4. G. I. Vorovitskaya, S. N. Khramtsova, and V. S. Shapot, Vestn. Akad. Med. Nauk SSSR, No. 3, 29 (1982).
5. Yu. A. Umanskii, in: Immunology of Chemical Carcinogenesis [in Russian], Kiev (1975), p. 104.
6. A. Boyum, Scand. J. Clin. Lab. Invest., 21, 97 (1968).
7. K. Burton, Biochem. J., 62, 315 (1956).
8. E. R. Giblett, A. J. Amman, D. W. Wara, et al., Lancet, 1, 1010 (1975).
9. E. R. Giblett, J. E. Anderson, F. Cohen, et al., Lancet, 2, 1067 (1972).
10. B. S. Handerwerger and R. H. Schwartz, Transplantation, 18, 544 (1974).
11. N. K. Jerne and A. A. Nordin, Science, 140, 405 (1963).
12. S. M. Johnson, M. E. North, and G. L. Asherson, Lancet, 1, 168 (1977).
13. M. N. Julius, E. Simpson, and L. A. Herrenberg, Eur. J. Immunol., 3, 645 (1973).
14. K. Ogawa, K. Tominaga, S. Taoka, et al., Gann, 69, 471 (1978).
15. E. M. Shevach, L. Ellman, J. M. Davie, et al., Blood, 39, 1 (1972).

HEPATOMA 27 AND RAT COLONIC EPITHELIAL CELLS HAVE THE SAME PREKERATIN PROTEIN PROFILE

S. M. Troyanovskii, V. A. Krutovskikh, and G. A. Bannikov

UDC 616.36-006-008.939.6:616.348-018.73-0080939.6]-074

KEY WORDS: hepatoma 27, colon, prekeratin proteins.

The prekeratins are a family of related proteins found in the composition of intermediate filaments of epithelial cells. One special feature of the prekeratins, which distinguishes them from proteins of the intermediate filaments of other types of cells (vimetin, desmin, proteins of neurofilaments and glial filaments), is their biochemical heterogeneity. It has also been shown that the prekeratin profiles of different epithelia also differ [8]. It has been suggested that the different profiles of these proteins determines, at least partially, differences in the morphological reactions of different epithelial cells [7].

However, for various reasons the scale of this heterogeneity and of the differences in prekeratins in different cells have so far proved difficult to estimate. This is due above all to the high degree of homology of the different prekeratins, which makes the use of polyclonal antisera for the characterization of individual proteins of this family difficult. In some cases the increased heterogeneity of the prekeratins can be attributed to partial proteolysis of these proteins during isolation.

By using monoclonal antibodies which we obtained against various antigenic determinants of prekeratin and also a number of biochemical techniques, we showed in this investigation that under conditions when isolation of prekeratins is not accompanied by proteolytic degradation, their profile is the same in colonic epithelial cells and in cells of a transplantable hepatoma.

EXPERIMENTAL METHOD

Prekeratin was isolated from the liver and colon of noninbred rats. In addition, primary cultures of transplantable rat hepatoma 27, obtained from the collection of tumor strains, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, were used. For electrophoretic investigation of proteins, cells (or pieces of organs) were either dissolved immediately in buffer for electrophoresis (cell lysates) or prekeratin was isolated from them beforehand. Prekeratin was isolated by the standard method [5], which consists of successive extraction of the tissue homogenate, first with a 1% solution of Triton X-100, and then with 1.5 M KCl. Prekeratin proteins with molecular weights of 40-60 kilodaltons (kD) are sedimented by this treatment in the residue. The technique of polyacrylamide gel elec-

Laboratory of Mechanisms of Carcinogenesis and Laboratory of Carcinogenic Substances, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.
(Presented by Academician of the Academy of Medical Sciences of the USSR, N. A. Kraevskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 8, pp. 220-223, August, 1984. Original article submitted August 11, 1983.

0007-4888/84/9808-1111$08.50

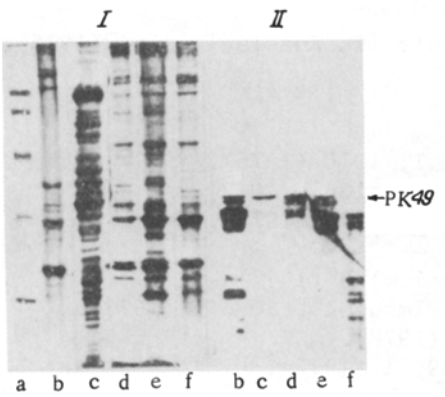

Fig. 1

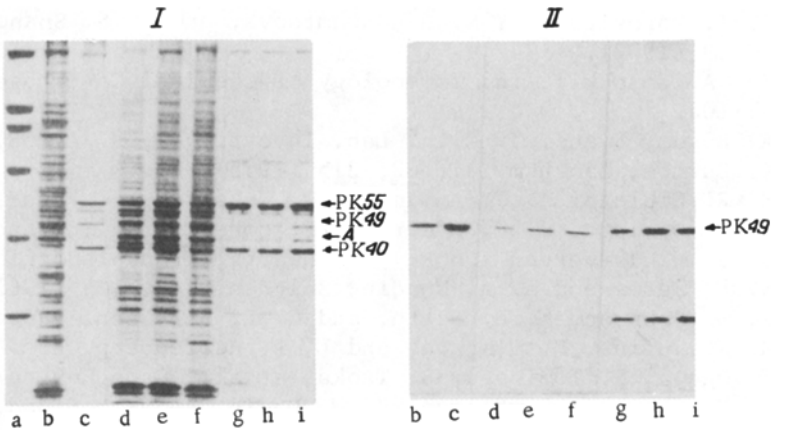

Fig. 2

Fig. 1. Degradation of hepatic prekeratin during isolation of filaments: I) staining for protein with Coomassie P250; II) immunoautoradiography with antibodies of clone C12 from the same specimens as in I. a) Markers, from top to bottom; 200, 97.4, 66, 45, and 29 kD; b) preparation of hepatic prekeratin; c) liver lysate; d) residue after extraction with 1% solution of Triton X-100 (1 h, 4°C); e) residue after extraction with 1.5 M KCl (12 h, 4°C); f) residue after extraction with 1.5 M KCl (12 h, 37°C). Arrow indicates PK49.

Fig. 2. Prekeratin proteins of hepatoma 27 and colonic epithelium. I) Staining for protein with Coomassie P250; II) immunoautoradiography with antibodies of clone C12 from the same specimens as in I. a) Markers (the same as in Fig. 1); b) hepatome 27 (lysate); c) hepatome 27 (prekeratin preparation); d-f) lysates of different fractions of colonic enterocytes (fractions 2, 5, and 8 respectively); g-i) prekeratin preparations from same fractions of erythrocytes (2, 5, and 8). A) Actin.

trophoresis in the presence of sodium dodecylsulfate (PAG-SDS) and of immunoautoradiography was described by the writers previously [1, 2]. Peptide mapping was carried out by the method in [3]: the track of one specimen, separated in PAG-SDS in one direction, was cut out of the gel slab and placed on top of 10% concentrating gel. A solution of protease V8 from *Staphylococcus aureus* (25 μg/ml) in the top gel buffer with 20% glycerol, 0.1% SDS, and 20 mM EDTA, was layered above it. After concentration of the specimen on the boundary between the top and bottom gels the current was increased from 10 to 30 mA. The peptides were separated in an acrylamide gradient gel (from 15 to 25%).

Enterocytes were separated from the colonic mucosa by slow rotation (60-120 RPM) of the colon, turned inside out and fitted over a dialysis tube filled with phosphate buffer, in a 27 mM solution of sodium citrate in phosphate buffer [3, 4]. By gradual stripping of the epithelium, different cell fractions (from six to nine) were obtained, containing enterocytes at different stages of differentiation — from the top to the bottom of the crypt.

The method of obtaining monoclonal antibodies of hybridoma clones A1 and C12 and their preliminary characterization were described by the writers previously [2].

EXPERIMENTAL RESULTS

A preparation of intermediate liver filaments is quite complex in this protein composition (Fig. 1b) and, besides prekeratin, it evidently contains proteins of the extracellular matrix, the actin cytoskeleton, and chromatin also. Staining of such a preparation, separated in PAG-SDS and transferred to a nitocellulose filter, with antibodies of clone C12 revealed a group of proteins with mol. wt. of under 49 kD (Fig. 1b). Similar staining of the liver lysate revealed only one protein with mol. wt. 49 kD (Fig. 1c). The mobility of this protein in PAG-SDS corresponded to that of the protein with highest molecular weight from the preparation of intermediate filaments. The most likely explanation of this disparity is that during isolation of the intermediate filaments preparation from the liver, they underwent partial proteolysis. In fact, when the liver was homogenized in 1% Triton X-100 solution, several minor low-molecular-weight proteins appeared on electrophoresis (Fig. 1d). Their number increased considerably in the next stage (extraction in 1.5 M KCl). If this treatment was carried out at 37°C, the proteins reacting with antibodies of clone C12 with mol. wt. of

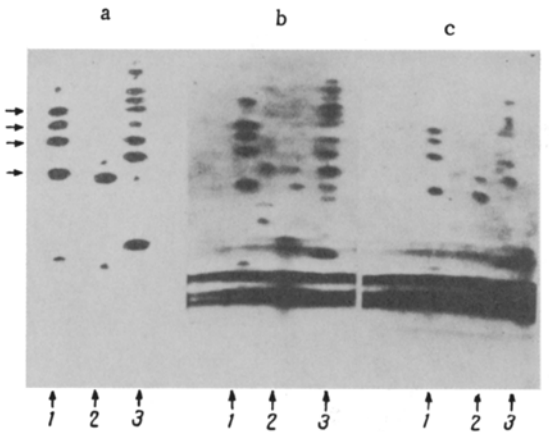

Fig. 3. Peptide maps of prekeratin proteins of hepatoma 27 (a) and colonic epithelium (b), obtained with the aid of protease V8. Gel stained with Coomassie P250. Scheme of distribution of peptides for three prekeratins of these cells shown on right: 1) PK55; 2) PK49; 3) PK40. Arrows indicate peptides of bovine albumin and protease V8.

over 43 kD disappeared (Fig. 1b). Thus there is probably only one protein in the liver with mol. wt. of 49 kD (PK49), which reacts with antibodies of clone C12. By the use of protease inhibitors such as phenylmethylsulfonyl fluoride, chemostatin, and trasylol (all from Sigma, USA) it was impossible to prevent degradation of PK49 during its isolation from the liver.

Similar isolation of prekeratin from cultured hepatoma 27 cells led to somewhat different results. In preparations obtained from these cells only a few proteins were found after staining with Coomassie: three major proteins with mol. wt. of 55, 49, and 40 kD and one minor protein with mol. wt. of 53 kD respectively (Fig. 2c). In these preparations, unlike those from the liver, virtually no proteins with other molecular weights were present. Thus proteins with mol. wt. of 55, 53, 49, and 40 kD, because of their biochemical criteria (solubility and molecular weight), are most probably prekeratins (PK55, PK53, PK49, and PK40 respectively). Only one of them, namely PK49, was labeled during immunoautoradiography with antibodies of clones C12 and A1 (Fig. 2b, c). Consequently, during isolation of prekeratins from hepatoma 27 cultures, their proteolytic degradation was much less severe than when they were isolated from the liver. The intensity of destruction probably depends on the presence of endogenous proteases, whose activity is not blocked by the inhibitors used, in the cells. Such degradation may lead to overestimation of the heterogeneity of the prekeratins. For instance, besides prekeratin proteins with mol. wt. of about 55, 53, and 49 kD, other prekeratins with mol. wt. of about 45 kD or less have been described in the rat liver [5]. According to our data, some of them may be breakdown products of prekeratins with higher molecular weight.

Proteins analogous to the prekeratins of hepatoma 27 (mol. wt. 55, 53, 49, and 40 kD) were found in preparations from the liver also (Fig. 1b), but because of serious contamination by proteins of other intracellular structures and fragments of prekeratins (as was demonstrated for PK49), it is difficult to use this preparation for biochemical research and, in particular, to study the peptide maps of these proteins. Accordingly we studied prekeratin proteins from cells of hepatocyte origin, specifically prekeratins of hepatoma 27. Peptide maps using protease V8 show that proteins PK55, PK49, and PK40 differ from one another: this protease did not reveal any identical peptides (Fig. 3). In other words, these proteins are not fragments of each other. The minor protein PK53 was not clearly visible in the maps obtained, and for that reason no definite conclusions can be drawn regarding its kinship with PK55, PK49, and PK40.

Three types of cells could be identified by the light microscope in films from the isolated fractions: absorbent — highly differentiated cells lining the mouths of the crypts (Fig. 4a), goblet — cells differentiated in another direction, whose secretion stains with mucicarmine (Fig. 4c), and poorly differentiated cells — small round cells located in the lower part of the crypt (Fig. 4a). The fraction of cells of each of the three types differed greatly in different fractions: fractions I and II contained about 80% of absorbent cells, fractions IV and V about 60% of goblet cells, and fractions VIII and IX more than 90% of poorly differentiated cells. Immunoautoradiography with antibodies of clone C12 after electrophoresis of lysates of all the cell fractions of the enterocytes revealed prekeratin protein PK49 in them (Fig. 2d-i). Isolation of prekeratin preparations from the cells of these fractions led to degradation of PK49 by a much lesser degree than in the case of their isolation from the liver: besides PK49, only one low-molecular-weight protein, reacting with antibodies of clone A1 and C12, with mol. wt. of 28 kD could be found in the isolated preparations. Electrophoresis in PAG-SDS, followed by staining for protein, showed that besides PK49 the preparations

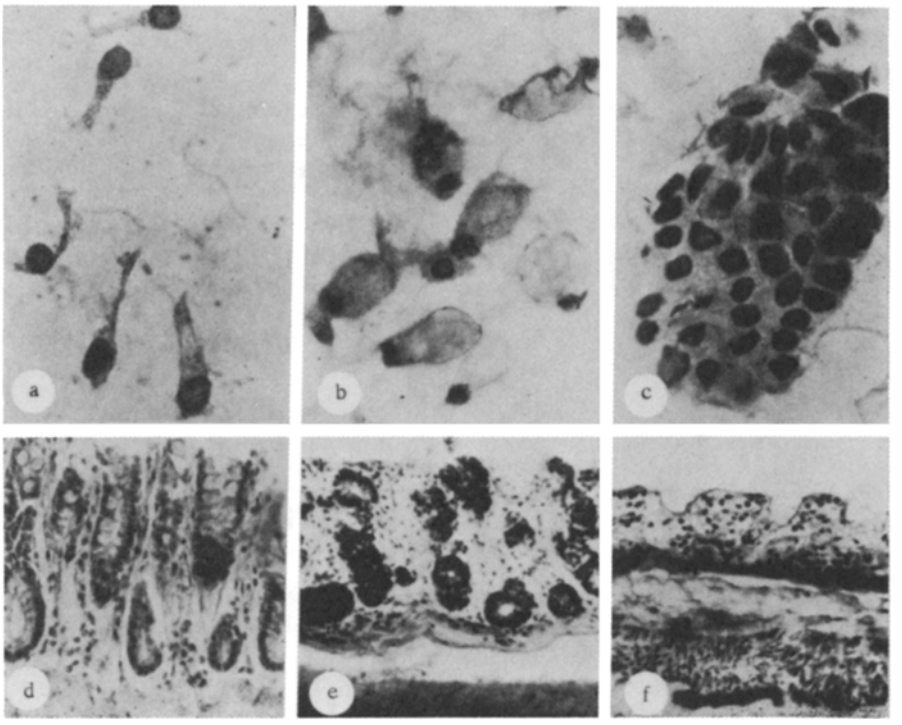

Fig. 4. Different fractions of colonic enterocytes. a) Absorbent cells in film from fraction I. Hematoxylin, 1250 ×; b) histological structure of colonic mucosa after removal of two fractions. Hematoxylin and eosin, 300 ×; c) goblet cells in film from fraction IV. Hematoxylin and mucicarmine, 1250 ×; d) histological structure of mucosa after removal of fraction IV. No goblet cells present. Hematoxylin and eosin, 300 ×; e) poorly differentiated enterocytes from fraction VII. Hematoxylin, 1250 ×; f) state of colonic mucosa after isolation of enterocytes. Basement membrane preserved. Hematoxylin and eosin, 400 ×.

of intermediate filaments of all cell fractions contained a further three proteins, corresponding to the analogous proteins of hepatoma 27 (mol. wt. 55, 53, and 40 kD); the ratio between the intensities of the bands of each of these proteins in the gel, moreover, was roughly the same in all fractions of colonic epithelium and in hepatoma 27 cells (Fig. 2). Peptide maps of colonic prekeratins, moreover, coincided with the corresponding maps of hepatoma 27 proteins (Fig. 3).

The presence of four prekeratins with the same molecular weights in a total preparation of rat colonic epithelium was demonstrated previously [6]. It was shown on a model of differentiation of the epidermis [8] that cells of the basal and apical layers of the epidermis and cells of hair follicles and sweat glands have different prekeratin compositions. It has been suggested that this is connected with morphological differences in the above-mentioned cells [7, 8]. The results now obtained show that such morphogenetically different cells as hepatoma 27 and colonic epithelial cells contain the same complement of prekeratins. Moreover, it follows from the results that most epithelial cells of the crypt of the large intestine synthesize the same prekaratin. Cells of different branches of entodermal differentiation (hepatocytes, goblet cells, colonic absorbent cells) thus contain the same complement of major prekeratin proteins: PK55, PK53, PK49, and PK40. Further investigations are needed to determine the universality of these prekeratins for intermediate filaments of other branches of entodermal differentiation and to study whether analogous proteins exist in epithelial cells of other branches of differentiation (ectodermal and mesodermal). Answers to these questions are necessary in order to understand both the role of intermediate filaments in the cells and the significance of the various prekeratin proteins in the diagnosis of different types of normal and malignant epithelia.

LITERATURE CITED

1. S. M. Troyanovskii and G. A. Bannikov, Tsitologiya, 23, 545 (1981)
2. T. A. Brasitus, Anal. Biochem., 123, 364 (1982).
3. D. P. Chopra, K. Y. Yeh, and R. W. Brochman, Cancer Res., 41, 168 (1981).
4. W. W. Franke, H. Denk, R. Kalt, et al., Exp. Cell Res., 131, 299 (1981).
5. W. W. Franke, S. Winkes, C. Gund, et al., J. Cell Biol., 90, 116 (1981).
6. E. Lazarides, Nature, 283, 249 (1981).
7. R. Moll, W. W. Franke, B. Volc-Platzer, et al., J. Cell Biol., 95, 285 (1982).
8. B. Schaffhausen and T. L. Benjamin, J. Virol., 40, 184 (1981).

PRODUCTION OF MONOCLONAL ANTIBODIES TO Lyt-2,3 ANTIGEN

A. V. Filatov, A. V. Chervonskii, and B. D. Brondz

UDC 612.112.94.017.1

KEY WORDS: monoclonal antibodies, antigens of T cells.

Mouse Lyt-2 and Lyt-3 antigens are membrane markers of T cells which appear in the course of their maturation in the thymus, are expressed in T killers and T suppressors, and are essential for the realization of their functions [4]. In particular, monoclonal antibodies (MCA) against Lyt-2,3 antigens block the cytotoxic action of T killers [14] and inhibit proliferation of T cells in mixed lymphocyte culture [6]. Lyt-2 and Lyt-3 molecules, with molecular weights of 34-38 and 30 kilodaltons respectively, are expressed on the surface of the same cells, their chains are covalently linked, and they form mono-, di-, and tetramers [11]. Several hybridomas producing MCA against Lyt-2 antigen are known [5, 7, 9]. Lyt-3 antigen has received less study.

This paper describes the production and properties of MCA against Lyt-2,3 antigens.

EXPERIMENTAL METHOD

Mice of lines CBA, BALB/c, C57BL/6, DVA/2, and AKR were obtained from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR, and of lines SJL and C58 from the Research Laboratory of Experimental Biology and Medicine, Academy of Medical Sciences of the USSR. AKR mice were immunized by a single intravenous injection of $2 \cdot 10^7$ thymocytes from CBA mice. Seven days later, $5 \cdot 10^7$ spleen cells of the immune mice were hybridized by means of 50% polyethylene-glycol (mol. wt. 1500 daltons, from Schuchardt, West Germany) with 10^7 mouse myeloma P3-X63-Ag 8.653 (X63) cells [3]. After hybridization the cells were transferred in DMEM medium with 10% embryonic calf serum, 4 mM L-glutamine (all from Flow Laboratories, England), 100 units/liter gentamicin (from Farmakhim, Bulgaria), 10^{-4} M hypoxanthine, $4 \cdot 10^{-7}$ M aminopterin, and $1.6 \cdot 10^{-5}$ M thymidine (all from Sigma, USA), into 96-well plates (3040, from Falcon, USA), seeded beforehand with peritoneal macrophages (10^4 cells per well). The presence of antibodies in the culture fluid (CF) was determined 10-14 days later in a two-stage complement-dependent cytotoxicity test [2], using rabbit complement (from Cedarline, Canada). During mass treatment $5 \cdot 10^7$ cells were incubated in 5 ml of complement (1:10) for 1 h at 37°C. Dead cells were removed by centrifugation in a Ficoll—Hypaque density gradient. Treatment of the cells with anti-Thy-1,2 serum (from Searle, England), diluted 1:20, was used as the positive control.

The indirect immunofluorescene test was carried out by incubating 10^6 cells in 50 µl of CP and, after washing, in 50 µl of rabbit antimouse antibodies labeled with fluorescein isothiocyanate (FITC N. F. Gamaleya Institute of Epidemiology and Microbiology). Fluorescence was recorded on a 50-H flow cytometer (Ortho, USA).

Laboratory of Physical Methods of Investigation, Research Institute of Immunology, Ministry of Health of the USSR. Laboratory of Immunochemistry of Tumors, Research Institute of Carcinogenesis, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR R. V. Petrov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 8, pp. 223-226, August, 1984. Original article submitted September 5, 1983.

