

Isolation and Characterization of the Two Subpopulations of Cells with Different Lethalities from Zajdela Ascitic Hepatoma

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ABSTRACT

Two distinct subpopulations of cells, light (L-cells) and heavy (H-cells), have been isolated and characterized from a rat ascitic tumor, the Zajdela ascitic hepatoma. These two populations have been separated by Percoll density gradient centrifugation and studied by flow cytometry. The two populations, in addition to their difference in buoyant densities, show characteristically different profiles for DNA and RNA contents, nonspecific esterase activity, and surface amino group distribution. The DNA distribution in the two types of cells clearly shows that the H-cells are rapidly proliferating while the L-cells are quiescent. Studies on the two groups of cells after colchicine treatment also confirm this conclusion. The H-cell induced tumors kill the host animals rapidly while the L-cell induced tumors regress in about 3 months. The H- and L-cells from the Zajdela tumor form a convenient experimental system to study the marked difference in the progression of tumors induced by these cells, possible differences in gene expression in regressing and nonregressing tumors and the interactions between the subpopulations with a view to delineate molecular events governing tumor progression and tumor heterogeneity.

INTRODUCTION

Compelling evidence obtained over the last several years has shown that tumors are often composed of complex populations of cells which differ significantly from one another in several of their properties. Tumor cells, isolated even from a single source, show differences in many characteristics such as proliferating rates, metastasizing potential, drug sensitivity, clonogenic potential, and cell differentiation features. Although the reasons for these differences are not clearly understood, they have been attributed to the general instability of the malignant phenotype, implying that tumors during their growth may give rise to cells which could be clonally interrelated but are phenotypically distinct (1-15). Despite these observations, results on experiments correlating possible differences in the tumorigenic properties of the clonally related, yet phenotypically distinct, subsets of tumor cells are with a few exceptions (2) meager. Such experimental systems showing differences in their tumorigenic properties would be valuable in designing experiments for studying the differences in gene expression leading to immunological or other phenotypic manifestations, generating information that could provide means for the control of the tumor.

A successful understanding of the heterogeneity in tumor cell populations depends on the efficiency of the separation methods used to isolate the subpopulations and the sensitivity of the criteria used for identifying the differences between such populations. A number of reports are available in the literature where subpopulations of cells in solid tumors or leukemias have been separated and characterized (13, 16-19). Ascitic tumor cells, however, appear to be relatively poorly characterized in terms of tumor heterogeneity, although they form a convenient experimental model system to study the problem (20).

We report in this communication the development of an

experimental system in the ZAH,² a chemically induced tumor adapted to grow in the peritoneal cavity of the rat, where the kinetic and tumorigenic properties of subpopulations of cells derived from the same tumor are distinctly different. Subpopulations of these cells were first isolated on a density gradient of Percoll and then studied by flow cytometry. The cells were further characterized by examining the differences in the growth potentials of the two types of cells. We provide evidence that these populations have different proliferative capacities by showing (a) their sensitivity to colchicine *in vivo* and (b) the difference in the growth rates of the tumors caused by them. We show also that the two populations induce tumors with different lethalities in the host animal. Based on chromosomal studies it is shown that the two groups of cells could be clonally related phenotypic variants.

MATERIALS AND METHODS

Tumor

The ZAH tumor was maintained by serial transplantation in Wistar rats from tumor-bearing animals originally obtained from Dr. F. Zajdela, Institute de Radium, Orsay, France. The colony of rats was propagated by sibling mating.

Stains and Chemicals

DAPI and FDA were from Sigma Chemical Co., St. Louis, MO; AO was from Eastman Kodak Co., Rochester, NY; Fluorescamine was from Hoffman LaRoche, Basel, Switzerland; and Percoll was from Pharmacia, Uppsala, Sweden.

Cell Separation Method

Ascitic fluid (about 30 ml/animal) was obtained from 5-7-day-old tumors. The cells were pelleted, washed three times with normal saline, and then taken up in saline to give a suspension of 2×10^7 cells/ml. They were separated by isopycnic gradient centrifugation. Continuous Percoll gradients were made according to the manufacturer's instruction manual using 55% stock isoosmotic Percoll solution and centrifuging in an SM24 rotor of Sorvall RC5B centrifuge at $30,000 \times g_{\text{average}}$ for 30 min. Approximately 1×10^7 cells were layered on each gradient and the tubes were spun in swing-out buckets in a tabletop centrifuge at $400 \times g$ for 15 min. The cells corresponding to the separate bands (see Fig. 1) were collected from top by careful removal of the required bands with a Pasteur pipet and washed with saline by centrifugation at 2000 rpm for 5 min in an SS34 rotor. The heavy and light bands were rebanded two more times before they were processed according to the requirements for individual experiments. There were minor disturbances of the bands during the collection of cells from the first gradient, but after the third centrifugation the bands were distinct. Chromosome preparations were made by using standard air drying methods from both L- and H-cells.

Tumor Propagation with Separated Cells

Cells from the heavy (H) and light (L) bands were collected, washed three times with normal saline (0.15 M NaCl), and adjusted to 2×10^6 cells/ml. Aliquots (0.5 ml) of the suspensions of the separated cells

² The abbreviations used are: ZAH, Zajdela ascitic hepatoma; DAPI, diaminodiphenylindole; FDA, fluorescein diacetate; AO, acridine orange; PBS, phosphate-buffered saline; MC, modal channel.

CELL SEPARATION ON PERCOLL GRADIENTS

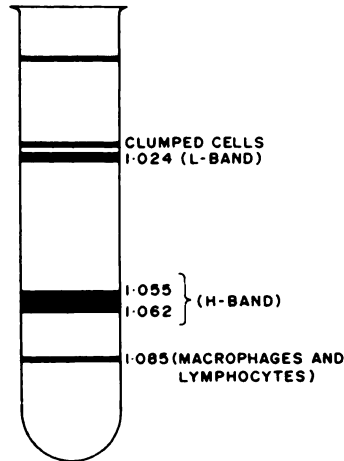


Fig. 1. Separation profile of ZAH on a 55% Percoll gradient. The densities of each band were measured by standard density marker beads. Figures shown against individual bands correspond to isopyknic densities (g/ml).

were injected i.p. into individual animals in 2 different groups of 5–10 animals each. These animals were kept under observation for a period of 3 months or until they died.

Colchicine Administration in Tumor Bearing Rats

Colchicine (Loba Chemie, India) in saline (10 μ g/ml) was injected into an animal with a 5–6-day-old tumor. Samples of cells were taken from the peritoneal cavity, prior to injection of the drug and 5 h after colchicine injection; cells were stained with DAPI and the fluorescence was measured cytofluorimetrically as outlined below.

Staining Protocols

DAPI. The staining methodology followed was according to the method of Thornthwaite *et al.* (21). Cells were suspended in a solution of DAPI (10 μ g/ml) made in 146 mM PBS (with 1.0 mM CaCl_2 and 0.5 mM MgCl_2), 0.6% Nonidet P-40, and 2.0% fetal calf serum. The cells were incubated in the dark, at room temperature, for about 5 min and then used for fluorescence measurements.

AO. Staining with AO was carried out according to the method of Andreef *et al.* (22). A suspension of 2×10^5 cells (in 0.2 ml of saline) was mixed with 0.4 ml of a solution containing 0.08 N HCl, 0.15 M NaCl, and Triton X-100 (0.1%, v/v). After 1 min, 1.2 ml of a solution containing 0.2 M Na_2HPO_4 , 0.1 M citric buffer (pH 6.0), 1 mM sodium EDTA, 0.15 M NaCl, and AO (8 μ g/ μ l) were added to the mixture. The addition of AO in the presence of chelating agents (EDTA, citrate) results in the denaturation of cellular RNAs which then fluoresce at a wavelength of 580 nm while DNA remains in its native double helical state and fluoresces in the region of 520–540 nm.

FDA. Staining was done according to the method of Baisch (23). Approximately 5×10^6 cells were suspended in 10 ml PBS (pH 7.3) containing 1.0% fetal calf serum. To this was added a 10- μ l solution of FDA (1 mg/ml) in acetone. The cells were gently vortexed and incubated at 37°C for about 15 min and then FDA fluorescence was measured by cytofluorimetry.

Fluorescamine. To stain the surface NH_2 groups of the cells, fluorescamine was used as a specific dye. The method of staining was according to the method of Hawkes *et al.* (24). About 1×10^6 cells were washed twice with 0.1 M borate buffer (pH 8.4) and suspended in 0.5 ml of the same buffer. To this were added 10 μ l of a solution of fluorescamine made in acetone (10 mg/ml). Staining was allowed for about 30 s followed by the addition of 2 ml of glucose in PBS for fluorescence measurements. The cells were resuspended in borate buffer for fluorescence measurements.

Fluorescence Measurements

The fluorescence yields from the different staining protocols were recorded by the flow cytofluorimeter ICP11 (Phywe, Gottingen, Federal Republic of Germany). For fluorescence measurements of DAPI and fluorescamine labeled cells BG38 and UG1 were used as excitation filters and 3-73 (Corning International) as barrier filters. For FDA and AO BG38 and KP490 were used as excitation filters and LP520 and KP540 (green emission) and OG550 and DM580 (red emission) were used as barrier filters. Cytofluorimetry data were analyzed according to the method of Baisch *et al.* (25).

RESULTS

Density Gradient Centrifugation. Upon separation of ZAH cells in 55% continuous Percoll gradient, 4 major bands were obtained. The position of these bands and their densities, as determined by density marker beads (Pharmacia), are shown in Fig. 1. The topmost band contained mainly clumps of cells which did not enter the gradient. The next two bands from the top contained tumor cells; the lighter band (designated L) corresponds to a density of 1.024 g/ml while the heavier band (designated H) showed a density range of 1.055–1.062 g/ml. The lowermost band had a density of about 1.085 g/ml and was composed of macrophages and lymphocytes. Viability counts of cells from the H- and L-bands were carried out before each experiment and both groups of cells were 95–100% viable.

Survival of Animals Given Injections of L- and H-Cells. The tumors induced by the H- and L-cells showed distinct differences in the survival pattern of the hosts, as shown in Fig. 2. All the animals given injections of H-cells died within 18 days of injection, while those injected with L-cells showed longer periods of survival; 40% of these animals survived for periods over 3 months after which the tumors regressed. It was also observed that the H-cell induced tumors grew faster than the L-cell induced tumors. The former had a shorter latent period and its growth rate (increase in weight per animal per day) was more than that in the latter group.

DNA Distribution Profiles. The DNA distribution in ZAH cells was studied by staining the cells with (a) DAPI and (b) AO (green fluorescence). Fig. 3 shows the results of fluorescence measurements using DAPI staining. The unseparated population was found to contain three subpopulations of cells in terms of DNA distribution (Fig. 3a). Based on the relative distribution

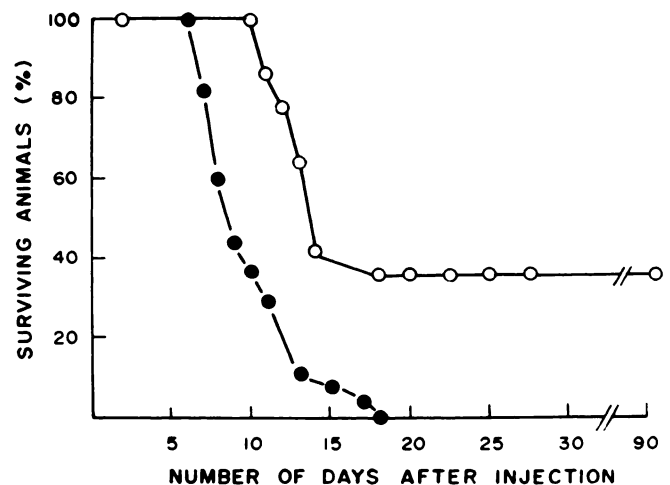


Fig. 2. Survival pattern of animals after injection of light and heavy cells into Wistar rats. The percentage survival values shown are mean values obtained from 3 independent experiments, using 10 animals each for L- and H-tumors in each set of experiments. O, animals with L-cell induced tumors; ●, animals with H-cell induced tumors.

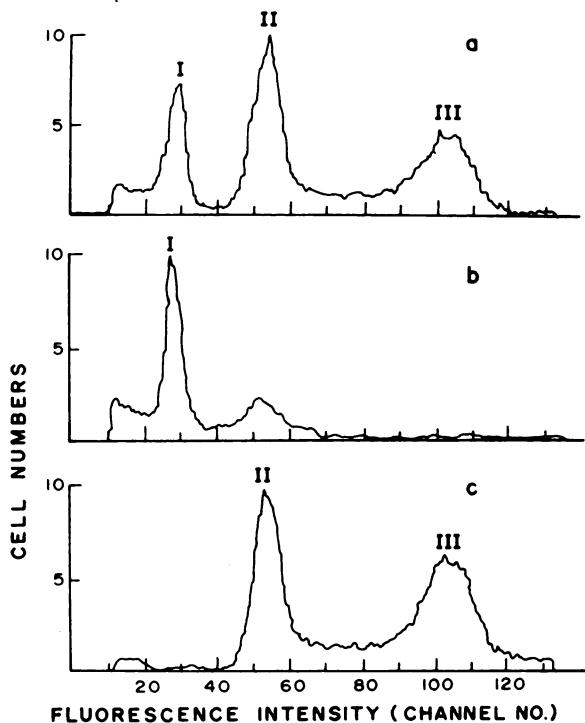


Fig. 3. DNA distribution profiles of unseparated and separated ZAH cells after staining with DAPI (see text). The fluorescence intensity is shown in channel numbers. *a*, unseparated cells (total cells scanned, 35,334); *b*, L-cells (total cells scanned, 14,438); *c*, H-cells (total cells scanned, 43,248). Numbers on ordinate, actual numbers of cells divided by 100.

of cells and according to the analysis of cell cycle stages as identified by DNA cytofluorimetry by Baisch *et al.* (25) and Darzynkiewicz *et al.* (26), peaks I and II were assigned to the two major subpopulations of the tumor (both of G₁ phase) while peak III was assigned to the G₂ + M cells from the populations of cells in peak II. Since the DNA content of peak II cells (modal channel MC52) is about double that of peak I (MC26), it is likely that peak II contains some cells from the G₂ + M fraction of the peak I cells. The fluorescence profiles of the separated cells (Fig. 3, *b* and *c*) show that the H-band cells exhibit only peak II and peak III while the L-band cells show mainly peak I (G₁ phase) with a very small peak at channel 52. The small peak at channel 52 could arise from the G₂ + M fraction of peak I cells and probably a small amount of H-band cells.

Experiments using acridine orange (green fluorescence) also show DNA distribution profiles similar to those obtained using DAPI stained cells (data not shown). These results show that the tumor contains two populations of cells, one (H-cells) having the majority of proliferating cells with double the amount of DNA compared to the other (L-cells) which appears to have a negligible proportion of dividing cells.

RNA Distribution Profile. The RNA distribution patterns of these cells, as shown by the red fluorescence of acridine orange, also show the presence of two types of cells in the tumor (Fig. 4). In the unseparated population two peaks are identifiable, one at modal channel 10 and the other at modal channel 37. In the separated cells, the L-band contains cells with low fluorescence intensities (MC18) and the H-band contains cells with higher fluorescence intensities (MC40). The heterogeneous distribution of RNA in the H-band cells would suggest that they have a larger fraction of proliferating cells as compared to the L-band cells which show greater uniformity in their RNA distribution (22).

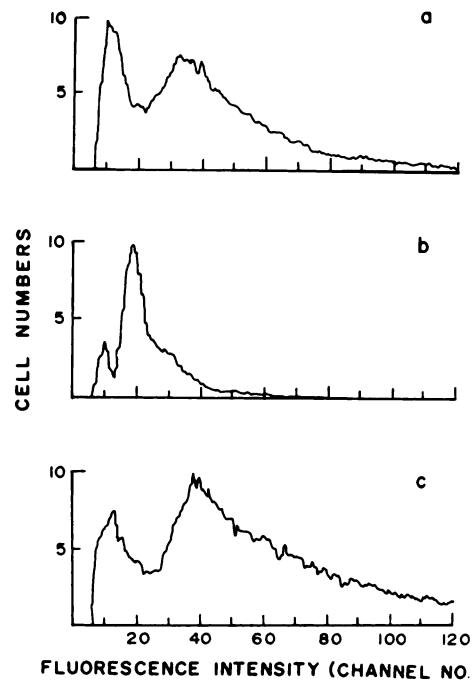


Fig. 4. RNA distribution profiles of separated and unseparated ZAH cells as studied by red fluorescence by acridine orange staining. *a*, unseparated cells (total cells, 32,838); *b*, L-cells (total cells, 14,036); *c*, H-cells (total cells, 41,470). Numbers on ordinate, actual numbers of cells divided by 100.

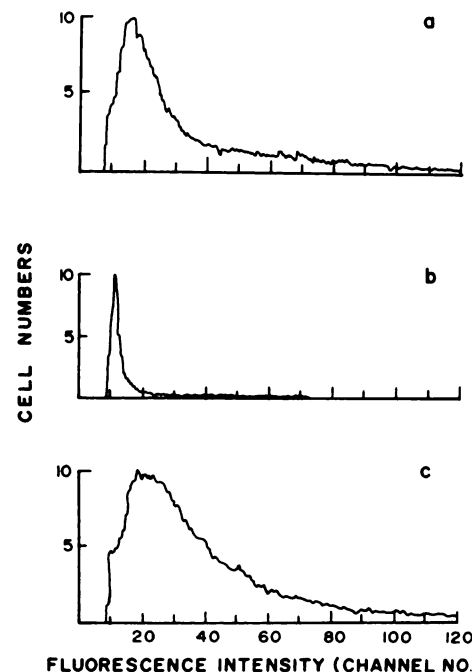


Fig. 5. Fluorescence emission profiles after FDA staining on unseparated and separated ZAH cells. *a*, unseparated cells (total cells, 25,536); *b*, L-cells (total cells, 3,732); *c*, H-cells (total cells, 39,791). Numbers on ordinate, actual numbers of cells divided by 100.

Nonspecific Esterase and Surface Amino Group Distribution. The heterogeneity observed in the DNA and RNA contents of ZAH cells was also reflected in the distributions of the surface amino groups and the nonspecific esterases. When the unseparated cells were stained with fluorescein diacetate, which acts as the substrate for the cellular esterase, we find a unimodal (MC17) distribution of cells (Fig. 5). However, this distribution is skewed towards higher intensities indicating the presence of a significant number of cells with higher enzyme activity. After separation, the cells with higher enzyme activity appear exclu-

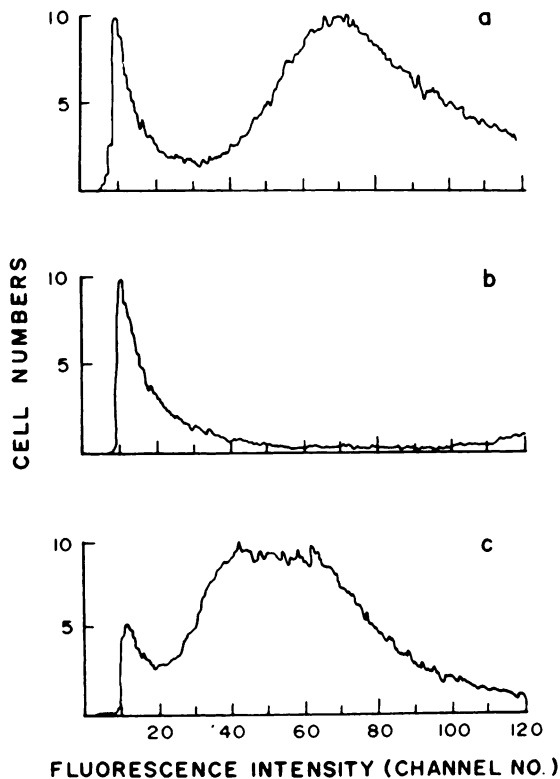


Fig. 6. Surface amino group distribution in ZAH cells after staining with fluorescamine. *a*, unseparated cells (total cells, 72,730); *b*, L-cells (total cells, 17,028); *c*, H-cells (total cells, 66,006). Numbers on ordinate, actual numbers of cells divided by 100.

sively in the H-band while those in the L-band show a low level of esterase activity (Fig. 5, *b* and *c*).

Analysis of fluorescamine stained cells gives the pattern of surface amino group distribution on the cells. In the unseparated population the majority of cells show (Fig. 6) a higher amount of surface NH_2 groups (MC70). There is, in addition, another narrow peak at MC13. The H-band cells show a pattern qualitatively similar to that of the unseparated cells while the L-band cells show only the narrow peak.

Modal Chromosome Numbers. Chromosomal studies by G-banding of ZAH cells have shown that the tumor cells contain a submetacentric marker chromosome.³ A comparison of metaphase plates from L- and H-cells shows that the marker chromosome is present in both the cell populations. The modal chromosome number of the H-cells was 58 within the range of 52 to 63, while that of the L-cells was 52 within the range of 48 to 62. It is, however, important to note that the total number of metaphases in L-cells is low (L-cells being primarily quiescent cells; see below), but the definite presence of the submetacentric marker chromosome in both L- and H-cells would suggest that the two subpopulations are clonally related and are likely to be phenotypic variants.

Colchicine Sensitivity of L- and H-Cells. In order to ascertain the proliferative potentials of L- and H-cells their sensitivity to colchicine was examined and the results are shown in Fig. 7. Fig. 7A shows the typical DNA distribution pattern of the L- and H-cells before colchicine treatment. After colchicine treatment (Fig. 7*b*) the intensities of peaks II and III diminish but peak I remains unchanged indicating that colchicine treatment affects only the H-cells. This conclusion is further supported by the results presented in Fig. 7, *c* and *d*, where recordings of

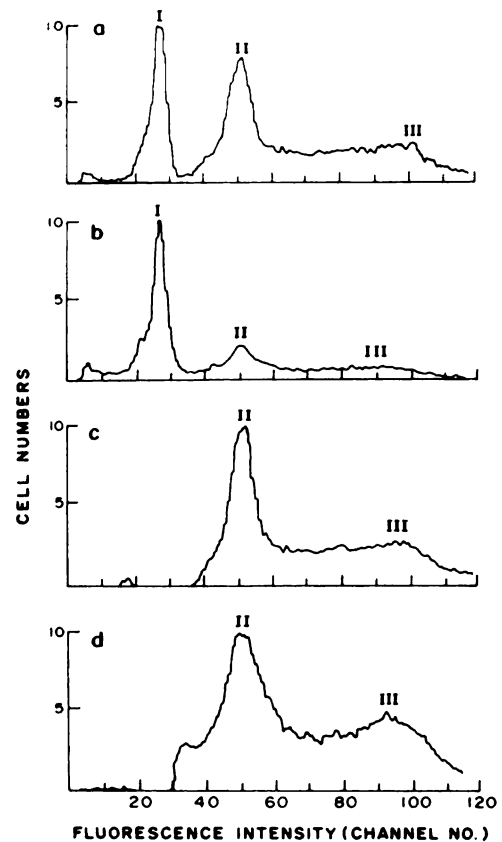


Fig. 7. Flow cytometric profiles of DAPI stained unseparated tumor cells before and after colchicine treatment. *a*, cells before colchicine treatment (total cells scanned, 31,699); *b*, cells after colchicine treatment (total cells, 13,800); *c*, cells before colchicine (after masking peak I; total cells, 30,621); *d*, cells after colchicine treatment (after masking peak I, total cells, 42,104). Numbers on ordinate, actual numbers of cells divided by 100.

the same samples were done after masking peak I in order to clearly bring out the distribution of H-cells after colchicine treatment. A comparison of the distribution of cells shows that colchicine treatment results in the accumulation of 20% more cells in peak III (the $G_2 + M$ fraction of the H-cells), thereby showing that the H-cells represent the majority of the proliferating cells of the original tumor while the L-cells are relatively quiescent.

Interconversion of L- and H-Cells. Tumors (9–15 days old), induced separately by H- and L-cells, when examined by cytofluorimetry after DAPI staining showed distinct characteristics. The H-cell induced tumors exhibit a profile (Fig. 8*c*) similar to that of the original tumor (Fig. 3*a*) with three peaks corresponding to L-cells (peak I, modal channel, MC26) and H-cells (peaks II and III, MC52 and MC98). However, the fraction of L-cells in the original tumor (Fig. 3*a*, peak I) is larger than that present in the H-cell induced tumor (Fig. 8*c*, peak I). The fraction of H-cells, on the other hand, is larger in the H-cell induced tumor (Fig. 8*c*, peak III) compared to that present in the original tumor (Fig. 3*a*, peak III). This indicates (a) a higher percentage of dividing cells in the H-cell induced tumor and (b) a regeneration of L-cells in an H-cell induced tumor. It should be noted that peak I in Fig. 8*c* appears only from samples of tumors that are at least 11 days old, suggesting that regeneration of L-cells occurs only after a certain period. L-cell induced tumors, on the other hand, fall into two distinct groups. Group I exhibited a unimodal distribution of cells (Fig. 8*a*) at modal channel 26 corresponding to the fluorescent peak from L-cells of the original tumor (Fig. 3*a*). Group I animals survived for a period

³ J. K. Roy and M. R. Das, manuscript in preparation.

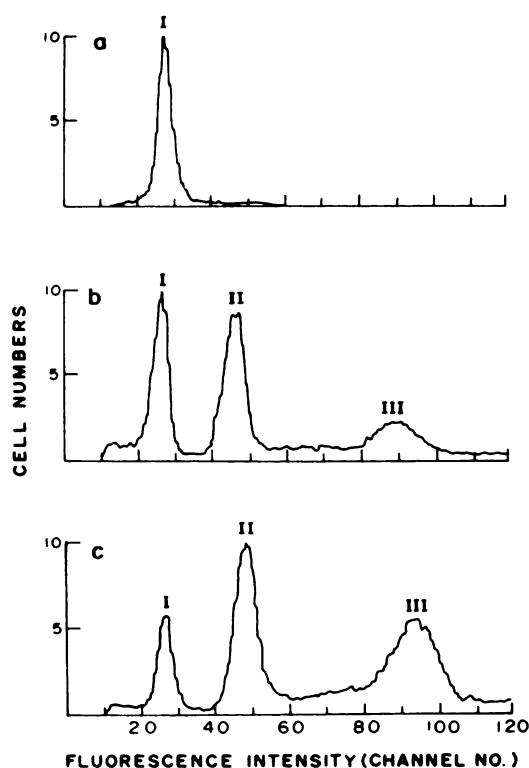


Fig. 8. DNA distribution patterns of L- and H-cell induced tumors. *a*, L-cell induced tumor group I, where animals survived for more than 3 months (total cells scanned, 6,295); *b*, L-cell induced tumor group II, where animals died by 18 days (total cells, 26,822); *c*, H-cell induced tumor (total cells, 35,354). Numbers on ordinate, actual numbers of cells divided by 100.

beyond 3 months after which the tumors regressed. Tumor cells from this group of animals formed a single band on Percoll gradient. The DNA distribution of group II animals is shown in Fig. 8*b* and it shows a clear regeneration of peaks II and III as found in the original tumor (Fig. 3*a*) indicating the regeneration of H-cells in the L-cell induced tumor. Group II animals invariably died within 18 days after injection with the L-cells. Cells from this group formed distinct L- and H-bands on Percoll gradients.

DISCUSSION

The basis of the heterogeneity of cell populations in tumors is poorly understood. An insight into the mechanism(s) by which cells from a single tumor acquire a diversity of properties is crucial in understanding the biology of tumors, their progression, and also clinical management.

With the aim of understanding some of these differences we have isolated two subpopulations from ZAH on the basis of differences in their buoyant densities. The difference in buoyant densities between the L- and H-cells could arise from the increased nucleic acid contents in H-cells compared to L-cells, both types of cells being of comparable sizes. These subpopulations of cells show distinct differences in fluorescent intensities when different markers were used for estimating their DNA and RNA contents, nonspecific esterases and surface amino groups. Planimetric analysis of the DNA distribution profiles in Fig. 3 shows that approximately 54% of the proliferating cells are in the H-band while the L-band contains only about 5% dividing cells. Furthermore, the heterogeneous and high intensity fluorescence of RNA in H-cells, in contrast to the uniform and low intensity fluorescence in L-cells (Fig. 4), would suggest that L-cells are quiescent and more differentiated while the H-cells are poorly differentiated and rapidly proliferating

(27–29). This conclusion is further substantiated by the higher level of esterase activity present in H-band cells (Fig. 5) because a high level of activity of nonspecific esterase has been shown to be associated with proliferating cells (30). Likewise, with fluorescamine the H-cells exhibit a much higher fluorescence compared to L-cells in conformity with earlier observations (31) which demonstrate a higher level of activity for transformed cells. The two groups of cells also differ in their sensitivity to colchicine treatment, the H-cells showing a higher rate of proliferation. While both kinds of cells are tumorigenic, tumors induced by the H-band cells progress rapidly and are invariably lethal, but those induced by the L-band cells progress at a slower rate and a significant fraction ($\approx 36\%$) of such tumors regress in about 3 months.

The presence of the submetacentric marker chromosome in both L and H cells and the general similarities including the modal chromosome numbers between these cells show that both the populations are cytogenetically similar. It is possible that there could still be small (although significant) differences in the genotypes of these two populations which we have not yet been able to detect. Thus at present the two cell types appear to be phenotypic variants similar to earlier *in vitro* observations on the divergence of phenotypes from tumor stem cells (2, 7, 9, 11, 13, 14, 32, 33).

The appearance of L-cells in the H-cell-induced tumors due to the proliferation of contaminating L-cells in the original inoculum of H-cells can be ruled out by estimating the expected percentage of L-cells in an 11-day-old tumor (the earliest when L-cells become detectable in H-cell-induced tumors). Even if one assumes a 10% contamination of L-cells in the H-band inoculum, taking into account the percentage of dividing cells in the L-cell population (Fig. 3), only 1% cells would be expected to be present after 11 days in the H-cell-induced tumor. It may be noted that the assumption of 10% cross-contamination of cells after a three stage separation is purely hypothetical because there is no detectable presence of such contamination as can be seen from flow cytofluorimetric profiles of the separated samples. The experimental value for L-cells (Fig. 8*c*) on the other hand is close to 8%. The observed result can be explained only on the basis of regeneration of L-cells from H-cells.

Although contamination of proliferating H-cells in the L-cell inoculum could result in a sizable increase in the H-cell population, on the basis of quantitative comparison as mentioned above, such contamination is unlikely. Furthermore, as much as 36% of the animals inoculated with L-cells do not develop tumors containing H-cells, although the same preparations were used for inoculating animals developing group I and group II types of tumors induced by L-cells.

In most of the experimental systems thus far examined change in metastatic potential has been used successfully as the major criterion for variability in cell populations. It has also been observed that there is less variability of subpopulations of cells under *in vivo* compared to *in vitro* conditions (3, 8, 34, 35). In addressing the question of variability in terms of its origin and its dynamic nature two important considerations are the frequency with which the events occur and information on kinetic interconversion (1, 3–6, 8, 10, 12, 14). Our results, although not quantitative at present with regard to kinetic data, show several interesting features. H-cells appear to be potential sources of proliferating cells (P) and the L-cells those of quiescent (Q) cells. From our data what is clear is that the rate of interconversion from H to L is much greater than the reverse conversion. Secondly, the conversion from L to H does not take

place in one-third of the animals given injections of separated L-cells and hence the system can be used to look for extramutational or epigenetic factors deciding generation of variability (3, 6, 8, 12). From available information, the proliferating nature of H-cells and the quiescent nature of L-cells would argue that perhaps one of the dependent factors in the generation of variability could be the rate of cell division. It may also be pointed out that our experimental system makes use of "lethality" as a biological criterion for variability and provides an independent system in addition to systems making use of metastatic potential alone as a parameter to study variability.

In conclusion, several points may be made on the studies discussed above. The physically distinct nature (as supported by careful cytofluorimetric analysis) of the two subpopulations of cells from ZAH differing widely in their tumorigenic properties are demonstrated by unambiguous experimental evidence. Our observations suggest that the two subpopulations of cells are phenotypic variants differing in their state of differentiation. The system that we have developed is extremely useful for designing experiments to answer major questions on the factors responsible for tumor regression and the molecular basis of such regression. It provides a useful experimental system to study differential gene expression, antigenic differences, and the mechanism(s) of interconversion of subpopulations from the same tumor differing in their tumorigenic properties.

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