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Effect of cadmium on immune responses and enzyme activities in *BALB/c* mouse

2. Humoral immune responses

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카드뮴이 *BALB/c* 마우스의 면역반응 및 효소활성에 미치는 영향

2. 체액성 면역반응

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초 록 : 카드뮴이 마우스의 체액성 면역능에 미치는 영향을 평가하고자 *BALB/c* 마우스를 대상으로 0, 25, 50, 100 및 200ppm의 카드뮴이 첨가된 음료를 6~7주간 자유급식시킨 후 면양적혈구로 면역시켜 마우스의 항체생산능에 대한 카드뮴의 영향을 평가하였다.

1. 1차 면역동물 비장세포중 용혈반형성 세포수는 대조군에 비하여 모든 실험군에서 유의하게 감소하였다($p < 0.01$). 그러나 2차 면역동물 비장세포중 용혈반형성 세포수는 대조군에 비하여 실험군에서 다소 증가하였다.

2. 2차 면역반응에서의 SRBC에 대한 총항체가는 대조군에 비하여 실험군에서 증가하는 경향을 보였으며, IgG 항체반응은 50ppm 투여군을 제외하고 모든 실험군에서 조금씩 증가하였다.

3. 혈청내 IgG_1 및 IgE 농도는 대조군에 비하여 모든 실험군에서 증가하였는데 특히 IgG_1의 증가가 지명하였다.

이상의 결과를 전보와 연계시켜 보면 카드뮴이 농도에 따라서는 생체내의 세포성 면역에서 중요한 역할을 하는 대식세포와 T세포 아군의 기능 또는 분포도 등을 변화시킴으로써 항체생산능에 영향을 미칠 수 있음을 시사한다.

Key words : cadmium, humoral immune respones, *BALB/c* mouse.

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Introduction

Cadmium(Cd) has been widely used in industry, as an anticorrosive agent for steel iron, copper, brass, and other alloys, and as a stabilizer for paint, pigments, batteries, and plastics[1,2]. Cd is a potential hazard in industrialized and urbanized societies[3,4]. The major hazards of Cd would be resulted from exposure to dust or fumes containing high concentrations of the metal, or from Cd-contaminated food[1].

There is also accumulating evidence for immune alteration in animals exposed to Cd. Cd exposure has been reported variously to stimulate[5~7], to suppress[8~12], and to have no effect on the immune responses[13]. These diverse effects have been related to the dose, route, time of administration with respect to antigen stimulation, length of exposure to Cd and strains of Cd intoxicated animals[13,14]. Meanwhile, numerous investigators have reported that under certain circumstances Cd expourse suppresses primary humoral immunity[8,11,21], but whether or not the secondary humoral immune response is equivocal[15,9,15]. And also, the proliferative responses of splenocytes to various mitogens were decreased [15,16] or increased[7,17~19] by various heavy metals. As described above, there are a lot of controversy about the effects of Cd on humoral immune responses.

In this manuscript, therefore, the effects of Cd on the humoral immune responses such as plaque forming cell(PFC) responses in primary or secondary immune response, hemagglutination titers, and concentration of IgG_1 and IgE in antiserum against sheep red blood cells(SRBC) were investigated.

Materials and Methods

Animals : Male *BALB*/c mice, 6 to 8 weeks of age and weighing 17 to 25g, were obtained from the Korea research institute of chemicla technology(Taejeon). Animals were housed five to six per cage, maintained at ambient temperatures of 20 to 23℃, and fed commercial rodent chow pellet(Samyang Co.) *ad libitum*.

Cadmium treatment : Cadium chloride($CdCl_2$) was provided by Sigma(C-3141) Mice received distilled water alone or water supplemented with 25, 50, 100 or 200ppm of $CdCl_2$ *ad libitum* for 6 to 7 weeks.

Immunization with SRBC : SRBC was obtained by venipuncture and was stored in Alsever's solution at 4℃. The SRBC was then washed 3 times with phosphate buffered saline(PBS, pH 7.2) just before use.

Experimental design : Mice were treated p.o. with varying concentrations of $CdCl_2$(0, 25, 50, 100 or 200ppm) for 6~7 weeks and divided into two groups by immunization schedule. One group of mice was singly immunized with 0.2ml of 2% SRBC in PBS i.p. on day 38 of Cd(primary immunization group). And the other group of mice was immunized with 0.2ml of 2% SRBC on day 29 of Cd and boostered on day 42(secondary immunization group). Blood were drained by puncture of orbital venous plexus and spleens were collected from mice of pirmary immunized group on day 4 of immunization, and from mice of secondary immunized grooup on day 7 of SRBC-booster.

Spleen cell suspension : Spleens were removed and single cell suspensions were prepared after lysing RBC with distilled water. The culture medium(RPMI-1640) was supplemented with penicillin(100IU/ml), streptomycin(100μg/ml), 2mM L-glutamine, 25mM Hepes buffer, and 5% fetal calf serum(FCS, Gibco). Viability was estimated by trypan blue dye exclusion test.

Analysis of plaque forming cells(PFC) :

IgM antibody response : The number of PFC was measured according to a modification of cunningham and Szenberg[20]. Briefly, three pieces of double-sided tape, 1.25cm wide, were laid across a clean microscope slide(75×25mm), dividing it into two equal areas. Two clean coverslips(25mm square) were placed on the tape such that two edges of each coverslip were firmly attached to the tape forming two shallow chambers. The single cell suspensions of spleen were prepared at 1.5×10^6 cells/ml as described above. To 40μl of the cell suspension, 40μl of fresh 1 : 3 diluted guinea pig serum and 40μl of 25% SRBC were added. The 40μl of this mixture was delivered into the each chamber, The chambers sealed with paraffin were incubated at 37℃ for 1 hr. The plaques were counted with light microscope. The number of

PFC per 2×10^4cells/chamber was calculated.

IgG antibody response : The same procedure was used for assay of the secondary response except that 25% SRBC suspension was reacted with goat anti-mouse IgG(H+L, Jackson Immunoresearch) diluted 1/500 for 30 min prior to assay.

Titration of antibodies to SRBC[13] : Seven days after secondary immunization, blood was obtained by puncture of orbital venous plexus of mice, serum was separated and complement was inactivated in 56℃ for 30 min.

Total hemagglutination(HA) antibody response : Twofold dilutions of the serum were prepared with PBS, pH 7.2, as diluent using multimicropipette. An equal volume(25μl) of a 2% SRBC suspension in PBS was added, and the tray was left at 37℃ for 2 hrs. The agglutination pattern was recorded, ang the titer was defined as the reciprocal of the highest dilution of the serum giving agglutination.

2-mercaptoethanol(2-ME) resistant HA antibody response : After titration of total HA antibody as above, 25μl of 0.15M 2-ME was added, shaked well and reincubated at 37℃ for 2 hrs. The agglutination titer was defined.

ELISA for IgG_1 and IgE[28] : To detect indirectly the concentrations of IgG_1 and IgE in the IgG-rich antiserum, the sandwithc ELISA was used. Briefly, primary antibodies (rabbit anti-mouse IgG_1, sheep anti-mouse IgE, Serotec) diluted appropriately in carbonate bicarbonate buffer(pH 9.6) was added into each well of 96 well EIA/RIA plate(Costar) and incubated overnight at 4℃. Then, the plate was washed three times with PBS-tween 20 and dried. And the pooled sera of each group diluted appropriately(1/16) with PBS-tween 20 were added in each well and incubated for 2 hrs at 37℃. Then, the plate was washed and dried. Peroxidase conjugated secondary antibodies(sheep anti-mouse IgG_1-peroxidase), rat anti-mouse IgE peroxidase, Serotec.) were added in each well and incubated for 1 hr at 37℃. Then, orthophenylenediamine containing substrate indicator solution was added and reacted for 10 min at room temperature. Finally this reaction was stopped by adding 4N H_2SO_4 and absorbance of each well at 490nm was measured.

Statistical analysis : The statistical significance of data between unexposed control group and Cd-exposed group was estimated by Student's paired t-test.

Results

Splenic direct IgM and IgG antibody response : The direct IgM and IgG antibody responses of the splenocytes against SRBCs were investigated. For direct IgM antibody response, mice were immunized with 0.2ml of 2% SRBC i. p. on day 38 of Cd exposure. On the other hand, in secondary immunization group for IgG antibody response, the first and second immunization were performed on day 29 and 42 of Cd, respectively. Four days after primary of 7 days after secondary immunization, the PFC assay was carried out. Due to the change in number of splenocytes induced by Cd exposure, results were expressed both as PFC/2×10^4 cells and PFC/spleen.

As shown in Table 1 and Table 2, direct IgM antibody responses were significantly decreased in all Cd-fed groups as compared with control, but in total splenocytes, IgG responses of Cd groups were slightly increased although PFCs in definite number of splenocytes were similar to that of control.

HA titers : Total HA titers and 2-ME resistant IgG titers were investigated at 7 days after the second immunization. As shown in Table 3, total HA titers were increased in all Cd-fed groups, especially in 100ppm Cd group. IgG titers were increased slightly except for 50ppm Cd group.

Concentrations of IgG_1 and IgE : The concentrations of IgG_1 and IgE in IgG-rich antiserum were detected indirectly by using sandwitch ELISA. As shown in Table 4, concentrations of IgG_1 were increased in all Cd-fed groups, especially 50, 100 and 200ppm groups. The IgE concentration were also increased in all cd-fed groups, especially 25, 50 and 100ppm Cd groups.

Discussion

At present, cadmium(Cd), a by-product of zinc and lead mining and smelting, is very important metal with many applications. It is used mainly in electroplating or galvanizing because of its noncorrosive properties[1,2]. It is also used as a

able 1. Effect of $CdCl_2$ on direct IgM PFC response

Groups	PFC/2×10^4 cells	PFC×10^3/spleen
0	37.67±8.04	452.73±96.69
25	11.57±3.51**	160.39±48.58**
50	12.50±4.04**	206.00±66.54**
100	9.00±3.21**	127.44±45.43**
200	9.33±4.93**	138.32±73.02**

Mice were administered with 0, 25, 50, 100 or 200ppm of Cd in drinking water. The mice were immunized with 0.2ml of 2% SRBC on day 38 of Cd administration.
PCf was assayed 4 days after primary immunization.
Each value represents the mean±SD.
Asterisks indicate values significantly different from control(df=p <0.01).

Table 2. Effect of $CdCl_2$ on IgG PFC response

Groups	PFC/2×10^4 cells	PFC×10^3/spleen
0	15.44±4.45	187.57±53.99
25	15.68±5.98	219.79±82.96
50	11.44±5.52	188.60±86.07
100	18.75±8.45	265.49±119.61
200	13.67±4.38	202.53±64.85

The mice were primed with 0.2ml of 2% SRBC on day 29 of Cd and boostered on day 42 of Cd.
PFC was assayed 7 days after the second immunization. Each value represents the mean±SD.

Table 3. Effect of $CdCl_2$ on the antibody production of mice boostered with SRBC

Groups	PFC/2×10^4 cells	PFC×10^3/spleen
0	8.35±0.14	5.60±0.22
25	8.38±0.14	5.63±0.63
50	8.46±0.10	5.50±0.27
100	8.80±0.27**	5.90±0.65
200	8.67±0.41	5.83±0.58

The mice were primed with 0.2ml of 2% SRBC on day 29 of Cd and boostered on day 42 of Cd. Antibody titers were assayed 7 days after the second immunization.
Each value represents the mean±SD.
Asterisks indicate values significantly different from control(df=4, **p<0.01).

Table 4. Effect of $CdCl_2$ on IgG_1 and IgE production in mice

Groups	Immunoglobulins	
	IgG_1	IgE
25	105.5[a]	105.7
50	118.7	107.3
100	117.4	108.7
200	107.2	100.4

The mice were primed with 0.2ml of 2% SRBC on day 29 of Cd and boostered on day 42 of Cd. Seven days after the second immunization, antiserum against SRBC(IgG-rich antiserum) was harvested.
[a]:Each value indicates the mean percentage of control.

color pigment for paints and plastics, and cathod material for nickel-cadmium batteries. Cd has been recognized as one of the most toxic pollutants due to its ability to induce severe alterations in various organs and tissues following either acute or chronic exposure[1,2,4].

In a recent year, the effects of Cd on immune responses have also been widely investigated. However, there are a lot of controversy about the effect of Cd on immune responses including immunostimulation[5-7], immunosuppression[8-12] and no effect cd on the humoral immune responses of BALB/c mice, when adult male *BALB*/c mice(6~8 weeks) received distilled water only or water containing 25, 50, 100 or 200ppm $CdCl_2$ for 6~7 weeks. Under the present conditions of exposure and dose, total PFCs in the primary immune response were significantly decreased in all Cd administered groups(Table 1). However PFCs in the secondary immune response were not suppressed but even slightly enhanced(Table 2). This assertion in supported by data showing increase of CD_4^+ cells[29] which has considerably more responsible for IgG response[9]. Previous study reported that oral exposure of Cd to mice for several weeks enhanced proliferative responses of lymphocyte to various mitogens[19] or caused polyclonal activation of B cell thereby producing antinuclear antibodies[22,25]. And also, it is possible that lymphoid cells responding to Con A and PHA as well as those participating in the antibody response to SRBC are more resistant than other lymphocyte populations to the toxic effects of Cd accumulation. Thus, their proportion would be increased in the spleen of mice exposed to Cd[7].

On the other hand, total and 2-ME resistant HA titers of secondary immune responses were slightly increased in Cd-fed mice(Table 3). These results supported by evidences that in Cd-fed mice blood B cells increase[18,23], low level of Cd ($\sim 10^{-6}$M) exists which has mitogenic effect[14,23,25], the activity of leucine aminopeptidase is reduced thereby decreasing or prolonging the break down of antibody and/or

nucleic acid from cells killed or injured by the Cd increase which can enhance antibody synthesis by acting as adjuvants[26]. This present study showed that IgG titers of Cd-fed mice in the secondary immune response were slightly increased except for 50ppm Cd group and concentrations of IgG_1 and IgE in antiserum were significantly increase. It is well-known that glucocorticoids both *in vitro* and *in vivo* induce T cells to produce predominantly interleukin(IL)-4 and IL-4 is required in culture for production of IgG_1 and IgE[27,28]. Therefore, it is possible that in Cd-fed mice the level of glucocorticoids is increased[7,14,23] and it modifies the lymphocyte distribution of blood and spleen[23], especially T-helper cells into spleen[29]. Subsequently, they might promote IL-4 production enabling enhance IgG_1 and IgE from activated mouse B cells.

The results of this study and previous report suggest that Cd is able to modify the immune responses by altering the distribution of T cell subpopulations and function of macrophage which play a major role in the celluar immune responses, and the humoral immune responses may be related with these alterations.

Summary

This study was designed to investigated the effects of cadmium(Cd) feeding on the humoral immune responses such as PFC-responses and production of immunoglobulins in *BALB*/c mice. The results obtained were summarized as follows;

1. Total PFCs of direct IgM antibody response were significantly decreased in all Cd-fed goups, whereas total PFCs of IgG antibody response were slightly increased.

2. In secondary immunization, total HA titers were increased in all Cd groups as compared with control, especially in 100ppmm group and also IgG titers were slightly increased except for 50ppm group.

3. The levels of IgG_1 were increased to 5.5% 18.7%, 17. 4% and 7.2% in 25, 50, 100 and 200ppm groups as compared with control, respectively. And also the levels of IgE were increased to 5.7%, 7.3%, 8.7% and 0.4% in those of Cd groups, in order. Conclusively, concentrations of IgG_1, and IgE were increased in all Cd groups.

Based on the results of this study and previous report, it was shown that Cd might affect humoral immune responses by modifying the distribution and function of cells playing in the cellular immune response.

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References

1. Frank EG, Jerome JP. *Introduction to environment toxicology*. Elsevier New York, 31~42, 1980.
2. Goyer RA. Toxic effects of metals. In : Klaassem CD, Amdur MO, Doull J(eds), *Casarett and Doull's Toxicology*. 3rd ed., Macmillian Publishing Co. New York, 592-596, 1986.
3. Yost KJ. Some aspects of cadmium flow in the U.S. *Environ Health Perspect*, 28: 5-16, 1979.
4. Kostic MM, Ognjanovic B, Dimitrijevic S, *et al.* Cadmium-induced changes of antioxidant and metabolic status in res blood cells of rat *in vivo* effects. *Eur J Haematol*, 51; 86~92, 1993.
5. Koller LD, Roan JG. Effects of lead and cadmium on mouse peritoneal macrophages. *J Reticuloendothel Soc*, 21: 7-12, 1977.
6. Fujimaki H, Murakami M, Kubota K. *In vitro* evaluation of cadmium induced augmentation of the antibody response. *Toxicol Appl Pharmacol*, 62: 288-293, 1982.
7. Malave I, De Ruffino D. Altered immune response during cadmium administration in mice. *Toxicol Appl Pharmacol*, 74: 46-56, 1984.
8. Graham J, Miller F, Daniels PE, *et al.* Influence of cadmium, nickel and chromium on primary immunity in mice. *Environ Res*, 16: 77-87, 1978.
9. Koller LD, Exon JH, Roan JG. Antibody suppression by cadmium. *Arch Environ Health*, 30: 598-601, 1975.
10. Lawrence DL. Heavy metal modulaton of lymphocyte activities. I. *In vitro* effects of heavy metals on primary humoral immune responses. *Toxicol Appl Phar-*

macol, 57: 439-451, 1981.

11 Koller LD. Immunosuppression produced by lead, cadmium and mercury. *Am J Vet Res,* 34: 1457-1458, 1973.

12. Stelzer KJ, Pazdernik TL. Cadmium-induced immunotoxicity. *Int J Immunopharmacol,* 5: 541-548, 1983.

13. Wesenberg GBR, Wesenberg F. Effect of cadmium on the immune response in rats. *Environ Res,* 31: 413-419, 1983.

14. Ohsawa M, Sato KM, Takahashi K, *et al.* Strain differences in cadmium-mediated suppression of lymphocyte proliferation in mice. *Toxicol Appl Pharmacol,* 84: 379-388, 1986.

15. Thomas PT, Ratajczak HV, Aranyi C, *et al.* Evaluation of host resistance and immune function in cadmium-exposed mice. *Toxicol Appl Pharmacol,* 80: 446-456, 1985.

16. Gaworski CL, Sharma RP. The effects of heavy metals on[^{3}H] thymidine uptake in lymphocyte. *Toxicol Appl Pharmacol,* 46: 305-313, 1978.

17. Koller LD, Roan JG, Kerkvliet NI. Mitogen stimulation of lymphocytes in CBA mice exposed to lead and cadmium. *Environ Res,* 19: 177-188, 1979.

18. Ohsawa M. Immunotoxicity of cadmium. In: Hashimoto K and Minami M, ed. *Seminars of toxicity mechanisms-1,* Center for Acadmice Publications Tokyo. 57-63, 1987.

19. Mullers G, Gillert KE, Karuse C, *et al.* Effects of cadmium on the immune system of mice. *Experientia,* 35: 909-910, 1978.

20. Cumningham AJ, Szenberg A. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology,* 14: 599-600, 1968.

21. Bozelka BE, Burkholder PM, Chang LW. Cadmium, a metalic inhibitor of antibody-mediated immunity in mice. *Environ Res,* 17: 390-400, 1978.

22. Ohsawa M, Takahashi K, Otsuka F. Induction of antinuclear antibodies in mice orally exposed to cadmium at low concentrations. *Clin Exp Immmunol,* 73: 98-102, 1988.

23. Ohsawa M, Sato K, Takahasi K, *et al.* Modified distribution of lymphocyte subpopulations in blood and spleen from mice exposed to cadmium. *Toxicol lett,* 19: 29-35, 1983.

24. Khansari DN, Murgo AJ, Faith RE. Effects of stress on the immune system. *Immunol Today,* 5: 170-175, 1990.

25. Shenker BJ. Matarazzo WJ, Hirsch RL, *et al.* Trace metal modification of immunocompetence, I. Effect of trace metals in the cultures on *in vitro* transformation of B lymphocytes. *Cell Immunol,* 34: 19-24, 1977.

26. Jones RH, Williams RL, Jones AM. Effects of heavy metal on the immune response. Preliminary findings for cadmium in rats. *Proc Soc Exp Biol Med,* 137: 1231-1236, 1971.

27. Thomson AW. The cytokine handbook. *Interleukin-4. Academic Press,* 119-148, 1991.

28. Ochel M, Vohr HW, Pferffer C, *et al.* IL-4 is required for the IgE and IgG_1 increase and IgG_1 autoantibody formation in mice treated with mercuric chloride. *J Immunol,* 146: 3006-3011, 1991.

29. Yoon CY, Kim TJ, Song HJ. Effect of cadmium on immune responses and enzyme activities of BALB/c mice 1. Cellular immune responses. *Korean J Vet Res,* 35: 543-552, 1995.