

# Mercury exposure as a model for deviation of cytokine responses in experimental Lyme arthritis: HgCl<sub>2</sub> treatment decreases T helper cell type 1-like responses and arthritis severity but delays eradication of *Borrelia burgdorferi* in C3H/HeN mice

C. Ekerfelt,\*† M. Andersson,\*†  
A. Olausson,\*† S. Bergström<sup>§</sup> and  
P. Hultman<sup>†‡</sup>

\*Division of Clinical Immunology, †Unit of  
Autoimmunity and Immune Regulation and

‡Division of Molecular and Immunological  
Pathology, Department of Molecular and Clinical  
Medicine, Faculty of Health Sciences, University  
Hospital, Linköping, Sweden, and §Department of  
Microbiology, University of Umeå, Umeå, Sweden

Accepted for publication 25 June 2007

Correspondence: C. Ekerfelt, Division of Clinical Immunology, Department of Molecular and Clinical Medicine, Faculty of Health Sciences, University Hospital, SE-581 85 Linköping, Sweden.

E-mail: Christina.Ekerfelt@imk.liu.se

## Introduction

Lyme borreliosis is a complex infection caused by the bacterium *Borrelia burgdorferi* and related species. The clinical characteristics of the disease differ depending on the subspecies and the geographical location [1,2]. A common manifestation in Europe is neuroborreliosis, whereas arthritis is a more general outcome in the United States [1,2]. The course of the illness differs among individuals, ranging from an asymptomatic infection [3–5] to so-called 'chronic borreliosis', i.e. symptoms persisting for more than 6 months despite adequate antibiotic treatment [6–10].

The mechanisms leading to chronic borreliosis are not known and remain a subject of controversy [11]. Current

## Summary

Lyme borreliosis is a complex infection, where some individuals develop so-called 'chronic borreliosis'. The pathogenetic mechanisms are unknown, but the type of immune response is probably important for healing. A strong T helper cell type 1 (Th1)-like response has been suggested as crucial for eradication of *Borrelia* and for avoiding development of chronic disease. Many studies aimed at altering the Th1/Th2 balance in Lyme arthritis employed mice deficient in cytokine genes, but the outcome has not been clear-cut, due possibly to the high redundancy of cytokines. This study aimed at studying the importance of the Th1/Th2 balance in murine *Borrelia* arthritis by using the Th2-deviating effect of subtoxic doses of inorganic mercury. Ninety-eight C3H/HeN mice were divided into four groups: *Borrelia*-infected (Bb), *Borrelia*-infected exposed to HgCl<sub>2</sub> (BbHg), controls exposed to HgCl<sub>2</sub> alone and normal controls. Mice were killed on days 3, 16, 44 and 65 post-*Borrelia* inoculation. Arthritis severity was evaluated by histology, spirochaetal load determined by *Borrelia* culture, IgG2a- and IgE-levels analysed by enzyme-linked immunosorbent assay (ELISA) and cytokine-secreting cells detected by enzyme-linked immunospot (ELISPOT). BbHg mice showed less severe histological arthritis, but delayed eradication of spirochaetes compared to Bb mice, associated with increased levels of IgE (Th2-induced) and decreased levels of IgG2a (Th1-induced), consistent with a Th2-deviation. Both the numbers of Th1 and Th2 cytokine-secreting cells were reduced in BbHg mice, possibly explained by the fact that numbers of cytokine-secreting cells do not correlate with cytokine concentration. In conclusion, this study supports the hypothesis that a Th1-like response is required for optimal eradication of *Borrelia*.

**Keywords:** arthritis, *Borrelia burgdorferi*, cytokines/interleukins, immunoglobulins

hypotheses include (i) long-term persistence of the spirochaete (despite treatment with antibiotics) [12–14], (ii) an aberrant immune response leading to chronic inflammation (possibly including post-infectious autoimmunity) [15,16] or (iii) genomic differences among subspecies of *B. burgdorferi* [17,18]. Irrespective of which of these hypotheses holds true, the establishment of a certain quality of the immune response against *Borrelia* is of crucial importance for resolution of the disease.

The *Borrelia*-specific T cell response in patients with Lyme borreliosis is predominantly a T helper cell type 1 (Th1)-like response [19–26], especially in target organs such as joints and the central nervous system [22,25,27–29]. Mice of the BALB/c strain eradicate the spirochaetes and resolve arthritis

more rapidly than C3H/HeN mice, a phenomenon associated with a stronger initial Th1-like response in BALB/c mice. The stronger Th1 response in BALB/c mice was down-regulated due to the ensuing Th2-like response, in contrast to the gradual increase in the Th1-like response in C3H/HeN mice [30]. Studies on the importance of Th1/Th2 responses using mice with targeted deletions of Th1- or Th2-associated cytokine genes have shown divergent results [31–35]. This may be due to the high degree of functional redundancy, whereby silencing of a single cytokine gene may be compensated for by other cytokine genes.

Exposure to subtoxic doses of mercury leads to a Th2 deviation in most mouse strains [36–42], which is a robust way of influencing the Th1/Th2 balance [43]. This model has been used previously to assess the importance of the Th1/Th2 balance in murine infections [44]. For example, resistance to *Leishmania major* in mice is dependent upon an adequate initial interferon (IFN)- $\gamma$  production, whereas an early interleukin (IL)-4 production results in a non-healing phenotype [45,46], resembling our working hypothesis for Lyme borreliosis [47–49]. Treatment with HgCl<sub>2</sub> prior to *L. major* infection is associated with increased production of IL-4 and IgE, and causes increased foot pad swelling and parasite burden [44].

In the present study we used exposure to mercury as a means of inducing Th2-deviation in *Borrelia*-susceptible C3H/HeN mice to test the hypothesis that a strong Th1-like response is required early in infection for optimal eradication of *Borrelia* and to avoid chronic inflammatory Th1-like responses, leading potentially to chronic borreliosis. Our results support the hypothesis that a Th1-like response is crucial for eradication of *Borrelia* spirochaetes.

## Materials and methods

### Mice

Ninety-eight female C3H/HeN mice, susceptible to *Borrelia* infection, were obtained from Taconic M&B (Ry, Denmark). The mice were housed in groups of seven in steel-wire cages under a 12-h light/12-h dark cycle at the Animal Unit, Faculty of Health Sciences, Linköping University, Linköping with *ad libitum* access to pellets (Type R 36, Lactamin, Vadstena, Sweden). The mice were 9–10 weeks old at the onset of the experiment. Mice were divided into four groups: 28 normal controls, 14 mercury-treated controls (Hg), 28 infected with *Borrelia* only (Bb) and 28 treated with mercury and infected with *Borrelia* (BbHg).

### Treatment with mercury

Forty-two mice were exposed to mercury at non-toxic doses (approximately 190  $\mu$ g Hg/kg body weight/day) by dissolving 10 mg/l HgCl<sub>2</sub> (Fluka Chemie, Buchs, Switzerland) in the drinking water, given *ad libitum*, for 14 days. Fresh solutions

were given once a week. Treatment with Hg started simultaneously in the group to be treated with Hg only and in the group also to be treated with *B. burgdorferi*. Treatment with Hg continued for 3 more days after the day of inoculation of *Borrelia*, in total 14 days.

### *Borrelia* spirochaetes

*B. burgdorferi* N40, a proven infectious strain [50], was grown in Barbour Stoenner Kelly (BSK II) medium supplemented with 6% normal rabbit serum (Department of Microbiology, Umeå University, Sweden) at 34°C for 72 h prior to infection.

### *Borrelia* infection

Fifty-six C3H/HeN mice, 28 of which were not exposed to mercury, were anaesthetized with Isofluran (Forene®; Abbott Scandinavia AB, Solna, Sweden) and injected subcutaneously at the tail root with 10<sup>5</sup> *B. burgdorferi* N40 in 100  $\mu$ l BSK II. In the 28 BbHg mice the inoculation took place 11 days after onset of Hg treatment.

### Experimental plan

Seven animals from each of the Bb, BbHg and normal control groups were killed on days 3, 16, 44 and 65 post-immunization (p.i.) with *Borrelia* spirochaetes. The mice exposed to mercury only (Hg) were killed on days 3 and 65. On the due day the mice were anaesthetized with Isofluran (Forene®; Abbott Scandinavia AB), a blood sample for Ig analysis was obtained by retroorbital puncture, and the mice were killed by cervical dislocation. Ear and tibiotarsal joints were taken for evaluation of spirochaetal load. Inguinal lymph nodes were taken for cytokine analysis.

### Clinical evaluation

From day 0 of mercury exposure, 11 days before inoculation with *B. burgdorferi*, all mice were weighed and the average thickness of tibiotarsal joints of both hind legs were measured using an electronic digital calliper (Biltema AB, Helsingborg, Sweden) every other day. Measurements were taken at the thickest portion of the joint with the joint extended. Data are reported as the change in swelling. Swelling was defined as increase in joint diameter at the actual day compared with the diameter before onset of treatment.

### Histopathology of the tibiotarsal joints

The left rear tibiotarsal joint was taken from each mouse for histology. Samples were fixed in formaldehyde 4% (Histolab Products AB, Göteborg, Sweden), decalcified, embedded in paraffin, and sections were stained with haematoxylin and eosin. Slides were analysed blinded by one of the authors

(P. H.) and scored for neutrophil infiltration, mononuclear cell infiltration, synovia thickness and cartilage hyperplasia. The scores for all parameters were: 0, normal; 0.5, minimal; 1, slight; 2, moderate; and 3, severe.

### Spirochaetal load

#### *Culture of Borrelia spirochaetes*

To determine the extent of spirochaetal dissemination, biopsies from both ears, the left kidney, the right tibiotarsal joint and blood were cultured in BSK II medium supplemented with 100 µg/ml phosphomycin and 50 µg/ml rifampicin (both purchased from Sigma-Aldrich, Steinheim, Germany) at 34°C. The cultures were centrifuged at 900 g for 10 min at room temperature (RT) and counted in a Bürker chamber by phase contrast microscopy after 5 days.

#### Cytokine analyses

The lymph nodes were ground through a sterile strainer into Click's medium (Sigma, St Louis, MO, USA) supplemented with 0.04 mM β-mercaptoethanol (Sigma), 200 µM L-glutamine (Sigma-Aldrich), 100 U penicillin/ml (Invitrogen, Stockholm, Sweden) and 100 µg streptomycin/ml (Invitrogen). The suspension was centrifuged at 4°C at 400 g for 10 min. The cells were washed once in Click's medium, centrifuged as described above, resuspended in Click's medium with 10% fetal calf serum (FCS) (Sigma-Aldrich, Steinheim, Germany), referred to hereafter as 10% Click's medium, counted under a phase contrast microscopy and diluted to a density of  $1 \times 10^6$  lymphocytes/ml in 10% Click's medium. To analyse the cytokine production for mouse IL-4, IL-12p70 and IFN-γ, enzyme-linked immunospot assay (ELISpot<sup>PLUS</sup>) kits (Mabtech AB, Nacka Strand, Sweden) were used according to the manufacturer's instructions. Cell suspension (100 µl containing  $1 \times 10^5$  cells) with 10% Click's medium (50 µl) or Osp-enriched fraction (OF) of *B. burgdorferi* (50 µl), prepared as described previously [51], were added to each well and incubated in 5% CO<sub>2</sub> at 37°C for 2 days. Cell cultures were run in duplicate. Stimulation with OF was used to evaluate the number of *Borrelia*-specific cytokine-producing cells. The mitogen concanavalin A (ConA, 40 µg/ml, Sigma) was used as a positive control, while wells with 10% Click's medium only, without cells, worked as negative controls. The spots were counted by dissection light microscopy. ConA stimulation always elicited strong responses for all cytokines. No spots were found in the negative control wells.

#### Immunoglobulin analyses

Blood samples were taken for assessment of serum IgE and IgG2a concentration to determine the Th1/Th2 status [52]. Blood was centrifuged at 600 g for 10 min at room

temperature (RT), the serum removed and frozen in -70°C. IgE analyses were performed by enzyme-linked immunosorbent assay (ELISA) as described previously [53] and IgG2a was analysed utilizing a commercial IgG2a kit (Bethyl Laboratories Inc., Montgomery, TX, USA).

#### Statistical analysis

All data presented over time are medians. The spread of data is shown as interquartile range. Data were examined by non-parametric Mann-Whitney rank sum test. *P*-values less than 0.05 were considered significant and *P*-values  $> 0.05 < 0.1$  were considered a trend.

#### Ethics

The Ethics Committee for Animal Experimentation in Linköping approved this study.

### Results

#### Clinical signs

##### *Weight*

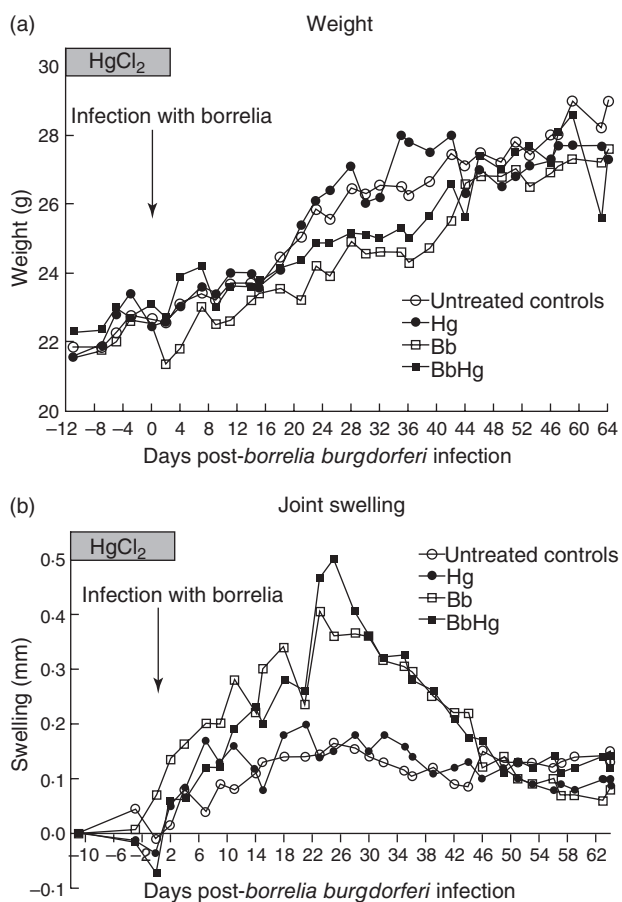
Normal control mice and mice treated with HgCl<sub>2</sub> alone had similar weight curves with a steady weight gain during the experiment, whereas Bb and BbHg mice showed a smaller weight gain compared with normal control mice and mice treated with HgCl<sub>2</sub> only (Fig. 1a, most pronounced in Bb *versus* controls on day 11 p.i., *P* = 0.004; in BbHg *versus* controls on day 36 p.i., *P* = 0.021). There were no differences in weight between Bb and BbHg mice at any time during the experiment. After day 44 p.i. the weight curves converged for all four groups of mice.

##### *Joint diameter*

In both Bb and BbHg mice the swelling of the right hind tibiotarsal joints started to increase on day 9 p.i. and reached a maximum on days 23–28 (*P* < 0.001 for both Bb and BbHg compared to controls, Fig. 1b). The swelling started to decline in both groups after day 28 and was not significantly different from the normal control mice on day 44 p.i. The difference in swelling was not significant (*P* > 0.05) between the Bb and BbHg groups at any time. The group treated with HgCl<sub>2</sub> alone showed no significant difference in joint diameter compared with the normal control group.

#### Histopathology

Histological assessment of the left rear tibiotarsal joints in the BbHg group of mice showed no or only minimal infiltration of inflammatory cells, no increased tendon sheath



**Fig. 1.** Weight (a) and joint swelling (b) in C3H/HeN mice in the four groups of mice during the experiment. Normal controls; Hg, controls exposed to HgCl<sub>2</sub>; Bb, infected with *Borrelia burgdorferi* N40; BbHg, infected with *B. burgdorferi* N40 and exposed to HgCl<sub>2</sub> prior to infection. Joint swelling was calculated as the difference between the joint measure for each day and the first measure. Data represent median values.

thickness and no cartilage hyperplasia on day 16 p.i. (Fig. 2c, Table 1). In contrast, at this time all mice in the Bb group showed a slight–severe infiltration of mononuclear cells, increased synovial thickness, and in many instances slight or moderate infiltration of neutrophils and cartilage hyperplasia (Fig. 2b, Table 1). The difference was significant between Bb and BbHg mice for all parameters except infiltration of neutrophils (Table 1). On days 3, 44 and 65 p.i. no differences were found between the Bb and BbHg groups ( $P > 0.05$ ). On day 65 p.i., histological assessment showed normal joints in five of seven mice in both the Bb and BbHg groups, whereas two mice in each group still showed some infiltration of mononuclear cells, increases in tendon sheath thickness and cartilage hyperplasia (data not shown).

### Spirochaetal load

Spirochaetes were detected in cultures from both ear(s) and the right rear hind joint in Bb as well as BbHg mice on days 16 and 44 p.i. (data not shown). On day 44 p.i., BbHg mice showed a trend towards higher spirochaete burden in both ear and joint compared to Bb mice ( $P = 0.061$  and  $P = 0.063$ , respectively) (Fig. 3). No spirochaetes were detected in cultures taken on days 3 or 65 p.i. (data not shown). Spirochaetes were not detected in cultures from kidney or blood at any time during the experiment.

### Immunoglobulin isotypes

#### Serum IgE

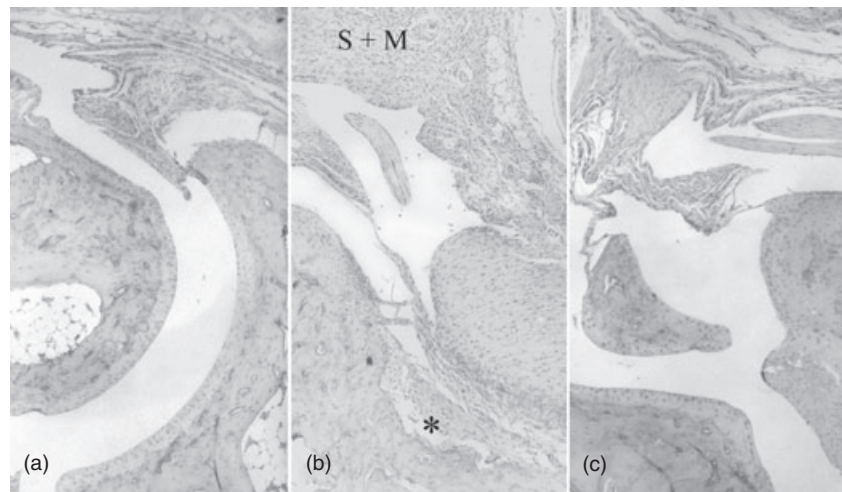
Mice treated with Hg alone had ninefold higher serum IgE levels compared to normal control mice on day 3 p.i. ( $P = 0.001$ ). The increase in BbHg mice was 12-fold compared to normal controls as well as the Bb mice ( $P = 0.001$ , Fig. 4a). On day 16 p.i., the increase of IgE in both Bb and

**Table 1.** Histopathological assessment of arthritis severity in tibiotarsal joint sections from *Borrelia burgdorferi*-infected C3H/HeN mice 16 and 44 days after infection.

	16 days p.i.			44 days p.i.		
	Bb <i>n</i> = 7	BbHg <i>n</i> = 7	<i>P</i> -value	Bb <i>n</i> = 7	BbHg <i>n</i> = 7	<i>P</i> -value
Neutrophils infiltration	0 (0–2)	0 (0–0)	n.s.	0 (0–2)	0 (0–0)	n.s.
Mononuclear cell infiltration	1 (1–3)	0 (0–0.5)	$P = 0.001$	0 (0–1)	1 (0–1)	n.s.
Synovia thickness	2 (1–3)	0 (0–0.5)	$P = 0.001$	1 (1–2)	2 (0–3)	n.s.
Cartilage hyperplasia	1 (0–1)	0 (0–0)	$P = 0.026$	0 (0–1)	1 (0–1)	n.s.

Median values (range in parenthesis) of scores are given. The scores for all parameters ranged from 0 (normal) to 3 (severe). p.i., post-immunization, Bb, *B. burgdorferi*-infected mice, BbHg, *B. burgdorferi*-infected mice treated with HgCl<sub>2</sub>. *P*-values refer to comparisons using the Mann–Whitney *U*-test; n.s.: non-significant.

**Fig. 2.** Histology from the right hindleg joint of C3H mice. (a) Normal control mouse. Ordinary synovia and preserved cartilage–bone interface. (b) Sixteen days after inoculation with *Borrelia burgdorferi*. Hyperplasia of synovia (S) with massive infiltration of mainly mononuclear inflammatory cells (M). Cartilage destroyed with superficial erosion of the bone structure (\*). (c) Sixteen days after inoculation of *B. burgdorferi* and 27 days after onset of mercury treatment. Minimal synovial hyperplasia with scant inflammatory cells and preserved cartilage–bone interface.

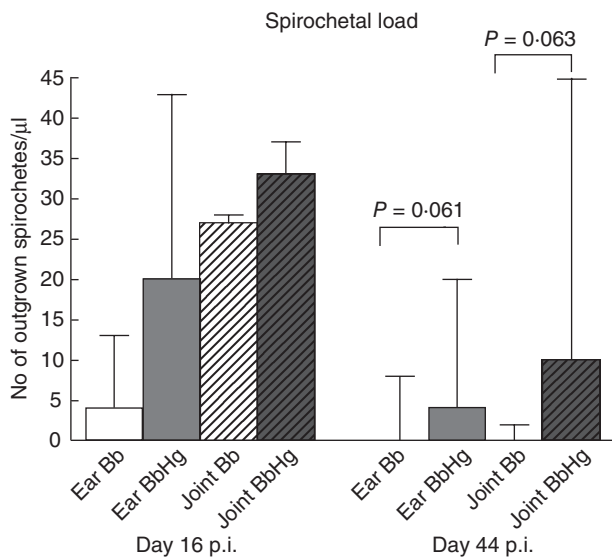


BbHg mice was three- to fourfold compared to normal controls ( $P = 0.018$  and  $P = 0.003$ , respectively). On day 44 p.i. the IgE level was twofold higher in BbHg and Bb mice ( $P = 0.038$ , Fig. 4a) compared to normal controls, i.e. there was no difference between the BbHg and Bb groups. No further differences in IgE levels were seen.

#### Serum IgG2a

Bb, BbHg and Hg mice showed a twofold increase in serum IgG2a level 3 days p.i. compared with normal controls

(Fig. 4b). On day 16 p.i. the serum IgG2a level was increased sixfold in both Bb and BbHg mice compared to normal controls (both  $P = 0.017$ ). On day 44 p.i. the increase was five- and 10-fold in Bb and BbHg mice compared with the normal controls, respectively ( $P = 0.004$  and  $P = 0.001$ , respectively, Fig. 4b), i.e. the level in Bb mice was twice as high as in BbHg mice ( $P = 0.004$ , Fig. 4b). On day 65 the mice treated with Hg alone showed no increase in serum IgG2a, while Bb and BbHg mice showed a seven- and fivefold increase compared to the normal controls ( $P = 0.001$ , respectively).



**Fig. 3.** Effect of mercury exposure on spirochaetal load. Numbers of *Borrelia* spirochaetes in ear and tibiotarsal joint from C3H/HeN mice 16 and 44 days after experimental infection with *Borrelia burgdorferi* N40. Bb, mice infected with *B. burgdorferi*; BbHg, mice infected with *B. burgdorferi* and treated with HgCl<sub>2</sub> prior to infection. Data represent median values from seven mice in each group. Error bars indicate interquartile range.  $P$ -values refer to comparisons using the Mann–Whitney  $U$ -test.

#### Cytokines

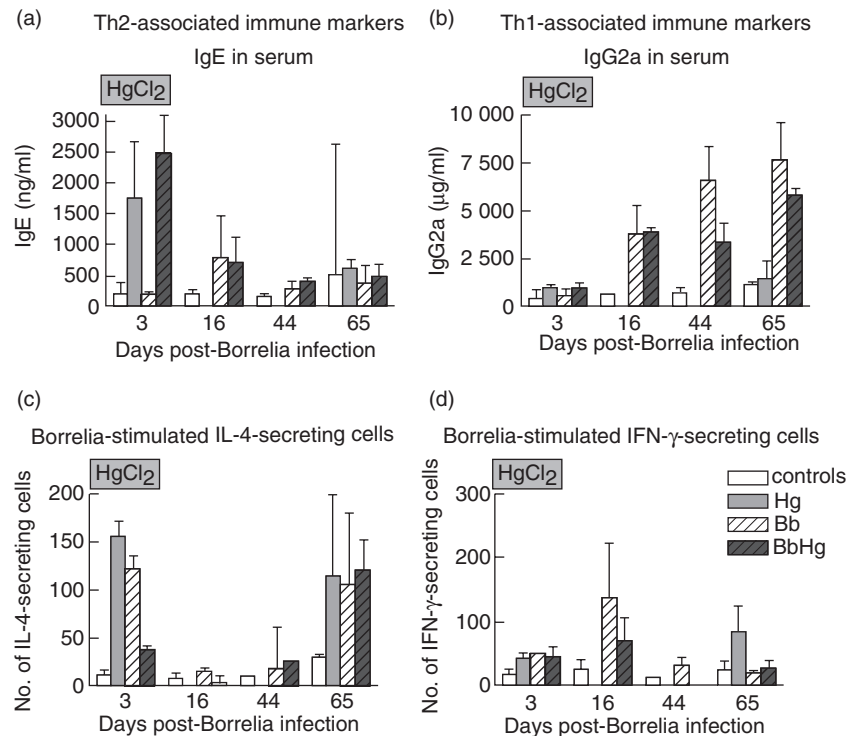
##### IL-4

The number of *Borrelia*-stimulated IL-4-producing lymph node cells was 14-fold higher in mice treated with Hg only for 14 days (3 days p.i.) compared to normal control mice ( $P = 0.002$ , Fig. 4c). On days 3 and 16 p.i., Bb mice showed a three- to fourfold higher number of lymph node cells secreting IL-4, both regarding the spontaneous (data not shown) and the *Borrelia*-stimulated secretion, compared to BbHg mice (Fig. 4c, day 3 p.i.  $P = 0.002$  and  $P = 0.004$  for spontaneous and *Borrelia*-stimulated secretion, respectively; day 16 p.i.,  $P = 0.032$  and  $P = 0.012$ , respectively). No further differences between the groups were seen in the number of IL-4-secreting cells.

##### IFN- $\gamma$

On day 3 p.i., the lymph node cells from mice treated with Hg only showed a two- to threefold increase in the number of IFN- $\gamma$ -producing cells both spontaneously and after *Borrelia* stimulation compared to normal control mice ( $P = 0.003$ ). The number of IFN- $\gamma$ -secreting cells (spontaneous as well as *Borrelia*-induced) in mice treated

**Fig. 4.** Effect of mercury exposure on T helper 1 (Th1)/Th2 responses in C3HHeN mice infected experimentally with *Borrelia burgdorferi*. Serum levels of (a) IgE (Th2) and (b) IgG2a (Th1), numbers of *Borrelia*-stimulated (c) interleukin-4- (Th2), (d) interferon- $\gamma$ -secreting cells (Th1) in 100 000 lymphocytes from inguinal lymph nodes. Control, normal mice not infected with *B. burgdorferi*; Hg, mice treated with HgCl<sub>2</sub> not infected with *B. burgdorferi*; Bb, mice infected with *B. burgdorferi*; BbHg, mice infected with *B. burgdorferi* and treated with HgCl<sub>2</sub> prior to infection. Data represent median values from seven mice in each group. Error bars indicate interquartile range.



with Hg only 3 days p.i. (after 14 days Hg treatment, Fig. 4d) was similar to that in Bb and BbHg mice 3 days p.i. Bb mice showed increased numbers of spontaneously IFN- $\gamma$ -secreting cells compared with normal controls on day 16 p.i. ( $P = 0.004$ , data not shown) and an increased number of *Borrelia*-induced IFN- $\gamma$ -secreting cells compared with BbHg mice on day 16 p.i. ( $P = 0.048$ , Fig. 4d). No further differences were seen between the groups.

#### IL-12p70

No difference was observed between the different groups with respect to spontaneous or *Borrelia*-induced IL-12p70-secreting cells 3 days p.i., or in spontaneous or *Borrelia*-induced IL-12p70-secreting cells 16 days p.i. ( $P > 0.05$ , data not shown). There was, however, a trend towards increased numbers of IL-12p70-secreting cells in Bb compared with BbHg mice on day 16 p.i. ( $P = 0.073$ , data not shown). The number of IL-12p70-secreting cells could not be assessed on day 44 p.i. due to low numbers of lymphocytes. There were no differences between the groups on day 65 p.i.

#### Discussion

The findings of this study indicate that mice exposed to HgCl<sub>2</sub> prior to *Borrelia* infection had increased spirochaetal load but decreased joint inflammation compared with normal, *Borrelia*-infected mice. Although the increase in spirochaetal load did not reach statistical significance, similar strong trends for increase was seen in both ear-tissue and

joints, together supporting a real increase. The increase in spirochaetal load in BbHg mice correlated with reduced levels of IgG2a and increased levels of IgE. Because IgG2a secretion in mice is an effector response generated by Th1 [54] and IgE switching is dependent on the Th2-cytokines IL-4 [55] and IL-13 [56], the *Borrelia*-induced effector response was Th2-deviated in mercury-treated mice. Thus, our findings indicate that the Th1 response is important for eradication of *Borrelia*.

When examining the course of *Borrelia* infection, BbHg mice initially showed a mercury-induced Th2-type effector response (IgE), which decreased gradually after completion of mercury treatment. This was associated with a delayed Th1-response compared with Bb mice, reflected both in the number of IFN- $\gamma$ -secreting cells and the following Th1-induced isotype switch to IgG2a, which most probably explains the similarly delayed eradication of *Borrelia* in BbHg mice. The different kinetics of IgE and IgG2a responses seen in the current study, i.e. a rapid increase of IgE following HgCl<sub>2</sub>-treatment which then declines, and a later increase of IgG2a that gradually increases, has been reported previously [57].

Although significantly less severe histopathology was recorded in BbHg compared to Bb mice on day 16 p.i. ( $P = 0.001$  for both infiltration of mononuclear cells and synovia thickness, respectively), strongly indicating a decreased inflammatory response, the increase in joint swelling (Fig. 1b) was not significantly different between the two groups. The failure to detect differences in joint swelling may possibly be explained by technical difficulties in the

measurement of joint diameter using a calliper, handling mice not anaesthetized, leading to a high variability.

So far, no established model of murine chronic borreliosis in mice is available. Thus, all mice recovered at the end of the experiment, making it impossible to draw any conclusions regarding the mechanisms in development of chronic borreliosis. It should, however, be noted that the reparative response in the joints, measured as cartilage hyperplasia, was increased in Bb mice compared to BbHg mice on day 16 p.i., suggesting a more beneficial response for resolving the disease in Bb mice. In the current study the histopathology showed a more severe *Borrelia*-induced arthritis in mice not exposed to mercury, tallying with a more efficient eradication of *Borrelia* compared to mercury-treated mice. The delicate balance between eradication of the microbe and protection of the host from immune-mediated tissue damage has been illustrated previously in studies of *Borrelia* infection. Mice lacking Th1-like responses showed less severe symptoms but a much higher spirochaetal burden than wild-type mice, while mice with decreased Th2-like responses developed more severe arthritis [31,58]. The present study also partly corroborates a previous study where pregnancy, considered to be associated with a Th2 shift [59,60], was shown to markedly reduce arthritis severity in *Borrelia*-infected mice [61]. The reduced severity was associated with a slight reduction in IFN- $\gamma$  and markedly increased IL-4.

The decreased inflammatory response recorded in the joints of BbHg mice compared to Bb mice on day 16 p.i. corresponded with reduced numbers of cytokine-secreting cells in BbHg mice on the same occasion. This is in accordance with the observed reduction in clearance of spirochaetes in BbHg mice detected on day 44 p.i. However, the decreased numbers of *Borrelia*-induced cytokine-secreting cells in BbHg mice included both cells secreting IL-4 (Th2) and IFN- $\gamma$  (Th1). In addition, there was a trend for decreased numbers of IL-12p70-secreting cells (Th1-inducing). This does not tally with the persistence of Th1 (IgG2a) and Th2 (IgE) type of immunoglobulins on day 16 p.i. The discrepancy between the *in vivo* observations of serum immunoglobulin isotypes, showing an initial Th2 response which after completion of mercury treatment shifted gradually to Th1, and the *in vitro* observations of cytokine secretion may be explained by the fact that the number of cytokine-secreting cells does not correlate with the amount of cytokines secreted [62,63], i.e. the cytokine secretion varies between the cells. The reason for using ELISPOT, which detects the number of cytokine-secreting cells instead of measuring cytokine concentration in cell culture supernatants by, for example, ELISA, was that ELISPOT has outstanding sensitivity in the measurement of IL-4, which is extremely difficult to detect [62]. Nevertheless, the serum levels of IgG2a and IgE, which are indicators of cytokine-generated effects, clearly indicated a Th2-deviation also at the cytokine level in BbHg mice. We consider the *in vivo* results on serum

immunoglobulin isotypes, which are linked to *in vivo* secretion of cytokines [54,55], as the best measurement of the Th1/Th2 balance.

The rebound of IL-4-secreting cells on day 65 in all groups except the untreated controls is surprising. Speculatively, this time-point would reflect a return to homeostasis in the Th1/Th2 balance, which in *Borrelia*-infected mice was Th1-dominated during the eradication of *Borrelia*, and thus suppressed Th2-responses. Borreliol lipoproteins are known to be highly immunogenic, inducing unspecific activation of numerous cell types [64], e.g. secretion of cytokines, which would also explain the response in the uninfected HgCl<sub>2</sub>-treated group. The untreated non-infected group did not, however, show any significant cytokine response to *Borrelia* antigen on any occasion, which may indicate that the HgCl<sub>2</sub> treatment *per se* also causes hyperreactivity of the Th2-type to highly immunogenic antigens after the initial immunodeviating effects has declined.

In conclusion, this study indicates that mice exposed to HgCl<sub>2</sub> prior to infection with *B. burgdorferi* have decreased arthritis severity but reduced elimination of *Borrelia* spirochaetes, which was associated with an initial mercury-induced Th2-type response and a delayed Th1-type response compared to unexposed *Borrelia*-infected mice. Our findings support the hypothesis that Th1-type responses are required for optimal eradication of *Borrelia*.

## Acknowledgements

We would like to thank Klara Martinsson MSc and Christer Bergman, Research Engineer, Division of Molecular and Immunological Pathology, Faculty of Health Sciences at Linköping University, for excellent guidance and help with the analyses of immunoglobulin isotypes and with histological analyses, respectively. This study was supported by grants from the Swedish Research Council branch of Medicine to C. E. (grant no. 14631), to S. B. and to P. H. (grant no. 9453).

## References

- 1 Nadelman RB, Wormser GP. Lyme borreliosis. *Lancet* 1998; **352**:557–65.
- 2 Stanek G, Strle F. Lyme borreliosis. *Lancet* 2003; **362**:1639–47.
- 3 Berglund J, Eitrem R, Ornstein K *et al.* An epidemiologic study of Lyme disease in southern Sweden. *N Engl J Med* 1995; **333**:1319–24.
- 4 Ekerfelt C, Masreliez C, Svenvik M *et al.* Antibodies and T cell reactivity to *Borrelia burgdorferi* in asymptomatic population: a study of healthy blood donors in an inland district in the south-east of Sweden. *Scand J Infect Dis* 2001; **33**:806–8.
- 5 Ekerfelt C, Forsberg P, Svenvik M *et al.* Asymptomatic *Borrelia* seropositive individuals display the same incidence of *Borrelia*-specific interferon-gamma (IFN- $\gamma$ )-secreting cells in blood patients with clinical *Borrelia* infection. *Clin Exp Immunol* 1999; **115**:498–502.

- 6 Kaiser R. Variable CSF findings in early and late Lyme neuroborreliosis: a follow-up study in 47 patients. *J Neurol* 1994; **242**:26–36.
- 7 Nocton JJ, Steere AC. Lyme disease. *Adv Int Med* 1995; **40**:69–115.
- 8 Oschmann P, Dorndorf W, Hornig C *et al.* Stages and syndromes of neuroborreliosis. *Neurology* 1998; **245**:262–72.
- 9 Vrethem M, Hellblom L, Widlund M *et al.* Chronic symptoms are common in patients with neuroborreliosis – a questionnaire follow-up study. *Acta Neurol Scand* 2002; **106**:205–8.
- 10 Berglund JL, Stjernberg K, Ornstein K. 5-y follow-up study of patients with neuroborreliosis. *Scand J Infect Dis* 2002; **34**:421–5.
- 11 Stricker RB, Lautin A, Burrascano JJ. Lyme disease: point/counterpoint. *Exp Rev Anti Infect Ther* 2005; **3**:155–65.
- 12 Frey M, Jaulhac B, Piemont Y *et al.* Detection of *Borrelia burgdorferi* DNA in muscles of patients with chronic myalgia related to Lyme disease. *Am J Med* 1998; **104**:591–4.
- 13 Oksi J, Marjamaki M, Nikoskelainen J *et al.* *Borrelia burgdorferi* detected by culture and PCR in clinical relapse of disseminated Lyme borreliosis. *Ann Med* 1999; **31**:225–32.
- 14 Preac-Mursic V, Weber K, Pfister HW *et al.* Survival of *Borrelia burgdorferi* in antibiotic treated patients with Lyme borreliosis. *Infection* 1989; **17**:355–9.
- 15 Hemmer B, Pinilla C, Gran B *et al.* Cross-recognition of self antigens by *Borrelia burgdorferi*-specific T cells derived from the cerebrospinal fluid of a patient with CNS Lyme disease. *VII International conference on Lyme borreliosis and other emerging tick-borne diseases*, Munich, Germany, 1999.
- 16 Sigal LH. Immunologic mechanisms in Lyme neuroborreliosis: the potential role of autoimmunity and molecular mimicry. *Semin Neurol* 1997; **17**:63–8.
- 17 Wagner EF, Hanna N, Fast LD *et al.* Novel diversity in IL-4 mediated responses in resting human naive B-cell versus germinal center/memory B cells. *J Immunol* 2000; **165**:5573–9.
- 18 Wang G, Ojaimi C, Wu H *et al.* Disease severity in a murine model of Lyme borreliosis is associated with the genotype of the infecting *Borrelia burgdorferi sensu stricto* strain. *J Infect Dis* 2002; **186**:782–91.
- 19 Forsberg P, Ernerudh J, Ekerfelt C *et al.* The outer surface proteins of Lyme disease *Borrelia spirochaetes* stimulate T cells to secrete interferon-gamma (IFN-gamma): diagnostic and pathogenic implications. *Clin Exp Immunol* 1995; **101**:453–60.
- 20 Oksi J, Savolainen J, Pene J *et al.* Decreased interleukin-4 and increased gamma interferon production by peripheral blood mononuclear cells of patients with Lyme borreliosis. *Infect Immun* 1996; **64**:3620–3.
- 21 Yin Z, Braun J, Neure L *et al.* T cell cytokine pattern in the joints of patients with Lyme arthritis and its regulation by cytokines and anticytokines. *Arthritis Rheum* 1997; **40**:69–79.
- 22 Ekerfelt C, Ernerudh J, Bunikis J *et al.* Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN- $\gamma$  predominates over IL-4 secretion in response to outer surface proteins of Lyme disease *Borrelia spirochetes*. *J Neuroimmunol* 1997; **79**:155–62.
- 23 Ekerfelt C, Ernerudh J, Forsberg P *et al.* Augmented intrathecal secretion of interferon- $\gamma$  in response to *Borrelia garinii* in neuroborreliosis. *J Neuroimmunol* 1998; **89**:177–81.
- 24 Widhe M, Ekerfelt C, Forsberg P *et al.* IgG subclasses in Lyme borreliosis: a study of specific IgG subclass distribution in an IFN- $\gamma$  predominated disease. *Scand J Immunol* 1998; **47**:575–81.
- 25 Grusell M, Widhe M, Ekerfelt C. Increased expression of the Th1-inducing cytokines interleukin-12 and interleukin-18 in cerebrospinal fluid but not in sera from patients with Lyme neuroborreliosis. *J Neuroimmunol* 2002; **131**:173–8.
- 26 Widhe M, Jarefors S, Ekerfelt C *et al.* *Borrelia* specific IFN- $\gamma$  and IL-4 secretion in blood and CSF during the course of human Lyme borreliosis: relation to clinical outcome. *J Infect Dis* 2004; **189**:1881–91.
- 27 Wang W-Z, Fredriksson S, Sun J-B *et al.* Lyme neuroborreliosis: evidence for persistent up-regulation of *Borrelia burgdorferi*-reactive cells secreting interferon- $\gamma$ . *Scand J Immunol* 1995; **42**:694–700.
- 28 Granström M. Borrelios/Lyme disease – serologi/diagnostik. Information Från Läkemedelsverket [Information from the Medical Products Agency] 1998; **9**:98.
- 29 Gross DM, Steere AC, Huber BT. T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis. *J Immunol* 1998; **160**:1022–8.
- 30 Kang I, Barthold SW, Persing DH *et al.* T-helper-cell cytokines in the early evolution of murine Lyme arthritis. *Infect Immun* 1997; **65**:3107–11.
- 31 Anguita J, Rincón M, Samanta S *et al.* *Borrelia burgdorferi*-infected, interleukin-6 deficient mice have decreased Th2 responses and increased Lyme arthritis. *J Infect Dis* 1998; **178**:1512–5.
- 32 Brown CR, Reiner SL. Experimental Lyme arthritis in the absence of interleukin-4 or gamma interferon. *Infect Immun* 1999; **67**:3329–33.
- 33 Christe M, Rutti B, Brossard M. Cytokines (IL-4 and IFN- $\gamma$ ) and antibodies (IgE and IgG2a) produced in mice infected with *Borrelia burgdorferi sensu stricto* via nymphs of *Ixodes ricinus* ticks or syringe inoculations. *Parasitol Res* 2000; **86**:491–6.
- 34 Christopherson JA, Munson EL, England DM *et al.* Destructive arthritis in vaccinated interferon gamma-deficient mice challenged with *Borrelia burgdorferi*: modulation by tumor necrosis factor alpha. *Clin Diagn Lab Immunol* 2003; **10**:44–52.
- 35 Satoskar AR, Elizondo J, Monteforte GM *et al.* Interleukin-4-deficient BALB/c mice develop an enhanced Th1-like response but control cardiac inflammation following *Borrelia burgdorferi* infection. *FEMS Microbiol Lett* 2000; **183**:319–25.
- 36 Bagenstose LM, Salgame P, Monestier M. Cytokine regulation of a rodent model of mercuric chloride-induced autoimmunity. *Environ Health Perspectives* 1999; **107**:807–10.
- 37 Goldman M, Druet P, Gleichmann E. Th2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. *Immunol Today* 1991; **12**:223–7.
- 38 Häggqvist B, Hultman P. Murine metal-induced autoimmunity: baseline and stimulated cytokine mRNA expression in genetically susceptible and resistant strains. *Clin Exp Immunol* 2001; **126**:157–64.
- 39 Johansson U, Sander B, Hultman P. Effects of murine genotype on T cell activation and cytokine production in murine mercury-induced autoimmunity. *J Autoimmun* 1997; **10**:347–55.
- 40 Mathieson PW. Mercury: god of Th2 cells? *Clin Exp Immunol* 1995; **102**:229–30.
- 41 Ochel M, Vohr HW, Pfeiffer C *et al.* 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.
- 42 Prigent P, Saoudi A, Pannettier C *et al.* Mercuric chloride, a chemical responsible for T helper cell (Th)2-mediated autoimmunity in brown Norway rats, directly triggers T-cells to produce interleukin-4. *J Clin Invest* 1995; **96**:1484–9.



- 43 Havarinasab S, Hultman P. Organic mercury compounds and mercury. *Autoimmun Rev* 2005; **4**:270–5.
- 44 Bagenstose LM, Mentink-Kane MM, Brittingham A *et al*. Mercury enhance susceptibility to murine leishmaniasis. *Parasite Immunol* 2001; **23**:633–40.
- 45 Morris L, Trout AB, McLeod KS *et al*. Interleukin-4 but not gamma interferon production correlates with the severity of murine cutaneous leishmaniasis. *Infect Immun* 1993; **61**:3459–65.
- 46 Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. *Ann Rev Immunol* 1995; **13**:151–77.
- 47 Jarefors S, Janefjord CK, Forsberg P *et al*. Decreased up-regulation of the interleukin-12Rb2-chain and interferon- $\gamma$  secretion and increased number of forkhead box P3-expressing cells in patients with a history of chronic Lyme borreliosis compared with asymptomatic individuals. *Clin Exp Immunol* 2006; **147**:18–27.
- 48 Jarefors S, Karlsson M, Forsberg P *et al*. Reduced numbers of interleukin-12 secreting cells in patients with Lyme borreliosis previously exposed to *Anaplasma phagocytophilum*. *Clin Exp Immunol* 2005; **143**:322–8.
- 49 Sjöwall J, Carlsson A, Vaarala O *et al*. Innate immune responses in Lyme borreliosis: enhanced tumour necrosis factor- $\alpha$  and interleukin-12 in asymptomatic individuals in response to live spirochaetes. *Clin Exp Immunol* 2005; **141**:89–98.
- 50 Barthold SW, Beck DS, Hansen GM *et al*. Lyme borreliosis in selected strains and ages of laboratory mice. *J Infect Dis* 1990; **162**:133–8.
- 51 Bergström S, Sjöstedt A, Dotevall L *et al*. Diagnosis of Lyme borreliosis by an enzyme immunoassay detecting immunoglobulin G reactive to purified *Borrelia burgdorferi* cell components. *Eur J Clin Microbiol Infect Dis* 1991; **10**:422–7.
- 52 Hovden AO, Cox RJ, Madhun A *et al*. Two doses parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. *Scand J Immunol* 2005; **62**:342–52.
- 53 Havarinasab S, Lambertsson L, Qvarnström J *et al*. Dose–response study of thimerosal-induced murine systemic immunity. *Toxicol Appl Pharmacol* 2004; **194**:169–79.
- 54 Finkelman FD, Holmes J, Katona IM *et al*. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Ann Rev Immunol* 1990; **8**:303–33.
- 55 Kuhn R, Rajewski K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science* 1991; **254**:707–10.
- 56 Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003; **21**:425–56.
- 57 Johansson U, Hansson-Georgiadis H, Hultman P. The genotype determines the B cell response in mercury-treated mice. *Int Arch Allergy Immunol* 1998; **116**:295–305.
- 58 Anguita J, Persing DH, Rincón M *et al*. Effect of anti-interleukin 12 treatment on murine Lyme borreliosis. *J Clin Invest* 1996; **97**:1028–34.
- 59 Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* 1997; **18**:478–82.
- 60 Wegmann TG, Lin H, Guilbert L *et al*. Bidirectional cytokine interactions in the maternal–fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; **14**:353–6.
- 61 Moro MH, Björnsson J, Marietta EV *et al*. Gestational attenuation of Lyme arthritis is mediated by progesterone and IL-4. *J Immunol* 2001; **166**:7404–9.
- 62 Ekerfelt C, Ernerudh J, Jenmalm MC. Detection of spontaneous and antigen-induced human IL-4 responses *in vitro*; comparison of ELISPOT, a novel ELISA and real time RT–PCR. *J Immunol Meth* 2002; **260**:55–67.
- 63 Rönnelid J. Reactivity to collagen type II and C1q in rheumatic diseases. Stockholm, Sweden: Department of Medicine, Rheumatology Unit, Karolinska Institute, 1997.
- 64 Talkington J, Nickell SP. *Borrelia burgdorferi* spirochetes induce mast cell activation and cytokine release. *Infect Immun* 1999; **67**:1107–15.