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# CHROMOSOMALLY DEPLETED INTERSPECIFIC HYBRID CELL CLONES SELECTED WITH CYTOTOXIC ANTISERA: Utilization in the Study of Control of Murine Leukemia Virus Host-Range

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A chromosomally stable mouse-Chinese hamster hybrid cell line was subjected to five rounds of selection with cytotoxic antisera raised in rabbits against either the parental mouse 3T3 cells or the parental Chinese hamster Wg-1 cells. Routine karyological analysis of clones isolated at each stage of serum selection revealed that treatment with either serum resulted in a limited loss of chromosomes (compared to the untreated hybrid cell cultured in parallel) and that the pattern of chromosome loss could not be correlated with the particular antiserum used for selection. However, more detailed analysis with the SSC-formamide C-banding technique, which identifies chromosomes containing a mouse centromere region, demonstrated that while large-scale chromosome loss was not achieved as a result of antiserum selection, the limited loss of chromosomes did, in fact, reflect a specific depletion of chromosomes in response to treatment with cytotoxic antiserum. Specific chromosomal elimination was shown to occur as early as the first round of antiserum treatment. Antigenic analysis of the serum-selected clones revealed a quantitative decrease in the expression of the species-specific surface antigens selected against, but no qualitative loss of antigens was detected. The results suggest that treatment with cytotoxic antiserum may select for clones that have lost specific chromosomes bearing genes regulating the expression of species-specific surface antigens, rather than for those demonstrating large-scale depletion of chromosomes bearing the corresponding structural genes. Some of these chromosomally depleted hybrid cell clones have been used (along with pseudotype viruses containing the genome of vesicular stomatitis virus within the envelope of murine leukemia virus, VSV [MuLV]), to study the mechanisms regulating MuLV replication in Chinese hamster cells. The results indicate that the restriction of MuLV replication in Chinese hamster cells operates at two levels: (a) an inability to adsorb to or penetrate Chinese hamster cells; and (b) an additional intracellular block which is dominant in the mouse-Chinese hamster hybrid cell clones examined. This latter block is presently under study.

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## INTRODUCTION

One of the features of interspecific hybrids which has greatly contributed to their usefulness in a wide variety of investigations has been the observation that, in certain species combinations, chromosomes of one parent tend to be preferentially lost with continued growth in vitro, thus allowing close study of a small number of chromosomes of this parent in a genetic environment dominated by the other parent. This property has been exploited to greatest advantage in gene-mapping studies (1). Nevertheless, recent work indicates that even in those cases where preferential chromosome loss appears most strongly established, such as the depletion of human chromosomes in human-mouse hybrid cells (2, 3), this property need not be universal (4–6). Furthermore, with some cell combinations, different authors have reported conflicting preferential chromosome loss; thus, mouse-Chinese hamster hybrids have been reported as having a tendency preferentially to lose either mouse (7), or hamster (8–11), or neither (12, 13) set of chromosomes. For obvious reasons, it would be highly desirable to have available techniques for the controlled selective depletion of chromosomes of one parental type in interspecific hybrids which demonstrate little spontaneous loss, or for reversing the preferential direction of elimination in hybrids in which chromosome loss is usually observed. Techniques effective in the former case have been described, including the concomitant loss of chromosomes accompanying the specific elimination of a chromosome metabolically selected against (13), as well as the preferential loss of chromosomes of the parent subjected to X- or $\gamma$-irradiation or bromodeoxyuridine (BUdR)–labeling before fusion (12). Note, however, that irradiation of mouse cells prior to fusion with human cells was not effective in abrogating the usual pattern of human chromosome elimination (14).

Preliminary experiments previously reported (15) suggested the possibility of using specific cytotoxic antiserum as a selective procedure for the elimination of chromosomes of either parent in interspecific hybrids. Given the potential widespread applicability of such a technique, as well as our interest in the surface properties of somatic cell hybrids, we have attempted to confirm and extend this approach with a line of mouse-Chinese hamster hybrid cells which had a stable karyotype with little depletion of chromosomes of either parent. The serum-selected clones obtained have, in fact, been shown to contain fewer chromosomes of the parental type selected against. Furthermore, this specific elimination of chromosomes was accompanied by the quantitatively decreased expression of the species-specific surface antigens selected against. Lastly, two of these serum-selected clones, as well as the parental cells and the original unselected hybrid cell line, have been utilized in preliminary studies of the mechanism(s) involved in restricting the growth of Moloney murine leukemia virus (M-MuLV) in Chinese hamster cells.

## METHODS

### Cells

The parental mouse cells, 3T3 TK$^-$ (thymidine kinase deficient), resistant to $10^{-4}$ M BUdR, are clonal derivatives of mouse 3T3 cells (16), and were received from Dr. G. Marin, Laboratory of Molecular Embryology, Naples, Italy. The parental Chinese hamster cells, Wg-1 HGPRT$^-$ (hypoxanthine-guanine phosphoribosyl transferase deficient), resistant to $10^{-5}$ M 6-thioguanine (TG), are a subclone of the DON Wg-3 HGPRT$^-$ cell

line originally isolated by Westerveld et al. (17), and were obtained from Professor G. Pontecorvo at this institute. The Wg-1 X 3T3 stable hybrid cell line, X13 RS2a (14), was subsequently subcloned (G. Pontecorvo, unpublished observations), and one such subclone, HYB 50(2)B, was studied in detail.

### Cell Culture

3T3 $TK^-$ cells were maintained in Dulbecco's modification of Eagle's medium (18) supplemented with 10% heat-inactivated calf serum ($DC_{10}$), antibiotics, and $10^{-4}$ M BUdR. Wg-1 $HGPRT^-$ cells were grown in $DC_{10}$ supplemented with $10^{-5}$ M TG, and HYB 50(2)B in either $DF_{10}$ (10% fetal calf serum) or HAT medium (19), consisting of $DF_{20}$ supplemented with $10^{-4}$ M hypoxanthine, $10^{-6}$ M methotrexate (or aminopterin), $4 \times 10^{-5}$ M thymidine, and $10^{-5}$ M glycine, which selects for cells containing both TK and HGPRT.

### Rabbit Antisera

Antisera were raised against the 3T3 $TK^-$ mouse cells and the Wg-1 $HGPRT^-$ Chinese hamster cells in New Zealand white rabbits by a modification of a previously described protocol (15). Cells used for immunization were harvested with 0.02% ethylene diaminetetraacetate (EDTA), washed once with phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Rabbits were inoculated in four subcutaneous (sc) sites six times at weekly intervals with between 2 and $20 \times 10^7$ cells per inoculation.

Animals were test bled 10 days after the last inoculation ("postimmune" sera), boosted sc with $5 \times 10^7$ cells after a further 4 weeks, and then bled again ("postboost" sera). Sera were collected aseptically, heated at 56°C for 30 min, and stored at –20°C. Before use, sera were absorbed twice with the heterologous parent cells (EDTA harvested, once PBS washed) at a ratio of $\geqslant 10^7$ cells/0.1 ml serum. Absorptions were carried out for 24 hr at 4°C. Additional serum absorptions will be described in Results.

### Serological Assays

**$^{51}$Cr-release cytotoxicity assay.** A modification of the procedure described by Sanderson (20) was used to titer the cytotoxic antisera. Briefly, target cells were harvested with EDTA and washed once with PBS, viability was ascertained by trypan blue exclusion, and the cells were resuspended in $DC_5$ to $10^7$ viable cells/ml. Labeling was carried out with 100 $\mu$Ci $^{51}$Cr (Radiochemical Center, Amersham, Bucks, England; 100–300 mCi/mg)/$10^7$ viable cells/ml for 45 min at 37°C in a $CO_2$ incubator. The cells were then washed three times with $DC_5$, viability was determined again, and the cells were resuspended in $DC_5$ to $2 \times 10^6$ viable cells/ml and placed on ice. 0.1 ml of this cell suspension was mixed with 0.1 ml of appropriate serum dilutions and 0.1 ml of guinea pig complement (1/10 dilution of guinea pig serum in Veronal-buffered saline [Oxoid Ltd., London, England] supplemented with 0.1% bovine serum albumin [VBS-BSA] ). Controls without serum or C′ were brought up to volume with VBS-BSA. Maximum release of $^{51}$Cr was determined by adding 0.2 ml of 1% SDS to a 0.1-ml aliquot of the cell suspension. All mixtures were incubated for 30 min at 37°C after which 1 ml of cold $DC_5$ was added to each tube and the cells were pelleted. The radioactivity in 1-ml aliquots of the supernatant was then determined in a Nuclear-Enterprises (Reading, England) $\gamma$-irradiation counter. The end point is taken as the serum dilution yielding 10% specific release (21), where specific

release = experimental –C′ control/SDS maximum release –C′ control.

**Membrane immunofluorescence (MIF).** The indirect MIF procedure has already been described in detail elsewhere (22). Target cells were harvested with EDTA, and rabbit serum and fluorescein-conjugated goat anti-rabbit γ-globulin (Cappel Laboratories, Downington, Pa.) were used at dilutions of 1:4 and 1:10, respectively. Results are expressed in terms of a fluorescence index (FI), with staining considered significant when the FI $\geqslant 0.20$ ([22]; see footnote 1, Table III).

**Colony inhibition assay.** The method utilized has been previously described (15). In brief, cells were harvested with EDTA, washed twice with VBS-BSA and resuspended to $10^6$ cells/ml in VBS-BSA. 0.1 ml of the cell suspension was then incubated with 0.1 ml of the appropriately diluted antiserum for 20 min at 37°C in a shaking waterbath, followed by the addition of 0.8 ml of guinea pig C′ (1:10 dilution) and a further incubation for 1 hr at 37°C. At completion of this incubation, 0.2-ml aliquots from each sample were plated in 50-mm dishes (three dishes per sample) in $DF_5$ and colonies were fixed, stained, and counted from 7 to 14 days after seeding.

### Selection of Hybrid Cells Resistant to Cytotoxic Antiserum

A slightly modified colony inhibition assay was used in order to select serum-resistant hybrids, with between 0.1 and 0.3 ml of the final reaction mixtures being plated in 50-mm tissue culture dishes (NUNC, Roskilde, Denmark). Five cycles of serum selection were performed with each antiserum with 2–4 surviving colonies picked after each treatment (see Results). At every round of selection, no more than one colony was picked from any plate so as to ensure the independence of each clone. The cloned survivors were grown in $DF_5$, their resistance to HAT medium was confirmed, and then they were stored in liquid nitrogen. For identifying selected clones, M and H indicate selection with anti-3T3 (mouse) or anti-Wg-1 (hamster) antisera, respectively, the number of cycles of antiserum treatment is denoted by the following digit, and the individual clone is identified by a small letter; e.g., H-4-b represent clone b isolated after four cycles of selection of HYB 50(2)B with anti-Wg-1 serum. The complete selection procedure is presented in detail in Results (see Fig. 4).

### Chromosome Analysis

The procedure for general karyotyping has been described elsewhere (23); 15–40 metaphases were examined for each cell clone. A modification of the C-banding technique of Dev et al. (24), which specifically stains mouse centromeres, was used to identify mouse chromosomes, as has already been reported for interspecific hybrids between mouse and a variety of other cell types (25, 26). Briefly, cells at approximately 50% confluence were incubated for 40 min in medium containing 0.1 μg/ml colcemid (Grand Island Biological Co., Slough, England), collected with 0.25% trypsin, treated for 8 min with 0.07 M KCl, and fixed, by centrifugation, in three changes of 3:1 (v/v) methanol-acetic acid. The cell suspension was then droppered onto clean dry slides and air dried. One to seven days after preparation, slides were incubated for 7.5 min at 65°C in a solution of 95 ml formamide (neutralized with concentrated HCl):5 ml of 20 × SSC(3.0 M NaCl, 0.3 M $Na_3C_6H_5O_7$), pH 7.0, rinsed in tap water, and stained for 40 min in 10% Gurr's Giemsa

R66 in Gurr's pH 6.8 buffer (Gurr, Searle Diagnostic, High Wycombe, England). Metaphases (between 15 and 30 for each cell line) were examined double blind to avoid bias, and chromosomes were classified as telocentric or metacentric and as mouse (C-band at centromere) or hamster (no C-band) (see Figs. 1–3). Statistical analysis was performed on the differences between the means using the Student's t test.

### Viruses

The Moloney strain of murine leukemia virus (M-MuLV) was a gift of Dr. J. Hartley, National Institutes of Health, Bethesda, Md. Stocks of this virus were prepared on either BALB/3T3 cells (clone A31), or on the mouse cell line SC-1, kindly provided by Drs. W. P. Rowe and J. Hartley. Leukemia virus titers were determined by the XC plaque test (27). Wild-type vesicular stomatitis virus (VSV) was kindly provided by Dr. J. Zavada, Czechoslovakia Academy of Science, Prague.

It has recently been shown (28–32) that superinfection by VSV of cells infected with either avian or murine leukemia viruses yields a small proportion of progeny VSV that is resistant to neutralization by VSV antiserum. Members of this resistant fraction of VSV possessed the antigenicity, host range, and interference properties of the RNA tumor virus, and are called phenotypically mixed virions or pseudotypes. Because these pseudotypes have all the known envelope properties of the leukemia virus, they can be used to distinguish between extracellular and intracellular blocks to RNA tumor virus replication. To produce such VSV (MuLV) pseudotypes, secondary BALB/c mouse embryo cells (embryos taken at 15–18 days of gestation) were seeded in $DF_{10}$ at $2 \times 10^6$ cells/90-mm dish. The following day the cells were pretreated with DEAE-Dextran (25 $\mu$g/ml) for 1 hr. The DEAE-Dextran was then removed, and M-MuLV was added at a multiplicity of infection (moi) of 1. Three to four days later, the infected cells were transferred to fresh dishes and infected with VSV at an moi of 1. VSV was allowed to grow at 37°C for 12–18 hr, at which time the cells and medium were harvested and the cell debris was removed by low-speed centrifugation. The VSV plaque assay was performed as described previously (28). Wild-type VSV was neutralized with hyperimmune sheep antiserum supplied by Dr. J. Zavada (29). Neutralization was performed at 4°C for 15 hr with antiserum diluted 1:20 in PBS (a concentration which completely neutralizes high-titer stocks of wild-type VSV); controls were mixed with an equal volume of PBS.

### Materials

BUdR, TG, thymidine, and hypoxanthine were obtained from the Sigma Chemical Co., St. Louis, Mo., methotrexate was from Lederle Laboratories, Pearl River, N.Y., and calf and fetal calf serum were from Flow Laboratories, Irvine, Scotland.

## RESULTS

### Production of Rabbit Anti-3T3 and Anti-Wg-1 Antisera

Antisera were raised in rabbits against 3T3 $TK^-$ and Wg-1 $HGPRT^-$ parental cells by the protocol outlined in Methods. The titers of these sera, as assayed by colony inhibition and $^{51}Cr$-release cytotoxicity, are presented in Table I. Although none of the sera demonstrated any activity against the heterologous parent cell, all sera utilized for hybrid selection were nevertheless absorbed twice with the opposite parental cells. Because of their higher titer, the post boost sera 95 (anti-3T3) and 96 (anti-Wg-1) were used in

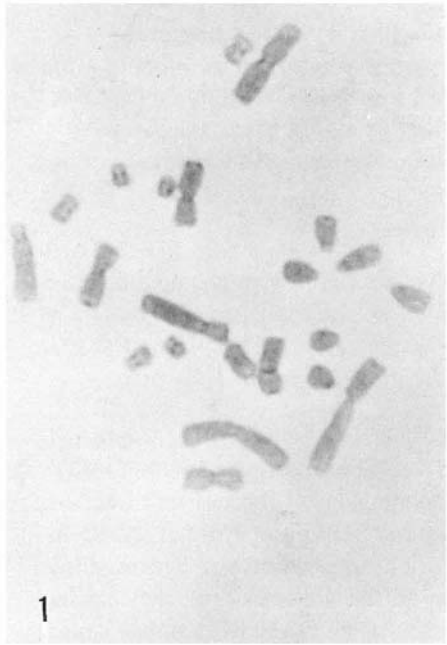


Fig. 1. A metaphase from a Wg-1 HGPRT$^-$ Chinese hamster cell treated with formamide-SSC and stained with Giemsa. The chromosomes are uniformly pale in staining with no differentiation of the centromere.

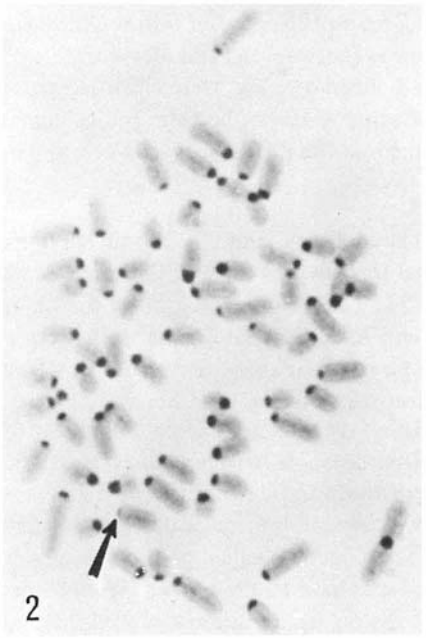


Fig. 2. A metaphase from a 3T3 TK$^-$ mouse cell treated with formamide-SSC and stained with Giemsa. Note the intensely stained centromere region. The arrow indicates a mouse chromosome with little C-banding material at the centromere.

subsequent hybrid selection experiments.

For antisera raised against xenogeneic cells, both sera 95 and 96 have relatively low cytotoxic activity. Nevertheless, at low serum dilutions (1:2–1:8) both demonstrated extensive killing of the specific target cells. To minimize cell survivors in the colony inhibition selection procedure, it was decided to use the sera at dilutions of 1:4–1:8 (usually 1:4). Preliminary tests with the HYB 50(2)B cells as targets confirmed the absence of any prozone effect at this high serum concentration, which could have resulted in artifically high target cell survival.

### Serum Selection of Mouse-Chinese Hamster Hybrid Cells

**Karyotypic analysis.** Sera 95 and 96 were used to select five rounds of mouse-Chinese hamster hybrid cell clones, beginning with the stable HYB 50(2)B cell line, as diagramed in Fig. 4. It can be seen that the degree of target cell killing decreased with subsequent cycles of serum selection, suggesting a decreased sensitivity of isolated survivor clones to the antisera with increasing serum treatments. To determine whether such a phenomenon was correlated with the loss of chromosomes of the appropriate type, i.e. of that parent type against which the antiserum treatment was directed, the parental cell lines, the initial hybrid, and all isolated survivor clones were karyotyped (Fig. 5). Several

points should be emphasized: (a) The nonselected parental HYB 50(2)B hybrid carried in culture for 9 months demonstrated relatively little spontaneous chromosome loss. (b) The major chromosome loss occurred during the first serum selection, with subsequent serum selections resulting in the gradual elimination of a small number of additional chromosomes, although marked chromosome loss also occurred during the third selection with anti-3T3 serum. (c) Chromosomes lost early were primarily telocentric, with metacentric chromosome elimination occurring mainly during the later serum selections. (d) Up to the third round of treatment, hybrids selected with anti-Wg-1 serum had slightly fewer chromosomes, but after this stage the pattern was reversed and the anti-3T3 serum-selected clones contained smaller numbers of chromosomes. (e) In general, the karyograms of the clones selected with the two different sera did not appear to differ markedly, but as will be discussed below, this conclusion, based upon general karyotypes, can be misleading. (f) Selection with anti-3T3 serum apparently led to conditions favoring chromosomal instability or lack of cytokinesis, as judged by the isolation of "2s" clones after the third round of anti-3T3 serum treatment, which were not seen in the late rounds of anti-Wg-1 selection (data not shown).

The above karyotypic analysis of the serum-selected hybrid clones clearly demonstrated that a large-scale specific depletion of chromosomes did not occur in response to treatment with either the antimouse or antihamster serum. Nevertheless, it was equally apparent that chromosome loss had occurred in the serum-selected clones which did not take place in the untreated HYB 50(2)B cells. This suggested that some species-specific chromosome loss may have occurred, but gross chromosomal analysis was not sensitive enough to detect this. The relatively similar karyotypes of the clones selected with either antimouse or antihamster serum would thus be coincidental and reflect masking of any small-scale specific chromosome elimination. To examine this point, the parental cells, the untreated HYB 50(2)B hybrid cells carried for 15 months in culture, and the clones obtained after one, three, and five selections with either serum were examined by the formamide-SSC C-banding technique in order to specifically identify the number of mouse and hamster chromosomes present. These results are presented in Table II.

The data indicate that although large-scale chromosome elimination was not achieved as a result of antiserum selection, the small-scale loss of chromosomes did, in fact, reflect the specific depletion of chromosomes in response to treatment with the appropriate cytotoxic antiserum. This is demonstrated by the statistically significant decrease ($P < 0.0005$) in mouse chromosomes after only one round of anti-3T3 treatment (M-1-a) relative to the first round anti-Wg-1-selected cells (H-1-a), as well as the significantly lower content ($P < 0.0005$) of hamster chromosomes of the latter. While it is clear that mouse chromosomes continued to be eliminated by subsequent treatments with anti-3T3 antiserum (compare M-1-a vs. M-3-a and M-5-c, $P < 0.0005$; M-3-a vs. M-5-c, $P < 0.05$), the situation was somewhat more complicated with the anti-Wg-1-selected clones, probably because of the higher spontaneous loss of hamster chromosomes (see below). Thus, the number of hamster chromosomes, which differed significantly between the antimouse and antihamster first round clones, were not significantly different at the third round (compare H-3-a vs. M-3-a); nevertheless, they were again significantly decreased by the fifth cycle of selection (H-5-a vs. M-5-c, $P < 0.0005$).

Several additional points deserve comment. The statistical analysis of the differences

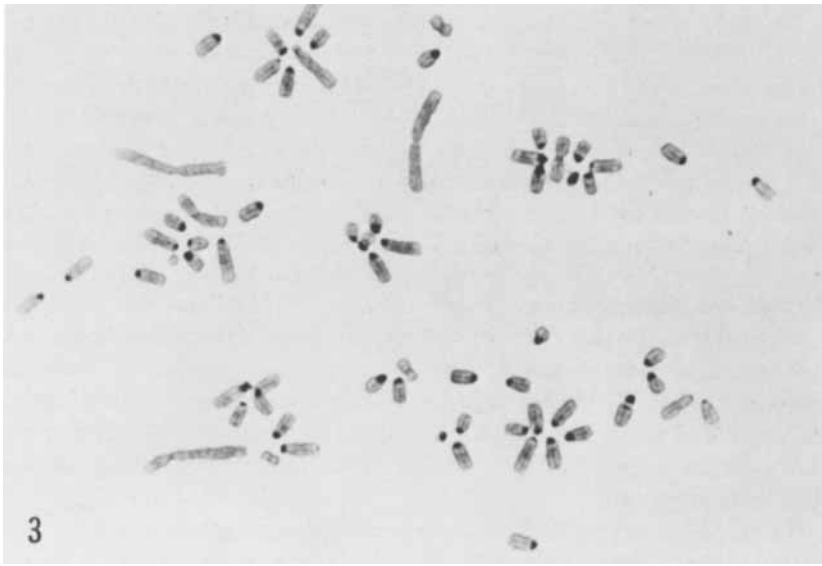


Fig. 3. A metaphase from a M-5-c mouse-Chinese hamster hybrid cell treated with formamide-SSC and stained with Giemsa. C-banding analysis indicates 52 mouse telocentric chromosomes, 5 hamster telocentrics, and 8 hamster metacentrics.

**TABLE I. Production of Rabbit Anti-3T3 and Anti-Wg-1 Antisera**

| Serum no. | Raised against | Description[1] | Target cell | Titer[2] Colony inhibition | Titer[2] $^{51}$Cr-release |
|---|---|---|---|---|---|
| 41 | – | Preimmune | 3T3 | < 1/2 | < 1/5 |
| | | | Wg-1 | < 1/2 | < 1/5 |
| 83 | 3T3 | Postimmune | HYB 50(2)B | 1/8 | – |
| | | | 3T3 | 1/15 | 1/18 |
| | | | Wg-1 | < 1/2 | < 1/5 |
| 95 | 3T3 | Postboost | HYB 50(2)B | 1/18 | – |
| | | | 3T3 | 1/32 | 1/36 |
| | | | Wg-1 | < 1/2 | < 1/5 |
| 42 | – | Preimmune | 3T3 | < 1/2 | < 1/5 |
| | | | Wg-1 | < 1/2 | < 1/5 |
| 84 | Wg-1 | Postimmune | HYB 50(2)B | 1/22 | – |
| | | | 3T3 | < 1/2 | < 1/5 |
| | | | Wg-1 | 1/25 | 1/35 |
| 96 | Wg-1 | Postboost | HYB 50(2)B | 1/30 | – |
| | | | 3T3 | < 1/2 | < 1/5 |
| | | | Wg-1 | 1/40 | 1/67 |

[1] See Methods for immunization procedure and for description of the end point of the $^{51}$Cr-release cytotoxicity assay.

[2] End point of colony inhibition assay equals dilution yielding 50% cell survival relative to control treated with C′alone.

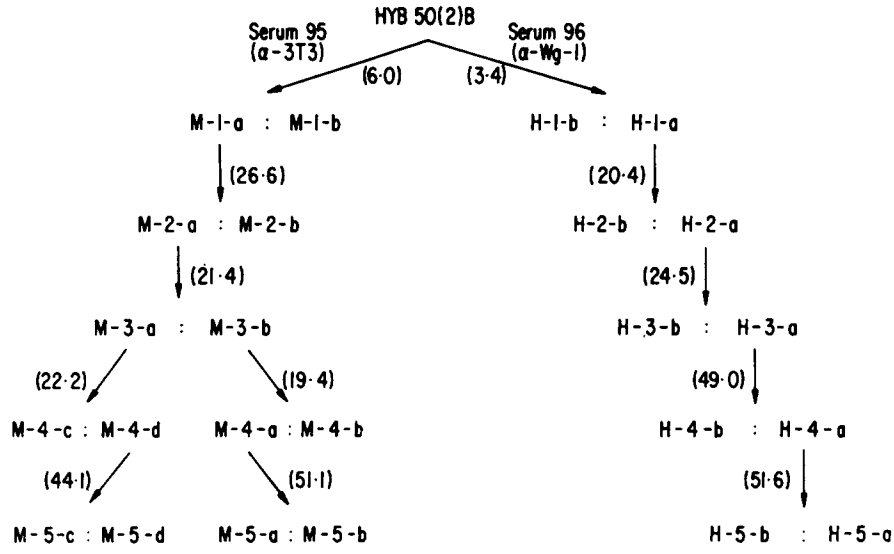


Fig. 4. Flow sheet diagraming derivation of serum-selected mouse-Chinese hamster hybrid clones. Numbers in parentheses represent percent cell survival in that round of selection, calculated on the basis of controls treated with normal rabbit serum + C′.

between the chromosomal constitution of the serum-selected clones and the untreated HYB 50(2)B cells presented in Table II is complicated by the fact that the first round selected clones, M-1-a and H-1-a, were examined by C-banding analysis after a very short period of in vitro culturing, while the third and fifth round selected clones had been in culture for a period comparable to the untreated control HYB 50(2)B cells (~15 months) before analysis by C-banding. Thus, the most valid comparisons of M-1-a and H-1-a are with each other, while the other serum-selected clones, which are better controlled for spontaneous chromosome loss due to long-term culturing, can be compared relative to each other and the untreated HYB 50(2)B cells.

For unknown reasons, treatment with either antiserum resulted in a significant nonspecific loss of hamster chromosomes (compare third and fifth round selected hybrids vs. control HYB 50(2)B cells; for all $P < 0.0005$), which also accounts for the significant drop in total chromosome count. In contrast, mouse chromosomes were lost only in response to antimouse serum treatment and not after antihamster antiserum selection. Note, however, that significant loss of mouse chromosomes did occur as a result of extended growth "in vitro" (compare HYB 50(2)B with H-1-a, $P < 0.0005$). The statistically significant increase in hamster metacentrics seen after five rounds of anti-3T3 serum selection relative to three rounds of treatment with this serum ($P < 0.01$) possibly reflects a chromosome duplication event, demonstrating one of the limitations of this approach. In addition, there may have been a slight overestimation of hamster telocentrics in the various hybrid cell clones, since approximately 40% of parental mouse 3T3 $TK^-$ cells contained 1–3 chromosomes (overall mean = 0.58 ± 0.31) lacking a C-band (Fig. 2, Table II);

**TABLE II. C-Banding Analysis of Parental Cells, Untreated Hybrid, and Serum-Selected Hybrid Cell Clones**

| Cell clone | Selected with serum no.[1] | No. of metaphases examined | Karyotype (Mean ± SD) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mouse | | | Hamster | | | Total | | |
| | | | Telo. | Meta. | Total | Telo. | Meta. | Total | Telo. | Meta. | Total |
| 3T3 TK$^-$ | – | 26 | 65.11±2.49[2] | 0.19±0.24 | 65.30±2.55 | – | – | – | 65.11±2.49[2] | 0.19±0.24 | 65.30±2.55 |
| Wg-1 HGPRT$^-$ | – | 28 | – | – | – | 5.54±0.16 | 16.50±0.18 | 22.04±0.09 | 5.54±0.16 | 16.50±0.18 | 22.04±0.09 |
| HYB 50(2)B (15 months) | – | 26 | 53.31±3.06 | 0.30±0.68 | 53.61±3.04 | 7.04±1.91 | 12.46±2.21 | 19.50±2.83 | 60.34±3.69 | 12.76±2.52 | 72.95±4.67 |
| M-1-a | 95 | 26 | 55.27±3.18 | 0.04±0.21 | 55.31±3.18 | 8.39±1.90 | 11.61±1.30 | 20.00±2.69 | 63.66±3.41 | 11.65±1.30 | 75.27±3.70 |
| H-1-a | 96 | 32 | 59.00±2.09 | – | 59.00±2.09 | 8.09±1.30 | 9.03±1.06 | 17.12±1.38 | 67.09±2.21 | 9.03±1.06 | 76.12±2.23 |
| M-3-a | 95 | 22 | 51.86±2.10 | 0.14±0.35 | 52.00±2.12 | 4.64±1.29 | 7.45±1.18 | 12.09±1.41 | 56.50±2.41 | 7.59±1.22 | 64.14±2.77 |
| H-3-a | 96 | 17 | 54.24±1.64 | – | 54.24±1.64 | 5.24±1.48 | 8.00±1.12 | 13.24±1.67 | 59.48±2.32 | 8.00±1.12 | 67.48±2.27 |
| M-5-c | 95 | 21 | 50.25±1.17 | 0.85±0.36 | 51.10±1.42 | 4.50±1.17 | 8.25±1.00 | 12.75±1.24 | 54.75±1.56 | 9.10±1.03 | 63.91±1.79 |
| H-5-a | 96 | 28 | 54.05±1.66 | 0.09±0.30 | 54.14±1.71 | 4.45±1.10 | 6.29±0.78 | 10.74±1.25 | 58.50±2.15 | 6.38±0.80 | 64.88±2.40 |

[1] 95 = anti-3T3 serum, 96 = anti-Wg-1 serum.

[2] Includes 0.58 ± 0.31 telocentrics not demonstrating C-band.

this could account for the higher number of (non-C-banded) telocentrics classified as hamster in the nontreated HYB 50(2)B hybrid than are present in the parental Wg-1 hamster cells. Nevertheless, the C-banding analysis serves to emphasize the danger of using only total chromosome counts in studies of this type and has shown that, despite a limited loss of chromosomes in response to cytotoxic serum, a portion of this elimination (on the order of approximately three chromosomes) is specific. This technique also allows the detection of a significant change which is restricted to a particular class of chromosomes, as illustrated by the antihamster selected H-5-a cells. Compared to the antimouse selected M-5-c cells, there is a significant decrease in hamster metacentrics ($P < 0.0005$), but no significant difference in hamster telocentrics (Table II). Note also that while the difference in total chromosome count is not statistically significant, this is an artifact caused by the significant differences in total telocentrics and total metacentrics (for both, $P < 0.0005$) being in opposite directions and thus canceling each other out. Furthermore, the C-banding analysis reveals that the significant depletion of chromosomes in the untreated HYB 50(2)B cells due to growth in vitro relative to the short-term cultured M-1-a ($P < 0.025$) and H-1-a ($P < 0.0005$) cells is essentially restricted to the telocentrics.

**Antigenic analysis.** Given the demonstration that specific chromosome loss could be selected for with mouse-Chinese hamster somatic cell hybrids by multiple treatments with cytotoxic antisera, it was essential to determine whether this depletion was accompanied by the loss of cell surface antigens of the appropriate parental type. This was examined by serum absorption studies, as monitored by indirect MIF with unfixed cells, using the third round selected M-3-a and H-3-a clones (Table III). It can be seen that M-3-a and H-3-a demonstrate reduced expression of mouse and hamster surface antigens, respectively, despite the absence of a statistically significant difference in hamster chromosomes between the third round clones selected with antimouse and antihamster antiserum (Table II). However, since considerable nonspecific loss of hamster chromosomes had occurred after three rounds of treatment with either serum, and given the significant specific depletion of hamster chromosomes after only one cycle of selection (H-1-a vs. M-1-a), it is probable that specific loss of hamster chromosomes was evident at the third round of selection, but was masked in our analysis by the nonspecific loss of hamster chromosomes from both the antimouse and antihamster serum-selected clones.

Most importantly, the effect on antigen expression is quantitative rather than qualitative, since the residual activity of both sera against unselected HYB 50(2)B cells subsequent to two cycles of absorption with either serum-selected hybrid could be totally removed by a further two absorptions with the same hybrid. Further evidence of a specific effect on antigen expression is the similar target cell reactivity of M-3-a and HYB 50(2)B cells with anti-Wg-1 serum and of H-3-a and HYB 50(2)B cells with anti-3T3 serum, indicating that no general depression in the expression of all surface antigens has occurred. It also appears that the hybrid HYB 50(2)B cells do not contain all of the antigens present on the parental 3T3 and Wg-1 cells, since the homologous serum absorbed four times with either selected hybrid still reacted with the parental cells while no longer staining HYB 50(2)B cells. However, further absorption cycles would be necessary to conclusively rule out a quantitative difference here, as well. In any event, it is clear that diminished antigenic expression has occurred in response to multiple cycles of antiserum selection of the HYB 50(2)B mouse-Chinese hamster hybrid cells.

Fig. 5, part 1

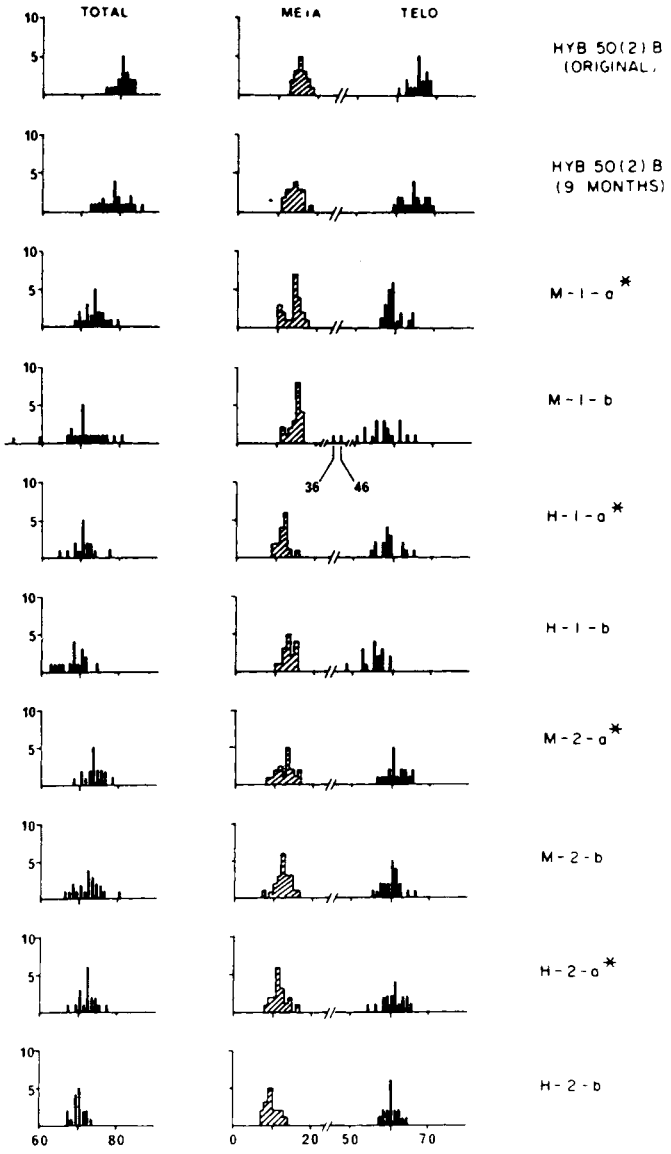
TOTAL
MEIA
TELO
HYB 50(2) B (ORIGINAL)
HYB 50(2) B (9 MONTHS)
M-1-a*
M-1-b
H-1-a*
H-1-b
M-2-a*
M-2-b
H-2-a*
H-2-b
10
5
36
46
60
80
0
20
50
70

Fig. 5, part 2

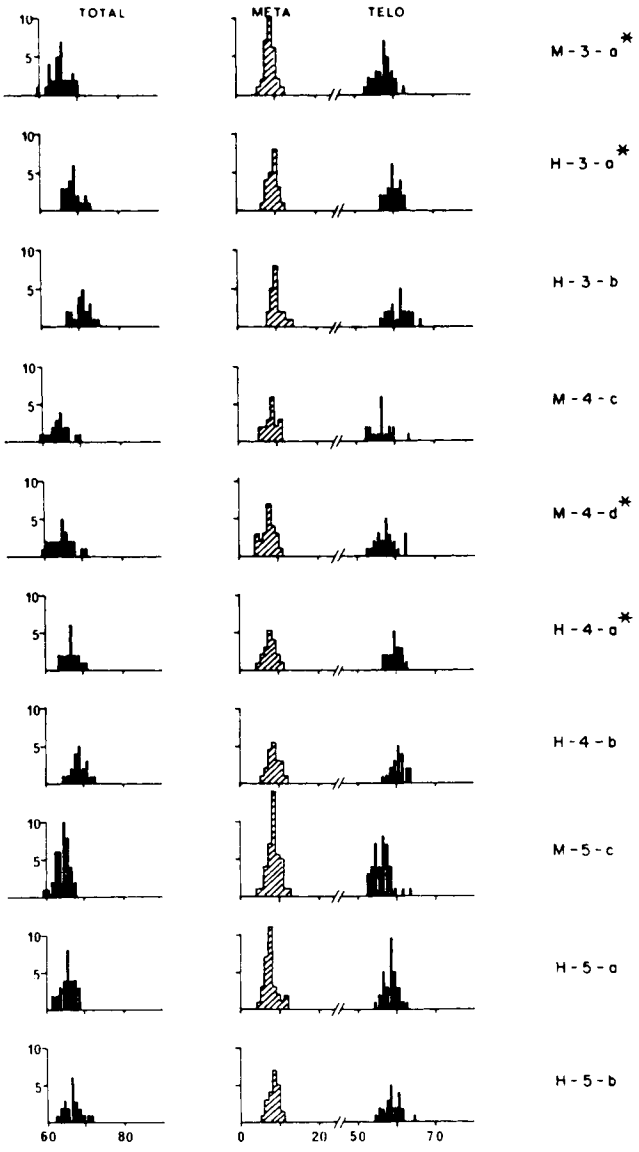


Fig. 5. Karyograms of untreated HYB 50(2)B hybrid cells (at start of experiment and after 9 months in culture) and serum-selected clones. Left-hand figure represents total chromosome count and right-hand figure indicates metacentric (cross-hatched) and telocentric (solid) chromosomes. *Designates clones used for subsequent round of serum selection (see Fig. 4). For simplicity, those clones demonstrating a 2s mode are not included.

### Use of Depleted Hybrid Cell Clones in Studies of M-MuLV Restriction in Chinese Hamster Cells

The third round selected clones, M-3-a and H-3-a, as well as the parental nonserum-treated hybrid cell line, HYB 50(2)B, have been utilized to investigate the mechanisms which control the replication of M-MuLV in Chinese hamster cells. The parental mouse (3T3 $TK^-$) and Chinese hamster (Wg-1 $HGPRT^-$) cells and serum-selected clones M-3-a and H-3-a were infected with M-MuLV, and, as shown in Table IV, only the mouse cells were capable of supporting MuLV replication. This inability of M-MuLV to replicate in the mouse-hamster hybrid cells could be due to an extracellular block (absorption/penetration) and/or to some later intracellular block in viral replication. To distinguish between these possibilities, the properties of phenotypically mixed virions of VSV, containing the genome of VSV and the envelope properties of M-MuLV, were exploited. The VSV (MuLV) pseudotype viruses, obtained as described in Methods, were shown to possess the expected host range and interference properties: (a) ability to plaque on mouse cells (Table IV), and (b) inability to replicate in chicken cells or in mouse embryo cells chronically infected with M-MuLV (data not shown).

As indicated in Table IV, both the serum-selected clones, as well as the unselected HYB 50(2)B hybrid cells, supported the replication of VSV after infection with the VSV (MuLV) pseudotype virus as or more efficiently than did the 3T3 cells (note that the ratio between titers in the presence and absence of VSV-neutralizing antiserum represents the efficiency of pseudotype virus infection). Nevertheless, no progeny VSV was produced after pseudotype infection of the Wg-1 cells. These results indicate that the restriction of MuLV replication in Chinese hamster cells operates at two levels: (1) an inability to absorb to or penetrate Chinese hamster cells, reflected by the failure of the pseudotype virus to infect Wg-1 cells; and (2) an additional intracellular block which continues to function in mouse-Chinese hamster cells, since these cells support VSV replication after pseudotype infection, while not allowing the replication of M-MuLV itself.

## DISCUSSION

The data presented in this report indicate that while the specific elimination of chromosomes from stable somatic cell hybrids by repeated selection against surface antigenic markers with cytotoxic antiserum has been achieved, the scale on which this has occurred is too small to support its application as a general technique for extensive chromosome depletion. However, it should be realized that with other interspecific combinations (or even with other mouse-Chinese hamster clones), more potent cytotoxic antisera, and different selection conditions, it may be possible to obtain much more marked elimination of the appropriate chromosomes.

Although we have considered the HYB 50(2)B mouse-Chinese hamster hybrid cell clone to be nondepleted, it is clear from the data presented (see Table II and Fig. 5) that some spontaneous chromosome loss has occurred. The original hybrid clone, X13 RS2a, karyotyped within 3 weeks of isolation, contained an essentially complete complement of both sets of chromosomes (ref. 14; Pontecorvo, personal communication). Approximately seven chromosomes were eliminated during the subcloning procedure, with minimal additional loss during a further 9 months in culture. However, after another 6 months in

culture, both telocentrics and metacentrics were reduced by a further three to four chromosomes. Thus, while the hybrid utilized for these investigations is relatively stable, some chromosome loss, primarily telocentric, but of both mouse and hamster origin, did occur as a result of in vitro manipulations. The enhanced nonspecific loss of hamster chromosomes after three and five cycles of anti-3T3 treatment (compare HYB 50(2)B vs. M-3-a and M-5-c, Table II) remains unexplained.

The rationale behind these experiments was that in the face of selective pressure directed against surface antigens, surviving clones could be isolated which had lost at least some of these antigens and, hopefully, the chromosomes carrying the corresponding structural genes. However, several alternative mechanisms can be envisaged which would allow clones to survive the treatment with cytotoxic antiserum plus C′ and which would tend to reduce the magnitude of any potential chromosome loss. First, since the expression of surface antigens can differ during various stages of the cell cycle (33, 34), some of the surviving cells may have been those with diminished antigen expression at the time of incubation with serum and C′. Second, some of the surface antigens against which the rabbits responded when immunized with the parental cells may be present in a masked configuration ("cryptic") on the surface of the hybrid cells; numerous examples of antigen masking have been reported in other cell systems (34–36). The apparent absence of certain mouse and hamster antigens on the unselcted HYB 50(2)B hybrid cells (Table III) could possibly be due to such a cryptic configuration. Third, as judged by their relatively weak cytotoxicity, the sera employed in this study may have reacted only against a limited range of species-specific antigens, thereby restricting the number of chromosomes being selected against. This would be especially important if the structural genes coding for such

**TABLE III. Antigenic Analysis of Third Round Serum-Selected Hybrid Cell Clones by Membrane Immunofluorescence[1]**

| Serum No.[2] | Raised against | Absorbed with[3] | Fluorescence index | | | | |
|---|---|---|---|---|---|---|---|
| | | | HYB 50(2)B | 3T3 | Wg-1 | M-3-a | H-3-a |
| 95 | 3T3 | – | 0.86 | 0.88 | – | 0.96 | 0.90 |
| | | M-3-a (2x) | 0.42 | 0.39 | – | 0.12 | 0.37 |
| | | M-3-a (4x) | 0.03 | 0.36 | – | 0.01 | 0.02 |
| | | H-3-a (2x) | 0.40 | 0.50 | – | 0.07 | 0.17 |
| | | H-3-a (4x) | 0.00 | 0.35 | – | 0.02 | 0.03 |
| 96 | Wg-1 | – | 0.82 | – | 0.89 | 0.97 | 1.00 |
| | | M-3-a (2x) | 0.40 | – | 0.49 | 0.09 | 0.15 |
| | | M-3-a (4x) | 0.04 | – | 0.27 | 0.05 | 0.07 |
| | | H-3-a (2x) | 0.46 | – | 0.59 | 0.39 | 0.14 |
| | | H-3-a (4x) | 0.02 | – | 0.35 | 0.01 | 0.02 |

[1] Membrane immunofluorescence technique is described in Methods.
Fluorescence index (FI) = $\frac{\%\text{ negative control cells} - \%\text{ negative test cells}}{\%\text{ negative control cells}}$
calculated on the basis of the heterologous parent cell; staining of the test cells is considered significant when FI $\geq$ 0.20 (22).

[2] All sera used at dilution of 1:4.

[3] Both sera were preabsorbed twice with heterologous parental cells plus additional absorptions as indicated (number of absorptions in parentheses); see Methods for absorption procedure.

**TABLE IV. Plating of Moloney MuLV and VSV (MuLV) Pseudotype Virus on Mouse, Chinese Hamster, and Interspecific Hybrid Cells**

| Virus | Cell type | Anti-VSV Ab | Titer (/ml) MuLV (XC) | Titer (/ml) VSV (PFU) | Titer + Ab / Titer − Ab | Relative plating efficiency[1] |
|---|---|---|---|---|---|---|
| M-MuLV | 3T3 TK$^-$ | – | $> 10^5$ | | – | – |
| | Wg-1 HGPRT$^-$ | – | 0 | | – | – |
| | M-3-a | – | 0[2] | | – | – |
| | H-3-a | – | 0[2] | | – | – |
| VSV (MuLV) | 3T3 TK$^-$ | – | | $1.2 \times 10^8$ | $6.75 \times 10^{-5}$ | 1.00 |
| | | + | | $8.1 \times 10^3$ | | |
| | Wg-1 HGPRT$^-$ | – | | $1.5 \times 10^8$ | $<10^{-8}$ | < 0.00015 |
| | | + | | 0[3] | | |
| | HYB 50(2)B | – | | $3.4 \times 10^6$ | $1.06 \times 10^{-4}$ | 1.57 |
| | | + | | $3.6 \times 10^2$ | | |
| | M-3-a | – | | $1.3 \times 10^7$ | $5.46 \times 10^{-4}$ | 8.09 |
| | | + | | $7.1 \times 10^3$ | | |
| | H-3-a | – | | $3.3 \times 10^7$ | $1.97 \times 10^{-4}$ | 2.92 |
| | | + | | $6.5 \times 10^3$ | | |

[1] 3T3 TK$^-$ normalized to 1.00. [2] Occasional syncytia. [3] Occasional plaques.

antigens are widely distributed on different chromosomes, as has been previously suggested from work with interspecific mouse-human hybrids (2). It should also be noted that not all surface antigens need act as transplantation-type antigens (i.e. susceptible to C′ -dependent lysis), which would further restrict the range of selective pressure afforded by our colony inhibition procedure.

Last, and probably most relevant to the present results, is the possibility that treatment with multispecific antiserum may, in fact, preferentially select for the elimination of chromosomes bearing regulatory genes for a series of antigens rather than of chromosomes containing individual structural genes. The limited specific loss of chromosomes which we have obtained, as well as the quantitative, but not qualitative, alteration in antigen expression by the serum-selected hybrids is in accord with such a mechanism. The inability to detect qualitative loss of surface antigens also argues against the forced elimination of a few chromosomes bearing several structural genes for such markers. Coordinate control of the degree of expression of mouse H-2 antigens in intraspecific hybrids has been reported after the fusion of TA3-Ha carcinoma (characterized by low H-2 antigen expression) with normal mouse fibroblasts (37, 38). It is of interest that increased antigen expression, as measured by serum absorption, did not always correlate with increased sensitivity to cytotoxic antiserum (38), an observation extremely relevant to the present report.

Since we were dealing with multispecific antisera, no attempt has been made to ascertain whether specific chromosomes were eliminated in response to serum selection. Such an analysis requires antiserum of limited specificity and is most suitable with hybrids containing a restricted number of the chromosomes under study, as has been carried out by Puck et al. (39) with mouse-human hybrid cells and a human antigen sensitive to serum-mediated cytotoxicity.

The ability to obtain limited, but specific, depletion of chromosomes from stable interspecific hybrid cell lines represents a potentially powerful tool with application to a wide variety of systems. As demonstrated in this paper, we have already utilized some of the serum-selected mouse-Chinese hamster hybrid cell clones in preliminary studies of the host-range restriction which prevents MuLV growth in Chinese hamster cells. The results presented indicate that the extracellular block in the Wg-1 hamster cells to M-MuLV replication is a recessive trait in mouse-Chinese hamster hybrid cells and suggests that the presence of a specific receptor at the cell surface is required for MuLV adsorption/penetration. Furthermore, the results using the VSV (MuLV) pseudotype virus indicate the existence of a postpenetration intracellular block to M-MuLV replication in the hybrid cells, presumably due to the presence of an inhibitory function coded for by the hamster cell genome. Such a function may be specific for Chinese hamster cells since other workers have reported both mouse-rat (40) and mouse-human (6) hybrids to be fully permissive for M-MuLV replication. However, in these cases, it may be that the gene(s) coding for the intracellular block was absent due to spontaneous chromosome loss. It will be of interest to determine whether continued selection of the hybrid cells with cytotoxic anti-hamster serum can lead to the isolation of hybrid clones permissive for M-MuLV replication or, conversely, whether further selection with antimouse antiserum will lead to hybrid clones restrictive for pseudotype infection due to loss of the gene(s) coding for the M-MuLV cell surface receptor.

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