Transforming Growth Factor β (TGF-β)-dependent Inhibition of T Helper Cell 2 (Th2)-induced Autoimmunity by Self–Major Histocompatibility Complex (MHC) Class II–specific, Regulatory CD4+ T Cell Lines

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Summary

Autoreactive anti–MHC class II T cells are found in Brown Norway (BN) and Lewis (LEW) rats that receive either HgCl₂ or gold salts. These T cells have a Th2 helper cell phenotype in the former strain and are responsible for Th2-mediated autoimmunity. In contrast, T cells that expand in LEW rats produce IL-2 and prevent experimental autoimmune encephalomyelitis, a cell-mediated autoimmune disease. The aim of this work was to investigate, using T cell lines derived from HgCl₂-injected LEW rats (LEWHg), the effect of these autoreactive T cells on the development of Th2-mediated autoimmunity. The five LEWHg T cell lines obtained protect against Th2-mediated autoimmunity induced by HgCl₂ in (LEW × BN)F₁ hybrids. The lines produce, in addition to IL-2, IFN-γ and TGF-β, and the protective effect is TGF-β dependent since protection is abrogated by anti-TGF-β treatment. These results identify regulatory, TGF-β–producing, autoreactive T cells that are distinct from classical Th1 or Th2 and inhibit both Th1- and Th2-mediated autoimmune diseases.

Mercuric chloride or gold salts induce in Brown Norway (BN), in (Lewis [LEW] × BN)F₁ hybrids, and in susceptible mice, a transient Th2-dependent B cell polyclonal activation (1–4) responsible for an increase in serum IgE concentration and for the production of various autoantibodies including anti-DNA and antilaminin antibodies (for review see reference 1). These latter antibodies are associated with the occurrence of an autoimmune glomerulonephritis as observed in some patients treated with gold salts (5). T cells that recognize either self-MHC class II molecules or an ubiquitous self-peptide presented by MHC class II molecules play an important role in the induction of B cell polyclonal activation in this model (6, 7). In contrast, HgCl₂ provokes in LEW rats, a nonantigen-specific suppression and protects from autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) to which this strain is otherwise highly susceptible. Autoreactive anti-self-MHC class II T cells have also been detected in HgCl₂-injected LEW rats (8). Autoreactive T cell lines have been derived from gold-injected BN rats and HgCl₂-injected LEW rats (LEWHg T cell lines) by repeated stimulations with syngeneic APCs. In both strains, these anti-MHC class II T cell lines are RT1.B- (mouse IA equivalent), but not RT1.D- (mouse IE equivalent) restricted (7, 8). Whereas T cell lines derived from BN rats produce IL-4 and are able to passively transfer autoimmunity into CD8-depleted naive BN rats (7), the LEWHg T cell line, derived from HgCl₂-injected LEW rats, produces IL-2 and IFN-γ and protects LEW rats against EAE, a Th1-mediated autoimmune disease, by inducing regulatory CD8+ T cells (8).

The aim of this study was to assess the effect of adoptive transfer of LEWHg T cell lines on the course of Th2-mediated autoimmunity induced by HgCl₂ in (LEW × BN)F₁ hybrids. We show that (a) the lines produce TGF-β in addition to IL-2 and IFN-γ, (b) adoptive transfer of the lines prevents HgCl₂-induced autoimmunity, and (c) this protection is abrogated after anti-TGF-β mAb administration.

Materials and Methods

Rats

BN and LEW rats were obtained from Charles River (Rouen, France) and maintained in our facilities. F₁ hybrids were obtained by crossing BN male and LEW female rats in our animal house.

1 Abbreviations used in this paper: AU, arbitrary units; BN, Brown Norway; EAE, experimental autoimmune encephalomyelitis; LEW, Lewis; LEWHg, HgCl₂-injected LEW rats.
Culture Medium

RPMI 1640 (Biochrom KG, Berlin, Germany) was supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml), nonessential amino acids (0.1 mM), L-glutamine (2 mM; Gibco, Paisley, U.K.), sodium pyruvate (1 mM; Biochrom KG), and 2-ME (5 × 10⁻⁵ M); Sigma Chemical Co., St. Louis, MO).

mAbs and FAC S® Analysis

R73, W3/25, OX8, OX81, and B10.H2 mAbs are mouse IgG1 mAbs that recognize rat TCR-α/β chains (9), CD4 (10), CD8 (11), rat IL-4 (12), and thryoglobulin, respectively. The IgG1 1D11.16 and the IgG2a 2G.7 anti-TGF-β mAbs were provided by Dr. W. Waege (Celtrix Pharmaceuticals, Santa Clara, CA; 13) and by Dr. C.M. Melief (University of Leiden, Leiden, Netherlands, 14), respectively. The IgG1 DB.1 and DB.12 anti-IFN-γ mAbs were provided by Dr. P.H. Van der Meide (15) and B10.H2 mAb by Dr. D. Glotz (Paris, France). The W3/25 and OX8 hybridomas were obtained from the Public Health Laboratory Service (Oxford, U.K.); Dr. T. Hunig (Würzburg, Germany), and Dr. D. Mason (Medical Research Council, Oxford, U.K.) provided the R73 and OX81 hybridomas, respectively. Ascites and purified antibodies were prepared as described (16).

The phenotype of cells was determined by double staining; cells were incubated with FITC-labeled W3/25 and biotinylated-mAbs were provided by Dr. P.H. Van der Meide (15) and Dr. D. Mason (Medical Research Council, Oxford, U.K.) provided the R73 and OX81 hybridomas, respectively. Ascites and purified antibodies were prepared as described (16).

The five LEWHg T cell lines (LEWHg A, B, C, D, and E) used in this study have been previously described (8). They were derived from lymph node cells of five distinct LEW rats injected with HgCl₂ for 7 d; they are CD4⁺/CD8⁻ TCR-α/β⁺, proliferate in the presence of syngeneic APCs, and are MHC class II (R T1.B)-restricted. The LEWOVA T cell line was also a CD4⁺/CD8⁻ TCR-α/β⁺ T cell line derived from the draining lymph nodes of a LEW rat immunized 10 d before with 100 μg OVA in CFA. Except when otherwise mentioned, T cell lines were stimulated repeatedly every 2 wk as follows: 2 × 10⁶ cells/well were cultured in 24-well flat-bottomed plates (Nunc, Kamstrup, Denmark), in a 6% CO₂ incubator in the presence of normal irradiated (3,000 rads) syngeneic lymphocytes (5-10 × 10⁶/well), in a volume of 1 ml/well culture medium supplemented with 10% heat-inactivated FCS and 10% Con A supernatant (8). The LEWOVA T cell line was stimulated with 50 μg/ml OVA (Sigma Chemical Co.).

Cytokine Assays

T cell lines were stimulated with irradiated thymocytes as described above, in the absence of Con A supernatant; 24-, 48-, 72-, 96-, and 120-h culture supernatants were collected and stored at −20°C. IL-2 production was assessed using proliferation of the IL-2-dependent CTL-L-2 cell line (7). INF-γ production was determined by two-site sandwich ELISA (7). Values were expressed as U/ml IFN-γ referring to a standard curve constructed using serial dilutions of recombinant purified rat IFN-γ (a gift from Dr. P. van der Meide, Vrije U Nisvrest, Amsterdam, Netherlands). IL-4 detection was based upon the ability of this cytokine to upregulate MHC class II molecule expression on B cells (12). The assay was performed, in the presence or in the absence of the OX 81 anti-rat IL-4 mAb (50 μg/ml), by fluorometry. R ecombinant rat IL-4, (provided by Dr. D. Mason, Medical Research Council, CIU, Oxford, U.K.) was used as a positive control.

TGF-β was measured by ELISA (R&D Sys., Inc., Abingdon, U.K.) according to the manufacturer’s instructions. To demonstrate that TGF-β was produced by T cells and not by APCs, the LEW Hg T cell line was also stimulated as follows: 96-flat-bottomed wells were precoated with rabbit anti–mouse Ig antibodies (DAKOPATTS, Copenhagen, Denmark) overnight; the LEW Hg T cell line was then added (5 × 10⁴/well), together with mouse anti-rat TCR-α/β mAb (50 μg/ml) as described elsewhere (9). Supernatant was collected after 24, 48, and 96 h. Cells were also cultured for 72 h to assess the proliferative response. The anti-rat TCR-α/β mAb was replaced in control cultures by the B10.H 2 mAb.

Experimental Design

HgCl₂ Injections and Follow-Up of Murine D i s e a s e. Rats were injected with HgCl₂ (100 μg/100 g body wt, subcutaneously, 3 times/wk) during 4 to 6 wk (6). All the rats were bled once a week for determination of serum IgE concentration, antilaminin, and occasionally, anti-DNA antibody titers as previously described (17, 18). Kidneys were processed at the time of killing for immunofluorescence studies, using FITC-conjugated sheep anti-rat IgG antiserum as previously described (7). The intensity of fluorescence was scored on a scale from 0 to 4 in blind study by two independent examiners.

Adoptive Transfer of T Cell Lines. T cell lines (LEWHg A, LEWHg B, LEWHg C, LEWHg D, and LEWHg E) were stimulated for 3 d with syngeneic irradiated thymocytes only or LEWOVA with syngeneic irradiated thymocytes plus ovalbumin. T cell lines (10⁷ cells) were then intraperitoneally injected into naive F1 rats 2 wk before the first HgCl₂ injection.

Thymectomy, Anti-CD8, and Anti-TGF-β Treatment. Rats were thymectomized at 8 wk of age (19), allowed to recover for 3 wk, and then injected with the LEWHg T cell line; 15 d later, rats were injected with HgCl₂. In addition, some rats received the anti-CD8 (OX8) mAb or the control isotype-matched B10.H2 mAb (500 μg intraperitoneally, once a week for 1 mo) from the first HgCl₂ injection (8). The effect of TGF-β neutralization was assessed using the anti-TGF-β mAbs 1D11.16 and 2G.7 (2 mg intraperitoneally on days −2, 0, 3, 6, and 9 with respect to the first HgCl₂ injection).

Statistical Analysis

Serum IgE concentration, antilaminin, and anti-DNA antibody titers were compared among the different groups by using nonparametrical tests. The number of rats per group with kidney-bound IgG was compared by chi-square analysis.

Results

Description of T Cell Lines. As previously described (8), the five LEWHg T cell lines used in this study are CD4⁺/CD8⁻ TCR-α/β⁺ and proliferate in the presence of normal syngeneic MHC class II+ APCs. They all produced IL-2 and INF-γ (Table 1) with a peak at 24 h for IL-2 and 48 or 72 h for INF-γ. The LEWHg, but not the LEWOVA,
Table 1. Cytokines Produced by the LEWHg and LEWOVA T Cell Lines

| Line (N.o. of exp.) | IL-2* (cpm × 10³) | IFN-γ (U/ml) | TGF-β (ng/ml) |
|---------------------|-------------------|-------------|-------------|
| LEWHgA (8)          | 12 ± 3.5          | 15 ± 9      | 2.3 ± 0.9   |
| LEWHgB (2)          | 8 ± 6             | 10 ± 13     | 1.7 ± 0.9   |
| LEWHgC (2)          | 12 ± 7            | 13 ± 15     | 0.6 ± 0.5   |
| LEWHgD (2)          | 8 ± 12            | 2.4 ± 5     | 0.15 ± 0.3  |
| LEWHgE (2)          | 9.6 ± 15          | 13 ± 15     | 0.6 ± 0.5   |
| LEWOVA (5)          | 10.5 ± 1.5        | 12 ± 3      | <0.1        |
| Unstimulated lines  | 0.5 ± 0.1         | 0           | <0.1        |

Results are expressed as mean ± SD when five or eight experiments were performed; otherwise individual values were given.

*The background value of CTLL-2 cells was 700 ± 150 cpm.

T cell lines produced TGF-β (Table 1) with a peak at 96 or 120 h of stimulation. None of these cytokines were detected in supernatants from irradiated APCs only, or in supernatants of resting T cell lines (Table 1). To eliminate a role for APCs in producing TGF-β, the LEWHgA T cell line was stimulated by plate-bound anti-TCR mAb in the absence of APCs. In such conditions, the LEWHgA T cell line proliferated (index 3 ± 0.5) and produced TGF-β (1.72 ± 0.05 ng/ml). No proliferation and no TGF-β secretion were observed when the anti-TCR mAb was replaced by the control B10.H2 mAb.

The supernatant from the stimulated LEWHgB T cell line increased MHC class II expression on B cells (mean fluorescence intensity of 51 versus 10 for B cells cultured alone), and this increase was abolished by addition of the anti-IL-4 OX81 mAb (mean fluorescence intensity of 14) suggesting that this line produced IL-4. None of the other LEWHg or LEWOVA T cell lines produced detectable amounts of IL-4 in this assay (not shown).

A new MHC class II LEWHg T cell line prevented HgCl₂-induced autoimmunity in F1 rats. Transfer of the LEWHgA T cell line at the time of the first HgCl₂ injection attenuated the autoimmune manifestations, but did not completely prevent the polyclonal activation of B cells nor renal IgG deposition (data not shown). By contrast, HgCl₂-induced immunopathological manifestations were abrogated or considerably reduced in rats that received the LEWHg T cell lines 15 d before the first HgCl₂ injection. In these rats, antilaminin antibody titer was much lower as compared to that of HgCl₂-injected rats (P < 0.005) and did not differ from that observed in normal rats (Fig. 1, A and B). Similar results were obtained concerning anti-DNA antibodies (not shown). Serum IgE concentration dramatically decreased when compared to HgCl₂-injected rats (P < 0.01), but remained higher than in normal rats (Fig. 1, C and D). The number of rats with glomerular IgG deposits was reduced in those who received the LEWHg T cell lines (from 60 to 19% depending upon the line, versus 100% in rats that received HgCl₂ only; P < 0.01); when considering rats that still exhibited IgG glomerular deposits, the intensity of immunofluorescence was greatly diminished when compared to rats injected with HgCl₂ only (Fig. 2). Transfer of the LEWOVA T cell line had no effect on HgCl₂-induced autoimmunity; these rats behaved as rats injected with HgCl₂ only with respect to serum IgE concentration, antilaminin antibody titer (Fig. 1, A and C), and glomerular IgG deposits (Fig. 2).

CD8⁺ C cells and Recent Thymic Emigrants Are Not Involved in the Protection. Since we had previously observed that the LEWHg T cell line protects from EAE in a CD8-dependent manner (8), the potential role of CD8⁺ cells was addressed in this present model. To achieve CD8⁺ cell depletion, rats were thymectomized and injected with the anti-CD8 mAb. These rats displayed <1.5% CD8⁺ cells in spleen and lymph nodes at the time of killing, compared to 15 ± 3% in control rats. In these rats, HgCl₂ injections induced similar, or even more severe, manifestations than in control rats injected with HgCl₂ (Fig. 3, A and B). Transfer of the LEWHg T cell line inhibited HgCl₂-induced antilaminin antibody production (Fig. 3 A), increase in serum...
IgE concentration (Fig. 3 B), and glomerular IgG deposits (not shown) whether rats were thymectomized and treated with the anti-CD8 mAb or not. These results show that neither CD8\(^+\) cells nor recent thymic emigrants were involved in the protection.

Protection of Mercury-induced Autoimmunity by LEWHg CD4\(^+\) T Cell Lines Is TGF-\(\beta\)-dependent. We next examined whether the inhibition of mercury disease induced by transfer of LEWHg T cell lines depends on the production of TGF-\(\beta\) in vivo. Interestingly, blocking TGF-\(\beta\) by administration of 1D11-16 mAb at the time of mercury disease induction completely abrogated the LEWHgA-mediated protective effect (Fig. 4). This is exemplified by the restoration of autoantibody production (Fig. 4 A), IgE synthesis (Fig. 4 B), and the reappearance of kidney Ig deposits to levels comparable to those observed in HgCl\(_2\)-injected control rats (not shown). Administration of a different TGF-\(\beta\)-specific mAb (2G.7) also prevented the protection induced by the transfer of LEWHgA T cells (Table 2). Treatment with anti-TGF-\(\beta\) mAb alone did not influence HgCl\(_2\)-induced autoimmune syndrome.

To demonstrate that TGF-\(\beta\)-dependent inhibition of Th2-mediated autoimmunity is not a unique property of LEWHgA T cell line, we tested the effect of anti-TGF-\(\beta\) mAb administration on the protection induced by the LEWHgA and two other self-MHC class II reactive T cell lines. LEWHgA, LEWHgB, and LEWHgC T cell lines have a comparable regulatory potential when transferred to F1 rats before induction of HgCl\(_2\)-induced disease (Table 2). Administration of the anti-TGF-\(\beta\) 2G.7 mAb at the time of disease induction completely reverted the protective effect induced by the transfer of LEWHg T cell lines as shown by the complete restoration of the disease criteria including loss of body weight (Table 2).

Taken together our data show that three autoreactive anti-self-MHC class II T cell lines independently derived from HgCl\(_2\)-injected LEW rats prevent Th2-mediated autoimmune disorders in (LEW \(\times\) BN)F1 rats, and that their strong immunoregulatory potential is dependent on the production of TGF-\(\beta\).

**Discussion**

This study shows that five autoreactive anti-self-MHC class II T cell lines derived from distinct HgCl\(_2\)-injected LEW rats protect susceptible (LEW \(\times\) BN)F1 rats from HgCl\(_2\)-induced, Th2-mediated autoimmunity. These lines produce TGF-\(\beta\) that is responsible for the protection observed.

An increase in serum IgE concentration, although considerably less than in controls, persisted in HgCl\(_2\)-injected...
rats transferred with the LEW Hg A T cell lines. This was possibly due to the recently described direct effect of HgCl₂ on IL-4 gene transcription (20).

A autoreactive anti-MHC class II T cells that may enhance or suppress immune responses have been described in normal situations and are responsible for the autologous mixed lymphocyte reaction (see review in reference 21). Similar cells may also participate in the peripheral control of pathogenic autoreactive T cells. Thus, autoreactive anti-MHC class II T cell lines derived either from diabetic biobreeding rats, or from nondiabetic NOD mice prevent autoimmune diabetes when transferred to diabetes-prone biobreeding rats (22) or NOD mice (23), respectively. HgCl₂ has been recently shown to induce a polyclonal activation of T cells in both BN and LEW rats (24), and thus, probably allows, among other T cells, the expansion of autoreactive anti-MHC class II T cells, from precursors present in normal animals (25).

The mechanism of action of these autoreactive, regulatory T cells is not yet completely understood. We will first discuss the role of cytokines and then the nature of cells involved in protection. Our results show that protection is provided by TGF-β. We cannot rule out that other cytokines produced by these lines also play a role in the regulation observed, but neutralization of TGF-β alone abrogated the regulatory effect of the line indicating that this cytokine was of major importance. The regulatory T cell lines described in other systems (26) were not tested for their ability to produce TGF-β, except for those derived from animals orally tolerized with myelin basic protein that were able to prevent EAE in a TGF-β-dependent manner (27–29). However, there is now large evidence that TGF-β plays a major role in controlling the emergence of several autoimmune or inflammatory diseases (30–39).

A second point to be discussed concerns the nature of the cells involved in the protection. One possibility is that the T cell lines are responsible by themselves for the effect through TGF-β production. Powrie and co-workers have shown that autoaggressive and regulatory T cells coexist in normal rats and mice (40, 41). The autoaggressive Th1-like subset induces severe immunopathological manifestations, such as colitis in mice, when transferred into immunocompromised recipients. The regulatory Th2-like subset can, however, be pathogenic and, in addition, prevents manifestations induced by the former subset. Powrie et al. have shown in mice that this protection was due to TGF-β, and not to IL-4 (42). The LEW Hg T cell lines might belong to this regulatory subset, but interestingly, do not have a Th2-like phenotype since they produce IL-2 and IFN-γ, but no detectable IL-4 with a biological assay except for one T cell line. Taken together, these results identify self-reactive T cells producing, in addition to TGF-β, either Th1- or Th2-associated cytokines, with a strong regulatory potential in both Th1- and Th2-mediated autoimmune diseases. Another nonexclusive possibility is that the T cell line recruits other T cells that participate in protection. In this respect, CD8⁺ T cells are crucial in the protective effect of the LEW Hg A CD4⁺ T cell line towards EAE in LEW rats (8). CD8⁺ cells play no role in the protection of F1 rats from HgCl₂-induced autoimmunity since protection was still observed in rats profoundly depleted of CD8⁺ cells after thymectomy and anti-CD8 mAb treatment. The difference in CD8⁺ cell requirement in both situations could be due to the different genetic make up of the recipients or to differences in experimental models. That the LEWHgA T cell line had to be injected 2 wk before the first HgCl₂ injection is compatible with a recruitment of CD4⁺ cells by the T cell line. If recruited cells play a role in protection
against HgCl₂-induced autoimmunity, they could resemble three previously described regulatory T cell populations (a) The first possibility is recent thymic emigrants, CD4⁺, or CD8⁺ T cells that have been converted into regulatory cells by the autoreactive T cells selected on thymic epithelium in an IL-4–independent manner (43). It is unlikely that recruited recent thymic emigrant cells were at play in our model since thymectomized rats injected 3 wk later with the LEW.HgA T cell line and injected with HgCl₂ were still protected. (b) The recruited T cells could be similar to the antidiotypic or antiergotypic T cells described by Lohse et al. (44). (c) They could also be related to regulatory cells guided to tolerance in the so-called infectious tolerance model (45). These hypotheses are currently being tested.

To conclude, the fact that HgCl₂ can induce the expansion, among other T cells, of a subset of regulatory T cells that may recognize ubiquitous peptides in the context of MHC class II molecules is probably of importance for our understanding of the regulatory circuits involved in autoimmunity. Depending upon the strain tested, those T cells could produce different cytokines: self MHC class II–reactive T cells developing in BN rats produce IL-4 and induce Th2-mediated disorders, whereas T cells expanding in LEW rats preferentially produce TGF-β responsible for down-regulation of autoimmunity.

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