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Cytogenetics and the major histocompatibility complex in a proliferating microcell-mediated hybrid clone

Yan Yongshan

Institute of Genetics, Academia Sinica, Beijing, China

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Summary. By way of a microcell fusion, three chromosomes from a B82HTQ2 (TK^-) cell were introduced into a PG19 ($HGPRT^-$) cell. Analysis of this hybrid clone showed that the transferred chromosomes restored a positive HGPRT status but failed to produce heterozygosity for the major histocompatibility complex (H-2). The three chromosomes also proved stable in both long term culture in vitro and tumor testing in mice. It is suggested that the method could prove useful in correcting genetic defects or in introducing new genetic characteristics without the introduction of the genes coding for major histocompatibility antigens. The surface structure of the microcells was studied by scanning electron microscope. The optimum for induction of the microcells from B82HTQ2 cells and its' purification were reported here. Frequency of the sister chromatid exchange (SCE) of the hybrid cells and their sensitivity to mitomycin C (MMC) were also examined.

Key words: Somatic cell fusion – Microcell-mediated hybrid – H-2 – SCE – Gene complementation

Introduction

Since it was discovered that hybrid cells derived from inter- and intraspecific somatic cell fusion by inactivated Sendai virus are viable and frequently undergo chromosome segregation and preferential loss, studies of somatic cell genetics by way of gene mapping and complementation have been greatly advanced. In intraspecific hybrids, however, this advance has been hampered because segregation and loss of the parent chromosomes is very slow (Ringerzt et al. 1976). Recently, this problem has been overcome by fusing microcells to whole cells (Wonton et al. 1981). Each of these microcells carries a few chromosomes and thus offers an efficient vehicle for transferring a small part of a donor genome into a recipient cell. In this paper the method is used for mouse cells- to transfer three B82HTQ2 chromosomes into a whole PG19 cell. The intraspecific hybrid thus formed was studied from the point of view of cytogenetics, H-2, stability of donor chromosomes in both continuous subculture in vitro and tumor testing in vivo, and their ability to survive in HAT medium.

Materials and methods

Cell lines

PG19 cells ($HGPRT^-$) (Jonasson et al. 1977) were used as recipients and B82HTQ2 (TK^-) cells (derived from B82 cells) (Littlefield 1966) as donors. In order to test if PG19 cells could produce any revertants, 1×10^8 PG19 cells were inoculated into HAT (hypoxathin, aminopterin, thymidine) selective medium (Littlefield 1964).

Microcell preparation

B82GTQ2 cells grew in Minimal Essential Medium (MEM) with 10% foetal calf serum (FCS) for 48 h. Colcemid (Gibco Diagnostic) was added to a final concentration of 0.2–0.3 μg/ml, followed by further culture for between 24 and 96 h. The cells were removed from the flasks with trypsin/ethylenediaminetraacetic acid (EDTA) and enucleated basically using the Wigler and Weinstein (1975) method. Briefly, this consisted of washing in MEM with 5% FCS and re-suspending in 1.5 ml prewarmed 12.5% Ficoll (made up in MEM containing 10 μg cytochalasin B (CB)/ml, 0.5% dimethyl sylforide and 10% FCS) with a fine needle before ultracentrifuging at 35 C° at 3×10^4 rpm for 1 h. The whole gradient content was washed in MEM four times and resuspended in 2 ml of 0.3% bovine serum albumin (BSA). For microcell purification, this suspension was added to the top of a 1–3% BSA gradient in MEM contained in a siliconized tube (total volume 50 ml at pH 7.2

prepared 2 h before) and left to sediment at unit gravity either at room temperature or at 4 C°. The cells were allowed to disperse for 3.5 h at the higher temperature and for 7–9 h at the lower. Subsequently, the upper 15–18 ml in the tube contained pure microcells and this volume was either removed, the microcells washed three times in MEM and resuspended in 2 ml of Hank's solution at 4 C° for cell fusion, or it was fractionated with a Density Gradient Fractionator (2.5 ml/min), the microcells washed as above and then fixed in a 3:1 mixture of methanol: glacial acetic acid. Giemsa staining was used to test for their purity.

Cell fusion

1. Whole cell fusion between B82HTQ2 and PG19 as a control

Approximately $1-2\times10^6$ cells of each were resuspended together in 0.2–0.5 ml Hank's solution (pH 7.0). 0.2 ml of UV-inactivated Sendai virus (2×10^3 HAU) was then added. The mixture was shaken to clump the cells and they were kept at 4 C° for 30 min and then at 37 C° for another 40 min. After this, 10 ml of MEM with 10% FCS was added and the diluted suspension was seeded into several flasks containing more of this medium. Twenty-four hours after cell fusion, this medium was exchanged for the HAT medium. Ten days later, hybrid clones were picked out in HT (HAT without aminopterin) using a ring technique and then grown on in MEM with 10% FCS.

2. Cell fusion between the microcells of B82HTQ2 and whole PG19

Approximately 3×10^6 PG19 cells were mixed with 3×10^6 of the microcells. The cell fusion and the hybrid clone procedure were carried out as described above.

Chromosome analysis

The cells were pretreated with 0.05–0.1 μg colcemid per ml for 40–60 min, exposed to 0.075 M potassium chloride as hypotonic treatment for 13 min and then fixed in 3:1 methanol/glacial acetic acid. After 60–120 min, air-dried preparations were made on precleaned slides. These slides were aged for 3–6 days before Giemsa banding, using a modification of the combined ASG/trypsin technique described by Gallimore and Richardson (1973). This involved incubation in 2×SSC at 63–65 C° for 40–70 min, drying through methanol, treating with 0.02% trypsin (Difco 1:250) in PBS for 80–100 s at 4 C° or for 40 s at room temperature, washing in tap water, and staining for 4–6 min in a 1:5 solution of R66 Giemsa (Gurr) made up in 0.005 M phosphate buffer at pH 6.8. The banded chromosomes were identified, using the system recommended by the Committee on Standardized Genetic Nomenclature for Mice (1972).

Tests for the stability of the transferred B82HTQ2 chromosomes in the hybrid in vitro and in vivo and their ability to survive in HAT medium

For the in vitro test, the hybrid cells were continually subcultured for six months in MEM and then analysed cytogenetically. They were then subcultured in HAT medium. For in vivo testing, the hybrid cells were washed in PBS at least four times and then injected subcutaneously into the subcapular region of nude mice. New born, syngenetic ($C_3H\times C_{57}BL_6$) mice were sublethally irradiated at the rate of $1-8\times10^6$ cells per mouse. When tumors had grown to approximately 1 cm diameter they were explanted and subcultured in MEM. The cultures were analysed cytogenetically by conventional chromosome counting and by G-banding. They were then subcultured in HAT.

Scanning electron microscopy

A cell fusion of B82HTQ2 microcells and PG19 cells was prepared as previously described. The mixture of cell fusion was incubated in MEM (10% FCS) at 4 C° in 2.5% glutaraldehyde (pH 7.4) containing 0.1 M sodium cacodylate and 0.1 M sucrose and allowed to settle overnight onto poly-I-lysine treated coverslips at 4 C°. They were then dehydrated in a graded alcohol series, transferred to liquid carbon dioxide and critical point dried. The attached cells were finally sputter-coated with a layer of gold and examined in a Jeol 100CX microscope operated at 40 KV.

Identification of histocompatibility antigen $H\text{-}2^k$ of the B82HTQ2

The parental lines and the hybrid cells were subcultured in MEM with 10% FCS for two days, detached with 0.02% EDTA, washed three times in PBS and twice in DAB/2% NCS/azide. Each sample was divided into three aliquots and centrifuged at 1,500 rpm for 5 min. These cell pellets were incubated for 1 h at 4 °C with a 50 μl volume of either monoclonal anti-$H2^k$ antibody (antibody 27/R9), monocloned anti-rat thymocyte antibody (antibody W3/25) or with medium DAB/2% NCS/10 mM azide. They were then further incubated for 1 h at 4 °C with 50 μl RAM-Fitc containing 10% normal rat serum (Williams et al. 1977). They were finally washed twice, resuspended in medium and examined in a fluorescence-activated cell sorter (Becton Dickinson FACS II). (All the H-2 tests were kindly provided by Dr. D. W. Mason, Oxford University, Immunology Unit, UK.)

SCE frequency of the hybrid cells and their sensitivity to MMC

PG19 and hybrid M58-1 cells were subcultured in MEM containing 10% new born calf serum (NCS) at 37 °C for 24 h, MMC (Kyowa, Japan) was then added into the culture medium to obtain a final concentration of 3, 10 and 40 ng/ml for 2 h. BrdU (Sigma) was then added to a final concentration of 30 μg/ml for another 48 h under dark conditions. The chromosome preparation and the SCE analysis were performed as described in our previous paper (Yan Yongshan et al. 1985). Examination of spontaneous SCE frequencies was carried out as above except that MMC was not added into the medium.

Results

Microcells can be induced in B82HTQ2 cell cultures by colcemid but the treatment has to be controlled since its addition causes cells to progressively detach from the flask surface. After 96 h few cells remain. It was found that 0.3 μg/ml for 38–40 h or 3.0 μg/ml for 24 h produced a sufficient number of microcells before detachment became a problem. The method of separation and purification used in the experiment ensured that only pure microcells were included in the gradient fraction subsequently used for cell fusion. For example, Fig. 1 illustrates the comparative nuclear size differences in a sample of the population obtained from the upper 15–18 ml fraction as against that obtained from the bottom of the gradient after a 3.5 h unit gravity separation at room temperature. Similarly, Table 1 shows the distribution of microcells recovered from

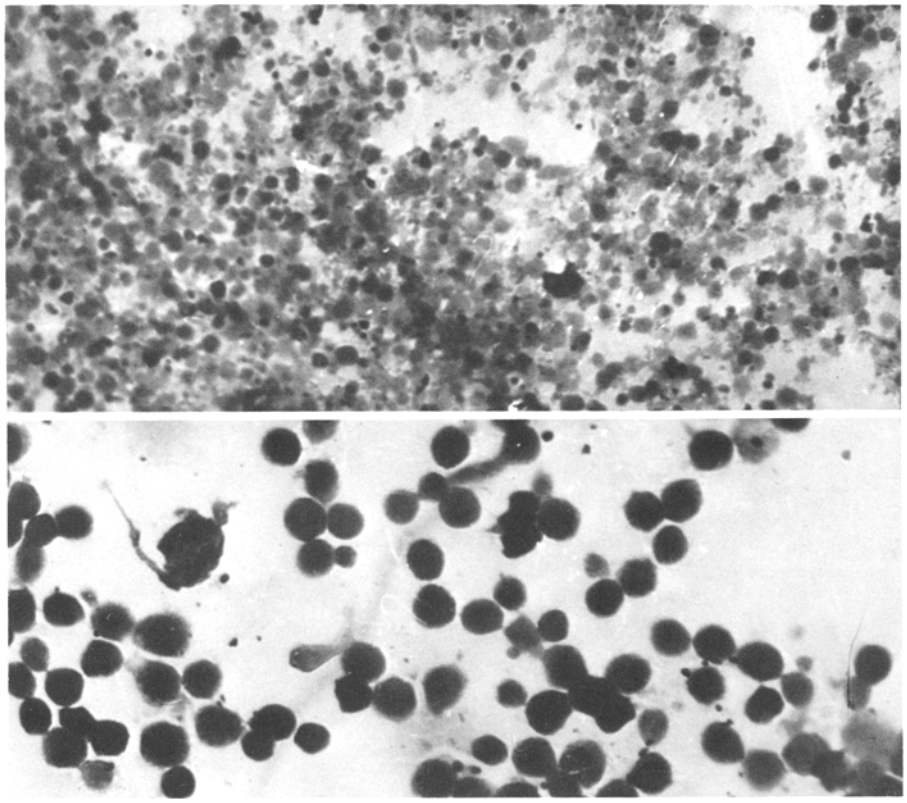


Fig. 1. Pure microcells of B82HTQ2 cells (*upper*) and their nuclei collected from bottom of a 1–3% BSA gradient

Table 1. Gradient fractions of 1–3% BSA for microcells (4ml/tube)

Tube (no.) from top of gradient	Microcells	Mini cells (karyoplasts)
1	+	–
2	+ +	–
3	+ +	–
4	+ + +	–
5	+ + +	+
6	+	+ +
7	+	+ +

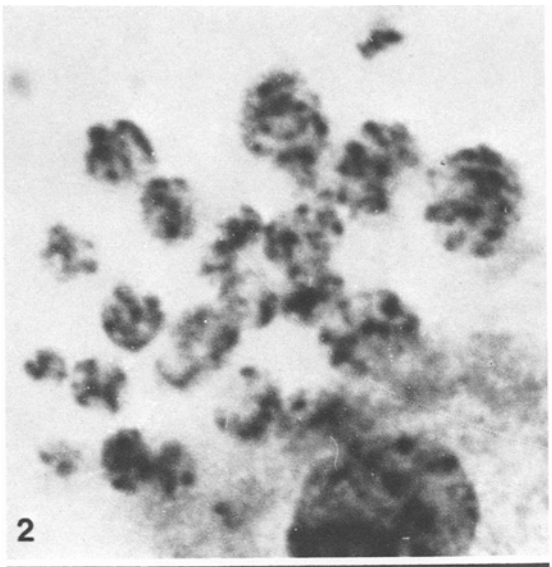
In 40 fields (300× microscope) no microcell or mini cell could be found: –; 1–20 microcells or mini cells/field +; 21–50/field + +; > 50/field + + +

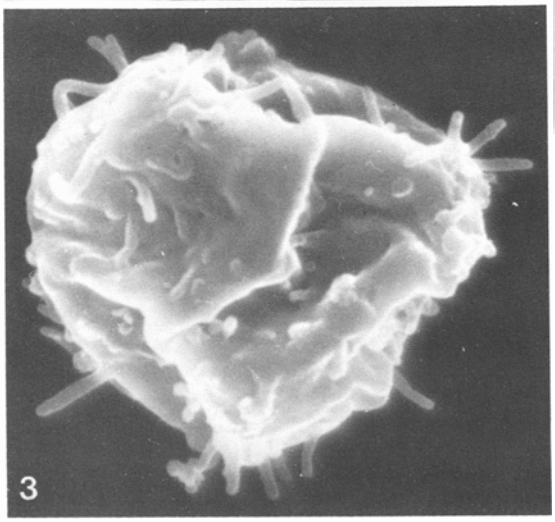
sheer descending fractions after a 7 h unit gravity at 4 °C.

The pure microcells (Fig. 1) contained some cytoplasm and stained either intensely or weakly with Giemsa. The cast majority were observed to be in the interphase stage and in a sample of several hundred scored, only two were seen to have one, two or a group of chromosomes surrounded by a nuclear membrane (Fig. 2). Fine details of the microcell surface were clearly observed by scanning electron microscopy; they were seen to be totally enveloped by an intact cytoplasmic membrane with fingerlike protuberances (Fig. 3) or microvilli similar to those found in intact cells.

One month after the fusion of the B82HTQ2 microcells to whole PG19 cells, five HAT-resistant clones were recovered. All five exhibited the fibroblast morphology of PG19, which differs markedly from the epithelial one of B82HTQ2 (Fig. 4). One of those clones was selected for study and called M58-1.

In this experiment the mean chromosome number of the parent cell lines was found to be 38 for PG19 and 49 for B82HTQ2 whereas that in M58-1 was 41 and for the PG19/B82HTQ2 whole cell fusion it was 88, the sum of the two (Fig. 6). G-banding of M58-1 cells (Fig. 5) revealed the expected complement of an whole PG19 cell together with three additional chromosomes named as B1 (a centric fusion between chromosome 5 and 14), B2 (a centric fusion between chromosome X and 19) and B3 (a centric fusion between chromosomes 19). The G-banding of the chromosomes also showed that PG19 cells have a karyotype of X0 (Fig. 5) the same as that reported in previous paper (Jonasson et al. 1977). M58-1 cells, even after six months in continuous subculture in MEM, retained its mean chromosome number (Fig. 7) and moreover, in the G-banded sample analysed, the frequencies of B1, B2 and B3 chromosomes in M58-1 cells were very high, at a range





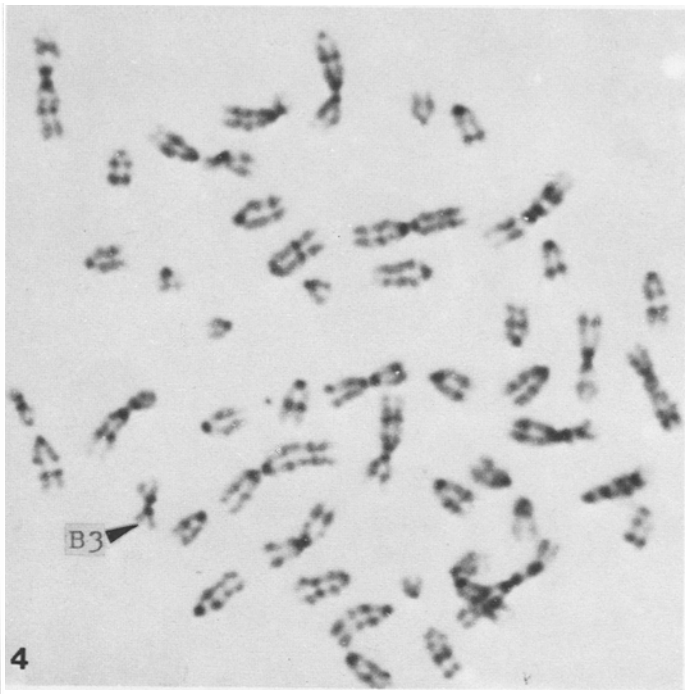


Fig. 2. Microcell containing one, two or a group of chromosomes

Fig. 3. Photograph from a scanning electron microscope of a B82HTQ2 microcell (×7,500)

Fig. 4. G-banding pattern of B82HTQ2 cells

Table 2. Stability of B1, B2 and B3 chromosomes in induced tumors

Tumors	B1	B2	B3	Survival in HAT
T1	+	–	+	+
T4	+	+	+	+
T7	+	–	+	+
T7-2	+	+	+	+
T7-3	+	+	+	+
T8	+	+	+	+
T10	+	–	+	+
T13	+	+	+	+

+ either cells had the chromosomes mentioned in the table or cells could grow in HAT, otherwise as –

of 97–99%. The hybrid cells were also found to grow well in HAT medium. None of PG19 cells could grow in HAT medium.

Tumors arose in $C_3H \times C_{57}BL/6$ approximately six weeks after injecting the M58-1 cells. Nine tumors were explanted from the nine mice mentioned above, and were named T1, T4, T7, T7-2, T7-3, T8, T10, T6 and T13. Two of them retained the same chromosome number as the injected cells, the remainder showed a loss of between one and three chromosomes (Fig. 7). All tumors maintained their ability to grow in HAT. G-banding analysis showed that six of the tumors had kept all the three chromosomes considered to be of B82HTQ2 origin but the remainder had lost B2 chromosome (Table 2).

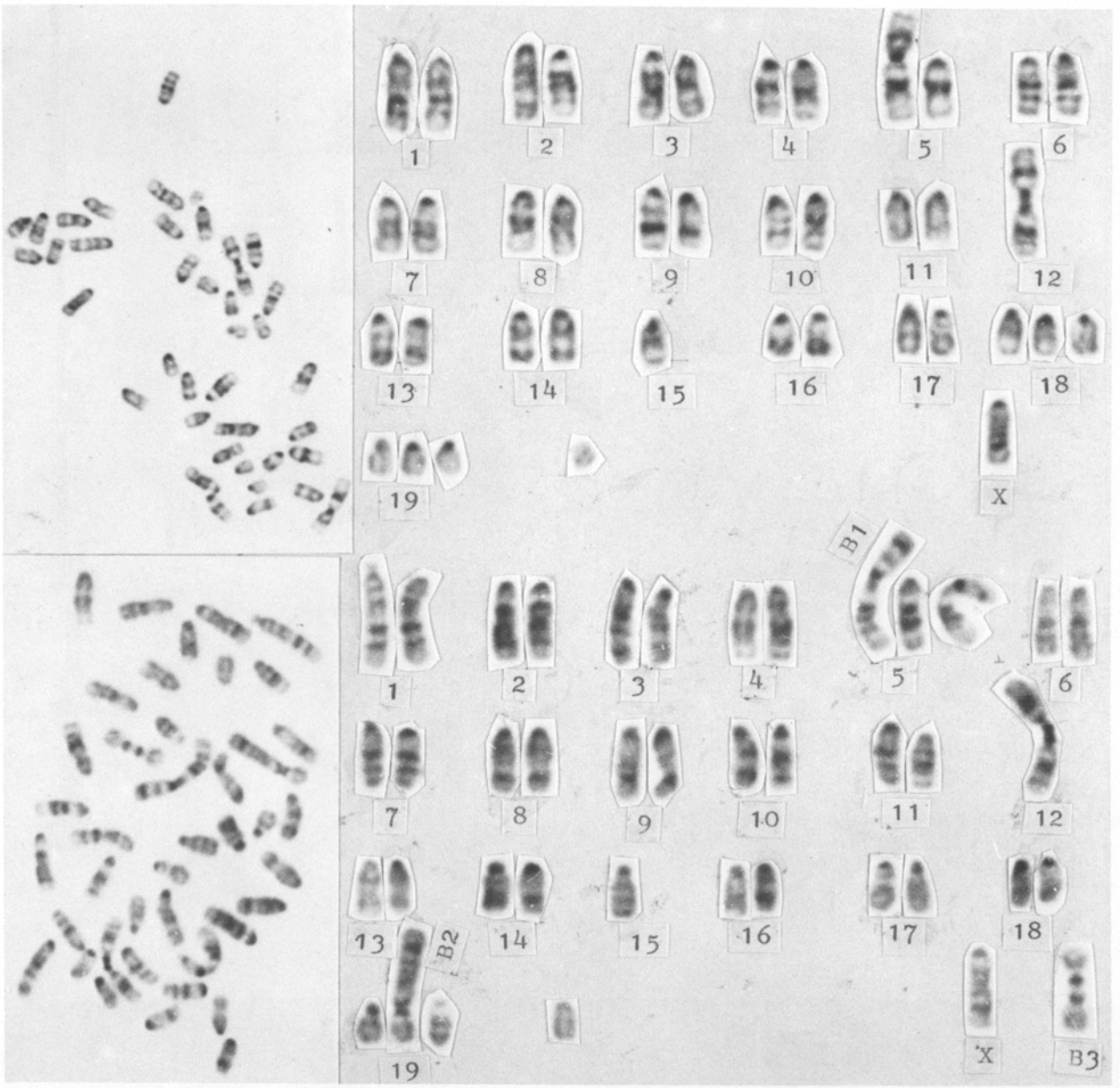


Fig. 5. G-banding patterns of PG19 cells (*upper*) and M58-1 cells (*lower*)

The cell sorter fluorescence histograms obtained from the analysis of the major histocompatibility complex, H-2, are shown in Fig. 8. It is clear that B82HTQ2 cells carry the antigen H-2^k, M58-1 and PG19 do not carry antigen H-2^k. PG19 and M58-1 cells show a tendency to bind immunoglobulin non-specifically.

The spontaneous SCE frequencies and the sensitivity to MMC of both PG19 and M58-1 cells are shown in Fig. 9–10. There is no significant difference between PG19 and M58-1 cells in both spontaneous SCE frequencies and the sensitivity to MMC.

Discussion

Micronucleated cell induction increases with length of colcemid treatment but at the same time there is a decrease in the number of intact cells adhering to the bottom of the flask. In this experiment, 0.3 μg/ml of colcemid for 38–40 h at 37 C° gave optimal conditions for producing large numbers of viable microcells in the B82HTQ2 cell line. The finding of a few microcells containing one, two or a small group of chromosomes provides cytological proof for the nuclear contents of

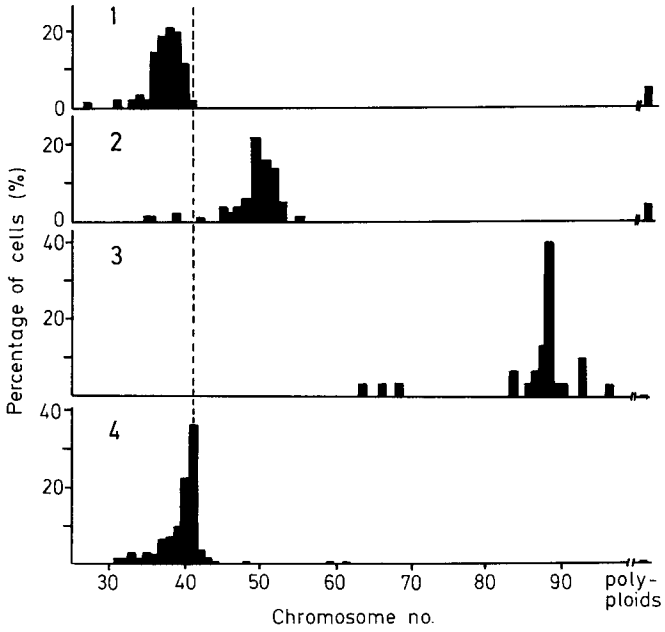


Fig. 6. Chromosome distribution for PG19, B82HTQ2 and their hybrids at the 2nd passage. *1* PG19; *2* B82HTQ2; *3* Hybrid derived from the whole cell fusion; *4* M58-1

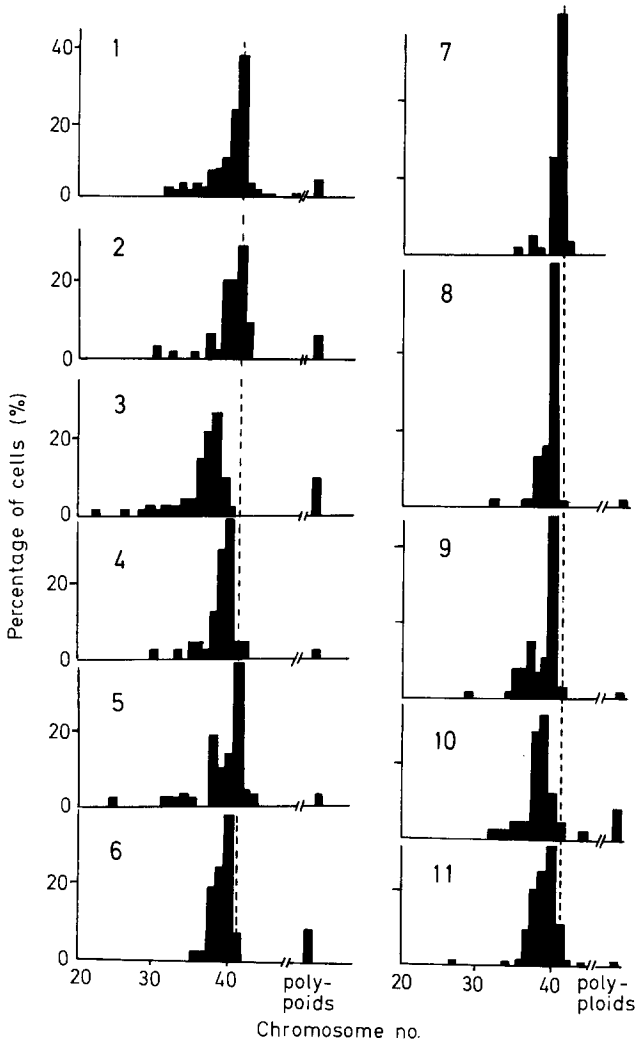


Fig. 7. Chromosome distribution for M58-1 cells *1* M58-1 (at 2nd passage); *2* M58-1 subcultured in vitro for six months; *3–11* Induced tumors T1, T7, T3, T7-2, T7-3, T4, T8 and T6, respectively

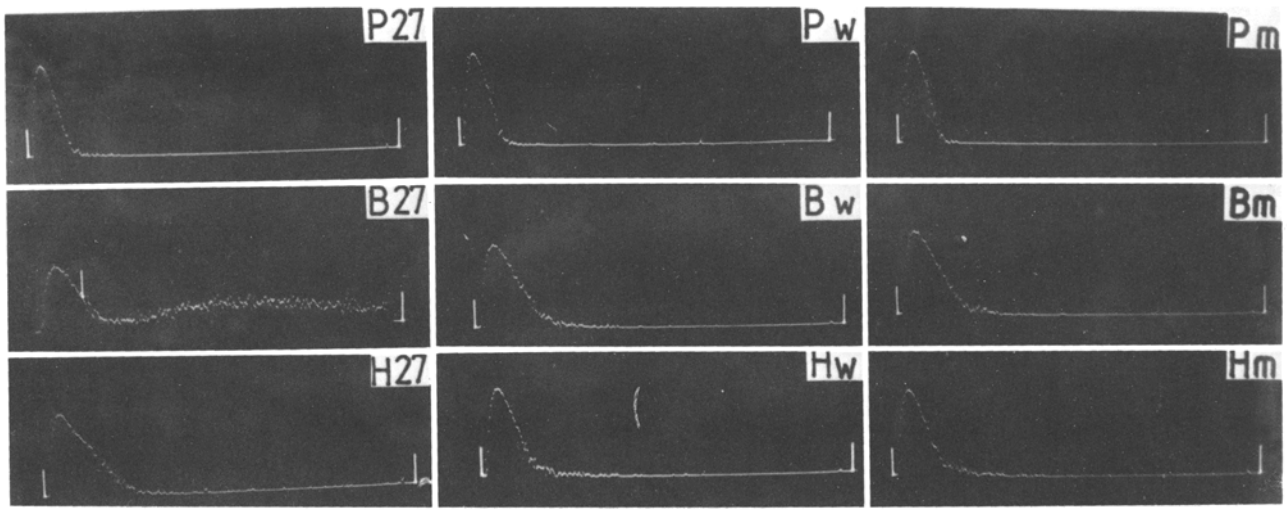


Fig. 8. Photograph of a Cell Sorter for the H-2^k antigen. *Abscissa:* fluorescence intensity/cell; *Ordinate:* logarithm of cell number. P27, Pw, Pm = PG19 cells plus H-2^k (27), W3/25, medium, respecitvely. B27, Bw, Bm = B82HTQ2 cells plus H-2^k (27), W3/35, medium, respecitively. H27, Hw Hm = M58-1 cells plus H-2^k (27), W3/25, medium, respectively

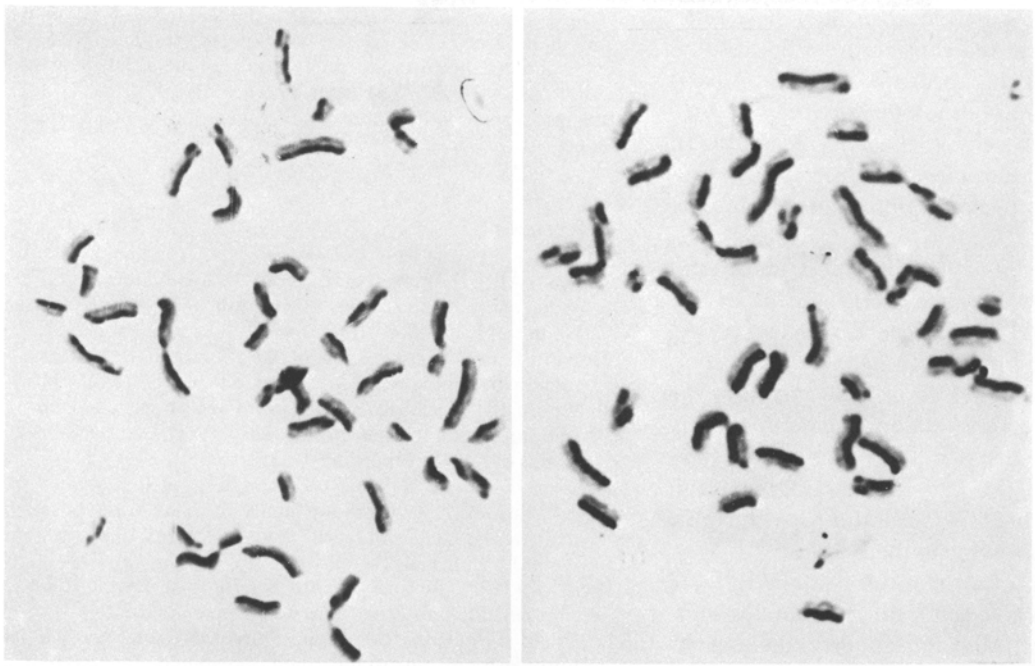


Fig. 9. SCE patterns of PG19 cells (*left*) and M58-1 cells (*right*)

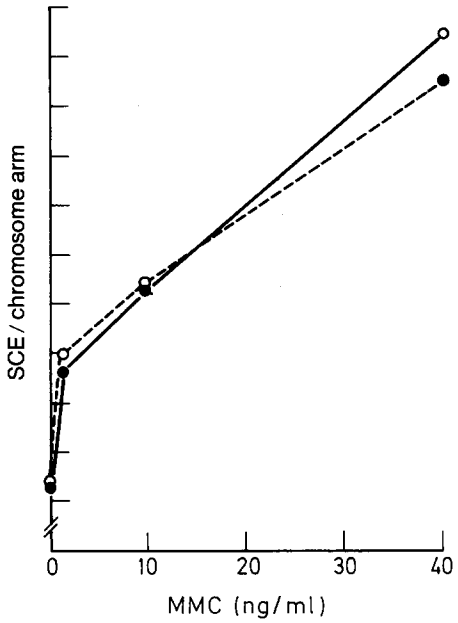


Fig. 10. Spontaneous SCE frequency and sensitivity to MMC for PG19 and M58-1 cells. ●——● PG19; ×- - - - × M58-1

microcells and supports the microspectrophotometer DNA determination of Ege and Ringertz (1976) in which DNA values equivalent to a content of one or two chromosomes were recorded.

As mentioned above, chromosome segregation and loss of the intraspecific hybrids are very slow. However, M58-1 cells at secondary passage have only 41 chromosomes and should derive from the fusion between a B82HTQ2 microcell and a PG19 cell, not from while cell fusion. The ability of this hybrid to grow in HAT implied that one of the three was an X chromosome, or part of an X chromosome, because the gene in the mouse coding for HGPRT is X linked (Epstein 1972; Jonasson et al. 1977). Indeed, G-banded analysis of M58-1 cells confirmed the presence of two X chromosomes, one of which fused at it's centromere to a chromosome 19 (in B2 chromosome). Since the PG19 cell line has a karyotype of X0 and has not produced a single revertant in several years (Jonasson et al. 1977 and our results here), one of the two X chromosomes found in M58-1 cells, doubtlessly, came from a microcell of B82HTQ2 cells. The transferred X chromosome restored a positive HGPRT status. Previous work (Jonasson et al. 1977) and our observations demonstrated that the PG19 cell has two chromosomes no. 14, and there are three chromosomes no. 5 in quite a number of PG19 cells. In M58-1 cells, however, there are three chromosomes no. 14, one of which fused to a chromosome no. 5 (in B1 chromosome). The chromosome no. 14 in B1 could come from the B82HTQ2 microcell. The B3 chromosome is same as that found in B82HTQ2 cells (Fig. 4). Our results thus show that three chromosomes of B82HTQ2 cells were transferred into a PG19 cell by means of microcell fusion.

The three transferred chromosomes are stable in both continuous culture in vitro and tumor testing in mice, and also when subjected to growth in HAT medium. It is clear that there are three ways for transferred

exogenous chromosomes stabilized in their host cells: in most cases the transferred chromosomes do not fuse to the chromosomes of the host cells; secondarly parts of the transferred chromosomes integrate into the chromosomes of the host cells (Sekiguchi et al. 1975); thirdly, the transferred chromosomes fuse to the chromosomes of the host (e.g. in present paper the B1 chromosome).

Since PG19 cells can grow in C_3H mice and B82HTQ2 cells in $C_{57}BL/6$ mice, PG19 and B82HTQ2 cells carry H-2^b and H-2^k, respectively (Klein 1975). The results from the analysis of H-2 show that in common with PG19 cells, the hybrid cells do not carry the H-2^k antigen. In mice, this major histocompatibility complex is located in the middle portion of chromosome no. 17 (Klein 1975), and in view of the result obtained, the original B82HTQ2 cell did not contribute any copies of this chromosome to the hybrid.

Through the fusion of a microcell containing three chromosomes with a whole cell leading to the transmission of one genetic trait and not another, this experiment demonstrates the potential of introducing small amounts of an exogeneous genome into recipient cells without the transfer of the gene coding for the histocompatibility antigen. This method could prove useful for the correction of genetic defects, the control of cellular gene expression and in studies concerned with graft acceptance.

SCE represents the interchange of DNA replication products at apparently homologous loci within a replicating chromosome. As is well known, MMC is one of DNA cross-linking agents. Most of damage caused by MMC to chromosomal DNA is obviously repaired without detectable changes in chromosome morphology. SCE contains information about both chromosomal DNA breakage and repair. MMC may reflect the action of basic cellular DNA repair procedures (Latt 1974; Carrano et al. 1978). There is no significant difference in both spontaneous SCE frequency and sensitivity to MMC measured by SCE frequencies between PG19 and M58-1 cells that might indicate that the chromosomes of M58-1 cells are quite stable.

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