

Regulatory Role of OX22^{high} T Cells in Mercury-induced Autoimmunity in the Brown Norway Rat

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Summary

The monoclonal antibody OX22 defines a functional split within CD4⁺ T cells in the rat, with OX22^{high} cells mainly producing interleukin 2 (IL-2) and interferon γ and responsible for delayed-type hypersensitivity responses, and OX22^{low} cells mainly producing IL-4 and -5 and responsible for providing B cell help. There are reciprocal interactions between OX22^{high} and OX22^{low} cells, and it has been suggested that the OX22^{low} subset has a role in the prevention of autoimmunity. We have used OX22 in vivo to define the role of these subsets in mercuric chloride-induced autoimmunity in the Brown Norway rat. In this model, there is polyclonal B cell activation and animals develop widespread tissue injury. Treatment of thymectomized animals with OX22 led to a profound reduction in the number of OX22^{high} T cells in the peripheral blood. OX22-treated animals consistently developed more severe tissue injury than controls given an irrelevant antibody of the same isotype. Control animals pretreated with broad spectrum antimicrobial drugs showed milder tissue injury, but this protective effect of antimicrobials was lost in OX22-treated animals. Transfer of naive T cells to OX22-treated animals provided protection, but if T cells were depleted in vitro of OX22^{high} cells before transfer, this effect was lost. These data provide evidence for a protective immunoregulatory role for OX22^{high} T cells in mercuric chloride-induced autoimmunity.

The functional split of T cells defined by the cell-surface markers CD4 and CD8 is well-known, with helper/inducer T cells defined by expression of CD4 and suppressor/cytotoxic T cells defined by CD8. There has been considerable recent progress in the characterization of functionally distinct T cell subpopulations within these major categories. In the mouse, CD4⁺ cells can usually be subdivided on the basis of the cytokines they secrete, with one subset (Th1) predominantly producing IL-2 and IFN- γ , and a second subset (Th2) predominantly producing IL-4 and IL-5 and responsible for providing B cell help (1). There is evidence of a similar functional split within CD4⁺ T cells in the rat (2, 3) and in humans (4). A number of phenotypic markers have been used to define subsets of CD4⁺ T cells, and one of the best studied of these is the CD45 molecule, also known as the leukocyte common antigen (5–7). CD45 occurs in several different isoforms that are derived by differential splicing of at least three variable exons situated near the 5' end of the gene (8). A number of mAbs are available that recognize specific CD45 isoforms denoted CD45RO, CD45RA, CD45RB, and CD45RC (9). In the rat, the mAb MRC-OX22 binds to a high molecular weight isoform (CD45RB and/or CD45RC) and defines subpopulations of CD4⁺ T cells with different functions (2, 10). OX22^{high} CD4⁺ cells produce IL-2 and IFN- γ , but little IL-4, whereas OX22^{low}

CD4⁺ cells provide B cell help, producing more IL-4 with less IL-2 and IFN- γ (2, 10). Transfer of OX22^{high} CD4⁺ T cells into nude athymic rats has been reported to induce a severe wasting disease with inflammation in a number of organs, whereas transfer of OX22^{low} CD4⁺ T cells has no such effect (11). Furthermore, if unfractionated CD4⁺ T cells were transferred, i.e., containing a mixture of approximately two-thirds OX22^{high} and one-third OX22^{low}, the nude recipients remained well, indicating that there is an interaction between the two subsets, with the OX22^{low} population apparently able to inhibit the deleterious effect of the OX22^{high} cells (11).

Mercuric chloride (HgCl₂) is a T cell-dependent polyclonal B cell activator that induces autoimmunity in susceptible rodent strains. In the Brown Norway (BN)¹ rat, HgCl₂ induces a self-limiting autoimmune syndrome with autoreactive CD4⁺ T cells (12, 13), hypergammaglobulinemia predominantly affecting the IgE class (14), and a number of IgG autoantibodies including antibodies to glomerular basement membrane (GBM) (15). We have recently reported (16) that HgCl₂-treated BN rats develop widespread tissue injury, par-

¹ Abbreviations used in this paper: BN, Brown Norway; GBM, glomerular basement membrane.

ticularly affecting the gut, skin, and liver, with some features resembling GVHD. We have examined the effect of OX-22 treatment in vivo on the autoimmune response induced in BN rats by HgCl₂. Our results provide evidence for a protective regulatory role of OX22^{high} T cells in this experimental model.

Materials and Methods

Experimental Model. BN rats were obtained from OLAC (Bicester, UK) and a breeding colony established. For all experiments, animals were used in age- and sex-matched groups. Rats were given HgCl₂ diluted in distilled water by five subcutaneous injections over a 7-d period, each injection consisting of 1 mg/kg HgCl₂. Animals were weighed regularly and serial blood samples were taken from a tail vein for analysis of lymphocyte subsets (heparinized blood) and for separation of serum for assay of anti-GBM antibodies. Where antimicrobial pretreatment was used, animals were given tylosin (Tylan[®]; Eli Lilly Italia, Florence, Italy) 10 mg/kg per day subcutaneously for 5 d, metronidazole (Torgyl[®]; May & Baker, Dagenham, UK) 20 mg/kg per day subcutaneously for 5 d, and ivermectin (Ivomec[®]; Merck, Sharp & Dohme, Hoddesdon, Herts, UK) 200 µg/kg subcutaneously on the first and fifth days. For the in vivo mAb treatment, animals were given mAbs by daily intravenous injection for 5 d immediately before starting HgCl₂.

Anti-GBM Antibody Assay. Sera were assayed for anti-GBM antibody by a solid phase RIA as described previously (17). Briefly, a collagenase digest of normal rat glomeruli was coated onto microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). After washing, the plates were incubated with test or control sera and bound antibody detected with goat anti-rat IgG (Sigma Immunochemicals, Poole, Dorset, UK) radiolabeled with ¹²⁵I by the chloramine T method. Sera were assayed in triplicate and the mean result expressed as a percentage of a strongly positive reference serum.

In Vivo mAb Treatment. Thymectomies were performed at age 3–4 wk by suction via a partial sternotomy wound under ether anesthesia. Animals were allowed to recover for at least 2 wk and then given mAbs 200 µg daily for five consecutive days by intravenous injection via a tail vein. The mAbs were produced in tissue culture or ascites, purified on protein A or by ion-exchange chromatography, then dialyzed into PBS and filter sterilized. Test animals received MRC OX22 or MRC OX8, and control animals received an isotype-matched (IgG1) mouse myeloma protein with an undetermined specificity, derived from a mineral oil-induced plasmacytoma (MOPC 21; Sigma).

Flow Cytometry. Blood was taken from the tail into heparinized tubes and PBL preparations made by density centrifugation on Ficoll-Hypaque (Pharmacia, Milton Keynes, UK). All dilutions were in PBS containing 1% BSA (British Drug House, Poole, Dorset, UK), and all incubation stages were at 4°C for 45 min. Cells were plated at 5 × 10⁵ cells per well in a microtiter plate and stained with MRC OX22 (10 µg/ml) followed by FITC-conjugated sheep anti-mouse Ig that had been adsorbed with rat serum proteins (Sigma), diluted 1 in 250. Double-staining analysis was also performed on PBLs, using biotinylated OX52 (pan-T cell) (18) (10 µg/ml) as the second antibody and streptavidin-PE (Serotec Ltd., Oxford, Oxon, UK) (diluted 1 in 250) as the second detection reagent. For analysis of the cells used in the cell transfers, first antibodies were OX22 and OX52, and detection was with FITC-conjugated sheep anti-mouse Ig. Cells were analyzed using a flow cytometer (Epics Profile; Coulter, Luton, UK). Appropriate controls were incorporated to assess autofluorescence and nonspecific staining.

Cell Transfers. Cell transfers were administered on day 10 after starting HgCl₂. Nine syngeneic naive BN rats were used as spleen cell donors. Spleens were removed into tissue culture medium (IMDM; Gibco, Paisley, Scotland), red cells lysed with Tris-buffered ammonium chloride, and single cell suspensions prepared by sieving. Nylon wool columns were used to prepare purified T cells, which were then pooled and counted. One portion of these was kept for cell transfers, and one portion was subjected to depletion in vitro of OX22^{high} T cells. To achieve this, T cells were incubated in a 96-well plate at 4 × 10⁶ cells/well with 50 µg OX22, washed twice, and then incubated with magnetic beads coated with sheep anti-mouse IgG (Dynal AS, Oslo, Norway), using a 10:1 bead/target ratio. A magnet was used to extract cells bound to the beads. Remaining cells were washed, resuspended in tissue culture medium, and infused intravenously into HgCl₂-treated animals (4 × 10⁷ cells per animal). Aliquots were kept at each stage for analysis by flow cytometry.

Histological Methods. Animals from each group were killed at various stages of the experiment and immediate necropsies performed. Organs were fixed in buffered formalin, routinely processed, and embedded in paraffin wax. 3–4 µm sections were evaluated for histological alterations with haematoxylin–eosin stain. All sections were examined by an experienced histopathologist (S. Thiru) without knowledge of the experimental groups. Severity of inflammatory changes in the gut, liver, and lung was graded for each animal according to an arbitrary scale: 0, normal; 1, mild; 2, mild to moderate; 3, moderate to severe; and 4, severe. These scores were then added up to give a total tissue injury score for each animal, with a possible maximum score of 12.

Statistical Analysis. Serial data on anti-GBM antibody titers and body weight were analyzed by repeated measures analysis of variance using a computer program (PC+; SPSS Inc., Chicago, IL). Histological data were analyzed by the permutation test for two independent samples (19).

Results

Experimental Design. The results are reported of four experiments involving a total of 96 animals (summarized in Fig. 1). In the first experiment (*n* = 15), five rats received OX22, five received OX8 to deplete CD8⁺ T cells (20), and five received no mAb. In the second experiment (*n* = 27), nine rats received OX22, nine received OX8, and nine received A5, an irrelevant mAb of the same isotype (21). These experiments were designed to analyze the effect of these mAbs on the immunoregulation of the autoantibody response (20), and so detailed histological analysis was not carried out on all animals. However, the external appearances of the animals were scored according to an arbitrary scale to assess the severity of the mucocutaneous inflammation, and in experiment 2, histology was obtained from nine of the animals (three in each group). Taking experiments 1 and 2 together, there was a significant difference between the groups in terms of external scores (*p* = 0.04, Kruskal-Wallis analysis of variance), with the OX22 group having more severe inflammation and the OX8 group not being significantly different from controls. The histological analysis of nine animals in experiment 2 showed that the OX22-treated animals had more severe tissue injury than the controls (*p* < 0.05) and the OX8 group (*p* < 0.05). The controls and the OX8 group were not significantly different.

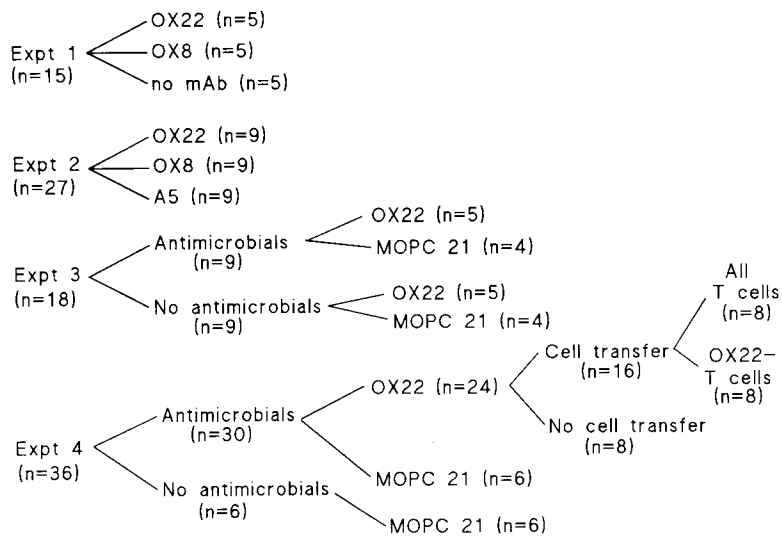


Figure 1. Experimental design.

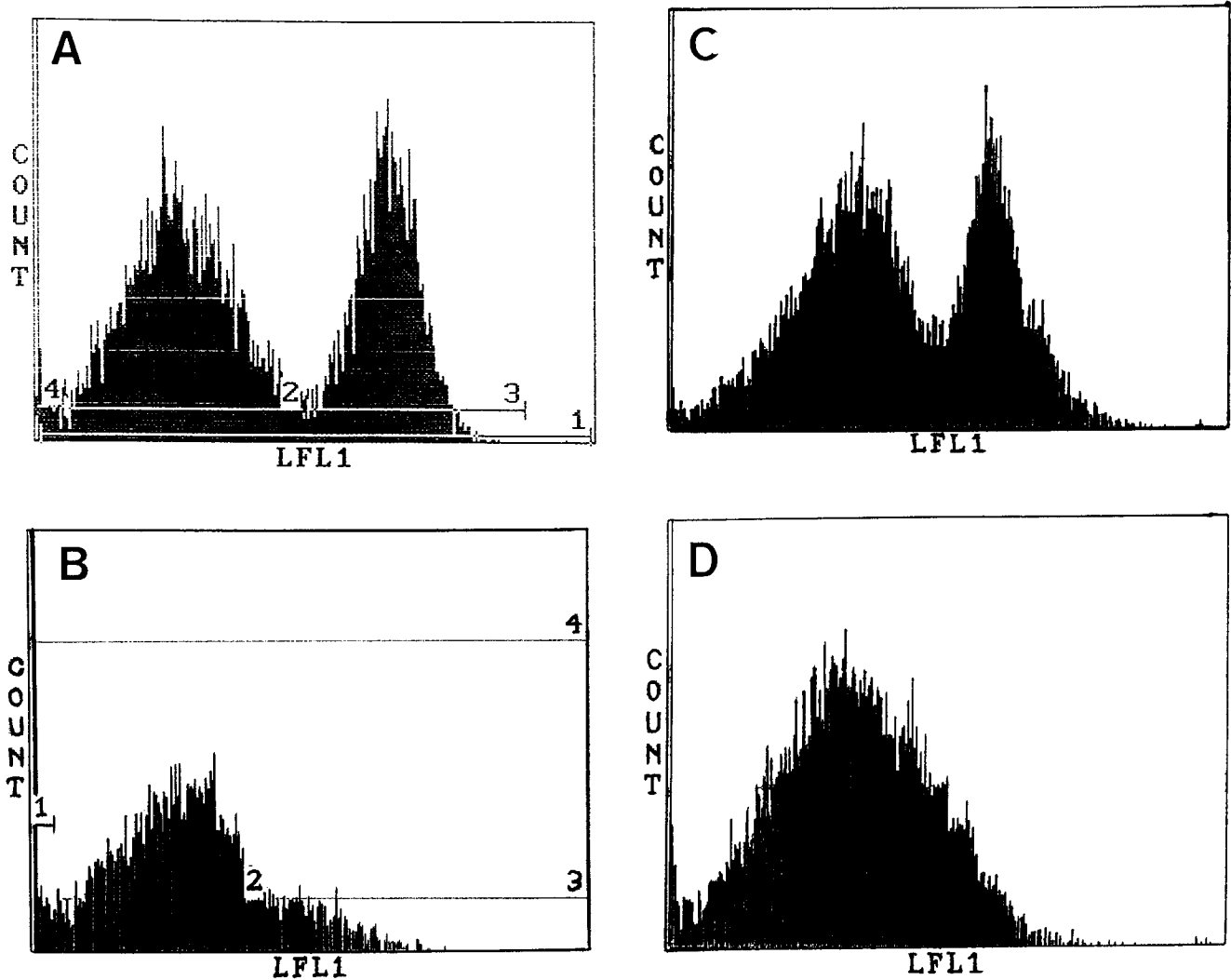


Figure 2. Flow cytometry analysis. (A) PBLs from untreated animal, stained with OX22 (LFL1). (B) PBLs from OX22-treated animal, stained with OX22 (LFL1). (C) Nylon wool-separated T cells, stained with OX22 (LFL1). (D) T cells after in vitro depletion of OX22^{high} cells, stained with OX22 (LFL1).

Having observed that OX22 treatment exacerbated tissue injury, we designed the third and fourth experiments to confirm and extend this observation. In the third experiment ($n = 18$), nine animals were pretreated with antimicrobials and nine were not. In each group of nine, five received OX22 and four received control mAb. In the fourth experiment ($n = 36$), 30 animals were pretreated with antimicrobials and six were not. In the pretreated group, six received control mAb and 24 received OX22. The OX22-treated animals were further subdivided into three groups of eight for the cell transfers: eight received unfractionated T cells, eight received OX22-depleted T cells, and eight received no cell transfer.

Flow Cytometry Analysis. PBLs from untreated animals gave a typical profile of staining with OX22, with two peaks of positive-staining cells, designated OX22^{high} and OX22^{low}, typically accounting for 55–65% and 30–40% of total PBLs, respectively (Fig. 2 A). Using double staining, there was a population of OX22^{high} T cells which accounted for 35–45% of total PBLs (data not shown). Animals receiving control mAb showed no difference from untreated animals. OX22-treated animals showed loss of the OX22^{high} peak (Fig. 2 B) and reduction of the double-staining OX22^{high} T cells to 10% or less of the total. Flow cytometric analysis of the spleen cells used in the cell transfers showed that 96–97% of the nylon wool-separated cells were OX52 positive, i.e., a relatively pure population of T cells. This cell preparation contained OX22^{high} and OX22^{low} cells (Fig. 2 C), and after in vitro depletion with OX22, the OX22^{high} population had been removed (Fig. 2 D).

Tissue Injury: External Appearances. As we have reported previously (16), rats developed severe inflammation of mucocutaneous junctions by 10–12 d after HgCl₂. In the two initial experiments in which various anti-T cell mAbs were used, it was a consistent observation that the external inflammation was most marked in animals that had received OX22. As also reported previously (16), antimicrobial pretreatment greatly reduced the severity of the mucocutaneous inflammation, but this was less apparent in the OX22-treated animals, in which the protective effect of antimicrobials was overcome by the exacerbating effect of OX22 treatment.

Tissue Injury: Body Weight. In the fourth experiment, animals were weighed sequentially. Animals not pretreated with antimicrobials showed significant weight loss compared with pretreated animals ($p = 0.002$), with a mean weight loss of 5.9% of baseline body weight in the nonpretreated group compared with a mean weight gain of 2.4% of baseline in the pretreated animals. There was a trend to greater weight loss in the OX22-treated animals than in the controls, but this did not quite reach statistical significance ($p = 0.06$).

Tissue Injury: Histology. In the third and fourth experiments, the differences between the tissue injury seen in the various groups were quantified using the histological scoring system. In the third experiment, OX22-treated animals had more severe tissue injury than controls ($p < 0.0005$, Fig. 3). There was no significant protective effect of antimicrobial pretreatment in this experiment (data not shown). Fig. 4 shows

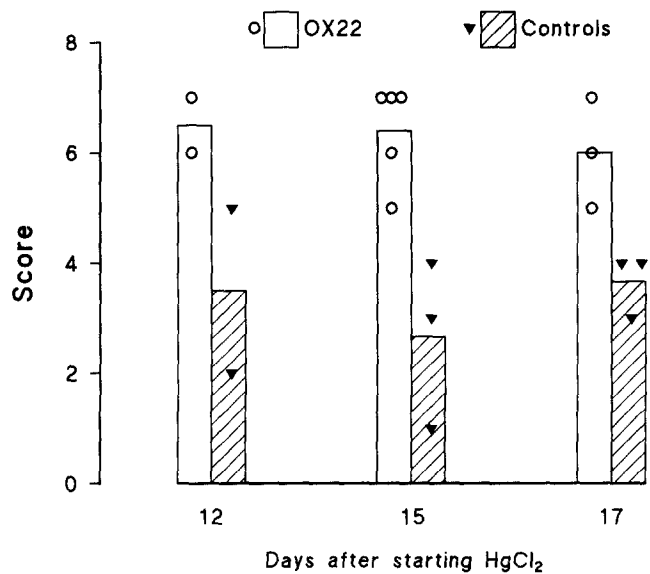


Figure 3. Experiment 3: effect of OX22 on tissue injury score. OX22 compared with control mAb, $p < 0.0005$.

the effect of OX22 treatment and antimicrobial treatment in experiment 4. OX22-treated animals had more severe tissue injury than animals receiving control mAb (Fig. 4, first bar compared with third bar at each time point, $p < 0.005$). Within the groups of animals receiving control mAb, antimicrobial pretreated animals had milder tissue injury than animals not pretreated (Fig. 4, first bar compared with second bar, $p = 0.039$). Animals pretreated with antimicrobials and then given OX22 were not significantly different from animals not pretreated with antimicrobials (Fig. 4, second bar com-

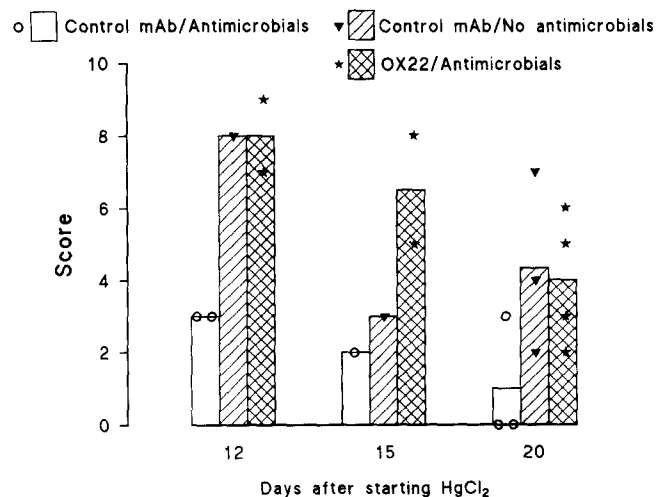


Figure 4. Experiment 4: tissue injury score. Effect of OX22 (first bar compared with third bar at each time point, $p < 0.005$); antimicrobial pretreatment (first bar compared with second bar, $p = 0.039$); and the interaction between OX22 and antimicrobials (second bar compared with third bar, $p = 0.32$).

pared with third bar, $p = 0.32$), indicating that OX22 treatment overcame the protective effect of antimicrobial pretreatment. Fig. 5 shows histological data for the cell transfer recipients: animals receiving unfractionated T cells had milder tissue injury than animals receiving T cells depleted in vitro of OX22^{high} cells ($p = 0.007$).

Anti-GBM Antibody Titers. In none of the experiments was there any significant difference in anti-GBM titer between OX22-treated animals and controls (Fig. 6). As reported previously (16), antimicrobial pretreatment did not affect anti-GBM titer.

Discussion

From this series of experiments, we conclude the following: treatment of thymectomized rats with OX22 in vivo leads to loss of OX22^{high} T cells. Animals treated with OX22 before receiving HgCl₂ develop more severe tissue injury than animals receiving a control mAb, and the protective effect of antimicrobial pretreatment in this experimental model is overcome by OX22 administration. OX22 treatment does not affect anti-GBM autoantibody levels. Transfer of unfractionated T cells from naive animals to OX22-treated animals reduces the severity of tissue injury, but transfer of T cells depleted of OX22^{high} cells confers less protection. Taken together, these data provide evidence for the hypothesis that OX22^{high} T cells play a protective immunoregulatory role in mercuric chloride-induced autoimmunity.

The epitope recognized by OX22 is found on macrophages and B cells as well as T cells (10, 22). This widespread distribution might have been expected to decrease the chances of administered OX22 antibody affecting a particular T cell subset. However, our data clearly demonstrate the loss of OX22^{high} staining T cells. We cannot be sure whether this represents depletion of the OX22^{high} T cell subset or simply modulation of the OX22 target from the cell surface. The

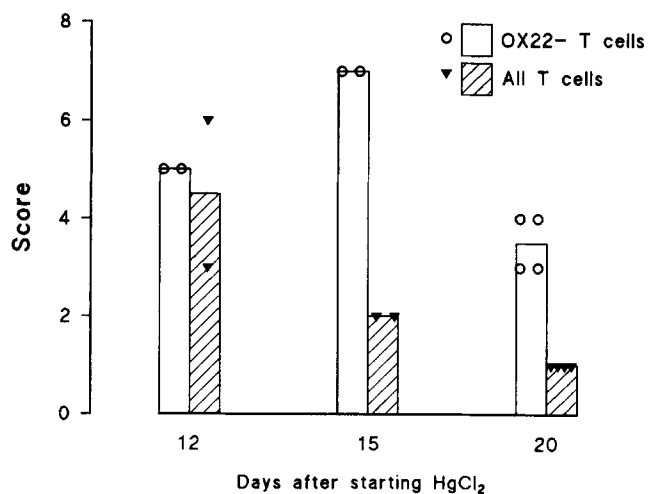


Figure 5. Experiment 4: tissue injury score in cell transfer recipients (OX22⁻, transfer of T cells depleted in vitro of OX22⁺ cells; all T cells unfractionated, $p = 0.007$).

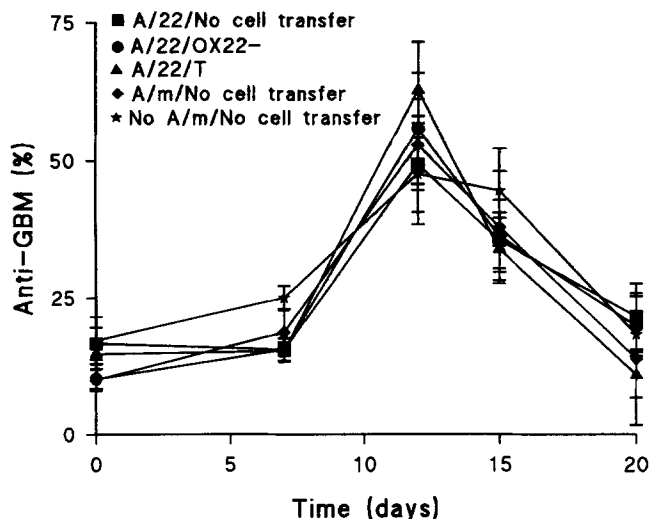


Figure 6. Experiment 4: serial anti-GBM antibody levels (mean \pm SE of the mean). (A) Antimicrobial pretreatment; (22) OX22 treatment in vivo; (m) mouse IgG (control mAb); (OX22⁻) transfer of T cells depleted in vitro of OX22⁺ cells; (T) transfer of unfractionated T cells.

distinction between these possibilities is not critical since a number of in vitro studies have shown that antibodies directed against various CD45 epitopes can have functional effects in the absence of cell killing (23–27). Our data show functional effects of OX22 in vivo, irrespective of the precise fate of the OX22^{high} T cell subset. Since CD8⁺ T cells are also OX22^{high}, treatment with OX22 in vivo would be expected to affect these cells, as well as the OX22^{high} subset of CD4⁺ T cells. The data from experiments 1 and 2 indicate that OX8 treatment in vivo, which effectively depletes CD8⁺ T cells (20), does not worsen tissue injury. We have conducted numerous experiments in which CD8⁺ T cells were depleted (20), and we have never observed any exacerbation of tissue injury as a result of this treatment. Indeed, in many cases, OX8-treated animals seemed to have less severe tissue injury (our unpublished observations). Depletion of CD8⁺ T cells by OX22 treatment cannot therefore explain our observations, and by implication, the effects of OX22 treatment in vivo are likely to be due to depletion of OX22^{high} CD4⁺ T cells.

In the experiments alluded to earlier (11), it was the OX22^{high} subset of T cells that led to tissue injury in nude rats, and the OX22^{low} subset which were protective. This has led to the suggestion (2) that OX22^{low} CD4⁺ T cells play a role in the prevention of autoimmunity in vivo. Our data on HgCl₂-induced autoimmunity indicate that in this model the roles of these subsets are reversed, with the OX22^{low} subset having a deleterious effect and the OX22^{high} subset playing a protective role. It has been postulated that HgCl₂-induced autoimmunity reflects preferential activation of Th2-type CD4⁺ T cells, since there is polyclonal B cell activation and hyper-IgE (28). In the mouse, IL-4 (predominantly a Th2 product) is a key cytokine in HgCl₂-induced autoimmunity (28, 29). In the rat, IL-4 is mainly produced by the OX22^{low} subset of T cells (2). Since it is known that

there are reciprocal interactions between OX22^{high} and OX22^{low} T cells (11), if HgCl₂ preferentially activates OX22^{low} (i.e., Th2-type) cells, it is perhaps not surprising that depletion of OX22^{high} (i.e., Th1-type) cells should lead to exacerbation of tissue injury in this model. There is evidence that the respective roles of OX22^{high} and OX22^{low} cells in alloimmune reactions are more akin to the roles we postulate in HgCl₂-induced autoimmunity, with the OX22^{high} subset having a “suppressor” role (30) and the OX22^{low} subset being effector cells (31). The protective effect of OX22^{high} cells which we observed may be strain specific. BN rats have anomalous T cell subsets, with a small CD8⁺ population (20), and a high ratio of OX22^{low} to OX22^{high} CD4⁺ T cells compared with other strains.

Genetic factors play an important part in determining the response to HgCl₂. In the rat, MHC and background genes are both involved in determining the response to HgCl₂ (32–34). The MHC haplotype that confers susceptibility is RT-1ⁿ (32). In resistant strains of rat, HgCl₂ induces immunosuppression, such that induction of organ-specific autoimmune diseases, e.g., experimental allergic encephalomyelitis (35) or Heymann nephritis (36), in rat strains which are normally fully susceptible, is prevented by prior treatment with HgCl₂. Susceptibility to HgCl₂-induced immunosuppression is also under the influence of the MHC, with RT-1^l being the key haplotype (37). A hypothesis which would fit in with the importance of the balance between Th1 and Th2 cells is that in susceptible strains, Th2 cells are preferentially activated by HgCl₂, whereas in resistant strains it is a Th1 response that predominates (28). There is evidence in the mouse that the MHC genotype determines whether Th1 or Th2 cells are activated by HgCl₂, with a mainly Th1-type response in H-2^d mice and a Th2-type response in H-2^s mice (28). Responsiveness in the mouse to HgCl₂ is linked to the MHC class II A^s molecule, and coexpression of the other class II molecule (the E molecule) in an A^s strain decreases responsiveness (34). There are also examples in the rat in which responses channelled via the two class II molecules appear to have opposing immunoregulatory effects (38, 39). Whether this applies to the response to HgCl₂ in the rat is unknown, but clearly there is ample precedent for the differential activation of regulatory subsets by different MHC molecules, both between and within particular haplotypes.

The effector mechanisms mediating tissue injury in this model are not well-defined. A direct pathogenic role for autoantibodies seems unlikely since in our experiments autoantibody titers were not affected by OX22 treatment or antimicrobials, so that there was a dissociation between severity of tissue injury and level of autoantibody. A role for granulocytes is suggested by the infiltration of tissues by these cells and the development of a leucocytoclastic vasculitis (16). OX22^{low} cells have a particular propensity to recruit cells of

the granulocyte lineage (40), so this could provide one mechanism for the deleterious role of OX22^{low} cells. As indicated earlier, depletion of CD8⁺ T cells *in vivo* may be associated with a protective effect, indicating that CD8⁺ T cells may play a role as effectors. Alternatively, the tissue injury may be directly mediated by OX22^{low} T cells. Although it is known that OX22^{high} T cells mediate some forms of GVHD (10), there is evidence that in so-called “syngeneic” GVHD, the key effector cell has an OX22^{low} phenotype (41, 42). The tissue injury we observed has some similarities with GVHD (16), and since our animals had not received a graft, the model may more closely resemble syngeneic GVHD, which is thought to be autoimmune mediated (43).

The protective effect of antimicrobial pretreatment is also of interest. The severity of tissue inflammation in GVHD is influenced by microbial load, both in experimental animals (44) and in humans (45), with pathogen-free individuals developing much less severe disease. Furthermore, in human diseases characterized by necrotizing leucocytoclastic vasculitis similar to that seen in our animals, antibiotics alone may have useful therapeutic effects (46), and tissue injury is known to be exacerbated by intercurrent infection (47). One possible mechanism for an effect of infection is illustrated by the recent demonstration that infection with a nematode can break T cell tolerance, apparently preferentially affecting the IL-4-producing (Th2-type) subset (48). In our experience, the protective effect of antimicrobials is somewhat variable. For instance, in the experiments described here, the effect was seen in the fourth experiment but not in the third. This may simply reflect variation in the microbial load of different batches of animals. Antimicrobials would not be expected to have any effect if the pathogen load of the animals is low.

HgCl₂-induced autoimmunity in the BN rat has features in common with a number of human autoimmune diseases (summarized in reference 49). At present, the treatment of human autoimmunity usually involves the use of nonspecific immunosuppressive drugs. If certain lymphocyte subsets have deleterious roles and others are protective, therapy could be improved by using reagents such as mAbs to specifically target the damaging subset. At present, little is known about the role of subsets of CD4⁺ T cells in humans, but this is an area where further advances could lead to the design of more specific forms of immunotherapy for human disease.

In summary, we have presented evidence that tissue injury in BN rats with HgCl₂-induced autoimmunity is exacerbated by *in vivo* depletion of OX22^{high} T cells, and that restoration of this population by cell transfer provides protection. Thus, OX22^{high} T cells seem to play an important protective immunoregulatory role in this model. This finding has implications for the understanding of interactions between Th1- and Th2-type cells in the rat, and may also have relevance to human autoimmunity.

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