CELL STRUCTURE AND FUNCTION 25: 41–46 (2000)

Different Responses of Polyploidized V79 Cells after Removal of Two Drugs, Demecolcine and K-252a

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ABSTRACT. **To examine whether or not cells polyploidized by different mechanisms behave in a different manner after drug removal, V79 Chinese hamster cells were assessed by flow cytometry (FCM) after their polyploidization by demecolcine and K-252a, inhibitors of spindle-fiber formation and protein kinase, respectively. Cell cycle analysis of DNA histograms of V79 cells before and after the drug release was performed. With both drugs, the ploidy of V79 cells increased just after the drug removal and was maintained for a week. A difference was evident 10 days after the release. Tetraploid cells were the main population from 10 to 18 days after the release of K-252a, but not demecolcine. Cell cycle parameters were almost the same in pseudo diploid and tetraploid V79 cells, except for the tetraploid S phase which was 2h longer.**

Key words: cell cycle analysis/V79 cells/polyploidization/K-252a/demecolcine

V79 Chinese hamster lung cells were polyploidized at the same rate by demecolcine and K-252a, inhibitors of protein kinases and spindle-fiber formation in M phase, respectively (Fujikawa-Yamamoto *et al*., 1993), but their morphology differed between multi- and mono-nuclei detected in the cells polyploidized by demecolcine and K-252a, respectively (Fujikawa-Yamamoto *et al*., 1999b). It is of interest to determine whether or not the cells polyploidized by different mechanisms behave in a different manner after the drug removal.

A relationship between the rate of DNA synthesis and DNA content has been reported for Chinese hamster cells by Graves and McMillan (1984) wherein the duration of S phase is almost constant regardless of the DNA content. This was supported by the finding of increased BrdU uptake in polyploid CHO cells (Takanari *et al*., 1985). Although several studies have reported a constant duration of the S phase in the polyploidization of cultured cells (Brenneisen *et al*., 1994; Fujikawa-Yamamoto *et al*., 1997b; Graves *et al*., 1984; Jordan *et al*., 1996; Usui *et al*., 1991; Watters *et al*., 1994; Zhang *et al*., 1996), except for Meth-A cells (Fujikawa-Yamamoto *et al*., 1997b), the cell cycle parameters, that is, the duration of G_1, S and G_2/M phases, of polyploid cells in a steady state of growth have not been studied well because of difficulty in achieving such a state.

In this study, the behavior of V79 cells polyploidized by demecolcine and K-252a was examined by flowcytometry (FCM) for about one month after the removal of the drugs and the cell cycle parameters determined for the pseudo diploid and tetraploid.

Materials and Methods

Cells

V79 (Chinese hamster lung cell line) cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C as a monolayer culture in Leibovitz's L15 : Ham's F10 mixture (7:3) supplemented with 10% fetal calf serum (M.A. Bioproducts, Walkersville, Md, USA), streptomycin (100 μg/ml) and penicillin (50 units/ml). The cells were cultured at low density.

Drug treatment and subculture

Exponentially growing V79 cells were plated in Petri dishes (90 mm diameter, Nalge Nunc International, IL, USA) at a density of about 2×10^5 cells/dish, and the medium was changed 24 h after seeding. Twelve hours thereafter, the cells were exposed to K-252a (800 nM, Funakoshi, Tokyo Japan) or demecolcine (270 nM, Sigma, MO, USA) for 96 h. The cells were then released from the

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drug exposure by changing the medium. To obtain independent results, six dishes were prepared. Every 72 h, the V79 cells in 2 dishes were subcultured by 1/2 or 1/4 through two washes with $PBS^{(-)}$ (divalent cation-free phosphate-buffered saline) and trypsinization (0.17% trypsin and 30 mM EDTA), and the residual cells were prepared for flow cytometry and cell growth measurements. This subculture cycle was started at 0, 24 and 48 h after the drug removal.

Cell preparation for flow cytometry and cell counting

V79 cells obtained through subculture were fixed with 20% ethanol, and then incubated with 0.5 ml of 0.25% RNase (Type II-A, Sigma) for 3 h at 4°C. At this step, the cells were counted by a hemocytometer. Immediately before the measurements, the cells were stained with PI (propidium iodide, 7.5×10^{-5} M) and red fluorescence was examined by means of FCM. Under these staining conditions, the signal due to residual double stranded RNA is negligible and the relative intensity of the red fluorescence corresponds to the DNA content (Krishan, 1975).

Flow cytometry

The fluorescence from individual cells was measured using a FACSORT (Becton Dickinson Immunocytometry Systems, USA). The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm was detected using a photomultiplier tube. The relative intensity of red fluorescence (FL2H) was measured and DNA histograms were obtained.

Cell cycle analysis

FCM data of FL2H, signals of red-fluorescence intensity through a logarithmic amplifier, for 10000 cells were input to CASL, a software for cell cycle analysis of DNA histograms on a log scale, through a transfer software, "FACS to ASCII" (free ware), and the DNA histograms were decomposed to cell fractions depending on the DNA content (Fujikawa-Yamamoto, 1999a). CASL is written on Mathematica (Ver. 2.2) with a personal computer (Quadra 840AV, Macintosh) and can analyze DNA histograms with 2c to 128c DNA content. The algorithm is similar to Fried's method (Fried *et al*., 1976; Fried, 1977) except that normal distribution functions having the same half-width instead of same CV (coefficient of variation) value (Fried *et al*., 1976; Fried, 1977) are used as components.

Chromosome analysis

Exponentially growing V79 cells in Petri dishes (60 mm diameter, NUNC) were exposed to demecolcine at a concentration of 270 nM for 48h (for polyploidization) or 1h (for control). The cells were trypsinized and swelled by 75 mM KCl, fixed with Carnoy fixing solution (CH_3OH:CH_3COOH=7:3) and dropped onto slide glasses. The cells were stained with Giemsa solution to take photographs of the chromosomes. Chromosome number was counted from the photographs. Lymphocytes of a male Chinese hamster were used for the control of karyotyping. Karyotype analysis was performed by a Karyovision (Sumitomo Kinzoku, Tokyo, Japan).

Results

To examine the retention of DNA ploidy, changes in DNA histograms were measured for V79 cells released from exposure to K-252a (Fig. 1) or demecolcine (Fig. 2). V79 cells were polyploidy at 96 h after the addition of K-252a or demecolcine. In V79 cells exposed to K-252a, however, a 4c peak was observed till 96 h after the drug addition, suggesting an important role for protein kinase in cell-cycle progression. After K-252a removal, the ploidy of the V79 cell population increased for 8 days, and was tetra ranging from 4c to 8c for 10 to 18 days. The fraction of diploid cells then increased gradually (Fig. 1). After demecolcine removal, in contrast, the diploid fraction increased, altering the decrease of hyperploidy from 4 days after the drug removal (Fig. 2).

To examine the growth of V79 cells after the release from drugs, cell numbers were counted, corrected for the subcultures and plotted against days after the drug removal (Fig. 3). The cell number decreased till 8 days after the drug removal, and then increased. Doubling times of cell population between 10 and 18 days were about 24 and 26 h for demecolcine and K-252a, respectively. We concluded that the doubling time was 24 and 26 h for pseudo diploid and tetraploid V79 cells under these experimental conditions. The growth of control V79 cells subcultured every day is also plotted in Figure 3 and the doubling time was 24 h. Note that frequent subculturing tends to elongate the doubling time and to eliminate dead cells from the cell popula-

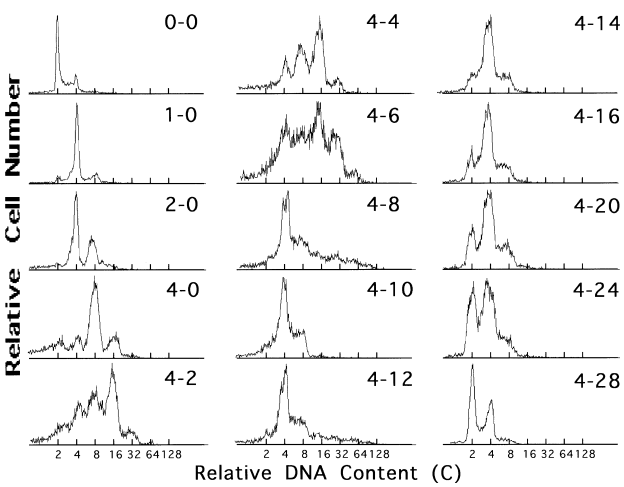

Fig. 1. Changes in DNA fluorescence histograms of V79 cells after the removal of K-252a. Exponentially growing V79 cells were exposed to K-252a at a concentration of 800 nM for 4 days. The cells were released from the drug and cultured again with a 3 day-interval subculture. Paired numerals in the histogram represent the time (day) after the drug addition and the removal, respectively. The abscissa represents the relative DNA content (c, complement).

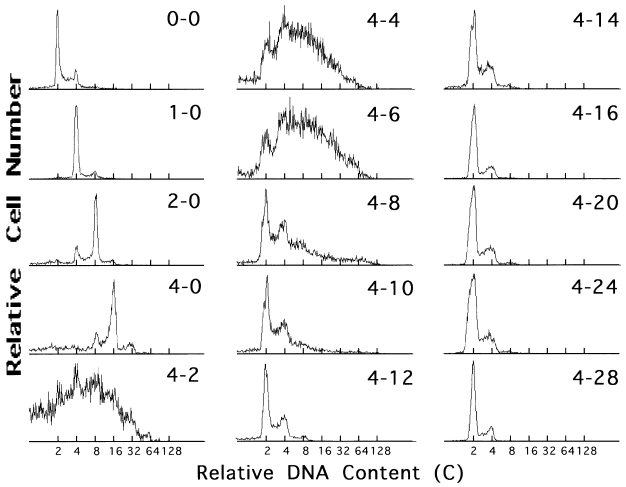

Fig. 2. Changes in DNA fluorescence histograms of V79 cells after the removal of demecolcine. Exponentially growing V79 cells were exposed to demecolcine at a concentration of 270 nM for 4 days. The cells were released from the drug and cultured again with a 3 day-interval subculture. Paired numerals in the histogram represent the time (day) after the drug addition and the removal. The abscissa represents the relative DNA content (c, complement).

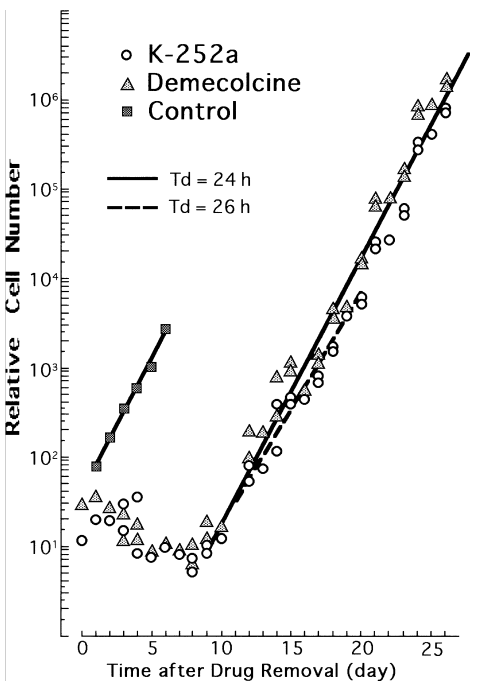

Fig. 3. Changes in relative cell number (growth curves) of V79 cells after the removal of K-252a (open circle) and demecolcine (closed triangle). Exponentially growing V79 cells were exposed to K-252a and demecolcine for 4 days at concentrations of 800 and 270 nM, respectively. The cells were released from the drug and cultured again with a 3 day-interval subculture. Closed squares represent the control which has been subcultured every day. The abscissa represents the time (day) after the drug removal. Solid and broken lines were drawn to facilitate understanding, and correspond to 24 and 26 h of doubling time, respectively.

tion.

To examine the changes in phase-fractions of polyploid V79 cells, cell cycle analysis was applied to the DNA histograms in Figures 1 and 2. A representative example of output from the cell cycle analysis software, "CASL", is shown in Figure 4. In Figure 4, panels A, B, C and D represent an experimental histogram of a V79 cell population at 18 days after K-252a release, an ideal histogram with the same fraction of each component in the S phase, a finally synthesized histogram and cumulated histograms, respectively. The ideal histogram (B of Fig. 4) was drawn to facilitate understanding.

Figure 5 represents the changes in the ploidy fraction of V79 cells after the removal of K-252a (upper panel) and demecolcine (lower panel). In Figure 5, the six squares correspond to the cell fractions of 2C + 2S, 4C + 4S, 8C + 8S, 16C + 16S, 32C + 32S and 64C + 64S + 128C in the order from the top, where C and S means G_2/M/G_1 and S phase, respectively, and the numbers indicate ploidy. Open bars in the squares represent the S fractions. A large S fraction was observed for about 7 days after the drug removal, suggesting a slow rate of DNA synthesis.

To determine cell cycle parameters of pseudo diploid and tetraploid V79 cells in a steady state of growth, the duration of G_1, S and G_2/M phase was calculated for the cell population 12 days after the demecolcine and K-252a removal (Table I). Note that pseudo diploid and tetraploid cells were the main population in DNA histograms at those times for demecolcine and K-252a, respectively. A remarkable difference was observed in the S phase duration in that it was about 2 h longer in pseudo tetraploidy than in pseudo diploidy.

Discussion

K-252a, a potent inhibitor of protein kinases C and I (Zollner, 1993), and demecolcine polyploidize many cultured cells, though their role in polyploidization is still unknown (Zong *et al.*, 1994, Zong *et al.*, 1995). V79 cells were

Table I. CELL CYCLE PARAMETERS OF PSEUDO DIPLOID AND TETRAPLOID V79 CELLS

	pseudo diploidy			pseudo tetraploidy		
doubling time	24h			26h		
phase	G_1	S	G_2/M	G_1	S	G_2/M
franction*	0.411	0.453	0.134	0.383	0.496	0.121
	(0.365)	(0.402)	(0.119)	(0.251)	(0.325)	(0.079)
duration** (hour)	8.0	11.6	4.4	8.0	13.7	4.3

* Phase fractions of diploid and tetraploid cells were determinined omiting those for other ploidy in the V79 cell population at 12 days after demoolcine and K-252a removal, respectively. Numbers in parenthesis represent the franction of the total cell pupulation.

** Phase duration was calculated through conventional equations Watanabe and Okada, 1967) using doubling time instead of cycle time.

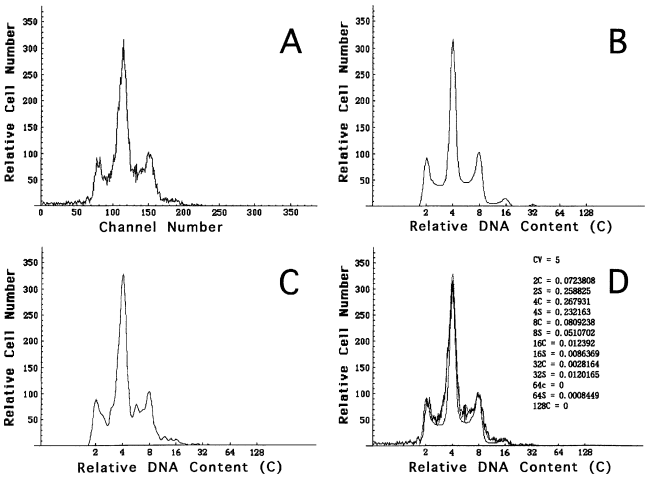

Fig. 4. Representative outputs of CASL (Cell-cycle Analysis on a Scale of Log). Panels A, B, C and D represent an experimental histogram for V79 cells at 18 days after K-252a removal, an ideally synthesized histogram, a finally synthesized histogram and cumulated histograms, respectively. The ideally synthesized histogram (panel B) was drawn to facilitate understanding and represents a histogram synthesized with the same fractions of S phase components, meaning a constant rate of DNA synthesis.

polyploidized by K-252a exhibiting a nuclear morphology that was mainly multi-lobed mononuclear (Fujikawa-Yamamoto, *et al*., 1999b). Demecolcine (Colcemid) prevents the formation of spindle fibers in M phase and induces polyploidization and multi-nucleization in V79 cells (Fujikawa-Yamamoto *et al*., 1999b). Ten days after K-252a and demecolcine removal, the main population became tetraploidy and diploidy, respectively. The different ploidy alterations after the drug removal may be caused by structural differences in the nucleus.

At this stage, just how the population of higher polyploid V79 cells is reduced till 8 days after the drug removal is unknown. The polyploidy above 8C may divide to lower polyploidy (Fujikawa-Yamamoto *et al*., 1993) or may be eliminated through death (Zong *et al*., 1998). Though apoptosis has been reported in spontaneous polyploidized Meth-A cells (Fujikawa-Yamamoto *et al*., 1997a) and in drug-induced highly polyploidized cells (Zong *et al*., 1998), the cell division of highly polyploided V79 cells into many cells was observed by videography (Fujikawa-Yamamoto *et al*., 1993). The facts that the tetraploid cells were a major population for about 10 days under several subcultures and that they grew with the same rate of the diploidy in the term proved the cell division of tetraploid cells.

It is of interest that the doubling time was almost the same in diploid and tetraploid V79 cells. The elongation of 2 hours might be within an experimental error to 26 h of the doubling time, or be attributable to the double content of DNA. Though two rates of DNA synthesis, constant and double-increase, have been reported for polyploid cells, the doubling time increased with the ploidy in both cases (Fujikawa-Yamamoto *et al*., 1997b, Graves and McMillan, 1984). Equivalence in doubling time and in the phase fractions suggests that the tetraploid cells have double the content of all the elements of diploid V79 cells. If the intracellular content increases with the ploidy of V79 cells, a spatial increase would cause a delay in the transport of materials in highly polyploid cells. A gradual decrease of the cell population from higher polyploidy would support this explanation.

To confirm the integrity of polyploidized V79 cells, the chromosomes of demecolcine-derived polyploid V79 cells were examined (Fig. 6). Figure 6 shows the karyotyping charts and chromosome number distribution of V79 cells in the presence of demecolcine. Though the number of chro-

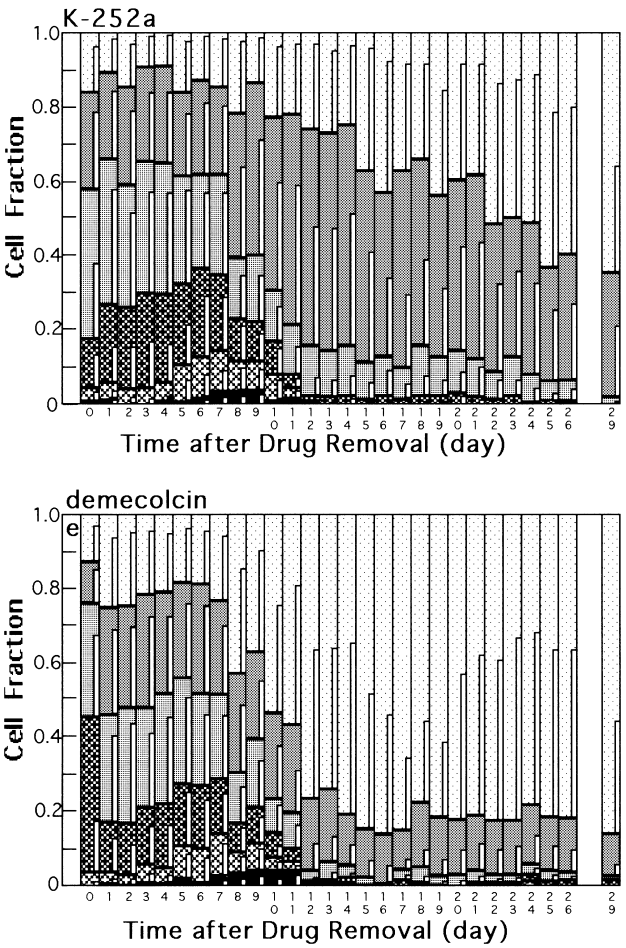

Fig. 5. Changes in ploidy fractions of V79 cells after the removal of K-252a (upper panel) and demecolcine (lower panel). Exponentially growing V79 cells were exposed to K-252a and demecolcine for 96 h. The cells were released from the drug and cultured again with a 3 day-interval subculture. The DNA histograms were analyzed by CASL and the phase fractions were plotted against days after the drug release. Squares of □, ▨, ▤, ▩, ▦ and ■ represent fractions of 2C + 2S, 4C + 4S, 8C + 8S, 16C + 16S, 32C + 32S and 64C + 64S +128C, respectively. Where C and S represent the peak-fraction and the S-phase-fraction, respectively. Open bars represent the S phase fractions.

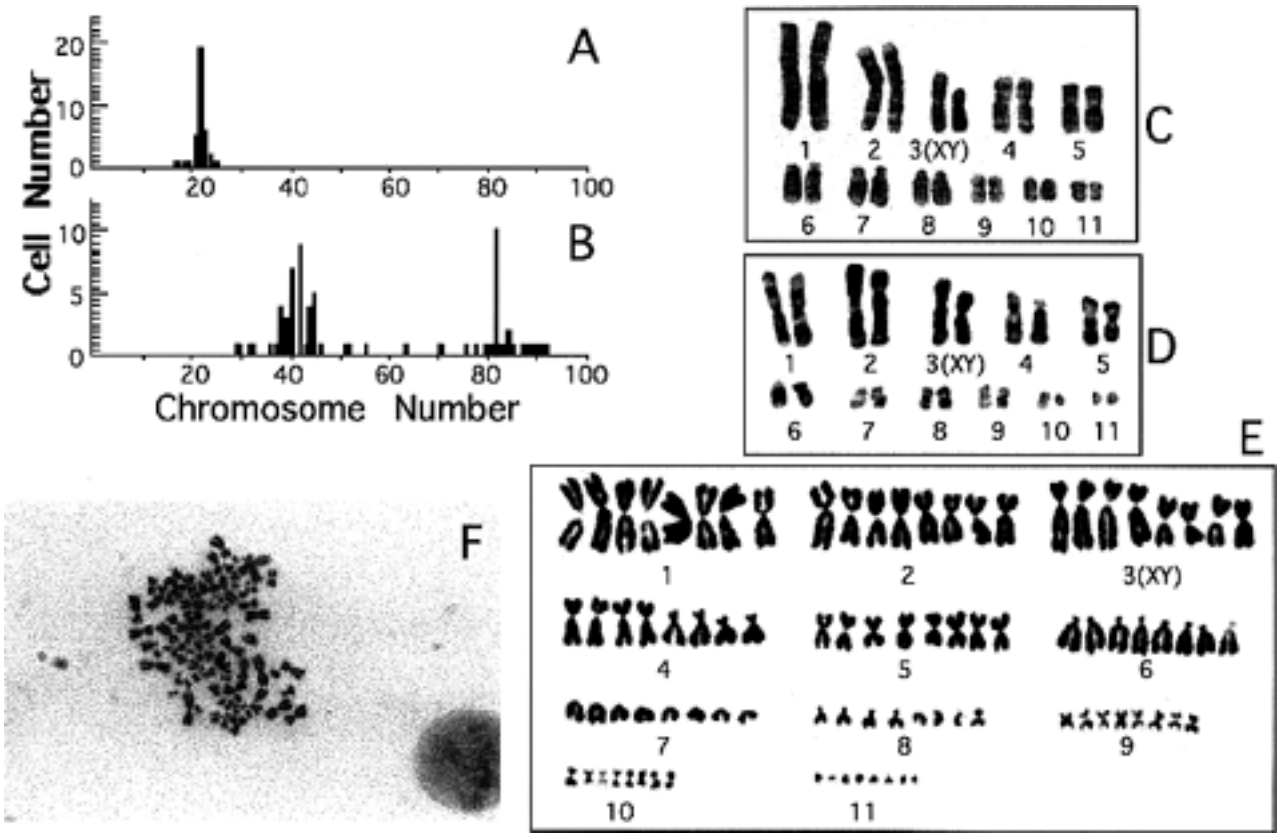

Fig. 6. Histograms of chromosome number (panels A and B) and karyotyping charts (panels D and E) for V79 cells exposed to demecolcine. Exponentially growing V79 cells were exposed to demecolcine at the concentration of 270 nM for 1 and 48 h. The chromosomes were stained with Giemsa solution. Panels A and B represent histograms of chromosome number for V79 cells on 1 and 48 h exposure to demecolcine, respectively. Panels D and E represent karyotyping charts of a cell from panels A and B, respectively. Panel F is the original photograph of panel E. Panel C is obtained from lymphocytes of a male Chinese hamster.

mosome is distributed widely at higher ploidy and the karyotyping of V79 cells is much different from that of Chinese hamster lymphocyte, 11 groups of 8 homologous chromosomes were identified, suggesting the integrity of chromosome number in polyploid V79 cells.

Acknowledgements. This study was supported in part by a grant for High-Technology Research Center Project by the Ministry of Education of Japan and Kanazawa Medical University (H1-99). The author wish to thank Dr. Mashio Kitatani and Dr. Mamoru Ozaki who helped with karyotyping of Chinese hamster and V79 cells. Two male Chinese hamsters were donated by the animal experimental center of Kanazawa University.

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(*Received for publication, December 3, 1999*

and in revised form, December 21, 1999)