Jpn. J. Cancer Res. 80, 271–276, March 1989

Human Monoclonal Antibodies against Cytokeratin 18 Generated from Patients with Gastric Cancer

Tsutomu Abe,[1,3] Masayuki Fukumoto,[1] Keiko Tsuchiya,[1] Kentaro Kuramochi,[1] Tadaaki Furuta,[1] Shinji Togoh,[2] Kiyoshi Nishiyama[2] and Shuji Tsuchiya[2]

[1]*Medical Science Laboratory, Asahi Chemical Industry Co., Ltd., 2-1 Samejima, Fuji, Shizuoka 416 and* [2]*Second Department of Surgery, School of Medicine, Yokohama City University, 3-4 Urafune-cho, Minami-ku, Yokohama 232*

By co-culturing regional lymph node B-cells and HAT-sensitive mutant cells obtained from RPMI-1788 cells, no less than 20,000 Epstein-Barr (EB)-transformed colonies were obtained from 32 patients with gastric cancer. From B-cell cultures generating antibodies reactive with gastric cancer tissues as well as cultured gastric cancer cells, two EB-transformed cell clones termed C418-59 and C1218-39 were isolated. Both of them produced human IgM-class antibodies, termed Mab418-59 and Mab1218-39, respectively. Both antibodies reacted with an antigen with a molecular weight of 45 kd existing in gastric cancer MKN-45, MKN-1, and Kato-III cells, and also with all of 4 adenocarcinomas of the stomach in paraffin sections. The antigen recognized by both antibodies was identified as a kind of cytoskeletal protein, cytokeratin 18. In this study, it was confirmed that B-cell clones generating autoantibodies against cytokeratin 18 were present in some patients with gastric cancer.

Key words: Human monoclonal antibody — Cytokeratin 18 — Tissue polypeptide antigen

The isolation of B-cell clones generating human autoantibodies against cancer-related antigens has started a new era in cancer immunology. In the past seven years, human monoclonal antibodies reactive with cancer-related antigens have been produced in many laboratories by human-human hybridoma,[1)] human-mouse hybridoma,[2-4)] and Epstein-Barr (EB) virus transformation[5)] techniques.

A kind of intermediate filament cytoskeletal protein, cytokeratin 18 (CK 18), is significantly increased in adenocarcinoma, and CK 18 has some antigenic sites to which autoimmune reactions are readily induced. Moreover, a tumor-related antigenic substance, tissue polypeptide antigen (TPA) originally described by Björklund and Björklund[6)] was identified as a mixture of proteolytic derivatives of three cytokeratins, 8, 18, and 19.[7-9)]

We tried to isolate B-cell clones generating autoantibodies against gastric cencer-related antigens, using a newly developed EB transformation technique that involves co-culturing lymphocytes and HAT-sensitive B-cells in a selection medium. We report here that two EB-transformed clones generating human IgM-class antibodies against CK 18 were obtained from lymph node cells of patients with gastric cancer, and both antibodies also reacted with a TPA-like soluble antigen existing in protein-free culture medium of gastric cancer cells.

[3] To whom all correspondence should be addressed.

MATERIALS AND METHODS

Cell cultures 8-Azaguanine-resistant, HAT-sensitive B-cells, ATCC CRL8118, selected from RPMI-1788 (ATCC CCL156) cells, and three gastric cancer cells, MKN-45, MKN-1, and Kato-III, were cultured in RPMI-1640 containing 10% (v/v) fetal calf serum (FCS) at 37°C under 5% CO_2 in air.

EB transformation of human B-cells in lymph nodes Regional lymph nodes of a patient with gastric cancer without metastasis were obtained from a pathological tissue sample. A single cell suspension of lymphocytes from lymph nodes was mixed with ATCC CRL 8118 B-cells at a ratio of 1:2, and the cells were transferred into RPMI-1640 containing 20% (v/v) FCS, 10^{-4} *M* hypoxanthine, 4×10^{-7} *M* aminopterin, 1.6×10^{-5} *M* thymidine, and 10^{-4} *M* levamisole (HATL medium). The medium (0.1 ml) containing 10^4 lymphocytes was poured into each well of 96-well microtestplates (Costar, Cambridge, MA), and incubated in a CO_2 incubator under 5% (v/v) CO_2 in air. Half of the spent medium was replaced with fresh medium every 4 days.

Selection of clones generating monoclonal antibodies reactive with cultured cancer cells A mixture of gastric cancer MKN-45, MKN-1 and Kato-III cells (5×10^4 cells each) was poured into each well of the U-shaped microtestplates (Costar), and 50 μl of cultured media from wells containing growing colonies was transferred into each well containing target cancer cells, then gently mixed and incubated at 20°C for 1 h. The microtestplates were centrifuged and the supernatants were removed.

Then, the microtestplates were washed twice with 20 m*M* phosphate buffer solution containing 0.14 *M* NaCl and 0.2% (w/v) bovine serum albumin, pH 7.4 (PBS-0.2% BSA), and 50 μl of PBS-0.2%BSA containing 5 μg/ml of a mixture (1:1) of biotinylated goat anti-human κ-chain and biotinylated goat anti-human λ-chain antibody (Tago, Burlingame, CA) was added into each well of the microtestplates. The contents of each well were gently mixed and the plates were incubated at 20°C for 30 min, then washed twice with PBS-0.2%BSA. A 50 μl aliquot of a biotin-avidin complex solution, ABC Reagent (Vector Laboratories, Burlingame, CA) was added to each well of the microtestplates, and gently mixed. The plates were incubated at 20°C for 30 min, and washed twice with PBS-0.2%BSA. Substrate solution (0.1 ml) composed of 20 m*M* Tris-HCl buffer, pH 7.4, 0.5 mg/ml of 3,3′-diaminobenzidine (DAB) and 0.02% (v/v) H_2O_2 was added. After reaction at 20°C for 10 min, the target cancer cells, which appeared brown, were observed under an inverted microscope.

Immunoperoxidase staining of paraffin-embedded tissue sections of gastric cancer The formalin-fixed and paraffin-embedded tissues were cut to approximately 4 μm, deparaffinized with xylene, and hydrated with a decreasing graded series of ethanol. Then the slides were immersed for 10 min in methanol-H_2O_2 [1 ml of 30% (w/v) H_2O_2 in 100 ml of absolute methanol] and finally immersed in PBS. The human monoclonal antibody solution containing 1–5 μg/ml antibody was added onto the slide and incubated for 30 min at room temperature. After thorough washing of the slide, peroxidase-labeled goat anti-human IgM antibody (Tago) dissolved in PBS-1%BSA was added and incubated for 30 min at room temperature.

Cloning of transformed cells Cloning of cells generating a monoclonal antibody was performed by the limiting dilution method. One-tenth milliliter of RPMI-1640 containing 20% (v/v) FCS, the cells (3 cells per ml), and 0.1% (v/v) sheep red blood cells were poured into each well of 96-well microtestplates (Costar). This procedure was performed twice.

Purification of human IgM The B-cell clone generating IgM was cultured in a serum-free medium, HB101 (Hana Biologics, Berkeley, CA). Five liters of culture medium was filtered through a Plasmaflo membrane (Asahi Medical, Tokyo) to separate cells and cell debris, then loaded onto a column containing 300 ml bed volume of Q-Sepharose (Pharmacia) equilibrated with 20 m*M* Tris-HCl buffer solution, pH 7.4. The column was washed with 1 liter of 20 m*M* Tris-HCl buffer solution containing 0.14 *M* NaCl, pH 7.4 and then IgM fractions were eluted with 20 m*M* Tris-HCl buffer solution containing 0.4 *M* NaCl, pH 7.4. IgM fractions were dialyzed against 5 m*M* Tris-HCl buffer solution, pH 7.4. Then the precipitated IgM was dissolved again in 20 m*M* Tris-HCl buffer solution containing 0.4 *M* NaCl, pH 7.4, and finally purified by gel filtration chromatography using Sephacryl S-400 (Pharmacia).

Preparation of 45 kd antigen and 37 kd soluble antigen The antigen preparation was extracted from gastric cancer MKN-45 cells in the presence of 8% (w/v) trichloroacetic acid and 0.5% (w/v) sodium dodecyl sulfate (SDS), and the insoluble components were removed by centrifugation. The soluble fraction was dialyzed against PBS containing 0.05% (v/v) Tween 20 and used as the antigen preparation.

The soluble antigen was prepared from protein-free culture medium of MKN-45 cells after two days of culture in RPMI-1640 as described by Chan *et al.*[10] The culture medium was filtered to remove cells and gross debris, then concentrated with a Diaflo PM10 (Amicon, Danvers, MA) and dialyzed against PBS.

Gel electrophoresis and immunoblotting One-dimensional polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed as described by Laemmli.[11] Two-dimensional PAGE was performed according to O'Farrell[12] and Achtstaetter *et al.*[13] Polypeptides were separated by isoelectric focusing [ampholyte ranges of pH 3.5–10 and pH 4–6 were mixed at a ratio of 8:2, and 8.5 *M* urea, 2% (v/v) NP-40 and 2% (v/v) 2-mercaptoethanol were dissolved in the gel] in the first dimension and SDS-PAGE in the second dimension. Then the polypeptides were electroblotted onto a nitrocellulose membrane (Bio-Rad, Richmond, CA) as described by Towbin *et al.*[14] The membrane was incubated with human monoclonal antibodies or murine anti-CK 18 antibody PKK3 (Labsystems, Helsinki), and visualized by using the corresponding peroxidase-labeled secondary antibodies and a DAB solution. The electrophoresis calibration kit (for low-molecular-weight proteins, Pharmacia) was used as calibration standards.

RESULTS

EB transformation of B-cell clones generating antibodies reactive with cultured gastric cancer cells and gastric cancer tissues Mixtures of lymphocytes in regional lymph nodes without metastasis obtained from 32 patients with gastric cancer and the HAT-sensitive B-cells were co-cultured in the HATL medium as described in "Materials and Methods." From no less than 20,000 wells containing growing cells, two wells were selected. IgM-class antibodies existing in both wells reacted with each of gastric cancer MKN-45, MKN-1 and Kato-III cells. The two clones were isolated as described in "Materials and Methods" and termed C418-59 and C1218-39.

Table I shows the characteristics of these clones and the generated antibodies, termed Mab418-59 and

Table I. Characteristics of B-cell Clones and Generated Monoclonal Antibodies

B-cell clone	C418-59	C1218-39
Karyotype	diploid	diploid
Doubling time	18 h	20 h
Antibody generated by B-cell clone	Mab418-59	Mab1218-39
Antibody class	IgM-λ	IgM-κ
Amount of antibody[a]	20–50 μg/ml	1–2 μg/ml
Antigen specificity[b]	45 kd (CK 18)	45 kd (CK 18)
	37 kd	37 kd

a) Antibodies were measured in a 1-day culture of 10^6 transformed cells in 1 ml.
b) The molecular weight was estimated by SDS-PAGE.

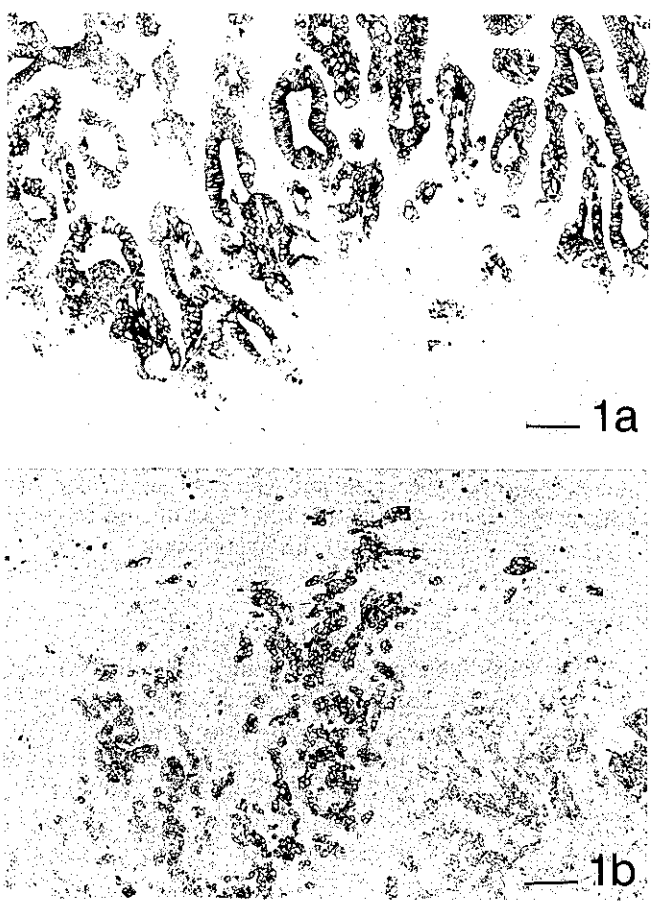

Fig. 1. Immunoperoxidase staining of gastric cancer tissue sections, well-differentiated adenocarcinoma (a) and poorly-differentiated adenocarcinoma (b), with Mab418-59. The cancer tissues were fixed in formalin and embedded in paraffin. Bar = 100 μm.

Mab1218-39, respectively. Clone C418-59 has been maintained for more than two years and produces 20–50 μg/ml IgM in a culture medium. Clone C1218-39 was obtained from another patient with gastric cancer. It has also been maintained for more than two years, but the antibody production is only 1–2 μg/ml IgM in the culture medium. Both clones showed the diploid karyotype. Signet ring cell carcinoma was pathologically diagnosed from tissue samples from both patients.

Immunoperoxidase staining of paraffin-embedded tissue sections of gastric cancer Mab418-59 was confirmed to be capable of staining all of 4 adenocarcinomas of the stomach in paraffin sections by using the immunoperoxidase technique. Typical staining of well-differentiated adenocarcinoma and poorly-differentiated adenocarcinoma with Mab418-59 is shown in Figs. 1a and 1b. Mab1218-39 gave identical results, but the staining was weaker than that with Mab418-59. Sections of autologous cancer tissues which were pathologically diagnosed as signet ring cell carcinoma were found to be indistinctly stained with both autoantibodies (data not shown).

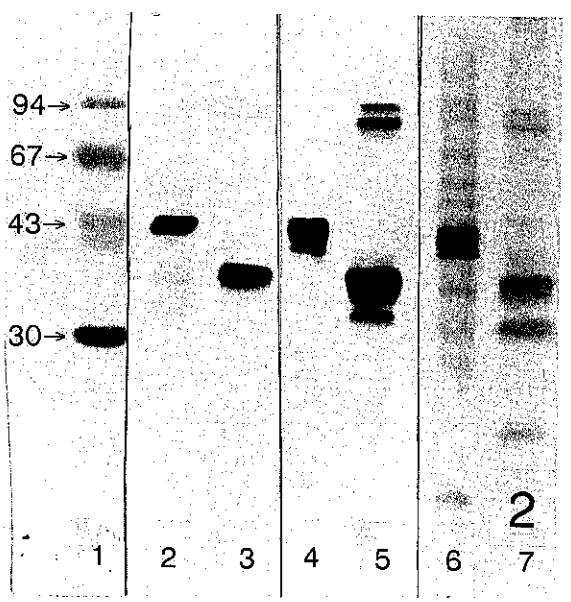

Fig. 2. One-dimensional immunoblots of antigens defined by three monoclonal antibodies. Extracts from MKN-45 cells (lanes 2, 4 and 6) and the concentrated protein-free culture medium of MKN-45 cells (lanes 3, 5 and 7) were defined by Mab418-59 (lanes 2 and 3), Mab1218-39 (lanes 4 and 5), and murine anti-human CK18 antibody PKK3 (lanes 6 and 7). The positions of molecular weight markers are indicated (lane 1, stained with amido black). Molecular markers were 97 kd; bovine serum albumin, 67 kd; ovalbumin, 43 kd; carbonic anhydrase, 30 kd.

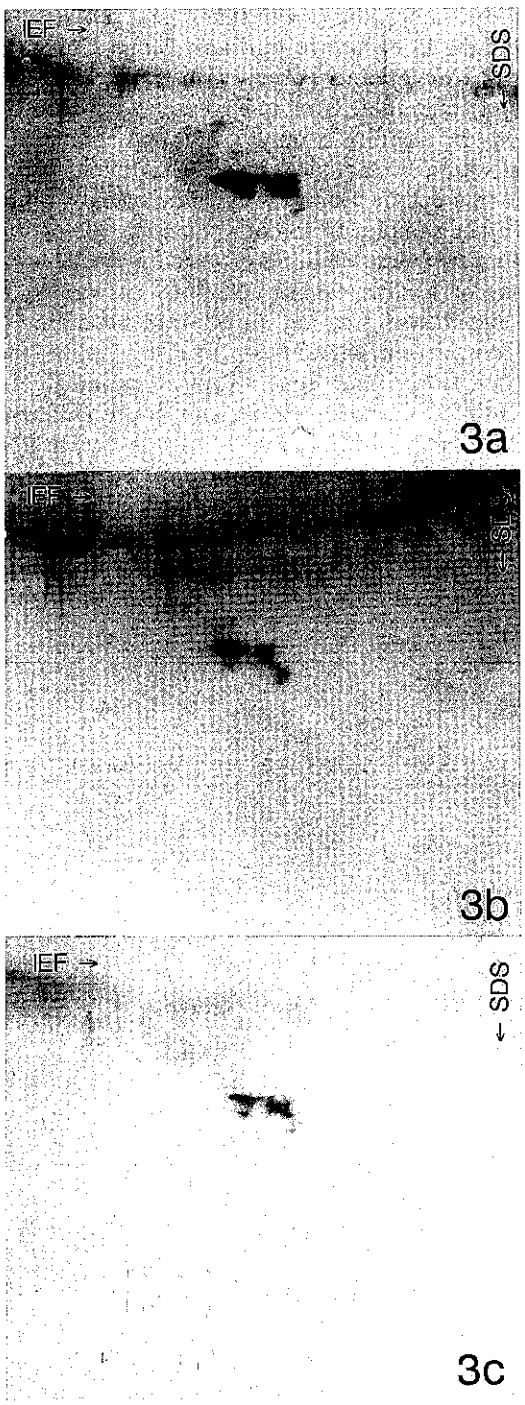

Fig. 3. Two-dimensional immunoblots of antigens defined by three antibodies. Extracts from MKN-45 cells were separated by isoelectric focusing (first dimension, IEF) and SDS-PAGE (second dimension, SDS). Polypeptides separated by two-dimensional electrophoresis were transferred onto nitrocellulose papers and defined by Mab418-59 (a), Mab1218-39 (b), and murine anti-human CK18 antibody PKK3 (c).

Purification of Mab418-59 Clone C418-59 was transferred into a serum-free medium HB101 (Hana Biologics), and Mab418-59 (>90% pure, data not shown) was purified from the culture medium of the C418-59 cells as described in "Materials and Methods." SDS-PAGE in the presence of 2-mercaptoethanol confirmed its purity and that Mab418-59 was composed of heavy chains (μ) and light chains (λ) with molecular weights of approximately 70 kd and 30 kd, respectively. The purified preparation of Mab418-59 was used for the following characterization of antigens.

Antigen identification The extracts from gastric cancer MKN-45 cells were separated by one-dimensional SDS-PAGE and identified by Western blotting. A component of 45 kd in the presence or absence of 2-mercaptoethanol was stained with both of Mab418-59 and Mab1218-39, and its mobility was similar to that of CK 18 (identified with murine anti-CK 18 monoclonal antibody PKK3[15]), as shown in Fig. 2. Mab418-59 showed the highest specificity to the 45 kd antigen among them. The 45 kd antigen was also extracted from MKN-1 and Kato-III cells (data not shown). Moreover, western blots with these antibodies following two-dimensional PAGE confirmed that the 45 kd antigen was identical with human CK18 (Figs. 3a, 3b, and 3c).

These antibodies also reacted with the soluble substance existing in the protein-free culture medium of MKN-45 cells (Fig. 2). The molecular weight of the soluble antigen was estimated at 37 kd by SDS-PAGE. Furthermore, it was confirmed by gel chromatography that the soluble antigen was present in physiological conditions as a high-molecular-weight complex with a molecular weight of approximately 700 kd (data not shown).

Our results indicate that there are some B-cell clones that selectively proliferate and generate autoantibodies against CK 18 or its degraded peptides in the regional lymph nodes from patients with gastric cancer.

DISCUSSION

It was considered that the antigenic sites recognized by both monoclonal antibodies are situated on human CK 18. A human cytoskeletal protein, CK 18 is one of the 19 different cytokeratin polypeptides of human tissue origin classified by Moll *et al.*[16] Human CK 18 is known to be not a cancer-specific substance and it exists widely in simple epithelial tissues as well as in adenocarcinoma. The amino acid sequence predicted from a human CK 18 cDNA was recently defined.[7, 17, 18] CK 18 did not show uniform migration on two-dimensional gel electrophoresis (Fig. 3). Although the observed polymorphism of cytokeratins is considered to be partly due to variety of phosphorylation sites and numbers, the relation between

the polymorphism and the cancer-related transformation is still undefined.

A soluble antigen was also observed in protein-free culture medium of MKN-45 cells. The existence of the 37 kd antigen may be explained as follows. Proteolytic derivatives of cytokeratins exist in the culture media of breast cancer MCF-7, BT-20, T47-D and SK-Br3 cells.[10] Moreover, a 37 kd protein released from a colon cancer cell line SLu-1 was likely to be derived from 45 kd protein.[19] The properties of the 37 kd antigen resembled those of a cancer-related substance, tissue polypeptide antigen (TPA) observed in cancer patients' sera.

Although it has been generally understood that all components of intermediate filaments are cytoplasmic proteins, a result inconsistent with this hypothesis was recently reported. An IgG anti-keratin autoantibody identified in the serum of a Brazilian pemphigus foliaceus patient was considered to be capable of recognizing an extracellular domain of a kind of cytokeratin, CK 10.[20] It was suggested that CK 10 is not a cytoplasmic protein but a transmembrane protein.

A human monoclonal antibody reacting with CK 18 was also obtained from axillary lymph node cells from a patient with breast cancer.[4] Furthermore, a human-mouse hybridoma 1G8 was obtained from cell fusion of mouse myeloma NS-1 cells and human spleen cells previously co-cultured with a human lung adenocarcinoma cell line A549, and a human IgM-class antibody immunoprecipitating with a complex of 46 kd and 58 kd cellular components of A549 was generated.[21] We considered that the 1G8 antigen complex was composed of CK 18 and some other cytoskeletal protein(s). These findings suggest that autoimmune reactions against cytokeratins are readily inducible in man.

Although the mechanism of transformation is still obscure, EB virus-related nuclear antigen was observed in the HAT-sensitive cells, and EB virus is easily released from EB-transformed B-cells when the cells are treated with various chemical substances. Moreover, since the stemline number of the HAT-sensitive B-cells was hypoploid (mode 45), we considered that the growing cells having diploid karyotype must be EB-transformed from normal lymph node cells. Levamisole (10^{-4} *M*) was found to accelerate the transforming rate of lymph node cells about 2-fold. The efficiency of transformation was estimated at one transformed cell per 5×10^3 to 10^5 lymphocytes (not B-cells). This EB-transformation method should be widely applicable to cloning of B-cells producing other kinds of monoclonal antibodies.

ACKNOWLEDGMENTS

We thank Mr. T. Kaneda and Mr. J. Shin for their skillful technical assistance.

(Received November 4, 1988/Accepted January 26, 1989)

REFERENCES

1) Sikora, K., Alderson, T., Ellis, J., Phillips, J. and Watson, J. Human hybridomas from patients with malignant glioma. *Br. J. Cancer,* **47,** 135–145 (1983).
2) Schlom, J., Wunderlich, D. and Teramoto, Y. A. Generation of human monoclonal antibodies reactive with mammary carcinoma cells. *Proc. Natl. Acad. Sci. USA,* **77,** 6841–6845 (1980).
3) Nowinski, R., Berglund, C., Lane, J., Lostrom, M., Bernstein, I., Young, W. and Hakomori, S. Human monoclonal antibody against Forssman antigen. *Science,* **210,** 537–539 (1980).
4) Cote, R. J., Morrissey, D. M., Houghton, A. N., Thomson, T. M., Daly, M. E., Oettogen, H. F. and Old, L. J. Specificity analysis of human monoclonal antibodies reactive with cell surface and intracellular antigens. *Proc. Natl. Acad. Sci. USA,* **83,** 2959–2963 (1986).
5) Irie, R. F., Sze, L. L. and Saxon, R. E. Human antibody to OFA-1, a tumor antigen, produced *in vitro* by Epstein-Barr virus-transformed human B-lymphoid cell lines. *Proc. Natl. Acad. Sci. USA,* **79,** 5666–5670 (1982).
6) Bjöklund, B. and Bjöklund, V. Antigenicity of pooled human malignant and normal tissues by cytoimmunological technique. Presence of an insoluble, heat-labile tumor antigen. *Int. Arch. Allergy Appl. Immunol.,* **10,** 153–183 (1957).
7) Laube, R. E., Bosch, F. X., Romano, V., Zimbelmann, R., Höfler, H. and Frank, W. W. Cytokeratin expression in simple epithelia, III. Detection of mRNAs encoding human cytokeratins nos. 8 and 18 in normal and tumor cells by hybridization with cDNA sequences *in vitro* and *in situ. Differentiation,* **33,** 69–85 (1986).
8) Lüning, B. and Nilsson, U. Sequence homology between tissue polypeptide antigen (TPA) and intermediate filament (IF) proteins. *Acta Chem. Scand.,* **37,** 731–733 (1983).
9) Weber, K., Osborn, M., Moll, R., Wiklund, B. and Lüning, B. Tissue polypeptide antigen (TPA) is related to the non-epidermal keratins 8, 18 and 19 typical of simple and non-squamous epithelia: re-evaluation of a human tumor marker. *EMBO J.,* **3,** 2707–2714 (1984).
10) Chan, R., Possitto, P. V., Edwards, B. F. and Cardiff, R. D. Presence of proteolytically processed keratins in the culture medium of MCF-7. *Cancer Res.,* **46,** 6353–6359 (1986).
11) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685 (1970).

12) O'Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, **250**, 4007–4021 (1975).
13) Achtstaetter, T., Hatzfeld, M., Quinlan, R. A., Parmelle, D. C. and Frank, W. W. Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. *Methods Enzymol.*, **134**, 355–371 (1987).
14) Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354 (1979).
15) Virtanen, I., Miettinen, M., Lehto, V-P., Kariniemi, A-L. and Paasivuo, R. Diagnostic application of monoclonal antibodies in intermediate filaments. *Ann. N. Y. Acad. Sci.*, **455**, 635–648 (1985).
16) Moll, R., Franke, W. W., Schiller, D. L., Geiger, B. and Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell*, **31**, 11–24 (1982).
17) Romano, V., Hatzfeld, M., Magin, T. M., Zimbelmann, R., Frank, W. W., Maier, G. and Ponstingl, H. Cytokeratin expression in simple epithelia, I. Identification of mRNA coding for human cytokeratin no. 18 by a cDNA clone. *Differentiation*, **30**, 244–253 (1986).
18) Oshima, R. G., Millán, J. L. and Caceña, G. Comparison of mouse and human keratin 18: a component of intermediate filaments expressed prior to implantation. *Differentiation*, **33**, 61–68 (1986).
19) Hollmann, M., Staab, H. J., Spindler, E., Sproll, M., Anderer, F. A. and Fortmeyer, H. P. Monoclonal antibody-defined circulating human tumor-associated antigen with epitope shared by cytokeratins. *Biochem. Biophys. Res. Commun.*, **128**, 34–39 (1985).
20) Diaz, L. A., Sampaio, S. A. P., Martins, C. R., Rivitte, E. A., Macca, M. L., Roscoe, J. T., Takahashi, Y., Labib, R. S., Patel, H. P., Mutasim, D. F., Dugan, E. M. and Anhalt, G. J. An autoantibody in pemphigus serum, specific for the 59 kD keratin, selectively binds the surface of keratinocytes: evidence for an extracellular keratin domain. *J. Invest. Dermatol.*, **89**, 287–295 (1987).
21) Ho, M-K. Production of human monoclonal antibodies by *in vitro* immunization. *In* "Human Hybridoma,"ed. A. J. Strelkauskas, pp. 23–38 (1987). Marcel Dekker, Inc., New York.