Mercury-induced Autoreactive Anti-class II T Cell Line Protects from Experimental Autoimmune Encephalomyelitis by the Bias of CD8$^+$ Antiergotypic Cells in Lewis Rats

By Maria Castedo, Lucette Pelletier, Jérôme Rossert, Régine Pasquier, Henri Villarroya,* and Philippe Druet

*From INSERM U28, Hôpital Broussais, 75674 Paris Cedex 14; and *INSERM U134, Hôpital Salpêtrière, 75651 Paris Cedex 13, France*

Summary

Brown-Norway (BN) rats injected with $HgCl_2$ develop a systemic autoimmune disease associated with a polyclonal B cell activation, due to autoreactive T cells specific for self-class II molecules, while Lewis (LEW) rats injected with $HgCl_2$ do not exhibit autoimmunity and develop a non-antigen-specific, CD8-mediated immunosuppression assessed by a depression of T cell functions, and a protection against experimental autoimmune encephalomyelitis (EAE). Resistance to $HgCl_2$-induced autoimmunity is not due to these suppressor cells since treatment with an anti-CD8 monoclonal antibody (mAb) did not allow autoimmunity to appear. The absence of autoimmunity in this strain could result from the absence of autoreactive T cells, or from quantitative or qualitative differences of these cells between susceptible and resistant strains. In the present study, we show that CD4$^+$ anti-class II T cells are present in $HgCl_2$-injected LEW rats and are as frequent as in BN rats when assessed by limiting dilution analysis. LEW CD4$^+$ autoreactive T cell lines were derived. They proliferated in the presence of normal class II-bearing cells, secreted interleukin 2, and did not induce B cells to produce immunoglobulins. Transfer of one of these lines, LEW Hg A, into normal LEW rats led to the appearance of CD8$^+$ cells responsible for a non-antigen-specific immunosuppression that induced complete protection from EAE. Immunosuppression was abrogated after treatment with an anti-CD8 mAb. In vitro, CD8$^+$ cells from rats injected with the LEW Hg A T cell line proliferated in the presence of activated T cells whatever their origin. We conclude that $HgCl_2$ induces CD4$^+$ autoreactive T cells that proliferate in the presence of class II$^+$ cells in susceptible BN as well as in resistant LEW rats. But while these cells collaborate with B cells to produce autoantibodies in BN rats, they initiate in LEW rats a suppressor circuit involving antiergotypic CD8$^+$ suppressor cells.

Brown-Norway (BN)[1] rats injected with $HgCl_2$ develop a lupus-like systemic autoimmune disease in the context of a polyclonal B cell activation (1, 2), due to autoreactive T cells specific for self-class II molecules (3). In contrast, Lewis (LEW) rats injected with $HgCl_2$ do not develop autoimmunity but exhibit a non-antigen specific CD8-mediated immunosuppression (4). This immunosuppression is mediated by non-antigen-specific CD8$^+$ cells responsible for depression of T cell functions (4). $HgCl_2$-injected LEW rats are protected against Heymann's nephritis (5) and experimental autoimmune encephalomyelitis (EAE) (6, 7). Protection is not absolute, probably due to the emergence of contrasuppressor cells (7). Until now, the way these suppressor cells were induced and their specificity were unknown. Since anti-class II T cells were present in BN rats injected with $HgCl_2$ (3) and since in another situation anti-class II T cells have been shown to induce suppressor cells (8), it was tempting to look for the presence of anti-class II T cells in $HgCl_2$-injected LEW rats and to assess their possible connection with the mercury-induced suppressor cells observed in that strain.

We show here that anti-class II T cells are detectable in LEW rats injected with $HgCl_2$, with a frequency similar to that found in susceptible BN rats. Autoreactive anti-class II CD4$^+$ T cell lines have been derived from LEW rats injected with $HgCl_2$. One of these lines has been transferred in naive LEW rats and it acted as a suppressor inducer line. It triggered the appearance of CD8$^+$ antiergotypic and per-

[1] *Abbreviations used in this paper:* BN, Brown-Norway; BP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; LDA, limiting dilution analysis; LEW, Lewis; PPD, purified protein derivative.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/04/0881/09 $2.00
Volume 177 April 1993 881-889

haps antiidiotypic suppressor cells. These autoreactive anti–class II T cells differed from those found in $HgCl_2$-injected BN rats by their inability to help in vitro or in vivo B cells to produce antibodies.

Materials and Methods

Rats. LEW and BN rats were initially obtained from the Centre de Selection et d'Elevage des Animaux de Laboratoire (Orléans-La Source, France), and spontaneously hypertensive rats from Iffa Credo (Lyon, France). These strains were then maintained by brother-sister mating in our facilities. 8–12-wk-old males were used.

Antigens. Myelin and myelin basic protein (BP) were prepared as described elsewhere (6). Purified protein derivative (PPD) from *Mycobacterium tuberculosis* was obtained from Statens (Serum Institut, Copenhagen, Denmark). OVA was obtained from Sigma Chemical Co. (St. Louis, MO).

Injections of $HgCl_2$, Immunization with OVA, and EAE Induction. $HgCl_2$ was subcutaneously injected at a dose of 100 μg/100 g body weight, three times a week, as described elsewhere (1). LEW rats were immunized with 100 μg of OVA (Sigma Chemical Co.) emulsified in CFA in the rear footpads and killed after 10 d. EAE was induced as described (5). Briefly, myelin was suspended in PBS at a concentration of 10 mg/ml; the solution was emulsified in an equal volume of CFA containing 0.2 mg/ml *M. tuberculosis* (H-37RA; Difco Laboratories, Detroit, MI); 0.25 ml of this emulsion was injected subcutaneously in the two rear footpads.

mAbs and FACScan® Analysis. W3/13, W3/25, OX8, OX39, OX6, OX17, and OX22 mAbs were initially obtained from the Public Health Laboratory Service collection (Oxford, UK) as cells. Ascites and purified antibodies were prepared as described (9). They recognize, respectively, all T cells (10), $CD4^+$ (11), $CD8^-$ (11), IL-$2R^-$ cells (12), MHC class II IA molecules (13), MHC class II IE molecules (14), and CD45R molecules (15). MARK-1 (mouse anti-rat κ light chain) mAb was kindly provided by H. Bazin (Louvain, Belgium). B10H2, a mouse anti-thyroglobulin IgGl mAb was kindly provided by Dr. D. Glotz (Hôpital Broussais, Paris, France), and was used as an irrelevant mAb. Cells to be tested were stained by indirect immunofluorescence with a fluoresceinated goat $F(ab')_2$ anti–mouse Ig antibody (Caltag, San Francisco, CA) adsorbed on normal rat serum. All antibodies were used 1:100 diluted. The cells were analyzed with a FACScan® (Becton Dickinson & Co., Sunnyvale, CA); a minimum of 10,000 cells were counted.

Serum IgE Concentration and Renal Immunofluorescence. Serum IgE concentration was determined by ELISA as described elsewhere (16), and Ig deposits in the glomeruli were looked for as previously (1).

Cell Culture Medium. Culture medium was RPMI 1640 (Seromed, Berlin, Germany) supplemented with penicillin (100 U/ml; Gibco, Paisley, UK), streptomycin (100 μg/ml), nonessential amino acids (0.1 mM), L-glutamine (2 mM), sodium pyruvate (1 mM; Biochrom KG, Berlin, Germany), 2-ME (2×10^{-5} M; Sigma Chemical Co.), and 2% heat-inactivated normal rat serum or 10% in the case of limiting dilution analysis (LDA).

Limiting Dilution Analysis. T cell subsets and B cells were purified by panning (3). The purity of $CD4^+$, Ig^+, and $CD8^+$ cells was assessed as described (3), and was found to be 97, 80, and 98% respectively. LDA was performed as described elsewhere (3). Briefly, $CD4^+$ or $CD8^-$ T cells from LEW rats injected with $HgCl_2$ for 4, 6, 14, or 30 d were cultured at an input from 1,000–80,000 cells/well in culture medium supplemented with 15% Con A supernatant obtained from rat spleen cells as described elsewhere (3), in the presence of normal or autologous 3,000-rad irradiated T- or B-enriched cells as stimulator cells. 24 wells were seeded for each cell concentration. Cultures were performed in 96-well U-bottomed microtiter plates (Nunc, Kamstrup, Denmark) for 7 d in a 6% CO_2 incubator. [^{3}H]Thymidine was added 24 h before the end of the culture. Background values were obtained by omitting stimulator cells in the culture. A culture was considered positive when the number of counts per minute was superior to mean background plus 3 SD. In some experiments, stimulator cells were preincubated with the OX6, OX17, W3/25 or OX8 mAbs (50 μg/ml) for 90 min at 4°C and washed before use. The frequency of negative wells for each cell concentration was semilogarithmatically plotted as a function of the number of cells/well. Experimental results were analyzed according to Taswell (17), and were shown to fit a single-hit Poisson's distribution, at least for the initial part of the curves. Results were expressed as the mean reciprocal frequencies obtained from six rats on each day.

Proliferative Responses of $CD8^+$ Cells from LEW Rats Injected with $HgCl_2$. Purified $CD8^+$ cells (2×10^5/well) from normal LEW rats or from LEW rats injected with $HgCl_2$ for 7, 14, or 30 d were cultured in culture medium supplemented with 3% Con A supernatant alone, or in the presence of 2×10^5 irradiated (3,000 rad) $CD4^+$ or $CD8^+$ cells from normal or autologous rats, in 96-well flat-bottomed microculture plates (Costar, Cambridge, MA) for 4 d. [^{3}H]Thymidine (1 Ci/mmol = 37 GBq/mmol; 0.4 μCi/well) (Dositek, Saclay, France) was added 24 h before the end of the culture. Thymidine uptake was measured as described elsewhere (3). The results were expressed as cpm [^{3}H]TdR.

Preparation of T Cell Lines. T cell lines were obtained from LEW rats injected with $HgCl_2$ for 7 d by culturing 0.5×10^6 lymph node cells in 96-well U-bottomed microtiter plates (Nunc), in a 6% CO_2 incubator in the presence of normal irradiated (3,000 rad) syngeneic thymocytes (5–10 $\times 10^6$/ml), under a volume of 0.2 ml/well in culture medium without Con A supernatant. After 4–6 d, half of the medium was removed and replaced by culture medium containing 10% Con A supernatant. 7–10 d later, cells were stimulated again with thymocytes. Exactly the same procedure was then followed after this second round of stimulation.

To obtain an anti-OVA T cell line (LEW OVA), LEW rats were immunized with 100 μg OVA, and 10 d later lymph node cells were stimulated in the presence OVA (50 μg/ml). Con A supernatant was added after 4–6 d as described above, and a second round of antigenic stimulation was performed 10 d later exactly as the first one. The culture was maintained on a basis of one stimulation every 2 wk. Phenotype of the cells was assessed after three, five, and eight stimulations.

Proliferative Response of T Cell Lines. T cell lines from the $HgCl_2$-injected rats were cultured (5×10^4 cells under a volume of 200 μl) in 96-well flat-bottomed microculture plates (Costar), alone or in the presence of various stimulator cells (5×10^6/ml) for 3 d. In some experiments, stimulator cells were preincubated with various mAbs (50 μg/ml) as described in LDA experiments. The proliferative response of the LEW OVA T cell line was assessed by culturing 5×10^4 cells in the presence of irradiated (3,000 rad) syngeneic thymocytes (10^5/ml) and OVA (50 μg/ml) for 3 d. 24 h before the end of the culture, cultures were pulsed with 1 μCi [^{3}H]thymidine/well and processed as described (3). Results were expressed as cpm [^{3}H]TdR.

Titration of IL-2 in Culture Supernatants. Supernatants were collected after 3 d of culture and assayed 1:4 diluted on triplicate cultures of 10^4 IL-2-dependent mouse CTLL-2 cells per well, in 96-well flat-bottomed microculture plates. The CTLL-2 cultures were incubated for 24 h, pulsed during the last 12 h with [^{3}H]thymidine, and processed as above. Human rIL-2 (Cetus Corp., Emeryville,

CA) was used as a positive control. Results were expressed as cpm [^{3}H]TdR.

Ig Production In Vitro. Unseparated spleen cells from normal LEW rats were layered over Ficoll-Hypaque and RBC were eliminated by density centrifugation. Cocultures were performed by adding 5×10^4 purified T cells, preactivated by autologous APC for 3 d, to 2×10^5 spleen cells under a final volume of 0.2 ml/well in 96-well flat-bottomed microculture plates for 12 d. T cells alone and spleen cells alone were cultured as controls. 12 replicate wells of each culture combination were set up. 3 d after the beginning of the culture, 100 μl of supernatant medium was removed in each well, and was replaced by fresh medium containing 3% Con A supernatant as a source of IL-2. At the end of the culture, supernatants were removed, pooled, and assayed for Ig production by ELISA (18).

Transfer Experiments. LEW rats were intravenously injected with 5×10^7 cells from one of the T cell lines (LEW Hg A) derived from $HgCl_2$-injected LEW rats or from the LEW OVA T cell line, and simultaneously immunized with myelin. Rats transferred with T cells were intraperitoneally injected or not with the OX8 anti-CD8 or B10H2 mAbs as irrelevant mAbs (0.5 mg/rat per injection on days 0, 10, and 19). It was verified at the time of death that OX8-injected rats were depleted of $CD8^+$ cells (3% of lymph node cells expressed CD8 molecules vs. 18% in LEW rats injected with B10-H2 mAb). Rats have also been transferred with 5×10^7 LEW OVA cells cultured for 3 d with BP (50 μg/ml) or with the same number of thymocytes cultured in the presence of BP in the same conditions. Rats were examined daily to assess neurological manifestations. These manifestations were scored on a 0–4 scale: 0, no clinical signs; 1, flaccid tail; 2, ataxia; 3, hind limb paralysis; and 4, moribund or dead (6, 7).

Rats were killed on day 21 because paralyzed rats recover at that time. Lymph node cells were prepared as described (2). Cells (2×10^5/well) were cultured alone, in the presence of Con A (2.5 μg/ml) for 2 d, in the presence of irradiated (3,000 rad) lymph node cells from normal BN rats (10^6/ml) for 4 d, or in the presence of BP or PPD (50 μg/ml) for 3 d. Various numbers of lymph node cells from rats transferred with LEW Hg A cells were added at the beginning of the culture, and the percentage of inhibition of the proliferative response was calculated.

The proliferative response was also performed in the presence of 2×10^5 irradiated (3,000 rad) LEW Hg A or LEW OVA cells, previously activated or not, or in the presence of 20% supernatant obtained after culturing the lines for 3 d in the presence of their antigen, or in the presence of 20% Con A supernatant. Cells were cultured for 4 d in 96-well flat-bottomed microculture plates. [^{3}H]Thymidine was added 24 h before the end of the culture, and thymidine uptake was measured as described above.

The occurrence of manifestations similar to those triggered by $HgCl_2$ in BN rats were looked for in the different groups by measuring serum IgE concentration every 1 wk until death, and by examining kidneys at the time of death for the presence of Ig deposits.

Statistical Analysis. Data were compared by using Student's *t* or nonparametric Mann Whitney or Wilcoxon tests.

Results

Autoreactive Anti–class II T Cells Are Present in LEW Rats Injected with $HgCl_2$. $CD4^+$ T cells obtained from LEW rats injected with $HgCl_2$ for 4, 6, 14, or 30 d proliferated in the presence of syngeneic B-enriched cells. The frequencies of these autoreactive T cells were not statistically different between days 4 and 30 (Fig. 1). Mean reciprocal frequencies of autoreactive T cells were 34.0 ± 10.3, 24.2 ± 12.5, 16.0 ± 4.8, and 22.2 ± 3.3 × 10^3, at days 4, 6, 14, and 30, respectively (Fig. 1). The ability of B-enriched cells to stimulate autoreactive T cells was similar whether they came from normal LEW rats or from autologous rats exposed to $HgCl_2$ (Fig. 1). T cells did not respond when B cells were preincubated with the OX6 anti–class II IA mAb (Fig. 1). Preincubation of B cells with the anti–class II IE, the anti-CD4, or the anti-CD8 mAbs did not modify the frequency of autoreactive T cells (not shown). Normal or autologous T cells were unable to act as stimulator cells (Fig. 1). $CD4^+$ T cells from normal LEW rats did not respond to autologous B cells (Fig. 1) showing that, within the limits of the method, autologous mixed lymphocyte reaction was not observed in normal rats.

$CD8^+$ Cells from LEW Rats Injected with $HgCl_2$ Proliferate in the Presence of Autologous $CD4^+$ Cells. Since non–antigen-specific $CD8^+$ suppressor cells and autoreactive $CD4^+$ cells were found concomitantly in LEW rats injected with $HgCl_2$, a connection between these two populations was sought. To test whether $CD8^+$ cells were stimulated by $HgCl_2$-activated $CD4^+$ T cells, purified $CD8^+$ lymph node cells from LEW rats injected with $HgCl_2$ were cultured in the presence of irradiated autologous purified $CD4^+$ cells or in the presence of $CD4^+$ cells from normal LEW rats. $CD8^+$ cells from LEW rats injected with $HgCl_2$ for 7 d proliferated in the presence of autologous $CD4^+$ cells but not in the presence of $CD4^+$ cells from normal LEW rats (Fig. 2) or $CD8^+$ cells whether they originated from normal

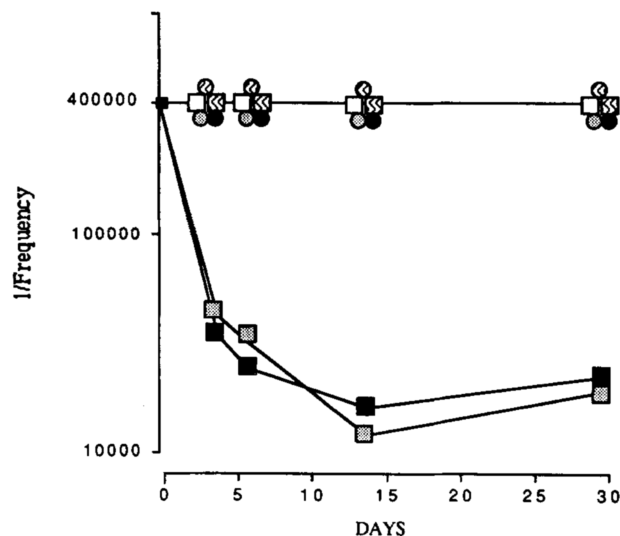

Figure 1. Mean reciprocal frequencies of autoreactive anti–class II $CD4^+$ T cells in $HgCl_2$-injected LEW rats. $CD4^+$ T cells from rats injected with $HgCl_2$ for 4, 6, 14, and 30 d were cultured in the presence of syngeneic B cells from autologous (■) or normal (▩) rats, or of syngeneic T cells from autologous (●) or normal (○) rats. B cells pretreated with the OX6 mAb were also used as stimulator cells; they were obtained either from autologous (⊘) or from normal (▨) rats. $CD4^+$ T cells from LEW rats injected with the control solution were cultured in the presence of normal syngeneic spleen cells (□).

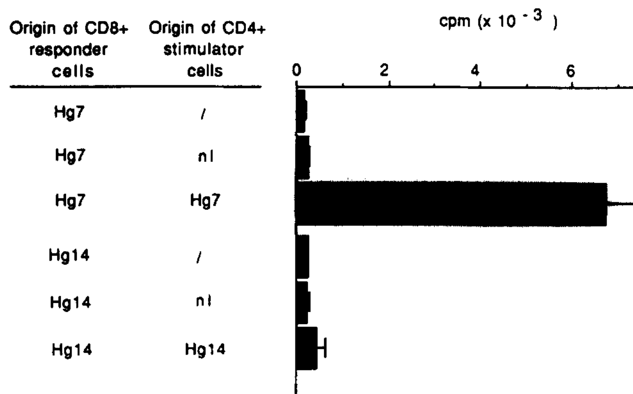

Figure 2. Proliferative response of $CD8^+$ lymph node cells from LEW rats injected with $HgCl_2$ for 7 (Hg7) or 14 d (Hg14) in the presence of irradiated $CD4^+$ cells from normal (*nl*) or $HgCl_2$-injected (*Hg7* or *Hg14*) rats. Results are mean of five experiments.

Table 1. *IL-2 Production by LEW Hg and LEW OVA T Cell Lines*

CTLL-2	Supernatant from:	cpm
+	–	310 ± 115
+	LEW Hg A	18,795 ± 380*
+	LEW Hg B	15,425 ± 875*
+	LEW Hg C	14,695 ± 1,218*
+	LEW OVA	17,880 ± 1,112*
+	rIL-2‡	26,545 ± 9,754*

Supernatant from T cell lines was added to CTLL-2 cells, and thymidine incorporation was measured after 24 h.
* $p < 0.001$ when compared with control culture.
‡ rIL-2 was added at a concentration of 5 U/ml.

or autologous rats (not shown). The mean reciprocal frequency of these $CD8^+$ cells was $45 \pm 12 \times 10^3$. $CD8^+$ cells from LEW rats injected with $HgCl_2$ for 14 (Fig. 2) or 30 d (not shown) were unable to proliferate whatever the stimulator cells tested, although the increase in the number of $CD8^+$ cells was still observed (not shown) as previously reported (4).

Characterization of Autoreactive T Cell Lines Derived from LEW Rats Injected with $HgCl_2$. We have studied the possibility that cells activating the $CD8^+$ suppressor cells were $HgCl_2$-induced autoreactive anti–class II T cells. To test this hypothesis, we developed autoreactive T cell lines from $HgCl_2$-treated LEW rats. Seven autoreactive T cell lines were derived from seven rats (LEW Hg A through LEW Hg G). After three stimulations, all the lines had the same phenotype, i.e., $W3/13^+$ $CD4^+$ $CD8^-$ Ig^- $CD45R^-$$IL\text{-}2R^+$. This phenotype remained stable after 10 mo of culture. These cell lines produced IL-2 after stimulation with irradiated syngeneic thymocytes (Table 1; and not shown). As a control, a T cell line was obtained from LEW rats immunized with OVA (LEW OVA) that had the same phenotype and also produced IL-2 when stimulated with OVA (Table 1). Fig. 3 *a* shows the proliferative response of LEW Hg A cells in the presence of various stimulator cells. They proliferated in the presence of LEW syngeneic thymocytes, but not in the presence of allogeneic BN or SHR thymocytes. The response was completely abolished when the stimulator cells were preincubated with an anti–class II IA mAb (OX6), while the response was not modified when the stimulator cells were preincubated with an anti–class II IE (OX17) or with the other IgG1 mAbs (W3/25 or OX8; not shown). Normal syngeneic spleen cells were also efficient at stimulating this T cell line, and preincubation with the OX6 mAb also inhibited the T cell response (not shown). Similar results were obtained with the other LEW Hg lines (not shown). This suggests that the LEW Hg T cell lines recognize either IA class II molecules or a self-peptide in the context of IA class II molecules. As shown in Fig. 3 *b*, LEW OVA T cells proliferated in the presence of OVA, and this proliferative response was completely abolished when cultures were performed with stimulator cells preincubated with the anti–class II OX6 mAb, suggesting that the LEW OVA T cell line recognizes a peptide derived from OVA in the context of class II molecules.

Anti–class II T Cell Lines Are Unable to Provide B Cell Help. LEW Hg A, B, and C lines were tested for their ability to trigger B cell differentiation. The addition of LEW Hg A T cells to syngeneic spleen cells as a source of B cells did not modify the baseline production of Ig by these cells (Ig

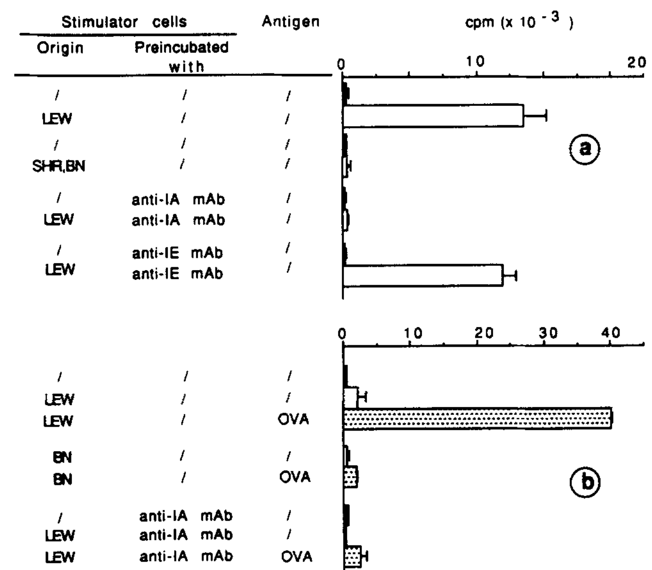

Figure 3. Specificity of the LEW Hg A T cell line. (*a*) Proliferative response of LEW Hg A T cells cultured alone (■) or in the presence of APC (□). Cultures were also performed in the presence of syngeneic APC preincubated with the OX6, anti–class II IA, or the OX17, anti–class II IE mAb. (*b*) Proliferative response of LEW OVA T cells cultured alone (■), in the presence of APC (□), or in the presence of OVA (50 μg/ml) and APC (▤), preincubated or not with the OX6 mAb. Results are the mean of five experiments.

concentration: 115 ± 25 ng/ml in cocultures vs. 95 ± 40 ng/ml in cultures containing spleen cells only). Similar results were obtained with LEW Hg B and C T cells.

All seven attempts to derive autoreactive anti–class II T cell lines from normal LEW rats failed. This might be related to the very low frequency of such cells in normal rats as shown by LDA.

The Autoreactive Anti–class II LEW Hg A T Cell Line Protects LEW Rats from EAE. It has been previously shown that $HgCl_2$-induced $CD8^+$ suppressor cells were able to protect LEW rats from the appearance of autoimmune diseases such as EAE (7). To test the role of anti–class II T cells in the initiation of the suppressor circuit, we transferred the LEW Hg A T cell line in rats immunized with myelin. As shown in Table 2, all the LEW rats immunized with myelin developed a severe EAE, while the immunized rats transferred with preactivated LEW Hg A cells were protected. Rats were not protected when they received nonactivated LEW Hg A T cells. The protection was completely abolished after treatment of the recipients with the OX8 anti-CD8 mAb. Injections of the irrelevant B10-H2 mAb did not abrogate LEW Hg A–mediated suppression (Table 2). LEW OVA cells had no effect on the course of EAE whether they were cultured in the presence of OVA or BP before transfer. Injection of thymocytes alone cultured in the presence of BP did not protect recipients from EAE (Table 2), showing that LEW Hg A-induced suppression was not due to contaminating thymocytes.

The Autoreactive Anti–class II LEW Hg A T Cell Line Triggers Non–Antigen-specific $CD8^+$ Suppressor Cells. Responses to BP or PPD, but also to the Con A mitogen or to allogeneic antigens of lymph node cells from rats transferred with the LEW Hg A T cell line, were inhibited when compared with the responses of LEW rats immunized with myelin alone (Fig. 4). This suppression was CD8 mediated since in vivo depletion of $CD8^+$ cells restored, at least in part, all the responses. Treatment with an irrelevant mAb did not modify

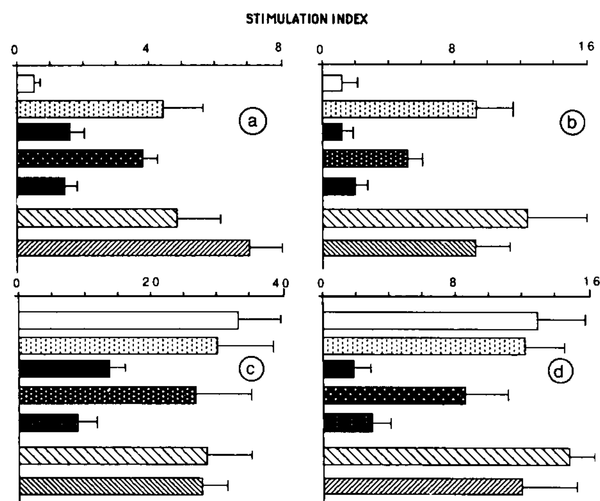

Figure 4. Non–antigen-specific, CD8-dependent immunosuppression induced by transfer of the LEW Hg A T cell line in normal LEW rats. Proliferative responses to BP (*a*), PPD (*b*), Con A (*c*), or lymph node cells from BN rats as an alloantigen (*d*) of lymph node cells from LEW rats. LEW rats were normal (□), immunized with myelin (▩), immunized with myelin and transferred with 5 × 10^7 LEW Hg A T cells whether they were treated with an anti-CD8 mAb (■), an irrelevant mAb (■), or not (■), or immunized with myelin and transferred with 5 × 10^7 LEW OVA T cells preactivated with OVA (▧) or BP (■). Stimulation index = cpm of stimulated cells/cpm of unstimulated cells. Results are mean of six experiments.

Table 2. *Effect of Transfer of T Cell Lines on Actively Induced EAE*

Cells transferred	In vitro challenge with:	mAb in vivo	Clinical score
–	–	–	2, 2, 3, 3, 4, 4
LEW Hg A	APC	–	0, 0, 0, 0, 0, 0
LEW Hg A*	–	–	1, 2, 2, 3, 3
LEW Hg A	APC	OX8	1, 2, 3, 3, 3
LEW Hg A	APC	B10-H2	1, 0, 0, 0, 0
LEW OVA	BP	–	3, 2, 3, 3
LEW OVA	OVA	–	3, 4, 2, 4
APC‡	BP	–	2, 2, 3, 4, 4

APC are syngeneic thymocytes. OX8 is an anti-CD8 mAb; B10-H2 was used as an irrelevant mAb. Cells transferred were activated for 3 d with the appropriate stimulus.
* In this culture, LEW Hg A cells were kept for 7 d after the end of stimulation in medium containing Con A supernatant as a source of IL-2.
‡ Rats were transferred with thymocytes incubated with BP (50 μg/ml) as a source of myelin-bound APC.

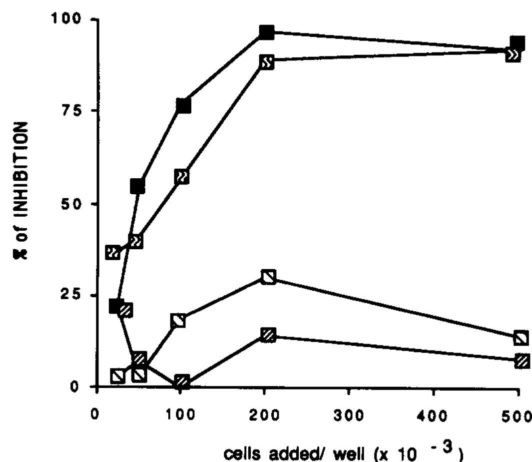

Figure 5. Inhibition of the proliferative response to BP of lymph node cells from LEW rats immunized with myelin by various numbers of heterologous lymph node cells. Lymph node cells assessed for suppression came from LEW rats immunized with myelin and transferred with the LEW Hg A T cell line (■), injected in addition with an anti-CD8 mAb (▨), or an irrelevant mAb (▩), or from rats transferred with the LEW OVA T cell line (▧).

LEW Hg A–mediated non-antigen-specific suppression. Injection of LEW OVA cells did not modify the proliferative responses to BP, PPD, Con A, or allogeneic antigens as compared with the responses of rats immunized with myelin alone (Fig 4).

Lymph node cells from rats transferred with LEW Hg A T cells suppressed in a dose-dependent manner the proliferative responses to BP (Fig. 5) and to PPD (not shown) of lymph node cells from LEW rats immunized with myelin alone. Treatment with the OX8 mAb of LEW rats transferred with LEW Hg A cells inhibited this suppressive effect while treatment with the irrelevant B10H2 mAb did not. Lymph node cells from rats transferred with LEW OVA cells had no effect on these aforementioned responses.

Lymph Node Cells from LEW Hg A Recipient Rats Recognize Activated T Cells. To assess the specificity of cells from rats transferred with the LEW Hg A T cell line, lymph node cells were cultured in the presence of various stimulus, 21 d after the transfer. They proliferated significantly in the presence of the activated LEW Hg A T cell line ($p < 0.005$) and also, but to a lesser extent, in the presence of the activated LEW OVA T cell line ($p < 0.01$), (Table 3), but not in the presence of autologous APC (not shown), when compared with lymph node cells cultured alone. The proliferative response was greatly reduced when LEW Hg A stimulator cells were not activated, and was abolished when nonactivated LEW OVA cells were used (Table 3). LEW spleen cells activated by Con A, but not normal spleen cells, can also stimulate cells from rats transferred with the LEW Hg A T cell line. Supernatant from Con A–activated spleen cells or from LEW Hg A–activated cells only induced a marginal proliferative response (Table 3). Lymph node cells from rats transferred with the LEW Hg A T cell line and treated with the anti-CD8 mAb exhibited the same pattern of responses but with much less intensity. Lymph node cells from rats transferred with the LEW OVA T cell line responded to activated LEW OVA T cells, and less to activated LEW Hg A T cells or resting LEW OVA T cells, and not at all to resting LEW Hg A cells. They did not respond to OVA (not shown).

None of the LEW rats transferred with the LEW Hg A T cell line, whether or not they were immunized with myelin and whether or not they were treated with the OX8 mAb, exhibited an increase in serum IgE concentration or renal immune deposits (not shown). These data show that the LEW Hg A T cell line fails to promote autoimmunity.

Table 3. *Proliferative Responses of Lymph Node Cells from Rats Transferred with the LEW Hg A or the LEW OVA T Cell Line*

Cells transferred	Treatment with anti-CD8 mAb	Stimulus	cpm
LEW Hg A	–	–	225 ± 157
LEW Hg A	–	LEW Hg A*	17,568 ± 9,236
LEW Hg A	–	LEW Hg A‡	4,790 ± 3,678
LEW Hg A	–	Sn LEW Hg A	523 ± 145
LEW Hg A	–	Sn Con A	986 ± 163
LEW Hg A	–	LEW nl	722 ± 251
LEW Hg A	–	LEW nl§	8,892 ± 270
LEW Hg A	–	LEW OVA*	5,655 ± 2,436
LEW Hg A	–	LEW OVA‡	498 ± 242
LEW Hg A	+	–	356 ± 175
LEW Hg A	+	LEW Hg A*	3,442 ± 1,364
LEW Hg A	+	LEW Hg A‡	1,021 ± 498
LEW Hg A	+	Sn LEW Hg A	778 ± 298
LEW Hg A	+	Sn Con A	1,225 ± 625
LEW Hg A	+	LEW OVA*	1,798 ± 293
LEW Hg A	+	LEW OVA‡	512 ± 131
LEW OVA	–	–	945 ± 198
LEW OVA	–	LEW Hg A*	3,225 ± 354
LEW OVA	–	LEW Hg A‡	1,045 ± 512
LEW OVA	–	LEW OVA*	8,990 ± 1,693
LEW OVA	–	LEW OVA‡	3,629 ± 355

Sn, supernatant.
* Cell lines were activated for 3 d with the appropriate stimulus before they were used as stimulator cells.
‡ Stimulator cells were used 7 d after the last stimulation.
§ Stimulator cells were normal LEW lymph node cells (LEW nl) stimulated for 2 d with Con A.

Discussion

This work shows that $HgCl_2$ induces in LEW rats the appearance of anti–class II autoreactive $CD4^+$ T cells and of antiergotypic $CD8^+$ suppressor cells. Autoreactive anti–class II $CD4^+$ T cell lines have been derived. One of them was transferred that induced $CD8^+$ suppressor T cells responsible for a non–antigen-specific suppression and for prevention of EAE.

Autoreactive anti–class II $CD4^+$ T cells are likely responsible for the autoimmune disease induced by $HgCl_2$ in BN rats, as suggested by transfer experiments (19). The $CD4^+$ T cells described here in LEW rats recognized self–class II molecules as in BN rats; their frequency was similar in both strains. Because these $CD4^+$ T cells were unable to promote autoimmunity in LEW rats, even when CD8 depleted, they probably differ at the functional level from those observed in BN rats. Two different $CD4^+$ T cell subsets have been described in mice according to the cytokines produced: Th1 cells produce IL-2 and IFN-γ, and are mainly involved in delayed-type hypersensitivity, while Th2 cells produce IL-4, IL-5, and IL-10, and are mainly involved in B cell help (20). There are convincing arguments supporting a role for Th2 cells in $HgCl_2$-induced autoimmunity in rats (21) and mice (22). The autoreactive T cell lines obtained in LEW rats produced IL-2 and were unable to help B cells in vitro for Ig production, suggesting that they belong to the Th1-like subtype. It has been demonstrated that human Th1 clones may

have a cytotoxic effect on APC (23) that could induce suppression. We have verified that the LEW Hg A T cell line had no such cytotoxic effect (not shown).

The characteristics of our autoreactive lines were very similar to those of the autoreactive cells detected in $HgCl_2$-injected LEW rats. They were IA but not IE restricted. In $HgCl_2$-injected LEW rats, autoreactive T cells and suppressor T cells appeared concomitantly, and $CD8^+$ cells were able to proliferate in the presence of autologous $CD4^+$ cells but not in the presence of $CD8^+$ cells. The LEW Hg A T cell line also induced, after transfer, the appearance of $CD8^+$ suppressor cells, which proliferated in the presence of the transferred $CD4^+$ line. In $HgCl_2$-injected, as in transferred, rats, $CD8^+$ cells were able to downmodulate various T cell functions and to prevent EAE (7). One apparent difference between suppression induced by $HgCl_2$ or by transfer of the LEW Hg A T cell line concerns the appearance of contrasuppressor cells, which were probably responsible for the partial protection towards EAE in $HgCl_2$-injected LEW rats while all the rats transferred with the line were protected. Studies are in progress to look for the presence of such cells in rats transferred with the LEW Hg A T cell line and to establish their possible connection with the suppressor cells.

The $CD8^+$ suppressor cells induced by the transfer of the line or after $HgCl_2$ injections proliferated in the presence of $CD4^+$ T cells. In the latter situation, as reported in another system (24), APC were not required; whether APC are important or not in the former situation is presently under study. Results obtained in rats transferred with the LEW Hg A T cell line showed that lymph node cells from these animals recognized far better this T cell line when preactivated. They also recognized, although less, the activated, irrelevant, anti-OVA T cell line or Con A–activated spleen cells but not resting cells. According to Lohse et al. (25), these data demonstrate that the LEW Hg A T cell line triggers $CD8^+$ antiergotypic cells, defined as cells that recognize a T cell activation marker. Therefore, the induction of antiergotypic cells is not restricted to T cell vaccination (25). CD8-depleted lymph node cells from rats transferred with the LEW Hg A T cell line still proliferated to the same stimuli as undepleted lymph node cells, but with much less intensity; this residual proliferation could be due to the remaining $CD8^+$ cells, or to $CD8^-$ antiergotypic cells (25). LEW Hg A T cell line–induced antiergotypic cells did not proliferate either in the presence of culture supernatant from activated LEW Hg A cells, or from Con A–activated cells, which suggests that these cells did not respond to lymphokines only but to a membrane antigen. The nature of this antigen remains to be determined. It could be an adhesion molecule (25) or peptides derived from the trimolecular complex (class II molecule, TCR, and peptide), expressed in the context of class I molecule as proposed by Janeway (26). The fact that lymph node cells from rats transferred with the LEW Hg A T cell line proliferated far better in response to activated LEW Hg A cells than to the other activated cells tested suggests either that activated LEW Hg A cells are better stimulators or that LEW Hg A–induced suppressor cells are heterogeneous. This is reminiscent of T cell vaccination where antiidiotypic (27–32) and, to a lesser degree, antiergotypic (25) suppressor T cells are able to protect against T cell–mediated diseases.

The way the $CD8^+$ suppressor cells act in our model remains to be determined. A cytotoxic effect on T cells or APC is unlikely since lymph node cells from rats transferred with the LEW Hg A T cell line are unable to kill LEW Hg A cells, autologous spleen cells, or lymph node cells from normal LEW rats immunized with myelin and challenged in vitro (not shown). They could interfere with signaling pathways of encephalitogenic effector cells (33) or by releasing cytokines such as IL-4 (34) or TGF-β (35). This latter hypothesis could explain the non–antigen-specific immunosuppression as shown for a suppressor line obtained from a nonobese diabetic mouse that was diabetes resistant (36). Works are in progress to obtain suppressor T cell lines to answer these questions.

It has already been reported that anti–class II T cells, able to polyclonally activate B cells, might trigger the appearance of suppressor cells able to suppress immune responses to several exogenous antigens (8). These results, as ours, could be explained by the emergence of antiergotypic and/or antiidiotypic cells. It could also explain results concerning the control of autoreactive anti–class II T cells by suppressor cells (37–39). Anti–class II T cells, initially described as a consequence of an immune response directed against nominal antigens (40), also exist as normal elements of the immune system (37, 39). Anti–class II T cells activated during an immune response would trigger suppressor cells that downmodulate in turn every autoreactive activated cell, whatever their specificity. Thus, $HgCl_2$-induced suppressor cells could represent an exacerbation of a physiological process controlling autoreactive cells that escape tolerance in periphery.

To seek chemicals that mimic mercury effects in LEW rats could be an interesting approach for therapeutics of multiple sclerosis in humans. Indeed, this demyelinating disease, of which EAE is the best experimental model, has been shown to be associated with a defect of suppressor inducer cells (41). Such treatments, by restoring normal regulatory circuits, would be more interesting than T cell vaccination since they do not require heavy technology, and since they could be applied to different autoimmune diseases.

We thank Drs. Pascal Poncet and C. Carnaud for the fruitful discussion.

This work was supported in part by a grant from Rhône-Poulenc-Rorer. Maria Castedo is a recipient of a grant from the Ministère de la Recherche et de l'Espace.

Address correspondence to Maria Castedo, INSERM U28, Hôpital Broussais, 96 rue Didot, 75674 Paris Cedex 14, France.

Received for publication 4 November 1992.

References

1. Druet, E., C. Sapin, E. Günther, N. Feingold, and P. Druet. 1977. Mercuric chloride-induced anti-glomerular basement membrane antibodies in the rat. Genetic control. *Eur. J. Immunol.* 7:348.
2. Hirsch, F., J. Couderc, C. Sapin, G. Fournier, and P. Druet. 1982. Polyclonal effect of $HgCl_2$ in the rat, its possible role in an experimental autoimmune disease. *Eur. J. Immunol.* 12:620.
3. Rossert, J., L. Pelletier, R. Pasquier, and P. Druet. 1988. Autoreactive T cells in mercury-induced autoimmunity. Demonstration by limiting dilution analysis. *Eur. J. Immunol.* 18:1761.
4. Pelletier, L., R. Pasquier, J. Rossert, and P. Druet. 1987. $HgCl_2$ induces nonspecific immunosuppression in LEW rats. *Eur. J. Immunol.* 17:49.
5. Pelletier, L., M. Galceran, R. Pasquier, P. Ronco, P. Verroust, J. Bariety, and P. Druet. 1987. Down-modulation of Heymann's nephritis by mercuric chloride. *Kidney Int.* 32:227.
6. Pelletier, L., J. Rossert, R. Pasquier, H. Villarroya, M.-F. Belair, M.-C. Vial, R. Oriol, and P. Druet. 1988. Effect of $HgCl_2$ on experimental allergic encephalomyelitis in Lewis rats. $HgCl_2$-induced down-modulation of the disease. *Eur. J. Immunol.* 18:243.
7. Pelletier, L., J. Rossert, R. Pasquier, H. Villarroya, R. Oriol, and P. Druet. 1991. $HgCl_2$-induced perturbation of the T cell network in experimental allergic encephalomyelitis. II. In vivo demonstration of the role of T suppressor and contrasuppressor cells. *Cell. Immunol.* 137:379.
8. Sano, K., I. Fujisawa, R. Abe, Y. Asano, and T. Tada. 1987. MHC-restricted minimal regulatory circuit initiated by a class II-autoreactive T cell clone. *J. Exp. Med.* 165:1284.
9. Pelletier, L., J. Rossert, R. Pasquier, M.-C. Vial, and P. Druet. 1990. Role of CD8+ T cells in mercury-induced autoimmunity or immunosuppression in the rat. *Scand. J. Immunol.* 31:65.
10. Barclay, A.N. 1981. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues. *Immunology.* 42:593.
11. Brideau, R.J., P.B. Carter, W.R. McMaster, D.W. Mason, and A.F. Williams. 1980. Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* 10:609.
12. Paterson, D.J., W.A. Jefferies, J.R. Green, M.R. Brandon, P. Cortese, M. Puklavec, and A.F. Williams. 1987. Antigens of activated rat lymphocytes including a molecule of 50000 Mr restricted to CD4 positive T-blasts. *Mol. Immunol.* 24:1281.
13. McMaster, W.R., and A.F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* 9:426.
14. Fukumoto, T., W.R. McMaster, and A.F. Williams. 1982. Mouse monoclonal antibodies against rat major histocompatibility antigens. Two Ia antigens and expression of Ia and class I antigens in rat thymus. *Eur. J. Immunol.* 12:237.
15. Spickett, G.P., M.R. Brandon, D.W. Mason, A.F. Williams, and G.R. Woollett. 1983. MRC-OX22, a monoclonal antibody that labels a new subset of T lymphocytes and reacts with the high molecular weight form of the leukocyte-common antigen. *J. Exp. Med.* 158:795.
16. Sapin, C., F. Hirsch, J.-P. Delaporte, H. Bazin, and P. Druet. 1984. Polyclonal IgE increase after $HgCl_2$ injections in BN and LEW rats: a genetic analysis. *Immunogenetics.* 20:227.
17. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* 126:1614.
18. Guéry, J.C., E. Druet, D. Glotz, F. Hirsch, C. Mandet, E. De Heer, and P. Druet. 1990. Specificity and cross-reactive idiotypes of anti-glomerular basement membrane autoantibodies in $HgCl_2$-induced autoimmune glomerulonephritis. *Eur. J. Immunol.* 20:93.
19. Pelletier, L., R. Pasquier, J. Rossert, M.-C. Vial, C. Mandet, and P. Druet. 1988. Autoreactive T cells in mercury-induced autoimmunity. Ability to induce the autoimmune disease. *J. Immunol.* 140:750.
20. Mossmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.
21. Dubey, C., B. Bellon, and P. Druet. 1991. TH1 and TH2 dependent cytokines in experimental autoimmunity and immune reactions induced by chemicals. *Eur. Cytokine Net.* 2:147.
22. Ochel, M., H.W. Vohr, C. Pfeiffer, and E. Gleichmann. 1991. IL-4 is required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride. *J. Immunol.* 146:3006.
23. Del Prete, G.F., M. De Carli, M. Ricci, and S. Romagnani. 1991. Helper activity for immunoglobulin synthesis of T helper type I (Th1) and Th2 human T cell clones: the help of Th1 clones is limited by their cytolytic capacity. *J. Exp. Med.* 174:809.
24. Vandenbark, A.A., G. Hashim, and H. Offner. 1989. Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. *Nature (Lond.).* 341:541.
25. Lhose, A.W., F. Mor, N. Karin, and I.R. Cohen. 1989. Control of experimental autoimmune encephalomyelitis by T cells responding to activated T cells. *Science (Wash. DC).* 244:820.
26. Janeway, C.A. 1989. Immunotherapy by peptides? *Nature (Lond.).* 341:482.
27. Cohen, I.R. 1986. Regulation of autoimmune disease. Physiological and therapeutic. *Immunol. Rev.* 94:5.
28. Lider, O., N. Karin, M. Shinitzky, and I.R. Cohen. 1987. Therapeutic vaccination against adjuvant arthritis using autoimmune T cells treated with hydrostatic pressure. *Proc. Natl. Acad. Sci. USA.* 84:4577.
29. Lider, O., T. Reshef, E. Beraud, A. Ben-Nun, and I.R. Cohen. 1988. Anti-idiotypic network induced by T cell vaccination against experimental autoimmune encephalomyelitis. *Science (Wash. DC).* 239:181.
30. Sun, D., A. Ben-Nun, and H. Wekerle. 1988. Regulatory circuits in autoimmunity: recruitment of counterregulatory $CD8^+$ T cells by encephalitogenic $CD4^+$ T cell line cells. *Eur. J. Immunol.* 18:1993.
31. Jingwu, Z., M. Schreurs, R. Medaer, and J.C.M. Raus. 1992. Regulation of myelin basic-protein-specific helper T cells in

multiple sclerosis: generation of suppressor T cell lines. *Cell. Immunol.* 139:118.

32. Lohse, A.W., E. Mor, T. Reshef, K.H. Meyer zum Büschenfelde, and I.R. Cohen. 1990. Inhibition of mixed lymphocyte reaction by T cell vaccination. *Eur. J. Immunol.* 20:2521.
33. Hisatsune, T., A. Enomoto, K.-I. Nishijima, Y. Minai, Y. Asano, T. Tada, and S. Kaminogawa. 1990. CD8+ suppressor T cell clone capable of inhibiting the antigen- and anti-T cell receptor-induced proliferation of Th clones without cytolytic activity. *J. Immunol.* 145:2421.
34. Bloom, B.R., P. Salgame, and B. Diamond. 1992. Revisiting and revising suppressor T cells. *Immunol. Today.* 13:131.
35. Karpus, W.J., and R.H. Swanborg. 1991. CD4+ suppressor cells inhibit the function of effector cells of experimental autoimmune encephalomyelitis through a mechanism involving transforming growth factor-β. *J. Immunol.* 146:1163.
36. Pankewycz, O.G., J.-X. Guan, and J.F. Benedict. 1992. A protective NOD islet-filtrating CD8+ T cell clone, I.S.2.15, has in vitro immunosuppressive properties. *Eur. J. Immunol.* 22:1017.
37. Nagarkatti, P.S., M. Nagarkatti, L.W. Mann, L.A. Jones, and A.M. Kaplan. 1988. Characterization of an endogenous Lyt2+ T-suppressor-cell population regulating autoreactive T cells in vitro and in vivo. *Cell. Immunol. Immunopathol.* 112:64.
38. Romain, P.L., C. Morimoto, J.F. Daley, L.S. Palley, E.L. Reinherz, and S.F. Schlossman. 1984. Reactivity of inducer cell subsets and T8-cell activation during the human autologous mixed lymphocyte reaction. *Clin. Immunol.* 30:117.
39. Nagarkatti, P.S., E.C. Snow, and A.M. Kaplan. 1985. Characterization and function of autoreactive T-lymphocyte clones isolated from normal unprimed mice. *Cell. Immunol.* 94:32.
40. Faherty, D.A., D.R. Johnson, and M. Zauderer. 1985. Origin and specificity of autoreactive T cells in antigen-induced populations. *J. Exp. Med.* 161:1293.
41. Morimoto, C., D.A. Hafler, H.L. Weiner, N.L. Letvin, M. Hagan, J. Daley, and S.F. Schlossman. 1987. Selective loss of the suppressor-inducer T-cell subset in progressive multiple sclerosis. *N. Engl. J. Med.* 316:67.