IL-2 may be a limiting factor precluding lymphocytes from genetically resistant mice from responding to HgCl₂

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Abstract

It is unclear how HgCl₂ causes autoimmune disorders in genetically predisposed rodents. We investigated the cytokine profile induced by HgCl₂ in vitro, and found a high frequency of IL-2-secreting cells in splenocytes from susceptible A.SW and BALB/c mice, whereas the frequency was low in cells from resistant DBA/2 mice. More IL-2-secreting cells were induced in splenocytes from the high responder A.SW mice than in cells from the intermediate responder BALB/c mice. Unexpectedly, a similar level of IL-4 production was induced in splenocytes from BALB/c and DBA/2 mice. IL-4 production was high in unstimulated cells from A.SW mice and was further increased by HgCl₂. IFN-γ-secreting cells were detectable in splenocytes from all three strains after activation by HgCl₂. The highest frequency of IL-10-secreting cells was found in splenocytes from A.SW mice after activation, whereas the frequency was lower in cells from BALB/c mice, followed by cells from DBA/2 mice. We showed that neutralizing anti-IL-2 antibody profoundly inhibited the in vitro response to HgCl₂. In contrast, antibodies against IL-4, IFN-γ and IL-10 did not significantly affect the response of splenocytes from either A.SW or DBA/2 mice. The addition of IL-2 into cultures enhanced the proliferative response to HgCl₂ in splenocytes from DBA/2 mice to a level comparable with that in cells from BALB/c mice. We found no evidence for the suggestion that HgCl₂ induces a T_h 1/T_h 2 imbalance in resistant/susceptible strains. We conclude that IL-2 may be a limiting factor precluding lymphocytes from resistant mice from responding to HgCl₂.

Introduction

Repeated injections of subtoxic doses of HgCl₂ can induce a systemic autoimmune disease in certain strains of rat and mouse. The immunopathological alterations include lymphocyte hyperplasia, increased serum Ig levels, autoantibody production and deposition of immune complexes in tissues (1). It has been well documented that HgCl₂ induces a T cell-dependent polyclonal activation of B cells (2–6), but the mechanism is not well understood. Since cationic mercury is capable of non-specifically binding to the sulphhydryl group and many other chemical groups (7), mercury may modify proteins and the novel modified molecules may somehow trigger an immunological dysfunction. We and others have previously described that a very low dose of HgCl₂ (10 µM) was able to activate murine T lymphocytes to proliferation and transformation in a primary in vitro culture (8,9). Moreover, we showed that lymphocytes from different mouse strains differed in their capability of responding to HgCl₂ (9). CD4⁺ T cells from susceptible mice were preferentially activated, suggesting that T_h 1 cells play a crucial role for the immunological effects caused by HgCl₂ and may define the ability of different mouse strains to respond to HgCl₂ (9,10).

A characteristic feature of this experimental model is that the induction of autoimmunity is under stringent genetic control. Some of the susceptibility genes have been mapped to the MHC region, but non-MHC genes are also involved. The Brown–Norway rats that carry RT1n genotype are responders, whereas the Lewis rats that carry RT1ν genotype are non-responders (11–13). A.SW mice and other H-2k mouse strains
are highly susceptible to HgCl$_2$-induced autoimmunity (14–16). BALB/c mice (H-2$^b$) are also responders, and exhibit hypergammaglobulinemia and tissue immune complex deposits (16–18). DBA/2 mice carry the same H-2 genotype as BALB/c mice, but do not display any autoimmune manifestations after treatment with HgCl$_2$ (14–16, 19). Studies have shown that the unresponsive state of resistant strains is not due to immunosuppression mediated by CD8$^+$ T cells (10,20). It also seems unlikely that a specific TCR repertoire—shaped by MHC or minor lymphocyte stimulating antigens and other self-ligands—predisposes to the development of autoimmunity after activation by HgCl$_2$ (21).

Immune responses may be divided into two categories based on the pattern of cytokine secretion by $T_h$ cells: IFN-γ-dominant $T_h$1-type response and IL-4-dominant $T_h$2-type response (22). Although IL-2 has been regarded as a cytokine characteristic of $T_h$1, it is essential for promoting T cell proliferation and is therefore an important factor in both $T_h$ responses (23). The two types of $T_h$ response have antagonistic effects on each other (24). Skewing towards $T_h$1- or $T_h$2-type responses has been shown to play an important role in governing the susceptibility of an individual to certain infections and autoimmune diseases (25,26). Goldman et al. postulated that an imbalance between $T_h$1- and $T_h$2-type responses might account for the different consequences of HgCl$_2$ treatment in resistant and susceptible strains (27). In order to further explore the mechanism, a better knowledge of the pattern of cytokine secretion in response to HgCl$_2$ in different strains is required. In the present paper, we investigated the cytokine profile induced by HgCl$_2$ in vitro at different times of culture. The high responder A.SW strain, the intermediate responder BALB/c strain and the non-responder DBA/2 strain were studied. We tested our hypothesis that IL-2 might be a limiting factor that precludes lymphocytes from resistant strains to responding to HgCl$_2$.

Methods

Mice and stimulus

Female A.SW, BALB/c and DBA/2 mice either obtained from our animal facilities at Stockholm University or purchased from Harlan (Bicester, UK) or Charles River (Uppsala, Sweden) were used at the age of 7–11 weeks. A stock solution of HgCl$_2$ (Merck, Darmstadt, Germany) prepared in physiological saline was filter sterilized (0.22 µm filter, Costar, Cambridge, MA) and was added into cultures to achieve a final concentration of 10 µM.

Cell culture

Single splenocytes were prepared and suspended at a density of 5 x 10$^6$/ml in RPMI 1640 medium (Life Technologies, Gaithersburg, MD), buffered with 10 mM HEPES buffer solution (Life Technologies) and supplemented with 10% heat-inactivated human AB serum (Sabbatsberg Hospital, Stockholm, Sweden), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Cell suspensions were seeded in 24- or 96-well flat-bottom culture plates (Costar) with 1.5 or 0.2 ml/well respectively. The cells were incubated at 37°C for the indicated time period in a humidified atmosphere containing 5% CO$_2$.

Detection of cytokine-secreting cells

Cytokine-secreting cells were detected by ELISPOT assay (28). The capture antibodies, biotinylated detective antibodies and streptavidin–alkaline phosphatase conjugate were purchased from PharMingen (San Diego, CA). HgCl$_2$-stimulated and unstimulated cells were harvested from 24-well plates after 2, 22, 46, 70 or 94 h of culture and counted. The cells were then spun down, and the supernatants were kept and used to resuspend the cells to an appropriate volume. The cells resuspended with their original culture medium (the supernatants) were then plated into the nitrocellulose-backed 96-well filtration plates (Millipore, Sundbyberg, Sweden) pre-coated with anti-IL-2 (10 µg/ml), anti-IL-4 (5 µg/ml), anti-IFN-γ (15 µg/ml) or anti-IL-10 (10 µg/ml) antibodies. After incubation for 20 h at 37°C in a humidified atmosphere containing 5% CO$_2$, the plates were thoroughly washed and corresponding biotinylated anti-IL-2 (2 µg/ml), anti-IL-4 (0.5 µg/ml), anti-IFN-γ (1 µg/ml) and anti-IL-10 (1 µg/ml) antibodies were added. After overnight incubation at 4°C, the plates were washed and incubated with 1:1000 diluted streptavidin–alkaline phosphatase conjugate for another 2 h at room temperature. Spots representing single cytokine-secreting cells were then developed by the BCIP/NBT substrate kit (BioRad, Hercules, CA) in the dark and were counted with the aid of a dissecting microscope. The X63-Ag8.653 cell lines transfected with IL-2 or IL-4 cDNA genes (29; a kind gift from Dr F. Melchers, Basel Institute of Immunology, Basel, Switzerland) and BALB/c splenocytes stimulated by 2.5 µg/ml of concanavalin A (Con A; Pharmacia, Uppsala, Sweden) were used as positive controls for IL-2-, IL-4-, IFN-γ- and IL-10-secreting cells respectively. The frequency of cytokine-secreting cells was calculated and presented as numbers per well of the original culture in 24-well plates.

Neutralizing anti-IL antibodies and other reagents

Neutralizing anti-IL-2, anti-IL-4 and anti-IL-10 antibodies (rat IgG) purchased from PharMingen were used. Neutralizing antibody against IFN-γ (rat IgG) was purchased from either Pharmingen or Biosource International (Camarillo, CA). Supernatants from rat lymphocytes (5 x 10$^6$/ml) stimulated by 2 µg/ml of Con A for 40 h and a supernatant from IL-2-transfected X63-Ag8.653 cell line were kindly prepared by our technician Ms Sirkka Hellman and used as sources of IL-2.

Flow cytometry

Cells (~1 x 10$^6$) suspended in 100 µl of cold (4°C) Earl’s balanced salt solution (Life Technologies) supplemented with 2% FCS (Life Technologies) were incubated for 15–20 min with FITC-conjugated anti-CD8α/53-6.7 antibody (Becton Dickinson, Mountain View, CA) and phycoerythrin-conjugated anti-CD4/GK1.5 antibody (Becton Dickinson) respectively, and kept cold and in the dark. After two washes, the stained cells were analyzed on a FACSScan cytometer (Becton Dickinson) equipped with an argon-ion laser and interfaced with a Hewlett-Packard Model 310 computer by means of a GPII interface device. The cytometer was calibrated by AutoCOMP Software using CaliBRITE beads (Becton Dickinson). Data
were collected in a four-parameter list mode with linear amplification for forward scatter, linear scale for side scatter, and logarithmic scale for green and red fluorescence, and were processed with the FACScan Research Software (version 2.1). Viable lymphocytes were counted after gating on forward and side scatter. The frequency data are presented using total lymphocytes for the denominator. Large activated cells were gated based on forward and side scatter as described previously (9).

**DNA synthesis**

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{^{3}{H}}\text{Thymidine (Amersham, Amersham, UK) was pulsed into cultures to achieve a final concentration of } 2 \times 10^{-6} \text{ Ci/ml. The cells were harvested 8 h later using a Skatron cell harvester (Lier, Norway). The incorporated radioactivity was counted by a 1450 MicroBeta Plus Liquid Scintillation Counter (Wallac, Turku, Finland) and was given as c.p.m.}
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**Statistics**

Statistical evaluation was performed using two-tail Student’s t-test. Results were considered statistically significant at the level of \( P < 0.05 \).

**Results**

High frequency of IL-2-secreting cells was found in splenocytes from susceptible strains after activation by HgCl\(_2\).

Splenocytes from A.SW, BALB/c and DBA/2 mice were cultivated in the presence or absence of HgCl\(_2\) as described in Methods. The cells were harvested at different times of culture, and IL-2-, IL-4-, IFN-\(\gamma\) and IL-10-secreting cells were enumerated using the ELISPOT assay.

**IL-2.** IL-2-secreting cells were induced by HgCl\(_2\) in splenocytes from all three strains, peaking at 22 h of culture (Fig. 1a). A high frequency of IL-2-secreting cells was found in splenocytes from susceptible A.SW and BALB/c mice, whereas the frequency was low in cells from resistant DBA/2 mice. More IL-2-secreting cells were induced in splenocytes from the high responder A.SW mice than in cells from the intermediate responder BALB/c mice. Our data suggest that the susceptibility to HgCl\(_2\)-induced autoimmunity is associated with the capability of IL-2 production principally by T\(_h\) cells under the stimulation of HgCl\(_2\).

**IL-4.** A considerable number of cells were found to secrete IL-4 spontaneously in the beginning of culture. While this background declined after 22 h, an increase of IL-4 production in response to HgCl\(_2\) became detectable and persisted until the last time point we checked (Fig. 1b). Unexpectedly, IL-4-secreting cells were strongly induced by HgCl\(_2\) in splenocytes from resistant DBA/2 mice. More IL-2-secreting cells were induced in splenocytes from the high responder A.SW mice than in cells from the intermediate responder BALB/c mice. Our data suggest that the susceptibility to HgCl\(_2\)-induced autoimmunity is associated with the capability of IL-2 production principally by T\(_h\) cells under the stimulation of HgCl\(_2\).

**IFN-\(\gamma\).** As depicted in Fig. 1(c), HgCl\(_2\) induced some IFN-\(\gamma\)-secreting cells in cell cultures of all three strains, but the frequency was low. Almost no background production was observed in unstimulated cultures. Thus, HgCl\(_2\) induced detectable production of IFN-\(\gamma\) in splenocytes from both susceptible A.SW and BALB/c mice and resistant DBA/2 mice.
IL-2 is a limiting factor in Hg-non-responder mice

IL-10. Since IL-10 plays a major role as a regulator of immune and inflammatory responses, the production of IL-10 was also studied. As shown in Fig. 1(d), IL-10-secreting cells were induced by HgCl₂ in splenocytes from all three strains, peaking at 70 h of culture. The highest frequency of IL-10-secreting cells was found in cells from A.SW mice, whereas the frequency was lower in cells from BALB/c mice, followed by cells from DBA/2 mice.

Neutralizing anti-IL-2 antibody profoundly inhibited the response to HgCl₂

We have shown that HgCl₂ failed to induce a Th1-type response, which is characterized by predominant production of IFN-γ and lack of IL-4 production, in splenocytes from resistant DBA/2 mice. We further investigated the importance of IL-2, IL-4 and IFN-γ by examining the effects of neutralizing antibodies against these cytokines on the response to HgCl₂. Antibodies at concentrations of 0.1, 0.5 (data not shown), 1, 5 (data not shown), 10 (data not shown) and 40 µg/ml were added into cultures, and the proliferative response to HgCl₂ was measured by determining DNA synthesis. We found that the addition of anti-IL-2 antibody at a concentration as low as 1 µg/ml profoundly inhibited the response to HgCl₂ in splenocytes from A.SW mice (Fig. 2a). The response of splenocytes from DBA/2 mice was also inhibited by this antibody (P < 0.01). In contrast, anti-IL-4 antibody failed to influence the response to HgCl₂ in splenocytes from either A.SW or DBA/2 mice (Fig. 2b). Similar results were obtained with anti-IFN-γ antibody (Fig. 2c).

Neutralizing anti-IL-10 antibody at concentrations of 0.2, 5 and 40 µg/ml was also examined. As depicted in Fig. 2(d), the addition of this antibody did not significantly affect the response to HgCl₂ in splenocytes from either A.SW or DBA/2 mice.

The addition of IL-2 into cultures enabled splenocytes from resistant mice to respond to HgCl₂

To test our hypothesis that IL-2 is a limiting factor in resistant strains that prevents a response to HgCl₂, we investigated whether the addition of exogenous IL-2 into cultures would make splenocytes from resistant DBA/2 mice responsive to HgCl₂. Supernatants from both Con A-stimulated rat splenocytes and IL-2-transfected X63-Ag8.653 cell line were used as sources of IL-2. As shown in Fig. 3, the addition of IL-2 enhanced the response to HgCl₂ in splenocytes from DBA/2 mice as determined by thymidine incorporation, to a level comparable with the response of splenocytes from BALB/c mice. No significant amplification of the response to HgCl₂ was observed in IL-2-supplemented cultures of splenocytes from BALB/c (Fig. 3) or A.SW (data not shown) mice, indicating that endogenous sources of IL-2 may be sufficient to fully promote a response to HgCl₂ in cells from susceptible strains.

We have previously shown that blast transformation of CD4⁺ T cells is a characteristic of the response to HgCl₂ in cultures of splenocytes from susceptible mice (9). Therefore, the effect of IL-2 on T-cell transformation induced by HgCl₂ was studied. As expected, CD4⁺ T cells from resistant DBA/2 mice were induced to transform by HgCl₂ when exogenous IL-2 was supplemented, resembling a response of CD4⁺ T cells from susceptible BALB/c mice (Fig. 4a). The addition of IL-2 had a marginal effect on the transformation of CD8⁺ T cells (Fig. 4b).

Discussion

IL-2 was originally identified as a T cell growth factor since it was found to be obligatory for T cell activation and expansion.
IL-2 is a limiting factor in Hg-non-responder mice

Fig. 3. Supplement of exogenous IL-2 enhanced the response to HgCl₂ in splenocytes from DBA/2 mice. Splenocytes (5×10⁶/ml) from DBA/2 and BALB/c mice were cultivated in the presence (triangles) or absence (circles) of 10 µM of HgCl₂. Supernatants from Con A-stimulated rat splenocytes (Con A sup) and IL-2-transfected X63-Ag8.653 cell line (IL-2 sup) at various concentrations were added at the beginning of culture. On the indicated days, [³H]thymidine was pulsed and radioactivity uptake was counted. Mean c.p.m. ± SD from triplicate cultures are shown and are representative of two (BALB/c strain) or four (DBA/2 strain) experiments.

(30). It is now known that IL-2 plays a central facilitative role in immune responses, promoting the proliferation of most subsets of lymphocytes (23). Sufficient IL-2 must be present in order to drive the expansion of lymphocytes that initially responded to the stimuli. The marginal activation of CD4⁺ T cells by HgCl₂ observed in cultures of splenocytes from DBA/2 mice (9,10) suggests the possibility that IL-2 is a limiting factor preventing lymphocytes from this resistant strain from responding to HgCl₂. We showed in the present study that: (i) the capability of lymphocytes from different mouse strains to produce IL-2 after activation by HgCl₂ corresponded with the susceptibility of these strains to HgCl₂-induced autoimmunity, (ii) neutralization of IL-2 was able to profoundly inhibit the response to HgCl₂ and (iii) supplement of exogenous IL-2 enabled splenocytes from resistant mice to respond to HgCl₂ to a level comparable with cells from susceptible mice. Our findings indicate that IL-2 is a critical factor and may determine the outcome of a response to HgCl₂ in different individuals. The possible role of IL-2 in the development of HgCl₂-induced autoimmunity is supported by the finding that cyclosporin A, an agent known to mainly inhibit endogenous IL-2 production (31), was capable of preventing the autoimmune development caused by HgCl₂ (32). It is possible that the production of this cytokine is somehow limited in genetically resistant strains, failing to promote a response that leads to autoimmune manifestations.

One of the hallmarks of HgCl₂-induced autoimmune alterations is hypergammaglobulinemia mainly affecting IgG1 and IgE isotypes, suggesting that IL-4 could be an important mediator of the T cell-dependent activation of B cells. Indeed, a number of studies demonstrated that IL-4 expression was induced by HgCl₂ in susceptible strains, and was responsible for IgG1 and IgE production (33–37). It has been postulated that HgCl₂ induces a Th2-type response in susceptible strains, but a Th1-type response in resistant strains (27). However, our data indicated that there was no significant difference between susceptible and resistant mouse strains with regard to the production of IL-4 and IFN-γ by lymphocytes after stimulation with HgCl₂ in vitro. We found no evidence to support the proposal that HgCl₂ induces an imbalance between Th1- and Th2-type responses in resistant and susceptible strains.

Hultman et al. recently showed that injections of HgCl₂ induced the production of IL-2, IL-4 and IFN-γ in susceptible mice, whereas none of these cytokines were induced in resistant mice (38). Similar data were also reported in rats by Mathieson et al. (39). Therefore, there is no evidence that HgCl₂ preferentially promotes a Th1-type response in genetically resistant animals. Neutralization of IFN-γ neither enhanced the response to HgCl₂ (the present study) nor converted the resistant mice susceptible to HgCl₂-induced autoimmunity (40), again suggesting that the unresponsive state of resistant strains is not due to IFN-γ-mediated suppression of a Th2-type response observed in susceptible strains.

A Th2-type response characterized by IL-4-dependent IgE production was suppressed in susceptible mice by administration of either neutralizing anti-IL-4 antibody or recombinant IFN-γ, nevertheless, these mice were not protected from autoimmune disease caused by HgCl₂ (33,40). Consistent with these findings, it was reported recently that HgCl₂ was able to induce autoantibody production and tissue immune complex deposits in IL-4-deficient mice (41 and Hu et al., manuscript submitted). Thus, a Th2-type response is not necessary for HgCl₂-triggered development of autoimmunity.
IL-2 is a limiting factor in Hg-non-responder mice

Quite unexpectedly, the study from Kono et al. showed that IFN-γ knockout H-2^k mice had low autoantibody levels and essentially normal immunohistology following administration of HgCl_2, indicating that the induction of autoimmunity might be IFN-γ dependent (41).

We showed previously that HgCl_2 preferentially activated CD4^+ T cells from susceptible mice; however, CD4^+ T cells from resistant mice might not be completely unresponsive to HgCl_2 (9,10). Our findings—that some IL-2 were produced from resistant mice might not be completely unresponsive to HgCl_2—it is unlikely that IL-10 plays a major inhibitory role in this model. It is possible that, as suggested by Druet et al., regulatory CD4^+ T cells and transforming growth factor-β secreted by these cells are involved in down-regulation of the response to HgCl_2 in resistant strains (45).

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**Abbreviations**

- Con A: concanavalin A

**References**

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