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THE EFFECT OF SELECTED METALS ON MARROW CELLS IN CULTURE

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SUMMARY

The differential effects produced in culture by silver, platinum, and stainless steel were studied. After 24 h, mouse marrow cultured on silver showed a significant decrease in the percentages of immature granulocytic and erythroid cells, and a significant increase in the percentage of mature granulocytic cells. The effect, which was not observed at 72 h, was unrelated to the equilibrium potential assumed by silver.

INTRODUCTION

Implanted bi-metallic rods consisting essentially of platinum and silver soldered end to end have been shown capable of producing cellular growth responses in host animals. SMITH has shown that the bi-metallic rods can initiate limb regeneration in adult frogs[1], and BECKER has shown the same response in immature rats[2]. More recently BAKER *et al.* showed that the bi-metallic rods can enhance the healing of hyaline cartilage defects in rabbits[3]. The electrical characteristics of the bi-metallic rods extend over the range of 50–400 mV, and 0.02 to 0.1 nanoamp/mm^2 [4], indicating that the biological growth response has a very low threshold. The stimuli produced by the bi-metallic rods or the individual metals are unknown. Although the distinctions are not rigorous, one could imagine either a chemical or electrochemical basis for the observed effects. The increase in the use of implants of inorganic materials for prosthetic devices presents the same problem from the opposite perspective. Testing is usually confined to tissue tolerance, with little consideration of the possibility of specific cellular effects which may affect either the host or the efficacy of the device over its lifetime. An initial step in consideration of these questions *in vitro* is the study of the cellular effects produced by individual metals. We report here observations concerning the relative effect on mammalian marrow cell morphology produced in tissue culture by three implantable metals; namely, silver, platinum and stainless steel. Bone marrow was chosen because of the variety of cell types it con-

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tains and because it is the site of many experimental and prosthetic implants. We include also some electrochemical measurements made under culture environmental conditions.

METHODS

Metal disks

Marrow cells were incubated directly on 16 mm diameter metal disks which were cut from 0.010 in. flat stock with a metal punch. The silver and platinum had a nominal purity of 99.9%, and the stainless steel was industrial grade No. 302. The disks were sanded with a graded series of abrasives and polished with platinum tripoli. The aim was to prepare silver, platinum and stainless steel disks with surfaces as nearly alike as possible, hence care was taken to standardize the preparative procedure. The silver and stainless disks were used once and discarded. The platinum disks were used the first time and then heated to cherry red and taken through the preparative steps described above before being used a second time. All disks were steam autoclaved before use.

Tissue culture

Mature Swiss Ha ICR female mice were used as the source of marrow cells. The mice were sacrificed with ether and the femur and tibia were scraped clean of soft tissue. The ends of each bone were removed and the medullary canals of the resulting tubes of bone were aspirated with McCoy's media containing 20% fetal calf serum. A disk (the substrate) was placed at the bottom of the central well of a sterile disposable plastic culture dish. The marrow cell suspension was diluted to the desired concentration and then pipetted into the central well where the cells were allowed to settle. The standard cell innoculum was 10^7 cells/dish, and viability was verified by dye exclusion tests employing trypan blue. The cells were incubated for either 1 or 3 days at 37° in a 5% CO_2 atmosphere. At the end of the incubation period, the disk was removed and flushed with fresh media thereby resuspending the cells. The cells were allowed to settle for 1 h under culture environmental conditions onto a glass cover slip. The cover slip was then removed, dried immediately under forced air and prepared for microscopic study with the cells *in situ*.

Initially, a variety of other recovery techniques were employed, including trypsin, collagenase, versene, and mechanical scraping. No particular method gave superior results and therefore the method described was ultimately adopted.

Our primary purpose was to determine whether there existed any differential effect between the metals. There is therefore no culture substrate which is a control in the ordinary sense. Nevertheless it seemed worthwhile to include for study a more common cell culture substrate. Therefore in one set of cultures, the metal disks were replaced by glass cover slips which were treated exactly as the metal disks in that following incubation, the cells were flushed and allowed to settle on another cover slip before being prepared for study. In an additional set of cultures, after incubation, the cells were fixed *in situ* without being deposited onto a second cover slip. All cell

preparations were stained with Wright-Giemsa. A minimum of 400 nucleated cells per preparation are counted.

Metal wire

The same metals in the form of 0.010 inch diam. wire were employed to measure the spontaneous potentials associated with the metals. They were cleaned with acetone to remove drawing oils, washed thoroughly in distilled water and steam autoclaved before use. The wires were insulated with polyethylene tubing, leaving an exposed length of 5 mm, which penetrated the solution. Open circuit potentials were taken with respect to a calomel reference electrode employing an electrometer amplifier having an input impedence of 10^{13} ohms. The measurements were made in the same media and under the same conditions as that employed in the cell cultures, except for the absence of cells. The reference electrode was isolated from the experimental flasks by two intermediate chambers, one containing a balanced salt solution and the other, the culture media. Connections were made with small-bore polyethylene tubing.

RESULTS AND DISCUSSION

In order to statistically compare the various preparations it was necessary to group the observed cell types. The percentages of myeloblasts and promyelocytes were grouped and are termed progranulocytes. The percentages of neutrophilic myelocytes and neutrophilic metamyelocytes and all mature forms were grouped and are termed neutrophils. The third and fourth groups are composed of the eosinophils and the lymphocytes. The entire nucleated erythroid series including the pronormoblast, basophilic normoblast, polychromatophilic normoblast, and the normoblast, are summed

TABLE I

RELATIVE PERCENTAGES AND STANDARD DEVIATIONS OF MARROW CULTURED ON METAL

The number of preparations counted are indicated in parentheses.

Time in culture	*Cell type*	*Substrate*		
		Silver (10)	Platinum (12)	Stainless (10)
1 Day	Progranulocytes	1.8 ± 1.0	3.4 ± 1.9	3.7 ± 2.9
	Neutrophils	75.7 ± 7.8	72.3 ± 15.7	69.4 ± 6.2
	Eosinophils	4.7 ± 2.2	2.8 ± 1.2	2.9 ± 1.3
	Lymphocytes	8.9 ± 7.6	7.4 ± 8.4	8.6 ± 9.0
	Normoblasts	6.8 ± 2.2	12.0 ± 7.5	14.1 ± 6.4
	Fibroblasts	1.8 ± 1.3	1.2 ± 1.7	1.0 ± 0.6
		Silver (10)	Platinum (10)	Stainless (9)
3 Days	Progranulocytes	2.7 ± 1.7	1.1 ± 1.7	2.8 ± 2.2
	Neutrophils	83.1 ± 9.1	88.8 ± 5.6	89.3 ± 4.2
	Eosinophils	8.1 ± 10.7	2.6 ± 3.0	1.5 ± 1.5
	Lymphocytes	0.5 ± 0.7	0.8 ± 0.9	0.9 ± 0.9
	Normoblasts	0.8 ± 0.9	1.8 ± 2.9	0.5 ± 0.8
	Fibroblasts	4.6 ± 3.8	5.6 ± 2.0	4.5 ± 2.1

under the term normoblast. The sixth significant cell group is the fibroblast. The relative percentages of the six groups of cells as a function of the metal substrate at 1 day and at 3 days are given in Table I. We found that the total number of cells that survived the culture period and recovery procedure did not materially vary with the substrate. Differences were found on day 1; by day 3 the spontaneous changes which occur in culture had overwhelmed any differences between the metals. The data in Table I were analyzed for statistical significance and the results are given in Table II

TABLE II

COMPARISION OF SIGNIFICANCE ($P < 0.05$) BETWEEN METALS

Non-zero entry indicates significance by the two-tailed *t*-test. *Plus* or *minus* sign indicates whether the first listed metal had a higher or lower percentage than the second listed metal.

Cell group	*Metals compared*	*Time of culture*	
		1 Day	*3 Days*
Progranulocytes	Silver-platinum	(−)	0
	Silver-Stainless	(−)	0
	Platinum-Stainless	0	0
Neutrophils	Silver-Platinum	0	0
	Silver-Stainless	(+)	0
	Platinum-Stainless	0	0
Eosinophils	Silver-Platinum	(·)	0
	Silver-Stainless	(·)	0
	Platinum-Stainless	0	0
I ymphocytes	Silver-Platinum	0	0
	Silver-Stainless	0	0
	Platinum-Stainless	0	0
Normoblasts	Silver-Platinum	(—)	0
	Silver-Stainless	(-)	0
	Platinum-Stainless	0	0
Fibroblasts	Silver-Platinum	0	0
	Silver-Stainless	0	0
	Platinum-Stainless	0	0

where all combinations of the metals are listed. Of the twelve comparisons involving silver at day 1, significant differences were found in seven cases. In contrast, platinum never differed from stainless steel. None of the effects observed at day 1 persisted at day 3. The relative percentages of lymphocytes and fibroblasts were independent of all the metals at both 1 day and day 3. Lymphocytes are among the cells least likely to adhere to a surface, and fibroblasts are among those cells most likely to do so. This observation therefore indicates that the differential effect of silver is not due to a difference in silver's adhesive properties. In contrast, the effect arises from the chemical or electrochemical activity of silver.

The percentages of fibroblasts shown in Table I at both 1 and 3 days are lower than those we generally observed for marrow cultures on glass. This could have resulted from either fibroblastic inhibition by the metallic surfaces, or from the mere adhesion of the fibroblasts to the metallic surfaces. Both alternatives would produce

lower fibroblasts percentages when the cells were resuspended. The question could not satisfactorily be resolved in the case of the metals because the fibroblasts that were not resuspended, if any, were unobservable by light microscopy. We chose therefore to examine fibroblastic adhesion employing glass. Table III shows the effect of resuspension on cell percentages for cells cultured on glass. As can be seen, most fibroblasts adhere to the glass and resist suspension, resulting in much lower fibroblast percentages in the resuspended cell population. On the basis of this result we tentatively conclude that fibroblast adherence accounts for the low fibroblast percentages of the resuspended cells cultured on metal.

TABLE III

THE EFFECT OF RESUSPENSION ON RELATIVE PERCENTAGES FOR CELLS CULTURED ON GLASS

In column A, the cells were resuspended in the same manner as the cells cultured on metal. In column B the cells were fixed *in situ*. The number of preparations counted is indicated in parentheses. Values are mean ± S.D.

Time in culture	*Cell group*	*Glass substrate*	
		A (20)	B (24)
1 Day	Progranulocytes	3.6 ± 1.8	1.0 ± 1.0
	Neutrophils	71.0 ± 0.3	43.0 ± 13.6
	Eosinophils	3.2 ± 2.1	4.4 ± 2.7
	Lymphocytes	17.9 ± 9.3	4.0 ± 4.8
	Normoblasts	3.2 ± 3.2	17.2 ± 10.2
	Fibroblasts	1.0 ± 0.8	29.2 ± 19.5
3 Days	Progranulocytes	2.7 ± 1.7	1.0 ± 1.4
	Neutrophils	88.3 ± 5.7	34.3 ± 26.9
	Eosinophils	4.2 ± 2.4	0.9 ± 1.6
	Lymphocytes	0.4 ± 0.4	0.0
	Normoblasts	0.0	0.4 ± 0.8
	Fibroblasts	4.4 ± 3.2	63.0 ± 28.5

If the cells cultured on metal (Table I) are compared at day 1 to those cultured on glass (Table III) marked differences can be seen in the lymphocyte and normoblast populations. If the metals and the resuspended glass cultures are compared, the metallic lymphocyte percentages are lower, and the metallic normoblast percentages are higher. The situation is reversed when the metals are compared to the glass cultures fixed *in situ*. In either case, it seems reasonable to conclude that these cell types are most affected by the metals as compared to glass. At day 3 there is no significant difference between the metal and glass cultures.

Returning to the differential effect produced by silver at day 1 (Table II), we have concluded that the effect is probably chemical or electrochemical. Processes can occur at the metal solution interface which give rise to an electric potential between the metal and the solution. To assess the role of the electrode potential, the equilibrium potential between each metal and a calomel reference electrode was measured in the culture medium for 24 h. The results, expressed with respect to the standard

hydrogen electrode, are given in Table IV. The values were all positive, and the silver and stainless steel values were sufficiently close to seemingly preclude the solution potential as a significant factor in the differential effect of silver. On this basis we believe that the chemical activity of the silver ion is responsible for the effect associated with silver.

TABLE IV

EQUILIBRIUM POTENTIALS WITH RESPECT TO HYDROGEN AT THE END OF 24 h

The results are averages and standard deviations of triplicate measurements.

Metal	*Equilibrium potentials (mV)*
Platinum	431 ± 31
Stainless Steel	182 ± 19
Silver	136 ± 40

From Table II it can be seen that for silver the percentages of immature granulocytic cells were decreased and the percentages of mature granulocytic cells were increased. To the extent observed, the erythroid series followed the same pattern. Neither the fibroblasts nor the lymphocytes were selectively affected by silver, although in the case of the lymphocytes we observed so few immature forms that it was not possible to determine whether the ratio of immature to mature cells was altered by silver. These observations may indicate that silver accelerates the maturational process of the granulocytic and erythroid series. They are also consistent with the possibility that silver is toxic to the immature cells of both series because such an effect would remove them from the intact, non-degraded cell population and yet not produce a significant change in the total number of cells recovered. The data do not enable us to conclusively discriminate between these two alternatives, although since the mature granulocytes cultured on silver do show a percentage increase, we believe the former is more probable.

We conclude that of the metals tested, only silver had a specific effect upon the cellular population of mammalian bone marrow cultures. Silver either causes the early maturation of the primitive cells of the granulocytic and erythroid series, or is selectively toxic to them. On the basis of the equilibrium potential measurements we suspect the chemical activity of the silver ion rather than the potential assumed by the silver causes the differential effect of silver. We believe that the commonly used implanted materials, even though they are considered non-toxic, may have similar specific effects which could be cumulative. It is suggested that the methyl-methacrylate plastics be evaluated in this light. It is possible that other metallic cations may have specific cellular effects which may be of therapeutic value in disorders of the bone marrow and that a search for these effects would be worthwhile.

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