Regeneration of the Mammalian Liver III. Electrokinetics of Replicating Cells Treated with Anti-Mitotic Antibiotics.* (30993)

BARRY L. ZARET,† LORRAINE R. WEST AND FREDERICK F. BECKER‡
(Introduced by B. W. Zweifach)
Department of Pathology, New York University School of Medicine, New York City

Many tumor cells have been demonstrated to possess an increased total surface electrostatic charge when compared with normal cells of their tissue of origin. This phenomenon is determined by measurement of their electrophoretic mobility under standard conditions(1). Of interest, therefore, was the finding of Eisenberg *et al*(2) and others(3) that the mammalian liver cell (mitotically inactive in the normal state) assumes, upon entering preparation for cell division, an elevated electrophoretic mobility approximating that of tumor cells. These findings gave rise to the hypothesis that there is a direct relationship between the surface ionogenic characteristics of cells and their ability to divide. However, it has been demonstrated recently that the action of the enzyme neuraminidase reduces the mobility of such tumor cells while failing to affect the stimulated liver cell(4,5). This suggests that the specific surface factors or components responsible for the total surface-charge of malignant and replicating normal cells may be dissimilar.

It is, therefore, of interest to determine the nature of the surface alterations in a cell of transient mitotic activity such as the replicating liver and to compare them with those of tumor cells. We have attempted to prevent the increase in electrophoretic mobility of the liver cell which usually follows its stimulation to cell division by partial hepatectomy(6), by blocking induced ribonucleic acid synthesis with actinomycin D and protein synthesis with puromycin. In addition, actinomycin D was administered to animals during the rapid growth phase of the Novikoff ascites hepatoma to compare the effects of this agent upon the surface characteristics of normal and malignant cells.

Despite the failure of either of these antibiotics to prevent the assumption of an increased electrophoretic mobility by the replicating liver cell, a selective surface alteration of a portion of the tumor cell population was demonstrated.

Materials and methods. The selection of animals, operative procedures and other manipulations have been described(6). The 70% hepatectomy was that of Higgins and Anderson(7) and in all discussions, the operation will be taken as time zero.

Cell suspensions were prepared by the method of Jacob and Bhargava(8) with modification. The animals were anesthetized with ether, the liver perfused *via* the inferior vena cava above the renal veins utilizing a #18 needle, with 0.02 M sodium citrate in calcium free Locke's solution. The perfusate was placed from 10 to 100 cm above the animal being slowly raised during the perfusion. When the liver blanched the needle was reinserted into the portal vein and the perfusion continued until the fluid draining from the inferior vena cava was clear. In general this required 25 to 40 cc of perfusate. The right posterior lobe was always used. The cells were then washed in 1% sodium chloride 3 times at 500 rpm, 10°C for 5 minutes on a #269 head, International Centrifuge PR-2. The cells were finally suspended in 1% sodium chloride at a concentration of 0.25-0.70 $\times 10^6$ cells/ml.

The electrophoretic mobility of these cells was determined according to the method of Angers and Rottino(9). Measurements were performed in 1% saline or Michaelis Buffer adjusted to pH 7.0 and were carried out at 25°C. Ten measurements were made on each specimen.

Actinomycin D was generously supplied by Merck Sharp and Dohme. It was suspended

* Aided by Grant E-355 from Am. Cancer Soc.

† This study was performed during a USPHS medical student summer fellowship.

‡ Career Scientist, Health Research Council of City of New York.

in sterile water to a concentration of 100 μg/ml and administered intravenously. All of the partially hepatectomized rats received 200 μg of actinomycin D at time of operation. Sham operated controls received 200 μg of actinomycin D intravenously and were sacrificed 6 hours later. The tumor bearing rats received 200 μg 18 hours and 4 hours prior to sacrifice.

Puromycin was suspended in phosphate buffered saline to a concentration of 400 mg% and 8 mg was administered intraperitoneally 2 hours prior to operation. An additional 4 mg was administered 1 hour prior to operation and again at the time of partial hepatectomy.

Results. The electrophoretic mobility of the liver cells of sham operated animals remained constant throughout the postoperative period and was identical to that of liver cells from normal animals. As early as 3 hours following partial hepatectomy the electrophoretic mobility of the liver cells had increased by 11% and a maximal increase of 28% was determined at 20 hours, the latest period studied. The variation of response from cell to cell decreased with the length of time between operation and measurement. Although only measurements of single, apparently viable cells were included in results, similar determinations were obtained from 2-5 cell clumps. Differences in the number of washes used caused little change in results (Table I).

TABLE I. Effect of AMD and Puromycin on Liver and Tumor Cells.

Procedure	No. of rats	A.EP.M., μ/s/v/cm	% NI
Sham	3	1.20 ± .08	100
Sham + AMD	2	1.08 ± .03	90
Hep. 3h	2	1.33 ± .23	111
Hep. 3h AMD	2	1.49 ± .02	124
Hep. 3h purom.	4	1.35 ± .31	112
Hep. 6h	3	1.38 ± .18	115
Hep. 6h + AMD	3	1.39 ± .02	116
Hep. 12h	2	1.51 ± .05	126
Hep. 12h + AMD	2	1.47 ± .00	123
Hep. 20h	2	1.54 ± .03	128
Asc. Tum.	2	1.51 ± .02	126
Asc. Tum. + AMD	2	1.51 / 1.14	126/95

AMD = actinomycin D; Hep. = 70% hepatectomy; purom. = puromycin; Asc. Tum. = ascites tumor (Novikoff); A.EP.M. = avg electrophoretic mobility in micra per second per volt per cm.

Actinomycin D decreased the mobility of control liver cells without an apparent effect upon viability. Neither actinomycin D nor puromycin in significant doses prevented the assumption of increased cell mobility after partial hepatectomy at any of the periods studied. Viability of the liver cells was not apparently affected (Table I).

Novikoff ascites tumor cells demonstrated an electrophoretic mobility similar to that maximally achieved by liver cells during their preparation for cell division. Little variation in measurement from cell to cell was detected despite moderate size variation of the tumor cells. When administered to the animals during the log phase of tumor growth (as determined by animal weight gain) actinomycin D produced a significant variation in the measured electrophoretic mobility of the tumor cell population. Two cell groups of approximately equal size were detected, one of which retained the electrophoretic mobility of the untreated tumor cell and another which assumed a mobility similar to that of the actinomycin-treated normal liver cells. These populations could not be distinguished morphologically under the experimental conditions, but all of the cells appeared to be viable as judged by phase-microscopic morphology.

Discussion. Based mainly on the results obtained previously from the study of tumor cells, it has been suggested that the total surface electrostatic charge reflects to some extent the mitotic activity or mitotic potential of nucleated cells(1). The sudden increase in electrophoretic mobility of normal liver cells when stimulated to cell division by partial hepatectomy offered strong support to the view that a high anionic surface charge was consistent with mitotic activity regardless of cell type(2). The rough correlation between a return to normal surface electropotential and the cessation of mitotic activity was also consistent with this view.

The high total surface charge of tumor cells has been repeatedly reported to be related to a neuraminidase susceptible factor (4,10). This enzyme sensitivity has also been demonstrated in several tissue culture lines whether they were derived from normal or malignant cells(10). Although there is agree-

ment that the normal liver cell during interphase is resistant to neuraminidase activity, conflicting results have been obtained with the liver cell during its preparation for cell division. Fuhrmann *et al*(4) have reported resistance to such activity while more recently Chaudhuri and Lieberman(11) have reported susceptibility.

Again, this latter study reports data contrary to the above results in that actinomycin D prevented the assumption of increased electrophoretic mobility. The only procedural differences which might explain such contrary data was the use by Chaudhuri and Lieberman of immature rats and a different method of liver cell preparation. The method of liver cell preparation used in the presently reported study produced a pure parenchymal cell suspension with minimal cell alteration.

It has been reported recently that under electron microscopy, the stimulated hepatocyte undergoes widespread anatomic alteration of its surfaces including outpouchings and loss of cytoplasm, loss of microvilli and of intercellular substances(12). Such changes seem adequate to produce an altered surface charge by baring new anionic groups. Restoration of the normal surface with progression of the process of cell division would reestablish the normal electrostatic charge.

At present, therefore, the nature of the surface charge change detected in liver cells during its preparation for cell division is unclear. The dose of actinomycin D administered in our own experiments should have eliminated synthesis of new protein *via* the induction of ribonucleic acid, and puromycin should have directly halted protein synthesis (13,14). In view of these findings and the morphologic alterations of the surface it is suggested that the assumption of high electrostatic charge of the liver cell is the result of reorientation of protein molecules already present at the surface or the secretion of substances already available in the cell through a membrane possessing increased permeability.

A portion of the tumor cell population was also resistant to the effects of actinomycin D. However, it is interesting to note that the non-dividing liver cell and a significant percentage of the tumor cells were susceptible to the effects of this agent. Both of these cell types would then appear to be dependent upon the continued synthesis of a proteinaceous surface component for maintenance of their usual surface charge. Although we have no information as to the nature of the tumor cells involved, it is possible to speculate that it is the non-dividing tumor cell in which the total surface charge is dominated by a more constant turnover of surface components. Neuraminidase susceptibility can still be used to distinguish such non-dividing cells since all tumor cells seem equally affected by enzyme treatment.

Summary. Following amputation of approximately 70% of a rat's liver, there ensues a period of active nucleic acid synthesis followed by a wave of cell division. Surface alterations occur during the preparation for mitosis which result in an increase of electrophoretic mobility and it has been suggested that cell division is dependent upon surface charge. The present study demonstrates that the assumption of increased electrostatic charge during the preparation for cell division is not the result of newly synthesized protein and in accord with previous work indicates that cell division is independent of surface charge. However, it was suggested that the total surface charge of interphase cells is related to the constant synthesis of surface proteins.

The authors wish to express their appreciation to Dr. John Angers, without whose help the study could not have been performed.

1. Ambrose, E. J., James, A. M., Lowick, J. H. B., Nature, 1956, v177, 576.

2. Eisenberg, S., Ben-Or, S., Doljanski, F., Exp. Cell Res., 1962, v26, 451.

3. Ruhenstroth-Bauer, G., Fuhrmann, G. F., Z. Naturforsch., 1961, v16b, 252.

4. Fuhrmann, G. F., Granzer, E., Kubler, W., Ruoff, F., Ruhenstroth-Bauer, G., ibid., 1962, v17b, 610.

5. Bucher, N. L. R., Int. Rev. Cytol., 1963, v15, 245.

6. Becker, F. F., Am. J. Path., 1963, v43, 497.

7. Higgins, G. M., Anderson, R. M., Arch. Path., 1931, v12, 186.

8. Jacob, S. T., Bhargava, P. M., Exp. Cell Res., 1962, v27, 453.

9. Angers, J., Rottino, A., Blood, 1961, v17, 119.
10. Simon-Reuss, I., Cook, G. M. W., Seaman, G. V. F., Heard, D. M., Cancer Res., 1964, v24, 2038.
11. Chaudhuri, S., Lieberman, I., Biochem. & Biophys. Res. Comm., 1965, v20, 303.
12. Lane, B. P., Becker, F. F., Am. J. Path. in press.
13. Malay, G. F., Lorenson, M. G., Malay, F., Biochem. & Biophys. Res. Comm., 1965, v18, 364.
14. Gorsky, I., Alzawa, Y., Mueller G. C., Arch. Biochem. & Biophys., 1961, v95, 508.

Received November 29, 1965. P.S.E.B.M., 1966, v121.

Comparative Immunology. Hemolytic Complement in Amphibia.* (30994)

DONALD W. LEGLER† AND E. EDWARD EVANS
Department of Microbiology, University of Alabama Medical Center, Birmingham

The participation of complement in immune reactions results from the sequential interaction of a number of components. Complement activity has been studied and characterized in a variety of mammalian and avian systems, but detailed evidence for its presence in lower vertebrates has been fragmentary. A notable exception was the work of Cushing(1,2) which described the hemolytic complement of the carp and bullfrog in some detail, and included a comprehensive review of the earlier literature. More recently, an investigation of complement components of certain bony fish, elasmobranchs and cyclostomes has been reported(3).

The present study was undertaken to investigate various aspects of the phylogeny of the complement system as observed in representative species of amphibia. Certain physical and chemical properties of these amphibian sera were compared with those of a reference guinea pig complement system.

Materials and methods. Three species of amphibia were studied: *Bufo marinus* (marine toad), *Rana pipiens* (leopard frog), and *Necturus maculosus* (mud puppy). Ten specimens from each group were maintained for a minimum of 7 days prior to bleeding in a 25°C controlled temperature environment. Bleeding was accomplished by cardiac puncture, and the individual samples were allowed to clot for one hour at room temperature, followed by two hours at 10°C. After centrifugation at 0-5°C, the sera were maintained in an ice bath. All quantitative procedures were initiated within 4 hours of time of bleeding, since it has been observed that a marked lability to storage, even at low temperatures, characterizes certain of these lower vertebrate sera.

Quantitative estimates of complement levels were determined graphically by plotting the C'H50 for each individual serum, as well as for pooled sera, utilizing the standard technique outlined in Kabat and Mayer(4). The standardized erythrocyte suspension was optimally sensitized with an appropriate dilution of rabbit antibody, and optical densities of the cell lysates and controls were recorded in a Beckman DU spectrophotometer at a wave length of 541 mμ. Duplicate dilutions were carried out for test samples as well as positive and negative controls, using pH 7.4 isotonic veronal buffer containing 0.00015 M calcium ions and 0.005 M magnesium ions. Samples were incubated for one hour at 30°C, followed by one hour at 35°C. This incubation procedure was found to be optimal for amphibian sera, and was used for all experiments unless otherwise noted.

Qualitative assays for natural hemolytic activity were performed with washed erythrocyte suspensions from a variety of species without addition of heterologous antibody. Degree of hemolysis was scored visually. Each tube contained: 0.1 ml serum dilution, 0.1 ml erythrocyte suspension in a concentra-

* Supported by USPHS Service Grant AI-02693.
† Conducted during tenure of a fellowship on Grant DE-7 from Nat. Inst. of Dental Research.