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Spontaneous Fusion between Ehrlich Ascites Tumor Cells and Host Cells *in vivo*: Kinetics of Hybridization, and Concurrent Changes in the Histocompatibility Profile of the Tumor after Propagation in Different Host Strains*

P. K. LALA, V. SANTER and K. S. RAHIL

Department of Anatomy, McGill University, 3640 University Street, Montreal, Canada

Abstract—*Evidence of spontaneous fusion between Ehrlich ascites tumor (EAT) cells and host cells* in vivo *was derived from the appearance of host-specific* T_6 *chromosome markers in EAT cells after propagation in* CBA/HT_6 *mice. After* 1 year *of weekly passages, these hybrids lost some unidentified chromosomes, so that their modal chromosome number reverted to the original stem line mode of* 68. *Simultaneously, their incidence rose to a peak of* 18% *with no further change over a* 4 year *period. This increase was not explained by immunoselection pressure. Presumptive host cell nuclei in polynucleate cells in the EAT were of lymphocyte, monocyte and macrophage type. Since host–host cell heterokaryons were also noted, the observed tumor–host cell fusion may be a non-selective process. Nevertheless, this may be of biological importance, since concurrent changes in the histocompatibility of the EAT were noted after propagation in different host strains. While the original EAT line (EAT-0) maintained in* CF_1 *mice was highly tumorigenic by the subcutaneous (s.c.) route in* CF_1 *but not in* CBA/HT_6 *mice, i.p. propagation of the tumor in the latter strain (as two lines called EAT-2 and EAT-3) caused a progressive loss in s.c. tumorigenicity for* CF_1 *mice but a concurrent gain in tumorigenicity for* CBA/HT_6 *mice. When EAT-3 was propagated into* $(CBA/HT_6 \times C57Bl/6)F_1$ *hybrids, this tumor (now called EAT-4) retained s.c. tumorigenicity for the* F_1, *but gradually lost its tumorigenicity for* CBA/HT_6 *mice and remained nontumorigenic for the C57Bl/6 strain. Radioautographic examination of surface H-2 antigens using a sandwich labeling with monospecific or polyvalent antisera revealed the presence of k and b antigens on EAT-0 cells as well as somatic cells of* CF_1 *mice in which EAT-0 was maintained. EAT-3 long maintained in* CBA/HT_6 $(H\text{-}2^k)$ *mice showed the presence of k but not b antigens. After a propagation in* F_1 $(H\text{-}2^{kb})$ *mice, EAT-4 showed a moderate decline in the level of k but a small regain of b antigens, more evident after neuraminidase treatment. Subcutaneous tumors labeled more heavily for the host haplotype than the corresponding ascites tumors. The direction and time course of histocompatibility changes in the EAT were best explained by tumor–host cell fusion and chromosome loss.*

INTRODUCTION

EHRLICH ascites tumor (EAT) of the mouse has been subjected to numerous studies in our laboratory, primarily aimed at understanding the control mechanisms in tumor growth [1–9] as well as characterizing the host cell subsets invading the tumor [9–11]. While analysing the frequency distribution of chromosome numbers of tumor cells in the EAT line maintained *in vivo* in our laboratory, we incidently observed that the karyotopic constitution of a small proportion of cells in that distribution could possibly be explained by

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hybridization between the tumor cells and host cells. This suspicion was confirmed by the use of chromosome markers and supported by cytological studies. This phenomenon of spontaneous tumor–host cell fusion *in vivo* was mentioned by us in passing in an earlier paper [6] and some preliminary findings were reported in abstract form [12].

Several other laboratories have reported the evidence for some spontaneous tumor–host cell fusion *in vivo* in a number of tumor–host systems [13–22]. However, the long-term fate of these hybrids *in vivo* or their possible influence on the biological properties of the tumor have not been adequately examined. The present report is the outcome of a series of long-term studies on the Ehrlich ascites tumor aimed at understanding (1) the kinetics of tumor–host cell fusion *in vivo* and the karyotypic evolution of the fusion products, (2) the fate of the fusion products *in vivo*, (3) the nature of the participant host cell types involved in this fusion process, and finally (4) the possible biological consequences of this phenomenon *in vivo*. These objectives were achieved by (1) an identification of the hybrids in metaphase with the aid of marker chromosomes specific for the tumor or the host, (2) a direct morphological examination of polynucleate cells within the tumor, and (3) by an evaluation of possible changes in the histocompatibility profile of tumor cells after intraperitoneal propagation in different host strains. Histocompatibility profile was examined in two ways: (a) tumorigenic ability of EAT cells when transplanted subcutaneously in different host strains and (b) a simultaneous assessment of the presence and density of a number of known H-2 antigens on the surface of EAT cells, using a sandwich radio-labeling technique followed by quantitative radioautography. Our results from these studies suggest that tumor–host cell fusion *in vivo* may have important implications for the tumor–host relationship.

MATERIALS AND METHODS

Animals

Female mice of the outbred CF_1 strain (Canadian Breeding Farms, St. Eustache, PQ) or the following inbred strains (Jackson Laboratories, Bar Harbor, ME) at 12–16 weeks of age were used for tumor transplantation: CBA/H and CBA/HT_6 ($H\text{-}2^k$ genotype), C57Bl/6 ($H\text{-}2^b$) and $(CBA/HT_6 \times C57Bl/6)F_1$ ($H\text{-}2^{kb}$). Although the CF_1 strain mice were derived from a closely bred stock, their H-2 genotype is unknown. CBA/HT_6 mice carry two T_6 marker chromosomes in somatic cells as part of their karyotype.

Tumor

Ehrlich ascites tumor (EAT) used in the present study was initially obtained from Dr. T. Hauschka (Roswell Park Memorial Institute) by the Cancer Research Unit of the University of California, San Francisco around 1962, but no karyotype record is available. It was initially grown by us in random bred Swiss Webster mice and from 1967 propagated in random bred CF_1 strain Swiss mice, resulting in no changes in cell cycle parameters [5] or apparent distribution of chromosome numbers. The modal number has remained at 68, including 1–5 (2.4 on the average) large metacentric markers and often (less constant) one minute marker. The postmitotic cell has a near tetraploid amount of DNA [2]. The tumor line long maintained in CF_1 mice by a weekly i.p. transfer of 10^6 cells is called EAT-0. New lines were established by interstrain transfer of 10^6 tumor cells by the i.p. route and maintained further by a weekly passage of the same number of cells within the new mouse strain. The history and nomenclature of the various EAT lines generated in this manner are presented in Table 1.

Chromosome preparation

Colcemid (courtesy of CIBA, Montreal) was injected i.p. directly into the ascites tumor at a dose of 0.05 μg/g body weight 3 hr prior to harvesting tumors for metaphase spreads, using a modification of the air-drying method of Rothfels and Siminovitch [24]. Slides were stained with acetic orcein and mounted for microscopy. In well spread metaphases, the total number of chromosomes was scored and the marker chromosomes were identified directly under the microscope with the aid of an image-tracing device (Wild Zeichenapparat) at a table level magnification of 1200. Randomly selected, well spread metaphase plates were photographed for a detailed analysis of karyotypes. The distribution of chromosome numbers in the various EAT lines or the incidence of tumor cell metaphases showing T_6 markers (single or double) at various generations of passage in the CBA/HT_6 strain was based on an examination of 50 metaphase plates per point. Chromosomes indicative of T_6 morphology were never seen in the EAT maintained in the CF_1 or CBA/H strain. In double blind

Table 1. *Ehrlich ascites tumor lines maintained intraperitoneally in different mouse strains*

EAT line	Origin	Strain of maintenance
EAT-0	EAT in Swiss Webster mice	CF_1, since 1968
EAT-1	EAT-0	CBA/H, since 1971
EAT-2	EAT-0	CBA/HT_6, since 1971
EAT-3	EAT-2, cycled once as subcutaneous tumor in CBA/HT_6 mice at 78th passage	CBA/HT_6, cycled in 1973
EAT-4	EAT-3	$(CBA/HT_6 \times C57Bl/6)F_1$, since 1975

studies, a false positive was scored only on a single occasion. Great care was taken to avoid confusion with the autosomes numbered 19, which may show some subterminal constriction but are larger than the T_6 chromosomes, or minute markers in this EAT line, which are somewhat smaller and do not show subterminal constriction.

Cytology

Smears of EAT-0 as well as EAT-2 at different ages were stained with MacNeal's tetrachrome for cytological examination of interphase cells with single or multiple nuclei, as well as mitotic figures. Uninucleate tumor cells were easily distinguishable from most host cells (e.g., lymphocytes, polymorphs, monocytes and macrophages) on the basis of morphological criteria as reported earlier [10]. Tumor cell nuclei were large, had several nucleoli and a distinctive chromatin pattern. Nuclear identity in polynucleate cells was established according to these criteria of distinctive nuclear morphology only when such identification was unequivocal. In uncertain cases, polynucleate cells were scored as 'unidentified'.

Histocompatibility bioassay by subcutaneous transplantation

In the course of our studies with EAT we found that this tumor is universally accepted by the intraperitoneal route in all mouse strains tested, but in contrast, fatal takes of EAT transplanted by the subcutaneous (s.c.) route were far from universal [9]. Amongst a number of explanations, histocompatibility differences between the host and the tumor remained a strong possibility. Thus, it was felt that transplantation by the s.c. route might provide a classical bioassay for possible histocompatibility changes in the EAT after propagation in a new mouse strain. To test this, the following tumor–host combinations were employed: EAT-0 cells, long maintained in the ascites form in CF_1 mice, were tested subcutaneously in the CF_1 as well as CBA/HT_6 mice. EAT-2 cells maintained for various passage generations in the ascites form in CBA/HT_6 mice were tested in CF_1 as well as CBA/HT_6 mice. Since CF_1 is an outbred strain with unknown histocompatibility antigens, EAT long maintained in CBA/HT_6 strain (EAT-3) was transferred into F_1 mice produced by mating CBA/HT_6 females with C57Bl/6 males, and then maintained i.p. in the F_1 strain to generate the EAT-4 line (Table 1). EAT-4 cells at various passage generations in the F_1 strain were then examined for fatal s.c. tumor takes in the F_1 as well as in both parental strains, viz. CBA/HT_6 and C57Bl/6 mice.

Subcutaneous transplantation of EAT cells

10^7 tumor cells (0.1 ml vol), obtained as a routine from 7 day old ascites tumors, were inoculated on to the anteromedial aspect of the left thigh. This dose was selected on the basis of our earlier experience on tumor growth kinetics [6, 9]. Tumor growth, rejection or regression were recorded by early palpation of the injection site and a measurement of the external tumor diameter (average of the longest and shortest diameters) with a pair of calipers when tumors were palpable. These records were initially maintained daily, and then at intervals of twice a week until death. Tumor-free animals at the end of 160 days were tested for possible immunity against an intraperitoneal challenge of 10^6 EAT cells taken from the various EAT lines.

Purification of tumor cells

In some experiments, tumor cells freed from

contaminant host leukocytes by a sucrose density gradient centrifugation technique reported by us earlier [23] were used for subcutaneous transplantation. The resultant purity of tumor cells was more than 99%; the minor contamination was due to large macrophages, accounting for less than 1% of the cell population.

Examination of the presence and the density of H-2 antigens

Intraperitoneal propagation of EAT cells into a different host strain was found to result in progressive changes in the tumorigenicity of EAT cells by the s.c. route: a gradual loss of tumorigenicity for the original strain, and a gradual gain in tumorigenicity for the new host strain. To test whether these resulted from alterations in the major histocompatibility (H-2) antigens on the EAT cell surface, H-2 antigens were examined in the following EAT lines (Table 1): EAT-0 at 500–600 passages in CF_1 mice, EAT-3 at 158–203 passages in CBA/HT_6 mice and EAT-4 at 20–50 passages in $(CBA/HT_6 \times C57Bl/6)F_1$ mice. All ascites tumors were examined at 7 days of age. In some experiments, s.c. tumors at 14–21 days of growth after inoculation of 10^7 ascites tumor cells in the same mouse strain were examined simultaneously. Normal thymocytes were used as the control tissue. A sandwich immunolabeling technique was applied to single cell suspensions followed by radioautography, as described below.

(a) *Preparation of cell suspensions.* EAT cells in 1–2 ml ascites fluid, collected with 100 U of heparin were washed in excess of Hank's balanced salt solution (HBSS; GIBCO, New York) and incubated for 2 hr at 37°C with 2 changes in 14 ml HBSS to allow elution of loosely bound cell surface Ig. This step of removing naturally bound Ig was essential for the immunolabeling technique described later. In the case of subcutaneous tumors, the viable cortical part was dispersed mechanically and viable single cell suspension freed from clumps and debris was prepared as reported earlier [25] prior to incubation in HBSS. Thymocytes were prepared in the same manner except for the incubation step. For surface labeling, all cells were finally resuspended in ice-cold Eagle's minimal essential medium (GIBCO) containing 10% FCS (henceforth to be called FEM) at a concentration of 20×10^6 cells/ml.

(b) *Treatment of cells with neuraminidase or sodium periodate.* In some surface labeling experiments tumor cells were pretreated with these agents in an attempt to modify the cell surface coat, and thus make the cell membrane antigens more accessible. Neuraminidase from *Vibrio cholerae* (Calbiochem, La Jolla, CA) at 50U/ml and $NaIO_4$ at 0.002 M were prepared in HBSS. Cells and reagents were equilibrated at 37°C for 10 min, then 1 ml containing 20×10^6 cells was mixed with 1 ml of reagent and incubated for 60 min at 37°C. Control cell suspensions were incubated with 1 ml HBSS. Preliminary experiments had established that there was no loss of cell viability under these conditions. After incubation, each suspension was diluted to 12.5 ml with ice-cold FEM. The cells were washed twice with FEM, and resuspended in 1 ml FEM.

(c) *Antisera.* Polyvalent anti-$H\text{-}2^k$ (C57Bl/6 anti-CBA/HT_6) and anti-$H\text{-}2^b$ (CBA/HT_6 anti-C57Bl/6) sera used in some experiments were raised in this laboratory. The minimal titre for 90% cytotoxicity on thymocytes was 1:128 for both sera. Monospecific antisera against the K or D end of the H-2 complex, used in most of the experiments, were obtained through the courtesy of Dr. J. G. Ray, National Institute of Allergy and Infectious Diseases, Bethesda, MD. They are listed in Table 2. An IgG-rich fraction of goat anti-mouse IgG was obtained from Meloy Laboratories, Springfield, VA. Normal mouse serum (NMS) was obtained by cardiac puncture of normal C57Bl/6 mice aged at least 12 weeks. The serum was harvested, pooled, and stored at −20°C. All sera were inactivated at 56°C for 30 min before use.

(d) *Sandwich labeling procedure.* Aliquots of 0.1 ml, containing 2×10^6 cells in FEM, were incubated for 30 min at 0°C with 0.1 ml of a 1/20 dilution of either normal mouse serum or anti-H-2 serum. The cells were washed twice through discontinuous gradients of FCS in MEM (50%, 75%, 100% FCS) to remove unbound protein, then incubated for 30min at 0°C with 0.1 ml of ^{125}I-anti-IgG at a final concentration of 10 μg/ml. The cells were washed through a further 2 FCS gradients, and smeared on gelatin-subbed slides. The goat anti-mouse IgG was radio-iodinated by a modification of the chloramine-T method [26], resulting in a specific activity of 5–6 μCi/μg protein.

(e) *Radioautography.* This was done as reported earlier [11] using a 4 day exposure time. Radioautographic grain counts were de-

Table 2. Monospecific antisera directed against K or D end specificities of the H-2 complex (from Catalogue of Mouse Alloantisera, NIAID)

Catalogue number	Specificity of antiserum	Lymphocytotoxic titre (^{51}Cr release)
D3b	K^k	>1:2500
D5bAF	D^k	>1:4000
D33	K^b	>1:1400
D2	D^b	1:200

termined for 300–500 cells per smear under oil immersion. Based on background grain count distribution on cell-free areas, small lymphocytes with $\geqslant 6$ grains and tumor cells with $\geqslant 11$ grains were scored as labeled.

(f) *Statistical analysis.* One-way analysis of variance and '*t*'-test analysis were carried out on a Model 9830A calculator (Hewlett-Packard Canada Ltd., Pointe Claire, PQ) using the 'Stat-Pac' programme. A change was considered significant if $P<0.05$.

RESULTS

Evidence of tumor–host fusion in vivo: *karyotypic evolution and the fate of the fusion products*

The frequency of chromosome numbers in EAT-0 at metaphase showed a small cluster distinct from the distribution around the mode (68) or the numbers indicative of polyploid cells (Fig. 1A).The chromosome numbers in this cluster were close to 100. It was suspected that these might represent hybrids between the stem line (with 68 mode) and diploid host cells (with 40 chromosomes) present within the tumor. To test this, we transferred 10^6 EAT-0 cells i.p. from the CF_1 to the CBA/HT_6 strain. This resulted in fatal tumors in all cases. During the first passage generation of EAT-2 the distribution of chromosome numbers did not show any appreciable difference from that of EAT-0 (Figs. 1B and C), but occasional metaphases in the 95–110 chromosome group showed one or two host-specific T_6 markers in addition to the tumor-specific metacentric markers (Table 3). At later passages the distribution remained closely similar (Figs. 1D and E), but an increasing proportion of metaphases in the group containing 95–110 chromosomes became T_6+ve (Table 3). T_6-bearing metaphases were not seen in the group having 60–80 chromosomes (modal number 68, possibly representing 'stem line' cells) during

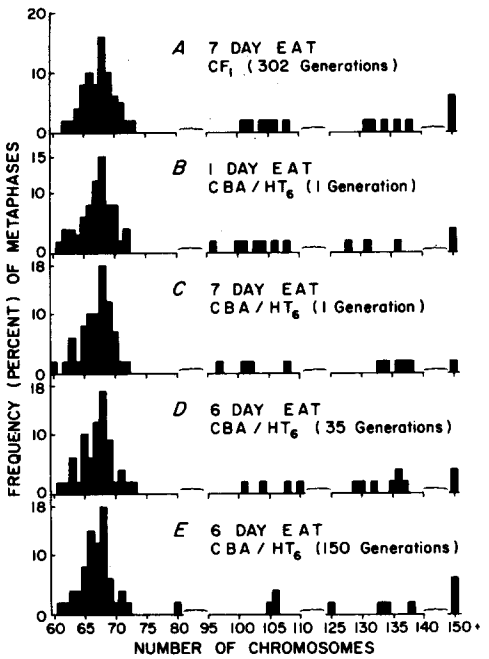

Fig. 1. Distribution of chromosome numbers in the EAT lines: (A) *EAT-0 long maintained in CF_1 strain,* (B) *and* (C) *At first passage of EAT-2,* (D) *and* (E) *At 35th and 150th passages of EAT-2.*

early passages, but following their appearance (at 16th passage), their incidence within this group increased slowly and reached a maximum (~18%) at the 65th passage, i.e., after just over a year of weekly propagation in the CBA/HT_6 strain. There was no further rise even after 4 years of passage (Table 3).

The early appearance of T_6 markers in the EAT-2 metaphases with 95–110 chromosomes confirms that these represent early products of host–tumor cell fusion retaining nearly complete sets of parental chromosomes. All metaphases in this group became T_6+ve by the 16th passage in CBA/HT_6 hosts. If this group in the EAT-0 also represented host–tumor cell hybrids, then such hybrids when transferred into CBA/HT_6 mice were slowly replaced by new products of host–tumor cell fusion during this period. Thus, hybrids with complete par-

Table 3. Frequency of T_6 positive karyotypes amongst metaphases grouped by chromosome numbers in the EAT-2 after various numbers of passages in CBA/HT_6 mice

EAT-Line	No. of passages	Tumor age (days)	60–80 group	95–110 group	125–140 group	150+ group	Total
EAT-0	302	7	0/37	0/6	0/5	0/2	0/50
EAT-2	1	1	0/38	1/7	0/3	0/2	1/50
	1	7	0/40	1/4	0/5	0/1	1/50
	3	6	0/39	2/5	0/4	0/2	2/50
	16	6	1/41	3/3	0/4	0/2	4/50
	21	6	2/38	3/3	1/6	0/3	6/50
	35	5	5/40	2/3	1/6	0/1	8/50
	35	6	4/37	4/4	1/7	0/2	9/50
	58	9	5/37	4/4	1/6	0/3	10/50
	65	6	7/38	3/3	2/7	0/2	12/50
	86	7	6/31	2/2	1/4	1/3	10/50
	137	6	5/39	2/2	1/6	1/3	9/50
	150	6	5/40	3/3	1/4	1/3	10/50
	216	7	5/40	2/2	1/6	1/2	9/50

ental sets of chromosomes may not survive indefinitely.

A later appearance of T_6+ve metaphases, and a subsequent increase in their incidence within the 'stem line' EAT-2 cells can only be explained by the loss of some chromosomes from those containing complete parental sets. The kinetics of karyotypic conversion of the latter into the former set of cells can be followed more closely in Fig. 2. It took 58 passages (i.e., just over a year) for the hybrids to attain a stable 'stem line' which may have offered some survival advantage.

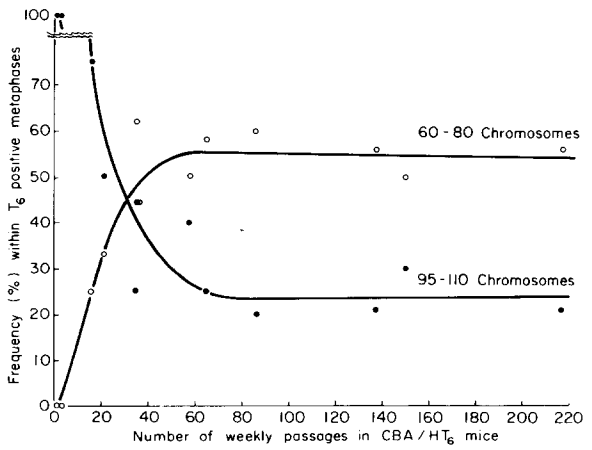

Fig. 2. Frequency of two groups of metaphases (with 60–80 and 95–110 chromosomes) amongst T_6+ve cells at various passage generations of EAT-2 in CBA/HT_6 mice.

The incidence of tumor-specific metacentric marker chromosomes remained essentially similar for all the EAT lines (Table 4) and also for T_6+ve cells in EAT-2, indicating that they may be essential for the tumor life cycle. Their numbers ranged from 1 to 5, with a median of 2 in cells excluding polyploids.

Figure 3 shows the overall incidence of T_6+ve (1 or 2 markers) tumor cell metaphases at various passage generations of EAT-2 in the CBA/HT_6 strain. This incidence increased slowly but significantly to a maximum of about 18% at 50–57 passages, followed by a plateau. A minor decline apparent thereafter was not statistically significant. This was within the limits of the inter-tumor variation, at the same or different tumor ages within a single passage. However, since the possibility of elimination of the T_6 marker from some hybrids cannot be excluded, the observed frequency of T_6+ve tumor cells may reflect the minimal incidence of hybrids.

Attempts to establish the specific identity of chromosomes lost from the hybrids in the EAT-2 prior to settling back to the stem line karyotype were unsuccessful. However, all or most missing chromosomes in the latter karyotypes (when compared with the karyotypes with 95–110 chromosomes) were indistinguishable from normal mouse chromosomes. Since strain differences for chromosomes other than the T_6 were not identifiable, their origin (i.e., tumor or host) remains unknown.

The slow rise of karyotypically identifiable hybrids in the EAT-2 with prolonged passage could be explained either by a pressure of immuno-selection or some other reason. To test the former possibility, EAT-1 long maintained in CBA/H mice (55 passages after initial transfer from CF_1 mice) was transferred into syngeneic histocompatible CBA/HT_6

Table 4. Frequency (per cent) of tumor cell metaphases (excluding polyploids) showing different numbers of metacentric markers

EAT line	Number of passages	Number of metacentric markers per metaphase 1	2	3	4	5
EAT-0	302	6	66	12	12	4
EAT-1	31	5	68	11	14	2
EAT-2	150	8	70	10	12	0
EAT-2, pooled T_6+ve cells	1–216	4	72	10	14	0

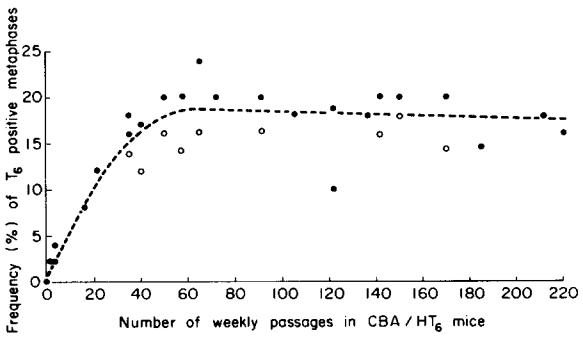

Fig. 3. Overall percentage of T_6+ve tumor cell metaphases at various passage generations of EAT-2 in the CBA/HT_6 strain. Multiple points with same symbols indicate different animals; different symbols indicate different tumor age within the same passage generation.

mice with the assumption that this would not impose an immunoselection pressure. Despite this, a similar increase in the incidence of T_6 +ve metaphases (to 12–18%) was observed after about 50–60 passages in this strain (Table 5), indicating that this increase was

Table 5. Incidence of T_6+ve metaphases in the EAT when transferred from CBA/H to CBA/HT_6 strain mouse

Number of passages	%T_6+ve tumor cell metaphases
0	0, 0
12	4, 6
50	12, 16
52	18, 14, 14
60	14, 16, 18

unrelated to an immuno-selection pressure *in the ascites form* of the tumor.

Figures 4 and 5 illustrate some metaphase spreads including CBA/HT_6 marrow karyotype and T_6+ve tumor–host cell hybrids from various passages of EAT-2.

Smears of EAT-0 and EAT-2 were examined for morphological evidence of cell fusion and chromosome loss. An evaluation of the incidence of polynucleate cells (bi- or multinucleate) and the nuclear identity in such cells was attempted by scoring at least 5000 cells per animal (Table 6). The incidence increased with tumor age in both strains. A majority of polynucleate cells in most tumors showed only tumor type nuclei (Fig. 6a). Only a minor fraction of the binucleate or multinucleate cells had nuclei with distinctive morphology of both tumor and host counterparts. The latter included lymphocyte-type (Figs. 6b and c), as well as monocyte- or macrophage-type nuclei (Fig. 6d); granulocyte-type nuclei were never seen. A significant proportion showed only host type nuclei (Figs. 6e–g). Macrophage–macrophage homokaryons were more frequent in older tumors, in which macrophages were more abundant and showed some tendency to form clusters (Fig. 7e). When multiple nuclei were present in a single cell (Fig. 7a), precise nuclear identity in most cases could not be established. It is conceivable that bi- or multinucleate cells could develop in two ways—fusion or endomitosis. Cells possessing tumor as well as host nuclei could only result from fusion. Cells containing more than one host nucleus could also result from fusion alone, since ^{3}H-thymidine labeling of EAT showed no local proliferation of lymphocytes, monocytes or polymorphs and only very insignificant proliferation of macrophages [10]. Cells containing more than one tumor nucleus might, however, arise from fusion or endomitosis. When mitotic cells were examined, co-existence of a mitotic with a typical interphase nucleus was never observed. However, occasional cells, encountered very infrequently, showed a mitotic nucleus coexisting with a pulverized-appearing nucleus, indicating the fusion between a mitotic cell and an interphase cell in DNA synthesis

Table 6. Incidence (mean of 3 animals per interval) of various types of polynucleate cells for every 1000 uninucleate cells in ascites tumors

EAT-Line	Tumor age (days)	Uninucleate cells		Total	Binucleate cells				Multinucleate cells
		Tumor	Host		Tumor–Tumor	Tumor–host	Host–host	Unidentified	
EAT-0, 321 passages	1	142	858	3.8	2.0	0.6	0.2	0	0.8
	4	906	94	8	5.4	1.2	0.4	1	1.8
	9	913	87	10.6	7.2	1.2	0.8	1.4	3.4
	13	890	110	16	11.4	1.6	1.2	1.8	5.6
EAT-2, 98 passages	2	649	351	4.6	3.0	0.8	0.4	0.4	1.2
	8	919	81	11.6	8.4	1.4	0.6	1.6	3.2

(S phase). The pulverized appearance represents premature chromatin condensation in an S-phase nucleus induced by the mitotic nucleus within the fusion product [27]. Occasional binucleate cells with nuclei in different mitotic stages indicative of asynchronous mitosis were also seen (e.g., Fig. 6i). Similarly, asynchronous DNA synthesis, indicated by the presence of labeled and unlabeled nuclei within a single cell 1 hr following i.p. ^{3}H-thymidine injection was also occasionally encountered (Fig. 6b). Furthermore, morphological suggestion of chromosome loss was provided by the presence of occasional anaphase figures that showed one or more small chromosome clusters excluded from the main spindle (Fig. 6h). These possibly appeared as micronuclei observed in some telophases and interphase cells (Figs. 7b–d).

Changes in subcutaneous tumorigenicity of EAT cells after intraperitoneal propagation in a different host strain

Subcutaneous tumorigenicity of EAT-0 cells. The growth characteristics of s.c. transplants of 10^7 EAT-0 cells long maintained in CF_1 mice into identical strain hosts could be distinctly classified into 3 categories (Fig. 8 and Table 7):

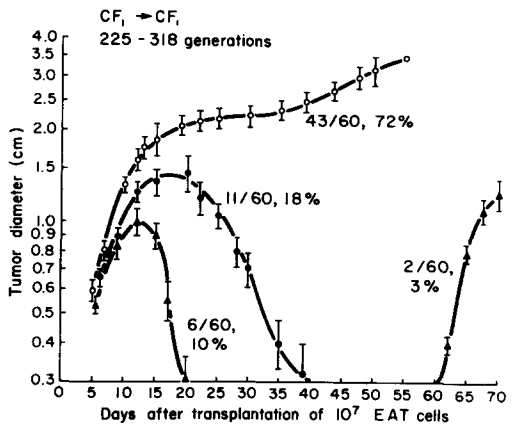

Fig. 8. Growth characteristics of subcutaneous transplants of 10^7 EAT-0 cells (at 225–318 passages) in CF_1 mice. Vertical bars are the standard errors of the mean in each group. Transplants were assigned to distinct groups according to the growth criteria described in the text. For recurring tumors, the onset of the growth curve (extreme right) indicates the median recurrence time.

(a) fatal continued growth (72%); tumors attained large sizes (maximal dia. of 2–4 cm), killing the hosts between the fifth and eighth week, (b) delayed regression (18%) following growth to an appreciable size (1–2 cm peak dia.) for an average of 3 weeks. The regression phase lasted for an average of another 3 weeks. A small proportion (3% of

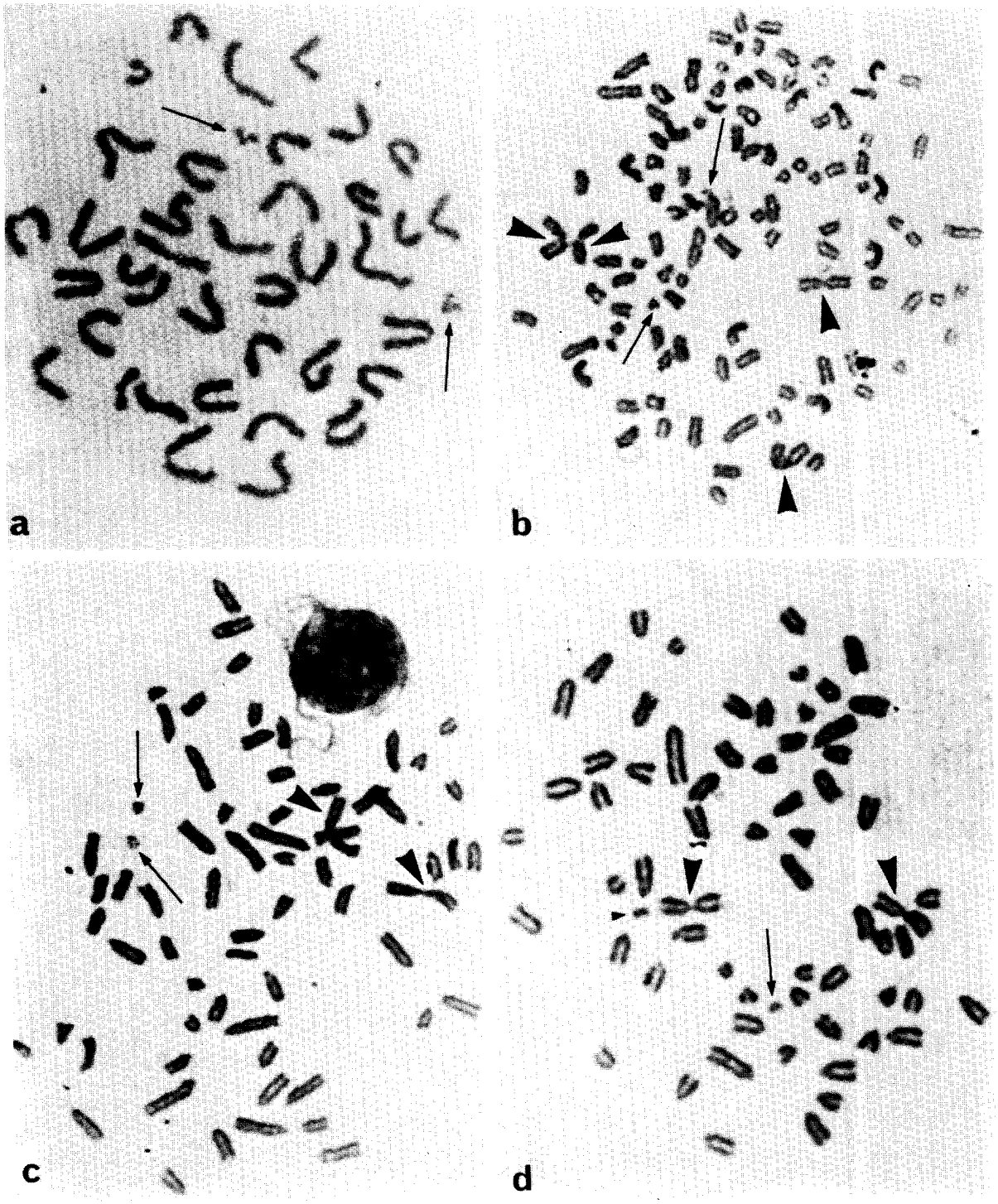

Fig. 4. *Metaphase spreads illustrating normal CBA/HT*$_6$ *host cell and tumor host cell hybrids in EAT-2: tumor-specific large metacentric markers are indicated by thick arrowheads, and minute markers by thin arrowheads, and host-specific* T_6 *markers by thin arrows.* a—*bone marrow cell from the CBA/HT*$_6$ *host showing* 40 *chromosomes including two* T_6 *markers.* b—*EAT cell from the* 3*rd passage,* 6-day *old tumor with* 108 *chromosomes including* 4 *large metacentric and* 2 T_6 *markers. This is a hybrid which possibly contains the complete sets of parental chromosomes.* c—*EAT cell from the* 16*th passage,* 6-day *old tumor with* 67 *chromosomes including* 2 *metacentric and* 2 T_6 *markers. This hybrid must have lost some chromosomes.* d—*EAT cell from the* 21*st passage,* 6-day *old tumor with* 66 *chromosomes including* 2 *metacentric,* 1 *minute and* 1 T_6 *marker.*

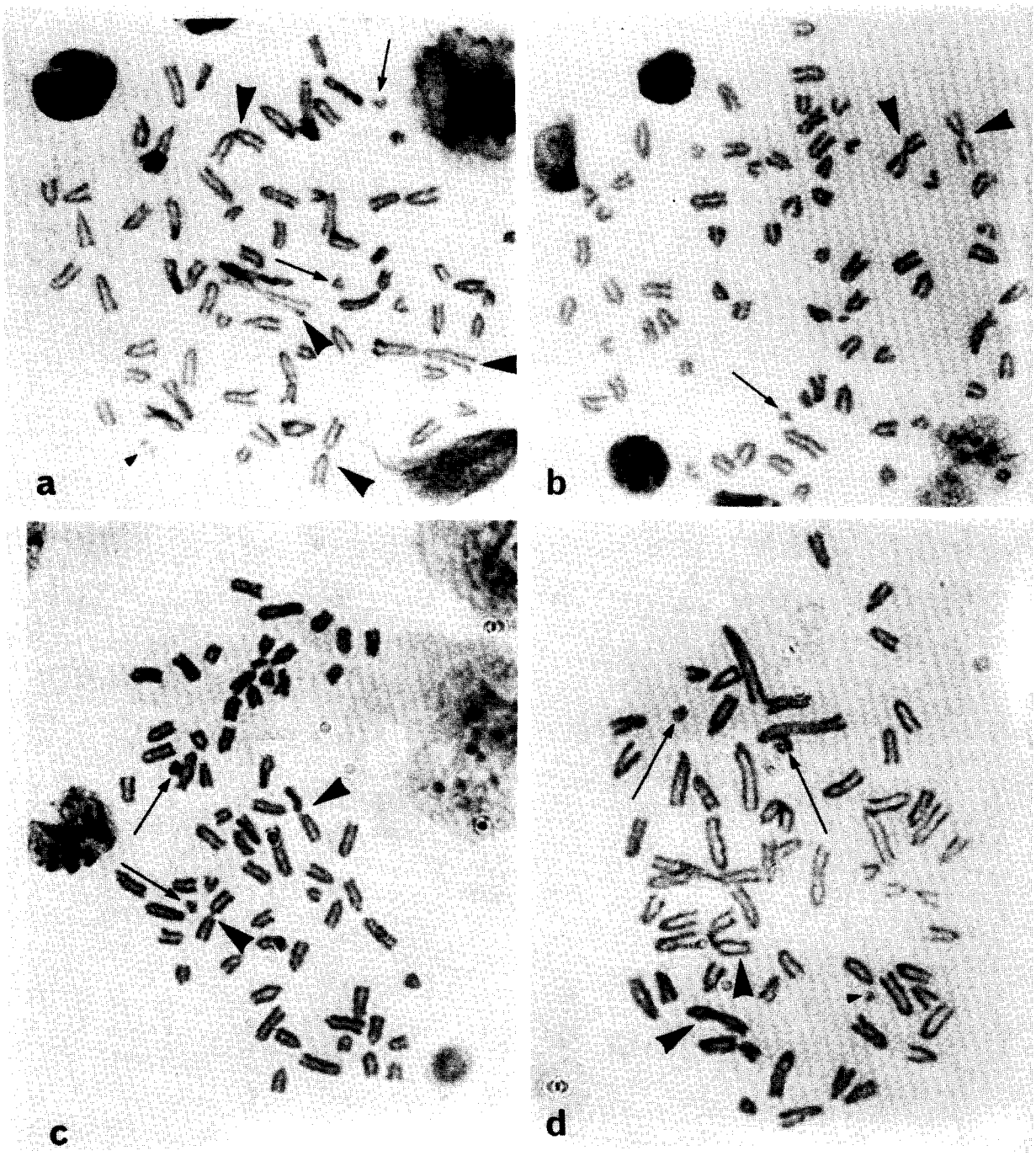

Fig. 5. *Metaphase spreads from EAT-2 at various passages. Keys same as in Fig.* 4. a—*EAT cell from the* 35*th passage*, 5-day *old tumor with* 68 *chromosomes including* 4 *metacentric*, 1 *minute and* 2 T_6 *markers.* b—*EAT cell from the* 58*th passage*, 9-day *old tumor with* 66 *chromosomes including* 2 *metacentric and* 1 T_6 *markers.* c—*EAT cell from the* 86*th passage*, 7-day *old tumor with* 65 *chromosomes including* 2 *metacentric and* 2 T_6 *markers.* d—*EAT cell from the* 150*th passage*, 6-day *old tumor with* 63 *chromosomes including* 2 *metacentric*, 1 *minute and* 2 T_6 *markers.*

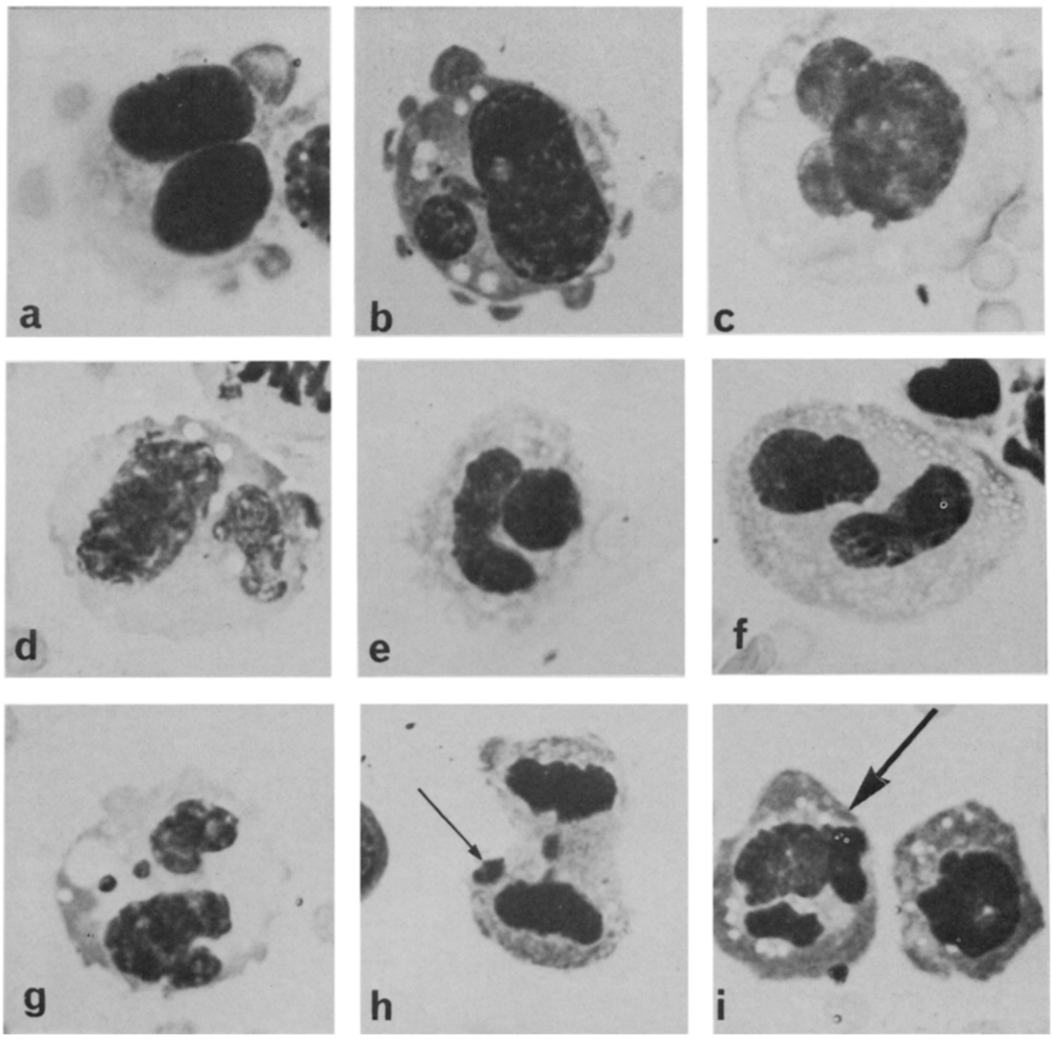

Fig. 6. *Cytological preparations from EAT-0 and EAT-2:* a—*A binucleate cell containing two tumor type nuclei. This happens to be from a radioautograph of* 7-day *old EAT smeared* 1 hr *after i.p.* ^{3}H-*thymidine injection. Both nuclei are labeled but the micrograph focuses at the level of the nuclei rather than silver grains.* b—*A binucleate cell containing one tumor nucleus (right) and the other (left) lymphocyte type nucleus. This cell is sampled from the same tumor as in Fig.* 6a, *and the nuclei are in asynchronous DNA synthesis. The tumor nucleus is labeled and the host nucleus is unlabeled (micrograph focused at the level of the nuclei rather than silver grains).* c—*A trinucleate cell with one tumor type and two lymphocyte type (left) nuclei.* d—*A binucleate cell showing one tumor type nucleus (left) and the other having the morphology of a monocyte (right).* e—*A binucleate macrophage–lymphocyte heterokaryon.* f—*A binucleate macrophage–macrophage homokaryon.* g—*A binucleate cell indicating macrophage–macrophage (or monocyte) fusion. Two pyknotic chromatin masses appear as cytoplasmic inclusions.* h—*An anaphase figure showing segregation of a small chromosome cluster (arrow) from the pair of main chromosome masses joined by the spindle.* i—*Mitotic cell on the left exhibits two nuclei in asynchronous mitosis: one in prophase, the other in anaphase (darker chromatin masses).*

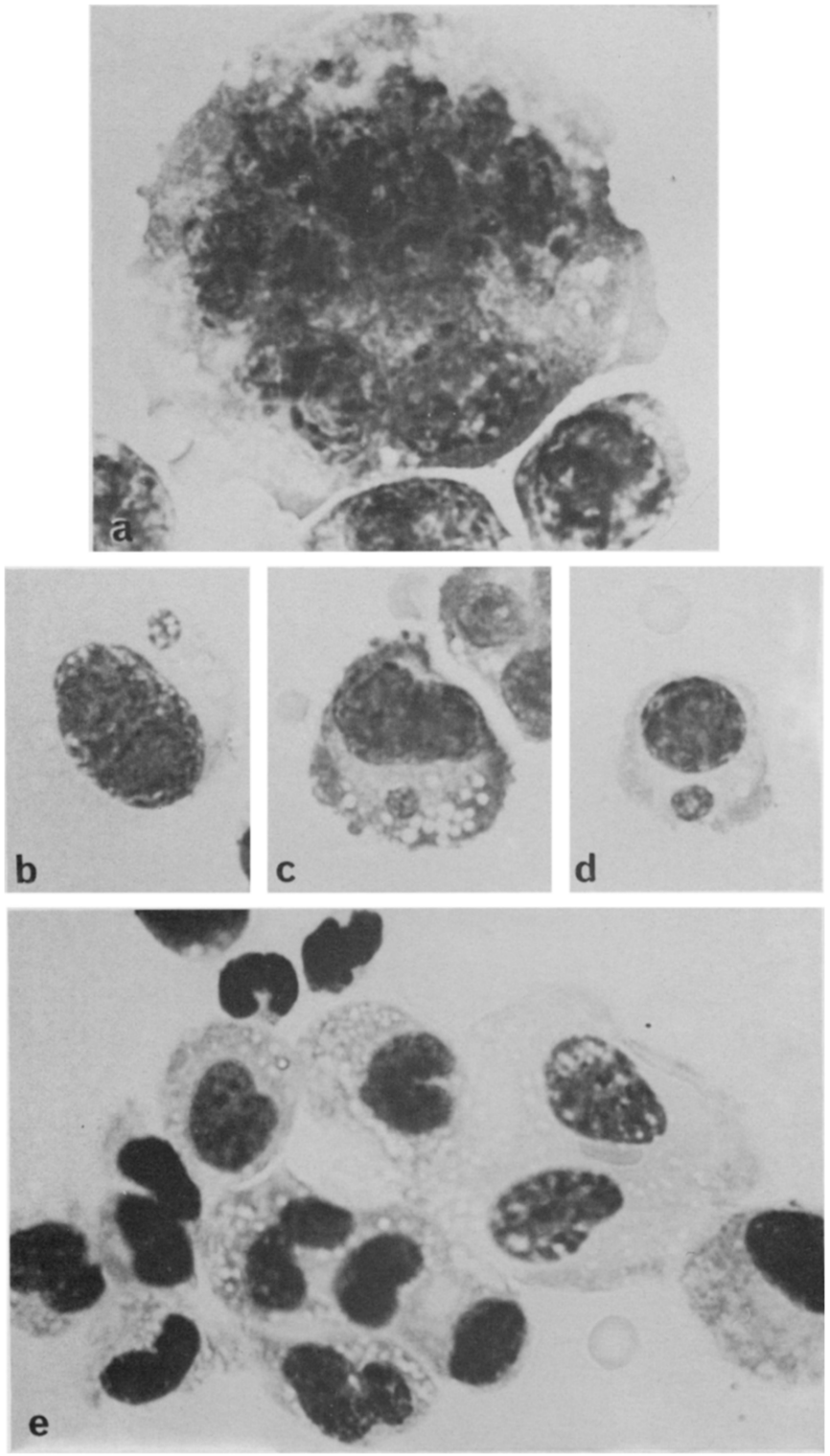

Fig. 7. Cytological preparations from EAT-0: a *A multinucleate giant cell (top).* b, c *and* d—*Interphase cells containing micronuclei. These possess distinct nuclear envelopes.* e —*A cluster of macrophages adherent to one another from a smear of unmanipulated* 9-day *old ascites tumor.*

Table 7. Subcutaneous tumorigenicity of 10^7 cells from various EAT lines

EAT line	No. of i.p. passages when tested	Recipient strain for s.c. transplant	Growth characteristics of s.c. transplants: No. of recipients	Nondetectable take	Prompt regression	Delayed regression	Fatal continued growth	Fatal local recurrence	Total incidence of fatal take
EAT-0	225–318	CF_1	60	0	6 (10%)	11 (18%)	43 (72%)	2 (3%)	75%
	265–286	CBA/HT_6	25	9 (36%)	16 (64%)	0	0	0	0%
EAT-2	21	CBA/HT_6	6	0	0	6 (100%)	0	0	0%
	35	CBA/HT_6	20	0	0	16 (80%)*	4 (20%)	1 (5%)	25%
	56–130	CBA/HT_6	70	0	0	53 (76%)*	17 (24%)	7 (10%)	34%
	160–232	CBA/HT_6	60	0	0	45 (75%)	15 (25%)	4 (7%)	32%
	10	CF_1	16	0	2 (13%)	5 (31%)	9 (56%)	0	56%
	58–85	CF_1	23	10 (44%)	7 (30%)*	3 (13%)	3 (13%)	1 (4%)	17%
	120–232	CF_1	36	16 (44%)	11 (31%)	6 (17%)*	3 (8%)	1 (3%)	11%
Fractionated EAT-0†	328	CBA/HT_6	20	6 (30%)	12 (60%)	2 (10%)	0	0	0%
Fractionated EAT-2†	135	CBA/HT_6	20	0	0	15 (75%)	5 (25%)	2 (10%)	35%
EAT-3	79‡	CBA/HT_6	20	0	0	8 (40%)*	12 (60%)	6 (30%)	90%
	162–171‡	CBA/HT_6	20	0	0	14 (70%)*	6 (30%)	1 (5%)	35%
	162–171‡	CF_1	25	10 (40%)	7 (28%)	6 (24%)*	2 (8%)	1 (4%)	12%

*Including fatal recurrences listed on the last but one column.
†Free from host-derived cells.
‡With an intervening s.c. passage after 78 i.p. passages.

the total transplants) showed fatal tumor recurrence at the same site following a complete regression. The median recurrence time, when first palpable, was 62 days from the day of transplantation. (c) Prompt regression (10%) following transient growth to a peak of 0.7–1.2 cm diameter at mid-second week; tumors disappeared at the end of the third week. When an identical number of EAT-0 cells from the ascites tumors long established in the CF_1 strain were transplanted s.c. in CBA/HT_6 strain hosts, the findings were in marked contrast (Fig. 9 and Table 7). There was no

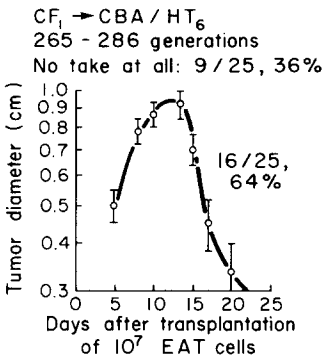

Fig. 9. Growth characteristics of subcutaneous transplants of 10^7 EAT-0 cells (at 265–286 passages) in CBA/HT_6 mice. Keys same as in Fig. 8.

palpable tumor take in 36% of the animals, and in the rest the tumors regressed promptly after transient growth similar to group (c) above.

Subcutaneous tumorigenicity of EAT-2 cells. Intraperitoneal propagation of the tumor in CBA/HT_6 strain mice after an initial transfer from the CF_1 mice caused a remarkable difference in growth characteristics of the tumor when transplanted by the s.c. route in either mouse strain. Transplants of EAT-2 cells progressively became more tumorigenic in the CBA/HT_6 strain (Table 7 and Fig. 10). At 21st passage, all produced palpable takes, resulting in maximum tumor sizes of 1–2 cm in diameter during the 3rd week, but all regressed during the fourth to the sixth week, similar to category (b) above. At 35th passage, all s.c. transplants resulted in tumors of appreciable size, of which 20% grew until the animals died; in the rest there was a delayed regression, but fatal recurrences were noted in 5% of the transplants. Data from the 56th, 71st, 73rd, 100th and 130th passages were very much comparable with one another, and

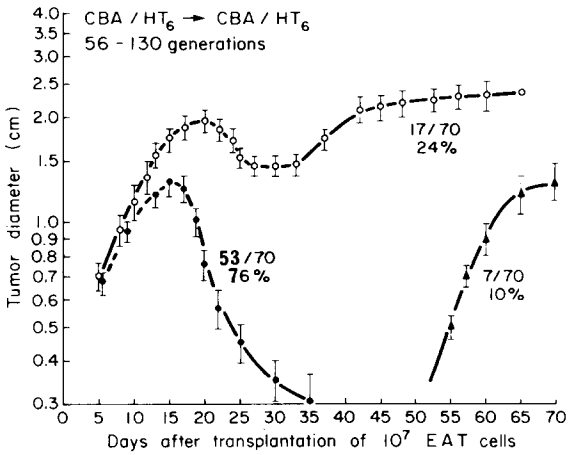

Fig. 10. Growth characteristics of subcutaneous transplants of 10^7 EAT-2 cells (at 56–130 passages) in CBA/HT_6 mice. Keys same as in Fig. 8.

were pooled (Fig. 10, Table 7). Moderate to large-sized tumors resulted from all transplants. However, continued growth leading to mortality occured in 24%; peak tumor sizes in this category (1.8–3.5 cm diam.) and host survival time (5–9 weeks) were comparable with the respective values for EAT-0 transplants in CF_1 mice. Tumors in the remainder reached a peak size of 1–2 cm in diameter and regressed slowly. For some, the regressing phase lasted up to the eighth week. However, a significant proportion (10%) showed a fatal recurrence (median recurrence time 55 days), so that the overall mortality was 34% in these transplants. No further changes in s.c. tumorigenicity for CBA/HT_6 mice (160–232 passages of EAT-2 in Table 7) were noticeable.

With increasing number of i.p. passages of EAT-2 in CBA/HT_6 mice, EAT-2 cells progressively lost their ability to produce fatal s.c. tumors in CF_1 mice (Table 7). The incidence of fatal tumor takes dropped to 56% (cf. 75% in the case of EAT-0) after as few as 10 passages. After 58–85 passages, this dropped to 17%; no palpable tumors were noted in 44% of the recipients, 30% showed prompt regression, 13% showed delayed regression and only 13% showed continued growth leading to death (Table 7, Fig. 11). No further significant changes in the growth characteristics of the s.c. transplants were noted in CF_1 mice after 120–232 passages of EAT-2 in CBA/HT_6 mice (Table 7).

Figure 12 summarizes the data on fatal s.c. tumorigenicity of the EAT-2 for CF_1 and CBA/HT_6 mice with increasing number of

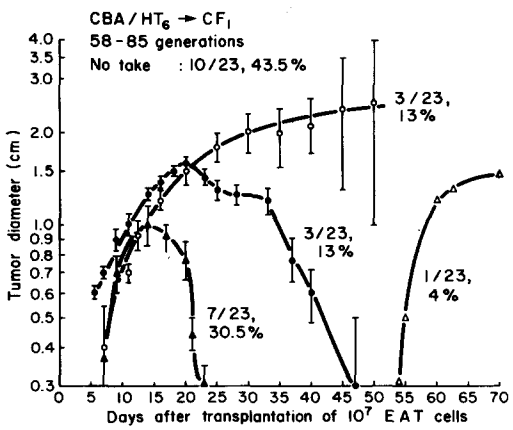

Fig. 11. *Growth characteristics of subcutaneous transplants of* 10^7 *EAT-2 cells (at* 58–85 *passages) in* CF_1 *mice. Keys same as in Fig* 8.

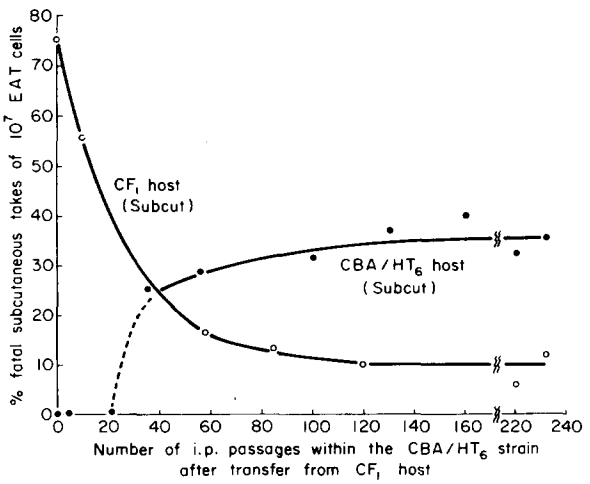

Fig. 12. *Fatal subcutaneous tumorigenicity (including fatal recurrences) of* 10^7 *EAT-2 cells for* CF_1 *and* CBA/HT_6 *strain mice at different i.p. passages in* CBA/HT_6 *mice.*

i.p. passages in CBA/HT_6 mice. They also include fatal local recurrences. It may be concluded that a progressive loss of s.c. tumorigenicity of EAT-2 for CF_1 mice was associated with a concurrent gain in tumorigenicity by the same route for the CBA/HT_6 mice. These findings cannot be explained on the basis of the presence of contaminant mononuclear leukocytes in the EAT inoculum which might help rejection in an allogeneic transfer (owing to homograft response) or help tumor growth during a syngeneic transfer by some immuno-stimulatory mechanism, as proposed by Prehn and his associates [28]. Firstly, changes were found to be progressive with increasing number of passages, whereas no change in the host cell concentration was noted in the EAT. Secondly, s.c. inoculation of EAT-0 or EAT-2 cells free from contaminant leukocytes prepared by the sucrose gradient centrifugation technique gave results which were identical to those obtained with unfractionated EAT (Table 7).

Subcutaneous tumorigenicity of EAT-3 cells. The time course for the gain in the s.c. tumorigenicity of EAT-2 for CBA/HT_6 mice (Fig. 12) after i.p. propagation in this mouse strain was similar to that for the increase in the incidence of identifiable tumor–host cell hybrids which had lost some chromosomes within the ascites form of the tumor. Such a parallel relationship may be due to some growth advantage of these hybrids in the s.c. form of tumor development. As a first step to test this possibility, s.c. passage was employed as a means for selecting 'tumorigenic' cells from the EAT-2. A solid s.c. tumor in its plateau phase of growth in the CBA/HT_6 mouse produced by inoculation of 78th passage EAT-2 cells was harvested, and 10^6 viable cells were injected i.p. into CBA/HT_6 mice to produce ascites tumors. 10^7 cells from such ascites tumors were transplanted by the s.c. route again into similar strain hosts. These inoculations proved to be highly tumorigenic (Table 7, Fig. 13). Sixty per cent of the

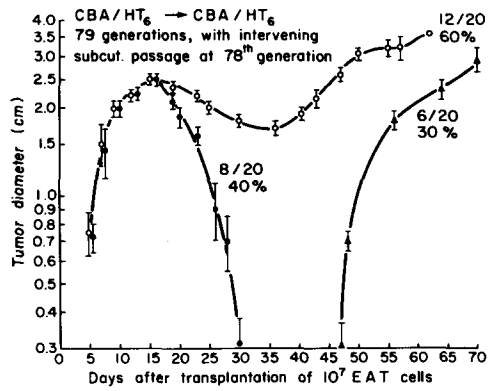

Fig. 13. *Growth characteristics of s.c. transplants of* 10^7 *EAT-3 cells in* CBA/HT_6 *mice immediately following a cyclic (i.p.→s.c.→i.p.) passage in* CBA/HT_6 *mice. Keys same as in Fig.* 8.

transplants showed fatal continued growth and the remainder regressed slowly. Amongst the latter, 6 out of 8 showed fatal recurrence. Thus the overall incidence of fatal tumor take was 90%. However, an enrichment of the cells highly tumorigenic for s.c. growth seen initially after the cyclic (ascites→solid→ascites) passage was not long

sustained during the ascites form of propagation. For example, after 84–93 i.p. passages following the s.c. detour, i.e., after a total of 162–171 passages of EAT-3, the s.c. tumorigenicity of these cells for CBA/HT_6, mice dropped to the level exhibited by EAT-2 cells that did not pass through a s.c. detour (Table 7). At these intervals, the s.c. tumorigenicity of these two EAT lines was also very similar for CF_1 mice (Table 7). Furthermore, the incidences of T_6 chromosome-bearing hybrid cells 14–20%) observed in long propagated EAT-3 were also similar to those for EAT-2.

Subcutaneous tumorigenicity of EAT-4 cells. EAT-4 was generated by i.p. propagation of EAT-3 cells into (CBA/HT_6 × C57B1/6)F_1 mice. This allowed a testing of histocompatibility of EAT cells by way of s.c. transplantation in a parent–F_1 hybrid combination. The results of s.c. inoculation of EAT-4 cells (at various i.p. passage generations in the F_1) into CBA/HT_6, C57B1/6 and F_1 mice are presented in Fig. 14. While the tumorigenicity

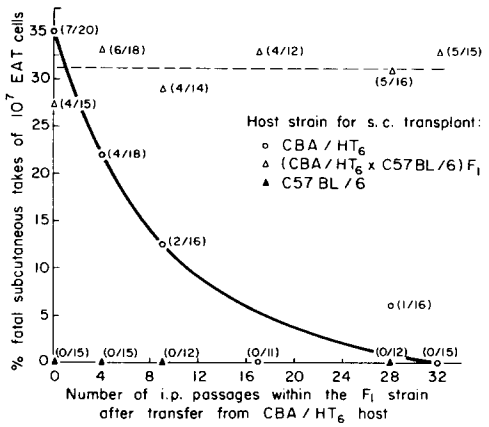

Fig. 14. Fatal subcutaneous tumorigenicity (including fatal recurrences) of 10^7 EAT-4 cells at different i.p. passages in F_1 mice for the F_1 and parental strain mice. Numbers in parentheses indicate number of mice which died of s.c. tumors out of the total number which received s.c. transplants.

for the F_1 strain remained unchanged, with levels similar to that of EAT-3 for CBA/HT_6 mice, the tumorigenicity for the CBA/HT_6 parent declined with time, and that for the C57B1/6 parent remained at the zero level.

Immunity against intraperitoneal challenge with various EAT lines in survivors rejecting subcutaneous transplants of various EAT line cells

10^6 EAT cells taken from various EAT lines were injected i.p. into the survivors of different mouse strains subjected to a single s.c. transplant of 10^7 EAT cells from various lines in the above experiments. A dose of 10^6 EAT cells i.p. causes fatal ascites tumors in 100% of the conventional, unimmunized animals irrespective of the mouse strain or EAT line used in this study. Thus, this dose was considered adequate for testing the presence of long-lasting immunity against EAT cells. As shown in Table 8, most of the mice which survived the s.c. transplants remained immune for 160 days or longer to an i.p. challenge of the EAT cells irrespective of the EAT lines tested.

Effect of intraperitoneal propagation of EAT in different host strains on the H-2 antigen profile of tumor cell surface.

Labeling of normal thymocytes. The sandwich labeling method for H-2 antigens was tested on normal thymocytes (Fig. 15). As expected,

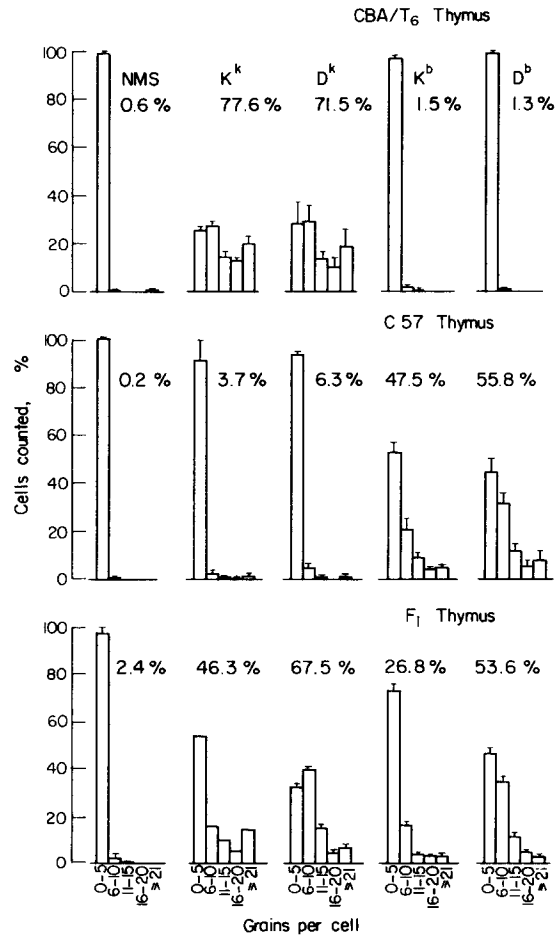

Fig. 15. Labeling of normal CBA/HT_6, C57BL/6 and F_1 thymocytes with monospecific anti-H-2 sera, showing the specificity of the labeling. NMS does not label. Cells with ≥6 grains were counted as labeled: percentages refer to the labeling index. The bars represent standard error of the mean for 3–6 experiments.

Table 8. Results of i.p. challenge with 10^6 *EAT cells from various EAT lines in long term survivors of subcutaneous transplants of various EAT line cells* (>160 days *post-inoculation*)

Survivor mouse strain	EAT-line used for s.c. transplant	EAT-line used for i.p. challenge	No. of fatal ascites tumors/No. challenged
CF_1	EAT-0	EAT-0	2/9
CF_1	EAT-2	EAT-2	1/15
CF_1	EAT-2	EAT-0	2/16
CBA/HT_6	EAT-0	EAT-0	1/10
CBA/HT_6	EAT-0	EAT-2	1/15
CBA/HT_6	EAT-2	EAT-2	4/71
CBA/HT_6	EAT-2	EAT-0	5/62
CBA/HT_6	EAT-3	EAT-3	1/10
CBA/HT_6	EAT-4	EAT-4	0/10
$(CBA/HT_6 \times C57Bl/6)F_1$	EAT-4	EAT-4	1/9
		EAT-4	2/8
C57Bl/6	EAT-4	EAT-0	2/8
		EAT-0	1/14

CBA/HT_6 thymocytes are heavily labeled by both anti-H-2^k sera, and are unlabeled by anti-H-2^b sera. Conversely, C57Bl/6 thymocytes are labeled by antisera against H-2^b but not H-2^k, while F_1 cells are labeled by antisera against both H-2^k and H-2^b. The variation in the degree of specific labeling by different antisera probably reflected differences in their potency (see Table 2). If NMS or an unrelated antiserum, anti-H-$2K^d$, was used, labeling was negligible. Strong specific labeling (91–95% of cells) was also seen with polyvalent anti-H-2^k and anti-H-2^b. Thymocytes from outbred CF_1 mice showed somewhat weaker but specific labeling for both H-2^k and H-2^b haplotypes with polyvalent as well as monospecific antisera (results not shown); such labeling was abrogated by prior absorption of the sera with CBA thymocytes.

Labeling of tumor cells. Line EAT-0, maintained in outbred CF_1 mice, labeled with all 4 monospecific antisera, as shown in Fig. 16. These cells also labeled with polyvalent anti-H-2 sera (results not shown). EAT-3, long maintained in CBA/T_6 mice after transfer from EAT-0, labeled heavily with both anti-H-2^k sera, but showed negligible binding of anti-H-2^b sera. Furthermore, as shown in Table 9, cells of line EAT-3 could absorb much of the labeling activity of anti-H-2 sera for CBA/T_6 thymocytes, confirming the presence of H-2^k antigens on EAT-3 cells. Line EAT-4, maintained in F_1 mice after transfer from line EAT-3, bound anti-H-2^k sera but to a much lower extent than EAT-3, while EAT-4 also showed weak labeling with anti-H-$2K^b$ and H-$2D^b$. It is clear from Fig. 16

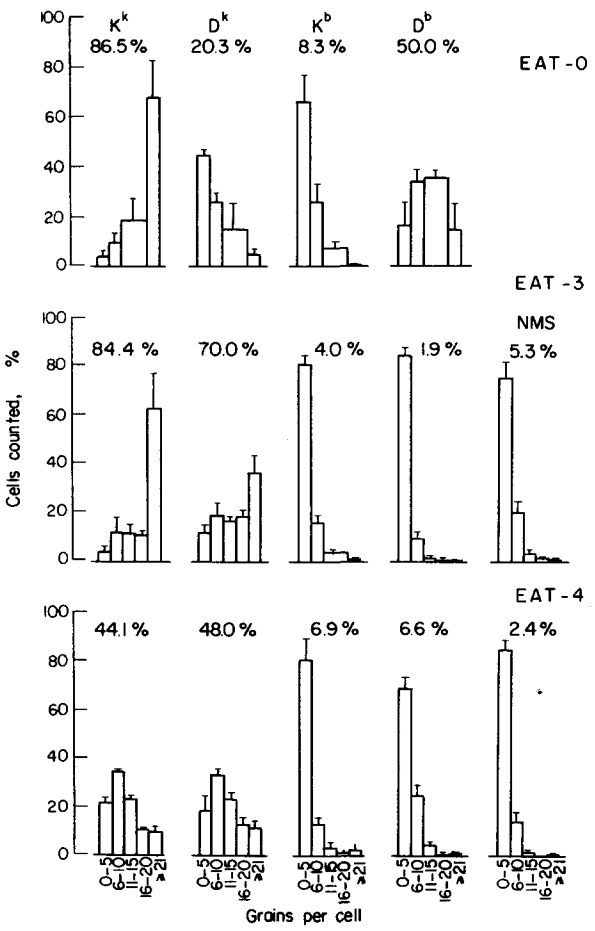

Fig. 16. Labeling of EAT cell lines with monospecific anti-H-2 sera: EAT-0 at 500–600 passages in CF_1 mice, EAT-3 at 158–203 passages in CBA/HT_6 mice, and EAT-4 at 20–50 passages in F_1 mice. NMS gives negligible labeling. Cells with ⩾11 grains were counted as labeled; percentages refer to the labeling index. The bars represent standard error of the mean for 3–8 experiments.

Table 9. Effect on labelling of normal CBA/HT$_6$ thymocytes with H-2^k antisera of a single prior absorption with EAT-3 cells

Antiserum	% of cells					% of cells* labeled
	0–5 grains	6–10 grains	11–15 grains	16–20 grains	⩾21 grains	
H-2^k unabsorbed	28.0	40.0	18.4	9.4	4.2	72.0
H-2^k absorbed	55.2	41.6	3.0	0	0.2	44.8
H-2K^k unabsorbed	19.6	29.4	12.2	14.2	24.6	80.4
H-2K^k absorbed	41.8	23.0	11.2	11.2	12.5	57.9

*Cells with ⩾6 grains are counted as labeled.

Table 10. Labeling of tumor cells grown intraperitoneally and subcutaneously in the same host strain

Target cell	Antiserum	% of cells					% of cells labeled*
		0–5 grains	6–10 grains	11–15 grains	16–20 grains	⩾21 grains	
EAT-3, ascites	H-2^k (polyvalent)	40.6	31.8	16.0	6.8	4.8	27.6
EAT-3, s.c.		15.8	29.6	16.6	7.2	30.8	54.6
EAT-4, ascites	H-2K^b	37.4	37.8	16.4	5.0	3.4	24.8
EAT-4, s.c.		1.0	7.0	16.0	13.0	63.0	78.0
EAT-4, ascites	H-2K^k	0.4	2.6	4.2	6.6	86.2	97.0
EAT-4, s.c.		1.0	5.0	8.0	16.0	70.0	94.0
EAT-4, ascites	H-2D^k	43.0	39.6	12.8	2.4	2.2	17.4
EAT-4, s.c.		23.3	31.0	15.3	13.0	17.3	45.6
EAT-4, ascites	H-2D^k	15.4	34.0	27.4	13.4	9.8	50.6
EAT-4, s.c.		9.0	30.2	25.8	12.8	22.2	60.8

*Cells with ⩾11 grains are counted as labeled.

Table 11. Effect on labeling of thymocytes and tumor cells of a single prior absorption of antisera

Antiserum	Target	% reduction after absorption
K^k	CBA/T$_6$ thymus	90.3
absorbed with	EAT-0	79.9
CBA thymus	EAT-3	16.7
	EAT-4	73.1
D^k	CBA/T$_6$ thymus	48.2
absorbed with	EAT-0	72.5
CBA thymus	EAT-3	25.3
	EAT-4	74.8
K^b	C57 thymus	59.0
absorbed with	EAT-0	58.2
C57 thymus	EAT-4	70.0
D^b	C57 thymus	97.0
absorbed with	EAT-0	46.4
C57 thymus	EAT-4	65.6

that transfer of EAT from one host strain to another of different H-2 constitution, followed by maintenance in the latter strain, causes substantial changes in the H-2 labeling profile of the tumor cells. These differences were significant for all the antisera tested. Cells of line EAT-3 and EAT-4 grown s.c. as solid tumors in CBA/HT_6 and F_1 mice respectively, showed both higher labeling indices and more intense labeling than did the same tumors grown i.p., as shown in Table 10.

To test whether the labeling of tumor cells was H-2 specific, and not caused by antibodies against MuLV antigens, as suggested by Klein [29, 30], the antisera were absorbed once with normal thymocytes of the appropriate haplotype before incubation with either thymocytes or tumor cells. As seen in Table 11, such absorption abolished 48–91% of the labeling of thymocytes, and caused a significant but more variable reduction in labeling of the tumor cells. This suggests that a large proportion of the labeling by unabsorbed sera must be specific for H-2 antigens.

Effects of neuraminidase and periodate on labeling. Pretreatment of tumor cells with neuraminidase or $NaIO_4$ was used to see if any exposure of H-2 antigens would be effected by the removal of surface coat material. Such treatment did not result in any significant labeling of EAT cells exposed to NMS only, rather than anti-H-2 sera. As shown in Fig. 17, labeling of EAT-3 cells with anti-H-$2K^k$ or anti-H-$2D^k$ is increased, both in labeling index and in intensity, after either neuraminidase or $NaIO_4$. With EAT-4, labeling is increased by neuraminidase pretreatment only.

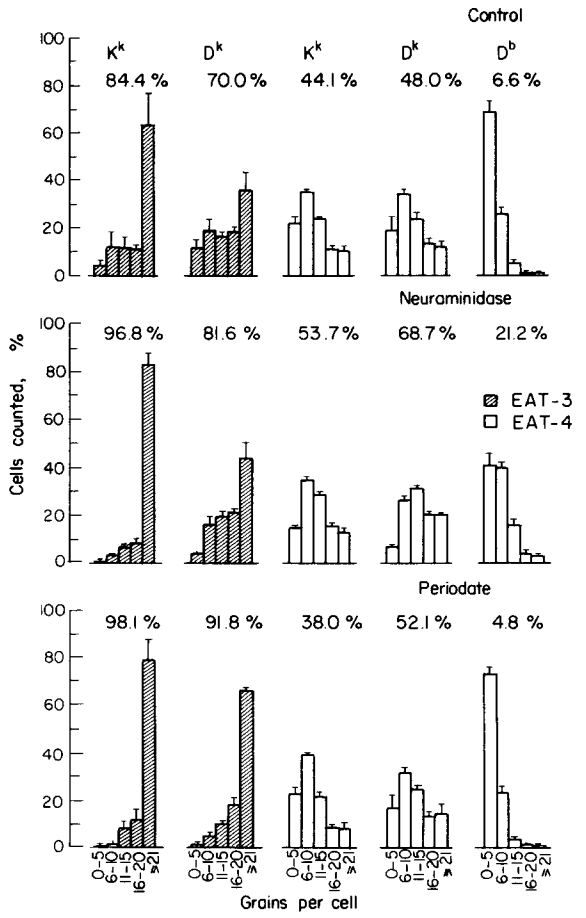

Fig. 17. The effect of pretreatment with neuraminidase or $NaIO_4$ *on labeling of EAT cells with monospecific anti-H-2 sera. Control preparations were preincubated with medium alone. Hatched bars = EAT-3; open bars = EAT-4. Cells with* $\geqslant 11$ *grains were counted as labeled; percentages refer to the labeling index. The bars represent standard error of the mean of 3–7 experiments.*

DISCUSSION

Our studies reveal that a slow, spontaneous hybridization between tumor cells and host-derived cells takes place continuously *in vivo* in the Ehrlich ascites tumor. Historically, the origin of the tumor goes back to the turn of the century in an unknown mouse strain [31]. Its genealogy has been reviewed by Hauschka and Levan [32], who performed extensive karyological studies on single cell clones derived from this tumor. Since its origin the tumor has passed through various hands in various mouse strains; thus possibly no two lines are alike. Nevertheless, indirect findings suggestive of tumor–host cell fusion have been reported for other EAT lines [13]. Bi- and multinucleate cells showing asynchronous DNA synthesis reported for another EAT line [33] and in the ascites fluid associated with a human ovarian carcinoma [34] may also indicate cell fusion. Evidence for tumor–host cell fusion has been provided for a number of experimental tumors [14–22]. Thus, this phenomenon may be more widespread than is suspected.

Our study in the EAT shows that the spontaneous tumor–host cell hybrids having nearly complete sets of parental chromosomes have a limited life. The survival value of hybrids appears to increase by the elimination of some unidentified chromosomes resulting in karyotypes with a similar chromosomes mode to the original 'stem line'. Such a chromosome constitution, including the metacentric markers, appears to be optimal for the growth of the present EAT line even after transfer into a

new host strain. Our findings on chromosome loss are consistent with the results reported from Karolinska and Oxford in a chain of studies designed to analyse malignancy by virally-induced fusion of various mouse malignant cell lines with non-malignant lines *in vitro* [35–39]. The resulting hybrid cells had little tumorigenic ability *in vivo*, so long as they retained nearly complete sets of parental chromosomes; occasional tumors resulted from selective overgrowth of some cells that had eliminated certain specific but unidentified chromosomes. In contrast, fusion of identical tumor cell lines with a highly malignant line resulted in tumorigenic hybrids retaining most of the chromosomes [40]. It was postulated that some somatic cell chromosomes in the hybrids can suppress malignancy, and their removal permits a reversion to the malignant phenotype.

The mechanism of chromosome elimination by the hybrids is rather poorly understood. One possible pathway is exclusion during segregation at mitosis of some chromosomes, which might then appear as micronuclei, as observed in the present case. It is not known whether such micronuclei are destined for attrition, or whether they can replicate any further,

Nuclei tentatively identified as host components were of the lymphocyte, monocyte and macrophage types. We showed earlier that the mononuclear cells within the EAT are newly-formed cells selectively recruited from blood [10], derived to a large extent from the bone marrow [41]. These findings agree with the report of Wiener *et al.* [21] that the host counterpart of the hybrids found in another ascites tumor originated from the bone marrow. Host–tumor cell fusion seems to be a non-selective process, since we found polynucleate cells with all the possible nuclear counterparts, including host–host types. Since EAT cells (including the present lines) are known to contain free virus particles, a viral role in the fusion process remains a possibility.

Despite the fact that host–tumor cell fusion may be an incidental phenomenon, the outcome of such fusion may be relevant to the tumor–host relationship. Somatic mating may provide one mechanism of karyotypic evolution in tumors most suited for tumor cell survival in an adverse host environment. In the present case, a slow but significant rise in the T_6+ve metaphases with prolonged passage of EAT-2 in CBA/HT_6 mice indicates some *in vivo* selection of hybrids. Since this happened regardless of the previous life history of the EAT (i.e., whether maintained in histo-incompatible CF_1 or histocompatible CBA/H mice), it seems unlikely that improved histocompatibility of the hybrids was the sole basis for an increase in their incidence; other unknown mechanisms may be involved. However, the growth advantages of the hybrids over other cells within the EAT-2 did not appear to be too high. Even after 4 years of weekly passage, the proportion of T_6+ve cells remained close to 18% of the dividing population. If one assumes that T_6 is a stable marker for the hybrids, and thus reflects their true incidence, then there must be a mechanism allowing some form of steady state balance between the 'stem line' cells derived from the hybrids and the original 'stem line' cells. The other possibility is that most or all the cells in the stem line are finally replaced by the products of hybridization, but not been identified because of the instability of the T_6 marker.

Our EAT and its sublines, as well as EAT lines tested in other laboratories, are capable of producing fatal ascites tumors when transplanted i.p. into any mouse strain. Such universal transplantability, however, does not apply in the case of the s.c. route of transfer, as we have shown, and as also reported for other ascites tumors [42]. The s.c. tumorigenicity of the EAT changed progressively after i.p. propagation into a different mouse strain. It acquired significant virulence by the s.c. route against the propagating host strain, but lost its virulence against the original strain. These progressive and bidirectional changes in the s.c. tumorigenicity can be best explained by two types of antigenic changes in the tumor: (a) a loss or masking of some existing antigen(s) and (b) an acquisition or unmasking of some new antigen(s) on the tumor cell surface. The time-course of such antigenic changes was very similar to that observed for the increase in the incidence of identifiable tumor–host cell hybrids which had lost some chromosomes. Thus, the observed hybridization can account for the acquisition of some new antigen(s), and the chromosome loss for the loss of some existing antigen(s). These findings are somewhat similar to those reported by Chen and Watkins [43], except for the fact that they used hybrids between EAT cells and CBA embryo fibroblasts artificially fused *in vitro*. These hybrids were lethal to Swiss mice by the intraperitoneal route, but non-lethal to the same animals by the subcutaneous route. However, unlike ours, their EAT line both before and after hybridization

was tumorigenic to CBA mice by the subcutaneous route. This may have resulted from differences in the EAT lines, possibly owing to different host strains used for maintenance.

Since CF_1 is a non-inbred strain, the crucial histocompatibility bioassay was provided by the parent–F_1 hybrid system. EAT-4 originated from EAT-3 and was maintained in $(CBA/HT_6 \times C57Bl/6)F_1$ mice. It retained s.c. tumorigenicity for the F_1 mice at the same level as that shown by EAT-3 cells in CBA/HT_6 mice, but progressively lost its tumorigenicity for the original parental strain, CBA/HT_6, from which it was derived. Furthermore, it remained completely non-tumorigenic by the s.c. route for the other parental strain, C57Bl/6. These findings can only be explained by an acquisition of some host histocompatibility components by the EAT-4 during its propagation in the ascites form in the F_1 mice—again consistent with the findings of tumor–host cell fusion.

Host cell contamination of the ascites cells as a basis for changes in s.c. tumorigenicity after interstrain transfer has already been excluded, as stated in the Results. Another variant of this explanation was proposed for Sarcoma 180, which was strain-non-specific by the i.p. route, but specific by the s.c. route for mouse strains carrying H-2^d [44]. It was proposed that in an allogeneic s.c. transplant, blood vessels were derived from the donor rather than host cells and that the host mounted a homograft response against the vessels. This hypothesis can be discarded in our case on the same grounds. Some form of genetic resistance [45], unrelated to H-2 disparity, to s.c. EAT transplants may explain strain differences in tumorigenicity of the EAT, but cannot explain the alterations in its tumorigenicity after propagation into a different host strain.

Our findings of long-lasting immunity to all the EAT lines in survivors which have rejected s.c. transplants of various EAT line cells suggest that all these lines share some common antigen, although maintained in different host strains. This agrees with other reports [43, 46] of effective immunization of mice against EAT by inoculating hybrids between EAT cells and normal allogeneic or xenogeneic cells. Similar immunity can also be produced against syngeneic tumor cells after inoculation of semi-allogeneic hybrid cells [47].

Final evidence for changes in the H-2 antigens on EAT cells propagation in different host strains is provided by the immunolabeling technique. We have shown that the sandwich method enables sensitive and specific detection of H-2 antigens on the cell surface, with radioautographic grain count distribution providing a relative measure of the extent of antibody binding by individual cells. Absorption studies suggest that the labeling was specific, and not due to the presence of antiviral antibodies in the anti-H-2 sera. Since the glycoprotein surface coat may render H-2 antigens inaccessible, e.g., in the case of TA-3(Ha) mammary tumor line as opposed to the TA-3(St) line [48], H-2 antigens on EAT cells were also examined after treatment with neuraminidase or periodate to modify the surface coat [49, 50]. Pretreatment with the former was found to increase the specific labeling of EAT cells, thus increasing the sensitivity of the assay.

It appears that the number of passage generations in a given host strain influences the apparent H-2 haplotype of EAT cells, since the labeling of the EAT sublines varied in specificity and degree according to the host strain. Presumably the tumor cells acquire the H-2 antigens of the host by fusion with host cells during prolonged passage. Depending on which chromosomes are lost following fusion, the hybrid cell may express H-2 antigens, or fail to do so because of an EAT gene product which suppresses surface antigen expression [51]. Thus in the tumor cell population there may exist hybrids which express H-2, and others which do not, despite the presence of chromosome 17. Furthermore, the loss of one set of chromosome 17 from the hybrid may cause the loss of the H-2 antigen from one parental cell but expression of the other. This mechanism of origin of 'isoantigenic variants' has been reported after forced propagation of a hybrid between two tumor lines having different H-2 antigens into a parental strain mouse [52].

Thus our original line, EAT-0, possesses both H-2^k and H-2^b which are also detectable on somatic cells of CF_1 mice, in which the line is maintained; after many passages in a strain carrying H-2^k only, the level of H-2^k is increased for both K and D end determinants and H-2^b determinants are lost, to give the labeling pattern exhibited by EAT-3. After transfer from the H-2^k host and propagation in an F_1 host with both H-2^k and H-2^b, the cells of EAT-4 show a decline in H-2^k and a small gain in H-2^b, which is further enhanced after neuraminidase treatment. These findings adequately explain the concurrent changes in s.c. tumorigenicity, if one postulates that at

the s.c. site there is some growth advantage of the iso-antigenic variants derived from tumor–host cell fusion and chromosome loss. This view is supported by the findings that s.c. EAT cells show increased labeling for the host-specific H-2 haplotype, compared to the ascites EAT cells in the same mouse strain.

Further work is needed to identify (1) the mechanism promoting a slow but significant rise in the incidence of hybrids after i.p. transfer of the EAT into a syngeneic strain, (2) the mechanism of reversion of s.c. virulence of EAT-3 cells for CBA/HT_6 mice after prolonged i.p. passage to the level of that of EAT-2, and (3) the mechanism of protection of i.p. allotransplants of EAT despite the expression of H-2 antigens. The presence of blocking factors and nonspecific immunosuppressive molecules in the ascites fluid has been implicated in some reports [42, 53]. Finally, it is not yet known whether the observed host–tumor cell fusion and the accompanying alterations in the biological properties of the tumor represent a natural phenomenon, or a laboratory artefact created by repeated transplantation of tumor cells. Whether this may be one of the mechanisms leading to changes in the tumor cell phenotype during the development of a spontaneous tumor remains to be investigated.

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