antigen (Fig. 6A). It was also found that the nuclear Tris extracts of HeLa and Namalwa that had not been induced by NDV failed to compete with 38-kDa antigen (Fig. 6B). The small competition observed at higher concentration of Namalwa (uninduced) nuclear Tris extract probably reflects nonspecific binding to the antibody.

The 38-kDa antigen in the phosphocellulose fraction was separated from other proteins by SDS–PAGE. It was excised, electrophoretically eluted, labeled with ^{125}I, reduced, *S*-carboxymethylated and digested with trypsin and mapped as described in Materials and Methods. Seven major peptides were observed in the tryptic peptide map of the 38-kDa antigen (Fig. 7).

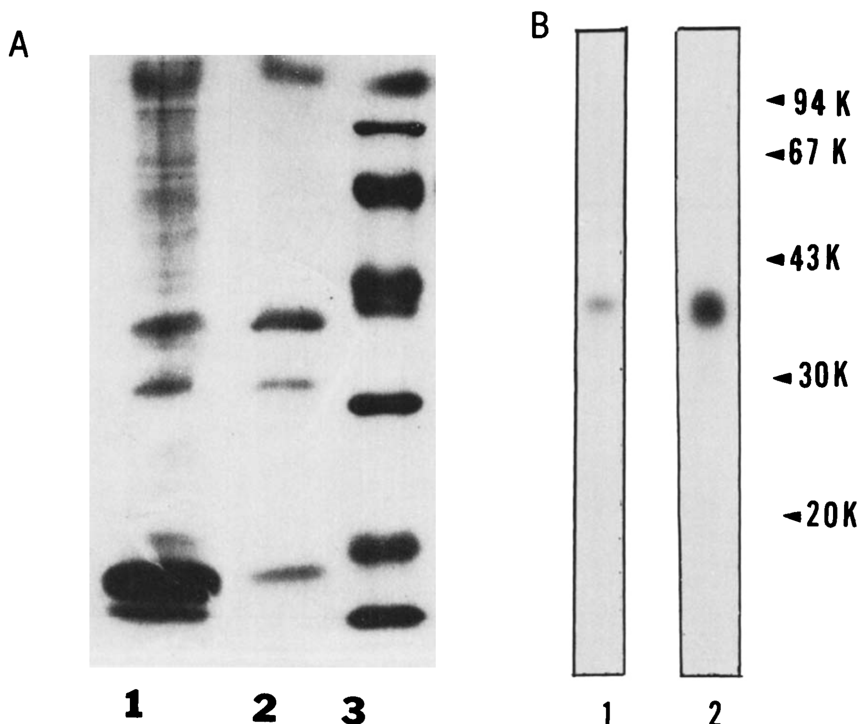

FIG. 3. Identification of Namalwa RNP antigen transferred to nitrocellulose. (A) Coomassie blue-stained gel of electrophoretically separated proteins. Lane 1, active HAP fraction; Lane 2, phosphocellulose fraction; Lane 3, Low-molecular-weight standards (Pharmacia Fine Chemicals; phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin of molecular weight 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20 kDa, and 14.4 kDa, respectively). (B) Localization of immunoreactive antigen on nitrocellulose sheet by "Western" blotting method using IPA as described in Materials and Methods. Lane 1, active HAP fraction and Lane 2, phosphocellulose fraction. Each sheet was incubated with anti-DE-0.15 *M* antibody.

Discussion. It was reported from this laboratory that the Tris extracts of HeLa and Namalwa nuclei contain antigens which are not found in normal liver (5, 15). In these studies, both anti HeLa nucleolar and anti-DE-0.15 *M* antibodies were used to identify the antigens. In the present study, the antigen(s) in the RNP fraction of Namalwa nuclear Tris extract were studied using anti-DE-0.15 *M* antibody. This antibody was found to give bright nucleolar fluorescence in HeLa and other tumor cells but not in human liver (5).

The antigen was purified from RNP by combination of hydroxylapatite and phosphocellulose chromatography (Figs. 1, 2). Two immunological methods (9, 16) were used to identify the antigen. The ELISA was used to identify the chromatographic fractions containing the antigen(s) and "Western Blotting" was used to identify the individual antigen(s). One antigen with a molecular weight of 38 kDa was detected using the anti-DE-0.15 *M* antibody (Fig. 3B).

The SDS–PAGE of phosphocellulose fractions resolved the 38-kDa protein as a distinct band well separated from other protein bands (Fig. 3A). Thus, it offered a convenient method for purifying the antigen by electrophoretic elution of the excised band.

To determine whether the antigen was present in normal liver, the liver nuclear proteins were extracted with 3 *M* NaCl or 9 *M* urea. Most of the human liver antigens common to Namalwa antigens could be extracted by these methods. "Western" blotting showed that the 38-kDa antigen is present in Namalwa but not in liver extracts (Fig. 5). It was possible that the crude liver nuclear extracts contain relatively small amounts of the antigen which could have escaped detection. This

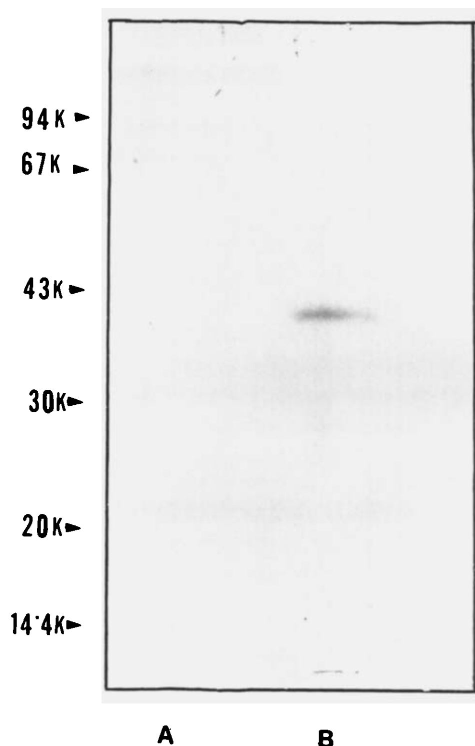

FIG. 4. Immunolocalization of antigen from phosphocellulose fraction of Namalwa RNP on nitrocellulose. The phosphocellulose fraction reactive against anti-DE-0.15 *M* antibody was separated by SDS–PAGE, transferred to nitrocellulose sheets as described in Materials and Methods. (A) Incubated with preimmune rabbit antibody. (B) Incubated with anti-DE-0.15 *M* antibody.

possibility was ruled out because no activity was demonstrated in the HAP fractions of liver RNP extract by ELISA assay.

Further evidence of the absence of 38-kDa antigen in liver was obtained by ELISA competition assays. The advantage with this method was that it permits the reaction to take place in a more native state without SDS treatment; half-maximal optical density was at 20–30 ng. The results indicated that the liver extracts did not contain antigens related to the 38-kDa protein of Namalwa cells. The Tris extracts of Namalwa nuclei and the phosphocellulose fraction which contained the antigen compared quantitatively (Fig. 6A). The purified 68-kDa antigen from the 100,000*g* supernatant of Tris extract of Namalwa nuclei also did not compete with the 38-kDa antigen (Fig. 6A). This result shows that the 38-kd antigen is not immunologically related to the HuAg 68 kDa (closed circles) which is composed of both the major, pI 6.3, and minor, pI 6.1 components. ELISA competition assays with nuclear Tris extracts of HeLa and Namalwa that had not been induced by NDV showed that the antigen is not present in these cells (Fig. 6B). Thus, the antigen appears in Namalwa cells as a result of induction by NDV.

Hightower *et al.* (17) showed that the NDV virus contains six different polypeptides designated L (150–200 kDa), G, (75 kDa), N and G_2 (56 kDa), a 53-kDa protein and a membrane protein, M (41 kDa). The 38-kDa antigen reported above is dissimilar from all these proteins with the possible exception of protein M. It differs from protein M in that: (a) protein M requires high salt (1 *M*) and detergent to remain in solution (18) whereas the 38-kDa antigen is soluble in 0.3–0.4 *M* NaCl and requires no detergent for solubilization. (b) The

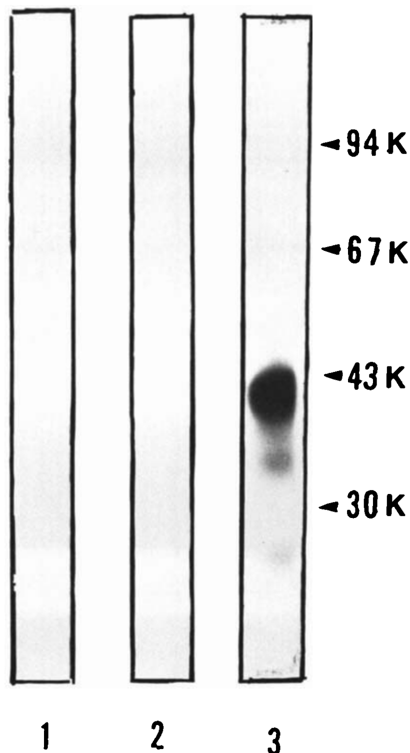

FIG. 5. Immunolocalization of proteins on nitrocellulose sheets. The proteins separated by SDS–PAGE, transferred to nitrocellulose, and incubated with anti-DE-0.15 *M* antibody as described in Materials and Methods. Lane 1, 9 *M* urea extract and lane 2, 3, *M* NaCl extract of normal human liver nuclei. Lane 3, active HAP fraction of Namalwa RNP.

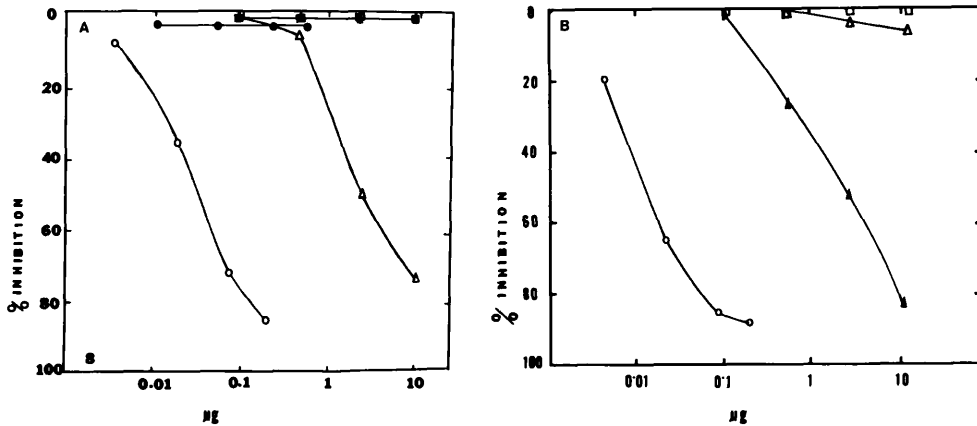

FIG. 6. ELISA competition assay of various extracts with 38-kDa antigen of Namalwa RNP for anti-DE-0.15 *M* antibody. Each microtiter well was coated with 40 ng of phosphocellulose fraction containing 38 kDa as the only antigen. Six-hundred nanograms of the antibody were incubated with various extracts before being applied to the wells. (A) (○), phosphocellulose fraction of Namalwa RNP; (△), total Namalwa nuclear Tris extract; (●), 68 kDa/6.3 antigen of Namalwa nuclear Tris extract (▲), Tris and (□), 9 *M* urea extracts of normal human liver nuclei. (B) (○), phosphocellulose fraction of Namalwa (induced) RNP; (▲), total Tris extract of Namalwa (induced) nuclei; (△), total Tris extract of Namalwa (uninduced) nuclei, (□), total Tris extract of HeLa nuclei.

overall amino acid compositions of M protein and 38-kDa antigen differed in that the M protein contained more histidine, threonine, proline, methionine, and leucine whereas the 38-kDa protein contained more serine and had a reversed arginine–lysine ratio (19).

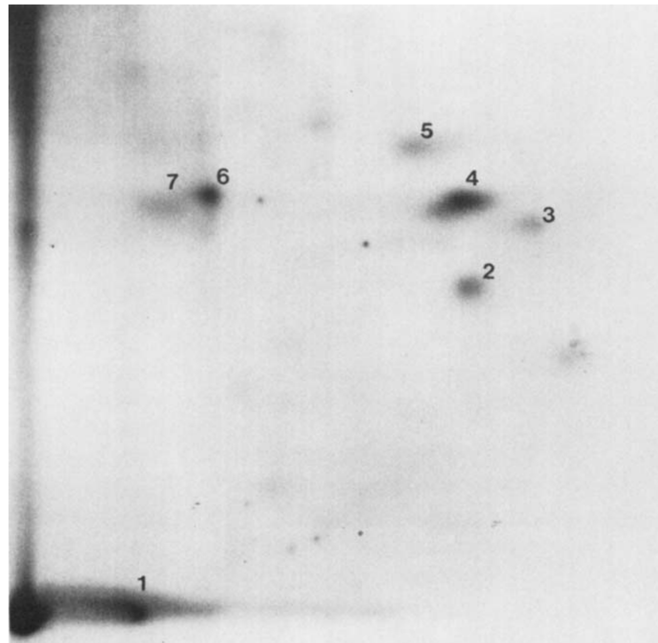

FIG. 7. Tryptic fingerprint of 38-kDa antigen. The ^{125}I-labeled 38-kDa antigen was digested with trypsin and fractionated by electrophoresis and chromatography on cellulose sheet as described in Materials and Methods.

Two other proteins with molecular weights of 66 kd and 36 kDa are induced by NDV infection of the cells (20). The 36-kDa protein is present in minute amounts. There is very little information about the structure and function of this protein (21). In contrast, the 38-kDa antigen is one of the major proteins of RNP extract of Namalwa nuclei.

Accordingly, the Namalwa antigen is not a likely product of the NDV or a constituent of the virus. It seems probable that the antigen is a cellular product which results from the viral infection. It is possible that the antigen may be a precursor of interferon or related in some way to the synthesis of interferon. Further studies on its structure and function will be necessary to establish its role in these induced cells.

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Received July 26, 1982. P.S.E.B.M. 1983, Vol. 172.