Short Communication

Leptin-Deficient Mice Are Protected from Accelerated Nephrotoxic Nephritis

Ruth M. Tarzi,* H. Terence Cook,[†] Ian Jackson,[‡]
Charles D. Pusey,* and Graham M. Lord[‡]

From the Departments of Renal Medicine and Transplantation,*
Histopathology,[†] and Immunology,[‡] Faculty of Medicine, Imperial
College, Hammersmith Hospital, London, United Kingdom

Leptin is an adipose tissue-derived hormone that signals nutritional status to the hypothalamus. Recent evidence indicates that leptin modifies proinflammatory immune responses and may provide a key link between nutritional deficiency and immune dysfunction. To study the influence of leptin deficiency on immune-mediated renal disease, susceptibility to accelerated nephrotoxic nephritis was examined in leptin-deficient C57BL/6-*ob/ob* mice and C57BL/6 wild-type controls. The model was induced with sheep anti-mouse glomerular basement membrane antibody injected to mice preimmunized against sheep IgG, and mice were sacrificed 8 days after induction of disease. The leptin-deficient *ob/ob* mice were strongly protected from glomerular crescent formation, macrophage infiltration, glomerular thrombosis, and albuminuria in this model. Our findings suggest that leptin is required for the induction and maintenance of immune-mediated glomerulonephritis, and that blockade of the leptin axis might provide an attractive therapeutic possibility in human autoimmune disease. (Am J Pathol 2004, 164:385–390)

Leptin, the *obese* gene product, is a hormone produced mainly by adipocytes and is a central mediator of the neuroendocrine pathways involved in the control of food intake, basal metabolism, and reproductive function.¹ Leptin-deficient *ob/ob* mice display obesity, diabetes, reduced activity, reduced metabolic rate, and infertility. *Ob/ob* mice also have impaired cell-mediated immunity and a propensity to develop Th2 immune responses, similar to starved animals and malnourished humans.² Leptin replacement reverses the immunodeficiency and lymphoid atrophy associated with acute starvation in rodents.³ Leptin modulates cognate T-cell-mediated immune responses by signaling through the long isoform of

the leptin receptor (Ob-Rb) expressed on the cell surface of CD4⁺ T lymphocytes. Leptin also modulates phagocyte function, suggesting that leptin has an additional role in controlling the innate immune response.^{4–7}

Most forms of glomerulonephritis are immunologically mediated. Accelerated nephrotoxic nephritis is a model of immune complex glomerulonephritis, in which an immune response is raised to foreign anti-glomerular basement membrane antibody deposited in the glomerulus. This leads to a proliferative glomerulonephritis, characterized by albuminuria, leukocyte infiltration, glomerular capillary thrombosis, glomerular crescent formation, and renal impairment. Previously, investigators have demonstrated that CD4⁺ T-cell responses, especially Th1 responses, are important in mediating disease in this model.^{8–10} Leukocytes, in particular macrophages, are involved in the effector phases of nephrotoxic nephritis.¹¹ In this study, we have investigated the role of leptin deficiency on susceptibility to accelerated nephrotoxic nephritis, using *ob/ob* mice on a C57BL/6 background. Leptin-deficient mice were strongly protected from histological renal injury in this model. The protection was associated with a global reduction in humoral immune responses in two of three experiments performed. However, in one of the three experiments, *ob/ob* mice developed an immune response to the sheep IgG that was as strong as wild-type (WT) mice. These mice were still protected from disease, indicating that defects in effector responses of the *ob/ob* mice were also present. These results suggest that blockade of the leptin axis may provide a therapeutic approach in the treatment of immune-mediated glomerulonephritis.

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Address reprint requests to Graham Lord, M.D., Ph.D., Research Fellow, Harvard School of Public Health, Department of Immunology and Infectious Diseases, 651 Huntington Ave., Boston, MA 02115-6017. E-mail: glord@hsph.harvard.edu.

Materials and Methods

Mice

Male mice, 6 to 10 weeks old, were used throughout. Homozygous obese mice on a C57BL/6 background (*ob/ob*) and C57BL/6 controls (WT) were purchased from Harlan UK (Bicester, UK). All mice were housed with free access to food and water, according to institutional guidelines.

Preparation of Nephrotoxic Globulin and Induction of Accelerated Nephrotoxic Nephritis

A sheep was immunized with murine glomerular lysate initially in complete and then incomplete Freund's adjuvant, and serum was obtained with monthly and terminal bleeds. A γ -globulin-enriched fraction of immune serum was prepared by ammonium sulfate precipitation, after first heat inactivating complement activity. The precipitate was extensively dialyzed against phosphate-buffered saline (PBS), sterile filtered, and stored at -70°C . The endotoxin content of the nephrotoxic globulin was undetectable, measured using a Biowhittaker 1000-QCL LPS assay kit (Biowhittaker, Walkersville, MD).

Mice were immunized intraperitoneally with 0.2 mg of sheep IgG in a 50:50 mix with complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). In two initial experiments (experiments 1 and 2), 5 mg of nephrotoxic globulin (NTS) was injected intravenously to both WT and *ob/ob* mice 5 days after immunization ($n = 6/\text{group/experiment}$). In the third experiment (experiment 3), weight adjusted doses of NTS were given (0.15 mg/g = 3.75 mg for WT and 5 mg for *ob/ob*). The mice were monitored clinically, and 8 days after NTS injection, mice were anesthetized with intraperitoneal midazolam and fentanyl, and exsanguinated before harvesting the kidneys.

Histological Studies and Quantitative Immunofluorescence

Kidneys were fixed for 2 hours in Bouin's solution, transferred to 70% ethanol, processed to paraffin, and stained with periodic acid-Schiff (PAS) reagent. Samples were assessed by an observer blinded to the experimental group. Glomerular thrombosis was assessed by scoring individual glomeruli for PAS-positive material as follows: grade 0, no PAS-positive material; grade 1, 0 to 25% of glomerular cross-section PAS-positive; grade 2, 25 to 50%; grade 3, 50 to 75%; grade 4, 75 to 100%. Fifty glomeruli were scored per kidney and the mean glomerular thrombosis score was calculated for each mouse. Glomerular crescents were defined as glomeruli containing two or more layers of cells in Bowman's space (50 glomeruli counted per kidney, converted to percentage crescents).

For immunofluorescence, kidneys were embedded in OCT (CellPath, Powys, UK), snap-frozen in isopentane cooled with liquid nitrogen, and stored at -70°C . Sections of 5 μm were fixed in acetone for 10 minutes.

Glomerular mouse and sheep IgG was visualized by direct immunofluorescence using fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Fc-specific) and fluorescein isothiocyanate-conjugated monoclonal mouse anti-sheep IgG clone GT34 (Sigma). To quantitate immunofluorescence, sections were examined at $\times 100$ magnification using an Olympus BX4 fluorescence microscope (Olympus Optical, London, UK) and a Photonic Science Color Coolview camera (Photonic Science, Robertsbridge, UK). Samples from each experiment were stained on the same occasion and measured together. Images of the sections were captured by the image analysis equipment and the total pixel intensity for each glomerulus was measured and averaged for 20 glomeruli per section. The arbitrary units of fluorescence (AFU) correspond to the mean pixel intensity for 20 glomeruli for each mouse.

For immunoperoxidase staining for cell markers, kidneys were fixed for 4 hours in periodate-lysine-paraformaldehyde solution, washed overnight in 13% sucrose in PBS, and frozen in isopentane cooled with liquid nitrogen. Sections of 5 μm were stained for macrophages with FA11 (monoclonal rat anti-mouse CD68; Serotec, Oxford, UK) and CD4 with GK1.5 (BD Pharmingen, San Diego, CA), using a three-layer immunoperoxidase technique as previously described.¹²

Serum Creatinine, Albumin, and Albuminuria

Serum creatinine and albumin were measured in the Department of Clinical Chemistry, Hammersmith Hospital, London, UK. Serum albumin was analyzed using the bromocresol green method and serum creatinine was measured using an Olympus AU640 autoanalyzer. Individual 24-hour collections of urine were performed using metabolic cages, with free access to food and water. Albuminuria was assessed by radial immunodiffusion against a rabbit anti-mouse albumin. Briefly, a gel was prepared containing rabbit anti-mouse albumin (Sigma). Urine samples or mouse albumin standards were loaded into wells in the gel and incubated for 24 hours at 4°C . The gel was washed, dried, and stained with Coomassie blue, and the albumin concentration of the urine was calculated with reference to the standard curve.

Measurement of the Murine Anti-Sheep IgG Immune Response

Serum mouse anti-sheep IgG levels were measured by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Nunc Maxisorb; Fisher Scientific, Loughborough, UK) were coated overnight at 4°C with 100 $\mu\text{g}/\text{ml}$ of sheep IgG (Sigma). After blocking for 1 hour with 3% bovine serum albumin in PBS/1% bovine serum albumin/Tween 20 (ICN Biomedicals Inc., Aurora, OH) diluted serum samples were incubated for 1 hour at 37°C . For each ELISA a range of dilutions of a known positive standard sample was run in parallel. Goat anti-mouse IgG (Fc-specific) conjugated to alkaline phosphatase (Sigma) was used as a secondary and the OD₄₅₀ was

Table 1. Baseline Parameters of Unmanipulated Mice Expressed as Median (Range)

	C57BL/6	C57BL/6 <i>ob/ob</i>
Number of mice	6	6
Age (weeks)	9	9
Weight (g)	25 (25–29)	40 (38–42)*
Albuminuria (mg/24 hours)	0.03 (0.03–0.06)	0.11 (0.04–0.33)*
Serum albumin (g/l)	28 (26–31)	35 (34–38)*
Serum creatinine (μ mol/L)	42 (37–55)	56 (42–67)
Macrophages/gcs	0.04 (0.02–0.11)	0.02 (0.00–0.05)
CD4+ T cells/gcs	0.02 (0.00–0.04)	0.03 (0.00–0.06)

* $P < 0.01$ compared with wild type, Mann-Whitney *U*-test.

measured using *p*-nitrophenyl phosphate as the substrate (Sigma). Activity of the samples was measured with reference to nonsaturated portion of the standard curve.

Measurement of Serum Leptin Levels

Serum leptin levels were measured using a commercially available kit (Quantikine M mouse leptin immunoassay; R&D systems, Minneapolis, MN). Briefly, a sandwich ELISA was performed by adding standard dilutions or diluted samples to wells coated with polyclonal anti-leptin antibodies. After washing, an enzyme-linked polyclonal antibody specific for mouse leptin was added to the wells. After washing an enzyme substrate was added and after 30 minutes the OD at 450 nm was read using an ELISA plate reader. The manufacturer reported the mean level of leptin for a group of male mice to be 1500 pg/ml (range, 0 to 3200 pg/ml).

Statistics

Results are expressed as median (range). Nonparametric tests of significance were applied throughout. For comparing two groups, the Mann-Whitney *U*-test was used. GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze the data. Differences were considered significant when $P < 0.05$.

Results

Baseline Renal Parameters of *ob/ob* Mice

The *ob/ob* mice were ~30% heavier than the WT mice at the initiation of the experiments (6 to 10 weeks old), $P < 0.01$. Most of the *ob/ob* mice were diabetic (glycosuria on urine dipstick). There was also a statistically significant minor increase in albuminuria in the *ob/ob* mice ($P < 0.01$), although the absolute levels were low (Table 1). In addition, there was a trend to a higher baseline serum creatinine in the *ob/ob* mice ($P = 0.06$) and serum albumin levels were significantly higher in the *ob/ob* mice than the WT controls ($P < 0.01$). However, the renal histology of the *ob/ob* mice was normal by light microscopy of

Figure 1. Histological and functional disease in *ob/ob* mice and WT C57BL/6 controls at day 8 after induction of nephritis with weight-adjusted doses of NTS (experiment 3). **A:** Glomerular thrombosis score ($n = 50$ glomeruli per kidney); **B:** percentage of glomerular crescents; **C:** macrophages/glomerular cross-section; **D:** albuminuria (mg/12 hours); **E:** serum albumin (g/L); and **F:** serum creatinine (μ mol/L). The *ob/ob* mice were strongly protected from glomerulonephritis.

PAS-stained sections, and there was no glomerular macrophage or CD4 T-cell infiltration (Table 1).

Leptin-Deficient Mice Were Protected from Accelerated Nephrotoxic Nephritis

Leptin-deficient *ob/ob* mice were strongly protected from histological injury at day 8 after the induction of accelerated nephrotoxic nephritis in all three experiments performed. In the first two experiments (experiments 1 and 2), 5 mg of nephrotoxic globulin (NTS) was given to both WT and *ob/ob* mice. The *ob/ob* mice showed protection from glomerular thrombosis and glomerular crescents in both of these experiments ($P < 0.05$ experiment 1 and $P < 0.01$ experiment 2). To exclude any underdosing of NTS because of the higher body mass of the *ob/ob* mice, a third experiment (experiment 3) was performed, using weight-adjusted doses of NTS (3.75 mg of NTS to WT mice and 5 mg of NTS to *ob/ob* mice). Histological and functional parameters from experiment 3 are shown in Figure 1. Leptin-deficient *ob/ob* mice were strongly protected from histological injury (Figure 1, A to C, and Figure 2, A and B). Urine collections taken from days 2 to 3 after nephritis induction showed protection of the *ob/ob* mice from albuminuria ($P < 0.05$) (Figure 1D), and the *ob/ob* mice were also protected from hypoalbuminemia at the termination of the experiment (Figure 1E). Differences in serum creatinine at day 8 failed to reach statistical significance (Figure 1F), but serum creatinine readings in mice are an insensitive marker of renal disease because of low muscle bulk. There was no significant difference in the glomerular infiltrate of CD4-positive T cells between WT and *ob/ob* mice [WT median 0.16 CD4 T cells/glomerular cross-section (gcs) (range, 0.04 to 0.26), and *ob/ob* 0.16 cells/gcs (range, 0.08 to 0.3)].

Quantitation of sheep IgG deposited on the glomerular basement membrane at day 8 showed no significant difference in experiments 1 and 2, in which 5 mg of NTS was given to each mouse. However, in experiment 3, in

which a higher dose of NTS was given to the *ob/ob* mice (5 mg), than the WT mice (3.75 mg) there was greater fluorescence for sheep IgG from the *ob/ob* glomeruli than the WT glomeruli ($P < 0.01$), reflecting the higher dose of NTS given (Figure 2E). Despite this, the *ob/ob* mice were still strongly protected from disease.

Humoral Immune Responses and Deposited Glomerular IgG

As a measure of the humoral immune response, deposited glomerular mouse IgG was quantitated on frozen kidney sections from day 8 after nephritis induction. In experiments 2 and 3, there was significantly less deposited mouse IgG on the *ob/ob* glomeruli than the WT glomeruli ($P < 0.01$) (Figure 2F shows results from experiment 3). In accordance with this, serum-specific IgG responses against sheep IgG were also reduced in serum taken at day 8 in the *ob/ob* mice for these experiments (Figure 2G shows results from experiment 3).

Interestingly however, in experiment 1, five of six of the *ob/ob* mice developed a strong humoral immune response against sheep IgG, which was not significantly less than WT (Figure 3A). There was also no difference in the deposited glomerular mouse IgG between WT and *ob/ob* in this experiment [WT median 44 AFU (range, 22 to 55 AFU) and *ob/ob* 36 AFU (range, 10 to 54 AFU)]. The reason for the discrepancy of the immune response in this experiment compared with the other two experiments performed is not clear. Despite the fact that the *ob/ob* mice developed a strong immune response in this experiment 1, they were still strongly protected from histological injury compared with WT (Figure 3; B to D), with only one of six *ob/ob* mice developing appreciable thrombosis, crescents, or macrophage infiltration, compared with five of six WT mice. These results imply that as well as defects in antibody responses, there may be additional defects in innate effector responses in *ob/ob* mice leading to reduced glomerular damage.

Studies of the immune responses of *ob/ob* mice have described a Th2 pattern of T-cell immune responses, including decreased interferon- γ production and increased interleukin-4 production. We were unable to obtain sufficient *in vitro* proliferation of splenic T cells in response to the sheep IgG antigen to measure cytokine responses. Subclass switching of IgG production to IgG1 rather than IgG2a is another feature of a Th2 response. The subclass humoral responses to sheep IgG were

Figure 2. **A** and **B**: Representative PAS-stained glomeruli from WT and *ob/ob* mice, respectively, day 8 after induction of nephritis. The WT kidneys showed glomerular hypercellularity, capillary thrombosis, and crescent formation (**A**), while the *ob/ob* kidneys were virtually normal (**B**). **C** and **D**: Glomerular direct immunofluorescence for mouse IgG showing deposition of greater quantities of IgG in the WT glomerulus (**C**) than the *ob/ob* glomerulus (**D**). **E**: Quantitative immunofluorescence for sheep IgG at day 8 after induction of nephritis (AFU). The mice were given weight-adjusted doses of NTS and more sheep IgG was seen on the *ob/ob* glomeruli than the WT glomeruli. **F**: Quantitative immunofluorescence for glomerular mouse IgG at day 8 confirming greater quantities in WT glomeruli than *ob/ob* glomeruli. **G**: Serum levels of mouse total IgG specific for sheep IgG at day 8 after the induction of accelerated nephrotoxic nephritis (arbitrary ELISA units).

Figure 3. Day 8 after induction of accelerated nephrotoxic nephritis with 5 mg of NTS (experiment 1). *Ob/ob* mice were protected from glomerular thrombosis and crescents despite an equivalent humoral immune response to WT. **A:** Serum levels of mouse IgG specific for sheep IgG at day 8 after induction of accelerated nephrotoxic nephritis (arbitrary ELISA units). **B:** Glomerular thrombosis score ($n = 50$ glomeruli per kidney); **C:** percentage of glomerular crescents; **D:** macrophages/glomerular cross-section.

therefore measured in serum taken at day 8. Mouse IgG2a levels against sheep IgG were low in both WT and *ob/ob* mice (1:50 dilution used) and there was no significant difference between the groups in all three experiments (data not shown). IgG1 levels were relatively higher (1:2000 serum dilution), but were significantly

lower in the *ob/ob* group than the WT group for experiments 2 and 3 ($P < 0.01$, not shown). There was no significant difference in the specific IgG1 levels between WT and *ob/ob* in experiment 1. Therefore, the *ob/ob* mice did not show a Th2 shift of specific antibody subclass production (that is relatively higher IgG1 levels and lower IgG2a levels than WT) in these experiments.

Leptin Levels during Glomerulonephritis Induction

Leptin levels have been reported to rise during acute inflammation, such as autoimmune encephalitis and bacterial peritonitis.^{7,13,14} To investigate serum leptin levels during nephritis induction, levels were measured at baseline, after immunization, and after injection of NTS to eight male WT mice. However, leptin levels remained within the normal range throughout disease induction, and so leptin may be playing a permissive rather than a regulatory role.

Discussion

The results shown here clearly demonstrate that leptin-deficient *ob/ob* mice were strongly protected from histological renal injury and albuminuria in accelerated nephrotoxic nephritis. The protection of the *ob/ob* mice cannot be explained purely by the larger size of these mice, as weight-adjusted doses of nephrotoxic globulin were given, but the *ob/ob* mice were still strongly protected from nephritis compared with WT mice.

In two of three experiments, there was a global reduction in anti-sheep antibody responses and deposited glomerular mouse IgG, which may account for the protection from disease. However, in one experiment, *ob/ob* mice developed an equivalent humoral immune response to WT mice. Despite this, only one of six of the mice in this experiment developed histological injury. This implies defects in both innate effector responses and humoral responses in the *ob/ob* mice. *In vitro* experiments have certainly demonstrated defects in phagocytosis and cytokine production by *ob/ob* and *db/db* macrophages.^{4–6}

Similar reductions in humoral immune responses to those found here were shown in a model of antigen-induced arthritis.¹⁵ This impaired humoral response could be secondary to a direct effect of leptin signaling on B cells and/or to an indirect effect on T-cell function, as CD4⁺ T cells are critical regulators of antibody production. Ob-Rb mRNA is expressed by both murine B and T lymphocytes.^{2,15} There is strong evidence for an effect of leptin on T-cell survival, proliferation, and function.^{2,3} However, the evidence for whether leptin exerts a direct effect on B cells is more circumstantial. It has previously been noted that diabetic mice (deficient in Ob-Rb, the long isoform of the leptin receptor) have reduced numbers of circulating and bone marrow B cells.¹⁶

Several studies have shown that leptin deficiency predisposes to Th2 rather than Th1 immune responses.^{2,17,18} *In vitro* studies showed that as well as promoting T-cell

allogeneic responses in general, leptin increased interferon- γ production, and reduced interleukin-4 production.² In an antigen-induced arthritis model, T-cell proliferation and interferon- γ production by T cells derived from local lymph nodes were reduced in the *ob/ob* mice compared with WT.¹⁵ Splenic T-cell proliferation was also reduced in a model of experimental autoimmune encephalitis, with additional evidence of a Th2 shift in immune responses.¹⁷ We were unable to measure T-cell proliferative responses in our model, as we failed to obtain reproducible T-cell proliferation in response to sheep IgG *in vitro*.

The presence of hyperglycemia and high cortisol levels in *ob/ob* mice, occurring as a consequence of obesity rather than a primary effect of leptin, may also affect the immune response. However, previous experiments with food restriction showed that reducing plasma levels of cortisol and glucose in the *ob/ob* mice did not reverse the immune defects, whereas leptin replacement restored a normal immune response.^{2,3}

During the development of nephritis in WT mice, leptin levels remained within the normal range. This is in contrast to experimental autoimmune encephalitis, in which levels were seen to surge several days before disease development.¹⁴ However, this model is different to the accelerated nephritis model in that disease develops throughout several weeks. It may be that in our model the timing of the blood samples did not detect a transient surge. It is also possible that in the nephritis model, leptin plays a permissive rather than a regulatory role. In this way, as long as leptin is present, disease may occur, although the absolute levels may not be critical.

In summary, these investigations show that leptin-deficient *ob/ob* mice were strongly protected from autoimmune glomerulonephritis, associated with defects in humoral immunity, and probable additional defects in the innate immune response. We have previously suggested that falling leptin levels signal falling nutrient availability and cause suppression of energy expensive immune responses.¹⁹ Furthermore, subtle changes in leptin levels may be pathogenic in the development and maintenance of autoimmunity.²⁰ Therefore, the data presented in this article further supports the hypothesis that blockade of the leptin axis is an attractive therapeutic possibility in the treatment of autoimmune diseases.

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