[CANCER RESEARCH 30, 1174–1184, April 1970]

Cellular Resistance to Actinomycin D in Chinese Hamster Cells *in Vitro*: Cross-Resistance, Radioautographic, and Cytogenetic Studies[1]

June L. Biedler and Hansjörg Riehm[2]

Division of Drug Resistance, Sloan-Kettering Institute for Cancer Research, and Department of Pediatrics, Memorial Hospital for Cancer and Allied Diseases [H. R.], New York, New York 10021

SUMMARY

Several actinomycin D (AD)-resistant sublines maintained at 0.1, 1.0, and 10.0 μg/ml AD and exhibiting an increase in resistance up to 2500-fold were developed *in vitro* from Chinese hamster cells.

Dose-response data for sensitive and resistant sublines demonstrated that AD-resistant cells were cross-resistant, in decreasing order, to mithramycin, vinblastine, vincristine, puromycin, daunomycin, demecolcine, and mitomycin C. In general, the greater the cross-resistance to an agent, the greater its molecular weight. Increase in resistance to AD of a graded series of sublines was accompanied by proportional decrease in sensitivity to vincristine and daunomycin, and several experimentally derived daunomycin-resistant cell lines also exhibited increased resistance to AD.

In radioautographic experiments, it was found that degree of resistance was inversely related both to degree of nuclear labeling by AD-^{3}H and to inhibition of uridine-5-^{3}H incorporation by the antibiotic.

Karyotype analysis revealed that chromosomal alterations, once established, were stable and apparently were not specifically related to the resistant state.

These investigations support the hypothesis that the development of resistance to AD in Chinese hamster cells is due to qualitative difference in cell membrane, resulting in decreased permeability to AD and other compounds.

INTRODUCTION

Cellular resistance to AD[3] has been developed experimentally and studied in bacterial populations (19, 22) and mammalian cell populations (9, 10, 23) *in vitro*. Polsinelli *et al.* (19) found that decreased permeability of a resistant strain of *Bacillus subtilis* to radioactive AD was associated with a markedly reduced rate of autolysis and genetic transformability. Modification of cell wall as the probable basis of resistance was further substantiated with the demonstration by Slotnick and Sells (22) of wild type sensitivity to AD of protoplasts from 100- and 1000-fold-resistant strains of *B. subtilis*.

The occurrence of natural resistance to AD within both phyla has been documented. Natural resistance of *Escherichia coli* was reported by Hurwitz *et al.* (13). Haywood and Sinsheimer (11) and Mach and Tatum (17) showed that protoplast suspensions of *E. coli* were sensitive to the antibiotic, while Leive (16) found that even a 2-min exposure of intact organisms to EDTA rendered them sensitive, thus implicating the cell wall as barrier to the drug. Wong *et al.* (24), working with green monkey kidney cells, found them 10 to 100 times more resistant to the effects of AD than HeLa and mouse embryo cells. It is known that animal neoplasms may be naturally resistant to the action of AD. For example, Schwartz *et al.* (21) compared a resistant, hydrocarbon-induced transplantable tumor with the highly sensitive Ridgway osteogenic sarcoma of mouse and found that the latter tissue retained tritiated drug for a longer period. On a cellular level, however, the basis for differential sensitivity was not clear. Similarly, Kessel and Wodinsky (15), in an *in vivo* study of several mouse leukemias, observed that the greater the sensitivity of the tumor line to AD, the greater its uptake and retention during the first hours after drug administration. The nature of AD resistance in mammalian cells selected in culture by intermittent exposure to antibiotic was investigated by Goldstein *et al.* (9, 10). HeLa cells maintained at 0.4 μg/ml showed markedly reduced incorporation of AD-^{3}H by both radioautographic and scintillation counting techniques, and altered permeability of cell or nuclear membrane was postulated.

In the present study, described in part in preliminary communications (4, 6), several sublines of Chinese hamster cells with different levels of resistance to AD were developed so that possible quantitative relationships between drug sensitivity and uptake of tritiated antibiotic could be investigated. Experimental results indicate correspondence between degree of resistance and autoradiographic labeling by AD-^{3}H and tend to support the hypothesis that modification(s) affecting the plasma membrane of the cell, resulting

[1]This investigation was supported in part by funds from USPHS Grant CA-08748.

[2]Present address: Kinderklinik der Freien Universität, Heubnerweg 6, 1 Berlin 19, Germany.

[3]The abbreviations used are: AD, actinomycin D; DM, daunomycin; ED_{50}, 50% effective dose; VCR, vincristine.

Received July 28, 1969; accepted October 23, 1969.

in decreased permeability to the drug, is the primary mechanism of resistance to AD.

Since the AD-resistant lines exhibited cross-resistance to DM, among other agents, it seemed of interest to investigate whether resistance between the 2 antibiotics was reciprocal. Preliminary studies of 1 DM-resistant line (DC-3F/DM I) and a doubly resistant subline (DC-3F/AD IV/DM) indicate that resistance to DM is accompanied by increased resistance to AD.

MATERIALS AND METHODS

Cells and Culture Medium. Line Dede, derived from normal female Chinese hamster lung tissue, was established in 1954 by Dr. T. C. Hsu. It was obtained in 1965, was twice cloned, and is designated DC-3F. CLM-7 is a fibroblastic line derived from bone marrow of a normal male Chinese hamster. It was established *in vitro* and cloned once in 1966. Both lines have chromosome numbers in the diploid range and grow as monolayer cultures on glass.

Eagle's minimal essential medium supplemented with 20% fetal calf serum was used throughout.

Development of Resistant Sublines. Drug-resistant sublines were selected in the presence of AD or DM by stepwise increases in concentration of drug. For the CLM-7 series, CLM-77/AD III was cloned from the same cell population used to initiate the other AD-resistant sublines derived from CLM-7 and was thus derived independently of CLM-7/AD XV. Chart 1 shows the plan of development of the resistant lines. After sublines were established at maintenance concentrations of drug, they were transferred routinely every 7 days with 2 medium changes with fresh drug.

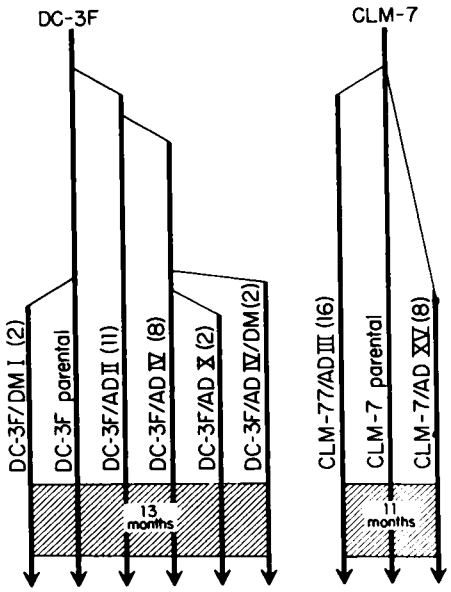


Chart 1. Schematic representation of derivation of drug-resistant sublines of parental DC-3F and CLM-7 cell lines. *Number in parentheses*, number of months each subline was exposed to AD or DM before experiments described in text were started; *cross-hatched area*, duration of the experimental period for each series of cell lines.

Assay of Drug Sensitivity. For determination of sensitivity of parental lines and resistant sublines to the selective agents used as well as to other chemical agents, an *in vitro* assay system was utilized. All resistant sublines were grown without drug for 10 to 15 days and cultures were transferred at least twice prior to assay. Milk dilution bottles containing graded concentrations of drug or no drug were inoculated with 5×10^5 ($\pm 0.3 \times 10^5$) cells. Duplicate cultures were set up at each of the concentrations, 4 to 6/assay, and there were 3 or more control cultures without drug. For each agent and each cell line assays were performed at least twice, with the exceptions noted below. The number of cells per bottle was determined at 72 (±2) hr by means of a Coulter Model F counter. For counting, cultures were rinsed in order to remove unattached cells and cellular debris, trypsinized, and then pipetted sufficiently to produce a single cell suspension as viewed microscopically. Drug sensitivity is recorded as the average number of cells in treated cultures/-average number in controls, as a percentage value for each concentration. The dose effective in inhibiting and/or killing 50% of the cell population by the end of the 3-day period is reported as the ED_{50} value.

Data Analysis. With the assumption of linear response, a line was fitted to the drug sensitivity data by the method of least squares (X = log dose of drug; Y = percentage of "no drug" control) with the aid of a computer, and log ED_{50} was obtained. Significance of difference of response to drugs between sensitive and resistant sublines, *i.e.*, cross-resistance, was based on the t test. Replicate determinations of ED_{50} for each subline provided the degree of variation on which the test was based. The "within replicate" determination was pooled for parental and resistant subline. Thus the degrees of freedom for each t test was the sum of the "number of replicate experiments minus 1" for sensitive and resistant subline.

Radioautography. Cells were grown on No. 2 glass coverslips in 35-mm plastic plates in a humidified air-CO_2 mixture for 3 days and were in exponential growth phase at time of addition of tritiated compounds. Previous to preparation for radioautographic study, resistant sublines were maintained for 10 to 15 days without drug. Treated and control plates were reincubated at 37° for the 1- or 2-hr experimental period, after which coverslips were transferred to a small rack. Since preliminary experiments indicated that degree of nuclear labeling by AD-^{3}H was strongly influenced by methods of rinsing and fixation, a standardized procedure was used. Coverslips were rinsed for 1 min in complete growth medium, followed by 30 sec in balanced salt solution. After fixation in 70 and 100% methanol, 5 min each, and drying, coverslips were rinsed for 30 min in a 37° tap water bath. Coverslips were mounted on glass slides, allowed to dry for 2 days, and dipped in Kodak NTB liquid emulsion (diluted 1:1 with distilled water) at 45°. Slides were stored at 4° for 14 days. After exposure, preparations were developed in Kodak D-19 developer for 4 min at 18°, rinsed, fixed for 8 min in Kodak rapid fixer with hardener, and given 3 distilled water rinses of 5 min each. Slides were

dried, stained in Mayer's hematoxylin for 8 min, and rinsed in distilled water.

Depending on density, the mean number of grains per nucleus of 200 to 1000 cells was determined. Average number of background grains was determined from estimates of nuclear size and subtracted from nuclear grain counts. Counting was performed by 2 or 3 independent observers on coded, randomized slides.

Population Doubling Time. Estimation of growth rate of parental and resistant sublines was made from total cell counts of 5 to 6 sets of duplicate cultures over a 24 to 72-hr period, when cells were in exponential growth phase. At least 2 determinations were carried out for each cell line, and the values, never differing by more than 1.5 hr, were averaged. Resistant sublines were grown without drug for 10 to 15 days, conditions thus being consistent with those for drug assay and autoradiographic procedures.

Karyotype Studies. Preparation of metaphase cells for chromosome observation was carried out by standard procedures of acetic alcohol fixation and acetic orcein staining of air-dried coverslip preparations of cells previously subjected to Colcemid (0.5 μg/ml) and 0.2% NaCl solution. Although karyotypes of the sublines were monitored frequently, the observations reported here were made after sublines were grown in presence of maintenance concentrations of drug for at least 100 days, and thus represent stable and characteristic chromosome patterns.

Source of Chemical Agents. Actinomycin D (Lyovac Cosmegen), hydrocortisone (Hydrocortone phosphate), and nitrogen mustard (Mustargen HCl) were obtained from Merck, Sharp and Dohme, Inc., Rahway, N. J.; vinblastine (Velban) and vincristine (Oncovin) from Eli Lilly and Co., Indianapolis, Ind.; mithramycin from Charles Pfizer and Co., Inc., Brooklyn, N. Y.; daunomycin from Farmitalia Research Laboratories, Milan, Italy; demecolcine (Colcemid) from Ciba Pharmaceutical Products, Inc., Summit, N. J.; novobiocin (Albamycin) from the Upjohn Company, Kalamazoo, Mich.; and amethopterin (methotrexate) from Lederle Laboratories, Pearl River, N. Y. Other compounds were obtained from the chemical file of the Sloan-Kettering Institute.

AD-^{3}H (specific activity, 3.38 Ci/mmole) and uridine-5-^{3}H (specific activity, 8.0 Ci/mmole) were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Thymidine-methyl-^{3}H (specific activity, 2.0 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Drug Resistance and Cross-Resistance of Parental and AD- and DM-Resistant Sublines. For both strains of Chinese hamster cells, DC-3F and CLM-7, resistance to AD as related to selective concentration of drug was established to a similar degree (Table 1). In general, the higher the maintenance level of AD the greater the disparity between this and sensitivity to AD in terms of ED_{50}. However, the resistant sublines grew well in the presence of antibiotic and appeared healthy. All sublines were maintained in selective concentration of drug for at least 2 months before the experiments described here were carried out. As indicated by the population doubling times (Table 2), there was a tendency for the resistant cells, even when maintained without drug, to grow at a slower rate than parental cells.

The response of parental cells and all resistant sublines to AD, VCR, and DM was determined by the 3-day assay procedure. As demonstrated in Chart 2 for the DC-3F sublines sensitive or resistant to AD alone, there was high reproducibility of response; a straight line represents the data well. With these 3 agents, the slopes for parental and resistant populations were similar. The experimentally determined ED_{50} values for the selective agents used and the calculated "degree of resistance" based on the values obtained for AD, VCR, and DM are shown in Table 1.

As resistance to AD increased there was decrease in sensitivity to both DM and VCR. When the ED_{50} values for

Table 1

Drug concentrations at which resistant lines maintained, response of cell lines to drug in terms of ED_{50}, and degree of resistance and cross-resistance

Cell line	Maintenance concentration		ED_{50} (μg/ml)	Degree of resistance[a] to			Population doubling time (hr)[b]
	Drug	μg/ml		AD	VCR	DM	
DC-3F			0.0024[c]	1	1	1	13.0
DC-3F/AD II	AD	0.1	0.20	81	51	9.4	15.0
DC-3F/AD IV	AD	1.0	0.91	376	189	29	14.5
DC-3F/AD X	AD	10.0	5.94	2450	556	76	15.0
CLM-7			0.0015[c]	1	1	1	12.5
CLM-77/AD III	AD	0.1	0.12	83	107	124	16.5
CLM-7/AD XV	AD	1.0	0.61	415	131	51	16.5
DC-3F			0.016[d]	1	1	1	
DC-3F/DM I	DM	2.0	5.31	102	163	326	17.0
DC-3F/AD IV/DM	DM	5.0	6.66	1960	330	409	18.0

[a] Resistance is expressed as ratio of ED_{50} values for resistant line to parental line; ratios were calculated as antilog of difference between log ED_{50} values obtained by computer.
[b] See "Materials and Methods."
[c] ED_{50} for AD.
[d] ED_{50} for DM.

Table 2

Relative response of parental DC-3F and AD-resistant subline DC-3F/AD IV to chemical agents

Agent	M.W.	No. of replicate experiments		ED_{50} for DC-3F		ED_{50} ratio[a] (DC-3F/AD IV: DC-3F)	Significance[b]
		DC-3F	DC-3F/AD IV	μg/ml	Molar		
Mithramycin	1089[c]	2	2	0.068		670	$p < 0.01$
AD	1255.5	4	6	0.0024	1.9×10^{-9}	376	$p < 0.01$
Vinblastine sulfate	929.9	2	2	0.0043	4.6×10^{-9}	239	$p < 0.01$
Vincristine sulfate	923.0	2	3	0.020	2.1×10^{-8}	189	$p < 0.01$
Puromycin	471.5	2	2	1.47	3.1×10^{-6}	84	$p < 0.01$
DM	527.5	4	4	0.016	3.1×10^{-8}	29	$p < 0.01$
Demecolcine	371.4	2	2	0.013	3.4×10^{-8}	18	$p < 0.01$
Mitomycin C	334.3	3	3	0.019	5.6×10^{-8}	3.1	$0.01 < p < 0.05$
Proflavine sulfate	325.3	3	2	0.14	4.2×10^{-7}	2.9	N.S.[d], $p > 0.05$
Novobiocin	612.7	2	1	91.8	1.5×10^{-4}	1.9	N.S., $p > 0.05$
5-Bromodeoxyuridine	307.1	2	2	18.3	6.0×10^{-5}	1.2	N.S., $p > 0.05$
4-Nitroquinoline-*N*-oxide	190.2	2	2	0.023	1.2×10^{-7}	1.1	N.S., $p > 0.05$
Amethopterin	454.5	2	2	0.012	2.6×10^{-8}	1.1	N.S., $p > 0.05$
6-Mercaptopurine	152.2	2	2	0.44	2.9×10^{-6}	0.5	N.S., $p > 0.05$
Hydrocortisone	486.4	4	2	219.0	4.5×10^{-4}	0.4	N.S., $p > 0.05$
Nitrogen mustard	192.5	3	4	0.35	1.8×10^{-6}	0.4	N.S., $p > 0.05$

[a] See Table 1, Footnote *a*.
[b] See "Materials and Methods."
[c] Tentative molecular weight, by kind permission of Dr. K. V. Rao of the John L. Smith Memorial for Cancer Research, Charles Pfizer & Co., Inc, Brooklyn, N.Y.
[d] N.S., not significant.

AD are plotted against the values for DM and VCR, respectively (Chart 3), a linear relationship is observed for the DC-3F series. Disparity of relative response of the CLM-77/AD III subline is shown in Table 1; cross-resistance to DM and to VCR was approximately the same as resistance to the selective agent itself.

When a resistant cell line was developed in the presence of DM, a similar pattern of cross-resistance was obtained, although reduction in sensitivity to AD was higher than would be expected on the basis of results with sublines DC-3F/AD IV. Reciprocity of resistance between AD and DM is further exemplified by the doubly resistant subline DC-3F/AD IV/DM, derived from DC-3F/AD IV and grown in the presence of DM. An increase in resistance to DM of approximately 11-fold was accompanied by a 6-fold increase in resistance to AD (Table 1).

These observations, suggesting a common mode of resistance to 3 agents for which dissimilar mechanisms of action have been postulated (1, 8, 20), prompted investigation of a wider series of chemical agents. Dose-response data obtained for parental DC-3F and the 376-fold AD-resistant subline DC-3F/AD IV are shown in Table 2. Agents are listed in order of decreasing ED_{50} ratios. Thus, in addition to VCR and DM, the subline was also cross-resistant to the antibiotics mithramycin, puromycin, and mitomycin C and to the alkaloids vinblastine and demecolcine.

It may be seen that for this group of compounds exhibiting cross-resistance there is a direct relationship between degree of resistance and molecular weight. In general, the greater the molecular weight, the higher the cross-resistance of the DC-3F/AD IV subline. With the exception of amethopterin, those compounds to which the parental and AD-resistant sublines exhibited similar sensitivities were either relatively biologically inactive or of relatively low molecular weight.

Autoradiography of Sensitive and Resistant Cells with AD-^{3}H and Tritiated Nucleic Acid Precursors. For determination of uptake of AD and its effect on incorporation of DNA and RNA precursors, sublines were exposed to AD-^{3}H at 2.66 and 5.32 μCi/ml and to uridine-^{3}H and thymidine-^{3}H as outlined in Tables 3 and 4. It is evident that as resistance to AD increased (Table 1), uptake of AD-^{3}H in terms of mean number of grains per nucleus was diminished (Table 3). For the DC-3F series, this relationship was confirmed in experiments with double the amount of tritiated drug (2.0 μg/ml), resulting in an approximate 2-fold increase in labeling. Uptake is inversely proportional to degree of resistance, as illustrated in Chart 4.

Although most of the grains were present in the nucleus, the greater the uptake of AD-^{3}H, the higher is the number of cytoplasmic and background grains (Figs. 1 to 8). For example, with 2.0 μg/ml AD-^{3}H, the mean background counts were 10.0, 5.5, 2.3, and 1.4 for DC-3F, DC-3F/AD II, DC-3F/AD IV, and DC-3F/AD X, respectively, suggesting that the high number of background grains is due to extraction of AD-^{3}H from the cells and contamination of the preparations during rinsing and fixation procedures. Autoradiograms of highly resistant populations, such as DC-3F/AD X and DC-3F/AD IV/DM, exposed to 1.0 μg/ml AD-^{3}H, in which there were essentially no labeled cells (Table 3), showed a grain distribution similar to those of emulsion controls.

A short exposure to a high concentration of AD completely inhibited incorporation of uridine-^{3}H into sensitive parental cells, as demonstrated in Table 3. It can be seen

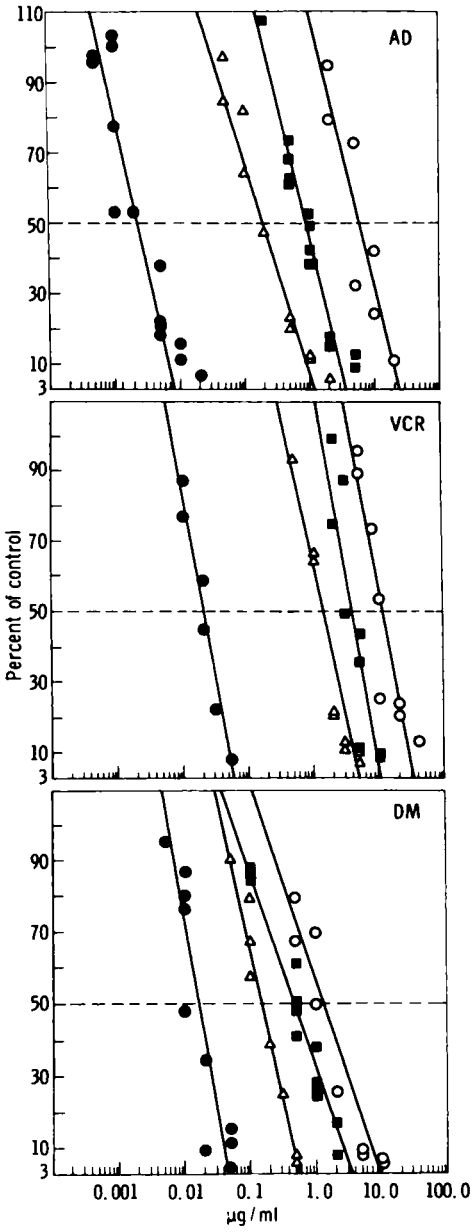


Chart 2. Response of parental DC-3F and AD-resistant sublines to various concentrations of AD, VCR, and DM. Each curve was calculated as an average of the fitted lines obtained in replicate experiments, based on the average ED_{50} and the average slope. The number of replications for DC-3F and DC-3F/AD IV in each subline-drug combination is indicated in Table 2; the remaining sublines were done in duplicate. •, DC-3F; △, DC-3F/AD II; ▪, DC-3F/AD IV; ○, DC-3F/AD X.

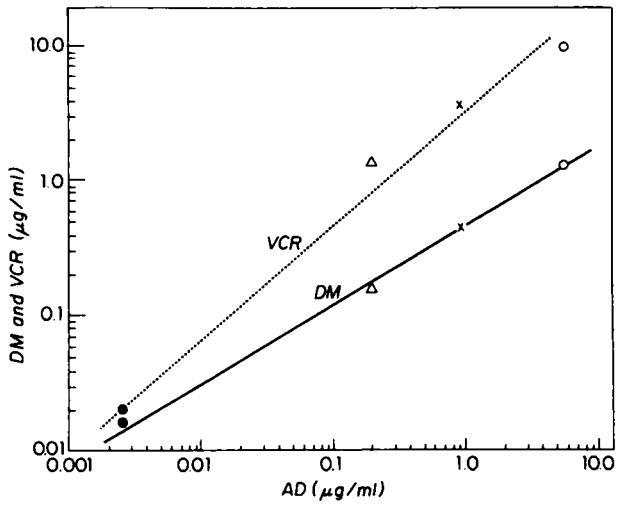


Chart 3. ED_{50} of AD plotted against ED_{50} of VCR and of DM for parental DC-3F and AD-resistant sublines. •, DC-3F; △, DC-3F/AD II; X, DC-3F/AD IV; ○, DC-3F/AD X.

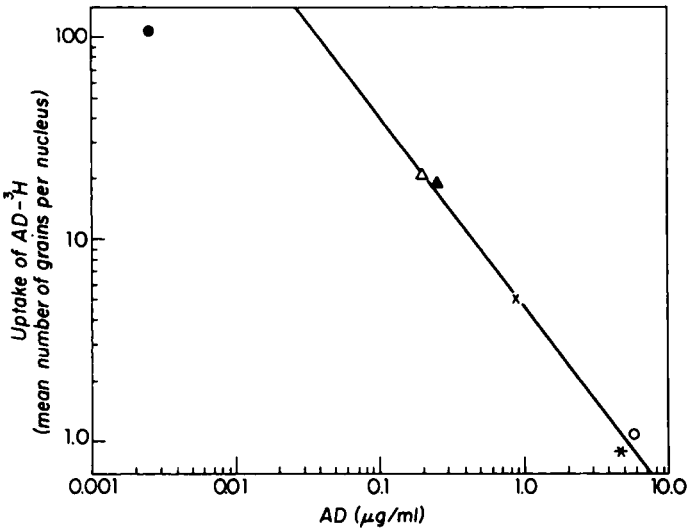


Chart 4. Relationship between sensitivity to AD in terms of ED_{50} and mean number of grains per nucleus in autoradiograms of cells exposed to 2.0 μg/ml AD-^{3}H (see Table 3) for parental DC-3F and sublines resistant to AD and DM. •, DC-3F; △, DC-3F/AD II; ▲, DC-3F/DM I; X, DC-3F/AD IV; ○, DC-3F/AD X; *, DC-3F/AD IV/DM.

that the degree of inhibition for the resistant sublines is approximately proportional to degree of sensitivity to AD. An exception is CLM-77/AD III, in which inhibition is less than would be expected on basis of resistance. In contrast, in the presence of antibiotic at similar drug levels and exposure periods, incorporation of thymidine-^{3}H was inhibited slightly or not at all, as indicated by autoradiographic data shown in Table 4.

Cytogenetic Studies. Determination of the number of chromosomes per cell was made for the AD-resistant sublines and the parental lines several times during the course of *in vitro* culture and drug exposure. As seen in Table 5, the modal numbers for the resistant sublines were 22, the diploid chromosome number of the Chinese hamster, or 23. The percentage of metaphases in the tetraploid or higher polyploid range varied from 1 to 6 for all lines.

Either visual or photographic analysis was carried out on most of the 500 cells included in Table 5, as well as on small samples of cells at other times during cultivation, in order to characterize the sublines with respect to stable

Table 3

Uptake of AD-^{3}H or uridine-^{3}H in absence and presence of AD as demonstrated in radioautograms of parental and drug-resistant cells

Cell cultures were exposed to AD-^{3}H for 2 hr and to uridine-^{3}H for the final hr of a 2 hr exposure to AD.

	Mean no. of grains/nucleus				
	AD-^{3}H		2.0 μCi/ml uridine-^{3}H		%
Cell line	1.0 μg/ml	2.0 μg/ml	Alone	10.0 μg/ml AD	inhibition
DC-3F	54.5	108.9[a]	87.7[a]	−0.9[a]	100
DC-3F/AD II	9.5	20.6[a]	115.6[a]	8.4[a]	93
DC-3F/AD IV	1.2	5.1[a]	40.4[a]	23.1[a]	43
DC-3F/AD X	−0.2	1.1[a]	41.1[a]	31.2[a]	24
DC-3F/DM I	10.1	19.0	14.1	−0.4	100
DC-3F/AM IV/DM	0.3	0.9	24.4	15.4	37
			0.5 μCi/ml uridine-^{3}H		
			Alone	10.0 μg/ml AD	
CLM-7	39.0		25.7	0.3	99
CLM-77/AD III	4.6		28.1	17.0	40
CLM-7/AD XV	0.4		24.2	12.6	48

[a]Average of values from 2 separate experiments.

Table 4

Frequency of cells showing incorporation of thymidine-^{3}H in absence and presence of AD

Cell cultures were exposed to AD for the final 2 hr and to thymidine-^{3}H for the final hr before fixation. Cells with 5 or more grains/nucleus were considered labeled; at least 1000 cells/subline were scored.

	% labeled cells	
	2.0 μCi/ml thymidine-^{3}H	
Cell line	Alone	10.0 μg/ml AD
DC-3F		
DC-3F	64.1	54.8
DC-3F/AD II	37.8	40.2
DC-3F/AD IV	51.4	49.9
DC-3F/AD X	27.0	27.0
	2.0 μCi/ml thymidine-^{3}H	
	Alone	1.0 μg/ml AD
CLM-7	55.5	49.4
CLM-77/AD III	56.4	63.0
CLM-7/AD XV	24.1	23.2

chromosomal alterations. A representative karyotype for each cell line is shown in Fig. 9; chromosomes are arranged according to the system of Hsu and Zenzes (12). Parental DC-3F was characterized by an abnormal No. 6 chromosome present in all resistant lines, and the heterochromatic X was absent in over 90% of cells. Other variations were infrequent. DC-3F/AD II showed miscellaneous numerical variations in more than 50% and inconsistent structural variations in about 25% of cells. In later passages, an additional No. 10-11 chromosome was usually present. DC-3F/AD IV had 1 or possibly 2 abnormal No. 1 chromosomes in 88% of metaphases, and a small fraction showed a deleted No. 7-8 chromosome, as well as a deleted No. 2 chromosome. DC-3F/AD X, exposed to the highest concentration of AD, had 5 to 6 structurally abnormal chromosomes, as depicted in Fig. 9, in all metaphases observed.

Parental CLM-7, karyotypically normal in early culture passages, possessed an additional No. 10-11 chromosome in most cells and sometimes a No. 9 chromosome, when examined at 14 months. After 20 months, a No. 7-8 chromosome appeared to have broken at or near the centromere region, the small arm being retained in about 50% of cells (Fig. 9). CLM-77/AD III, exposed to 0.1 μg/ml AD for 1.5 years, showed 6 to 7 consistent structural abnormalities, as seen in Fig. 9. In all cells analyzed, an additional chromosome classified as a deleted No. 9 was present, and sometimes an additional No. 10-11 chromosome was present.

Thus, for the DC-3F series, the higher the selective concentration of drug the greater the frequency of structurally altered chromosomes. Both resistant sublines of CLM-7 showed a variety of deleted and translocated chromosomes. The degree of consistent alteration contrasts with the relatively normal and stable karyotypes of the parental lines, where deviations were chiefly numerical. Since in previous studies (3, 5, 7) an association between resistance to amethopterin and specific chromosomal abnormalities was established, it seemed pertinent to examine the present material for a possible relationship between resistance to AD and specific karyotypic change. In a comparison of the 3 independently derived sublines DC-3F/AD X, CLM-77/AD III, and CLM-7/AD XV, no common abnormality could be detected.

DISCUSSION

Cross-resistance studies with AD-resistant mammalian cells

Table 5

Distribution of chromosome numbers in parental and AD-resistant cell lines

Cell line	AD (μg/ml)	Duration of exposure to AD (mo.)[a]	No. of chromosomes							No. of metaphases counted
			20	21	22	23	24	25	26	
DC-3F[b]	0		2	43	5					50
			5	43	2					50
DC-3F/AD II	0.1	14	1	11	20	8				40
		21	1		3	41	5			50
DC-3F/AD IV	1.0	11		6	29	5				40
DC-3F/AD X	10.0	4		12	26	2				40
		8		4	35	11				50
CLM-7[c]	0				4	22	14			40
					3	5	20	20	2	50
CLM-77/AD III	0.1	18			6	20	13	1		40
CLM-7/AD XV	1.0	6		4	28	14	3	1		50

[a]Number of months exposed to maintenance concentration of AD.
[b]Counts were carried out after 10 and 17 months, respectively, of growth *in vitro* after subcloning in 1965.
[c]Counts were carried out after 14 and 20 months, respectively, of growth since establishment *in vitro*.

are few. Subak-Sharpe (23) found that a BHK21 subline derived in the presence of AD and showing a 5-fold increase in resistance was also 5 times more resistant to puromycin than was the parental, untreated line. The naturally resistant green monkey kidney cells described by Wong *et al.* (24) were also relatively resistant to puromycin. The papers of Kessel *et al.* (14) and Kessel and Wodinsky (15) reported that mouse leukemias resistant to vinblastine and to a terepthalanilide derivative were each cross-resistant to AD and DM, while mice bearing a VCR-resistant line and treated with AD showed only a moderate increase in mean survival time as compared to the parental leukemia. The present data concur with and extend these observations, demonstrating cross-resistance between AD and a variety of antibiotics and alkaloids inhibitory to cell growth at comparatively low concentration.

It is evident from the relative response of the parental and AD-resistant sublines of the DC-3F series to AD, VCR, and DM (Chart 3) that degree of cross-resistance is proportional to degree of AD resistance itself. Such a result may be accounted for by a common cellular attribute controlling response to the 3 agents. An explanation satisfactorily fitting the results of uptake studies with tritiated drug is that of reduced permeability of the cells to AD and concomitantly and nonspecifically reduced permeability to other chemical agents.

The correspondence between molecular weight and degree of cross-resistance to biologically active agents is notable. It would appear that modification of the surface membrane of cells, after prolonged exposure to AD, may make molecular weight an important factor in penetration of certain chemical agents into resistant cells (Table 2).

In conformance with data concerning the mechanism of action of AD in biological systems (20), results of the radioautographic experiments performed with tritiated nucleic acid precursors suggest that high concentrations of AD for short periods of time markedly affect RNA, but not DNA, synthesis in sensitive parental Chinese hamster cells. For the highly resistant cell line DC-3F/AD X, in contrast, exposure to a concentration of antibiotic approximately twice the ED_{50} resulted in the least inhibition, suggesting that comparatively little antibiotic reached the active site within the cell.

In this *in vitro* study of highly resistant Chinese hamster cell lines obtained by direct exposure to selective agents, cellular resistance is clearly due to inability of AD to be transferred through the surface membrane. Whether certain other mechanisms, such as altered DNA binding affinity or drug degradation processes, were additionally or alternatively operative was not tested experimentally. The hypothesis of Kessel and Wodinsky (15) that lack of retention of drug in resistant cells may be the determinant of drug response could not be confirmed. However, differences in biological systems and experimental techniques may contribute to this fundamental discrepancy. Autoradiography with AD-^{3}H has certain inherent difficulties based on the solubility of the antibiotic in water, alcohol, and other substances. Once standardized, the procedures used in this study gave reproducible results. Moreover, it was of interest to note the location and intensity of background grains in autoradiographic preparations of the various sensitive and resistant sublines. For sensitive cells, there was positive correlation between cell density and number of background grains. For highly resistant cells, in contrast, the number of background grains was low and independent of cell density. These observations indicate that during preparation of autoradiograms some intracellular drug was extracted and remained in the vicinity of the cells, whereas in highly resistant cells there was little or no tritiated drug to be extracted.

Consistent with the finding that resistance is correlated with reduced drug uptake is the karyotypic stability of the resistant sublines. It is likely that chromosomal abnormalities occurred before full resistance to maintenance concentration of AD was reached and that drug-induced modification of

karyotype was infrequent thereafter. That AD can produce dose-dependent chromosome breakage was demonstrated by Ostertag and Kersten (18), while Arrighi and Hsu (2) found metaphase chromosome abnormalities, including breakage, in Chinese hamster cells; interaction between antibiotic and DNA was postulated.

The present investigations suggest that the resistance to AD developing in Chinese hamster cells exposed to various concentrations of antibiotic is quantitatively related to decreased penetration of drug into the cell. Studies are in progress to assess further the possibility and biological consequence of altered cell membrane. In preliminary experiments (4), it was found that the surfactant Tween 80 markedly increased uptake of AD in resistant cells, while equivalent concentrations, in terms of growth-inhibitory effect, of a variety of other chemical agents did not.

ACKNOWLEDGMENTS

We are indebted to Dr. Isabel M. Mountain for statistical evaluation and computer analysis of data. Discussions with Dr. Herbert S. Schwartz were very helpful. The excellent assistance of Miss Francesca R. Marshall, Miss Marion Linczer, and Miss Barbara A. Spengler is gratefully acknowledged.

REFERENCES

1. Armstrong, J. G. Vincristine (NSC-67574) Symposium—Summary and Prospectives. Cancer Chemotherapy Rept., *52:* 527–535, 1968.
2. Arrighi, F. E., and Hsu, T. C. Experimental Alteration of Metaphase Chromosome Morphology: Effect of Actinomycin D. Exptl. Cell Res., *39:* 305–308, 1965.
3. Biedler, J. L., Albrecht, A. M., and Hutchison, D. J. Cytogenetics of Mouse Leukemia L1210. I. Association of a Specific Chromosome with Dihydrofolate Reductase Activity in Amethopterin-treated Sublines. Cancer Res., *25:* 246–257, 1965.
4. Biedler, J. L., and Riehm, H. Reduced Cellular Resistance to Actinomycin D in the Presence of a Detergent. Proc. Am. Assoc. Cancer Res., *10:* 6, 1969.
5. Biedler, J. L., Schrecker, A. W., and Hutchison, D. J. Selection of Chromosomal Variant in Amethopterin-resistant Sublines of Leukemia L1210 with Increased Levels of Dihydrofolate Reductase. J. Natl. Cancer Inst., *31:* 575–601, 1963.
6. Biedler, J. L. and Schwartz, H. S. Resistance to Actinomycin D: Mechanism, Cross-Resistance, and Karyotype of Mammalian Cell Lines. In Vitro, *4:* 155, 1968.
7. Biesele, J. J., Biedler, J. L., and Hutchison, D. J. The Chromosomal Status of Drug Resistant Sublines of Mouse Leukemia L1210. *In:* Genetics and Cancer, pp. 295–307. Austin, Texas: University of Texas Press, 1959.
8. Di Marco, A. Daunomycin and Related Antibiotics. *In:* D. Gottlieb and P. D. Shaw (eds.), Antibiotics, Vol 1. Mechanism of Action, pp. 190–210. New York: Springer-Verlag, Inc., 1967.
9. Goldstein, M. N., Hamm, K., and Amrod, E. Incorporation of Tritiated Actinomycin D into Drug-sensitive and Drug-resistant HeLa Cells. Science, *151:* 1555–1556, 1966.
10. Goldstein, M. N., Slotnick, I. J., and Journey, L. J. *In Vitro* Studies with HeLa Cell Lines Sensitive and Resistant to Actinomycin D. Ann. N. Y. Acad. Sci., *89:* 474–483, 1960.
11. Haywood, A. M., and Sinsheimer, R. L. Inhibition of Protein Synthesis in *E. coli* Protoplasts by Actinomycin-D. J. Mol. Biol., *6:* 247–249, 1963.
12. Hsu, T. C., and Zenzes, M. T. Mammalian Chromosomes *in Vitro*. XVII. Idiogram of the Chinese Hamster. J. Natl. Cancer Inst., *32:* 857–869, 1964.
13. Hurwitz, J., Furth, J. J., Malamy, M., and Alexander, M. The Role of Deoxyribonucleic Acid in Ribonucleic Acid Synthesis, III. The Inhibition of the Enzymatic Synthesis of Ribonucleic Acid and Deoxyribonucleic Acid by Actinomycin D and Proflavin. Proc. Natl. Acad. Sci. U. S., *48:* 1222–1230, 1962.
14. Kessel, D., Botterill, V., and Wodinsky, I. Uptake and Retention of Daunomycin by Mouse Leukemic Cells as Factors in Drug Response. Cancer Res., *28:* 938–941, 1968.
15. Kessel, D., and Wodinsky, I. Uptake *in Vivo* and *in Vitro* of Actinomycin D by Mouse Leukemias as Factors in Survival. Biochem. Pharmacol., *17:* 161–164, 1968.
16. Leive, L. Actinomycin Sensitivity in *Escherichia coli* Produced by EDTA. Biochem. Biophys. Res. Commun., *18:* 13–17, 1965.
17. Mach, D., and Tatum, E. L. Ribonucleic Acid Synthesis in Protoplasts of *Escherichia coli*: Inhibition by Actinomycin D. Science, *139:* 1051–1052, 1963.
18. Ostertag, W., and Kersten, W. The Action of Proflavin and Actinomycin D in Causing Chromatid Breakage in Human Cells. Exptl. Cell Res., *39:* 296–301, 1965.
19. Polsinelli, M., Cifferri, O., Cassani, G., and Albertini, A. Mechanism of Resistance to Actinomycin in *Bacillus subtilis*. J. Bacteriol., *88:* 1567–1572, 1964.
20. Reich, E., Franklin, R. M., Shatkin, A. J., and Tatum, E. L. Effect of Actinomycin D on Cellular Nucleic Acid Synthesis and Virus Production. Science, *134:* 556–557, 1961.
21. Schwartz, H. S., Sodergren, J. E., and Ambaye, R. Y. Actinomycin D: Drug Concentrations and Actions in Mouse Tissues and Tumors. Cancer Res., *28:* 192–197, 1968.
22. Slotnick, I. J., and Sells, B. H. Actinomycin Resistance in *Bacillus subtilis*. Science, *146:* 407–408, 1964.
23. Subak-Sharpe, H. Biochemically Marked Variants of the Syrian Hamster Fibroblast Cell Line BHK21 and Its Derivatives. Exptl. Cell Res., *38:* 106–119, 1965.
24. Wong, K. T., Baron, S., Levy, H. B., and Ward, T. G. Dactinomycin: Relative Resistance of Green Monkey Kidney Cell Cultures to Its Action. Proc. Soc. Exptl. Biol. Med., *125:* 65–67, 1967.

Figs. 1 to 5. Photomicrographs of sensitive and resistant cells exposed to 2.0 μg/ml AD-^{3}H for 2 hr. Mayer's hematoxylin, X 1200.

Fig. 1. Parental DC-3F cell line.

Fig. 2. Subline DC-3F/AD II.

Fig. 3. Subline DC-3F/AD IV.

Fig. 4. Subline DC-3F/AD X.

Fig. 5. Subline DC-3F/DM I.

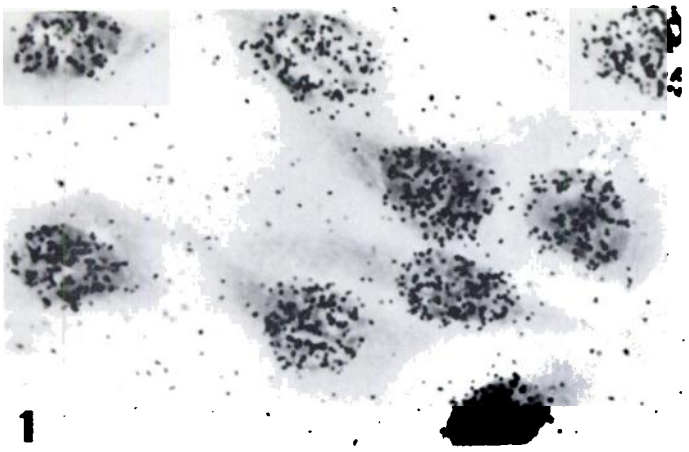
Figs. 6 to 8. Photomicrographs of sensitive and resistant cells exposed to 1.0 μg/ml AD-^{3}H for 2 hr. Mayer's hematoxylin, X 1200.

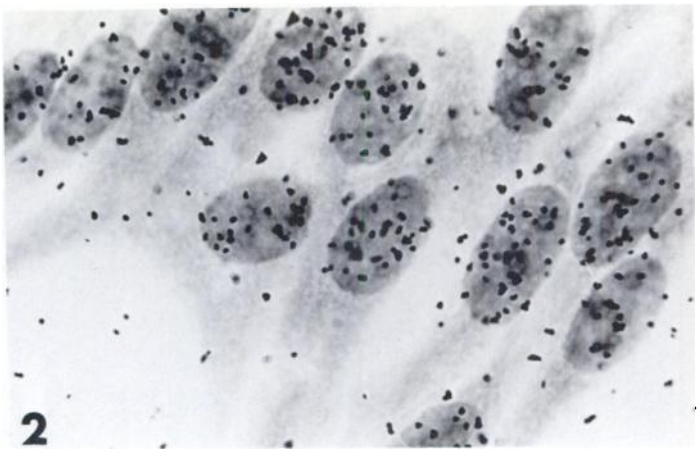
Fig. 6. Parental CLM-7 cell line.

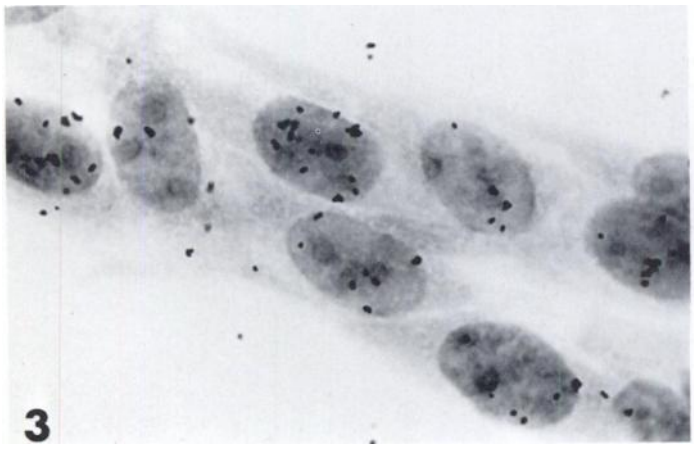
Fig. 7. Subline CLM-77/AD III.

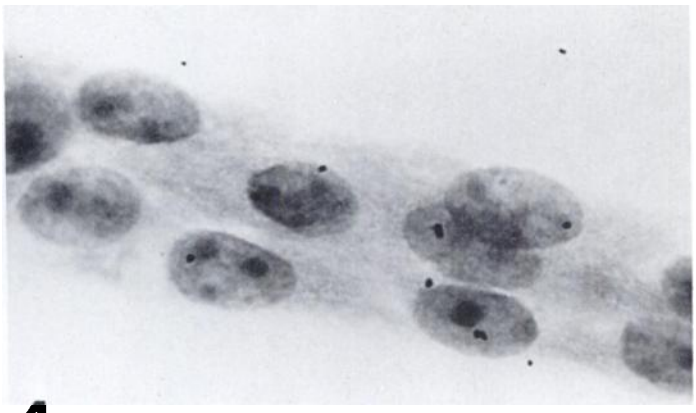
Fig. 8. Subline CLM-7/AD XV.

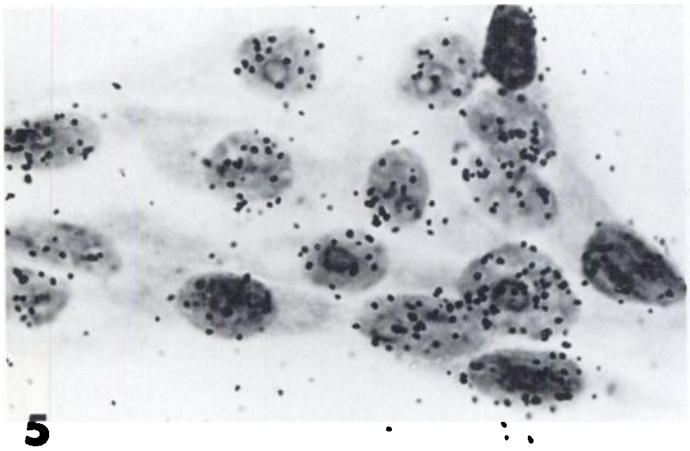
Fig. 9. Karyotypes of parental and AD-resistant sublines. Deleted and translocated chromosomes are indicated by *arrows* and designated *d* and *t*, respectively. Additional and absent chromosomes are indicated by *ad.* and *ab.*, respectively. Acetic orcein, X 2000.

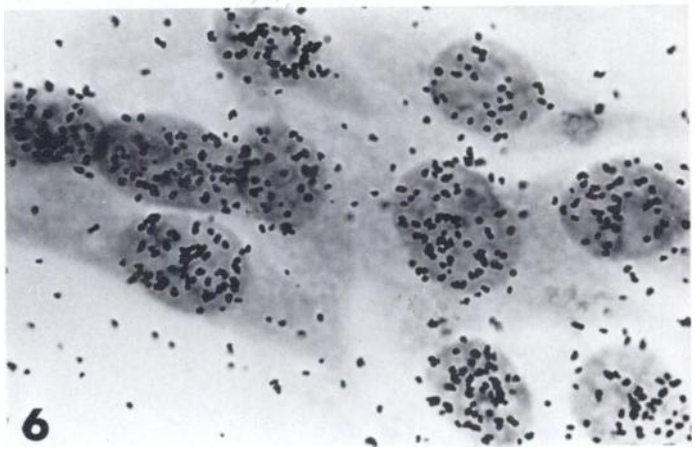


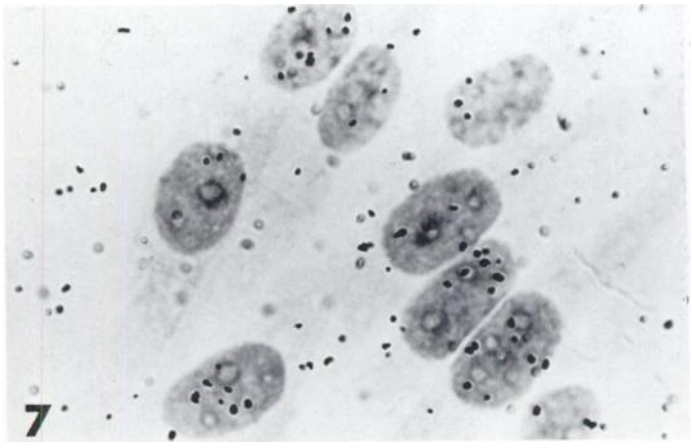


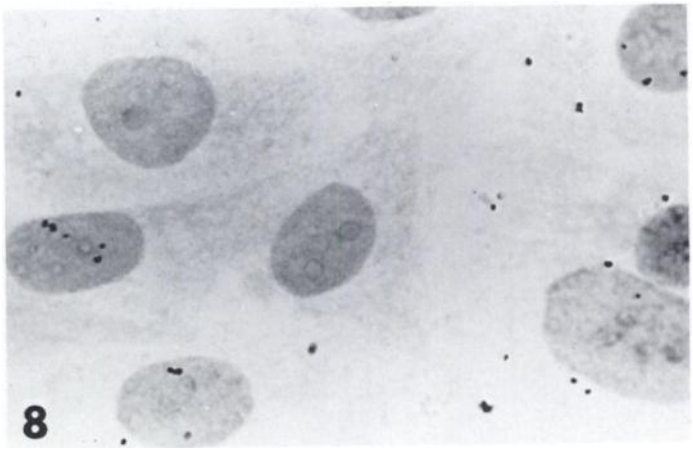












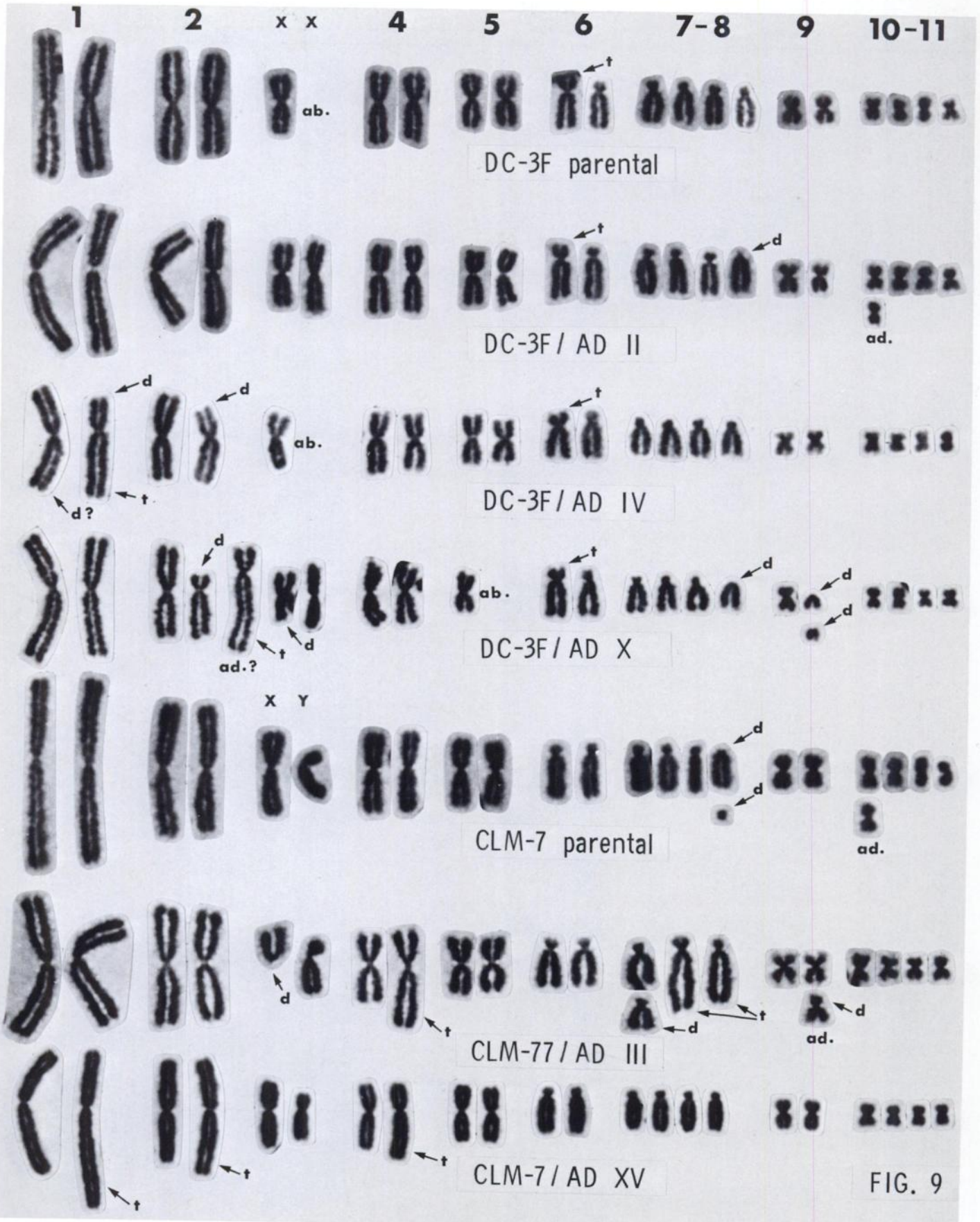


FIG. 9