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# Oncogenic Transformation and Cell Lysis in C3H/10T½ Cells and Increased Sister Chromatid Exchange in Human Lymphocytes by Nickel Subsulfide[1]

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## ABSTRACT

We have scored frequency of transformation and appearance of the oncogenic marker "long microvilli" in mouse embryo fibroblast C3H/10T½ cells in culture for the compound $Ni_3S_2$. Furthermore, we have scored for $Ni_3S_2$-induced sister chromatid exchange in cultured human lymphocytes. Nickel subsulfide in moderate doses caused morphological transformation to type I, II, and III foci and induced long microvilli on the cells in the transformed cultures, demonstrating oncogenic transforming ability. Higher doses led to cell lysis after a lag period. The carcinogenic potency of $Ni_3S_2$ in this system was not as strong as that of methylcholanthrene.

$Ni_3S_2$ increased the sister chromatid exchange frequency in human lymphocytes in a marginal, not dose-dependent way. The increased sister chromatid exchange may indicate that the carcinogenic effect of $Ni_3S_2$ is genetic rather than epigenetic.

## INTRODUCTION

Workers in nickel refineries have an increased risk for pulmonary and nasal carcinomas (9, 11-13, 20, 31, 32). A study in Norway showed that workers employed in a nickel refinery for 3 years or more had an increased (7:1) risk of lung cancer and a 40:1 risk for cancer of the nasal sinuses (22). The incidence increased dramatically with the number of years employed; for workers employed 25 years or more, the incidence was 166:1 for cancer of the nasal sinuses.[3] Kreyberg (15) emphasized that tobacco smoking was probably a contributing factor to the development of pulmonary carcinomas also in nickel workers and pointed out that cigarette smoke and nickel dust probably act synergistically in the production of lung cancers.

Torjussen *et al.* (35) showed that nickel concentrations in nasal mucosa, plasma, and urine of process workers were signficantly higher than in non-process workers. Furthermore, clinical and pathological-anatomical studies showed that epithelial dysplasia occurred exclusively in nickel process workers, but several years after the first nickel exposure (36). The incidence of dysplasia increased with age and duration of the exposure. The carcinogenic activity of the nickel subsulfide dust was considered to be weak, because latency time was long and relatively few nickel workers developed carcinomas (36). On the basis of our previous findings (26-28) that long microvilli serve as oncogenic markers in mouse embryo fibroblast C3H/10T½ cells, Boysen *et al.* (2) sought the same marker in abnormal epithelial cells and demonstrated that long microvilli were indeed present in such cells in nasal dysplasia in nickel workers. Paralleling the epidemiological study by Boysen *et al.* (2), we sought information on whether the C3H/10T½ cells exposed to $Ni_3S_2$ in culture would develop the same oncogenic marker upon transformation.

Animal experiments have proved that $Ni_3S_2$ is a potent carcinogen (38). Furthermore, nickel subsulfide has been found to exert a morphological transforming effect in cell culture studies (7, 8) and to cause embryonic mortality in rats (33). Nickel compounds are genotoxic in both plant and animal cells. On the chromosomal level, turbagenic (3) as well as clastogenic effects have been found (18, 37), and point mutation at the *HPRT* locus has been induced in hamster cells (17).

In light of the above-mentioned facts, it is noteworthy that nickel compounds have been negative in all bacterial mutagenesis tests (30).

We have tested $Ni_3S_2$ with respect to transformation of mouse embryo fibroblast C3H/10T½ cells in culture, particularly the induction of long microvilli (26, 28). Furthermore, since any genotoxicity of $Ni_3S_2$ in human cells has not yet been reported, we decided to examine whether this compound could induce SCE[4] in human lymphocytes *in vitro*. Positive results were found in both systems.

## MATERIALS AND METHODS

**Cell Source and Culture Conditions.** The origin and method of culture of the C3H/10T½ cells were the same as described previously (24). The medium in the cell cultures was changed every 3.5 days. Throughout the experiments, the medium contained heat-inactivated 10% fetal calf serum.

Human peripheral lymphocytes were drawn from the same healthy male donor.

**Transformation and Scoring of Morphological Transformation.** The transformation system and scoring of the 3 types of transformed foci were essentially as reported elsewhere (23). The C3H/10T½ cells were seeded at 500 cells/ml in 5-ml cultures in series of 12 Petri dishes. The series were exposed to the compound for a period of 24 hr starting 24 hr after the cells were seeded. The cultures were then washed 3 times with medium. The medium was subsequently changed every 3.5 days and allowed to grow for 6 weeks and were then fixed and stained with Giemsa. Solvent medium with acetone alone was used in the control series. The final concentration of acetone was 0.7%. The transformed foci after adding the test substances (see below) were counted after 6 weeks. The number of foci represents the mean of 11 cultures in the $Ni_3S_2$ series and the mean of 10 cultures in the MCA and the *N*-hydroxyphenacetin series. The type I focus is composed of tightly packed cells. The type II focus displays massive piling up into multilayers. The type III focus is composed of highly polar, fibroblastic, multilayered arrays of densely stained cells.

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[4] The abbreviations used are: SCE, sister chromatid exchange; MCA, 20-methylcholanthrene.

**Scanning Electron Microscopy.** Preparation of cells for scanning electron microscopy and evaluation of micrographs were as reported previously from our laboratory (28). Nonsynchronized cells in the subconfluent, late logarithmic phase of growth were used. After 6 weeks in postconfluent cultures, one dish was picked at random from each series, trypsinized, passaged once to cultures with coverslips, prepared for scanning electron microscopy, and quantitatively evaluated as reported previously (27). The amount and appearance of microvilli were quantitatively evaluated at ×2500. One hundred strictly randomly chosen cells were analyzed in each series except in the *N*-hydroxyphenacetin series where 87 cells were evaluated.

**Substances and Doses Used.** The $Ni_3S_2$ was generously given us by Dr. F. William Sunderman of the University of Connecticut, Farmington, Conn., and was assayed for transforming effects in series of 12 cultures at 0.001, 0.01, 0.1, 1.0, 10.0, and 100.0 μg/ml. The solubility of $Ni_3S_2$ is so low that the concentrations given in Tables 1 and 2 are rather fictitious as regards dissolved material. It appeared that precipitation of the $Ni_3S_2$ indeed occurred at all dose levels tested. On the other hand, the presence of $Ni_3S_2$ particles available for phagocytosis will depend on the amount of $Ni_3S_2$ added to the culture. MCA was from Sigma Chemical Co., St. Louis, Mo., and was assayed at 1.0 μg/ml. We have previously reported (10) on the transforming ability of the drug *N*-hydroxyphenacetin. For comparison of carcinogenic activity, we also assessed the microvilli pattern induced by this compound against the pattern induced by MCA and by $Ni_3S_2$. *N*-Hydroxyphenacetin was a gift of Dr. Elizabeth Weisburger and Dr. Snorri S. Thorgeirsson, National Cancer Institute, Bethesda, Md. MCA and *N*-hydroxyphenacetin were dissolved in acetone. In the control series, acetone only was added to the medium.

**Chromosome Preparations from Human Lymphocytes.** Whole-blood cultures from the same healthy donor were established and harvested as described previously (4). The 5-bromodeoxyuridine (Sigma) concentration was 5 μg/ml. $Ni_3S_2$ was added at 24 or 48 hr prior to harvest. Differential staining of chromatids were made according to the method of Alves and Jonasson (1). The number of SCE's was counted in 30 cells from each culture. The results were statistically evaluated by the *t* test.

## RESULTS

**Transformation Assays.** With $Ni_3S_2$ at doses of 0.001, 0.01, and 0.1 μg/ml, transformation to type I, II, and III foci took place. At the higher concentrations, the cells first appeared to grow normally for 3 weeks, but then lysis occurred, most rapidly in the series with the highest concentration (100 μg/ml) of the $Ni_3S_2$ dust. Thus, in the series at 1.0, 10.0, and 100.0 μg/ml, no transformed foci could be observed. In repeated transformation experiments with $Ni_3S_2$, this apparently delayed cytotoxic effect by $Ni_3S_2$ was reproduced.

The transformation effect by the metal compound $Ni_3S_2$ was compared to transformation effects by the polycyclic hydrocarbon MCA and to that of *N*-hydroxyphenacetin (10). The data are presented in Table 1.

**Scanning Electron Microscopic Evaluation of Cells.** We observed a high concentration of short microvilli in all the transformed series. The oncogenic marker, long microvilli, was observed in the $Ni_3S_2$ series, was seen to a smaller extent in the MCA series but did not appear in the *N*-hydroxyphenacetin series. The results are presented in Table 1.

**SCE in Human Lymphocytes.** The results of the SCE analysis are given in Table 2. Treatment for 48 hr with doses of 1 to 100 μg/ml increased the SCE frequency. A dose of 1 mg/ml was too toxic to permit cell division. We observed statistically significant effects on the SCE in human lymphocytes after exposure to $Ni_3S_2$ for a 48-hr period. In our study, the 24-hr exposure to $Ni_3S_2$ during the last cell cycle before harvest did not increase the SCE frequency in most cultures. The significant effects on the SCE found after 48 hr treatment are then the sum of the exchanges taking place during each cell cycle.

## DISCUSSION

In culture, the carcinogenic activity of particulate $Ni_3S_2$ has been related to its uptake in Syrian hamster embryo cells and in Chinese hamster ovary cells, which actively phagocytized the $Ni_3S_2$ particles (5). The carcinogenic potency of nickel subsulfide has been found to be proportional to its cellular uptake (6). Oskarsson *et al.* (19) have shown that $Ni_3S_2$ was gradually lost in the form of solubilized nickel and sulfur from the site of injection. Nonsolubilized particles were also lost, apparently by phagocytizing reticuloendothelial cells (19).

In their study on Syrian hamster fetal cells, Costa *et al.* (7, 8) found transformation (10%) among the cells that had been exposed to nickel subsulfide for 6 to 8 days. After 2 weeks, the morphological transformation that had been defined by tightly packed, piled-up colonies was observed. Such pattern of cell growth could be prevented, however, by admixture of manganese dust with $Ni_3S_2$.

We also found a concentration-dependent delayed cytotoxic effect by $Ni_3S_2$, which is not easy to explain. No similar cytotoxic effect has been observed for any other compound that we have tested.

In the mouse embryo fibroblast cells, type I, II, and III transformed foci could be observed 6 weeks after exposure to low or moderate doses of the nickel compound. This seems to be the first time a metal compound has been shown to induce transformation in the C3H/10T½ cell system, but this morphological transformation is consistent with the results in hamster fetal cells (7, 8).

We have previously reported that formation of microvilli on the one hand and the growth pattern morphology on the other hand appear to be independent (27). Our present results further substantiate this view since the occurrence of microvilli was dependent on the dose of $Ni_3S_2$, whereas no such effect appeared with respect to formation of type I, II, and III foci over a 100-fold concentration range. This suggests that in our system the quantitation of actual oncogenic transformation should include assessment of the frequency of type I, II, and III focus formation and the occurrence and concentration of long microvilli.

Clones of $Ni_3S_2$-transformed Syrian hamster fetal cells have been shown to be able to grow in soft agar and to produce malignant tumors in mice (8). In animal studies, application of nickel subsulfide to respiratory tract epithelium resulted in widespread atrophy, focal epithelial necrosis (38), and induction of carcinomas. We have not tried to transplant our transformed cells to animals to prove oncogenic potential, but the induction of long microvilli may be an index of oncogenic transformation by $Ni_3S_2$, as shown previously for 7,12-dimethylbenz(a)anthracene (25, 28).

Based on this criterion, the oncogenic potential is already developing in the $Ni_3S_2$ and in the MCA series. Since these cultures were at passage 2 after transformation and since the transformed foci had not been cloned (and the cells evaluated thus constitute a mixture of normal and transformed cells), high

Table 1

*Transformation of C3H/10T½ cells by nickel subsulfide*

The formation of transformed foci was determined in mouse embryo fibroblast C3H/10T½ cells which had been exposed to $Ni_3S_2$. In a reference experiment, for which the data are included for reasons of comparison, cultures had been exposed to MCA and *N*-hydroxyphenacetin. The concentration of microvilli on the cell surface was described in 4 classes: none (−); few (+); many (++) (still possible to count); and innumerable (+++) (counting no longer possible). The ratio between the number of cells displaying short or long microvilli is shown in each of the 3 categories.

| Series | Concentration (μg/ml) | Transformed foci/culture dish | | | Plates with foci | Microvilli | | | | Microvilli (short/long) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Type I | Type II | Type III | | +++ | ++ | + | − | +++ | ++ | + |
| $Ni_3S_2$ | | | | | | | | | | | | |
| 1 | 0 | 0 | 0 | 0 | 0/11 | 1 | 8 | 40 | 51 | 1/0 | 8/0 | 40/0 |
| 2 | 0.001 | 2.6 | 1.7 | 1.1 | 11/11 | 2 | 14 | 38 | 46 | 1/1 | 14/0 | 38/0 |
| 3 | 0.01 | 3.0 | 0.9 | 0.9 | 11/11 | | | | | | | |
| 4 | 0.1 | 1.8 | 1.0 | 0.7 | 11/11 | 7 | 36 | 40 | 17 | 1/6 | 32/4 | 39/1 |
| 5 | 1.0 | Confluent layer Some lysis | | | | | | | | | | |
| 6 | 10.0 | Few cells, lysis | | | | | | | | | | |
| 7 | 100.0 | Complete lysis | | | | | | | | | | |
| MCA | | | | | | | | | | | | |
| 8 | 1.0 | 2.8 | 3.0 | 2.5 | 10/10 | 15 | 41 | 16 | 28 | 14/1 | 41/0 | 16/0 |
| *N*-Hydroxyphenacetin | | | | | | | | | | | | |
| 9 | 50.0 | 2.8 | 0 | 1.6 | 10/10 | 2 | 22 | 30 | 33 | 2/0 | 22/0 | 30/0 |

Table 2

*SCE[b] in human lymphocytes after treatment with various doses of $Ni_3S_2$*

| Dose (μg/ml) | Experiment 1 (24 hr) | Experiment 2[a] (48 hr) | Experiment 3 | | Experiment 4 | |
|---|---|---|---|---|---|---|
| | | | 24 hr | 48 hr | 24 hr | 48 hr |
| 0 | 6.2 ± 3.4[b] | 7.3 ± 2.2 | 6.6 ± 2.9 | | 6.3 ± 2.6[a] | |
| 0.001 | 6.4 ± 1.8 | 6.2 ± 2.3 | | | | |
| 0.01 | 6.7 ± 3.3 | 7.7 ± 2.4 | | | | |
| 0.1 | 5.7 ± 2.6 | 7.0 ± 2.1 | | | | |
| 1 | 5.8 ± 2.4 | 9.0 ± 2.9[c] | | 6.1 ± 2.7 | 7.6 ± 2.2[a,d] | 8.6 ± 3.1[a,c] |
| 10 | 5.5 ± 2.4 | 9.9 ± 3.3[c] | | 6.2 ± 2.5 | 7.4 ± 3.1[a] | 7.9 ± 2.7[a,d] |
| 30 | | | | | | 8.4 ± 2.8[c] |
| 100 | | | 6.3 ± 2.3 | 9.4 ± 3.7[d] | 7.4 ± 2.3 | |
| 1000 | | | No mitosis | No mitosis | | |

[a] Average of 2 parallel cultures.
[b] Mean ± S.D.
[c] $p < 0.0005$.
[d] Significantly different from control (*t* test, $p < 0.001$).

fractions of microvilli carrying cells in the cell populations cannot be expected.

The fraction of the cell population displaying microvilli increased with passage in culture both in the $Ni_3S_2$-exposed series and in the MCA- and N-hydroxyphenacetin-exposed ones. Boysen *et al.* (2) identified the various epithelial changes in nasal mucosa of nickel workers by the size and shape of superficial cells and changes in surface markers such as microvilli. Epidemiological studies indicated that dysplastic alterations might precede cancer (36), since dysplasia exclusively were found in nickel workers. Boysen *et al.* (2) showed that cells with long microvilli could be observed in such dysplasia.

Thus, our findings of morphological transformation and induction of long microvilli in our mouse embryo fibroblast system appear to mimic nicely the *in vivo* human situation after exposure to nickel subsulfide.

As regards genotoxicity of nickel compounds, the main effect seems to be turbagenic, observed as chromatid gaps, spindle abnormalities, and lagging chromosomes, although chromosome breakage is also found (18, 34, 37). The induction of mutation at the *HPRT* locus in Chinese hamster V79 cells (17) may be a direct effect of $Ni^{2+}$ ions upon the DNA. $Ni^{2+}$ ions have been found to increase the misincorporation of bases during DNA synthesis with *Escherichia coli* polymerase (29), which may indicate that the effect of the metal is upon the DNA processing machinery rather than upon the molecule itself. No chromosome damage was observed in male albino rats after exposure to $NiSO_4$ (16) or in human lymphocytes and fibroblasts after exposure to nickel powder and nickel oxide (21).

The SCE test has generally been considered to reveal a direct effect upon the DNA, but other mechanisms may also operate, since the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate, which does not damage the DNA, has been found to increase the SCE frequency in V79-4 Chinese hamster lung fibroblasts (14).

Although statistically significant by *t* test, the observed increase in SCE induced by nickel subsulfide is marginal and not dose dependent. We suggest that this is due to phagocytized $Ni_3S_2$ particles, which may supply the cell with nickel ions and $Ni_3S_2$ molecules. The genotoxic effect of $Ni_3S_2$ may then be the result of a general toxification of cellular functions affecting processes with genetic consequences, in this case SCE.

In nickel refinery workers in whom plasma concentrations of nickel measured up to 4.2 ng/ml, no increase in SCE frequency was found in their lymphocytes (39). This is consistent with our findings.

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