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Pretreatment of lymphocytes with mercury *in vitro* induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile

H. HU, M. ABEDI-VALUGERDI & G. MÖLLER *Department of Immunology, Arrhenius Laboratories for Natural Sciences, Stockholm University, Sweden*

SUMMARY

Mercury can induce autoimmune disease in susceptible mouse strains. We found that *in vitro* mercuric chloride induced a high proliferative response in spleen lymphocytes from mercury-susceptible SJL mice, but a low response in resistant mice, such as C57BL/6 (H-2^b), A/J (H-2^a) and CBA (H-2^k) mice. However, a high proliferative response was obtained with lymphocytes from all tested low-responder mice by pretreating them *in vitro* for 1–3 days with mercuric chloride and then wash away the excess mercury. Both $CD4^+$ and $CD8^+$ T cells were activated in the restored response, but $CD4^+$ T cells was the major responding cell population, as in high-responder mice. We also measured the cytokine production at the protein level after mercury stimulation *in vitro*. We found that in mercury stimulation the different culture conditions resulted in different patterns of cytokine production. The continuous presence of mercury induced interleukin-2 (IL-2) and interferon-γ, but not IL-4 production in spleen cells from both high- and low-responder mice. In contrast, by pretreating the cells with mercury and then washing, spleen cells from both high and low-responder mice produced IL-4. Our results suggest that spleen cells from both mercury-susceptible and -resistant mice have the potential to respond to mercury *in vitro* and produce both Th1- and Th2-type cytokines. But the mercury-induced cytokine profile can shift depending on the conditions for activation.

INTRODUCTION

Mercuric chloride can induce autoimmune diseases in susceptible rats and mice.[1,2] Both T and B cells are polyclonally activated,[3–6] and autoantibodies, such as antinuclear (ANA) and antinucleolar (ANolA) antibodies, are induced.[7–9] The susceptible animals finally develop autoimmune glomerulonephritis due to antiglomerular basement membrane (GBM) autoantibodies and to immune complex deposition.[6,9,10]

Mercury-induced autoimmunity is not an all-or-none phenomenon. Studies have shown that mercury injection induces an increased major histocompatibility complex (MHC) class II molecule expression in both susceptible and resistant animals.[8,11,12] Using the activation marker CD45RB^{lo}, it has been found that $CD4^+$ T cells of both susceptible and resistant mice are activated to the same extent *in vivo*.[13] This suggests that the lymphocytes from resistant animals have the potential to respond to mercury, but the response is regulated by unknown mechanism(s). Resistance to mercury *in vivo* has been suggested to be due to either a $CD8^+$ T cell-mediated non-antigen-specific immunosuppression[12,14–16] or a Th1-type response in resistant animals.[17–19] However, neither does the depletion of $CD8^+$ T cells in resistant animals result in autoimmunity[12] nor does an anti-interleukin-2 receptor (IL-2R) monoclonal antibody therapy change abnormalities caused by mercury.[17] Our previous studies have shown that *in vitro* mercury induces a high proliferative response in susceptible mice, in which $CD4^+$T cells are preferentially activated, while in resistant mice, the response was low and only $CD8^+$ T cells were transformed.[20] It is not known what mechanism(s) are responsible for the low-responsiveness in lymphocytes from resistant mice *in vitro*. In this study, we aimed to study further the regulation of mercury-induced response *in vitro*.

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Correspondence: Dr H. Hu, Department of Immunology, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden.

MATERIALS AND METHODS

Animals

BALB/c (H-2^d), C57BL/6 (H-2^b), DBA/2 (H-2^d) and A/J (H-2^a) were bred in our animal facilities at the Department of Immunology, Stockholm University. SJL (H-2^s) and CBA (H-2^k) mice were purchased from Harlan (Bicester, UK) and B & K Universal AB (Stockholm, Sweden), respectively. All mice were used at 6–8 weeks of age.

Stimuli and reagents

Stock solution of mercuric chloride ($HgCl_2$) (Merck, Darmstadt, Germany) was prepared in physiological saline at

a concentration of 1×10^{-3} M and filter sterilized (0·22 μM, Costar, Cambridge, MA). Anti-IL-2, anti-IL-4 antibodies (both capture and detection) were purchased from Pharmingen (San Diego, CA). Anti-interferon-γ (IFN-γ) antibodies (capture and detection) were made from hybridoma cell lines R4-6A2 (American Type Culture Collection; ATCC, Rockville, MD) and AN18 (ATCC), respectively.

Cell preparation and culture conditions

Spleen cells from different mouse strains were washed twice in Earle's balanced salt solution (BSS) and cultured at a concentration of 3×10^6 cells/ml in RPMI-1640 medium containing 10% Fetal calf serum (FCS), $NaHCO_3$ (0·075%), penicillin/streptomycin (50 IU/50 μg), HEPES (10 mM) and L-glutamine (2 mM) in flat-bottom 96-well plates in a final volume of 0·2 ml/well.

An appropriate concentration of mercuric chloride (10 μM) was added to the medium at the start of the cultures. In mercury-washing-away experiments, after different days' culture with mercuric chloride (10 μM), the cells were washed twice with BSS and the living cells were recovered by lympholyte-M gradient (Cedarlane Laboratories Ltd, Canada). The cells recovered on day 1 or day 2 were incubated till day 3 and the cells recovered on day 3 were incubated till day 4 (3×10^6/ml; 0·2 ml/well; 96-well plate). Cells were cultured at 37° in a 5% CO_2 incubator.

[^{3}H]Thymidine-incorporation assay

[^{3}H]Thymidine (Amersham International, Amersham, UK) was added (2 μCi/ml) to the cultures (3×10^6 cells/ml; 0·2 ml/well; 96-well plate) at different times. After 6 h the cells were harvested (Semi-automative cell harvester, SKATRON, Lier, Norway) and the incorporated radioactivity was analysed by liquid scintillation counting in a β-counter (1218 Rackbeta Liquid Scintillation Counter; LKB Wallac, Bromma, Sweden).

Immunofluorescence staining

Cells from each well of the cultures were washed twice and approximately 10^6 cells were suspended in 100 μl fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of rabbit anti-immunoglobulin antibody (Dakopatts, Sweden), phycoerythrin (PE)-conjugated rat anti-mouse CD4 antibody (Becton Dickinson, Mountain View, CA) and FITC-conjugated rat anti-mouse CD8 antibody (Becton Dickinson) appropriately diluted in BSS containing 0·2% sodium azide. The samples were incubated for 20 min on ice and washed twice before analysis. For the fluorescence-activated cell sorter (FACS) analysis, the gate was set to exclude the dead cells.

Cytokine detection

In ELISPOT assay, cells were added (3×10^6/ml; 0·1 ml/well) to duplicate or triplicate wells of nitrocellulose plates (Millipore AB, Stockholm, Sweden) coated with anti-IL-2 (10 μg/ml), anti-IL-4 (15 μg/ml) or anti-IFN-γ (15 μg/ml) antibodies. After different time-period incubation, plates were washed and corresponding biotinylated detection antibodies (1 μg/ml) were added and incubated for 2–4 h at room temperature, washed, and incubated with streptavidine-alkaline-phosphatase (-ALP) for 90 min. BCIP/NBT substrate solution (Bio-Rad, Richmond, CA) were added and incubated for 1–2 h until dark spots emerged. Spots were counted in a dissection microscope (×40).

Statistical analysis

Results were subjected to statistical analysis by using the Student's *t*-test.

RESULTS

Only a short period of contact between mercury and lymphocytes is sufficient to induce a proliferative response

It has been proposed that mercury ions bind to self proteins, modify them and lead to activation of autologous T cells which then induce polyclonal activation of B cells.[21] By autoradiography, it has been shown that mercury ions bind to the membranes of lymphocytes as early as 15 min after isotope addition, and a stronger binding occurs after 48 h.[22] This suggests that a short period of contact between mercury ions and lymphocytes would be sufficient for activation. Previously we have shown that *in vitro* mercuric chloride induces a strong proliferative immune response in BALB/c mice (Fig. 1a).[20] In this study we cultured the spleen lymphocytes from BALB/c mice with mercuric chloride for different periods of time and washed cells with BSS to remove the excess mercury. The recovered cells were then cultivated for one or two additional days in the absence of mercury. We found that incubation with mercuric chloride for 1 day was sufficient to induce a significant proliferative response of spleen lymphocytes measured on day 3 (Fig. 1b). Interestingly when mercury was washed away from 2 or 3 days' culture, a much greater proliferative response was induced compared with the response in which mercuric chloride was present during the entire culture period (Fig. 1a, b). This higher proliferative response was observed in another mercury-susceptible strain, SJL mice, which was also proved to be a high-responder to mercury stimulation *in vitro* (Fig. 1c, d).

Mercury-exposed lymphocytes from low-responder mice become high responders after removal of excess mercury by washing

Our previous studies have shown that mercury induces a low proliferative response of spleen lymphocytes from DBA/2 mice, which are known as a mercury-resistant mouse strain.[20] Nevertheless, in such cultures, there were $CD8^+$ cells transformed by mercury stimulation.[20] Findings from other laboratories also suggest that lymphocytes from mercury-resistant mice may have a potential to respond to mercury.[11–13] To study this issue further, we tested some other mercury-resistant mice such as C57BL/6, A/J and CBA mice and found that the proliferative responses were low (Fig. 2a–c), as in DBA/2 mice (Fig. 2d).[20] Since a much higher proliferative response was induced in high-responder mice after the removal of excess mercury from the medium, we performed analogous experiments with lymphocytes from low-responder mice. Spleen lymphocytes from C57BL/6, A/J, CBA and DBA/2 mice were cultured with mercuric chloride for 3 days and thereafter washed with BSS. The recovered cells were cultured for one additional day and thymidine incorporation was measured. As shown in Fig. 2(a) to (d), high proliferative responses were found in cells from all tested mice. The restored response was higher in DBA/2 and CBA mice than in C57BL/6 and A/J mice. In low-responder mice, incubation with mercury for 1 day was also sufficient to induce a significant proliferation of spleen lymphocytes (not shown).

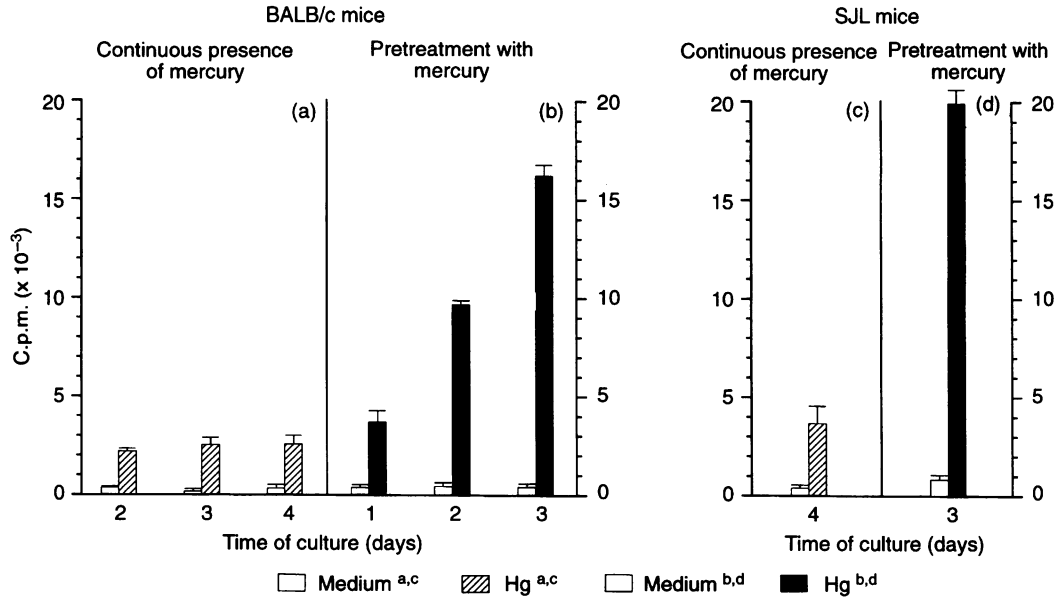

Figure 1. (a,c) Spleen cells (3×10^6/ml) from BALB/c or SJL mice were incubated in the continuous presence of mercuric chloride. [^{3}H]Thymidine was added (2 μCi/ml) on the days indicated and the cells were pulsed for 6 h. Each column represents the mean c.p.m. of triplicate cultures. (b,d) Spleen cells (3×10^6/ml) from BALB/c or SJL mice were preincubated with mercuric chloride. At different time-periods, the cells were washed twice with BSS and the living cells were recovered on a lympholyte-M gradient. The cells recovered on day 1 or day 2 were incubated till day 3 and the cells recovered on day 3 were incubated till day 4. On day 3 or day 4 [^{3}H]thymidine was added (2 μCi/ml) and the cells were pulsed for 6 h. Each column represents the mean c.p.m. of triplicate cultures. Results were representative of three separate experiments.

Phenotype of the responding cells in the restored response in low-responder mice

We have previously shown that stimulation by mercuric chloride activated both CD4$^+$ and CD8$^+$ T cells in high-responder mice, while in low-responder mice, only CD8$^+$ T cells responded.[20] We analysed the phenotype of the responding cells in the restored response in low-responder mice by FACS analysis. In DBA/2 mice, after washing away the mercuric chloride and culturing the recovered cells for one additional day, the number of transformed CD4$^+$ T cells increased substantially (Fig. 3a), as in SJL mice (Fig. 3b), while the number of transformed CD8$^+$ T cells increased only marginally, though it was statistically significant ($P<0{\cdot}05$). The number of transformed CD8$^+$ T cells from SJL mice increased more clearly (Fig. 3b).

Cytokine production of spleen cells exposed to mercury with and without washing

To investigate further the differences between removal and continuous presence of mercury in mercury-induced responses, we analysed the cytokine production in these two different situations. As shown in Fig. 4(a) and (b), the continuous presence of mercury induced IL-2 and IFN-γ producing cells on day 1 in spleen cells from both high- and low-responder mice. The number of IFN-γ-producing cells slightly increased till day 2 (Fig. 4a, b), whereas no IL-4-producing cells were induced (Fig. 4a, b). The profiles of cytokine-producing cells were the same on day 3 as on day 2 in both high- and low-responder mice (not shown). In contrast, after washing away the mercury on day 1 and continuing the incubation to day 3, we found an induction of IL-4-producing cells in spleen cells from both high- and low-responder mice (Fig. 5a, b). There was also an increase of IL-2 producing cells, which were likely to be the remaining IL-2-producing cells activated on day 1 (Fig. 5a, b). However, the number of IFN-γ-producing cells decreased below control level (Fig. 5a, b), suggesting an inhibitory effect on IFN-γ production after washing. Spleen cells from both high- and low-responder mice showed the same cytokine production profile.

DISCUSSION

In the primary immune response stimulated by mercuric chloride *in vitro*, we found that the activation of cells did not require the continuous presence of mercury ions in the medium. At the optimal mercury concentration for stimulation, although the continuous presence of mercury induced a cell proliferation in lymphocytes from high-responder mice, suppressive effects were seen in high- and particularly in low-responder mice (compared with washing-away experiments). Even in concanavalin A (ConA) stimulation, the mercury at such an optimal concentration actually suppressed the ConA-induced cell proliferation before the response reached a peak (not shown). It seems that the continuous presence of mercury has a general suppressive effect *in vitro*. After excess mercury was removed, we found that lymphocytes from genetically determined mercury low-responder strains became high responders. Since the cells in medium control also went through washing-out procedure, the proliferation of lymphocytes in mercury washing-away experiments was due to the exposure to mercury during the pretreatment.

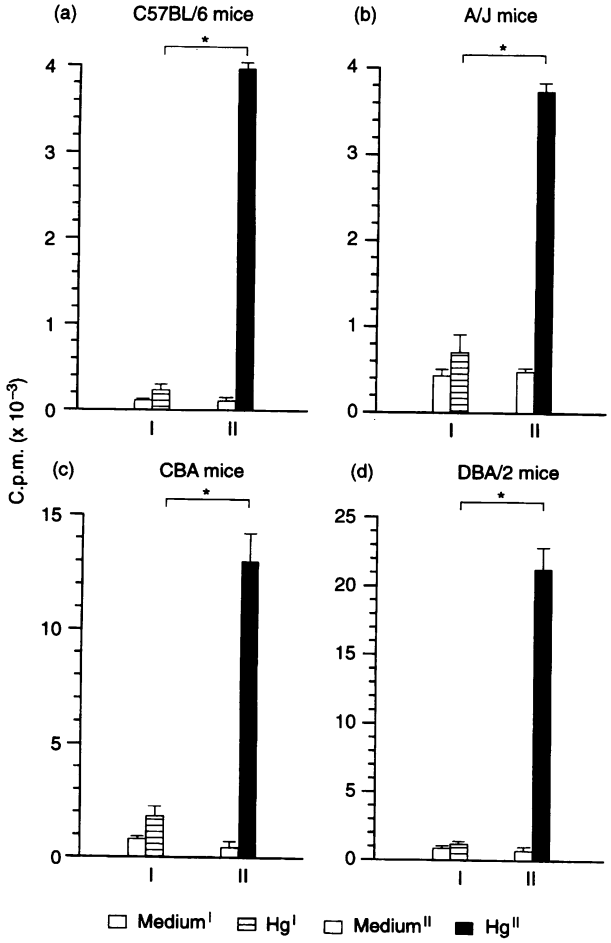

Figure 2. Treatment I: Spleen cells (3×10^6/ml) from C57BL/6, A/J, CBA and DBA/2 mice were incubated in the continuous presence of mercuric chloride. [^{3}H]Thymidine was added (2 μCi/ml) on day 4 and the cells were pulsed for 6 h. Each column represents the mean $\pm$ SD c.p.m. of triplicate cultures. Treatment II: Spleen cells (3×10^6/ml) from C57BL/6, A/J, CBA and DBA/2 mice were pre-incubated with mercuric chloride. After culture for 3 days, the cells were washed twice by BSS. The living cells were recovered by lympholyte-M gradient and incubated for one additional day. On day 4 [^{3}H]thymidine was added (2 μCi/ml) and the cells were pulsed for 6 hr. Each column represents the mean $\pm$ SD c.p.m. of triplicate cultures. In both treatments I and II, results were representative of three separate experiments. Significant differences of mercury stimulation between I and II were calculated by Student's *t*-test; $^*P < 0{\cdot}05$.

The degree of low-responsiveness to mercury varies among the low-responder mouse strains. *In vivo* studies on A/J mice have shown that these mice develop antinuclear autobodies after mercury treatment, but they do not have renal mesangial deposits.[9] CBA mice, having H-2A and H-2E regions identical with A/J mice, are resistant to induction of both antinuclear antibodies and the formation of immune complex deposits induced by mercury.[9] C57BL/6 and DBA/2 are resistant mouse strains with regard to nearly all *in vivo* parameters except contact dermatitis after exposure to mercury chloride.[19,23] Although the formation of immune complex deposits and autoimmune glomerulonephritis are referred to as *in vivo* susceptibility to the injection of mercuric chloride,[9,10] in terms of responding capacity, low-responder mice do produce antinuclear autoantibodies or develop contact dermatitis.[9,19,23] Our results of restored response are consistent with the *in vivo* responding capacities of low-responder mice. It suggests that spleen lymphocytes from both *in vivo* mercury-susceptible and -resistant mice have a potential to respond to mercury *in vitro*. But in some way the response to mercury is highly regulated in different mouse strains.

It has been postulated that the Th1/Th2 cytokine profile plays an important role in mercury-induced autoimmunity, i.e. a preferential Th2-type response in susceptible animals but a Th1-type response in resistant animals.[13,18,19,24,25] *In vivo*, autoreactive anti-MHC class II CD4$^+$ T cells have been detected in both susceptible BN and resistant LEW rats, and it is found that LEW autoreactive CD4$^+$ T cells secret IL-2 and do not help B cells to produce immunoglobulin, which is believed to belong to Th1.[12] *In vitro*, mercury stimulation induces IFN-γ mRNA in spleen cells from both BN and LEW rats, whereas IL-4 mRNA is exclusively induced in spleen cells from BN rats.[25]

In the current *in vitro* study in mice, we used an ELISPOT assay to detect cytokine production at the protein level. (Due to the low frequency of positive cells ($< 1/1500$), we were not able to do the intracellular staining and double staining to tell the phenotype of the cytokine-producing cells.) We found that mercury-induced cytokine profiles were the same in spleen cells from both susceptible and resistant mice, either IL-2 and IFN-γ production in the continuous presence of mercury, or IL-4 production when excess mercury was washed away after pretreatment. In contrast to the *in vitro* cytokine production pattern in mercury-model in rats (as mentioned above), our results demonstrate first, that lymphocytes from both susceptible and resistance mice have the potential to produce Th1- and Th2-type cytokines after mercury stimulation *in vitro*, and second, that after washing, the cytokine profile can shift.

The washing process is assumed to wash away toxic metabolic products and make improve the cells' survival. Since cytokine production has been shown to be augmented in preactivated cells,[26–28] it is conceivable that the washing process does not change the cytokine production pattern but enhances the production of original ones. However, this was not the case in mercury-stimulation. It seems that in the continuous presence of mercury, the excess mercury favoured induction of IL-2 and IFN-γ production but a suppressed cell proliferation, while after washing, the bound mercury preferentially induced IL-4 production and an enhanced cell proliferation. The suppressed IFN-γ production could be due to the induced IL-4 production.[29,30] The addition of anti-MHC class II antibodies almost completely blocked the cytokine production in both situations (unpublished observation), suggesting that the mercury-induced cytokine production is not due to non-specific effects of mercury on cells. The washing process did not merely result in a low concentration of mercury, since the continuous presence of a low concentration of mercury (down to 0·1 μM) did not induce any of the three cytokines or cell proliferation (not shown).

Our *in vitro* results showed that for mercury stimulation, at least two distinct conditions existed. Although high- and low-responders behaved essentially identically under the same *in vitro* conditions, the cytokine production patterns of the two conditions were reciprocally different. With regard to the *in vivo* mercury situation, very little is known about the

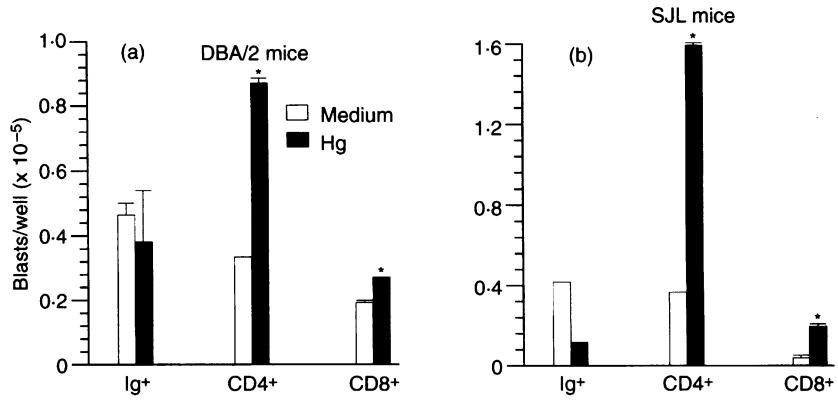

Figure 3. Spleen cells (3×10^6/ml) from DBA/2 or SJL mice were preincubated with mercuric chloride. After culture for 3 days, the cells were washed twice with BSS. The living cells were recovered by lympholyte-M gradient and incubated for one additional day. On day 4 the cells were washed and stained for the phenotype analysis of immunoglobulin (Ig), CD4 and CD8 by FACS. The gate was set to analyse only the large transformed cells. Each column represents the mean ± SD of two experiments. Significant differences between mercury stimulation and medium control were calculated by Student's *t*-test. $*P < 0{\cdot}05$.

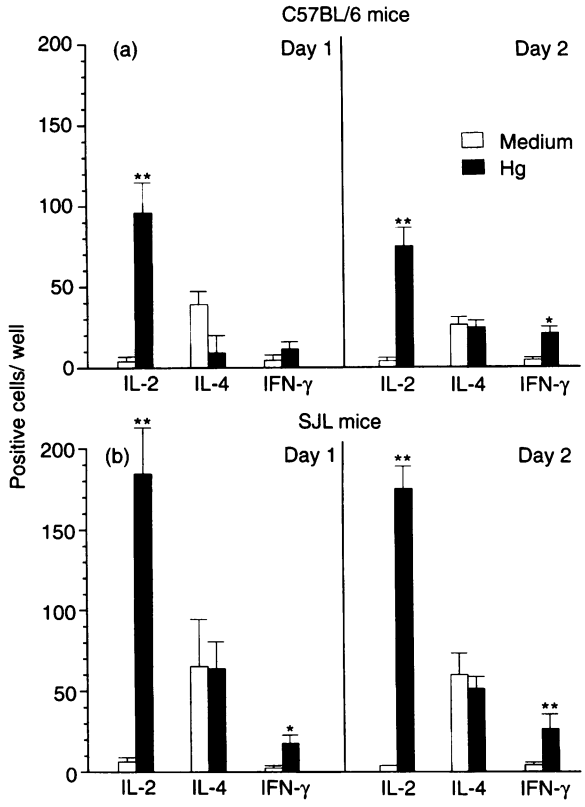

Figure 4. Spleen cells from C57BL/6 or SJL mice were incubated with mercuric chloride in an ELISPOT plate (3×10^6/ml; 0·1 ml/well) coated with different anticytokine antibodies. After different time-periods of culture, the cytokine production was measured as described in the Materials and Methods. Each column represents the mean of duplicate or triplicate cultures. Results were representative of three separate experiments. Significant differences between mercury stimulation and medium control were calculated by Student's *t*-test. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$.

mercury microenvironment to which lymphocytes are exposed after mercury injection. Considering the capacity of each mouse strain to transport, absorb and delete the mercury ions, we would assume that local mercury environment might differ in different mouse strains. It is likely that mercury injection results in two different *in vivo* situations in high- and low-responder mice, which lead to distinct consequences, i.e. a Th1-type response in resistant mice and a Th2-type response, that causes the autoimmunity, in susceptible mice. In rats, it has been recently found that IFN-γ and IL-4 were up-regulated with a different time–course.[31] In mice *in vivo*, a strong increase of IL-4 mRNA was detected in mercury-treated high-responder mice, but only a weak increase in low-responder mice.[13] However, the more complete cytokine production has not been studied. The finding that treatment with anti-IL-4 monoclonal antibody reduced the titers of IgG1 ANolA, but increased those of IgG2A, IgG2B and IgG3 ANolA,[8] indicates that the initial autoimmune process might have other cytokines/mechanisms involved as well. Recent studies have revealed that the manipulation of lymphocyte accessory counterreceptor interactions could affect the course of mercury-induced autoimmune disease *in vivo*.[32] In rats it has also been shown that mercury treatment induces different patterns of adhesion and expression of costimulatory molecules in susceptible and resistant strains,[33] suggesting the different effects of mercury *in vivo*.

Previous studies have shown that in low-responder mice only $CD8^+$ T cells respond to mercury.[20] In our experiments, the phenotype analysis of the restored response in low-responder mice demonstrated that both $CD4^+$ and $CD8^+$ T cells were activated, but $CD4^+$ T cells more than $CD8^+$ T cells. Evidence has shown that the major IFN-γ-producing cells are $CD8^+$ T cells, whereas the major IL-4-producing cells are $CD4^+$ T cells.[26] Since $CD4^+$ T cells were the major responding cell population after washing (Fig. 3), it is expected that IL-4 production was induced (Fig. 5a, b). The mechanism by which that activation of $CD4^+$ T cells but not $CD8^+$ T cells from low-responder mice is specifically suppressed in the continuous presence of mercuric chloride during *in vitro* culture is not known.

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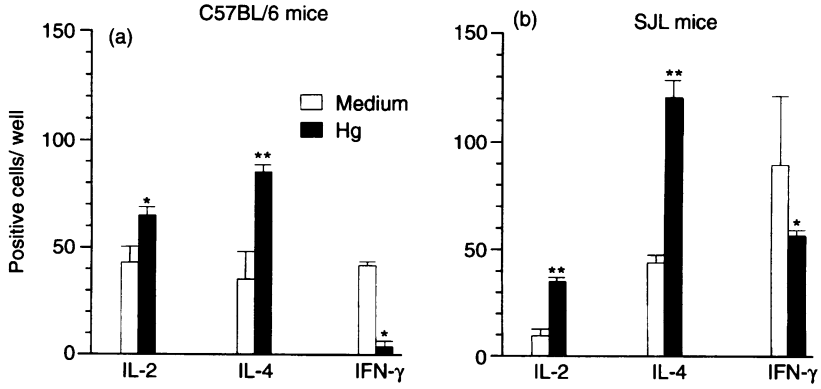

Figure 5. Spleen cells from C57BL/6 or SJL mice were preincubated with mercuric chloride for 1 day. Afterwards cells were washed twice with BSS and then incubated in an ELISPOT plate (3×10^6/ml; 0·1 ml/well) coated with different anti-cytokine antibodies for another 2 days. The cytokine production was measured as described in the Materials and Methods. Each column represents the mean of duplicate or triplicate cultures. Results were representative of three separate experiments. Significant differences between mercury stimulation and medium control were calculated by Student's *t*-test. $*P<0·05$; $**P<0·01$.

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