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Measurement of Permeability of the Cell Membrane to Water

ABSTRACT—The water permeability was measured for blood cells from normal subjects and for blood cells from patients with leukemia. The method of measurement of the kinetics of water influx employed a stopped flow apparatus. The method of calculation of results was based on irreversible thermodynamics. The measurements and calculations were both performed with the assistance of a computer. The water permeability of the leukemic cells was decreased from normal, and the values were more variable than normal. Leukemia changes the structure of the cell membrane.

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Cancer represents a derangement of the normal functions of a cell. This derangement may affect the physiological properties of the cell membrane. Cancer of the blood (leukemia) may affect the membrane properties of circulating blood cells. If there is such an alteration, it might be useful in predicting the response of a patient to chemotherapy, and hence, in selecting an effective anti-leukemic drug.

Such an approach to the problem of predicting of drug therapy is suggested in the acute leukemias for several reasons. There are more drugs which are effective against acute leukemias than against other cancers, such as solid tumors. Lymphatic leukemia is very drug sensitive compared to other types of leukemias. Sampling neoplastic cells is simpler for leukemias than solid tumors.

One of the goals of oncology is to improve the chemotherapy of malignant disease, such as leukemia. Often, drug effectiveness is evaluated on the basis of statistical considerations, using data from large scale clinical trials with many drugs. The oncologist needs a procedure that could help predict which drug, if any, would have the greatest chance for success in the treatment of a specific patient. Ideally, the diagnostic procedures should work in vitro with micro-samples and produce results promptly. There is need for such a predictive test in acute myelogenous leukemia for example. There is a large number of candidate drugs, and all of them cannot be tried on a patient before a successful one is found. Not only may the patient not survive long enough for several drugs to be tested, but the effect of a particular drug may be influenced by prior chemotherapy.

For a drug to be effective at the cellular level in a lymphoproliferative disease, such as leukemia, the drug must enter the cell. Entrance may be affected by the normal permeability of the cell membrane to the drug as well as the effect of the disease on the cell membrane permeability. Penetration of the drug through the cell membrane determines the response of a cell to the drug, e.g., as methotrexate. If methotrexate enters, the cells respond to the drug in cases of animal leukemia and human leukemia. Kessel (1969) reported that alkalating agents, busulfan and nitrogen mustard, entered cells, and this entrance was associated with the response of the cell to the drug. Kessel (1971) tested platelets and granulocytes from humans with leukemia of unspecified type. If the drug affected the capacity of the cells to incorporate labeled uridine or thymidine into RNA, then the drug administration was effective against the leukemia.

To be effective, anti-leukemia drugs must be taken up and retained within leukemic blast cells. While within the cells, they should have the maxium effect on the cell. Vincristine is a drug useful in the treatment of acute leukemia, and its efficacy is determined by the permeability of the cell wall. Complex procedures are necessary, involving assay of the radiosiotopically labeled drugs, to measure the concentration of the drug within the cell. We hope to study one of the factors (membrane permeability) upon which successful drug therapy depends. This paper describes our approach to measuring membrane permeability.

Samples from human volunteers

Samples of peripheral blood were obtained from human volunteers or from patients with several types of acute leukemia. Two milliliters of blood were collected in EDTA (ethylenediaminetetracetate) anticoagulant. One volume of cells was mixed with 99 volumes of human blood plasma of the same type and group as that of the patient. Three milliliters of the resulting 1% cell suspension were placed in one syringe of a stopped flow apparatus, and a "challenging" solution was placed in the other syringe. The challenging solution was typed plasma which either was undiluted or was diluted with distilled water to a known hypotonic concentration. All samples of plasma and distilled water were degassed with a vacuum pump before using. The appearance of selected samples of cells was examined microscopically before the sample entered the stopped flow apparatus and after the cells had been through the instrument.

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Instrumentation

A cell suspension was placed in one syringe, and a "challenging" solution was placed in a second syringe. The syringes were placed in an Aminco-Morrow stopped flow apparatus. To perform an experiment, compressed air forced aliquots from each syringe through a "T"-shaped mixer into an optical cuvette, and the flow was stopped. Simultaneously, an oscilloscope trace was initiated. Monochromatic light shown through the optical cuvette. The light was multiply scattered by the cells, and the beam emerging from the cuvette fell on the photocathode of a photomultiplier. The photomultiplier signal was amplified and appeared at the beam deflection plates in a storage oscilloscope. Water moved from the hypotonic suspension medium into the blood cells, increasing the cell volume. When the blood cell volume changed, the amount of light scattering varied, and the phototube signal changed accordingly. A linear calibration related phototube signal to erythrocyte volume at equilibrium. Selected curves traced on the scope phosphor were photographed. The kinetics of cell volume change, and hence of water movement, were calculated from measurements of the photograph.

Distilled water was mixed with distilled water to provide a reference trace. The response of the system indicated whether the operation of the apparatus was satisfactory. Instrument response was checked also by mixing two aliquots of a latex particle suspension, noting the resulting oscilloscope trace, and comparing this trace with that obtained on previous days.

Procedure of experiment

Experiments were performed in which erythrocytes were challenged with a hypotonic plasma. The per cent optical transmission was recorded as a function of time. Points were selected on the recorded curve, and the coordinates of the points were measured with a traveling microscope, and/or with a coordinate reader. The coordinate reader had two transparent cursors which move in perpendicular directions, and each cursor bore a cross-hair. Each cursor was connected to a potentiometer across which a stable and constant D–C voltage was maintained. A fraction of the potentiometer voltage was tapped, and this fraction was proportional to the distance ot the cursor from a reference point. The voltages tapped from the two potentiometers were the input signals to two channels of a mini-computer. The computer was programmed to convert the two analog voltages to digital signals, display the point defined by these two coordinates on the screen of a cathode ray tube (to help detect an operator's mistakes), format, and punch the digital values of the coordinates of the points into paper tape. Another program, used subsequently, read the paper tape, printed the data, and used calibration constants to scale the values to those of the original photograph. The program calculated the logarithms of the ordinates. A method of data reduction was used (Frost and Pearson) that did not require knowledge of the asymptote which the curve approached. This elimination of the need to estimate the unknown asymptote removed a source of potential error from the calculations. However, this gain is at the cost of being able to use only half the data points for the regression analysis. The program performed a linear regression on the points. It calculated the pseudo-first order rate constant from the slope of the regression line, the corresponding half time for the cell volume change, and the osmotic filtration coefficient of the cell membrane. This calculation was based on a method described by Farmer and Macey.

The data set was edited by removing the measurements made during the first 150 milliseconds (during which the phototube signal did not represent cell volume change only). The remaining data were fitted by the equation: $y = \text{sum}\ (B_i \exp(-K_i\ t)) + A$, in which i was 1, 2, and 3 in successive calculations.

Reproducible and nonreproducible data photos

If erythrocytes were mixed with hypertonic medium, a single phase of volume change was usually seen. The first 150 milliseconds of data on photographs usually was not reproducible. This was interpreted as being due to an artifact of mixing. The curve at times greater than 150 milliseconds was reproducible. This portion of the curve was measured. If the measured points were plotted on semilogarithm paper, a single exponential usually fitted the experimental curve. The values calculated for the osmotic filtration coefficient for cells from normal donors were reproducible and were about 0.6 microns per minute per atmosphere. The curves for the cells for patients with leukemia were more variable. Some of these curves had the same general shape as the curves from normal cells, but the associated value of the half-time of cell swelling was longer, and the value of the osmotic filtration coefficient was generally lower than normal (3/4 of normal in acute lymphocytic leukemia to 1/5 normal in acute myelogenous leukemia). Some of the experiments produced curves which were the mirror image of the expected swelling curves, and we cannot explain these.

Membrane changes observed

Malignancy changes properties of the cell membrane. Ambrose found that if the membranes of fibroblasts from normal subjects touched, they formed an adhesion in the region of contact. Fibroblasts from patients with sarcoma lost this ability to form stable intercellular lesions. Sanel and Serpick studied monocytes from patients with acute monocytic or monomyelocytic leukemia. They found membrane bonding between cells by "zipperlike" junctions. The junctions exhibited reciprocal interdigitations of plasmalemmal extrusions and areas of intimate contact between surface membranes of the adjoining cells. These areas of contact were different from sites of adhesion. They were larger and they showed a specialized architecture, consisting of facing, rigidly parallel membranes of the two cell surfaces separated by an electron-lucent space 150 to 200 angstroms thick. If stained with uranyl acetate, the junctions appeared as two confronting trilaminar unit membranes separated by perpendicular, ladder-like intercepts 30 to 50 angstroms in diameter, evenly spaced 90 to 100 angstroms apart. Sanel and Serpick surmised that "the induction or unmasking of unique, highly specialized structural complexes in these leukemic cells... may be related to neoplastic modifications of the plasma membrane or its extraneous coat". The zipperlike junctions were found in vitro. The altered membrane bonding was not demonstrated in vivo, but it might occur. The high incidence of infection with Pseudomonas aeruginosa or similar organisms in leukemic patients is consistent with altered cell membranes in vivo.

Kanno (1966) reported that such zipperlike junctions were associated with selective permeability. Therefore, altered membrane permeability might be expected in

monocytic or myelocytic leukemia. However, none of the above authors measured membrane permeability.

Altered membrane glycoproteins could contribute to changes in permeability of outer and inner membranes of tumor cells. Such altered membrane glycoprotein has been reported by a number of workers. Klein et al. examined the lymphocytes of an 82-year-old male with chronic lymphocytic leukemia. They reported that cells from chronic lymphocytic leukemics have unusual surfaces. IgM heavy chain (mu chain) and kappa light chain immunoglobulins were found on the cell surfaces in amounts sufficient to be shown by fluorescein-labeled antibodies and fluorescence microscopy. About 80,000 IgM molecules were present on each lymphocyte. Klein found no evidence that the immunoglobulins come from other sources than the leukemic cell itself. The leukemia cell synthesized its surface immunoglobulins. If these molecules were located at sites where transmembrane movement occurred (as if they moved from cell interior to exterior through "pores") then their presence might help to explain an altered membrane permeability of lymphocytes from patients with leukemia.

Preud 'Homme and Seligman studied lymphocytes from patients with leukemia. They found gamma-, kappa-, and lambda, and usually also mu-chain determinants on lumphocytes from patients with chronic lymphocytic leukemia. These determinants made the surfaces of these cells immunologically different from the surfaces of normal lymphocytes.

Rubin and Schultz corroborated this finding. Further, they found a specific defect in ribosonal RNA processing in lymphocytes of patients with chronic lymphocytic leukemia. This reflected defective "T" cells (thymus-derived lymphocytes). They regarded chronic lymphocytic leukemia as a fundamental abnormality in lymphopoiesis.

Holt et al., studied the surfaces of normal and leukemic lymphocytes from patients with chronic lymphocytic leukemia. They found that the surface of the leukemic lymphocyte was considerably more resistant to ionic etching than normal cells. This was due to loss of the physical hardness and consequent increased resilence of the lymphocyte surface. This alteration of the properties of the surface was also evident from other measurements. They suggested that alteration of phospholipid metabolism may produce a micro-injury in the membrane and may be partly responsible for the observed weakness of the membrane of the leukemic lymphocyte.

Thomson found that lymphocytes from patients with chronic lymphocytic leukemia were unusually resistant to hypotonic shock. This implies that the membrane permeability to water was reduced.

Burrows reported that lymphocytes from patients with chronic lymphocytic leukemia show decreased osmotic fragility. The time to lysis was increased over normal, indicating decreased membrane permeability to water.

Hemplin reported that normal human leukocytes had values of the osmotic filtration coefficient (L_p) of 0.83 microns per minute per atmosphere. Leukocytes from patients with leukemias had the following significantly different values for L_p chronic lymphocytic leukemia: 0.57;, acute myelogenous leukemia: 0.47; chronic myelocytic leukemia: 0.45. He found that the following factors affected the value of L_p : age of donor, volume of the cell, volume of osmotically inactive material in the cell, and % granulocytes in the granulocyte-lymphocyte mixture. Hempling did not report examining erythrocytes.

Solomon has associated the water permeability with a pore size. This drop in permeability in leukemia can be interpreted as a dramatic decrease in membrane pore size. It raises the following questions:

In which cell is pore size changed?
In which leukemias is pore size changed?
Does pore size decrease in cell leukemias?
If so, does it affect the progress of the disease?
When, in the course of the leukemia, does the pore size decrease?
In order for an anti-leukemia drug to be effective, must it be less than a certain critical size to enter the cell?
Would entrance of the drug be potentiated by another drug which may increase membrane permeability?

The answers to these questions await further research.

The composition of the cell suspension solutions and of the challenging solutions varied from laboratory to laboratory. No protein was present in the cell resuspension medium or the challenging solution in most cases. Cells may crenate in the absence of protein in solution, even if the solution is isotonic. Other workers usually determined the value of the osmotic filtration coefficient from a few samples of cells.

In the reports found, it was not clear if the cells were taken from patients prior to the initiation of chemo- or radiation therapy, during, or after therapy, or in remission. If the value of membrane permeability changed during the course of the disease, the studies in the literature did not monitor these changes. Rather, any such changes would appear as excess variation in the measured value of membrane permeability. The sampling schedule should be coordinated with the treatment schedule, and permeability measurements need to be interpreted in the context of the patient's clinical status.

Combined with the other appropriate tests, the measurements of the cell membrane permeability might supply useful information in the treatment of certain leukemias, as giving diagnostic evidence for monitoring these effects of drugs on the cells, predicting the effectiveness of drugs on the patient's disease, or predicting the onset of a leukemic crisis.

Osmotic filtration measured

We applied the technique to detecting a change from the normal value of the osmotic filtration coefficient of the cell membrane associated with acute leukemias. The value of the osmotic filtration coefficient was measured for erythrocytes from leukemic patients. The results from these measurements indicated a decrease in the osmotic filtration coefficient of erythrocyte membranes from patients with acute leukemias. The values were significantly decreased below normal. This finding is not inconsistent with the hypothesis that disease effects the function and structure of the cell membrane. The procedure may be helpful to the oncologist; for example, in the selection of drug-responsive patients. The procedure might enable the evaluation of the probable results of drug therapy, or the use of drug combinations.

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