CELLULAR GROWTH CONTROL: PROPERTIES OF A UNIQUE ADHERING DERIVATIVE OF L5178Y CELLS

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SUMMARY: A new cell line derived from L5178Y murine lymphoma cells with unique properties is described. The cells, L5178Y Adh, grow simultaneously in suspension culture and attached to a substratum but not in contact with each other. Limitation of growth of the cells in either the suspended state or attached to substratum is not linked to a limiting surface or cell density in a manner analogous to "contact inhibition." The adhering and non-adhering cells grow with different doubling times. Cell size analysis indicates that initial gravity settling of the inoculum determines which cells attach. Contact of these cells' external surface with substratum may then initiate events which ensure attachment of adhering populations.

Recent investigations have made it clear that the induction of cells to perform their specific functions may be initiated by events occurring at the cell surface. The mode of growth of mammalian cells in culture falls into one of two categories (1). Fibroblasts, e.g., generally grow as a monolayer attached to each other and to a substratum of serum-coated glass or polystyrene, whereas lymphoid cells grow as a suspension of discrete cells attached neither to each other nor to any substratum. Cells grown in monolayers are termed "contact inhibited" when a limiting cell density on the culture vessel surface is reached (2). Cells can be released from contact inhibition by alterations in the growth medium (3) or by neoplastic transformation (4), forming multiple layers at the surface of the vessel (i.e., cells become the substratum).

Some cells normally grown as monolayers may be grown in "spinner" cultures, in which case they remain suspended as long as they are provided with mechanical agitation (usually stirring). These cells usually grow in clumps in suspension and upon cessation of the mechanical agitation usually adhere to the culture vessel. We describe here a cell line isolated from L5178Y murine lymphoma with unique properties which make it fall into neither of these two major categories. The new cells, L5178Y Adh, grow both in suspension and attached to a substratum simultaneously under the same medium and culture

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conditions. The only difference between the two populations is attachment to a substratum. Cells that attach to the substratum ultimately undergo a morphologic differentiation not seen in the suspended population. This work is of importance because recent evidence suggests that tumor cell populations are heterogeneous with regard to various biological properties (5). This may be due to acquired genetic lability which permits the sequential selection of variant subpopulations produced as a result of that genetic instability, and indeed human tumors may evolve by clonal selection (6).

MATERIALS AND METHODS

Cell cultures. L5178Y cells have been maintained in vitro in this laboratory for 7 years. L5178Y Adh cells were from Dr. D. Kessel, Michigan Cancer Institute, Detroit, Michigan. These cells were derived from a clone of L5178Y cells originally acquired from our laboratory. RPMI 1640 medium was purchased from Grand Island Biological, Grand Island, New York. Donor horse serum (Biocell Laboratories, Los Angeles) was added to the final concentration of 10%.

Kinetic studies. Stationary phase L5178Y Adh cells were used as the inoculum for all growth studies. Two different conditions were studied. First, only adhering cells were inoculated. In this case, the liquid contents of the glass flask with a growing surface of 250 cm^2 was discarded, leaving only the cells attached to the glass. The medium was replaced with Tris basic buffered salt solution plus 0.125% trypsin and incubated at 25° for 5 min. The trypsinate was centrifuged at 650 x *g* for 5 min and resuspended in fresh medium. A sample was taken for counting and size determination. Sizing was performed with a Coulter counter and a Model II Channelizer. The cell suspension was diluted with fresh medium, and polystyrene T-flasks (Falcon Plastics) of 25 cm^2 growing surface area were inoculated uniformly with approx. 1.25×10^6 cells in 9.0 ml of medium. In the second condition (non-adhering cells), cells in the liquid content of the medium served as the inoculum; 1.25×10^6 cells in 9.0 ml of medium were inoculated as above.

In either condition, at indicated times after inoculation, three flasks were individually sampled for counting and size analysis. The medium was removed and replaced with 4.5 ml of 0.1 M potassium phosphate buffer, pH 7.0. The growing surface was scraped with a rubber policeman and the buffer removed to a separate tube. Another 4.5 ml of buffer was added, the scraping was repeated, and the resultant suspension was pooled with the first. The samples were centrifuged at 650 x *g* at room temperature and resuspended in 30 ml of "Isoton" for counting and sizing.

Morphology. L5178Y Adh cells were grown in 60 mm diameter polystyrene Petri dishes at 37° in a 5% CO_2 atmosphere, inoculated with adhering cells; each petri dish contained a glass cover slip. For phase microscopy the cover slip was removed and examined; suspended cells were observed as wet mounts.

Aqueous two-phase polymer partitioning. Samples from cultures grown in either condition (1 or 2 above) were partitioned in dextran and polyethylene glycol, as described previously (7,8) except that the final NaCl concentration was 0.025 M rather than 0.05 M.

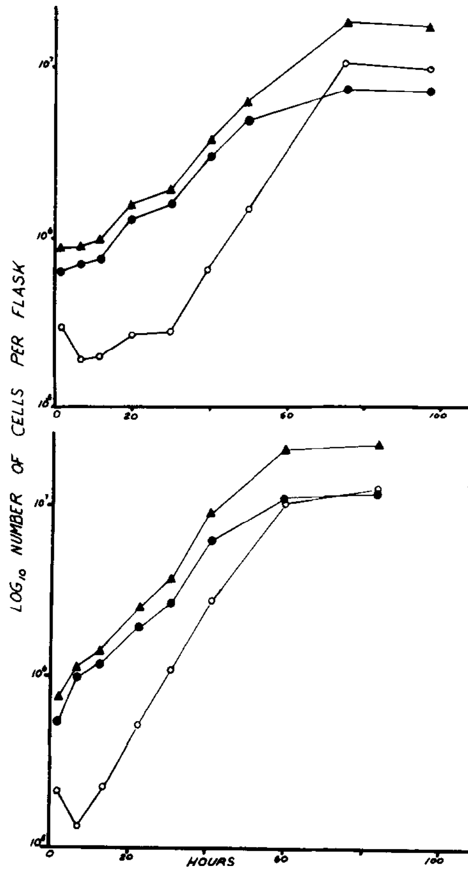

Fig. 1. Growth kinetics of L5178Y Adh in RPMI 1640 + 10% horse serum: cell number vs. time. Top, inoculum--adhering cells; bottom, inoculum--suspended cells. Open circles, cells recovered as suspension; closed circles, cells recovered attached to plastic; triangles, complete population (sum of adhering and suspended). Time is hours post-inoculum; i.e., 0 hr represents initial inoculum.

RESULTS AND DISCUSSION

Growth kinetics of L5178Y Adh medium are shown in Fig. 1. In both cases stationary cultures were used for the inoculum, as described above. When adhering cells were the inoculum (Fig. 1 top) the total population had a lag phase of 12 h, a doubling time of 13 h, and entered stationary phase approx. 20 h after inoculum transfer. Cells settle to the culture flask bottom rapidly, and settling is almost complete by 2 h after transfer. The settling process is complete by 7-12 h (Fig. 1 top). These cells that are suspended have an apparent doubling time of 8-9 h. The number of cells suspended surpasses the number of cells adhering at approx. 70 h after transfer.

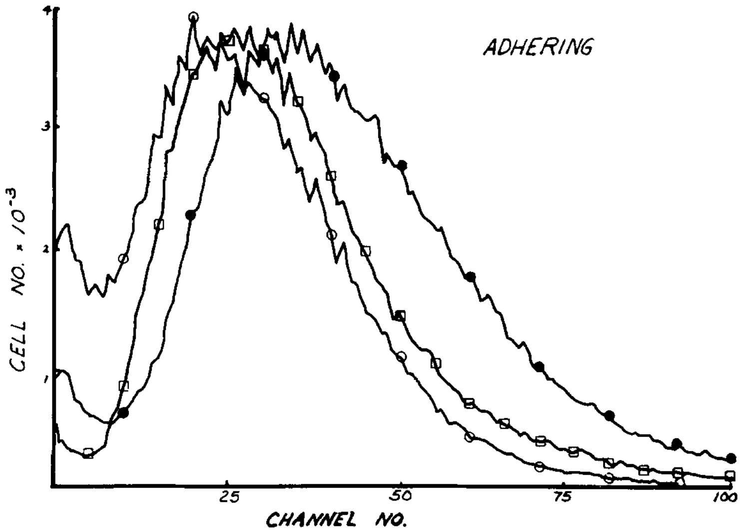

Fig. 2. Size distribution of L5178Y Adh as a function of time: adhering cells inoculated--number of cells vs channel number. Each channel represents a window of 16 μm^3 beginning with channel 1 equal to 176 μm^3. Each curve is an actual tracing of the size distribution. The symbols are included only for identification and do not represent individual points: Squares, initial inoculum; open circles, cells recovered suspended 2 hr post-inoculation; closed circles, cells recovered adhering 2 hr post-inoculation.

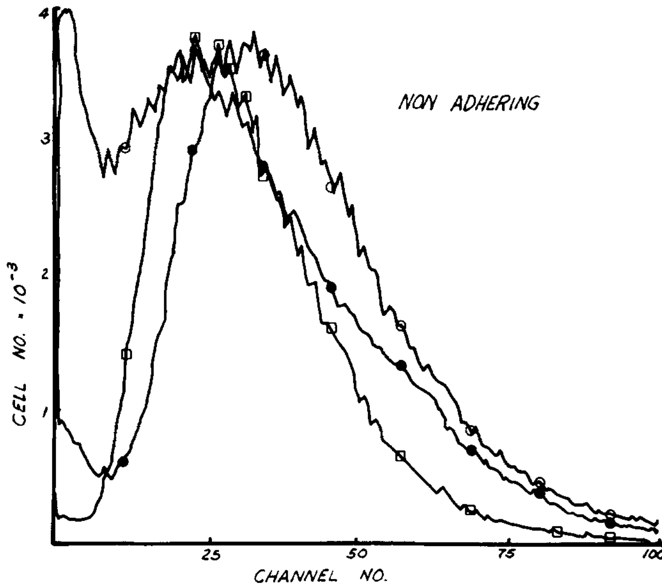

Fig. 3. Size distribution of L5178Y Adh as a function of time: non-adhering suspended cells inoculated--number of cells vs. channel number. Symbols are as given in Figure 2.

When suspended non-adhering cells (condition two above) were the inoculum (Fig. 1 bottom), no lag phase occurred. The total population had a doubling time of 13 h. The adhering and suspended cells have apparent doubling times of 12-13 h and 8-9 h, respectively, and are therefore comparable with those of Fig. 1 top. The settling-out of the suspended non-adhering cell inoculum is similar to the case when adhering cells are the inoculum (Fig. 1).

Cell size as a function of time was studied. The same pattern of size distribution occurred as a function of time regardless of whether adhering (condition 1) or suspended (condition 2) cells were the inoculum (Figs. 2 and 3).

Figs. 2 and 3 each depict the size distribution of the initial inocula, cells adhering and cells remaining suspended, 2 h after inoculation. The initial inoculum in Fig. 2 (adhering cells, condition 1) has a modal volume of 608 μm^3, and cells adhering 2 h post this inoculum have a modal volume of 720 μm^3, and the cells remaining suspended 2 h post inoculum, 544 μm^3. The corresponding volumes for Fig. 3 (when suspended cells--condition 2--are used as the inoculum), are 576, 704, and 544 μm^3. Simply put, these data show that even though the modal volumes of the initial inoculum vary somewhat whether adhering or suspended cells serve as the inoculum, after 2 h cells suspended or attached have similar modal volumes from both inocula. In Table 1 are modal volumes for cells that attach or for suspended cells under either condition of inoculation (1 or 2 above). The data show that the post-inoculation settling process is almost complete by 2 h after inoculation.

Photomicrographs of adhering and suspended non-adhering cells in exponential phase (48 h after transfer of suspended cells) are given in Fig. 4. Interestingly, 5% of the adhering exponential cells resemble fibroblasts; i.e., they have discrete processes not seen in the nonadhering cells. Granular bodies at the cell periphery are present on both suspended and adhering cells throughout the growth cycles. In both adhering and suspended cells in the exponential phase, unequal outgrowths are observed to be pinching off from the cells.

Results of the time course study of aqueous two-phase polymer partitioning are given in Table II. The data represent the means of four observations of each of 10 independent partitions. Partition ratio is expressed as the fraction of the total cell number which is recovered from the top phase (7) after partitioning. As has been pointed out by others and by us previously (7,8), it is difficult to totally express mechanistically what

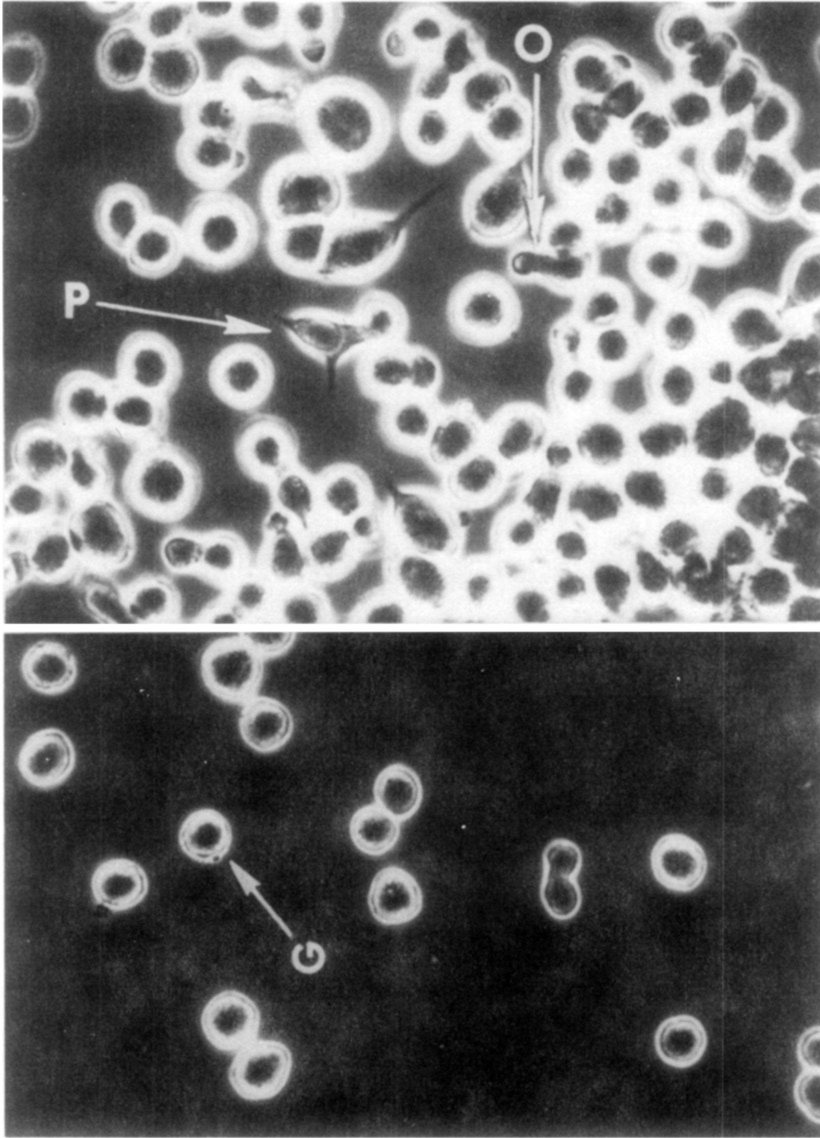

Fig. 4. Photomicrographs of exponential cells. Top (a), adhering cells; bottom (b), nonadhering cells. The letters in the photomicrographs represent: P, processes; G, peripheral granules; O, outgrowths. These photomicrographs are representative of some 200 taken, all of which were illustrative of what was observed in morphological monitoring of the cells.

differences in partitioning ratio mean. Rather the data in Table II are presented merely to indicate that, because the ratio is so different at the various time points, the external periphery of the cells is indeed different when the cells are manifesting themselves by growth in suspension or in an attached mode.

Table I. Modal Volume of Suspended and Adhering Cells as a Function of Time Post-incubation

Adhering inoculum			Suspended inoculum		
Time post transfer (h)	Modal volume* (μm^3)		Time post transfer (h)	Modal volume (μm^3)	
	Adhering	Suspension		Adhering	Suspension
0	...	608 ± 27	0	...	512 ± 27
2	720 ± 24	480 ± 24	2	688 ± 24	528 ± 28
7	672 ± 23	592 ± 25	7	688 ± 24	400 ± 21
12	702 ± 26	656 ± 26	12	720 ± 20	528 ± 31
20	720 ± 16	692 ± 20	22	704 ± 21	560 ± 24
30	592 ± 27	592 ± 19	30	672 ± 12	560 ± 18
40	592 ± 24	560 ± 19	42	592 ± 19	544 ± 17
50	592 ± 25	528 ± 27	67	320 ± 19	320 ± 17
60	560 ± 19	464 ± 29	90	272 ± 20	240 ± 20
75	368 ± 15	400 ± 25			
96	320 ± 14	210 ± 14			

* Modal volumes represent the volume for the mode of the population as determined on the Coulter Counter channelizer; i.e., it is that volume which occurs maximally in the frequency distribution of volumes.

We describe here characteristics of a cell line that can grow either attached to a substratum or as a suspension. The most interesting fact of this finding is that either cell subline can give rise to the other. Kinetic and size analyses suggest that the factor determining which cells settle out from the inoculum and which cells attach to the substratum (whether it be attached cell inoculum or suspended cell inoculum) is the cell volume or density. Once cells attach to the substratum they proceed to grow with apparent kinetics similar to the parent line (a suspension culture). The less dense cells, which remain suspended (initially approx. 20% of the total), have a very rapid doubling time (8-9 h) and eventually outgrow the adhering cells. This is to be expected since the space for growth on the monolayer is more limiting than that of the suspension. It has been observed in bacterial systems that dividing cells trapped on a substratum can, under certain circumstances, drop their new daughters from the substratum (9). This does not seem to be the case in the present system (see Fig. 1).

Both kinetic and size analysis show that the behavior of the cell population throughout the growth cycle is not dependent upon the origin of the inoculum; i.e., inocula taken from either the non-adhering or adhering compartments give rise to comparable growth kinetics, settling-out behavior, and size distributions with time. Photomicrographs, however, clearly indi-

Table II. Time Course of Aqueous Two-Polymer Partition of Adhering and Suspended Cells

Time (h post-transfer)	Partition ratio*	
	Suspended cells	Adhering cells
2	0.49	0.47
12	0.22	0.16
24	0.33	0.54
36	0.39	0.59
48	0.40	0.47
60	0.36	0.51

* Fraction of total cells added which is recovered from the top phase. In this case suspended cells were used as the inoculum.

cate that the suspended cells differ from the adhering cells. Although clumps of cells are observed in the micrographs of the adhering state (Fig. 4), size analysis (Table I) indicates that the clumps are readily dispersed to individual cells when they are scraped from the substratum. This is not a condition usually associated with monolayer cultures where large nondispersible aggregates are encountered.

If both types of inoculum give rise to comparable behavior and if the suspended cells are vastly different, both morphologically and by aqueous polymer partition analysis, from the adhering cells, what then initiates this differentiation--especially in the light of the fact that density appears to be the only determinant of which cells adhere initially? The most likely answer is that cell surface-to-substratum contact, i.e., contact with exogenous substratum, sets in motion events that determine the fate and mode of cell growth.

The present work demonstrates a cell line with remarkable ability to react to its environment in terms of growth control. Furthermore the work illustrates the heterogenicity of a malignant cell population--a population phenomenon which may obtain in vivo (5,6). Finally, the results are of extreme importance for the phenomenon of cell external surface to genome communication and for epigenetic control of cellular events.

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