Analysis by Computer-controlled Cell Sorter of Friend Virus-transformed Cells in Different Stages of Differentiation

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Summary

In most systems involving cellular differentiation and cellular transformation the biological process is non-synchronous and the sample heterogeneous. In order to answer some of the basic questions about the control mechanisms of cellular changes and the order in which they proceed one must have access to homogeneous classes of cells. Friend virus transformed erythroid cells which are stably maintained in tissue culture can be chemically induced to differentiate and are thus very advantageous for *in vitro* studies (1–3).

With such a system the questions which we pose are a) the reversibility of the differentiation process; b) the order of steps in the production of specialized messenger RNA; c) the time of shut-off of undifferentiated messenger production; d) the relationship of viral RNA production to the differentiation process; e) the onset and extent of specific protein synthesis; f) the correlation of DNA metabolism with the timing or course of events. By using a computer-controlled cell separator we can select live cells on the basis of their macromolecular content, membrane properties (using a new parameter, fluorescence emission anisotropy), and size (4, 5, 34). Thus with proper probes as described here, we are able to select

cells at different stages in their differentiation and can begin to attack the questions posed above.

Materials and Methods

The Instrument

Systems for separating suspensions of living mammalian cells on the basis of spectroscopic properties present or induced in the cells have been developed in several laboratories (4–7). Our instrument differs from the others in that it is controlled on-line by a computer and thus a) facilitates the use of numerous detectors (up to five) for both light scattering and fluorescence, b) allows the simultaneous separation of cells into four categories to be performed on the basis of complex functions of the measured signals (such as the fluorescence anisotropy shown here) and c) generates, displays and stores frequency distributions of the number of cells having any measured property or the combination of several properties. These features make the instrument ideally suited to the selection of cells from complex biological mixtures.

Figure 1 shows a schematic diagram of the instrument and its general features. The aqueous suspension of cells exits from an inner nozzle 50 microns in diameter and is narrowed into a thin stream by the colaminar flow of the sheath liquid

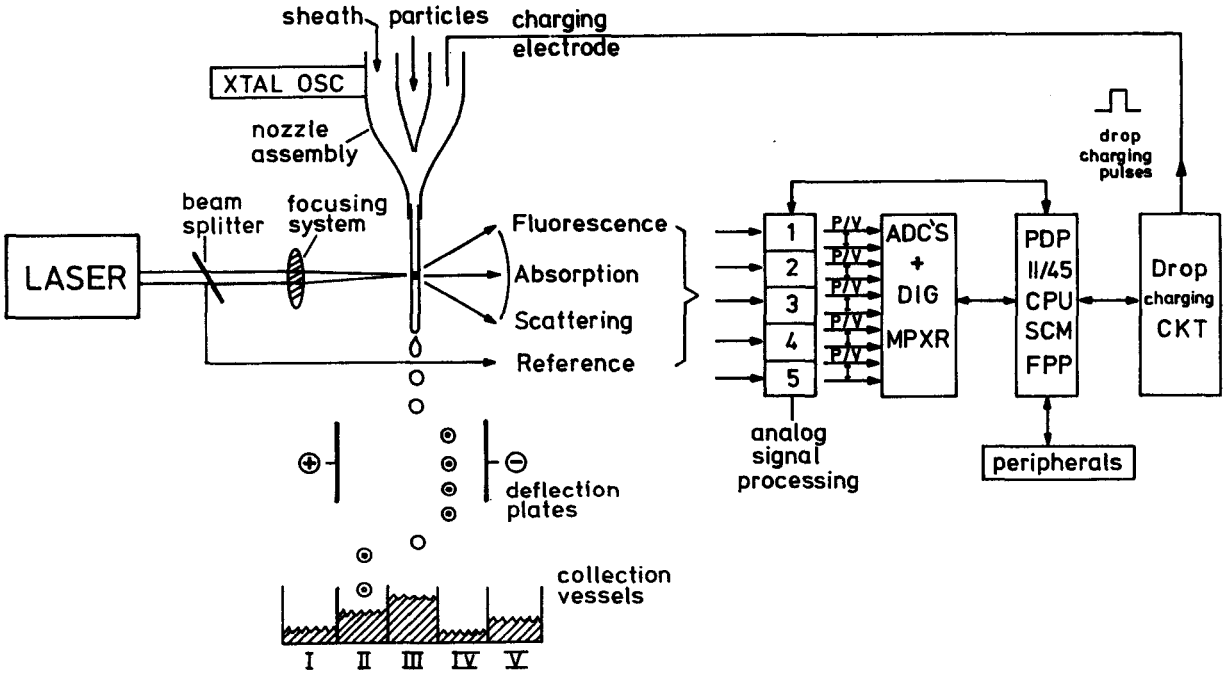


Fig. 1: Schematic diagram of the computer-controlled analyzer-sorter. The flow system leading to the nozzle is omitted. Excitation and detection are represented symbolically; the actual components used are described elsewhere (4, 22). The five independent analog processing modules can accept signals from up to five different detectors, usually a combination of two independent fluorescent detectors and 2 or 3 light scattering detectors and a laser reference signal. The 10 peak or valley (P/V) and integral (I) output signals derived from each cell at the time of intersection with the laser beam are digitized by parallel analog to digital convertors (A2C's) and multiplexed into the central processor (CPU, Digital Equipment Corporation PDP11/45) (taken from reference 4).

such that individual cells follow one another. These streams pass through the outer nozzle (also 50 microns in diameter) into air and just below the tip the fluid column intersects a focussed laser beam. As each cell passes through the laser beam a spectroscopic "fingerprint" is registered by the detectors and recorded by the computer. That is, the cells scatter light according to their size, membrane properties, and internal structures. They emit inherent fluorescence if excited in the ultraviolet. More generally, specific fluorescence proportional to the content of macromolecules in the cell or on its surface can be elicited by the use of fluorescent probes. In all cases the magnitudes of the optical signals from an individual cell are measured and converted to a proportional voltage by the various detectors for the light-scattering and fluorescence. These voltages are simultaneously recorded by the computer. Thus each cell is assigned to a particular category by the combination of this information according to freely programable algorithms.

For example, the DNA content of a cell can be determined by the direct proportionality it has to the fluorescence of bound acriflavin after Feulgen treatment or to the fluorescence of bound Hoechst benzimidazole dyes in living cells.[1] The number of antigen binding sites can be determinded by the fluorescent signal from labelled antibody bound to cells and likewise the number of lecitin or hormone binding sites can be measured directly from the fluorescence of labelled hormone or lectin bound to the cells. In addition to determination of content by the absolute fluorescence intensity of the labelled cells one can derive information about the environment of a fluorescent molecule, e. g., the fluidity of the plasma membrane, by measurement of polarized fluorescence emission or anisotropy (5, 34).

Because a crystal oscillator is imposed on the stream the liquid column breaks into droplets at a constant distance from the tip of the nozzle. At the precise moment when the cell reaches the place in the stream where it will be trapped in a droplet the computer gives an electrical charge to the stream, the magnitude and polarity depending upon the category to which the cell has been assigned. Thus the droplet containing the desired cell is given a known charge. The droplets pass through an electrical field established between two metal plates and are deflected according to their charge. Four deflected and the undeflected streams are collected. In addition, the spectroscopic information from each cell is processed by the computer and displayed as the cumulative frequency or number of cells plotted against the size of a particular signal or combination of signals.

Automated cell separators can select living cells sterilely at speeds up to several thousand cells per second with high purity. The fluorescent probes can include, besides those alluded to above: fluorogenic substrates for intracellular enzymes and non-covalent or covalent dyes which equilibrate nonspecifically, e. g. in the cytoplasm as for the purpose of size measurement. Since cells are separated on the basis of an expression of a specific cell function or structure it is a selection related directly to their biological state as opposed to conventional gradient centrifugation or electrophoretic methods which separate on combined gross physical properties often less related to biological function.

[1] D. Arndt-Jovin, unpublished results.

The cell system

The Friend virus or spleen focus-forming virus transformed cells are erythroid cells which are arrested in the proerythroblast stage and can be maintained permanently in uncloned or cloned culture (3, 8–10). If aprotonic solvents such as dimethylsulfoxide (DMSO) or dimethoxyethane (2, 3, 11) or short chain fatty acids such as butyric acid (12, 13) are added to the growth medium the cells resume the process of erythroid differentiation which had been blocked by the transformation event. However all cells are not equally responsive to the inducing stimulus (17, 20). During the 2–4 cell divisions required to reach the stage of non-dividing late erythroblast or non-nucleated cell containing 25 % of its soluble cytoplasmic protein as hemoglobin, a number of morphological and biochemical changes in the cells can be observed. Generalized RNA synthesis decreases while mRNA for proteins specific to the differentiated state such as globin increase dramatically (8, 9, 14, 15, 16, 17). The increased production of viral RNA and the activation and release of an endogenous spleen focus-forming virus complex are observed in virus positive cell lines and in some virus negative ones, but no activation of the Friend helper virus takes place (10, 17, 18). These processes are coordinated temporally with the synthesis of globin mRNA (10, 17, 18). In some other virus negative cell lines virus release appears to be inhibited but a 10-fold increase in other endogenous virus-like intracisternal A particles can be observed (10, 17, Krieg *et al.* unpublished observations). The *de novo* synthesis of hemoglobin (3, 19) and the appearance of spectrin in the membrane[2] occur later in differentiation and are characteristic for all the cell lines.

Tissue culture. The isolation and characterization of erythroleukemia cell lines F4N and B8 from DBA/2 mice have been described (3, 8, 10). The lines at a cell density of about 1×10^6 were induced to differentiate by treatment of F4N at 1–1.2 % and B8 at 1.5–2 % DMSO.

Antibody to the H-2 mouse histocompatibility antigens. Antibody was prepared as previously described (5) and used at 1:2 dilution in phosphate buffered saline (PBS). The binding of the antibody to erythroleukemia cells was visualized using fluoresceinated rabbit antimouse IgG as described (5).

Concanavalin A. The methods for the purification and the radioactive and fluorescent labelling of Concanavalin A (Con A) for use in subsequent binding studies to the cells have been described (5).

Labelling of cells with DPH. 1,6-diphenyl-1, 3, 4-hexatriene (DPH) was obtained from Aldrich and a fresh dispersion in PBS was prepared daily as described by Shinitzky and Inbar (29). The methods for labelling cells with DPH and measuring its fluorescence emission anisotropy have been described (5, 34.)

Results

We have investigated various properties of the differentiating Friend cells with the help of the cell separator and correlated them with other biochemical changes as is discussed in more detail elsewhere (5, 21). Table I summarizes the properties

[2] H. Eisen, manuscript in preparation.

TABLE I

MOUSE SPLEENIC CELLS TRANSFORMED WITH FRIEND ERYTHROLEUKEMIA VIRUS
CELLULAR PROPERTIES WHICH CHANGE AFTER DMSO INDUCTION

PROPERTY	CHANGE	BASIS FOR SELECTION IN CELL SEPARATOR
CELL SIZE	DECREASES OVER 2-3 CELL CYCLES	YES, LIGHT SCATTERING
LECTIN BINDING	EARLY: INCREASED AGGLUTINABILITY	NO
	LATE: INCREASED IN NUMBER OF BINDING SITES	YES, FLUORESCENT-LABELLED LECTIN
MEMBRANE VISCOSITY	INCREASES WITH DIFFERENTIATION	YES, ANISOTROPY OF DPH FLUORESCENCE
ANTIGENIC DETERMINANT	H-2 HISTOCOMPATIBILITY ANTIGEN DECREASES	YES, FLUORESCENT ANTIBODY TO THE H-2 LOCUS
SYNTHESIS OF MACROMOLECULES	DNA, CELL CYCLE KINETICS	YES, ACRIFLAVIN-FEULGEN OR HOECHST 33342 STAINING
	SPECIFIC mRNA	NO
	MEMBRANE PROTEIN, SPECTRIN, INCREASES	YES, FLUORESCENT ANTIBODY TO SPECTRIN
	HEMOGLOBIN PRODUCTION	CORRELATES WITH HIGH SPECTRIN CONTENT AND HIGH ANISOTROPY
VIRUS	VIRAL RNA	NO
	VIRUS PRODUCTION	YES, FLUORESCENT ANTIBODY TO VIRAL ANTIGENS

of the cells which change upon induction and how they can be probed by the cell separator. The following results show representative data for 3 surface phenomena of the cells.

a) *Lectin binding:* The mobility of the lectin binding sites for Con A on Friend Virus transformed cells seems to change during the first day of differentiation as measured by the increased agglutinability of the cells (21) and coincides with a decrease in membrane permeability 6 hours after addition of DMSO. However, measurements of the number of binding sites for the lectin assayed both by binding of ^{125}I-labelled Con A (shown in figure 2) and by fluorescence of bound fluoresceinated Con A indicate that no net increase in the number of lectin binding sites occurs until later in differentiation. Although the difference in number of binding sites is only 2-fold which normally would be difficult to see due to the rather broad distribution of absolute values for individual cells, some enhancement of the difference can be achieved by taking advantage of the fact that cells decrease

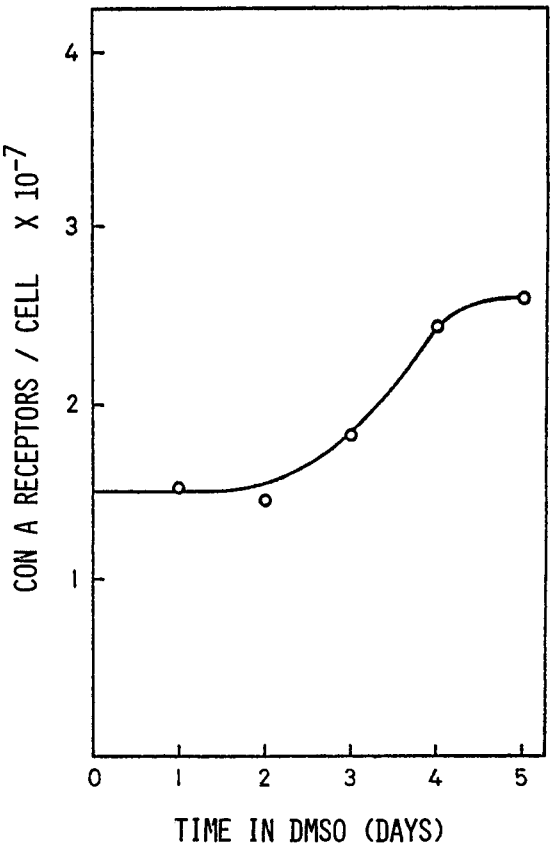


Fig. 2: Increase in Con A receptors per cell for Friend virus transformed cells with time after induction with DMSO. Each point represents the specific binding of ^{125}I-labelled Con A obtained by incubating 10^6 cells in 1 ml PBS from cultures induced in 1 % DMSO for 0–5 days with 150 µg of Con A for 15 minutes and washing through a gradient. Details of labelling and assay procedures have been published (5, 21).

in size as they differentiate. In figure 3 we see such a frequency distribution of the ratio of the fluorescence signal to the light scattering signal (the number of lectin binding sites divided by a function of the cell diameter (22, 23)) for an uninduced and 6-day DMSO unduced population of cells.

b) *H-2 binding sites:* The mature mouse erythrocyte has fewer H-2 histocompatibility antigen sites than the precursor cells as demonstrated by cytotoxicity measurements (24). Thus we can expect and do see a decrease in the number of sites when living cells tagged with fluorescent antibody are measured and sorted in the cell separator. Figure 4 shows a fluorescence micrograph of the antibody complex bound to living uninduced cells and figure 5 the measurement of this antibody binding on individual cells with the cell separator. As expected the mean signal size of the fluorescent cell population decreases with time after induction of the cultures by DMSO.

c) *Membrane fluidity:* The transport of small molecules in induced Friend virus cells is very different from the non-differentiating precursor (8 and unpublished observations). Such effects may reflect changes in the membrane permeability. Additionally, there is considerable evidence in the hemopoietic system for a large increase in the rigidity of the cell membrane between stem cells and erythrocytes. This rigidity can be demonstrated by the fact that lectins and antibodies do not

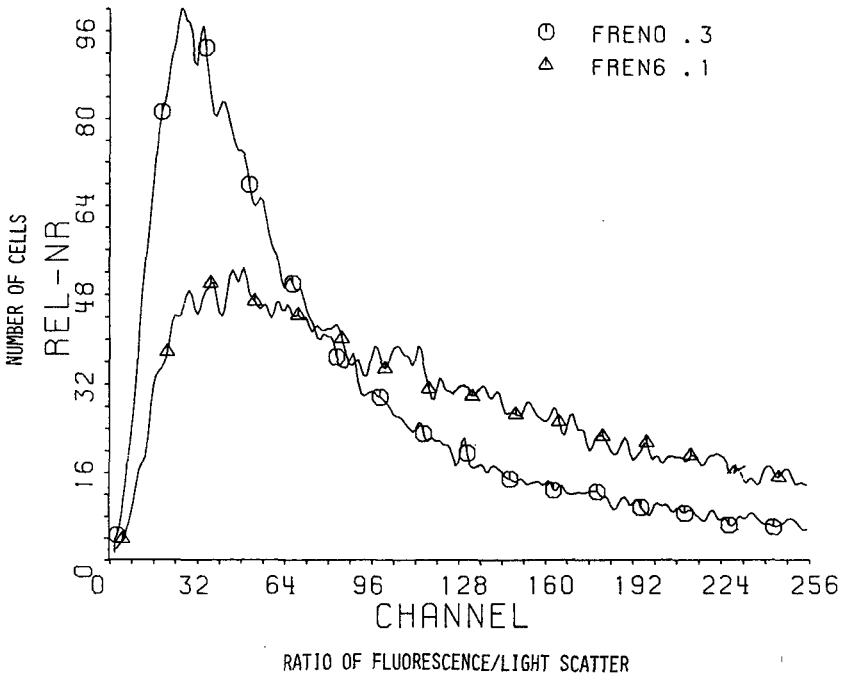


Fig. 3: Change in number of Con A receptors per cell as measured by fluorescence. Fluoresceinated Con A was bound to cells and the ratio of the fluorescence to light scattering signals for each cell monitored in the cell separator (represented by the abscissa). This parameter is larger with increasing number of Con A receptors per cell and/or decreasing cell size. The data are plotted as frequency distributions with relative number of cells for each signal ratio given on the ordinate. A total of 3 x 10^4 cells from an uninduced culture, -O-, and a 6-day DMSO induced culture, -△-, were measured. The induced culture has a population of resistant cells in a stage of outgrowth as well as differentiating cells.

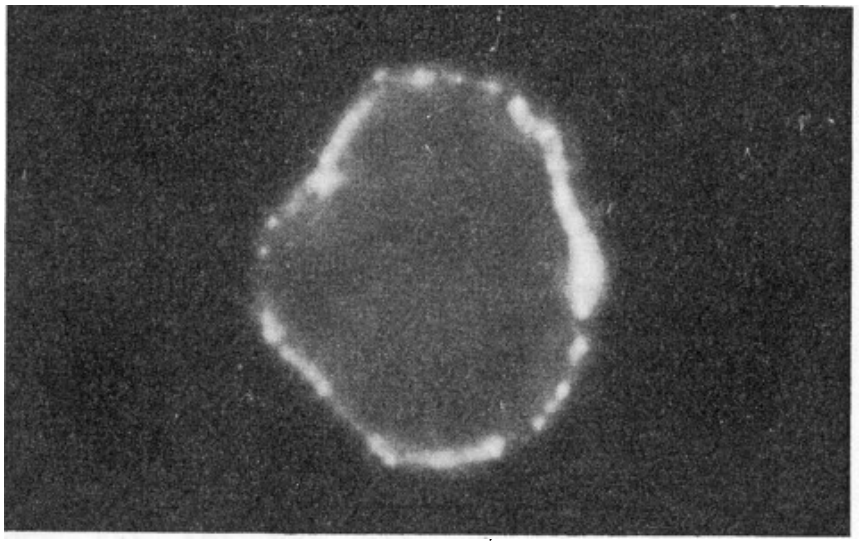


Fig. 4: Immunofluorescence demonstration of H-2 histocompatibility antigen on Friend virus transformed cells. Fluoresceinated rabbit anti-mouse IgG was reacted against anti H-2 mouse IgG bound to uninduced Friend cells. The fluorescence micrograph was taken on a Zeiss epi-illuminated fluorescence microscope, excitation below 480 nm and emission above 530 nm, Kodak Plus X film.

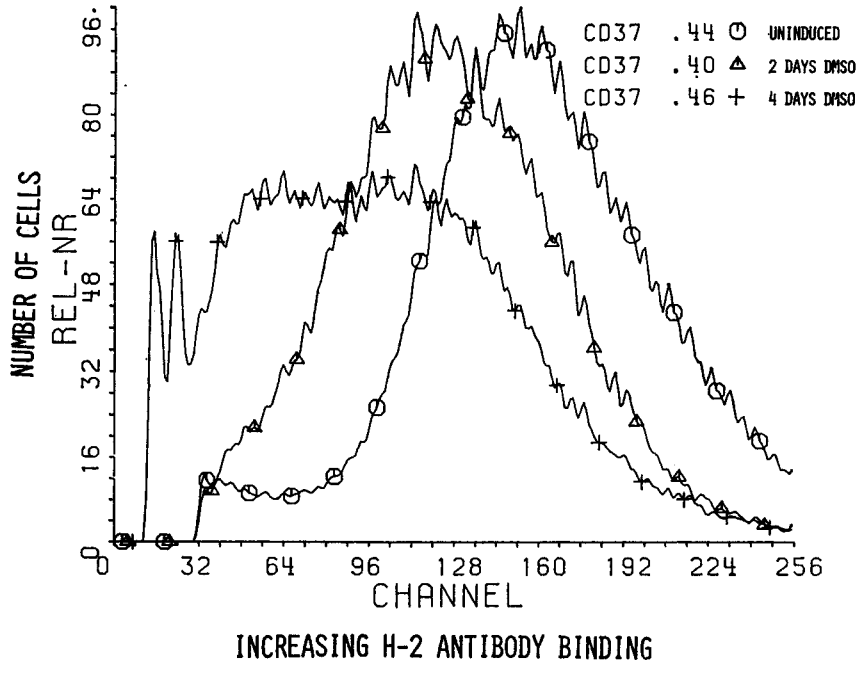


Fig. 5: Measure of the number of H-2 antigen sites in differentiating Friend cells. Indirect fluorescent antibody to H-2 was quantitated in the cell separator for populations of Friend cells with excitation at 488 nm and emission above 530 nm. The data are plotted as frequency distributions with the abscissa indicating increasing antibody binding and the ordinate the number of cells normalized to 10^5. -0-, uninduced cells; -△-, 2 days of 1 % DMSO; -+-, 4 days of 1 % DMSO.

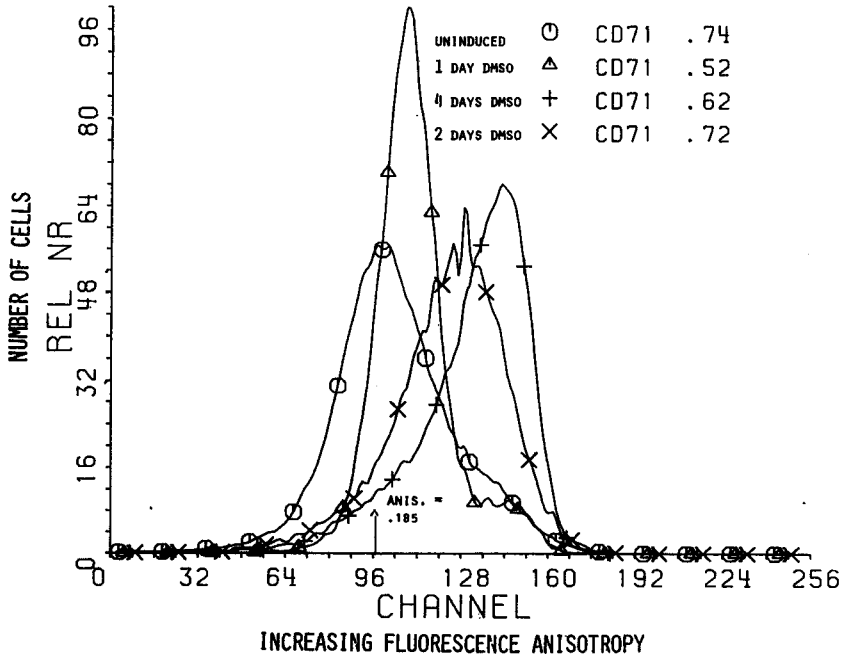
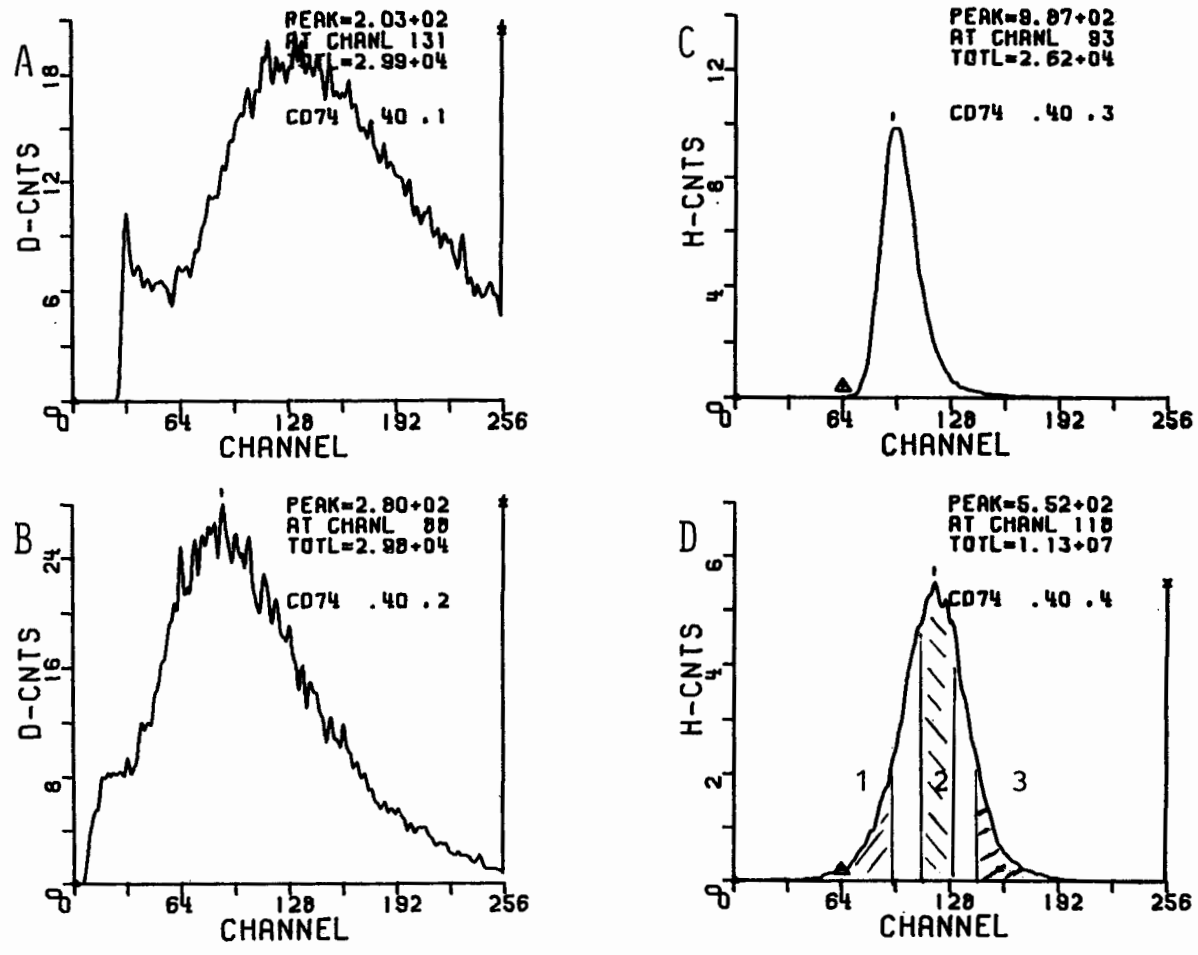


Fig. 6: Increase in fluorescence anisotropy with differentiation. The polarized fluorescence of DPH bound to Friend cells was measured in the cell separator and real-time anisotropy functions determined. Frequency distributions for populations of uninduced, -0-; 1 day, 1.2 % DMSO induced, -△-; 2 days induced, -x-; and 4 days induced, -+-, are shown on a single plot. Increasing anisotropy (correlated with increasing membrane viscosity) is plotted on the abscissa and cell number normalized to 10^5 cells on the ordinate.



PROPERTY	CUT 1	CUT 2	CUT 3
ANISOTROPY	.10-.15	.17-.20	.22-.29
MICROVISCOSITY (POISES)	.9-1.7	2.1-2.8	4.2-14
SIZE (MICRONS)	12	10	7
SPECTRIN CONTENT	-	-/+	+
BENZIDINE STAINING	-	-	+
GLOBIN mRNA	(-)	(+)	(+)

Fig. 7: Correlation of properties of induced Friend cells sorted on fluorescence anisotropy. The frequency distributions A–D were measured on DPH stained Friend cells after induction in DMSO for 2 days. Plot A is the distribution of the apparent parallel fluorescence intensities; plot B, that of the apparent perpendicular fluorescence intensities; plot C, that of the ratio I_A/I_B; and plot D, that of the fluorescence anisotropy. Cells sorted on the anisotropy according to the indicated cuts can be correlated with other properties as shown in the table.

cap or form large patches in erythrocytes whereas the undifferentiated cells are capable of capping. Studies of the lipid composition of erythrocytes reveal a high cholesterol to phospholipid ratio (25), a condition in model membranes and cells which reduces fluidity (30). Under some conditions, a direct measure of local membrane fluidity can be obtained by measuring the depolarization of fluorescence of a dye situated in the membrane (5, 29, 30, 34). The degree of depolarization is dependent upon the extent to which the excited molecules are displaced from their original orientation during the lifetime of their excited state. The displacement is directly proportional to the hydrodynamic properties of the medium and temperature. Thus the emission polarization (or anisotropy) can be used as probes of membrane viscosity, i. e., fluidity (26–28).

The molecule DPH has been shown to be bound in the hydrocarbon layer of biological membranes and thus functions as a probe of the viscosity of that region (5, 29, 30). The cells continue to grow normally after treatment with the dye. The amount of dye taken up by individual cells is variable but one is concerned primarily with the emission polarization, a function independent of quantity to a first approximation.

Friend virus transformed cells induced with DMSO appear to have increasingly rigid membranes as is shown by the increasing anisotropy of the dye in the cell membranes correlated with time after induction in figure 6. We have been able to demonstrate that the DMSO itself does not appear to alter the dye's emission and one can detect the growth of resistant cells in the population by the appearance at late induction times of a peak of cells with anisotropy like that seen in uninduced populations. In a 2–3 day DMSO induced population there is a broad distribution of cell stages. Separation of the cells on the basis of their anisotropy allows the correlation with other biological properties and the further outgrowth of the homogeneous populations so derived. Figure 7 shows how such a heterogeneous population can be separated and the fractions correlated with cellular functions. The ability to regrow the populations provides us with a means of determining the reversibility of differentiation by assaying for the production of globin messenger in the separated and then regrown cells. These experiments are in progress.

Discussion

The erythroleukemia Friend virus transformed cell is not only a good model for controlled differentiation but may serve to answer some basic questions concerning the role of viruses in leukemias. The fact that one can release these cells from the transformed state and initiate a seemingly normal differentiation provides the means for studying the fate of the transforming virus. Hopefully, such results may suggest means for probing viral etiologies in human leukemias. In addition, the question of reversibility of differentiation although well documented in plants (31) has not been conclusively established for higher animals and is particularly important in any understanding of the mechanism of induction and the mode of treatment for leukemias. As described here with the Friend system, one can select large numbers of cells on the basis of some differentiated function or property with the cell separator and then study the specific mRNA's and specific functional

proteins after further culture with and without the inducing agent for differentiation. With this technique it is possible to chronicle the sequence of events leading to reticulocyte-like cells and to compare the process in several cell lines. Once this information is compiled we hope to look at the *in vivo* hemopoietic system with some of these probes to see how comparable the *in vitro* and normal processes are.

The extension of automated cell-separation techniques beyond the quantitation of macromolecular content to obtaining separation on the basis of cellular structure or organization has been described here. We have shown that a complete gradation of differentiating cell stages can be obtained by separating on the basis of the fluorescence emission polarization of a dye, DPH, which appears to bind the plasma membrane and monitor fluidity changes therein.

DPH has been used to monitor the fluidity of membranes of leukemic cells (29) and their lower cholesterol content (32, 33) has been implicated as a factor in their neoplastic behaviour. Our preliminary work on leukemic lymphocytes using the DPH probe with the cell separator indicates that membrane fluidity may be correlated with the nature and state of the disease.

It is probable that the technique of fluorescent anisotropy separation using appropriate fluorescent probes will enable us to look at numerous processes in biological systems which result in changes in cellular permeability or membrane structure, such as response to hormones, infection by virus, other types of differentiation, or transformation.

Thus it would appear that flow systems capable of separating cells on the basis of spectroscopic properties may soon play a larger role in diagnosis of leukemia (e. g. by use of specific fluorescent antibodies, as has been described by Dr. M. Greaves at this symposium), in the recognition of preleukemia states (by perhaps changes in membrane structure or function), and in the early diagnosis of relapse. Flow systems with their rapid screening of statistically significant populations (usually 1000 cells per second) and high sensitivity (laser excitation) should provide a means for large screening programs once enough specific probes are developed.

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Abbreviations
DMSO = dimethylsulfoxide.
Con A = concanavalin A.
DPH = 1,6-diphenyl-3,4,5-hexatriene.