CHARACTERIZATION OF TWO HeLa SUBLINES: TCRC-1 PRODUCES REGAN ISOENZYME AND TCRC-2, NON-REGAN ISOENZYME

ROBERT M. SINGER and WILLIAM H. FISHMAN. From the Tufts Cancer Research Center and the Department of Pathology (Oncology), Tufts University School of Medicine, Boston, Massachusetts 02111

INTRODUCTION

HeLa Cells have been found to produce a placental form of alkaline phosphatase (1, 8, 17) which is very similar to the Regan isoenzyme, a carcinoplacental antigen found in cancer patients (7–9).

In order to investigate the nature of gene expression of carcinoplacental proteins, HeLa cells were cloned, and a subline (HeLa TCRC-1), monophenotypic for the Regan isoenzyme was selected. The properties of another line, HeLa TCRC-2 (HeLa 12-2), discovered by Kelly et al. (14) were studied with regard to non-Regan isoenzyme.

MATERIALS AND METHODS

Culture Methods

Cells are maintained in monolayer cultures in Eagle's minimum essential medium with Earle's salts, modified for suspension culture[1] (Grand Island Biological Co., Grand Island, N.Y., catalog no. 165G),

[1] Cells have been adapted to grow in monolayer and spinner culture, and are maintained on spinner culture media routinely. No gross difference in morphology or growth has been noticeable due to prolonged growth on low calcium media.

supplemented with calf serum (final concentration is 10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml). The medium contains sodium bicarbonate at a final concentration of 0.1%.

Cultures are passaged weekly. Cell viability is determined using trypan blue (16), and 200 × 10^3 viable cells are added to 30-ml Falcon tissue culture bottles (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) in 5-ml growth media. The media is changed every other day. Cultures were found to be negative for mycoplasma contamination using a kit produced by Flow Laboratories, Inc., Rockville, Md.

Cells

The wild-type HeLa cells and HeLa TCRC-2 were kindly furnished by Dr. Robert Rustigian from the Brockton V. A. Hospital, Brockton, Mass.

Cloning

Selection of HeLa cell clones for high levels of Regan and non-Regan isoenzymes of alkaline phosphatase is accomplished using the method described by Maio and DeCarli (15). Monodispersed suspensions were plated out at very low density (100 cells per 100-mm Petri dish). When colonies reached a diameter of about 2 mm, a mixture of *p*-nitrophenyl phosphate and agar at pH 9.4 was poured over the plates. Colonies positive for alkaline phosphatase appeared as brilliant yellow in 2–5 min. Approximately 6% of the colonies appeared positive. These positive colonies were removed and placed in culture until there were ample cell numbers to allow propagation of the cell lines.

The first property determined was heat stability. 83% of the colonies were heat stable at 65°C for 5 min. The specific activities of these heat stable colonies varied from 0.003 to 0.75 (μmol/min/mg protein). HeLa TCRC-1 was selected from this group on the basis of high levels of heat-stable alkaline phosphatase which is shown by this report to be Regan isoenzyme.

RESULTS

A summary of the properties of the two clones TCRC-1 and TCRC-2 appears in Table I.

The alkaline phosphatase of TCRC-1 has the amino acid inhibition characteristics, heat stability, and antigenic sites of placental alkaline phosphatase, whereas that of TCRC-2 possesses the characteristics of non-Regan isoenzyme (6, 11, 13).

As can be seen from Fig. 1 *a*, placental and HeLa TCRC-1 alkaline phosphatases form lines of identity against antisera to placental alkaline phosphatase and no "spurs" are apparent. From Fig. 1 *b* in which the central well contains antisera to HeLa TCRC-1, it can be seen that lines of identity lacking spurs are formed between HeLa TCRC-1 and placental alkaline phosphatase, and no precipitin line is visible between rabbit antibody to HeLa TCRC-1 alkaline phosphatase and HeLa TCRC-2. Thus, TCRC-1 produces alkaline phosphatase which is antigenically indistinguishable from that of the placenta and can be referred to as a Regan-producing cell line.

These HeLa cell lines respond differently to prednisolone treatment (Table I). Prednisolone elevates alkaline phosphatase activity in HeLa TCRC-1 after 24 h, and a maximum elevation occurs on day 4. On the other hand, the hormone exerts no significant effect for the first 6 days on

TABLE I

Some Characteristics of HeLa Clones

	HeLa TCRC-1	HeLa TCRC-2	HeLa parent	Ref.*
Alkaline phosphatase				
In vitro				
Specific activity (μmol/min/mg protein)	0.75	3.2	0.09	6
L-Phenylalanine inhibition	73.1%	0	76.5%	11
L-Homoarginine inhibition	11.5%	77.5%	36.8%	10
Heat inactivation (5 min at 65°C)	10.9%	100%	5%	6
Reaction to placental antibody	+	−	+	13
In culture				
Prednisolone effect (24 h) on specific activity	+175%	0%	+500%	
β-Glucuronidase (μg phenolphthalein/h/mg)	32	5.8	ND	4
Chromosome number	67–69	64	68–69	1

* Refers to reference number which describes method used.
ND, not determined.

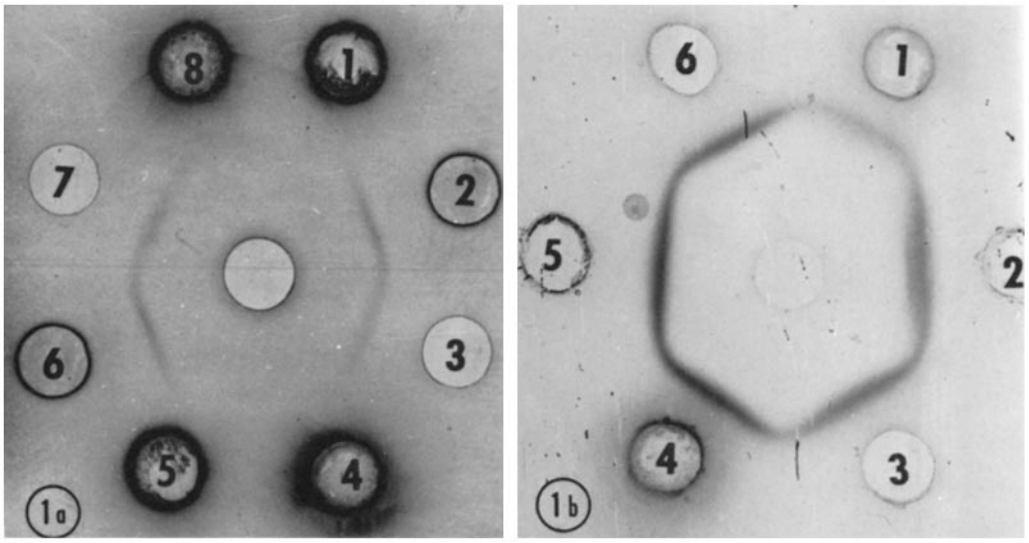

FIGURE 1 *a* and *b* Ouchterlony double-diffusion plates. Plates are prepared as 1.5 agarose (Seakem, Marine Colloids) in 0.2 sodium barbitol buffer pH 8.6. Visualization of phosphohydrolase activity is by the method of L. Fishman (3). (*a*) Central well contains rabbit antisera to purified human placental alkaline phosphatase (dilution 1:50). Wells 2 and 6 contain HeLa TCRC-1 alkaline phosphatase. Wells 3 and 7 contain purified human placental alkaline phosphatase. Wells 1, 4, 5, and 8 contain butanol extract of HeLa TCRC-2. (*b*) Central well contains rabbit antisera to purified HeLa cell alkaline phosphatase (2). Wells 1 and 4 contain purified HeLa TCRC-1 alkaline phosphatase. Wells 2 and 5 contain unpurified HeLa TCRC-1 alkaline phosphatase. Well 3 contains unpurified human placental alkaline phosphatase. Well 6 contains purified human placental alkaline phosphatase.

the enzyme activity of HeLa TCRC-2 and thereafter suppresses the activity.

Base level and prednisolone-induced enzyme of HeLa TCRC-1 have been compared electrophoretically on a Beckman microzone cell (Beckman Instruments, Inc., Fullerton, Calif.) in the presence and absence of antibody to placental alkaline phosphatase as described by Inglis et al. (13). Both the induced and the base level enzyme migrate similarly on the cellulose acetate membrane. They both fail to migrate in the presence of the antibody to placental alkaline phosphatase.

Experiments indicate that in addition to the aforementioned properties, these clones are quite different with respect to β-glucuronidase activity. As seen in Table I, HeLa TCRC-1 has significant levels of β-glucuronidase activity while HeLa TCRC-2 has low enzyme activity.

The chromosome number of HeLa TCRC-1, the Regan line, ranges from 59 to 71, with most of the population having between 67 and 69 chromosomes. There does not appear to be a clear modal chromosome number. The chromosome number of HeLa TCRC-2 ranges from 59 to 72 with a modal chromosome number of 64 (Table I). The HeLa parent strain is quite aneuploid, with a modal chromosome number of 68 or 69. These karyotypes, and the associated phenotypes show no signs of instability thus far (6 mo).

DISCUSSION

The many HeLa variants which have been under investigation should be mentioned. Thus, it has been shown, for example, that HeLa S3 cells from three different sources were very different with respect to chromosome number and enzyme levels (1). HeLa A, HeLa 229, and $HeLa_{65}$ undergo glucocorticoid hormone induction (1, 12). On the other hand, it has also been reported that $HeLa_{75}$, $HeLa_{71}$ (12), and HeLa G do not have elevated alkaline phosphatase levels in the presence of hydrocortisone (1). What has been missing in these other studies has been a thorough characterization of the isoenzyme species.

With respect to chromosome number, neither of our two cell lines has the karyotypic characteristics of HeLa strains studied by Bottomley et al. (1). However, we have found, as they have, that

phenotypic expression of alkaline phosphatase seems to be related to chromosome number. Experiments are now in progress to determine the stability of chromosome number and phenotypic expression of alkaline phosphatase.

With regard to the enrichment of TCRC-1 with β-glucuronidase, current studies are directed to evaluating the significance of this finding in relation to expression of Regan isoenzyme and to its well-known existence in neoplastic tissue (5, 18).

This research has been supported in part by Grants-in-aid from the National Cancer Institute, National Institutes of Health (CA-12924; CA 13332; K6-CA-18, 453), John A. Hartford Foundation, New York, and the Institutional Grant IN-23-0 of the American Cancer Society, New York.

Received for publication 9 August 1973, and in revised form 20 November 1973.

REFERENCES

1. Bottomley, R. H., A. L. Trainer, and M. J. Griffin. 1969. Enzymatic and chromosomal characterization of HeLa variants. *J. Cell Biol.* **41**:806.
2. Doellgast, G. J., and W. H. Fishman. 1973. Chromatography of placental isoenzymes of alkaline phosphatase on amino acid-sepharoses: Salt effects. *Fed. Proc.* **32**(3):582. (Abstr.).
3. Fishman, L. 1973. Acrylamide disc gel electrophoresis of alkaline phosphatase of human tissues, serum and ascites fluid using Triton X-100 in the sample and gel matrix. *Biochem. Med.* In press.
4. Fishman, W. H. 1967. Determination of β-glucuronidases. *Methods Biochem. Anal.* **15**:77–145.
5. Fishman, W. H., J. R. Baker, and P. R. F. Borges. 1959. Localization of β-glucuronidase in some human tumors. *Cancer.* **12**:240.
6. Fishman, W. H., W. A. Bardawil, H. G. Habib, C. L. Anstiss, and A. Green. 1972. The placental isoenzyme of alkaline phosphatase in sera of normal pregnancy. *J. Clin. Pathol. (Lond.).* **57**:65.
7. Fishman, W. H., N. R. Inglis, and S. Green. 1971. Regan isoenzyme: A carcinoplacental antigen. *Cancer Res.* **31**:1054.
8. Fishman, W. H., N. R. Inglis, S. Green, C. L. Anstiss, N. K. Ghosh, A. E. Reif, R. Rustigian, M. J Krant, and L. L. Stolbach. 1968. Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. *Nature (Lond.).* **219**:697.
9. Fishman, W. H., N. R. Inglis, L. L. Stolbach, and M. J. Krant. 1968. A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res.* **28**:150.
10. Fishman, W. H., and H. G. Sie. 1970. L-Homoarginine; an inhibitor of serum "bone and liver" alkaline phosphatase. *Clin. Chim. Acta.* **29**:339.
11. Ghosh, N. K., and W. H. Fishman. 1968. Purification and properties of molecular-weight variants of human placental alkaline phosphatase. *Biochem. J.* **108**:779.
12. Ghosh, N. J., A. Rukenstein, R. Baltimore, and R. P. Cox. 1972. Studies on hormonal induction of alkaline phosphatase on HeLa cell cultures. Kinetic, thermodynamic and electrophoretic properties of induced and base-level enzymes. *Biochim. Biophys. Acta* **286**:175.
13. Inglis, N. R., D. T. Guzek, S. Kirley, S. Green, and W. H. Fishman. 1971. Rapid electrophoretic microzone membrane technique for Regan isoenzyme (placental type alkaline phosphatase) using a fluorogenic substrate. *Clin. Chim. Acta* **33**:287.
14. Kelly, V., R. Rustigian, E. Mansfield, D. Ellis, N. R. Inglis, and W. H Fishman. 1970. Repression of an alkaline phosphatase isoenzyme in cultured human cells. Abstracts of the International Association for Dental Research, Chicago, Ill. 48th General Meeting, New York. 227.
15. Maio, J. J., and L. L. DeCarli. 1962. Distribution of alkaline phosphatase variants in a heteroploid strain of human cells in tissue culture. *Nature (Lond.).* **196**:600.
16. Pappenheimer, A. M. 1917. Experimental studies upon lymphocytes. I. The reactions of lymphocytes under various experimental conditions. *J. Exp. Med.* **25**:633.
17. Robson, E. B., and H. Harris. 1965. Genetics of the alkaline phosphatase polymorphism of the human placenta. *Nature (Lond.).* **207**:1257.
18. Watts, C., and D. M. Goldberg. 1969. New observations on β-glucuronidase in human cervical cancer. *Eur. J. Cancer.* **5**:465.