Immortalization of Normal Human Kidney Epithelial Cells by Nickel(II)[1]

Guri Tveito, Inger-Lise Hansteen, Helge Dalen, and Aage Haugen[2]

Department of Toxicology, National Institute of Occupational Health, P. O. Box 8149 Dep, 0033 Oslo 1 [G. T., A. H.]; Department of Occupational Medicine, Telemark Hospital, 3600 Porsgrunn [I-L. H.]; and Laboratory of Clinical Electron Microscopy, University of Bergen, 5021 Bergen [H. D.], Norway

ABSTRACT

The occupational and environmental hazards of nickel exposure are of great concern in environmental medicine. Nickel workers have increased risk of cancer of the nose, lung, larynx, and possibly the kidney. In the present investigation we have studied the effects of nickel ions on fetal human kidney cortex explants. The explants were continuously exposed to 5 μg/ml NiSO$_4$. After 70–100 days in culture foci of phenotypically altered cells appeared. Immortalized cell lines were established and demonstrated to be of human epithelial origin. Tumorigenicity was not induced, but the cells demonstrated decreased requirement for serum, increased plating efficiency and saturation density, and formation of colonies in soft agar. Chromosome changes in the treated cells were observed. Worth mentioning are change in ploidy (3n) and abnormalities of chromosomes 1, 7, 9, 11, 13, 14 and 20; increased numbers of chromosome 17; and loss of normal chromosomes 20 and 22.

INTRODUCTION

Cancer arises in a multistep fashion and particular chemical, physical, and biological agents are major etiological factors (1–3). The malignant transformation of human cells in culture by carcinogenic agents permits analysis of alterations and evolution in cell properties that may be representative of cancer cells. Since most human tumors are of epithelial origin it is important to study such cell systems. Furthermore, we know very little about the similarities and differences between human and rodent cells in their response to chemical carcinogens *in vitro*. Such comparisons are needed to extrapolate more accurately from experimental data in animals to human assessment. There is some information on human nonepithelial cells, but there is little knowledge available concerning epithelial cells (4, 5). Human cells and particularly epithelial cells are refractory to *in vitro* transformation by chemical carcinogens. However, efforts to investigate the progression of events that lead human cells of epithelial origin to become neoplastic in response to carcinogenic agents have been aided by the recent development of tissue culture systems for propagation of human epithelial cells (6–8). There are few reports on the establishment of normal kidney proximate tubule epithelial cells in culture (9–11).

Nickel is a potent carcinogen in experimental animals, and epidemiological studies among workers in certain nickel industries suggest a connection between nickel exposure and tumors in the respiratory tract and possibly the kidney (12–14). We have utilized primary human fetal kidney epithelial cell culture to determine the ability of this environmental carcinogen to transform normal cells of human epithelial origin. The term "transformed" will be used to indicate that the cells have taken on one or more characteristics of tumor cells (15).

MATERIALS AND METHODS

Chemicals. Chemicals were purchased as follows: epidermal growth factor and fibronectin, Collaborative Research, Inc., Waltham, MA;

transferrin, ethanolamine, phosphoethanolamine, 3,3',5-triiodothyronine, epinephrine, insulin, hydrocortisone, polyvinylpyrrolidone, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and trypsin inhibitor, Sigma Chemical Co., St. Louis, MO; Vitrogen and gentamicin, Flow Laboratories, Irvine, United Kingdom; FCS[3] and Colcemid, Gibco, Paisley, Scotland; and fluorescein isothiocyanate-conjugated antirabbit IgG, Bio Yeda, Rehovot, Israel.

Cell Culture and Treatment of NHKE Cells. Human fetal kidneys were obtained from midtrimester therapeutic abortions. Consent for the experimental use was given by Aker Hospital, Oslo, Norway. After abortion the kidneys were immersed in ice-cold L-15 medium and transported to the laboratory. The cortex was dissected free, cut into small (0.2-cm²) pieces, placed on the scratched surface of culture dishes to hold the explants in place, and inoculated in α-MEM medium (Gibco) with supplements (see below). Primary cultures were propagated by the explant outgrowth culture procedure. After 1–2 weeks a patch of epithelial cells had grown out 0.5–1.0 cm around the kidney cortex explant. The explants were then transferred to a new dish to initiate additional outgrowth cultures.

The procedure for nickel treatment was as follows. Fetal human kidney explants were exposed continually to 5 μg/ml NiSO$_4$. The kidney epithelial cells grow off the nickel-exposed and control explants and established primary cultures. After 14 days the explants were replanted into new dishes containing medium with 5 μg/ml NiSO$_4$ to produce second outgrowths while the primary explant outgrowths were changed to medium without NiSO$_4$. This replanting process was repeated 10 times. Foci of phenotypically altered cells appeared in primary cultures from the fifth outgrowth after 70–100 days of continuous exposure to NiSO$_4$. Clones were selected from these cultures on the basis of phenotypical alterations.

Media. NHKE cells were cultured in α-MEM medium supplemented with epidermal growth factor (10^{-5} g/liter), insulin (5×10^{-3} g/liter), hydrocortisone (3.4×10^{-6} g/liter), 3,3',5-triiodothyronine (6.7×10^{-9} g/liter), transferrin (5×10^{-3} g/liter), and 5% FCS. IHKE cells were cultured in the same medium; however, FCS concentration was reduced to 1%.

Growth Rates. Measurement of growth rates was performed for NHKE and IHKE cells in α-MEM. The cells were inoculated into 35-mm tissue culture dishes at a density of 5000 cells/cm² dish. Four to 6 days later the cells were counted to obtain R(PD/D) (6).

Determination of Saturation Density. The saturation density of each cell population was determined in α-MEM in conjunction with growth rate measurements.

Electron Microscopy. Cell cultures designated for electron microscopy were grown to confluency in Petriperm dishes, in which the bottoms consist of 25-μm-thick Teflon membranes. The cell monolayers were double-fixed *in situ* at room temperature for 2 h in 2% glutaraldehyde followed by 1 h in 1% OsO$_4$. Both fixatives were made up in 0.1 M cacodylate buffer (pH 7.2) supplemented with 0.1 M sucrose (vehicle osmolality, 300 mOsmol (16)). The cell cultures were dehydrated in ascending concentrations of ethanol and embedded in Epon 812 (17). After polymerization and reembedding the monolayers were sectioned parallel with the growth substratum on a Reichert ultramicrotome. The ultrathin sections were contrasted with uranyl acetate (18) and lead citrate (19) and studied in a Philips 300 transmission electron microscope operated at 60 kV.

Immunofluorescence Microscopy. Rabbit anti-keratin antibody was generously provided by Dr. Susan Schlegel. Immunofluorescence microscopy of the cells was performed according to the double antibody technique described by Sun and Green (20).

Chromosomal Preparations. Metaphase chromosome spreads were prepared using standard procedures. Subconfluent cultures of the cell line to be analyzed were treated for 2 h with Colcemid, harvested by trypsination, and pelleted by low speed centrifugation. The resulting cell pellet was resuspended in 0.075 M KCl for 5–10 min and fixed by resuspension three times in methanol:glacial acetic acid (3:1). Thereafter fixed cells were "dropped" onto clean microscopic glass slides. Some slides were stained with conventional Giemsa solution for chromosome counting while other slides were processed for G-banding (21).

Soft Agar Growth Assay. A 2-ml base layer of 0.5% (w/v) agar (Agar Noble; Difco, Detroit, MI) in α-MEM was added to a 60-mm Costar Petri dish; 1×10^4 freshly trypsinized IHKE cells were incubated in 0.5 ml α-MEM. Subsequently, the cell suspension was mixed with 1.5 ml of the above agar medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies larger than 8 cells were scored after 10–14 days by counting 20 random fields corresponding to 2.3% of the total dish area.

Nude Mice. To assay for tumorigenicity 1×10^7 IHKE cells were injected s.c. into athymic nude mice.

RESULTS

NHKE cells grown out from the explant could be seen after 3–4 days in culture. After 1–2 weeks, patches of epithelial cells had grown out 0.5–1 cm around the kidney cortex explant. NHKE cells growing out from the explant were polygonal (Fig. 1A). In dense areas the cells showed a more cuboidal pattern. Multicellular hemicysts known as domes were formed in confluent NHKE cell cultures (Fig. 1B). Domes result from fluid accumulation between the cell layer and the culture dish (22).

A number of cell lines were obtained from nickel-treated cultures. IHKE-1 cells were pleomorphic with stellate-fibroblastoid morphology and varied in size and shape. (Fig. 1C).
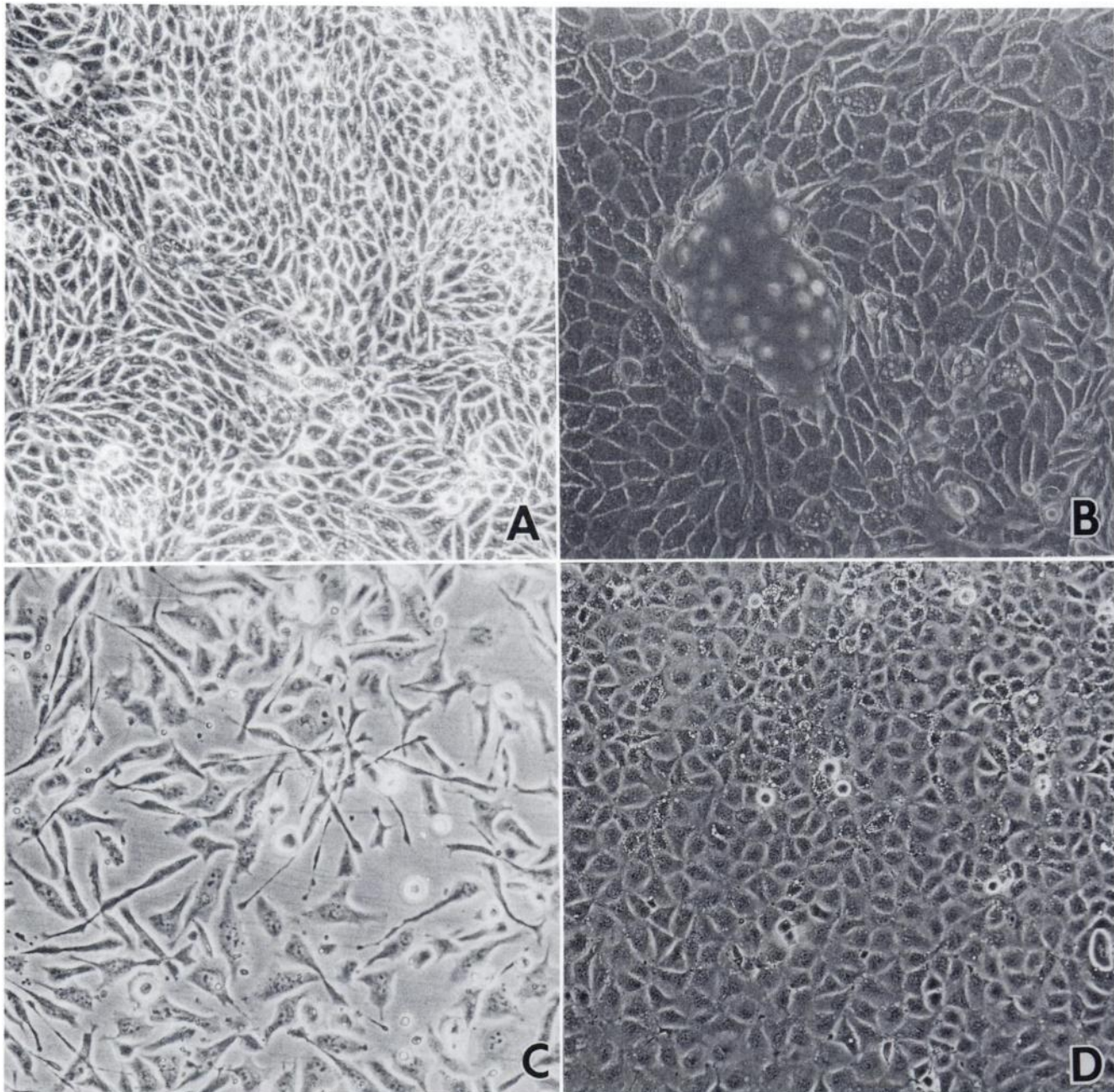

Fig. 1. Phase contrast photomicrographs of (A) outgrowth of NHKE cells from kidney explant, (B) confluent monolayer of NHKE cells showing dome formation, (C) IHKE-1, and (D) IHKE-2. × 500.

IHKE-2 cells had a flat more uniform morphology (Fig. 1D). The cell lines have an apparently unlimited life span, and each line has been subcultivated for more than 130 passages with no evidence of decreased proliferative capacity. Keratin, an epithelial marker, was revealed by indirect immunofluorescence with antiserum to whole keratin (Fig. 2). In both NHKE and IHKE cells accumulation of filaments were seen in the perinuclear region. As revealed by electron microscopy the cells contained keratin filaments (tonofilaments) and desmosomes, characteristic features of epithelial cells (Fig. 3A). These intermediate filaments, constituting the cytoskeletal framework of the cell, were arranged in fibrils or in an irregular network. Desmosomes, from which keratin filaments radiated into the cytoplasm, linked adjacent cells to each other (Fig. 3B). During cytoplasmic spreading the desmosomes were apparently first established at sites where slender surface processes came into contact with the adjacent cell surface (Fig. 3C).

In Vitro Growth Properties. The *in vitro* growth properties of the IHKE cells (24th passage) were examined and compared with those of the normal ones. The serum requirement for the growth of NHKE and IHKE cells was determined. The growth rate for NHKE cells was lower than for IHKE cells in α-MEM medium supplemented with 5% FCS. The serum dependence of NHKE and IHKE cell growth is shown in Fig. 4. Only IHKE cells were able to grow in a serum-free medium. IHKE cells are maximally stimulated at 1% FCS, while the growth of NHKE cells increased up to a concentration of 5% FCS.

The chemically transformed cells were characterized by quantitative differences in growth properties, such as saturation density and soft agar colony-forming efficiency (Table 1). The saturation densities of IHKE cells (24th passage) were approximately 2 times higher than that of the untreated NHKE cells. Furthermore, the IHKE cells grew in soft agar with a colony-forming efficiency of 0.05, whereas the untreated cells were unable to grow in soft agar. Tumorigenicity was not induced.

Chromosome Abnormalities Induced by NiSO₄. Five cultures, NHKE 1–5, were analyzed after 6 passages in culture. The results showed a wide variation in chromosome number. The untreated control culture, NHKE 1 in Fig. 5, had mainly cells with the normal chromosome count, 46,XX, while the majority of cells in the NHKE 2–5 cultures had chromosome counts between 70 and 86. Only a few cells revealed very high counts.
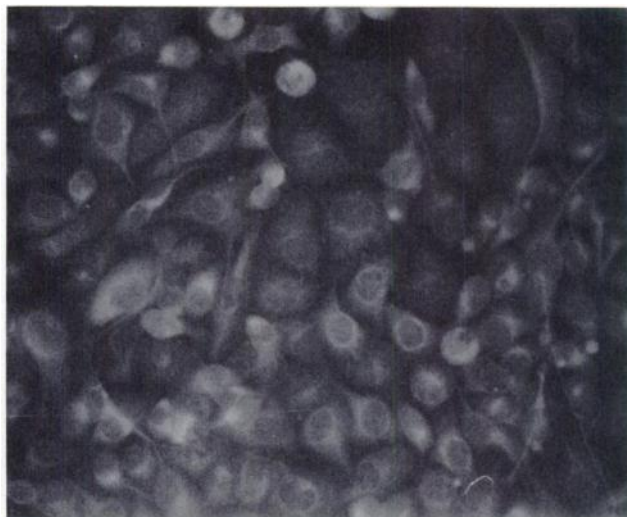
The chromosomes involved in marker chromosome formation, dicentrics, tricentrics, rings, and exchanges, were noted and the mean number of rearrangements per chromosome per cell was calculated. The results are presented in Fig. 5. Most rearrangements were found in NHKE 3 and NHKE 5 involving mainly chromosomes 2, 7, 9, 19, and 22. Double minutes were mainly seen in NHKE 4.

Other cells were cultured up to the 24th passage (IHKE) before processing for chromosome studies and growth in soft agar. Two cell lines were apparent, one with 39–40 chromosomes and one with 71–86 chromosomes with loss or gain of chromosome groups as indicated in Fig. 6. Most cells contained double minutes, tiny single fragments, and marker chromosomes as reported in Table 2.

Four cell clones were selected in agar and cultured for 37 to 42 passages before another chromosome study was performed. All 4 clones, IHKE 1–4, were immortalized and had chromosome counts around the triploid (3n) number (Table 2). Common for all IHKE clones were many marker chromosomes and gain of chromosomes in groups 16 and 17 and loss in groups 14, 15, 20, and 22 (Fig. 6). In addition there was loss or gain of slightly different groups of chromosomes in the different lines. Some of the chromosome losses were not consistent in all cells and were probably due to rupture of the cell membrane.

All cell clones contained some of the same marker chromosomes as the IHKE cells reported in Table 2. New marker chromosomes were formed in the four clones, mostly involving different chromosomes in each clone.

DISCUSSION

Studies of neoplastic transformation in epithelial cells are critical to an understanding of human cancer since more than 80% of human tumors are epithelial in origin. The kidney is a target organ for chemical carcinogenesis. The kidney epithelial cells are constantly exposed to high concentrations of chemicals. The results of the present study demonstrate that Ni(II) induces *in vitro* transformation of NHKE cells. The cell lines are unambigously of human epithelial origin. Keratin has been shown to be the structural protein component of cytoskeletal intermediate filaments in the epithelial cells (23). In our studies we used antisera against whole keratin. All cells stained positively for cytokeratin confirming their unmodified epithelial nature. Furthermore, the cell lines have human chromosomes and retain the epithelial characteristic desmosomes.

Increased saturation density as well as the ability to grow in soft agar appeared to be acquired properties of the immortalized cells. However, the cells did not form tumor in athymic nude mice. Thus, although immortal, they likely do not represent neoplastic transformation, *i.e.*, capable of forming a benign or malignant tumor in susceptible animal host. It has been found that an important step in the establishment of a malignant cell line *in vitro* is rescue from senescence or immortalization (24–28). Human cells are highly refractory to changes in the mechanisms that limit their *in vitro* life span (4, 5). We have previously reported that an extended life span was observed in the nickel-treated bronchial epithelial cultures (29). However, the cells after a while senesce in culture, and they did not grow in soft agar.

The occupational and environmental hazards of metal exposure are of great concern. Nickel compounds have been implicated as important occupational carcinogens. Certain types of cancer appear to be more frequent among workers in nickel refineries (lung, nasal sinuses, larynx, and kidney carcinomas)

Fig. 2. Indirect immunofluorescence for keratin. The example shown is IHKE-2.
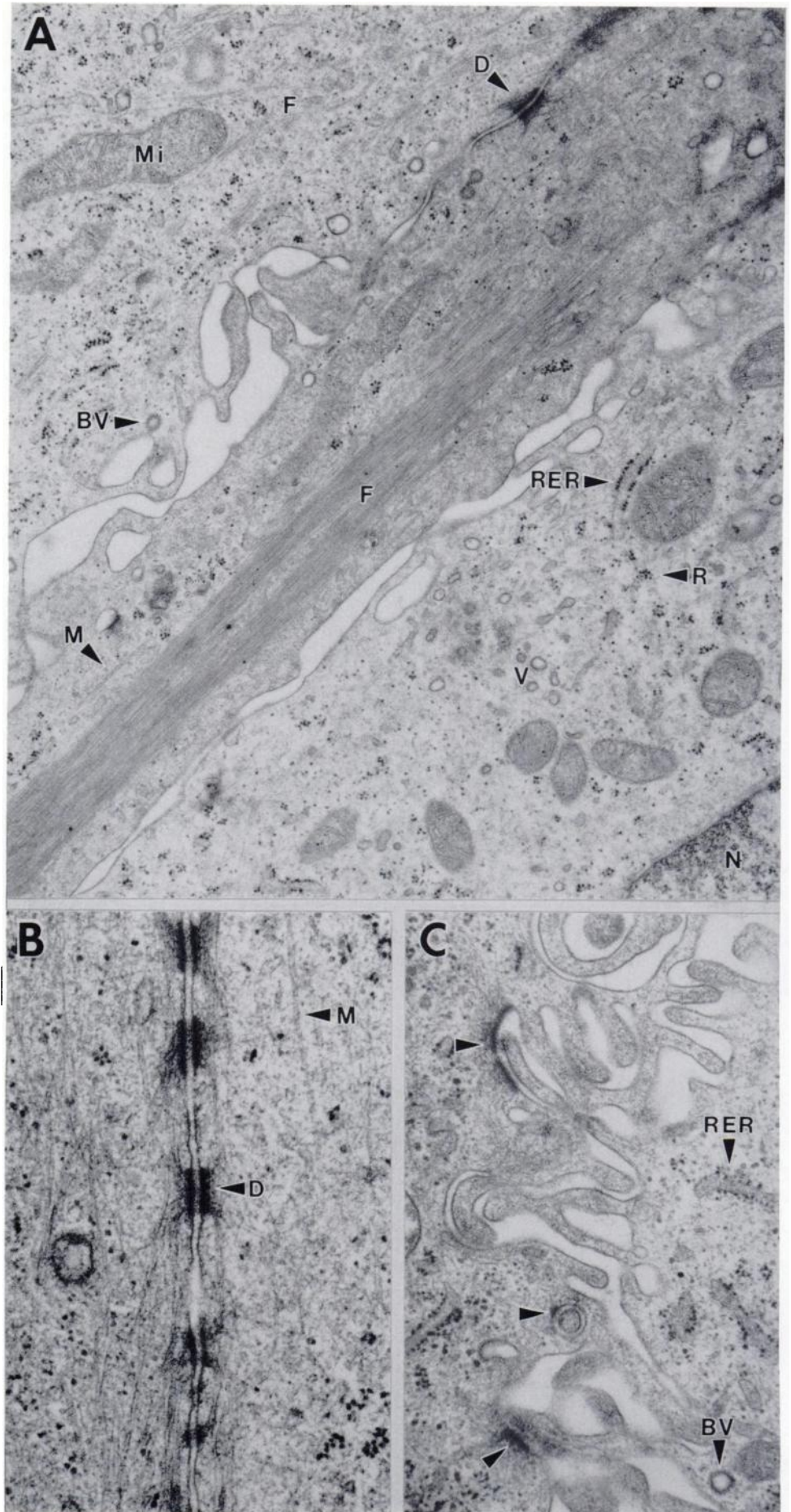
Fig. 3. Electron micrographs of cultivated human kidney cells sectioned parallel with the growth substratium. In *A*, various cell organelles including mitochondria (*Mi*), free (*R*), and membrane-attached ribosomes (*RER*) and pinocytotic (*V*) and bristle-coated (*BV*) vesicles are dispersed throughout the cytoplasm. The cytoskeletal elements are represented by short segments of microtubulus (*M*) and abundant keratin filaments (*F*). The latter structures, arranged as fibrils or as irregular networks, are attached to the plaques of the desmosomes (*D*). *N*, nucleus; × 22,000. In *B*, numerous spot desmosomes (*D*) link adjacent cell membranes to each other. Note the short microtubular segment (*M*) and that the desmosomes are interconnected by keratin filaments. × 30,000. *C*, spot desmosomes are apparently formed at the sites where slender surface processes come into contact with the adjacent cell surface (*arrowheads*). *RER*, rough surfaced endoplasmic reticulum. × 40,000.
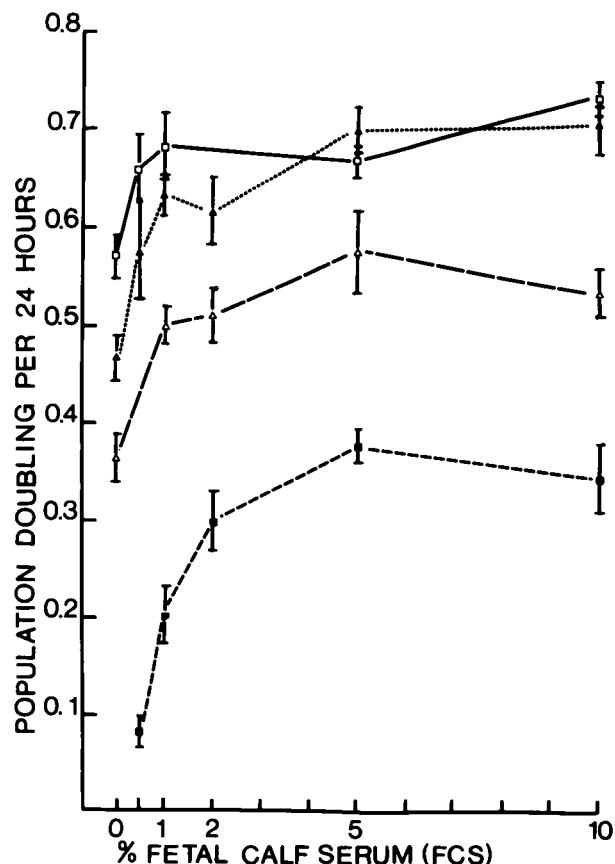
Fig. 4. Growth rate dose responses of NHKE (■) IHKE-1 (□), IHKE-2 (▲), and IHKE-3 (△).

appeared, while double minutes and single fragments occurred in almost all cells.

The four cell clones had many chromosomal rearrangements in common but also differed from the original cell line and from each other both in gain or loss of chromosomes and in amount of marker chromosomes. Some of the chromosome loss is due to rupture of the cell membrane, indicating that the chromosome pattern within the clone is more constant than it was possible to demonstrate. Acquisition of new marker chromosomes occurred, but to a lesser degree than in the primary cultures. The chromosomal changes demonstrated here with great changes in chromosome number and structure towards selection of more stable clones with less rearrangements are not an uncommon finding in cytogenetic changes in response to carcinogenic treatment both *in vivo* and *in vitro*.

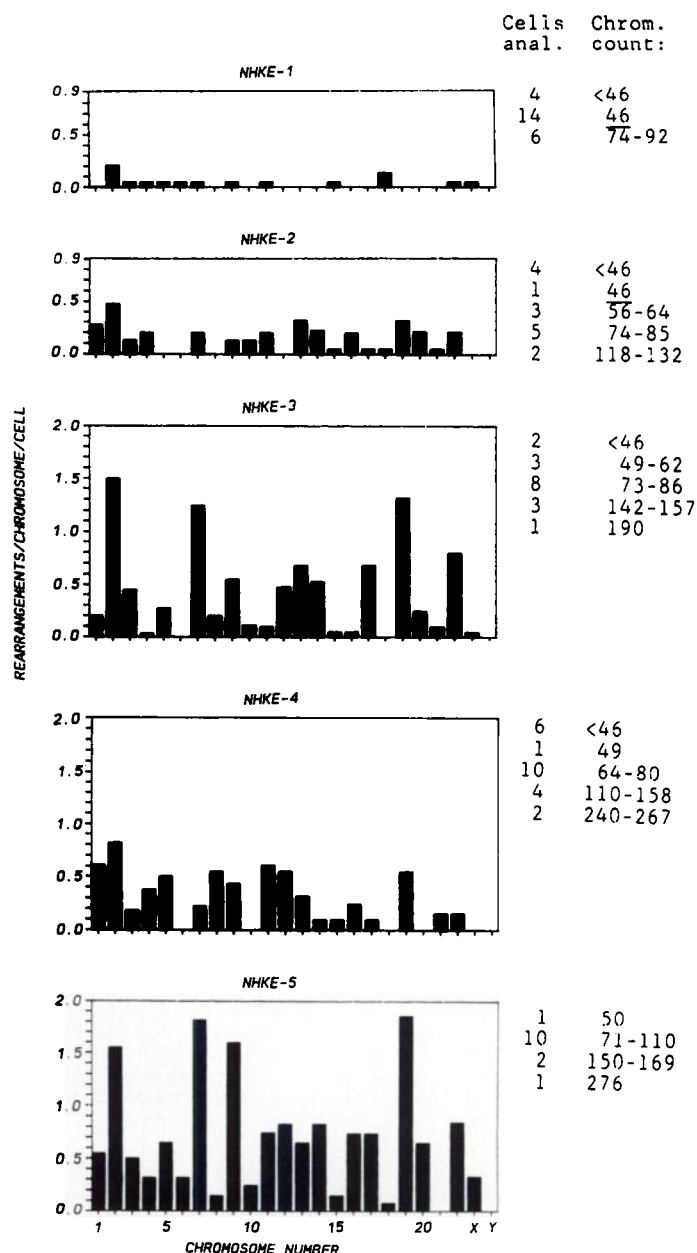Our present findings have led us to conclude that nickel

Table 1 *Saturation density, cloning efficiency in soft agar, and transplantability*

Cells	Life span	Saturation density (×10⁵/ cm²)	Cloning efficiency (%)	Transplantability
NHKE	Limited	0.4	<0.0006	0/5
IHKE	Unlimited	0.9	0.05	0/5

(12–14, 30, 31). Previous studies have demonstrated the genotoxic effects of nickel compounds. Experimentally, nickel subsulfide induces renal carcinomas following intrarenal injections into rats (32), and nickel carbonate has been shown to induce protein-DNA cross-links in rat kidney *in vivo* (33) and in cultured mammalian cells (34). The nickel ion is thought to be the ultimate carcinogenic form of nickel and has been shown to be an efficient transforming agent for animal cells in culture (35). However, nickel is inactive in gene mutation assays (36). Nickel ions are known to bind to phosphate group of DNA, but nickel also has an affinity for purine and pyrimidine bases (37). NHKE cultures 2–5 underwent considerable chromosomal changes both numerically and structurally compared to the untreated control culture NHKE 1. Some changes occurred spontaneously in control cultures usually as polyploidization (2n×2). Marker chromosome formation was not found in the untreated cultures. During further cultivation of the cells, the chromosome picture changed; the chromosome number was stabilized around the triploid count (3n) with loss or gain of the same chromosomes. Chromosomes with more complicated rearrangements such as dicentrics, tricentrics, rings, etc., dis-

	Cells anal.	Chrom. count:
NHKE-1	4	<46
	14	46
	6	74-92
NHKE-2	4	<46
	1	46
	3	56-64
	5	74-85
	2	118-132
NHKE-3	2	<46
	3	49-62
	8	73-86
	3	142-157
	1	190
NHKE-4	6	<46
	1	49
	10	64-80
	4	110-158
	2	240-267
NHKE-5	1	50
	10	71-110
	2	150-169
	1	276

Fig. 5. Chromosome changes in four groups of explants exposed *in vitro* to NiSO₄ (NHKE-2, NHKE-3, NHKE-4, NHKE-5) and one group of unexposed explants (NHKE-1). The results are presented as the mean number of rearrangements per chromosome per cell.

Table 2 *Number of cells with different types of structurally rearranged chromosomes (marker chromosomes) in the different cell clones studied*

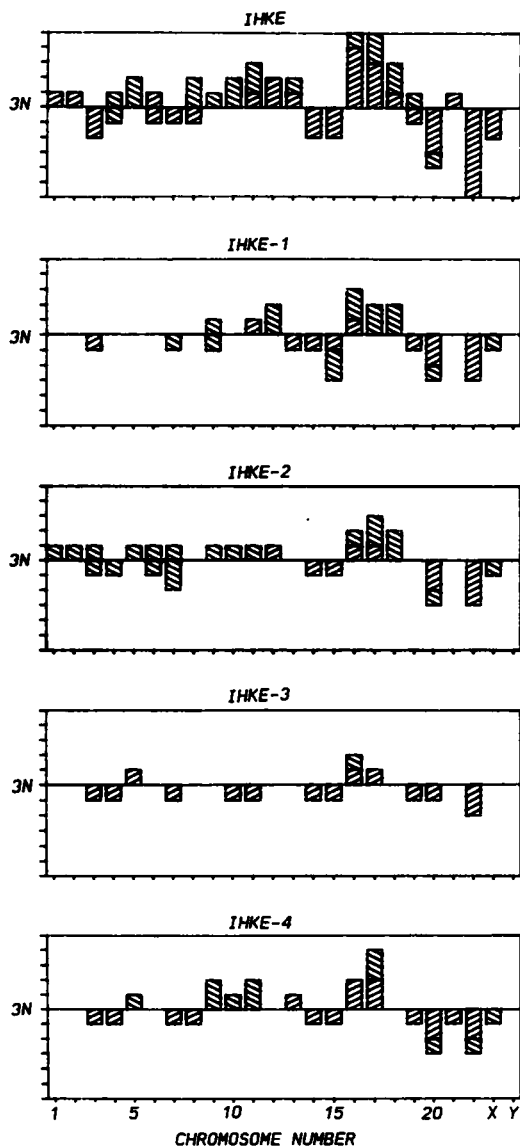| Cell clone	No. of cells studied	Chromosome no. around	Double minutes	Single fragments	Marker chromosomes involving											
					Inversions						Translocations			Iso 11q	Dicentrics	
					7p	1q	1p	7 or 9	22q−	14q+	20:13	20:22	21:21		2q+	20:X
IHKE	17	3n	15	14	17	7	7	2	10	4						
	4	2n	3	4			1									
IHKE-1	7	3n	7	2	7			7	7		7		3		4	2
IHKE-2	14	3n	10	5	14	1	1	1	5		2	2				
IHKE-3	6	3n	5	2	6	4	3	2		1				6		
IHKE-4	10	3n	8	1	10	3	1		1			3				

Fig. 6. Chromosome number distribution around the triploid number (3n) with gain or loss of certain chromosomes demonstrated for the original cell line IHKE at the 24th passage and for 4 different clones (IHKE-1, IHKE-2, IHKE-3, and IHKE-4) selected from this line in agar and then cultured from 37 to 42 passages before the chromosome analyses. Gain or loss of one chromosome in all cells studied (■); gain or loss of one chromosome in some of the cells (▨).

treatment of NHKE cells results in chromosome aberrations and immortality, but not to tumorigenicity. Experiments are in progress to determine whether immortalized IHKE cells transfected with oncogenes undergo progressive changes that result in neoplastic transformation and tumors in nude mice.

REFERENCES

1. Miller, E. C. Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. Cancer Res., 38: 1479–1496, 1978.
2. Foulds, L. Natural history of cancer. J. Chron. Dis., 8: 2–37, 1958.
3. Land, H., Parada, L. F., and Weinberger, K. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (Lond.), 304: 596–602, 1983.
4. DiPaolo, J. A. Relative difficulties in transforming human and animal cells in vitro. J. Natl. Cancer Inst., 70: 3–8, 1983.
5. Harris, C. C. Human tissues and cells in carcinogenesis research. Cancer Res., 47: 1–10, 1987.
6. Lechner, J. F., Haugen, A., McClendon, I. A., and Pettis, E. W. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. In Vitro (Rockville), 18: 633–642, 1982.
7. Tsao, M. C., Walthall, B. J., and Ham, R. G. Clonal growth of normal human epidermal keratinocytes in a defined medium. J. Cell Physiol., 110: 219–229, 1982.
8. Taub, M. (ed.). Tissue Culture of Epithelial Cells. New York: Plenum Publishing Corp., 1985.
9. Gilbert, S. F., and Migeon, B. R. Renal enzymes in kidney cells selected by D-valine medium. J. Cell Physiol., 92: 161–168, 1977.
10. Taub, M., and Livingston, D. The development of serum-free hormone-supplemented media for primary kidney cultures and their use in examining renal functions. Ann. NY Acad. Sci., 372: 406–421, 1981.
11. Gibson-D'Ambrosio, R. E., Samuel, M., Chang, C. C., Trosko, J. E., and D'Ambrosio, S. M. Characteristics of long-term human epithelial cell cultures derived from normal human fetal kidney. In Vitro (Rockville), 23: 279–287, 1987.
12. Doll, R., Mathews, J. D., and Morgan, L. G. Cancers of the lung and nasal sinuses in nickel workers. Br. J. Ind. Med., 34: 102–105, 1977.
13. Magnus, K., Andersen, A., and Høgetveit, A. C. Cancer of respiratory organs among workers at a nickel refinery in Norway. Int. J. Cancer, 30: 681–685, 1982.
14. Lessard, R., Reed, D., Maheux, B., and Lambert, J. Lung cancer in New Caledonia, a nickel smelting island. J. Occup. Med., 20: 815–817, 1978.
15. Schaeffer, W. I. Usage of vertebrate, invertebrate and plant cell, tissue and organ culture terminology. Tissue Cult. Res., 17: 19–23, 1983.
16. Ericsson, J. L. E., Brunk, U. T., and Arborgh, B. Fixation. In: J. V. Johannessen (ed.), Electron Microscopy in Human Medicine, Vol. 1, pp. 99–115. New York: McGraw-Hill Book Company, 1978.
17. Luft, J. H. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol., 9: 409–414, 1961.
18. Watson, M. L. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol., 4: 475–479, 1958.
19. Reynolds, E. S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17: 208–212, 1963.
20. Sun, T. T., and Green, H. Keratin filaments of cultured human epidermal cells. J. Biol. Chem., 253: 2053–2060, 1978.
21. Seabright, M. A rapid banding technique for human chromosomes. Lancet, 2: 971–972, 1971.
22. Leighton, J., Estes, L. W., Mansukhani, S., and Brada, Z. A cell line derived from normal dog kidney (MOCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. Cancer (Phila.), 26: 1022–1028, 1970.
23. Franke, W. W., Appelhaus, B., Schmid, E., Freudenstein, C., Osborn, M.,

and Weber, K. Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to pre-keratin. Differentiation, *15:* 7–25, 1979.

24. O'Brien, W., Stenman, G., and Sager, R. Suppression of human growth by senescence in virally transformed human fibroblasts. Proc. Natl. Acad. Sci. USA, *83:* 8659–8663, 1986.

25. Rhim, J. S., Trimmer, R., Arnstein, P., and Huebner, R. J. Neoplastic transformation of chimpanzee cells induced by adenovirus type 12– simian virus 40 hybrid virus. Proc. Natl. Acad. Sci. USA, *78:* 313–317, 1981.

26. Pater, A., and Pater, M. M. Transformation of primary human embryonic kidney cell to anchorage independence by a combination of BK virus DNA and the Harvey-*ras* oncogene. J. Virol., *58:* 680–683, 1986.

27. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T., and Nose, K. Multi-step process of neoplastic transformation of normal human fibroblasts by ⁶⁰Co γ-rays and Harvey sarcoma virus. Int. J. Cancer, *37:* 419–423, 1986.

28. Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res., *48:* 1904–1910, 1988.

29. Lechner, J. F., Tokiwa, T., McClendon, I. A., and Haugen, A. Effects of nickel sulfate on growth and differentiation of normal human bronchial epithelial cells. Carcinogenesis (Lond.), *5:* 1697–1703, 1984.

30. Peto, J., Cucle, H., Doll, R., Hermon, C., and Morgan, L. G. Respiratory cancer mortality of Welsh nickel refinery worker. IARC Sci. Publ., *53:* 37–46, 1984.

31. Roberts, R. S., Julian, J. A., Muir, D. C. F., and Shannon, H. S. Cancer mortality associated with the high-temperature oxidation of nickel subsulfide. IARC Sci. Publ., *53:* 23–35, 1984.

32. Sunderman, F. W., Jr., Maenza, R. M., Hopfer, S. M., Mitchell, J. M., Allpass, P. R., and Damjanov, I. Induction of local cancer in rats by intrarenal injection of nickel subsulfide. J. Environ. Pathol. Toxicol., *2:* 1511–1527, 1979.

33. Ciccarelli, R. B., Hampton, T. H., and Jenette, K. W. Nickel carbonate induces DNA-protein crosslinks and DNA stand breaks in rat kidney. Cancer Lett., *12:* 349–354, 1981.

34. Patierno, S. R., and Costa, M. DNA-protein crosslinks induced by nickel compounds in intact cultured mammalian cells. Chem.-Biol. Interact., *55:* 75–91, 1985.

35. DiPaolo, J. A., and Casto, B. C. Quantitative studies of *in vitro* morphological transformation of Syrian hamster cells by inorganic metal salts. Cancer Res., *39:* 1008–1013, 1979.

36. Biggart, N. W., and Costa, M. Assessment of the uptake and mutagenicity of nickel chloride in *Salmonella* tester strains. Mutat. Res., *175:* 209–215, 1986.

37. Eichorn, G. L., and Shin, Y. A. Interaction of metal ions with polynucleotides and related compounds. XII. The relative effects of various metal ions on DNA helicity. J. Am. Chem. Soc., *90:* 7323–7328, 1968.