IgM antibody production in mice intraperitoneally injected with zirconium oxychloride

S SHIMA, K MORITA, S TACHIKAWA, T ITO, H KURITA, T YOSHIDA, Y KATO, Y YAMAMOTO

From the Department of Public Health, School of Medicine, Fujita-Gakuen Health University, and Department of Serology, College of Medical Technology, Fujita-Gakuen University, Toyoake, Aichi 470-11, Japan

Abstract The effect of zirconium (Zr) on the humoral immune response was studied by measuring the level of IgM-plaque forming cells (IgM-PFC) against sheep red blood cells (SRBC) in the spleen of C57 BL mice intraperitoneally injected with zirconium oxychloride. Two experiments, a single injection of zirconium oxychloride of 1/5, 1/10, 1/50, and 1/100 of the LD50 for intraperitoneal injection and continuous injection of 1/20, 1/40, and 1/80 of the LD50 every other day for two or four weeks in mice, were carried out. In the case of a single injection zirconium oxychloride was intraperitoneally injected on days −1, 0, +1, +2, and +3 in relation to SRBC immunisation. The following conclusions may be drawn from this study: (1) Zr was shown to have an adjuvant like activity in relation to the humoral immune response, at least to IgM antibody production; (2) this effect was recognised not only with a single injection with Zr but also after continuous injection; (3) a single injection of Zr was more effective when the mice were treated with Zr 24 hours before or after SRBC immunisation; and (4) with regard to an injected dose of Zr, it was shown that a lower dose (1/50, 1/100 of the LD50 for a single injection and 1/40, 1/80 of the LD50 for continuous injection) led to a more enhanced level of IgM-PFC than a higher dose (1/5, 1/10 of the LD50 for a single injection, and 1/20 of the LD50 for continuous injection).

Zirconium (Zr) is widely present as a trace element in mammalian tissue but its physiological function is not clear. The metal is used primarily in the atomic energy industry because of its low cross section for neutron capture, its good strength to weight ratio, and its high degree of heat and corrosion resistance. Elsewhere it has been applied in the electronic and ceramic industries and in the manufacture of medical instruments. In Japan its demand in the form of the oxide has steadily increased in the ceramic industry in recent years. On the other hand, it is still not clear whether exposure to zirconium dusts in workers is harmful or not. Several studies have been carried out in man or in experimental animals,1−4 but there is much left to learn concerning the details of the potential toxicity of Zr and it is necessary to obtain clearer and more extensive knowledge on the biological activity, potential toxicity, and immunotoxicity of Zr.

To clarify the effect of Zr on the immune system, IgM antibody production in mice intraperitoneally injected with zirconium oxychloride was studied.

Materials and methods

Experimental Animals

C57 BL/10SnSlc male mice (body weight: 18–20 g, 6–10 weeks old) obtained from Chubu Science KK in Aichi, Japan, were used in this study. A total of 205 animals was divided into several experimental subgroups.

Antigen and Complement

Sheep red blood cells (SRBC) were used as the antigen. Packed SRBC (Nippon Bio-Test Laboratories, Inc) were washed in saline three times and adjusted to a concentration of 10% suspension in Eagle's MEM medium (Nissui Seiyaku Co Ltd, Tokyo). Fresh serum collected from guinea pigs was used as complement. The serum was absorbed with 10% SRBC at 0°C for 20 minutes before use.

Zirconium Solution for Injection in Mice

Zirconium oxychloride (ZrOCl2 8H2O) was used for injection; it was dissolved in saline and sterilised by millipore filter (0.22 μ).

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PREPARATION OF MOUSE SPLEEN CELLS
Cells were prepared by the method of Yahara and Edelman as follows. The spleen was teased in saline, pH 7.2, and filtered through a stainless wire mesh. The filtrate was centrifuged at 800 rpm for 10 minutes. Pellets were washed twice with saline and exposed to distilled water to eliminate the erythrocytes. Cell suspensions were adjusted to a concentration of 1 × 10⁶ cells/ml in Eagle's MEM medium.

MEASUREMENT OF IgM-PFC IN MOUSE SPLEEN AGAINST SRBC
IgM antibody production against SRBC was assayed by the method of haemolytic plaque formation as follows. Splenic cells were collected from mice immunised with 10% SRBC four days earlier and cell suspensions were prepared as mentioned above. Then 100 µl of a mixture containing 0-4 ml of a suspension of 1 × 10⁶ cells/ml spleen cells, 0-05 ml of 50% SRBC, and 0-05 ml of guinea pig serum was applied with a pipette to the side of the chamber for incubation. The chamber was sealed with heated paraffin. After incubation at 37°C for 60 minutes the number of plaque forming cells in the chamber was counted.

IgM ANTIBODY PRODUCTION AGAINST SRBC IN MICE INTRAPERITONEALLY INJECTED ONCE WITH ZIRCONIUM OXYCHLORIDE
A total of 125 mice was used in this experiment. The mice were divided into five groups of 25 mice each and each group was subdivided into four Zr injected groups and one control group of five mice each. The four Zr groups were intraperitoneally injected once with zirconium oxychloride in a dose of 34 mg/kg, 17 mg/kg, 3.4 mg/kg, and 1.7 mg/kg body weight (about 1/5, 1/10, 1/50, and 1/100 of the LD₅₀ for intraperitoneal injection) on days, −1, 0, +1, +2, and +3 in relation to SRBC injection, respectively. The controls were injected with saline at the same pH as the Zr solution for the same periods. Spleen cells were collected from the four Zr groups and one control group four days after SRBC injection. IgM antibody production was determined by measuring IgM-PFC in 1 × 10⁶ spleen cells as above.

IgM ANTIBODY PRODUCTION IN MICE INTRAPERITONEALLY INJECTED WITH ZIRCONIUM OXYCHLORIDE EVERY OTHER DAY FOR TWO AND FOUR WEEKS
Eighty mice were divided into two groups of 40 and each group was subdivided into three Zr injected groups and one control group of 10 mice each. The three Zr groups were intraperitoneally injected with zirconium oxychloride (8.5 mg/kg, 4.25 mg/kg, 2.125 mg/kg body weight, about 1/20, 1/40, and 1/80 of the LD₅₀ for intraperitoneal injection) every other day for two and four weeks, respectively. The controls were injected with saline of the same pH as the Zr solution for the same periods. IgM antibody production in the spleen of mice in each group was determined as above.

Results

IgM antibody forming cells in the spleen of mice against SRBC were determined by measuring the corresponding PFC in 1 × 10⁶ spleen cells. The rate of IgM antibody production of mice injected with Zr one day before SRBC immunisation is shown in fig 1 with individual PFC of each group. The mean PFC in each group showed an increase compared with that of the control. Of the Zr groups, the three treated with 1/5 (p < 0.05), 1/50 (p < 0.01), and 1/100 (p < 0.05) of the LD₅₀ were statistically significant. The incidence of mice in each Zr subgroup showing an increase of more than mean plus 2 standard deviations (M + 2 SD) PFC of the controls was 40% (2/5) for treatment with 1/5 of the LD₅₀, 20% (1/5) for 1/10, 100% (5/5) for 1/50, and 80% (4/5) for 1/100 of the LD₅₀.

The mean PFC in each group injected with Zr

![Graph](http://oem.bmj.com/)

**Fig 1** IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride one day before SRBC immunisation. Results are shown with IgM-PFC in 1 × 10⁶ cells/ml of spleen cells. A: control group, B: group treated with 1/5 of LD₅₀ for intraperitoneal injection, C: group treated with 1/10 of LD₅₀, D: group treated with 1/50 of LD₅₀, E: group treated with 1/100 of LD₅₀.
Table 1  IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride every other day for two weeks. Results are given as number of IgM-PFC and mean ± SD

<table>
<thead>
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<th>Zr dose (LD50) injected</th>
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<th>1/80</th>
<th>1/40</th>
<th>1/20</th>
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<tr>
<td>Zr-M</td>
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<td>3479</td>
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<td>IgM-PFC</td>
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<td></td>
<td>712</td>
<td>972</td>
<td>3264</td>
<td>2630</td>
</tr>
</tbody>
</table>

M 1190 2561* 1761† 1545†
SD 435 761 420 381

*Significant at p < 0.05.
†Not significant.

Table 2  IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride every other day for four weeks. Results are given as number of IgM-PFC and mean ± SD of each group in 1 × 10^6 cells/ml of spleen cells

<table>
<thead>
<tr>
<th>Zr dose (LD50) injected</th>
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<th>1/80</th>
<th>1/40</th>
<th>1/20</th>
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<td>2067</td>
<td>2627</td>
<td>3338</td>
<td>3714</td>
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</tbody>
</table>

M 2622 4275* 4907† 2731†
SD 336 646 943 427

*Significant at p < 0.01.
†Not significant.

IgM antibody production in mice intraperitoneally injected with zirconium oxychloride simultaneously with SRBC was also higher than that of the control. Overall, the results from the two groups treated with 1/50 and 1/100 of the LD50 were statistically significant (p < 0.01) from the controls. The incidence of mice showing an enhanced PFC formation of more than M + 2 SD of the control was 100–80% for treatment with 1/50–1/100 of the LD50.

The results for each subgroup injected with Zr one day after SRBC injection showed a similar increase to those of the subgroup injected with Zr simultaneously with SRBC. These two groups showed a remarkably enhanced response compared with the controls (p < 0.01).

In the groups treated with Zr two or three days after SRBC injection, the mean PFC of the two subgroups injected with 1/50 and 1/100 of the LD50 was remarkably higher than that of the controls, and the former was statistically significant (p < 0.05). On the other hand, mice in the subgroups injected with 1/5 and 1/10 of the LD50 often showed a decrease by comparison with the controls.

The results of the groups injected with Zr every other day for two and four weeks are shown in tables 1 and 2 with individual PFC and mean ± standard deviation (M ± SD) of each group. The mean PFC in each subgroup injected with Zr every other day for two weeks showed an increase over that of the control mice. This tendency was apt to be increased as the injected dose decreased (1/80 > 1/40 > 1/20 of the LD50), but only the group injected with 1/80 of the LD50 was statistically significant (p < 0.05) from the controls. The incidence of mice in each Zr subgroup showing an increase of more than M + 2 SD PFC of the control was 70% (7/10) for injection with 1/20 of the LD50, 20% (2/10) for 1/40, and 10% (1/10) for 1/20 of the LD50.

With respect to IgM-PFC in each group injected with Zr every other day for four weeks, the two groups injected with 1/40 and 1/80 of the LD50 showed a significantly enhanced response (p < 0.01). The group injected with 1/20 of the LD50, however, showed only a slight decrease that was not statistically significant from the controls. The incidence of mice showing an enhanced PFC response of more than M + 2 SD of the control was 100% for injection with 1/80–1/40 of the LD50.

Discussion and conclusion

The effects on human health of exposure to metals and metal compounds are mainly concentrated on the respiratory system. It has been indicated in many excellent studies in man and in experimental animals that the onset of lung diseases due to exposure to metal dusts may be closely related to effects on the immune system. Moreover, it has been reported that man is especially sensitive to beryllium, platinum, and nickel. On the other hand, studies
on the effects on the humoral immune response, such as the production of IgM antibody are few. In the present study the direct effect of Zr on IgM antibody production against SRBC was evaluated by studying the changes of IgM-PFC in spleen cells of mice intraperitoneally injected with zirconium oxychloride. The evaluation of IgM antibody production against SRBC in mice was carried out by a comparison with controls because the determination of PFC in the

*Fig 2* IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride simultaneously with SRBC. Results are shown with IgM-PFC in $1 \times 10^6$ cells/ml of spleen cells. (Groups A–E, see fig 1.)

*Fig 3* IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride one day after SRBC immunisation. (Groups A–E, see fig 1.)

*Fig 4* IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride two days after SRBC immunisation. (Groups A–E, see fig 1.)

*Fig 5* IgM antibody production against SRBC in mice intraperitoneally injected with zirconium oxychloride three days after SRBC immunisation. (Groups A–E, see fig 1.)
IgM antibody production in mice intraperitoneally injected with zirconium oxychloride

spleen of mice may be influenced by aging changes in the mice, certain conditions in PFC measurement and so on.

As shown in figs 1–5, the IgM response of mice injected once with Zr before or after SRBC immunisation was relatively enhanced in the case of treatment with low doses such as 1/50–1/100 of the LD50 for intraperitoneal injection. In the case of treatment with high doses such as 1/5–1/10 of the LD50 only those mice treated with 1/5 of the LD50 one day before SRBC injection showed a statistically enhanced response, the others showing a response similar to that of the controls. Concerning the incidence of mice with an increased IgM-PFC formation of more than M + 2 SD of the controls, mice injected with 1/50–1/100 of the LD50 showed a 60–100% response, and mice injected with 1/5–1/10 of the LD50 showed a 20–40% response. The results of the single injection experiment suggest that Zr may allow the development of an enhanced humoral immune response, at least the production of IgM antibody, and that there may be an optimal dose for enhancing the IgM immune response. It may also be more effective when Zr is injected 24 hours before or after SRBC immunisation.

An enhanced response of IgM antibody production against SRBC was also observed in mice injected with Zr every other day for two or four weeks. In general, it is thought that biotoxic effects on mice due to continuous injection with Zr for two to four week periods differ from those due to a single injection with Zr, and that the former is more effective than the latter. Despite continuous injections for two or four weeks, however, an enhanced response was recognised when mice were injected with low doses such as 1/40–1/80 of the LD50. These data suggest that Zr may have an adjuvant like activity in relation to humoral immune response. On the other hand, suppressed responses were often recognised in the case of mice injected with 1/20 of the LD50 for four weeks. This may be due to some effects induced by continuous injection with doses larger than the amount which may be excreted by the mice.

On the basis of these findings, the response of IgM antibody production due to Zr exposure is thought to depend on the physiological balance between the injected dose of Zr and the ability to excrete the metal. Moreover, it is suggested that long term exposure to low levels of Zr dust in the workplace of Zr industries may enhance the humoral immune response, or at least the IgM immune response, and may induce a state of hypersensitivity in exposed workers. Therefore, studies in man or in experimental animals exposed to Zr over a longer period should be carried out. It is of particular interest that Zr may act as an adjuvant like activity in inducing a prolonged antibody production, although there is much left to be clarified concerning the details of its activity.

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