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PROTEIN (14-3-2) BY HUMAN NEUROBLASTOMA
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PRODUCTION OF A NERVOUS-SYSTEM-SPECIFIC
PROTEIN (14-3-2) BY HUMAN NEUROBLASTOMA
CELLS IN CULTURE

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Cultured cell lines which continue to produce organ-specific products or exhibit differentiated physiological characteristics have been established from neoplasms of a variety of tissues. Recently functional clonal cell culture lines which produce the nervous-system-specific protein S-100^{1,2} have been isolated from glial tumors^{3,4}. These glial cultures have been extremely valuable in analyzing the regulation of accumulation of S-100 in growing cultures⁵, the synthesis and degradation of this protein⁶, and the regulation of several enzymes by hormones and neurotransmitters⁷.

Clonal cell lines derived from a spontaneously occurring mouse neuroblastoma, Cl300, have also been isolated⁸⁻¹⁰. Many of these clones exhibit electrophysiological¹¹⁻¹³, morphological^{14,15}, and biochemical¹⁶⁻¹⁸ properties of neurons. Cell hybridization studies with Cl300 clones have lead to a tentative model describing some aspects of neuronal differentiation^{19,20}. Although several reports of the establishments of cell strains from human neuroblastomas exist²¹⁻²³, such cells have not been extensively exploited for physiological and biochemical studies, in contrast to the mouse Cl300 neuroblastoma clones. In this report we describe the production of a nervous-system-specific protein, 14-3-2²⁴, by the human neuroblastoma line IMR-32²².

The purified 14-3-2 protein antigen was the generous gift of Dr. Blake Moore. Antisera to 14-3-2 were prepared in albino New Zealand female rabbits. Each rabbit received three sets of toe pad and intradermal injections. Each injection, given at weekly intervals, contained 5 mg of beef-brain 14-3-2 and 5 mg of methylated bovine serum albumin in complete Freund's adjuvant. Three weeks after the last intradermal immunization a 5 mg booster of 14-3-2 was given by ear-vein injection. The animals were bled from the ear on the

sixth and seventh days after the boost. Microcomplement fixation²⁵ titers against the homologous antigen were 1:1000 and 1:1200.

Extracts of mouse tissues were used to demonstrate organ-specificity. A single precipitin line formed between mouse brain extracts and antisera to bovine 14-3-2 protein. No precipitin line formed with extracts of liver, kidney, spleen, or heart. Cross-reacting material in mouse brain was detectable (80% complement fixation at peak) at concentration of soluble protein as low as 1-2 µg/ml with an antiserum dilution of 1:350 (homologous titer, 1:1000). No cross-reacting material could be detected by complement fixation in extracts of mouse liver, kidney, spleen, or heart at protein concentrations up to 1000 µg/ml (the highest tested). Serological demonstration of organ specificity of 14-3-2, first reported by Moore and Perez²⁴, has thus been confirmed on independently prepared antisera.

The human cell line IMR-32 first isolated by Tumilowicz et al.,²² was obtained from the American Type Culture Collection, Rockville, Maryland. Mouse C1300 neuroblastoma clones NB-41⁸ and N18¹⁴, as well as HeLa cells are currently carried in our laboratory. All cells were grown on 100 mm Falcon tissue culture plates in Nutrient Mixture F10²⁶ (Grand Island Biological) supplemented with 10% fetal calf serum, except N18, which was grown on Dulbecco's Modified Eagle's Medium (Grand Island Biological) containing 10% fetal calf serum. Medium was changed twice weekly in most cases, except as noted. Cells were grown at 37° in a 95% air-5% CO₂ humidified incubator. Cultures were passed by trypsinization (0.1% trypsin in Saline A²⁷). Cell number was determined by hemocytometer count; protein was measured by the method of Lowry et al.²⁸

Stationary phase IMR-32, HeLa, NB-41 and N18 cells were analyzed by microcomplement fixation for the presence of 14-3-2 protein. HeLa cells were included as a control culture derived from a non-neural human tumor. An extensive complement fixation reaction between extracts of IMR-32 cells and antisera to beef-brain 14-3-2 protein is demonstrable (Fig. 1). At the same antiserum dilution there is only a slight reaction with the HeLa extract, occurring at a protein concentration 30-fold greater than that of the IMR-32 neuroblastoma extract. Complement fixation reaction at such high concentrations of total-protein are difficult to interpret, and may well be due to nonspecific effects. The possible production of a small amount of this protein by cultured HeLa cells cannot be discounted at present, however. Cultured cells derived from a human neuronal tumor thus contain easily detectable amounts of 14-3-2 protein, while cells of a non-neuronal origin contain at best marginal levels of this antigen, in agreement with analysis of tissue extracts. Extracts of mouse neuroblastoma C1300 clones N18 and NB-41 do not react with antiserum to 14-3-2 protein at appropriate dilutions for the detection of mouse brain 14-3-2 protein.

In several cell culture systems the synthesis of an organ-specific protein does not occur while the cells are in exponential growth, but only after the culture has reached confluency. This type of regulatory phenomenon exists in the C₆ glial cell line. Accumulation of S-100 protein does not occur in rapidly dividing cells. Only in confluent stationary phase cells does the amount of the S-100 protein increase⁵. To determine whether a similar regulatory phenomenon for the nervous system-specific protein 14-3-2 occurs in IMR-32 neuroblastoma cells, we measured the amount of this protein at various points in the growth curve. The doubling time of the cells was about 25 hours (Fig. 2). This time is somewhat faster than that originally described²². In contrast to the results obtained for S-100 protein in C₆

cells, 14-3-2 appears to accumulate in growing IMR-32 cells. The amounts of 14-3-2 and total cell protein increase proportionally. The relative amount of this nervous system specific marker is constant in rapidly dividing and stationary phase IMR-32 cells. Cell division and production of 14-3-2 protein are clearly not mutually exclusive phenomena in IMR-32 neuroblastoma cells.

During the course of this work, Drs. M. Goldstein and B. Moore informed us of similar complement fixation results with IMR-32 cells and with NJB neuroblastoma cells, a strain isolated by Dr. Goldstein. We have also demonstrated the presence of 14-3-2 protein by complement fixation in NJB cells generously provided by Dr. Goldstein.

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Fig. 1 Complement fixation reaction between antiserum to beef-brain 14-3-2 protein and extracts of IMR-32 and HeLa cells. Stationary monolayers were washed twice in Tris-HCl buffer (0.01M), pH 7.4, containing 0.15 M NaCl. Cells from a single 100 mm plate were scraped with a rubber policeman into one ml of this buffer and frozen until assay. Cell suspensions were thawed and sonicated for two five-second intervals with a Bronwill Biosonic III Sonifier at an intensity setting of 30, using a microprobe. The sonicated suspension was centrifuged at 40,000 xg for 10 minutes. Sonication solubilized approximately 70% of the total protein. Microcomplement fixation reactions were performed in a final volume of 1.2 ml, using a 1:300 dilution of antisera to beef-brain 14-3-2 protein (homologous titer, 1:1000). IMR-32 extract (O); HeLa extract (●).

Fig. 2 Production of the nervous-system-specific protein 14-3-2 by the IMR-32 neuroblastoma strain. Cells were grown in nutrient mixture F-10 in 100 mm Falcon plates. Medium was changed at 48 hour intervals. Cell number per plate (O) was determined by hemocytometer count of suspensions of trypsinized cells. At the times indicated plates for total protein and 14-3-2 protein analysis were harvested (as described in the legend to Fig. 1) and frozen. Total protein per plate (●) and 14-3-2 C' units per plate (□) were determined after all samples had been harvested. The latter value, used because of the serological cross-reaction between beef brain 14-3-2 protein and the heterologous antigen in IMR-32 cells, is defined as that amount of cross-reacting antigen in IMR-32 cells required to give 40% fixation in the antibody excess region of a complement fixation reaction with antiserum to beef-brain 14-3-2 protein used at a dilution of 1:280 (for homologous bovine 14-3-2 protein

this value is 38 μg). 14-3-2 C' units per plate are calculated as
[reciprocal of the dilution of IMR-32 cell extract required to give 40%
fixation] X [volume of the extract]. 14-3-2 C' units/ μg total protein val-
ues are illustrated by the filled squares (■).