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The effect of cell monolayer density on the cytotoxicity of metal ions which are released from dental alloys

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Abstract. The effect of cell density (number of cells per unit area of a monolayer culture) on the *in vitro* cytotoxicity of metal ions which are known to be released from dental materials was investigated. The effects of cell density (1) may explain previous discrepancies in *in vitro* tests, (2) may be important in wound healing where cell density changes over time, and (3) may help clarify the mechanisms of cytotoxicity of metal ions. Balb/c 3T3 fibroblasts were plated at cell densities ranging from 10,000-80,000 cells/cm^2 and were exposed to 8 concentrations of 10 different metal ions. After 24 h, the succinic dehydrogenase activity and DNA synthesis were measured to quantify the cytotoxic effect. Higher cell densities markedly reduced the sensitivity of these fibroblasts to all metal ions except Al^{+3} and Zn^{+2}, but the magnitude of the reduction was metal dependent. In addition, the DNA synthesis was inhibited more than the succinic dehydrogenase activity for all metal ions except Zn^{+2}. The unique effect of cell density on each metal ion supported the hypothesis that the effect was not simply caused by a dilution of the number of metal ions per cell. Given these results, the effect of cell density should be carefully selected in *in vitro* cytotoxicity tests.

INTRODUCTION

Current *in vitro* tests for dental alloy cytotoxicity are not always in agreement with each other or with *in vivo* tests. For example, Craig and Hanks (1990) have measured the cytotoxicity of 29 dental alloys. Ten of these alloys were also tested by Wataha *et al.* (1992), and there was significant disagreement among the test results for 4 of the alloys. The cytotoxicity of metal ions which comprise dental alloys has also been measured, and the potencies of these ions vary with the method used to measure them. For example, Jowett *et al.* (1988) found that Zn^{+2} was not toxic at 20 parts per million (ppm) in organ culture, but Leirskar and Helgeland (1981) found that 10 ppm of Zn^{+2} caused a 96% cell death rate in cultured human epithelial cells, and Wataha *et al.* (1991b) found that less than 5 ppm caused 100% cell death in cultured fibroblasts. The usefulness of *in vitro* cytotoxicity tests for metals and metal ions depends upon the resolution of these types of discrepancies.

The number of cells per unit area of a monolayer culture (cell density) may be one factor which influences the cytotoxic response of cells to dental alloys or their released metal ions. Takimoto and Takeda (1991) recently showed that the cytotoxicity of some metal ions to L929 fibroblasts varied with cell density. However, these investigators did not evaluate several metals, such as Ag, Cd, Ga, Ti, V, and Zn, which are important to dental alloys. In addition, they did not use a cell type with a growth rate which slows at high cell densities (contact inhibition), and did not use a toxicity parameter which is cell-density sensitive. The evaluation of this type of cell line is important because it more accurately imitates *in vivo* cell behavior.

The purpose of this investigation was to measure the effects of cell density on the cytotoxic response of fibroblasts to metal ions which are released from dental alloys. This information is important for several reasons. First, it may resolve discrepancies in *in vitro* tests for the cytotoxicity of metal ions or dental alloys, as discussed previously, and may provide a basis for selecting cell density in these types of tests. Second, cell density effects may be important in wound healing around implanted metals where the cell density changes during the healing process. Finally, this information may help clarify the mechanisms of cytotoxicity of these metal ions.

MATERIALS AND METHODS

Table 1 lists the range of concentrations and the sources for the 10 metal ions studied. These metal ions were selected based on previous investigations which have shown they are released from dental alloys (Brune, 1986; Muller *et al.*, 1990; Geis-Gerstorfer *et al.*, 1991, Wataha *et al.*, 1991a). Metal ions were chosen rather than dental alloys because metal ions can be evaluated individually, and their concentrations and durations of exposure can be controlled. Aqueous solutions of the metal ions were prepared from sulfate or chloride salts at concentrations which allowed them to be added to the cell cultures in 20 µL aliquots and to provide the final concentrations listed in Table 1. Control solutions contained the sodium salts of the anions. These final concentrations were chosen based on previous research which established the potency of these ions at one cell density (Wataha *et al.*, 1991b). These concentrations include those which are released from alloys in *in vitro* cytotoxicity tests.

Balb/c 3T3 fibroblasts (ATCC #CCL 163, clone A31) were used in these experiments because they are well characterized, exhibit no tumorogenicity, and are contact inhibited (American Type Culture Collection, 1992). The properties of contact inhibition and lack of tumorogenicity are desirable because they more closely mimic properties of cells *in vivo*. The cell-culture medium consisted of Dulbecco's Modified Eagle Medium, 3% NuSerum, 28 mmol/L HEPES (N-2-hydroxyethylpiperazine-

TABLE 1: METAL ION CONCENTRATIONS AND SOURCES		
Metal Ion	Conc. Range Tested (μmol/L)*	Source
Ag^{+1}	0.9-14	Ag_2SO_4
Al^{+3}	370-3000	$AlCl_3$
Cd^{+2}	0.45-3.6	$CdCl_2$
Co^{+2}	1.7-680	$CoCl_2$
Cu^{+2}	7.9-470	$CuCl_2 \cdot 6H_2O$
Ga^{+3}	14-720	$Ga(NO_3)_3$**
Ni^{+2}	8.5-510	$NiCl_2 \cdot 6H_2O$
Ti^{+4}	210-1700	$TiCl_4$**
V^{+3}	2.0-100	VCl_3**
Zn^{+2}	1.5-60	$ZnCl_2 \cdot 6H_2O$

*Indicates lowest and highest concentrations of metal ions tested. Controls were solutions of the sodium anions of the metals (with no metals present).
** Obtained from atomic absorption standard solution.

N'-2-ethanesulfonic acid, pH = 7.2), penicillin (125 units/mL), streptomycin (125 μg/mL), gentamycin (10 μg/mL), and glutamine (2 mmol/L). The medium was sufficiently buffered to prevent changes in pH of more than 0.05 units during the addition of the metal ion solutions. Also, there was no evidence of precipitation upon addition of the metal ions using this medium. Cells were maintained at pre-confluent densities before the experiments were run.

Four cell densities were used: 10,000, 20,000, 40,000, and 80,000 cells/cm^2. These densities were chosen to provide environments for metal ions ranging from no confluence during the exposure (10,000 cells/cm^2) to confluence before the addition of the metal ions (80,000 cells/cm^2). These environments were verified using light microscopy and measurement of cellular mitochondrial function and DNA synthesis.

Succinic dehydrogenase (SDH) activity and DNA synthesis were used to assess the cytotoxic effect. SDH activity was measured as described previously by the conversion of MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) to its formazan salt (MTT-f) (Pearse, 1972) and was chosen because it is an indicator of cellular mitochondrial activity and does not shut down with confluence. DNA synthesis was measured as described previously by the incorporation of tritiated thymidine (3H-Tdr) (Wataha *et al.*, 1991b). This method was chosen because synthetic activity is reduced as the cell monolayer reaches confluence. Preliminary studies verified these properties in cultures without metal ions (Fig. 1). In Fig. 1a, SDH activity leveled off at higher cell densities (40,000 or 80,000 cells/cm^2) after 48 h, indicating reduced cell growth. Lower starting cell densities did not become dense enough in 72 h to level off. There was no evidence of a drop in SDH activity during these times. In Fig. 1b, DNA synthesis dropped off at high cell densities (40,000 or 80,000 cells/cm^2), but increased throughout the period for lower cell densities.

Cells were added at the specified cell densities in 96-well culture trays and were incubated for 24 h at 37°C, 95% relative humidity, and 5% CO2. The metal ions were then added to the wells, and the cultures were incubated for an additional 24 h.

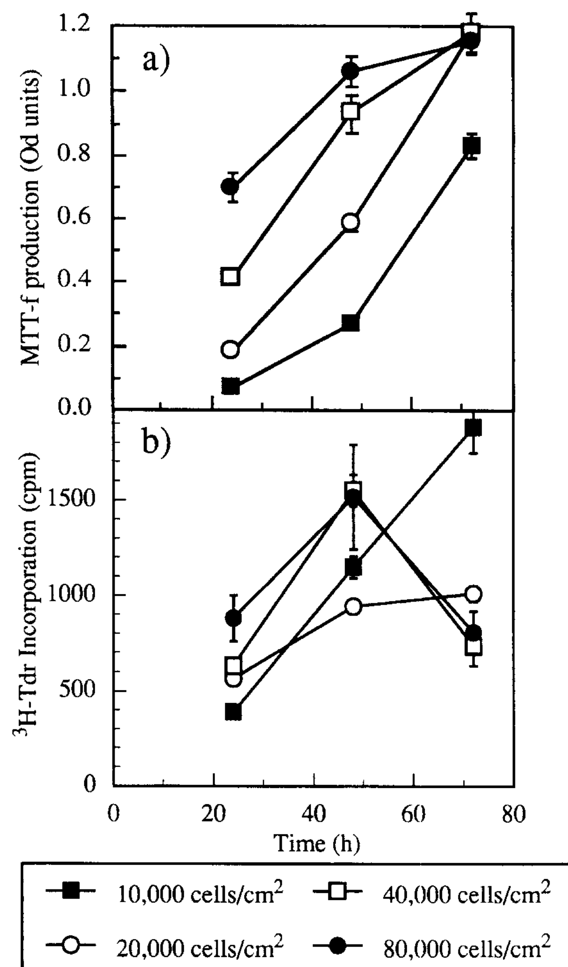

Fig. 1. **A graph showing: (a) SDH activity, measured by MTT-formazan production, and (b) DNA synthesis, measured by ^{3}H-Tdr incorporation, of cell monolayers plated at different cell densities and followed for 72 h. Error bars represent three standard deviations of the mean for n = 8.**

There were 8 replicates per concentration, and 8 concentrations per metal ion. Several experiments were repeated to verify inter-experimental precision.

The concentrations required to reduce SDH or DNA activity by 50% (TC50 values) for each cell density were calculated graphically by plotting the activities vs. metal ion concentration, and interpolating the TC50 concentration. Errors in the TC50 concentrations were also determined from these plots. Additional plots were then constructed of the TC50 values vs. the cell density for each metal ion. The percent change in the TC50 values between 10,000 and 80,000 cells/cm^2 was calculated. The TC50 values for SDH and DNA activities for each metal were compared using Student's t-tests (p = 0.05). The TC50 values for the different cell densities were compared using ANOVA and Tukey intervals (p = 0.05).

RESULTS

Table 2 summarizes the effects of cell density on the cytotoxicity of the metal ions measured by SDH activity, whereas Table 3 shows these effects for DNA synthesis. Some of these data are shown graphically in Fig. 2 for Co^{+2}, Ga^{+3}, and Zn^{+2}, which typify the types of behavior seen among the metal ions used in these

TABLE 2: 50% TOXICITY CONCENTRATIONS (TC50'S) OF METAL IONS MEASURED BY SDH ACTIVITY (in μmol/L)

Monolayer Density (cells/cm^2)	Ag^{+1}	Al^{+3}	Cd^{+2}	Co^{+2}	Cu^{+2}	Ga^{+3}	Ni^{+2}	Ti^{+4}	V^{+3}	Zn^{+2}
10,000	8.5*	>2970	2.3	44	130	36	230	690	8.2	14
20,000	12.8	>2970	2.5	71	170	86	270	810	12	12
40,000	15.3	>2970	3.3	87	240	370	360	1130	19	13
80,000	>16	>2970	3.9	98	255	930	500	1170	24	13

*Errors (3 standard errors of the mean) were $\leq$10% of the mean values at $p = 0.05$ and $n = 8$, determined graphically. Vertical lines connect values which were not statistically different at $p = 0.05$.

TABLE 3: 50% TOXICITY CONCENTRATIONS (TC50'S) OF METAL IONS MEASURED BY DNA SYNTHESIS (in μmol/L)

Monolayer Density (cells/cm^2)	Ag^{+1}	Al^{+3}	Cd^{+2}	Co^{+2}	Cu^{+2}	Ga^{+3}	Ni^{+2}	Ti^{+4}	V^{+3}	Zn^{+2}
10,000	6.4*	2000	0.4	5.0	25	19	36	190	6.0	14
20,000	8.7	1260	0.7	5.0	46	43	15	190	7.4	12
40,000	-	1050	1.1	5.0	79	89	15	250	14	12
80,000	-	1930	1.7	5.0	94	170	32	490	8.8	15

*Errors (3 standard errors of the mean) were $\leq$10% of the mean values at $p = 0.05$ and $n = 8$, determined graphically. Vertical lines connect values which were not statistically different at $p = 0.05$.

experiments. Increases in cell density from 10,000 to 80,000 cells/cm^2 increased the $TC50_{SDH}$ of Co^{+2} by over two times, indicating that the Co^{+2} was less toxic at the higher cell densities (Fig. 2a, Table 2). The effect began to level off at cell densities above 40,000 cells/cm^2. Similar SDH responses were seen for Cu^{+2}, Ti^{+4} and V^{+3} (Table 2). In the Co^{+2} system, the cytotoxicity as measured by DNA synthesis was not influenced by cell densities between 10,000 and 80,000 cells/cm^2.

Fig. 2b shows that both SDH activity and DNA synthesis were less sensitive to Ga^{+3} at cell densities above 20,000 cells/cm^2 because they showed higher TC50 values, although the effect was more pronounced for SDH activity. Unlike Co^{+2}, the effects did not seem to level off in the range of cell densities used in these experiments. Ni^{+2} was similar to Ga^{+3} in its effect on SDH activity (Table 2). Fig. 2c shows that Zn^{+2} cytotoxicity was not significantly influenced by cell density in the range of concentrations which were tested.

The $TC50_{SDH}$ values for nearly all metal ions increased at higher cell densities (Table 2), although the magnitude of the effects depended upon the metal. Exceptions were Zn^{+2} which exhibited no effect, and Al^{+3}, which did not exhibit a 50% reduction in SDH activity at any of the Al^{+3} concentrations. The effect of cell density on metal ion cytotoxicity as measured by DNA synthesis varied more among the metal ions (Table 3). Although Cd^{+2}, Cu^{+2}, Ga^{+3}, and Ti^{+4} showed higher $TC50_{DNA}$ values as the cell density increased, these changes were less dramatic than for the $TC50_{SDH}$ values. Other metals showed different responses. Al^{+3} and Ni^{+2} showed reduced $TC50_{DNA}$ values at 20,000 and 40,000 cells/cm^2 with higher values at 10,000 and 80,000 cells/cm^2. V^{+3} showed an increase in $TC50_{DNA}$ values until 40,000 cells/cm^2, with a drop-off at 80,000 cells/cm^2. Ag^{+1} showed a small but statistically insignificant increase between 10,000 and 20,000 cells/cm^2, but did not cause 50% toxicity above these densities.

Table 4 shows the percentage change in the TC50 values when cell density was increased from 10,000 to 80,000 cells/cm^2. There was a wide variation among metals. The percentage change ranged from -7% for Zn^{+2} to +2500% for Ga^{+3}, although most ions showed increases between 70 and 200%. $TC50_{DNA}$ values were similar in their diversity. When the magnitudes of these effects for SDH activity and DNA synthesis were compared for each metal, there was no clear pattern evident. For Cd^{+2} , Cu^{+2} and Ti^{+4}, the DNA synthesis was more sensitive to cell density changes. For Ag^{+1}, Co^{+2}, Ga^{+3}, Ni^{+2}, and V^{+3}, the SDH activity was more sensitive. For Zn^{+2}, there was no difference.

Except for Zn^{+2}, the metal ions were more potent inhibitors of DNA synthesis than the SDH activity because the TC50 values were lower for DNA synthesis (Tables 2 and 3). The difference between the TC50 values for SDH activity and DNA synthesis did not increase dramatically with increasing cell density for Ag^{+1}, Cd^{+2}, Cu^{+2}, and V^{+3} (Tables 2, 3, and 4). For Co^{+2}, Ga^{+3}, Ni^{+2}, and Ti^{+4}, the difference between the TC50 values became larger as the cell density increased (Figs. 2a and 2b, Tables 2 and 3).

DISCUSSION

The results of the current study are essentially in agreement with the work of Takimoto and Takeda (1991) although detailed comparisons were difficult because of differences in cell line, methods of measuring cytotoxicity, and different metal ions. Both studies agree that cell density generally decreases the sensitivity of cells to metal ions as reflected in the higher TC50 values at high cell density (Tables 2 and 3). Furthermore, the potency of the ions as measured by SDH activity appeared to be similar in the two studies for Al^{+3}, Cu^{+2}, and Ni^{+2}. Potency reflects how toxic a substance is, with a high potency implying that a smaller amount is necessary to cause an equivalent toxic effect.

The mechanism by which higher cell densities decrease the sensitivity of fibroblasts to metal ions is not clear from these experiments. Presumably, this effect could stem from a simple decrease in the number of metal ions available per cell as the cell density increased. However, there are two arguments against this hypothesis. First, the effect is markedly different for each metal ion (Table 4). If the effect was caused by a dilution of the metal ions by the cells, then all metals should have shown a similar reduction in potency. Second, the number of metal ions available per cell in the current study ranged from about 10^{19} at 10,000 cell/cm^2 to 10^{18} at 80,000 cells/cm^2. Thus, it would seem that there was an ample supply of ions per cell throughout the range of cell densities tested.

Since the conversion of the MTT to its formazan salt is essentially an electrochemical reaction, there is some concern

TABLE 4: EFFECTS OF CELL DENSITY ON 50% TOXICITY VALUES (TC50'S)		
	% Change in TC50 Value*	
	SDH	DNA
Ag^{+1}	+80	+36
Al^{+3}		-3.5
Cd^{+2}	+70	+300
Co^{+2}	+120	0
Cu^{+2}	+95	+275
Ga^{+3}	+2500	+790
Ni^{+2}	+115	-11
Ti^{+4}	+70	+160
V^{+3}	+190	+50
Zn^{+2}	-7	+7

*Between 10,000 and 80,000 cells/cm^2. Errors (3 SEM) were approximately 15% of these values.

that the metal ions could reduce the MTT to the formazan in the absence of any enzymatic (SDH) activity. Although this phenomenon has been observed on the surface of solid metal samples (Craig and Hanks, 1988), it was not experienced in this study with metal ions, and there are several possible reasons it was not a problem. First, the medium containing the metal ions was removed before adding the MTT solution to the cells. Although some metal ions could be retained by the cells, the majority of the metal ions were removed during this step. Second, the test for SDH activity aligned well with other tests for cellular disfunction, including microscopic examination (Wataha *et al.*, 1991b). If the metal ions were directly reducing the MTT, the SDH test should have been substantially less sensitive because the conversion of MTT to its formazan would give the appearance of cell viability. Third, Co^{+2} is a component of the MTT incubation solution (Pearse, 1972), and does not reduce the MTT over a period of several weeks.

Since the current study clearly showed that increased cell density reduced the sensitivity of the Balb/c cells to most metal ions, the question to ask is: which cell density is most appropriate for *in vitro* assessments of materials which release these ions? High densities (80,000 cells/cm^2 or greater) might seem attractive since the effect of cell density levels off for some metals at these densities (*e.g.*, Fig 1a), and these densities are similar to *in vivo* conditions of intact tissues. However, the cell monolayers are often very fragile at high densities, and problems such as washing artifacts become acute. Furthermore, a monolayer culture is most reproducible and homogeneous when it is in the growth phase. Once confluence is approached, it is more difficult to assure reproducible properties which are important for *in vitro* screening tests (Freshney, 1987). Thus, when testing these materials *in vitro*, both high and low density cultures should probably be used with the understanding that each has its advantages and disadvantages.

The results of the current study help explain the disparities between the alloy toxicity studies of Craig and Hanks (1988) and Wataha *et al.* (1992). In all alloys which showed disparate results, significant levels of Ag^{+1} and Cu^{+2} were released into the medium (Wataha *et al.*, 1991a). Since cell density had significant effects

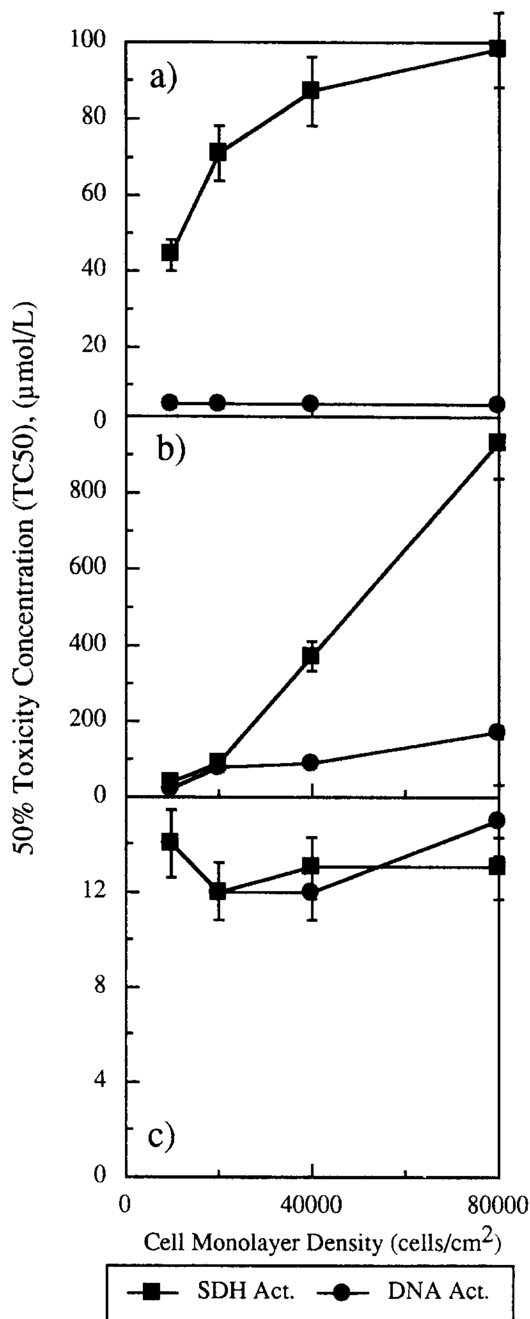

Fig. 2. **The effect of cell density on the 50% toxicity concentrations (TC50's) of (a) Co^{+2} (b) Ga^{+3}, and (c) Zn^{+2} for SDH activity and DNA synthesis. Error bars are 10% at p = 0.05 for n = 8.**

on the cytotoxicity of Ag^{+1} and Cu^{+2} (Table 2), it is possible that the lower toxicity observed by Craig and Hanks (1988) for these alloys was a result of the higher cell densities used in their experiments. The data in Table 2 would also be relevant to the cytotoxicity testing of other metals used in the mouth which release these elements. For example, cytotoxicity tests on gallium-based amalgams might be significantly influenced by cell density because of the dramatic effect cell density had on the apparent toxicity of gallium (Okabe *et al.*, 1992).

The results of the current study emphasize the importance of using cell monolayers of even density in cytotoxicity tests for materials which release metal ions. If a cell monolayer had gross

discrepancies in density, areas of the monolayer might exhibit inhomogeneous toxic responses. Since it can be difficult to produce even monolayers in culture, this is an important consideration (Ryan, 1989).

Since the responses of SDH and DNA synthesis are quite different (Tables 2 and 3), it is important to consider which parameter is most desirable to use in cytotoxicity testing. Although the DNA assay is more sensitive, it is more time consuming and more expensive to perform than the SDH activity test. In addition, the DNA test produces radioactive waste which must be properly disposed. Furthermore, the SDH activity sensitivity appears to be midrange among a variety of methods which are used to assess cytotoxicity (Wataha *et al.*, 1991b). Thus, for initial screening, the SDH activity would appear to be a more appropriate test.

Previous investigators have reported data which established the diverse behavior of metal ions in cell-culture (Kawahara *et al.*, 1968; Babich *et al.*, 1986; Wataha *et al.*, 1991b). The current study would support the previous findings, since cell density affects the cytotoxic behavior of each metal ion differently. Cell density affects cytotoxicity to different degrees, and the effects on SDH activity and DNA synthesis were not consistent among the metal ions (Table 4, Fig. 2). Given these unique responses, it is possible that these substances cause cytotoxicity via different mechanisms, or via common mechanisms which occur to different degrees.

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