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Induction of anti-nuclear antibodies in mice orally exposed to cadmium at low concentrations

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SUMMARY

Anti-nuclear antibodies (ANA) in serum were detected in male ICR mice fed drinking water containing 3, 30 and 300 ppm Cd as $CdCl_2$ for 10 weeks. In response to Cd exposure, ICR mice developed ANA of the IgG class giving nuclear patterns moderately stained by immunofluorescence or immunoenzyme method. Positive immunofluorescence staining of ANA was obtained in 50, 89 and 90% of ICR mice exposed to 3, 30 and 300 ppm Cd, respectively. Their spleen cells also showed an enhancement of antibody forming response to sheep red blood cells (SRBC) without the SRBC priming. When mice were primed with SRBC after exposure to Cd, however, a significant suppression of the antibody forming response was observed in mice fed 300 ppm Cd but not in those fed 3 ppm Cd. No significant differences in delayed-type hypersensitivity reaction to SRBC were observed between Cd-fed and control animals. Inbred BALB/c mice were less susceptible to the induction of ANA by Cd, as induced only in 300 ppm Cd-fed mice. Thus environmental exposure to Cd can induce ANA in ICR mice with a high susceptibility, presumably accompanied with a non-specific stimulation of antibody formation.

Keywords anti-nuclear antibodies cadmium chloride oral exposure mice

INTRODUCTION

Anti-nuclear antibodies (ANA), autoantibodies to nuclear constituents of normal cells, can be found in the sera of humans and animals under a variety of autoimmune conditions. Their pathogenic role has been demonstrated in glomerulonephritis associated with systemic lupus erythrematosus (SLE) in human and in SLE model mice. In animals, a definite induction of ANA is obtained in a susceptible strain of mice or rats administered with heavy metal compounds such as mercuric chloride ($HgCl_2$) (Weening, Fleuren & Hoedemaeker, 1978; Weening *et al.*, 1980; Robinson, Abraham & Balazs, 1984) and gold thiomalate (Robinson, Egorov & Balazs, 1983). Anti-glomerular basement membrane (GBM) antibodies are induced with glomerulopathy in Brown Norway rats injected with $HgCl_2$ (Druet *et al.*, 1977; 1978; Sapin, Druet & Druet, 1977). ANA are also demonstrated both in the serum (Weening *et al.*, 1978) and glomerular acid eluate (Weening, Hoedemaeker & Bakker *et al.*, 1981) of PVG/c rats with $HgCl_2$-induced immune complex glomerulopathy. These autoantibodies are, therefore, considered to be associated with pathogenesis of $HgCl_2$-induced glomerulopathy.

Cadmium (Cd) as chloride salt causes glomerular amyloidosis in rabbits injected chronically (Castano, 1971). Moreover, like mercury, Cd induces immune complex nephritis in Sprague-Dawley rats after oral exposure at 100 or 200 ppm Cd (Joshi *et al.*, 1981). A recent study has reported that autoantibodies against two components of GBM, laminin and type IV procollagen, were induced in Sprague-Dawley rats injected with or orally exposed to Cd (Bernard *et al.*, 1984). It is not known, however, whether ANA can be induced by Cd, in particular by oral exposure to low concentrations of Cd. On the other hand, Cd is known to be immunosuppressive in mice orally exposed to this metal, as demonstrated by host resistance against infectious agents (Koller, 1973; Thomas *et al.*, 1985) or antibody forming responses (Koller, Exon & Roan, 1975) or delayed-type hypersensitivity (DTH) reaction (Müller *et al.*, 1979) to sheep red blood cells (SRBC).

The present study was undertaken, therefore, to investigate inducibility of ANA and the dose-effect relation on immunostimulative (ANA induction) and immunosuppressive effects in mice orally exposed to Cd. We report here our findings that oral exposure to Cd at low concentrations induces ANA in ICR mice, not always accompanied with a suppression of immune responses to SRBC.

MATERIALS AND METHODS

Animals

Male mice of outbred ICR and inbred BALB/c strains were obtained from Charles River Japan Inc. (Atsugi, Japan).

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Animals at 6 weeks of age were fed with deionized water containing 3, 30 or 300 ppm Cd as $CdCl_2$ for 10 weeks. Control mice were given deionized water. All mice were given Rodent laboratory chow, CE-2 (Clea Japan, Inc., Tokyo, Japan). BALB/c mice were used only for the ANA detection.

Immunological assays

The effects of oral exposure to Cd on immune functions were investigated in ICR mice as follows: (a) delayed-type hypersensitivity (DTH) reaction to SRBC, (b) antibody forming response of spleen cells to SRBC, and (c) induction of serum ANA. DTH reaction to SRBC was measured by the footpad swelling test (Lagrange, Mackaness & Miller, 1974). Mice were sensitized i.p. with 10^8 SRBC at week 10 of the Cd exposure and challenged 4 days later i.d. in the right and left hind footpads with 2×10^8 SRBC and the corresponding volume of saline, respectively. Twenty-four hours later foodpad swelling was measured with a dial thickness gauge G-5 (Ozaki Mfg Co. Ltd, Tokyo, Japan). Antibody forming response of spleen cells to SRBC was assayed by the plaque forming cell (PFC) assay according to Cunningham & Szenberg (1968). The number of direct PFC was counted immediately at week 10 or 4 days after the immunization with 10^8 SRBC i.p. at week 10. Numbers of PFC were expressed as those per 10^6 viable spleen cells or per spleen.

Blood samples for serum ANA detection were collected by heart puncture from both ICR and BALB/c mice at week 10 of the Cd exposure. ANA in heat-inactivated sera at 56°C for 30 min were detected by indirect immunofluorescence staining procedure (Friou, 1957) with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Cappel Lab. Inc., Pa, USA) or indirect immunoenzyme staining procedure (Nakane & Pierce, 1967) with horseradish peroxidase conjugated rabbit anti-mouse IgG (Med. Biol. Lab. Co., Ltd, Nagoya, Japan) using 3,3′-diaminobenzidine tetrahydrochloride as enzyme substrate. The nuclear antigen source substrates were mouse liver cell line preparations fixed with ethanol on ice. The liver cell line (NCTC clone 1469 derivative) was kindly supplied by Japanese Cancer Research Resources Bank. FITC or peroxidase conjugated anti-mouse IgG serum was used at a working concentration of 1:20. Nuclear fluorescence or enzyme staining was graded from 0 (negative) to 4+ in intensity, and the staining pattern was noted. Sera with ANA titre more than 40 were represented as positive in results, because negative sera from normal mice were spontaneously graded from 0 to 1+ at a serum dilution of 1:20. Although the immunoenzyme staining was less sensitive than the immunofluorescence staining, similar results were obtained by the two ANA detection methods.

Determination of tissue cadmium

Cadmium in tissues such as blood, spleen, liver and kidney was determined by flame or flameless atomic absorption spectrometry after wet-ashing of the tissues with nitric acid.

RESULTS

General response to the Cd exposure

Toxic response of ICR mice exposed to Cd was investigated on changes in body weight and weights of organs such as liver, kidney, thymus and spleen. No significant differences in body weight and the organ weights between Cd-fed and control mice were observed, except a slight increase of kidney weight in mice fed 300 ppm Cd. The mice fed 3 and 30 ppm Cd ate and drank as much as controls. The mice fed 300 ppm Cd were also well in diet intake, but drank significantly less water ($4{\cdot}6 \pm 0{\cdot}5$ ml/mouse/day, mean ± s.d., $P < 0{\cdot}001$) after Cd exposure for 3 weeks than did controls ($7{\cdot}5 \pm 1{\cdot}0$ ml/mouse/day). All groups of mice were in good health with no clinical signs. Urinary proteins in spot urine of Cd-fed mice were comparable to those in controls, when estimated with urine test paper.

Table 1. Tissue cadmium concentrations (μg/g tissue) in ICR mice exposed to cadmium in the drinking water for 10 weeks (mean (s.e. in parentheses) of 9 to 11 mice)

	Concentration of Cd in the drinking water (ppm)			
Tissue	0	3	30	300
Liver	0·16 (0·01)	1·16 (0·07)	6·51 (0·51)	60·69 (2·70)
Kidney	0·68 (0·07)	3·25 (0·14)	11·91 (0·51)	65·50 (3·15)
Spleen	0·04 (0·01)	0·08 (0·01)	0·50 (0·03)	1·57 (0·15)
Blood	0·07 (0·02)	0·07 (0·01)*	0·10 (0·02)*	0·15 (0·02)

* Not significantly different from control values, at $P < 0{\cdot}05$. All other values in Cd-exposed groups are significantly different ($P < 0{\cdot}05$) from controls.

The results on tissue Cd concentration are shown in Table 1. In mice fed 3 ppm Cd there was no significant increase in blood Cd content and a slight increase in the spleen Cd content compared with controls, in spite of a marked accumulation of Cd in liver and kidneys which were the main storage organs of the metal.

Immune alterations

The effects of oral exposure to Cd on DTH reaction and the direct PFC response of spleen cells to SRBC in ICR mice are shown in Table 2. The swelling of footpads challenged with saline after priming with SRBC was negligible and no different between control and Cd-fed mice. DTH reaction on foodpad swelling was similar among all groups of mice, and any significant alterations by Cd exposure were not observed. However, the primary PFC response to SRBC after the immunization was reduced in Cd-fed mice. The reduction in PFC per 10^6 spleen cells was statistically significant in mice fed 300 ppm Cd. A tendency toward suppression of the primary PFC response was also observed in mice fed 30 ppm Cd but not those fed 3 ppm Cd. By contrast, the direct PFC per 10^6 spleen cells in unprimed mice was significantly enhanced in all groups of Cd-fed mice, even in mice fed 3 ppm Cd with trace amounts of Cd in their spleens. Their PFC per spleen also showed similar alterations with or without the immunization, respectively, although not statistically significant in 30 and 300 ppm Cd-fed mice because of a variation in recovery of viable spleen cells. Thus these findings indicate that an alteration in humoral immune function is a sensitive effect of oral exposure to Cd and seems to be primarily stimulative.

ANA induction

The results in Table 3 show apparent induction of ANA in sera of ICR mice exposed to Cd. ANA were detected by immunofluorescence staining in 50, 89 and 90% of ICR mice exposed to

Table 2. Alterations in immunological function in ICR mice exposed to cadmium in the drinking water for 10 weeks

	Concentration of Cd in the drinking water (ppm)			
	0	3	30	300
DTH reaction to SRBC*				
($\times 10^{-2}$ mm)	184 (12)	162 (15)	192 (12)	170 (12)
PFC response to SRBC†				
After priming with SRBC				
(PFC/10^6 spleen cells)	1010 (43)	1011 (81)	850 (96)	768 (67)‖
(PFC $\times 10^{-5}$/spleen)	1·99 (0·25)	2·31 (0·27)	1·54 (0·24)	1·61 (0·19)
Without priming				
(PFC/10^6 spleen cells)	40 (12)	104 (19)‡	102 (32)	145 (44)‡
(PFC $\times 10^{-3}$/spleen)	1·30 (0·28)	2·43 (0·29)§	1·90 (0·29)	1·90 (0·20)

* DTH reaction was determined by footpad swelling test. Footpad swelling is shown as the difference between the thicknesses of the footpads challenged with SRBC and saline, respectively. Results represent the mean (standard error) of eight to 10 mice.

† PFC response of spleen cells to SRBC was determined 4 days after priming with SRBC or immediately without priming at week 10. Results represent the mean (standard error) of five to seven mice.

The statistical significance between unexposed groups and those exposed to Cd was estimated by Student's *t*-test. ‡ $P<0.05$; § $P<0.01$; ‖ $P<0.001$.

Table 3. Induction of anti-nuclear antibodies (ANA) in ICR and BALB/c mice exposed to cadmium in the drinking water (number of positive mice/number tested)

	Concentration of Cd in the drinking water (ppm)			
	0	3	30	300
ICR	1/12 (8%)	5/10 (50%)*	8/9 (89%)†	9/10 (90%)†
BALB/c	1/10 (10%)	1/9 (11%)	2/10 (20%)	9/10 (90%)†

* $P<0.05$.

† $P<0.001$.

P values were calculated by Fischer's exact method.

3, 30 and 300 ppm Cd, respectively. The incidence of positive ANA in Cd-fed mice was significantly higher than that in controls (8%). Inbred male BALB/c mice were less responsive to ANA induction after oral exposure to Cd, which was apparent in mice fed 300 ppm Cd.

Cd-induced ANA gave rise to a pale but distinguishable rim-like staining pattern or a distinctive granular or diffuse staining pattern of nuclei of liver cells by immunofluorescence or the immunoenzyme method (Fig. 1). The rim-like pattern was predominant in Cd-induced ANA findings. ANA staining was usually moderate (1^+ to 3^+) at 1:40 serum dilution. Immunoenzyme staining findings on ICR positive sera and positive control sera obtained from MRL/l mice correspond well with immunofluorescence findings. The maximum ANA titre obtained from control ICR mice was 40, whereas ANA titres of positive sera obtained from Cd-fed ICR and BALB/c mice ranged from 80 to more than 320. Most of positive sera from Cd-fed mice gave a titre more than 160.

DISCUSSION

There is much evidence to suggest that environmental factors such as viruses and chemicals induce autoantibody formation (Alarcon-Segovia, 1984; Bigazzi, 1985). In the present study we showed that the oral exposure to low concentrations of $CdCl_2$ induced the production of ANA in ICR mice without any clinical manifestation. Induction of ANA was also found in Cd-fed BALB/c mice. However, they were less susceptible to the induction of ANA by Cd exposure when compared with ICR mice. This may be associated with genetic factor(s) in BALB/c mice responsible for a low frequency of spontaneous polyclonal B cell activation with ageing (Papoian, Pillarisetty & Talal, 1977) and/or a low responsiveness of splenic lymphocytes to Cd toxicity (Ohsawa *et al.*, 1986).

Previous studies on mice exposed to Cd in the same way had indicated that Cd suppressed *in vivo* humoral immune responses to SRBC in Swiss mice (Koller *et al.*, 1975) or cell-mediated immune responses to SRBC in CPRM-1 mice (Müller *et al.*, 1979). However, we observed a suppression of the primary antibody forming response, but not DTH reaction, in ICR mice exposed to Cd at higher concentrations. These variations may be due partly to the difference of mouse strain used. On the other hand the increase in antibody forming response to SRBC of unprimed spleen cells and ANA induction were significant in Cd-fed ICR mice. These indicators are most sensitive in dose-response relationships for demonstrating the immunomodulating effects of Cd on ICR mice. Our previous study has also shown that oral exposure of ICR mice to Cd for 10 weeks did not suppress but even slightly enhanced proliferative responses of their lymphocytes to mitogens, Concanavalin A (Con A) and lipopolysaccharide (LPS), and to allogeneic lymphocytes (Ohsawa *et al.*, 1982; Ohsawa, 1987). Therefore Cd could primarily stimulate such processes of immune response, includ-

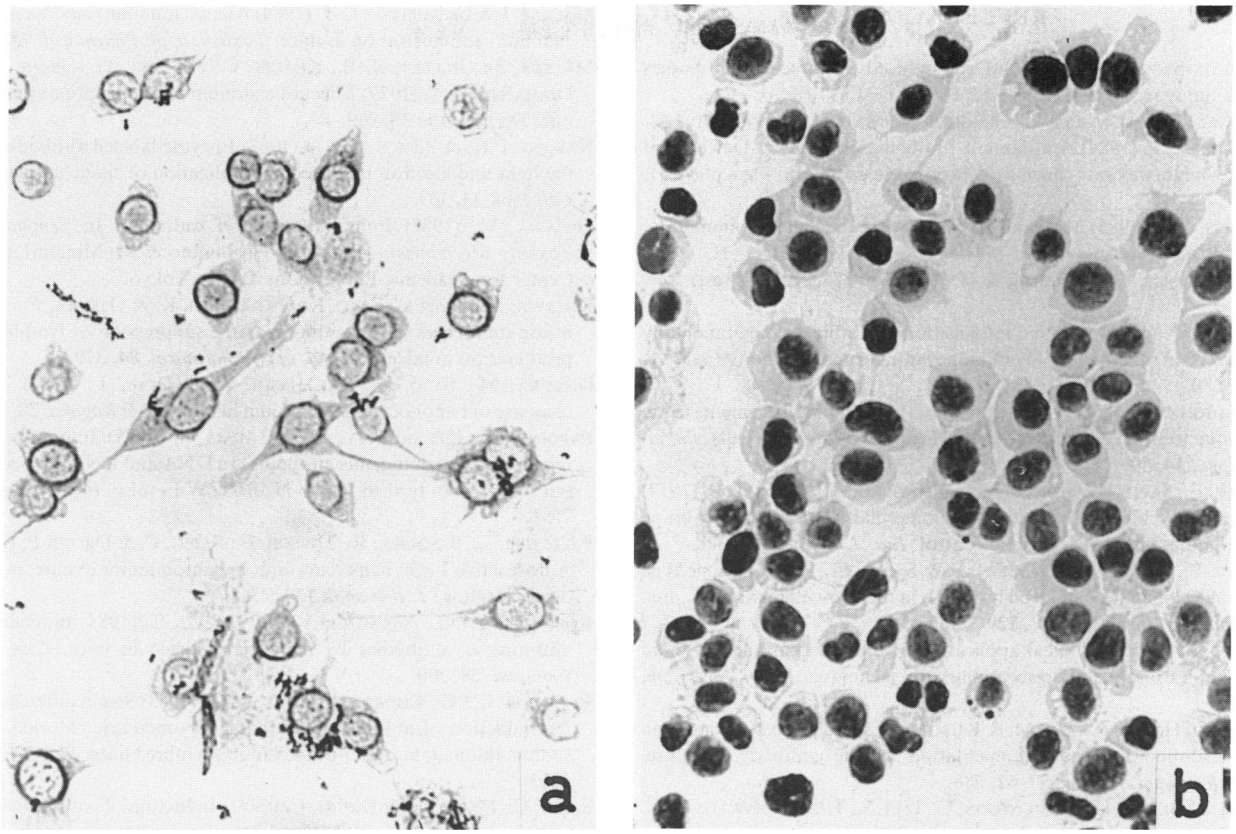

Fig. 1. Immunoenzyme nulcear staining with sera of Cd-fed ICR mice, showing (a) rim-like and (b) granular or diffuse patterns on mouse liver cell line preparation (× 200). Liver cell preparations were covered with mouse sera as the first and peroxidase-conjugated rabbit anti-mouse IgG as the second layer, followed by enzyme histochemical staining with hydrogen peroxide and 3,3′-diaminobenzidine.

ing induction of ANA. This suggestion is supported by direct evidence that *in vitro* treatment of Cd at priming with SRBC can stimulate anti-SRBC formation by cultured rat spleen cells (Fujimaki, Murakami & Kubota, 1982). The mechanism for the bidirectional effect of Cd on antibody formation dependent upon SRBC immunization in ICR mice is not yet known. Cadmium-induced immunosuppression might be more complicated.

Our preliminary findings indicate that ANA can be induced also in ICR mice which have received s.c. injections of $CdCl_2$. ANA can be induced irrespective of the route of Cd exposure, so this mouse model of $CdCl_2$-induced ANA is comparable to that of $HgCl_2$-induced ANA in ICR mice (Robinson *et al.*, 1984). It was shown by Weening, Hoedemaeker & Bakker (1981) that $HgCl_2$ decreased Con A activated suppressor function in the PVG/c rat. Hirsch *et al.* (1982) also showed that $HgCl_2$ enhanced both *in vivo* and *in vitro* PFC response to SRBC and TNP under the presence of T cells in the Brown Norway rat. They suggest that $HgCl_2$ acts as a polyclonal activator on spleen cells under the involvement of T cells. This has been supported by a more recent finding that $HgCl_2$ induces autoreactive T cells in the rat (Pelletier *et al.*, 1986), which presumably are responsible for the autoimmune response such as autoantibody induction. Cd stimulates mouse B lymphocytes *in vitro* (Shenker *et al.*, 1977). Our findings on the primary stimulation of immune response in Cd-fed mice also suggest that Cd causes polyclonal activation of B cells. On the other hand it has been shown that oral exposure of male C57BL/6 mice to 50 or 200 ppm Cd as $CdCl_2$ for 3 to 4 weeks resulted in a decrease of sodium periodate-activated suppressor function (Malaváe & De Ruffino, 1984). Therefore, like $HgCl_2$, the induction of ANA by $CdCl_2$ could be the result of either direct or indirect polyclonal activation of B cells. Besides Hg and Cd, gold sodium thiomalate can induce ANA production in mice (Robinson, Egorov & Balazs, 1983; Joseph *et al.*, 1986). Further studies are needed to investigate whether these heavy metals induce ANA production in part by a common mechanism.

In this study it is noted that ANA was induced by oral exposure to Cd at low concentrations. It is so far not clear whether Cd-induced ANA titre is enought to generate pathological effects such as immune-glomerulonephritis. The prolonged polyclonal activation of B cells by lipid A portion of LPS results in acceleration of the late life SLE of autoimmune model mice (Hang *et al.*, 1983). Therefore, ANA induction may be useful for predicting autoimmune manifestations in animals exposed to Cd, especially those with a genetic predisposition to autoimmune disease.

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