[Environmental Health and Preventive Medicine 3, 117-121, October, 1997]

*Original Article*

# The Influence of Beryllium on Cell Survival Rates in the *In-vitro* Culture System, on Intracellular DNA Synthesis and on SRBC-IgM Antibody Production Responses

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## Abstract

**Immunocytotoxicity of beryllium (Be) was evaluated by studying cell viability, intracellular DNA synthesis and SRBC-IgM response in an in-vitro culture system using non-sensitized spleen cells of a C57BL mouse. Be addition showed a suppressive effect on cell viability, an enhancing effect on DNA synthesis and on IgM antibody production. The suppressive effect on cell viability manifested itself markedly as the concentration of Be was increased or the culture time was prolonged. The DNA synthesis-enhancing effect was noted at a relatively low concentration of Be (not more than 10$\mu$M). The enhancing effect on the IgM response was related to Be concentration at not more than 20$\mu$M. The experimental results mentioned above speculate that the cytotoxicity of Be shows a conflicting pattern of enhancement or suppression according to the concentration used and that immunologically it has a modulating effect or an activating effect on the immunocompetent cells.**

**Key words:** Beryllium, Survival rate, DNA synthesis, SRBC-IgM antibody

## Introduction

Many metals such as cobalt, nickel, chromium, zirconium, platinum, mercury and beryllium (Be) are known as the so-called 'sensitizing metals' that cause allergic disorders to the living body[1,2]. These metals and metal compounds have been used ubiquitously in various fields with the development of frontier technology industries in recent years. On the other hand, environmental pollution by these metals is spreading and infiltrating into not only the workshop environment, but, also, into local communities and the livelihood environment of individuals, exerting an influence on health.

Looking closely at the immune toxicity of Be among these metals, we planned a series of experimental studies to elucidate the toxic influence on immunocompetent cells and the influence on the immune response in the living body, that is, immunogenicity, antigenicity and adjuvanticity of Be *in-vitro* and *in-vivo*.

As one step in that plan, we studied the influence of Be on cell survival rate, intracellular DNA synthesis and humoral immune response in the *in-vitro* culture system in an attempt to elucidate its immunocytotoxic characteristics.

## Materials and Methods

### Materials

Experimental animals used were C57BL/6CrS1c mice (males at 5 weeks of age, Charles River Japan, Inc.). Beryllium chloride ($BeCl_2$, Mitsuwa Chemical Co., Ltd) was dis-

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solved in a saline solution, sterilized by filtration and used as a metallic solution.

The medium was RPMI 1640 (Gibco Laboratories) containing 10% fetal calf serum (Cell Culture Laboratories), 100 U/ml penicillin (Meiji Seika Kaisha, Ltd.), 100 $\mu$g/ml streptomycin (Meiji Seika Kaisha, Ltd.), 0.1mM non-essential amino acid (Gibco Laboratories), 1mM sodium pyruvate (Gibco Laboratories) and $5\times10^{-5}$M 2-mercaptoethanol (Katayama Chemical Industries Co., Ltd).

For sheep red blood cells (SRBC) antigen sensitization, preserved sheep blood (Nippon Bio-Test Laboratories, Inc.) was washed with a saline solution. The solution was adjusted to a 10% level and 0.2 ml of this solution was intraperitoneally administered to the mouse. A mouse spleen cell suspension was prepared according to the method of Yahara et al[3] with some modification.

Methods

1) The influence of Be addition on the survival rate of mouse spleen cells.

To a 96-well microplate (FALCON, 3072) was added a spleen cell suspension ($1\times10^6$ cells/ml) 200$\mu$l, a Be solution (final concentration ; 0～160$\mu$M)10$\mu$l and a culture fluid 40$\mu$l. The mixture was cultured for 96 hours. The number of live cells was counted by the Trypan Blue staining every 24 hours after the inception of culture.

2) The influence of Be addition on DNA synthesis in mouse spleen cells.

To a 96-well microplate (FALCON, 3072) was added a spleen cell suspension ($1\times10^6$ cells/ml) 200$\mu$l, a Be solution (final concentration : 0～160 $\mu$M) 10$\mu$l and a culture fluid 40$\mu$l. The mixture was cultured for 72 hours. After culture, 9.25 kBq of $^3$H-thymidine ($^3$H-TdR) was added. After culture for 24 hours, the amount of $^3$H-TdR taken up into the cells was measured.

3) The influence of Be addition on the SRBC-IgM antibody production response of mouse spleen cells.

To a 35×10 mm dish for tissue culture (COSTAR, 3035) was added a spleen cell suspension ($1\times10^7$ cells/well, a primary reaction : non-sensitized mouse spleen cells, a secondary reaction : SRBC sensitized mouse spleen cells), a SRBC antigen solution ($1\times10^7$ cells/well), a Be solution (final concentration : 0～40$\mu$M) and cell culture fluid. The mixture was cultured for 96 hours. After completion of the culture, IgM-plaque forming cells (IgM-PFC) were measured according to the method of Cunningham et al[4].

## Results

1) The influence of Be addition on mouse spleen cell survival in an *in-vitro* culture.

The number of live cells on measurement was calculated in terms of percentage with the number of cells at the inception of the culture as 100, and the mean value±standard deviation (M±S.D.), thereof, was used to express the influence of Be as shown in Table 1.

The measurement was made in triplicate for a total of five measurements. The cell survival rate in the control group decreased gradually with an increase in the number of days of culture. According to changes in the mean value, about 20% of the cells died on the 1st day of culture. The increase in the number of dead cells was relatively slow up to the 4th day, during which about 73% was confirmed to be alive.

In the Be addition group, the influence of an increase in the number of days of culture was similar to the depression curve in the control group, but the influence was stronger from the 2nd day of culture onward. On the 1st day of culture, the cell survival rate with an increase in the amount of Be addition was within the normal variation range of the control group. From the 2nd day on, however, the number of live cells decreased evidently with an increase in the amount of Be added. The number of live cells showed a statistically significant decrease particularly in the 80 $\mu$M or more group on the 2nd day and in the 40 $\mu$M or more group on the 3rd and 4th days.

2) The influence of Be addition on mouse spleen cell DNA synthesis.

Be was added to spleen cells obtained from non-sensitized mice, and changes in the intracellular DNA synthesis were studied by measuring the amount of $^3$H-TdR taken up by the cells in a 4-day culture. The results expressed as percentage against the amount of $^3$H-TdR taken up (M±S.D. of cpm) in the control group are presented in Table 2. The measurement was made in triplicate for a to-

**Table 1 Survival viability (%) of mouse spleen cells cultured for 24, 48, 72 and 96 hours in the presence of various concentrations of beryllium chloride.**

| Be concentration in culture ($\mu$M) | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 0.0 | 80.2 ± 10.3 | 79.9 ± 8.5 | 75.6 ± 12.4 | 73.3 ± 16.2 |
| 10.0 | 75.1 ± 9.6 | 80.1 ± 8.8 | 67.0 ± 11.6 | 54.4 ± 13.4 |
| 20.0 | 82.7 ± 10.6 | 78.0 ± 10.1 | 58.1 ± 11.8 | 49.4 ± 10.5 |
| 40.0 | 75.2 ± 9.4 | 62.1 ± 12.4 | 43.1 ± 10.3* | 38.0 ± 11.5* |
| 80.0 | 77.7 ± 10.0 | 49.9 ± 8.7* | 40.5 ± 12.1* | 31.0 ± 10.0* |
| 160.0 | 75.7 ± 8.8 | 47.7 ± 8.4* | 34.1 ± 10.3* | 28.1 ± 8.0* |

The data is given in mean±S.D. of percentage in five experiments.
*Significant at $p<0.05$ compared with the control (0.0$\mu$M Be in culture).

**Table 2 Effects of beryllium chloride on the DNA synthesis of unsensitized mouse spleen cells.**

| Be concentration in culture ($\mu$M) | S.I. (%) |
|---|---|
| 0.0 | 100 ± 16 (218±34) |
| 5.0 | 144 ± 35* |
| 10.0 | 174 ± 51* |
| 20.0 | 111 ± 33 |
| 40.0 | 74 ± 35 |
| 80.0 | 58 ± 28* |
| 160.0 | 45 ± 23* |

The effect of different concentrations of beryllium chloride was tested following the incorporation of $^3$H-TdR in the DNA of spleen cells cultured for 96 hours. The mean stimulation index (S.I.) of five experiments each performed as triplicate cultures is given in M±S.D. of S.I. of the control and for the control also in cpm (in parenthesis). *Significant at $p<0.01$ compared with the contorol (0.0 $\mu$M Be in culture).

**Table 3 Effects of beryllium chloride on SRBC-IgM antibody production in splean cells from unsensitized mice *in-vitro*.**

| Be concentration in culture($\mu$M) | Number of PFC in $1 \times 10^6$cells/ml |
|---|---|
| 0.0 | 155 ± 43 |
| 0.5 | 851 ± 312 |
| 1.0 | 570 ± 149* |
| 5.0 | 529 ± 31* |
| 10.0 | 364 ± 65* |
| 20.0 | 219 ± 35 |
| 40.0 | 104 ± 73 |

Results are shown with M ± S.D. of IgM PFC in $1\times10^6$ cells/ml of spleen cells. *Significant at $p<0.01$ compared with the control (0.0 $\mu$M Be in culture).

**Table 4 Effects of beryllium chloride on SRBC-IgM antibody production in spleen cells from SRBC-sensitized mice *in-vitro*.**

| Be concentration in culture($\mu$M) | Number of PFC in $1 \times 10^6$cells/ml |
|---|---|
| 0.0 | 800 ± 137 |
| 0.5 | 857 ± 73 |
| 1.0 | 1,097 ± 192 |
| 5.0 | 1,137 ± 184* |
| 10.0 | 1,177 ± 57* |
| 20.0 | 1,244 ± 345* |
| 40.0 | 1,003 ± 67 |

Results are shown with M±S.D. of IgM PFC in $1\times10^6$ cells/ml of spleen cells. *Significant at $p<0,01$ compared with the control (0.0 $\mu$M Be in culture).

tal of 5 measurements.

As for the influence of Be addition, DNA synthesis increased at 20 $\mu$M. Particularly, the increase at 5 and 10 $\mu$M was statistically significant. At 40 $\mu$M or more, a suppressive tendency was shown. Suppressive changes at 80 and 160 $\mu$M were statistically significant.

3) The influence of Be addition on SRBC-IgM antibody production response of mouse spleen cells *in-vitro*.

Spleen cells obtained from non-sensitized mice (primary reaction) and SRBC-sensitized mice (secondary reaction) were used. The results expressed as M±S.D. of IgM-PFC in spleen cells $10^6$ are presented in Table 3 (primary reaction) and in Table 4 (secondary reaction).

In the primary reaction, the reaction was strongest at 0.5 $\mu$M and decreased according to an increase in the amount of Be added. Overall, an increase in the antibody production response was seen at concentrations up to 20.0 $\mu$M. The increase was statistically significant particularly at 1.0, 5.0 and 20.0 $\mu$M. At 40 $\mu$M, a suppressive reaction was shown, but there was no statistical difference.

In the secondary reaction, the reaction was increased at any concentration measured, and the increase was statistically significant at 5.0, 10.0 and 20.0 $\mu$M.

## Discussion

In order to elucidate the cytotoxicity, particularly immunocytotoxic effect of Be, we studied the influence of Be on the cell survival rate *in-vitro*, on intracellular DNA synthesis and on the SRBC-IgM antibody production response in a Be-added culture using mouse spleen cells.

Changes in the cell survival rate over time in the Be-added culture of mouse spleen cells were prominent compared with a slow decrease of the survival rate in the control group, and the influence of Be addition was clearly found. That is, the cell survival rate decreased significantly at 80$\mu$M or more from the 2nd day and at 40 $\mu$M from the 3rd day; the proportion of live cells at 40$\mu$M or more on the 4th day of the culture decreased to less than 52% of that in the control group. It is possible that essential components in cell culture fluid degenerated with Be addition, leading to the death of cultured cells or that Be taken up by cells exerted a toxic effect on the *in-vivo* metabolism of cells in one form or another. Which of these two possible factors strongly influenced the cell survival rate of this kind is the subject for future studies. In this respect, there are findings that the toxic effect of Be exerts an influence on the fluidity or permeability of the cell membrane and these are reports that Be inhibits the activity of various enzymes in the living body[5-9]. These are interesting findings in relation to the results of the present study.

Regarding the influence of Be on intracellular DNA synthesis, conflicting effects --- enhancement and suppression --- were observed depending on the Be concentrations in the culture fluid. That is, Be was considered to have the effect of increasing DNA synthesis at relatively low concentrations (not more than 20 $\mu$M), while a suppressive effect with an increase in Be concentrations was suggested. However, there is a strong possibility of this DNA synthesis suppressing effect being influenced by a decrease in the number of live cells with an increase in the amount of Be added. Quantitative changes in live cells as mentioned earlier may have some effect on this.

In light of the findings above, Be is considered to have a

toxic effect on intracellular metabolism but it has an enhancing effect on DNA synthesis. Regarding the effect of sensitizing metals on DNA synthesis, results of experiments with nickel, cobalt, chromium and mercury have been reported, and these metals are shown to have concentration-dependent enhancing effects on DNA synthesis[10-12]. These results have pointed out that the enhancing effect is found at a concentration of several $\mu$M, which is in favorable agreement with our experimental results. Furthermore, the culture time is considered to be closely concerned with this kind of enhancing effect, and many reports have stated that the optimal time was 48 hours to 72 hours. In the present experiment, we studied the influence of a 96-hour culture and found an enhancing effect over time. In this respect, it was suggested that the DNA synthesis enhancing effect of Be would be longer lasting compared with that of other sensitizing metals. As to the magnitude of action, it was almost equal to the results of experiments with other sensitizing metals.

Regarding the influence of Be on the SRBC-IgM antibody production response *in-vitro*, we studied Be addition at a concentration up to 40 $\mu$M, taking the results of the aforementioned two experiments into consideration. First, as for the influence of Be addition on the primary immune response using spleen cells of SRBC non-sensitized mice, Be showed the effect of enhancing the antibody production response up to 10 $\mu$M, and the lower the concentration of Be addition was the stronger the effect.

In the secondary immune response using spleen cells of SRBC sensitized mice, Be showed an enhancing effect in a concentration from 5.0 up to 20 $\mu$M. That is, it was suggested that Be has an adjuvant-like effect of enhancing the humoral immune response. Regarding such an adjuvant-like effect of metals, aluminum (AI) has formerly been known to have such an effect and it is used experimentally[13-14]. A mechanism has been pointed out whereby an antigen absorbed by aluminum hydroxide and aluminum phosphate stays at the site of administration, becomes susceptible to phagocytosis and tends to attain high immunogenicity.

It is also found that Be easily becomes hydroxide and phosphate and that soluble Be has cell agglutinating activity[15]. From the results of the present experiment, it can be surmised that the immunogenicity was enhanced by the mechanism of action similar to that found with A1. Furthermore, it is suggested that the synergistic action of the adjuvanticity and the lymphocyte activating effect of Be play a part in the enhancement of the humoral immune response by Be. Next, the degree of the enhancing effect according to the concentration of Be added showed a conflicting pattern in the primary and secondary reactions. In the primary reaction, a peak was found with a low Be concentration and the reaction decreased with an increase in the concentration, while in the secondary reaction the reaction tended to increase with an increase in the Be concentration. This is due possibly to the influence of whether or not these is SRBC sensitization in the cell group.

As above, we have made it clear that Be has an immunological action to activate the immunocytes and that the concentration of Be added is greatly concerned with it. In elucidating the mechanism of action of Be's immuno-cytotoxic reaction, it is indicated by these experimental results that demonstrating the action of Be as animmunomodulator may have an important immunological significance.

## Conclusion

The influence of Be addition on cell survival, DNA synthesis and SRBC-IgM antibody production response was studied in the mouse spleen cell culture system *in-vitro*, and as to the immunocy totoxicity of Be, the following conclusion was reached.

1. Be addition showed a suppressive effect on the cell survival rate. This suppressive effect manifested itself markedly according to the increase in Be concentration or the prolongation of the culture time.
2. The DNA synthesis-enhancing effect of Be was noted at relatively low concentrations (not more than 10 $\mu$M). As for the reaction intensity with Be addition, it was about twice that of the control group at the concentration of 10 $\mu$M.
3. Be addition had an enhancing effect of the IgM antibody production response. Be concentrations (not more than 20 $\mu$M) influenced this effect.

The experimental results mentioned above have made it clear that the cytotoxicity of Be shows a conflicting pattern of enhancement or suppression according to the concentration used and that immunologically it has an adjuvant-like effect and an activating effect on immunocytes.

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