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Altered Copper Metabolism in Cultured Cells from Human Menkes' Syndrome and Mottled Mouse Mutants

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Cultured cells of a variety of different types from human Menkes' syndrome patients and brindled mouse mutants exhibit similarly altered responses to changes in extracellular copper concentration. This suggests that the mutations in the mouse and human are very similar and that mutant gene expression is occurring in many different tissues. Intracellular copper levels are markedly elevated in mutant cells in normal medium and in medium containing a hundred-fold higher copper. Some cell lines from heterozygotes possess elevated copper levels. Elevated extracellular copper and zinc are significantly more toxic to mutant cells. Mutant cells exhibit normal rates of uptake of copper-64 over a 10-min period but abnormally high accumulation over 24 hr and low rates of efflux. Menkes' fibroblasts become saturated with copper-64 at lower extracellular concentrations than for normal fibroblasts. These data support the idea of enhanced intracellular binding in mutant cells.

KEY WORDS: copper; Menkes' syndrome; mottled mice.

INTRODUCTION

Menkes' syndrome in humans is a lethal X-linked recessively inherited disease

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in which copper metabolism is grossly disturbed. Danks *et al.* (1972) demonstrated decreased intestinal absorption of copper-64 with accumulation of an excess of copper in gut mucosal biopsies (Danks *et al.*, 1973). Hunt (1974) described a similar defect in intestinal copper transport in mottled mouse mutants.

The many clinical effects present in both the human and mouse mutants can be explained by defective function of copper-dependent enzymes as a result of overall copper deficiency. However, the failure of parenteral copper therapy to cure the disease indicated a more complex situation, and the mosaic skin changes in heterozygote humans (Volpintesta, 1974) and mice suggested expression of the mutation in many types of cells.

It is reasonable to expect that full understanding of the basic defect in these mutants will shed important light on normal processes of copper transport. The first evidence for the expression of the Menkes' mutation in cultured cells came from the observations of metachromasia in early-passage skin fibroblasts from hemizygotes and heterozygotes, when such cells were grown in media containing low copper concentrations (Danks *et al.*, 1973). Unusual sensitivity to high levels of copper and zinc in the medium was subsequently reported in a preliminary way (Danks, 1975, 1977; Camakaris *et al.*, 1976. Goka *et al.* (1976) showed that Menkes' fibroblasts possess markedly elevated copper levels when growing in normal medium, Horn (1976) demonstrated increased accumulation of copper-64 in Menkes' fibroblasts, and Beratis *et al.* (1978) and Chan *et al.* (1978) have recently reported similar findings using cultured human Menkes' fibroblasts.

This article presents results of many experiments relating to phenotypic expression of the Menkes' and mottled mutations in several different cell types and under various conditions of cultivation.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Fibroblasts were established from skin biopsies using standard procedures. Some Menkes' and potential heterozygote fibroblast lines were supplied by Professor Ferguson-Smith, Glasgow, and Professor André Boué, Paris. Professor Boué and Dr. Nina Horn, Denmark, supplied some of the Menkes' amniotic cell lines. Basal medium, fetal calf serum, and versene-trypsin were purchased from Commonwealth Serum Laboratories, Melbourne. Fibroblasts were grown in Eagle's basal medium (BME) buffered with 20 mM Hepes and containing 10% fetal calf serum (final medium referred to as FbGM). BME supplemented with 5% fetal calf serum is referred to as FbMM. FbMM contained between 0.05 and 0.07 μg/ml copper. Amniotic cells were grown in

BME supplemented with 20% fetal calf serum. The same batch of fetal calf serum was used in all experiments involving metal toxicity studies or incubations prior to measuring cellular copper levels. The copper concentration in this serum was 0.4 μg/ml.

Continuous lymphoid cell lines (CLCs) were established in our laboratory by transformation with EB virus and were grown in RPMI 1640 buffered with 20 mM Hepes and supplemented with 20% fetal calf serum. (This medium had a copper content of between 0.20 and 0.23 μg/ml.)

Mouse kidney epithelial cells and mouse lung fibroblasts were established from 6-day-old mice and mouse fetal cells from fetuses in the last 2 days of gestation. The procedure of "cold trypsinization" as described by Paul (1975) was used to prepare cell suspensions from tissues. The mouse cells were grown in BME containing 20% fetal calf serum.

Mice

Mice used were the brindled variants of the Mottled series of sex-linked alleles. Affected males die at 14 days postpartum. Original mating pairs of these mice were kindly sent to us by Dr. David Hunt. Mice were fed *ad libitum* on Mecon rat and mouse cubes which contained 16.7 μg/ml dry weight copper and acidified water containing 2.5 μg/ml copper. The symbol used for male hemizygotes is $Mo^{br/y}$, for heterozygotes $Mo^{br/+}$.

Copper Estimations on Cells

Fibroblast monolayers in 25-cm^2 flasks were washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS). The cells were detached with versene-trypsin and washed twice in PBS, washed once in 0.9% NaCl, and finally resuspended in 0.5 ml of 0.9% NaCl. The cells were uniformly dispersed and 20 μl of the suspension was immediately removed and thoroughly mixed with 20 μl of 0.4% trypan blue; aliquots were then transferred into an improved Neubauer hemocytometer for counting of cells. Cell viability as assessed by trypan blue exclusion was always greater than 90%. The cells were then collected as a pellet and lysed in either 50 or 100 μl of concentrated nitric acid (BDH "Aristar" grade). After 24 hr at room temperature the cell lysate was vortexed and 1 μl replicates were assayed for copper using a Varian-Techtron CRA-90 atomic absorption spectrophotometer, containing the BC-6 automatic background subtract module. CLCs were washed three times in PBS and once in 0.9% NaCl, and cells were counted and copper was estimated as described above. Copper content was expressed as μg Cu per 10^6 cells. Precision of the method as expressed by the coefficient of variation was 10.5%.

All glassware was acid washed and thoroughly rinsed in deionized water.

Metal Toxicity Studies

Cells were seeded into microplates (Linbro FB-16-24-TC) in 2 ml of FbGM. After confluent monolayers had been obtained, the medium was changed to FbMM and the cells were incubated in this medium for 5 days. The metal salts were diluted in FbMM, and stock solutions were made of each concentration to be tested. The FbMM in the wells was replaced with FbMM containing metal, and cells were examined at 24-hr intervals. Trypan blue was used to estimate cell viability. Viable cells are defined in these studies as those which exclude trypan blue.

Measurement of Rates of Uptake of Copper-64 into CLCs

A Menkes' and a normal continuous lymphoid cell line were grown in RPMI 1640 plus 20% fetal calf serum. Cell viability was estimated using trypan blue. The cells were spun down, washed once in RPMI 1640, and finally resuspended in RPMI 1640 at a concentration of 10^6 viable cells/ml. Copper-64 chloride was purchased as a sterile solution in isotonic saline from the Australian Atomic Energy Commission. The specific activity of the stock solution was 5μCi/μg Cu. Copper-64 was added to the medium to give final total copper concentrations of 0.26 and 0.82 μg/ml. The cell suspensions were incubated for 10 min at 37 C in a shaker bath. Duplicate 0.5-ml aliquots were removed into ice baths at zero time and at 10 min. The cells were washed in Hanks' balanced salt solution and then counted in a Packard gamma counter. Background and blank values were subtracted and corrections were made for the decay of copper-64.

Studies on Copper-64 Accumulation in Cultured Cells

Fibroblasts were grown to confluency in 25-cm^2 flasks containing FbGM. The medium was changed to fresh FbGM containing the desired concentration of copper-64. The cells were incubated for 24 hr at 37 C and harvested. In the efflux experiment two flasks of a normal and of a Menkes' fibroblast line were incubated in FbGM containing 1.4 μg/ml copper at a specific activity of 5.7 μCi/μg Cu. One flask of each was harvested after 24 hr, while in the other flask the medium was changed to fresh FbGM not containing copper-64 and the cells were incubated for a further 24 hr prior to harvesting. Fibroblasts were harvested as described above except that after trypsinisation they were washed twice in PBS containing 10% fetal calf serum. Cells were counted in a hemo-

cytometer as described previously and radioactivity was estimated in the cell pellet without prior cell lysis.

Continuous lymphoid cell lines were seeded into RPMI 1640 containing 20% fetal calf serum at a concentration of 10^6 viable cells/ml and copper-64 was added at the desired concentration. Cells were incubated at 37 C for 24 hr, washed three times in PBS containing 10% fetal calf serum, and counted in a hemocytometer as described previously; radioactivity was estimated in the cell pellet without prior cell lysis.

All cell pellets together with copper-64 standards were counted in an LKB Ultrogamma 1280 counter using programmed decay correction (half-life of copper-64 is 12.8 hr) and automatic background subtraction.

RESULTS

Cultured human and mouse cells derived from normals, male hemizygotes, and female heterozygotes were tested for their response to normal and elevated levels of copper in the medium. All tests were carried out in confluent nongrowing cultures (incubated in FbMM) in order to minimize variability introduced by differing growth rates of cells, differing degree of confluency, and different cell cycle status.

Sensitivity to Cytotoxic Effects of Copper and Other Divalent Cations

Skin fibroblasts from Menkes' patients were far more sensitive than normal fibroblasts to the toxic effects of 8.7 μg/ml copper added to the medium. After 3 days' incubation of five different Menkes' cell lines a range of between 35% and 65% cell death was obtained, whereas using five different normal cell lines the range was 0–10%. The increased rate of cell death of a Menkes' line vs. a normal line is clearly illustrated by Fig. 1. The toxic response to copper was first manifested as a distortion in cell shape indicating loss of integrity of the cell membrane. Cell death occurred subsequently and is defined in our studies as the inability of a cell to exclude the dye trypan blue. The toxic effect was ameliorated for both normal and mutant cells by doubling the histidine concentration in the medium (Fig. 1), histidine being a very good chelator of copper. When incubated in FbMM not containing added copper, there was no evidence of toxicity in either Menkes' or normal cells. Table I shows that the level of copper causing 50% cell death at various times after copper addition was always lower for Menkes' fibroblasts. Table II shows that cultured kidney epithelial cell lines from brindled mouse mutants displayed a similar phenomenon when compared to normal cells. The mouse kidney epithelial cells were more sensitive than human fibroblasts to copper toxicity.

The observed copper toxicity of all cells was dependent on many factors,

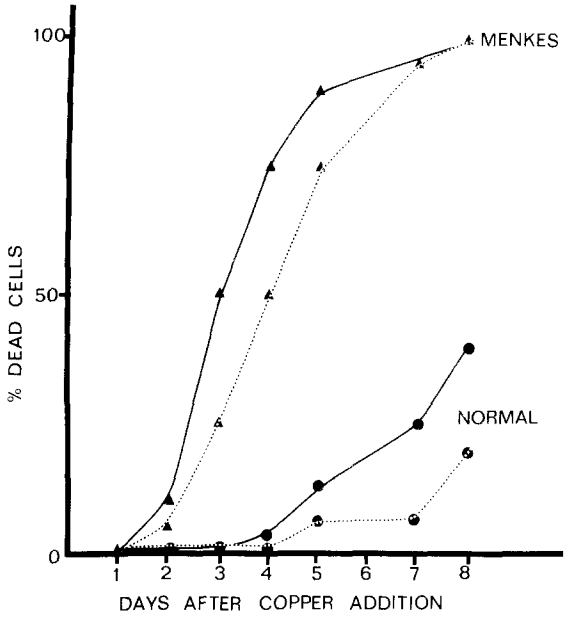

Fig. 1. Cytotoxic effect on Menkes' and normal fibroblasts at various times following addition of 8.7 μg/ml Cu (added as $CuSO_4$) to FbMM (contains 5×10^{-5} M histidine) or FbMM+His (contains 10^{-4} M histidine). ▲——▲, Menkes' fibroblasts in FbMM; ▲····▲, Menkes' fibroblasts in FbMM+His; ●——●, normal fibroblasts in FbMM; ●····●, normal fibroblasts in FbMM+His.

and reproducibility depended on standardization of these. Reduction in fetal calf serum concentration, decrease in *p*H, reduction in degree of cell confluency, or addition of copper to cells immediately following an active growth phase all resulted in increased sensitivity of cells to copper. Increasing histidine concentration (Fig. 1) or using a culture medium containing a higher concentration of amino acids (e.g., medium 199) decreased sensitivity. Some of the factors which increased sensitivity probably did so by increasing the concentration of uncomplexed copper in the medium and *vice versa*. Copper in the form of cupric chloride was less toxic than cupric sulfate for both normal and Menkes' fibrolasts. Using 8.7 μg/ml copper, the time taken for 50% cell death was 15% less for cupric sulfate than for cupric chloride. Such differential toxicity between these two copper salts was also observed *in vivo* when brindled mouse mutants were injected with high doses of copper (Mann *et al.*, unpublished data). The reduced toxicity of copper in the chloride form

Table I. Concentration of Copper (μg/ml) Which Resulted in 50% Cell Death of a Normal and a Menkes' Human Fibroblast Cell Line at Various Times After Addition of $CuSO_4 \cdot 5H_2O$ to FbMM

	Day 6	Day 17	Day 24	Day 27
Normal	>8.7	6.9	5.6	5.0
Menkes'	6.9	5.6	4.4	3.8

Table II. Concentration of Copper (μg/ml) Which Resulted in 50% Cell Death of a Normal and a Brindled Mutant Mouse Kidney Epithelial Cell Line at Various Times After Addition of $CuSO_4 \cdot 5H_2O$ to FbMM

	Day 1	Day 2	Day 3
Normal	>6.3	6.3	5.6
$Mo^{br/y}$	4.4	1.9	1.3

may be due to its stronger Lewis acidity and associated ability to form complex anions.

The specificity of the increased cytotoxic response of mutant cells to copper was tested by examining the response to other divalent cations. Normal and mutant human fibroblasts showed similar sensitivity to elevated levels of Co^{2+} and Mn^{2+}. However, Menkes' fibroblasts showed increased sensitivity to the toxic effects of elevated Zn^{2+} levels in the medium. The toxic response to zinc was different in nature from that for copper, suggesting that the mechanisms of cytotoxicity were different. Maximum cytotoxicity was observed within 24 hr after zinc addition to the medium, whereas copper cytotoxicity occurred gradually over a number of days. Cells often retained a fibroblastoid shape, but nuclei became pyknotic. Reproducible results were obtained only if confluent cultures were incubated in FbMM for at least 5 days prior to addition of zinc. Growing cells were much more sensitive to zinc, possibly because zinc transport may be linked to cell cycle status as zinc is required for DNA synthesis. It can be seen from Table III that 15 μg/ml zinc sulfate was a lethal concentration for Menkes' fibroblasts compared to 20 μg/ml for normal fibroblasts. Doubling the histidine concentration in the medium reduced the cytotoxic effect of zinc for both Menkes' and normal fibroblasts, although Menkes' cells still remained more sensitive to zinc. Brindled mouse kidney epithelial cells were also more sensitive to zinc with 17.5 μg/ml zinc sulfate, causing 100% cell death in the three mutant lines tested

Table III. Percent Viable Human Fibroblasts 24 hr After Addition of Varying Concentrations of $ZnSO_4 \cdot 7H_2O$ to Normal FbMM (Contains 5×10^{-5} M Histidine and 1.5 μg/ml $ZnSO_4 \cdot 7H_2O$ Equivalent) and to FbMM Containing 10^{-4} M Histidine (FbMM + His)[a]

	FbMM + 15 μg/ml	Fbmm + His + 15 μg/ml	FbMM + 17.5 μg/ml	FbMM + His + 17.5 μg/ml	FbMM + 20 μg/ml
Normal	78 ± 22 (5)	98 ± 3 (3)	75 ± 12 (6)	86 ± 15 (4)	6 ± 5 (5)
Menkes'	7 ± 3 (3)	95 (1)	2 ± 4 (9)	5 ± 0 (2)	2 ± 2 (7)

[a] Results are expressed as mean ± SD with number of observations in parentheses.

compared to only 10–20% death in three normal mouse kidney epithelial cell lines.

Intracellular Copper Levels

Intracellular copper levels were measured in normal and mutant cells as another index of response to normal and elevated copper levels in the medium.

Copper levels were measured in a large number of normal, mutant, and heterozygote human fibroblast lines following 14 days' incubation in FbMM and FbMM plus 6 μg/ml copper. These conditions which involve long-term incubation of confluent cells under nongrowing conditions should minimize any variation in copper uptake and accumulation due to a difference in growth rate of cells. All cell lines tested showed elevated intracellular copper levels when incubated in medium containing added copper (Table IV). Menkes' fibroblasts contained approximately thirteenfold higher levels than normal cells in FbMM and sevenfold higher levels in FbMM plus 6 μg/ml copper. Menkes' fibroblasts in FbMM contain 3 times more copper than normal cells in copper-loaded medium. The difference in copper levels between Menkes' and normal cells was found to be greatest after 14 days' incubation. There was no significant difference in copper levels or viability in a normal line between passage 6 and passage 15 and in a Menkes' line between passage 13 and passage 21. Three of nine obligate heterozygote cell lines and six of 14 potential heterozygote lines showed copper levels which were more than 2 SD higher than the mean for normal cells when incubated in FbMM plus 6 μg/ml copper. Three of the six potential heterozygote lines with elevated levels came from mothers who had already borne one Menkes' child while a fourth was from a grandmother of an affected child.

Random X-chromosome inactivation makes heterozygotes a mosaic of normal and mutant cell populations. Therefore, a small skin biopsy may

Table IV. Copper Levels in Human Cells in Normal Medium and "Copper-Loaded" Medium[a]

Cell line	Normal medium	"Copper-loaded" medium
Normal fibroblasts[b]	0.023 ± 0.013 (12)	0.113 ± 0.074 (12)
	0.009–0.045	0.037–0.23
Menkes' fibroblasts[b]	0.282 ± 0.091 (6)	0.770 ± 0.156 (6)
	0.175–0.405	0.54–1.02
Obligate heterozygote fibroblasts[b]	0.07	0.29[c]
	0.08	0.68[c]
	0.04	0.35[c]
	0.02	0.125
	0.02	0.04
	0.02	0.035
	0.004	0.025
	0.03	0.11
	0.03	0.05
Potential heterozygote fibroblasts[b]	0.03	0.28[c]
	0.062	0.342[c]
	0.10	0.474[c]
	0.05	0.509[c]
	0.04	0.386[c]
	0.03	0.270[c]
	—	0.117
	—	0.16
	0.02	0.041
	0.03	0.079
	0.01	0.065
	0.02	0.238
	0.01	0.132
	0.027	0.087
Normal amniotic[b]	0.013 ± 0.014 (9)	0.142 ± 0.070 (8)
	0.005–0.05	0.064–0.23
Menkes' amniotic[b]	0.278 ± 0.059 (5)	1.147 ± 0.284 (3)
	0.20–0.35	0.84–1.40
Normal continuous[d] lymphoid	0.002 ± 0.002 (3)	0.024 ± 0.005 (3)
	0.001–0.004	0.019–0.029
Menkes continuous[d] lymphoid	0.024 ± 0.004 (3)	0.057 ± 0.010 (3)
	0.021–0.029	0.047–0.067

[a] Copper concentration is expressed as $\mu g/10^6$ cells. Results are presented as mean ± SD with number of observations in parentheses. The range is also given. Measurements on individual cultures were averaged, and these averages were used in calculating means for each category.

[b] Incubated in FbMM (normal medium) or FbMM + 6 μg/ml copper ("copper-loaded" medium) for 14 days.

[c] These heterozygote lines possessed significantly elevated copper levels.

[d] Incubated in RPMI 1640 + 20% fetal calf serum (normal medium) or RPMI 1640 + 20% fetal calf serum + 6 μg/ml copper ("copper-loaded" medium) for 3 days.

happen to sample mainly (or only) normal cells or mainly (or only) mutant cells. This may explain the wide range of copper levels found in heterozygote cell lines (one line had levels as high as in Menkes' hemizygote cells while others had normal levels).

Menkes' human amniotic cell lines and Menkes' human continuous lymphoid cell lines (Table IV) also expressed the copper accumulation phenotype, providing evidence that the mutation is likely to be expressed *in vivo* by a variety of different cells.

The copper accumulation phenotype in both normal and "copper-loaded" medium was also expressed in kidney epithelial cell lines, lung fibroblasts, and fetal cells derived from the brindled mouse mutants (Table V), providing further evidence for the homology of the human and mouse diseases and for a widespread expression of the mutation in the body. As in the case of some heterozygote human fibroblasts, kidney epithelial and lung fibroblast cells derived from heterozygotes had copper levels intermediate between those of normal and mutant cells (Table V).

Copper-64 Uptake and Accumulation

In order to further investigate the copper accumulation phenotype, studies were carried out with the radioactive copper isotope, copper-64. Rates of uptake of copper-64 into human continuous lymphoid cell lines over 10 min were similar in normal and mutant cells. In medium containing 0.26 μg/ml copper, uptake in normal cells was 34 pg copper per 10^6 cells while in mutant cells it was 40 pg per 10^6 cells. Similarly in medium containing 0.82 μg/ml copper, the values were 82 and 90 for normal and mutant cells, respectively.

Table V. Copper Levels in Mouse Cells After 2 Days' Incubation in FbMM + 1.3 μg/ml Copper[a]

Cell line	FbMM	FbMM + 1.3 μg/ml Cu
Normal lung fibroblasts	0.019 ± 0.010 (3)	0.055 ± 0.031 (4)
Mo$^{br/y}$ lung fibroblasts	0.071 ± 0.011 (3)	0.342 ± 0.06 (3)
Mo$^{br/+}$ lung fibroblasts	—	0.31
Normal kidney epithelial	0.009 ± 0.005 (6)	0.028 ± 0.007 (4)
Mo$^{br/y}$ kidney epithelial	0.061 ± 0.04 (4)	0.335 ± 0.133 (3)
Mo$^{br/+}$ kidney epithelial	—	0.248 ± 0.09 (2)
Normal fetal	0.014 ± 0.008 (4)	0.041 ± 0.01 (3)
Mo$^{br/y}$ fetal	0.30	0.145 ± 0.013 (2)

[a] Copper concentration is expressed as μg/10^6 cells, and results are presented as mean ± SD with number of observations in parentheses.

These findings make it unlikely that copper accumulation in Menkes' cells is due to a primary defect which increases the rate of uptake across the cell membrane. Copper may accumulate in Menkes' cells because of enhanced intracellular binding following normal entry. To test this further, copper-64 accumulation in cells was measured over a 24-hr period. Table VI shows that Menkes' fibroblasts accumulate 15 times more copper-64 than normal cells after 24 hr and Menkes' continuous lymphoid cells accumulate 9 times more copper-64 than normal (Table VI). In the presence of 1.5 μg/ml extracellular copper Menkes' fibroblasts accumulate 17 times more copper than normal, whereas at 7.6 μg/ml Menkes' fibroblasts accumulate 4 times more copper than normal cells (Table VII). There was no significant cell death in either the Menkes' or normal cells in these experiments. These data indicate that the intracellular copper accumulation being observed is a saturable binding process and that a higher degree of saturation is occurring at lower concentrations of extracellular copper in the case of Menkes' fibroblasts. However, this experiment does not provide information about the nature of the intracellular copper-binding component(s) in the normal and mutant fibroblasts.

In another experiment it was shown that when the cells were incubated in copper-64 for 24 hr and then a 24-hr "chase" was performed in medium not containing copper-64, only 2% of the isotope was released by Menkes' cells

Table VI. Copper-64 Accumulation in Human Fibroblasts and Continuous Lymphoid Cell Lines (CLCs) after 24 hr Incubation[a]

Cell line	ng Cu/10^6 cells
Normal fibroblasts[b]	4 ± 1 (7)
Menkes' fibroblasts[b]	60 ± 19 (4)
Normal CLCs[c]	1 ± 0.2 (3)
Menkes' CLCs[c]	9 ± 3 (3)

[a] Counts have been converted to ng Cu taken up by 10^6 cells in 24 hr. Results are expressed as mean ± SD with number of observations in parentheses.

[b] Medium was FbMM containing 1 μg/ml copper of specific activity 6.7 μCi/μg Cu.

[c] Medium was RPMI 1640 + 20% fetal calf serum containing 0.45 μg/ml copper of specific activity 4.4 μCi/μg Cu.

Table VII. Copper-64 Accumulation in Human Fibroblasts (Two Different Normal Lines and Two Different Menkes' Lines) After 24 hr Incubation in FbGM Containing Different Copper Concentrations[a]

Cell line	FbGM containing 1.5 μg/ml Cu	FbGM containg 7.6 μg/ml Cu
Normal	5	50
Normal	5	70
Menkes'	100	260
Menkes'	70	200

[a] Counts have been converted to ng Cu taken up by 10^6 cells in 24 hr.

whereas normal cells released 78%. This indicates defective efflux of copper from Menkes' fibroblasts.

DISCUSSION

Cells cultured from human Menkes' patients and brindled mouse mutants exhibited similar responses to variations in the copper concentration in the medium, lending further support to the idea of homology of the mutations in the two species.

At usual copper concentrations in the culture medium the intracellular levels in mutant cells were markedly elevated. At a 100 times higher external copper concentration the intracellular copper concentration increased in both normal and mutant cells, but the level in the mutant cells remained well above that in the normal cells. As all cells were harvested by trypsinisation, the copper levels measured presumably did not include copper in the outer proteinaceous coat which cultured mammalian cells possess. Beratis *et al.* (1978) also reported the inability of trypsin to remove copper-64 from cultured fibroblasts. The copper accumulation phenotype is exhibited by many different types of cultured cells from both the human and mouse mutants, indicating the widespread expression of these mutations. Recent *in vivo* studies on the brindled mouse mutant support the idea of the expression of the mutation in many tissues (Mann *et al.*, 1979). Horn (1976) has observed increased accumulation of copper-64 in human Menkes' amniotic cells and has used this as a method of intrauterine diagnosis of Menkes' syndrome. We have successfully used a measurement of total copper levels in amniotic cells as an alternative method of intrauterine diagnosis. The finding of elevated levels in some heterozygotes has provided a test for carriers of Menkes' syndrome, subject to the limitations imposed by cell mosaicism as a result of random X-chromosome inactivation. However, culturing cells from different biopsy

sites should increase the detection frequency. Another factor which may cause a decrease of the copper levels in cultured heterozygote cells may be if normal cells have a growth advantage in culture. Some preliminary evidence for this was obtained in an experiment where known normal and known Menkes' fibroblasts were mixed in equal proportions and grown for several passages. The copper concentration of the cell population was observed to fall with increasing number of passages. Confirmation of this will require experiments with several different Menkes' and normal lines.

Another phenotype exhibited by cultured cells from both the human and mouse mutants is that of increased sensitivity to the cytotoxic effects of both copper and zinc. Beratis *et al.* (1978) and Chan *et al.* (1978) have also observed increased sensitivity of human Menkes' fibroblasts to elevated extracellular copper. However, it is pertinent to attempt to correlate intracellular copper levels in normal and mutant cells with cytotoxicity as it seems reasonable to expect increasing cytotoxicity with increasing intracellular copper. However, from our data human Menkes' fibroblasts at certain intracellular copper levels appear to be more resistant to the cytotoxic effects of copper. In normal medium Menkes' fibroblasts have an intracellular copper level of 0.28 μg Cu/10^6 cells and do not exhibit cytotoxic effects, yet normal fibroblasts in "copper-loaded" medium possess intracellular levels of 0.11 μg Cu/10^6 cells, and they do show cytotoxic effects. It is only when Menkes' fibroblasts are incubated in "copper-loaded" medium where the intracellular levels increase to 0.77 μg Cu/10^6 cells that they exhibit more cytotoxicity than normal cells. Earlier studies (Danks *et al.*, 1973) had shown that early-passage cells cultured from Menkes' hemizygotes and heterozygotes exhibited metachromasia in media containing low copper concentrations. A feasible explanation for this cytopathic phenomenon in those studies was that it was an indication of defective cellular metabolism as a result of copper deficiency, although proof of this will require estimation of copper-dependent enzyme activities. All these data suggest that Menkes' fibroblasts have very narrow tolerance limits for copper. At low copper concentrations in the medium they show signs of copper deficiency while at moderately elevated copper levels in the medium they exhibit cytotoxicity.

These data are compatible with the hypothesis proposed by Danks (1975, 1977) that the fundamental defect in human Menkes' syndrome and in the mottled mouse mutant involves an increase in the affinity for copper of an intracellular copper-binding protein, one of whose functions is the extrusion of copper from cells. This would mean that at low copper concentrations in the medium the altered protein binds most of the intracellular copper, resulting in diminished copper availability to copper-dependent enzymes. In medium containing usual copper concentrations this protein becomes saturated with copper and some copper becomes available for copper-depen-

dent enzymes. Cells appear healthy, even though they have abnormally high intracellular copper levels, as most of the copper is bound to the altered protein—at similar intracellular copper levels normal cells exhibit cytotoxicity presumably due to a higher proportion of toxic ionic copper. In "copper-loaded" medium, as all binding sites on the mutant molecule are saturated and extrusion of copper is being interfered with, ionic copper accumulates with toxic effects. As there is no evidence in Menkes' syndrome or in the mottled mouse mutants of abnormalities in zinc metabolism the toxic response to unphysiologically high levels of zinc may merely indicate that the molecule concerned can bind zinc when zinc is present in high concentrations. Presumably, if the mutant protein is already saturated with copper, this is not possible and uncomplexed zinc then becomes toxic. Clearly the molecule concerned could be a metallothionein. Chan *et al.* (1978) have discussed the possibility of an altered metallothionein in Menkes' cells.

Studies on rates of uptake into human continuous lymphoid cell lines over a short time demonstrated no differences between normal and Menkes' cells, which suggests that an enhanced rate of uptake across the cell membrane is not the reason for intracellular copper accumulation. The decreased rates of efflux of copper from Menkes' cells and the finding that Menkes' fibroblasts become saturated with copper-64 at lower concentrations of extracellular copper than normal cells support the idea of copper retention in mutant cells due to enhanced intracellular binding. Beratis *et al.* (1978) observed a linear relationship between external copper-64 concentration and the intracellular levels accumulated in both Menkes' and normal fibroblasts. This difference in results could be due to their use of growing cells and possibly different times of incorporation. Incorporation rates may be influenced by cells growing at different rates, and therefore in our studies nongrowing cells were used.

Alternative explanations to the one discussed above are an increased rate of synthesis of a major copper-binding protein, or increased stability of a major copper-binding protein secondary to a mutation affecting an enzyme which normally degrades this protein and releases bound copper or an enzyme necessary for specific release of copper from sites where it is bound.

The finding that the mutation in human Menkes' syndrome and in mottled mouse mutants is expressed in cultured cells should facilitate purification of the altered protein from mutant cells. Furthermore, the altered response of mutant cells to variations in external copper concentration should permit a study of the normal role of this protein in intracellular copper metabolism.

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