Loss of Neoplastic Properties in Vitro

II. Observations on KB Sublines


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SUMMARY

Ten sublines of the KB cell line obtained from a number of different laboratories were reexamined with respect to chromosome number and heterotransplantability to the Syrian hamster cheek pouch. Twelve clonal cultures derived from 2 of these sublines were similarly examined.

These sublines differed from one another and from the KB cell line originally studied, which was heterotransplantable with inocula of circa 10 cells. Only 2 of the sublines could be heterotransplanted with inocula of less than $10^4$ cells; 6 were heterotransplantable with inocula of $10^4$ to $10^6$ cells; and 2 failed to heterotransplant, even with inocula of $10^6$ cells. Similar differences in heterotransplantability were observed among the clonal derivatives of 2 of these sublines. Although the modal numbers of these sublines and clonal derivatives were basically similar, ranging from 73 to 80, a few lines had a chromosome of sufficiently distinctive morphology to serve as a marker. There was, however, no apparent correlation between these minor karyotypic differences and heterotransplantability. Possible differences between nonheterotransplantable and heterotransplantable sublines with respect to the pattern of variability in cytoplasmic protein and cytoplasmic ribonucleic acid (RNA) content per cell remain to be studied in detail.

The KB cell line, originally isolated in 1955 from a human epidermoid carcinoma (2), has been used extensively for the bioassay of potential anti-tumor agents, particularly in laboratories participating in the screening programs of the Cancer Chemotherapy National Service Center (17). Heterotransplantation studies done in 1955 (13) and subsequently (8–11) with the KB subline then carried at The Children's Cancer Research Foundation (CCRF) showed that implantation of circa 10 cells resulted in progressive, invasive growth in the cheek pouch of the Syrian hamster. In 1961, however, a KB subline then in use 1 of the cooperating bioassay laboratories failed to survive and grow, even when inocula of $10^6$ cells were similarly heterotransplanted to the cheek pouch. The cytochemical properties of this relatively nonmalignant subline were found to resemble those of populations of normal cells and to differ from those of the heterotransplantable KB cell line, as well as other cell lines derived from neoplastic tissue (1). Since parallel studies indicated similar biologic and cytochemical alterations in sublines derived from Sarcoma 180 (6), a number of KB sublines and clones of KB sublines were reexamined with respect to chromosome number, cytochemical characteristics, and their capacity to grow when heterotransplanted to the cheek pouch of the Syrian hamster. The results of these studies are reported here.

MATERIALS AND METHODS

Ten sublines of the original KB culture were obtained from laboratories to which the cell line had been distributed originally, with such information as was available concerning their culture history since receipt in the respective laboratories. These sublines were then cultivated as monolayer cultures in Eagle's minimal medium (3, 4, 18) supplemented with 10% whole calf serum.
TABLE 1
SOURCE OF KB SUBLINES STUDIED IN SYRIAN HAMSTER CHEEK POUCH

<table>
<thead>
<tr>
<th>Code</th>
<th>Source</th>
<th>Date obtained from source</th>
<th>Prior cultural history at source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Original	E</td>
<td>H. Eagle, NIH</td>
<td>1955</td>
<td>BME, 10% human serum, circa 1 yr</td>
</tr>
<tr>
<td>E</td>
<td>H. Eagle, Albert Einstein College of Medicine</td>
<td>1963</td>
<td>MEM, 10% calf serum, 3 yr</td>
</tr>
<tr>
<td>V</td>
<td>M. M. Vincent, Microbiological Associates</td>
<td>1962</td>
<td>Clonal isolate from suspension culture, 2 × BME, 10% horse serum, 6 mo</td>
</tr>
<tr>
<td>I</td>
<td>R. Schlesinger, University of St. Louis</td>
<td>1962</td>
<td>2 × BME, 10% horse serum, 1 yr</td>
</tr>
<tr>
<td>MARS</td>
<td>R. Schlesinger, University of St. Louis</td>
<td>1962</td>
<td>2 × BME, 10% calf serum, 1 yr</td>
</tr>
<tr>
<td>MACA</td>
<td>R. Schlesinger, University of St. Louis</td>
<td>1962</td>
<td>Suspension culture, 2 × BME, 3 mo</td>
</tr>
<tr>
<td>162</td>
<td>R. Schlesinger, University of St. Louis</td>
<td>1962</td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>R. Dulbecco, California Institute of Technology</td>
<td>1962</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>V. H. Bonifas, University of St. Louis</td>
<td>1962</td>
<td>Clonal isolate from suspension culture, 2 × BME, 10% horse serum, 6 mo</td>
</tr>
<tr>
<td>A'</td>
<td>P. S. Thayer, Arthur D. Little, Inc.</td>
<td>1962</td>
<td>BME, 10% calf serum, circa 3 yr</td>
</tr>
<tr>
<td>U</td>
<td>C. G. Smith, The Upjohn Company</td>
<td>1961</td>
<td>BME, 10% calf serum, circa 4 yr</td>
</tr>
</tbody>
</table>

* The Children’s Cancer Research Foundation subculture of this line was first heterotransplanted in 1955 (cf. Ref. 13), and upon subsequent occasions (cf. Refs. 8—10). A new subculture behaved similarly when heterotransplanted in 1960 (cf. Ref. 11), after 5–6 yr in BME, 10% human serum.

BME, basal medium, Eagle (cf. Ref. 3).

MEM, minimum essential medium (cf. Ref. 4).

PPLO, pleuropneumonia-like organisms.

2 ×, twice concentrated.

Subculture of KB-U subline.

Peptone supplements added to medium at various times (cf. Ref. 25).

Quantitated inocula for heterotransplantation were prepared from 5-day cultures in log-phase growth, and each dilution was implanted into both cheek pouches of each of 6 Syrian hamsters, as previously described (11). Three of each group of 6 hamsters were “conditioned” with cortisone acetate, 2–3 mg, administered s.c. at the time of implantation, and twice weekly thereafter (11, 12). The implanted cheek pouches were examined for the development of nodules (11), and histologic studies were done on all cheek pouches in which nodules developed. Each of the 22 sublines were examined in at least 2, and several in as many as 6, separate heterotransplantation experiments.

Chromosome analyses were done on the same in vitro passage used for the preparation of inocula for heterotransplantation, according to a modification of the method described by Todaro et al. (29). Monolayer cultures were incubated 4 hr in a serum-free medium containing 0.01 μg/ml of vincaleukoblastine (Velban) and harvested by trypsinization and centrifugation. The cells were resuspended in 0.7% sodium citrate (pH 7.0) and centrifuged, then washed in Carnoy’s solution (3:1 methanol:acetic acid) for 15–30 min. They were then spread and air-dried on coverslips, stained with 1% acetic orcein, and mounted. The range and modal chromosome number were determined by analysis of at least 100 well-spread metaphase cells.

The species of origin of each of these 22 sublines was determined by immunofluorescence with labeled antibody preparations of known specificity and sensitivity, as described elsewhere (24, 27). Monolayer cultures were trypsinized and washed in buffered saline, and 0.1 ml of cell suspension (3–4 × 10^6 cells/ml) was mixed with 0.1 ml of labeled antibody. The mixture was agitated for 30 min and rewash in buffered saline, and a drop of the final sediment was sealed under a coverslip for examination by fluorescence microscopy. Brilliant surface fluorescence with dilutions of labeled antibody known (by previous titration) to cause specific fluorescence was interpreted as evidence of species specificity.

**RESULTS**

The designations and other available data on the sublines used in these studies are summarized in Table 1. All of these sublines were identified as being of human stock cultures were frozen and preserved in liquid nitrogen as described by Stulberg et al. (26, 28). Twelve clones were derived, as described elsewhere (19), from 2 of these sublines. Subcultures of each of these 22 sublines were sent to CCRF for heterotransplantation studies and chromosomal analyses and to The Child Research Center of Michigan for species identification by immunofluorescence (24, 27).
TABLE 2
DIFFERENCES IN HETEROTRANSPLANTABILITY TO SYRIAN HAMSTER CHEEK POUCH AMONG VARIOUS KB SUBLINES

<table>
<thead>
<tr>
<th>Subline</th>
<th>No. of cells in inoculum$^a$</th>
<th>Chromosome counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10, NC</td>
<td>10, NC</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E (CCRF)$^b$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mars</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V-HP$^c$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IIB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>162</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MACA</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>U</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ C, cortisone acetate-conditioned; NC, normal hamsters; +, growth; 0, no growth (cf. text and Ref. 11).

$^b$ KB-E-Original, 1955 (cf. Table 1, Refs. 8–11, 13). CCRF, Children's Cancer Research Foundation. The modal numbers of 73–80 now found in these KB cultures are to be compared with the modal number of 83 reported for a KB culture by Hsu and Moorhead (15) in 1957, only 2 years after its original isolation.

$^c$ Retitrated following reinoculation in culture after 34 serial passages in hamster cheek pouch. Cf. text.

$^d$ Bimodal, with secondary peak at 76 chromosomes.

$^e$ In circa 50% of implanted cheek pouches.

It is evident that all of these KB sublines differed significantly from the subline studied originally, which had produced progressively invasive tumors even in normal hamsters with the smallest inoculum used (circa 10 cells) (8–11). Indeed, by the arbitrary criteria of "malignancy" described elsewhere (11) (i.e., the capacity of 10$^4$ cells or less to survive and grow progressively in the cheek pouches of normal hamsters), or the capacity of 10$^5$ cells or less to survive and grow progressively in the cheek pouches of cortisone acetate-conditioned hamsters), only 4 of these 10 KB sublines could be classified as "neoplastic": MARS, E, V, and IIB. Larger inocula were required for the progressive growth of the other sublines, and 2 sublines failed to survive and grow$^d$ even after the implantation of the largest inoculum used (10$^4$ cells, Table 2). A similar loss of capacity to survive and grow progressively in the hamster cheek pouch has been observed in cell lines derived from Sarcoma 180, as described elsewhere (6).

Clonal derivatives were isolated from 2 of these sublines, KB-162 and KB-MARS. Those derived from KB-162 differed somewhat from the parent subline with respect to heterotransplantability to the hamster cheek pouch (Table 3). Of those isolated from KB-MARS, 3


differences might have been demonstrable with more finely interpolated inocula (11).

It is of interest that 4 cell lines that produced cheek pouch tumors with inocula of 10$^4$–10$^5$ cells all contained the soluble specific antigen described by McKenna et al. (21) in HeLa cells and some human tumors, whereas this antigen was not demonstrable in 3 cell lines that failed to produce cheek pouch tumors when 10$^4$ cells were implanted in cortisone acetate-conditioned animals (J. M. McKenna and G. E. Foley, to be published).
(MARS-6A, -2A, and -3A) produced nodules only with significantly larger inocula, while 1 (MARS-1OA) produced nodules only in cortisone acetate-conditioned hamsters. Two others (MARS-7A and -8A) failed to survive and grow in hamster cheek pouches, even after the implantation of $10^6$ cells (Table 3). All of the clones studied in this manner were less "malignant" than the parent culture from which they were derived, requiring inocula of 10–1000-fold greater for the production of a cheek pouch tumor. This suggests that the "malignant"
properties of the parent culture depend primarily upon the presence of a highly neoplastic cell, which constitutes a minor fraction of the total population. Other evidences of biologic heterogeneity in populations of neoplastic cells have been described (14, 20, 23). More recently, Klein-smith and Pierce (16), using an in vivo cloning technic, described differences in the capacities for growth, differentiation, and the formation of embryoid bodies among individual cells derived from the same populations of embryonal carcinoma cells.

In addition to the varying capacity of these sublines to survive and grow in the hamster cheek pouch, there were distinct histologic differences in the nodules produced. Figs. 1 and 2 are histologic sections of cheek pouches of cortisone acetate-conditioned hamsters implanted with \(10^6\) cells of KB-MARS-6 and \(10^6\) cells of KB-14, respectively, and are representative of the progressively growing, invasive nodules resulting from the implantation of cell lines of neoplastic origin. In contrast, Figs. 3 and 4 are sections of cheek pouches of cortisone acetate-conditioned hamsters implanted with \(10^4\) cells of KB-MARS-10 and \(10^4\) cells of KB-162, respectively, and are representative of the characteristic rejection of the implanted inoculum by the hamster.

The progressively growing, invasive tumors that developed following implantation of \(10^6\) cells of KB-V (Table 2) in normal hamsters were transplanted serially in hamster cheek pouches as trochar implants (12). After 34 consecutive serial passages at approximately 10-14-day intervals, the cheek pouch tumors were recultured as described elsewhere (7). Varying inocula prepared from 5-day cultures in log-phase growth were reimplanted in normal and cortisone acetate-conditioned hamsters. The results obtained with this cell line (KB-V-HP) were identical with those obtained with the original KB-V subculture (Table 2).

Most of the KB sublines exhibited a characteristic distribution of chromosome numbers; clones could be similarly distinguished from the parent sublines. The modal chromosome number ranged from 73 to 80 (Tables 2, 3) and in most instances represented more than 25% of each cell population. Two of the clones (MARS-5A and -9A, Table 3) were characterized by a bimodal distribution, and one subline (KB-V, Table 2) showed no distinct modal peak.

Although these KB sublines showed no large metacentric chromosomes than diploid human cells, there was a marked increase in the number of large acrocentric, medium metacentric, and smaller chromosomes. The KB-MARS and KB-162 sublines were characterized by a long acrocentric chromosome, which could serve as a “marker.” They differed markedly, however, in their heterotransplantability. Paradoxically, this chromosome was seen but infrequently in clones derived from KB-MARS. A large dicentric chromosome was characteristic of KB-MARS-8A. There was a high frequency of endoreduplication in KB-U, a nonheterotransplantable subline, which was not observed in other nonheterotransplantable populations, namely, KB-A (a subline of KB-U; cf. Table 1) and KB-MARS-7A and -8A (Tables 2, 3).

The instability of these populations is evidenced by a high percentage of chromosome breaks, occasional dicentric chromosomes, abnormal metaphase plates, and some endoreduplication. The actual level of tetraploidy probably is considerably higher than that reflected by the chromosome counts because of the difficulty of obtaining well-spread metaphase plates in cells with high chromosome numbers. These sublines may be in transition, and may well exhibit further changes with continued serial subculture.

**DISCUSSION**

It is evident that the KB sublines studied here differ from one another and from the KB subline originally studied with respect to their capacity to survive and grow progressively when transplanted to the cheek pouches of the Syrian hamster.

The basis for these developing differences is obscure. There was no regular correlation with either the distribution of chromosome units in the population or the presence of readily recognizable chromosomal markers. Further, 34 serial transplantations in the hamster cheek pouch of the tumor produced by implantation of the KB-V subline did not alter its ability to survive and grow when reisolated in vitro and retransplanted in the hamster cheek pouch. This suggests that heterotransplantability as measured by the minimal inoculum required for successful heterotransplantation is an intrinsic property of the subline at a given stage in its cultural history.

On the other hand, experiments with clonal derivatives of the KB-MARS and KB-162 sublines indicate that, at any one time, individual cells in a population may vary markedly with respect to their capacity to survive and grow in the hamster cheek pouch (Table 3). The isolation of clonal derivatives from KB-MARS that could not be heterotransplanted even at inocula of \(10^4\) cells indicates that nonheterotransplantable cell lines can be derived by selection (and/or biologic alteration) from an originally heterotransplantable culture during serial propagation in vitro. The possible analogy to alterations in bacterial “virulence” or “pathogenicity” has been discussed in a previous paper (6). Studies with embryonal carcinoma cells (16, 22, 23) indicate that they may differentiate spon-

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**Figs. 1, 2.—**Cheek pouches of cortisone acetate-conditioned hamsters 44 and 63 days after implantation of \(10^6\) cells of KB-MARS-6, and \(10^6\) cells of KB-14, respectively. H & E, X 255. Representative of progressively growing, invasive nodules resulting from implantation of cells of neoplastic origins.

**Figs. 3, 4.—**Cheek pouches of cortisone acetate-conditioned hamsters 62 days after implantation of \(10^6\) cells of KB-MARS-10, and \(10^6\) cells of KB-162. H & E, X 255 and 170, respectively. Representative inflammatory response to, and ultimate rejection of, implants similar to response to implantation of cells of normal origins.
taneously into normal-appearing adult somatic tissue, perhaps analogous to the changes in KB sublines in the course of their serial propagation.

Cytological studies (1) have indicated that hetero-
transplantable cell lines derived from neoplasms, including the KB cell line originally studied, differ from cell lines derived from normal tissues with respect to the pattern of variability in cytoplasmic protein and cytoplasmic RNA content per cell. As with the S-180 cell, the hetero-
transplantable KB lines similarly differ distinctly from nonheterotransplantable sublines with respect to the degree of variability in the cytoplasmic protein and cyto-
plasmic RNA content per cell (Fig. 2). Although these nonheterotransplantable cultures differ in this respect from those of normal human and mouse cell lines, respectively, they resemble the latter more closely than their heterotransplantable counterparts. The cytochemical heterogeneity characteristic of unselected populations of neoplastic cells (1) is of particular interest in view of the differences in the heterotransplantability of clonal deriva-
tives of KB-MARS. Cytochemical analyses of these clones are now in progress to determine the degree of correlation between their cytochemical characteristics and heterotransplantability.

Loss of neoplastic properties in the course of serial sub-
culture has now been described with several different cell lines of neoplastic origin (6). It is apparent that this change does not necessarily occur simultaneously in dif-
ter sublines of the same cell culture; and it is altogether probable that there are other associated changes consequent to serial propagation that have not yet been demonstrated. Antigenic variation is one possible difference that merits exploration and that may in fact be related to the developing differences in heterotransplantability re-
ported here (cf. Ref. 11). Since the KB cell line, as well as other established cell lines, is used widely in a number of different experimental situations, perhaps the relevant "known" biologic properties of such cell lines should be reexamined frequently.

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Cancer Res 1965;25:1254-1261.

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