Clin Exp Immunol 2003; **134**:202–209 doi:10.1046/j.1365-2249.2003.02303.x

Effects of deviating the Th2-response in murine mercury-induced autoimmunity towards a Th1-response

B. HÄGGQVIST & P. HULTMAN *Division of Molecular and Immunological Pathology (AIR), Department of Molecular and Clinical Medicine, Linköping University, Linköping, Sweden*

(Accepted for publication 4 September 2003)

SUMMARY

T-helper cells type 1 (Th1) and type 2 (Th2) play an important role in the pathogenesis of autoimmune diseases. In many Th1-dependent autoimmune models, treatment with recombinant interleukin-12 (rIL-12) accelerates the autoimmune response. Mercury-induced autoimmunity (HgIA) in mice is an H-2 regulated condition with antinucleolar antibodies targeting fibrillarin (ANoA), systemic immune-complex (IC) deposits and transient polyclonal B-cell activation (PBA). HgIA has many characteristics of a Th2 type of reaction, including a strong increase of IgE, but disease induction is critically dependent on the Th1 cytokine IFN-γ. The aim of this study was to investigate if a strong deviation of the immune response in HgIA towards Th1 would aggravate HgIA. Injections of both rIL-12 and anti-IL-4 monoclonal antibody (α-IL-4) reduced the $HgCl_2$-(Hg-)induced concentration of the Th2-dependent serum IgE and IgG1, but increased the Th1-dependent serum IgG2a. The IgG-ANoA developed earlier and attained a higher titre after combined treatment, and the ANoA titre of the IgG1 isotype decreased while the ANoA titre of the Th1-associated IgG2a, IgG2b and IgG3-ANoA isotypes increased. Treatment with rIL-12 alone increased the Hg-induced IgG2a and IgG3 ANoA titres, the PBA, and the IC deposits in renal and splenic vessel walls, while treatment with α-IL-4 + Hg inhibited renal but not splenic vessel wall IC deposits. We conclude that manipulating the cytokine status, by altering the Th1/Th2 balance, will influence autoimmune disease manifestations. This might be an important way of modulating human autoimmune diseases.

Keywords autoimmunity interleukin-12 anti-interleukin-4 mercury mice

INTRODUCTION

The paradigm of T helper cells type 1 (Th1) and type 2 (Th2) [1] is well established and plays a crucial role in autoimmune diseases [2]. For example, transfer of Th1 cells induces autoimmune disease in murine allergic encephalomyelitis [3] and insulin-dependent diabetes mellitus (IDDM) [4]. Other Th1-dependent autoimmune diseases in mice are collagen-induced arthritis (CIA) [5] and autoimmune myasthenia gravis [6]. Because of the reciprocal regulation of Th1 and Th2 cells, it has been proposed that activated Th2 cells may inhibit Th1-dependent autoimmune diseases (reviewed in [7]). In agreement with this, treatment with the Th2 cytokine IL-4 confers protection from IDDM development [8]. However, Th2 cells may, under certain conditions, cause diseases otherwise induced by Th1 cells [9,10]. Therefore, linking the Th1 or Th2 type of reaction to susceptibility or resistance to particular autoimmune conditions is an oversimplification.

In murine mercury-induced autoimmunity (HgIA), mice with a specific MHC haplotype (H-2^s) rapidly develop a systemic autoimmune disease characterized by antinucleolar antibodies (ANoA), specifically persistent antifibrillarin antibodies (AFA), systemic immune-complex (IC) deposits, transient polyclonal B-cell activation (PBA) and transient hyper-IgE condition [11,12]. Induction of AFA seems to be an antigen-specific reaction caused by modification of fibrillarin due to binding of Hg [13,14] and/or necrosis-induced proteolytic modification [15]. In support of an antigen-specific mechanism, Hg-induced AFA are dependent on $CD4^+$ cells [16], and T cells recognizing Hg-modified fibrillarin have been identified [14].

In 1991 Goldman *et al*. have suggested that HgIA was a prototypic Th2 disease [17]. Studies have been conducted in the mouse with the intention to attenuate or ameliorate HgIA by deviating the response towards Th1. Deviation has been accomplished by treatment with α-IL-4 [18] or recombinant interferon-gamma (rIFN-γ) [19]. The Th2-dependent parameters such as IgE

Correspondence: Per Hultman, Division of Molecular and Immunological Pathology (AIR), University Hospital, SE-581 85 Linköping, Sweden.

E-mail: perhu@imk.liu.se

are reduced efficiently by such deviation, but the ANoA response is not affected. By using mice with targeted mutations Kono *et al.* showed subsequently that IFN-γ but not IL-4 is required for induction of HgIA [20]. This implies that Th1 cells play a key role in the induction of HgIA.

Recently, we assessed the expression of mRNA for several cytokines during induction of HgIA. We found an early expression of Th1-derived IL-2 and IFN-γ that vanished rapidly. The latter might be due to the massive increase of IL-4 [21], which inhibits Th1 cells [22]. Our objective with the present study was to examine if a strong deviation towards Th1 would aggravate the autoimmune disease manifestations in HgIA. We used a treatment which has proved to be very efficient for ameliorating progressive cutaneous leishmaniasis in mice [23], a disease condition which shares with HgIA the dominance of Th2-associated cytokines [21]. We found that Th1 deviation accelerated several aspects of the HgIA condition.

MATERIALS AND METHODS

Animals

Female A.SW (H-2^s) mice were obtained from M&B A/S (Ry, Denmark). The mice were housed in steel-wire cages under 12-h dark/12-h light cycles and given type R36 pellets (Lactamin, Vadstena, Sweden) and drinking water *ad libitum*. All mice were 9–10 weeks old at onset of the experiment.

Substances used for treatment

$HgCl_2$ (Hg) of analytical grade (Fluka, Seelze, Germany) was dissolved and diluted to 10 mg/l in tap water. NaCl was a sterile, 0·9% solution. Rat IgG1 (RIg) (hybridoma IR 871) was a sterile solution, free from endogenous toxins, in 0·15 M phosphate buffered saline (PBS) (Technopharm, Paris, France). Monoclonal antibodies to IL-4 (α-IL-4 antibody) (clone 11B11 [24]) were obtained as a sterile solution in PBS from National Institute of Health (Bethesda, MD, USA). Recombinant interleukin-12 (rIL-12), Cat. 419-ML (R&D Systems Inc., Minneapolis, MN, USA) was freshly prepared as a 1-μg/ml solution in NaCl and aliquots for daily injections were kept at −70°C and thawed just prior to administration. Monoclonal antibodies to CD4 (α-CD-4 Ab) (clone GK1·5) were prepared as a 10-mg/ml solution in NaCl.

Treatment

Ten mg Hg/l was given *ad libitum* in the drinking water. Treatment with rIL-12 was started 18 h prior to Hg treatment and consisted of daily intraperitoneal (i.p.) injections of 0·2 μg rIL-12 for 10 days. Treatment with α-IL-4 consisted of 2 mg antibody given as i.p. injections 24 h prior to the start of Hg treatment and after 4 days of Hg treatment (Fig. 1). Controls were instead given 0·2 ml NaCl i.p. and 2 mg RIg i.p. The different treatment groups and the treatment schedule are described in Table 1 and Fig. 1.

In addition, in the pilot study, groups were given 1 mg α-CD-4 antibody i.p. 7 days prior to Hg treatment, in combination with rIL-12 + α-IL-4 treatment.

Blood and tissue sampling

Blood samples were obtained from the mice by weekly bleeding from the retro-orbital plexus (Fig. 1). Animals were sacrificed after 4 weeks and tissues from the spleen, kidney and liver were snap-frozen in isopentan-CO_2 for direct immunofluorescence and fixed in HistoCHOICE™ (Amresco Inc., Solon, OH, USA) for light microscopy.

Serum ANA

Indirect immunofluorescence was performed as described previously [25]. Briefly, sera diluted 1 : 30–1 : 20 480 were applied to monolayers of fixed Hep-2 cells (Binding Site Ltd, Birmingham, UK), and bound serum antibody detected by FITC-conjugated goat antimouse IgG antibodies (Sigma Chemical Company, St Louis, MO, USA) diluted 1 : 50. The presence and pattern of fluorescence were observed using an epi-illuminescence microscope (Nikon Instech Co. Ltd, Kanagawa, Japan). The titres of ANoA of the IgM, IgG1, IgG2a, IgG2b and IgG3 isotype were assessed similarly using FITC-conjugated goat anti(α)-mouse IgM, goat α-mouse IgG1, α-mouse IgG2a, α-mouse IgG2b and α-mouse IgG3 antibodies (SouthernBiotech, Birmingham, AL, USA) diluted 1 : 80, 1 : 50, 1 : 160, 1 : 80 and 1 : 50, respectively.

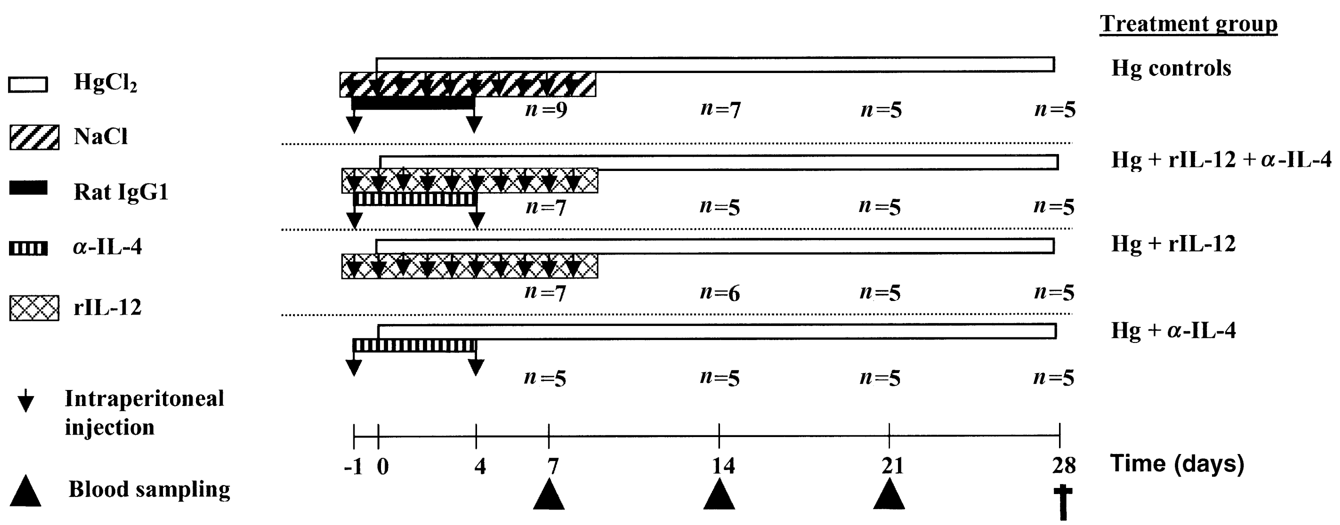

Fig. 1. Treatment schedule for Hg, rIL-12, α-IL-4 and control substances in the different groups.

Table 1. IgM-α-ssDNA and IgM-α-DNP antibody titre in the different treatment groups

Antibody titre[a]	Treatment group	Weeks after onset of treatment with Hg					
		n	1[b]	n	2[b]	n	3[b]
IgM-α-ssDNA	Hg controls	9	0·569 ± 0·210	7	0·544 ± 0·153	5	0·487 ± 0·094
	Hg + rIL-12 + α-IL-4	7	0·464 ± 0·065	5	0·715 ± 0·168	5	0·774 ± 0·116
	Hg + rIL-12	7	0·534 ± 0·205	6	0·875 ± 0·221[c]	5	1·031 ± 0·369[e]
	Hg + α-IL-4	5	0·422 ± 0·046	5	0·538 ± 0·090[d]	5	0·493 ± 0·079[f]
IgM-α-DNP	Hg controls	9	0·436 ± 0·176	7	0·423 ± 0·119	5	0·359 ± 0·076
	Hg + rIL-12 + α-IL-4	7	0·380 ± 0·076	5	0·663 ± 0·137[c]	5	0·656 ± 0·219[c]
	Hg + rIL-12	7	0·456 ± 0·070	6	0·715 ± 0·110[e]	5	0·708 ± 0·115[e]
	Hg + α-IL-4	5	0·384 ± 0·071	5	0·436 ± 0·092[f,g]	5	0·363 ± 0·073[f,g]

[a](OD_{405}). [b]Mean antibody titre ± s.d. Significantly different antibody titre when comparing groups using ANOVA followed by Tukey's post-test: [c]$P < 0·05$, *versus* Hg controls; [d]$P < 0·05$, *versus* Hg + rIL-12; [e]$P < 0·01$, *versus* Hg controls; [f]P 0·01, *versus* Hg + rIL-12; [g]$P < 0·05$, *versus* Hg + rIL-12 + α-IL-4.

Serum IgM-α-DNP and IgM-α-ssDNA antibodies
These methods have already been described [26]. For measuring anti-dinitrophenyl antibodies (α-DNP antibodies) the method described by Jachez [27] was used with minor modifications. Briefly, microtitre plate wells were coated with serum albumin conjugated with DNP, followed by serum diluted 1 : 100 with PBS-Tween 20 (0·1%) and bovine serum albumin (BSA) (1%). For detection alkaline phosphatase (ALP)-conjugated goat anti-mouse IgM antibodies (Sigma) were used.

IgM-α-ssDNA antibodies were measured by an enzyme-linked immunosorbent assay (ELISA), which is a modification by Johansson *et al*. [26] of the method described previously by Izui *et al*. [28]. Briefly, microtitre plate wells were coated with single-stranded DNA (ss-DNA) overnight followed by blocking with PBS-Tween 20 (0·2%) – BSA (1%). Serum diluted 1 : 150 was then applied and bound antibodies detected as described above. Pooled sera from autoimmune MRL-*lpr/lpr* mice were used as positive controls and from normal mice as negative controls.

Serum Ig isotype concentrations
For analysis of serum IgM [26] microtitre plates were coated with rat α-mouse IgM (clone LO-MM-3) monoclonal antibody (MoAb) (Technopharm, Paris, France). Following blocking, the wells were incubated with diluted serum, and bound IgM was detected using diluted horseradish peroxidase (HRP) conjugated rat antimouse IgM (clone LO-MM-3) MoAb (Technopharm). A standard curve was constructed by using mouse myeloma protein of the IgM isotype (clone MADNP-5, Technopharm). For measuring serum IgE, microtitre plate wells were coated with rat anti-mouse IgE MoAb (clone 23G3, Southern) overnight and then blocked with normal goat serum. Serum diluted 1 : 2·5–1 : 160 was added and HRP-conjugated antimouse IgE antibodies (clone 23G3, Southern) were used to detect bound IgE. A standard curve was constructed by using mouse IgE from an a-DNP hybridoma (clone SPE-7, Sigma).

For measuring serum IgG1, microtitre plate wells were coated with rat antimouse IgG1 MoAb (clone LO-MG1, Technopharm) overnight and then blocked with fat-free milk. Serum diluted 1 : 1000–4000 was added and HRP-conjugated antimouse IgG1 antibodies (clone LO-MG1-2, Technopharm) were used to detect bound IgG1. A standard curve was constructed by using mouse IgG1 from an a-DNP hybridoma (MADNP-1, Technopharm). For measuring serum IgG2a, microtitre plate wells were coated with rat antimouse IgG2a MoAb (clone R8-140, Pharmingen) overnight and then blocked with fat-free milk. Serum diluted 1 : 200 was added and ALP-conjugated antimouse IgG2a antibodies (clone R19-15, Pharmingen) were used to detect bound IgG2a. A standard curve was constructed by using purified mouse IgG2a (Pharmingen).

Tissue immune complex deposits
Cryostate sections were prepared from samples of the left kidney and examined by direct immunofluorescense as described before [16] using FITC-conjugated goat antimouse IgG antibodies (Southern) and anti-C3c antibodies (Organon-Technica, West Chester, PA, USA). The titre of immune reactants in the tissues was determined by serial dilution of the antibodies to 1 : 5120. The actual titre was defined as the highest dilution that still resulted in a specific staining. Deposits in vessel walls were graded as 0, absent; +1, scattered deposits; +2, moderate amount of deposits; +3, abundant deposits; +4, vessel walls filled with deposits. The slides were examined without knowledge of the treatment given.

Light microscopy
The tissue was dehydrated, cleared and embedded in paraffin blocks. Four-μm sections were cut in a microtome and stained with periodic acid Schiff reagent and periodic acid silver-methamine [29].

Statistical methods
ANOVA followed by Tukey's test was used for comparisons of results obtained by ELISA. The differences between the groups with regard to the presence and titre of ANoA and IC deposits were analysed with Fisher's exact test and the non-parametric Kruskal–Wallis test followed by Dunn's test. $P < 0·05$ was considered statistically significant.

RESULTS

Animal health
The animals showed no signs of disease during the experiment. A few mice died during or immediately after the blood samplings. Among the Hg controls two mice died at the blood samplings

after 1 and 2 weeks. Two mice died in the group given Hg + rIL-12 + α-IL-4 during the blood sampling after 1 week. In the group given Hg + rIL-12 one mouse died during the blood sampling after 1 week, and another mouse during the blood sampling after 2 weeks.

Pilot study using α-CD4 + rIL-12 + α-IL-4 to deviate the immune response in HgIA

We first used treatment with α-CD4 followed immediately by treatment with rIL-12 and α-IL-4 for modulating the Th1/Th2-balance in HgIA. The rationale for this therapy was to deplete existing $CD4^+$ cells and subsequently deviate developing Th0 cells into Th1 cells. However, this regimen abolished the induction of ANoA (data not shown), indicating that the development of new $CD4^+$ cells was not sufficient to support the induction of ANoA, known to be a reaction dependent on T cells and specifically $CD4^+$ cells [16]. However, treatment with rIL-12 combined with α-IL-4, without depleting $CD4^+$ cells, reversed the Th2 response to a Th1 response, as evidenced by suppression of IgE and IgG1 and increase of IgG2a, both with regard to the antigen-specific ANoA response and the total serum Ig isotype pattern (see below).

Effect of rIL-12 and/or α-IL-4 treatment on Hg-induced IgG ANoA

A.SW mice treated for 1 week with Hg in combination with rIL-12 + α-IL-4 showed a significantly accelerated IgG-ANoA response compared with the Hg controls, both with regard to the fraction of ANoA positive mice (100% compared with 22%) ($P < 0{\cdot}05$; Fisher's exact test) and the titre ($P < 0{\cdot}01$) (Fig. 2a). The IgG-ANoA titre also continued to be increased significantly after 2 weeks' treatment. rIL-12 is important for the acceleration of ANoA development because 71% of mice given Hg + rIL-12 but none of the mice given Hg + α-IL-4 showed IgG-ANoA after 1 week's treatment (Fig. 2).

Effect of rIL-12 and/or α-IL-4 treatment on the isotype pattern of Hg-induced ANoA

The only significant effect of treatment with α-IL-4 alone on Hg-induced ANoA compared with the controls was an increase of IgG2b-ANoA after 3 weeks (Fig. 2).

In contrast, after 2 and 3 weeks of treatment with Hg in combination with rIL-12 + α-IL-4 the IgG1-ANoA titre was significantly ($P < 0{\cdot}01$) reduced compared to the Hg controls (Fig. 2).

After 2 weeks of treatment with Hg and either rIL-12 alone or in combination with α-IL-4, the IgG2a-ANoA titre was significantly higher ($P < 0{\cdot}001$ and $P < 0{\cdot}05$, respectively), compared to the titre in the Hg controls. The same pattern was seen after 3 weeks, although the difference was significant ($P < 0{\cdot}05$) only in the group treated with rIL-12 + α-IL-4 (Fig. 2).

The IgG2b-ANoA titre induced by Hg was significantly ($P < 0{\cdot}05$) increased after 3 weeks in mice given either rIL-12 or α-IL-4, or rIL-12 + α-IL-4 compared with the Hg controls (Fig. 2). After 2 weeks of Hg treatment, the mean IgG3-ANoA titre was higher in mice given rIL-12 or r-IL-12 + α-IL-4 compared with the Hg controls, but the difference was significant for the group given rIL-12 only ($P < 0{\cdot}05$) (data not shown).

Effect of rIL-12 and/or α-IL-4 treatment on the Hg-induced polyclonal B-cell activation

During the 3 weeks the Hg controls showed a reduction of the mean values for the PBA markers (IgM-α-ssDNA and IgM-α-DNP and serum IgM) of 14–41%, but the differences between the three time-points were not significant. Mice receiving rIL-12 showed increasing mean levels of all PBA markers during the 3 weeks of Hg treatment (19–93%), and they were significantly different compared to Hg controls after 2 and 3 weeks (Tables 1 and 2). Treatment with Hg + α-IL-4 + rIL-12 increased the mean level of the PBA markers 40–72% during 3 weeks' treatment, and the differences were significant for serum IgM and IgM-α-DNP compared with Hg controls (Tables 1 and 2). Treatment with Hg + α-IL-4 caused a maximum increase of 19–35% in the mean level of PBA markers, which was significantly higher for serum IgM compared with the Hg controls but significantly lower for both IgM-α-ssDNA and α-DNP compared with the groups given rIL-12 ± α-IL-4 (Tables 1 and 2). Therefore, rIL-12 treatment alone but also in addition with α-IL-4 clearly enhanced the polyclonal B-cell activation in HgIA, while the effect of α-IL-4 alone was weak and lower than after treatment with rIL-12 ± α-IL-4.

Effects of rIL-12 + α-IL-4 treatment on Th1 and Th2 associated Ig isotypes in HgIA

As evidence of Th2 suppression, after 1 week the Hg-treated mice receiving combined treatment with rIL-12 + α-IL-4 showed a significantly reduced serum IgE concentration compared to Hg controls (Table 2). The mean IgE concentration in this group was lower than in the group given Hg + α-IL-4. Treatment with rIL-12 alone suppressed the IgG1 concentration, similar to α-IL-4 ± rIL-12, after 1 week, but lowered the mean IgG1 concentration more than the two other treatment modes after 2 and 3 weeks, indicating that rIL-12 had a distinct Th2-suppressing effect. However, in the group given rIL-12 alone the IgE concentration did not decrease significantly after 1 week, and showed a significant increase after 2 weeks compared with α-IL-4 ± rIL-12 (Table 2), which indicates a specific effect of rIL-12 on serum IgE. Combined treatment with rIL-12 + α-IL-4 gave rise to the highest mean concentration of IgG2a after 2 and 3 weeks, and it was significantly different compared with the other treatment modes after 2 weeks. We interpret this as a Th1 switching effect of rIL-12.

Tissue IC deposits

The mean titre of IgM and C3 in the mesangial deposits was higher in all treatment groups compared with the Hg controls, but the difference did not reach statistical significance (Table 3). Treatment with rIL-12 enhanced the deposits of IgG and C3 induced by Hg in the renal and splenic vessel walls (Table 3). Treatment with α-IL-4 abolished the induction of IC deposits in the kidney vessel walls, but caused no significant change in the splenic deposits. Treatment with rIL-12 + α-IL-4 attenuated Hg-induced IC deposits in the renal vessels but had no effect on IC deposits in the splenic vessels compared with Hg controls.

DISCUSSION

The major objective of this study was to investigate if an autoimmune disease condition (HgIA) with characteristics of a Th2 type of reaction, but dependent on the Th1 cytokine IFN-γ [20], is aggravated by deviating the immune response pattern strongly from Th2 to Th1. We found that the Th1 deviation, accomplished by combined treatment with rIL-12 + α-IL-4, aggravated the HgIA by causing an earlier development of IgG-ANoA and by inducing a higher titre of IgG-ANoA. The group receiving Hg + rIL-12 also showed accelerated ANoA development,

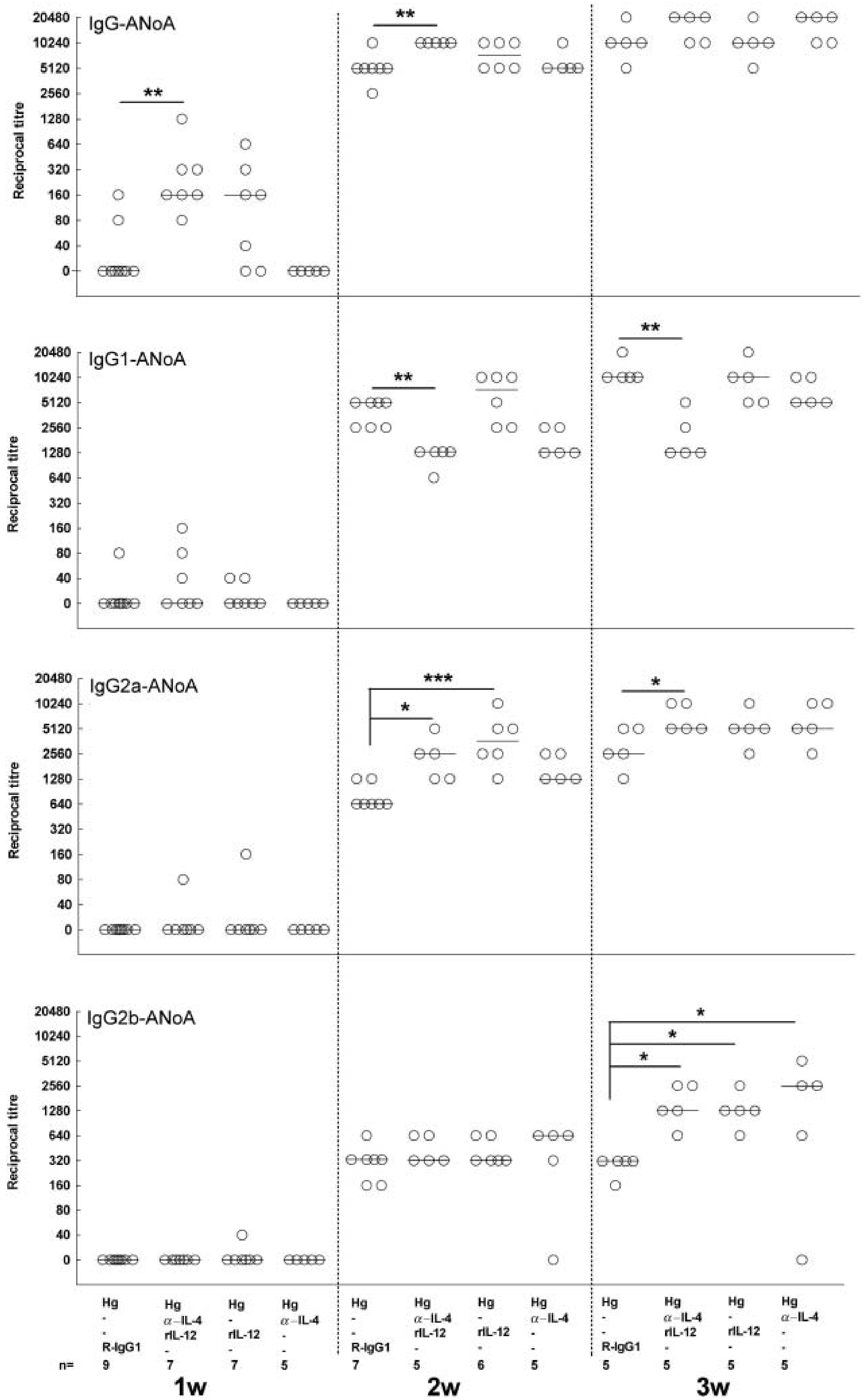

Fig. 2. The reciprocal titre of IgG, IgG1; IgG2a; IgG2b ANoA in the different groups 1, 2 and 3 weeks after onset of treatment. *, ** and *** denote significant differences ($P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively) compared with Hg controls as determined by Kruskal–Wallis test followed by Dunn's post-test.

Table 2. Serum Ig concentrations in the different treatment groups

Serum Ig conc.	Treatment group	Weeks after onset of treatment with Hg: *n*	1	*n*	2	*n*	3
IgE	Hg controls	9	29·55 ± 23·92	7	27·21 ± 25·53	5	14·64 ± 11·76
(μg/ml)	Hg + rIL-12 + α-IL-4	7	1·881 ± 1·302[b]	5	13·73 ± 11·71[d]	5	6·347 ± 7·859
	Hg + rIL-12	7	18·92 ± 8·957	6	53·55 ± 20·92	5	14·99 ± 10·35
	Hg + α-IL-4	5	6·671 ± 2·145[a]	5	15·70 ± 14·36[d]	5	4·467 ± 2·266
IgG1	Hg controls	9	0·786 ± 0·202	7	0·659 ± 0·078	5	0·779 ± 0·178
(mg/ml)	Hg + rIL-12 + α-IL-4	7	0·531 ± 0·118[b]	5	0·607 ± 0·066	5	0·637 ± 0·083
	Hg + rIL-12	7	0·571 ± 0·027[a]	6	0·563 ± 0·064[a]	5	0·558 ± 0·069
	Hg + α-IL-4	5	0·523 ± 0·091[a]	5	0·674 ± 0·043	5	0·712 ± 0·116
IgG2a	Hg controls	9	33·02 ± 9·210	7	22·17 ± 4·274	5	32·38 ± 7·044
(μg/ml)	Hg + rIL-12 + α-IL-4	7	25·86 ± 4·468	5	34·25 ± 4·524[c]	5	42·24 ± 14·56[d]
	Hg + rIL-12	7	23·35 ± 3·924[a]	6	24·68 ± 1·647[e]	5	23·37 ± 2·542
	Hg + α-IL-4	5	19·51 ± 1·673[b]	5	26·21 ± 2·167[e]	5	27·42 ± 4·638
IgM	Hg controls	9	0·193 ± 0·084	7	0·143 ± 0·025	5	0·113 ± 0·008
(mg/ml)	Hg + rIL-12 + α-IL-4	7	0·120 ± 0·014[a]	5	0·171 ± 0·025	5	0·169 ± 0·023[b]
	Hg + rIL-12	7	0·158 ± 0·017	6	0·189 ± 0·032[a]	5	0·189 ± 0·023[c]
	Hg + α-IL-4	5	0·129 ± 0·016	5	0·137 ± 0·019[d]	5	0·153 ± 0·025[a]

Significant differences in serum Ig concentration when comparing groups using ANOVA followed by Tukey's post-test. Values denote mean serum Ig concentration ± s.d. [a]$P < 0{\cdot}05$ *versus* Hg controls; [b]$P < 0{\cdot}01$ *versus* Hg controls; [c]$P < 0{\cdot}001$ *versus* Hg controls; [d]$P < 0{\cdot}05$ *versus* Hg + rIL-12; [e]$P < 0{\cdot}01$ *versus* Hg + rIL-12 + α-IL-4.

Table 3. Immune-complex deposits in A.SW mice treated mercuric chloride and rIL-12 and/or anti-IL-4 and controls

	Kidney glomerular mesangium			Vessel walls: Kidney				Spleen			
				IgG		C3		IgG		C3	
Treatment group[a]	IgG[b]	IgM[c]	C3[b]	n[d]	titre[c]	n[d]	titre[c]	n[d]	titre[c]	n[d]	titre[c]
Hg controls	320 ± 196	1·2 ± 0·45	832 ± 429	5/5[e]	1·0 ± 0	3/5	0·6 ± 0·55	4/5	2·2 ± 1·3	4/5	1·4 ± 0·89
Hg + rIL-12 + α-IL-4	160 ± 98	1·6 ± 0·89	2560 ± 1568	1/5[f]	0·2 ± 0·45[g]	1/5[f]	0·2 ± 0·45[g]	5/5	2·8 ± 0·45	5/5	1·4 ± 0·55
Hg + rIL-12	256 ± 88	2·0 ± 0	2816 ± 1402	5/5[h]	2·4 ± 0·55[i]	5/5[h]	2·6 ± 0·55[i]	5/5	3·8 ± 0·45[j]	5/5	3·0 ± 0
Hg + α-IL-4	144 ± 36	2·0 ± 0	1792 ± 701	0/5[k]	0	0/5	0	5/5	2·6 ± 0·55	5/5	0·8 ± 0·45

[a]See Fig. 1; [b]mean reciprocal titre ± s.d.; [c]mean titre ± s.d. (grading 0–4); [d]fraction of mice with immune complex deposits; e, significantly different compared with Hg + rIL-12 + α-IL-4 ($P < 0{\cdot}05$, Fisher's exact test); [f]significantly different compared with Hg + rIL-12 ($P < 0{\cdot}05$, Fisher's exact test); [g]significantly different compared with Hg + rIL-12 ($P < 0{\cdot}05$; Kruskal–Wallis and Dunn's post-test); [h]significantly different compared with Hg + α-IL-4 ($P < 0{\cdot}01$, Fisher's exact test); [i]significantly different compared with Hg + α-IL-4 ($P < 0{\cdot}01$, Kruskal–Wallis and Dunn's post-test); [j]significantly different compared with Hg controls ($P < 0{\cdot}05$, Kruskal–Wallis and Dunn's post-test); [k]significantly different compared with Hg controls ($P < 0{\cdot}01$, Fisher's exact test).

although the response was less homogeneous than after combined treatment. In contrast, mice given only α-IL-4 + Hg showed no acceleration of IgG-ANoA development and the titre was not increased, which is in accordance with previous observations [18], and concurs with findings in mice with a targeted mutation of the IL-4 gene [20]. Therefore, active deviation by the Th1-promoting IL-12 cytokine is crucial for aggravating the autoimmune response in HgIA.

With regard to the isotype pattern of the Hg-induced ANoA, our results after treatment with rIL-12 + α-IL-4 are in agreement with those observed after administration of α-IL-4 to Hg-treated mice, namely an increase in IgG2a, IgG2b and IgG3 ANoA titres, but a reduced titre of IgG1 ANoA [18]. The increase in IgG2a, IgG2b and IgG3 ANoA titres was also seen after treatment with rIL-12 + Hg (present study), which is in agreement with the effect of rIL-12 treatment in another antigen-specific response [30].

Our results are at variance with those of Bagenstose *et al.*, who reported that treatment with rIL-12 in combination with Hg suppressed the ANoA response of all IgG isotypes [31]. This discrepancy may be due to differences in the rIL-12 regimen, because we treated the mice with rIL-12 for 10 days, while Bagenstose *et al.* treated the mice for 4 days [31]. In addition, in the group to which we gave rIL-12 + α-IL-4, suppression of IL-4, a cytokine abundantly expressed in HgIA [21], might have allowed for a brisker development of Th1 cells, as IL-4 has an inhibiting effect on Th1 cells [22,32].

With regard to the effect of the serum immunoglobulin isotypes as markers of Th1/Th2 balance, injections of rIL-12 + α-IL-4 reduced the Th2-dependent serum IgE and IgG1 levels, indicating a Th2 suppression, and increased the Th1-dependent serum IgG2a, indicating a Th1 switching. Treatment with a-IL-4 alone had an expected suppressing effect on serum IgG1 and IgE concentrations [18]. Treatment with rIL-12 alone suppressed serum IgG1, as observed already in HgIA [31]. However, rIL-12 alone increased the serum IgE level, which is unexpected given the general assumption that rIL-12 down-regulates switching to both the IgG1 and IgE isotype [33]. Germann *et al.* showed that rIL-12 may fail to suppress or even enhance the effect of IL-4 on serum IgE, depending on both the strain and the dose of rIL-12 used [33]. Furthermore, Bagenstose *et al.* reported that rIL-12 enhanced Hg-induced IL-4 production in A.SW mice and caused an increased mean serum IgE level, although the difference was not significant compared with mice given Hg alone [31]. The stronger increase of IgE production in the present study might be related to the longer treatment time with rIL-12 in our study (see above). This might be related to the stimulating or even dominating effect on Th2 development which has been reported when IL-12 is present *in vitro* [34,35] or *in vivo* [33,36] together with IL-4.

With regard to other HgIA parameters, treatment with rIL-12 increased the renal and splenic vessel wall IC deposits. The mechanism behind the development of vessel wall deposits in HgIA is not known, but the splenic deposits are more stable and less affected by manipulating HgIA compared with the renal deposits [37,38]. This was also observed in the present study, because treatment with α-IL-4 inhibited the Hg-induced vessel wall IC deposits in the kidney but did not affect significantly the deposits in the spleen. Treatment with rIL-12 + α-IL-4 showed that the effect of IL-4 dominated, as the Hg-induced renal IC deposits were severely reduced, but the deposits in the spleen remained unchanged compared with Hg controls.

Interestingly, there was a co-variation between vessel wall IC deposits and PBA, another feature of HgIA [39]. Treatment with rIL-12 + Hg enhanced PBA compared with Hg controls, while α-IL-4 alone or in combination with rIL-12 did not affect consistently the degree of PBA induced by Hg. This co-variation between PBA and development of IC deposits has also been observed when the HgIA model is modified by other means, such as mercury species [40], dose or gender [37]. Because PBA induces antibodies of the IgM isotype, and the deposits in addition consist of IgG and C3, PBA must act as a co-factor.

Our main question, whether a strong deviation of the immune response towards Th1 in HgIA would aggravate disease manifestations, has been dealt with previously only indirectly in conjunction with studies aiming at down-regulating the Th2 response by inhibiting Th2 or stimulating Th1 [18,19,31]. Acceleration of the disease has not been found previously, although α-IL-4 treatment [18] augmented the IgG titre of ANoA induced by Hg. The observations in other Th1-dependent autoimmune disease models, such as experimental autoimmune myasthenia gravis [6], experimental arthritis [5] and autoimmune diabetes in non-obese diabetic (NOD) mice [41], indicate that treatment with IL-12 aggravates and/or accelerates disease manifestations. In these models the proposed mechanism includes a preferential expansion of IFN-γ secreting cells. While we did not measure IFN-γ secreting cells, a correlation exists between IFN-γ gene expression and the severity of HgIA [20].

Similarly, *in vivo* treatment of SJL (H-2^s) mice with rIL-12 aggravates experimental allergic encephalomyelitis (EAE) after adoptive transfer of antigen-stimulated lymph node cells [42]. However, in the EAE model the effect of rIL-12 treatment is IFN-γ independent [43]. These authors suggested that IL-12 plays an important role by suppressing IL-10 expression which would otherwise inhibit the development of autoimmune effector cells [43]. The concept of an immunoregulatory circuit comprising IL-12 and IL-10 which may modulate autoimmune diseases is interesting, as we have found previously that IL-10 expression is up-regulated in Hg-treated resistant A.TL mice [21]. This implies that IL-10 may play a role in maintaining the self-tolerance in resistant strains during Hg treatment.

We conclude that strong deviation of the immune response from Th2 towards Th1 using directly Th1-inducing cytokines does not provide protection from the development of HgIA, but instead accelerates and aggravates several of the disease parameters, including antinucleolar antibodies, vessel wall IC deposits and PBA.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Swedish Research Council, Branch of Medicine (project no. 9453). The technical assistance of Christer Bergman, Marie-Louise Eskilsson, Christina Karlsson and Elham Nikookhesal is gratefully acknowledged.

REFERENCES

1 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; **136**:2348–57.

2 Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. Immunol Res 1999; **20**:147–61.

3 Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol 1989; **124**:132–43.

4 Katz JD, Benoist C, Mathis D. T helper cell subsets in insulin-dependent diabetes. Science 1995; **268**:1185–8.

5 Germann T, Szeliga J, Hess H *et al.* Administration of interleukin 12 in combination with type II collagen induces severe arthritis in DBA/1 mice. Proc Natl Acad Sci USA 1995; **92**:4823–7.

6 Sitaraman S, Metzger DW, Belloto RJ, Infante AJ, Wall KA. Interleukin-12 enhances clinical experimental autoimmune myasthenia gravis in susceptible but not resistant mice. J Neuroimmunol 2000; **107**:73–82.

7 Lafaille JJ. The role of helper T cell subsets in autoimmune diseases. Cytokine Growth Factor Rev 1998; **9**:139–51.

8 Rapoport MJ, Jaramillo A, Zipris D *et al.* Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. J Exp Med 1993; **178**:87–99.

9 Pakala SV, Kurrer MO, Katz JD. T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. J Exp Med 1997; **186**:299–306.

10 Lafaille JJ, Keere FV, Hsu AL *et al.* Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. J Exp Med 1997; **186**:307–12.

11 Hultman P, Eneström S, Pollard KM, Tan EM. Antifibrillarin antibodies in mercury-treated mice. Clin Exp Immunol 1989; **78**:470–7.
12 Takeuchi K, Turley SJ, Tan EM, Pollard KM. Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. J Immunol 1995; **154**:691–9.
13 Pollard K, Lee D, Casiano C, Blüthner M, Johnston M, Tan E. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. J Immunol 1997; **158**:3521–8.
14 Kubicka-Muranyi M, Griem P, Lübben B, Rottmann N, Lührmann R, Gleichmann E. Mercuric chloride-induced autoimmunity involves upreglated presentation by spleen cells of altered and unaltered nucleolar self antigens. Int Arch Allergy Appl Immunol 1995; **108**:1–10.
15 Pollard KM, Pearson DL, Bluthner M, Tan EM. Proteolytic cleavage of a self-antigen following xenobiotic-induced cell death produces a fragment with novel immunogenic properties. J Immunol 2000; **165**:2263–70.
16 Hultman P, Johansson U, Dagnaes-Hansen F. Murine mercury-induced autoimmunity: the importance of T-cells. J Autoimmun 1995; **8**:809–24.
17 Goldman M, Druet P, Gleichmann E. A role for Th2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. Immunol Today 1991; **12**:223–7.
18 Ochel M, Vohr HW, Pfeiffer C, Gleichmann E. IL-4 is required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride. J Immunol 1991; **146**:3006–11.
19 Doth M, Fricke M, Nicoletti F, Garotta G, van Velthuysen M-L, Bruijn JA. Genetic differences in immune reactivity to mercuric chloride ($HgCl_2$): immunosuppression of H-2^d mice is mediated by interferon-gamma (IFN-γ). Clin Exp Immunol 1997; **109**:149–56.
20 Kono D, Balomenos D, Perason D *et al.* Systemic autoimmunity indiced by mercury is dependent on IFN-gamma but not Th1/Th2 imbalance. J Immunol 1998; **161**:234–40.
21 Häggqvist B, Hultman P. Murine metal-induced systemic autoimmunity. baseline and stimulated cytokine mRNA expression in genetically susceptible and resistant strains. Clin Exp Immunol 2001; **126**:157–64.
22 Nakamura T, Kamogawa Y, Bottomly K, Flavell RA. Polarization of IL-4- and IFN-gamma-producing $CD4^+$ T cells following activation of naive $CD4^+$ T cells. J Immunol 1997; **158**:1085–94.
23 Heinzel FP, Rerko RM. Cure of progressive murine leishmaniasis: interleukin 4 dominance is abolished by transient CD4 (+) T cell depletion and T helper cell type 1-selective cytokine therapy. J Exp Med 1999; **189**:1895–906.
24 Ohara J, Paul WE. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. Nature 1985; **315**:333–6.
25 Hultman P, Eneström S. Murine mercury-induced immune-complex disease: effect of cyclophosphamide treatment and importance of T-cells. Br J Exp Pathol 1989; **70**:227–36.
26 Johansson U, Hansson-Georgiadis H, Hultman P. Murine silver-induced autoimmunity: silver shares induction of antinucleolar antibodies with mercury, but causes less activation of the immune system. Int Arch Allergy Appl Immunol 1997; **113**:432–43.
27 Jachez B, Montecino-Rodriguez E, Fonteueau P, Loor F. Partial expression of the *lpr* locus in the heterozygote state. Immunology 1988; **64**:31–6.
28 Izui S, Kobayakawa T, Zryd MJ, Louis J, Lambert PH. Mechanism for induction of anti-DNA antibodies by bacterial lipopolysaccharides in mice. II. Correlation between anti-DNA induction and polyclonal antibody formation by various polyclonal B lymphocyte activators. J Immunol 1977; **119**:2157–68.
29 Eneström S, Hultman P. Immune-mediated glomerulonephritis induced by mercuric chloride in mice. Experientia 1984; **40**:1234–40.
30 Germann T, Bongartz M, Dlugonska H *et al.* Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. Eur J Immunol 1995; **25**:823–9.
31 Bagenstose LM, Salgame P, Monestier M. IL-12 down-regulates autoantibody production in mercury-induced autoimmunity. J Immunol 1998; **160**:1612–7.
32 Lee JD, Rhoades K, Economou JS. Interleukin-4 inhibits the expression of tumour necrosis factors alpha and beta, interleukins-1 beta and -6 and interferon-gamma. Immunol Cell Biol 1995; **73**:57–61.
33 Germann T, Guckes S, Bongartz M *et al.* Administration of IL-12 during ongoing immune responses fails to permanently suppress and can even enhance the synthesis of antigen-specific IgE. Int Immunol 1995; **7**:1649–57.
34 Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 1993; **260**:547–9.
35 Schmitt E, Hoehn P, Germann T, Rude E. Differential effects of interleukin-12 on the development of naive mouse $CD4^+$ T cells. Eur J Immunol 1994; **24**:343–7.
36 Wang ZE, Zheng S, Corry DB *et al.* Interferon gamma-independent effects of interleukin 12 administered during acute or established infection due to *Leishmania major*. Proc Natl Acad Sci USA 1994; **91**:12932–6.
37 Hultman P, Nielsen JB. The effect of dose, gender, and non-H-2 genes in murine mercury-induced autoimmunity. J Autoimmun 2001; **17**:27–37.
38 Hultman P, Eneström S. Dose–response studies in murine mercury-induced autoimmunity and immune-complex disease. 1992; 113:199–208.
39 Johansson U, Hansson-Georgiadis H, Hultman P. The genotype determines the B cell response in mercury-treated mice. Int Arch Allergy Appl Immunol 1998; **116**:295–305.
40 Hultman P, Hansson-Georgiadis H. Methyl mercury-induced autoimmunity in mice. Toxicol Appl Pharmacol 1999; **154**:203–11.
41 Trembleau S, Penna G, Bosi E, Mortara A, Gately MK, Adorini L. Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. J Exp Med 1995; **181**:817–21.
42 Leonard JP, Waldburger KE, Goldman SJ. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. J Exp Med 1995; **181**:381–6.
43 Segal BM, Dwyer BK, Shevach EM. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. J Exp Med 1998; **187**:537–46.