Arch Toxicol (1988) 62: 133–145

Archives of
Toxicology

Cadmium induced changes in cell organelles: an ultrastructural study using cadmium sensitive and resistant muntjac fibroblast cell lines

Muriel J. Ord,[1]* Simon D. Bouffler,[2] and Rakesh Chibber[1]*

[1] Biology Department, Southampton University, Bassett Crescent East, Southampton S09 3TU, United Kingdom
[2] CRC Mammalian Cell DNA Repair Research Group, Zoology Department, Cambridge University, Downing Street, Cambridge CB2 3EJ, United Kingdom

Abstract. A detailed electron microscopy study of cadmium sensitive and resistant muntjac fibroblast cell lines has identified a wide range of intracellular damage following exposure to cadmium. Damaged organelles included cell membrane, mitochondria, Golgi cisternae and tubular network, chromatin, nucleoli, microfilaments and ribosomes. Although cell membrane damage was generally the earliest indication of adverse cadmium action, particularly with continuous cadmium exposures, cells could tolerate extensive membrane loss. Mitochondrial distortion and some damage to Golgi was also tolerated. The turning point at which cadmium became lethal was generally marked by a cascade of events which included damage to both nuclear and cytoplasmic components. These results for fibroblasts are discussed and compared with damage reported in other types of cells.

Key words: Cadmium – Ultrastructure – In vitro – Nucleus – Cytoplasm – Muntjac

Introduction

Cadmium is one of the most widely studied of environmental pollutants. It is taken up readily by plants, animals and micro-organisms and, at high doses, can cause acute damage to cells, tissues and whole organisms (Friberg et al. 1971; Peterson and Alloway 1979; Page et al. 1986). It has a long biological half-life and is cumulative, so that it may build up to considerable burdens from repetitive low level doses (Bremner 1979; Hallenbeck 1984; Bernard and Lauwerys 1986; Nomiyama 1986). Though much is now known about protective mechanisms against cadmium poisoning, particularly by the universally occurring metallothioneins, (Webb 1979 a), there is still little agreement on the sensitivities of the different intracellular organelles to cadmium or on the effects of cadmium once protective mechanisms have been breached. Ultrastructural studies on animal tissues and cells in culture have done little to clarify the situation. While some suggest mitochondria and/or membrane may be the site of the primary lesion (Toury et al. 1985; Jamall and Sprowls 1987), others point to a nucleolar and/or RNA lesion (Sina and Chin 1978; Puvion and Lange 1980; Morselt et al. 1983; Dudley et al. 1984). Indeed, all the major cell organelles have been identified at some time as possible sites of cadmium damage.

The present morphological study has examined the range of intracellular lesions resulting from both acute and continuous cadmium exposures using a permanent line of Indian muntjac fibroblasts sensitive to continuous cadmium at levels as low as 0.1 μM, and three lines derived from it with resistance of up to 100 times that of the parent line. Organelles examined included cell membrane, mitochondria, Golgi, microfilaments, microtubules, autophagosomes, RER, free polysomes, nucleolus and chromatin, with differences noted in the timing of morphological changes and in the extent to which damage to each was tolerated or reversed. A comparison is made between the cadmium sensitive and resistant muntjac lines, and between damage to muntjac cells and other cell types reported in the literature.

Material and methods

Cell culture and treatment procedures. All experiments were carried out using Indian muntjac fibroblasts grown in monolayer: stock cultures in flasks (25 cm^2 surface area), experimental cells in 3.5 cm diameter dishes. Cells were cultured at 37°C in HEPES or bicarbonate buffered Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum, 2 mM glutamine, with 50 units/ml penicillin and 50 μg/ml streptomycin. Where appropriate, cadmium acetate was added at the same time as cells were changed to fresh medium, with stock solutions of cadmium prepared and stored in polyethylene bottles at 4°C. For light and transmission electron microscopy (TEM), exponentially growing cells were harvested by trypsinization, plated out in MEM at 10^5 cells/3.5 cm diameter dish and incubated for 24 h at 37°C to allow complete recovery from any changes caused by trypsin. Medium was subsequently replaced and cadmium added to half the dishes to give the dose levels indicated in Table 1; cultures were then returned to the incubator for 1, 5, (8), 12, (18), 24, 48 and (96) h (brackets indicate times used in only some experiments) before fixing in Karnovsky's solution. Each "concentration × time" combination in an experiment included two dishes for direct comparison, i. e. one with and one without cadmium. Experiments were repeated at least three times.

For cadmium survival curves, exponentially growing cells were harvested by trypsinization and plated out at

* Members of MRC Toxicology Research Unit, Carshalton, Surrey, SM5 4EF, United Kingdom
Offprint requests to: M. J. Ord

Table 1. Cadmium concentrations used to give acute or chronic dose levels for the four lines of muntjac. All "concentration × time" points were in pairs with fresh medium plus cadmium added to one dish and fresh medium only to the second. With continuous cadmium doses using cell lines normally cultured in 5 or 10 μM cadmium, each accompanying (or control) dish had the cadmium omitted

	M cells	CR5 cells	CCR5 cells	CCR10 cells
Acute doses	1 μM* (5,10 μM)	–	25 μM* (10,15 μM)	40 μM* (100 μM)
Chronic doses	–	5 μM	5 μM	10 μM

* General dose most useful for acute cadmium damage

Table 2. The series of events which followed change of muntjac fibroblasts to fresh medium. These were used to monitor cadmium induced changes in cell activity

Time after medium change	Activity	Cell line
10 min	Rapid increase in endocytosis	all lines
1 h	Extensive membrane blebbing	CCR5 & CCR10
1 h	Build up of fluid in vacuoles	M & CR5
1 h	Association between mitochonndria and RER	all lines
1 h	Reattachment of free ribosomes	all lines
5 h	Increase in Golgi associated vesicles	CR5, CCR5 & CCR10
5 h	Close contact between mitochondria and nuclear membrane	all lines
5–8 h	Loosening of the granular and fibrillar nucleolar elements	all lines
12 h	Increase in mitotic figures	all lines

Table 3. Parameters used as indicators of activity for individual organelles

Mitochondria	Close contacts with RER cisternae and, to a lesser extent, with other cell organelles
Golgi	Numerous wide diameter parallel cisternae with many associated vesicles
Nucleus	Increased nuclear membrane surface area (by identation) and increased contact with cytoplasmic organelles
Nucleolus	More open configuration with granular and fibrillar components well separated and nucleolar outline irregular
RER	Ribosomes attached over all ER area, cisternae wide and with opaque contents
Cell membrane	Numerous coated pits and internalized coated vesicles
Microfilaments	Dark and light bands across bundles within stress fibres
Autophagosomes	Decrease in fluid content, concentration of discarded organelles, change to residual or dense bodies

5×10^4 cells/6 cm dish. Following a 24 h recovery period, cadmium was added to their culture medium and the cells incubated at 37°C for 1, 5, 12 or 24 h. Treatment media were removed, cells were rinsed with phosphate buffered saline and fresh medium added. All cultures were returned to the incubator for 6 days. At the end of this period, cells from the different cell lines were trypsinized, counted and the ratios of mean cell number of treated cells to the relevant untreated cells, the surviving fraction, were calculated for each dish. All experiments were carried out with two or more replicate plates and repeated three times.

Four cell lines were used in the evaluation. The cadmium sensitive parent line (M cell), an immortal but anchorage dependent fibroblast obtained from Flow laboratories (ATCC no. CCL157), and three lines derived from it, which had become adapted to growth in the continuous presence of cadmium (Bouffler and Ord, unpublished work). These included: CR5 cells growing in 5 μM cadmium and surviving with continual repair and/or replacement of damaged components; CCR5 cells growing in 5 μM cadmium and CCR10 cells growing in 10 μM cadmium, both with doubling times similar to that of the parent line. Although toxicity curves show these cell lines to be 50–100 times more resistant to cadmium than the parent M cell, no significant increases in metallothionein have been detected in any of them (unpublished work).

Fixation, embedding and cutting procedures. Cells were fixed for light and TEM directly in their culture dishes, using a double fixation procedure as follows: medium was tipped off, cells were rinsed twice in phosphate-buffered saline, fixed for 1 h in Karnovsky's fixative (pH 7.2), rinsed in 0.1 M cacodylate buffer and fixed a further 1 h in 2% osmium tetroxide made up in 0.1 M cacodylate buffer. Fixed cells were loosened from the dish, blocked in agar (Ord and Smith 1982), dehydrated through increasing concentrations of ethanol and impregnated with Spurr resin (Spurr 1969). Blocks were cut at 80 nm with a Dupont diamond knife, stained in 2% uranyl acetate followed by lead acetate and examined with a Philips EM 300. Some sections were also cut at 1 μM with glass knives, stained with toluene blue and mounted in Spurr resin for examination with a Zeiss microscope at × 200–400.

Ultrastructural studies. Evaluations of cell damage were based on comparisons of the morphology of individual organelles in the presence and absence of cadmium and on changes in the parameters given in Tables 2, 3. Proof of irreversible damage to organelles was based on their appearance in secondary lysosomes for autophagocytosis, while the slowing of autophagocytosis itself was used as an indication of interference with Golgi and/or lysosomal activities. The timing and frequency of appearance of an organelle in autophagosomes gave further information about its sensitivity to cadmium. Changes contributing to lethal damage were confirmed by fixing separately firmly attached cells (majority viable), loosely attached cells (many non-viable) and detached cells (non-viable), and comparing their damage. No attempt has been made to give numerical values for damaged organelles, since sectioning introduced too great an error (Smith and Ord 1983). Where necessary, serial sections were used to confirm specific organelle forms. No attempt was made to obtain values from

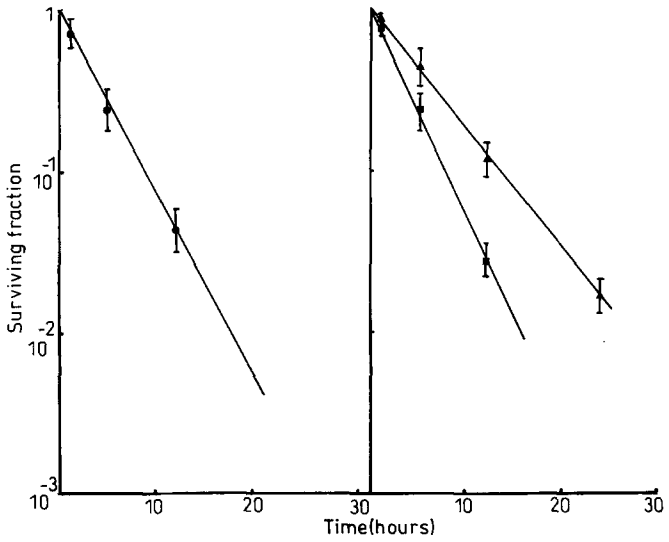

Fig. 1. Cell survival as a function of cadmium exposure time of M (—▲—), CCR5 (—■—), and CCR10 (—●—) cells at cadmium concentrations of 1 μM, 25 μM and 40 μM, respectively

morphometric studies, since the asymmetrical distribution of organelles in these cell lines made such data impossible to evaluate.

Results

Acute doses of cadmium inhibited cellular activities more rapidly in M cells than in CCR5 or CCR10 cells. The same cellular organelles were damaged but the extent of damage to an organelle at any given time differed considerably. In spite of such differences, however, the following series of events could be recognized in all three cell lines in response to the acute cadmium exposures of Table 1. (I) Damage to cell membrane was already extensive in all cells within 1 h of addition of cadmium. (II) Changes in mitochondrial morphology were prominent by 5 h. (III) Between 5 and 12 h cytoplasmic damage increased to include the membranes of many Golgi cisternae, while nuclear changes included a build up of peripheral heterochromatin and condensation of nucleolar components. (IV) Between 12 and 24 h acute doses of cadmium produced a cascade of cell damage which included condensation of stress fibre bundles, loss of ribosomes, loss of nucleolar structure, and condensation of chromatin. (V) By 48 h many cells had lost their membrane integrity and/or sustained extensive nuclear damage.

Comparison of these timings with the cytotoxicity curves of Fig. 1, suggested that membrane damage in itself was not responsible for cell death; i. e. if a cell was removed from cadmium after a 1 h exposure to a concentration which produced extensive membrane damage, that cell could recover completely. However, the rapid drop in cell viability when exposures were continued for 5 or 12 h (i.e. 25–50% and less than 10% survival, respectively) suggested that as damage spread to include cytoplasmic organelles and nuclear components cadmium damage became rapidly irreversible. Few cells recovered after the longer exposure periods where TEM examination showed that most cells contained extensive damage to both nuclear components and cytoskeletal elements.

The overlap in timing of damage for mitochondria, Golgi and nuclear components during the 5–12 h exposure period prevented a clear evaluation of damage to individual organelles at this critical period. Further information was gained from studying the damage pattern of CR5 cells. These cells grow continuously in 5 μM cadmium, surviving by repair and/or replacement of damaged organelles rather than damage prevention. As each change to fresh medium acted as a trigger for increased endocytosis, with apparent increase in cadmium intake, it was possible to determine the sequence of damage to organelles by examining the cells at a series of time intervals following medium change. These waves of damage showed that as with acute doses of cadmium to M, CCR5 and CCR10 cells, membrane discard was the first indication of damage. Large quantities of myelin bodies were already conspicious at 1 h, as were increases in RER and Golgi. By 5–12 h Golgi vesicle scatter, mitochondrial/RER dissociation and change in mitochondrial form suggested that the activity of both mitochondria and Golgi were temporarily affected. Autophagocytosis of some of these organelles indicated that their damage could be irreversible. Though division timings were only marginally delayed, the large number of chromosome aberrations and abnormal cytokineses suggested that cadmium could have a long-term effect on the cells (Bouffler and Ord, unpublished work). Recovery from each wave of damage appeared to take place during the 48–96 h period, when decreased endocytosis suggested decreased cadmium uptake.

The chief difference in the chronic damage patterns of CCR5 and CCR10 cells from that of CR5 cells, or from the acute damage patterns of M, CCR5 and CCR10 cells, was the decrease in internal cell damage. Membrane damage still appeared with each medium change and some mitochondrial and Golgi form or activity changes were present but the latter were markedly less than for CR5 cells or following acute cadmium damage. Cells continued to divide normally and little cell death could be detected in the cultures.

Details of normal and cadmium damaged organelles

Membrane. In the four Muntjac lines, as in other firbroblasts, there is continual internalization and recycling of cell membrane as medium is taken up by clathrin coated endocytotic vesicles (Figs. 2 A, C). The endocytosed fluid is collected in vacuoles or endosomes, and absorbed. In M cells, where fluid assimilation is slow, endosomes can reach up to 3 μM diameter and form a characteristic feature of the cell (Figs. 2 B). In CR5 cells, endosomes are smaller with fragile membranes, while in CCR5 and CCR10 cells they are less than 500 nm. There was no apparent increase or decrease in fluid uptake by any of the four cell lines when cadmium was present in the external medium. However, as vacuoles were used to collect solid components as well as fluids, i. e. as autophagosomes or secondary lysosomes, they rapidly filled with discarded cell organelles after acute cadmium poisoning (Figs. 2 D, F). The most common of these components, cell membrane, was present in the vacuoles as large and small myelin figures, singly or collected (Fig. 2 E). In all but M cells (where discarded membrane generally remained as large

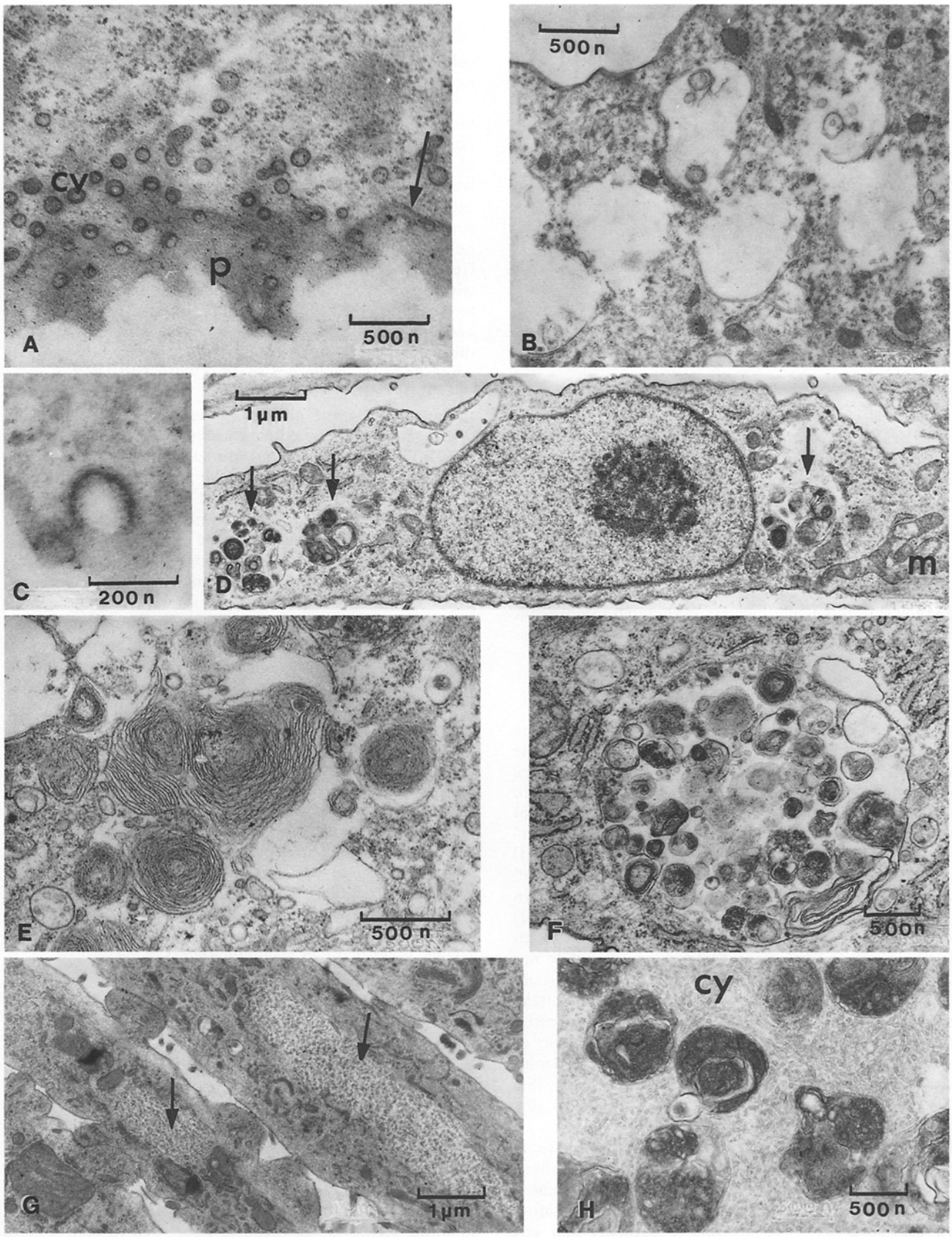

Fig. 2. Coated vesicles *(cv)* and cell membrane turnover in normal and cadmium-treated cells. **(A)** Cluster of cv's at membrane of ventral surface (↓) of a CR5 cell 12 h after fresh medium with 5 µM Cd. **(B)** Fluid filled vacuoles or endosomes of M cell 5 h after change to fresh medium. **C** Coated pit at membrane edge in CR5 cell. **D** Three large autophagosomes (↓) in a CR5 cell 12 h after change to fresh medium with 5 µM Cd; note large branched mitochondrion *(m)* at right. **E** Numerous myelin figures in a CR5 cell 5 h after change to fresh medium with 5 µM cadmium .**F** Autophagosome enlarged to show heterogeneity of contents; though CR5 cells tolerate cadmium, each change to fresh medium with 5 µM cadmium results in destruction of many organelles. **G** Granular autophagosomes (↓) in two adjacent CCR5 cells 5 h after exposure to cadmium at an acute level. This type of material has been identified as polysomes. **H** Residual bodies in cytoplasm *(cy)* of a CR5 cell 48 h after changing cell to fresh medium with 5 µM cadmium

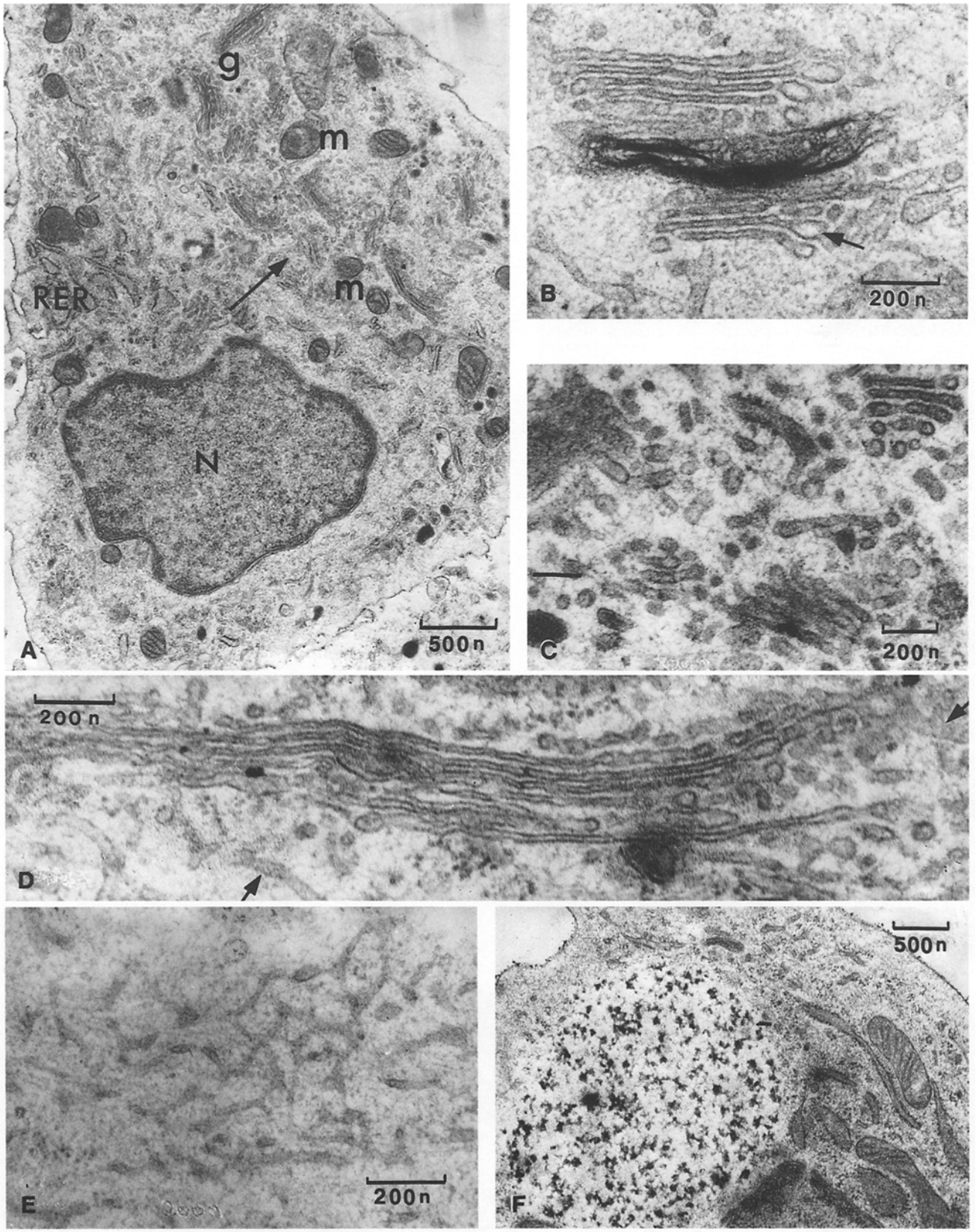

Fig. 3. The Golgi complex of cells exposed to acute doses of cadmium, or growing continuously in low levels of cadmium. **A–D** = Golgi stacks, **E, F** = peripheral tubular network. **A** Cross-section through the main body of a CCR5, a muntjac line growing continuously in 5 μM cadmium; Golgi stacks *g* and associated vesicles occupy a defined area to one side of the nucleus (N); mitochondria *m* intermingle with stacks but *RER* and free polysomes are excluded (av. stack width = 800–900 nm). **B** Three closely spaced stacks of Golgi cisternae with vesicles forming at outer edges → in a CCR5 cell 24 h after an acute dose of cadmium. The membranes of cisternae in the central area have been damaged and are beginning to form a myelin body (av. stack width = 500–800 nm, cisternae depth = approx. 13 nm). **C** Three stacks and scattered vesicles in a CCR5 cell 5 h after an acute cadmium dose; note narrow diameter of stacks (av. = 300–400 nm) and increased depth of cistenae (av. depth = 40 nm). **D** Golgi stack of a CCR10 cell (12 h after change to fresh medium with its normal culturing cadmium level of 10 μM) showing the increased width and decreased depth of the very active golgi cisternae; (stack width = 2000 nm; cisternae depth = 13 nm). Note beginning of tubular network at edges →. **E** Small area of the extensive tubular network of a CCR10 cell (average tubule diameter = 20 nm). **F** A vacuole with partially autophagocytosed tubular material in a CCR5 cell 24 h after an acute dose of cadmium. Such vacuoles are distinctive and initially tubules can be easily recognized

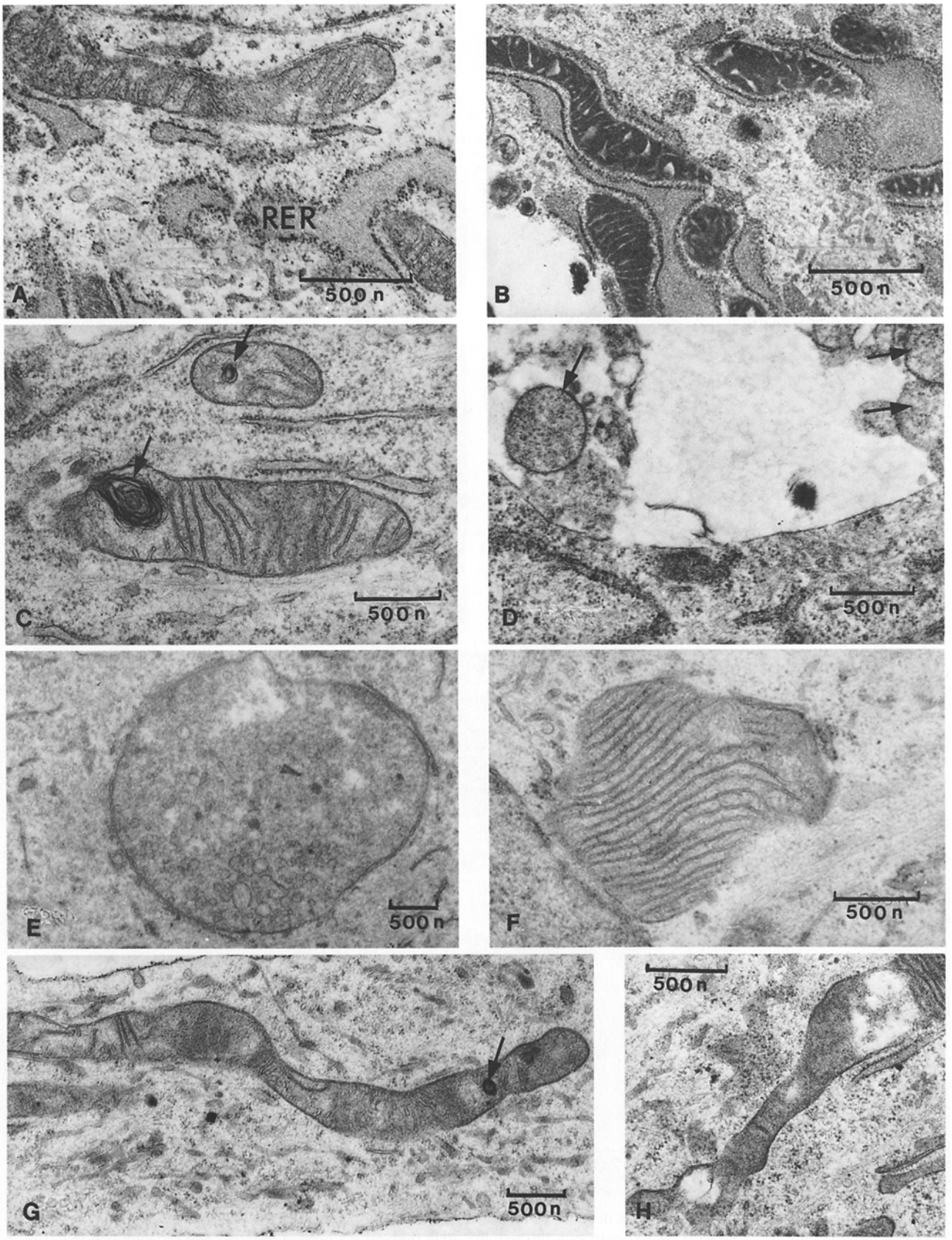

Fig. 4. Mitochondrial form changes in control and cadmium-treated cells. **A** Orthodox mitochondrion of an M cell 5 h after change to fresh medium. **B** Condensed mitochondria of a CCR5 cell in depleted medium. Note close association with *RER* in both **A** and **B**. **C** Two mitochondria of a CCR10 cell in which damaged cristae are forming myelin figures within the mitochondrial matrix, (↓). **D** Three discarded mitochondria (↓) in an autophagosome of a CR5 cell 1 h after change to fresh medium with 5 μM cadmium. **E** A large chunky mitochondrion with poor cristal formation in a CCR5 cell, and **F** a large chunky mitochondrion with excess cristal formation in a CCR10 cell; these formed 10–30% of the mitochondria of cells in low levels of cadmium. **G** Part of a long mitochondrion from a CCR10 cell 12 h after an acute dose of cadmium; note formation of a myelin figure at *arrow*. **H** A damaged mitochondrion with electron translucent areas in its matrix 12 h after an acute dose of cadmium

Table 4. Form changes in the mitochondria of M, CR5, CCR5 and CCR10 cells with acute and continuous doses of cadmium. The number of stars (*) gives an indication of the frequency of the form in the different cell lines, i.e. the more stars the more prominent the effect. "nil" indicates that this form was not observed with the dose regime indicated. Note that condensed mitochondria, as found with acute cadmium doses were more common when cadmium was added with depleted than with fresh medium

	Acute cadmium doses			Continuous cadmium doses	
	M cell	CR5	CCR5/CCR10	CR5	CCR5/CCR10
Inhibition of mitochondrial interactions	***	***	***	**	*
Translucent areas in matrix	***	**	*	*	nil
Destruction of cristae in situ	nil	*	***	*	**
Discard in autophagosomes	***	***	*	*	nil
Increased length and/or branching	*	**	nil	nil	nil
Chunky mitochondria with or without increased cristae	nil	nil	*	*	***
Condensed mitochondria with wide cristal spaces	*	*	*	In situations as expected for controls only	

irregular masses), autophagocytosis was well developed, the contents of autophagosomes being gradually degraded, condensed into residual bodies (Fig. 2 H) and ejected. Prior to condensation these contents served as proof of irreversible organelle damage to membrane (Fig. 2 E), ribosomes (Fig. 2 G), Golgi tubules (Fig. 3 F), mitochondria (Fig. 4 D) and microfilaments (Figs. 5 G, H), while the absorption process itself served as an indicator of continued cell activity. In the cadmium resistant CCR5 and CCR10 cells damage was confined almost entirely to membrane. When cadmium addition was accompanied by depleted medium rather than fresh medium the amount of discarded membrane in autophagosomes was markedly decreased, though discard of other organelles was similar to that found with fresh medium.

Golgi. The Golgi complex of the muntjac fibroblast consists of Golgi stacks with associated *cis* and *trans* vesicles and a tubular network. In M and CR5 cells Golgi stacks are scattered throughout the cytoplasm. In CCR5 and CCR10 cells, stacks are concentrated in an area to one side of the nucleus (Fig. 3 A), separated from, but surrounded by, RER and free polysomes. The Golgi stacks decrease in size and number in cultures with depleted medium; addition of fresh medium is followed by an increase in the number of stacks, in the diameter and number of cisternae and associated vesicles per stack but a decrease in cisternal depth. The tubular network (Fig. 3 E) is more variable, less prominent and less permanent in its presence, quantity and distribution than Golgi stacks, intermingling with other organelles in both cell body and peripheral cytoplasm. With acute cadmium doses, Golgi activity decreased by 5 h as recognized by decreased cisternae per stack, disorganization of associated vesicles (Fig. 3 C), and formation of myelin bodies from cisternae membrane within the stacks themselves (Fig. 3 B). The tubular network initially increased in the presence of cadmium and was only found in autophagosomes as cadmium damage reached lethal levels, (Fig. 3 F). Continuous exposure to low levels of cadmium resulted in an increase in all components of the Golgi complex, waves of damage after fresh medium causing only a temporary inhibition in its activity.

Mitochondria. The variability of mitochondrial forms within the normal growth conditions of control cells (Smith and Ord 1983) makes their use for evaluating toxic damage difficult, i.e. mitochondria of muntjac fibroblasts increased in length, decreased in width and changed from orthodox to condensed forms with depletion of medium (Figs. 4 A, B). Only a few form changes were considered reliable indicators of cadmium damage in the present experiments: (1) the appearance of large translucent areas in the mitochondrial matrix (Fig. 4 H); (2) the bundling of mitochondrial cristae into myelin figures within the mitochondrion itself (Fig. 4 C); (3) the disposal of mitochondria in autophagosomes (Fig. 4 D). Other form alterations which suggested activity changes but not necessarily damage were: (1) branching of the long cylindrical mitochondria (Fig. 2 D); (2) enlarged, but not apparently swollen mitochondria (Fig. 4 E): (3) condensation of mitochondrial matrix (as in Fig. 4 B), (4) change in matrix/cristal ratios or increase in width of intracristal spaces, (Figs. 4 F–G). The frequency of the different types of change are given in Table 4.

Ribosomes. All four muntjac lines have prominent interconnecting RER networks occupying large areas of cytoplasm; their cisternae can be wide or narrow and are generally filled with electron opaque material (see Figs. 4 A, B). Free polysomes are present but not abundant in M and CR5 cells, thought their numbers are greater in CCR5 and CCR10 cells. On addition of cadmium, RER activity initially increased in all cell lines. Even when cells showed marked mitochondrial and Golgi damage, RER still retained a full complement of ribosomes and free polysomes. When lethal levels of cadmium were reached free polysomes decreased and autophagosomes appeared with granular contents resembling polysomes (Fig. 2 G). Since only a few cells had appreciable quantities of single ribosomes even when lethality reached 100%, it was assumed that once ribosomes were damaged they were rapidly destroyed.

Cytoskeleton. The cytoskeleton of the muntjac fibroblast is composed of both microfilaments and microtubules tightly associated to form stress fibres (Fig. 5). Bundles from these reach down to the adhesive foci which attach the cell to the substratum. In the M and CR5 cells stress fibres form the most prominent component of the wide but thin stretches of peripheral cytoplasm, acting as pathways along which the endocytotic vesicles and possibly other organelles move (Fig. 5 D). In the CCR5 and CCR10 cells stress fi-

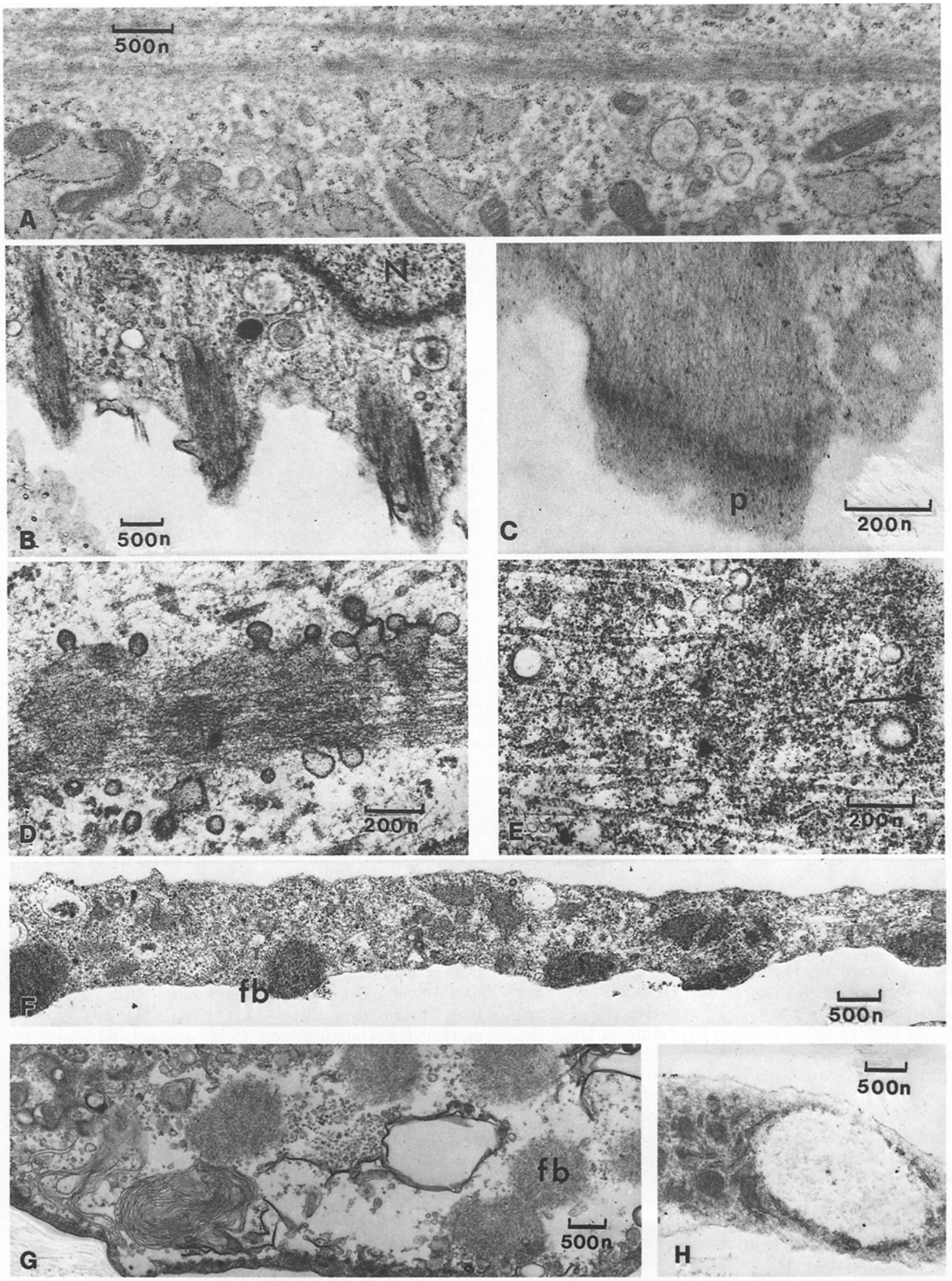

Fig. 5. Cytoskeletal elements in normal **A–D** and cadmium treated **E–H** cells. **A** Thin bands of stress fibres crossing dorsally above the nucleus (note the dark and light banding) and **B** three adhesive foci on ventral surface of an M cell; nucleus *(N)* at upper edge. **C** An adhesive plaque of a CR5 cell to show the bundle of microfilaments and external glycocalyx *(p)* at attachment site. **D** A stress fibre tract leading into a CR5 cell from an adhesive plaque (to right but out of picture); cell was in 4-day-old medium and the abundance of associated vesicles is an indication of their slower movement. **E** Stress fibre area in an M cell 12 h after an acute cadmium exposure; adhesive plaque is at right edge of picture →; microfilaments have disappeared leaving microtubules and associated coated vesicles only. **F** Four adhesive plaque areas in an M cell 12 h after exposure to acute levels of cadmium; floating or loosely attached cells frequently showed condensation of these fibre bundles *fb*. **G** Mixed content autophagosome with five discarded fibre bundles in an M cell 5 h after exposure to acute levels of cadmium. **H** One of many autophagosomes with pale flocculent contents believed to be microfilament components; this type of autophagosome generally appears well after other organelle damage, here in a CCR5 cell 48 h after an acute cadmium exposure

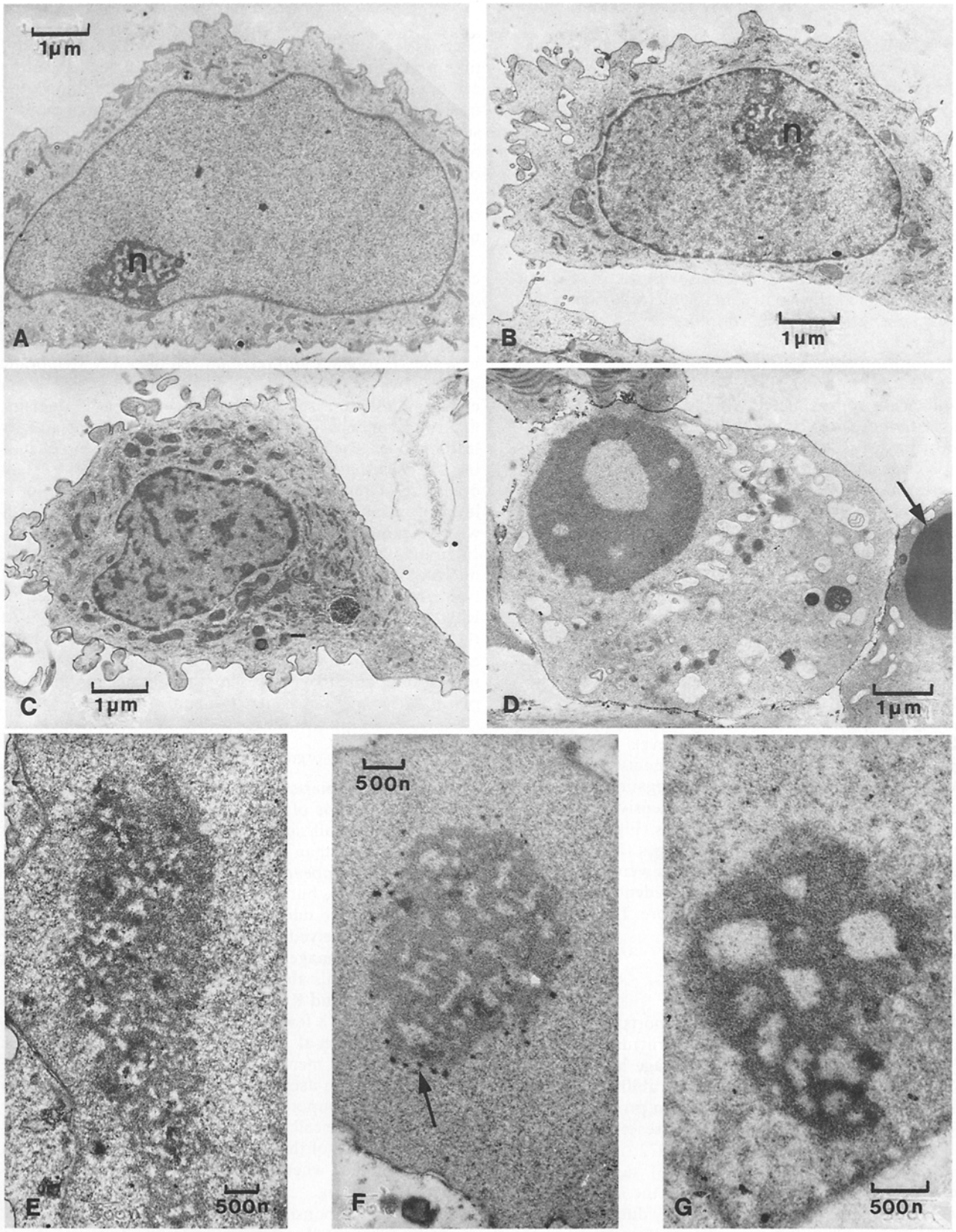

Fig. 6. Nuclei **A–D** and nucleoli **E–G** of normal and cadmium-exposed M cells. **A** A CR5 nucleus with clear nucleoplasm and single nucleolus *n*. **B** A CR5 nucleus 5 h after an acute dose of cadmium; opaque material in nucleoplasm does not necessarily indicate irreversible damage. **C** Nucleus of a CCR5 cell (12 h after exposure to an acute dose of cadmium) showing large amounts of heterochromatin (cut has just missed nucleolus). **D** necrotic nucleus of a CCR10 cell 48 h after exposure to an acute dose of cadmium; adjacent cell nucleus → is also irreversibly damaged. **E** Active nucleolus of an M cell 1 h after change to fresh medium; note looseness of granular and fibrillar components and irregularity of nucleolar perimeter. **F** Granules (↑) in the perinucleolar region in a CR5 cell 24 h after addition of 5 µM cadmium without fresh medium. **G** Condensed nucleolus of a CCR5 cell 24 h after an acute dose of cadmium; dense granular material has almost disappeared

bres are greatly reduced, micofilaments forming less organized tracts and networks. Cadmium damage to cytoskeleton, most easily seen in M and CR5 cells, was identified by the following changes: (I) the condensation of fiber bundles at adhesive foci (Fig. 5 F); (II) the separation of stress fibres into their individual filament and tubular components; (III) the loss of microfilaments leaving tract areas with microtubules only (Fig. 5 E); (IV) the appearance of bundles of microfilaments in mixed content autophagosomes (Fig. 5 G); (V) the appearance in peripheral cytoplasm of numbers of autophagosomes with pale flocculent contents (Fig. 5 H) coincident with the dissolution of stress fibres and believed to contain microfilament material. These changes, if severe, led to detachment of the cell from the substratum.

Nucleus. Over 90% of muntjac fibroblasts in culture have a single nucleus; approximately 80% of these have one nucleolus with loosely associated granular and fibrillar components (Fig. 6 E). During much of interphase the nucleoplasm is clear and there is little to no heterochromatin at the nuclear membrane (Fig. 6 A); during G2 small patches of chromatin are present in the nucleoplasm and in late G2 blocks of chromatin appear before the dissolution of the nuclear membrane. Damage by cadmium has been divided into two stages: stage 1, which occurred slowly and could be reversed; stage 2, which was irreversible and resulted in cell death. Stage 1 included: (I) decreased contact between cytoplasmic organelles and nuclear membrane; (II) accumulation of heterochromatin in nucleoplasm and at nuclear membrane (Figs. 6 B, C); (III) condensation of the nucleolar edges with the appearance of perinucleolar granules in a small number of cells (Fig. 6 F). These changes could be seen in cells exposed to low levels of cadmium and in cells in early stages of acute treatments. Stage 2: (I) severe nucleolar condensation with segregation or loss of granular component (Fig. 6 G); (II) extensive condensation of chromatin; (III) nuclear pyknosis (Fig. 6 D); (IV) rupture of the nuclear membrane. Changes (III) and (IV) represent necrotic damage and these cells were considered dead. Stage 1 changes were already evident by 5–12 h; stage 2 changes seldom appeared before 12–24 h after acute cadmium exposures.

Discussion

In spite of the considerable number of reports in the literature on cadmium damage at the ultrastructural level, no consistent pattern of cell organelle damage has emerged. This is not surprising, since cadmium's ability to inhibit enzyme systems in vivo, to replace zinc in protein sites, to interfere with calcium uptake and to react with lipids (Eichhorn 1975; Vallee and Ulmer 1972; Frazier and Kingsley 1976; Webb 1979 b; Kopp 1986), suggests that it would affect more than one cell site. If the sensitivity of each vulnerable site varied with its activity during the presence of cadmium, then different cells could sustain significantly different morphological damage. Consequently, different ultrastructural studies would not be expected to show the same patterns of damage, particularly where the cells involved are highly specialized or contain cadmium protective mechanisms.

Our in vitro study, though based on one cell type, has attempted to maximize the range of morphological changes recognizable at the EM level by using a series of time points to give a "moving picture" of damage, using cell lines with different cadmium sensitivities, using varied treatment regimes including acute and continuous exposures with high and low concentrations of cadmium and by varying the culture conditions to give different degrees of cell activity in the presence of cadmium. With these conditions we have been able to identify a wide spectrum of intracellular damage, some transitory, some more permanent and possibly contributing to cell death.

Cadmium-induced damage in muntjac fibroblasts occurred in the following chronological order. (1) Membrane damage was always the first visible sign of damage on addition of either acute or chronic levels of cadmium; it was independent of the cell's sensitivity to cadmium occurring in sensitive, tolerant and resistant cell lines. (2) Membrane damage was followed closely by mitochondrial form changes and decreased Golgi activity. (3) Golgi damage, nucleolar condensation and inhibition of nuclear activity leading to heterochromatin accumulation all occurred during the subsequent 12 h; differences in the relative extent or timing of this damage could be attributed to the dose level of cadmium used, the state and activity of the cells when exposed to it, or morphological differences in the cadmium resistant cell lines. (4) Nucleolar segregation, microfilament condensation and ribosome detachment from free polysomes or RER occurred at the borderline where cell survival was no more than a few percent. (5) At the lethal dose level extensive nuclear changes occurred, e.g. chromatin condensation and nuclear membrane rupture accompanied by extensive vacuolation of the cytoplasm and loss of outer membrane integrity.

Damage to specific organelles

In muntjac fibroblasts membrane discard was the most consistent indicator of cadmium damage. It appeared at all dose levels, at all sampling times and was more prominent with chronic than acute dosing. Visible indications of a lesion did not appear at the membrane itself, nor at the fluid uptake stage, but as an inhibition of membrane recycling. Membrane damage as a contributor to cadmium poisoning has received far less attention than nuclear or mitochondrial damage, yet molecular studies leave no doubt of cadmium's ability to react with membrane components (Frazier and Kingsley 1976; Webb 1979 b). Its absence from all but a few ultrastructural studies (Johansson et al. 1983; Toury et al. 1985) may be due to the use of cells with lower rates of membrane turnover or to the reversible nature of the lesion itself. Since membrane integrity can be of the greatest importance to the functioning and/or movement of some cell types, e.g. embryonic cells, the possible consequence of this lesion (even if reversible) should not be overlooked when evaluating factors contributing to cadmium poisoning.

The Golgi, responsible for glycosylation of lysosomal enzymes and new cell membrane, retained its morphological integrity until lethal cadmium levels were approached. However, a number of changes, including a decrease in the number of Golgi associated vesicles, the number and diameter of Golgi body cisternae and disarray among cisternae, suggested that its activity was affected earlier. Though these changes are indicative of decreased Golgi activity, it should be noted that they can occur in normal

cells under conditions which decrease overall cell activity (Ord 1979). A number of other investigators have reported changes connected with the Golgi complex, i.e. increases or decreases in Golgi activity, increases or decreases in lysosomal activity, increases in autophagosomes or dense bodies (Castano 1971; Nishizumi 1972; Hoffmann et al. 1975; Fowler et al. 1975; Richardson and Fox 1975; Johnsson et al. 1983). If lysosomal enzyme activities were inhibited, then cellular materials discarded in autophagosomes would not be recycled, so depriving the cell of valuable repair and rebuilding materials.

The fate of the mitochondrion with cadmium poisoning has been difficult to assess. Two levels of mitochondrial damage were recognized. (I) Lethal damage which was followed by discard through autophagocytosis; this was characteristic of acute cadmium dosing, and more common in the cadmium-sensitive M cells. (II) Non-lethal damage which resulted in a variety of reversible and irreversible form changes; this was more typical of the cadmium-resistant CCR5 and CCR10 cells under conditions of continuous exposure. Literature reports suggest that the mitochondrion is one of the organelles most consistently damaged by both acute and chronic cadmium exposure (Jacobs and Jacobs 1956; Castano 1971; Nishizumi 1972; Faeder et al. 1977; Ord and Al Atia 1979; Meiss et al. 1982; Toury et al. 1985). Indeed, in cells unprotected by metallothionein it has been presented as the site of the primary biochemical lesion (Jamall and Sprowls 1987). There is no clear support for considering mitochondrial damage as the primary lesion in the cadmium tolerant or resistant muntjac cells, though an energy lesion could certainly be considered as a contributory factor in assessing damage. The case for mitochondrial damage as the primary lesion is stronger for M cells, but as many types of damage are precipitated within a very short time the contribution of mitochondrial damage cannot be separated from that of nuclear, microfilament, ribosomal, Golgi and/or membrane damages.

RER activity increased with both acute and chronic cadmium dosing in CR5, CCR5 and CCR10 cells. The numbers of free polysomes increased in cadmium-treated CCR5 and CCR10 cells. Ribosomes detached only at lethal levels of cadmium, but once ribosome damage and/or detachment occurred, it was irreversible and the ribosomes were destroyed. There are numerous literature reports concerning cadmium damage which suggest changes in ribosomes, RER, SER and/or free polysomes but these present no consistent pattern of change (Stowe et al. 1972; Faeder et al. 1977; Kuliszeweki and Nicholls 1981; Meiss et al. 1982; Morselt et al. 1983; Dudley et al. 1984). Indeed, the stability of ribosomes in the presence of cadmium would be necessary to explain the fact that many cells produce cytosolic proteins as protective mechanisms against cadmium poisoning. If ribosomes were easily damaged, or their activity inhibited, protective proteins could not be synthesized at a time when they were most needed.

While damaged membrane could be replaced and much of the mitochondrial damage was tolerated, stress fibre damage with resultant detachment of cells from the substratum appeared irreversible. Like membrane, however, this type of damage has received scant attention in the literature, possibly because morphological signs of microfilament damage are part of a cascade of events leading to cell death. Damage to brush border of kidney and microvillae of intestinal mucosa has been noted (e.g. Castano 1971; Richardson and Fox 1975) but without mention of their microfilament structure. Teratogenic effects have been reported following cadmium exposure (reviewed by DeGraeve 1981) which could relate to microfilament and/or cell membrane damage but no appropriate study has been made to link teratogenesis with the integrity of these organelles during cell migration in embryo formation. In the present study the persistence of microtubules in stress fibre areas which had lost their microfilaments suggested that the microtubules were less sensitive to cadmium than the microfilaments. If so, then the different dependencies of cells on microfilaments and/or microtubules could be one factor contributing to a variable cadmium response among morphologically different cells.

Nuclear activity changes were indicated early in cadmium exposures, e.g. loss of nuclear/cytoplasmic interaction, reversible nucleolar changes and heterochromatin accumulation. Nuclear damage was prominent as lethal levels of cadmium were approached, e.g. segregation of nucleolar components, condensation of chromatin, loss of nuclear membrane integrity. Chromosome aberrations and abnormal mitoses occurred with both lethal and sublethal levels of cadmium; (these have been dealt with elsewhere, Bouffler and Ord, unpublished work). Nucleolar damage receives considerable attention in the literature. The studies of Puvion and Lange (1980) and Cervera et al. (1983) investigating the bases of nucleolar segregation and accumulation of perinucleolar granules present evidence for the involvement of some step(s) in transcription, RNA processing and/or RNA metabolism in cadmium-induced damage. They suggest that an RNA lesion is one of the most important effects of cadmium. Though perinucleolar granules did not show an accumulation in the muntjac fibroblast equal to that of the liver (Puvion and Lange 1980) or HeLa cell (Cervera et al. 1983), nucleolar condensation and subsequent segregation were similar; the lower numbers of perinucleolar granules may simply reflect a difference in RNA storage and/or processing in the muntjac cell. A number of other literature reports also indicate nuclear damage, frequently stressing nucleolar changes (Stowe et al. 1972; Webb et al. 1972; Hoffman and Niyogi 1977; Sina and Chin 1978; Enger et al. 1979; Morselt et al. 1983; Dudley et al. 1984).

There is no doubt that cadmium induces changes at many sites in the muntjac fibroblast and that in all probability both nuclear and cytoplasmic damage contribute to cell death. Proof of this, however, comes only with separation of damage to the two components. Though this is impossible using morphological studies, it has been accomplished using micrurgy techniques with Amoeba proteus, (Ord and Al Atia 1979). Amoeba proteus is a mobile cell which suffers a similar spectra of morphological damage as muntjac fibroblasts on exposure to cadmium. When nuclei were transfered between cadmium treated and control cells the resultant combinations of "cadmium treated nuclei in control cytoplasms" and of "control nuclei in cadmium treated cytoplasms" showed conclusively that both contained irreversible damage which contributed to their subsequent death.

In the present study, the wide range of changes with different cadmium exposures, and the time overlap of damage to a number of cell organelles, show clearly that cadmium can damage many different organelles. In addi-

tion, they show that the extent of damage to a particular organelle may change with differences in cell activity, with different cadmium levels and in the presence or absence of a protective mechanism. Comparisons of these results with literature-reported damage for other cell types suggests that the function and/or morphology of a cell may also influence the damage sustained by the different organelles. When environmental variables are added to the many variables contributed by the cell itself, it becomes possible to see why such difficulty has arisen in assessing the damaging capacity of cadmium, and in prediciting its potential danger to man or his environment.

Acknowledgements. The authors are greatly indebted to Mr Trevor Courtney for ever ready help and advice throughout the entire period of EM work.

References

Bernard A, Lauwerys R (1986) Effects of cadmium exposure in humans. In: Foulkes EC (ed) Cadmium. Handbook of Experimental Pharmacology, 80. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo: pp 135–177

Bremner I (1979) Mammalian absorption, transport and excretion of cadmium. In Webb M (ed) The chemistry, biochemistry and biology of cadmium. Elsevier/North-Holland Biomedical Press, Amsterdam: pp 175–193

Castano P (1971) Chronic intoxication by cadmium experimentally induced in rabbits. Pathol Microbiol 37: 280–301

Cervera J, Alamar M, Martinez A, Renau-Piqueras J (1983) Nuclear alterations induced by cadmium chloride and L-canavanine in HeLa S3 cells. Accumulation of perichromatin granules. J Ultrastruct Res 82: 241–263

DeGraeve N (1981) Carcinogenic, teratogenic and mutagenic effects of cadmium. Mutat Res 86: 115–135

Dudley RE, Svoboda DJ, Klaassi CD (1984) Time course of cadmium-induced ultrastructural changes in rat liver. Toxicol Appl Pharmacol 76: 150–160

Eichhorn GL (1975) Active sites of biological macromolecules and their interaction with heavy metals. In McIntyre AD, Mills CF (eds) Ecological toxicology research. Effects of heavy metal and organohalogen compounds. Plenum Press, New York, pp 123–142

Enger MD, Campbell EW, Ratliff RL, Tobey RA, Hildebrand CE, Kissane RJ (1979) Cadmium-induced alterations in RNA metabolism in cultures of Chinese hamster cells sensitive to and resistant to the cytotoxic effects of cadmium. J Toxicol Environ Health 5: 711–728

Faeder EJ, Chaney SQ, King LC, Hinners TA, Bruce R, Fowler BA (1977) Biochemical and ultrastructural changes in livers of cadmium-treated rats. Toxicol Appl Pharmacol 39: 473–487

Fowler BA, Jones HS, Brown HW, Haseman JK (1975) The morphologic effects of chronic cadmium administration on the renal vasculature of rats given low and normal calcium diets. Toxicol Appl Pharmacol 34: 233–252

Frazier JM, Kingsley BS (1976) Kinetics of cadmium transportation in the isolated perfused rat liver. Toxicol Appl Pharmacol 38: 583–593

Friberg L, Piscator M, Nordberg G (1971) Cadmium in the environment. CRC Press, Cleveland, Ohio

Hallenbeck WH (1984) Human health effects of exposure to cadmium. Experientia 40: 136–142

Hoffman DJ, Niyogi SK (1977) Metal mutagens and carcinogens affect RNA synthesis rates in a distinct manner. Science 198: 513–514

Hoffmann EO, Cook JA, DiLuzio NR, Coover JA (1975) The effects of acute cadmium administration in the liver and kidney of the rat. Lab Invest 32: 655–664

Jacobs EE, Jacobs M, Sanadi DR, Bradley LB (1956) Uncoupling of oxidative phosphorylation by cadmium ion. J Biol Chem 223: 147–156

Jamall IS, Sprowls JJ (1987) Effects of cadmium and dietary selenium on cytoplasmic and mitochondrial antioxidant defence systems in the heart of rats fed high dietary copper. Toxicol Appl Pharmacol 87: 102–110

Johansson A, Camner P, Jarstrand C, Wiernik A (1983) Rabbit alveolar macrophages after inhalation of soluble cadmium, cobalt, and copper: A comparison with the effects of soluble nickel. Environ Res 31: 340–354

Kopp J (1986) Cadmium and the Cardiovascular System. In Foulkes EC (ed) Cadmium. Handbook of experimental pharmacology, 80. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo: pp 195–280

Kuliszewski MJ, Nicholls DM (1981) The effect of cadmium on kidney ribosomes. ChemBiol Interact 33: 307–318

Meiss R, Robenek H, Rassat J, Themann H, Reichert B (1982) Ultrastructural alterations in the hepatic parenchyma of the rat following acute cadmium intoxication. Arch Environ Contam Toxicol 11: 283–289

Morselt AFW, Peereboom-Stegeman JHJC, Puvion E, Maarschalkerweerd VJ (1983) Investigation for the mechanism of cadmium toxicity at cellular level. Arch Toxicol 52: 99–108

Nishizumi M (1972) Electron microscopic study of cadmium nephrotoxicity in the rat. Arch Environ Health 24: 215–225

Nomiyama M (1986) The chronic toxicity of cadmium. In: Foulkes EC (ed) Cadmium. Handbook of experimental pharmacology, 80. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo: pp 101–133

Ord MJ (1979) The effects of chemicals and radiations within the cell: An ultrastructural and micrurgical study using Amoeba proteus as a single-cell model. Int Rev Cytol 61: 229–281

Ord MJ, Al-Atia GR (1979) The intracellular effects of cadmium: An experiment study using Amoeba proteus as a single cell model. In Webb M (ed) The chemistry, biochemistry and biology of cadmium. Elsevier/North-Holland Biomedical Press, pp 141–173

Ord MJ, Smith RA (1982) Correlation of mitochondrial form and function in vivo: microinjection of substrate and nucleotides. Cell Tissue Res 227: 129–137

Page AL, El-Amamy MM, Chang AC (1986) Cadmium in the environment and its entry into terrestrial food chain crops. In: Foulkes EC (ed) Cadmium. Handbook of experimental pharmacology, 80. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo: pp 33–74

Peterson PJ, Alloway BJ (1979) Cadmium in soils and vegetation. In: Webb M (ed) The chemistry, biochemistry and biology of cadmium. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 45–92

Puvion E, Lange M (1980) Functional significance of perichromatin granule accumulation induced by cadmium chloride in isolated rat liver cells. Exp Cell Res 128: 47–58

Richardson ME, SpiveyFox MR (1975) Dietary cadmium and enteropathy in the Japanese quail. Histochemical and ultrastructural studies. Lab Invest 31: 722–731

Sina JF, Chin B (1978) Cadmium modification of nucleolar ultrastructure and RNA synthesis in Physarum polycephalum. Toxicol Appl Pharmacol 43: 449–459

Smith RA, Ord MJ (1983) Mitochondrial form and function relationships in vivo: their potential in toxicology and pathology. Int Rev Cytol 83: 63–134

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43

Stowe HD, Wilson M, Goyer RA (1972) Clinical and morphologic effects of oral cadmium toxicity in rabbits. Arch Pathol 94: 389–405

Toury R, Stelly N, Boissonneau E, Dupuis Y (1985) Degenerative processes in skeletal muscle of Cd^{2+}-treated rats and Cd^{2+}-inhibition of mitochondrial Ca^{2+}-transport. Toxicol Appl Pharmacol 77: 19–35

Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium and lead. Ann Rev Biochem 41: 91–128

Webb M (1979 a) The metallothioneins. In: Webb M (ed) The chemistry, biochemistry and biology of cadmium, Elsevier/North-Holland Biomedical Press, Amsterdam, pp 195–283

Webb M (1979 b) Interactions of cadmium with cellular components. In: Webb M (ed) The chemistry, biochemistry and biology of cadmium. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 285–340

Webb M, Heath JC, Hopkins T (1972) Intranuclear distribution of the inducing metal in primary rhabdomyosarcomata induced in the rat by nickel, cobalt and cadmium. Br J Cancer 26: 274–278

Received August 31, 1987/Accepted February 16, 1988